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REGULAR ARTICLE

Metabolic profiling of benzoxazinoids in the roots and rhizosphere of commercial winter wheat genotypes

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Abstract

Background and objectives Integrated weed management in commercial wheat production is urgently needed due to increasing herbicide resistance and production costs. Benzoxazinoids (BXs), which include benzoxazinones and benzoxazolinones, are unique bioactive metabolites produced by certain members of the Poaceae including maize, wheat, rye and some dicots. BXs play important roles in plant defence and are causal agents of allelopathic interference. We investigated the role of genetics, environment and crop growth stage on BX abundance in the roots and rhizoplane of selected commercial wheat cultivars, and quantified their microbial transformation products (aminophenoxazinones) in roots and rhizosphere soils.

Methods Cultivar trials of competitive wheat (*Triticum aestivum* L.) genotypes were conducted in two moderate to low rainfall (449–572 mm) locations in southeastern Australia in 2015 and 2016. Replicated shoot, root, rhizoplane, and rhizosphere soil samples were collected for metabolic profiling at selected crop phenological stages, extracted and further analysed for known benzoxazinoid metabolites by liquid chromatography coupled with high resolution mass spectrometry.

Results Fifteen BXs and related microbially derived aminophenoxazinones were detected in wheat shoots, roots, rhizoplanes and rhizosphere soils in both years and locations. MBOA, HMBOA and HMBOA-Glc were the three most abundant BX metabolites in wheat tissues, with the heritage cultivar Federation producing the highest levels of MBOA. The phytotoxic aminophenoxazinones APO and AMPO were the most abundant BX microbial transformation products and were detected in wheat roots, rhizoplanes and rhizospheres. Abundance varied with cultivar, growth stage, location and year.

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Conclusions Microbially-produced aminophenoxazinones generated from both heritage and modern wheat root exudates were detected and quantified in rhizosphere soils, with abundance dependent on cultivar, growth stage, and season. Concentrations of microbial metabolites APO, AMPO, and AAPO were higher in the rhizosphere of young wheat seedlings in contrast to that of mature plants suggesting that phenoxazinone production was upregulated early in the season. Our findings demonstrate that BX metabolites at all life stages of wheat potentially undergo rapid biotransformation to aminophenoxazinones under field conditions, resulting in ecologically relevant concentrations sufficient for weed suppression by certain wheat cultivars.

Keywords Benzoxazinones · Aminophenoxazinones · Allelochemicals · Wheat · Soil microbiota · Weed suppression · Root exudation

Introduction

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

Crop competition and allelopathy are both well-documented mechanisms of plant interference under controlled conditions (Weston 2005). The combined effects of these processes determine the total weed suppressive potential of a crop cultivar, and research has recently 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). Numerous plant species produce and release phytotoxic secondary metabolites that inhibit germination or growth of neighbouring plants (Belz 2007; Macías et al. 2007). The production of such allelochemicals in cereal crops is influenced by environmental conditions as well as biological factors and consequently their release over time is often highly variable (Niemeyer 2009).

Benzoxazinoids (BXs) are a class of indole-derived plant defence chemicals containing compounds with a 2-hydroxy-2H-1,4-benzoxazin-3(4H)-one skeleton (Wouters et al. 2016; Supplementary Table S1). They are widespread in grasses, including key cereals 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, displaying a wide range of antifeedant, insecticidal, antimicrobial and allelopathic activities (Wouters et al. 2016). Highest concentrations are produced in young tissues of roots and shoots, where they are glucosylated and stored in vacuoles or exuded by roots (Schulz et al. 2019).

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, DIBOA, DIMBOA, DIBOA-Glc, DIMBOA-Glc, and dihexose derivative of DIBOA (DIBOA-Glc-Hex) are the most common hydroxamic acids in cereals (Adhikari et al. 2015; Tanwir et al. 2013).

The biosynthesis of BXs in cereal crop tissues and their biotransformation in the soil rhizosphere have been well documented and are summarised in Fig. 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

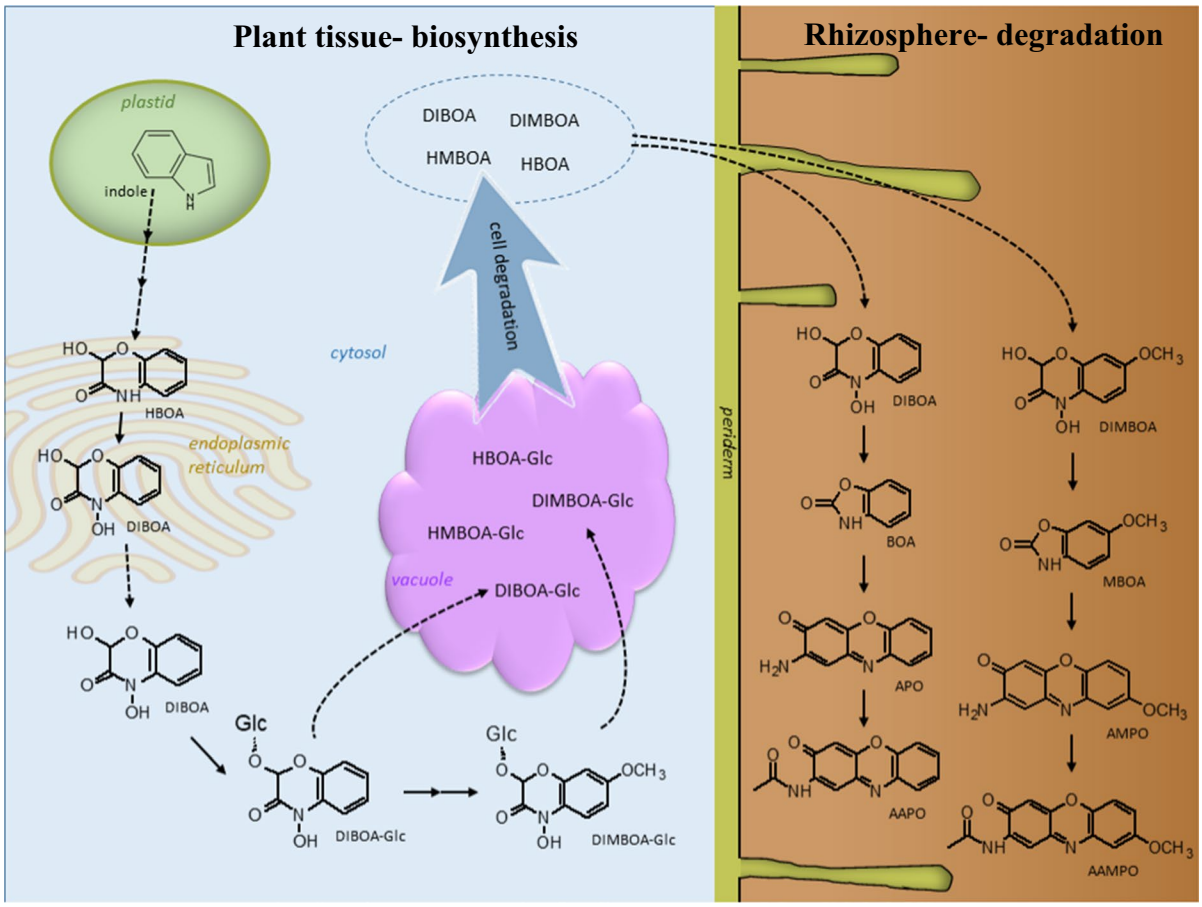


Fig. 1 Schematic illustration of biosynthesis of benzoxazinoids in graminaceous plant tissue and microbial production of aminophenoxazinones in rhizosphere soil. Benzoxazinoids include the benzoxazolinones (BOA and MBOA), hydroxamic acids (DIBOA and DIMBOA), and lactams (HBOA and HMBOA)

2-β-D-glucopyranosyloxy-4-hydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA-glucoside) (Frey et al. 2009; Schulz et al. 2013). 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).

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-2*H*-1,4-benzoxazin-3(4*H*) -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; Wu et al. 1999). 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; Wu et al. 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. 2013; Bertholdsson 2011). However, allelopathic interactions frequently 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). The soil is the main vehicle that mediates contact between allelochemicals, soil microbes and their target plants (Bertin et al. 2003). 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 (Weidenhamer 1996; Inderjit 2001; Blum 2006).

After entering the soil, DIMBOA and DIBOA are degraded to the BXs 6-methoxy-2-benzoxazolinone (MBOA) and 2-benzoxazolinone (BOA), respectively (Fomsgaard et al. 2006) (Supplementary Table S1). They are further transformed in the soil through microbial activity to the potently active bioherbicides 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-methoxyphenul) malonamic acid (HMPMA)] (Fomsgaard et al. 2004; Understrup et al. 2005; Villagrasa et al. 2009).

In maize and wheat shoots, DIMBOA is most abundant 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). The BX profile in cereals varies with plant part, development stage, cultivar, and growing conditions (Carlsen et al. 2009; Hanhineva et al. 2011). 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).

In addition to their role as plant defence compounds, BXs have been shown to modulate the soil microbiome in maize, where BX knock-out mutants impacted community structure of both bacteria and fungi (Kudjordjie et al. 2019). BXs have also been shown to attract the beneficial bacterial strain *Pseudomonas putidastrain* to the rhizosphere of maize plants (Neal et al. 2012). It is therefore likely that BXs enhance the overall fitness of plants producing those metabolites through cross-kingdom interactions (Schandry and Becker 2020), potentially contributing to their evolutionary success.

The use of metabolic profiling to assess presence and quantity of allelochemicals in the rhizosphere provides additional insight into the complex interplay between plants and their associated rhizosphere microorganisms (Weston et al. 2015). The importance of microbes in allelochemical transformation is evident and the interactions between such metabolites and biotic and abiotic components of the soil matrix impacts degradation, transport and phytotoxicity (Barto et al. 2011). Given our interest in selection of weed-suppressive cereal crops and allelochemical interactions in the rhizosphere, we performed a comprehensive series of field and metabolic studies with wheat, the most economically important cereal crop in Australia, in two locations over two successive cropping years. Our objectives were to 1) under Australian conditions, compare the abundance of BXs produced in selected commercial wheat cultivars, some of which are known to be more weed suppressive than others, using metabolic profiling; 2) investigate the role of genetics, environment and crop phenology on the production and release of BXs into rhizosphere soil and 3) identify and quantitate

aminophenoxazinones in roots, rhizoplanes and rhizospheres of various wheat cultivars. For each objective, commercial wheat cultivars were compared with the heritage cultivar Federation and winter cereal rye (*Secale cereale* L.), both of which are recognized as strongly suppressive to weeds.

Materials and methods

Field experimentation

In 2015 and 2016, replicated wheat field trials (four replicates per cultivar) were sown at two locations in moderate to low rainfall zones at Wagga Wagga (572 mm, average annual rainfall) and Condobolin (449 mm, average annual rainfall) NSW, respectively. Plots (12 m × 2 m) were seeded at identical planting densities with 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). Modern commercial wheat cultivars with variable time to maturity along with a grazing winter wheat cultivar and the heritage cultivar Federation, bred and released in Australia in 1901, were compared (Table 1). Federation is early-maturing and drought resistant and was included 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 kandosols, surface (0–10 cm) pH 6.4, typically planted for the commercial 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 rotationally planted to cereals and pasture legume crops. Both soils exhibited low inherent fertility and organic matter content. Standard commercial

Table 1 Wheat cultivars established in field trials performed in Condobolin and Wagga Wagga, NSW in 2015 and 2016

Cultivar	Breeder	Year of release	Main use	Growth characteristics
Condo	AGT[a]	2014	Grain	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
Espada	AGT	2008	Grain	Mid-season maturity. Good seedling vigour. Large grain with low screenings
Federation[b]	William Farrer	1901	Grain	The first Australian cultivar 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
Gregory	EGA[d]	2004	Grain	Excellent yield potential in early to mid-season sowing. Medium to slow maturity
Janz CL	AGT	1989	Grain	Widely adapted Clearfield® variety. Moderate seedling vigour. Medium–strong straw strength, with good lodging & shattering resistance
Livingston	AGT	2008	Grain	Early maturing cultivar
Wedgetail	EGA	2002	Dual purpose	Dominant winter wheat. Large grain size. Adapted to higher rainfall regions of the wheat belt
Grazer (cereal rye)[c]	EGA	2002	Dual purpose	Rapid growth with early vigour, grazing possible after emergence if tillering has occurred to anchor the plant

[a]Australian Grain Technologies

[b]bred by William Farrer in 1901

[c]Cereal rye was used as a control for the field trials

[d]Enterprise Grains Australia

practices to reduce weed populations including pre- and post-chemical control were applied. Initially soil samples were collected to evaluate the weed seedbank and predominant species are reported in Mwendwa et al. (2020).

All crops were established with seed that was generated in Wagga Wagga NSW from harvest the previous season. This potentially reduced seed variation that can adversely impact cultivar trials conducted with seed generated 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 existing weeds were controlled with glyphosate (Weedmaster® DST® 470 g L^{-1} glyphosate, Nufarm) at 960 g ha^{-1}.

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 or soil collection at both field locations. Samples consisted of tissue or soil collected from two sub-samples per plot (five plants per sub-sample, 10 plants per plot sample and randomly selected) which were later combined to form one composite sample of each tissue type for each cultivar and in each of four replicates (Mwendwa et al. 2016). In this case, we analysed a composite sample of shoots, roots or rhizosphere soil per plot at three selected stages of crop growth in 2015 and 2016 from four replicates selected from each experiment. 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 with ice for transportation to the laboratory and then stored at -80 °C until extraction.

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 and Duke 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, 034,925) and placed in 10 mL extraction cells (Buchi, Switzerland). The extraction was conducted under 1400 psi using 100% methanol, at temperature 35 °C (Krogh et al. 2006). 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 15 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).

UHPLC-QTOF-MS and QQQ-MS analysis

Metabolic profiling of the extracts for targeted secondary metabolites using purified standards for comparison 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 using similar methods as described in Fomsgaard et al. (2006) and Krogh et al. (2006). Quantification of BXs was performed using liquid chromatography coupled to quadrupole time-of-flight mass spectrometry in negative ion mode, and microbially-produced metabolites (APO, AAPO, AMPO, and AAMPO) were profiled in positive ion mode using liquid chromatography coupled to triple quadrupole mass spectrometry.

Benzoxazinoids analysis—UHPLC-QTOF-MS

Metabolic profiling of shoot and 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^{-1}, and nebuliser pressure was 35 psig. Sheath gas flow and temperature were 10 L min^{-1} and 250 °C, respectively. Full scan mass spectra were acquired over a range of 100–1700 *mz* at a rate of two spectra/second using negative ion mode.

Chromatographic separation was performed using a Synergi™ Polar-RP column (2.0 mm × 30 mm, 2.5 µm particle size) (Phenomenex, Torrance, CA USA) preceded by a guard column (2.0 × 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 was initiated with 100% A for 1 min, then ramped to 100% B over 1–4 min, held at 100% B for 30 s and then ramped back to 100% A over 0.1 min and held at 100% A from 4.6–9 min. 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 1.5 µg mL^{-1}). BX metabolite abundance was expressed in units of detector response as purified standards were generally unavailable for the majority of BX metabolites.

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 elution order based on similar analyses performed by Mwendwa et al. (2016) using standards in the Fomsgaard laboratory at Aarhus University in Denmark or with purified standards obtained from Fomsgaard. BOA and MBOA were purchased from Sigma-Aldrich (Castle Hill, NSW).

Aminophenoxazinone analysis—UHPLC-QQQ-MS

Targeted metabolic profiling of aminophenoxazinones in root tissue, rhizoplane and rhizosphere soil extracts 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 electrospray ionisation (ESI) 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 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 purified standards kindly provided by I. Fomsgaard, Aarhus University using positive ion mode. Due to their structural similarity, APO and AAPO were quantified using a single MRM experiment tracking the transition 213,185➔ m/*z*. AMPO was quantified in a separate MRM run tracking the transition 243➔ 228 m/*z*. AAMPO could not be consistently detected using this or any other 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 min, 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 aminophenoxazinone metabolites (APO, AAPO and AMPO) at a concentration of 3.3 µg mL^{-1}. The abundance of aminophenoxazinones is expressed in absolute concentrations in the extracts (as µM). It was not reasonable to express all sample concentrations per unit of plant tissue as rhizosphere samples, for example, were extracted from the surface of roots and soil samples varied slightly in texture and moisture content.

Statistical analysis

PCA plots were produced using Agilent Mass Profiler Professional v. B14.9 (Agilent, California, USA). 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.

Results

Benzoxazinoids and derivatives detected in plant tissue and rhizosphere soil

Fifteen unique BXs and their derivatives (hydroxamic acids, lactams, benzoxazolinones and aminophenoxazinones) were detected in shoots, roots, rhizoplane and rhizosphere soil extracts of wheat and rye (positive control) in both years and locations. Table 2 provides a summary of metabolites detected in plant tissue and soil. Aminophenoxazinones (APO, AAPO, AMPO) produced by microbial transformation were generally not detected in shoots but were present in abundance in root, rhizoplane and rhizosphere extracts. However, AAMPO was rarely detected, and even then, only in trace quantities. 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). 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 (distinguished by colour).

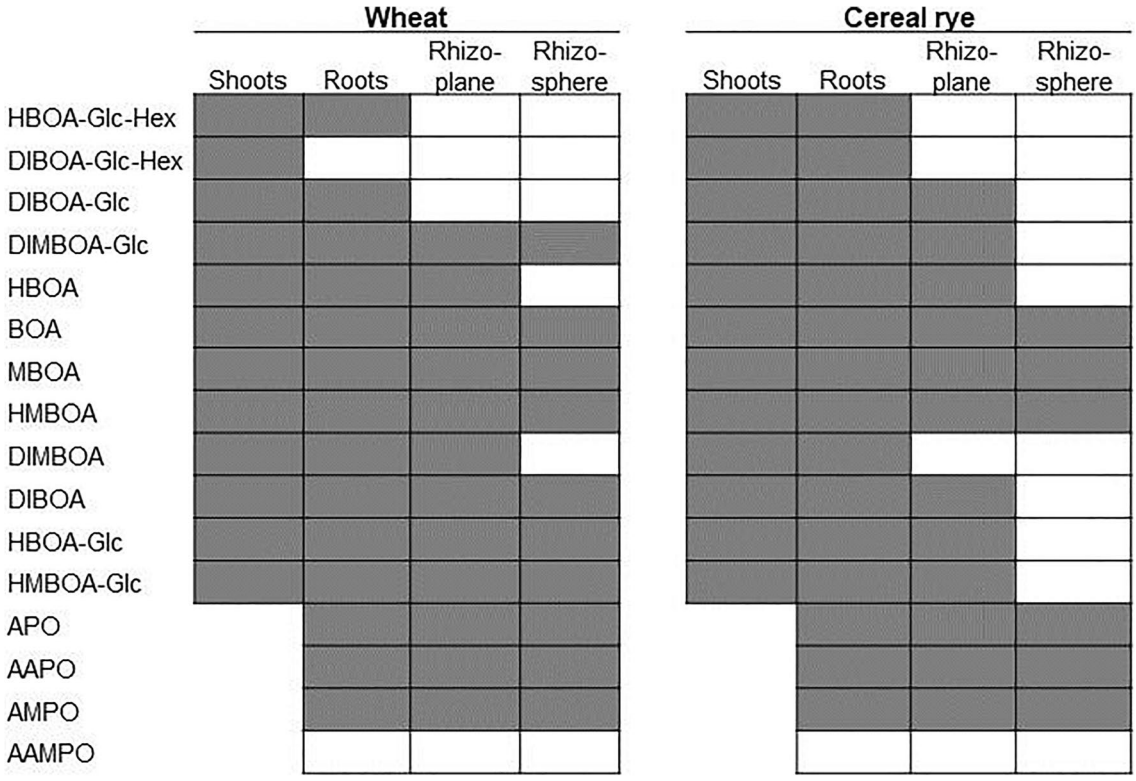
Benzoxazinoids and derivatives abundance in wheat tissues

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 growth stage, 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 highest in the 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 than in 2016, a year with above average rainfall in Condobolin ($P < 0.001$, Supplementary Table S3, Fig. 5). 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).

The abundance of BOA and MBOA in wheat roots, rhizoplane and shoots

BOA was detected in significant quantities in both wheat and cereal rye shoot, root, and rhizoplane samples. Remarkably, BOA abundance was seven times greater in rye shoots and three times greater in rye

Table 2 Summary of the BXs and derivative metabolites detected in all wheat cultivars and rye shoots, roots, rhizoplane and rhizosphere



Grey bars indicate positive detection of metabolites

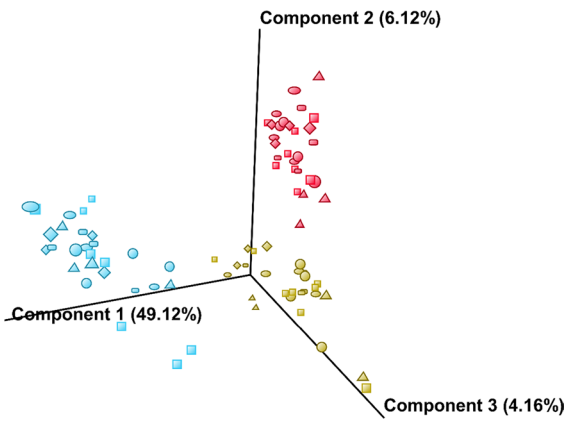


Fig. 2 A representative PCA plot of the metabolites detected in root, shoot and rhizoplane samples from wheat cultivars grown in Condobolin NSW in 2015 (negative ion mode LC MS QToF analysis). Different shapes or symbols represent each of the various (7) wheat cultivars, while different colours represent various tissue types (blue = shoot, yellow = root, and red = rhizoplane)

root and rhizoplane samples compared to the corresponding samples 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 shoot samples ($P < 0.001$, Supplementary Table S3) while BOA was present in highest abundance in the shoots of cereal rye ($P < 0.001$, Supplementary Table S4). MBOA abundance in root, rhizoplane and shoot samples differed significantly by cultivar ($P < 0.001$, Supplementary Table S3).

The abundance and concentrations of aminophenoxazinones in wheat roots, rhizoplane and rhizosphere

The aminophenoxazinones APO and AMPO were detected in abundance on the surface of wheat roots and rhizosphere soil samples and were the most

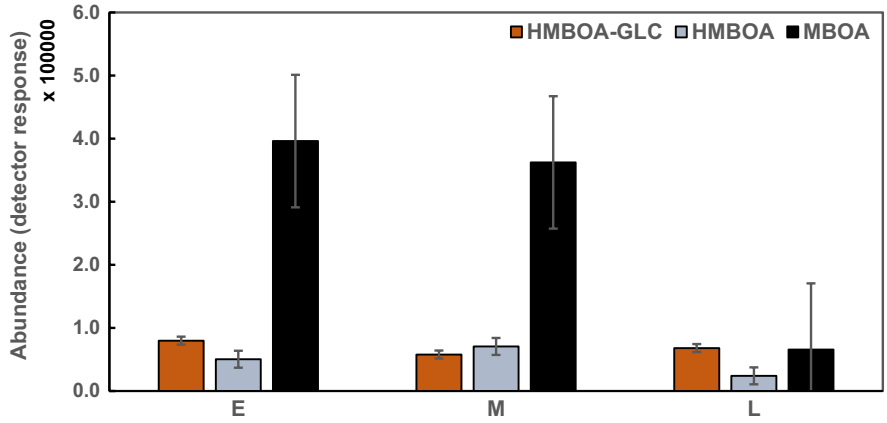


Fig. 3 The most abundant wheat BX metabolites detected in shoots and roots based on sampling time (crop growth stage). Values were averaged across year, location, cultivar and tissue. E = early (tillering), M = mid (vegetative) and L = late (mature post-flowering) phenological stages. Error bars indicate standard errors

abundant microbially transformed metabolites at the three crop growth stages monitored ($P < 0.001$, Supplementary Tables S5 & S6, Fig. 9). AAPO was less abundant than APO and AMPO at all crop growth stages and was detected only at very low levels. APO and AMPO were generally highest at the early vegetative crop growth stage. Cultivar differences in the abundance of APO and AMPO were also noted ($P < 0.001$, Supplementary Tables S5 & S6, Fig. 10); levels of these compounds were highest in cultivars Federation and Janz CL.

Significant differences were noted in the abundance of aminophenoxazinones in the roots, rhizoplane and rhizosphere by tissue, location and year ($P < 0.001$, Supplementary Tables S5, S6 & S7). Aminophenoxazinone 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 (Fig. 11). 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 (Fig. 11).

Not unexpectedly, the abundance of APO was dramatically higher in rye compared to wheat samples ($P < 0.001$, Supplementary Table S8) and was highest in rye root and rhizoplane samples ($P < 0.001$, Supplementary Table S8, Fig. 12). Rhizosphere soil samples showed lower levels of this metabolite in both wheat and rye.

Significant differences were noted in the concentrations of aminophenoxazinones in the roots, rhizoplane and rhizosphere by cultivar, tissue, location and year ($P < 0.001$, Fig. 13, Supplementary

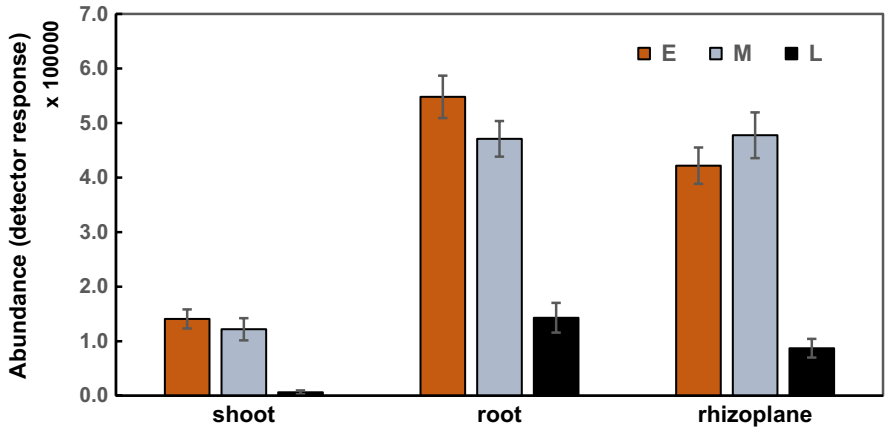


Fig. 4 MBOA abundance in extracts of wheat shoots (5 g), roots (5 g) and rhizoplanes (6 g) based on sampling time (crop growth stage). Values were averaged across year, location and cultivar [E = early (tillering), M = mid (vegetative) and L = late (mature post-flowering) crop phenological stages]. Error bars indicate standard errors

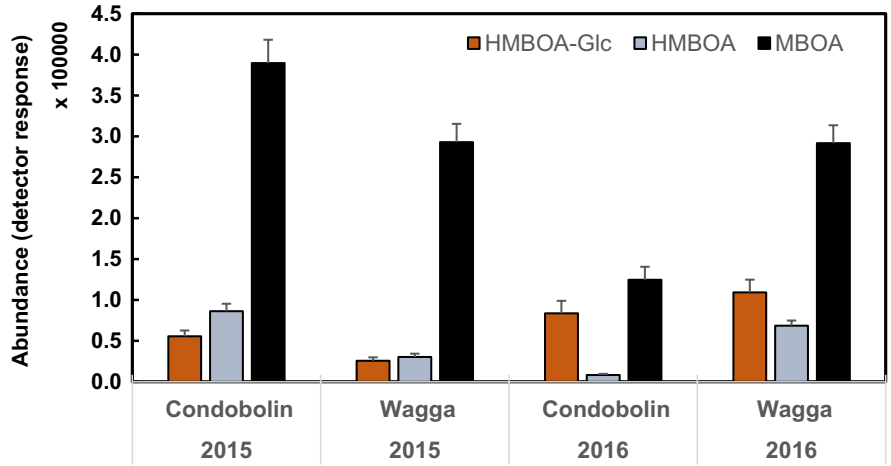


Fig. 5 Abundance of HMBOA-Glc, HMBOA and MBOA in extracts of wheat shoots (5 g), roots (5 g) and rhizoplane (6 g) samples for the two years and locations of the study. Values were averaged across cultivar, tissue and crop growth stage. Error bars indicate standard errors

Tables S9, S10 & S11). The concentrations of aminophenoxazinones in extracts of wheat rhizosphere samples, averaged over location, year, variety and growth stage, were AMPO, 7.7 µM; APO, 1.0 µM; and AAPO, 1.0 µM.

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 the 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 BXs has been well described in both monocots and dicots (Dick et al. 2012), and the related microbial biotransformation pathway of aminophenoxazinones 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 (Weston et al. 2015; 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),

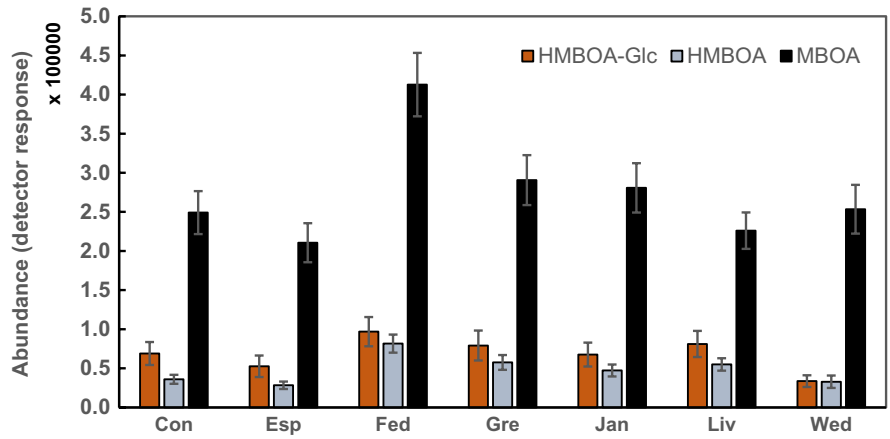


Fig. 6 Most abundant wheat BX metabolites based on cultivar (Con = Condo, Esp = Espada, Fed = Federation, Gre = Gregory Jan = Janz CL, Liv = Livingston, Wed = Wedgetail). Values were averaged across year, location, tissue and crop growth stage. Error bars indicate standard errors

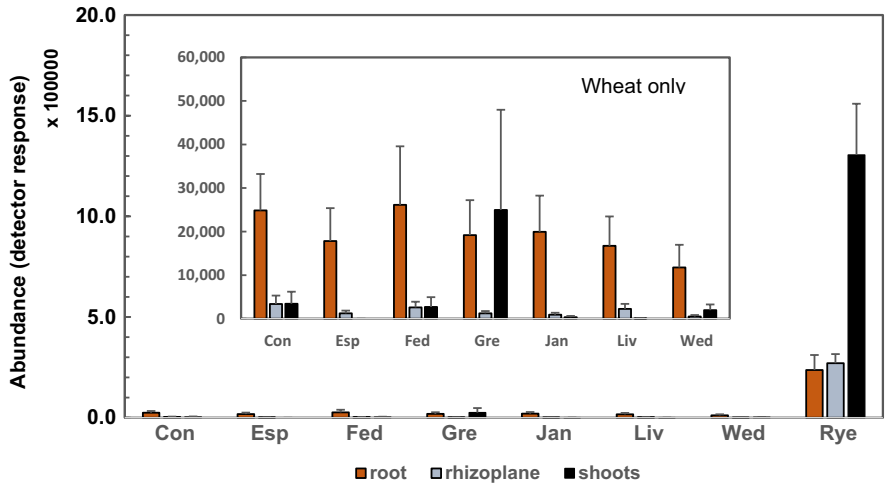


Fig. 7 The abundance of 2-benzoxazolinone (BOA) in wheat cultivars and rye (positive control) based on sample type and cultivar. Inset shows an expanded view of the abundance of BOA for wheat cultivars only (cultivars include Con = Condo, Esp = Espada, Fed = Federation, Gre = Gregory Jan = Janz CL, Liv = Livingston, and Wed = Wedgetail). Values were averaged over year, location, and crop growth stage. Error bars indicate standard errors

indicating potential for 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 performed by LC- MS QToF mass spectrometry. Fresh field samples were collected from replicated trials performed over two years and two 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 12 individual BX metabolites including BX glycosides, lactones, and hydroxamic acids in one rapid analysis using a polar reverse phase column for a remarkably large number (~2000) of replicated samples generated in this study.

In addition, we performed and optimised the subsequent analysis of related microbially transformed soil metabolites, the aminophenoxazinones, using a similar column and metabolic profiling system. We successfully detected three of four biotransformed aminophenoxazinones known to exhibit potent

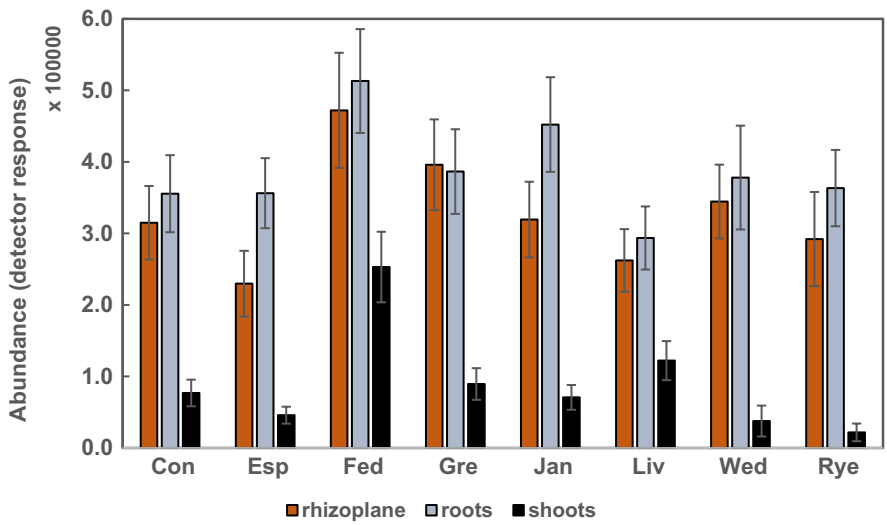


Fig. 8 The abundance of 6-methoxy-2-benzoxazolinone (MBOA) in wheat cultivars and rye (positive control) based on sample type and cultivar. (Cultivars include Con = Condo, Esp = Espada, Fed = Federation, Gre = Gregory Jan = Janz CL, Liv = Livingston, Rye = Cereal rye, Wed = Wedgetail). Values were averaged across the year, location, and crop growth stage. Error bars indicate standard errors

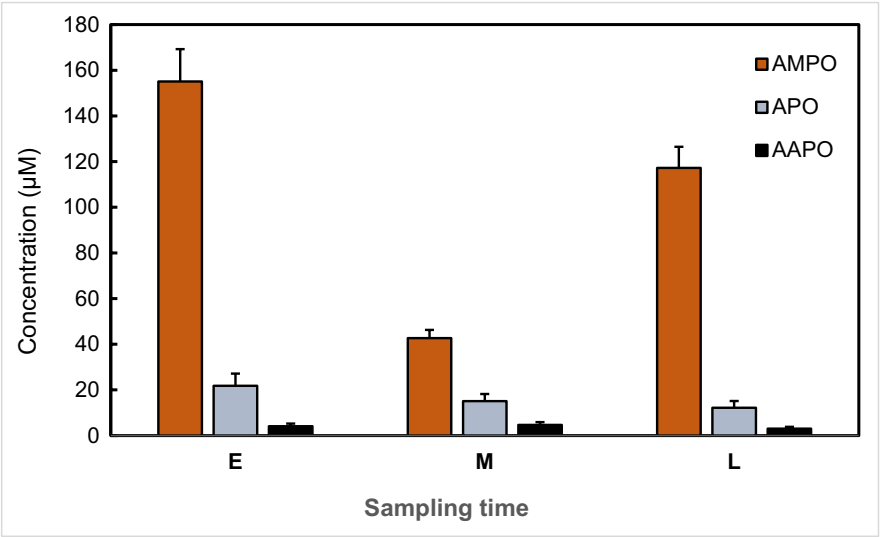


Fig. 9 The concentrations in extracts of all wheat tissues insert all wheat tissues (APO, AAPO and AMPO) in extracts of wheat across the growing season (indicating crop growth stage) [E = early (tillering), M = mid (vegetative) and L = late (mature post-flowering) crop phenological stages]. Values were averaged across year, location, cultivar, and tissue. Error bars indicate standard errors

activity as allelochemicals in the root and soil rhizosphere (Venturelli et al. 2015). Our findings show both qualitative and quantitative differences in production of BXs and their biotransformed metabolites (aminophenoxazinones) in Australian wheat cultivars and their 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. As this study utilised an unusually large and statistically relevant number of replicated tissue and soil samples to describe allelochemical production and transformation over time in various wheat genotypes under distinct soil and climatic conditions, the results of this study are unique and therefore of critical importance in describing actual allelochemical presence and persistence over time under various field conditions *in planta* and in the rhizosphere.

The concentrations reported of both benzoxazinones and aminophenoxazinones are of their concentrations in extracts rather than concentrations in tissues or soils, due to the differences in matrix type with each sample. Successful detection of these metabolites, which are present in trace concentrations, is dependent on efficient extraction in concentrations allowing for detection with instruments possessing adequate sensitivity. The actual concentrations should

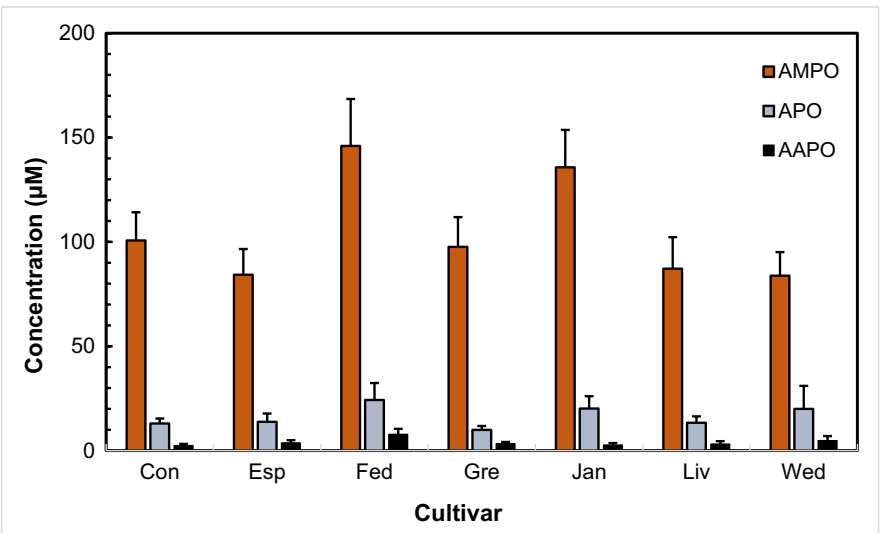


Fig. 10 The concentrations of aminophenoxazinones (APO, AAPO and AMPO) in extracts of the seven wheat cultivars (Con = Condo, Esp = Espada, Fed = Federation, Gre = Gregory Jan = Janz CL, Liv = Livingston,, Wed = Wedgetail). Values were averaged over year, location, tissue and crop growth stage. Error bars indicate standard errors

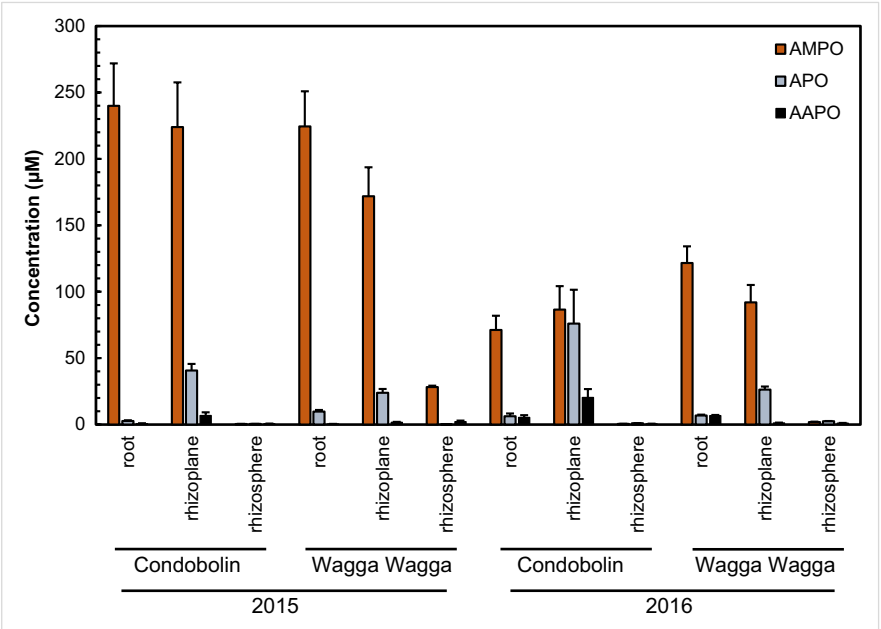


Fig. 11 The concentrations of aminophenoxazinones (APO, AAPO and AMPO) in the extracts of roots, rhizoplane and rhizosphere of wheat by location and year. Values were averaged across wheat cultivar, and crop growth stage. Error bars indicate standard errors

be reassessed under more highly controlled conditions to validate the ranges detected in this study. Losses due to transformation, degradation, inefficient extraction, retention in pipette or on glass are also likely to reduce the amounts of metabolites recovered compared to their actual, and likely higher, concentrations in the field. Due to the transient nature of many these metabolites, it is difficult to predict their exact role in plant-plant interactions. However, aminophenoxazinones have been reported as more stable than other BX metabolites (e.g. Macías et al. 2005a, b) but experimentation on their stability is limited.

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 2 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

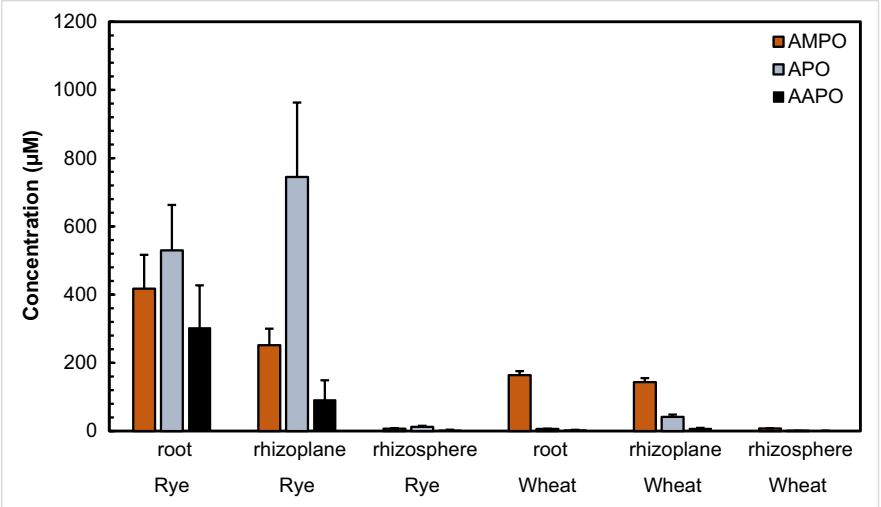


Fig. 12 The concentrations of APO, AAPO, and AMPO in the extracts of roots, rhizoplane and rhizosphere of rye and wheat (averaged over seven wheat cultivars, growth stage, year and location). Error bars are standard errors

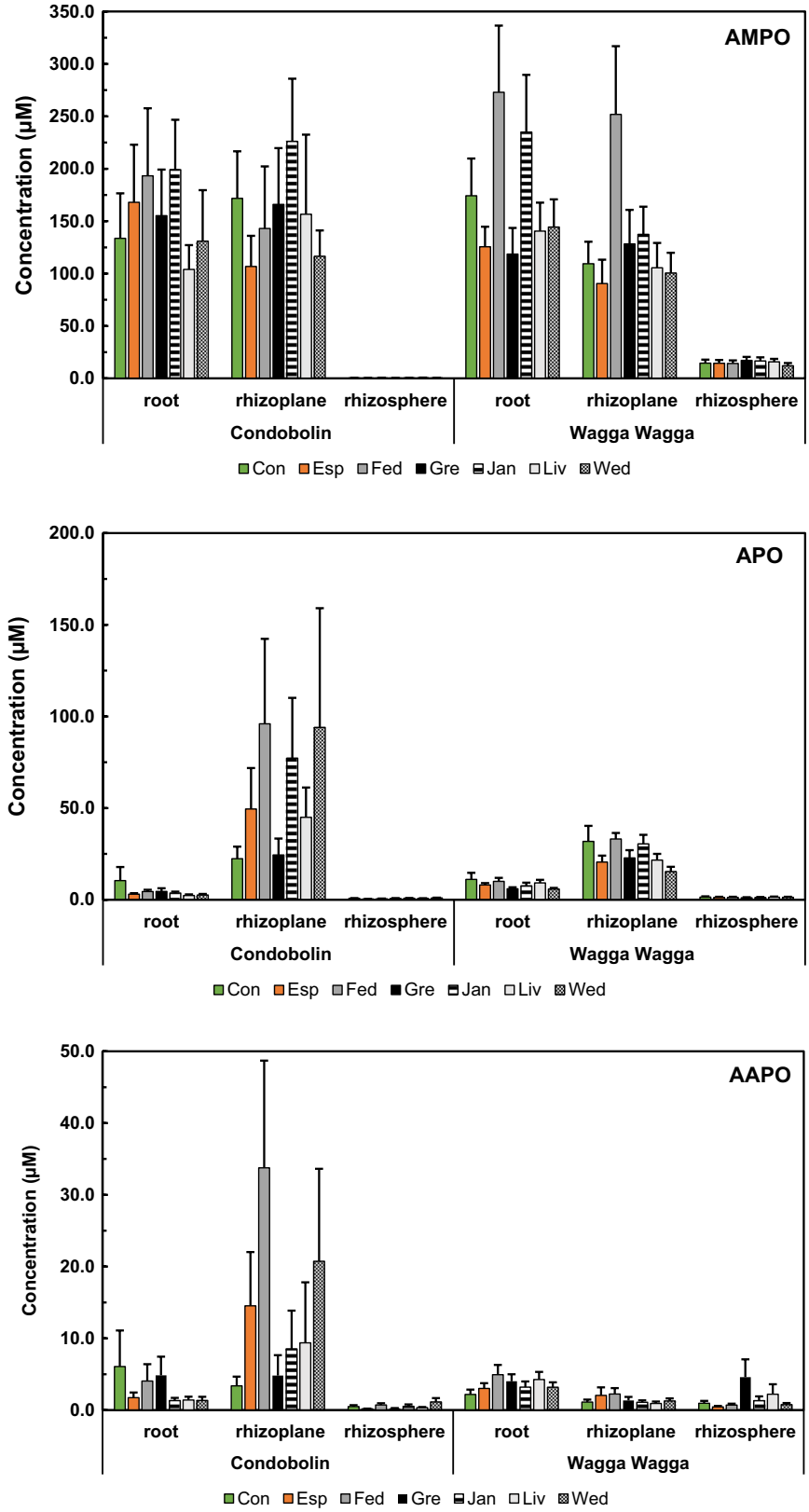


Fig. 13 The concentrations (μM) of AMPO, APO, and AAPO in the extracts of roots, rhizoplane and rhizosphere of wheat cultivars by location avaraged over the two years. Cultivars include Con = Condo, Esp = Espada, Fed = Federation, Gre = Gregory Jan = Janz CL, Liv = Livingston, Rye = Cereal rye, Wed = Wedgetail. Error bars are standard errors

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 BX 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 observed in soil and soil solutions (Woodward et al. 1978; Wu et al. 2000). DIMBOA is the precursor of MBOA, which is the catabolite detected in greatest abundance in field-grown wheat roots and rhizosphere samples collected at both locations and years in our study. Interestingly, production of DIMBOA by wheat has previously been 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 polygenic depending on the population (Simcox and Weber 1985). These findings suggest that selection for production of related allelochemicals in wheat is also feasible.

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. 2005a, b; Chen et al. 2010). The substantial concentrations of MBOA in rhizosphere soils of wheat reported here and by others also suggest strong potential for allelopathic interference by winter wheat produced under Australian conditions.

The sequence of 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 tenfold 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 ten-fold differences in the concentration of DIBOA among the shoot tissues of eight cultivars of rye. In this field study, the first of its kind in the southern hemisphere, DIMBOA and DIBOA were detected only in trace quantities, and then only in the shoots of the wheat cultivars.

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 BX 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 detected only trace quantities of DIMBOA in roots and rhizosphere soil.

However, MBOA was detected at up to four-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, likely via 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 whereas 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 finding hints that the recent selection for higher yield and semi-dwarf stature among other traits may 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 has been 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 BX production, particularly of MBOA, as observed among the cultivars evaluated in this study suggests that enhanced BX production may be amenable to selection 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 BX 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).

The abundance of BOA and MBOA in wheat roots, rhizoplane and rhizosphere

The BXs 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 BX 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 1955; Rice et al. 2005), which degrades to BOA (Fig. 1).

The detection of numerous BXs 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 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. 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 a 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.

The abundance and concentrations of aminophenoxazinones in wheat roots, rhizoplane and rhizosphere

The aminophenoxazinones 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 50% more abundant in the root and rhizoplane of certain wheat cultivars (Federation and Janz CL) (Fig. 10), and, interestingly, these cultivars have also been reported 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); this finding is not surprising 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 extracts of wheat sampled in this study was 20 µM, with > 300 µM maximal concentration for AMPO, both potentially high enough to exert significant deleterious effects on germinating seeds or neighbouring seedlings (Macías et al. 2006, 2014; Venturelli et al. 2015).

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 that the phytotoxicity of APO and AMPO were comparable to the specific activity of commercial herbicides (Macías et al. 2006; Venturelli et al. 2015). Aminophenoxazinones are also much more stable in the soil environment in contrast to BXs, particularly under dry soil conditions such as those typically found in Australia. Aminophenoxazinones 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 BX derivatives that are further transformed by soil microbiota to potent bioherbicides by selected wheat genotypes could potentially aid management of 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 associated with 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 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 APO and AMPO (Macías et al. 2006, 2009). The formation of APO from root-exuded DIBOA as well as the uptake 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 aminophenoxazinones in the soil under field conditions (Venturelli et al. 2015).

In the current study, we noted the presence of ecologically relevant concentrations of the aminophenoxazinone 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 50% 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 BXs and aminophenoxazinone metabolites in the plant 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 other location effects, as shown in Fig. 11. Further work to document heritability of BX expression in wheat is needed.

Potential role of soil microbial communities on BX metabolism

The presence of aminophenoxazinone metabolites detected in the soil rhizosphere in our experiment suggest that soil fungi and/or bacteria are involved in biotransformation of MBOA and BOA in Australia, but 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). In a concurrent study conducted at the research field site in Wagga Wagga used for this study, we observed that bacterial and fungal communities associated with the rhizosphere of a Brassicaceae (rapeseed) crop varied temporally, likely influenced by the exudation of secondary metabolites by living roots and leaf litter following senescence (Gurusinghe et al. 2019). Similarly, it is highly likely that cross-kingdom interactions between wheat roots and microbial taxa are further impacted at various growth stage of the plant. Further investigations are required to evaluate the interactions of specific wheat associated BXs with soil microbial communities present in Australian soils. A positive linear relationship between MBOA levels in the wheat rhizosphere and the presence of soil fungi/bacteria has been established in controlled environment experiments conducted using field-collected soil in China (Chen et al. 2010). In that study, DIMBOA significantly decreased the actinobacterial biomass at 1 h, while MBOA resulted in the decrease of actinobacterial biomass at 48 h 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 selects for a specific soil microflora in the plant rhizosphere.

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 BXs produced by wheat and its root exudates by soil microbiota under field conditions.

This work is the first to demonstrate the production of aminophenoxazinones in Australian soils, suggesting that weed suppression through allelochemical production might be boosted under field conditions for certain wheat cultivars. Our findings lead to a mechanistically informed field model for the molecular mode of action of aminophenoxazinones in the target plant and provide insights into allelochemical defence and competition strategies of BX-producing cereals. Further research on the relative abundance of BXs and their biotransformed metabolites is required to determine which Australian wheat cultivars or soils lead to the highest rate of weed suppression. Interestingly, BX and related aminophenoxazinone production was upregulated early in the season as opposed to late season, after crop maturity. Genes associated with plant defence are frequently upregulated in seedling crops and weeds, and our findings provide additional support for this model.

Both APO and AMPO were found in abundance in association with the roots and rhizosphere of certain cultivars, especially the heritage cultivar Federation, but were most abundant in years with lower soil moisture content. 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 aminophenoxazinones, 3) chemical signalling of plants in response to the environment and other plant and microbial populations is involved in allelopathic interference in cereals.

It is obvious from our findings that the study of plant/soil interactions requires the generation of key information to facilitate widespread adoption of competitive crops and their associated rhizospheres for weed suppression. Such research is to some extent limited by the lack of cost-effective, non-destructive sampling methods that would potentially allow for repeated sampling of plant metabolites or microbial communities in the rhizosphere over time (Mohney et al. 2009; Reiss et al. 2018). Therefore, we suggest additional research focused on root exudation and uptake, together with metabolite identification, transformation and persistence be considered when attempting to better characterize allelopathic interference.

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