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

James M. Mwendwa[1], Paul A. Weston[1], Inge Fomsgaard[2], Bente B. Laursen[2], William B. Brown[1], Hanwen Wu[1], Greg Rebetzke[3], Jane C. Quinn[1] and Leslie A. Weston[1]
[1] Graham Centre for Agricultural Innovation (NSW Department of Primary Industries and Charles Sturt University), Wagga Wagga, New South Wales 2678, Australia
[2] Department of Agroecology, Aarhus University, Forsøgsvej 1, DK-4200 Slagelse, Denmark
[3] CSIRO Plant Industry, P.O. Box 1600, Australian Capital Territory 2601, Australia
(jmwendwa@csu.edu.au)

Summary 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.

INTRODUCTION

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). 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.

MATERIALS AND METHODS

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.

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

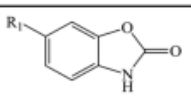
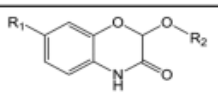
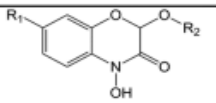
Benzoxazolinones		Lactams			Hydroxamic acids		
							
R_1		**R_1**	**R_2**		**R_1**	**R_2**	
H	BOA	H	H	HBOA	H	H	DIBOA
OCH_3	MBOA	H	Glc	HBOA-Glc	H	Glc	DIBOA-Glc
		OCH_3	H	HMBOA	OCH_3	H	DIMBOA
		OCH_3	Glc	HMBOA-Glc	OCH_3	Glc	DIMBOA-Glc
		H	Glc-Hex[a]	HBOA-Glc-Hex	H	Glc-Hex[a]	DIBOA-Glc-Hex

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-glucopyranosyloxy-1,4-benzoxazin-3-one; HMBOA-Glc, 2-β-D-glucopyranosyloxy-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-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. [a] Structure not fully elucidated (Adhikari *et al*. 2015, Tanwir *et al.* 2013).

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).

Secondary metabolite analyses Analytical standards for 10 BXs and 2 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.

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 ionization 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}

Table 1. Identification (based on molecular weight, MRM transition ion pair, and retention time on chromatographic column) of 10 known benzoxazinoids and 2 provisionally characterised benzoxazinoids analysed by Q Trap 4500 LC-MS/MS. The standards were mixed in groups of fours.

Standard	Acronym	Mass	m/z Values		Retention time/ min
			Q1 Mass (Da)	Q3 Mass (Da)	
A	BOA	135.12	133.901	41.933	12.98
A	MBOA	165.15	163.930	148.867	13.82
A	HBOA	165.15	163.916	107.929	10.69
A	HMBOA	195.17	193.902	122.901	11.78
C	HBOA-Glc	327.2867	325.9	163.9	8.79
C	HMBOA-Glc	357.3127	355.971	193.9	10.11
C	DIBOA	181.1455	179.888	133.976	10.80
C	DIMBOA	211.1715	210.1	164.3	14.00
L	DIBOA-Glc	343.2861	341.900	133.800	8.40
L	DIMBOA-Glc	373.3121	372.000	148.800	10.28
L	DIBOA-Glc-hex	505.43	504.000	133.900	4.90
L	HBOA-Glc-hex	489.43	488.100	163.900	4.90

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.

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).

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 ionization 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 ionization 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.

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 3).

Table 2. 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.

Metabolite	Mass	m/z Values		Q Trap 4500 Rt (min)	Triple Quad Rt (min)
		Q1 Mass (Da)	Q3 Mass (Da)		
BOA	135.12	133.901	41.933	12.98	11.75
MBOA	165.15	163.930	148.867	13.82	12.48
HBOA	165.15	163.916	107.929	10.69	9.56
HMBOA	195.17	193.902	122.901	11.78	10.45
HBOA-Glc	327.2867	325.9	163.9	8.79	7.17
HMBOA-Glc	357.3127	355.971	193.9	10.11	8.62
DIBOA	181.14548	179.888	133.976	10.80	ND*
DIMBOA	211.17146	210.1	164.3	14.00	ND*
DIBOA-Glc	343.2861	341.900	133.800	8.40	7.39
DIMBOA-Glc	373.3121	372.000	148.800	10.28	9.01
DIBOA-Glc-hex	505.43	504.000	133.900	4.90	5.33
HBOA-Glc-hex	489.43	488.100	163.900	4.90	3.99

Table 3. 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 detection limit.

Metabolite	Cereal rye				Gregory wheat				Wedgetail Wheat		
	Shoots	Roots	Rhizo	Soil	Shoots	Roots	Rhizo	Soil	Shoots	Rhizo	Soil
HBOA-Glc-Hex	0.43	0.08	<LDL	<LDL	0.94	0.02	<LDL	<LDL	0.05	<LDL	<LDL
DIBOA-Glc-Hex	1.85	0.00	<LDL	<LDL	0.01	<LDL	<LDL	<LDL	0.02	<LDL	<LDL
DIBOA-Glc	1477.97	0.02	0.033	<LDL	0.91	0.00	<LDL	<LDL	0.34	<LDL	<LDL
DIMBOA-Glc	7.75	6.65	0.003	<LDL	389.55	2.36	0.002	<LDL	5.08	0.007	0.010
HBOA	1.02	3.63	0.016	<LDL	0.06	1.17	0.002	<LDL	<LDL	0.001	<LDL
BOA	2.85	0.66	0.027	0.008	0.09	4.99	0.003	0.002	0.01	0.001	<LDL
MBOA	0.98	625.12	2.797	0.047	104.13	151.03	0.258	0.010	0.05	0.070	0.265
HMBOA	0.04	3.27	0.018	0.003	1.67	3.63	0.015	<LDL	0.01	0.003	0.003
DIMBOA	2.65	0.38	<LDL	<LDL	0.54	5.20	<LDL	<LDL	<LDL	<LDL	<LDL
DIBOA	314.97	0.06	0.004	<LDL	0.52	45.45	0.003	0.015	0.03	0.002	0.011
HBOA-Glc	5.32	2.69	0.037	<LDL	5.03	5.44	0.001	0.004	6.41	<LDL	<LDL
HMBOA-Glc	0.68	13.02	0.013	<LDL	23.30	5.23	0.006	0.338	3.70	0.016	0.011

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 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 Whitcomb 2010). Both instruments showed a similar level of sensitivity with relatively minor differences observed over metabolites surveyed (data not shown).

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 genotypes 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.

ACKNOWLEDGMENTS

We acknowledge the financial support of the Grains Research and Development Corporation (Projects UCS 00020, 00022, and 00023) and technical support of Graeme Heath, Shamsul Haque and Dom Skoneczny with respect to data collection, sampling and analyses.

REFERENCES

Adhikari, K.B., Tanwir, F., Gregersen, P.L., Steffensen, S.K., Jensen, B.M., Poulsen, L.K. and Fomsgaard, I.S. (2015). Benzoxazinoids: cereal phytochemicals with putative therapeutic and health-protecting properties. *Molecular nutrition and food research 59* (7), 1324-38.

Bertholdsson, N.O., Andersson, S.C. and Merker, A. (2012). Allelopathic potential of *Triticum* spp., *Secale* spp. and *Triticosecale* spp. and use of chromosome substitutions and translocations to improve weed suppression ability in winter wheat. *Plant Breeding* 131 (1), 75-80.

Browne, R.W. and Whitcomb, B.W. (2010). Procedures for determination of detection limits: application to high-performance liquid chromatography analysis of fat-soluble vitamins in human serum. *Epidemiology* 21 (4), S4-S9.

Krogh, S.S., Mensz, S.J., Nielsen, S.T., Mortensen, A.G., Christophersen, C. and Fomsgaard, I.S. (2006). Fate of benzoxazinone allelochemicals in soil after incorporation of wheat and rye sprouts. *Journal of Agricultural and Food Chemistry* 54, 1064-74.

Macías, F.A., Molinillo, J.M., Varela, R.M. and Galindo, J.C. (2007). Allelopathy—a natural alternative for weed control. *Pest Management Science* 63 (4), 327-48.

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.

Weston, L.A., Skoneczny, D., Weston, P.A. and Weidenhamer, J.D. (2015). Metabolic profiling: an overview—new approaches for the detection and functional analysis of biologically active secondary plant products. *Journal of Allelochemical Interactions* 1, 15-27.

Wu, H., Haig, T., Pratley, J., Lemerle, D. and An, M. (2000). Distribution and exudation of allelochemicals in wheat (*Triticum aestivum*). *Journal of Chemical Ecology* 26 (9), 2141-54.