Mechanisms of Weed Suppression in Wheat (*Triticum aestivum* L.) and Canola (*Brassica napus* L.) in Southeastern Australia

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Evaluation of Selected Commercial Wheat Cultivars for Canopy Architecture, Early Vigour, Weed Suppression and Yield in the Southern NSW  

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I hereby declare that this submission is my work and to the best of my knowledge and belief, understand that it contains no material previously published or written by another person, nor material which to a substantial extent has been accepted for the award of any other degree or diploma at Charles Sturt University or any other educational institution, except where due acknowledgement is made in the thesis. Any contribution made to the research by colleagues with whom I have worked at Charles Sturt University or elsewhere during my candidature is fully acknowledged.

I agree that this thesis be accessible for the purpose of study and research in accordance with normal conditions established by the Executive Director, Division of Library Services or nominee, for the care, loan and reproduction of thesis, subject to confidentiality provisions as approved by the University.

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James M Mwendwa (BSc, MSc)
Charles Sturt University, Wagga Wagga
March 2019
List of Abbreviations

AAMPO - 2-acetylamino-7-methoxy-3H-phenoxazin-3-on

AAPO- 2-acetylamino-3H-phenoxazin-3-one;

AMPO - 2-amino-7-methoxy-3H-phenoxazin-3-one;

ANOVA - Analysis of Variance

APO- 2-amino-3H-phenoxazin3-one;

APWMA- Australian Pesticides and Veterinary Medicines Authority

AUD – Australian dollar

BOA - benzoaxolin-2-one

BXs- Benzoxazinoids

DIBOA- 2,4-dihydroxy-1,4-benzoaxin-3-one

DIBOA-Glc- 2-β-D-glucopyranosyloxy-4-hydroxy-1,4-benzoaxin-3-one

DIBOA-Glc-hex - double-hexose derivative of DIBOA

DIMBOA- 2,4-dihydroxy-7methoxy-1,4-benzoaxin-3-on

DIMBOA-Glc- 2-β-D-glucopyranosyloxy-4-hydroxy-7-methoxy- 1,4-benzoaxin-3-one

ESI -electrospray ionisation

HBOA- 2-hydroxy-1,4-benzoaxin-3-one

HBOA- GLc- 2-β-D-glucopyranosyloxy-1,4-benzoaxin3-one

HBOA-Glc-hex -doublehexose derivative of HBOA

HMBOA- 2-hydroxy-7-methoxy-1,4-benzoaxin-3-one

HMBOA-Glc- 2-β-D-glucopyranosyloxy-7-methoxy-1,4-benzoaxin-3-one
IWM – integrated weed management

LAI- leaf area index

LC-MS – liquid chromatography mass spectrometry

LC-MS/MS - Liquid chromatography with tandem mass spectrometry

LSD - Least Significant Difference

MANOVA – Multivariate Analysis of Variance

MBOA- 6-methoxy-benzoxazolin-2-one

MRM - multiple reaction monitoring

MS- mass spectrometry

NDVI - normalised difference vegetative index

NSW- New South Wales

PAR - photosynthetically active radiation

PCDL- personal compound database and library

PLS – partial least squares

QQQ- triple quadrupole

QToF- quadrupole time of flight

RCB- randomised complete blocks

TIC - total ion chromatogram

UHPLC – ultra-high-pressure liquid chromatography
Acknowledgements

This thesis has been completed under the supervision of Prof. Leslie A. Weston, Dr William B. Brown, Dr Hanwen Wu, Prof. Jeffrey D. Weidenhamer and A/Prof. Jane C. Quinn. I would like to express my deepest appreciation for the direction, mentorship, scholastic guidance and encouragement of my supervisors. Thank you for challenging me every day and believing in me. To Prof. Weston, I am very grateful for the many discussions and for all the opportunities you provided me to improve my skills. To Dr. Brown, thank you for your technical input in setting up and monitoring field trials. Special thanks to Dr Paul A. Weston for his support with statistical analysis, LC-MS (QTOF and QQQ) analysis and data computation.

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Many others have supported me during my PhD study in different ways and capacities. Primarily, I would like to thank Mr Graeme Heath for his support and the countless hours we spent together in the field collecting samples. I am grateful for the Plant Interaction Research Group at Charles Sturt University including visiting scholars for their assistance with field experimentation, data collection and laboratory extractions. Special thanks to A/Prof M. B Bagherieh-Najjar, Dr Saliya Gurusinghe, Dr Xiaocheng Zhu, Dr Dominik Skoneczny, Dr Razia Shaik, Dr. Yuling Yin, Mr Vincent West and Mr
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Publications and Manuscripts

The following list is mostly a collection of manuscripts, conference proceedings, abstracts and posters resulting from the research of this PhD project.

1. Book Chapter


2. Journal Publications and Published Conference Proceedings


3. Conference Presentations


4. Abstracts of Oral Communications and Poster Presentations


5. Newsletter/Media Articles


Abstract

This project was designed to assess the use of competitive canola and wheat cultivars for weed suppression under southern Australian field conditions in an effort to apply IWM strategies for weed management and provide fundamental knowledge on the mechanisms underlying crop interference with broadacre weeds. Replicated canola and wheat cultivar field trials were conducted in Condobolin and Wagga Wagga in 2014-2016. Crop and weed growth were monitored at selected phenological growth stages by assessment of above-ground canopy traits including early vigour, canopy closure, crop height, crop and weed biomass, leaf area index (LAI), Normalised difference vegetation index (NDVI), and light interception. In addition, for wheat, shoot and root tissues and rhizosphere soil samples were collected for metabolic profiling. Extracts of plant tissue and soil were analysed for secondary metabolites associated with weed suppression using liquid chromatography-mass spectrometry, with a particular focus on benzoaxazinoids (BXs) and related microbially-produced metabolites.

Wheat cultivar and location influenced the production of wheat biomass, early vigour, leaf area index, light interception, crop height and yield, and weed number and biomass. Early crop vigour and biomass accumulation were strongly and inversely correlated with accumulation of weed biomass in both year and location, suggesting these traits were associated with weed interference. Accumulation of weed biomass was inversely related to early crop vigour, crop biomass, NDVI, height, LAI and the light interception at both locations.

Cultivar differences were also observed in canola canopy architecture and yield; early season biomass, light interception, LAI and crop vigour exhibited both year and location interactions. Cultivars with the greatest biomass, light interception, LAI, and visual vigour were most weed suppressive. Although crop and weed biomass accumulation differed significantly among cultivars for both location and year, weed biomass was strongly and inversely related to crop cultivar biomass production. Hybrid cultivars exhibited up to 50% less weed biomass in contrast to open-pollinated cultivars.

Metabolic profiling provided key information related to biosynthesis and release of metabolites associated with weed suppression in commercial field-grown wheat under standard production practices, in comparison to cereal rye and the heritage wheat cultivar Federation, both recognised for their ability to effectively suppress weeds. Up to 15 individual BXs including BX glycosides, lactones, and hydroxamic acids were detected.
in roots and soil. Both qualitative and quantitative differences in BXs were observed and were dependent on cultivar, crop growth stage, season and location.

Phytotoxic microbial metabolites (the phenoxazinones 2-amino-3H-phenoxazin3-one (APO), 2-actylamino-3H-phenoxazin-3-one (AAPO), 2-amino-7-methoxy-3H-phenoxazin-3-one (AMPO), 2-acetylamino-7-methoxy-3H-phenoxazin-3-on (AAMPO)) were detected and were transformed in the wheat rhizosphere from benzoxazolinones produced by roots and exudates through the action of soil microbiota in both locations. Abundance was dependent on cultivar, phenology, season and location. These findings first demonstrate that production of phenoxazinones can occur at ecologically important concentrations in Australian soils, such that weed suppression by certain wheat cultivars may be facilitated under field conditions. Further research is required to determine if 1) cultivars expressing production of BX metabolites including hydroxamic acids may be targeted for enhanced weed control through biosynthetic modification or 2) if soil microbial transformants may be regulated to encourage production of the potently active phenoxazinones.
Preface

This PhD research was fully funded and supported by the Grains Research and Development Corporation (GRDC) through project UCS 00020, 00022 and 00023. A GRDC top-up scholarship was awarded to James Mwendwa as part of UCS 00020. GRDC funding included scholarship, operating funds and partial salary for a technical position awarded to James Mwendwa who assisted with research performed on UCS 00020 and 00023. In addition, the Graham Centre for Agricultural Innovation provided funding to James Mwendwa through travel awards to attend and present research at both national and international conferences.

To fulfil the UCS 00020 milestones, field trials were performed in 2014-17 in the project “Weed Management in the Southern Region Mixed Farming Systems - Strategies to Combat Herbicide Resistance”. The key objectives of this study included 1) determination of novel chemical and non-chemical approaches for management of weeds in cereal and pulse crops, 2) evaluation of selected current commercial canola (Brassica napus L.) cultivars for above-ground weed competitive traits with and without the use of pre-emergent herbicides, and 3) evaluation of mechanisms of weed suppression in genetically diverse wheat (Triticum aestivum L.) cultivars, including competition for resources and allelopathy. Objectives 2 and 3 are described in research reported on in this thesis that was conducted primarily by James Mwenda and assisted by CSU colleagues.

To combat the challenges of environmental pollution and herbicide resistance, crop types which release natural herbicides or produce significant biomass to reduce weed growth (i.e. allelopathic or competitive crops) have been shown to play a useful role in integrated weed management strategies for broadacre crops. The key objectives of this PhD project were to 1) evaluate the mechanism(s) of weed suppression by both Australian canola and wheat cultivars in the field, grown commercially in the southern cropping region and 2) provide fundamental knowledge on the biochemical mechanism(s) underlying allelochemical interference of wheat on annual weeds.

Wheat and canola were selected for further study due to their importance in Australian agriculture and relative contribution to the agricultural economy. Wheat first came to Australia with European settlement in 1788, and considerable breeding has been performed to develop high yielding cultivars that are suitable for the Australian climate. It is Australia’s largest grain crop and accounts for ca. 90% of the total value of grain production, 3% of the total value of Australia’s exports, and 16% of Australia’s total farm
exports. Wheat also accounts for more than half of the broadacre acreage farmed in Australia. Canola is the dominant broadleaf break crop in the profitable and sustainable cereal cropping system in southern Australia and is mainly used in rotation with wheat and barley. A diverse selection of canola cultivars is commercially available, including those resistant to herbicides in both open-pollinated and hybrid cultivars to accommodate the needs of growers across diverse rainfall zones of Australia.

The combined effects of crop competition and chemical interference through allelopathy determine the total weed-suppressive potential of a given cultivar, and research groups worldwide have been working to improve such traits simultaneously to achieve maximum weed suppression. This project, therefore, evaluated selected diverse Australian canola and wheat cultivars for their ability to suppress annual weeds as assessed by early crop vigour, biomass, height, canopy photosynthetically active radiation, leaf area index, weed biomass and weed count in multi-location field experimentation throughout three years (2014-2016).

The field studies were complemented by laboratory experiments designed to identify the year, location and cultivar differences with respect to the production of phytotoxic plant secondary metabolites associated with weed suppression. Metabolic profiling of wheat shoots, roots and rhizosphere soil was performed to evaluate the potential for allelochemical interactions with weeds under field conditions. Phytotoxic phenoxazinones were previously detected in abundance in the soil surrounding certain suppressive cultivars, suggesting certain settings result in allelopathic tendencies of both wheat and cereal rye (Secale cereale L.) cultivars.

All fieldwork was performed at Condobolin NSW DPI Agricultural Research and Advisory Station (ARAS) and the Wagga Wagga Graham Centre Field Research Site, NSW. Initial ultra-high pressure liquid chromatography with tandem mass spectrometry (UHPLC-MS) method development was performed in the laboratory of A/Prof Inge Fomsgaard at Research Centre Flakkebjerg, Department of Agroecology, Aarhus University, Denmark in 2014 using benzoxazinoid standards obtained from the same laboratory. Advanced laboratory research and optimised analysis including plant tissue/soil extractions and UHPLC-MS/MS were performed in the laboratories of the National Life Sciences Hub at Charles Sturt University, Wagga Wagga, Australia.

This project involved countless hours spent collecting and extracting shoots, roots and soil for LC-MS analysis. Further months were spent analysing more than 1500
samples using both LC-QTOF and LC-QQQ mass spectrometry to characterise and quantify the benzoxazinoids and phenoxazinones produced by various wheat cultivars under Australian conditions. I strongly believe that this project, which combined agronomy, plant physiology, analytical chemistry and plant biochemistry to study plant interactions with weeds, has provided a strong insight into the biochemical defence mechanisms of wheat. Completion of this work also necessitated multidisciplinary collaboration with a diverse group of researchers working on the larger GRDC project, UCS 00020.

Conducting research on this GRDC-supported project has provided ample funding for project support and has allowed me, James Mwendwa, to explore a relatively unknown frontier in plant science, secondary metabolite production in the wheat rhizosphere under field conditions. In addition, I have gained a tremendous amount of knowledge about natural plant products, plant biochemistry and physiology, and I hope to use this knowledge to develop my interdisciplinary analytical skill set further.
General Introduction

Globally, weeds are one of the main constraints for yield loss in field crops. In fact, the potential yield loss due to weeds is much higher in grain crops than from other pests; overall, weeds produced the highest potential loss (34%), with animal pests and pathogens being less important (losses of 18 and 16%, respectively) (Oerke 2006; Jabran et al. 2015). Recently, herbicide resistance was estimated to cost AUD 187 million annually in additional herbicide treatment costs in Australia, on top of the costs of using extra integrated weed management practices (Llewellyn et al. 2016). Further, Llewellyn et al. (2016) estimated the overall cost of weeds to Australian grain growers at AUD 3.3 billion annually, which on average equates to AUD 146/ha in expenditure and yield losses.

Herbicides have typically been used as a simple and reliable technology for weed control. However, intensive and non-judicious use of herbicides has resulted in the widespread appearance of herbicide-resistant weeds (Manalil 2014). Herbicide-resistant weeds are on the rise across Australia, including an increasing number of crop weeds exhibiting resistance to multiple herbicides (Owen et al. 2013). To combat the challenges of environmental pollution and herbicide resistance, the development of crop cultivars which release natural herbicides or produce significant biomass to reduce weed growth (i.e. allelopathic or competitive crops) has been suggested (Jabran et al. 2015).

Both competition and allelopathy as mechanisms of plant interference have been well documented under controlled conditions but not necessarily in the field (Weston 2005). The combined effects of allelopathy and crop competition determine the total weed-suppressive potential of a given cultivar, and research groups worldwide have been working to improve both traits simultaneously to achieve maximum weed suppression (Bertholdsson et al. 2012; Worthington and Reberg-Horton 2013), particularly in cereal crops. However, the relationship between crop competition and allelopathy as mechanisms of plant interference has not been fully addressed in Australian grain crops.
Thesis outline

The thesis is divided into nine chapters consisting of seven manuscripts published, submitted or prepared for submission to peer-reviewed journals including two conference proceedings papers and one book chapter. Each experimental chapter includes the following sections: Abstract, Introduction, Materials and Methods, Results, Discussion, Acknowledgements and References.

The eight chapters are organised in two parts: 1) evaluation of crop competition and weed suppression by selected canola cultivars (2 chapters) and 2) mechanism of weed suppression in selected Australian wheat cultivars including the secondary metabolites involved in weed interference (4 chapters). Chapter 8 provides a general discussion of the main findings and recommendations for future research. The appendices comprise three sections a) three additional manuscripts, b) conference abstracts and c) oral communications and poster presentations as outputs from the research project. This thesis, as a series of manuscripts, results in some unavoidable repetition, and I ask for your understanding while reviewing this thesis.

Chapter 1 presents the review of related literature which has been published as a chapter in the “Integrated Weed Management for Sustainable Agriculture” by Burleigh Dodds Science Publishing Limited (UK). The chapter provides a broad review of the literature on competitive grain crops and cultural strategies for weed management, including the use of weed-suppressive cultivars, post-harvest crop residues and cover crops for management of the weed seedbank and eventual weed suppression.

This chapter also addresses essential factors influencing the effect of allelopathy on weeds, including soil and environmental conditions which limit or intensify the efficacy of allelochemicals. It is important to note that the case studies data in sections 6 and 7 of this chapter on the production of benzoazinoids in cereal crops and competitive cereal cultivars as a tool in integrated weed management are part of my research data completed as part of this PhD project.

Part one consists of Chapters 2 and 3. Chapter 2 is a manuscript published in the peer-reviewed Journal of Crop Protection. The chapter presents 1) findings on the evaluation of the impact of residues of several grain crops on winter and post-harvest summer annual weed establishment from 2012 to 2014 and 2) in-crop and post-harvest weed suppression in 2014-2015 using a genetically diverse set of canola cultivars, including those found to be highly weed-suppressive in the first trial.
Chapter 3 presents a further investigation on an evaluation of the diversity in competitive ability for weed suppression and yield tolerance to weed infestation by genetically diverse commercial Australian canola cultivars, with emphasis on assessment and impact of plant growth, including early vigour and crop canopy architectural traits. In addition, the competitive ability of canola cultivars in the presence and absence of pre-emergent herbicides under natural weed infestations in two locations typically receiving low to moderate rainfall is compared.

Part two consists of Chapters 4, 5, 6, and 7 which present results on the mechanism of weed suppression in selected Australian wheat cultivars. Chapter 4 is a short manuscript published in the peer-reviewed proceedings of the 20th Australasian Weeds Conference in 2016. Initial data is presented from the assessment in 2014-2015 of above-ground competitive traits of selected spring and winter wheat cultivars which are well adapted for the southern farming region. In Chapter 5, specific crop morphological traits were evaluated for their association with weed suppression in a two-year study conducted from 2015-2016 over two locations with the objective of evaluating genotypically diverse cultivars for enhanced weed suppression and yield tolerance in the low to moderate rainfall zone in southern Australia.

Chapter 6 is another short manuscript published in the peer-reviewed Proceedings of 20th Australasian Weeds Conference in 2016 and presents initial method development for metabolic profiling of benzoxazinoids in weed-suppressive and early vigour wheat cultivars using LC-MS QToF and LC-MS Qtrap mass spectrometry. Chapter 7 is a manuscript prepared for Plant and Soil. It presents the results of additional metabolic profiling with crucial information regarding crop metabolism and microbial conversion of secondary metabolites in the soil associated with weed suppression in commercial wheat cultivars.

Chapter 8 provides a general discussion of the entire research project and recommendations for future studies. The findings from all the chapters are summarised, and the implications for future research directions are discussed.

Finally, in Appendix A, other journal publications and conference proceedings which I have contributed to through participation in the trials and/or compilation of the data as part of training and skill development are presented. In addition, conference abstracts and posters are presented in Appendix B and C, respectively. Lectures and/or poster presentations at these conferences, workshops and seminars are also presented.
References


Objectives and Hypotheses

2.1 Research objectives

The resistance of weeds to herbicides is increasingly threatening sustainable grain crops production leaving growers with limited control options to effectively and economically manage the weeds. A diversified herbicide use pattern and integration of appropriate non-chemical methods are envisaged to reduce the pace of herbicide resistance development and environmental pollution. One such alternative is the widespread adoption of novel crop cultivars with increased competitiveness against annual weeds.

Four key findings have emerged from my literature review: 1) canola and wheat are species with complex genetics, complex responses to the environment, the ability to grow in a range of environmental conditions and are both subject to yield loss due to weed infestation; 2) past studies have not been particularly successful in separating weed competition from allelochemical interference mechanisms, particularly in the field; 3) allelopathy in wheat is not always intense, and field trials have not been particularly successful in demonstrating allelopathy in different environments, but it exists, and crop cultivar may play a key role; and 4) the separation of allelopathy from competitive crop interference in field and controlled environments has not been achieved for wheat (or many species, for that matter).

Therefore, this study proposed to perform both field and laboratory studies with the objective of 1) assessing the above-ground canopy competitive traits of selected genetically diverse Australian canola and wheat cultivars which are well adapted for the southern farming region of Australia and to 2) further assess and measure wheat secondary metabolites (BXs) involved in weed suppression in the soil rhizosphere. The use of new targeted and non-targeted metabolic profiling techniques using sensitive mass spectrometers provided the opportunity to probe more deeply into the secondary products of wheat and its rhizosphere that are related to plant defence and weed suppression.
2.2 Hypotheses

**Hypothesis 1: Weed suppression in canola and wheat is influenced by cultivar genetics and competitive ability**

Previous studies have identified traits for crop competitiveness including height, early vigour, tiller number, canopy leaf area/light penetration and root data. However, these past studies have not been successful in separating weed suppression as a result of crop competition from allelopathic mechanisms. Field trials were set up in low and medium rainfall areas (Condobolin & Wagga Wagga) to assess a range of canola and wheat cultivars for their yield potential and ability to impact weed populations.

Canopy traits (e.g., crop biomass, Normalized Difference Vegetation Index (NDVI), leaf area index (LAI), visual vigour ratings and photosynthetically active radiation (PAR) above and below the crop canopy) were assessed along with weed biomass. Crop growth vigour, root mass and dry matter were expected to show cultivar tolerance against depleted resources such as nitrogen. Crop and weed biomass, NDVI, PAR and LAI measurements data were intended to provide information on crop competitiveness against weeds. *This hypothesis is addressed in Chapters 2 and 3 (canola) and 4 and 5 (wheat).*

**Hypothesis 2: Environmental factors rather than cultivar genetics influence weed suppression or interference in the field.**

Trials were established in different seasons and two locations differing with respect to soil, in-crop rainfall and average daily temperatures. These environmental and seasonal factors were expected to have an impact on plant growth and thus to influence canopy traits. These factors could also impact the composition of plant allelochemicals and the amount of exudates produced by roots of the crop plants. LC-MS was used to identify and quantitate metabolite changes over time under various growth conditions. *This hypothesis is addressed in Chapters 2 and 3 (canola) and 4, 5 and 7 (wheat).*

**Hypothesis 3: Weed suppression in wheat is influenced by allelochemical interference through the direct release of allelochemical secondary compounds by wheat plants either by root exudates and/or microbial transformations.**

Different wheat cultivars may produce and exude different chemical profiles in the rhizosphere, thereby regulating microbial composition, diversity and activities – further affecting downstream nutrient cycling, metabolite degradation or transformation of non-toxic compounds to more active metabolites. Based on results from field
observations, metabolic profiling of wheat shoots, root exudates and soil rhizosphere was conducted with field-grown populations of selected cultivars to evaluate if allelochemical interactions are involved in successful interference with weeds or the production of secondary products important in plant defence immediately after germination as well as at crop maturity.

Although past literature suggests allelopathy in wheat may be limited in most commercial cultivars, metabolic profiling techniques were used to identify and quantitate targeted and non-targeted metabolites and allelochemical compounds responsible for allelopathy interference at different crop growth stages based on cultivar and environmental changes. In addition, a cultivar of rye and a heritage cultivar of wheat were included in replicated field trials, as both are known to be particularly weed suppressive and may also be potentially allelopathic. LC-MS QTOF (Agilent 6530) was used for both targeted and non-targeted analysis while LC-MS QQQ (Agilent 6470A) was used for quantitation of the metabolite compounds. This hypothesis was addressed in Chapters 6 and 7.
Chapter 1: Literature Review

The Use of Allelopathy and Competitive Crop Cultivars for Weed Suppression in Cereal Crops

Due to the rise of herbicide resistance, diverse weed management tools are required to ensure sustainable weed control. The aim of this manuscript was to review previous research studies on the use of allelopathy and competitive crop cultivars for weed suppression in cereal crops. In addition, I was invited to contribute a chapter in *Integrated Weed Management for Sustainable Agriculture* edited by R. Zimdahl.

This manuscript focuses on competitive cereal crops and cultural strategies for weed management, including the use of weed-suppressive cultivars, post-harvest crop residues, and cover crops for management of the weed seedbank and eventual weed suppression. It also addresses factors influencing the effect of allelopathy on weeds, including soil and environmental conditions which limit or intensify the efficacy of allelochemicals. The response of some weeds to secondary metabolites released by living cereal crops and/or crop residues (selectivity) is also reviewed. Finally, recommendations are given for future research, aiming to address the knowledge gap regarding the fate of these compounds in the environment and their role in important physiological processes in both plants and microbes in the soil rhizosphere.

I developed this manuscript through the mentorship and scholastic guidance from Prof. Weidenhamer and Prof Weston. They also edited the manuscript before and after submission for publication. Burleigh Dodds Science Publishing published the manuscript as a book chapter in *Integrated Weed Management for Sustainable Agriculture* edited by Emeritus Prof Robert L. Zimdahl

The use of allelopathy and competitive crop cultivars for weed suppression in cereal crops

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1 Introduction: key issues and challenges

Weeds are a persistent problem in agriculture, increasing production costs but reducing crop yields (Wu 2016). Worldwide, yield losses of approximately 34% are caused by crop weeds in broadacre crops and are higher than the losses caused by other crop pests (Jabran et al. 2015). Herbicides are the most widely used method to manage weeds in commercial crops. To date, weeds have globally evolved resistance to 23 of the 26 known herbicide sites of action and to 161 different herbicides (Heap 2017). Herbicide resistance in weeds restricts control options, thereby escalating economic loss and threatening agricultural sustainability (Wu 2016). This threat comes at a time when increasing global populations require greater agricultural productivity, and environmental concerns have resulted in significant restrictions on the use of some herbicides.
In Australia, herbicide resistance in both grasses and broadleaf weeds is on the rise, with resistance to multiple herbicides being reported for an increasing number of weeds (Owen et al. 2013). However, diversity in weed management tools could potentially provide sustainable weed control and slow the development of herbicide resistance in weeds. To combat the challenges of environmental pollution and herbicide resistance, crop types which release natural herbicides or produce significant biomass to reduce weed growth (i.e. allelopathic or competitive crops) are suggested (Jabran et al. 2015). Both competition and allelopathy as mechanisms of plant interference have been well documented under controlled conditions (Weston 2005). The combined effects of allelopathy and crop competition determine the total weed-suppressive potential of a given cultivar, and research groups worldwide have been working to improve both traits simultaneously to achieve maximum gains in weed suppression (Bertholdsson et al. 2012; Worthington and Reberg-Horton 2013), particularly in cereal crops.

1.1 Plant interference with weed growth

Plant interference can be defined as any physical or chemical mechanism that results in the reduction of plant growth over time due to the presence of another plant. Competition is usually described as the process whereby plants interfere with the growth of neighbouring plants by utilization or competition for growth-limiting resources, including light, nutrients or moisture (Weston and Duke 2003; Weston 2005). Highly competitive wheat (*Triticum aestivum* L.) cultivars typically have the ability to access better light, nutrients and water resources in a limited space, thus suppressing the growth and reproduction of neighbouring weed species (Bertholdsson 2011; Mwendwa et al. 2016a; Worthington et al. 2015). However, it has also been suggested that chemical interference (allelopathy) may prove to be as important as competition for resources in modulating plant community function and dynamics (Fernandez et al. 2016).

One strategy for integrated weed management is to use the inherent ability of many cereal crops to suppress weeds through a combination of high early vigour (competition) and allelopathic activity to further reduce weed interference (Bertholdsson 2005; Mwendwa et al. 2016a, b). Some crops including rice (*Oryza sativa* L.), sunflower (*Helianthus annuus* L.), sorghum (*Sorghum bicolor* (L.) Moench], wheat, rye (*Secale cereale* L.), maize (*Zea mays* subsp. *Mays* L.), barley (*Hordeum vulgare* L.), alfalfa (*Medicago sativa* L.) and *Brassica* spp. can exhibit strong allelopathic potential (Jabran and Farooq 2013). Allelopathic interference mechanisms are often difficult, if not
impossible, to distinguish from interference due to competition in a cropped field (Weidenhamer 1996; Weston 2005). Determination of the mechanism(s) associated with weed suppression is essential to determine if the use of crop cultivars for allelopathic and competitive weed suppression in cereal crops is going to provide sustainable solutions for weed management. Allelopathy and competition most likely act separately and interactively, and this may prove important for highly competitive crop cultivars.

Although allelopathic effects are mainly considered to be negative interactions, positive (stimulatory) interactions have also been reported depending on the mixture of allelochemicals in an extract, the target plant and the concentration tested (Eichenberg et al. 2014; Muzell Trezzi et al. 2016). Regardless of whether the interactions are positive or negative, maximizing the allelopathic potential of crops to reduce pest pressure and assist judicious nutrient management by crop sequences via crops or intercrops requires a better understanding of such interactions (Jabran and Farooq 2013).

1.2 Chapter overview

This chapter focuses on competitive cereal crops and cultural strategies for weed management, including the use of weed-suppressive cultivars, post-harvest crop residues and cover crops for management of the weed seedbank and eventual weed suppression. This chapter also addresses important factors influencing the effect of allelopathy on weeds, including soil and environmental conditions which limit or intensify the efficacy of allelochemicals. The response of some weeds to secondary metabolites released by living cereal crops and/or crop residues (selectivity) is also reviewed, since this ability may limit the use of some crop residues for weed control. Finally, recommendations for future research are required to address the knowledge gap regarding the fate of these compounds in the environment and their role in important physiological processes in both plants and microbes in the soil rhizosphere.

2. Competitive crops and cultural strategies in weed management

In Australia, Europe and North America, non-chemical methods for weed control such as harvest weed seed destruction and crop competition are being increasingly adopted across grain-growing areas and are proving successful in controlling weeds that escape pre-sowing herbicide control (Harker et al. 2011; Norsworthy et al. 2012). A diversified herbicide-use pattern and integration of appropriate non-chemical methods are envisaged to minimize the pace of herbicide resistance evolution (Manalil 2014). Cultural
weed management is also employed and refers in part to agronomic practices that use competitiveness of the crop to maximize crop growth while diminishing the growth and subsequent competitiveness of associated weeds or mechanical, biological or other practices to remove or restrict establishment of weeds in cropping areas (Vencill et al. 2012).

One alternative weed management strategy involves production of cereal crop cultivars with increased competitive ability with weeds. Crop competitive ability can be specified in terms of either crop tolerance against weeds or growth inhibition of weeds themselves by resource limitation and/or allelopathy (Bertholdsson 2010). Cultural strategies including crop rotation or successional planting, improved crop competition through cultivar selection and planting date, and optimized seeding rates are often considered but not always used by commercial producers (Beckie and Gill 2006). Crop sequences taking advantage of different planting times and production practices allow a variety of cultural techniques to be used to optimize crop competition with weeds at the expense of weed growth and reproduction (Vencill et al. 2012).

The ability to suppress weeds clearly appears to be cultivar dependent (Bertin et al. 2003; Wu et al. 2007). Crop cultivars with higher early vigour are generally capable of extracting more soil moisture which in turn enables them to maintain lower canopy temperatures on warm days (Zerner et al. 2008), an essential trait in dryland broadacre farming. Certain wheat cultivars will produce acceptable yields in the absence of herbicides under ideal conditions and suppress weeds (Andrew et al. 2015; Worthington et al. 2015; Mwendwa et al. 2016a).

Cultivar-dependent weed suppression has been observed in a variety of cereal crops. For instance, Seavers and Wright (1999) showed that the suppressive ability of oat (Avena sativa L.) cultivars at early stages of growth was greater than could be accounted for by canopy structure alone. The oat cultivars had low early ground cover and were the slowest of the three-cereal species (oats, barley, wheat) to develop a closed canopy, but their suppressive ability was higher throughout the growing season. In a recent study carried out at two ecologically different locations, differences in weed suppression by selected Australian commercial winter wheat cultivars were largely determined by crop architecture and phenology early in the growing season (Mwendwa et al. 2016a; Fig. 1).

Andrew et al. (2015) state that in comparison with aboveground canopy measurements, belowground traits have received relatively little attention in cereal crop–
weed interactions. This is partly due to the difficulties associated with measuring root traits, particularly when incorporating them into a screening protocol for new cultivars. It has been proposed that belowground traits determine the degree to which crop, and weeds share resource pools (Smith et al. 2010), meaning more tolerant cultivars may be those with belowground traits which avoid resource pool overlap (Andrew et al. 2015). However, at this stage there is relatively little information available on the root traits among cereal cultivars in relation to weed suppression, resulting in a knowledge gap in technical information to select cultivars for competitive ability based on root traits.

Competitive traits in cereal crops also vary in their effects between years (Coleman et al. 2001; Vandeleur and Gill 2004) and locations due to crop or weed species response to weather (Andrew et al. 2015). These variations may cause challenges in selecting the best cultivars. However, Lemerle et al. (1996) found that the best cultivars were generally consistent across years and sites, despite different weather patterns, and suggested that the similarity in soil type may have had a bearing on this. However, the lack of correlation between the yield of cultivars and weed-suppressive ability indicates that selection for weed suppression may not be equally efficient in all environments and that the performance of cultivars identified as highly weed suppressive may be affected by cultural practices such as planting date as well as environmental conditions (Worthington et al. 2015).

Development of wheat cultivars with increased inherent competitiveness against herbicide-resistant weeds is a potential supplement to in-crop herbicide use and, in some cases, an alternative management strategy, particularly for organic producers (Weston 1996). Competitive cultivars can better access light, nutrients and water resources in limited space, thus suppressing the growth and reproduction of nearby weed species (Worthington et al. 2015; Fig. 1). Our most recent three-year studies evaluated six replicates of 12 genetically diverse wheat cultivars in standard plots (12 × 2 m) grown in two ecologically different study sites. It was conducted using seed of each cultivar produced in the same location to eliminate variation associated with seed production differences. Results also showed that wheat cultivar competitive traits were influenced by both cultivar and environmental factors, as evidenced by clear differences in cultivar performance, yield and weed suppression among both locations. However, only a few selected cultivars, such as Condo (Fig. 1) performed well at both sites in all years (Mwendwa et al. 2016a).
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Figure 1 Differences in canopy closure between two wheat cultivars (cv. Condo (left) and cv. Espada (right)) at 113 days after emergence (DAE) at Wagga Wagga in 2015. Note the presence of greater canopy closure in the crop on the left (cv. Condo) which later resulted in significantly reduced weed biomass in this crop compared to the crop on the right (cv. Espada).

In Greece, the use of competitive cultivars alone resulted in a 50% reduction in the total amount of herbicides used for weed control in commercial wheat (Travlos 2012; Andrew et al. 2015). Thus, developing cereal cultivars with superior competitive ability against weeds can complement cultural methods for weed control while maintaining acceptable yields and suppressing weeds (Worthington and Reberg-Horton 2013; Andrew et al. 2015; Mwendwa et al. 2016a).

2.1. Breeding for more competitive crops

In the past century, it was thought that the competitive ability of wheat had been reduced by selection based on yield potential. Research has shown that ancient cereal cultivars or landraces are often more competitive with weeds than the higher yielding, semi-dwarf modern cultivars (Vandeleur and Gill 2004; Bertholdsson et al. 2012). Enhanced suppression was thought to be associated with crop height, root architecture or allelopathy. Today, selective breeding programmes for improvement of the competitive ability of modern cereal crops without compromising yielding ability exist. They focus on the incorporation of morphological traits that enhance early crop vigour (size of leaf 1 and 2) and light interception without affecting harvest index (Vandeleur and Gill 2004; Andrew et al. 2015).
To realize the potential of competitive crop cultivars as a tool in integrated weed management, a quick and simple-to-use protocol for assessment of the competitive potential of new cultivars is required. It is likely that assessment will not be based on a single trait, but the combined effect of multiple traits for interference (Bertholdsson 2011; Andrew et al. 2015). Because weed-suppressive ability is strongly and positively correlated with competitive wheat traits, including vigour and erect growth habit during tillering (Zadoks GS 29), high leaf area index (LAI) at stem extension (GS 31), plant height at tillering and stem extension (GS 29, 31), grain yield in weedy conditions and grain yield tolerance (Bertholdsson 2010; Worthington et al. 2015), these traits will be particularly important to incorporate into an assessment protocol. Our recent field studies of wheat competition attempted to incorporate an evaluation of many of the traits described above (Mwendwa et al. 2016a).

3. The effect of allelopathy on weed suppression

Allelochemicals, typically considered to be secondary plant metabolites, are produced by plants for a variety of purposes including chemical defence and communication. Along with microbial decomposition products, they are the active agents of allelopathy (Cheng and Cheng 2015). Cereal crops can frequently suppress weeds through the release of allelochemicals from intact roots of living plants and/or by decomposition of phytotoxic plant residues (Bertin et al. 2003; Belz 2007; Ferreira and Reinhardt 2010).

The incidence of growth inhibition of certain weeds and the induction of phytotoxic symptoms by plants and their residues have been well documented for many crops, including all major grain crops such as rice, rye, barley, sorghum and wheat (Belz 2007; Ferreira and Reinhardt 2010; Weston 1996), and is density, location and weed dependent (Weston 1996). Varietal autotoxicity occurs when plants of a given cultivar release chemical substances that inhibit or delay germination and growth of the same cultivar (Wu et al. 2007).

3.1. Allelochemical production and mode of action

Allelochemicals tend to exhibit mechanism(s) of action in plants that differ from the sites targeted by herbicides (Muzell Trezzi et al. 2016). Duke and Dayan (2011) note that some of the most potent phytotoxins are synthesized by microbes. A few phytotoxins...
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or allelochemicals share molecular target sites with synthetic herbicides, but molecular target sites for allelochemicals are frequently unidentified or unique. This implies that the development of allelopathic crop cultivars may not be severely affected by existing herbicide resistance in weed populations. In addition, allelochemicals are present in all plant species and tissues and are typically released into the rhizosphere by a variety of mechanisms, including decomposition of residues, volatilisation and root exudation (Weston 2005). Expression of allelopathic traits may allow plants to strengthen their defence system against biotic and abiotic stressors, and aid in regulating nutrient transformation (Jabran and Farooq 2013).

Plant secondary metabolites are essential for the interaction of plants with their biotic environment, help attract pollinators or seed dispersers, act in defence against natural enemies and inhibit potential competitors (Muzell Trezzi et al. 2016). The ability of a plant to produce and release allelopathic metabolites into the environment and/or to tolerate the presence of allelochemicals released by neighbouring plants including weeds can be crucial to the ability of a species to survive and reproduce (Bertholdsson et al. 2012, Worthington et al. 2015). Allelopathy is generally regulated by a dynamic mixture of allelochemicals and their metabolites, which is affected by cultivar and developmental stage of the producing plant, the environment, cultivation and the rate of chemical or microbial degradation in the rhizosphere (Belz 2007; Mwendwa et al. 2016a).

Bertholdsson (2005, 2011) has noted that the weed-suppressive ability of today’s commercial cereal crop wheat is typically lower than that of other commercially produced cereals including rye, oats and barley. It has been suggested that selection for enhanced suppression may improve these traits substantially through focused breeding. Two factors are likely to be important for expression of weed-suppressive ability: 1) early season biomass production by the crop and 2) potential allelopathic activity of the crop (Bertholdsson 2011).

For example, cereal rye is thought to be allelopathic due to the presence of phytotoxic benzoazinoids (BXs) whose biosynthesis is developmentally regulated, with the greatest accumulation in young shoot tissue. Concentration is influenced by cultivar and environment (Schulz et al. 2013). In wheat seedlings, BX biosynthesis begins shortly after germination and reaches a maximum of 7–10 days (Argandoñaa et al. 1981; Bertholdsson et al. 2012), but in rye, synthesis was highest 60 days following germination (Burgos and Talbert 2000; Bertholdsson 2012). Therefore, periods of maximal
allelochemical production vary with crop and field location and should be targeted and monitored to achieve optimal weed suppression over time.

BXs are characteristic secondary compounds produced not only by rye but also by several other species of Poaceae, including maize, triticale (*Triticeae* *Wittm. ex A. Camus.*), and wheat, and some dicots belonging to the *Acanthaceae*, *Scrophulariaceae*, and *Lamiaceae* (Schulz et al. 2013). The BXs present in wheat, barley and rye, and their suppressive effects on weeds, pest and diseases are of great interest in sustainable agriculture (Bertholdsson et al. 2012; Tanwir et al. 2013). Rye produces numerous BXs but [2,4-dihydroxy-1,4(2H) benoxazin-3-one (DIBOA) and 2(3H)-benzoxazolinone (BOA)] are generally associated with its allelopathic potential. Other important allelochemicals have also been reported (Jabran et al. 2015). These products are also associated with enhanced human health, especially the potential suppression of prostate cancer (Adhikari et al. 2015; Steffensen et al. 2016).

Application of BOA to developing weed or crop seedlings has been shown to significantly affect the transcriptome, proteome and metabolome of germinating seedlings, resulting in inhibition of both germination and growth, sometimes even death of sensitive species and up-regulation of plant defence and stress genes (Reigosa et al. 1999; Reigosa and Pazos-Malvido et al. 2007). The inhibition of germination and reduction of seedling growth were observed in many plant species exposed to BOA and DIBOA. Often, radicles and root tips were more affected than shoots. Both DIBOA and BOA inhibited the emergence of barnyard grass (*Echinochloa crus-galli* L. Beauv.), cress (*Lepidium sativum* L.) and lettuce (*Lactuca sativa* L.) when applied to Petri dish bioassays and in field soil, indicating their potential to cause allelopathic inhibition of plant growth (Barnes and Putnam 1986, 1987; Schulz et al. 2013).

In barley, allelochemical toxicity increased after the release of BXs by roots, between day 0 and day 6 (in a lab seed-after-seed protocol). The allelopathic potential of barley root exudates was also dependent on the receiver weed species (Bouhaouel et al. 2015). Plant part and rhizosphere location (distance from root) also affected BX concentration in wheat and rye (Mwendwa et al. 2016b) with the release of these allelochemicals dependent upon cultivar and environmental conditions. For example, in rye, cultivars with varying contents of BOA and DIBOA were identified (from 0.52 to 1.15 mg/g dry tissue). Typically, those with the highest content of BXs were the most phytotoxic in laboratory assays (Schulz et al. 2013).
While BXs are important allelochemicals present in both wheat and rye, and their suppressive effects on weeds, pest and diseases are of great interest in sustainable agriculture (Carlsen et al. 2009, Bertholdsson et al. 2012), they do not occur in sorghum or rice, which produce other highly active allelochemicals (Schulz et al. 2013). The chemistry implicated in allelopathic interactions of wheat and rye is thus believed to be based on the activity of benzoazolinones, a class of BXs, in contrast to the diverse chemistry produced by other cereals including rice (Duke et al. 2005; Wang and Kong 2013) and sorghum (Ferreira and Reinhardt 2010; Weston et al. 2013).

Studies performed with suppressive rice have found that the synthesis of two compounds phytotoxic to barnyard grass [a flavone (5, 7, 4-trihydroxy-3, 5-dimethoxyflavone) and a cyclohexanone (3-isopropyl-5-acetoxy-cyclohex-2-en-1-one)] is induced in rice plants by the presence of weeds (Kong et al. 2004; Duke et al. 2005). The mechanism of this induction is unknown. In addition, momilactone A and B have been identified as potent allelochemicals in rice seedlings (Kato-Noguchi et al. 2008; Kato-Noguchi and Ino 2005; Kato-Noguchi and Peters 2013), and its production has been shown to be enhanced by the presence of barnyard grass seedlings in cultivated rice. Induction of sorgoleone production in sorghum has also been reported following exposure of sorghum seedlings to velvetleaf (Abutilon theophrasti Medik.) extracts and exposure to weeds (Dayan 2006; Weston et al. 2013). Understanding how to induce allelochemical expression in field crops is critical to maximising allelopathic activity. Therefore, additional research is needed to determine environmental effects on allelochemical production.

There is evidence that the allelopathic potential of barley has been impaired by modern breeding which has emphasised selection for other traits, including yield. Using a bioassay with perennial ryegrass (Lolium perenne L.) as the model weed (receiver), Bertholdsson (2005) showed that the allelopathic activity measured as root growth inhibition of perennial ryegrass decreased 14–31% in the Nordic barley germplasm collection since the start of selection and breeding over 100 years before. Differences in the sensitivity of cultivars and ecotypes may be due to different weed species-dependent strategies that have evolved to cope with allelochemicals (Schulz et al. 2013). However, the allelopathic activity of barley is still considered to be generally high, in contrast to other cereals such as wheat. Despite the negative effects of selective breeding for other traits, it may contribute to the effectiveness of barley as a cover crop (Bertholdsson 2010).
Sorghum’s allelopathic properties were first suggested by reports of reduced growth of crops grown in rotation with sorghum (Weston et al. 2013). Its use as green manure or a cover crop for suppressing the growth of weeds is likely also related to its allelopathic properties. Certain species of Sorghum, such as Sudangrass (Sorghum × drummondi Nees ex Steud.), are very allelopathic, making them practical to grow as weed-free monocultures (Ferreira and Reinhardt 2010). Sorghum-Sudangrass hybrids (Sudex) are also often used as green manures in the nursery and horticultural cropping systems. In recent years, sorghum phytotoxicity and allelopathic interference have been well described in greenhouse and laboratory settings. Many observations of weed suppression in diverse locations and with various sorghum plant parts and residues have been reported (Weston et al. 2013).

A diverse group of sorghum allelochemicals, including numerous phenolics, a cyanogenic glycoside (dhurrin) and a hydrophobic p-benzoquinone (sorgoleone) were isolated and identified in recent years from sorghum shoots, roots, root exudates and soil, as our capacity to analyse and identify complex secondary products in trace quantities in the plant and in the soil rhizosphere has improved (Weston et al. 2013; 2015; Weidenhamer 2005). Sorgoleone (2-hydroxy-5-methoxy-3-[(8´Z, 11´Z)-8´, 11´, 14´pentadecatriene]-p-benzoquinone) was identified as the most phytotoxic compound produced by sorghum. It occurs as a mixture of several hydroquinones as root exudates and inhibits growth and establishment of many weeds (Ferreira and Reinhardt 2010; Weston et al. 2013). The allelochemicals present in sorghum tissues also vary with plant part, age and cultivar evaluated (Weston et al. 2013).

4. The effect of soil and environment on plant metabolites (allelochemicals)

4.1. Overview

In recent years, hundreds, if not thousands, of wheat cultivars have been screened for their weed-suppressive potential on several weed species in laboratory studies (Wu et al. 2000; Duke et al. 2005). Related wheat species such as Triticum durum Desf., T. spelta L. and Aegilops speltoides Tausch., as well as triticale and rye, have been considered as possible sources of allelopathic germplasm, but despite the fact that some have exhibited strong allelopathic potential, these sources have been relatively under-investigated (Belz and Hurle 2005; Duke et al. 2005; Schulz et al. 2013). In general, phytotoxicity has
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primarily been attributed to root exudates or secretions or decomposing residues (Duke et al. 2005).

Root exudates represent one of the largest direct inputs of plant chemicals into the rhizosphere, and therefore also likely represent the largest source of allelochemical inputs into the soil (Bertin et al. 2003). Wheat root exudation, as measured by excretion of phenolics in agar media, was shown to vary with wheat accession (Wu et al. 1999, 2000). Analysis by multiple regression showed that accessions with strong allelopathic potential were associated with the production of significantly higher levels of allelochemicals in shoots and roots (Wu et al. 1999, 2000, 2001).

However, actual weed suppression in the field is dependent on the behaviour of these compounds in the soil. To understand an allelopathic interaction process within a given soil, the process must be viewed and understood within the specific environmental constraints of that soil (Blum 2006). Upon release from a source organism, the soil is the main vehicle that mediates contact between allelochemicals and their target plants (Muzell Trezzi et al. 2016). Therefore, assessment of allelopathy must account for the effect of soil on the behaviour and activity of allelochemicals (Teasdale et al. 2012; Blum 1995, 2006). In addition, allelopathy should be assessed in a range of soil types as several climatic and edaphic factors affect soil microflora (Inderjit 2005).

The role of soil microorganisms in chemically mediated interactions between plants is poorly understood, and appropriate methodologies are needed to assess the role of soil microbial ecology in allelopathy. Soil properties including organic matter, reactive mineral surfaces, ion exchange capacity, inorganic ions and abiotic and biotic factors of the soil environment significantly influence allelochemical activity (Inderjit 2001; Blum 2006). Microorganisms can chemically alter the allelochemicals released into an ecosystem, highlighting their key role in chemical plant-plant interactions and suggesting that allelopathy is likely to shape the vegetation composition and participate in the control of ecological biodiversity (Fernandez et al. 2013). For example, allelochemicals released into the environment inhibited germination and growth of neighbouring plants by altering their metabolism or affecting their soil community mutualists (Fernandez et al. 2016). Wang et al. (2013) further suggested that the shift in the microbial community composition induced by barnyard grass might generate positive feedback in rice growth and reproduction in a given paddy system.
However, the mechanism of the release of the associated allelochemicals from residues and by root exudation and their interaction with the soil microbial community are not well understood. Therefore, additional research on their release mechanisms is suggested due to the complexity of these interactions. Currently, we are evaluating the effect of soil microbial communities on the activity of cereal and other plant residues in the suppression of weeds in projects funded by the Australian Grains Research and Development Corporation. Allelochemicals are altered or degraded by soil microbes over time in the field; however, soil microbes can also degrade residues to release additional metabolites (Weston and Duke 2003). Therefore, studies on microbial influence on allelochemical activity will result in fundamental knowledge about the degradation of plant residues and associated metabolites in various soil types and the role of soil microbial communities in phytotoxicity.

Under the appropriate environmental conditions, phytotoxins may be released into the environment in sufficient quantities to affect the growth of neighbouring plants (Weidenhamer 1996; Weston 1996). For example, Bertholdsson (2010) found that highly allelopathic spring wheat lines derived from a cross between allelopathic and non-allelopathic parents suppressed weed biomass 24% more than the non-allelopathic parent in a dry year and 12% more in a wet year. However, the effects of temperature and drought on the toxicity of allelochemicals, their rates of microbial breakdown and other factors could have a major effect on the efficacy of these compounds in controlling weed growth in extreme environments such as those encountered in Australia.

According to Einhellig (1996), allelopathy is strongly coupled with exposure to other crop stressors, including insects and disease, temperature extremes, nutrient and moisture variables, radiation and herbicides. These specific stressors can enhance both allelochemical production and toxicity, thus increasing the potential for allelopathic interference. Therefore, environmental effects on the release of allelochemicals or residue degradation and release over time are critical to study under controlled conditions.

4.2. Analysis of allelochemicals in plant and soil

Precise metabolic profiling of allelochemicals in the plant and, at the same time, in the soil rhizosphere could provide strong insight into the dynamics of the release of bioactive metabolites following incorporation of plant material or living root exudates into the soil (Krogh et al. 2006; Weston et al. 2015). Chen et al.’s (2010) study quantified
DIMBOA and MBOA in the wheat rhizosphere and analysed the soil microbial community structure. MBOA rather than DIMBOA was found in the wheat rhizosphere, and its concentration varied with cultivars, plant densities and growth conditions. Recent studies have incorporated the use of metabolomics to study release rates of various metabolites from plant to soil or aboveground environment. These studies have shown that certain plant species not only compete for resources; they produce allelochemicals which further interfere with plant growth. Work in the Mediterranean forests has shown the effect of allelochemicals and competition on seedling growth of local trees (Fernandez et al. 2016). We suggest similar studies be done in agroecosystems for cereal production.

Studies reported by Rice et al. (2012) and Teasdale et al. (2012) demonstrated relatively low concentrations of the most toxic BX compounds, 2-aminophenoxazin-3-one (APO), DIBOA and DIMBOA from incorporated rye residues when cover crops were soil incorporated, whereas the less toxic compounds, BOA and MBOA, and the nontoxic compounds, HBOA and HMBOA, were predominant BX species in amended soils. Growth assays with lettuce and smooth pigweed (*Amaranthus hybridus* L.) species showed inhibition whether rye residue was left on the surface or incorporated in soil during the first two weeks after rye applications; however, there were not sufficient concentrations of any BX in the soil to explain these effects (Rice et al. 2012). This suggests that the activity of BXs may depend on interactions of mixtures and the soil environment as influenced by soil microbiota. Hence inconsistent results for bioassays using only single applications of pure compounds to test for allelopathy activity are often observed.

In addition, Teasdale et al. (2012) removed soil from beneath a field site maintained with coverage of rye residue and assayed this soil in pots but observed little phytotoxicity against the weeds encountered in the field. This could be due to degradation of allelochemicals over time under field conditions. When BOA and MBOA were exogenously added to soils to maintain extractable levels of up to 10 µg g⁻¹ soil (100–500 times higher than measured BX in field soils), no significant inhibition of pigweed was observed (Teasdale et al. 2012). The Teasdale findings support the hypothesis that the activity of BXs may depend on interactions of mixtures and the soil environment as influenced by soil microbiota. Rhizosphere soil microbes can potentially contribute to the allelopathic potential of plants through positive feedback (Wu et al. 2015) or through direct biotransformation of BXs resulting in the production of
aminophenoxazinone compounds APO and AMPO (Macías et al., 2006, 2009). APO and AMPO are more phytotoxic and are comparable to the specific activity of commercial herbicides (Macías et al. 2006).

However, Rice et al. (2012) found that movement of these compounds into the soil column was minimal, with more than 70% of BOA and 97% of MBOA remaining in the top 1 cm of soil profiles, and complete dissipation was noted in less than 24 hours. Because communication between plants and other organisms below ground drives community dynamics (Inderjit 2005), these results suggest that the movement and the activity of these compounds in the soil may be facilitated by specific dynamics mediated by microbes and the soil medium through chemical signalling, as the presence of these metabolites at significant concentrations in the upper soil profile had no effect on weeds. In addition, this also suggests that rainfall or conditions affecting soil bioavailability of these metabolites may be required for allelopathic activity.

During degradation in the soil, different metabolites accumulate in a manner that is both concentration and soil type dependent. The differences may be related to the density and diversity of microorganisms associated with the plant and those in soil (Glenn et al. 2001, Schulz et al. 2013) or the relative mobility of allelochemicals in the soil which is associated with their chemical structure, including their polarity and lipophilicity. Wheat seedlings have been shown to be capable of detecting the presence of competing weeds and responding by increasing MBOA production in the rhizosphere (Chen et al. 2010). Metabolites released from the crop plants depend on the crop species and may in turn also influence the microflora associated with the root system (Schulz et al. 2013). However, for allelopathic interference to occur, significant and dynamic concentrations of particular allelochemicals are required for uptake by plants or microbial communities, so solubility in the soil/water in field soils is critical.

For instance, Chen et al. (2010) found there was a positive linear relationship between the MBOA level in the wheat rhizosphere and soil fungi/bacteria. When DIMBOA was applied to soil, MBOA concentrations were increased, resulting in enhanced levels of soil fungi, suggesting that DIMBOA and MBOA could affect soil microbial community structure to their advantage through the change in fungal population structure and growth. Microorganisms are not only responsible for the degradation of allelochemicals in soil, but the common mycorrhizal network of soil can also be involved in transporting compounds away from the zones of highest microbial
activity and expanding the zones of bioactivity for allelochemicals in soil to areas some distance away from the plant (Barto et al. 2011, 2012). Sorting out the dynamics of complex mixtures of allelochemicals in soil remains a major challenge.

Various analytical approaches including HPLC, GC, MS and MS/MS can be used in concert to analyse the presence of plant metabolites in tissue and in the soil. Both environmental and microbial factors can affect the degradation of plant metabolites in the soil and thereby affect their efficacy. In non-sterilized soil, for instance, DIBOA showed a half-life of 43 hours. However, APO, the final microbial degradation product of DIBOA, has a low mineralisation rate and, therefore, a half-life greater than 90 days (Macías et al. 2005).

In addition, it is up to one thousand times more active as a growth inhibitor than DIBOA or BOA due to its half-life in the soil. Therefore, its build up over time in the soil may be correlated with allelopathic activity in contrast to the presence of temporal BXs typically released by plants and rapidly degraded. In contrast, some flavonoid glycoside molecules exuded by rice plants can suffer high rates of mineralization by soil microorganisms, resulting in accumulation of aglycosylated compounds. These flavonoid glycosides and aglycosides have a half-life of 2 and 30 hours, respectively, suggesting the potential for greater allelopathic activity associated with the presence of the second group of compounds (Muzell Trezzi et al. 2016), which are also often more biologically active (Weston and Mathesius 2013).

In summary, to better understand allelopathy, three main areas require focused research in the future: 1) the role of soil microorganisms in chemically-mediated interactions between plants; 2) the development of new analytical tools to enable detection and quantification of low, dynamic concentrations of metabolites and their resulting catabolites; and 3) experimental designs to elucidate the underlying dynamics of complex mixtures of allelochemicals in soil.

5. Use of crop residue mulches and cover crops in weed suppression

5.1. Crop residues as mulches in weed suppression

Crop residues, when present in uniform and dense stands under conservation farming can suppress weed seedling emergence, delay emergence and allow the crop to gain an initial advantage in terms of early vigour (Chauhan et al. 2012). For instance, the
seedling emergence rate of littleseed canarygrass (*Phalaris minor* Retz.) was reduced for wheat planted no-till compared with conventional ploughing and sowing (Franke et al. 2007; Bajwa et al. 2015).

Crop residues interfere with weed development and growth in several ways that include physical and chemical effects including the alteration of soil physical, chemical and biological characteristics based on two possible sources of allelochemicals: secondary metabolites can be released directly from crop litter, or they can be produced by microorganisms that use plant residues as a substrate (Ferreira and Reinhardt 2010). McCalla and Norstadt’s (1974) review on phenolic acids showed that levels required for strong phytotoxicity to successive crops often greatly exceed those typically observed in the soil following residue degradation.

This suggests that a combination of factors may be associated with weed suppression, including physical presence of mulch or stubble and the presence of allelochemicals, which are most often rapidly degraded once a threshold concentration for inhibition is reached. The inclusion of specific crops or cultivars with allelopathic properties in the cropping rotation may result in more effective weed management. Weston and Duke (2003) reported that cereal rye residues apparently reduce weed seed germination and seedling growth by shading, lowering soil temperature, moderating diurnal temperature fluctuations and acting as a physical barrier to prevent light from reaching the soil surface. In addition, rye and its residues also release secondary metabolites that accumulate near the soil surface to further inhibit weed seed germination and growth (Weston and Duke 2003).

There are multiple approaches to the management of weeds in crop residues or stubble depending upon environmental conditions and management. Teasdale et al. (2012) reported that surface rye residue was highly inhibitory to small-seeded broadleaf seedlings throughout an experimental period of 4 weeks. Residues left on the soil surface have generally led to decreased soil temperature fluctuations and reduced light penetration, both of which have been shown to reduce weed germination (Liebman and Mohler 2001; Schulz et al. 2013). Gavazzi et al. (2010) found that grass weeds were reduced by 61%, whereas broadleaf weeds were reduced by 96% when rye mulch was used in a no-tillage system. The level of weed suppression was dependent on weed species and the thickness of the mulch layer, with an exponential relationship between mulch biomass and weed emergence (Schulz et al. 2013).
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Trends in wheat yield responses to conservation cropping in Australia were analysed using data from 33 medium (3–5 year) and long-term (>5 years) agronomic experiments. The overall effect of tillage (direct-drilled vs cultivated) was small in all regions (−0.18 to +0.06 t ha⁻¹), while stubble retention (stubble retained vs stubble burnt) reduced yield in all regions (−0.31 to −0.02 t ha⁻¹). Reduced early seedling growth of direct-drilled crops was a major factor underlying the yield response at most sites, and yield reduction was rarely associated with the lack of available water or nitrogen (Kirkegaard 1995; Kirkegaard et al. 2014). However, the reduction of early seedling growth and in yield was attributed to reduced soil temperature associated with the presence of residues or the effects of either autotoxicity and/or phytotoxicity associated with residues (Kirkegaard et al. 2014). Further studies on these field-based interactions are required to determine best stubble management strategies and crop choice for the subsequent season and are now the subject of additional research in Australia.

Crop residues retained on the soil surface have been reported to decompose more slowly than residues incorporated in the soil, which may result in a slower release rate but longer supply of allelochemicals (Kruidhof et al. 2009). For example, when the residue material is retained on the soil surface, effective weed control has been observed for 4–8 weeks after mulching (Gavazzi et al. 2010; Weston et al. 2014). Previous studies also reported that the concentration of BX compounds released from rye and wheat residues in soil peaked 1–4 days after stubble incorporation in soil and declined to negligible amounts within 10 days (Krogh et al. 2006).

However, in the absence of incorporation, it is difficult to predict how long allelochemicals may be released from residues on the soil surface. When stubble remained on the soil surface, stubble build-up leading to poor seed/soil contact resulted in increased numbers of grass weeds but reduced broadleaves (Scott et al. 2010). This is thought to be due to both the physical presence of residues and release of allelochemicals over a 60-day period following harvest/kill of the cover crop. In Australian broadacre cropping regions, crops are planted 5–6 months after harvest into the remaining crop stubble (Weston et al. 2014). This practice could potentially reduce the physical effects on crop seedlings as adequate decomposition of the stubble may occur before sowing the following season, but chemical interference in Australia is often mediated by the presence of rainfall or soil moisture.
Teasdale and others have shown that incorporated residues of rye inhibited lettuce and pigweed growth for approximately two weeks after incorporation (Teasdale et al. 2012). Emerging weed seedlings are sometimes effectively controlled by allelopathic mulches through leaching or timed release of allelochemicals; however, a well-established weed flora is difficult to eradicate this way (Farooq et al. 2013). In either case, both allelopathy and competitive weed-suppressive ability are complex, quantitatively inherited traits that are heavily influenced by environmental factors (Worthington and Reberg-Horton 2013). In some cases, with residue incorporation, it is difficult to determine if suppression is due to physical and/or chemical suppression of weeds.

To date, studies have not been able to elucidate the independent contributions of these traits to weed suppression. The soil has proven to be an important barrier in allelopathic interactions because allelochemicals must survive transit through the soil in sufficient concentrations to affect target plants (Barto et al. 2012) and allelochemicals may serve as attractants to common soil microbes (Shi et al. 2011). Further research on these interactions is required to gain a better understanding of the mechanism of the release and activity of allelochemicals in the soil.

In addition, the choice of species or cultivars to utilize for desired weed suppression, or the weed biotype encountered in field settings, may affect the ability of the plant or its residues to interfere with plant growth (Weston and Duke, 2003). For example, Barnes and Putnam (1987), as well as Gavazzi et al. (2010), found that broadleaf weeds were approximately 30% more sensitive to DIBOA and BOA compared with grass weeds. Further studies have indicated that larger-seeded weed species are less sensitive to allelochemicals (Weidenhamer et al. 1987; Tabaglio et al. 2008) and that seed size and mass affect selective suppression of weeds with crop residues (Liebman and Davis 2000).

The quantity of crop residues also varies among crops. For example, oilseeds and pulses typically produce less biomass than cereals. In rain-fed areas, the crop biomass will also depend on the amount and pattern of rainfall. Therefore, depending on the region, crop and rainfall, the effects of crop residue on the weed population will vary (Chauhan et al. 2012). Campiglia et al. (2015) reported that various mulch strips caused differences in weed species composition dominated by perennial ruderal weeds in mulched areas, while in tilled soil weed flora was dominated by annual weeds.
5.2. Use of cover crops to suppress weeds

Cover crops provide another strategy to minimise weed populations while maintaining seasonal vegetative ground cover to prevent soil erosion. A cover crop is usually a ‘noncash’ crop that can be grown before, or in the case of a living mulch or smother crop, with a cash crop so that vegetative cover remains on the field for as long as possible during the year (Melander et al. 2005). Cover crops provide several advantages. They typically assist producers to meet conservation-tillage requirements for year-round vegetation cover; aid in soil erosion prevention; improve soil structure and, often, organic matter content; protect plants in sandy areas from sand-blow injury; fix nitrogen if the cover crop is a legume; and possibly suppress weed emergence and growth (Melander et al. 2005; Norsworthy et al. 2011; Vencill et al. 2012). Cover crops are frequently used in temperate or subtropical crop production areas where moisture is not limiting production, such as the United States, Canada, Brazil and Queensland or northern Australia.

Suppression of weeds by cover crops depends partly on biomass production of the crop (Vencill et al. 2012). In a field study conducted in the southern United States, rye, crimson clover (Trifolium incarnatum L.), hairy vetch (Vicia villosa Roth.), barley and a mixture of the four species suppressed the emergence of eastern black nightshade (Solanum ptycanthum Dun.). Crimson clover inhibited the emergence of eastern black nightshade beyond what could be attributed to physical suppression alone. The emergence of yellow foxtail [Setaria glauca (L.) Beauv.] was inhibited by rye and barley but not by the other cover crops or the cover crop mixture (Creamer et al. 1996). The use of cover crops to suppress weeds is also influenced by the type of weed, environmental factors and farming system.

A field study by Gavazzi et al. (2010) examined the allelopathic effects of rye cover crop on grass and broadleaf weeds in maize grown with two tillage systems (no-tillage, conventional tillage) at three nitrogen rates (0, 250, 300 kg N ha$^{-1}$). Mulching significantly reduced the density of grass and broadleaf weeds by 61% and 96%, respectively. Linear regressions between the concentrations of DIBOA and DIBOA-glycoside in the rye mulch and weed inhibition (%) were statistically significant, with $R^2$ values of 0.59 and 0.65 for grass and broadleaf weeds, respectively. Another study reported that white mustard (Sinapis alba L.) reduced both seedling establishment, by 51–73%, and biomass, by 59–86%, of small-seeded annual broadleaf weeds in a greenhouse...
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In addition, under field conditions white mustard was also the most effective cover crop, reducing weed survival 21–57%.

Other factors influencing the effect of cover crops in weed suppression include season, location, type of cover crop, soil cover and density of the resulting mulch. For instance, Dorn et al. (2015) sowed cover crops directly after harvesting cereals and before next year’s main crop (grain maize or sunflower). The presence of cover crops caused a 96–100% reduction of weed dry matter at the four sites managed under integrated production, while effects were lower at the four sites managed under organic production, ranging from 19 to 87%. Cover crops that covered soil quickly and produced greater dry matter provided the greatest weed suppression. However, their weed-suppressing effect was difficult to predict and was typically dependent on the year of the investigation, experimental site, cover crop species, the speed of soil cover in autumn and the density of the resulting mulch layer in spring.

Altieri et al. (2011) and Schulz et al. (2013) assessed the effects of various combinations of rye, hairy vetch, fodder radish (*Raphanus raphanistrum* subsp. *Sativus* (L.) Domin.), black oats (*Avena strigosa* Schreb.) and ryegrass (*Lolium multiflorum* Lam.) in reducing winter and summer weed populations in bean crops. Results indicated that the best cover crop mixtures included a significant proportion of rye, vetch and fodder radish (Schulz et al. 2013). The main advantages of these mixtures were the generation of higher crop biomass, improved spectrum of target weeds, broader adaptability to pedo-climatic conditions, complementary effects on soil quality (N fixation for legumes, nematocidal effect and improved soil tilth and soil structure) (Altieri et al. 2011, Schulz et al. 2013).

Bradow and Connick (1990) showed that cover crops would release volatile germination inhibitors, so if the crop is disked or killed by rolling, a pulse of volatiles will be released, sufficient to provide effective weed control in the subsequent vegetable crop (Altieri et al. 2011).

6. Case studies: production of benzoxazinoids in cereal crops

In recent experimentation using metabolic profiling in wheat tissues to examine metabolite levels of diverse BXs representing several chemical groups, lactams and hydroxamic acids predominated and were detected in various plant parts (Fig. 2). Fourteen BXs and their derivatives have been reported in tissues, rhizoplane and rhizosphere bulk soil including their respective BX glycosides (Adhikari et al. 2015;
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Tanwir et al. 2013; Mwendwa et al. 2016b). When produced in great quantities, BXs are typically stored as glycosides which are enzymatically converted to aglycone forms under stress conditions (Tanwir et al. 2013). Their biosynthesis involves nine enzymes thought to form a linear pathway leading to the storage of DIBOA and DIMBOA as the glucoside conjugates (Dutartre et al. 2012). The aglycones and their derivatives are thought to be responsible for the phytotoxic effects of rye residues, but they may also act in concert with other compounds, such as ferulic acid and related phenolics, luteoline glucuronides, β-phenyllactic acid and β-hydroxybutyric acid (Schulz et al. 2013).

The concentrations of BOA and DIBOA varied depending on plant organ, age, cultivar and the fertilisation regime, as well as on temperature, water supply, photoperiod, UV irradiation and light intensity (Niemeyer 2009; Schulz et al. 2013). Selected Australian wheat cultivars were also at the same time evaluated for their ability to suppress annual weeds in the field. Metabolites were extracted from the plant tissues and soil rhizosphere and profiled in the Liquid chromatography mass spectrometry quadrupole-time of flight (LC-MS QToF). There was a clear difference in the distribution and abundance of metabolites in wheat tissues and on the root surface or rhizoplane depending on cultivar, growth stage and time of harvest (Mwendwa et al. 2016b; Fig. 3). With increasing age of wheat seedlings in the field, metabolite levels increased in roots but generally remained stable in shoots, up to 75 days following seeding.

Figure 2 Chemical structures of the BXs most commonly found in cereal grains and bakery products. BOA, benzoxazolin-2-one; MBOA, 6-methoxy-benzoxazolin-2-one; HBOA, 2-hydroxy-1,4-benzoxazin3-one; HMBOA, 2-hydroxy-7-methoxy-1,4-benzoxazin-3-one; HBOA-Glc, 2-β-d-glucopyranosylxy1,4-benzoxazin-3-one; HMBOA-Glc, 2-β-d-glucopyranosylxy-7-methoxy-1,4-benzoxazin-3-one; HBOA-Glc-Hex, double-hexose derivative of HBOA; DIBOA, 2,4-dihydroxy-1,4-benzoxazin-3-one; DIMBOA, 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one; DIBOA-Glc, 2-β-d-glucopyranosylxy4-hydroxy-1,4-
benzoxazin-3-one; DIMBOA-Glc, 2-β-d-glucopyranosyloxy-4-hydroxy-7-methoxy-1,4-benzoxazin-3-one; DIBOA-Glc-Hex, double-hexose derivative of DIBOA. *Structure not fully elucidated (Adhikari et al. 2015; Tanwir et al. 2013).

For example, wheat cv. Condo exhibited a higher relative abundance of DIMBOA-Glc and HMBOA-Glc in its roots in July compared to June, when it was 10 weeks old. In addition, the wheat cultivars and cereal rye (cv. Grazer) showed significantly higher levels of MBOA and BOA in the soil rhizoplane in July compared to June, showing that as the plant matures, it is releasing higher concentrations of allelochemicals into the soil and rooting zone. The distribution of BX secondary metabolites in wheat cultivar tissues suggested differential production of certain key bioactive metabolites among cultivars. Interestingly, Condo, the most weed-suppressive cultivar in aboveground field assessments conducted in several years of field experimentation (Fig. 1) also exhibited the greatest abundance of the four major BXs in its root tissues and on the rhizoplane or root surface (Fig. 3). Further metabolic analysis of wheat tissue, rhizoplane and rhizosphere bulk soils is currently underway to evaluate the potential role of these metabolites, as well as microbially altered metabolites such as APO and AMPO, in weed interference by various wheat cultivars.

Generally, BOA and MBOA are more stable in field soil than DIBOA and DIMBOA, and rapid soil degradation is associated with the microbial activity (Schulz et al. 2013). For example, when MBOA was placed in a previously sterilized soil, its concentration did not change over a period of 4 days (Macías et al. 2004). MBOA, an intermediate in the degradation pathway from DIMBOA to 2-amino-7-methoxy-3H-phenoxazin-3-one (AMPO), was resistant to biodegradation in the soil (Chen et al. 2010). However, AMPO was the final degradation product observed for DIMBOA in non-sterile soils (Macías et al. 2004). BX concentration in wheat and rye and release to the soil rhizosphere are therefore significantly affected by plant tissue and rhizosphere location (distance from root) (Mwendwa et al., 2016b), with the release and toxicity of these allelochemicals being influenced by cultivar and environmental conditions (Weidenhamer 1996; Weston 1996).
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Figure 3 The relative abundance (each bar represents an ion having a specific mass-to-charge ratio (m/z), and the length of the bar indicates the relative abundance of the ion) of four main benzoazinoid metabolites (BXs) detected in the wheat cultivar tissue and rhizoplane in June, 25 days after crop emergence (DAE) and July (57 DAE) 2016 (Mwendwa et al. 2016b).

Studies on the structural requirements for phytotoxicity on the benzoazinone skeleton revealed that the oxygen atom at N-4 is crucial for the phytotoxic effect, since all 4-hydroxy benzoazinones (DIBOA, DIMBOA, D-DIBOA and D-DIMBOA) are more active than the corresponding lactams (HBOA, HMBOA, D-HBOA, and D-HMBOA) (Macías et al. 2006; Schulz et al. 2013). In a comprehensive screening of the activity of many BXs and related compounds, Macías et al. (2005, 2006) found that APO was the most active compound requiring 0.05–0.10 mM to inhibit root length elongation of several species by 50%. In contrast, DIBOA and DIMBOA were less inhibitory requiring 0.5 mM for similar activity while BOA and MBOA were least phytotoxic, requiring 1.0 mM. HBOA and HMBOA had minimal inhibitory activity. Teasdale et al. (2012) reported that the most toxic BX compounds – APO, DIBOA and DIMBOA – were present at relatively low levels, compared to the less toxic compounds, BOA and MBOA, and the non-toxic compounds, HBOA and HMBOA, were the predominant BX species in amended soils. This suggests that concentrations of these metabolites are dynamic in the soil rhizosphere and are dependent on soil microbial community and local environment.

Together these findings suggest that allelopathic activity is dependent upon the presence of a mixture of bioactive compounds at concentrations that result in actual
growth inhibition of specific species and that these metabolites are persistent for sufficient periods of time to result in toxicity. However, the specific activity, rate of degradation and persistence of BXs and associated microbially produced metabolites need to be further explored individually and in mixtures, for them to be used effectively for weed and pest suppression in sustainable agriculture. Clearly their activity in soil varies from that observed in laboratory assays.

7. Case studies: competitive cereal cultivars as a tool in integrated weed management

Several plant traits associated with early wheat vigour (early canopy cover, greater leaf width and tiller number) have been reported to be positively correlated with competitive crop ability (Mwendwa et al. 2016a; Vandeleur and Gill 2004; Zerner et al. 2008). Worthington et al. (2015) demonstrated that weed-suppressive ability was correlated with competitive traits, including vigour and erect growth habit during tillering (Zadoks GS 29), high LAI at stem extension (GS 31), plant height at tillering and stem extension (GS 29, 31), grain yield in weedy conditions, and grain yield tolerance.

Recent studies have shown that early leaf area formation in cereal crops is an important indicator of their weed-suppressive ability (Coleman et al. 2001). Mwendwa et al. (2016a) reported that wheat (cv. Federation and Condo) and cereal rye (cv. Grazer) cultivar crops with the highest LAI were also the most weed suppressive. Cultivar light interception was also highly positively correlated to LAI at 57 days after crop emergence ($r^2 = 0.97, P < 0.001$; Fig. 4).

Bertholdsson (2005, 2011) used early crop mass as an indicator of vigour in wheat and barley and found it to be one of two traits (along with allelopathy) that significantly contributed to the suppression of *Lolium perenne* L. and volunteer *B. napus* (canola) across all years of study. Bread wheat and durum wheat cultivars that were more competitive against *L. rigidum* also had high vigour, acquiring higher biomass at the seedling stage (Lemerle et al. 1996). Mwendwa et al. (2016a) demonstrated that crop phenology early in the season might be particularly important to weed suppression throughout the season and crop yields (Fig. 5).
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As previously stated, some cereal cultivars have been reported to produce high yields in the absence of herbicides under ideal conditions while also suppressing weed populations (Andrew et al. 2015; Worthington et al. 2015; Mwendwa et al. 2016a). Table 1 shows significant differences in weed suppression and yield of six wheat cultivars and cereal rye over two growing seasons. Although, rye was the most suppressive in both years, some wheat cultivars such as cv. Condo, Espada and Janz CL were equally weed suppressive and high yielding.

This suggests that breeding cultivars with early growth vigour and competitive ability may potentially affect weed suppression and reduce weed propagule numbers in the seedbank at harvest. For example, in 2014 Condo produced 21% greater yield than Gregory and reduced weed biomass by 10% compared to Gregory. In addition, in 2015 Condo produced 10% greater yield than Gregory and reduced weed biomass by 97%.

![Image](insert-image-url)

**Figure 4** The relationship between wheat cultivar ground surface PAR light interception and leaf area index at 57 DAE in Wagga Wagga in 2015 ($r^2 = 0.97; P < 0.001$) from Mwendwa et al. 2016a.
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Figure 5 Differences in early crop vigour, canopy closure and weed infestation between control treatment; rye (cv. Grazer; left), wheat (cv. Gregory; middle and cv. Federation, right) 80 days after crop emergence at Wagga Wagga (NSW, Australia) field trials in 2016. There are visible weeds in Gregory but not in Federation.

Table 1 Differences in weed biomass (g m\(^{-2}\)) 110 and 130 days after crop emergence (DAE) respectively and crop yield (t ha\(^{-1}\)) at field trials Wagga Wagga (NSW, Australia) in 2014 and 2015. In some cases, low weed biomass was associated with high yielding ability, as in cv. Condo

<table>
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<th>Year</th>
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<tr>
<td></td>
<td>Biomass (130 DAE) g/m(^2)</td>
<td>Yield t/ha</td>
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<td>Rye</td>
<td>1.1</td>
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<td>Janz</td>
<td>2.5</td>
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<td>Gregory</td>
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<td>Mace</td>
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<td>Livingstone</td>
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<td>Espada</td>
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<td>Condo</td>
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<td>LSD 5%</td>
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* P-Value: NS= not significant, * P<0.05, ** P<0.01, *** P<0.001

Plant height has also been identified as one of the traits most commonly associated with competitiveness (Vandeleur and Gill 2004). While lower yielding in weed-free situations, taller cultivars were typically better tolerators of weed pressure and suppressors of weed growth (Lemerle et al.1996). Although the advantages of plant height
in terms of shading weeds are clear, it alone cannot explain variation in competitive ability (Andrew et al. 2015).

In organic fields, increased plant height and early maturity were associated with reduced weed biomass, while strong early season vigour was related to increased yield, increased spikes m$^{-2}$ and reduced weed biomass (Mason et al. 2007). However, the timing of emergence also influenced light interception, as the same weed species may be relatively tall or short depending on the emergence time relative to the crop. It is important to consider these weed and cultivar-specific traits when selecting a cultivar for weed suppression (Mwendwa et al. 2016a).

8 Summary and future trends

8.1. Summary: how research can contribute to enhanced and sustainable crop production

In Europe, Australasia and North America, non-chemical methods for weed control such as harvest weed seed destruction and crop interference, or competition are being increasingly adopted across the grain-growing areas and are proving successful in controlling weeds that escape pre-sowing herbicide control (Norsworthy et al. 2012). The use of cereal crops with both strong competitive ability and allelopathic effects to control weeds will be a key step towards diversification of weed control and management tools that could both provide sustainable weed control and reduce chances of herbicide resistance development in weeds (Jabran et al. 2015). However, one of the great challenges to the effective use of allelopathy as a tool for weed management is the lack of knowledge about the active allelopathic constituents, their toxicity and their availability in the soil. This lack of knowledge has led to scepticism about the utility of this approach.

As knowledge of the active chemistry of allelopathic crops grows, their allelopathic potential can be further exploited through the selection of cultivars with improved weed-suppression capability. Similarly, the allelopathic potential of crops can be further strengthened through conventional breeding and the use of modern tools of biotechnology and genomics. Improving the allelopathic potential of crops against weeds, insect pests and disease pathogens through conventional breeding, molecular genetics and biotechnology offer promise for effective pest suppression (Jabran and Farooq 2013). In addition, metabolic profiling of secondary metabolites in soil and plant tissues will provide important physiological information regarding crop competitive traits and
biosynthesis and activity of related allelochemicals that may be important in long-term weed suppression in crops (Weston et al. 2015; Mwendwa et al. 2016b).

Breeding of BX-resistant cereal crops with high BX content, as well as a better understanding of the soil persistence of these compounds and their microbial metabolites will be important areas of future research. Knowledge about the selectivity of the BXs in managing diverse populations of weeds and influence of cultural practices on weed management through crop suppression may provide greater impetus to include allelopathic cereal cultivars in broadacre and/or organic cropping systems for weed control (Schulz et al. 2013). However, climatic and edaphic factors clearly influence soil microflora and allelochemical activity, and therefore allelopathy should be assessed in a range of soil types. To fully understand interference by allelopathic crops one must understand soil microbial ecology and evaluate the roles of soil microorganisms in chemically mediated interactions between plants (Inderjit 2005).

A better understanding of allelopathic effects in field situations (soil, climate and agronomic conditions), and of the dependency on cultural practices (cultivar, mixtures, fertilization level, conventional tillage or no-till systems, timing, and system of termination), may also provide the opportunity to profitably include cereal crops as cover crops in both broadacre and organic cropping systems and to use them as a complementary strategy in weed management (Schulz et al. 2013). The recommendation of Dorn et al. (2015) to support weed management in conservation-tillage systems by use of locally adapted cover crops with a rapid establishment, good soil coverage and high dry matter production is logical. However, additional post-emergent weed management measures coupled with diverse crop rotations are likely to be needed for reliable weed control on farms.

From an agronomic perspective, selectivity between crop and weeds is another important consideration that should be addressed. Studies regarding the effect of cover crop residues on weed flora generally concern the amount of aboveground biomass and reduction in the number of the weeds, while limited information regarding the variation in tolerance of weed species exists in the literature (Campiglia et al. 2015). In addition, although high residue biomass may facilitate weed suppression, it often interferes with planting and establishment of the crop, or with crop growth by raising the soil C/N ratio, leading to lack of N availability in crops (Schulz et al. 2013). Therefore, the management
of cereal cover crops and/or the use of allelochemicals in weed management must be optimized to provide maximal weed suppression and a limited or no effect on crops.

8.2. Future research trends

The current knowledge of cereal crop allelopathy shows some striking deficits that should be considered in future research. One of the primary deficits is the lack of knowledge about the dynamics of these metabolites in the soil, and whether soil concentrations are sufficient to inhibit germination and subsequent growth of target weeds. For example, Macias et al (2004) demonstrated that the degradation of DIMBOA by soil microbiota occurred rapidly to AMPO which remained stable and phytotoxic even after 3 months in the soil. This suggests the need for more detailed studies to understand how organic materials, mineral reactivity, ionic interchanging capacity, inorganic ions, and specific microbial and fungal activities further influence allelochemicals in the environment.

To better understand and improve the role of allelopathy against weeds, we recommend that future research focuses on three main areas: 1) the role of soil microorganisms in chemically mediated interactions between plants; 2) the application of new analytical tools such as high-resolution mass spectrometry coupled to HPLC to enable detection and quantification of low dynamic concentrations of metabolites and the resulting degradation compounds; and 3) experimental designs to elucidate the underlying dynamics of complex mixtures of allelochemicals in soil (Weston et al. 2015).

Research addressing these issues will help resolve the role of BXs in the allelopathic activity of cereal grains such as wheat and rye. Most studies indicate that the allelopathic activities of these crops are associated with the production of higher BX concentration, yet the amounts found in soil seem inadequate in most cases to account for the observed toxicity. The effect of BXs on soil microbial ecology is a particularly important question, as is the question of whether soil microbes in the presence of soil available BXs may generate other as yet unidentified phytotoxins.

Therefore, systematic screening for crop detoxification strategies and their differences in crop cultivars will help to unravel various detoxification pathways in plants and microbes. Cultivar-dependent variation in BX composition in cereal rye root exudates has rarely been investigated, and the pathways of exudation and potential for long-distance transport of BXs are unknown (Schulz et al. 2013). Further studies on the
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chemical signalling of plants in response to other plant and microbial populations and directed allelochemical delivery to target plants are required to fully elucidate the role of allelochemicals in the rhizosphere.

9. Where to look for further information

Further information on allelopathy in cereal crops including physiological processes, ecological implications, non-chemical weed management and biological weed and pest control; practices and environmental impact can be found in the following books:


10. References


The use of allelopathy and competitive crop cultivars for weed suppression in cereal crops


Glenn, A. E., Hinton, D. M., Yates, I. E. and Bacon, C. W. (2001). Detoxification of corn antimicrobial compounds as the basis for isolating Fusarium verticillioides and
The use of allelopathy and competitive crop cultivars for weed suppression in cereal crops

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Tanwir, F., Fredholm, M., Gregersen, P. L. and Fomsgaard, I. S. (2013). Comparison of the levels of bioactive benzoazinoids in different wheat and rye fractions and the transformation of these compounds in homemade foods. *Food Chemistry 141*(1), 444–50.


Part 1: Mechanisms of Weed Suppression in Canola (*Brassica napus* L.)

Weed-suppressive commercial canola cultivar GT-50 at flowering (above) and post-harvest (below)
Chapter 2

The Weed Suppressive Ability of Selected Australian Grain Crops; Case Studies from the Riverina Region in New South Wales

The purpose of this chapter is to provide a comparison of the ability of various dual-purpose grazing or non-grazing grain crops and their residues to suppress weeds until subsequent planting the following year season. The grain crops included canola, wheat, barley, triticale and oats. Canola was one of the most weed suppressive both in crop and post-harvest. Further evaluation of weed suppression by selected canola cultivars both in-crop and post-harvest was performed in field experiments over two years using both standard and recently released canola cultivars (experiment two).

The manuscript transitions between an earlier GRDC supported project at CSU (which I was involved in while working for Central West Farming Systems Inc) and the current PhD research by presenting the findings on the evaluation of the impact of residues of several grain crops on winter and post-harvest summer annual weed establishment from 2012 to 2014. This data is presented in experiment one. These results provided information used to design trials for the current study which included further and detailed investigations on the competitive ability of selected cultivars of canola and wheat. Canola data from 2014-2015 is presented and discussed based on experiment two.

All the authors contributed to the organisation, editing and review of the manuscript. In addition, Dr. Brown was involved in establishing and monitoring the canola crop in experiment two while Drs. L. A. and P. Weston analysed the data from the first experiment and provided support in computing the data from the second experiment. The manuscript was published in the peer-reviewed Journal of Crop Protection as described below.

The weed suppressive ability of selected Australian grain crops; case studies from the Riverina region in New South Wales

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Abstract

Herbicide resistance in both grasses and broadleaf weeds is on the rise across Australia, with an increasing number of cropping weeds experiencing resistance to multiple herbicides. One contributing factor to this issue is the adoption of conservation agriculture (CA). CA is a system of residue management that avoids the use of cultivation for the establishment of annual broadacre crops. Another contributing factor is poor management of herbicide mode of action strategies in broadacre farming. One key tool for integrated weed management (IWM) strategies is the use of competitive grain crop cultivars and postharvest crop residues, which can effectively suppress, or delay weed seedling emergence and provide an initial advantage for the crop in terms of early weed suppression. The ability of various dual-purpose grazing or non-grazing grain crops and their residues to suppress weeds until subsequent planting the following year was compared in two successive field experiments in the Riverina region of New South Wales (NSW), Australia. We evaluated 1) the impact of residues of several grain crops on winter and postharvest summer annual weed establishment from 2012 to 2014 and 2) in-crop and post-harvest weed suppression in 2014-2015 using a genetically diverse set of canola cultivars, including those found to be highly weed-suppressive in the first trial. Replicated field trials were established in Wagga Wagga, in a moderate rainfall zone (mean 572 mm/year) from 2012 to 2015 using commercially available crop cultivars. Differences in in-crop weed infestation and post-harvest crop fallows associated with grain crop cultivar and species were observed in each of three years. Significant weed suppression associated with grazing and non-grazing wheat residues was observed after harvest, with grazing
wheat exhibiting significant suppression of fleabane and witchgrass up to 130 days post-harvest. Grazing and non-grazing canola provided strong and significant suppression of fleabane and witchgrass for up to 140 days following harvest. Grazing cereal cultivars were generally more suppressive of weeds than non-grazing cultivars. Early vigour and ability to intercept light and accumulate biomass resulted in suppression of incrop weed growth in canola trials, with GT-50 the most weed suppressive canola cultivar. Weed biomass differed with cultivar in both years and appeared to be inversely related to early crop vigour, suggesting the importance of crop biomass in regulating weed competition in the crop. Cultivars CB Taurus and GT50 were consistently the most weed suppressive when residues remained in plots 150 days post-harvest. These results indicate that the establishment of certain species and cultivars of grain crops may effectively suppress weed growth both in-crop and post-harvest, in the absence of post-emergent herbicides. In addition, the choice of canola cultivar for desired weed suppression impacts the subsequent ability of the crop and its residues to successfully interfere with weed growth.

**Keywords:** weed suppressive, Crop residue, Post-harvest, Dual-purpose, Herbicides, Conservation agriculture

1. Introduction

Since 1975, producers in Australian dryland agricultural systems have relied heavily on herbicides for weed control in broadacre cereal crops such as wheat (*Triticum aestivum* L.). However, during the 1990s, the phenomenon of herbicide resistance in weeds of prevalent cereal crops has increased at an alarming rate (Pannell et al., 2004), especially for common weeds such as annual ryegrass (*Lolium rigidum* G.), wild radish (*Raphanus raphanistrum* L.) and wild oats (*Avena fatua* L.) (Scott et al., 2010). To date, this trend has not changed as herbicide-resistant weeds are still on the rise across Australia, including an increasing number of weeds displaying resistance to multiple herbicides or herbicide families (Owen et al., 2013).

A key factor in farmer preference for management practices is the capacity to maintain or improve crop yields and profitability. Conservation agriculture (CA) is a system of residue management that avoids the use of cultivation for the establishment of annual broadacre crops. This system maintains crop residues on the soil surface with minimal soil disturbance over time and has clear benefits and costs for dryland and irrigated mixed farming systems of south-eastern Australia (Weston et al., 2014; Scott et al., 2010; Kirkegaard et al., 2014). The benefits arising from the ease of crop
management, energy/cost/time savings, and soil and water conservation have led to widespread adoption of CA, particularly on large farms, where producers harness the tools of modern science: highly-sophisticated machines, potent agrochemicals, and biotechnology (Giller et al., 2015).

Three important principles of CA include the use of minimal soil disturbance through tillage operations, permanent residue cover, and rotation of primary crops (Chauhan et al., 2012; Giller et al., 2015). The first two principles are interdependent since a protective residue or mulch, for example, cannot be well maintained when the soil has been thoroughly tilled. Thus, CA is deemed to be practised only when all three principles are carefully and precisely applied (Derpsch et al., 2014).

Weed management in CA can be more challenging than in conventional agriculture because there is limited weed seed burial in CA and frequent infestation of perennial weeds (Chauhan et al., 2012). In addition, herbicides may prove to be less effective for weed management because soil-applied herbicides are generally not incorporated in CA soils, resulting in reduced efficacy on the soil surface (Chauhan et al., 2012). This reduced efficacy has potentially contributed to increased herbicide resistance in weeds of Australian broadacre crops including annual ryegrass, wild radish and wild oats (Scott et al., 2010). However, in comparison, weed seeds that remain present on the soil surface are typically more prone to desiccation and greater predation by insects, especially ants (Chauhan et al., 2012).

Crop residues, when present in uniform and dense stands under CA, can effectively suppress or delay weed seedling emergence and provide an initial advantage for the crop in terms of early weed suppression (Chauhan et al., 2012). For instance, wheat planted in no-till conditions reduced the seedling emergence rate of littleseed canarygrass (Phalaris minor R.), when compared with conventional ploughing and sowing (Bajwa et al., 2015).

In addition, crop residues can also interfere with weed development and growth through alteration of soil physical, chemical, and biological characteristics based on two possible sources of allelochemicals: secondary metabolites released directly from crop litter or those produced by microorganisms that use plant residues as a substrate (Ferreira and Reinhardt, 2010). For example, McCalla & Norstadt’s (1974) review of phenolic acids, which are released from many cereal crop residues, showed that levels required for
strong phytotoxicity to successive crops often greatly exceed those later observed in the soil following residue degradation by microorganisms.

Previous studies have also shown the temporal impacts of crop mulches and residues on weed germination, establishment and weed management over time. In particular, cereal and grain residues including those of wheat, rye (Secale cereal L.), triticale (x Triticosecale Wittm. ex A. Camus.), oats and barley (Hordeum vulgare L.) as well as canola residues have been studied for their ability to suppress weeds when used as cover crops into which broadacre crops are subsequently planted (Liebl et al., 1992; Putnam et al., 1983; Weston, 1990, 2005). In Australian broadacre cropping regions, crops are planted up to 5 to 6 months after harvest into the remaining crop stubbles (Weston et al., 2014). We are particularly interested in the ability of selected grain crops to suppress weeds both in crop and in fallow, due to the presence of associated remaining crop stubble.

Another alternative weed management practice for Australian cereal crops is the use of cereal crop cultivars with increased competitiveness. The competitive ability of a crop can be specified either in terms of crop tolerance to weeds or growth inhibition of weeds by resource competition (Bertholdsson, 2010). Competitive cultivars often have better access to light, nutrients, and water resources in limited space due to crop architectural traits, thus suppressing the growth and reproduction of nearby weed species (Worthington et al., 2015).

The competitive ability of wheat is influenced by a range of plant attributes such as height, tiller number, and light interception by the canopy or light interception at the soil surface. Previous studies have shown that, increasing plant height improved bread and durum wheat's ability to tolerate and suppress oats while several plant traits associated with early wheat vigour (early canopy cover, greater leaf width and tiller number) were also positively correlated with crop competitive ability (Vandeleur and Gill, 2004; Zerner et al., 2008).

In a recent study carried out at two ecologically different locations, differences in weed suppression by selected Australian commercial winter wheat cultivars were largely determined by crop architecture and phenology early in the growing season (Mwendwa et al. 2016). In Greece, the use of competitive cultivars alone has been demonstrated to reduce herbicide usage by 50% in wheat (Travlos, 2012; Andrew et al., 2015). Thus, developing grain cultivars with superior weed competitive ability could complement
cultural methods by maintaining acceptable yields and suppressing weed populations (Worthington and Reberg-Horton, 2013; Andrew et al., 2015).

Increased competitive ability among canola cultivars has generally been attributed to early seedling emergence, seedling vigour, rapid root growth and rate of leaf expansion, early root and shoot biomass accumulation and canopy closure and plant height (Beckie et al., 2008; Asaduzzaman et al., 2014b) and our findings generally substantiate the reports of these authors, with canola cultivar impacting weed number and total weed biomass (Weston et al., 2014). Recent studies have shown that the ranking of cultivars for competitiveness against weeds is strongly influenced by seasonal conditions, with some cultivars consistently more competitive than others (Lemerle et al., 2014).

The purpose of this study was to compare the ability of various dual-purpose grazing or non-grazing grain crops and their residues to suppress weeds until subsequent planting the following year in the Riverina region of NSW Australia. In addition, based on results from the first experiment, further evaluation of weed suppression in canola and canola residues after harvest was conducted in field experiments over two years using both standard and recently released canola cultivars.

To assess the impact of crop cultivars on weed suppression in-crop and the potential of crop residues to suppress weeds after harvest, experiments were performed over three years in a low-input grain production system with moderate winter rainfall (572 mm) without irrigation. Specifically, these experiments established crops without the use of pre-emergence herbicides in commercially cropped sites in order to compare and evaluate subsequent weed suppression provided by crop residues without the confounding effects of residual herbicides.

2. Materials and methods

2.1. Crop establishment

2.1.1. Experiment 1

To evaluate the impact of crop residues on winter and summer annual weed establishment, identical experiments with similar cultivars of wheat, oats (Avena sativa L.), barley (Hordeum vulgare L.), triticale (Triticosecale Wittm. ex A. Camus), cereal rye (Secale cereal L.) and canola (Brassica napus L.) treatments were established in 2012,
2013 and 2014 at adjoining different sites at the Graham Centre Field Site in Wagga Wagga, NSW (Table 1). The field site had an average yearly rainfall of approximately 572 mm. Previous cropping history included wheat and canola rotations.

Crops were sown on 30 May, 31 May and 15 May in 2012, 2013 and 2014, respectively, as randomised complete blocks (RCB) with four replicates in 2 x 12 m plots of moderate chemical fertility and water-holding capacity red kandosol soils. Plots were planted using a cone seeder with 22 cm row spacing and pre-plant application of diammonium phosphate (DAP) at standard commercial rates of 50 kg ha\(^{-1}\) to all crops.

2.1.2. Experiment 2

Results obtained from the first experiment showed that certain canola cultivars were strongly weed suppressive, with one cultivar suppressing over 90% of weeds compared to a non-planted control or plots containing no residues post-harvest. The second experiment was designed to further evaluate a genetically diverse set of canola cultivars for enhanced suppression both in-crop and postharvest. Replicated canola field trials were established at the Graham Centre Field Site in 2014 and 2015 (Table 2) to evaluate canola cultivars for weed suppression.

The 2014 experiment was established on 25 April as RCB with six replicates while the 2015 experiment was established on 2 May, at an adjacent site in the same paddock using a similar design. All experiments were conducted on fine red clay loam sodosols which were previously used in commercial production of cereals, canola and/or lucerne. No pre- or post-emergence herbicides were used in these experiments in order to evaluate canola plant and residue activity on weeds in the absence of confounding effects. Field sites were selected for areas that had similar levels of established weeds to those in commercial production, meaning that existing weed pressures at these sites were not excessive.

Cultivar selection included material that had previously exhibited in-field competitive traits in previous studies (Lemerle et al., 2014; Weston et al., 2014). In addition, two newly released commercially available hybrid canola cultivars marketed as greater biomass producers with weed suppression potential were added in 2015: Hyola 600RR and Hyola 725RT.

Canola was sown between 3.0 and 4.0 kg ha\(^{-1}\) (depending on the cultivar seed weight) to achieve a target population of 45 plants m\(^{-2}\). Standard sowing practices were
used in all years, including knifepoint and press wheel planter, application of Jubilee fungicide at 300 mL100 kg⁻¹ of fertiliser (active constituent: 500 g L⁻¹ flutriafol) impregnated onto 70 kg ha⁻¹ MAP (Incitec Pivot Fertilisers). Plots of 2 x 12 m were planted using a cone seeder with a 22 cm row spacing, and the experiment was repeated three times in successive years.

2.2. Crop husbandry

2.2.1. Experiment 1

A post-emergent herbicide application of clethodim 240 @ 400 mL ha⁻¹ (active constituent: 240 g L⁻¹ clethodim) for canola and tralkoxydim 400 WG herbicide @ 500 g ha⁻¹ (active constituent: 400 g kg⁻¹ tralkoxydim) for winter cereal crops was applied after the final in-crop weed count and in August 2012 only, to manage large numbers of winter annual grasses before harvest.

2.2.2. Experiment 2

In both years, residual crop stubble before planting was removed by burning if necessary and no herbicide was applied. Application of insecticide at 2 L ha⁻¹ (Lorsban 500 EC e active constituent: chlorpyrifos at 500 g L⁻¹) and fungicide at 150 mL ha⁻¹ (Talstar 250 EC- active constituent: bifenthrin at 250 g L⁻¹) were used around and on all the canola plots at the appropriate timing. Top dressing was done at 50 kg ha⁻¹ N urea (46-0-0).

2.3. Data collection and harvest

In both experiments, harvest was performed in all experiments between 15 November and 15 December as crops matured, using a small plot harvester. The yield was measured as harvested grain (kg ha⁻¹).

2.3.1. Experiment 1

To determine in-crop weed suppression, weed numbers were recorded in September and November (at crop grain fill and before harvest, respectively) and for post-harvest weed suppression in January and March/April of each year using a 0.5 x 10 m rating zone centred in each plot; in this area two 0.5 m² quadrats were also evaluated for weed numbers and biomass by cutting weeds at the soil surface and obtaining a dry weight
for each treatment. Residual stubble biomass was assessed by cutting stubble at the soil surface and obtaining a dry weight for each treatment.

2.3.2. Experiment 2

Data collected each year included crop phenological characteristics, percentage light interception using light ceptometry (AccuPAR LP-80 Ceptometer, Decagon Devices®) to measure PAR (photosynthetically active radiation) both above and below the crop canopy, biomass cuts of crop and weeds (g m⁻²), visual vigour ratings (0 = poor, 10 = excellent), yield (kg ha⁻¹) and post-harvest weed suppression (0 = poor, 10 = excellent). All crop and weed biomass data were collected in each plot at the soil surface within a 50 x 50 cm quadrat with two subplots collected per plot. Weed counts were monitored as in experiment 1. All data collection was performed in association with critical plant developmental stages of stem elongation, flowering, maturity and post-harvest stubble residue assessment. Data collection focused upon those traits leading to both in-crop and potential post-harvest suppression of weeds.

Table 1: Crops, cultivars and seeding rates evaluated in Wagga Wagga in 2012, 2013 and 2014.

<table>
<thead>
<tr>
<th>Crop Species</th>
<th>Scientific name</th>
<th>Crop Cultivar</th>
<th>Grazing or Non-grazing</th>
<th>Seeding Rate kg/ha</th>
</tr>
</thead>
<tbody>
<tr>
<td>wheat</td>
<td><em>Triticum aestivum</em> L.</td>
<td>Wedgetail</td>
<td>grazing</td>
<td>60</td>
</tr>
<tr>
<td>wheat</td>
<td><em>Triticum aestivum</em> L.</td>
<td>EGA Gregory</td>
<td>non-grazing</td>
<td>60</td>
</tr>
<tr>
<td>oats</td>
<td><em>Avena sativa</em> L.</td>
<td>Graza</td>
<td>grazing</td>
<td>60</td>
</tr>
<tr>
<td>oats</td>
<td><em>Avena sativa</em> L.</td>
<td>Mitika</td>
<td>non-grazing</td>
<td>60</td>
</tr>
<tr>
<td>barley</td>
<td><em>Hordeum vulgare</em> L.</td>
<td>Urambie</td>
<td>grazing</td>
<td>60</td>
</tr>
<tr>
<td>barley</td>
<td><em>Hordeum vulgare</em> L.</td>
<td>Buloke</td>
<td>non-grazing</td>
<td>60</td>
</tr>
<tr>
<td>triticale</td>
<td><em>Triticosecale</em> Wittm. ex A. Camus.</td>
<td>Tobruk</td>
<td>grazing</td>
<td>80</td>
</tr>
<tr>
<td>rye</td>
<td><em>Secale cereal</em> L.</td>
<td>Cereal rye</td>
<td>grazing and grain</td>
<td>60</td>
</tr>
<tr>
<td>canola</td>
<td><em>Brassica napus</em> L.</td>
<td>CB Taurus</td>
<td>grazing</td>
<td>6.5</td>
</tr>
<tr>
<td>canola</td>
<td><em>Brassica napus</em> L.</td>
<td>Hyola 50</td>
<td>non-grazing</td>
<td>3.4</td>
</tr>
</tbody>
</table>

2.4. Statistical analysis

For both experiments, trial randomization and design and data analysis were performed using Agricultural Research Manager (ARM) version 9.0, a statistical software package by GDM (Gylling Data Management Inc., 2014). Grain harvest was performed at crop maturity each year using a small plot harvester. Statistical analysis of data was performed by ANOVA for randomised experiments with six replicates using GenStat statistical program (VSN, 2016); significant differences were separated using LSD (0.05). Data transformations were performed using the square-root ((x + c) ^0.5) function in
GenStat statistical windows program to correct data homogeneity and normality prior to analysis when required.

Table 2: Canola cultivars planted in 2014 and 2015 trials in Wagga Wagga, NSW.

<table>
<thead>
<tr>
<th>Cultivar - 2014</th>
<th>Cultivar - 2015</th>
<th>Type</th>
<th>Description of end use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyola 50</td>
<td>Hyola 50</td>
<td>Hybrid/conventional</td>
<td>grain canola</td>
</tr>
<tr>
<td>CB Taurus</td>
<td>CB Taurus</td>
<td>Hybrid/conventional</td>
<td>Grain/ grazing canola</td>
</tr>
<tr>
<td>GT-50</td>
<td>GT-50</td>
<td>Hybrid/ Roundup ready</td>
<td>grain canola</td>
</tr>
<tr>
<td>AV Opal</td>
<td>AV Opal</td>
<td>Hybrid/conventional</td>
<td>grain canola</td>
</tr>
<tr>
<td>Barossa</td>
<td>Barossa</td>
<td>Hybrid/conventional</td>
<td>grain canola</td>
</tr>
<tr>
<td>46C76 (ATR Bonito)</td>
<td>ATR Bonito</td>
<td>Open pollinated/ Triazine tolerant</td>
<td>grain canola</td>
</tr>
<tr>
<td>-</td>
<td>Hyola 600 RR</td>
<td>Hybrid/ Roundup ready</td>
<td>grain canola</td>
</tr>
<tr>
<td>-</td>
<td>Hyola 725 RT</td>
<td>Hybrid/ Dual tolerant</td>
<td>grain canola</td>
</tr>
</tbody>
</table>

3. Results and Discussion

3.1. Impact of crop cultivar residue on in-crop and post-harvest weed suppression (experiment 1)

Experimental results in 2012-13, 2013-14 and in 2014-15 were similar in terms of yield obtained for each crop cultivar and weed infestation within crop before harvest as assessed by weed biomass and number (data not presented). However, post-harvest weed suppression provided by crop residues of various crop/cultivars was substantially impacted by moisture availability and rainfall, depending on the year. Major in-crop weeds encountered in all years included flaxleaf fleabane (*Conyza bonariensis* L.), annual ryegrass, and bromegrass (*Bromus* spp.), but in 2012, annual ryegrass management was more problematic and required one post-emergent application of an appropriate selective grass herbicide for control in August due to higher rainfall received in late winter.

In 2013, 2014, in-crop weed pressures were moderate. In 2013-2014, fumitory (*Fumaria muralis* Sond. Ex W.D.J. Koch.) was more prevalent, and summer/autumn annuals included flaxleaf fleabane and witchgrass (*Panicum capillare* L.), which germinated following adequate post-harvest rainfall (Fig. 1). The yield of cereal and canola crops generally ranged from 2.3 to 4.0 t ha\(^{-1}\) in all three years depending on the crop, and yields obtained were similar to those of commercial producers. In general, dual-purpose crops for grain and/or grazing generally produced significantly reduced yields...
(by up to 2- to 3-fold) compared to standard grain crops. Canola yields ranged from 1.5 to 2.5 t ha\(^{-1}\) and were also similar to district averages.

![Graph showing cultivar differences in remaining crop stubble for fleabane and witchgrass counts](image)

Figure 1: Cultivar differences in remaining crop stubble for fleabane (P < 0.01, LSD 1.0) and witchgrass (NS, LSD 1.1) counts (plants per m\(^2\)) in 2013 trial plots at Wagga Wagga. Data was square root transformed to improve homogeneity. Bars represent mean values ± standard error. Weed counts were performed in March 2014 post-harvest to the crop 260 DAE (days after crop emergence) or 90 days after harvest; Control areas with no residues averaged 12 and 20 plants per m\(^2\) for fleabane and witchgrass, respectively.

Following harvest, the presence of crop residues of all crop cultivars resulted in significantly greater in-fallow weed suppression, with 50-100% reductions in weed biomass in comparison to uncropped controls with no crop residues. Weed infestation 60 and 120-150 days after harvest showed that crop residue significantly reduced fleabane and witchgrass pressure. Flaxleaf fleabane and witchgrass were the major summer weeds infesting plots after harvest in all years. In 2012-13, although there were greater populations of both witchgrass and fleabane, there were no significant differences in weed numbers per plot regardless of crop residue at 60 and 120 days after harvest. This may have been due to higher than normal rainfall after harvest. By late March 2013, 120 days after harvest, witchgrass predominated in cereal plots, but grazing canola and canola plots contained few, if any, witchgrass seedlings.

In 2013-14, greatest suppression of post-harvest fleabane (P < 0.01, LSD 1.0) seedlings was observed in grazing and nongrazing canola, grazing wheat and oat stubbles (Fig. 1); witchgrass populations were not significantly different among plots. In 2014-15,
grazing and non-grazing barley, grazing wheat and oat significantly suppressed fleabane establishment ($P < 0.001$, LSD 0.1) while the suppression of witchgrass was again not significantly different (LSD 2.2) among cultivars (Fig. 2). In both years, oat and grazing wheat stubble treatments were consistently weed suppressive, especially against fleabane, while triticale was most suppressive against witchgrass.

![Figure 2: Cultivar differences in remaining crop stubble for fleabane ($P < 0.001$, LSD 0.1) and witchgrass (NS, LSD 2.2) counts (plants per m$^2$) in 2014 trial plots at Wagga Wagga. The data was transformed by square root to improve homogeneity. Bars represent mean values ± standard error. The weed counts were performed post-harvest in late January 2015, 50 days after crop harvest or 220 DAE (days after crop emergence).](image)

In 2012-13, grazing and non-grazing canola plots were nearly weed-free for 90 days or more post-harvest. In 2013/14, this period was extended, presumably because of low rainfall. Fleabane was the only weed of significance that established in post-harvest grazing canola stubble; numbers were low in March 2012 and 2013 likely due to low summer rainfall received. In contrast, witchgrass numbers in May 2014 (120-150 days after harvest) were increased, but canola (both cultivars), grazing wheat (Wedgetail) and oat plots remained cleaner than other cereal plots, with up to 75% less witchgrass biomass and 50% decreases in weed seedling numbers in contrast to other treatments (Fig. 1).

In January of both 2013 and 2014, grazing barley, wheat and both canola cultivars showed limited weed infestation 45-60 days after harvest. Although weed counts were significantly different among treatments in 2012 and 2013, it was evident that grazing and hybrid canola plots remained relatively weed free for a period of 90-140 days post-harvest in both years. In 2015, increased summer rainfall received in January 2015 stimulated weed seed germination and weed establishment by late January. However, unlike 2014, grazing and non-grazing barley, oat and grazing wheat stubble treatments
were significantly ($P < 0.001$) more weed suppressive than grain and grazing canola (Fig. 2). The presence of higher soil moisture may have resulted in less suppression of canola cultivars in 2015.

Crop residues remaining in plots were assessed following crop harvest. Significant differences were noted in post-harvest crop residue levels in all three years (Fig. 3), with the greatest postharvest residue retained on the soil surface of oat, barley and rye plots, where residue levels were up to two-fold times higher ($P < 0.001$) than those retained in canola plots. However, both grain canola (Hyola 50) and grazing canola (CB Taurus), despite having lower remaining stubble biomass compared to the cereal crops were more weed suppressive. It is important to note that canola plots were predominantly fleabane free in March and April of 2013 and CB Taurus was also witchgrass free up to 5 months following harvest, in contrast to cereal plots retaining greatest levels of residual stubble.

In the 2014 trial, visual weed suppression ratings were taken on January 31, 2015, 50 days after harvest. Plots were rated for percent weed control of flaxleaf fleabane and witchgrass (Fig. 4) and total weed suppression (Fig. 5). Similar to 2013 and 2014, the two most prevalent weeds present were flaxleaf fleabane and witchgrass. Significant differences were noted in control/suppression of fleabane, with Mitika grain oats and both the barleys exhibiting the poorest suppression, similar to findings observed in 2013 and 2014 (data not shown). Suppression of witchgrass was also poorest in Mitika oats, Wedgetail grazing wheat, and Buloke barley. The greatest weed suppression in 2014 trials was found in the Graza oats, Grazer rye and the grain canola, Hyola 50 (Figs. 4 and 5).

Establishment of summer annual weeds was up to ten-fold lower in plots containing crop residues than individual control plots without stubble (data not shown). Some crops, including rye, grazing and cereal barley, grazing and cereal wheat and grazing and grain canola, were clearly more suppressive of in-crop weeds, likely due to reduced light penetration at the soil surface, a consequence of competitive canopy architecture. Previous studies have demonstrated that wheat cultivars showing greater competitive ability once established can potentially assist in reducing weed growth and subsequent seed production, thus reducing the potential for crop yield loss (Zerner et al., 2008).
Figure 3: Differences in post-harvest cultivar stubble biomass (g/m²) remaining in the test plots of A) 2012 (LSD 114.3, P < 0.001), B) 2013 (LSD 267.0, P < 0.001) and C) 2014 (LSD 127.6, < 0.001) trials at Wagga Wagga. Bars represent mean values ± standard error.

However, after harvest, dried stubble residues alone remained on the soil surface, with no residual herbicide residues present, and stubble residue amount remaining was crop-cultivar dependent. Residues are typically the source of allelochemicals, and nutrients released over time to the soil rhizosphere due to residue decomposition (Weston, 2005). In addition, residues present on the soil surface can alter moisture availability and soil microbial interactions (Weston and Duke, 2003). The suppressive effect of residues on weeds, particularly certain residues of grazing cultivars, further suggests that the genetic variability existing in cereals crop cultivars may be used to improve their
allelopathic potential by selective breeding strategies (Bertin et al., 2003; Worthington and Reberg-Horton, 2013).

Figure 4: Differences in post-harvest cultivar residue weed suppression (%) of fleabane (LSD 27.4, P < 0.001) and witchgrass (LSD 28.2, P < 0.001) from 2014 trials in late January 2015 at 50 days after crop harvest (220 DAE-days after crop emergence). Bars represent mean values ± standard error.

Figure 5: Differences (LSD 2.2, P < 0.001) in post-harvest remaining cultivar residue on summer visual weed suppression rating (0 = poor, 10 = excellent) 2014 trials in late January 2015, 50 days after harvest (220 DAE-days after crop emergence). Bars represent mean values ± standard error.
Findings related to weed suppression associated with the presence of decomposing foliar residues and roots suggests the potential role of allelochemicals produced by residue decomposition of the canola stubble in addition to the physical impact of canola residues. Previous studies have demonstrated that crop residues interfere with weed growth through alteration of soil physical, chemical, and biological characteristics based on two possible sources of allelochemicals: secondary metabolites that are released directly from crop litter, including roots, or those produced by microorganisms that use plant residues as a substrate (Ferreira and Reinhardt, 2010).

A considerable literature has shown the presence of significant levels of glucosinolates and isothiocyanates in canola residues and stubble leachates, likely important in weed suppression (Al-Khatib et al., 1997; Gardiner et al., 1999). In addition, the synergistic effects of sinapyl alcohol, p-hydroxybenzoic acid and 3, 5, 6, 7, 8-pentahydroxy flavones exuded by some canola cultivars may play a key role in canola allelopathy against weeds (Asaduzzaman et al., 2015).

Previous studies have shown the temporal impacts of crop mulches and residues on weed germination, establishment and management over time. In particular, cereal and grain residues including those of wheat, rye, triticale, oats and barley as well as canola residues have been studied for their ability to suppress weeds when used as cover crops into which broadacre crops are subsequently planted (Liebl et al., 1992; Putnam et al., 1983; Weston, 1990, 2005).

Up to 95% control of economically important broadleaf weeds and grasses has been reported when significant residues remain on the soil surface. This is thought to be due to both the physical presence of residues and release of allelochemicals over a 60-day period following harvest/kill of the cover crop (Weston et al., 2014; Scott et al., 2010). In either case, both allelopathy and competitive weed suppressive ability are complex, quantitatively inherited traits that are heavily influenced by environmental factors (Worthington and Reberg-Horton, 2013). To date, studies have not generally been able to demonstrate the independent contribution of these traits to weed suppression.

Clear and significant differences in weed infestation were observed in-crop and also in post-harvest crop fallows and were associated with grain crop cultivar and weed species evaluated in all three years of the first trial. Crops were produced in soils with low to moderate weed infestation and in the absence of residual herbicides. Crops
generally proved to be both competitive with weeds during their establishment and growth as evidenced by reduced numbers of weeds in crop plots compared to borders without grain crops (data not presented).

However, weed suppression ability was strongly cultivar rather than species dependent (Bertin et al., 2003; Wu et al., 2007). Therefore, the use of grain cultivars with superior competitive ability against weeds should complement current cultural methods for weed control by maintaining acceptable yields and suppressing weed populations (Worthington and Reberg-Horton, 2013).

3.2. Impact of canola cultivars on in-crop and post-harvest weed suppression (experiment 2)

Weed biomass differed with cultivar in both years and appeared to be inversely related to early crop vigour (Figs. 6, 10-12), suggesting the importance of crop biomass in regulating weed competition in the crop. GT-50 was the most weed suppressive canola cultivar in both years while Barossa, ATR Bonito and Hyola 725RT were least weed suppressive in-crop. Weed biomass was ten-fold higher in 2014 than in 2015, with high weed numbers likely associated with greater soil moisture availability in 2014.

In addition, early cultivar biomass was highly correlated with reduced weed biomass. In 2014, due to the exceptional growth of weeds resulting in high weed biomass, there was a strong and negative correlation ($r^2 = 0.51$, $P < 0.001$) between early cultivar growth vigour and weed biomass (Fig. 7). In 2015, there was a tendency toward reduced weed biomass with early canola vigour, but this correlation was not significant ($r^2 = 0.19$, NS) (Fig. 8). These trends suggest the importance of early crop vigour in competitive ability against weeds; these findings are in agreement with previous studies showing that canola crop competitiveness assessed by weed dry matter suppression at flowering was negatively correlated with crop dry matter accumulation (Lemerle et al., 2010).
Figure 6: A). The differences in 2014 Canola cultivar biomass taken at 70 (LSD 70.6, P < 0.05), 130 (LSD 296.3, NS), 190 (LSD 285.1, P < 0.001) DAE and the cultivar impact on weed biomass at 130 (LSD 118.7, P < 0.001) DAE and B) differences in 2015 canola cultivar biomass taken at 70 (LSD 41.2, P < 0.001), 125 (LSD 167.2, P < 0.5), 180 (LSD 260.7, P < 0.05) DAE and the cultivar impact on weed biomass at 180 DAE (LSD 20.7, P < 0.5). The error bars are calculated startdar error.

Based on previous studies, the competitive response of canola cultivars, assessed by weed biomass suppression, was positively correlated ($r^2 = 0.52$, P < 0.01) with early-season crop biomass accumulation (Beckie et al., 2008; Asaduzzaman et al., 2014a). The current study also demonstrated a similar relationship between early-season crop biomass...
accumulation and weed biomass suppression in 2014. The reduction of weed biomass at
and/or before flowering has significant benefits for lower weed seed production and
reduced seedbank replenishment (Lemerle et al., 2014).

The Hyola 600RR cultivar exhibited moderate weed suppression despite
demonstrating high early growth vigour and biomass. In comparison, CB Taurus and AV
Opal did not produce high early biomass in either year (Fig. 6) but demonstrated strong
weed suppression ability (Figs. 6 and 9). This suggests that weed suppression in these
cultivars may be due to traits other than crop competition alone, such as allelopathy. Our
results support those of a previous study which showed that AV Opal and other cultivars
were weed suppressive and potentially allelopathic at various sowing times
(Asaduzzaman et al., 2014a).

Brassica spp. have been shown to exhibit allelopathic potential associated with
increased concentration of glucosinolates and isothiocyanates produced in foliage (Al-
Khatib et al., 1997; Gardiner et al., 1999; Asaduzzaman et al., 2014c). Asaduzzaman et al.
(2014c) reported that the allelopathic activity of canola, as measured by reduction in
annual ryegrass root and shoot growth, increased with canola crop seedling densities.
However, the Australian cultivar Av-Opal and the breeding line Pak85388-502
suppressed root length of ryegrass more than other cultivars, even at low densities. This study demonstrated the enhanced weed suppression potentially associated with the production of bioactive secondary metabolites by several canola cultivars in comparison to the effects of crop competition.

![Graph showing regression analysis](image)

Figure 8: The regression ($r^2 = 0.19$, NS) between the mean of canola cultivar early biomass (70 DAE) and weed biomass at 180 DAE at Wagga Wagga in 2015. Each data point in the graph is a mean of data from six replicates.

Additionally, the synergistic effects of sinapyl alcohol $p$-hydroxybenzoic acid and 3, 5, 6, 7, 8-pentahydroxy flavones exuded by some canola cultivars may play a role in canola weed suppression (Asaduzzaman et al., 2015). Brassica spp. have received consideration due to allelopathic potential associated with crop residues and/or plant extracts (Norsworthy et al., 2011). In addition, previous studies demonstrated that canola hybrids were more competitive than open-pollinated canola cultivars (Harker et al., 2011) as the hybrid cultivars were generally taller, more vigorous, had a denser canopy, and were also more weed suppressive than open-pollinated cultivars (Beckie et al., 2008). Hyola 50 and GT-50 were typically most suppressive in our studies and produced denser canopies and greater biomass in both years as compared to other hybrids or open-pollinated cultivars.
Fig. 9 show crop visual ratings based on cultivar growth vigour in 2014 and 2015, respectively. Certain canola cultivars with high visual ratings such as GT-50, Stego and Hyola 50 demonstrated reasonable weed suppression. However, CB Taurus was an exception, demonstrating strong weed suppression and lower early vigour (Figs. 9 and 10). This suggests that weed suppression in CB Taurus may be due to a combination of factors, including potential allelopathic interference with weeds.

![Figure 9](image)

Dominant weeds in 2014 included fumitory, flaxleaf fleabane and ripgut bromegrass (*Bromus diandrus* Roth.) as opposed to annual bluebell (*Wahlenbergia gracilis* G. Forst.) in 2015. Interestingly there were no significant differences in weed count among cultivars (Table 3). In addition, there was no positive correlation observed between weed biomass and weed numbers per plot. We observed that numerous small weeds were established across plots and biomass per plant was highly variable.

Visual ratings for weed suppression provided by canola residues on summer annual weeds was cultivar dependent (Table 4, Fig. 12). CB Taurus and GT-50 were generally weed suppressive, ranking among top four cultivars, and suppressed both witchgrass and common lamb's quarters (*Chenopodium album* L.) in 2014 and 2015. In contrast, AV Opal and ATR Bonito performed most poorly against summer weeds in 2014 and 2015, respectively.

The current study has demonstrated that canola exhibits cultivar-dependent effects on summer annual weeds in both incrop and post-harvest evaluations. The canola cultivars CB Taurus and GT-50 were consistently weed suppressive, but results were not
always significant, depending on weed evaluated and time of rating. Interestingly, ATR Bonito ranked as the most weed suppressive cultivar in 2014 but was least suppressive in 2015 at 220 DAE; the high level of weed may have influenced this and pathogen infestation observed in ATR Bonito in 2015, both potentially associated with ample rainfall.

Figure 10: CB Taurus canola plot from the side (Left) and above (right) at Wagga Wagga, NSW - July 2014.

Figure 11: GT-50 canola plot from the side (Left) and above (right) at Wagga Wagga, NSW - July 2014.
Canola is a major oilseed crop in Australia but weed infestations can greatly reduce yield and quality. Cultivars exhibiting a high level of competition with weeds offer opportunities to reduce herbicide inputs through integrated weed management (Harker et al., 2011). In addition, the prospects of herbicide resistance in weed species necessitate the search for alternative weed control options, such as canola interference through crop competition and allelopathy (Asaduzzaman et al., 2014a, 2014b). In the current study, some canola cultivars demonstrated strong weed suppressive abilities resulting in reduced weed biomass and numbers in crop and post-harvest.

Post-harvest residue performance with respect to weed suppression is also clearly influenced by weed species encountered. Hyola 50 and CB Taurus generally performed well in terms of weed suppression when compared to other cereal residues but were occasionally outperformed by other hybrid canola cultivars (Weston et al., 2014). The incorporation of white mustard or rapeseed residues into soil resulted in suppression of small-seeded weeds such as shepherd's purse (*Capsella bursa-pastoris* L.), pineapple weed (*Matricaria discoidea* DC.), and common chickweed (*Stellaria media* L.) more so than large-seeded weeds such as ladysthumb (*Persicaria maculosa* S.F.Gray.) and Pennsylvania smartweed (*Polygonum pensylvanicum* L.) (Al-Khatib et al., 1997).
Table 3: Weed counts m$^{-2}$ (mean of six replicates) in 2014 and 2015 based upon days after crop emergence

<table>
<thead>
<tr>
<th>Canola cultivar</th>
<th>2014</th>
<th>2015</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days after emergence</td>
<td>40</td>
<td>70</td>
</tr>
<tr>
<td>ATR Bonito</td>
<td>207</td>
<td>186</td>
</tr>
<tr>
<td>AV Opal</td>
<td>214</td>
<td>156</td>
</tr>
<tr>
<td>Barossa</td>
<td>178</td>
<td>157</td>
</tr>
<tr>
<td>CB Taurus</td>
<td>208</td>
<td>147</td>
</tr>
<tr>
<td>GT-50</td>
<td>182</td>
<td>123</td>
</tr>
<tr>
<td>Hyola 50</td>
<td>194</td>
<td>141</td>
</tr>
<tr>
<td>Hyola 600RR</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Hyola 725RT</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>LSD 0.05</td>
<td>54.0</td>
<td>48</td>
</tr>
<tr>
<td>P Value</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

The competitive ability of specific canola cultivars is likely associated with a genetic as well as an environmental component of variance, and selective breeding will be required for its maintenance or improvement, as demonstrated in cereals (Bertholdsson, 2011). Combining competitive cultivars of any species with optimal agronomic practices that facilitate crop health will generally enhance cropping system sustainability and allow growers to extend the life of their valuable herbicide tools (Harker et al., 2011). However, the feasibility of breeding for strongly competitive cultivars of canola depends on the ability of plant breeders to incorporate plant competitive traits without detracting from other desirable traits such as grain yield, quality or disease resistance (Lemerle et al., 2014). In Canada, canola competitiveness was improved by the choice of cultivar and the use of higher seeding rates (Beckie et al., 2008).

Crop competitiveness can be optimized to reduce weed growth and reproduction through farming practices that allow implementation of a variety of cultural techniques such as sowing crops with different planting dates and production timelines (Vencill et al., 2012). In addition, the ability of crops to suppress weeds appears to be strongly cultivar dependent (Bertin et al., 2003; Wu et al., 2007). However, over the past century, the competitive ability of wheat cultivars has likely been reduced as the selection is typically based on the potential to achieve high yields. Older cultivars or landraces have often been shown to be lower yielding but more competitive with weeds than the higher
yielding, semi-dwarf modern cultivars (Vandeleur and Gill, 2004; Bertholdsson et al., 2012).

Table 4: Ratings of weed suppression (0 = poor and 10 = Excellent) by cultivar stubble remaining after harvest in 2014 (100-130 days after harvest = 270 or 330 DAE) and 2015 (50-100 days after harvest or 220 or 270 DAE).

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>270 DAE</th>
<th>300 DAE</th>
<th>Cultivar</th>
<th>220 DAE</th>
<th>270 DAE</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATR Bonito</td>
<td>6.8</td>
<td>5.3</td>
<td>CB Taurus</td>
<td>8.2</td>
<td>4.5</td>
</tr>
<tr>
<td>CB Taurus</td>
<td>6.3</td>
<td>4.8</td>
<td>Hyola 600RR</td>
<td>7.0</td>
<td>3.5</td>
</tr>
<tr>
<td>GT-50</td>
<td>5.0</td>
<td>4.7</td>
<td>Hyola 50</td>
<td>6.7</td>
<td>2.8</td>
</tr>
<tr>
<td>Barossa</td>
<td>5.2</td>
<td>3.8</td>
<td>GT-50</td>
<td>6.3</td>
<td>2.8</td>
</tr>
<tr>
<td>Hyola 50</td>
<td>4.3</td>
<td>4.2</td>
<td>Hyola 725RT</td>
<td>5.5</td>
<td>2.8</td>
</tr>
<tr>
<td>AV Opal</td>
<td>4.2</td>
<td>3.8</td>
<td>AV Opal</td>
<td>5.2</td>
<td>2.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Barossa</td>
<td>4.7</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ATR Bonito</td>
<td>4.0</td>
<td>2.7</td>
</tr>
<tr>
<td>LSD 0.05</td>
<td>2.9</td>
<td>2.7</td>
<td>LSD 0.05</td>
<td>2.3</td>
<td>1.4</td>
</tr>
<tr>
<td>P value</td>
<td>NS</td>
<td>NS</td>
<td>P value</td>
<td>0.05</td>
<td>NS</td>
</tr>
</tbody>
</table>

4. Conclusions

Our results show that establishment of certain cereal crop or canola cultivars may effectively result in enhanced in-crop and post-harvest weed suppression, with or without the use of post-emergent herbicides during the growing season, especially when considering common summer weeds which are problematic postharvest, such as fleabane and witchgrass.

Cultivar and/or crop choice may thus be an economical form of weed management due to competition by the crop and possibly other factors, such as the production of allelochemicals by decomposing crop residues. As the amount of crop biomass remaining on the soil surface post-harvest in our experiments was not well correlated with weed suppression, results suggest that other factors, including the presence of allelochemicals in suppressive canola cultivars, may be associated with weed suppression post-harvest.

Further investigation is therefore required under Australian conditions to determine the mechanisms of weed suppression associated with canola and its residues, particularly the identification of allelochemical(s) associated with post-harvest weed suppression in field soils. This has not yet been achieved. Ideally, cultivars with both
allelopathic and competitive interference ability would be useful in Australian cropping systems with long periods of summer fallow.

In southern Australia, where significant seasonal and locational differences also impact performance, certain cereals and their residues, including grazing wheat and oat, as well as canola cultivars CB Taurus, GT-50 and Hyola 50, were superior in terms of weed suppression in-crop and post-harvest to barley, rye or triticale which generally produced higher crop biomass and residue loads.

In Australia, factors such as plant density and cultivar (hybrid and non-hybrid) are also important non-chemical control options as herbicide resistance increases (Lemerle et al., 2011). This suggests that the choice of cultivar, combined with optimal agronomic practices, will play an important role in optimising sustainable canola cropping systems for Australia. However, the potential ability of certain wheat and canola cultivars to interfere with weed establishment and growth while maintaining grain yield and quality in the presence of weeds should be further investigated, in larger field trials under variable environments.

5. References


Chapter 3

Evaluation of Selected Commercial Canola Cultivars for Early Vigour, Weed Suppression and Yield in Southern New South Wales

Building on the previous study (Chapter 2) the objective of this study was to further investigate on the diversity in the competitive ability for weed suppression and yield tolerance to weed infestation by genetically diverse commercial Australian canola (referred to as canola in this chapter) cultivars, with a clear emphasis on assessment and impact of plant growth, including early vigour and crop canopy architectural traits. In addition, a comparison in the competitive ability of canola cultivars in the presence and absence of pre-emergent herbicides under natural weed infestations in two locations typically receiving low to moderate rainfall was made.

All the authors contributed to writing and editing the manuscript. In addition, Dr. Brown was involved in establishing and monitoring the canola crop while Dr. P. Weston provided support in the statistical analysis of the data. Prof. Bagherieh-Najjar provided support with using R to perform PLS analysis and modelling. Other contributors to this study are included in the acknowledgement.

This manuscript has been submitted to Weed Research, and it is currently under review.

Evaluation of selected commercial canola cultivars for early vigour, weed suppression and yield in southern New South Wales


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Abstract

The potential of canola cultivars to suppress weed growth while maintaining optimal grain yield and quality is not well understood under field conditions. This study examined a diverse range of commercial canola cultivars for their inherent ability to suppress weeds and exhibit yield tolerance of weed competition with and without pre-emergent herbicide treatment. Canola competitive ability was compared over two growing seasons in both Condobolin and Wagga Wagga, New South Wales in both herbicide-treated and untreated plots, with natural weed infestations closely simulating commercial production practices. Cultivar differences were observed in canola canopy architecture and yield; however, early season biomass, light interception, leaf area index and visual vigour ratings exhibited both year and location interactions. Cultivars with the highest biomass, light interception, leaf area indices, and visual vigour were also the most weed suppressive. Although crop and weed biomass accumulation differed significantly among cultivars for both location and year, weed biomass was inversely related to cultivar biomass in both locations. Hybrid Hyola and GT-50 cultivars exhibited up to 50% less weed biomass in contrast to the other cultivars. Pre-emergent herbicide applications contributed to higher crop yield in both locations and years. Given the consistent aboveground competitive ability of certain canola cultivars, our findings suggest that cultivar, environment and seasonal effects may significantly contribute to cultivar biomass accumulation and reduced weed biomass. Cultivar selection may also allow for improved management of the weed seedbank with strategic use of both selective and non-selective herbicides.

Keywords: Brassica spp; herbicide; weed-suppressive; photosynthetically active radiation; crop residue
1. Introduction

*Brassica* crops are currently produced worldwide for oil, food and feed purposes, and represent significant economic value due to their nutritional, medicinal, bioindustrial, biocontrol and crop rotational properties (Ahuja et al. 2010). In Australia canola (*Brassica napus* L.) is a dominant broadleaf break crop in the profitable and sustainable cereal cropping system (Seymour et al. 2012). A diverse selection of canola cultivars is commercially available, including those resistant to herbicides in both open-pollinated and hybrid cultivars to accommodate the needs of growers across diverse rainfall zones (Kirkegaard et al. 2012; Zhang et al. 2016).

Herbicide tolerance is an important feature of canola germplasm in Australia, with five herbicide tolerance systems to choose from: conventional (CV) – no extra herbicide tolerance; triazine tolerant (TT) – tolerant to selected triazine herbicides such as simazine and atrazine; Clearfield® (CL) – tolerant to imidazolinones, which are effective in controlling many broadleaf and grass weeds in canola; Roundup Ready® (RR) – tolerant to glyphosate; and combined triazine and glyphosate tolerant (Dual-tolerant - RT®) (Kirkegaard et al. 2012; Zhang et al. 2016; Preston et al. 2016). However, each of these systems has weaknesses in weed management as observed with respect to the management of annual ryegrass.

In recent reports from Australia as well as North America, hybrid canola cultivars generally produced higher yields of better quality oils, with significant variation in grain yield observed between weedy and weed-free plots, depending on crop cultivar, weed infestation and crop phenology (Beckie, 2007; Lemerle et al. 2014; Preston et al. 2016). In Australia, canola cultivar has been found to be a critical factor in determining yield potential. Significant differences in yield responsiveness between hybrid and open-pollinated canola cultivars have been noted, the magnitude of which was moderated by seasonal rainfall or soil moisture availability. Hybrid cultivars typically out-yielded open-pollinated canola in favourable environments where rainfall was less limiting and the growing season was longer (Kirkegaard et al. 2012; Zhang et al. 2016).

In South Australia, cultivar-dependent differences in crop vigour and biomass production have been found, with the open-pollinated varieties ATR Stingray less competitive and ATR Bonito moderately competitive relative to the highly competitive hybrid variety Hyola 559TT (Kleemann et al. 2017). In Canada, analysis of replacement series and derivation of relative crowding coefficients, based on shoot dry weight or leaf
area, indicated that hybrid canola cultivars were approximately twice as competitive as open-pollinated cultivars when weed interference was relatively high (Zand and Beckie, 2002). Based on these studies, in moderate to high rainfall zones, hybrid canola cultivars appear to be most suitable for both crop competition against weeds and rotations in integrated weed management (IWM) programs.

In addition to economic considerations around cost of establishment and yield potential of hybrid cultivars, increasing incidence of herbicide resistance in weeds and perceived effects of pesticides on the environment and human health have led producers in Canada and the EU to consider a more integrated approach to weed management to reduce their heavy dependence on herbicides (Beckie, 2007; Zare et al. 2012). IWM, which involves greater reliance on non-chemical weed management tactics such as crop interference, is therefore worthy of greater consideration in other areas of global importance for canola production.

One IWM approach under consideration is the selection and breeding of crop cultivars possessing traits associated with weed suppression (Zare et al. 2012). However, canola cultivars, in contrast to wheat or other commercially available cereals, vary substantially in their ability to interfere with weed growth and establishment. Numerous morphological, physiological and metabolic plant traits influence the ability of canola to secure resources (light, water, nutrients, space) from unrelated but competing neighbours (inter-specific competition) or from other canola plants (intra-specific competition) (Asaduzzaman et al. 2014a; Lemerle et al. 2017).

Canola crop interference with weed establishment is typically influenced by competition for resources as well as allelopathic tendencies in the crop, both of which favour growth (Asaduzzaman et al. 2014a) and performance of the crop. In Canada, newly released canola hybrids evaluated under field conditions have exhibited greater competitiveness for resources than open-pollinated cultivars (Harker et al. 2011). In Australia, Preston et al. (2016) reported that switching from an open-pollinated cultivar to a hybrid reduced annual ryegrass seed set by up to 50%. Also, Lemerle et al. (2014) reported hybrids to be more competitive and higher yielding. Therefore, the incorporation of hybrid and high vigour canola cultivars in broadacre rotations offers a simple opportunity to reduce annual ryegrass seed set by providing extra competition early in the season (Preston et al. 2016) and thus may allow for an overall reduction in herbicide usage.
Cultural practices, including crop density, arrangement, planting date and choice of cultivar, also impact the crop’s ability to compete with weeds (Mohler, 2001). In addition, rotational crop choice, intercropping, seeding rate, row spacing, and fertiliser placement can influence the competitiveness of the crop or the weed, or both (Swanton et al. 2015). Maintaining optimal establishment by use of competitive canola cultivars is thus critical to minimise long-term weed seedbank replenishment and subsequent yield loss due to weed infestation (Lemerle et al. 2016).

A key aspect of effective IWM programs is that they are employed across years incorporating a range of chemical and non-chemical controls (Kleemann et al. 2016). Therefore, information on the factors influencing competitive ability and reliability of canola cultivars for effective weed suppression and their interaction with herbicide programs is critical for the adoption of competitive cultivars as part of an IWM system. Previous field studies conducted in the Riverina region of NSW by Lemerle et al. (2010; 2011; 2014; 2016) and Asaduzzaman et al. (2014a-c) focused on weed suppression based on cultivar choice, biomass accumulation through variable planting density, and competitiveness against Lolium rigidum L. (ryegrass) in particular as well as volunteer wheat (Triticum aestivum L.).

This multi-year study was therefore designed as a logical next step to further examine diversity in competitive ability for weed suppression and yield tolerance to weed infestation by genetically diverse commercial Australian cultivars, with an emphasis on assessment and impact of plant growth, including assessment of early vigour and crop canopy architectural traits over time. We also sought to compare the competitive ability of canola cultivars in the presence and absence of pre-emergent herbicides under natural weed infestations in two locations typically receiving low to moderate rainfall, Condobolin and Wagga Wagga, New South Wales (NSW).

2. Materials and Methods

Replicated field trials were established at the Graham Centre Field Site (Wagga Wagga, NSW) and NSW Department of Primary Industries Agricultural Research and Advisory Station (Condobolin, NSW) in 2014, 2015 and 2016. The two locations occur in the moderate and low rainfall zones respectively, in New South Wales (NSW), with Wagga Wagga receiving 572 mm rainfall and Condobolin 440 mm annually. At Wagga
Wagga, field trials were conducted on fine red clay loam sodosols, surface pH 6.4, which were previously planted commercially for the production of cereals, canola and/or lucerne (*Medicago sativa* L.). At Condobolin, soils were predominantly red gradational, and red-brown earth sodosols with surface pH 7.0 and were previously rotated among cereals and pasture legume crops. Both soils exhibited low inherent fertility and organic matter content and were maintained using standard commercial practices to reduce weed populations.

Trials were sown in both field sites in close proximity to the previous trial sites following cereal rotations on April 25, May 2 and May 14 at Wagga Wagga and May 6, May 15 and May 17 at Condobolin in 2014, 2015 and 2016, respectively. Sowing rates ranged from 3.0 to 4.0 kg seed ha\(^{-1}\) based on seed weight to achieve a target population of 45 plants m\(^{-2}\) in each plot. Standard sowing practices were used in all years, including knifepoint and press wheel planter, and seed treatment with flutriafol fungicide at 150 g 100 kg\(^{-1}\) of fertiliser (*Jubilee*, 500 g a.i L\(^{-1}\), flutriafol SC, Adama Essentials) impregnated onto 70 kg ha\(^{-1}\) MAP (Incitec Pivot Fertilisers). Individual plots measured 2 × 12 m and were planted using a calibrated cone seeder with a 22 cm row spacing.

In all years, residual crop stubble was burnt before planting. Insecticide application of chlorpyrifos at 1 kg ha\(^{-1}\) (*Lorsban* 500 EC, chlorpyrifos at 500 g a.i L\(^{-1}\), EC, Dow AgroSciences) and bifenthrin at 37.5 g ha\(^{-1}\) (*Talstar* 250 EC, bifenthrin at 250 g a.i L\(^{-1}\), EC, FMC Australasia) were made as needed on all plots at appropriate timing to reduce pest populations. Plots were top dressed with 50 and 70 kg ha\(^{-1}\) N urea (46-0-0) at the appropriate timing at both locations in 2015 and 2016, respectively.

### 2.1. 2014 trial

As commercial canola cultivars are frequently updated, the cultivars selected for field trials included several older cultivars known for their weed-suppressive traits as well as several newer hybrids and open-pollinated cultivars that were commercially available in Australia at the time (Table 1). In 2014, trials were established without herbicides to evaluate the inherent ability of eight canola cultivars to establish and perform in the presence of natural weed populations. A randomised complete block design with six replicates was used.
Aboveground crop biomass, early vigour, total weed numbers and weed biomass were measured in two 50 × 50 cm quadrats per plot at four critical growth stages: early growth (30-40 days following establishment), vegetative growth (60-70 days following establishment), flowering and crop harvest. Biomass was obtained by removing plant material at the soil surface and weighing it after drying at 40 °C for 5 days in a forced air oven. Vigour of the crop was estimated visually on a rating scale from 0 (= poor) to 10 (= excellent). Weed suppression was also visually estimated at 60-70 days after harvest using a scale of 0 (= no suppression) to 10 (= 100% suppression). All results are presented as days after crop emergence (DAE). Weed counts were monitored in-crop and after harvest by counting identified weeds in two 50 × 50 cm quadrats per plot.

2.2. 2015-2016 Trial

In 2015 and 2016, a split-plot experimental design was employed, with trifluralin herbicide (Tri) (+ or -) as the whole plot and cultivar as the sub-plot. TriflurX (Trifluralin 480 EC, 480 g a.i L⁻¹ trifluralin EC, Nufarm) was applied and incorporated lightly immediately before sowing at 960 g ha⁻¹ in all treated main plots. Five cultivars from the 2014 trials and two additional hybrid cultivars (Hyola 600RR, Hyola 725 RT) and open-pollinated (ATR Bonito) representing the latest herbicide-tolerant systems technology, were evaluated (Table 1). Canola stand, crop and weed biomass, and weed count were measured as described above. The normalized difference vegetation index (NDVI), photosynthetically active radiation (PAR) light interception, leaf area index, and yield were also measured. Leaf area index and light interception (%) at the base of the crop canopy was measured as the absorption of PAR using a light ceptometer (AccuPAR LP-80 Ceptometer, Decagon Devices®), while NDVI crop readings were performed using a handheld sensor (GreenSeeker 505®, Trimble® NTech Industries Inc.). Grain was harvested at crop maturity using a small plot harvester.
2.3. Data analysis

Trial design, randomisation and initial data analyses were performed using Agricultural Research Manager (ARM) version 9.0 (Gylling Data Management Inc., 2014). Comprehensive statistical analysis of selected data sets was performed using GenStat (VSN International, 2018) for ANOVA with means separated using LSD (0.05 confidence level). Data were transformed using the square-root \((x+c)^{0.5}\) function to correct for homogeneity of variances and normality before analysis as required. Partial least square (PLS) regression analysis was performed using R Studio (R Core Team 2017).

Partial least squares (PLS) regression (Abdi, 2003; Kvalheim, 2010) was performed to attempt to develop a predictive linear model for weed suppression by canola cultivars based on weed dry biomass as the response variable and selected crop canopy traits as the predictive variables. Numerous crop or canopy traits were used as predictors in the model to visualise the relationships between the dependent variable (dry weed biomass) and the most influential predictive variables among crop traits including crop biomass, PAR light interception, leaf area index, visual vigour ratings and NDVI taken at different crop growth stages.
Table 1. Canola cultivars and commercial use for field trials performed in Condobolin and Wagga Wagga, NSW in 2015 and 2016.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Type</th>
<th>Year in trial</th>
<th>Commercial use</th>
<th>Growth characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2014</td>
<td>2015-16</td>
<td></td>
</tr>
<tr>
<td><strong>Stego</strong></td>
<td>Hybrid/ forage brassica</td>
<td>x</td>
<td>grazing</td>
<td>Late maturing. Medium to high rainfall areas 500 -700mm. Can be grazed multiple times.</td>
</tr>
<tr>
<td><strong>Pioneer 46C76</strong></td>
<td>OP^[1] / conventional</td>
<td>x</td>
<td>grain</td>
<td>Mid to late season maturing, open-pollinated cultivar. Tall height and adapted for medium to high rainfall areas</td>
</tr>
<tr>
<td><strong>Hyola 971CL</strong></td>
<td>Hybrid/ Clearfield</td>
<td>x</td>
<td>grain/grazing</td>
<td>Late maturing, winter graze and grain hybrid. Good grain yield and very high in biomass for very high rainfall zones.</td>
</tr>
<tr>
<td><strong>Hyola 50</strong></td>
<td>Hybrid/ conventional</td>
<td>x</td>
<td>x</td>
<td>grain</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Mid to mid-early maturing hybrid. Widely adapted for medium rainfall areas but currently replaced by newer cultivars. Has excellent vigour</td>
</tr>
<tr>
<td><strong>CB Taurus</strong></td>
<td>Hybrid/ conventional</td>
<td>x</td>
<td>x</td>
<td>grain/grazing</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Late maturing cultivar with vernalisation requirement for flowering, generally performs well with high winter rainfall. Excellent early vigour and growth.</td>
</tr>
<tr>
<td>Variety</td>
<td>Type</td>
<td>HT</td>
<td>ST</td>
<td>Crop</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------------------------</td>
<td>----</td>
<td>----</td>
<td>-----------</td>
</tr>
<tr>
<td><strong>GT-50</strong></td>
<td>Hybrid/ Roundup Ready (RR®)</td>
<td>x</td>
<td>x</td>
<td>grain</td>
</tr>
<tr>
<td><strong>AV Opal</strong></td>
<td>OP/ conventional</td>
<td>x</td>
<td>x</td>
<td>grain</td>
</tr>
<tr>
<td><strong>Barossa</strong></td>
<td>Hybrid/ conventional</td>
<td>x</td>
<td>x</td>
<td>grain</td>
</tr>
<tr>
<td><strong>CATR Bonito</strong></td>
<td>OP/ Triazine tolerant (TT®)</td>
<td>x</td>
<td></td>
<td>grain</td>
</tr>
<tr>
<td><strong>Hyola 600RR</strong></td>
<td>Hybrid/ Roundup Ready (RR®)</td>
<td>x</td>
<td></td>
<td>grain</td>
</tr>
<tr>
<td><strong>Hyola 725RT</strong></td>
<td>Hybrid/ Dual herbicide tolerant (RT®).</td>
<td>x</td>
<td></td>
<td>grain</td>
</tr>
</tbody>
</table>

^OP = Open pollinated, ^GT = Glyphosate tolerant, ^ATR = Atrazine resistant, ^RT= Roundup Ready and Atrazine tolerant
3. Results

Monthly rainfall received during the growing season is reported in Table 2 for both locations from 2014 to 2016. Given the variable rainfall conditions across years, canola cultivars also exhibited variable performance under the less than optimal (2014), optimal (2015) and higher than optimal (2016) rainfall conditions experienced. With the above average in-crop rainfall in 2016, there was greater weed establishment and pressure as assessed by weed counts and higher total weed biomass in 2016 at both sites. At Condobolin, *Crassula* spp. L. (stocercrop) was the dominant weed, consistent with results of a field survey performed by Lemerle et al. (1996). At the Wagga Wagga site, *Fumaria* spp. L. (fumitory), *Hyacinthoides non-scripta* (L.) Chouard ex Rothm. (bluebell), *Papaver* spp. L. (poppy) and *Hordeum murinum* L. (barleygrass) were the dominant weeds, all of which are commonly encountered in the mixed cropping zone of the Riverina region in south-eastern Australia (Broster et al. 2012).

Table 2. The distribution of rainfall (mm) by month and the total in-crop rainfall for the Wagga Wagga and Condobolin Canola field trial sites in 2014, 2015 and 2016.

<table>
<thead>
<tr>
<th></th>
<th>Jan</th>
<th>Feb</th>
<th>Mar</th>
<th>Apr</th>
<th>May</th>
<th>Jun</th>
<th>Jul</th>
<th>Aug</th>
<th>Sep</th>
<th>Oct</th>
<th>Nov</th>
<th>Dec</th>
<th>In-crop</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Wagga</strong></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td><strong>Wagga</strong></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2014</td>
<td>13.2</td>
<td>27.2</td>
<td>63.4</td>
<td>56.6</td>
<td>47.0</td>
<td>81.0</td>
<td>24.2</td>
<td>10.6</td>
<td>36.6</td>
<td>22.0</td>
<td>47.0</td>
<td>29.0</td>
<td>325.0</td>
</tr>
<tr>
<td>2015</td>
<td>89.0</td>
<td>41.2</td>
<td>2.0</td>
<td>56.4</td>
<td>18.8</td>
<td>66.0</td>
<td>60.0</td>
<td>98.8</td>
<td>22.7</td>
<td>10.0</td>
<td>92.2</td>
<td>30.2</td>
<td>424.9</td>
</tr>
<tr>
<td>2016</td>
<td>58.6</td>
<td>20.0</td>
<td>42.6</td>
<td>10.8</td>
<td>102.1</td>
<td>99.7</td>
<td>86.8</td>
<td>68.1</td>
<td>178.0</td>
<td>79.1</td>
<td>28.0</td>
<td>53.5</td>
<td>652.6</td>
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<tr>
<td><strong>Long-term average</strong></td>
<td>37.8</td>
<td>36.9</td>
<td>37.6</td>
<td>38.5</td>
<td>43.9</td>
<td>50.8</td>
<td>49.4</td>
<td>48.2</td>
<td>48.8</td>
<td>51.4</td>
<td>41.1</td>
<td>41.2</td>
<td>372.1</td>
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</table>

<table>
<thead>
<tr>
<th></th>
<th>Jan</th>
<th>Feb</th>
<th>Mar</th>
<th>Apr</th>
<th>May</th>
<th>Jun</th>
<th>Jul</th>
<th>Aug</th>
<th>Sep</th>
<th>Oct</th>
<th>Nov</th>
<th>Dec</th>
<th>In-crop</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Condobolin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td><strong>Condobolin</strong></td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2014</td>
<td>35.2</td>
<td>46.7</td>
<td>104.5</td>
<td>28</td>
<td>27.6</td>
<td>57.4</td>
<td>9.2</td>
<td>22.2</td>
<td>11</td>
<td>11.5</td>
<td>17.7</td>
<td>88.6</td>
<td>184.6</td>
</tr>
<tr>
<td>2015</td>
<td>59.2</td>
<td>35.9</td>
<td>0.2</td>
<td>64.7</td>
<td>11.6</td>
<td>31.8</td>
<td>41.2</td>
<td>42.3</td>
<td>6.8</td>
<td>65.2</td>
<td>67.3</td>
<td>28.5</td>
<td>330.9</td>
</tr>
<tr>
<td>2016</td>
<td>80.9</td>
<td>0</td>
<td>21.8</td>
<td>21.4</td>
<td>60.2</td>
<td>161.5</td>
<td>37.1</td>
<td>43.4</td>
<td>143.2</td>
<td>31.6</td>
<td>40.8</td>
<td>56.7</td>
<td>539.2</td>
</tr>
<tr>
<td><strong>Long-term average</strong></td>
<td>47.2</td>
<td>43.4</td>
<td>41.4</td>
<td>30.8</td>
<td>35.5</td>
<td>33.3</td>
<td>35.7</td>
<td>33.8</td>
<td>32.4</td>
<td>47.5</td>
<td>39.7</td>
<td>41.9</td>
<td>288.4</td>
</tr>
</tbody>
</table>
3.1. 2014 trial

Cultivars differed significantly in early (vegetative) and final (at maturity) \( P < 0.01 \) biomass production at both sites (Table 3) with differences associated with site \( P < 0.001 \) and cultivar by site interactions \( P < 0.01 \). However, only at Condobolin were cultivar differences in biomass at the flowering stage noted \( P < 0.05 \) with 46C76 and 971CL producing lower biomass compared to Hyola 50, Stego and Barossa. At early vegetative growth stage non-hybrid commercial cultivars (e.g. 46C76 and Barossa) produced lower biomass compared to GT-50, Hyola 50 and Stego at Wagga Wagga while at Condobolin 46C76 and CB Taurus produced lower biomass compared to Hyola 50.

Based on visual vigour ratings, early growth and growth at flowering trends among cultivars were different, with significant site by cultivar interaction \( P < 0.001 \). At flowering stage GT-50, Stego and Hyola 50 typically exhibited strong early vigour by visual assessment in both locations, which roughly correlated with early biomass assessment (Table 3). In addition to these cultivars, 971CL had strong early stage vigour especially at Wagga Wagga. Early cultivar biomass accumulation was strongly correlated with reduced weed biomass \( (r^2 = 0.68, P < 0.001) \) at Wagga Wagga with GT-50 and Stego being most weed suppressive (Fig. 1). In general, weeds did not accumulate adequate biomass for assessment during the growing season in Condobolin.

Weed counts were not significantly different among cultivars and sites except at Condobolin at crop maturity \( (P < 0.001) \). Weed counts obtained in Condobolin at this time consisted primarily of many small stonecrop seedlings that established before crop canopy closure, after which weed growth was greatly reduced. CB Taurus had the highest weed count among all cultivars.
The relationship between canola cultivar early biomass (g m$^{-2}$) taken at 70 DAE and weed biomass (g m$^{-2}$) at Wagga Wagga in 2014 (Condobolin data not presented). Each data point represents the mean of six replicates. The dotted line shows the fitted regression.

\[
y = -1.7028x + 573.72
\]

\[r^2 = 0.68\]
Table 3. Average canola dry biomass (g m⁻²), visual vigour rating (0 = poor, 10 = excellent) and weed count (plants m⁻²) taken at different crop growth stages (early, flowering, maturity) at Condobolin and Wagga Wagga sites in 2014.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Condobolin</th>
<th></th>
<th></th>
<th>Wagga Wagga</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Crop biomass</td>
<td>Vigour</td>
<td>Weed count</td>
<td>Crop biomass</td>
<td>Vigour</td>
<td>Weed count</td>
</tr>
<tr>
<td></td>
<td>E  F  M</td>
<td>E  F  M</td>
<td>E  F  M</td>
<td>E  F  M</td>
<td>E  F  M</td>
<td>E  F  M</td>
</tr>
<tr>
<td>46C76</td>
<td>291 595 616</td>
<td>4.3 6.3</td>
<td>21 27 0</td>
<td>198 371 1155</td>
<td>4.8 4.2</td>
<td>207 186 55</td>
</tr>
<tr>
<td>971CL</td>
<td>323 656 590</td>
<td>5.0 6.7</td>
<td>25 36 0</td>
<td>270 361 826</td>
<td>7.0 4.6</td>
<td>181 179 53</td>
</tr>
<tr>
<td>AV Opal</td>
<td>361 697 620</td>
<td>4.8 7.0</td>
<td>14 25 0</td>
<td>247 385 978</td>
<td>5.5 4.6</td>
<td>214 156 74</td>
</tr>
<tr>
<td>Barossa</td>
<td>283 922 590</td>
<td>3.3 6.1</td>
<td>19 21 0</td>
<td>156 466 1119</td>
<td>4.5 3.6</td>
<td>178 157 51</td>
</tr>
<tr>
<td>CB Taurus</td>
<td>146 788 443</td>
<td>2.2 3.2</td>
<td>15 23 37</td>
<td>227 497 879</td>
<td>4.8 3.5</td>
<td>208 147 59</td>
</tr>
<tr>
<td>GT-50</td>
<td>373 759 693</td>
<td>6.3 8.9</td>
<td>38 19 0</td>
<td>292 557 1298</td>
<td>7.0 5.8</td>
<td>182 123 43</td>
</tr>
<tr>
<td>Hyola 50</td>
<td>425 878 474</td>
<td>5.5 8.0</td>
<td>22 15 0</td>
<td>250 531 1253</td>
<td>6.7 6.3</td>
<td>194 141 63</td>
</tr>
<tr>
<td>Stego</td>
<td>370 893 579</td>
<td>5.3 7.9</td>
<td>24 17 1</td>
<td>269 614 901</td>
<td>7.7 5.7</td>
<td>238 178 73</td>
</tr>
<tr>
<td>LSD</td>
<td>101 202 136</td>
<td>0.8 0.4</td>
<td>22.0 24.3 12.3</td>
<td>70.6 296.3 170</td>
<td>1.5 1.0</td>
<td>53.6 47.6 35.9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>P value^A</th>
<th>Condobolin</th>
<th></th>
<th></th>
<th>Wagga Wagga</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NS  NS  ***</td>
<td>NS  NS  ***</td>
<td></td>
<td>NS  NS  ***</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

^A P value *** = P < 0.001, ** = P < 0.01 * = P < 0.05, NS = not significantly different, E = vegetative, F = Flowering, M = maturity
Table 4: Comparisons between canola cultivar average crop and weed dry biomass (g m\(^{-2}\)) taken at early and flowering crop growth stages at Condobolin and Wagga Wagga field trial sites in 2015 and 2016 seasons. All means presented in this table are an average of six replicates.

<table>
<thead>
<tr>
<th>Year-2015</th>
<th>Condobolin</th>
<th>Wagga Wagga</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cultivar</strong></td>
<td><strong>Crop/E(^a)</strong></td>
<td><strong>Crop/F</strong></td>
</tr>
<tr>
<td>ATR Bonito</td>
<td>62.0</td>
<td>290</td>
</tr>
<tr>
<td>AV Opal</td>
<td>71.6</td>
<td>299</td>
</tr>
<tr>
<td>Barossa</td>
<td>51.8</td>
<td>330</td>
</tr>
<tr>
<td>CB Taurus</td>
<td>13.8</td>
<td>299</td>
</tr>
<tr>
<td>GT-50</td>
<td>89.8</td>
<td>333</td>
</tr>
<tr>
<td>Hyola 50</td>
<td>64.4</td>
<td>254</td>
</tr>
<tr>
<td>Hyola 600RR</td>
<td>66.9</td>
<td>339</td>
</tr>
<tr>
<td>Hyola 725RT</td>
<td>45.1</td>
<td>314</td>
</tr>
<tr>
<td><strong>LSD</strong></td>
<td>20.2</td>
<td>116.9</td>
</tr>
<tr>
<td><strong>P value(^b)</strong></td>
<td>***</td>
<td>NS</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Year-2016</th>
<th>Condobolin</th>
<th>Wagga Wagga</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cultivar</strong></td>
<td><strong>Crop/E(^a)</strong></td>
<td><strong>Crop/F</strong></td>
</tr>
<tr>
<td>ATR Bonito</td>
<td>92.7</td>
<td>598.5</td>
</tr>
<tr>
<td>AV Opal</td>
<td>45.0</td>
<td>600.9</td>
</tr>
<tr>
<td>Barossa</td>
<td>69.2</td>
<td>602.0</td>
</tr>
<tr>
<td>CB Taurus</td>
<td>68.4</td>
<td>570.6</td>
</tr>
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<td>GT-50</td>
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<td>827.6</td>
</tr>
<tr>
<td>Hyola 50</td>
<td>66.3</td>
<td>995.1</td>
</tr>
<tr>
<td>Hyola 600RR</td>
<td>77.7</td>
<td>1027.5</td>
</tr>
<tr>
<td>Hyola 725RT</td>
<td>76.6</td>
<td>817.4</td>
</tr>
<tr>
<td><strong>LSD</strong></td>
<td>29.2</td>
<td>237.2</td>
</tr>
<tr>
<td><strong>P value(^b)</strong></td>
<td>**</td>
<td>***</td>
</tr>
</tbody>
</table>

\(^a\)Crop growth stages: E = early, vegetative, F = flowering

\(^b\)P values: *** = P < 0.001, ** = P < 0.01 * = P < 0.05, NS = not significantly different
3.2. 2015 and 2016 field trials

3.2.1. Cultivar and weed dry matter (biomass)

Cultivar influenced both crop and weed biomass accumulation in both years \((P < 0.001)\) and locations \((P < 0.001)\) (Table 4, Fig. 2). In 2015, AV Opal and Hyola 600RR had higher early above-ground biomass production at Wagga Wagga while AV Opal and GT-50 at Condobolin. Conversely, at flowering Hyola 600RR and Hyola 50 exhibited greatest above-ground biomass accumulation in both locations in 2016 while CB Taurus and Barossa produced the least.

As in 2014, weed biomass was inversely related to cultivar biomass accumulation at both sites, with a coefficient of determination \((r^2)\) of 0.52 \((P < 0.001, \text{Fig. 2})\) at Condobolin and 0.44 \((P < 0.001\) at Wagga Wagga in 2015. In 2016 the above average rainfall resulted in greater weed establishment and better crop growth, particularly in Wagga Wagga. The coefficient of determination was higher at Wagga Wagga \((r^2 = 0.66, P < 0.001)\) and lower at Condobolin \((r^2 = 0.24, P < 0.01, \text{Fig. 3})\). In this two-year field trial, Hyola 600RR and Hyola 50 were consistently the most weed suppressive canola cultivars in both locations and GT-50 at Condonolin only, reducing weed infestation by 20-50\% while AV Opal was among the most weed suppressive in 2015 only at both locations. CB Taurus and Hyola 725RT occasionally performed as well in weed suppression depending on the year and location (Table 4).
Figure 2: The relationship between mean canola cultivar early biomass at 70 DAE and weed biomass at flowering (150 DAE) (g m$^{-2}$) at Condobolin and Wagga Wagga in 2015. Each data point represents the mean of six replicates. The linear lines show the fitted regression.
3.2.2. Modelling weed suppression using canola canopy traits

Cultivar differences in early crop vigour, PAR light interception, leaf area index and normalised difference vegetation index ($P < 0.001$; Table 5) were observed, with both year and location interactions highly significant ($P < 0.001$). Ranking of cultivars based on each of these traits suggested that the cultivars with the highest biomass, light interception as measured by PAR, leaf area and early vigour were also clearly the most weed suppressive (see also Fig. 2, Table 3 and 4).

When interpreting the models, the angle between the predictors and the dependent variable is an approximation of the correlation between the variables (Fig 4). A small angle indicates the variables are positively correlated, an angle of $\sim 90^\circ$ indicates the
variables are not correlated, and an angle close to 180° indicates the variables are negatively correlated (Abdi, 2003; Kvalheim, 2010).

In 2015 the model prediction differed in accordance with crop growth stage. In Wagga Wagga at early crop growth (70 DAE), visual vigour at 20 and 50 DAE were negatively correlated ($r^2 = 0.29$) with weed dry biomass according to PLS regression (Fig. 4A). In addition, plant count, NDVI and dry crop biomass indicate an angle closer to 90° suggesting that these predictors are uncorrelated with weed biomass (Abdi 2003; Kvalheim 2010). At flowering stage (115 DAE) in Condobolin, NDVI, early crop biomass, crop height, crop plant counts, and early crop vigour were all negatively correlated with weed biomass with a model fit of $r^2 = 0.34$ (Fig. 4B).

In contrast, in 2016 in exceptionally wet soil conditions at Wagga Wagga PAR light interception and LAI (70 DAE) were highly and positively correlated with dry weed biomass at the crop vegetative growth stage (Fig. 4C). LAI and PAR light interception at 50 DAE were also positively correlated with weed biomass. In Condobolin at 160 DAE (crop maturity), the model had a fit of $r^2 = 0.56$ with only NDVI (115 DAE) being negatively correlated with weed biomass. However, crop plant count, crop vigour (40 DAE) and NDVI (60 DAE) were highly positively correlated with weed biomass, and the remaining predictors indicated positive correlation with weed biomass (Fig. 4D).

In addition, Figure 5 shows strongly positive correlations between cultivar dry biomass at 120 DAE and photosynthetically active radiation interception at 100 DAE at Wagga Wagga ($r^2 = 0.91$, $P < 0.001$) and Condobolin ($r^2 = 0.54$, $P < 0.001$). Based on the ranking in the current study, the cultivars that accumulated higher early biomass also had higher PAR light interception ability, greater leaf area index and greater early growth vigour (Table 5) especially at Wagga Wagga. Overall, the PLS model analysis demonstrates that weed biomass was inversely related to crop biomass in 2015 at both locations, but this relationship did not hold in 2016, a year of high rainfall at both locations.
Notes; wbio 70 = weed biomass at 70 DAE, wbio 115 = weed biomass at 115 DAE, wbio 160 = weed biomass at 160 DAE

Figure 4: Correlation circle of partial least squares (PLS) regression, illustrating the correlations of the dependent variable (dry weed biomass) predicted by several independent variable crop canopy traits at Wagga Wagga (A, C) and Condobolin (B, D) taken at 70, 115 and 160 DAE respectively in 2015 and 2016. The independent predictors include (A) X1 = plant count, X2 = vigour 20 DAE, X3 = vigour 50 DAE, X4 = NDVI 60 DAE, X5 = crop dry biomass 70 DAE (B) X1 = plant count, X2 = vigour 40 DAE, X3 = NDVI 60 DAE, X4 = crop dry biomass 70 DAE, X5 = crop height 70 DAE X6 = Leaf area index 115 DAE, X7 = PAR light interception % 115 DAE, X8 = crop dry biomass 115 DAE. (C) X1 = crop dry matter 70 DAE, X2 = plant count, X3 = vigour 50 DAE, X4 = PAR light interception % 50 DAE, X5 = Leaf area index 50 DAE, X6 = Leaf area index 70 DAE, X7 = PAR light interception % 70 DAE, X8 = NDVI 60 DAE. (D) X1 = plant count, X2 = vigour 40 DAE, X3 = NDVI 60 DAE, X4 = crop dry biomass 70 DAE, X5 = crop height 70 DAE, X6 = Leaf area index 115 DAE, X7 = PAR light interception % 115 DAE, X8 = crop dry biomass 115 DAE, X9 = vigour 60 DAE, X10 = crop height 115 DAE and X11 = NDVI 115 DAE.
Figure 5: The positive correlations between mean canola cultivar dry biomass (g m\(^{-2}\)) at 120 DAE and photosynthetically active radiation (PAR) interception (%) at 100 DAE at A) Wagga Wagga \(r^2 = 0.91\) \(P < 0.001\) and B) Condobolin \(r^2 = 0.54\), \(P < 0.001\). Each data point represents the mean of six replicates. The dotted line shows the fitted regression.

For Wagga Wagga:
\[
y = 14.498x - 323.57
\]
\[
r^2 = 0.9107
\]

For Condobolin:
\[
y = 5.4621x + 318.28
\]
\[
r^2 = 0.5374
\]
Table 5. Canola cultivar differences in visual vigour ratings, leaf area index (LAI), PAR light interception (%), and normalised difference vegetation index (NDVI) at Condobolin and Wagga Wagga in 2015 and 2016.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Early crop vigour rating</th>
<th>Early leaf area index (LAI)</th>
<th>PAR Light interception</th>
<th>Early crop NDVI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Condobolin</td>
<td>Wagga Wagga</td>
<td>Condobolin</td>
<td>Wagga Wagga</td>
</tr>
<tr>
<td>ATR Bonito</td>
<td>3.3</td>
<td>4.3</td>
<td>7.5</td>
<td>6.8</td>
</tr>
<tr>
<td>AV Opal</td>
<td>4.0</td>
<td>3.2</td>
<td>8.7</td>
<td>6.8</td>
</tr>
<tr>
<td>Barossa</td>
<td>2.8</td>
<td>3.5</td>
<td>7.0</td>
<td>5.7</td>
</tr>
<tr>
<td>CB Taurus</td>
<td>1.0</td>
<td>1.5</td>
<td>6.7</td>
<td>4.7</td>
</tr>
<tr>
<td>GT-50</td>
<td>3.3</td>
<td>5.5</td>
<td>8.5</td>
<td>8.8</td>
</tr>
<tr>
<td>Hyola 50</td>
<td>2.8</td>
<td>3.2</td>
<td>9.0</td>
<td>7.7</td>
</tr>
<tr>
<td>Hyola 600RR</td>
<td>3.2</td>
<td>5.2</td>
<td>9.3</td>
<td>8.7</td>
</tr>
<tr>
<td>Hyola 725RT</td>
<td>3.0</td>
<td>4.0</td>
<td>7.3</td>
<td>7.5</td>
</tr>
<tr>
<td>LSD</td>
<td>0.7</td>
<td></td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>P valueA</td>
<td>***</td>
<td></td>
<td>*</td>
<td></td>
</tr>
</tbody>
</table>

A P value: *** = P < 0.001, ** = P < 0.01, * = P < 0.05, NS = not significantly different. Visual ratings were taken at 60-70 days following crop establishment and were on a 0 to 10 scale where 0= poor or no vigour and 10= excellent vigour. All means presented in this table are an average of six replicates.
3.2.3. Grain yield versus weed competition

In 2015 and 2016 grain yield differed with respect to herbicide and canola cultivar at both Wagga Wagga and Condobolin ($P < 0.001$, Table 6). There was no significant herbicide treatment by cultivar interaction effect at either location or year. At both locations, yield was significantly ($P < 0.001$) higher at Condobolin and Wagga Wagga for all trifluralin-treated plots in comparison to untreated plots, by an average of 30-100%. The Round-Up Ready hybrid cultivars (GT-50, Hyola 600RR) and Hyola 50 yielded up to 40% greater yields in comparison to the Dual-tolerant cultivar (Hyola 725RT) in both herbicide-treated and untreated plots.

Significant interactions between cultivar and location ($P < 0.001$) were noted, with cultivars GT-50, Hyola 600RR and Hyola 50 generally producing somewhat higher yields at Wagga Wagga than Condobolin at in both years. This may be due to the longer growing season at Wagga Wagga and/or ability to sow the crop earlier in the season. Moreover, the interactions between location and year were significant ($P < 0.001$), with four out of eight cultivars (Control) producing higher yields at Condobolin in 2016, suggesting the impact of weeds on crop yield.

Table 6. Canola cultivar yield (t ha$^{-1}$) in-control or untreated and trifluralin treated plots at Condobolin and Wagga Wagga field trials in 2015 and 2016.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control Trifluralin</td>
<td>Control Trifluralin</td>
<td>Control Trifluralin</td>
<td>Control Trifluralin</td>
</tr>
<tr>
<td>ATR Bonito</td>
<td>0.9 1.1</td>
<td>1.6 1.7</td>
<td>1.2 1.3</td>
<td>0.8 1.6</td>
</tr>
<tr>
<td>AV Opal</td>
<td>0.9 1.2</td>
<td>1.3 1.5</td>
<td>1.3 1.6</td>
<td>0.7 1.5</td>
</tr>
<tr>
<td>Barossa</td>
<td>1.1 1.0</td>
<td>1.1 1.1</td>
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<td>0.6 1.5</td>
</tr>
<tr>
<td>CB Taurus</td>
<td>0.2 0.3</td>
<td>1.4 1.5</td>
<td>0.3 0.4</td>
<td>0.4 1.0</td>
</tr>
<tr>
<td>GT-50</td>
<td>1.4 1.6</td>
<td>1.7 1.8</td>
<td>1.7 1.9</td>
<td>1.1 2.0</td>
</tr>
<tr>
<td>Hyola 50</td>
<td>1.4 1.4</td>
<td>1.6 1.7</td>
<td>1.7 1.8</td>
<td>1.0 1.6</td>
</tr>
<tr>
<td>Hyola 600RR</td>
<td>1.3 1.5</td>
<td>1.4 1.6</td>
<td>1.6 2.0</td>
<td>1.2 1.8</td>
</tr>
<tr>
<td>Hyola 725RT</td>
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<td>1.1 1.2</td>
<td>1.3 1.5</td>
<td>0.9 1.5</td>
</tr>
<tr>
<td>Mean</td>
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<td>1.3 1.5</td>
<td>0.8 1.6</td>
</tr>
<tr>
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<td>0.09</td>
<td>0.09</td>
<td>0.2</td>
</tr>
<tr>
<td>$P$ value$^a$</td>
<td>***</td>
<td>**</td>
<td>***</td>
<td>***</td>
</tr>
</tbody>
</table>

$^a$P value: *** = $P < 0.001$, ** = $P < 0.01$ * = $P < 0.05$
3.2.4. Post-harvest weed suppression ratings

Differences in post-harvest weed suppression ratings between cultivars \((P < 0.001)\), location \((P < 0.001)\) and year \((P < 0.001)\) were noted (Table 7, Fig. 6). In addition, the interaction between cultivar, location and year was significant \((P < 0.001)\). In 2015 the most weed suppressive canola crop residues were present in Hyola 600RR and Hyola 725RT at Condobolin while at Wagga Wagga CB Taurus and Hyola 600RR residues performed more successfully in terms of weed suppression. Hyola 50 (Condobolin) and Barossa (Wagga Wagga) residues provided the least weed suppression. In 2016 AV Opal and CB Taurus residues were more weed suppressive at both locations with Hyola 725RT and ATR Bonito (Condobolin) and Barossa (Wagga Wagga) proving least suppressive.

In contrast to pre-harvest results, CB Taurus and Hyola 600RR crop residues consistently provided greater weed suppression in both years at both locations while Barossa residues were least suppressive. However, the impacts of dried crop residues were season and location dependent and were difficult to predict, as differential residue decomposition over time was noted.

Figure 6: Representative images of canola cultivar A) GT-50 and B) CB Taurus post-harvest in summer 2015 at Condobolin, showing the suppressive impact of the crop residues on summer weeds in the absence of herbicide.
Table 7: Differences in post-harvest visual weed suppression ratings (0 = poor, 10 = Excellent) for canola cultivars at Condobolin and Wagga Wagga trial sites in 2015 and 2016 taken at 60- and 70-days post-harvest, respectively. The cultivars are ranked according to the average suppression rating score across the site-years.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Condobolin</th>
<th></th>
<th>Wagga Wagga</th>
<th></th>
<th>Mean</th>
</tr>
</thead>
<tbody>
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<td>4.5</td>
<td>5.5</td>
<td>6.0</td>
</tr>
<tr>
<td>Hyola 600RR</td>
<td>6.5</td>
<td>9.2</td>
<td>3.5</td>
<td>3.5</td>
<td>5.7</td>
</tr>
<tr>
<td>AV Opal</td>
<td>4.7</td>
<td>9.6</td>
<td>2.8</td>
<td>4.0</td>
<td>5.3</td>
</tr>
<tr>
<td>Hyola 725RT</td>
<td>5.5</td>
<td>9.0</td>
<td>2.8</td>
<td>3.5</td>
<td>5.2</td>
</tr>
<tr>
<td>GT-50</td>
<td>4.8</td>
<td>9.2</td>
<td>2.8</td>
<td>3.3</td>
<td>5.1</td>
</tr>
<tr>
<td>ATR Bonito</td>
<td>4.3</td>
<td>9.0</td>
<td>2.7</td>
<td>3.8</td>
<td>5.0</td>
</tr>
<tr>
<td>Hyola 50</td>
<td>4.0</td>
<td>9.5</td>
<td>2.8</td>
<td>3.8</td>
<td>5.0</td>
</tr>
<tr>
<td>Barossa</td>
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<td>9.2</td>
<td>2.5</td>
<td>2.8</td>
<td>4.8</td>
</tr>
<tr>
<td>LSD</td>
<td>0.4</td>
<td>0.5</td>
<td>0.6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*P value: *** = *P < 0.001, ** = *P < 0.01 * = *P < 0.05
4. Discussion

4.1. Cultivar and weed dry matter (biomass)

Early cultivar biomass accumulation was strongly and negatively related with weed biomass in 2014, 2015 (Condobolin) and 2016 (Wagga Wagga) indicating cultivar dependent competitive ability against weeds in both locations and years. The most weed-suppressive cultivars included GT-50, Hyola 600RR and Hyola 50, with CB Taurus and Barossa being consistently less competitive during the growing season. These findings support those of previous studies that suggested that the competitive response of canola cultivars was positively correlated with early-season crop biomass accumulation (Beckie et al. 2008a; Asaduzzaman et al. 2014b). In this case, hybrid cultivars Hyola 50 and GT-50 were exceptionally competitive with weeds, likely due to the formation of dense canopies with an associated accumulation of greater above-ground biomass as compared to other cultivars (Mwendwa et al. 2018).

The inverse relationship between cultivar and weed biomass at both sites with coefficients of determination ($r^2$) of 0.68, 0.44 and 0.66 (2014-2016, Wagga Wagga) and 0.52 and 0.24 (2015-2016, Condobolin) clearly suggests the relative importance of early crop vigour and rapid canopy closure to subsequent weed suppression. This relationship was relatively consistent over the years and location with some slighter seasonal variations. These findings tend to support previous results in Canada and Australia showing an inverse correlation between weed biomass with early season canola biomass accumulation (Beckie et al. 2008a; Asaduzzaman et al. 2014b; Mwendwa et al. 2018).

Of note, at Condobolin and Wagga Wagga (in 2014 and 2015), cultivar CB Taurus demonstrated reduced biomass accumulation while exhibiting significantly higher weed suppression in contrast to other cultivars at flowering and crop maturity. Additionally, CB Taurus performed similarly in 2016 at the flowering stage in both locations. These findings suggest that suppression by CB Taurus may be due to traits other than crop competition alone and could possibly be associated with an allelopathic interference mechanism. Previous studies showed that open-pollinated cultivars such as AV Opal established at various sowing times were competitive with weeds despite producing somewhat reduced in-crop biomass (Asaduzzaman et al. 2014b).

Asaduzzaman et al. (2014d) suggested that enhanced weed suppression in several open-pollinated cultivars was potentially associated with the production of bioactive
secondary metabolites rather than crop competition. In this case, root extracts contained more total secondary metabolites than shoot extracts, but fewer bioactive metabolites, including glucosinolates and isothiocyanates, were noted in root extracts or exudates in laboratory studies under controlled conditions (Gardiner et al. 1999; Asaduzzaman et al. 2015). However, the impact of canola allelochemicals on the suppression of weeds under field conditions requires further investigation in field conditions to determine if production of potential phytotoxins is related to aboveground crop competitiveness, or to the production of allelochemicals that may be released into the soil over time, including those following harvest.

4.2. Modelling weed suppression using canola canopy traits

PLS regression is a technique that reduces the predictors to a smaller set of uncorrelated components and performs least squares regression on these components, instead of on the original data. PLS regression is especially useful when predictors are highly collinear, or when there are more predictors than observations (Mevik and Wehrens 2007). The interrelatedness of plant characteristics associated with canopy structure and weed competitiveness in grain crops [i.e. plant height, early canopy closure, LAI, vertical leaf orientation, rapid biomass accumulation at the early crop growth stage, high shoot dry matter, large root biomass and root volume (Sardana et al. 2017)] makes PLS regression analysis particularly well-suited to characterise relationships between crop plant characteristics and weed suppression.

The current study suggests that negative PLS regression correlation relationships in 2015 between some of the cultivar traits related to canopy architecture/light interception and weed biomass at both locations account for significant reductions in weed biomass over various crop growth stages (vegetative vs flowering) in years with typical rainfall. Surprisingly, the 2016 model fits at both locations showed a positive relationship between crop and weed growth. This suggests that in a year when soil moisture is not limiting, the competitive advantage of the crop is nullified, indicating that the lack of water availability is potentially a major contributor to weed suppression in Australian dryland canola systems.

Hybrid cultivars are often taller, more vigorous, establish a denser canopy, and can be more weed competitive than open-pollinated (Zand and Beckie, 2002) and other older hybrid cultivars. Lemerle et al. (2014) also reported that hybrid canola cultivars reduced weed biomass at flowering by up to 50%, in contrast to open-pollinated cultivars,
and estimated a significant reduction of weed seeds in the weed seedbank. Similar studies performed in Canada indicated that hybrid canola cultivars were approximately twice as competitive as open-pollinated cultivars when weed interference was relatively high (Zand and Beckie, 2002; Harker et al., 2003, 2011).

In addition, crop cultivars possessing traits such as fast germination, quick growth, high biomass and leaf area have a competitive advantage over weeds (Sardana et al. 2017). Our data suggest that there is a strong relationship between cultivar biomass accumulation and PAR light interception (light absorption efficiency of the leaves), which may contribute to cultivar competitive ability and weed suppression in some years, albeit not 2016. However, further assessment to determine the contribution of each of these physiological and morphological traits is required, and experiments under different watering regimes to influence soil moisture availability are required.

One potential explanation for the strong competitive ability of plants with a well-developed canopy is that this adaptation gives them an advantage in exploring, capturing, and exploiting available resources. For example, in a mixed canopy of crop and weeds, light capture efficiency is a function of light interception, and is determined by leaf area index, height, and light absorption efficiency of the leaves (i.e. leaf angular orientation, thickness, vertical leaf area distribution) (Swanton et al. 2015) sufficient to shade weeds at all stages of the growth cycle. In addition, strong interference with weed establishment should likely also include other traits such as early growth, and flowering, allelopathic tendencies and rapid root growth for nutrients (N, P, K, S) and water uptake (Lemerle et al. 2014; 2016). A truly competitive cultivar should maintain its yield under relatively low weed interference pressure while reducing the growth and seed production of weeds against which it is competing.

Interactions between cultivar and other cultural practices such as seeding rate and timing of herbicide application also clearly influence canola competitiveness. However, in canola, the recommended seeding rate of 40 plants/m² was optimal for weed suppression regardless of cultivar (Lemerle et al. 2016). We recommend further studies, particularly those under various soil moisture regimes, to develop models that may guide plant breeders in selecting weed competitive canola cultivars. It would appear that under typical moisture-limiting production conditions, similar to those experienced in 2015, canola cultivars that rapidly establish and obtain early canopy closure along with high early biomass accumulation could be most effective in limiting weed growth and biomass
accumulation. Additional work is required to determine the morphological, physiological and metabolic traits associated with strongly competitive canola cultivars, as well as cultivar interactions with other agronomic interventions.

4.3. Cultivar grain yield vs weed competition

The significantly higher yield in 2015 and 2016 at both locations for all herbicide-treated plots in comparison to the control indicates that pre-emergent herbicide applications were important in managing early weed competition against the crop and thus contributed significantly to higher yield potential in canola, especially in years when soil moisture was not limiting. Trifluralin was typically applied prior to sowing and targeted grass weeds including annual ryegrass, barley grass and brome grass as well as susceptible broadleaf species. The effect of weed competition on crop yield is driven by variables such as the density and type of weeds that emerge with or before the crop (Swanton et al. 2015), herbicide treatment and rainfall as demonstrated in the current study. However, cultivar biomass and seed yield response to weed interference are often correlated with the weed-suppressive ability (Beckie et al. 2008a).

The consistently lower yields in the untreated plots demonstrate the impact and importance of understanding the time of weed emergence relative to the crop and the need to manage the weeds either at pre-sowing by herbicide application or early crop establishment to potentially reduce weed interference early in the season. Weed tolerance refers to the ability of the crop to maintain high yields despite weed competition, while weed-suppression refers to the ability of the crop to suppress weed growth through competition. Under both situations, the presence of weeds results in reduced crop productivity (Sardana et al. 2017). In addition, cultivars exhibiting a high level of competition with weeds offer opportunities to reduce herbicide inputs through integrated weed management (Harker et al. 2011).

Canola hybrids have been reported to produce significantly higher yields and perform more competitively in contrast to open-pollinated cultivars under high weed pressures, but limited differences in yield were noted when weed interference was lower (Harker et al. 2011; Lemerle et al. 2014; 2016). Results of the present study revealed that the most recently released hybrid cultivars generally outperformed other hybrid and open-pollinated cultivars under dryland conditions where rainfall was limiting.

However, yields were highest in years receiving greatest rainfall and under conditions in which the growing season was lengthened as observed in Condobolin in
In this case, weed biomass and weed numbers were also significantly increased in contrast to seasons receiving less rainfall. In southeastern Australia, the choice of cultivar is important to maximise yield, and clearly, great variation in yield potential can be experienced by producers depending on year and location.

4.4. Post-harvest weed suppression ratings

Physical or chemical effects associated with the presence of canola residues on the soil surface may result in greater weed suppression associated with certain cultivars, and these could be due to allelopathic effects of residues, altered soil microbiota, or differences associated with soil moisture retention beneath the residues (Fig. 6). Our findings evaluating soil moisture retention in previous experiments with canola residues suggested that limited differences exist in soil moisture retention beneath various cultivars in such summer fallows (data not presented). However, additional experimentation is required to verify which environmental factors are critical for continued weed suppression in crop mulches under hot, and typically dry, summer fallow conditions.

Previous studies have shown that retained residues of cereal crops (especially wheat) were up to two-fold times higher in biomass than those retained in canola (CB Taurus cv.) plots (Weston et al. 2014; Mwendwa et al. 2018). However, both grain canola (Hyola 50) and grazing canola (CB Taurus), despite having lower remaining stubble biomass compared to the cereal crops, were more weed suppressive. Additionally, AV Opal and other canola cultivars with limited crop residues have been shown to be weed suppressive and potentially allelopathic at various sowing times (Asaduzzaman et al. 2014b). Other studies have also reported allelopathic potential associated with crop residues and/or plant extracts in Brassica spp. (Norsworthy et al. 2011). Canola residues suppressed nearly all Panicum capillare L. (witchgrass.) and most Conyza spp. (fleabane) growth for up to 4 months following harvest (Weston et al. 2014).

Our findings suggest that competitive ability of canola during the cropping phase and post-harvest is associated with the morphological, physiological and metabolic traits present in newer cultivars such as Hyola 600RR. However, canola allelopathy or residue phytotoxicity potentially occurs independently of such competitive traits impacting aboveground morphological growth and phenology of the crop (Asaduzzaman et al. 2014a). Further field studies to evaluate rates of residue decomposition and release of
Phytotoxins/allelochemicals are required to ascertain the reasons for prolonged weed suppression in canola stubble.

5. Conclusions

Clearly canola cultivar competitiveness is strongly influenced by cultivar, location and seasonal conditions which vary by year. However, some cultivars, particularly hybrids such as GT-50 and Hyola 600RR were consistently more competitive or weed tolerant than others regardless of the site and season. This study demonstrated that diverse cultivar-dependent competitive traits such as early growth vigour, biomass production, absorption of photosynthetically active radiation (due to increased leaf area and/or canopy structure) and production and retention of crop residue impacted weed establishment and total weed biomass.

Our results also showed that the choice of cultivar offers potential as a tool for maintaining suitable grain yield in the presence of weeds while potentially delaying the development of herbicide resistance through efficacious weed suppression. Therefore, competitive crops are potentially an important part of IWM (Harker et al. 2011) and can be a cost-effective means for weed suppression in dryland canola production.

We suggest that canola pre-breeding programs strongly consider selection for morphological, physiological and phenological traits associated with competitive and vigorous cultivars, as well as other weed suppressive properties. We further suggest that development of predictive models, performed under different soil moisture regimes, will aid in the design of more effective breeding programs for weed suppression in grain crops particularly in areas with limited soil moisture availability.

6. Conflicts of interest

The authors declare no conflicts of interest.

7. Acknowledgements

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Richard McCallum (NSW DPI Condobolin ARAS), Graeme Heath and the Plant Interaction Research Group at Charles Sturt University for their assistance with field experimentation and data collection.

8. References


Part 2: Mechanisms of Weed Suppression in Wheat

(*Triticum aestivum* L.)

Investigating annual weed establishment in wheat crop for weed suppression at vegetative growth stage of wheat and after canopy closure at Wagga Wagga trial site.
Chapter 4

Field Evaluation of Australian Wheat Genotypes for Competitive Traits and Weed Suppression

Following on from the study on weed suppressive ability of grain crops (Chapter 3), some wheat cultivars were significantly more weed suppressive than others. Due to the importance of wheat to Australian agricultural economy, further studies were performed to evaluate the competitive ability of selected Australian wheat cultivars. Above ground competitive traits of selected superior Australian spring and winter wheat cultivars were assessed and are discussed herein. Differences in weed suppression were largely impacted by crop architecture and phenology early in the growing season.

Chapter 4 is a published conference proceedings paper that was editorially reviewed. It presents the initial wheat field data from Condobolin and Wagga Wagga trial sites for 2014 and 2015. Both modern and traditional commercial cultivars were evaluated including dual purpose cultivars such as Wedgetail and Whistler which are grown in most regions of Australia.

All the authors contributed to writing and editing of the manuscript. However, Dr. Brown, Dr. Haque and Mr. Heath were also involved in establishing and monitoring the field trials. In addition, they provided technical support during sampling. Other contributors to this study are included in the acknowledgement.

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Field evaluation of Australian wheat genotypes for competitive traits and weed suppression

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Abstract

In 2014 and 2015, replicated field trials were performed at commercial paddocks in moderate to low rainfall zones at Wagga Wagga and Condobolin NSW, respectively. In 2014, a total of 11 winter wheat cultivars (Triticum aestivum L.) representing four major breeding family lines grown in Australia were evaluated with 13 cultivars assessed in 2015. At each site, crop and/or weed growth were monitored at various stages of growth: early season (tillering), vegetative, grain filling, harvest and post-harvest. Significant differences between wheat cultivar and location were observed for crop biomass, early vigour, leaf area index (LAI), weed number, weed biomass, canopy architecture and yield in both 2014 and 2015. Differences in weed suppression were largely impacted by crop architecture and phenology early in the growing season. Cultivar competitive traits were also influenced by both cultivar and environmental factors, as shown by clear differences in cultivar performance, yield and weed suppression among both locations. Cultivars Condo and Espada were superior performers for yield and weed suppression in both locations and years analysed. This data supports the concept that choice of wheat cultivar can prove to be a cost-effective means of weed management.

Keywords Weed suppression, canopy architecture, phenology, propagules, weed seedbank.
1. Introduction

Herbicide resistance in both grasses and broadleaf weeds is on the rise across Australia, with an increasing number of cropping weeds experiencing resistance to multiple herbicides (Owen et al. 2013). Globally, weeds have evolved resistance to 22 of the 25 known herbicide modes of action and to 160 different herbicides (Heap 2016).

Highly competitive wheat cultivars typically have the ability to access better light, nutrients, and water resources in a limited space, thus suppressing the growth and reproduction of neighbouring weed species (Bertholdsson 2011, Worthington et al. 2015). In Greece, the use of competitive wheat cultivars alone has reduced the use of herbicides by 50% for weed management (Travlos 2012, Andrew et al. 2015). However, in the past century, the competitive ability of wheat has been typically reduced by selection based on yield potential. Older cultivars or landraces have been shown to be more competitive with weeds than the higher yielding, semi-dwarf modern cultivars (Bertholdsson et al. 2012).

To realise the potential of competitive crop cultivars as a tool in integrated weed management, a quick and simple-to-use protocol for assessing their competitive potential is required as it is likely that selection will not be based on a single trait but will need to capture the combined effect of multiple traits (Bertholdsson 2011, Andrew et al. 2015). In this study, both field and laboratory experiments were performed with the overall objectives of 1) assessing the competitive traits of selected superior Australian winter wheat cultivars which are well adapted for the southern farming region, 2) assessing the impact of environmental factors associated with location and year, including soil moisture and temperature, on weed suppressive ability of wheat, 3) identifying and quantifying key wheat metabolites associated with weed suppression and 4) evaluating weed suppression by remaining wheat stubble post-harvest.

2. Materials and methods

In 2014 and 2015 field trials were sown at two locations in moderate to low rainfall zones at Wagga Wagga (572 mm) and Condobolin (449 mm) NSW, respectively. Plots were seeded with six replications in a randomized complete block design. Eleven wheat cultivars representing four major genetic families of winter wheat commercially grown in Australia were selected for evaluation, plus one cultivar of winter cereal rye.
(Secale cereale) as a known suppressive control. In 2015, two additional cultivars, Trojan and Federation, were included. Trojan is a recently released cultivar, and Federation is an older heritage cultivar bred and released in 1901 and widely used until 1970. Soils were typical Aeolian fine red clays with clay content varying with soil depth.

At sowing, replicated soil samples were taken from each block to evaluate the weed seedbank in the glasshouse. At Condobolin, the crop was sown on 6th and 15th May at 33 cm spacing while at Wagga Wagga the crop was sown on 20th and 22nd May at 25 cm spacing for 2014 and 2015 respectively. A knife point and press wheel planter was used. Fertilizer was applied at 70 kg ha⁻¹ MAP (Incitec Pivot Fertilisers). Equal plant density of each cultivar was established in each trial (target 120 plants m⁻²) by assessment of seed weight per volume when seeding to reduce confounding effects for enhanced evaluation of cultivar competitiveness. No pre- or post-emergent herbicides were applied at either site during wheat production.

All data collection was performed at the critical plant developmental stages of stem elongation, flowering, maturity as well as post-harvest stubble residue assessment. Data collected each year included crop phenological characteristics, percentage light interception using light Ceptometer (AccuPAR LP80 Ceptometer, Decagon Devices®) to measure PAR (Photosynthetically Active Radiation) both above and below the crop canopy, NDVI (Normalised Difference Vegetative Index) readings (GreenSeeker® 505 handheld sensor and Trimble Recon PDA) to monitor crop biomass production, biomass cuts of crop and weeds (gm⁻²), visual vigour ratings (0 = poor, 10 = excellent), yield (kg ha⁻¹) and post-harvest weed suppression(0 = poor, 10 = excellent).

Crop and weed biomass data were collected within a 50 × 50 cm quadrat in each plot at the soil surface with two subplots collected per plot. Grain harvest was performed before 15th December in each year and each location, using a small plot harvester. Yield was measured as harvested cereal grain. Statistical analysis of data was performed by ANOVA for randomised block experiments with six replicates using GenStat (VSN, 2016); significant differences were separated using LSD (0.05).
3. Results and discussion

At each location, cultivar differences were significant for crop growth parameters, including early vigour, leaf area index, NDVI, and crop biomass. Weed numbers and weed biomass were also significantly affected by crop cultivar at each location. Figures 1 and 2 depict cultivar differences in early vigour and weed suppression at Wagga Wagga. Tables 1 and 2 reflect weed suppression provided by various wheat cultivars in terms of weed biomass taken at approximately 100 days after crop emergence (DAE) at both locations in 2014 and 2015.

Based on the results from 2014 and 2015 growing season, Condo, Janz CL and Espada demonstrated enhanced crop competitive ability against weeds in contrast to other cultivars. At Wagga Wagga in 2015 (Figure 2 and Table 1), Federation, Condo and Janz CL cultivars demonstrated strongest early vigour in terms of wheat growth at 50 days after establishment (DAE) and subsequently provided the greatest weed suppression at 110 DAE. Similar trends in weed suppression were also observed in 2014; Janz CL, Espada, Corack and Condo were highly weed suppressive (Table 1 and Fig 1) although there were no significant differences between the cultivars in weed suppression. Federation and Trojan were introduced in 2015.

Crop competitive ability can either be specified in terms of crop tolerance against weeds or growth inhibition of weeds by resource competition (Bertholdsson 2010). Previous studies have found that cultivars with higher early vigour are generally capable of extracting more soil moisture which in turn enables them to maintain lower canopy temperatures on warm days (Zerner et al. 2008) which is essential in dryland broadacre farming. Certain wheat cultivars under ideal conditions will produce acceptable yields while suppressing weed populations (Worthington et al. 2015, Andrew et al. 2015).
Figure 1: Wheat cultivar biomass taken 60 DAE (LSD 17.8; P <0.001) and 130 DAE (LSD 25.7; P <0.001) and weed biomass (LSD 10.9; P= NS) taken at 130 DAE at Wagga Wagga in 2014 arranged by weed biomass

Figure 2: Wheat cultivar biomass taken 50 DAE (LSD 151; P <0.001) and 110 DAE (LSD 106; P <0.001) and weed biomass (LSD 2.9; P <0.5) taken at 110 DAE at Wagga Wagga in 2015 arranged by weed biomass
Condo and Espada were amongst the three most weed suppressive cultivars and also produced the highest grain yields (4.0 and 3.9 t ha\(^{-1}\) in 2014 and 5.4 and 5.6 t ha\(^{-1}\) in 2015 respectively; data not presented). Recent studies have also shown that certain cultivars successfully reduced the economic burden of weeds over time by resisting yield loss (Vandeleur and Gill 2004) while reducing weed seedbank inputs through successful suppression. Lemerle et al. (2001) also observed that cultivars which showed competitive yield advantage also suppressed *L. rigidum*.

At Condobolin in 2014, Mace was highly weed suppressive but was not significantly different from Espada, Condo, Janz CL and Livingstone. Gregory was significantly less weed suppressive than other cultivars evaluated (Table 2). In 2015, Trojan, Condo, Scout and Espada were most weed suppressive. The ability to suppress weeds appears to be strongly cultivar dependant (Wu et al. 2001; Bertin et al. 2003). However, environmental factors such as moisture availability and daily temperature also play a key role in influencing the performance of specific cultivars in a particular season as shown by locational performance differences.

<table>
<thead>
<tr>
<th>Rank</th>
<th>Cultivar</th>
<th>2014 g m(^{-2})</th>
<th>Rank</th>
<th>Cultivar</th>
<th>2015 g m(^{-2})</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Rye</td>
<td>1.1</td>
<td>1</td>
<td>Rye + Federation</td>
<td>0.0</td>
</tr>
<tr>
<td>2</td>
<td>Janz CL</td>
<td>2.5</td>
<td>2</td>
<td>Condo</td>
<td>0.1</td>
</tr>
<tr>
<td>3</td>
<td>Espada</td>
<td>3.3</td>
<td>3</td>
<td>Janz CL</td>
<td>0.3</td>
</tr>
<tr>
<td>4</td>
<td>Condo</td>
<td>5.1</td>
<td>4</td>
<td>Espada</td>
<td>0.6</td>
</tr>
<tr>
<td>5</td>
<td>Gregory</td>
<td>5.7</td>
<td>5</td>
<td>Trojan + Mace</td>
<td>1.2</td>
</tr>
<tr>
<td>6</td>
<td>Livingstone</td>
<td>7.3</td>
<td>6</td>
<td>Livingstone</td>
<td>3.1</td>
</tr>
<tr>
<td>7</td>
<td>Mace</td>
<td>13.5</td>
<td>7</td>
<td>Gregory</td>
<td>3.2</td>
</tr>
<tr>
<td>P – value</td>
<td>NS</td>
<td></td>
<td></td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>LSD 0.05</td>
<td>10.9</td>
<td></td>
<td></td>
<td>2.9</td>
<td></td>
</tr>
</tbody>
</table>
Table 2: Weed biomass (g m\(^{-2}\)) taken 130 and 109 DAE at Condobolin in 2014 and 2015 respectively.

<table>
<thead>
<tr>
<th>Rank</th>
<th>Cultivar</th>
<th>g m(^{-2})</th>
<th>Rank</th>
<th>Cultivar</th>
<th>g m(^{-2})</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>Mace</td>
<td>0.03</td>
<td>1</td>
<td>Trojan</td>
<td>4.9</td>
</tr>
<tr>
<td>2</td>
<td>Espada</td>
<td>0.04</td>
<td>2</td>
<td>Condo</td>
<td>7.6</td>
</tr>
<tr>
<td>3</td>
<td>Condo</td>
<td>0.06</td>
<td>3</td>
<td>Livingstone</td>
<td>11.6</td>
</tr>
<tr>
<td>4</td>
<td>Janz</td>
<td>0.06</td>
<td>4</td>
<td>Espada</td>
<td>11.9</td>
</tr>
<tr>
<td>5</td>
<td>Livingstone</td>
<td>0.07</td>
<td>5</td>
<td>Federation</td>
<td>12.4</td>
</tr>
<tr>
<td>6</td>
<td>Rye</td>
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<td>6</td>
<td>Janz</td>
<td>21.1</td>
</tr>
<tr>
<td>7</td>
<td>Gregory</td>
<td>0.70</td>
<td>7</td>
<td>Gregory</td>
<td>44.3</td>
</tr>
</tbody>
</table>

P-value: *
LSD 0.05: 0.5
LSD 30.5

The competitive ability of wheat is influenced by a range of plant attributes such as height, tiller number, and light interception by the canopy or light interception at the soil surface. If one can limit light interception at the soil surface, the germination and establishment of weeds can often be significantly reduced. In recent experimentation, increasing plant height improved bread and durum wheat’s ability to tolerate and suppress oats. Several plant traits associated with early wheat vigour (early canopy cover, greater leaf width and tiller number) were also positively correlated with crop competitive ability (Vandeleur and Gill 2004, Zerner et al. 2008).
High leaf area index is known to be important in light interception (Figures 2 and 3). Federation, Condo and Grazer rye had the highest leaf area index of all crops and cultivars in Wagga Wagga, and this trait is most closely associated with early vigour growth and crop competitive ability (Vandeleur and Gill 2004, Zerner et al. 2008), but is not always selected for in current commercial lines and cultivars. Previous studies have also reported that early leaf area development is an important contributor to weed competitive ability (Coleman et al. 2001).

In Wagga Wagga in 2015 cultivar light interception was highly positively related to leaf area index at 50 days after crop emergence ($r^2 = 0.97$, $P < 0.001$; Figure 2). A similar relationship was also seen at Condobolin in 2015 (Figure 3) with cultivar light interception being highly positively related to leaf area index at 100 DAE ($r^2 = 0.89$, $P < 0.001$). However, after this time there was no significant relationship between these traits, suggesting crop height and other growth characteristics were important in light interception. For example, Federation and Condo cultivars in Wagga Wagga at 50 DAE were weed suppressive, having less than 50% of photosynthetically active radiation reach the soil surface compared to Wedgetail which had over 70% reach the soil surface. This

![Figure 2: The relationship between wheat cultivar ground surface PAR light interception and leaf area index at 50 DAE in Wagga Wagga in 2015 ($r^2 = 0.97$, $P < 0.001$).](image)

\[ y = 47.33x + 7.2778 \]
\[ R^2 = 0.96 \]
suggests that the canopy of Federation and Condo successfully intercepted more light than did Wedgetail while allowing less to reach the soil surface, thereby influencing weed establishment (Table 1 and 2).

Figure 3: The relationship between wheat cultivar ground surface PAR light interception and leaf area index at 100 DAE in Condobolin in 2015 ($r^2 = 0.89$; $P < 0.001$).

Plant height was also identified as one of the traits most commonly associated with competitiveness (Vandeleur and Gill 2004). However, timing of emergence also influences light interception, as the same weed species may be relatively tall or short depending on the emergence time relative to the crop. It is important to consider these cultivar competitive traits at breeding and sowing.

We have demonstrated that crop phenology early in the season may be particularly important in impacting overall weed suppression and the subsequent weed seedbank at the time of harvest. Initial studies over two years in two locations in the moderate to low rainfall zone show that the choice of wheat cultivar can prove to be a cost-effective means of weed management and may potentially impact weed propagule numbers in the subsequent seedbank.
4. Acknowledgements

We acknowledge the financial support of the Grains Research and Development Corporation (Projects UCS 00020, 00022, 00023) and technical support of the CSU weed research team in performing these studies.

5. References


Chapter 5:

Evaluation of Selected Commercial Wheat Cultivars for Canopy Architecture, Early Vigour, Weed Suppression and Yield in the Southern NSW

In chapter 5 we present a more detailed analysis of the competitive ability of wheat against annual broadacre weeds in southern Australia. In comparison to Chapter 4, additional wheat morphological traits were evaluated for their association with weed suppression in a replicated study over two locations and two years with the objective of evaluating genotypically diverse cultivars for enhanced weed suppression and yield tolerance in the low to moderate rainfall zone in southern Australia. In addition, an older heritage cultivar (Federation) known for its ability to suppress weeds and more recently released high yielding modern cultivars were included in the cultivar comparison to increase the robustness of the data, and identify traits associated with weed suppression.

Significant differences between wheat cultivar and location and year were observed for crop biomass, early vigour, leaf area index, PAR light interception, crop height, weed number, weed biomass, and yield. Differences in weed suppression were largely impacted by crop architecture and phenology early in the growing season; key traits included leaf area index (LAI) and the ability to achieve early canopy closure through vigorous early growth and rapid formation of crop biomass, all contributing to intense shading at the soil surface. Cultivar competitive traits were influenced by both cultivar (cultivar) and environmental factors, as shown by clear differences in the growing environment at both locations each year, as well as cultivar dependent biomass accumulation, yield and weed suppression.

All the authors contributed in writing and editing the manuscript. In addition, Dr. Brown was involved in establishing and monitoring the field trials while Dr. L. and P. Weston provided support in the statistical analysis of the data. Other contributors to this study are included in the acknowledgement. Chapter 5 has been prepared as a manuscript for publication and has been submitted to Crop and Pasture Science Journal and is currently under review.

Evaluation of selected commercial wheat cultivars for canopy architecture, early vigour, weed suppression and yield in southern NSW

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Abstract

In 2015 and 2016, replicated field trials were performed to evaluate mechanisms of weed suppression in diverse Australian wheat (Triticum aestivum L.) cultivars in moderate to low rainfall zones at Wagga Wagga (572 mm) and Condobolin (449 mm) New South Wales, respectively. In both years, thirteen wheat cultivars representing four major genetic families of winter wheat commercially grown in Australia were selected for evaluation including the heritage cultivar Federation, and one cultivar of winter cereal rye (Secale cereale L.) as known weed-suppressive controls. At each trial crop and weed growth were monitored at various phenological stages including early growth, vegetative, flowering, grain-fill and harvest. Significant differences between wheat cultivar and location were observed for crop biomass, early vigour, leaf area index (LAI), weed number, weed biomass, canopy architecture and yield in each year. Differences in weed suppression were largely impacted by crop architecture and phenology early in the growing season, particularly leaf shape and the ability to achieve early canopy closure. Cultivar competitive traits were also clearly influenced by both cultivar and environmental factors, as shown by differences in cultivar performance, yield and weed suppression by season and location. Cultivars Condo, Espada and Janz were superior performers in terms of weed suppression and yield in both locations and years. Our results suggest that establishment of competitive wheat cultivars can result in effective suppression of weed growth (up to 90% or greater) in the absence of post-emergent
herbicides, and the choice of cultivar could constitute a cost-effective and sustainable weed management tool.

**Keywords:** Weed suppression, canopy architecture, phenology, photosynthetically active radiation, weed seedbank.

### 1. Introduction

Weeds are a persistent problem in cereal crops, increasing production costs while reducing crop yields (Wu, 2016). Worldwide, yield losses of approximately 34% are caused by weeds among the major food crops and are typically higher than losses due to other crop pests (Jabran et al., 2015). Herbicides are the most widely used tools to manage weeds in commercial crops, but weeds have now evolved resistance to 23 of the 26 known herbicide sites of action and to 163 different herbicides across the globe (Heap, 2018) limiting options for chemical control. Herbicide resistance of weed species also restricts control options, thereby escalating economic losses and threatening agricultural sustainability (Wu, 2016). This threat comes at a time when increasing global populations require greater agricultural productivity for food sustainability, and environmental concerns have resulted in significant restrictions on the use of some herbicides.

In Australia, herbicide resistance in both grasses and broadleaf weeds is on the rise, with resistance to multiple herbicides reported for an increasing number of cropping weeds (Owen et al., 2013; Bajwa et al., 2016). Llewelyn et al. (2016) estimated that the cost of additional herbicides due to herbicide resistance was AUD 187 million on top of the costs of using extra integrated weed management practices. They also reported the overall cost of weeds to Australian grain growers as AUD 3.3 billion annually, which equates to AUD 146/ha in expenditure and yield losses of ca. 2.76 million tonnes of grain annually. Therefore, crop cultivars which reduce weed growth (i.e. allelopathic or competitive crops) are an attractive option because they offer useful options that are environmentally friendly for no additional in-crop weed management costs (Andrew et al., 2015).

Crop competition and allelopathy are well-documented mechanisms of plant interference under controlled conditions (Weston, 2005). The combined effects of both determine the total weed-suppressive potential of a crop cultivar, and research has been undertaken to improve both competitive features and allelopathic potential.
simultaneously to achieve maximum gains in crop weed suppression (Bertholdsson et al., 2012, Worthington & Reberg-Horton, 2013). For example, competitive wheat cultivars have been demonstrated to result in a 50% reduction in application of recommended levels of herbicides (Travlos, 2012). However, despite obvious efficacy, the relationship between wheat crop competition and allelopathy as mechanisms of plant interference has not been fully elucidated in this crop.

Enhanced above-ground crop competition can be achieved using competitive crop species/cultivars in combination with cultural practices such as increased seeding rates, narrow row spacing, altered row orientation, ploughing, crop rotation and delayed drilling (Llewellyn et al., 2007; Bajwa et al., 2016). Developing wheat cultivars with superior competitive ability against weeds should, therefore, complement cultural methods for weed management while maintaining acceptable yields and suppressing weed populations (Worthington & Reberg-Horton, 2013; Andrew et al., 2015). In addition, the incorporation of morphological traits that enhance early crop vigour, namely size of leaf 1 and 2, light interception without affecting harvest index, plant height at tillering and stem extension, grain yield in weedy conditions, grain yield tolerance (Bertholdsson, 2010; Worthington et al., 2015) and crop tillering ability (Fradgley et al., 2017) are all important factors to consider in selective breeding programs for improving the competitive ability of modern wheats without compromising their yielding ability (Vandeleur & Gill, 2004; Andrew et al., 2015).

A crop's ability to both suppress weed growth and tolerate weed competition should be a key consideration when taking an agroecological approach to weed management (Fradgley et al., 2017). Additionally, precise information on agronomic factors such as increased crop seeding rate or choice of variety for enhancing competitive crop ability in different environments (Lemerle et al., 2001; van der Meulen & Chauhan, 2017) is required to inform final cultivar choice. In this study, field experiments were performed to assess the competitive traits of selected commercial Australian winter and spring wheat cultivars with a focus on above-ground interactions. Specific crop morphological traits were evaluated for their association with weed suppression in a two-year study over two locations with the objective of evaluating genotypically diverse cultivars for enhanced weed suppression and yield tolerance in the low to moderate rainfall zone in southern Australia.
2. Materials and methods

2.1 Site description and experimental design

In 2015 and 2016, replicated wheat field trials were sown at two locations in moderate to low rainfall zones at Wagga Wagga (572 mm) and Condobolin (449 mm) NSW, respectively. Plots were seeded with six replications in a randomised complete block design. In both years, thirteen wheat cultivars representing four major genetic backgrounds of winter and spring wheat commercially grown in Australia were selected for evaluation, plus one cultivar of winter cereal rye as a positive suppressive control (Table 1).

Treatments included wheat cultivars with both short and moderate time to maturity along with two grazing winter wheat cultivars, Whistler and Wedgetail (Table 1). Other notable cultivars included the recently released cultivar Trojan, as well as Federation, an older heritage cultivar bred and released in 1901 and widely used until 1970. Federation is considered to be both early-maturing and drought resistant and was included for comparison of its inherent weed suppressive abilities and upright growth habit. Experimental sites were established at proximity to each other at each location in both 2015 and 2016 following a canola rotation at each site.

At Wagga Wagga, field trials were conducted on fine red clay loam sodosols, surface pH 6.4, that were previously planted commercially for the production of cereals, canola and/or Lucerne (Medicago sativa L.). At Condobolin, soils were predominantly red gradational, and red-brown earth sodosols with surface pH 7.0 and were previously rotated among cereals and pasture legume crops. Both soils exhibited low inherent fertility and organic matter content and were maintained using standard commercial practices to reduce weed populations.

2.2 Crop establishment

At sowing, replicated composite soil samples (10 cm core by 5 cm depth) were taken from each block to evaluate the weed seedbank present at experimental initiation as suggested by Menalled and Schonbeck (2011). Samples were taken every two meters along two diagonal transects in each replicate and samples bulked. Weed seedbanks were evaluated in a glasshouse experiment conducted for six months with continued flushes of weed seed emergence being counted, recorded and uprooted to allow for further weed seed germination (Table 3).
All crops were established with seed that was generated in Wagga Wagga NSW from harvest the previous season using standard practices for pest control and fertiliser application to potentially eliminate any cultivar variation due to seed variability resulting from production at different locations. At Condobolin, the crop was sown on 15th and 17th May at 33 cm spacing, typical for drier soils, while at Wagga Wagga the crop was sown on 22nd and 14th May at 25 cm spacing for 2015 and 2016, respectively, due to rainfall differences among the regions. Cultivars were established at equal plant density (target population of 120 plants m$^{-2}$) in each trial.

Seeding was performed in both locations with a knife-point and press wheel planter. Fertiliser was applied at 70 kg ha$^{-1}$ diammonium phosphate (DAP) (Incitec Pivot Fertilisers) treated with 400 ml ha$^{-1}$ Flutriafol (Intake® Hioad Gold 200 g ha$^{-1}$ Flutriafol, Crop Care). Before sowing, all established weeds were controlled with glyphosate (Weedmaster® DST® 470 g L$^{-1}$ Glyphosate, Nufarm) at 960g ha$^{-1}$.

2.3 Crop assessments and data collection

Data collection was performed at each location at critical plant developmental stages of crop establishment including stem elongation, flowering, and maturity. Data were collected each year on various crop phenological characteristics. Light Ceptometer (AccuPAR LP- 80 Ceptometer, Decagon Devices®) was used to measure PAR (photosynthetically active radiation) both above and below the crop canopy. NDVI (normalised difference vegetative index) readings (GreenSeeker® 505 handheld sensor and Trimble® Recon PDA, NTech Industries Inc.) were obtained to monitor crop biomass production. Other assessments included biomass cuts of crop and weeds (g m$^{-2}$), visual vigour ratings (0 = poor, 10 = excellent), yield (kg ha$^{-1}$) and post-harvest weed suppression (0 = poor, 10 = excellent).

Crop and weed biomass data were collected within a 50 × 50 cm quadrat in each plot at the soil surface with two quadrats randomly collected per plot. Grain harvest was performed before 15th December in each year and location, using a small plot harvester. The yield was measured as harvested cereal grain in t/ha.
2.4 Data analysis

Trial design, randomisation and data analyses were performed using Agricultural Research Manager (ARM) version 9.0 (Gylling Data Management Inc., 2014). Comprehensive statistical analysis of selected data sets was later performed using GenStat (VSN, 2018) for ANOVA and MANOVA with means separated using LSD (0.05 confidence level). Data were transformed using the square-root \((x+c)^{0.5}\) function to correct for homogeneity and normality before analysis as required. Partial least square (PLS) regression analysis was performed using R Studio (R Core Team 2017).

Partial least squares (PLS) regression (Abdi 2003; Kvalheim 2010) was performed using the plsdepot package of R to develop a predictive linear model for weed suppression by wheat cultivar based on weed dry biomass as the response variable and selected crop canopy traits as the predictive variables. Numerous crop or canopy traits were used as predictors in the model to visualise the relationships between the dependent variable (dry weed biomass) and the most influential predictive variables among crop traits including crop biomass, PAR light interception (% of PAR light intercepted by crop canopy), leaf area index, visual vigour ratings and NDVI taken at different crop growth stages.
**Table 1.** Wheat cultivars and commercial use for field trials performed in Condobolin and Wagga Wagga, NSW in 2015 and 2016.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Breeder</th>
<th>Main use</th>
<th>Growth characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Condo</td>
<td>AGT¹</td>
<td>grain</td>
<td>Early maturity adapted to low–medium rainfall areas. Similar in maturity to Livingston. Has consistently yielded well in low-medium rainfall environments and offers excellent physical grain quality. A tall plant type with medium straw strength. Released in 2014.</td>
</tr>
<tr>
<td>Corack</td>
<td>AGT</td>
<td>grain</td>
<td>Derived from Wyalkatchem, an early-maturing cultivar with high straw strength. Could be suitable for a wheat-on-wheat situation, low rainfall environments or late sowings.</td>
</tr>
<tr>
<td>Espada</td>
<td>AGT</td>
<td>grain</td>
<td>Mid-season maturity. Best performance in medium to high yield potential areas. Good seedling vigour. Produces large grain with low screenings.</td>
</tr>
<tr>
<td>Federation²</td>
<td>William Farrer</td>
<td>grain</td>
<td>The first specifically Australian variety that is both rust- and drought-resistant (release 1901). Early maturing, high-yielding and drought-tolerant with strong straw. Awnless and broad adaptation.</td>
</tr>
<tr>
<td>Grazer (Cereal rye)³</td>
<td>EGA⁴</td>
<td>dual purpose</td>
<td>Winter growing cereal and performs well on lighter soils. Rapid growth with early vigour and grazing possible four weeks after emergence if tillering and the secondary root system development has occurred to anchor the plant.</td>
</tr>
<tr>
<td>Gregory</td>
<td>EGA⁴</td>
<td>grain</td>
<td>Excellent yield potential in early to mid-season sowings in northern and southern Australia. Medium to slow maturity.</td>
</tr>
<tr>
<td>Janz CL</td>
<td>AGT</td>
<td>grain</td>
<td>Widely adapted main season Clearfield® variety. Moderate seedling vigour. Medium–strong straw strength, with good lodging and shattering resistance.</td>
</tr>
<tr>
<td>Livingston</td>
<td>AGT</td>
<td>grain</td>
<td>Early maturing cultivar. Livingston is derived from a cross involving Sunvale and was released as a higher-yielding alternative for Ventura in areas that are not constrained by acid soils.</td>
</tr>
<tr>
<td><strong>Mace</strong></td>
<td>AGT</td>
<td>grain</td>
<td>Mace has broad adaptation, consistently high relative yield under a wide range of conditions, and is less susceptible to downgrading at receival due to black point, pre-harvest sprouting or screenings losses than many other cultivars.</td>
</tr>
<tr>
<td><strong>Scout</strong></td>
<td>LongReach(^5)</td>
<td>grain</td>
<td>Mid-season maturity. High-quality grain with low screenings and high-test weight. Medium to long coleoptile with good early vigour. Contains the CSIRO Transpiration Efficiency gene, which confers improved water use efficiency.</td>
</tr>
<tr>
<td><strong>Suntop</strong></td>
<td>AGT</td>
<td>grain</td>
<td>A main season line with stable yields in areas with both low to high yield potential. More rapidly maturing than Gregory but similar in maturity to Janz with outstanding disease resistance and wide adaptation.</td>
</tr>
<tr>
<td><strong>Trojan</strong></td>
<td>LongReach</td>
<td>grain</td>
<td>Mid–long-season maturity suited to the medium-high rain zone. Short–medium plant height at maturity with good straw strength.</td>
</tr>
<tr>
<td><strong>Wedgetail</strong></td>
<td>EGA</td>
<td>dual purpose</td>
<td>Dominant winter wheat. Early sowing cultivar. Large grain size. Adapted to higher rainfall regions of the wheat belt.</td>
</tr>
<tr>
<td><strong>Whistler</strong></td>
<td></td>
<td>dual purpose</td>
<td>Early maturing winter wheat cultivar. Relatively low ‘cold requirement’ and heads prematurely when sown early as a dual-purpose crop.</td>
</tr>
</tbody>
</table>

\(^1\)Australian Grain Technologies, \(^2\)bred by William Farrer in 1901, \(^3\)Cereal rye was used as a control in all the field trials, \(^4\)Enterprise Grains Australia, \(^5\)LongReach Plant Breeders
3. Results

3.1. Rainfall and weed seedbank

Monthly rainfall received during the growing season is reported in Table 2 for both locations from 2014 to 2016. Given the variable rainfall conditions across years, wheat cultivars also exhibited variable performance under the average (2015) and above average (2016) rainfall conditions experienced. Possibly, due to the above average in-crop rainfall in 2016, significantly greater weed pressure was observed based on weed count biomass assessments at both sites (Table 4). At Condobolin and Wagga Wagga, weed counts and biomass increased by 84.8 and 82.3% and 94.8 and 97.0%, respectively in 2016 compared to 2015.

The dominant weed observed in Condobolin plots was stonecrop (*Crassula* spp. L.), similar to the results of a past field survey performed by Lemerle *et al.* (1996). Other common weeds at Condobolin included annual ryegrass (*Lolium rigidum* Gaud.) capeweed (*Arctotheca calendula* (L.) Levyns) and bluebell (*Hyacinthoides non-scripta* (L.) Chouard ex Rothm.). At the Wagga Wagga site, in-crop weeds included fumitory (*Fumaria* spp. L.), bluebell, capeweed, poppy (*Papaver* spp. L.), annual ryegrass and barley grass (*Hordeum murinum* L.), all of which are commonly encountered in the mixed cropping zone of the Riverina region in south-eastern Australia (Broster *et al.*, 2012). Other weeds present at lower densities in Wagga Wagga included stonecrop and fleabane (*Conzya bonariensis* (L.) Cronq.).

### Table 2: Monthly rainfall (mm) and the total in-crop rainfall for the Wagga Wagga and Condobolin canola field trial sites in 2014, 2015 and 2016 from Australian government bureau of meteorology.

<table>
<thead>
<tr>
<th></th>
<th>Jan</th>
<th>Feb</th>
<th>Mar</th>
<th>Apr</th>
<th>May</th>
<th>Jun</th>
<th>Jul</th>
<th>Aug</th>
<th>Sep</th>
<th>Oct</th>
<th>Nov</th>
<th>Dec</th>
<th>In-crop</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Wagga Wagga</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2014</td>
<td>13.2</td>
<td>27.2</td>
<td>63.4</td>
<td>56.6</td>
<td>47.0</td>
<td>81.0</td>
<td>24.2</td>
<td>10.6</td>
<td>36.6</td>
<td>22.0</td>
<td>47.0</td>
<td>29.0</td>
<td>325.0</td>
</tr>
<tr>
<td>2015</td>
<td>89.0</td>
<td>41.2</td>
<td>2.0</td>
<td>56.4</td>
<td>18.8</td>
<td>66.0</td>
<td>60.0</td>
<td>98.8</td>
<td>22.7</td>
<td>10.0</td>
<td>92.2</td>
<td>30.2</td>
<td>424.9</td>
</tr>
<tr>
<td>2016</td>
<td>58.6</td>
<td>20.0</td>
<td>42.6</td>
<td>10.8</td>
<td>102.1</td>
<td>99.7</td>
<td>86.8</td>
<td>68.1</td>
<td>178.0</td>
<td>79.1</td>
<td>28.0</td>
<td>53.5</td>
<td>652.6</td>
</tr>
<tr>
<td><strong>Long-term average</strong></td>
<td>37.8</td>
<td>36.9</td>
<td>37.6</td>
<td>38.5</td>
<td>43.9</td>
<td>50.8</td>
<td>49.4</td>
<td>48.2</td>
<td>48.8</td>
<td>51.4</td>
<td>41.1</td>
<td>41.2</td>
<td>372.1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Jan</th>
<th>Feb</th>
<th>Mar</th>
<th>Apr</th>
<th>May</th>
<th>Jun</th>
<th>Jul</th>
<th>Aug</th>
<th>Sep</th>
<th>Oct</th>
<th>Nov</th>
<th>Dec</th>
<th>In-crop</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Condobolin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2014</td>
<td>35.2</td>
<td>46.7</td>
<td>104.5</td>
<td>28</td>
<td>27.6</td>
<td>57.4</td>
<td>9.2</td>
<td>22.2</td>
<td>11</td>
<td>11.5</td>
<td>17.7</td>
<td>88.6</td>
<td>184.6</td>
</tr>
<tr>
<td>2015</td>
<td>59.2</td>
<td>35.9</td>
<td>0.2</td>
<td>64.7</td>
<td>11.6</td>
<td>31.8</td>
<td>41.2</td>
<td>42.3</td>
<td>6.8</td>
<td>65.2</td>
<td>67.3</td>
<td>28.5</td>
<td>339.9</td>
</tr>
<tr>
<td>2016</td>
<td>80.9</td>
<td>0</td>
<td>21.8</td>
<td>21.4</td>
<td>60.2</td>
<td>161.5</td>
<td>37.1</td>
<td>43.4</td>
<td>143.2</td>
<td>31.6</td>
<td>40.8</td>
<td>56.7</td>
<td>539.2</td>
</tr>
<tr>
<td><strong>Long-term average</strong></td>
<td>47.2</td>
<td>43.4</td>
<td>41.4</td>
<td>30.8</td>
<td>35.5</td>
<td>33</td>
<td>35.7</td>
<td>33.8</td>
<td>32.4</td>
<td>47.5</td>
<td>39.7</td>
<td>41.9</td>
<td>288.4</td>
</tr>
</tbody>
</table>

*Long-term average of last 100 years*
Table 3 presents the mean weed germination counts of the soil cores from both trial sites collected each year at sowing. The most prevalent weeds at Wagga Wagga were windmill grass (*Chloris* *spp.* Sw.), bluebell and fumitory, as well as Hillman’s panic grass (*Panicum Hillmanii* L.), fat hen (*Chenopodium album* L.) and sow thistle (*Sonchus* *spp* L.). At Condobolin, stonecrop and witchgrass (*Panicum capillare* L.) were the most prevalent in the weed seedbank samples and others observed included shepherd's purse (*Capsella bursa-pastoris* (L.) Medik.), fat hen and windmill grass.
Table 3: The mean glasshouse seedbank weed germination counts of the top soil from Condobolin and Wagga Wagga trial sites taken at crop sowing in 2015 and 16.

<table>
<thead>
<tr>
<th>Wagga Wagga</th>
<th>First count</th>
<th>Second count</th>
<th>Third count</th>
<th>Fourth count</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>19</td>
<td>21</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>86</td>
<td>90</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>21</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>6</td>
<td>56</td>
<td>31</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>19</td>
<td>51</td>
<td>22</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>83</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>55</td>
<td>318</td>
<td>152</td>
<td>7</td>
</tr>
</tbody>
</table>

Condobolin

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
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<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>6</td>
<td>0</td>
<td>21</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>5</td>
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<td>3</td>
<td>2</td>
<td>0</td>
<td>9</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>91</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>46</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>8</td>
<td>10</td>
<td>4</td>
<td>6</td>
<td>3</td>
<td>4</td>
<td>23</td>
<td>4</td>
<td>187</td>
<td>25</td>
<td>7</td>
</tr>
</tbody>
</table>
3.2. Early crop biomass differences and weed biomass interference

Wheat cultivar often impacted both crop and weed biomass accumulation as well as weed count in both years ($P < 0.001$) and locations ($P < 0.001$) (Table 4). Cultivar differences in early crop biomass accumulation were significant at elongation growth stage at both locations and years, but no differences were observed at the booting/flowering growth stage at either location in 2016. The cultivars which were consistently most weed suppressive in both years and locations were Federation, Condo, Espada and Janz CL. The least suppressive cultivars were Gregory in 2015 and 2016 and Mace in 2016. Certain cultivars were weed-suppressive in one year and location but not both (e.g. Scout and Trojan at Condobolin and Mace and Wedgetail at Wagga Wagga in 2015).

At 100 days after seeding, the recently released cultivar Condo was 82.8 and 98.0% more weed-suppressive than the older industry standard cultivar Gregory in 2015 at Condobolin and Wagga Wagga, respectively. However, as higher in-crop rainfall (Table 2) resulted in higher weed populations in 2016 at both locations, Condo was only 45.4 and 72.0% more weed-suppressive than Gregory at Condobolin and Wagga Wagga, respectively. Federation, a heritage cultivar with early vigour and upright growth habit, was typically the most weed-suppressive cultivar in both locations and years in this experiment and in experimentation conducted previously at both sites.
Weed count and biomass were significantly different depending on the location ($P < 0.001$) with significant cultivar, year and location interactions. In addition, early crop biomass accumulation resulted in reduced weed biomass at both sites, a relationship that was inversely related (coefficient of determination ($r^2$) of 0.47 ($P < 0.001$, Fig. 1) at Condobolin and 0.87 ($P < 0.001$, Fig. 2) at Wagga Wagga, (Table 4).

**Figure 1**: The relationship between mean wheat cultivar early biomass and weed biomass at crop maturity before flowering at Condobolin in 2015 and 2016. Each data point represents the average of the two-year mean of six replicates for each year.

$y = -1.4777x + 208.79$

$R^2 = 0.4661$
3.3. Weed suppression by other crop canopy traits

Cultivar differences were observed for all parameters associated with crop growth and vigour including early vigour, PAR light interception (%), leaf area index, normalised difference vegetation index (NDVI) and plant height ($P < 0.001$), with significant cultivar, year and location interactions ($P < 0.001$; Tables 6 and 7 in supplementary 1). Ranking of cultivars based on these individual parameters indicated that cultivars with highest early growth vigour, light interception as measured by PAR, leaf area and plant height were also the most weed suppressive (Tables 6 and 7).

At the early crop growth stage, PAR light interception and leaf area index were positively related with weed suppression across the years and locations ($P < 0.001$). At Wagga Wagga, the coefficient of determination was $r^2 = 0.97$ and $0.94$ in 2015 and 2016, respectively, while at Condobolin $r^2$ was $0.89$ and $0.22$, respectively. The cultivars with the highest PAR light interception also exhibited higher leaf area indices as well as lower weed biomass (e.g. Federation and Condo, Tables 4, 6 and 7).
Table 4: Wheat cultivar differences in average crop biomass (g m⁻²) at crop elongation and booting growth stages, in-crop weed count (plants m⁻²) and weed dry biomass at crop maturity at Condobolin and Wagga in 2015 and 2016. Each data point is a mean of six replicates.

<table>
<thead>
<tr>
<th>Cultivar/year</th>
<th>Elongation crop dry biomass</th>
<th>Booting crop dry biomass</th>
<th>In-crop weed count</th>
<th>Weed dry biomass at crop maturity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Condo</td>
<td>Wagga</td>
<td>Condo</td>
<td>Wagga</td>
</tr>
<tr>
<td>Condo</td>
<td>44.7</td>
<td>152.3</td>
<td>720.7</td>
<td>122.7</td>
</tr>
<tr>
<td>Corack</td>
<td>45.5</td>
<td>131.9</td>
<td>531.4</td>
<td>109.7</td>
</tr>
<tr>
<td>Espada</td>
<td>42.8</td>
<td>119.0</td>
<td>486.8</td>
<td>114.2</td>
</tr>
<tr>
<td>Federation</td>
<td>63.7</td>
<td>105.7</td>
<td>765.1</td>
<td>123.7</td>
</tr>
<tr>
<td>Grazer- rye</td>
<td>78.4</td>
<td>156.0</td>
<td>737.1</td>
<td>168.3</td>
</tr>
<tr>
<td>Gregory</td>
<td>43.8</td>
<td>90.6</td>
<td>464.5</td>
<td>107.1</td>
</tr>
<tr>
<td>Janz CL</td>
<td>47.2</td>
<td>103.0</td>
<td>707.9</td>
<td>135.7</td>
</tr>
<tr>
<td>Livingstone</td>
<td>57.3</td>
<td>146.6</td>
<td>672.3</td>
<td>106.9</td>
</tr>
<tr>
<td>Mace</td>
<td>46.4</td>
<td>95.0</td>
<td>539.6</td>
<td>113.7</td>
</tr>
<tr>
<td>Scout</td>
<td>38.9</td>
<td>154.0</td>
<td>587.7</td>
<td>118.3</td>
</tr>
<tr>
<td>Suntop</td>
<td>51.6</td>
<td>157.3</td>
<td>639.6</td>
<td>116.4</td>
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<td>Trojan</td>
<td>46.3</td>
<td>126.5</td>
<td>489.4</td>
<td>95.5</td>
</tr>
<tr>
<td>Wedgetail</td>
<td>42.6</td>
<td>131.4</td>
<td>479.1</td>
<td>123.6</td>
</tr>
<tr>
<td>Whistler</td>
<td>39.3</td>
<td>95.7</td>
<td>531.4</td>
<td>89.8</td>
</tr>
<tr>
<td>LSD</td>
<td>17.8</td>
<td>35.6</td>
<td>188.8</td>
<td>38.5</td>
</tr>
</tbody>
</table>

*P value: *** = P < 0.001, ** = P < 0.01 * = P < 0.05, NS= not significant

aP value: *** = P < 0.001, ** = P < 0.01 * = P < 0.05, NS= not significant
3.4. Modelling for weed suppression using crop canopy traits

PLS regression was performed to develop a predictive linear model for weed suppression in commercial wheat cultivars based on weed dry biomass as the response variable and selected aboveground crop canopy traits (crop biomass, PAR light interception, leaf area index, visual vigour ratings, plant height and NDVI) as the predictive variables. When interpreting the results of such PLS modelling, the angle between the predictors and the dependent variable is a visual approximation of the correlation between the variables. A small angle indicates the variables are positively correlated, an angle of ~90° indicates the variables are not correlated, and an angle close to 180° indicates the variables are strongly negatively correlated (Abdi 2003; Kvalheim 2010).

In 2015, the model prediction differed in accordance with crop growth stage and impact in interfering with weed biomass at both locations (P < 0.001). In Condobolin in 2015 crop vigour (at early and vegetative), crop biomass (at vegetative and flowering), vegetative NDVI, crop height at maturity, LAI and PAR light interception (at maturity) were all negatively correlated with weed biomass ($r^2 = 0.21$, Fig. 3A). However, the most strongly inversely correlated with weed biomass were early crop vigour (X7), vegetative crop biomass (X12), vegetative NDVI (X8) and height at crop maturity (X11).

In addition, height at flowering, vigour rating and NDVI at crop maturity indicate an angle closer to 90° suggesting that these predictors were not correlated with weed biomass. The model was similar at Wagga Wagga, with vigour rating (early, vegetative), crop biomass (vegetative, flowering), crop height (flowering, maturity), LAI and PAR light interception (maturity) and NDVI (at vegetative, maturity) negatively correlated with weed biomass ($r^2 = 0.22$, Fig. 3B). But the most strongly negatively correlated predictors with weed biomass were early vigour rating (X7), crop biomass (vegetative-X14, flowering-X16) and NDVI (at vegetative-X8, maturity-X17). The vigour rating at maturity, LAI and PAR light interception at vegetative crop stage were not closely associated with weed biomass.

In 2016 the model predictions showed a stronger inverse correlation of most predictors with weed biomass ($r^2 = 0.51$ at Condobolin, $r^2 = 0.59$ at Wagga Wagga). At Condobolin, negatively correlated (angle close to 180 degrees) predictors included early vigour rating, crop biomass (vegetative, maturity), NDVI (maturity), LAI (vegetative, maturity) and height at crop maturity (Fig. 4A) while vigour rating (vegetative, maturity), NDVI (vegetative), height at flowering and PAR light interception (vegetative, maturity)
were positively correlated (small angle) with weed biomass. At Wagga Wagga, vigour rating (early, vegetative, maturity), crop biomass (vegetative, maturity), NDVI (vegetative), height (at flowering, crop maturity), LAI (vegetative, maturity) and PAR light interception at crop maturity were negatively correlated with weed biomass while only NDVI (maturity) and PAR light interception at crop vegetative stage were positively correlated with weed biomass (Fig. 4B). The predictors most inversely correlated with weed biomass at both locations in 2016 were crop vigour (early, vegetative), crop biomass (vegetative, maturity), height (maturity), NDVI and LAI at vegetative stage.

Overall, the PLS model analysis demonstrated that weed biomass was inversely related to early crop vigour, biomass, NDVI (except NDVI at vegetative growth stage at Condobolin in 2016), height, LAI and PAR light interception in 2015 and 2016 at both locations; however, this relationship was stronger in 2016 ($P < 0.001$). The predictor’s impact on weed biomass in both years and locations varied depending on the cultivar, year and location. However, despite these variations, early crop vigour, crop biomass, height and NDVI contributed to reduced weed biomass at various crop growth stages until harvest.

**Figure 3:** Correlation circle of partial least squares (PLS) regression, illustrating the correlations of the dependent variable (dry weed biomass) predicted by several independent variable crop canopy traits at Condobolin (A) and Wagga Wagga (B) taken at crop maturity in 2015. The independent predictors include (A) $X7=$ early vigour rating, $X8=$ vegetative NDVI, $X9=$ vegetative vigour rating, $X10=$ height at flowering, $X11=$ height at maturity, $X12=$ crop dry biomass-vegetative, $X13= Maturity$ vigour rating, $X14= crop$ dry biomass- flowering, $X15= Maturity$ NDVI, $X16= LAI- maturity$, $X17= PAR$ light interception- maturity; (B) $X7=$ early vigour rating, $X8=$ vegetative NDVI, $X9 =$ vegetative LAI, $X10=$ PAR light interception-vegetative, $X11=$ vegetative vigour rating, $X12= height$ at flowering, $X13= height$ at maturity, $X14= crop$ dry biomass- vegetative, $X15= Maturity$ vigour rating, $X16= crop$ dry biomass- flowering, $X17= Maturity$ NDVI, $X18= LAI- maturity$, $X19= PAR$ light interception- maturity.
Figure 4: Correlation circle of partial least squares (PLS) regression, illustrating the correlations of the dependent variable (dry weed biomass) predicted by several independent variable crop canopy traits at Condobolin (A) and Wagga Wagga (B) taken at crop maturity in 2016. The independent predictors for (A) and (B) include X7 = early vigour rating, X8 = vegetative NDVI, X9 = vegetative LAI, X10 = PAR light interception - vegetative, X11 = vegetative vigour rating, X12 = height at flowering, X13 = height at maturity, X14 = crop dry biomass - vegetative, X15 = maturity vigour rating, X16 = crop dry biomass-maturity, X17 = Maturity NDVI, X18 = LAI - maturity, X19 = PAR light interception - maturity.

3.5. Cultivar grain yield vs weed tolerance

In 2015 and 2016, grain yield differed between cultivars and locations (Table 5). At Wagga Wagga, there were significant differences in yield between cultivars and years while interestingly at Condobolin cultivar differences were only noted between years, with higher yields in 2016 than in 2015 for all cultivars. The commercial cultivars most recently released, Trojan and Condo, consistently produced higher yields over both years and locations than older or heritage cultivars. However, the oldest cultivar in the trial, developed in central NSW in the late 1800s, Federation, was consistently the most weed suppressive on analysis but yield potential was 10 to 55% less than recently improved cultivars. Condo, Espada and Janz CL consistently produced higher yield while suppressing weeds moderately to exceptionally well.
Table 5: Mean wheat cultivar grain yield (t ha\(^{-1}\)) and in-crop dry weed biomass (g m\(^{-2}\)) at Condobolin and Wagga Wagga respectively, in 2015 and 2016.

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<thead>
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<th>Site</th>
<th>Grain yield t ha(^{-1})</th>
<th>In-crop dry weed biomass (g m(^{-2}))</th>
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<td>Wedgetail</td>
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<td>3.4</td>
</tr>
<tr>
<td>Whistler</td>
<td>0.4</td>
<td>3.4</td>
</tr>
</tbody>
</table>

LSD 0.58 0.76 0.32 0.82 15.7 15.1 2.4 135.5

P valuea

\(P\) value: **** = \(P < 0.001\), ** = \(P < 0.01\) * = \(P < 0.05\), NS= not significant

There were significant differences in both years between cultivars in yield at Wagga Wagga (\(P < 0.001\)) but not at Condobolin. In 2015 at Condobolin, yield may have been negatively impacted by the sudden rise in daily maximum temperature to 35.2, 36.0 and 33.4\(^{0}\)C for three consecutive days at grain fill (Fig. 5). In 2016, there were clear differences in weed biomass over cultivars, with a coefficient of determination was \(r^2 = 0.58\), although yield did not differ between cultivars (Table 5). At Wagga Wagga in 2015, the coefficient of determination of reression was \(r^2 = 0.25\) (figure not presented, \(P < 0.001\)), with four cultivars being more weed-suppressive (Federation, Condo, Espada, Janz CL). In 2016, the same cultivars were also most weed-suppressive \((r^2 = 0.45; P < 0.001)\), with cultivar differences in yield and dry weed biomass (Table 5).
4. Discussion

4.1. Early crop biomass differences and weed biomass interference

Clear differences were identified in biomass accumulation between wheat cultivars in the current study indicative of an impact of location and seasonal differences between 2015 and 2016. Early cultivar biomass accumulation was strongly inversely correlated with weed biomass for both locations and years, indicating heritable competitive ability against weeds. Early vigour of a cultivar has been shown previously to be related to crop establishment and the rate at which aboveground biomass is produced and has been correlated with morphological leaf traits such as leaf area in the earliest phases of growth (Rebetzke and Richards, 1999). Christensen (1995) observed that faster-developing cultivars of spring barley were more weed suppressive, while Coleman et al. (2001) reported that faster development of leaf area could improve the ability of wheat to compete with weeds.

Consistent with findings in this study, early crop vigour, leaf area index and canopy height were clearly identified as the traits most strongly correlated with weed
suppression in cereals (Andrew et al., 2015). A greater leaf area index soon after emergence should also increase light interception to maximise crop growth rates and increase biomass and yield for late-sown wheat crops (Rebetzke and Richards, 1999). Seavers and Wright (1999) reported that competitive ability in wheat, oat, and barley cultivars against *Galium aparine* L. was associated with high overall leaf area, resistance to loss of tillers under competition pressure, greater height, and a faster rate of canopy development. In wheat, traits relating to leaf size, specific leaf area and rate of production vary between cultivars and have been linked to higher suppressive ability (Coleman et al., 2001; Zerner et al., 2008).

Similarly, Korres and Froud-Williams (2002) reported that increased crop height and rapid tillering capacity in winter wheat cultivars were associated with better weed suppression in plots containing a diverse weed flora. Seed mass and leaf width taken together showed a better fit for early vigour in wheat than seed mass or leaf width alone (Maydup et al., 2012). The contribution of these canopy traits highlights the importance of early rapid crop growth rate resulting in early biomass accumulation as a weed suppressive mechanism.

Based on our findings, further investigation and wheat breeding based on the traits involved in early crop vigour as demonstrated by early crop biomass accumulation might produce superior weed-suppressive cultivars. In the longer term, substantial genetic gain in yield may be achieved if breeders are able to produce cultivars with faster growth rates and greater biomass at maturity (Austin, 1999) because the breeding of wheat with greater early vigour has potential to increase water- and nutrient use efficiency, as well as to improve weed competitiveness to raise crop yields profitably (Zhang et al. 2014).

### 4.2. Weed suppression by other crop canopy traits

In this study, we have demonstrated cultivar differences in crop growth vigour visual rating, PAR light interception (%), leaf area index, NDVI, and plant height with cultivar, year and location interactions. The term ‘trait’ is used in ecology for a characteristic which may be used as a predictor of fitness in different environments (Andrew et al., 2015). However, in this study, the term ‘trait’ is used for any feature that is morphological, physiological or phenological and can be identified and measured at the level of the individual (Violle et al., 2007). Competitive crop canopy traits have been studied to determine their contribution to weed suppression in conventional agricultural
systems where fertiliser and water supply are not restricting plant growth. Highly competitive wheat cultivars typically can access better light, nutrients, and water resources in a limited area, thus suppressing the growth and reproduction of neighbouring weed species (Bertholdsson, 2011, Worthington et al., 2015). Plant traits associated with canopy structure and weed competitiveness in grain crops include plant height, early canopy closure, LAI, vertical leaf orientation, rapid biomass accumulation at the early crop growth stage, high shoot dry matter, large root biomass and root volume (Sardana et al., 2017).

The cultivars with the highest early growth vigour, light interception as measured by PAR, leaf area and plant height in this study, Federation and Condo, were also the most weed suppressive across all locations and years analysed. Didon and Hansson (2002) also demonstrated that the most weed-suppressive barley cultivars were those that intercepted the most PAR. This finding suggests that measuring PAR may present a simple and useful way to assess the suppressive ability of a cultivar for future trait identification for pre-breeding analysis.

Our study supports the hypothesis that several crop canopy traits contribute to superior cultivar weed competitive ability, and therefore single trait measurement is not often a good measure of competitive ability. Despite being lower yielding in weed-free situations, taller wheat cultivars have been previously reported to be better in tolerating weed pressure and suppressing weed growth (Vandeleur and Gill, 2004). Wicks et al., (2004) examined the ability of thirteen red winter wheat cultivars to suppress a mixture of annual weeds, revealing a negative correlation between total annual weed density and mature winter wheat height. Two of the shortest cultivars exhibited stronger suppressive abilities than the tall cultivars (Wicks et al., 2004) indicating that competitive ability cannot necessarily be attributed to a single trait (Andrew et al., 2015).

Other morphological traits contributing to superior competitive ability included greater leaf length and width, capacity for the light interception, and flag leaf length (Coleman et al., 2001; Vandeleur and Gill, 2004). Additionally, planophile leaf inclination and higher leaf area index were identified as weed suppressive attributes, owing to increased capacity for light interception (Drews et al., 2009). Therefore, a combination of several crop canopy morphological traits contributes to crop competitive ability such as height, light interception, leaf area and inclination. In our study, height was not an exceptionally strong predictor of weed suppression for most cultivars, as today’s wheat cultivars are all semi-dwarf and have similar height. However, Federation,
the tallest cultivar in the trials, was exceptionally weed suppressive over both year and location. Unfortunately, it also possesses a tendency to lodge, but this was not observed even in years with high moisture at either location.

Traits that are consistently expressed under different environmental conditions may have the potential to be applied as heritable selection criteria for further breeding of weed competitive crop cultivars in wheat (Lemerle et al., 2001). Leaf traits observed at early stages of plant growth have a high heritability, suggesting that early vigour may be selected for in breeding programmes for future better weed-suppressive wheat cultivars (Rebetzke and Richards, 1999; Coleman et al., 2001). For example, leaf width was the morphological parameter most closely correlated with early vigour ($r^2 = 0.764, 0.821,$ and $0.769$ for leaves 1, 2, and 3, respectively) at 31 days after planting (Maydup et al., 2012). A similar outcome is identified in this study in which cultivars with higher early growth vigour and biomass accumulation were more weed suppressive at 30 – 60 days after crop emergence.

4.3. Modelling for weed suppression using crop canopy traits

Data generated in this study investigated multiple interactions both at a plant and environmental level. In order to analyse this complex dataset, a novel model was generated that utilised partial least squares (PLS) regression as the statistical model. PLS regression is a technique that reduces the predictors to a smaller set of uncorrelated components and performs least squares regression on these components instead of on the original data. This analysis is particularly useful when predictors are highly collinear, or when there are more predictors than observations (Mevik and Wehrens 2007). The interrelatedness of plant characteristics associated with canopy structure and weed competitiveness in grain crops [i.e. plant height, early canopy closure, LAI, vertical leaf orientation, rapid biomass accumulation at the early crop growth stage, high shoot dry matter, large root biomass and root volume (Sardana et al. 2017)] makes PLS regression analysis particularly well-suited to characterise relationships between crop plant characteristics and weed suppression.

PLS regression was performed to develop a predictive linear model for weed suppression in winter and spring wheat based on weed dry biomass as the response variable and selected crop canopy traits as the predictive variables. In 2015 and 2016, the PLS model showed an inverse relationship between some of the cultivar traits related to
canopy architecture/light interception and weed biomass at both locations. This relationship was stronger in 2016, with in-crop above average rainfall at both locations. This suggests that in a year when soil moisture is not limiting, the competitive advantage of the wheat crop is dramatically increased, suggesting lack of soil moisture is a major contributor to weed suppression in Australian dryland wheat production farming systems.

To our knowledge, this is the first Australian study to model aboveground crop canopy traits in determining wheat cultivar competitive ability against weeds. The PLS model developed suggests that early crop growth vigour, crop biomass, NDVI, height, LAI and PAR light interception (%) negatively impacted weed biomass with the most inversely correlated predictors in both years and locations being crop vigour (early, vegetative), crop biomass (vegetative, maturity), height (maturity), NDVI at vegetative stage. This suggests that the most competitive cultivars such as federation, Condo and Janz had early canopy closure due to early growth vigour and biomass accumulation resulting into early shading against the weeds.

In addition, seasonal changes may impact the crop weed-suppressive competitive ability as the model coefficient of determination was higher and more than double in 2016 at both locations (Condobolin- 21 vs 51, Wagga Wagga- 22 vs 59) when above average rainfall was received. The model outcomes also suggest that early crop biomass is not a standalone trait, but a combination of other cultivar traits such as leaf area index, crop height, tillers and early canopy closure impact the ability to suppress weed growth. A better understanding of the interaction between these plant characteristics will assist breeders in developing more weed suppressive wheat crop cultivars in the future. Our current comparative studies with recently developed early vigour cultivars from CSIRO wheat breeder Greg Rebetzke further suggests that early vigour (the ability of the crop to shade the soil by canopy architectural traits) before crop maturity (by 100 days after seeding) clearly impacts weed suppression (Rebetzke et al. 2018), and selection for such traits can result in enhanced weed suppression with respect to today’s commercial cultivars.

4.4. Cultivar grain yield versus weed tolerance

Crop competitive ability can be separated into two components: 1) the competitive effect or weed-suppressive ability, i.e. the capacity of the crop to reduce weed growth and reproductive success through interference, and 2) the competitive response or weed
tolerance; i.e. the ability of the crop to yield despite the presence of weeds (Lemerle et al., 2006; Watson et al., 2009; Bertholdsson, 2010). In the current study, some newer cultivars (e.g. Trojan) yielded higher despite being less weed suppressive. This suggests the competitive ability of the current commercial cultivars in weed tolerance. However, mechanisms of weed tolerance may be independent of suppressive traits (Callaway, 1992; Jordan, 1993). For example, Fradgley et al. (2017) reported that taller varieties of oats (Avena sativa L.) tended to be more weed tolerant but not necessarily more suppressive.

The wide use of semi-dwarf wheat cultivars has resulted in increased harvest index of modern cultivars, with shorter plants and higher grain yield than older varieties (Austin, 1999) just as we demonstrated in this study. However, Richards et al. (2002) reported that the shorter plant height is often associated with reduced early vigour, a pleiotropic and undesired effect of the high grain yield performance of modern semi-dwarf varieties. By contrast, in this study, we have demonstrated that some modern semi-dwarf wheat cultivars have high early growth vigour and, also enhanced weed-suppression (e.g. Condo). Similarly, Wicks et al. (2004) demonstrated that two short modern cultivars had higher weed suppression than tall heritage cultivars. Further studies are required to differentiate crop interference and to determine the relationship between weed suppression and weed tolerance in wheat cultivars.

A key finding of this study was that the heritage cultivar Federation was one of the most weed suppressive cultivars examined. Vandeleur and Gill (2004) examined 14 historical wheat cultivars ranging in release date from 1860 to 1994, to determine the impact of crop breeding on the competitive ability to suppress weeds. Using oat as the weed, there was a significant positive linear relationship ($r^2 = 0.81$, $P < 0.01$) between the year of cultivar release and crop yield loss, suggesting an inferior competitive ability in modern cultivars compared to their ancestral counterparts. The older cultivars not only provided superior weed suppression but were also more tolerant of weeds as indicated by smaller yield loss. Similarly, older cultivars or landraces have been shown to be more competitive with weeds than the higher yielding, semi-dwarf modern cultivars (Bertholdsson et al., 2012; van der Meulen & Chauhan, 2017). When sown at the same crop density heritage crop stands had, on average, lower weed biomass (56%) than modern crop stands (Lazzaro et al. 2017) indicating superior weed suppression. These findings suggest that re-examination of the value of some heritage wheat cultivars should be undertaken to give more options to the producer toolbox for wheat production in every changing climate condition.
Clearly improvements in yield potential and crop architecture have occurred with breeding for Australian dryland conditions. Condo, an Australian cultivar released in 2014, ranked second overall after Federation in weed suppressive ability while producing more than double the yield of Federation at Wagga Wagga in both years. Condo also showed similar yield to other high yielding cultivars whilst exerting greater weed-suppressive ability. Worthington et al. (2015) recently discovered that weed suppressive ability was correlated with crop competitive traits in wheat, including vigour and erect growth habit during tillering (Zadoks GS 29), high LAI at stem extension (GS 31), plant height at tillering and stem extension (GS 29, 31), grain yield in weedy conditions, and grain yield tolerance. This suggests that although the competitive weed ability of wheat has clearly been reduced by selection based on yield potential (Bertholdsson et al., 2012), there are some newer cultivars that could be used for enhanced weed management due to their weed suppressive ability while maintaining higher yield for the future. Weed suppression with competitive wheat varieties can be a cost-effective and sustainable weed management tool for growers to prevent weed propagules from entering the weed seedbank. However, to gain producer adoption, grain quality and yield attribute much also be exceptional.

5. Conclusions

Our results clearly show that the establishment of competitive wheat cultivars can result in effective suppression of weed growth (up to 90% or greater) in the absence of post-emergent herbicides. Significant differences between wheat cultivar and location and year were observed for crop biomass, early vigour, leaf area index, PAR light interception, crop height, weed number, weed biomass, and yield. Differences in weed suppression were largely impacted by crop architecture and phenology early in the growing season, particularly leaf area and the ability to achieve early canopy closure through early vigour and rapid formation of crop biomass contributing to shading at the soil surface. Cultivar competitive traits were also influenced by both cultivar and environmental factors, as shown by clear differences in cultivar performance, yield and weed suppression at each location and year. Cultivars Condo, Espada and Janz were superior performers in terms of weed suppression and yielding potential in both locations and years while Federation was the most weed-suppressive overall.
Overall our study results suggest that weed suppression may be most strongly associated with crop competitive ability early in the season, before boot stage and flowering. In addition, the choice of wheat cultivars for the desired yield and weed suppression impacts the subsequent ability of the crop to interfere with weed growth successfully and can prevent future weed propagules from entering the weed seedbank. Therefore, when applying IWM strategies for weed management through the use of minimal amounts of herbicides, some wheat cultivars may be a potential tool for maintaining suitable grain yield in the presence of weeds while reducing the use of herbicides and delaying the development of herbicide-resistant weeds.

We suggest that wheat pre-breeding programs strongly consider selection for morphological, physiological and phenological traits associated with competitive and vigorous cultivars, as well as other weed suppressive properties such as the production of allelopathic compounds. We further suggest that the development of predictive models, performed under different soil moisture regimes, will aid in the design of more effective breeding programs for weed suppression in cereal crops.

6. Acknowledgements

We acknowledge the financial support of the Grains Research and Development Corporation of Australia through project UCS 00020, 00022, and 00023 which provided project funding and post-graduate scholarship support for James Mwendwa. We thank Richard McCullum (NSW DPI Condobolin ARAS), M. B Bagherieh-Najjar (Department of Biology, Golestan University, Iran), Graeme Heath and the Plant Interaction Research Group at Charles Sturt University for their assistance with field experimentation and data collection.
7. References


### 7. Supplementary 1

**Table 6:** Wheat cultivar differences in visual vigour ratings, PAR light interception (%), leaf area index (LAI), normalised difference vegetation index (NDVI) and plant height (cm) taken at the vegetative and flowering stage of the crop respectively, at Condobolin and Wagga Wagga in 2015.

<table>
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<tr>
<th>Year</th>
<th>Site Cultivar</th>
<th>Vigour visual rating</th>
<th>PAR light interception (%)</th>
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<td></td>
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*V = vegetative growth stage, *F= flowering growth stage, *P value: *** = P < 0.001, ** = P < 0.01 * = P < 0.05, NS= not significant*
Table 7: Wheat cultivar differences in visual vigour ratings, PAR light interception (%), leaf area index (LAI), normalised difference vegetation index (NDVI) and plant height (cm) taken at the vegetative and flowering stage of the crop respectively, at Condobolin and Wagga Wagga in 2016.

<table>
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<tr>
<th>Site</th>
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<th>Plant height (cm)</th>
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<td>F&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>8.10</td>
</tr>
<tr>
<td>*P value&lt;sup&gt;b&lt;/sup&gt;</td>
<td>NS</td>
<td>***</td>
<td>**</td>
<td>***</td>
<td>**</td>
</tr>
</tbody>
</table>

<sup>a</sup>V = vegetative growth stage, <sup>b</sup>F= flowering growth stage, **P value: *** = P < 0.001, ** = P < 0.01 * = P < 0.05, NS= not significant
Chapter 6

Metabolic Profiling for Benzoxazinoids in Weed-Suppressive and Early Vigour Wheat Cultivars

In chapters 4 and 5 I have presented data describing the mechanism of weed suppression in certain Australian wheat cultivars. Suppression was associated with numerous above-ground wheat canopy traits. However, in addition to understanding the contribution of these traits to the mechanism of weed suppression in wheat, it was equally important to also understand the contribution of allelochemical interference with weeds and the consequent production of secondary metabolites for weed suppression. At each experimental site, shoots, roots, rhizoplane and bulk rhizosphere soil samples were collected at various crop phenological sampling times and extracted for metabolites.

In this published conference proceedings paper, I present a method for metabolite profiling to specifically analyse for numerous wheat produced BX secondary metabolites previously associated with weed suppression. The analysis was performed using liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (UHPLC-ESI MS QToF and LC-MS Qtrap).

Chapter 6 is a short manuscript published in the peer-reviewed Proceedings of 20th Australasian Weeds Conference in 2016 and outlines the method used for metabolic profiling of benzoxazinoids (BXs) in weed-suppressive and early vigour wheat cultivars using both the LC-MS QToF and LC-MS Qtrap. Results obtained showed reasonable sensitivity and detected substantial quantities of nearly all metabolites profiled in both shoot and root tissues, and several others in bulk and rhizosphere soils.

All the authors contributed to the writing and editing of the manuscript. A/Prof. Fomsgaard and Bente Laursen conducted a short course in LC-MS spectrometry which I attended and kindly provided standards of the BX metabolites for quantification. In addition, Dr. Brown was involved in establishing and monitoring the field trials.

Metabolic profiling for benzoxazinoids in weed-suppressive and early vigour wheat genotypes

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Abstract

Replicated wheat (Triticum aestivum L.) cultivar trials were performed in commercial fields in moderate to low rainfall zones at Wagga Wagga (572 mm) and Condobolin (449 mm) NSW in 2014 and 2015. At each experimental site, crop and/or weed growth were monitored at selected growth stages including tillering, vegetative, grain filling, harvest and post-harvest to the crop. In addition, shoots, roots, rhizoplane and bulk rhizosphere soil samples were collected. All shoot and root samples were extracted in methanol using an automated Buchi high-pressure extractor while soil samples were extracted using a rotary shaking method. Extracts were profiled for unique secondary plant products including benzoxazinoids (BXs) using liquid chromatography coupled to mass spectrometry. Metabolic profiling of wheat cultivar shoots, roots, and soils resulted in detection of up to 14 BXs including BX glycosides and other metabolites of interest. Both qualitative and quantitative differences in BXs were observed and were cultivar and location dependent. Plant part and rhizosphere location (distance from root) also impacted BX concentration. Additional metabolic profiling is now underway and will provide crucial information regarding crop metabolism and biosynthesis of metabolites associated with weed suppression in commercial wheat cultivars.

Keywords Weed suppression, metabolomics, residue, competition, resource allocation.
1. Introduction

Benzoxazinoids are important allelochemicals present in wheat, barley and rye, and their suppressive effects on weeds, pests and diseases are of great interest in sustainable agriculture (Bertholdsson et al. 2012). BXs are known to play important roles in plant defence against herbivory, in plant interactions including allelopathy (Wu et al. 2000, Macías et al. 2007), and are considered to be natural pesticides (Tanwir et al. 2013). When produced in excessive amounts, BXs are stored in the form of glucosides which are enzymatically converted to aglycone forms under stress conditions (Tanwir et al. 2013). BXs comprise a number of chemical groups, the most important being lactams and hydroxamic acids (Figure 1).

Precise metabolic profiling of allelochemicals in the plant and at the same time in the soil rhizosphere could provide strong insight into the release of bioactive metabolites following incorporation of plant material or living root exudates into the soil (Krogh et al. 2006, Weston et al. 2015). Therefore, we evaluated selected diverse Australian wheat cultivars for their ability to suppress annual weeds under field conditions. At the same time, we also assessed their respective plant parts and rhizosphere soils for the presence of bioactive allelochemicals associated with weed suppression over two growing seasons.

2. Material and methods

2.1. Establishment of field trials

In 2014 and 2015, field trials were sown at two locations in NSW: Condobolin, considered to be a low rainfall (mean 449 mm) region and Wagga Wagga, a moderate rainfall (mean 572 mm) region. Plots were seeded with six replications in a randomised complete block design. Eleven wheat cultivars representing four major genetic families of winter wheat commercially grown in Australia were selected for evaluation, plus one cultivar of winter cereal rye (Secale cereale L.), as a known weed-suppressive control.

In 2015, two additional cultivars (Trojan and Federation) were included. Trojan is a recently released cultivar, and Federation is an older heritage cultivar bred and released in 1901 and widely used until 1970. Soils were typical Aeolian fine red clays with the clay content varying with soil depth.
2.2. Cultivar sampling and sample extraction

Shoots, roots, rhizoplane and bulk rhizosphere soil from around the roots were sampled at four strategic times based on crop growth stage which included early (tillering), vegetative (stem elongation), flowering and maturity (grain fill) growth stages. Approximately 5 g of sampled shoots and roots tissue were collected in a factorial experiment. Samples (taken from 4 replicates × 7 cultivars × 2 locations × 4 sample types) were each extracted in methanol using an automated high-pressure extractor (Weston et al. 2015). Rhizoplane and bulk soil samples were extracted by shaking as per Krogh et al. (2006). Following extraction, samples were filtered (0.22 µm filter) before storage in amber HPLC vials. Metabolic profiling of the extracts for targeted secondary metabolites was accomplished using an LC-MS/MS Q Trap 4500 Mass spectrometer (AB SCIEX) and validated using an Agilent 6410 LC-MS QQQ (Agilent).

2.3. Secondary metabolite analyses

Analytical standards for 10 BXs and two provisionally characterised BXs, as listed in Table 1, were gifted by the Fomsgaard lab in Denmark. Analytical grade methanol and acetonitrile were obtained from Rathburn (Walkerburn, Scotland). Glacial acetic acid was obtained from Baker (Griesheim, Germany). HPLC water used for the solvent mobile phase was purified with a Milli-Q Millipore purification system.

<table>
<thead>
<tr>
<th>Benzoazolinones</th>
<th>Lactams</th>
<th>Hydroxamic acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>R₁ R₂</td>
<td>R₁ R₂</td>
<td>R₁ R₂</td>
</tr>
<tr>
<td>H BOA</td>
<td>H H HBOA</td>
<td>H H DIBOA</td>
</tr>
<tr>
<td>OCH₃ MBOA</td>
<td>H Gle HBOA-Gle</td>
<td>H Gle DIBOA-Gle</td>
</tr>
<tr>
<td>OCH₃ H HMBOA</td>
<td>OCH₃ H DIMBOA</td>
<td></td>
</tr>
<tr>
<td>OCH₃ Gle HMBOA-Gle</td>
<td>OCH₃ Gle DIMBOA-Gle</td>
<td></td>
</tr>
<tr>
<td>H Gle-Hex* HBOA-Gle-Hex</td>
<td>H Gle-Hex* DIBOA-Gle-Hex</td>
<td></td>
</tr>
</tbody>
</table>

Figure 1: Chemical structures of the BXs most commonly found in cereal grains and bakery products. BOA, benzoxazolin-2-one; MBOA, 6-methoxy-benzoxazolin-2-one; HBOA, 2-hydroxy-1,4-benzoxazin-3-one; HMBOA, 2-hydroxy-7-methoxy-1,4-benzoxazin-3-one; HBOA-Glc, 2-β-D-glucopyranosylxy-1,4-benzoxazin3-one; HMBOA-Glc, 2-β-D-glucopyranosyloxy-7-methoxy-1,4-benzoxazin-3-one; HBOA-Glc-Hex, doublehexose derivative of HBOA; DIBOA, 2,4-dihydroxy-1,4-benzoxazin-3-one; DIMBOA, 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3- one; DIBOA-Glc, 2-β-D-glucopyranosyloxy-4-hydroxy-1,4-benzoxazin-3-one; DIMBOA-Glc, 2-β-D-glucopyranosyloxy-4-hydroxy-7-methoxy-1,4-benzoxazin-3-one; DIBOA-Glc-Hex, double-hexose derivative of DIBOA. *Structure not fully elucidated (Adhikari et al. 2015, Tanwir et al. 2013).
2.4. LC-MS/MS Detection and analysis – Q-Trap 4500 LC-MS/MS

Methanolic extracts were further diluted in methanol by 4–400 times depending on sample type. The diluted samples were analysed using electrospray ionisation and multiple reaction monitoring (MRM) mode using an Applied Biosystems 4500 Q Trap LC-MS (Nærum, Denmark). The chromatographic column used for the BX analysis was a Synergi Polar RP-80A with a 250 × 2 mm internal diameter and a 4 µm particle size (Phenomenex). The instrument and the compound-dependent parameters were optimised using analytical standards (Adhikari et al. 2015). Metabolites were analysed using negative ion mode in conjunction with optimal instrument parameters.

Nitrogen was used as a collision and source gas. The instrument and compound-dependent MS/MS parameters were optimised using flow injection analysis of individual authentic compounds. Two mobile phases (A, 7% acetonitrile in water; and B, 78% acetonitrile in water, each containing 20mM of acetic acid) were used in a linear gradient system as follows: 0–1 min, 92:08; 1–3 min, 90:10; 3–13 min, 30:70; 13–14 min, 10:90; 14–16 min, 10:90; 16–17 min, 100:0; 17–23 min, 100:0 of A:B at a flow rate of 300 µL min⁻¹ with an injection volume of 10 µL. Twelve BXs were identified based on comparisons of mass spectra and the selectivity of the LC separation producing matching peaks at the predetermined retention times for the authentic standards listed in Table 1.

<table>
<thead>
<tr>
<th>Standard</th>
<th>Acronym</th>
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<th>m/z Values</th>
<th>Retention time/min</th>
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<td></td>
<td></td>
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<td>Q1 Mass (Da)</td>
<td>Q3 Mass (Da)</td>
</tr>
<tr>
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<td>BOA</td>
<td>135.12</td>
<td>133.901</td>
<td>41.933</td>
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<tr>
<td>A</td>
<td>MBOA</td>
<td>165.15</td>
<td>163.930</td>
<td>148.867</td>
</tr>
<tr>
<td>A</td>
<td>HBOA</td>
<td>165.15</td>
<td>163.916</td>
<td>107.929</td>
</tr>
<tr>
<td>A</td>
<td>HMBOA</td>
<td>195.17</td>
<td>193.902</td>
<td>122.901</td>
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<tr>
<td>C</td>
<td>HBOA-Glc</td>
<td>327.2867</td>
<td>325.9</td>
<td>163.9</td>
</tr>
<tr>
<td>C</td>
<td>HMOA-Glc</td>
<td>357.3127</td>
<td>355.971</td>
<td>193.9</td>
</tr>
<tr>
<td>C</td>
<td>DIBOA</td>
<td>181.1455</td>
<td>179.888</td>
<td>133.976</td>
</tr>
<tr>
<td>C</td>
<td>DIMBOA</td>
<td>211.1715</td>
<td>210.1</td>
<td>164.3</td>
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<tr>
<td>L</td>
<td>DIBOA-Glc</td>
<td>343.2861</td>
<td>341.900</td>
<td>133.800</td>
</tr>
<tr>
<td>L</td>
<td>DIBOA-Glc-hex</td>
<td>373.3121</td>
<td>372.000</td>
<td>148.800</td>
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<tr>
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<td>505.43</td>
<td>504.000</td>
<td>133.900</td>
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<tr>
<td>L</td>
<td>HBOA-Glc-hex</td>
<td>489.43</td>
<td>488.100</td>
<td>163.900</td>
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</table>
2.5. Calibration curves – Analyst AB SCIEX

Calibration curves for the 12 BX standards listed in Table 1 were prepared by serial dilution of the pure standard compounds. Initially, a 10 ppm standard solution was diluted in 25% acetonitrile to the desired final concentrations. The concentrations used for the standard curves ranged from 0.05 to 200 ng L\(^{-1}\) (200, 100, 50, 25, 12.5, 6.25, 1.56, 0.39, 0.19, 0.95 and 0.05) and calibration curves were fitted using quadratic regression with Analyst software (v. 1.5) from AB SCIEX. The data points were weighted by 1/x (\(r >0.99\) in all cases). The standard curve was generated by plotting the area of the integrated peak (y-axis) as a function of concentration (x-axis).

2.6. LC-MS/MS Detection and analysis – Agilent 6410 Triple Quad LC-MS/MS

The sample extracts were diluted with aqueous solvent by 1 to 5 times and were analysed via electrospray ionisation and multiple reaction monitoring (MRM) mode using an Agilent 6410 Triple Quad LC-MS/MS (Agilent Technologies). The C\(_{18}\) column (Phenomenex Synergi Polar RP-80A) possessed a 250 × 2.0 mm internal diameter and a 4 µm particle size (Phenomenex Australia Pty Ltd). The instrument and the compound-dependent parameters were optimised using analytical standards for BOA and MBOA. Negative mode ionisation was used in conjunction with the following instrument parameters: curtain gas, 11 psi; temperature, 350°C; ion source gas 1, 60 psi; ion source gas 2, 60 psi; interface heater, on; collision gas, medium; and ion spray voltage, −4500 V.

The compounds were grouped according to dwell time, and the MRM mass transitions were assessed for each molecule at the corresponding time of elution. The instrument and compound-dependent MS/MS parameters were optimised using flow injection analysis of individual authentic standards. Mobile phases were as follows: A, 7% acetonitrile in water; and B, 78% acetonitrile in water, each containing 20mM of acetic acid, in a linear gradient system (0–1 min, 92:08; 1–4 min, 90:10; 4–14 min, 30:70; 14–15 min, 10:90; 15–24 min, 10:90; 24–25 min, 100:0; 25–28 min, 100:0 of A:B) at a flow rate of 300 µL min\(^{-1}\) with an injection volume of 10 µL. The 12 BXs were identified based on comparisons of mass spectra with known analytical standards and corresponding retention times as listed in Table 2. The scanning time increased from 23 to 28 minutes per sample.
### Table 3: Retention time (Rt) comparison between the AB SCIEX Q Trap 4500 and Agilent Triple Quad 6410 LC-MS/MS identification of the key benzoxazinoid metabolites in the tissues of wheat cultivars and rye scanned previously in the Q Trap (based on molecular weight, MRM transition ion pair, and retention time on chromatographic column). ND* means not detected in the samples used.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Mass</th>
<th>m/z Values</th>
<th>Q1 Mass (Da)</th>
<th>Q3 Mass (Da)</th>
<th>Q Trap Rt (min)</th>
<th>Triple Trap Rt (min)</th>
<th>Quad Rt (min)</th>
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<tr>
<td>BOA</td>
<td>135.12</td>
<td>133.901</td>
<td>41.933</td>
<td>12.98</td>
<td>11.75</td>
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<tr>
<td>MBOA</td>
<td>165.15</td>
<td>163.930</td>
<td>148.867</td>
<td>13.82</td>
<td>12.48</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HBOA</td>
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<td>10.69</td>
<td>9.56</td>
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<tr>
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<td>122.901</td>
<td>11.78</td>
<td>10.45</td>
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<tr>
<td>HBOA-Glc</td>
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<td>163.9</td>
<td>8.79</td>
<td>7.17</td>
<td></td>
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<tr>
<td>HMBOA-Glc</td>
<td>357.3127</td>
<td>355.971</td>
<td>193.9</td>
<td>10.11</td>
<td>8.62</td>
<td></td>
<td></td>
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<tr>
<td>DIBOA</td>
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<td>179.888</td>
<td>133.976</td>
<td>10.80</td>
<td>ND*</td>
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<tr>
<td>DIMBOA</td>
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<td>164.3</td>
<td>14.00</td>
<td>ND*</td>
<td></td>
<td></td>
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<tr>
<td>DIBOA-Glc</td>
<td>343.2861</td>
<td>341.900</td>
<td>133.800</td>
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<td>7.39</td>
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<tr>
<td>DIMBOA-Glc</td>
<td>373.3121</td>
<td>372.000</td>
<td>148.800</td>
<td>10.28</td>
<td>9.01</td>
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<td></td>
</tr>
<tr>
<td>DIBOA-Glc-hex</td>
<td>505.43</td>
<td>504.000</td>
<td>133.900</td>
<td>4.90</td>
<td>5.33</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HBOA-Glc-hex</td>
<td>489.43</td>
<td>488.100</td>
<td>163.900</td>
<td>4.90</td>
<td>3.99</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### 3. Results and discussion

Metabolic profiling of wheat secondary metabolites from roots, rhizoplane and bulk rhizosphere soil using LC-MS Q Trap targeted analysis of BXs resulted in detection of all the BX compounds evaluated. The BXs were identified at varying concentrations, depending on cultivar and plant part or soil type analysed (Table 4). Using chromatographic conditions described in the methodology, and the use of cereal rye as a positive control (rye extracts are known to contain high concentrations of BXs), well-resolved peaks for the 12 BX compounds evaluated were obtained with comparable retention times using both mass spectrometers and similar chromatography methods (Table 2).

Limit of detection (LOD) was determined by considering the signal to noise (S/N) ratio of 3:1 for the strongest mass transition with respect to the background noise for each metabolite and sample type. The detection limit is the lowest quantity of a substance that can be distinguished from the absence of that substance; a blank value (Browne and
Both instruments showed a similar level of sensitivity with relatively minor differences observed over metabolites surveyed (data not shown).

Table 4: Detection of targeted secondary metabolites (BXs) in cereal rye (Secale cereale) and wheat (Triticum aestivum L.) cultivar (Gregory and Wedgetail) shoot and root tissues, rhizoplane (root surface) and bulk soil surrounding field-grown living roots sampled in July 30, 2014 as assessed with AB SCIEX Q Trap 4500 LC-MS/MS. The root sample results for Wedgetail are not presented as they have not been analysed yet. The <LDL means lower than the detection limit.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Cereal</th>
<th>Rye</th>
<th>Gregory wheat</th>
<th>Wedgetail</th>
<th>Wheat</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Shoots</td>
<td>Roots</td>
<td>Rhizo</td>
<td>Soil</td>
<td>Shoots</td>
</tr>
<tr>
<td>HBOA-Glc-Hex</td>
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<td>0.08</td>
<td>&lt;LDL</td>
<td>&lt;LDL</td>
<td>0.94</td>
</tr>
<tr>
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<td>0.00</td>
<td>&lt;LDL</td>
<td>&lt;LDL</td>
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</tr>
<tr>
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<td>0.033</td>
<td>&lt;LDL</td>
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</tr>
<tr>
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<td>6.65</td>
<td>0.003</td>
<td>&lt;LDL</td>
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</tr>
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<td>3.63</td>
<td>0.016</td>
<td>&lt;LDL</td>
<td>0.06</td>
</tr>
<tr>
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<td>0.66</td>
<td>0.027</td>
<td>0.008</td>
<td>0.09</td>
</tr>
<tr>
<td>MBOA</td>
<td>0.98</td>
<td>625.12</td>
<td>2.797</td>
<td>0.047</td>
<td>104.13</td>
</tr>
<tr>
<td>HMBOA</td>
<td>0.04</td>
<td>3.27</td>
<td>0.018</td>
<td>0.003</td>
<td>1.67</td>
</tr>
<tr>
<td>DIMBOA</td>
<td>2.65</td>
<td>0.38</td>
<td>&lt;LDL</td>
<td>&lt;LDL</td>
<td>0.54</td>
</tr>
<tr>
<td>DIBOA</td>
<td>314.97</td>
<td>0.06</td>
<td>0.004</td>
<td>&lt;LDL</td>
<td>0.52</td>
</tr>
<tr>
<td>HBOA-Glc</td>
<td>5.32</td>
<td>2.69</td>
<td>0.037</td>
<td>&lt;LDL</td>
<td>5.03</td>
</tr>
<tr>
<td>HMBOA-Glc</td>
<td>0.68</td>
<td>13.02</td>
<td>0.013</td>
<td>&lt;LDL</td>
<td>23.30</td>
</tr>
</tbody>
</table>

Results obtained showed reasonable sensitivity and the presence of substantial quantities of nearly all metabolites profiled in both shoot and root tissues, as well as bulk and rhizosphere soils. By profiling the level of these metabolites in soils surrounding living root systems, the role of BXs in weed suppression will be further examined. Future results using numerous wheat cultivars could provide strong insight into the resulting availability of these metabolites following their release by exudation and incorporation of plant material into the soil (Krogh et al. 2006), as well as the complex interplay between plants and their associated rhizosphere microorganisms, an area which is relatively understudied (Weston et al. 2015).
Targeted metabolic profiling in soil and plant tissues will provide important physiological information regarding crop competitive traits and biosynthesis and activity of related allelochemicals that may be important in long-term weed suppression in crop. Currently, profiling of large sample data sets from 2014 and 2015 growing seasons are underway, with differences between season, location, cultivar and plant part the subject of further analysis.

4. Acknowledgements

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5. References


Tanwir, F., Fredholm, M., Gregersen, P.L. and Fomsgaard, I.S. (2013). Comparison of the levels of bioactive benzoxazinoids in different wheat and rye fractions and the transformation of these compounds in homemade foods. *Food Chemistry* 141 (1), 444-50.


Chapter 7.

**Metabolic Profiling of Benzoxazinoids and Phenoxazinones in the Roots, Rhizoplane and Rhizosphere of Weed Suppressive Wheat Cultivars**

After LC-MS spectrometry method development for both LCMS QToF and QQQ (Chapter 6). Crop shoot, root, rhizoplane, and rhizosphere soil samples were collected for metabolic profiling from both locations in 2015 and 2016. The abundance of benzoxazinoids (BXs) in several wheat cultivars under Australian conditions were assessed and investigated the role of genetics, environment and crop phenology on abundance of BXs in the shoots, roots and rhizoplane of these wheat plants, quantified microbial transformation products of BXs in the roots and rhizosphere of wheat and characterised the microbial community in the rhizosphere of these plants.

In this chapter metabolic profiling data of wheat roots, rhizoplane and rhizosphere including the key findings are presented and discussed. Metabolic profiling provided clear insights into biosynthesis and release of BX metabolites associated with weed suppression in commercial wheat cultivars, in comparison with cereal rye and the heritage wheat cultivar Federation. Phytotoxic microbial metabolites (aminophenoxazinones) including APO, AAPO and AMPO, were detected and transformed from benzoxazolinones produced by wheat and its root exudates by soil microbiota under field conditions.

This manuscript has been co-authored by James Mwendwa and Dr. Paul Weston. P. Weston participated in metabolite analysis and quantitation as well as statistical analysis of the data. All the authors contributed to the editing and reviewing the manuscript. L.A. Weston assisted in design and data collection/analysis of all experiments performed. In addition, Dr. Brown was involved in establishing and monitoring the field trials. Other contributors to this study are included in the acknowledgement.

Chapter 7 has been prepared as a manuscript for publication in the Journal of Plant and Soil; it is currently under review by the authors before submission.

Metabolic profiling of benzoxazinoids and phenoxazinones in the roots, rhizoplane and rhizosphere of weed suppressive wheat cultivars

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Abstract

Background and objectives: Sustainable, integrated weed management in commercial wheat production is urgently needed due to increasing herbicide resistance and production costs. We assessed benzoxazinoid (BX) abundance in several wheat cultivars under Australian conditions, investigated the role of genetics, environment and crop phenology on BX abundance in shoots, roots and rhizoplane, quantified microbial transformation products (phenoxazinones) of BXs in the roots and rhizosphere and characterised the rhizosphere microbial community.

Methods: Cultivar trials of wheat (Triticum aestivum L.) were conducted in two moderate to low rainfall (449-572 mm) locations in 2015 and 2016. Plant shoot, root, rhizoplane, and rhizosphere soil samples were collected for metabolic profiling. Extracts were analysed for allelochemicals and metabolites associated with weed suppression using liquid chromatography coupled to high resolution mass spectrometers.

Results Fifteen BXs and their microbially derived phenoxazinones were detected in shoots, roots, rhizoplane, and rhizosphere soil of wheat in both years and locations. The three most abundant BX metabolites in wheat tissues were MBOA, HMBOA and HMBOA-Glc, with the heritage cultivar Federation producing the highest levels of MBOA. The phytotoxic phenoxazinones APO and AMPO were the most abundant microbial transformation products...
of BXs and occurred in the roots, rhizoplane and rhizosphere, with clear differences in production depending on cultivar, phenology, location and year.

**Conclusions** Microbially-produced phenoxazinones generated from modern commercial wheat root exudates were detected and quantitated in rhizosphere soils and compared to those produced by cereal rye and the heritage wheat cultivar Federation, both recognized for their potent ability to suppress weeds. Production of microbial metabolites APO, AMPO, and AAPO was higher in the rhizosphere of young wheat plants than that of mature plants after flowering. This is the first demonstration of BX biotransformation to phenoxazinones in Australia, at rates high enough to produce ecologically relevant concentrations sufficient to suppress weeds under field conditions in certain wheat cultivars.

**Keywords:** benzoxazinones, phenoxazinones, wheat, soil microbiota, weed suppression, root exudation.

### 1. Introduction

Weeds are a persistent problem in cereal crops, increasing production costs while reducing crop yields (Wu 2016). Worldwide, yield losses of approximately 34% are caused by weeds among the major food crops and are typically higher than losses due to other crop pests (Jabran et al. 2015). This suggests there is an urgent need for effective integrated weed management (IWM) strategies in commercial cereal production, especially wheat. In addition, the lack of new herbicide chemistry and the emergence of herbicide resistance in weeds of cereal crops points to the need for alternative weed management strategies (Jabran et al. 2015; Heap and Duke 2018). However, additional information on specific crop–weed interactions and cultivar traits contributing to weed suppression are needed to facilitate the development of IWM programs in wheat (*Triticum aestivum* L.).

Crop competition and allelopathy are well-documented mechanisms of plant interference under controlled conditions (Weston 2005). The combined effects of both processes determine the total weed-suppressive potential of a crop cultivar, and research has been undertaken to improve both competitive features and allelopathic potential simultaneously to achieve maximum gains in crop weed suppression (Bertholdsson 2012, Worthington and Reberg-Horton 2013). Many plant species produce and release bioactive secondary metabolites that inhibit germination or growth of neighbouring plants (Belz 2007;
Macías et al. 2007). The production and exudation of secondary plant metabolites or allelochemicals in cereal crops are influenced by environmental conditions as well as biological factors and are, consequently, highly variable over time (Niemeyer 2009).

Benzoxazinoids (BXs) are a class of indole-derived plant defence chemicals comprising compounds with a 2-hydroxy-2H-1,4-benzoxazin-3(4H)-one skeleton and their derivatives (Wouters et al. 2016; Supplementary Table S1). These phytochemicals are widespread in grasses, including important cereal crops such as maize (Zea mays L.), wheat and rye (Secale cereale L.) (Niemeyer 2009; Frey et al. 2009), as well as a few dicot species, and display a wide range of antifeedant, insecticidal, antimicrobial and allelopathic activities (Wouters et al. 2016). The most abundant BXs in cereal crops include the hydroxamic acids DIBOA and DIMBOA, the lactams HBOA and HMBOA, and the benzoxazolinones BOA and MBOA. Lactams exist either as glucosides or aglycones (Hanhineva et al. 2011; Pedersen et al., 2017). The most commonly occurring lactams are 2-hydroxy-1,4-benzoxazin-3-one (HBOA), 2-hydroxy-7-methoxy-1,4-benzoxazin-3-one (HMBOA), 2-β-D-glucopyranosyloxy-1,4-benzoxazin-3-one (HBOA-Glc), 2-β-D-glucopyranosyloxy-7-methoxy-1,4-benzoxazin-3-one (HMBOA-Glc), and the dihexose derivative of HBOA (HBOA-Glc-Hex). Similarly, the most common hydroxamic acids in cereals are DIBOA, DIMBOA, DIBOA-Glc, DIMBOA-Glc, and dihexose derivative of DIBOA (DIBOA-Glc-Hex) (Adhikari et al. 2015; Tanwir et al. 2013).

The biosynthesis of BXs in cereal crop tissues and their biotransformation in the soil rhizosphere has been well documented and is summarised in Figure 1. The synthesis of BXs, most intensively investigated in maize, is initiated by the conversion of indole-3-glycerol phosphate to indole in plastids. Subsequently, four cytochrome P450 dependent monooxygenases (BX2-BX5) convert indole to benzoxazinone by incorporation of oxygen. DIBOA-glucoside (2-β-D-glucopyranosyloxy-4-hydroxy-1,4-benzoxazin-3-one) is synthesized by glucosylation of DIBOA (2,4-dihydroxy-1,4(2H)-benzoxazin-3-one) at the 2-position (Dick et al. 2012) in the cytosol. The resulting glucoside is the precursor of 2-β-D-glucopyranosyloxy-4-hydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA-glucoside) (Frey et al. 2009; Schulz et al. 2013). The glucosides are then transported to vacuoles, where they are stored until cellular damage or decomposition occurs. Aglycones (e.g. DIBOA,
DIMBOA, etc.) are liberated upon hydrolysis of the glycosides and can be passively or actively transported outside the root (Villagrasa et al. 2009; Frey et al. 2009).

Figure 13: Schematic illustration of biosynthesis of benzoxazinoids in graminaceous plant tissue and microbial production of aminophenoxazinones in rhizosphere soil. Benzoxazinoids include the benzoxazolinones (BOA and MBOA), hydroxamic acids (DIBOA and DIMBOA), and lactams (HBOA and HMBOA).

According to Niemeyer (2009), hydroxamic acids are the most active class of BXs by virtue of a hydroxyl group bound to the heterocyclic nitrogen atom, although studies on pests of maize by Cambier et al. (2001) and Glauser et al. (2011) have shown that the methylated forms of hydroxamic acids are far more toxic than de-methylated. For example, 2-β-D-glucopyranosyloxy-7-methoxy-1H,1,4-benzoxazin-3(4H)-one (HDMBOA-Glc) was shown to have greater toxicity to Metopolophium dirhodum, an aphid, than DIMBOA-Glc (Cambier et al. 2001; Makowska et al. 2015). Among the BXs, the most effective phytotoxins
are DIBOA, DIMBOA and their degradation products BOA and MBOA (Tabaglio et al. 2008). The glucosylated benzoxazinoids are precursors of the aglycone BXs released over time from various plant tissues (Rice et al. 2012). Typically, glucosylated forms lose the glucose moiety and release the base structures HBOA, DIBOA, HMBOA, and DIMBOA when plant cells are damaged or disrupted (Fig. 1).

Owing to specific bioactivity against both microbial pests and weeds, BXs have been studied for their potential agronomic utility as natural herbicides in weed management, for example, by their incorporation as green manures in soil or use as cover crops (Mathiassen et al. 2006). Outside the Poaceae, BXs have been detected in the dicot families Acanthaceae, Ranunculaceae, Plantaginaceae, and Lamiaceae (Frey et al. 2009; Makowska et al. 2015). However, in contrast to other potentially toxic secondary metabolites, BXs have not yet been targets of selection in plant breeding programs (Niculaes et al. 2018).

Root exudates represent one of the greatest inputs of plant metabolites into the rhizosphere, and therefore likely represent the major source of allelochemical inputs as well (Bertin et al. 2003). Root exudation by wheat, as measured by excretion of phenolics into agar media, has been found to vary with accession (Wu et al. 1999; 2000). Analysis of laboratory results by multiple regression showed that wheat cultivar accessions with strong allelopathic potential produced significantly higher levels of allelochemicals in both shoots and roots than accessions with weaker allelopathic potential, but root exudates generally contained higher total levels of hydroxamic acids than did shoot extracts (Wu et al. 1999; 2000; 2001).

Numerous bioassays have been used to identify the potential contribution of BXs to weed suppression in cereals (Wu et al. 2000; Worthington et al. 2014; Bertholdsson 2011). However, allelopathic interactions are complex and often involve multiple compounds (Cheng and Cheng 2015) and may be mediated indirectly through biotransformation of exuded compounds by soil microbes; in fact, microbial degradation products may be significantly more potent than the original exuded precursors (Fomsgaard et al. 2004). This highlights the key role of microbes in plant-plant interactions mediated by chemicals. Upon release from a source organism, the soil is the main vehicle that mediates contact between allelochemicals and their target plants (Muzell Trezzi et al. 2016). As a result, soil properties including organic matter, reactive mineral surfaces, ion exchange capacity, inorganic ions
and abiotic and biotic factors of the soil environment significantly influence allelochemical activity (Inderjit 2001; Blum 2006).

After entering the soil, DIMBOA and DIBOA are degraded to the benzoxazolinones 6-methoxy-2-benzoxazolinone (MBOA) and 2-benzoxazolinone (BOA), respectively (Fomsgaard et al. 2006) (Supplementary Table S1). These benzoxazolinones are further transformed in the soil through microbial activity to aminophenoxazinones [i.e. 2-amino-3-H-phenoxazin-3-one (APO), 2-acetylamino-3-H-phenoxazin-3-one (AAPO), 9-methoxy-2-amino-3-H-phenoxazin-3-one (AMPO), and 2-acetylamino-9-methoxy-2-amino-3-H-phenoxazin-3-one (AAMPO)] (Table S1), acetamide [i.e. N-(2-hydroxyphenyl) acetamide (HPAA)] and corresponding malonamic acids [i.e. N-(2-hydroxyphenyl) malonamic acid (HPMA), and N-(2-hydroxyphenyl-4-methoxyphenyl) malonamic acid (HMPMA)] (Fomsgaard et al. 2004; Understrup et al. 2005; Villagrasa et al. 2009).

In maize and wheat, DIMBOA is the major benzoxazinoid while DIBOA is prevalent in cereal rye (Secale cereale) shoot tissue, and DIMBOA is found in the roots of all three species (Rice et al. 2005). Others have similarly confirmed that the benzoxazinoid profile in cereals varies with plant part, development stage, cultivar, and growing conditions (Carlsen et al. 2009; Hanhineva et al. 2011). In wheat, Chen et al. (2010) found that the concentration of the hydroxamic acid MBOA in the rhizosphere varied with cultivar, plant density, and growth conditions (Chen et al. 2010). Interestingly, biosynthesis of BXs is usually at its highest during the juvenile stage of plant growth, after which it declines and typically stabilises (Ebisui et al. 1998; Nomura et al. 2005, 2008). Other key factors influencing BX biosynthesis include photoperiod (Epstein et al. 1986), light intensity and the application of fertiliser (Manuwoto and Scriber 1985).

Metabolic profiling of allelochemicals in the rhizosphere can provide strong insight into the decomposition of plant material following its incorporation into the soil (Krogh et al. 2006) as well as the complex interplay between plants and their associated rhizosphere microorganisms, an area which is relatively understudied (Weston et al. 2015). The importance of microbes in the degradation of allelochemicals is now evident, and steady progress is being made in understanding how interactions between allelochemicals and biotic and abiotic components of the soil matrix affect degradation, transport and phytotoxicity (Barto et al. 2011).
Given our interest in weed suppressive cereal crops and the further evaluation of allelochemical interactions in their rhizosphere soils, we performed a comprehensive series of field and metabolic studies with wheat, the most economically important cereal crop in Australia, in multiple locations over two successive cropping years. Our objectives were to 1) compare the abundance of BXs produced in selected commercial wheat cultivars under Australian conditions, some of which are known to be more weed suppressive than others, using metabolic profiling by LC-MS; 2) investigate the role of genetics, environment and crop phenology on the production of BXs in wheat roots and their release into rhizosphere soil; 3) quantify the phenoxazinones (microbial transformation products of BX metabolites) in roots and rhizospheres of various wheat cultivars; and 4) characterise the microbial community present in the rhizosphere of selected wheat cultivars. In each case, results for commercial wheat cultivars were compared with the heritage cultivar Federation and winter cereal rye (*Secale cereale* L.), both of which are recognized as being strongly suppressive to weeds.

2. Materials and methods

2.1. Field experimentation

In 2015 and 2016, replicated wheat field trials (six replicates per cultivar) were sown at two locations in moderate to low rainfall zones at Wagga Wagga (572 mm, average rainfall per year) and Condobolin (449 mm, average rainfall per year) NSW, respectively. Plots (12 m x 2 m) were seeded at identical planting densities in six replications arranged in a randomised complete block design. In both years, seven wheat cultivars representing four major genetic backgrounds of winter and spring wheat commercially grown in Australia were evaluated, plus one cultivar of winter cereal rye (*Secale cereale* L.) as a positive suppressive control (Table 1).

Wheat cultivars included modern commercial wheat cultivars with both short and moderate time to maturity along with grazing winter wheat cultivar and the heritage cultivar Federation, bred and released in Australia in 1901 (Table 1). Federation is considered to be both early-maturing and drought resistant and was included for comparison because of its inherent weed suppressive abilities and upright growth habit (Mwendwa et al. 2018).
Experimental sites were established in close proximity at each location in both 2015 and 2016 following a canola rotation at each site.

At Wagga Wagga, field trials were conducted on fine red clay loam sodosols, surface (0-10 cm) pH 6.4, that were previously planted commercially for the production of cereals, canola and/or lucerne (*Medicago sativa* L.). At Condobolin, soils were predominantly red gradational, and red-brown earth sodosols with surface pH 7.0 and were previously rotated among cereals and pasture legume crops. Both soils exhibited low inherent fertility and organic matter content and were maintained using standard commercial practices to reduce weed populations.

All crops were established with seed that was generated in Wagga Wagga NSW from harvest the previous season to potentially eliminate any cultivar variation due to seed variability resulting from production at different locations. At Condobolin, the crop was sown on 15th and 17th May at 33 cm spacing, typical for drier soils, while at Wagga Wagga the crop was sown on 22nd and 14th May at 25 cm spacing for 2015 and 2016, respectively. Cultivars were established at equal plant density (target population of 120 plants m⁻²) in each trial.

Trials were sown in both locations with a knife-point and press wheel planter. Fertiliser was applied at 70 kg ha⁻¹ diammonium phosphate (DAP) (Incitec Pivot Fertilisers) treated with 400 ml ha⁻¹ Flutriafol (Intake® Hiload Gold 200 g ha⁻¹ Flutriafol, Crop Care). Before sowing, all established weeds were controlled with glyphosate (Weedmaster® DST® 470 g L⁻¹ Glyphosate, Nufarm) at 960 g ha⁻¹.
Table 1: Selected wheat cultivars based on above-ground crop competitiveness against weeds in field trials performed in Condobolin and Wagga Wagga, NSW in 2015 and 2016.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Breeder</th>
<th>Main use</th>
<th>Growth characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Condo</td>
<td>AGT¹</td>
<td>grain</td>
<td>Early maturity adapted to low–medium rainfall areas. Similar in maturity to Livingston. Excellent physical grain quality. A tall plant type with medium straw strength. Released in 2014.</td>
</tr>
<tr>
<td>Espada</td>
<td>AGT</td>
<td>grain</td>
<td>Mid-season maturity. Good seedling vigour. Large grain with low screenings.</td>
</tr>
<tr>
<td>Federation²</td>
<td>William Farrer</td>
<td>grain</td>
<td>The first Australian variety that is both rust- and drought-resistant (release 1901). Early maturing, high-yielding and drought-tolerant with strong straw. Awnless and improved baking quality and broad adaptation.</td>
</tr>
<tr>
<td>Grazer (Cereal rye)³</td>
<td></td>
<td>dual purpose</td>
<td>Rapid growth with early vigour and grazing possible four weeks after emergence if tillering and the secondary root system development has occurred to anchor the plant.</td>
</tr>
<tr>
<td>Gregory</td>
<td>EGA⁴</td>
<td>grain</td>
<td>Excellent yield potential in early to mid-season sowings. Medium to slow maturity.</td>
</tr>
<tr>
<td>Janz CL</td>
<td>AGT</td>
<td>grain</td>
<td>Widely adapted Clearfield® variety. Moderate seedling vigour. Medium–strong straw strength, with good lodging &amp; shattering resistance.</td>
</tr>
<tr>
<td>Livingston</td>
<td>AGT</td>
<td>grain</td>
<td>Early maturing cultivar.</td>
</tr>
<tr>
<td>Wedgetail</td>
<td>EGA</td>
<td>dual purpose</td>
<td>Dominant winter wheat. Large grain size. Adapted to higher rainfall regions of the wheat belt.</td>
</tr>
</tbody>
</table>

¹Australian Grain Technologies, ²bred by William Farrer in 1901, ³Cereal rye was used as a control in all the field trials, ⁴Enterprise Grains Australia.
2.2. Tissue and soil sampling

Shoots (green, healthy, mature expanded leaf tissue), roots (primary and secondary, collected at 10 to 20 cm depth), rhizoplane (root surface) and rhizosphere soil (associated with the plant rooting zone from 10 to 20 cm below soil surface) were randomly sampled by fresh tissue collection at both field locations. Sampling consisted of tissue collection from two sub-plots per plot (five plants per sub-plot per replicate, n=30) which were later combined to form one composite sample of each tissue type per replicate (n=6). In this case, we analysed 5 g shoots, roots or rhizosphere soil at three selected phenological stages of cereal growth in both 2015 and 2016. Sampling was thus performed at early vegetative growth (tillering stage), vegetative mid-season growth (stem elongation stage), and maturity (grain fill stage). All plant material and rhizosphere soils were immediately placed post-sampling in an insulated cooler packed with ice for transportation to the laboratory and then stored at -80 °C until extraction.

2.3. Sample extraction procedure

Frozen plant tissue was extracted with HPLC-grade methanol (Honeywell Burdick & Jackson, Muskegon, Michigan USA) using a pressurised solvent extraction system (E-916 Büchi, Switzerland) (Dayan et al. 2010). Nitrogen gas at high pressure facilitated rapid infiltration of the solvent into plant cells while limiting oxidation of secondary metabolites. Plant material (5 g of roots and shoots) was separately removed from the -80 °C freezer, thawed and mixed thoroughly with quartz sand, (particle size, 0.3–0.9 mm) (Büchi, Switzerland, 034925) and placed in 10 mL extraction cells (Buchi, Switzerland). The extraction was conducted under the following conditions: solvent - 100% methanol; temperature - 35 °C; pressure - 1400 psi. Samples were dried using a rotary evaporator (Multivapour P-6, Büchi, Switzerland) at 35 °C and reconstituted to 10 mL in methanol and stored in the dark at 4 °C.

Rhizoplane extraction was performed by placing 5 g of root tissue from each sample in 250 mL conical flasks with 50 mL methanol in a Ratek digital orbital shaker (Ratek Instruments, Australia) at 100 rpm for 20 min at room temperature. The suspension was filtered through Whatman No 1. filter paper, rotary evaporated to dryness and reconstituted as above.
Rhizosphere soils (6 g) were extracted in 250 mL conical flasks with 50 mL methanol in a rotary shaker at 125 rpm for 60 min at 40 °C. The soil suspension was filtered through Whatman No 1. Filter paper, rotary evaporated to dryness and reconstituted as above. All extracts including those of the rhizoplane and rhizosphere soils were filtered through 0.20 μm polytetrafluoroethylene (PTFE) syringe filters (Captiva Econofilter, Agilent Technologies, Australia) and stored in amber HPLC vials at 4 °C until further LC-MS analysis. Rhizosphere and rhizoplane extraction methods were based on those developed by Fomsgaard et al. 2006.

2.4. UHPLC-QTOF-MS and QQQ-MS analysis

Metabolic profiling of the extracts for targeted secondary metabolites was first performed (one injection per sample per replicate) using an LC-MS/MS Q Trap 4500 Mass spectrometer (AB SCIEX) and validated using an Agilent 6410 LC-MS QQQ (Agilent) during method development. Further analysis for secondary metabolites or allelochemicals associated with weed suppression was performed using liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (UHPLC-ESI MS QToF, Agilent 6530). Benzoxazinoids (BXs) were profiled in negative ion mode, and microbially-produced metabolites (APO, AAPO, AMPO, and AAMPO) were profiled in positive ion mode by LCMS QQQ (Agilent 6470A).

2.4.1 Benzoxazinoids analysis - UHPLC-QTOF-MS

Metabolic profiling of root tissue, rhizoplane and rhizosphere soil extracts was performed using an Agilent 1290 Infinity UHPLC system equipped with a quaternary pump, diode array detector (DAD), degasser, temperature controlled column (25 °C) and cooled auto-sampler compartments (4 °C) which were coupled to an Agilent 6530 quadrupole time-of-flight (QTOF) mass spectrometer (MS) with an Agilent Dual Jet Stream electrospray ionisation source (Agilent Technologies, Australia). Capillary voltage was set at 3500 V, and fragmenter voltage was 150 V. Nitrogen was used as the drying gas at a flow of 9 L/min, and the nebuliser pressure was 35 psig. Sheath gas flow and temperature were 10 L/min and 250 °C, respectively. Full scan mass spectra were acquired over a range of 100-1700 m/z at a rate of two spectra/second using negative ion mode.
Chromatographic separation was performed using a Synergi™ Polar-RP column (2.0 mm x 30 mm, 2.5 µm particle size) (Phenomenex, Torrance, CA USA) preceded by a guard column (2.0 x 3.0 mm, 2.5 µm particle size) of the same phase. The flow rate of the mobile phase was 0.3 mL min-1. Separation was obtained using a gradient of solvent A [92% water (Milli-Q, TKA-GenPure) + 7.0% HPLC-grade acetonitrile (RCI Labscan, Thailand)] amended with 0.1% (20 mM glacial acetic acid obtained from Baker, Griesheim, Germany) and solvent B [78% HPLC-grade acetonitrile (RCI Labscan, Thailand) + 21% water (Milli-Q, TKA-GenPure) amended with 0.1% (20 mM glacial acetic acid]. The solvent gradient began with 100% A for 1 min, then ramping to 100% B over 1-4 minutes, holding at 100% B for 30 seconds and then ramping back to 100% A over 0.1 min and holding at 100% A from 4.6-9 min. After every 20 samples, a quality control sample was analysed using a blank for comparison and a standard mixture of BX metabolites (BOA and MBOA at a concentration of 1.5mg/ml.

The target metabolite compounds were annotated based on matching molecular features extracted from chromatograms with Agilent Profinder (v. B.08) with a personal library of molecular formulas (Agilent PCDL Manager v. B.08.00), subject to matching the order of elution based on previous analysis performed by Mwendwa et al. (2016) using standards in the Fomsgaard laboratory at Aarhus University in Denmark.

2.4.2 Phenoxazinone analysis - UHPLC-QQQ-MS

Targeted metabolic profiling of root tissue, rhizoplane and rhizosphere soil extracts for phenoxazinones was performed using an Agilent 1290 Infinity UHPLC system equipped with a quaternary pump, degasser, temperature-controlled column (25 °C) and cooled auto-sampler compartments (4 °C) which were coupled to an Agilent 6470 Triple Quadrupole (QQQ LC-MS) mass spectrometer with a Jet Stream technology ionisation source (Agilent Technologies, Australia). Capillary voltage was set at 3500 V, fragmentor voltage was 135 V, dwell 200, collision energy 20 and cell accelerator voltage 5. Nitrogen was used as the drying gas at a flow of 9 L min⁻¹, and the nebuliser pressure was 35 psig. Sheath gas flow and temperature were 10 L min⁻¹ and 250 °C, respectively.

Multiple reaction mode (MRM) analyses were performed to quantitate the phenoxazinone metabolites (APO, AAPO, AMPO and AAMPO) using positive ion mode. APO and AAPO were quantified using a single MRM experiment tracking the transition
213→185 \textit{m/z} (because of the structural similarity of APO and AAPO, both molecules were detected by the method designed to quantify APO). AMPO was quantified in a separate MRM run tracking the transition 243→228 \textit{m/z}. AAMPO was not detected using this transition.

Chromatographic separation was performed using the same column as previously described. The flow rate of the mobile phase was 0.3 mL min\(^{-1}\). Separation was obtained using a gradient of solvent A [100% water (Milli- Q, TKA-GenPure) + 0.1% formic acid (LC–MS grade, LiChropur®, 98–100%, Sigma-Aldrich, USA)] and solvent B [5% water + 95 % HPLC-grade acetonitrile (RCI Labscan, Thailand) + 0.1% formic acid]. The gradient was initiated with 30% solvent B for 1 min, ramping up to 100% B over the next 5 minutes, holding at 100% for 30 s until ramping down to 30% B over the next 0.1 min. Every 20 samples, a quality control sample was analysed using a blank for comparison and a standard mixture of phenoxyazinone metabolites (APO, AAPO and AMPO) at a concentration of 3.3 µg ml\(^{-1}\).

2.5. Soil DNA extraction procedure

Genomic DNA was extracted from a composite soil sample consisting of 3 subplots per replicate which were combined to form two samples of 0.5 g each of field soil. The soil was collected from the rhizosphere (10-20 cm below the soil surface) of each of the seven wheat cultivars (immediately post-harvest) and a control using a Mo Bio Laboratories DNeasy® PowerSoil® DNA isolation kit (Mo Bio Laboratories, Carlsbad, CA, USA) following the manufacturer’s instructions. The DNA concentration was quantified using a Nanodrop 2000C spectrophotometer (Thermo Scientific, Waltham, MA, USA) spectrophotometer and the final concentration adjusted to 10 ng µl\(^{-1}\) with elution buffer.

2.6. Genomic and Meta-genomic Analysis

A total of fourteen 10 µl aliquots of the genomic DNA from each extraction was submitted to the Australian Genome Research Facility (AGRF, Brisbane, QLD Australia) for sequencing by Illumina Hi-Seq (using sequencing by synthesis; SBS), targeting the 27-519 region of bacterial 16S ribosomal RNA and the eukaryotic internal transcribed spacer (ITS)
2 region for fungi; Primer sequences utilised (universal degenerate primers) are listed in Table 2.

**Table 2: Primers used for Illumina sequencing of DNA extracted from soil.**

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>27F – Universal 16S</td>
<td>AGAGTTTGATCMTGGCTCAG</td>
<td>(Lutzoni Lab, 2014)</td>
</tr>
<tr>
<td>519R – Universal 16S</td>
<td>GWATTACCGCGGCKGCTG</td>
<td>(Lutzoni Lab, 2014)</td>
</tr>
<tr>
<td>1F – Universal ITS</td>
<td>CTTGGTCATTTAGAGGAAGTAA</td>
<td>(University of California Berkley), 2014</td>
</tr>
<tr>
<td>2R – Universal ITS</td>
<td>TGTGTTCTTCCATCGATG</td>
<td>(University of California Berkley), 2014</td>
</tr>
</tbody>
</table>

**2.7. Statistical analysis**

Data on compound abundance was log-transformed prior to analyses. Statistix v. 10 (Analytical Software, Tallahassee, FL, USA) was used to perform analysis of variance for metabolite abundance with means separated using LSD (0.05 confidence level). The standard error of the means was used to show the variability of the abundance. PCA plots were produced using Agilent Mass Profiler Professional v. B14.9.

Statistical analysis of genomic data was performed by AGRF using QIIME (Caporaso et al. 2010), and sequence alignments were developed with BLAST: Basic Logical Alignment Search Tool (National Center for Biotechnology Information, 2017) to generate data on bacterial and fungal Operational Taxonomic Units (OTUs).
3. Results

3.1. Benzoxazinoids and derivatives detected in plant tissue and rhizosphere soil

Fifteen unique BXs and their derivatives (hydroxamic acids, lactams, and benzoxazolinones) were detected in shoots, roots, rhizoplane and rhizosphere soil of wheat and rye (positive control) in both years and locations. Table 3 provides a summary of metabolites detected in plant tissue and soil. Phenoxazinones (APO, AAPO, AMPO) produced by microbial transformation were detected in trace quantities in the shoots but were present in abundance in root, rhizoplane and rhizosphere extracts. However, AAMPO was detected only in trace quantities or not at all in the root, rhizoplane and rhizosphere extracts. HBOA-Glc-Hex, DIBOA-Glc-Hex, DIBOA-Glc, DIMBOA-Glc were not detected in the rhizoplane or rhizosphere samples of wheat. DIMBOA was not detected in the soil rhizosphere of any of the wheat cultivars.
The relative distribution of metabolites detected in wheat roots, rhizoplane (root surface) and shoots clearly varied with tissue type, with unique metabolic profiles for each (Fig. 2). However, metabolite profiles for the seven wheat cultivars were quite similar, as indicated by the extensive overlap of cultivars (distinguished by shape symbols) in each tissue type profiled (as distinguished by colour).
3.2. Benzoxazinoids and derivatives abundance in wheat tissues

The three most abundant BX metabolites detected and identified in wheat tissues included MBOA and two of its derivatives, HMBOA and HMBOA-Glc. Significant differences in metabolite abundance were noted, based on growth stage, tissue type, location and cultivar. While HMBOA and HMBOA-Glc levels were similar over sampling time, MBOA was four times more abundant during the early and mid-season vegetative stages as opposed to crop maturity (post-flowering) ($P < 0.001$, Supplementary Table S3, Fig. 3). The
abundance of MBOA was higher in the shoot, root and rhizoplane samples at vegetative stages of growth ($P < 0.001$, Supplementary Table S3, Fig. 4) and was dependent on year, location and cultivar. For example, MBOA abundance was similar at Wagga Wagga in both 2015 and 2016 but was higher at Condobolin in 2015 in contrast to 2016, a year with above average rainfall in Condobolin ($P < 0.001$, Supplementary Table S3, Fig. 4). MBOA levels also differed significantly among cultivars, with the heritage cultivar Federation exhibiting the greatest abundance of MBOA ($P < 0.05$, Supplementary Table S3, Fig. 6).

Figure 15: The most abundant wheat BX metabolites detected based on sampling time and crop growth stage. Values were averaged across year, location, cultivar and tissue. E = early (tillering), M= mid (vegetative) and L = late (mature post-flowering) phenological stages. Error bars indicate standard errors.
Figure 16: MBOA abundance in wheat shoots, roots and rhizoplanes based on sampling time and crop growth stage. Values were averaged across year, location and cultivar [E = early (tillering), M= mid (vegetative) and L = late (mature post-flowering) crop phenological stages]. Error bars indicate standard errors.

Figure 17: MBOA abundance in wheat shoots, roots and rhizoplanes based on year and location. Values were averaged across, cultivar, tissue and crop growth stage. Error bars indicate standard errors.
Figure 18: Most abundant wheat BX metabolites based on cultivar (Con = Condo, Esp = Espada, Fed = Federation, Gre = Gregory Jan = Janz CL, Liv = Livingston, Wed = Wedgetail). Values were averaged across year, location, tissue, and crop growth stage. Error bars indicate standard errors.

3.3. The abundance of BOA and MBOA in wheat roots rhizoplane and rhizosphere

BOA was detected in significant quantities in both wheat and cereal rye roots, rhizoplanes and rhizosphere soils. Remarkably, BOA abundance was seven times greater in rye rhizosphere soils and three times greater in rye roots and rhizoplanes in contrast to roots and rhizosphere soils of all wheat cultivars ($P < 0.001$, Supplementary Table S4, Fig. 7). In contrast, MBOA abundance was similar in rye and wheat tissues but was highest in the heritage wheat cultivar Federation (Fig. 8). MBOA levels were higher in root and rhizoplane samples than in rhizosphere soils ($P < 0.001$, Supplementary Table S3) while BOA was present in highest abundance in the soil rhizosphere of cereal rye ($P < 0.001$, Supplementary Table S4). MBOA abundance in roots, rhizoplanes and rhizosphere soils differed significantly by cultivar ($P < 0.001$, Supplementary Table S3).
Figure 19: The abundance of 2-benzoxazolinone (BOA) in wheat cultivars and rye (positive control) based on sample type and cultivar. (Cultivars include Con = Condo, Esp = Espada, Fed = Federation, Gre = Gregory Jan = Janz CL, Liv = Livingston, Rye = Cereal rye, Wed = Wedgetail). Values were averaged over year, location, and crop growth stage. Error bars indicate standard errors.

Figure 20: The abundance of 6-methoxy-2-benzoxazolinone (MBOA) in wheat cultivars and rye (positive control) based on sample type and cultivar. (Cultivars include Con = Condo, Esp = Espada, Fed = Federation, Gre = Gregory Jan = Janz CL, Liv = Livingston, Rye = Cereal rye, Wed = Wedgetail). Values were averaged across the year, location, and crop growth stage. Error bars indicate standard errors.
3.4. The abundance of phenoxazinones in wheat roots rhizoplane and rhizosphere

APO and AMPO were detected in abundance on the surface of wheat roots and rhizosphere soils and were the most abundant microbial metabolites at the three crop growth stages monitored [early vegetative (tillering), mid-season (vegetative) and maturity (post-flowering)] ($P < 0.001$, Supplementary Tables S5 & S6, Fig. 9). AAPO was less abundant than phenoxazinones APO and AMPO at all crop growth stages and was detected only in trace quantities. APO and AMPO were generally highest at the early vegetative crop growth stage. Cultivar differences in the abundance of AMPO and APO were also noted ($P < 0.001$, Supplementary Tables S5 & S6, Fig. 10); in this case Federation, Janz CL and Wedgetail exhibited the greatest abundance in contrast to modern wheat cultivars.

Significant differences were noted in the abundance of phenoxazinones (APO, AMPO and AAPO) in the roots, rhizoplane and rhizosphere by tissue, location and year ($P < 0.001$, Supplementary Tables S5, S6 & S7). Phenoxazinone metabolite levels were higher in 2015 than 2016 at both locations, with the exception of APO, which was highest in the rhizoplane of Condobolin samples in 2016. In both years, AMPO levels were higher than APO in the roots, but abundance was similar in the rhizoplane. In addition, metabolite abundance varied with location in both 2015 and 2016 ($P < 0.001$, Supplementary Tables S5 & S6). In both locations, APO, AAPO and AMPO were detected in rhizosphere samples, but at significantly reduced abundance compared to root and rhizoplane samples.
Figure 21: The abundance of aminophenoxazinones (APO, AAPO and AMPO) in the roots, rhizoplane and rhizosphere of wheat by sampling time (indicating crop growth stage) \([E = \text{early (tillering)}, M = \text{mid (vegetative)} \text{ and } L = \text{late (mature post-flowering)} \text{ crop phenological stages}]. \) Values were averaged across year, location, cultivar and tissue. Error bars indicate standard errors.

Figure 22: The abundance of aminophenoxazinones (APO, AAPO and AMPO) in the roots, rhizoplane and rhizosphere of wheat by cultivar (Con = Condo, Esp = Espada, Fed = Federation, Gre = Gregory Jan = Janz CL, Liv = Livingston, , Wed = Wedgetail). Values were averaged over year, location, tissue and crop growth stage. Error bars indicate standard errors.
Figure 23: The abundance of aminophenoxazinones (APO, AAPO and AMPO) in the roots, rhizoplane and rhizosphere of wheat by tissue, location and year. Values were averaged across wheat cultivar, tissue and crop growth stage. Error bars indicate standard errors.

Not unexpectedly, the abundance of APO was up to 20-fold greater in rye in comparison to wheat samples ($P < 0.001$, Supplementary Table S8) and was highest in rye root and rhizoplane samples in contrast to rhizosphere soil ($P < 0.001$, Supplementary Table S8, Fig. 12).
Figure 24: The abundance of APO, AAPO, and AMPO in the root, rhizoplane and rhizosphere of rye and wheat (averaged over seven wheat cultivars). Error bars are standard errors.

3.5. Rhizosphere soil microbial diversity

The composition of bacterial and fungal OTU (Operational Taxonomic Units) is presented by relative abundance by phyla in various wheat cultivars (Figure S1 and S2) from soils collected in 2016 following wheat harvest. Cultivar differences were noted but in most cases were not statistically significant. In all samples, the Ascomycota predominated among the fungi, while among bacterial phyla the Actinobacteria and Proteobacteria predominated.
4. Discussion

The BX metabolite BOA in plants was first reported in cereal rye (Virtanen and Hietala, 1955), while the methoxy derivative MBOA was first isolated from wheat and maize (Virtanen et al. 1956). BOA and MBOA were later shown to be degradation products of DIBOA and DIMBOA (the 7 methoxy derivative) respectively, and their corresponding glucosides (Wahlroos and Virtanen, 1959). Since these initial studies, the biosynthesis of benzoxazinoids (BXs) has been well described in both monocots and dicots (Dick et al. 2012), and the related microbial biotransformation pathway of phenoxazinones has also been elucidated in both rye and wheat rhizospheres (Fomsgaard et al. 2006; Chen et al. 2010).

Numerous studies have more recently attempted to quantify the production of BX metabolites in wheat and correlate their presence with allelopathy or weed suppression. Allelopathy and competitive ability are complex, quantitatively inherited traits that are heavily influenced by environmental factors (Worthington and Reberg-Horton, 2013). Varietal rankings in wheat for weed suppressive ability are often inconsistent across growing seasons (Seavers and Wright, 1999) and study locations (Mokhtari et al. 2002; Worthington and Reberg-Horton, 2013), indicating strong genotype by environment interactions.

Despite noting cultivar and locational differences in weed suppression by wheat cultivars, associated metabolite profiling studies in wheat have typically reported on the production of a relatively few BX metabolites under controlled laboratory conditions (Belz and Hurle. 2005; Wu et al. 2000). This study, in contrast, describes the production of 15 key BX metabolites in field-grown Australian wheat cultivars and their rhizospheres, using a sensitive metabolic profiling system by LC- MS QToF mass spectrometry, with fresh samples obtained from replicated trials performed over multiple years and locations, using a collection of genetically diverse cultivars known to exhibit differential weed suppressive ability (Mwendwa et al. 2016). By optimising metabolic profiling of BXs in the wheat shoot, root, rhizoplane and soil extracts, we were able to detect numerous individual BX metabolites in one rapid analysis using a polar reverse phase column for the large number (~2000) of samples generated in this study.

In addition, we performed and optimised the subsequent analysis of related microbially transformed soil metabolites, the phenoxazinones, using the same column. Herein we successfully report on the detection and abundance of 15 BXs including BX
glycosides, lactones, and hydroxamic acids, as well as four biotransformed phenoxazinones known to exhibit potent activity as allelochemicals in the root and soil rhizosphere (Venturelli et al. 2016). Our findings indicate both qualitative and quantitative differences in BX and phenoxazinone production in Australian wheat cultivars and rhizospheres; notably, metabolite production in the field varied with wheat cultivar, crop phenology and location of production. Plant part and location in the rhizosphere (i.e. distance from the root) also impacted BX concentration. This study utilised a statistically relevant and very large number of replicated tissue samples to infer key biochemical findings obtained over the phenological development of various wheat genotypes under two distinct soil and climatic conditions over two years. This alone renders the results generated of critical importance to the study of allelopathic weed interference.

4.1. Benzoxazinoids and derivatives detected in plant tissue and rhizosphere soil

The profile of BX metabolites and derivatives exhibited a consistent and unique distribution within each wheat tissue type and associated rhizosphere (Table 3 and Fig. 2). Differential distribution was also observed by Wu et al. (2000), who reported that in laboratory assays, wheat allelochemicals were variably distributed in seedlings, with root extracts containing higher levels of specific BX metabolites than shoot extracts. BX metabolites in cereals are frequently stored as glucosides in considerable quantities in plant vacuoles, and over time and with disruption bioactive aglycones are released from their association with sugars by enzymatic activity upon environmental challenge (Hofman and Hofmanova 1969; Hashimoto and Shudo 1996). In aqueous solutions and hydrated soil, the benzoxazinoid aglycones are typically unstable and are transformed spontaneously to the benzoxazolinones BOA and MBOA (Hashimoto and Shudo 1996).

Interestingly, in our studies, DIMBOA was detected at very low abundance in shoots, roots and rhizoplane samples and not at all in rhizosphere soil, in contrast to the findings of Wu et al. (2000). This incongruence is likely due to the stability of DIMBOA in the relatively aseptic agar assays used by Wu et al. (2000) in direct contrast to the rapid degradation of DIMBOA typically noted in soil and aqueous solutions (Woodward et al. 1978; Wu et al. 2000). DIMBOA is the precursor of MBOA, which is the catabolite we detected in greatest abundance of any BX metabolite in field-grown wheat roots and rhizosphere samples.
collected at both locations and years. DIMBOA content in wheat was previously shown to be heritable (Niemeyer and Jerez 1997), and studies with maize inbred lines have shown that the accumulation of DIMBOA could be monogenic or polygenetic depending on the population (Simcox et al. 1985).

Numerous studies have reported on the phytotoxicity of benzoxazinoids and their metabolites with respect to weed germination and root growth. Specifically, DIMBOA and MBOA inhibit germination and radicle elongation of both monocots and dicots, with DIMBOA generally exhibiting higher specific activity than MBOA (Macías et al. 2005; Chen et al. 2010). The substantial concentrations of MBOA in rhizosphere soils of wheat reported here and by others suggest strong potential for allelopathic interference.

The sequence of reaction steps leading to the formation of DIBOA or DIMBOA is identical in maize, diploid and hexaploid wheat and wild barley (Hordeum lechleri (Steud.) Schenck) (Frey et al. 2009). The highest concentrations of DIMBOA and DIBOA were previously reported in graminaceous plants shortly after their germination and establishment and were then observed to decrease over time with plant maturity (Argandoña et al. 1980; Copaja et al. 1999). However, DIBOA and DIMBOA content levels in wheat may vary considerably between cultivars. Copaja et al. (1999) found up to 10-fold differences in the levels of DIMBOA among a large number of Chilean and British wheat cultivar leaves, mostly T. aestivum, while Burgos et al. (1999) reported over 10-fold differences in the concentration of DIBOA among the shoot tissues of eight cultivars of rye. In this study, DIMBOA and DIBOA were detected in trace quantities only in the shoots of the wheat cultivars.

4.2. The abundance of benzoxazinones and derivatives in wheat tissues

MBOA and two of its derivatives; HMBOA and HMBOA-Glc, were the most abundant BX metabolites identified in wheat tissues based on crop growth stage (Fig. 4) and cultivar (Fig. 6), with MBOA being four times more abundant than derivatives. Previously conducted field studies also noted that the concentration of benzoxazinone derivatives in the foliage of wheat was considerably higher at the early growth stages than later in the growing season at crop maturity, with DIMBOA being the most abundant (Mogensen et al. 2006;
Chen et al. 2010). In contrast to the findings of Wu et al. 2000, others have reported that concentrations of DIMBOA in roots were considerably lower than in the foliage at early vegetative growth stages but as the crop matured concentrations remained relatively consistent over time, resulting in a subsequently higher concentration in roots versus foliar tissues at later growth stages (Mogensen et al. 2006; Chen et al. 2010). In our study, as described above, we did only detect trace quantities of DIMBOA in roots or rhizosphere soil.

However, MBOA was detected at up to 4-fold or greater levels than DIMBOA, DIBOA HMBOA or its glucoside in the roots and rhizoplane in all wheat growth stages. This suggests that DIMBOA conversion to MBOA occurs rapidly and completely in Australian soils through hydrolysis, a result also noted in Danish soils by Fomsgaard et al. (2006). In rye, others have noted that methoxy-substituted compounds, DIMBOA-glucoside and MBOA, were prevalent in root tissue (Rice et al. 2005). In the current study, BOA was the most prevalent metabolite in rye roots and rhizosphere soil in contrast to all seven wheat cultivars where MBOA predominated in roots and soil, pointing to a significant cereal species difference in metabolite accumulation in the rhizosphere.

MBOA abundance also varied significantly with cultivar, with the heritage cultivar Federation producing consistently higher levels of MBOA than modern genotypes (Fig. 6). This suggests that the recent selection for higher yield and semi-dwarf stature among other traits may potentially be associated with reduced allelochemical production and potentially other competitive crop traits. Other reports have also suggested that heritage wheat cultivars or landraces often show enhanced weed suppressive ability when compared to modern cultivars (Bertholdsson, 2004; Vandeleur and Gill, 2004). Recently, there is renewed interest in Australia in breeding grain crops with improved weed suppression and competitive ability in response to the evolution and rapid expansion of herbicide-resistant weed populations and the need for sustainable weed management practices (Rebetzke et al. 2018). Variation in benzoxazinone production, particularly MBOA, as observed among the cultivars evaluated in this study suggests that this trait may be amenable to selection for enhancement by plant breeders and therefore should be further investigated as a means of producing cultivars with greater ability to out-compete weeds.

In previous field studies, DIMBOA concentration was influenced by environmental conditions including temperature (Gianoli and Niemeyer 1997) and light intensity (Åhman,
and Johansson, 1994). Hence, it is likely that cultural practices and environment also influence the production of DIMBOA and other benzoxazinone derivatives in wheat. The current study shows significant differences and interactions between genetics (cultivar) and environment (location and year effects), resulting in variable concentrations or expression of BX metabolites in the current study (Figs. 5 and 6).

4.3. The abundance of BOA and MBOA in wheat roots rhizoplane and rhizosphere

The benzoxazolinones BOA and MBOA are found both in planta and in the soil as degradation products of DIMBOA and DIBOA, respectively (Fomsgaard et al. 2006). MBOA was found at comparable levels in wheat cultivars and cereal rye, but BOA was the predominant benzoxazinone in rye. This is likely due to the fact that the main hydroxamic acid observed in wheat and maize is DIMBOA (which is rapidly hydrolysed to MBOA in soil), while in rye it is the demethoxylated analogue, DIBOA (Virtanen and Hietala, 1960; Rice at al. 2005), which degrades to BOA (Fig. 1).

The detection of numerous benzoxazolinones in the wheat rhizosphere in this study further confirms that a major route of allelochemical release into the environment is exudation from the roots of living plants into their immediate surroundings, i.e., the soil rhizosphere (Belz, 2007). Root exudates containing root-specific metabolites have critical ecological impacts on soil macro- and microbiota as well as on whole plants. Through the exudation of a diverse group of metabolites, roots and their exudates impact the soil microbial community in their immediate vicinity, support beneficial symbioses, alter the chemical and physical properties of the soil, and as in the case of BXs and related metabolites, influence resistance to pests and inhibit the growth of competing plant species (Bertin et al. 2003).

Chen et al. (2010) recently reported that MBOA was found in the rhizosphere of six wheat cultivars tested, but its concentration varied greatly with cultivars and growth conditions. In the current study, we report an abundance of MBOA in the roots, rhizoplane and rhizosphere of all cultivars with some cultivar differences noted. Past experiments showed that the MBOA concentration in the wheat rhizosphere increased with an increasing plant density and also weed infestation (Chen et al. 2010) suggesting production could be induced or elicited due to competitive interference. This is further supported by observations
from Lu et al. (2012) who noted that the synthesis and exudation of DIMBOA/MBOA in wheat seedlings appeared to be an active metabolic process influenced by the environment, particularly the presence of weeds. Numerous studies in rice (Kong et al. 2006) and sorghum (Dayan 2006) have shown that the production of allelochemicals is upregulated when crops are grown in the presence of competing weeds, again suggesting stress inducible response with respect to key defence secondary metabolites. However, the presence of a similar response in field-grown wheat should logically be investigated in diverse field conditions.

4.4. The abundance of phenoxazinones in wheat roots rhizoplane and rhizosphere

The phenoxazinones APO and AMPO were the most abundant microbial metabolites detected at the three crop growth stages monitored, while AAPO was detected only in trace quantities at all growth stages. The microbial metabolites APO and AMPO were up to 2-3-fold more abundant in the root and rhizoplane of certain wheat cultivars (Federation and Janz CL) (Fig. 10), and interestingly these cultivars were also noted to be considerably more weed suppressive than others (Mwendwa et al. 2018). Weeds have been shown to elicit enhanced allelochemical biosynthesis in competing crops, as occurs in plant defence induced by disease and insect attack (Belz, 2007; Chen et al. 2010). Consistent with the higher levels of BOA detected in the rhizosphere of rye compared to wheat (Fig. 7), the levels of its biotransformed derivative APO were also considerably higher in the root zone of rye (Fig. 12), which is expected given that APO results from microbial degradation of BOA. The highest concentrations of APO, the most phytotoxic of the microbial transformation products of the BXs (Macías et al. 2006; 2014), observed in the root zone of wheat sampled in this study was 4 uM, more than high enough to exert significant deleterious effects on neighbouring plants (Macías et al. 2006; 2014; Venturelli et al. 2015).

The allelochemicals APO and AMPO, as well as their precursors, have also been detected in plants in close proximity to DI(M)BOA donor plants (Macías et al. 2014). In vivo bioassays confirmed phytotoxicity of hydroxamic-acid-derived allelochemicals, such as APO and AMPO were comparable to the specific activity of commercial herbicides (Macías et al. 2006; Venturelli et al. 2015). Phenoxazinones are also much more stable in the soil environment in contrast to BXs. Phenoxazinones as allelochemicals are potent inhibitors of histone deacetylase activity and exert their activity through locus-specific alterations of
histone acetylation and associated gene expression (Venturelli et al. 2015). Hence, production of high levels of benzoxazinone derivatives that are further transformed by soil microbiota to potent bioherbicides by selected wheat genotypes could potentially reduce management issues associated with pests, diseases, and weeds (Mogensen et al. 2006) and also potentially reduce the need for synthetic herbicides in some circumstances (Weston and Duke, 2003).

The mode(s) of action of individual BX metabolites has been extensively studied previously (Macías et al. 2009; Sánchez-Moreiras et al. 2010; Schulz et al. 2013). In some cases, the activity has been related to the occurrence of necrosis, probably induced by early senescence processes in the oldest leaves that lead to an increase in oxidative activity when plants are treated with BOA, as well as a reduction in photosynthetic activity (Sánchez-Moreiras et al. 2010). APO has been shown to directly impact chromatin-modifying processes in cress (Arabidopsis thaliana L.) by inhibition of histone deacetylases (HDA) (Venturelli et al. 2015). A recent structure-activity study of a collection of BXs also showed that these compounds may inhibit the root growth of cress seedlings by inhibiting α-amylase activity (Kato-Noguchi et al. 2010).

Cipollini et al. (2012) observed that allelopathic plants can modify plant-microbe interactions, resulting in increased allelopathic effects through increasing the sensitivity of target plants to pathogens and favouring the growth of pathogenic or parasitic microbes when in the presence of high allelochemical concentrations. In addition, microbial communities can affect the allelopathic potential of a species or system in a more indirect way, such as the case of endophytic fungi that can stimulate allelochemical production by their host plants. Rhizosphere soil microbes can potentially contribute to the allelopathic potential of plants through positive feedback (Wu et al. 2015) or through direct biotransformation such as those resulting in the production of aminophenoxazinone compounds APO and AMPO (Macías et al., 2006, 2009). The formation of APO from root-exuded DIBOA as well as the absorption of APO by exposed plants has been confirmed (Krogh et al., 2006; Rice et al., 2012), but until now limited information has been reported about the rate of synthesis and the availability of various phenoxazinones in the soil under field conditions (Venturelli et al. 2015).
In the current study, we noted the presence of ecologically relevant concentrations of the phenoxazinone metabolites (APO AAPO and AMPO), produced by soil microbial transformation of BOA, MBOA and HMBOA, respectively, in both Condobolin and Wagga Wagga soils. APO and AMPO were up to 3-fold more abundant in the rhizospheres of certain wheat cultivars (Federation and Janz CL), and remarkably these cultivars were also previously noted to be significantly more weed suppressive under field conditions (Mwendwa et al. 2018). Our findings clearly confirm that allelochemical exudation and microbial transformation occur in Australian soils in association with commercial wheat production. However, the abundance of BX and phenoxazinone metabolites in the rhizosphere is dependent on factors such as crop species, cultivar, plant density, soil type and soil moisture availability (Belz and Hurle, 2005; Macías et al. 2014; Venturelli et al. 2015), as well as climatic conditions encountered as shown in Figure 11.

4.5. Preliminary data for rhizosphere microbial community diversity

The importance of microbes in the degradation of allelochemicals is clear, and recent progress in understanding how interactions between allelochemicals and biotic and abiotic components of the soil matrix affect degradation has been made (Fomsgaard et al. 2004; Barto et al. 2011; Cipollini et al. 2012). High throughput metagenomic DNA sequencing technologies offer cost-effective and reliable means to detect and identify soil microbiota, often to the genus or species level, and enable relative quantification of these entities between treatments. The gene encoding the ribosomal subunit 16S RNA of bacteria and the internal transcribed spacer (ITS) in fungi are of interest due to a combination of conserved regions and highly variable regions (Mignard and Flandrois 2006). In the current study, both 16S RNA and ITS were used to assess the composition of the microbiota involved in MBOA and BOA biotransformation in the rhizosphere of each cultivar through genomic and metagenomic analysis.

Based on the presence of aminophenoxazinone metabolites in the soil rhizosphere, either soil fungi and/or bacteria could be involved in biotransformation of the MBOA and BOA, and this process may not be unique to a particular group of microbes, as demonstrated in European studies which identified the role of actinomycetes (Gaeumannomyces, Plectosporium, Chaetosphaeria spp) and bacteria (Proteobacteria, Actinobacteria) in soil
transformation (Friebe et al. 1998; Fomsgaard et al. 2004; see Supplementary Table S2). Based on our initial metagenomics data, more specific studies are required to evaluate the DIMBOA, DIBOA, MBOA and BOA interactions with the existing soil microbial communities in Australian soils.

A positive linear relationship between the MBOA level in the wheat rhizosphere and the presence of soil fungi/bacteria has been established (Chen et al. 2010). DIMBOA significantly decreased the actinobacterial biomass at 1 h, while MBOA resulted in the decrease of the actinobacterial biomass at 48 hrs when compared to the uninoculated controls. However, both DIMBOA and MBOA application always resulted in increased soil fungal biomass (Chen et al. 2010), clearly suggesting that the presence of BX metabolites supports and nurtures a specific soil microflora in the plant rhizosphere site.

5. Conclusions

Metabolic profiling has provided clearer insights into the biosynthesis and release of BX metabolites associated with weed suppression in commercial field-grown wheat cultivars, in comparison with cereal rye and the heritage wheat cultivar Federation, both recognized previously for their potent ability to suppress weeds. Phytotoxic microbial metabolites (aminophenoxazinones) including APO, AAPO and AMPO, were detected and transformed from benzoxazolinones produced by wheat and its root exudates by soil microbiota under field conditions.

This work is the first to demonstrate that production of phenoxazinones occurs in Australian soils, such that weed suppression might be clearly impacted under field conditions for certain wheat cultivars (on their roots and in rhizosphere soils). Our findings lead to a mechanistically informed field model for the molecular mode of action of allelopathic aminophenoxazinones in the target plant and provide insights into allelochemical defence and competition strategies of BX producing cereals. Further research on their production, transformation, and parent molecules are required in the context of which Australian wheat cultivars or soils may be the most suppressive over time. We also found that phenoxazinone production was upregulated early in the season as opposed to late season, after crop maturity.
This is common for plant defence metabolites where genes associated with defence traits are upregulated in seedling cereals.

Both APO and AMPO were found in abundance in association with certain cultivars, especially the heritage cultivar Federation, but were more abundant in years with lower soil moisture content such as 2015 (data not presented). At this time further research is required to determine if: 1) genotypes expressing production of hydroxamic acids may be targeted for enhanced weed control through biosynthetic modification and plant breeding, 2) soil microbial transformants may be isolated and regulated to encourage production of the potently active phenoxazinones, 3) chemical signalling of plants in response to other plant and microbial populations and directed allelochemical delivery to target plants are required to fully mediate/elucidate the role of allelochemicals in the rhizosphere.

It is obvious that the study of such plant/soil interactions requires further research for widespread application in agricultural production worldwide. This is to some extent limited by the lack of non-destructive sampling methods that would potentially allow for repeated sampling of secondary plant metabolites in the root zone thus restricting the understanding of allelopathic interactions in intact plant communities. A recent study by Reiss et al. (2018) reported that silicone tubing micro-extraction may be useful in the measurement of these dynamics in the root zone of winter cereals throughout their growing season. Repeated destructive sampling over time, thereby generating large sample numbers, does address many of these issues but is labour intensive and costly. Therefore, we suggest further research in exudation and uptake dynamics, together with the dynamics of degradation and microbial transformation processes, must be considered in future studies aimed at the characterization of allelopathic phenomena.

6. Acknowledgements

We acknowledge a) the financial support of the Grains Research and Development Corporation (Projects UCS 00020, 00022, and 00023) b) Prof. Inge S. Fomsgaard, Aarhus University, Denmark for providing chemical standards for certain BXs and soil microbial metabolites (phenoxazinones), c) technical support of Graeme Heath, Dom Skoneczny, Xiaocheng Zhu, Saliya Gurusinghe Razia Shaik, Mr Vincent West and Mr Jack Wess with
respect to field sampling, data collection, extraction and analyses and d) Mrs Bente Laursen, Aarhus University, Denmark for technical support in liquid chromatography mass spectrometry.

7. References


Cambier, V., Hance, T., & De Hoffmann, E. (2001). Effects of 1, 4-benzoxazin-3-one derivatives from maize on survival and fecundity of Metopolophium dirhodum (Walker) on artificial diet. Journal of Chemical Ecology, 27(2), 359-370.


Chen, K. J., Zheng, Y. Q., Kong, C. H., Zhang, S. Z., Li, J., & Liu, X. G. (2010). 2,4-Dihydroxy-7-methoxy-1, 4-benzoxazin-3-one (DIMBOA) and 6-methoxy-benzoazolin-2-one (MBOA) levels in the wheat rhizosphere and their effect on the soil microbial community structure. Journal of Agricultural and Food Chemistry, 58(24), 12710-12716.


proximity to wild oat (*Avena fatua*) and flixweed (*Descurainia sophia*). *Weed Science*, 60(3), 360-365.


Tanvir, F., Fredholm, M., Gregersen, P. L. and Fomsgaard, I. S. (2013). Comparison of the levels of bioactive benzoxazinoids in different wheat and rye fractions and the transformation of these compounds in homemade foods. *Food Chemistry* 141 (1), 444-50


8. Supplementary data

**Supplementary Table S1: Reported structures of benzoxazinoids including the benzoxazolinones, hydroxamic acids, lactams and aminophenoxazinones.**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Systematic name</th>
<th>$R_1$</th>
<th>$R_2$</th>
<th>$R_3$</th>
<th>Molecular formula</th>
<th>Accurate mass</th>
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<tbody>
<tr>
<td>HBOA</td>
<td>2-hydroxy-2H-1,4-benzoxazin-3(4H)-one</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>C_{8}H_{7}NO_{3}</td>
<td>165.1468</td>
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<tr>
<td>HMBOA</td>
<td>2-hydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one</td>
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<td>H</td>
<td>OCH$_3$</td>
<td>C$<em>{9}$H$</em>{9}$NO$_{4}$</td>
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<tr>
<td>DIBOA</td>
<td>2,4-dihydroxy-2H-1,4-benzoxazin-3(4H)-one</td>
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<td>OH</td>
<td>H</td>
<td>C$<em>{8}$H$</em>{7}$NO$_{4}$</td>
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<tr>
<td>DIMBOA</td>
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<td>HBOA-glc</td>
<td>glucopyranosyloxy-2H-1,4-benzoxazin-3(4H)-one 2-β-D-glucopyranosyloxy-2H-1,4-benzoxazin-3(4H)-one</td>
<td>Glc</td>
<td>H</td>
<td>H</td>
<td>C$<em>{14}$H$</em>{17}$NO$_{8}$</td>
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</tr>
<tr>
<td>HMBOA-glc</td>
<td>glucopyranosyloxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one 2-β-D-glucopyranosyloxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one</td>
<td>Glc</td>
<td>H</td>
<td>OCH$_3$</td>
<td>C$<em>{15}$H$</em>{19}$NO$_{9}$</td>
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<td>DIBOA-glc</td>
<td>glucopyranosyloxy-4-hydroxy-2H-1,4-benzoxazin-3(4H)-one 2-β-D-glucopyranosyloxy-4-hydroxy-2H-1,4-benzoxazin-3(4H)-one</td>
<td>Glc</td>
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<td>H</td>
<td>C$<em>{14}$H$</em>{17}$NO$_{9}$</td>
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<tr>
<td>DIMBOA-glc</td>
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<td>Glc</td>
<td>OH</td>
<td>OCH$_3$</td>
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<tr>
<td>HBOA-glc-hex</td>
<td>dihexose derivative of HBOA</td>
<td>Glc-hex</td>
<td>H</td>
<td>H</td>
<td>C$<em>{20}$H$</em>{27}$NO$_{13}$</td>
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<tr>
<td>DIBOA-glc-hex</td>
<td>dihexose derivative of DIBOA</td>
<td>Glc-hex</td>
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<td>H</td>
<td>C$<em>{20}$H$</em>{27}$NO$_{14}$</td>
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<td>Compound</td>
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<td>Molecular Formula</td>
<td>Molecular Weight</td>
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</tr>
<tr>
<td>BOA</td>
<td>benoxazolin- 2(3H)-one</td>
<td>H - -</td>
<td>C&lt;sub&gt;7&lt;/sub&gt;H&lt;sub&gt;5&lt;/sub&gt;NO&lt;sub&gt;2&lt;/sub&gt; 135.1201</td>
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<tr>
<td>MBOA</td>
<td>6-methoxy-benoxazolin-2(3H)-one</td>
<td>OCH&lt;sub&gt;3&lt;/sub&gt; - -</td>
<td>C&lt;sub&gt;8&lt;/sub&gt;H&lt;sub&gt;7&lt;/sub&gt;NO&lt;sub&gt;3&lt;/sub&gt; 165.1461</td>
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<tr>
<td>APO</td>
<td>2-Amino-3H-phenoxazin-3-one</td>
<td>H NH&lt;sub&gt;2&lt;/sub&gt; -</td>
<td>C&lt;sub&gt;12&lt;/sub&gt;H&lt;sub&gt;8&lt;/sub&gt;N&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt; 212.2080</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AMPO</td>
<td>2-amino-7-methoxy-3H-phenoxazin-3(4H)-one</td>
<td>OCH&lt;sub&gt;3&lt;/sub&gt; NH&lt;sub&gt;2&lt;/sub&gt; -</td>
<td>C&lt;sub&gt;13&lt;/sub&gt;H&lt;sub&gt;10&lt;/sub&gt;N&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;3&lt;/sub&gt; 242.2340</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AAPO</td>
<td>acetylamino phenoxazin-3-one</td>
<td>H COCH&lt;sub&gt;3&lt;/sub&gt; -</td>
<td>C&lt;sub&gt;14&lt;/sub&gt;H&lt;sub&gt;10&lt;/sub&gt;N&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;3&lt;/sub&gt; 254.2450</td>
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<tr>
<td>AAMPO</td>
<td>2-acetylamino-7-methoxy-phenoxazin-3-one</td>
<td>OCH&lt;sub&gt;3&lt;/sub&gt; NH -</td>
<td>C&lt;sub&gt;15&lt;/sub&gt;H&lt;sub&gt;12&lt;/sub&gt;N&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;4&lt;/sub&gt; 284.2710</td>
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Supplementary Figure S1: Relative abundance by OTU (operational taxonomic units) of the fungal phyla in rhizosphere soil presented by wheat cultivar. The soil was collected following crop harvest in 2016.
Supplementary Figure S2: Relative abundance by OTU (operational taxonomic units) of bacterial phyla in rhizosphere soil presented by wheat cultivar. The soil was collected following crop harvest in 2016.
**Supplementary Table S2: The transformations of BOA, HBOA and MBOA in the soil by microbial activity as reported from previous studies**

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Tissue/soil</th>
<th>Specific activity</th>
<th>Microorganism</th>
<th>References</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>BOA to APO</td>
<td>Soil</td>
<td>phytopathogenic fungus.</td>
<td>1. <em>Avena sativa</em> root-colonising bacteria</td>
<td>Friebe et al. 1996 (1)</td>
<td>APO was shown to be 10 times more phytotoxic than BOA (Gagliardo and Chilton, 1992). Performed in a liquid growth medium (conc. of BOA 0.1 mM), incubated at 23 °C (Friebe et al. 1998).</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Friebe et al. 1996</td>
<td></td>
</tr>
<tr>
<td>BOA to APO</td>
<td>Soil</td>
<td>addition of the acetyl group in the molecule</td>
<td>Not recovered</td>
<td>Gents et al. 2005, Understrup et al. 2005 Friebe et al. 1996</td>
<td>The degradation of BOA to APO was concentration-dependent with low soil concentrations (400 μg kg⁻¹) yielding only one unidentified transformation product, while higher soil concentrations (400 mg kg⁻¹) yielded eight distinct transformation products, two of which were confirmed as APO &amp; AAPO.</td>
</tr>
<tr>
<td>APO to AAPO</td>
<td></td>
<td>by acetyl-transferases – hydrophobic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BOA to HPMA</td>
<td>Soil</td>
<td></td>
<td><em>Gaeumannomyces graminis</em> var. <em>graminis</em> and var. <em>tritici</em></td>
<td>Friebe et al. 1998 Fomsgaard et al. 2004</td>
<td>degraded BOA to N-(2- hydroxyphenyl) malonamic acid (HPMA) and MBOA to N-(2-hydroxyphenyl-4- methoxyphenyl) malonamic acid (HMPMA).</td>
</tr>
<tr>
<td>MBOA to HMPMA</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BOA to HPAA and HPMA</td>
<td>soil</td>
<td>APO is highly toxic to <em>F. verticillioides</em> and other organisms.</td>
<td><em>Fusarium verticillioides</em></td>
<td>Bacon et al. 2007</td>
<td>Maize pathogens such as <em>Fusarium verticillioides</em> are capable of detoxifying the benzoazolinones to 2-aminophenol (AP), which is converted to the less toxic N-(2-hydroxyphenyl) malonamic acid (HPMA) and 2-acetamidophenol (HPAA).</td>
</tr>
<tr>
<td>BOA to HPMA</td>
<td>Soil</td>
<td>an endophytic fungus</td>
<td><em>Fusarium moniliiforme</em></td>
<td>Yue et al. 1998</td>
<td>an endophytic fungus often present in maize cultivars, for degrading BOA and MBOA</td>
</tr>
<tr>
<td>MBOA to HMPMA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BOA MBOA</td>
<td>Soil</td>
<td></td>
<td><em>G. graminis</em> var. <em>avenae</em></td>
<td>Friebe et al. 1998 Fomsgaard et al. 2004</td>
<td>G. graminis var. avenae degraded BOA and MBOA and <em>Fusarium culmorum</em></td>
</tr>
<tr>
<td>Reaction</td>
<td>Soil</td>
<td>Fungi/Endophytes</td>
<td>Reference</td>
<td>Notes</td>
<td></td>
</tr>
<tr>
<td>----------</td>
<td>------</td>
<td>------------------</td>
<td>-----------</td>
<td>-------</td>
<td></td>
</tr>
<tr>
<td>BOA to HBOA</td>
<td>Soil</td>
<td>an endophytic fungus</td>
<td><em>Fusarium sambucinum</em></td>
<td>Zikmundova et al. 2002a Fomsgaard et al. 2004</td>
<td>Degraded BOA but no metabolites were detected for both. F. sambucinum was the endophytic fungus isolated from <em>A. tetragona</em>.</td>
</tr>
<tr>
<td>HBOA to APO, AAPO, NHAAP, and HAAP</td>
<td>Soil</td>
<td>endophytic fungi isolated from the roots of <em>A. tetragona</em></td>
<td><em>Plectosporium tabacinum</em>, <em>Gliocladium cibotii</em>, <em>Chaetosphaeria sp.</em>, <em>Fusarium sambucinum</em></td>
<td>Zikmundova et al. 2002a Fomsgaard et al. 2004</td>
<td>The cultures were incubated at 25°C in the dark. A sequential formation of APO, acetylation to AAPO, N-oxidation to 2-(N hydroxy) acetylamino-3H- phenoxazin-3-one (NHAAP), and finally the formation of 2-(2-hydroxyacetyl) amino-3H-phenoxazin-3-one (HAAP).</td>
</tr>
<tr>
<td>HBOA to AHPO, AAHPO, HHAAP, AAMPO</td>
<td>Soil</td>
<td>endophytic fungus isolated from the roots of <em>A. tetragona</em></td>
<td><em>Chaetosphaeria sp.</em></td>
<td>Zikmundova et al. 2002b Fomsgaard et al. 2004</td>
<td>These compounds were identified for the first time as 2-amino-7-hydroxyphenoxazin-3-one (AHPO), 2-acetylamino-7-hydroxyphenoxazin-3-one (AAHPO), 7-hydroxy-2-(2-hydroxyacetyl) amino-phenoxazin-3-one (HHAAP), and 2-acetylamino-7-methoxy-phenoxazin-3-one (AAMPO).</td>
</tr>
</tbody>
</table>
Supplementary Table S3: Analysis of variance of the abundance of MBOA (log transformed) in seven wheat cultivars.

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<th>Source</th>
<th>df</th>
<th>Sum of squares</th>
<th>Mean square</th>
<th>F</th>
<th>P</th>
</tr>
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<tbody>
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<td>Year</td>
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<td>110.702</td>
<td>37.17</td>
<td>&lt;0.001</td>
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<tr>
<td>Location</td>
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<td>122.078</td>
<td>40.99</td>
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<td>Cultivar</td>
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<td>42.29</td>
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<td>2.37</td>
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<td>Tissue</td>
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<td>301.214</td>
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<td>Cultivar<em>Tissue</em>Sampling</td>
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**Supplementary Table S4: Analysis of variance of the abundance of BOA (log transformed) in cereal rye and seven wheat cultivars.**

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<th>P</th>
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Supplementary Table S6: Analysis of variance of the abundance of AMPO (log transformed) in seven wheat cultivars.

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Supplementary Table S7: Analysis of variance of the abundance of AAPO (log transformed) in seven wheat cultivars.

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Supplementary Table S8: Analysis of variance of the abundance of APO (log transformed) in cereal rye and seven wheat cultivars.

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Investigating for root exudates of secondary metabolites from wheat root hairs at crop vegetative growth stage through a microscope
Chapter 8:

General Discussion

8.1. General discussion

The research encompassed in this thesis performed both field and laboratory studies with the objective of 1) assessing the above-ground competitive traits of selected superior Australian canola (Chapter 2 and 3) and wheat (Chapter 4 and 5) cultivars which are well adapted to the southern farming region in different locations and seasons and 2) assessed and quantified wheat secondary metabolites involved in weed suppression in the shoots, roots, rhizoplane and soil rhizosphere using new targeted and non-targeted metabolic profiling techniques (Chapter 6 and 7).

The research field studies demonstrated that diverse crop cultivar-dependent canopy competitive traits including 1) early growth vigour, 2) biomass production, 3) interception of photosynthetically active radiation (due to increased leaf area and/or canopy structure) and 4) production and retention of crop residue impacted weed establishment and total weed biomass in both canola and wheat.

In canola, it was demonstrated that cultivars with the greatest biomass, light interception, leaf area indices, and visual vigour were the most weed suppressive, with weed biomass being inversely related to cultivar biomass (Chapter 3). In wheat studies, it was further demonstrated that establishment of competitive wheat cultivars could result in effective suppression of weed growth (up to 90% or greater) in the absence of post-emergent herbicides, especially in a year when soil moisture was not limiting (Chapter 5). This finding suggests that the competitive advantage of the wheat crop is improved when soil moisture was not limiting in terms of production of above-ground biomass formation. Others have also found that several physiological plant traits negatively correlated with weed biomass, including early vigour, plant height and compact canopy architecture (Andrew et al. 2015; Zerner et al. 2016). These traits confer above-ground competition for light and resources, resulting in substantial suppression of weeds as demonstrated in the current study for both canola and wheat in Australian commercial farming systems.
Crop competitive traits have been thoroughly studied to determine their contribution to weed suppression in conventional agricultural systems where fertiliser and water supply are not restricting plant growth. Early vigour, leaf area index (LAI) and canopy height were identified as the traits most strongly correlated with weed suppression of cereals (Rebetzke and Richards, 1999; Andrew et al. 2015). In wheat, traits relating to leaf size, specific leaf area and rate of production vary between cultivars and have been linked to higher suppressive ability (Coleman et al. 2001; Zerner et al. 2008). In addition, recently Zerner et al. (2016) reported that mature crop height and early crop vigour were strongly correlated with improved weed suppression and tolerance against cultivated oats (Avena sativa L.) (a weed mimic) for 86 wheat cultivars at two sites over two growing seasons. In the current studies, differences in weed suppression were largely impacted by crop architecture and phenology early in the growing season, particularly growth vigour, leaf area and the ability to achieve early canopy closure.

Results of recent studies on weed suppressive cultivar choice in wheat and canola have been useful for growers wishing to manage weeds with an integrated approach instead of relying solely on herbicides; specifically, the choice of the canola and wheat cultivars for desired yield and weed suppression may clearly impact the subsequent ability of the crop to successfully interfere with weed growth. In addition, the choice of an effective cultivar can cost-effectively prevent future weed propagules from entering the weed seedbank. This suggests there are suitable Australian commercial canola and wheat cultivars that could be used as potential tools for maintaining suitable grain yield in the presence of weeds, while possibly reducing the use of pre-emergence herbicides and also delaying the development of herbicide-resistant weeds. For example, cultivars Condo and Janz CL were both high yielding and weed suppressive, in contrast to Federation, a heritage cultivar, which was strongly weed suppressive but not high yielding. Future wheat pre-breeding programs must strongly consider selection for morphological, physiological and phenological traits associated with competitive and vigorous cultivars for cost-effective weed suppression (Rebetzke et al. 2018).

There is also some general agreement that weed-suppressive ability in cereal crops is determined by a combination of the competitive and allelopathic properties of a crop (Worthington and Reberg-Horton, 2013; Bertholdsson, 2011). A recent study on weed suppressive potential of selected Canadian spring cereals by Reiss et al. (2018a) demonstrated that competitive traits such as leaf area index, crop height and early vigour, plus the production of benzoaxazinoids detected in the root zone were of equal importance.
to explain the variance of weed biomass at the field level. Another recent study investigating weed suppressive potential of 33 winter wheat, 24 winter rye and 11 winter triticale cultivars revealed a clear relationship between allelopathic and competitive traits, which together explained 62% of the variance in the data set when analysed via principal component analysis (Reiss et al. 2018b). In the current study, the most weed-suppressive cultivars (e.g. Federation, Janz CL) also produced the greatest amounts of the three major BXs in the root zone. However, it would appear that canopy architecture early in the season plays perhaps the most important role in effective weeds suppression in wheat in-crop.

The ability of a plant to produce and release allelopathic phytotoxic metabolites into the environment and/or to tolerate the presence of allelochemicals released by neighbouring plants including weeds can be crucial to the ability of a species to survive and reproduce (Bertholdsson et al. 2012, Worthington et al. 2015). For example, the inhibition of germination and reduction of seedling growth were observed in many plant species exposed to BOA and DIBOA (Macías et al. 2014). Often, radicles and root tips were more affected than shoots or hypocotyls by the presence of BX or phenoxazinone metabolites (Venturelli et al. 2015). Metabolic profiling conducted in this study provided key information regarding levels of secondary metabolites in various Australian wheat tissues and rhizosphere soils associated with weed suppression in commercial wheat cultivars in comparison with cereal rye and the wheat cv. Federation, both recognised for their potent ability to suppress weeds. Phytotoxic microbial metabolites (the phenoxazinones APO, AAPO, AMPO, AAMPO), which originate from benzoxazolinones produced and exuded by wheat and are subsequently transformed by soil microbiota, were detected in abundance in the rye rooting zone and also in that of certain wheat cultivars in this study (Chapter 7).

This is the first time that benzoxazinone-derived phenoxazinones have been detected in the rhizosphere and rhizoplane of Australian field-grown wheat; further research on the production of these compounds and their parent molecules in a broader range of cultivars and cereals would clearly be useful for selection of weed-suppressive cereal cultivars. A comparison of the production and persistence of phenoxazinones in Australian vs European soils would also be worthy for the study. In Australian red sodosols, the production of BXs and phenoxazinones was highest early in the season, a phenomenon which is commonly reported with respect to plant defence metabolites
where genes associated with defence traits are upregulated in seedlings vs mature plants (Argandoña et al. 1980; Copaja et al. 1999).

The allelochemicals APO and AMPO, as well as their precursors, have been previously detected in plants in proximity to DI(M)BOA donor plants (Macías et al. 2014). In vivo bioassays confirmed growth inhibitory activity for hydroxamic-acid-derived allelochemicals, such as APO and AMPO, comparable to that of commercial herbicides (Macías et al. 2006; Venturelli et al. 2015). These past findings have clearly established these metabolites as the agents responsible for the phytotoxic effects observed in cereal allelochemical interference. Further, these metabolites are potent inhibitors of histone deacetylases both in vitro and in vivo and exert their activity through locus-specific alterations of histone acetylation and associated gene expression (Venturelli et al. 2015).

In the current study, the aminophenoxazinone metabolites (APO and AMPO) were generated through soil microbial transformation of BOA, MBOA and HMBOA in the red sodosol soils in both Condobolin and Wagga Wagga; abundance was highest in Wagga Wagga soils in 2015 under relatively dry soil conditions. The microbial metabolites APO and AMPO were present at ecologically relevant concentrations in Australian soils and were ~10-fold more abundant in the root zone of certain wheat cultivars (Federation and Janz CL) which were also noted to be considerably more weed suppressive due to above-ground canopy architectural traits. Our findings are consistent with those of Venturelli et al. (2015) and others, and clearly point to the fact that allelochemical exudation and microbial transformation occur in challenging field conditions as well as in the laboratory.

The abundance of bioactive metabolites such as the phenoxazinones produced by wheat in the rhizosphere is dependent on multiple factors including species, cultivar and plant density (Belz and Hurle, 2005; Macías et al. 2014; Venturelli et al. 2015). The presence of soil microbes is also an important factor influencing the overall accumulation and impact of allelochemicals on plants. Soil bacteria including Pseudomonas spp. and actinomycetes, as well as diverse soil fungi are particularly adapted for rhizosphere colonisation by their ability to utilise diverse carbon sources present in root exudates (Friebe et al. 1998; Kiener 2003; Fomsgaard et al. 2004). This work is the first to demonstrate in Australia that production of phenoxazinones can occur at ecologically
important concentrations, such that weed suppression may be clearly impacted under field conditions, in certain wheat cultivars.

Precise metabolic profiling of allelochemicals in the plant and, at the same time, in the soil rhizosphere, provided strong insight into the dynamics of the release of bioactive metabolites following incorporation of plant material or living root exudates into the soil (Krogh et al. 2006; Weston et al. 2015). Tactics and approaches for manipulating the field environment to enhance survival, physiological behaviour and performance of these microbes might improve the allelopathic activity of a crop in the field (Newman et al. 1998). At this time further research in controlled environment experiments is required to determine if 1) cultivars expressing production of such hydroxamic acids may be targeted for enhanced weed control through biosynthetic modification or 2) if soil microbial transformants may be regulated to encourage production of the potently active phenoxyazinones.

8.2. Recommendations for Future Studies

This work performed in this thesis suggests that canola and wheat pre-breeding programs strongly consider selection for morphological, physiological and phenological traits associated with competitive and vigorous canola and wheat cultivars. For example, the wheat genotypic variation has been reported to be much greater for weed suppression than weed tolerance, suggesting a greater opportunity for the selection of improved weed suppression in wheat. However, strong positive correlation between weed suppression and tolerance ($r = 0.79$, $P < 0.001$) suggests that wheat cultivars selected on the basis of high weed suppression may also exhibit improved weed tolerance, consistent with the conclusions of Zerner et al. (2016).

I further suggest that the development of advanced predictive models such as PLS (partial least square) regression, generated with crops grown under different soil moisture regimes, will aid in the design of more effective breeding programs for weed suppression in grain crops. The interrelatedness of plant characteristics associated with canopy structure and weed competitiveness in grain crops [i.e. metabolite production, plant height, early canopy closure, LAI, vertical leaf orientation, rapid biomass accumulation at the early crop growth stage, high shoot dry matter, large root biomass and root volume] makes PLS regression analysis particularly well-suited to characterise relationships
between crop plant characteristics and weed suppression, especially when numerous interactions between factors may obscure patterns using simpler regression models.

Breeding of weed-resistant cereal crops with high BX content, as well as a better understanding of the soil persistence of these compounds and their microbial transformation products, are important areas of future research that will no doubt be addressed in Australia based on our findings. Knowledge of the selectivity of the BXs in managing diverse populations of weeds and the influence of cultural practices on weed management through crop suppression will also provide greater impetus to include allelopathic cereal cultivars in broadacre and/or organic cropping systems for weed control.

The results of the current study suggest a wealth of opportunities for further investigation into cereal crop allelopathy and crop-microbe interactions. For example, the lack of knowledge about the dynamics of secondary metabolites in the rhizosphere, and the soil concentrations required to inhibit germination and subsequent growth of target weeds are limitations that would be worthy targets of further studies. To better understand and improve the role of such allelochemicals, we recommend that future research focuses on three additional areas: 1) the role of soil microorganisms in chemically mediated interactions between plants; 2) the application of new analytical tools such as high-resolution mass spectrometry coupled to HPLC to enable detection and quantification of trace levels of metabolites and their resulting degradation compounds; and 3) experimental designs to elucidate the underlying dynamics of complex mixtures of allelochemicals in soil (Weston et al. 2015).

Research addressing these issues will help provide greater clarity of the role of BXs in the allelopathic activity of cereal grains such as wheat and rye in more detail and glucosinolates and isothiocyanates in canola. Most studies indicate that the allelopathic activities of cereal crops are associated with the production of higher BX concentration, yet in many cases, the levels of these compounds found in soil may be inadequate to account for the observed toxicity. Recovery of the metabolites pre and post-extraction will be important to account for. In the case of wheat, the production of BXs would be expected to be positively correlated with allelopathic activity if the BXs are successfully converted to active biotransformation products by soil microbes. Therefore, the effect of BX precursors on soil microbial ecology is a particularly important question, as is the
question of whether soil microbes in the presence of soil-available BXs may generate other, as yet unidentified, phytotoxins.

Cultivar-dependent variation in BX composition in cereal rye or barley root exudates has rarely been investigated, and the pathways of exudation and potential for long-distance transport of BXs are unknown (Schulz et al. 2013). In the future, I would like to participate in further studies on the chemical signalling of plants in response to other plant and microbial populations and directed allelochemical delivery to target plants in order to fully elucidate the role of allelochemicals in the rhizosphere of canola and wheat.

8.3. References


Appendices

Wagga Wagga weed suppressive wheat (above) and canola (below) trials in 2016
Other Journal Publications and Conference Proceedings

In Appendix A three publications presented which are either journal publication or conference proceedings. My contribution to these publications was through participation in the trials and/or compilation of the data as part of the training and skill development process both in the laboratory and field experimentation.

Appendix A1 is a manuscript published in the Journal of Experimental Botany. We studied the production and localisation of bioactive naphthoquinones (NQs) in the roots of Paterson’s curse (*Echium plantagineum* L.), an invasive endemic weed in Australia. I was involved in chemical extractions of shoots and roots. In addition, I participated in the microprobe experiments to assess NQs in rhizosphere and rhizosphere soil extraction using solid phase root zone extraction (SPRE) microprobes (5cm) prepared using silicone polydimethylsiloxane (PDMS).


Appendix A2 was a conference proceedings paper and presents initial data of the PhD research project on mechanisms of weed suppression by selected wheat cultivars. The paper targeted local grower and advisor audience.


Appendix A3 was a conference proceedings paper presented at Australasian Weeds Conference. I participated in this study while working for Central West Farming Systems Inc. we used the findings of the study to design the current PhD research focusing mainly on canola and wheat. We evaluated ten-grain crops and their residues for their ability to suppress both winter and summer annual weeds in the mixed cropping region of southern Australia due to their competitive abilities and the presence of residues remaining until the subsequent cropping season.

Appendix A1

Identification and Localization of Bioactive Naphthoquinones in the Roots and Rhizosphere of Paterson’s Curse (*Echium plantagineum*), a Noxious Invader

The manuscript “Identification and localization of bioactive naphthoquinones in the roots and rhizosphere of Paterson’s curse (*Echium plantagineum* L.), a noxious invader” was featured as the cover article for the special issue of the Journal of Experimental Botany.
Identification and localization of bioactive naphthoquinones in the roots and rhizosphere of Paterson’s curse (Echium plantagineum), a noxious invader

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Abstract

Bioactive plant secondary products are frequently the drivers of complex rhizosphere interactions, including those with other plants, herbivores and microbiota. These chemically diverse molecules typically accumulate in a highly regulated manner in specialized plant tissues and organelles. We studied the production and localization of bioactive naphthoquinones (NQs) in the roots of Echium plantagineum, an invasive endemic weed in Australia. Roots of E. plantagineum produced red-coloured NQs in the periderm of primary and secondary roots, while seedling root hairs exuded NQs in copious quantities. Confocal imaging and microspectrofluorimetry confirmed that bioactive NQs were deposited in the outer layer of periderm cells in mature roots, resulting in red colouration. Intracellular examination revealed that periderm cells contained numerous small red vesicles for storage and intracellular transport of shikonins, followed by subsequent extracellular deposition. Periderm and root hair extracts of field- and phytotron-grown plants were analysed by UHPLC/Q-ToF MS (ultra-high-pressure liquid chromatography coupled to quadrupole time of flight mass spectrometry) and contained more than nine individual NQs, with dimethylacrylshikonin, and phytotoxic shikonin, deoxyshikonin and acetylshikonin predominating. In seedlings, shikonins were first found 48h following germination in the root-hypocotyl junction, as well as in root hair exudates. In contrast, the root cortices of both seedling and mature root tissues were devoid of NQs. SPRE (solid phase root zone extraction) microprobes strategically placed in soil surrounding living E. plantagineum plants successfully extracted significant levels of bioactive shikonins from living roots, rhizosphere and bulk soil surrounding roots. These findings suggest important roles for accumulation of shikonins in the root periderm and subsequent rhizodeposition in plant defence, interference, and invasion success.
Keywords: Localization, periderm, plant secondary products, rhizosphere, shikonins, soil microprobes, SPRE, transport.

1. Introduction

1.1. Plant secondary products (PSPs) and root interactions

Although our knowledge of root structure and function has improved in recent years, the complex activities and interactions of roots in the soil rhizosphere and at the soil-root interface are often poorly described, and this is particularly true for invasive weeds. It has become increasingly evident that both root exudation and rhizodeposition in plants are responsive to biotic and abiotic stressors and are clearly important for the protection of sessile terrestrial plants, particularly in the immediate area surrounding living roots, otherwise known as the rhizosphere (Bertin et al., 2003; Badri et al., 2009; Watt and Weston 2009; Weston et al., 2012a). Over time, living roots accumulate and release bioactive plant secondary products (PSPs) from various root tissues, creating both physical and chemical barriers against penetration by plant pathogens, microbes and herbivores (Hutzler et al., 1998; Weston and Duke 2003; Callaway et al., 2008). Protective mechanisms used by living plants have recently been explored in some detail for selected crop or medicinal plants and their resulting cell suspension cultures (Weston et al., 2012a, 2013a; Yazaki, 2005; Ozgen et al., 2011; Wink, 2015).

The accumulation of PSPs in specialized tissues and organs in living roots and their potential role in rhizosphere defence have been documented for several crop and medicinal species. For example, the distribution of bioactive glucosinolates in the periderm of canola roots with respect to their role as soil fumigants and plant protectants was studied by McCully et al. (2008). In 2001, Czarnota et al. first described the role and mode of action of phytotoxic sorgoleone and related long chain hydroquinones produced by living sorghum root hairs as plant growth inhibitors, and also described localization and release of sorgoleone by living root hairs (Czarnota et al., 2003; Weston et al., 2012a, 2013a). The saponin avenicin was identified in oat root tips by Osbourn in 1996 and its activity and localization as an antifungal agent and plant protectant in roots was later described by Morrissey and Osbourn (1999). The role of flavonoids in legume roots, legume nodulation and rhizobium signalling processes has also been well documented and more recently flavonoids have been shown to mediate allelopathic interactions in the plant rhizosphere (Carlsen and Fomsgaard, 2008; Mathesius and Watt, 2011; Weston and Mathesius, 2013).
Phenolic constituents such as flavonoids are also important in structural plant protection (Harborne, 1999). Phenylpropanoid and flavonoid molecules accumulate in both guard cells and epidermal cells, on outer layers of organs, in waxes, or may be covalently linked to plant cell walls (Schnabl et al., 1989; Hrazdina and Jensen, 1992; Hutzler et al., 1998). Numerous studies have indicated a high degree of compartmentalization of phenylpropanoids and flavonoids, and enzymes responsible for their production. However, most root-produced compounds of interest remain to be evaluated in terms of their accumulation over time in various plant tissues and organs. As Hutzler et al. (1998) suggest, a basic understanding of the ecological function of phenolic compounds requires a simultaneous understanding of the structure of the compounds of interest, their biosynthetic pathways and regulation and also their tissue localization. Recent developments in microscopy techniques including confocal laser scanning microscopy (CLSM) have provided opportunities to study localization of PSPs, including phenolics, more precisely than the use of conventional brightfield and fluorescence microscopy. In particular, CLSM allows for identification of compounds of interest by studying specific fluorescence characteristics, including emission and absorption (Sheppard, 1993; Hutzler et al., 1998).

Currently, detailed information is very limited on the anatomy of invasive plant roots, the role of associated secondary products in plant protection, interference with plant growth and subsequent plant invasion. Although Callaway (2000), Callaway and Aschehoug (2000) and Callaway et al. (2008) outline the possible role of PSPs released by root exudates in plant invasion, the specific study of their localization in plant roots and the release mechanisms of allelochemicals by invasive plants have rarely been documented. However, PSPs are known to be important in influencing rhizosphere interactions among noxious weedy species, including those with neighbouring native plants as well as microbial associations (Hierro and Callaway, 2003; Stinson et al., 2006; Callaway et al., 2008; Inderjit et al., 2011).

Recent breakthroughs in the study of the plant rhizosphere have reported on root-associated microbiomes (Edwards et al., 2015) and the identification of novel microbial metabolites with activity as potent antibiotics or quorum sensing agents (Weston and Mathesius, 2013). However, fewer studies have actually documented the release of plant- or microbially-produced metabolites or ‘novel weapons’ influencing plant invasion success, particularly with respect to their localization in roots, the rhizosphere or in bulk soil (Weidenhamer and Callaway, 2010; Inderjit et al., 2011).
This is most certainly due to the difficulty in identification of trace quantities of PSPs in roots and the soil rhizosphere, and the fact that the soil rhizosphere interface can be an incredibly complex and dynamic matrix that is difficult to survey or potentially extract. The recent development of techniques that allow for dynamic profiling of non-polar to moderately polar root-produced PSPs in the soil rhizosphere with silicone tubing and solid phase root zone extraction has facilitated more precise and direct profiling of certain moderately polar to non-polar bioactive molecules released by living plant roots, as in the case of Sorghum bicolor and Tagetes erecta. These techniques have also allowed for consideration of spatial mapping of PSPs and their deposition within the living plant rhizosphere (Mohney et al., 2009; Weidenhamer et al., 2009).

1.2. Paterson’s curse (Echium plantagineum), an important invader in Australia

In Australia, Echium plantagineum L., commonly known as Paterson’s curse or salvation Jane, is a noxious weed infesting more than 30M ha of crop and rangeland (Piggin, 1982; Grigulis et al., 2001). It is native to the Iberian Peninsula, specifically the eastern regions of Spain and Portugal, and is now naturalized across much of southern Australia, parts of the Mediterranean, the USA and South Africa (Piggin, 1982; Weston et al., 2012b, 2013b). Introduced in the mid 1800s to Australia, the initial distribution of Paterson’s curse is likely associated with the frequent importation of Merino sheep, or as an accidental contaminant of pasture seed and hay (Zhu et al., 2014). In recent years its range has increased (Weston et al., 2013b) and it often dominates plant communities in poor, drought-prone soils to the extent that it costs the wool and meat industries more than A$250M per year in losses due to reduced livestock productivity (NRM South and the Southern Tasmanian Councils Authority, 2015).

1.3. Bioactive plant secondary products in Echium plantagineum

E. plantagineum produces significant quantities of several important PSPs including pyrrolizidine alkaloids in its leaves, stems, flowers and seeds that can cause liver, kidney and lung damage in mammals, eventually poisoning horses, sheep and cattle that have consumed sufficient quantities of foliage (Peterson and Jago, 1984; Colegate et al., 2005; Quinn et al., 2014; Skoneczny et al., 2015). However, in addition to pyrrolizidine alkaloids, E. plantagineum also produces unusual bright red-coloured naphthoquinones (NQs) in its roots. Analysis of many field-collected roots of E. plantagineum by the authors revealed that the outer layers of root tissue in the primary taproot or smaller fibrous secondary roots are very often pink or red due to the production of a mixture of bioactive, brightly coloured NQs known as shikonins (Weston et al., 2013b).
NQ content typically increases in roots of summer-collected plants in comparison to those sampled in winter or spring. In addition, geographically distinct populations of *E. plantagineum* collected from warm, dry roadside locations across New South Wales (NSW) at low elevations produced significantly (three to five-fold) higher concentrations of NQs than plants collected from similar sites with cooler average temperatures or higher elevations (Weston et al., 2013b).

Although the production of shikonins is unusual in higher plants, the roots of numerous members of the Boraginaceae, including species of *Alkanna*, *Arnebia* and *Lithospermum* as well as *Echium*, contain numerous shikonins (Papageorgiou et al., 1999; Sommer et al., 1999; Boehm et al. 2000). In the medicinal literature, these compounds are referred to as shikonins, alkannins or naphthazarins and have been the subject of numerous studies due to their activity as antioxidants, antihelminthics and purgatives, and as aids in wound-healing (Papageorgiou et al., 1999; Assimopoulou et al., 2006; Hu et al., 2006; Albreht et al., 2009). They have also been reported as curatives for prostate cancer due to their ability to induce cell apoptosis (Gara et al., 2015). Shikonins from the Boraginaceae specifically exhibit potent antibiotic activity against certain gram-negative bacteria (Papageorgiou et al., 1999). Strong antagonistic effects of shikonin and other naphthoquinones on other plants, insects, fungi and bacteria have also been observed; activity is likely associated with the potent inhibition of electron transport processes by NQs, particularly upon respiration, but cell division or other cellular processes may also be impacted (Binder et al., 1989; Brigham et al., 1999; Babula et al., 2009; Weston et al., 2012a, b). Both purified NQs, including shikonin and acetylshikonin and root extracts of field-grown Australian *E. plantagineum* showed potent activity on plant growth, in contrast to similar concentrations of extracts from Spanish plants (Garcia Duran et al., 2014, 2015).

In order to gain a more fundamental understanding of the ecological role of NQs in *E. plantagineum* root tissues and also the rhizosphere of this invasive plant, we employed both confocal and light microscopic imaging techniques to perform anatomical investigations of living roots of this weedy invader, along with UHPLC/Q-ToF MS (ultra-high pressure liquid chromatography coupled to quadrupole time of flight mass spectrometry) to perform metabolic profiling of root, root hair and soil extracts. We also utilized SPRE (solid phase root zone extraction) microprobes in the soil rhizosphere to profile PSPs of interest in the rhizosphere and bulk soil surrounding living plant roots to
further define the role of PSPs as potential drivers of plant/plant and plant/organismal interactions in the rhizosphere.

2. Materials and methods

2.1. NQ localization experiments in living plant tissue using confocal and light microscopy

_Echium plantagineum_ root tissues were collected from densely populated local field stands in Wagga Wagga, NSW, Australia (−35.0586°N, 147.3507°E) in 2014. At least five mature flowering specimens were collected for microscopic evaluation of NQs at various intervals from August to October 2014. On all occasions following collection, plants were placed in wet paper towelling to prevent dehydration and maintained at a temperature of 4 °C until evaluation under a microscope, which was typically performed within 1h of collection. _E. plantagineum_ seed was also collected from the same field location in Wagga Wagga, NSW, in 2013 and used to generate seedlings for time-course experimentation of NQ production under controlled environmental conditions. Seeds were germinated in June 2014 on Whatman No. 1 filter paper moistened with 5ml sterile deionized water in sterile 9-cm plastic Petri dishes containing 20 seeds per dish, with three replicates for each harvest time interval (n=60 seedlings). Harvest times were 12, 24, 48, 72, 96, 128 and 144h after experimental initiation. During incubation, dishes were sealed with parafilm and placed in a lighted incubator at 25/18 °C day/night temperatures with a 12-hour photoperiod; microscopic evaluation was performed immediately following each harvest.

Mature roots and seedling root and hypocotyl tissues were hand sectioned for examination using confocal microscopy (Nikon A1 Confocal TiE inverted microscope, excitation 488nm, emission 570–620nm for NQ/shikonin evaluation). In most cases, sections were directly examined without staining, however calcofluor white (1% aqueous solution) (Sigma Aldrich, Australia) was used on a few occasions for more pronounced staining of the cell wall. The fluorescence from calcofluor white was detected with an excitation of 405nm and emission from 425 to 475nm. The same confocal microscope was used for both hyperspectral analyses and imaging to compare _E. plantagineum_ fresh root periderm spectra with those generated with an ethanolic solution of pure shikonin (Sigma Aldrich, Australia) at a concentration of 1mg ml⁻¹. For spectral analyses, images were scanned sequentially with excitation wavelength of 405 and 488nm, with a wavelength interval of 6nm and emission range from 405–550 and 550–740nm, respectively. The two spectral scans were then automatically combined by Nikon
confocal NIS ver 4.10 to create the emission spectrum presented. For seedlings generated during the time course experiment, stereoscopic light microscopy using both a Nikon SMZ25 and a Leica M205FA was performed at each time interval with numerous sampled seedlings.

2.2. Microprobe experiments to assess NQs in rhizosphere and rhizosphere soil extraction

Solid phase root zone extraction (SPRE) microprobes (5cm) were prepared as per Weidenhamer et al. (2009) using silicone polydimethylsiloxane (PDMS) Silastic tubing (Fisher Scientific, USA), supported internally with fine stainless steel wire (22 gauge), as a probe for entrapment of nonpolar soil rhizosphere PSPs such as NQs. To fully evaluate whether microprobes could be used to entrap and collect NQs to intensify concentrations of these compounds in a rhizosphere setting, eight sets of three microprobes each were placed in contact with live roots collected from mature greenhouse-grown plants. Roots were sectioned into 5cm long pieces and placed in contact with microprobes for 1 or 12 hours in each of eight sterile Petri dishes containing moistened filter paper. After treatment, unexposed microprobes (those not subjected to root exposure and therefore serving as a negative control) and root-exposed microprobes were photographed. All unexposed controls and those microprobes exposed to roots for 12 hours were then extracted in HPLC grade 100% ethanol (VWR Chemicals, Australia) for 10min, followed by evaporation of extracts under a stream of N\textsubscript{2} gas for approximately 20min. The resulting dried extract was weighed and resuspended in ethanol, filtered through a 22 μm Millex syringe filter and subjected to UHPLC/Q-ToF MS analysis as described below.

Greenhouse-grown plants were propagated for 16 weeks in a soil mix containing 6:4 peat potting mix:sand (Scotts Co., Melbourne, Australia.). Seedlings were pre-germinated by imbibing in sterile water for 1 week using field-collected seed as described above in NSW, Australia; after 7 d, seedlings were transplanted into 1.5-l pots and were maintained in the glasshouse at 25/18 °C day/night temperatures at ~55% relative humidity. Plants were watered every other day by subirrigation and fertilized once per fortnight using a commercial liquid fertilizer (N:P:K=23:3.95:14, Aquasol Soluble Fertilizer, Australia). At 14 weeks of age, as plants began to flower, plants were sampled for rhizosphere-produced NQs using 5cm microprobes, as described above. Eight probes were placed equidistantly into each of six plant pots and were fully inserted into the soil surrounding the living plant, ~8cm from the centre of the plant rosette. The probes were
placed equidistantly around the plant at a distance of ~5cm from the taproot and were not in contact with the foliage of the plant.

In addition, soil media was collected separately from the rhizosphere (soil not adhering to plant roots but located around living plant root system) of each pot, thoroughly filtered to remove any small residual root pieces using a fine wire mesh screen (<1mm mesh holes), air-dried and extracted in 100% ethanol, filtered using a 22 μm Millex filter and subjected to analysis using an ion trap mass spectrometer for NQ detection.

2.3. Chemical extraction of field-collected root samples

Specimens of *E. plantagineum* were collected in the field from 21 locations across NSW and Australian Capital Territory, Australia, in 2013 and 2014. At each collection site, GPS coordinates were recorded, and five or more intact root specimens were collected from mature, flowering plants. Roots were carefully collected from field sites using a mattock to remove the majority of the root system without excessive damage to the taproot and main secondary roots. Excess soil was removed, and roots were placed in moist paper towelling and stored at 4 °C for 24h prior to extraction. Root periderm extracts were prepared by thinly peeling the coloured outer periderm layer only from taproots or primary roots using a sharp scalpel blade. To minimize the impact of plant-to-plant variation of extracts to be subjected to metabolic profiling, composite samples were prepared from 5–6 individual plant roots collected at each of 21 locations using ~0.2g of fresh periderm per plant to generate 1g total fresh weight of periderm tissue. Periderm peels (1g) were then extracted in 10ml of 100% HPLC grade ethanol (VWR Chemicals, Australia) for 14h in the dark, at room temperature, after placement on a slow orbital shaker at 120rpm. Following extraction, samples were filtered using a 22 μm Millex syringe filter, and 1ml of each extract was transferred into individual HPLC vials with duplicate samples available for replicated UHPLC/Q-ToF MS profiling ([Weston et al.](#), 2015). A similar protocol was used to extract fresh root periderm tissues of seedlings grown in controlled environments; in this case up to 0.25g of fresh tissue was extracted from 10 seedlings in ethanol to provide sufficient sample for further UHPLC/MS analysis.

2.4. UHPLC/Q-ToF MS analyses

Metabolic profiling of key NQs in root periderm extracts was performed using an Agilent 1290 Infinity UHPLC system equipped with quaternary pump, degasser, temperature controlled column and cooled autosampler coupled to an Agilent 6530
Quadrupole Time-of-Flight (QToF) mass spectrometer with Dual Agilent Jet Stream Electrospray Ionisation Source (Dual AJS ESI) (Agilent Technologies, Mulgarve, Australia) (Weston et al., 2015). Separation was achieved using a C\textsubscript{18} Poroshell column (2.1×100mm, 2.7µm) at 25 °C equipped with an SB-C\textsubscript{8} guard column (2.1×12.5mm, 5µm) (Agilent, Santa Clara, CA, USA) and a flow rate of 0.5ml min\textsuperscript{-1}. The column was equilibrated for 30min prior to analysis. Solvents used for extraction and UHPLC/MS were HPLC grade. Acetonitrile was obtained from Hipersolv (Tingalpa, Australia), formic acid (>99% purity) from Sigma (Castle Hill, Australia) and LC-MS water from Merck (Darmstadt, Germany). Separation of NQs was achieved using a gradient of mobile phase A (water+0.1% formic acid) and mobile phase B (95% acetonitrile+0.1% formic acid), starting with 50% B for 1min and reaching 100% B over 7min, continuing at 100% B until 10.50min, returning to 50% B over 0.1min and held at 50% B from 10.60–17.00min. (Skoneczny et al., 2014). The QToF was run and calibrated in negative ion mode, with nebulizer gas at 35 psig, capillary voltage at 3500V and fragmentor voltage at 135V. Nitrogen was used as drying gas at 250 °C at a flow of 9 l min\textsuperscript{-1}. Data were collected in negative ion mode using an extended dynamic range (2 GHz). Additional LC/MS-MS experimentation was performed for selected molecules of interest (Huang et al., 2010) using analytical standards of acetylshikonin (MW 330.1103; RT 5.75 min±0.7min), deoxyshikonin (MW 272.1048; RT 6.75±0.7min), dimethylacrylshikonin (MW 370.1416; RT 7.7 min±0.7min) purchased from ChemFaces (Wuhan, China) and shikonin (MW 288.0997; RT 3.51 min±0.7min) obtained from Biomol (Hamburg, Germany). All data were analysed using MassHunter software (ver. B.07, Agilent, Santa Clara, CA, USA).

2.5. Q-Trap HPLC/MS analyses

Further analyses of NQs in soil and soil microprobe extracts at trace concentrations were performed using Agilent 1200 series HPLC coupled to an ABSciex 3200 Q-Trap mass spectrometer (AB Sciex, Foster City, CA, USA) and using analytical standards (as described in the previous section) and HPLC grade solvents including methanol from Rathburn (Walkerburn, Scotland) and formic acid from Merck (Darmstadt, Germany). Separation was achieved using Kinetex XB-C\textsubscript{18} 2.1×100mm, 2.6 µm, 100 Å (Phenomenex, Macclesfield, UK) column with a gradient of solvent A (water, 0.02% formic acid) and solvent B (100% methanol, 0.02% formic acid). The gradient was initiated with 40% B for 1min and reached 98% B over 9min, continued at 100% B until 13.0min and returned to 40% B over 0.1min and held at 40% B from 13.1–18.50min.
Optimization of the MRM transitions for purified standards was performed using standards formulated at 5ppm (in 50:50 methanol:water) that were injected into the MS/MS interface using syringe infusion at 10 µl min⁻¹. To determine specific precursor and product ions for each standard, MS/MS was performed, and after optimization shikonins were evaluated in negative ion mode (Table 1). Data were analysed using Analyst 1.5 software (AB Sciex, Foster City, CA, USA).

**Table 1** Optimized values of compound dependent parameters for purified analytical standards of shikonin, deoxyshikonin, acetylshikonin and dimethylacrylshikonin obtained using ABSciex 3200 QTrap mass spectrometer (AB Sciex, Foster City, CA, USA).

<table>
<thead>
<tr>
<th>Compound name</th>
<th>Q1 (precursor ion)</th>
<th>Q3 (daughter ion)</th>
<th>Declustering potential</th>
<th>Entrance potential</th>
<th>Cell entrance potential</th>
<th>Cell exit potential</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deoxyshikonin</td>
<td>271.821</td>
<td>203.1</td>
<td>-40</td>
<td>-5</td>
<td>-28</td>
<td>-4</td>
</tr>
<tr>
<td>Shikonin</td>
<td>286.901</td>
<td>217.9</td>
<td>-25</td>
<td>-1</td>
<td>-18</td>
<td>-4</td>
</tr>
<tr>
<td>Acetylshikonin</td>
<td>328.923</td>
<td>269.1</td>
<td>-25</td>
<td>-5</td>
<td>-26</td>
<td>-4</td>
</tr>
<tr>
<td>Dimethylacrylshikonin</td>
<td>368.782</td>
<td>269.0</td>
<td>-20</td>
<td>-5</td>
<td>-20</td>
<td>-4</td>
</tr>
</tbody>
</table>

2.6. Statistical analysis

Abundance of deoxyshikonin, shikonin, acetylshikonin and dimethylacrylshikonin was analysed in 21 samples, representing 21 populations. Analysis of variance was performed on log transformed data in IBM SPSS statistics software (IBM Corp., NY, USA). Homogeneity of variances was assessed using Levene’s test prior to further analysis. Tukey HSD was used as a post hoc test to evaluate differences among metabolite levels averaged over populations.

3. Results and discussion

In both field and glasshouse raised *E. plantagineum* roots, shikonins were clearly identified in the outer one to two cell layers of newly formed periderm in mature and seedling taproots as well as secondary roots (Fig. 1 A–C). The periderm is defined as the protective outer cortical layer present in many roots and stems of dicots; this layer can also contain secondary plant products likely involved in plant protection (McCully *et al.*, ...
Shikonin presence was denoted by red colouration of the outer periderm and/or autofluorescence at 488nm, as reported by Papageorgiou et al. (1999).

Similar to localization of glucosinolates in canola periderm (McCully et al., 2008), shikonins were found only in outer periderm tissues and not interior root cortical tissues as observed by microscopy and evaluation by UHPLC/Q-ToF MS (Table 1). Shikonins are both UV absorptive and autofluorescent, and can be highly coloured, ranging from pink to red or purple, depending on concentration and pH. Confocal microspectrofluorimetry was therefore used to confirm the presence of shikonins in situ in mature periderm tissue of field-collected plants by scanning over an emission spectrum ranging from 405 to 740nm in comparison to a known standard of shikonin. Nearly identical spectroscopic results were obtained from both scans, suggesting that compounds present in mature periderm tissue of E. plantagineum are identical or closely related to the bioactive naphthoquinone shikonin (>98% pure standard of molecular weight=288.3) both in their unique colouration and their autofluorescence (Fig. 2). Upon closer examination of mature periderm cells under greater magnification, we clearly observed the presence of numerous small red-coloured vesicles in the interior of the cell (Fig. 1D, E), suggesting that incorporation into vesicles is a means of transport of PSPs such as shikonins in the cell, and likely also serves to protect intracellular organelles and processes against autotoxicity associated with the presence of naphthoquinones such as shikonins, which exhibit potent inhibition of respiration and electron transport processes (Babula et al., 2009; Weston et al., 2012a, 2013a).
**Fig. 1.** Localization of shikonins in *E. plantagineum*. Under bright field and confocal microscopy shikonins were bright red in colour. (A) Bright field image of mature taproot cross section showing the red-coloured periderm cells (arrow). Bar, 1mm. (B) Sequential scanning confocal image of mature secondary root cross section showing autofluorescence of the periderm tissue corresponding to shikonin localization (arrow). Bar, 250 µm. (C) Bright field image of mature taproot surface showing shikonin deposition in mature root. Bar, 250 µm. (D) Confocal image of a typical periderm cell of mature plant containing numerous small vesicles (arrows). Bar, 20 µm. (E) Confocal image of selected intact root epidermal cells from a 6-day-old seedling, showing numerous vesicles (arrows). Tissue was stained with calcofluor white (blue). Bar, 10 µm. (F) Sequential scanning confocal image of outer periderm cells of a mature plant, showing shikonins localized in extracellular areas (arrows). Tissue was stained with calcofluor white (blue). Bar, 10 µm. (G) Sequential scanning 3-D image of outer periderm cells of a mature plant, showing shikonins localized in extracellular areas (arrows). Tissue was stained with calcofluor white (blue). Width, 127.15 µm; height, 127.15 µm; depth, 28.50 µm. (H) Bright field image of root hair of 3-day-old seedling, showing root hair exudation of shikonins (arrows). Bar, 200 µm. (I) Confocal image of root hair in (H), showing numerous vesicles throughout the root hair (arrows). Bar, 10 µm.
Fig. 2. Spectral imaging of (A) pure shikonin in ethanol (concentration of 1mg ml\(^{-1}\) in ethanol) in comparison to (B) (black line) outer periderm cells of mature \textit{E. plantagineum} root containing shikonins using multiple spectral scans at excitation wavelengths of 405 and 488nm, and (B) (grey line) control spectral scan of root cortex which is devoid of shikonins. Respective fluorescence emission (peak maxima at ~560 and 620nm) is very similar for an analytical standard of shikonin and hyperspectral scan of root periderm \textit{in situ}.

Confocal analysis also revealed that shikonins accumulated in large quantities extracellularly by deposition outside of the cell in extracellular spaces over time or possibly in association with plant cell walls through covalent bonds (Fig. 1F, G). Phenylpropanoid and flavonoid molecules also accumulate in outer layers of plant organs, in waxes, or are even covalently linked to plant cell walls (Schnabl \textit{et al.}, 1989; Hrazdina and Jensen, 1992; Hutzler \textit{et al.}, 1998). It is possible that shikonins may play a role in structural integrity as well as exhibiting both phytotoxic and antimicrobial activity in the plant periderm and rhizosphere (Brigham \textit{et al.}, 1999; Garcia Duran \textit{et al.}, 2014). The extracellular deposition of shikonins suggests that they may play a role in structural integrity of the periderm layer over time and/or these PSPs are exported to prevent autotoxic build-up in the dynamic intracellular environment of a specialized periderm cell. Further labelling studies would help to determine if NQs are incorporated into the cell walls of periderm tissues or are just deposited in extracellular spaces.

Field and glass-house grown plant examination revealed that shikonins were also released by direct exudation in droplets which accumulated at the tips of living root hairs. We observed this phenomenon not only in mature plant root hairs but also in seedlings grown in Petri dishes within 48h of germination and radicle elongation. It is evident that considerable exudation occurs in seedlings as noted by the copious quantities of red-coloured exudates observed accumulating at the tips of living root hairs (Fig. 1H). Confocal analyses indicated the presence of numerous small vesicles present in living
root hair cells (Fig. 1I), which are likely associated with shikonin intracellular transport and exudation. Root hair exudation in *E. plantagineum* is strikingly similar to that observed in living sorghum roots, which release large quantities of sorgoleone accumulating in vesicles at the tips of living root hairs. In the case of non-polar long chain hydroquinones such as sorgoleone, exudation is reported to occur by direct extrusion through spaces or pores in the plasmalemma (Weston *et al.*, 2012b, 2013a). Further experimentation is required to determine if protein transporters are associated with the movement and deposition of moderately polar to non-polar shikonins to extracellular spaces, and to quantify the relative availability and abundance of these compounds on the surface of mature taproots and secondary roots which may not possess the large numbers of living root hairs found on seedlings of *E. plantagineum*. The living periderm is continually replaced in dicots, and over time significant rhizodeposition of shikonins may occur due to the continuous sloughing off and degradation of periderm tissue, as indicated for canola by McCully *et al.* (2008). This suggests that NQs could be continually replenished into the rhizosphere during the life cycle of a biennial such as *E. plantagineum*.

However, similar to sorgoleone exudation which increased in stressed plants, Brigham *et al.* (1999) noted that production of shikonins in root suspension cultures increased over time with exposure to stressors such as temperature and extracts containing fungal cell walls, which serve as elicitors of shikonin production. We also noted this correlation in field experiments which detected increased production of shikonins in plant roots that were collected from field sites experiencing greater temperature, lower elevation and likely drought stress (Weston *et al.*, 2013b).

Time course experiments performed in this study at short intervals following seed imbibition also yielded important information regarding phenological development of seedling root hairs and periderm tissues over time (Fig. 3A–E). Deposition of coloured shikonins was first observed in *E. plantagineum* seedlings at ~48h following imbibition of seed and consistently appeared in the root–hypocotyl junction. In addition, nearby numerous root hairs on the developing radicle were observed exuding copious quantities of bright red exudates as droplets at the tip of the root hair (Fig. 3C, F). When contrasting the production of shikonins in various locations along a single seedling root, we noted that the basal portion of the seedling root possessed a higher level of active root hairs and greater numbers of shikonin-producing periderm cells than did the acropetal portion of the same root at 72h following imbibition (Fig. 4A–D). With the presence of significant
quantities of shikonin in more mature periderm tissue and root hairs, we noted distinct autofluorescence due to the presence of these compounds in the basal portion of the root and not in the acropetal. The bright red autofluorescence observed in Fig. 4C was associated with the exudation of root hairs containing concentrated levels of shikonins.
Fig. 3. Time course experiment of *E. plantagineum* seedlings (a–e) at 24, 36, 48, 72 and 120h after germination. Note the location of shikonin production noted by red colouration as visualized on the radical and hypocotyl. Photos A–D correspond to seedlings at phenological stages a–d, respectively and photos E–F to radical and hypocotyl stages noted in panel e. (A) Seedling 24h after imbibition. (B) Seedling 36h after imbibition, showing newly emerging root hairs. (C) Seedling 48h after imbibition, showing shikonin localization in root primordial zone and numerous exuding root hairs. (D) Seedling 72h after imbibition, showing shikonin production in hypocotyl, radicle and root hairs. (E) Hypocotyl of seedling at 120h following imbibition, showing shikonin production in zone of differentiation between radicle and hypocotyl. (F) Radicle at 120h after imbibition, showing distinct mature root hairs producing shikonins and extensive shikonin accumulation in developing periderm. Bars, 500 µm (A); 200 µm (B–F).
**Fig. 4.** Radicle 72h after imbibition. (A) Bright field image of more mature (upper) radicle showing root hair exudation. (B) Bright field image of immature (lower) radicle, exhibiting no visible root exudation. (C) Fluorescent image of (A) with Texas red filter showing corresponding shikonin localization in same tissue sample. (D) Fluorescent image of (B) with Texas red filter showing absence of shikonin autofluorescence. Bars, 200 µm (A–D).

### 3.1. Screening of Australian populations of E. plantagineum

UHPLC/Q-ToF MS separation and detection resulted in successful metabolic profiling of numerous related and bioactive shikonins in field-grown plants; specifically, we detected and identified shikonins at significant and potentially bioactive as phytotoxins and antimicrobials ranging from 0.3 to 10ppm in periderm extracts resulting from direct ethanolic extraction from plants of 21 geographically distinct populations (Garcia Duran et al., 2014, 2015). Concentrations of shikonins present in intact periderm are estimated to be potentially higher in some periderm tissues, based on confocal experimentation and hyperspectral imaging studies, and estimation in intact tissues is dependent on rooting environment and plant maturity.

Although Weston et al. (2013b) previously reported the presence of acetylshikonin, deoxyshikonin and shikonin in root extracts using an Agilent LC/MS 6410 QQQ instrument, in this experiment using an Agilent 6530 UHPLC/Q-ToF MS, the presence of numerous additional (>9) shikonin derivatives in samples obtained from plants collected across southern Australia was more precisely noted using similar
methods to those reported in Skoneczny et al. (2014, 2016). By direct comparison with available purified standards of four derivatives, the abundance of three of the most bioactive NQs in periderm extracts including acetylshikonin, deoxyshikonin and shikonin (Garcia Duran et al., 2014) as well as dimethylacrylshikonin in all extracts was also assessed (Fig. 5A, B).

Fig. 5. Total ion chromatograms obtained using UHPLC/Q-ToF MS for a prepared mixture of analytical standards (A) in comparison to a standard periderm extract (B). Identified compounds include: 1, shikonin; 2, acetylshikonin; 3, deoxyshikonin; 4, dimethylacrylshikonin.

Despite variation among samples, we found a consistent pattern of compound abundance among root periderm extracts from mature field-collected plant populations (Fig. 6). Dimethylacrylshikonin was present in significantly higher abundance ($P<0.001$) in all field periderm extracts, while deoxyshikonin was present in significantly lower abundance ($P<0.001$) in all samples. Deoxyshikonin is thought to be the precursor of shikonin and is therefore likely rapidly converted to shikonin and also potentially numerous higher molecular weight derivatives, particularly dimethylacrylshikonin (Papageorgiou et al., 1999; Sommer et al., 1999). Shikonin, acetylshikonin and dimethylacrylshikonin were all highly active when assessed as either plant growth inhibitors or antimicrobials, with shikonin generally the most active (Weston et al., 2012b; Garcia Duran et al., 2014).
We have identified other NQ derivatives in root extracts of various *Echium* spp. in trace quantities, but their biological activity at this time is not known. Only one population (White Cliffs, NSW, Australia) showed greatly enhanced production of shikonins, specifically dimethylacrylshikonin, in contrast to the other 20 populations evaluated. Interestingly, plants in White Cliffs are typically exposed to high UV and summer temperatures and low average rainfall events, as this location borders the Australian outback, a large inland desert. The evaluation of gene expression underlying biosynthesis of dimethylacrylshikonin and other NQs in this population is now underway.

![Graph](image)

**Fig. 6.** Relative abundance of shikonins in periderm extracts collected from geographically distinct populations of *E. plantagineum* across NSW, Australia in 2012–2014. Samples were analysed using UHPLC/Q-ToF MS instrumentation using the tandem mass spectrometry approach in comparison to purified standards of the same NQ derivatives. Each sample was a composite of 5–6 individual collected from 21 populations and results were averaged for each compound of interest. Error bars denote standard error of the means, n=21. *, compound in significantly lower abundance (*P*<0.001); **, compound in significantly higher abundance (*P*<0.001).

### 3.2. Analysis of soil and microbe extracts

Using an HPLC/MS Q-Trap for sensitive analysis of NQs in extracts and soils, we developed a reliable multiple reaction mode (MRM) method that allowed detection of trace levels of selected shikonins in both soil and microprobe extracts. The main constituent found in microprobes brought into contact manually with living *E. plantagineum* roots or placed in soil of glasshouse-grown potted plants was acetylshikonin, observed in probe extracts at levels of ~0.9ppm and in ethanolic extracts of soil at levels of 2ppm using ethanolic extraction. Our limit of detection (LOD) of acetylshikonin in soil and microprobe samples subjected to potted soils and roots was ~0.3ppm. In all probe and soil extracts collected from potted soil rhizospheres
surrounding mature *E. plantagineum*, shikonin was the next most abundant compound (Table 1). Deoxyshikonin, dimethylshikonin and other shikonin derivatives were often below the limit of detection (LOD) in most samples (Table 2).

Interestingly, shikonin and acetylshikonin are typically the most phytotoxic of the shikonin type of NQs studied (Garcia Duran *et al.*, 2014, 2015), and acetylshikonin particularly appears to accumulate in highest concentrations in soil and periderm extracts over time. This may be associated with the relative stability of shikonin and acetylshikonin, in contrast to their precursor deoxyshikonin or larger molecular weight derivatives. However, these studies clearly show that NQs do accumulate in significant levels in the rhizosphere of living plants. Not only can shikonins be directly extracted from soil, small soil microprobes can be utilized to more accurately detect their presence within the living root system. Microprobes have been previously successfully employed for direct solid phase extraction of non-polar constituents such as the thiophenes or sorgoleones from soil (Weidenhamer *et al.*, 2009), and in this case the technique worked well to extract considerable quantities of moderately non-polar shikonin derivatives from soil, as reported by Weidenhamer *et al.* (2014). Entrapment of shikonins on microprobes was easily observed by the red or pink colour of microprobes placed in contact with living roots or removed from the rhizosphere (Fig. 7) of living potted plants.

**Table 2.** Presence of four bioactive shikonins identified by tandem MS and studied in different matrices in microprobe experimentation. LOQ, limit of quantification; LOD, limit of detection.

<table>
<thead>
<tr>
<th>Type of extract</th>
<th>Acetylshikonin</th>
<th>Deoxyshikonin</th>
<th>Dimethylscrylshikonin</th>
<th>Shikonin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Periderm</td>
<td>&gt;LOQ</td>
<td>&gt;LOQ</td>
<td>&gt;LOQ</td>
<td>&gt;LOQ</td>
</tr>
<tr>
<td>Silicone tubing</td>
<td>&gt;LOQ</td>
<td>&lt;LOD</td>
<td>&lt;LOD</td>
<td>&lt;LOD</td>
</tr>
<tr>
<td>Soil and silicone tubing</td>
<td>&gt;LOQ</td>
<td>&lt;LOD</td>
<td>&lt;LOD</td>
<td>=LOD</td>
</tr>
</tbody>
</table>
Fig. 7. SPRE microprobes consisting of PDMS tubing placed over stainless steel 22 gauge wire (Weidenhamer et al., 2009). Images A–C represent varying concentrations of NQs typically extracted by microprobes from the rhizosphere of *E. plantagineum* grown in pot experiments in the glasshouse. Bar, 1mm. Image C also appears visually identical to control probes and/or those not significantly exposed to root-infested soil.

The role of various root-produced naphthoquinones such as shikonins and their derivatives has been little described in the literature of higher plants under natural field settings, particularly those that are classified as invasive weeds of significance. Interestingly, we have found that *E. plantagineum* plants produced in several climatic zones in Spain contained >two-fold lower concentrations of shikonins than plants produced in field conditions across southern Australia (unpublished data; García Duran, 2014, 2015). These findings are similar to those of Thorpe et al. (2009), who detected greater bioactivity of *Centaurea maculosa* root exudates and bioactive PSPs on plants collected in invasive versus native ranges. We are currently further examining the role of environment and genetics upon regulation of NQ production in *Echium* spp. However, our findings here, in combination with our field and controlled environment experiments (García Duran et al., 2014; Skoneczny et al., in press) suggest that shikonins play an important role in plant protection against microbial invaders, insect herbivores and germinating plants in the rhizosphere of *E. plantagineum*. 
These studies utilized a combination of intricate approaches to study the localization and distribution of bioactive shikonins in the plant and rhizosphere directly surrounding living *E. plantagineum*. The reported biological activity and observed accumulation of shikonins and other NQ derivatives in the periderm and their subsequent deposition in the rhizosphere through exudation or tissue degradation are all suggestive of their potential importance as bioactive novel weapons of significance. This is likely to be of particular importance in monocultural stands under warm and dry conditions in Australia where shikonin production was noted to be enhanced. Studies are now underway to further evaluate and compare NQ and pyrrolizidine alkaloid production and genetics of *E. plantagineum* and other related species in Australia and their native range in the Iberian Peninsula.

4. Acknowledgements

The authors would like to express their appreciation to the Australian Research Council Discovery Program which funded this study through DP130104346 grant awarded to LAW, GMG and RMC and to the Graham Centre for Agricultural Innovation which supported a research award to DS and a research initiative award to LAW. JDW acknowledges support for this project from a 2014 Endeavour Research Fellowship sponsored by the Australian Federal Government through the Department of Education. We also acknowledge the support of Dr. Robert Woolley at Coherent Scientific who assisted with laser confocal imaging.

Footnotes

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Mechanisms of Weed Suppression by Wheat Cultivars

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Related GRDC Projects: UCS 00023, UCS 00022 UCS 00020

Keywords: wheat, weed suppression, metabolic profiling, metabolomics, residue, competition, resource allocation.

Take home messages:

- Field trials were performed in 2014-15 as part of GRDC Projects UCS 00023, UCS 00022, and UCS 00020 “Weed Management in the Southern Region Mixed Farming Systems - Strategies to Combat Herbicide Resistance” to evaluate mechanisms of weed suppression in genetically diverse wheat cultivars, including competition for resources and allelopathy.

- Replicated experiments were conducted with 12 wheat cultivars grown in both moderate to low rainfall zones at Wagga Wagga and Condobolin NSW, respectively.

- Wheat and weed growth was monitored at four stages of growth in each location; early season (tillering), grain filling stages, crop maturity (at harvest) and postharvest. Shoot, roots, rhizoplane and bulk soil from each wheat cultivar were profiled for unique secondary plant products including weed suppressive allelochemicals.

- Significant differences were observed among wheat cultivars with respect to early crop vigour, biomass, leaf area and canopy architecture and yield. Differences were also noted in visual weed suppression, weed count and weed biomass. Crop biomass and yield, as well as weed count and biomass, were both location and cultivar dependent.
• Targeted metabolite analysis of benzoxazinoids (allelochemicals) using LC-MS/MS Qtrap mass spectrometry showed metabolite differences over time between wheat cultivars and growth stage.

• Cultivar competitive traits are clearly influenced by both cultivar and environmental factors, as shown by differences in cultivar performance among the two locations. Cultivars that performed well in terms of crop biomass, yield and weed suppression in both locations included Espada, Condo and to a lesser extent, Janz.

• Additional methods development is now underway for the use of LC/MS QToF mass spectrometry to profile allelochemicals among roots, shoots and rhizosphere soils of various cultivars. This will provide important physiological information regarding crop competitive traits and biosynthesis and activity of related allelochemicals that may be important in long-term weed suppression in the crop.

1. Background

Herbicide-resistant weeds are on the rise across Australia, including an increasing number of cropping weeds experiencing resistance to multiple herbicides (Owen et al., 2013). For instance, glyphosate-resistant weeds across Australia now include annual ryegrass (*Lolium rigidum* Gaud.), barnyard grass (*Echinochloa oryzicola* Vasing.), liverseed grass (*Urochloa panicoides* P.Beauv.), windmill grass (*Chloris truncata* R.Br.), bromegrass (*Bromus inermis* Leyss.) and fleabane (*Conyza bonariensis* L.). As well, 103 populations of annual ryegrass resistant to glyphosate have been confirmed (Preston et al., 2010).

In comparison with pests and diseases, weeds have the potential to incur the greatest yield loss, through competition with the crop and decreasing yield quality, and can, therefore, incur high costs of control (Oerke, 2006). In Australia alone, weeds cost about $4 billion per annum in lost production, decreased quality and in control measures; weed control measures are estimated to be more than $700 million in the wheat industry (Sinden et al., 2004).

Development of wheat cultivars (*Triticum aestivum* L.) with increased inherent competitiveness against herbicide-resistant weed species is a potential non-chemical alternative to chemical weed control. To date, limited success has been achieved in Australia breeding cultivars for enhanced competitive ability, mainly because the complexity of weed suppression is influenced by many factors (Mokhtari et al., 2002;
Crop competitive ability can either be specified in terms of crop tolerance against weeds or growth inhibition of weeds by resource competition (Bertholdsson, 2010).

In addition, wheat cultivar (*Triticum aestivum* L.) shoot and root architectural traits likely play critical roles in crop weed suppression. Benzoazinoids are important allelochemicals present in wheat, barley and rye, and their suppressive effects on weeds, pest and diseases are of great interest in sustainable agriculture (Bertholdsson et al., 2012). The content of hydroxamic acids in wheat varies among species and cultivars and is dependent on plant age and organ assayed; younger leaf tissues produce higher levels of hydroxamic acids (Argandoña et al., 1981).

Crop tolerance through toleration of depleted resources and continuation of growth is measured by crop growth or dry matter accumulation whereas resource competition by suppressing weeds through rapidly depleting resources is measured by weed biomass or weed number. Competitive cultivars have the ability to better access light, nutrients, and water resources in limited space, thus suppressing the growth and reproduction of nearby weed species (Worthington et al., 2015).

Although cultivars with high competitive potential have been identified amongst cereal crops, competitiveness has not traditionally been considered a priority for breeding or farmer cultivar choice (Andrew et al., 2015). In Greece, the use of competitive cultivars alone has already been demonstrated to allow for a 50% reduction in total amounts of herbicides used for weed control in wheat (Travlos, 2012; Andrew et al., 2015). Thus, developing grain cultivars with superior competitive ability against weeds will complement cultural methods for weed control in maintaining acceptable yields and suppressing weed populations (Worthington & Reberg-Horton, 2013; Andrew et al., 2015).

To realise the potential of competitive crop cultivars as a tool in integrated weed management, a quick and simple-to-use protocol for assessing the competitive potential of new cultivars is required; it is likely that this will not be based on a single trait but will need to capture the combined effect of multiple traits (Andrew et al., 2015). A recent study has reported that weed suppressive ability was correlated with competitive traits, including vigour and erect growth habit during tillering (Zadoks GS 29), high leaf area index (LAI) at stem extension (GS 31), plant height at tillering and stem extension (GS
29, 31), grain yield in weedy conditions, and grain yield tolerance (Worthington et al., 2015).

Therefore, based on both field and controlled environment studies, the objectives of this study were to 1) assess the competitive traits of selected superior Australian winter wheat cultivars which are well adapted for the southern farming region, 2) assess the impact of environmental factors such as moisture and temperature on weed suppressive ability of wheat, 3) assess and measure wheat metabolites involved in weed suppression and 4) measure weed suppression by wheat stubble post-harvest. Selected Australian wheat cultivars were evaluated for their ability to suppress annual weeds in the field and controlled environments. Rye was used as a control.

2. Methodology

Field trials were sown on 6\textsuperscript{th} and 20\textsuperscript{th} of May 2014 at two different locations of low (Condobolin) and medium (Wagga Wagga) rainfall respectively in replicated (6) and randomised trials. Eleven wheat cultivars representing four major cultivars of winter wheat typically grown in Australia, plus one cultivar of rye known to be weed suppressive, were established for further study. The wheat cultivars included short and long maturing varieties and two cultivars of grazing wheat. The cultivars grown included Condo, Corrack, Gregory, Espada, Janz CL, Scout, Suntop, Livingston, Mace, Wedgetail, Whistler and Grazer rye.

At sowing, soil samples were taken from each replicate to determine the weed seedbank present at experimental initiation. Weed seedbanks were evaluated in the glasshouse over several months. At Condobolin site the crop was sown at standard 33cm spacing and at Wagga Wagga the crop spacing was a standard 25cm, suitable for these areas due to rainfall differences.

No pre- or post-emergent herbicides were used at either site. Sites possessed weed infestations typical of each region for commercial production; that is weed numbers were not particularly high in each location and reflected commonly encountered species including annual ryegrass (\textit{Lolium rigidum}), bromegrass (\textit{Bromus inermis} L.), witchgrass (\textit{Panicum capillare}), stonecrop (\textit{Crassula helmsii}), capeweed (\textit{Arctotheca calendula}), paterson’s curse (\textit{Echium plantagineum}), fleabane (\textit{Conyza bonariensis}), mustard (\textit{Sisymbrium orientale}), common lambsquarters (\textit{Chenopodium album}) and fumitory (\textit{Fumaria agrarian}).
During the growing season, dates for crop and weed assessment in both locations were 16-20th June 17-22nd July 18-23rd September 4-7th November 2014 and 13 – 30th January 2015. On each sampling date in 2014, crop growth visual vigour rating, crop biomass, weed count and biomass, shoots, roots, rhizosphere and bulk soil from around the roots were sampled based on crop growth stages. In 2015 information on weed suppression, weed counts, bulk soil and stubble were collected post-harvest from each plot. Crop and weed biomass cuts were performed using a 50cm x 50cm quadrat with two subplots per plot.

During the earlier pre-harvest sampling periods, crop canopy measurements were undertaken including normalized difference vegetation index (NDVI) using GreenSeeker® 505 Handheld Sensor c/w Trimble Recon PDA, photos and photosynthetically active radiation (PAR) and leaf area index (LAI) using light Ceptometer (AccuPAR LP-80 Ceptometer- Decagon Devices®). These parameters were assessed to gain valuable information regarding crop canopy architecture and photosynthetic efficiency as they related to crop growth and weed suppression.

Sampled shoots, stems and roots (taken from 4 replicates x 7 cultivars x 2 locations x 4 samples) were extracted in methanol using a Buchi high pressure extractor (Skoneczny et al., 2014; Weston et al., 2015) and stored at 4°C awaiting further analysis using the liquid (UPLC) column chromatography coupled with time of flight mass spectrometry (LC-MS QToF) to analyse, separate and identify targeted and non-targeted metabolites of interest based on relative abundance (Weston et al., 2015). Soil samples were stored at -80°C until for future analysis for hydroxamic acids (Fomsgaard et al., 2006; Krogh et al., 2006).

A metabolite profiling method was developed using LC-MS/MS Qtrap 4500 Mass spectrometer (AB SCIEX QTRAP® 4500). Benzoxazinoids and hydroxamic acids are key secondary metabolites of importance in cereal crops; they are known to play important roles in plant defence against herbivory and in plant interactions including allelopathy and are active as soil siderophores as well (Wu et al., 2000; 2002; Belz, 2007; Macías et al., 2007).

3. Results and discussion

Figure 1 and two below reflect cultivar differences in crop vigour and average biomass based upon three biomass cuts in July, September and November. Cultivar differences were significant at each location for parameters assessed. This indicated the
utility of performing experimentation with six replicates and two subplots per replicate in terms of ability to discern small differences in crop performance.

At Condobolin, Janz CL and Mace produced greater biomass while Wedgetail produced the lowest followed by Condo, Whistler and Gregory. At Wagga Wagga, Espada, Condo and Livingston produced greater biomass while Whistler, Wedgetail, Suntop and Gregory produced the lowest. In general, cultivars which produced higher biomass also ranked highly for vigour in June and July 2014. Vigour ratings plotted in Figures 1 and two were collected in June and July 2014.

![Wheat cultivar vigour rating and biomass Condobolin 2014](image)

**Figure 1:** Wheat cultivar visual vigour rating and biomass at Condobolin arranged in ascending order based upon average vigour rating (1 to 10) taken in June and July 2014.
Figure 2: Wheat cultivar visual vigour rating and biomass at Wagga Wagga arranged in ascending order based upon average vigour rating (1 to 10) taken in June and July 2014.

Figures 3 and four show weed biomass, count and yield differences among the cultivars at both locations. Average yields were 3.2 and 1.7 t/ha, respectively, in Wagga Wagga and Condobolin. Yield differences among locations reflect typical trends observed for overall yields in each region based on rainfall received and plant density at each location, with Wagga high-density plantings producing up to 2 times greater yields than Condobolin.

The cultivars have been arranged in ascending order based upon weed biomass (yellow bars). Espada, Janz CL and Whistler produced consistently lower weed counts in both locations. Whistler, Wedgetail and Janz CL produced lower grain yield while Espada produced highest yields in both locations. Condo also produced reasonable yields and limited weed biomass in both locations. Gregory and Mace produced high levels of weed biomass and weed numbers at both locations.
Figures 3 and 4 show wheat cultivar biomass and grain yield at both locations. The cultivars have been arranged in ascending order according to yield. Espada and
Condo produced the highest yields at Wagga Wagga with Livingston and Corrack yielding second best. At Condobolin Mace and Espada yielded the most grain with Livingston, Corrack, Suntop and Scout yielding the second highest. At Wagga Wagga, crop biomass was positively related to yield.

**Figure 5:** Wheat cultivar biomass and yield at Condobolin 2014

**Figure 6:** Wheat cultivar biomass and yield at Wagga Wagga 2014
Figures 7 and 8 show the differences in wheat cultivar biomass, weed count and biomass at both locations. The cultivars have been arranged in ascending order according to weed biomass grams per square meter (g/m²). Janz CL, Espada and Condo had lower weed biomass in both locations, and the poor performers were Gregory and Mace at Condobolin and Wagga Wagga respectively. At Wagga Wagga, there was a strong negative relationship between crop biomass and weed biomass.

![Wheat cultivar and weed biomass Condobolin 2014](image)

**Figure 7**: Wheat cultivar and weed biomass at Condobolin
Figures 9 and 10 show the differences in cultivar biomass, LAI and weed biomass at both locations. The cultivars are shown in ascending order based upon weed biomass. Wedgetail and Whistler had the highest LAI in both locations while Mace had the lowest.

Interestingly, in Condo, those factors which resulted in improved crop biomass resulted in higher weed biomass in the same cultivars. In this more extreme climatic location, the cultivars that generated more biomass also provided conditions and environment which favoured weed growth; this could potentially be due to canopy architecture and impact of shading on temperature and moisture availability beneath the canopy. However, in Wagga Wagga the converse was true; in less moisture limiting situations and under cooler temperatures, the cultivars which produced the highest biomass also suppressed weeds most significantly.
Figure 9: wheat cultivar biomass, LAI and weed biomass at Condobolin.

Figure 10: wheat cultivar biomass, LAI and weed biomass at Wagga Wagga.
4. Metabolite profiling

Preliminary results of metabolic profiling of wheat roots, rhizoplane and bulk rhizosphere soil secondary metabolites using LC-MS QTRAP targeted analysis of BXs show cultivar and growth stage metabolite differences (Figure 11 & 12) of Gregory and Wedgetail wheat. Gregory wheat and Grazer rye reflect the abundance differences in DIMBOA and DIBOA respectively as reported in previous studies (Adhikari et al. 2012; Tanwir et al. 2013). DIMBOA and DIMBOA-Glc are the major bioactive benzoaxinoids in maize and wheat; DIBOA/DIBOA-Glc are prevalent in rye aboveground tissue, whereas the methoxy-substituted compounds DIMBOA, DIMBOA-Glc and MBOA are prevalent in the root tissue (Rice et al., 2005). However, Wedgetail grazing wheat was similar to Rye in producing more DIBOA and DIBOA-Glc in comparison to that of Gregory wheat.

In July both Grazer (Rye) and Gregory produce more MBOA in their roots and shoots in Gregory but not in rye. Wedgetail had higher concentrations of DIBOA-Glc and DIBOA in September compared to rye and Gregory which is likely related to its unusual phenology. Wedgetail is late maturing wheat variety with winter growth habit.

![Graph showing metabolite concentrations](image)

**Figure 11:** Concentration of metabolites (µg/g) in the tissue of wheat and rye taken in July and September 2014
5. Conclusions

In year 1 of this experiment, we demonstrated that genetically diverse wheat cultivars performed differently in two locations with varying rainfall patterns. Significant differences in crop biomass, LAI, weed count and biomass between cultivars was also location dependent. These results show that although weed suppression in wheat is influenced by cultivar, the genotypic response in wheat is clearly influenced by environmental factors as well when it comes to growth, yield and weed suppression. However, certain cultivars were excellent performers in both locations in terms of weed suppression and crop yields, and these included Espada and Condo and to a lesser extent Janz.

Method development for secondary metabolite profiling and analysis using LC-MS/MS Qtrap in wheat has been completed, and metabolic analysis of wheat tissue and rhizosphere soils is underway to evaluate the role of these metabolites in weed interference. This method is being used for metabolomic analyses to evaluate both primary and secondary biosynthetic pathways operational in crop cultivars and to determine if these pathways influence weed suppression in either location over time. The
role of benzoxazinoids and hydroxamic acids in weed suppression will be further examined in detail through targeted metabolic profiling in both soils and plant tissue. Additional field experiments will be repeated over the next three years to determine the impacts of year and location upon wheat cultivar performance and weed suppression.

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Appendix A3

Comparison of Grain Crops and Their Associated Residues for Weed Suppression in the Southern Australian Mixed Farming Zone

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Abstract

We evaluated ten-grain crops and their residues for their ability to suppress both winter and summer annual weeds in the mixed cropping region of southern Australia due to their competitive abilities and the presence of residues remaining until the subsequent cropping season. The research was conducted in Wagga Wagga NSW, in 2012 and 2013 using replicated RCB designs. Trends in weed suppression among years were similar. Weed pressures and yields were moderate in 2012–13 and lower in 2013–2014, due to rainfall received. Yields averaged 2.3–4 tons ha−1 for all crops in both seasons. Winter weeds were well suppressed in the crop in 2013, but annual rye grass (Lolium rigidum Gaud.) was less well suppressed in 2012. Annual weeds established in the crop in 2013, but annual rye grass (Lolium rigidum Gaud.) was less well suppressed in 2012. Annual weeds established following harvest and included witchgrass (Panicum capillare L.) and flea-bane (Conyza spp.). Witchgrass was most suppressed in grazing wheat and canola stubbles, followed by hybrid canola stubbles in both years. Grazing canola residues suppressed nearly all witchgrass and most fleabane growth for up to 4 months following harvest. Soil analyses for weed suppressive allelochemicals potentially produced by canola residues were performed.

Keywords Conservation tillage, summer annual weeds, stubble tillage, grazing wheat, canola, barley, fleabane, witchgrass.
1. Introduction

Conservation tillage (CT) is a system of residue management that avoids the use of cultivation or tillage for the establishment of annual broadacre crops. This system maintains crop residues on the soil surface and minimises soil disturbance over time. It has several advantages in that its implementation generally results in reduced soil erosion due to wind and water, reduced operational expenses associated with cultivation or tillage, and conservation of water. Use of CT, also known as stubble tillage, may impact weed establishment, both during the cropping phase and post-harvest in the fallow phase (Liebl et al. 1992, Scott et al. 2010, Weston 1990). In CT, the emphasis is placed on the use of herbicides for both pre- and post-emergence weed management in the remaining stubble, rather than cultivation, potentially leading to increased herbicide resistance in weeds of broadacre crops including annual ryegrass, wild radish and wild oats (Scott et al. 2010).

Much experimentation has been performed in the USA, Europe, Australia and South America to document the impacts of CT upon crop yield and performance. However, less information is generally available about the longer-term impacts of stubble residues upon weed seed bank dynamics and weed infestation following adoption of CT systems in Australian cereal and grain crops. One of the most notable changes following use of CT is upon weed management because pre-plant tillage and cultivation does not occur (Liebman and Davis 2002). The adoption of CT has been reported by many investigators to result in increased numbers of annual grasses and perennial broadleaf weeds and decreases in annual broadleaf weeds over time (Liebl et al. 1992).

Although numerous international studies and those performed in WA and SA have shown increases in crop yields over time associated with stubble retention, other studies have shown reductions in crop yields in CT systems, particularly in high rainfall years (Scott et al. 2010). This may be associated with the presence of decomposing mulch and release of allelochemicals, nutrient unavailability, increases in soil-borne pathogens or unfavourable shifts in weed spectrum over time due to use of conservation tillage. To reduce key weeds associated with the production of broadacre crops by CT, some NSW producers have reverted to the burning of stubble and use of tillage. In contrast, if stubbles are allowed to remain on the soil surface, researchers have reported issues with stubble build-up leading to
poor seed/soil contact in future seeding events, and increased numbers of grass weeds associated with the presence of these residues (Scott et al. 2010).

Previous studies have shown the temporal impacts of crop mulches and residues on weed germination, establishment and weed management over time. In particular, cereal and grain residues including those of wheat (Triticum aestivum L.), rye (Secale cereal L.), triticale (× Triticosecale) oats (Avena sativa L.) and barley (Hordeum vulgare L.) as well as canola (Brassica napus L.) residues have been studied for their ability to suppress weeds when used as cover crops into which broadacre crops are subsequently planted (Liebl et al. 1992, Putnam et al. 1983, Weston 1990, 2005). Up to 95% control of economically important broadleaf weeds and grasses have been reported when significant residues remain on the soil surface. This is thought to be due to both the physical presence of residues and release of allelochemicals over a 60 day period following harvest/kill of the cover crop. In Australian broadacre cropping regions, crops are planted up to 5 to 6 months after harvest into the remaining crop stubbles. We are particularly interested in the ability of selected grain crops to suppress weeds both in crop and in fallow, due to the presence of associated remaining crop stubble.

The purpose of this study was, therefore, to examine and compare the ability of various grain crops and their residues to suppress weeds until subsequent planting the following year. Experiments were performed over 2 years in low input grain production systems with moderate winter rainfall (<550 mm) without irrigation. This experiment established crops without the use of pre-emergence herbicides in relatively clean commercially cropped sites in order to compare and evaluate subsequent weed suppression provided by crop residues without confounding effects of residual herbicides.

2. Materials and Methods

Identical experiments with similar crop/cultivar treatments were established in 2012 and 2013 at adjoining sites at the Graham Centre Field Site in Wagga Wagga NSW. Experiments were established on 30 and 31 May, respectively, as randomised complete blocks with four replicates in fertile red kandosol soils receiving average moderate yearly rainfall of ~550 mm. Plots of 2 × 16 m were designed to evaluate the impact of crop residues on winter and summer annual weed establishment. Previous cropping history included
precision seeded and harvested commercial wheat and canola. Plots were planted using a cone seeder with 22 cm row spacing and pre-plant application of diammonium phosphate (DAP) at standard commercial rates. A post-emergent herbicide application of clethodim for canola and tralkoxydim (other cereals) was applied in August 2012 only, to manage an infestation of annual winter grasses in and between plots.

Table 1: Crops, cultivars and seeding rates evaluated in Wagga Wagga in 2012 and 2013.

<table>
<thead>
<tr>
<th>Crop species</th>
<th>Crop cultivar</th>
<th>Grazing or non-grazing</th>
<th>Seeding rate kg ha⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat</td>
<td>Wedgetail</td>
<td>grazing</td>
<td>60</td>
</tr>
<tr>
<td>Wheat</td>
<td>EGA Gregory</td>
<td>Non-grazing</td>
<td>60</td>
</tr>
<tr>
<td>Oats</td>
<td>Graza</td>
<td>grazing</td>
<td>60</td>
</tr>
<tr>
<td>Oats</td>
<td>Mitika</td>
<td>Non-grazing</td>
<td>60</td>
</tr>
<tr>
<td>Barley</td>
<td>Urambie</td>
<td>Grazing</td>
<td>60</td>
</tr>
<tr>
<td>Barley</td>
<td>Buloke</td>
<td>Non-grazing</td>
<td>60</td>
</tr>
<tr>
<td>Triticale</td>
<td>Tobruk</td>
<td>Grazing</td>
<td>80</td>
</tr>
<tr>
<td>Rye</td>
<td>Ryecorn</td>
<td>Grazing</td>
<td>60</td>
</tr>
<tr>
<td>Canola</td>
<td>CB Taurus</td>
<td>Grazing</td>
<td>6.5</td>
</tr>
<tr>
<td>Canola</td>
<td>Hyola 50</td>
<td>Non-grazing</td>
<td>3.4</td>
</tr>
</tbody>
</table>

Harvest was performed between 15 Nov and 15 Dec as crops matured, using a small plot harvester. The yield was measured as harvested biomass and cereal grain. Weed numbers were recorded in Sept., Nov., Jan. and March/April of each year using a 0.5 m × 10 m rating zone centred in each plot; in this area, two 0.5 m² quadrats were evaluated for weed numbers and biomass as well as residual stubble biomass. Data were analysed by performing ANOVA for RCB experiments with four replicates; significant differences were separated using LSD (0.05) with differences indicated by letters over figure columns.

3. Results

Experimental results in 2012–2013 and 2013–2014 seasons were similar in terms of yield and weed suppression provided by remaining crop residues the subsequent year. Emerging weeds of significance did not differ among years, but in 2012 due to higher winter/spring rainfall, annual ryegrass management was more problematic and required one post-emergent application of an appropriate selective grass herbicide for control in August. In 2013, weed pressures were moderate. Weeds emerging in both years included the winter weeds annual ryegrass and fumitory (*Fumaria muralis* Sond. ex W.D.J.Koch) and
summer-autumn annuals included fleabane and witchgrass, which germinated following adequate rainfall.

The yield of cereal and grain crops generally ranged from 2.3–4 tons ha$^{-1}$ in 2012 and 2013 in Wagga, which was similar to that of regional producers. Weed pressures in crop were not great in 2013 and did not require the use of post-emergent herbicides for weed suppression. However, in 2012 weed pressures were higher, possibly due to greater timely rainfall amounts received (data not presented for weed numbers in the crop).

Crop residues of all types resulted in greater in fallow weed suppression, with 50 to 200% increases in weed management in comparison to uncropped borders with no residue, following crop harvest. Weed infestations were rated in January and again in March/April each year. Crop residue presence resulted in reduced fleabane and witchgrass pressures. Of the crops evaluated in 2012/13, greatest suppression of weed seedlings was initially observed in grazing and non-grazing wheat, grazing barley and grazing and non-grazing canola stubbles (Figures 1 and 2). Witchgrass was the major weed infesting plots by May 2013 and 2014. In 2014, grazing wheat and canola plus triticale suppressed witchgrass establishment most effectively. Fleabane was also present in both years; significant suppression of fleabane occurred in grazing wheat and canola plots (Figure 1).
Figure 1: Fleabane counts in plots in March 2014. Areas with no residues averaged 12 plants m$^{-2}$.

Figure 2: Witchgrass counted in plots in March 2014. Areas with no residues averaged 20 plants m$^{-2}$.
In 2012/13, grazing and non-grazing canola plots were nearly weed-free for 90 days or more following harvest. In 2013/14, this period was extended due to low rainfall. Fleabane was the only weed of significance established initially in post-harvest grazing canola stubble; numbers were low in March 2012 and 2013. By May 2014, witchgrass numbers in plots had increased, but canola plots and grazing wheat remained cleaner than other cereal plots, with up to 75% less witchgrass biomass and 50% decreases in seedling numbers than other treatments (Figure 2). In January of both years, up to 45 to 60 days post-harvest, grazing barley and wheat and as well as both canola types showed limited weed infestation. Although weed counts were sometimes not significantly different among treatments, it was visually evident that grazing and hybrid canola plots remained relatively weed free for a period of 90 to 140 days post-harvest in both years.

Residues remaining on the plot were assessed following crop harvest. Significant differences were noted in post-harvest crop residue levels in 2013 (Figure 3). In this case, canola, grazing canola, and grazing wheat had less residue remaining in the plot than grazing barley, rye and other cereals. We also measured soil moisture availability differences in selected plots in March 2013/14. There were no differences in soil moisture levels from 0 to 12 cm profiles among canola and barley treatments, even though weed infestation and crop stubble biomass remaining were quite different among treatments.
4. Discussion

We observed significant differences in weed infestation in crop (data not presented) and also in post-harvest crop fallows associated with grain crop cultivar and species evaluated. Crops were produced in soils with low to moderate weed infestation and in the absence of residual herbicides. Crops generally proved to be both competitive with weeds during their establishment and growth. In addition, remaining crop residues were suppressive to summer annual weed establishment, compared to borders without stubble. Some crops were clearly more suppressive of in crop weeds, including rye, barley, wheat and canola, likely due to reduced light at the soil surface and competitive canopy architectures. However, once harvest was performed, crop residues were all that remained on the soil surface, and amount remaining was crop/cultivar dependent. Residues are the source of allelochemicals and nutrients that are released over time to the soil rhizosphere from decomposition (Weston 2005). In addition, residue presence on the soil surface can alter moisture availability and soil microbial interactions (Weston and Duke 2003).

We observed significant weed suppression associated with grazing and non-grazing wheat residues, both pre- (data not presented) and post-harvest with grazing wheat exhibiting
significant suppression of fleabane and witchgrass up to 130 days post-harvest. Grazing and non-grazing canola provided strong and significant suppression of fleabane and witchgrass for up to 140 days following harvest. Interestingly, these crops did not have as much residue remaining on the soil surface as other less weed suppressive cereal crops. Grazing cultivars were generally more suppressive of weeds than non-grazing cereal cultivars evaluated. Soil analyses performed in late March indicated that moisture levels were not much different among treatments, especially in 2014 during an extended drought, indicating that differences in weed establishment were not likely associated with differences in soil moisture availability among treatments (data not presented). We are currently evaluating soil samples collected in both years for the presence of isothiocyanates (ITCs) and glucosinolates associated with weed suppression in Brassica species. (Siemens et al. 2002, Weston and Duke 2003); this will determine if canola presence and low weed infestation are associated with higher levels of suppressive secondary products.

5. Acknowledgements

We acknowledge the support of the Grains Research and Development Corporation and R. Squire, G. Heath, S. Hildebrand, A. Sheppard, B. Ryan, N. Weston and D. Skoneczny for their assistance.

6. References


Appendix B – Abstracts

This appendix is a collection of abstracts comprising the work that was generated during this PhD project and was either presented at conferences (including symposiums) and/or published in conference proceedings both national and international.

AB1. Royal Australia Chemical Institute (RACT) Symposium

Date and Venue - 28th September 2018, University of NSW

Metabolic Profiling for Benzoazinoids in Weed Suppressive and Early Vigour Wheat Cultivars

James M. Mwendwa1, Paul A. Weston1, Inge Fomsgaard2, William B. Brown1, Greg Rebetzke1, Jeffrey D. Weidenhamer4 and Leslie A. Weston1*

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Abstract

Replicated and randomised wheat (Triticum aestivum L.) cultivar trials were conducted in moderate to low rainfall zones at Wagga Wagga (572 mm) and Condobolin (449 mm) NSW, respectively in 2014-2016. At each experimental site, crop and/or weed growth were monitored at selected growth stages including tillering, vegetative, grain filling, harvest and after crop harvest. In addition, plant shoot and root tissues and rhizoplane, rhizosphere and bulk soil samples were collected for metabolomics profiling and biomass evaluation. Plant tissue samples were extracted in methanol using an automated Buchi high-pressure extractor1 while soil samples were extracted over 24h using a rotary shaker2. Extracts were filtered and specifically analysed for numerous secondary metabolites or allelochemicals associated with weed suppression using liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (UPLC-ESI MS QToF, Agilent 6530). Benzoazinoids (BXs) were profiled in negative ion mode, and related microbially-produced metabolites (APO, AAPO, AMPO, AAMPO) were profiled in positive ion mode2,3. Metabolic profiling resulted in detection of up to 20 individual BXs including BX glycosides, lactones, hydroxamic acids and related microbial metabolites of interest. Both qualitative and quantitative differences in BXs were observed and were cultivar-, growth stage- and
location-dependent. Plant part and rhizosphere location (distance from root) also impacted BX concentration. Microbially produced metabolites with phytotoxic activity were detected in rhizosphere soils. Metabolic profiling provided information regarding wheat metabolism, as well as the biosynthesis and release of metabolites associated with weed suppression in commercial wheat cultivars, in contrast to rye (*Secale cereale* L.) and a heritage wheat cultivar Federation, both recognized for their potent ability to suppress weeds. Certain wheat cultivars maintained high yield potential and were significantly more weed suppressive, depending on year and location, likely because of their vigorous early growth habit and canopy architecture as well as the release of BX and related microbially produced metabolites into the rhizosphere over time.

**Keywords:** Weed suppression, metabolomics, residue, competition, resource allocation.

*Table 5:* Microbial degradation products of BOA and MBOA in the soil including 2-amino-3H-phenoxazin-3-one (APO), 2-acetylamino-3H-phenoxazin-3-one (AAPO), 2-Amino-7-methoxy-3H-phenoxazin-3-one (AMPO) and 2-acetylamino-7-methoxy-3H-phenoxazin-3-one (AAMPO).

<table>
<thead>
<tr>
<th>BOA</th>
<th>APO</th>
<th>AAPO</th>
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<td><img src="image2" alt="APO" /></td>
<td><img src="image3" alt="AAPO" /></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>MBOA</th>
<th>AMPO</th>
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<tbody>
<tr>
<td><img src="image4" alt="MBOA" /></td>
<td><img src="image5" alt="AMPO" /></td>
<td><img src="image6" alt="AAMPO" /></td>
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</tbody>
</table>

**References**


AB2. The 21st Australasian Weeds Conference (21st AWC)

Date and Venue: 9-12 Sept 2018, Sydney, NSW

Metabolic Profiling for Benzoxazinoids in Weed Suppressive and Early Vigour Wheat Cultivars

James M. Mwendwa1, Paul A. Weston1, Inge Fomsgaard2, Bente B. Laursen2, William B. Brown1, Hanwen Wu1,3, Jane C. Quinn1, Jeffrey D. Weidenhamer4 and Leslie A. Weston1

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Abstract

Replicated and randomised wheat (Triticum aestivum L.) cultivar trials were conducted in moderate to low rainfall zones at Wagga Wagga (572 mm) and Condobolin (449 mm) NSW, respectively in 2014-2016. At each experimental site, crop and/or weed growth were monitored at selected growth stages including tillering, vegetative, grain filling, harvest and after crop harvest. In addition, shoots, roots, rhizoplane and bulk rhizosphere soil samples were collected for metabolomics profiling and biomass evaluation. Plant tissue samples were extracted in methanol using an automated Buchi high pressure extractor while soil samples were extracted using a rotary shaker. Extracts were filtered and specifically analysed for unique secondary metabolites or allelochemicals associated with weed suppression, specifically benzoxazinoids (BXs), using liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (UPLC-MS QToF). Metabolic profiling of wheat shoots, roots, and soils resulted in detection of up to 14 individual BXs including BX glycosides, lactones and hydroxamic acids of interest. Both qualitative and quantitative differences in BXs were observed and were cultivar-, growth stage- and location-dependent. Plant part and rhizosphere location (distance from root) also impacted BX concentration. Further metabolic profiling provided crucial information regarding crop metabolism, as well as the biosynthesis and release of metabolites associated with weed suppression in currently available commercial wheat cultivars, in contrast to weed suppressive rye (Secale cereale L.) and a heritage wheat cultivar Federation, both recognized for their potent ability to suppress weeds. We conclude that certain commercial wheat cultivars maintained high yield potential and were significantly more weed suppressive, depending on year and location, due to both their early growth habit and canopy architecture as well as the release of BX metabolites into the rhizosphere over time.

Keywords: Weed suppression, metabolomics, residue, competition, resource allocation.
AB3. The 21st Australasian Weeds Conference (21st AWC)

Date and Venue: 9-12 Sept 2018, Sydney, NSW

Field Evaluation of Selected Canola Competitive Cultivars for Suppression of Natural Weed Populations

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Abstract

In 2014-2016, replicated field trials were performed to evaluate mechanisms of weed suppression in Australian canola cultivars in moderate to low rainfall zones at Wagga Wagga (572 mm) and Condobolin (449 mm) NSW, respectively. In 2015-16, a split-plot design with and without trifluralin as the main plot and cultivar as the subplot was employed for trials; in this experiment, 8 cultivars including hybrid and open-pollinated cultivars were assessed. At each site, crop and weed growth were monitored at various phenological stages including early season, vegetative, grain-filling, harvest and post-harvest. Certain cultivars exhibiting early vigour and also the ability to intercept light due to leaf canopy structure were associated with increased suppression of in-crop weed growth in canola trials; in addition, improved post-harvest weed suppression was associated with the presence of remaining crop residues after harvest. Cultivars GT-50, Hyola 600RR and Hyola 50 were the most weed suppressive and consistently higher yielding in each year at both locations. CB Taurus and GT-50 provided higher weed suppression only after harvest when residues remained in plots for 150 days post-harvest. Pre-emergence trifluralin treatment resulted in improved crop yields in contrast to untreated plots for most, but not all, cultivars. In this case, the cultivars that possessed rapid early growth and vigour and significantly reduced light availability at the soil surface limited weed growth in the absence of trifluralin. Our results indicated that establishment of certain canola cultivars effectively resulted in enhanced in-crop and post-harvest weed suppression, with or without the use of post-emergent herbicides during the growing season, particularly for common spring and summer annual weeds which were problematic post-harvest.

Keywords: Weed suppression, canopy architecture, phenology, crop residue, annual weeds
AB4. International Society of Chemical Ecology - 34th Annual Meeting

Date and Venue: 12-18 Aug 2018, Budapest, Hungary

Metabolic Profiling for Benzoxazinoids in Weed Suppressive and Early Vigour Wheat Cultivars

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Abstract

Wheat (Triticum aestivum L.) cultivar trials were conducted in moderate to low rainfall zones at Wagga Wagga and Condobolin NSW in 2014-2016. At each site, crop and weed growth was monitored at tillering, vegetative, grain filling, harvest and post-harvest. Wheat roots, shoots, rhizosphere and bulk soils were collected from June to November for metabolomic profiling and biomass evaluation. Plant samples were extracted in methanol using an automated Buchi high-pressure extractor while soil samples were extracted by a rotary shaker. Extracts were analysed using untargeted analysis by UPLC-ESI MS QToF (Agilent 6530) and data analysed using Mass Profiler Professional (Agilent). Benzoxazinoids (BXs) were profiled using targeted analysis in negative ion mode while key microbially produced metabolites (APO, AAPO, AAMPO) were profiled in positive ion mode. Targeted metabolic profiling resulted in detection of up to 20 individual BXs including BX glycosides, lactones, hydroxamic acids and related microbial metabolites. Qualitative and quantitative differences in BXs were observed and were cultivar-, growth stage- and location dependent. Plant part and rhizosphere location (distance from root) also impacted BX concentration. Microbially-produced metabolites with phytotoxic activity were detected in rhizosphere soils. Metabolic profiling provided critical knowledge of seasonal impacts on wheat metabolism, as well as the biosynthesis and release of metabolites associated with weed suppression in commercial wheat cultivars, in contrast to rye (Secale cereale L.) and a heritage wheat cultivar Federation, both recognised for their potent ability to suppress weeds. Certain cultivars maintained high yield potential and were significantly more weed suppressive, depending on year and location, potentially due to their vigorous early growth habit, canopy architecture, and the release of BX and related microbially produced metabolites into the rhizosphere over time.

ORAL PRESENTATION; Session/symposium: New chemical structures/Omics in chemical ecology
AB5. International Society of Root Research (ISRR10)-

Exposing the Hidden Half- Date and Venue: 8-12 July 2018, Israel

Metabolic Profiling for Benzoxazinoids in Weed Suppressive and Early Vigour Wheat Cultivars

James M. Mwendwa¹, Paul A. Weston¹, Inge Fomsgaard², Bente B. Laursen², William B. Brown¹, Hanwen Wu¹,², Jane C. Quinn¹, Jeffrey D. Weidenhamer⁴ and Leslie A. Weston¹

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Abstract

Replicated and randomised wheat (Triticum aestivum L.) cultivar trials were conducted in moderate to low rainfall zones at Wagga Wagga (572 mm) and Condobolin (449 mm) NSW, respectively in 2014-2016. At each experimental site, crop and/or weed growth were monitored at selected growth stages including tillering, vegetative, grain filling, harvest and after crop harvest. In addition, shoots, roots, rhizoplane and bulk rhizosphere soil samples were collected for metabolomics profiling and biomass evaluation. Plant tissue samples were extracted in methanol using an automated Buchi high-pressure extractor while soil samples were extracted using a rotary shaker. Extracts were filtered and specifically analysed for unique secondary metabolites or allelochemicals associated with weed suppression, specifically benzoxazinoids (BXs), using liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (UPLC-MS QToF). Metabolic profiling of wheat shoots, roots, and soils resulted in detection of up to 14 individual BXs including BX glycosides, lactones and hydroxamic acids of interest. Both qualitative and quantitative differences in BXs were observed and were cultivar-, growth stage- and location-dependent. Plant part and rhizosphere location (distance from root) also impacted BX concentration. Further metabolic profiling provided crucial information regarding crop metabolism, as well as the biosynthesis and release of metabolites associated with weed suppression in currently available commercial wheat cultivars, in contrast to weed suppressive rye (Secale cereale L.) and a heritage wheat cultivar Federation, both recognised for their potent ability to suppress weeds. We conclude that certain commercial wheat cultivars maintained high yield potential and were significantly more weed suppressive, depending on year and location, due to both their early growth habit and canopy architecture as well as the release of BX metabolites into the rhizosphere over time.

Keywords: Weed suppression, metabolomics, residue, competition, resource allocation.
AB6. Mass Spectrometry Food & Environmental Symposium -
Use of LC/MS QQQ and QToF Technologies to Study Advanced Plant Interactions in Agricultural Systems Agilent Technologies Aug 31st, 2017, Melbourne, Australia

Metabolic Profiling of Benzoxazinoids in Weed-Suppressive Wheat Cultivars

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Abstract

Replicated and randomised wheat (\textit{Triticum aestivum} L.) cultivar trials were conducted in moderate to low rainfall zones at Wagga Wagga and Condobolin NSW, respectively from 2014 to 2016. At each experimental site, crop and/or weed growth were monitored at selected growth stages including tillering, vegetative, grain filling, harvest and after crop harvest. In addition, shoots, roots, rhizoplane and bulk rhizosphere soil samples were collected. All shoot and root samples were extracted in methanol using a Buchi automated high-pressure extractor, while soil samples were extracted overnight using a rotary shaker. Extracts were profiled for unique secondary plant products acting as allelochemicals for weed suppression, specifically benzoxazinoids (BXs), using liquid chromatography coupled triple quadrupole mass spectrometry (UPLC-MS QQQ). In addition, non-targeted metabolomics was performed to evaluate relative abundance of diverse metabolites using liquid chromatography coupled to a quadrupole time-of-flight mass spectrometry (UPLC-MS QToF) platform. Metabolic profiling of wheat shoots, roots, and soils resulted in detection of up to 14 BXs including BX glycosides, and other metabolites of interest, including BX precursors. Both qualitative and quantitative differences in BXs were observed and were cultivar-, growth stage- and location-dependent. Plant part and rhizosphere location (distance from root) also impacted BX concentration. The distribution of the secondary metabolites in wheat cultivar tissues suggests differential production of key bioactive metabolites as influenced by cultivar and growth stage. Further metabolic profiling provided crucial information regarding crop metabolism, as well as the biosynthesis and release of metabolites associated with weed suppression in currently available commercial wheat cultivars, in contrast to the cereal crop, rye (\textit{Secale cereale} L.) and the heritage wheat cultivar Federation, both known for their potent ability to suppress weeds. This presentation will focus on the results of three years of field experimentation at two locations and suggest which cultivars are best-suited for weed suppressive properties due to canopy architecture and allelopathic traits while maintaining high yield potential.

Keywords \textit{Weed suppression, metabolomics, residue, competition, resource allocation.}
Field Evaluation of Australian Canola Cultivars for In-crop and Post-Harvest Weed Suppression

James M. Mwendwa¹, William B. Brown¹, Graeme Heath¹, Jeffrey D. Weidenhamer² and Leslie A. Weston¹

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Abstract

In 2014-2016, replicated field trials were performed to evaluate mechanisms of weed suppression in Australian canola cultivars in moderate to low rainfall zones at Wagga Wagga and Condobolin NSW, respectively. In 2015-16, a split-plot design with and without trifluralin (Tri) as the main plot and cultivar as the subplot was employed for trials; 8 cultivars including hybrid and open-pollinated cultivars were assessed. At each site, crop and weed growth were monitored at various phenological stages including early season, vegetative, grain-filling, harvest and post-harvest.

Early vigour and ability to intercept light resulted in suppression of in-crop weed growth in canola trials, as well as post-harvest weed suppression associated with remaining crop residues. GT-50, Hyola 600RR and Hyola 50 were the most weed suppressive and consistently high yielding cultivars each year in both locations. CB Taurus and GT-50 provided greatest weed suppression when only residues remained in plots for 150 days post-harvest. Pre-emergence trifluralin treatment resulted in improved crop yields in contrast to untreated plots for most cultivars, but not all. In this case, these weed suppressive cultivars possessed rapid early crop growth and vigour and reduced light at the soil surface, potentially limiting weed growth in the absence of trifluralin.

Our results show that establishment of certain canola cultivars may effectively result in enhanced in-crop and post-harvest weed suppression, with or without the use of post-emergent herbicides during the growing season, especially when considering common spring and summer annual weeds which are problematic post-harvest. Therefore, canola cultivar choice may be an economical form of weed management due to competition by the crop and possibly other factors, such as production of allelochemicals by decomposing crop residues. Further investigation is underway to determine the allelopathic mechanisms in canola, particularly the identification of allelochemical(s) associated with post-harvest weed suppression in field soils.

Keywords: Weed suppression, canopy architecture, phenology, crop residue, allelochemical.
Field Evaluation of Australian Commercial Wheat Cultivars for Competitive Traits and Weed Suppression

James M. Mwendwa¹, William B. Brown¹, Graeme Heath¹, Hanwen Wu², Jane C. Quinn¹, Jeffrey D. Weidenhamer³ and Leslie A. Weston¹

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Corresponding author (jmwendwa@csu.edu.au)

Abstract

From 2014 to 2016, replicated field trials were performed to evaluate mechanisms of weed suppression in diverse Australian wheat cultivars in moderate to low rainfall zones at Wagga Wagga and Condobolin NSW, respectively. In 2014, a total of 11 winter wheat cultivars (Triticum aestivum L.) representing four major breeding family lines grown in Australia were evaluated; in 2015 and 2016, 13 cultivars were assessed including the heritage cultivar Federation. At each site, crop and weed growth were monitored at various phenological stages including early season, vegetative, grain-filling, harvest and post-harvest to the crop.

Significant differences between wheat cultivar and location were observed for crop biomass, early vigour, leaf area index (LAI), weed number, weed biomass, canopy architecture and yield in each year. Differences in weed suppression were largely impacted by crop architecture and phenology early in the growing season, particularly leaf shape and the ability to achieve early canopy closure. Cultivar competitive traits were also influenced by both cultivar and environmental factors, as shown by clear differences in cultivar performance, yield and weed suppression. Cultivars Condo and Espada were superior performers in terms of weed suppression and yielding potential in both locations and all years.

Our results were replicated over multiple locations and years and clearly suggest that establishment of competitive wheat cultivars can result in effective suppression of weed growth (up to 90% or greater) in the absence of post-emergent herbicides. This suggests that weed suppression may be associated with cultivar competitive ability and/or allelopathy. In addition, the choice of wheat cultivars for the desired yield and weed suppression impacts the subsequent ability of the crop to interfere with weed growth successfully and can prevent future weed propagules from entering the weed seedbank. Therefore, the choice of the wheat cultivar can provide cost-effective and sustainable weed management and is a useful tool.

Keywords: Weed suppression, canopy architecture, phenology, yield, weed seedbank.
Appendix B

AB9. The 8th World Congress of Allelopathy

International Allelopathy Society Marseille France July 2017

Metabolic Profiling for Benzoxazinoids in Weed Suppressive and Early Vigour Wheat Cultivars

Mwendwa J M1, Weston P A1, Fomsgaard I2, Laursen B B2, Brown W B1, Wu H1,3, Quinn J C1, Weidenhamer J D4, Shaik R1 and Weston L A1

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Abstract

Replicated and randomised wheat (Triticum aestivum L.) cultivar trials were conducted in moderate to low rainfall zones at Wagga Wagga and Condobolin NSW, respectively in 2014 to 2016. At each experimental site, crop and/or weed growth were monitored at selected growth stages including tillering, vegetative, grain filling, harvest and after crop harvest. In addition, shoots, roots, rhizoplane and bulk rhizosphere soil samples were collected. All shoot and root samples were extracted in methanol using Buchi automated high-pressure extractor, while soil samples were extracted using a rotary shaker. Extracts were profiled for unique secondary plant products acting as allelochemicals for weed suppression, specifically benzoxazinoids (BXs), using liquid chromatograph coupled to a triple quadrupole mass spectrometer (UPLC-MS QQQ). In addition, non-targeted metabolomics analysis was performed to evaluate relative abundance of diverse metabolites using a quadrupole time-of-flight mass spectrometry (UPLC-MS QToF) platform. Metabolic profiling of wheat shoots, roots, and soils resulted in detection of up to 14 BXs including BX glycosides and other metabolites of interest. Both qualitative and quantitative differences in BXs were observed and were cultivar-, growth stage- and location-dependent. Plant part and rhizosphere location (distance from root) also impacted BX concentration. The distribution of the secondary metabolites in wheat cultivar tissues suggests differential production of some key bioactive metabolites. Further metabolic profiling provided crucial information regarding crop metabolism, as well as the biosynthesis and release of metabolites associated with weed suppression in currently available commercial wheat cultivars, in contrast to weed suppressive rye (Secale cereale L.) and heritage wheat cultivars such as Federation, known for their potent ability to suppress weeds. This presentation will focus on the results of three years of field experimentation at two locations and predict which cultivars are best-suited for weed suppressive properties due to canopy architecture and allelopathic traits while maintaining high yield potential.

Keywords Weed suppression, metabolomics, residue, competition, resource allocation.
AB10. The 20th Australasian Weeds Conference (20th AWC)

Date and Venue: 11-15th Sept 2016, Perth, Western Australia

Field Evaluation of Australian Wheat Cultivars for Competitive Traits and Weed Suppression
James M. Mwendwa, William B. Brown, Shamsul Haque, Graeme Heath, Jane C. Quinn and Leslie A. Weston
Graham Centre for Agricultural Innovation (NSW Department of Primary Industries and Charles Sturt University), Wagga Wagga, NSW 2678, Australia

Abstract

In 2014 and 2015, replicated field trials were performed at commercial paddocks in moderate to low rainfall zones at Wagga Wagga and Condobolin NSW, respectively. In 2014, a total of 12 winter wheat cultivars (Triticum aestivum L.) representing 4 major breeding family lines grown in Australia were evaluated while 14 cultivars were assessed in 2015. The seed for each cultivar was produced on site in Wagga Wagga in 2014 in an effort to reduce variation associated with seed source. No herbicides were applied during experimentation; at each site, crop and/or weed growth were monitored strategically at various stages of growth: early season (tillering), vegetative, grain filling, harvest and post-harvest. Significant differences between wheat cultivar and location were observed for crop biomass, early vigour, leaf area index (LAI), weed number, weed biomass, canopy architecture and yield in both 2014 and 2015. Differences in weed suppression were largely impacted by crop architecture and phenology early in the growing season. Competitive growth traits such as early vigour, crop height, LAI and light interception acted in concert resulting in enhanced weed suppression associated with both canopy architecture and/or competition for resources. Cultivar competitive traits were also influenced by both cultivar and environmental factors, as shown by clear differences in cultivar performance, yield and weed suppression among both locations. Cultivars Condo and Espada were superior performers in both locations and years. Our data support the concept that the choice of the wheat cultivar can prove to be a cost-effective means of weed management.

Keywords: Weed suppression, canopy architecture, phenology, propagules, weed seedbank
Metabolic Profiling for Benzoazinoids in Weed Suppressive and Early Vigour Wheat Cultivars

James M. Mwendwa¹, Paul A. Weston¹, William B. Brown¹, Hanwen Wu¹, Greg Rebetzke², Jane C. Quinn and Leslie A. Weston
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Abstract

Replicated wheat cultivar trials were performed in commercial fields in moderate to low rainfall zones at Wagga Wagga and Condobolin NSW, respectively, in 2014 and 2015. Twelve winter wheat cultivars (Triticum aestivum L.) were evaluated in 2014, representing four major breeding family lines grown in Australia, while these and two additional cultivars were evaluated in 2015. At each experimental site, crop and/or weed growth were monitored strategically at selected growth stages including tillering, vegetative, grain filling, harvest and post-harvest to the crop. In addition, shoots, roots, rhizoplane and bulk rhizosphere soil samples were also collected. All samples were extracted in methanol using an automated Buchi high-pressure extractor. Soil samples were extracted by shaking with 1) 80% methanol and/or 2) 80% Methanol with 1% acetic acid. Extracts were profiled for unique secondary plant products including benzoazinoids (BXs) using liquid chromatography coupled to mass spectrometry. Significant differences between wheat cultivar and location were observed for weed number, weed biomass, early vigour, canopy architecture and yield. Metabolic profiling of wheat cultivar shoots, roots, and soils resulted in detection of up to 14 BXs including BX glycosides as well as other metabolites of interest. Both qualitative and quantitative differences in BXs were observed and were cultivar and location dependent. Plant part and rhizosphere location (distance from root) also impacted BX concentration. Additional metabolic profiling is now underway and will provide crucial information regarding crop metabolism and biosynthesis of metabolites associated with weed suppression in commercial wheat cultivars.

Keywords: wheat, weed suppression, metabolic profiling, metabolomics, residue, competition, resource allocation.
Metabolic Profiling for Benzoxazinoids in Weed Suppressive and Early Vigour Wheat Cultivars

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Abstract

Replicated and randomised wheat (*Triticum aestivum* L.) cultivar trials were performed in commercial fields in moderate to low rainfall zones at Wagga Wagga (572 mm) and Condobolin (449 mm) NSW in 2014, 2015 and 2016. At each experimental site, crop and/or weed growth were monitored at selected growth stages including tillering, vegetative, grain filling, harvest and post-harvest to the crop. In addition, shoots, roots, rhizoplane and bulk rhizosphere soil samples were collected. All shoot and root samples were extracted in methanol using an automated Buchi high-pressure extractor while soil samples were extracted using a rotary shaking method. Extracts were profiled for unique secondary plant products acting as allelochemicals for weed suppression, specifically the benzoxazinoids (BXs) (Fomsgaard et al. 2006) using liquid chromatography coupled to mass spectrometry. In addition, non-targeted metabolomics was performed to evaluate abundant constituents of interest using a UPLC-MS QToF platform. Metabolic profiling of wheat cultivar shoots, roots, and soils resulted in detection of up to 14 BXs including BX glycosides and other metabolites of interest (Adhikari et al., Tanwir et al., 2013). Both qualitative and quantitative differences in BXs were observed and were cultivar and location dependent. Plant part and rhizosphere location (distance from root) also impacted BX concentration. Further metabolic profiling is now underway and will provide crucial information regarding crop metabolism and biosynthesis of metabolites associated with weed suppression in currently available commercial wheat cultivars, in contrast to weed suppressive rye (*Secale cereale* L.) and historic cultivars such as Federation, known for their potent ability to suppress weeds.
Keywords: wheat, weed suppression, metabolic profiling, metabolomics, residue, competition, resource allocation.

References


![Figure 1: BOA (1,3-benzoxazol-2-one)](image1)

![Figure 2: MBOA (6-Methoxy-1,3-benzoxazol-2(3H)-one)](image2)
AB13. International Society of Root Research (ISRR-9)

Date and Venue: Roots Down Under - 6-9th Oct 2015, Canberra ACT

Mechanism of Weed Suppression in Weed Suppressive Wheat Cultivars

James Mwendwa¹, Leslie A. Weston¹ and William B. Brown¹

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Abstract

Australian wheat cultivars were evaluated for their ability to suppress weeds at two locations. Metabolic profiling of wheat roots, rhizoplane and bulk rhizosphere soils was then performed to evaluate primary and secondary metabolites to determine if allelochemical interactions or other factors are involved in successful weed interference.

Introduction

Wheat cultivar (Triticum aestivum L.) shoot and root architectural traits likely play critical roles in crop weed suppression. Benzoxazinoids are important allelochemicals present in wheat, barley and rye, and their suppressive effects on weeds, pest and diseases are of great interest in sustainable agriculture (Bertholdsson et al., 2012). The content of hydroxamic acids in wheat varies among species and cultivars and is dependent on plant age and organ assayed; younger leaf tissues produce higher levels of hydroxamic acids (Argandoña et al., 1981). We evaluated selected diverse Australian wheat cultivars for their ability to suppress annual weeds in the field and in controlled environments. Based on field and phytotron studies, metabolic profiling of wheat roots, rhizoplane and bulk rhizosphere soils was performed for both primary and secondary metabolites to evaluate if allelochemical interactions are involved in successful interference with weeds or if other factors critical to the production of secondary products are important in plant defence. Liquid chromatography coupled with mass spectrometry (LC-MS and LC-MS QTOF) was used to analyse, identify and quantify metabolites of interest through both targeted and non-targeted metabolic profiling.

Methods
Field trials were set up in April/May 2014 and 2015 in Condobolin and Wagga Wagga NSW. Roots, rhizoplane and bulk rhizosphere soil from around the roots were sampled at four strategic times based on the crop growth stage, including crop tillering, flowering, grain fill and post-harvest. Sampled roots and soil (taken from 4 replicates x 7 cultivars x 2 locations x 4 samples) were extracted in methanol using a Buchi high pressure speed extractor (Weston et al., 2015) and stored at 40C before analysis using LC-MS QTOF for targeted and non-targeted metabolites of interest (Weston et al., 2015). Soil samples were stored at -800C until analysis for hydroxamic acids (Fomsgaard et al., 2006; Krogh et al., 2006). Roots were dipped in methanol to harvest rhizoplane exudates and adherent soil followed by filtration with 0.22 µm syringe filters.

**Results and Discussion**

Field trials demonstrated significant differences between wheat cultivars in crop biomass, growth vigour, yield, leaf area index (LAI), weed suppression, weed count and biomass. Competitive cultivar traits are strongly influenced by both cultivar and environmental factors, as shown by differences in cultivar performance among the two locations representing low and moderate rainfall zones. Additional experimentation using metabolomics to profile primary and secondary metabolite differences among cultivars, locations, plant part and timing of collection is underway using LC-MS QTOF analysis for metabolite profiling. This will provide important information regarding crop physiological and biosynthetic differences that may impact crop competitive traits against common weed species. Metabolic profiling of allelochemicals in the rhizosphere could also provide strong insight into the resulting decomposition following incorporation of plant material into the soil (Krogh et al., 2006) and the complex interplay between plants and their associated rhizosphere microorganisms, an area which is relatively understudied (Weston et al., 2015).

**Conclusion**

Crop biomass, LAI, weed count and biomass was clearly location and cultivar dependent in 2014. Results show that although weed suppression in wheat was influenced by cultivar, the genotypic response was influenced by environmental factors, in terms of crop growth, yield and weed suppression. Cultivars Espada, Condo and to a lesser extent Janz showed a significant level of weed suppression in both locations in comparison to 9 other cultivars. Additional results will be obtained from metabolomic analyses performed over time to evaluate both primary and secondary biosynthetic pathways operational in crop cultivars.
and to determine if these pathways influenced weed suppression. The role of hydramic acids in weed suppression is also under examination through performance of targeted metabolic profiling in both soils and plant tissue.

References


Root Exudation of Lipophilic Naphthoquinones by Paterson’s Curse: A Clue to their Ecological Role?

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ABSTRACT

Roots of Paterson’s curse (Echium plantagineum) produce an array of coloured and colourless naphtho- and anthraquinones in their roots. Under certain environmental conditions, young roots produce large quantities of naphthoquinones in the outer layers of root periderm, and root hairs of seedlings exude droplets of dark red naphthoquinones. Polydimethylsiloxane (PDMS) microtubing is a useful tool to measure lipophilic allelochemicals in soil and has been used in several forms to probe the release of these compounds directly from Paterson’s curse roots, both in and out of soil growth media. Droplets of exudate have been collected directly from root hairs, and solid phase root zone extraction probes (constructed from silicone tubing mounted on a stainless-steel wire core) placed in the soil around Paterson’s curse plants have also been used to measure the release of these compounds from roots. Further metabolic profiling studies using LC-MS QToF (HPLC coupled to time of flight mass spectrometry) are underway to establish the specific composition of the root exudates and quantities of compounds being released. Our results demonstrate polydimethylsiloxane in various forms is a useful tool to monitor the release of nonpolar to moderately polar allelochemicals into soil. These naphthoquinones show potent antimicrobial, fungitoxic and phytotoxic activity due to their impact on electron transport and cellular respiration processes. Measurement of the dynamics of their release into the soil environment will be valuable in assessing the ecological role of these compounds.

Keywords: Allelopathy, diffusive sampling, Echium plantagineum, PDMS, rhizosphere, root exudation, soil analysis.

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Appendix C- Posters and Awards

This section presents posters and awards during the period of my research project. The posters were presented at various conferences and symposiums both local and international. The following list provides in detail the meetings (including dates and venues) were these posters were presented at from the earliest to the latest.

1. Royal Australia Chemical Institute (RACI) Natural Products Symposium Charles Sturt University – 4th October 2019 (Fig. C1)
2. Royal Australia Chemical Institute (RACI) Natural Products Symposium University of NSW - 28th September 2018 (Fig. C2)
3. The 21st Australasian Weeds Conference (21AWC), 9-12 September 2018, Sydney, NSW (Fig C2 and C3)
4. The 34th Annual Meeting of the International Society of Chemical Ecology 12-18 Aug 2018, Budapest, Hungary (Fig C2)
5. ISRR10- Exposing the Hidden Half- International Symposium organised by International Society of Root Research from 8-12 July 2018, Israel (Fig. C2)
6. The 8th World Congress allelopathy, Marseille, France 24-28 July 2017, organised by the International Allelopathy Society (Fig C3 and C4).
7. The 20th Australasian Weeds Conference (11-15 September 2016), Perth, Western Australia (Fig. C3 and C4)
8. RACI (Royal Australia Chemical Institute) Natural Products Symposium University of Wollongong Friday 30th September 2016 (Fig C4)
9. CSU Higher Degree Research (HDR) symposium in 2016 and 2017. The best poster prize was awarded for the canola poster “Field evaluation of Australian canola cultivars” (Fig. C2 and C3).
10. ISRR-9 Roots Down Under- International Symposium organised by International Society of Root Research from 6-9th October 2015, Canberra Australia (Fig. C5)
11. Presentation certificates at the 8th World Congress allelopathy, Marseille, France 24-28 July 2017, organised by the International Allelopathy Society (Fig C6, C7 and C8).
AC1: Metabolic profiling for benzoxazinoids and phenoazinones in early vigour weed suppressive wheat cultivars in roots, rhizoplane and rhizosphere.

Metabolic profiling for benzoxazinoids in weed suppressive and early vigour wheat genotypes.

Introduction

Wheat cultivar (Triticum aestivum L.) root and shoot architectural traits likely play critical roles in crop weed suppression. Benzoxazinoids (BXs) are allelochemicals present in wheat, barley and rye that exhibit suppressive effects on weeds, pests and pathogens. Biosynthesis of BXs in cereal crop tissues and their biotransformation in the soil rhizosphere has been well documented (Fig. 1, Mocia et al., 2019).

Results and Discussion

- There were significant differences between cultivars in canopy closure, crop biomass, leaf area index (LAI), weed suppression, weed biomass and yield (Mwenda et al., 2016), and parameters varied with site and season (data not shown).
- The relative distribution of metabolites detected in wheat roots, rhizoplane (root surface) and shoots varied with tissue type, with unique metabolic profiles for each tissue (Fig. 2).
- The three most abundant BX metabolites detected and identified in wheat tissues included NMOA and two of its derivatives, HMBOA and HMBOA-Glc with significant differences (P < 0.001) at growth stage, tissue type, location and cultivar (Fig. 3).

Materials and Methods

- Rapicoted and randomised wheat cultivar field trials were conducted in moderate to low rainfall zones at Wagga Wagga (572 mm) and Condobolin (440 mm) NSW.
- Roots, rhizoplane and bulk rhizosphere soil from around the roots were sampled at 4 strategic times based on crop growth stage.
- Roots were extracted using an automated Buchi high-pressure extractor (Weston et al., 2015).
- Rhizoplane and bulk soils were extracted as per Fornegard et al. (2008) and Krogh et al. (2008).
- Metabolic profiling was performed using an Agilent 6530 LCMS/MS QTOF for BXs and LCMS-QQQ for phenoxazinones.

Conclusions

- Differential production of BXs was observed at various crop growth stages, but typically declined with crop maturity.
- Phytotoxic microbial metabolites including APO, AAPO & AMPO, were detected & transformed from BXs produced by wheat & its root exudates by soil microbota under field conditions.
- Further research is required to: 1) enhance weed control through biosynthetic modification & genotype selection, 2) isolate & identify soil microbes associated with the production of potenty active phenoxazinones.

Acknowledgements

GRDC funding was received for project UC0002, 00022 and 00023. Technical support provided by Graeme Heath, Shamsul Haque, Diam Skonoczny, Xiaocheng Zhu, Ian Mentz and Richard McCauley.
Appendix C

AC2: Metabolic profiling for benzoazinoids in weed suppressive wheat cultivars shoots

Metabolic profiling for benzoazinoids in weed-suppressive and early vigour wheat genotypes

Introduction
- Wheat cultivar (Triticum aestivum L.) root and shoot architectural traits likely play critical roles in crop weed suppression.
- Benzoazinoids (Bx’s) are allelochemicals present in wheat, barley and rye that exhibit suppressive effects on weeds, pests and pathogens are of great interest in sustainable agriculture (Table 1).

Table 1: Classification of benzoazinoid compounds (Bx’s) found in cereals (Adihikari et al., 2016).

<table>
<thead>
<tr>
<th>Benzocinoids</th>
<th>Lactones</th>
<th>Hydroxyacids</th>
</tr>
</thead>
<tbody>
<tr>
<td>H</td>
<td>BOA</td>
<td>R</td>
</tr>
<tr>
<td>OC5, H</td>
<td>H</td>
<td>BOA-Gr</td>
</tr>
<tr>
<td>OC5</td>
<td>H</td>
<td>BOA-Gr</td>
</tr>
<tr>
<td>OC5</td>
<td>H</td>
<td>BOA-Gr</td>
</tr>
<tr>
<td>H</td>
<td>Glc</td>
<td>BOA-Gr-Hex</td>
</tr>
</tbody>
</table>

- Selected Australian wheat genotypes were evaluated for their ability to suppress annual weeds in the field and in controlled environments. Cereal rye (Secale cereale) was used as a positive control.
- Metabolic profiling of wheat and rye tissues, rhizosphere and rhizosphere soils was performed using LC-MS QTOF.

Materials and Methods
- Replicated and randomised wheat cultivar field trials were conducted in moderate to low rainfall zones at Wagga Wagga (572 mm) and Condobolin (449 mm) NSW.
- Roots, rhizosphere and bulk rhizosphere soil from around the roots were sampled at 4 strategic times based on crop growth stage.
- Roots were extracted using an automated Buchi high-pressure extractor (Winston et al., 2015).
- Rhizosphere and bulk soils were extracted as per Formoseg et al. (2008) and Krogh et al. (2006).
- Metabolic profiling was performed using an Agilent 6530 LCEC/MS QTOF.

Results and Discussion
- There were significant differences between cultivars in canopy closure (Fig. 1), crop biomass, leaf area index (LAI), weed suppression, weed biomass and yield (Mwendwa et al., 2016), and varied with site and season.

Figure 1: Canopy closure of wheat cultivar Condo (left) and Espada (right) at 113 days after crop emergence at Wagga Wagga. Note the more complete canopy closure of Condo.

Figure 2: The distribution of metabolites in wheat cultivar tissues in June and July 2018 at Wagga Wagga.
- Metabolic profiling of wheat shoots, roots, and soils resulted in detection of up to 14 individual Bx’s including BX glycosides, lactones and hydroxyacids of interest.
- Non-targeted analysis of Bx’s showed metabolite differences with sampling time and tissue type (Fig. 2). Total metabolites increased in the roots but remained relatively similar in the shoots.
- MBOA was the most abundant BX and HBOA the least abundant across cultivars (Fig. 3a).
- The relative abundance of the predominant BX (MBOA) in roots was higher at early (vegetative) and mid (flowering) crop growth stages at Wagga Wagga and Condobolin, respectively (Fig. 3b). The abundance of most Bx’s declined measurably at later growth stages.

Figure 3: The relative abundance of major metabolites in roots of wheat cultivars in 2015 and 2016 at Condobolin and Wagga Wagga at three times during the growing season.

Conclusions
- Differential production of Bx’s was observed at various crop growth stages, but typically declined with crop maturity.
- MBOA was the predominant BX observed in wheat and rye roots.
- Certain wheat cultivars were significantly more weed suppressive, depending on year and location, due to both their early growth habit and canopy architecture. However, further analysis is underway to understand how the release of BX metabolites (especially MBOA) into the rhizosphere over time contributes to weed suppression.

Acknowledgements
GRDC for sponsorship. Technical support: Dianne Heath, Shamsul Haque, Dem Skerlaci, Xiangtong Zhu, Jan Marz and Richard MCK Colson. NARS IV (FIP).

References
Field evaluation of Australian canola genotypes for in-crop and post-harvest weed suppression

Introduction
- Herbicide-resistant weeds are on the rise across Australia, including resistance to multiple herbicides or herbicide families (Owen et al., 2013).
- Differential weed suppression in the field associated with crop genotypes has been observed (Audacwaer et al. 2014).
- In Canada canola competitiveness was increased by choice of cultivar and use of higher seeding rates (Flaxer et al. 2003, Beeston et al. 2009).
- However, competitiveness and weed suppressive abilities of canola have not been determined in Australia (Zemere et al. 2014).
- We evaluated selected cultivars of canola architectural traits for weed suppression over time, with and without pre-emergence trifluralin treatment.

Figure 1: GT50 and AFR canola cultivar plots, trifluralin treated or untreated at pre-emergence showing different levels of weed infestation in-crop at 120 DAE in Wagga Wagga.

Materials and Methods
- In 2014 to 2015, replicated field trials were performed to evaluate mechanisms of weed suppression in selected Australian canola genotypes in moderate to low rainfall zones at Wagga Wagga and Condobolin NSW, respectively, in commercially managed paddocks.
- In 2015-16, a split-plot design with and without trifluralin (TR) as the main plot and cultivar as the sub-plot was employed for canola trials. 9 cultivars including hybrid and open-pollinated cultivars were assessed.
- At each site, crop and weed growth were monitored at various phenological stages including early season, vegetative, grain-filling, harvest and post-harvest, recorded as days after emergence (DAE).
- Data collection focused upon those traits leading to both in-crop and potential post-harvest suppression of weeds.

Preliminary Results and Discussion

Figure 2: Crop photosynthetically active radiation (PAR) light interception (%) measured at 30 DAE at Condobolin - (A) P20 (B) LT10 (C) LT10/GRDC.

Conclusions
- Selected canola cultivars have demonstrated enhanced in-crop and post-harvest weed suppression, which may reduce the need for use of post-emergent herbicides during the growing season.
- Canola cultivar choice may be an economical form of weed management due to competition by the crop and possibly other factors, such as production of allelochemicals by decomposing crop residues.
- Further investigation is a new avenue in Australian paddocks to determine the allelopathic mechanisms in canola, particularly the identification of allelochemical(s) associated with post-harvest weed suppression in field soils.

Acknowledgements
GRDC for sponsorship. Technical support; Shamul Raque, Ian More and Richard McCallum NSW DPI.

Figure 3: Canola cultivar biomass (g/plot at 90 DAE) (A) P20 (B) LT10 (C) LT10/GRDC and grain yield (kg/ha) for pre-emergent herbicide treated and untreated (P40, LT10/GRDC) at Condobolin.

- Early vigour and ability to intercept light and accumulate biomass rapidly (Fig. 2) resulted in suppression of in-crop weed growth in canola trials, as well as post-harvest weed suppression associated with remaining crop residue (Fig. 2 and 4).
- GT-50, Hyola 600R and Hyola 50 yielded the most and had the highest PAR light interception in both locations (Fig. 2 and 3).
- Cultivar GT-50 provided significantly greater weed suppression than Hyola 723RT at 63 days post-harvest regardless of herbicide treatment (Fig. 4).
Appendix C

AC4: Preliminary metabolic profiling for benzoazinoids in weed suppressive wheat cultivars

Figure 1: Classification of Benzoazinoid compounds found in cereals (Adhikari et al., 2015).

Figure 2: Preliminary metabolic profiling for benzoazinoids in weed suppressive and early vigour wheat genotypes

Introduction
- Wheat cultivar (Triticum aestivum L.) shoot and root architectural traits likely play critical roles in crop weed suppression.
- Benzoazinoids (BAs) are important allelochemicals present in wheat, barley and rye.
- BAs suppressive effects on weeds, pest and diseases are of great interest in sustainable agriculture (Fig 1).

Selected Australian wheat genotypes were evaluated for their ability to suppress annual weeds in the field and in controlled environments. Rye was used as a control.
- Developed a method for metabolic profiling of wheat shoots, roots, rhizosphere and bulk rhizosphere soils for secondary metabolites using LC-MS/MS to evaluate their role in weed suppression.

Materials and Methods
- Field trials April/May 2015 and 2016 at Condobolin and Wagga Wagga NSW.
- Roots, rhizosphere and bulk rhizosphere soil from around the roots were sampled at 4 strategic times based on crop growth stage.
- Shoots and roots were extracted using an automated extractor-Buch (Weston et al., 2015).
- Rhizosphere and bulk soils were extracted (Fonsgaard et al., 2006; Krogh et al., 2006).
- Metabolite profiling method was developed using LC-MS/MS Qtrap 4500 Mass spectrometer (AB SCIEX QTRAP 4500) and Agilent LCMS/MS QTOF.

Preliminary Results and Discussion
- Field trials demonstrated significant difference between wheat cultivars in crop biomass, growth vigour, yield, leaf area index (LAI), weed suppression, weed count and biomass.
- Based on field data, competitive cultivar traits are strongly influenced by both genotype and environmental factors (Fig 2).

Conclusion
- Weed suppression, crop growth and yield in wheat were influenced by genotype and environmental factors.
- The distribution of the secondary metabolites in wheat cultivar tissues suggest differential production of some key bioactive metabolites. Interestingly, Condo, the most weed suppressive cultivar had the greatest abundance of the four major BAs in the root.
- Further metabolic analysis of wheat tissue, rhizosphere and rhizosphere bulk soils is underway to evaluate the role of these metabolites in weed interference.

Acknowledgements

GRDC for sponsorship. Technical support, Graeme Hewitt, Shamsul Haque, Dom Skionczynski, Xiaohe Zhu, Ian Mice and Richard McCleary NSW DPI.

References

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Appendix C

AC5: Mechanism of weed suppression in weed suppressive wheat cultivars and preliminary metabolite profiling in shoots and roots

**Mechanism of weed suppression in weed suppressive wheat genotypes**

**Introduction**
- Wheat cultivar (*Triticum aestivum* L.) shoot and root architectural traits likely play critical roles in crop weed suppression.
- Bx's are important allelochemicals present in wheat, barley and rye.
- Bx's suppressive effects on weeds, pests and diseases are of great interest in sustainable agriculture (Fig. 1).

**Fig. 1: Classification of Benzoxazolinone compounds found in cereals** (Adhikari et al., 2016).

<table>
<thead>
<tr>
<th>Benzoxazolinone</th>
<th>Location</th>
<th>Hydroxylic oxide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bx</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2,6-DimBA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2,3,6-TrimBA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2,3-DimBA</td>
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<td></td>
</tr>
<tr>
<td>2-BA</td>
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</table>

- Selected Australian wheat genotypes were evaluated for their ability to suppress annual weeds in the field and in controlled environments. Rye was used as a control.
- Developed a method for metabolic profiling of wheat shoots, roots, rhizoplane and bulk rhizosphere soils for secondary metabolites using LC-MS/MS to evaluate their role in weed suppression.

**Materials and Methods**
- Field trials were conducted April/May 2014 and 2015 at Condobolin and Wagga Wagga NSW.
- Roots, rhizoplane and bulk rhizosphere soil from around the roots were sampled at 4 strategic times based on crop growth stages.
- Shoots and roots were extracted using an automated extractor-Buchi (Weston et al., 2015).
- Rhizoplane and bulk soils were extracted using shaking extraction (Finnegan et al., 2006; Krog et al., 2008).
- Metabolite profiling method was developed using LC-MS/MS Qtrap 4000 Mass spectrometer (AE SGEX QTRAP® 4500).

**Preliminary Results and Discussion**
- Field trials demonstrated significant differences between wheat cultivars in crop biomass, growth vigour, yield, leaf area index (LAI), weed suppression, weed count and biomass.
- Competitive cultivar traits are strongly influenced by both genotype and environmental factors (Fig. 2).

**Conclusion**
- Crop biomass, LAI, weed count and biomass was location and cultivar dependent.
- Weed suppression, crop growth and yield in wheat were influenced by genotype and environmental factors.
- Method development for secondary metabolite profiling and analysis in wheat has been completed and metabolic analysis of wheat tissue and rhizosphere soils is underway to evaluate the role of these metabolites in weed suppression.

**References**

**Fig. 3: Concentration of metabolites (Log10 pg/mL) in the tissue of wheat and rye taken in July and September 2014**

**Fig. 4: Concentration of metabolites (Log10 pg/mL) in the rhizoplane and rhizosphere bulk soil of wheat and rye taken in July and September 2014**

**Fig. 2: Picture of Trojan (Left) and Condor (right) wheat varieties at Condobolin AGS Sept 2015 shown at the same time set showing differences in canopy density.**
Congratulations to:

James Mwendwa

who received the prize for the best poster presentation at the Faculty of Science Higher Degree Research and Honours Symposium and Dinner on 10 & 11 August 2017 for his poster entitled:

"Field evaluation of Australian canola genotypes."

Professor Tim Wess
Executive Dean
Faculty of Science
10 August 2017

www.csu.edu.au

AC6: Award for the best poster presentation at the Charles Sturt University Faculty of Science Higher Degree Research and Honours Symposium 2017
AC7: Canola poster certificate of presentation at the 8th World Congress of Allelopathy 2017
AC8: Wheat poster certificate of presentation at the 8th World Congress of Allelopathy 2017
Appendix C

A353: Oral communications certificate for presenting metabolite profiling in selected wheat cultivars

Date: July 24th, 2017
Marseille, France

Wheat, Wellner, Jaffrey, West, Leslie
Mwendwa, James, Weston, Paul, Fomsgard, Håge, Larsen, Berent, Brown, William, Wu, Hanwen, Quin, Jane, Shanks

Has been presented by

"Genotypes"
Metabolic profiling for benzoxazolinoids in weed-suppressing and early vigour wheat

The oral communication entitled

8th World Congress of Allotropy