Loline alkaloids produced by *Epichloë occultans* in Australian *Lolium rigidum*

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Summary Annual ryegrass (*Lolium rigidum* Gaudin) is the most significant weed of southern Australian farming systems, causing an estimated $300 million in yearly losses to the grains industry (Pratley, pers. comm.). Annual ryegrass is frequently found in association with the endophytic fungus *Epichloë occultans* (C.D.Moon, B.Scott and M.J.Chr.). Fungal endophytes are known to provide ecological fitness benefits to many cool season grasses in part through production of different classes of bioactive compounds. However, it is unknown if *E. occultans* causes benefit or detriment to annual ryegrass through this association. This study examines the production of the most prevalent alkaloids known to be produced by *Epichloë* spp., the lolines, in 15 Australian ecotypes of annual ryegrass. Loline alkaloids are known to enhance host survival in other cool season grasses through protection from insect damage. A broad geographic selection of Australian biotypes of annual ryegrass naturalised to cropping areas based on endophyte presence in the seed was evaluated. Plants of each ecotype were grown and inspected for living endophyte presence before sampling. Loline alkaloids were assessed using GC-FID, including predominant natural variants of lolines; N-acetylloline (NAL), N-formylloline (NFL) and N-acetylnorloline (NANL). Total lolines measured in the plant samples ranged from 0–1200 ppm. Concentrations of NFL were consistently higher than NANL concentrations, whereas NAL was not detected in any of the samples. These results show the presence of lolines in infected *L. rigidum* in all areas sampled across southern Australia. The widespread production of loline alkaloids implies an ecological role in *L. rigidum*, likely associated with persistence of annual ryegrass through protection from herbivores.

Keywords *Epichloë occultans*, annual ryegrass, *Lolium rigidum*, endophyte, alkaloids.

INTRODUCTION

Annual ryegrass is a persistent weed of southern Australian farming systems for several reasons. The long history of use (*c.* 1880s) and a wide distribution in Australian agriculture has allowed it to spread and sustain local populations (Kloot 1983, Mullett 1919). The wide variety of environments it has adapted to in Australia reflects its phenotypic plasticity and is possibly associated with its genetic diversity. Annual ryegrass is a wind pollinated outcrossing species which may preserve its high genetic diversity through being self incompatible. Additionally, the species has evolved characteristics such as high seed production and seed dormancy, the latter allowing protracted emergence (McGowan 1970). Moreover, the evolution of herbicide resistance in annual ryegrass has led to key weed control strategies often being rendered ineffective (Broster and Pratley 2006).

To add to these challenges, Kirkby *et al*. (2011) discovered widespread infection of *Epichloë occultans* (formerly *Neotyphodium occultans*) (Leuchtmann *et al*. 2014) in annual ryegrass seed sampled from across Australia. *Epichloë* species are a class of fungal symbionts associated with many cool season grasses that often provide their grass hosts with elevated fitness and persistence. The magnitude of the symbiotic effect is unclear in annual ryegrass. However, the endophytes persistence, despite being imperfectly and exclusively vertically transmitted, implies an ecological advantage for the symbiosis.

Symbiotic associations between cool season grasses and fungal endophytes of *Epichloë* species have been reviewed extensively (Clay 1988, Schardl *et al*. 2007). Bacon *et al*. (1977) discovered the link between animal toxicosis in fescue and endophyte infection. The cause of the toxicosis was found to be ergot alkaloids produced by *Epichloë coenophialum* (Morgan-Jones and W. Gams). It was subsequently discovered that the removal of the endophyte caused the pasture to become less persistent, thus stimulating more research on fungal interactions and associated alkaloids. Other important classes of alkaloids were also found to be associated with endophyte infection (Clay 1988).

Four main classes of alkaloids are produced by *Epichloë* endophytes (Saikkonen *et al*. 2013):

Ergopeptine alkaloids, mainly ergovaline; Indole diterpene alkaloids, mainly lolitrem B and the Epoxy-janthtrems; Loline alkaloids, mainly N-acetylloline, N-formylloline and N-aceytlnorloline; Pyrropyrazine alkaloid peraminepermaine. The range of effects these alkaloids produce on herbivorous species has been reviewed extensively (Clay 1988, Faeth 2002, Schardl *et al*. 2007). Interestingly, the loline class of alkaloids were first discovered in *Lolium temulentum* L. harbouring the same endophyte as annual ryegrass, *E. occultans* (Hofmeister 1892), although the connection between the fungus and the alkaloids was not discovered until much later.

Annual ryegrass is currently a challenging weed problem for Australia. Endophyte infection is widespread and endemic in Australian annual ryegrass (Kirkby 2011). Given this perspective, we wished to determine if *E. occultans* produces alkaloids that may be contributing to the success of annual ryegrass, thus assisting its survival in a cropping setting.

MATERIALS AND METHODS

Biotypes Seed biotypes of annual ryegrass used in this study were sourced from the Herbicide Resistance Testing Service (HRTS) at Charles Sturt University. Biotypes were chosen for this experiment based on three criteria: geographic distinction, including biotypes from each southern Australian state; the amount and type of endophyte infection detected in seed at harvest; and age of seed sample, which is known to be associated with endophyte viability (Kirkby *et al*. 2011b). In total, 15 different biotypes were selected for the experiment (Table 1).

Seeds were pre-germinated in Petri dishes lined with Advantec (No. 2) filter paper with 3 mL of deionised water added. Ten plants of each biotype (Table 1) were transplanted into individual pots (150 mm diameter) containing a 50:50 mixture of sand and peat moss. Slow release fertiliser (15 g per pot, Osmocote, Scotts Co.) was added to each pot and plants were watered regularly as needed while being maintained in a protected outdoor area at ambient temperatures between May and October. To allow for sufficient plant material to be produced and to provide sufficient time for loline alkaloids to be expressed, plants were allowed to grow until early flowering.

Endophyte detection Preliminary examination of endophyte presence in seed was undertaken. Seed (1 g) from each biotype were separately placed in

Table 1. Biotype information, number of plants evaluated, concentration ranges and means (in brackets) of detected loline alkaloids in each biotype tested.

		Concentration range (mean in brackets)					
Location	n	NANL[A] [ppm]	StD[B]	NFL[C] [ppm]	StD[B]	Total [ppm]	StD[B]
Rand	4	23–48 (40)	11.5	41–103 (68)	25.9	64–151 (108)	35.7
Narromine	5	0–39 (20)	14.3	13–85 (41)	26.7	13–124 (61)	41.4
Cowra	4	7–119 (39)	54.3	17–179 (64)	77.1	21–298 (102)	131.2
Beckom	3	38–107 (78)	29.5	14–69 (47)	33.6	52–167 (125)	63.7
Curramulka	1	42	NA	105	NA	147	NA
Millicent	6	54–137 (88)	36.4	78–326 (183)	92.2	132–457 (270)	123.0
Epping Forest	6	0–53 (32)	18.3	0–73 (47)	26.6	0–117 (79)	43.1
Donald	8	0–105 (35)	24.8	0–73 (20)	43.2	0–170 (55)	65.6
Boort	1	43	NA	63	NA	106	NA
Lake Bolac	4	191–344 (284)	66.1	350–856 (634)	217.0	541–1200 (919)	282.2
Penhurst	6	23–305 (125)	109.7	54–850 (368)	352.8	77–1084 (493)	455.1
Hexham	1	250	NA	667	NA	917	NA
Cranbrook	6	35–89 (58)	22.2	47–166 (100)	45.6	82–244 (158)	66.4
Hyden	3	40–80 (62)	20.2	53–230 (143)	88.5	94–310 (204)	108.1
Corrigan	6	15–113 (57)	33.6	31–266 (146)	89.1	46–351 (203)	119.1
Mean		**71**	**79.4**	**157**	**229**	**263**	**284.8**

[A] n-acetylnorloline; [B] standard deviation; [C] n-formylloline.

McCartney bottles and soaked in 5% NaOH solution overnight to soften, after which seeds were rinsed with tap water to stop the process. Garners solution was then added and bottles were subsequently heated on a laboratory hotplate until boiling which was maintained for 10 min. Seed was then squashed onto microscope slides (Latch *et al*. 1987). For consistency with previous descriptions, *E. occultans* infection was confirmed for a biotype when rarely branched, highly convoluted hyphae, 1 to 2 µm in diameter with non-staining septa were found (Moon *et al*. 2000, Christensen *et al*. 2002).

Commercial Phytoscreen field tiller endophyte detection test kits from Agrinostics Ltd. Co. were also used for qualitative determination of *Epichloë* in tillers of annual ryegrass (Hill *et al*. 2014). After Chromogen solution addition and subsequent binding to the protein complex, a pink stain on the membrane is detected wherever *Epichloë* protein was present. Analytical procedures followed those outlined by immunoblot manufacturer (cat. #ENDO7973 for field tillers, Agrinostics Ltd. Co., Watkinsville, GA 30677, USA).

Sample preparation Two tillers per plant were evaluated for endophyte presence. Subsequently, samples were frozen and retained for chemical analysis. Only infected plant tillers were selected for determination of loline alkaloids. Individual plant samples consisted of one infected tiller, freeze dried and subsequently ground to pass through a 1 mm sieve.

Loline determination Loline alkaloids were measured using gas chromatographic methods (Baldouf *et al*. 2011). A sample of lyophilized grass tissue (100 mg) was extracted for 1 hour with 50 mL of 40% methanol/5% ammonia and 1 mL of 1,2-dichloroethane (containing 53.7 ng mL^{-1} 4-phenylmorpholine as internal standard, followed by centrifugation for 5 min at 8000 g. Supernatant was transferred to a glass GC vial via a 10 mm filter for GC FID (chromatography-flame ionization detector) analysis (Shimadzu GC2010Plus) equipped with a Restek capillary column (30 m × 0.25 mm × 0.25 µm film; Restek US Bellefonte, PA 16823, USA). The limit of quantitation using this technique was 25 mg g^{-1}.

RESULTS AND DISCUSSION

Assessment of two endophyte infected tillers from 10 plants per biotype resulted in the detection of between one to eight infected plants per biotype (Table 1). These results reveal the presence of loline alkaloids in all infected biotypes collected across southern Australia, although not all plants of every biotype contained lolines. Total loline concentrations ranged from 0 ppm to 1200 ppm (Table 1). Considerable variation in loline concentrations were detected within and between annual ryegrass biotypes in this study. Sugawara *et al*. (2006) found concentrations of NFL in *E. occultans* infected *Lolium multiflorum* Lam. ranged from 496 to 1031 ppm. Concentrations detected were also consistent with findings from studies of similar grass-fungal associations (Leuchtmann *et al*. 2000, Shiba and Sugawara 2008), although very limited literature exists on *E. occultans* infected *L. rigidum*.

NAL was not detected in any of the samples (results not shown). NAL's absence is consistent with *E. occultans* infected *L. rigidum* (Tepaske *et al*. 1993), although their evaluation was limited to one accession of *L. rigidum*. Different grass-fungal associations are reported to produce different alkaloid profiles (Leuchtmann *et al*. 2000). Specifically, NAL has been reported in grass-fungal associations such as *E. occultans* infected *Lolium persicum* (Boiss et Hohen.), *L. multiflorum* and *L. temulentum*. In contrast, the endophyte of *Lolium perenne* L., *Epichloë lolii* (Latch, M.J.Chr. & Samuels) has not been reported to produce loline alkaloids (Schardl *et al*. 2007). The concentrations of NFL we observed were consistently higher than and positively correlated with NANL concentration.

Lolines have broad spectrum insecticidal activity (Schardl *et al*. 2007), although their ecological impact in Australia is currently unknown. Artificial feeding assays have shown activity of NFL on rice leaf bug (*Trigonotylus caelestialium* Kirkhaldy) at concentrations as low as 50 µg g^{-1} (Shiba and Sugawara 2009), which were well within the detectable range in this study.

Living annual ryegrass plants in this study contained levels of alkaloids that would be potentially bioactive, and relatively high concentrations also exist in seed (Moore, unpublished data). Dissemination of endophyte in annual ryegrass occurs exclusively through seed (Moon *et al*. 2000) Protection of annual ryegrass seed from insect predation, including that of widespread granivorous ants, may assist in seed survival (McGowan 1969), and has been identified as a mechanism by which protection is provided by fungal endophytes in other associations (Knock *et al*. 1993). Anti-herbivory is one potential explanation for why *E. occultans* populations in Australian annual ryegrass are persisting despite imperfect vertical transmission.

This study presents the first comprehensive survey of loline alkaloids in annual ryegrass in Australia. The widespread detection of loline alkaloids in geographically diverse populations screened in this study implies an ecological purpose for their production, likely associated with protection from herbivory and increased persistence.

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