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Getting to the roots of black-grass: allelopathic interactions for control of *Alopecurus myosuroides*

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Abstract

Black-grass is a highly detrimental herbicide-resistant grass weed of arable agriculture in Europe. While cultural controls can partially mitigate black-grass, there remains great need for additional alternative control methods. A potential alternative approach to weed control is to harness the allelopathy (biochemical inhibition) of potent species. Cereals like wheat and rye synthesise and exude from their roots a class of compounds, benzoxazinoids, which have both plant-plant allelopathic potential, and other multi-kingdom inhibitory effects. Petri dish assays of benzoxazinoid compounds with documented allelopathic potential identified 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) and 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA) as promising compounds in inhibiting root development in black-grass populations, with inhibition unrelated to variability in herbicide resistance profile. Wheat is highly tolerant of these compounds, indicating species-specificity in their allelopathic potential. Crude cereal root exudates collected in hydroponic and axenic sand systems were also examined for allelopathic potential. Therein, wheat and rye exudates were confirmed as inhibitory, while a wider range of inhibitory potential was found in exudates of ancestor wheats. Subsequent chemical analyses of these exudates tentatively identified a suite of benzoxazinoid compounds present, with those related to DIMBOA exuded from modern wheat, and those related to DIBOA exuded from rye and ancestor wheats. Selection for DIMBOA synthesis may thus be an unintended consequence of wheat domestication. 2-hydroxy-1,4-benzoxazin-3-one (HBOA) was noted as another potential allelochemical, which was examined and confirmed as a potent black-grass inhibitor. Crude cereal exudates were capable of allelopathy towards black-grass in soil, but benzoxazinoids degraded quickly in many soils, reducing their inhibitory potential. This explains the greater efficacy of biological crop treatments exuding allelochemicals towards black-grass, in comparison to synthetic chemical treatments when applied in combination in glasshouse assays. Further dose-response analyses of the more persistent DIBOA-related compound 4-hydroxy-1,4-benzoxazin-3-one (D-DIBOA) identified that it is not an effective black-grass inhibitor, indicating that another allelochemical must be identified for the development of a pure

bioherbicide. Therefore, it is concluded that an allelopathic crop is a more realistic application of benzoxazinoid inhibitors for black-grass control, in combination with effective deployment of cultural control methods.

Publications from the work

- **Hickman DT,** Rasmussen A, Ritz K, Birkett MA, Neve P (2021) Allelochemicals as multi-kingdom plant defence compounds: towards an integrated approach. *Pest Management Science, 77*: (3): 1121-1131. https://doi.org/10.1002/ps.6076.

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Of course, I must finally credit the dedication of my loved ones through difficult times, without which none of this would have been possible.

Dedication

This thesis is dedicated to my grandparents, June Marina Davies and Ben Hickman, both of whom saw its start, but were unable to see its completion.

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Thesis Structure

This thesis is composed of a mixture of published and currently unpublished content in a loose paper format. Currently published content is denoted at the beginning of each chapter.

Chapter 1 is a summary of pertinent literature in the areas of weed science, black-grass, and allelopathy, as well as a review of multi-kingdom effects in phytotoxic allelochemicals.

Chapter 2 explores the allelopathic potential of benzoxazinoids, a group of secondary metabolites common in cereals, towards black-grass *in vitro*.

Chapter 3 describes the inhibitory effects of crude cereal root exudates towards black-grass in axenic systems.

Chapter 4 investigates the root exudate composition of a wide range of cereal root exudates to correlate the effects of pure benzoxazinoid compounds with crude exudate effects.

Chapter 5 assesses the maintenance of crude exudate and synthetic benzoxazinoid allelopathy in biologically active soils and their effects in combination in glasshouse assays.

Chapter 6 discusses the project in a wider context of contributions to the field of allelopathy, outstanding questions, and further novel approaches which could be used to improve its reputation in the wider scientific community.

Chapter 1

General Introduction and Literature Review:
Allelopathy as a multi-kingdom phenomenon

1. General Introduction and Literature Review- Selected content (predominantly Sections 1.5-1.9) published in Pest Management Science:

Hickman DT, Rasmussen A, Ritz K, Birkett MA, Neve P (2021) Allelochemicals as multi-kingdom plant defence compounds: towards an integrated approach. *Pest Management Science, 77*: (3): 1121-1131. https://doi.org/10.1002/ps.6076.

1.1. Black-grass as a weed

1.1.1. The prevalence of agricultural weeds

Agriculture has been disrupted by weeds since it was first practiced around 12,000 years ago. Weeds were traditionally controlled using hand-weeding, superseded by mechanical weeding during industrialisation (Timmons, 2005; Rueda-Ayala *et al.*, 2011). Mechanical weeding disrupts soil structure and the ecological community therein, and is more expensive than hand-weeding (Jabran *et al.*, 2015). An increasing world population in the late 19th and early 20th centuries led to agricultural intensification, which, following the Second World War, coincided with the development of selective synthetic herbicides (those which inhibit weeds but not crops) as a cheap and efficacious control method (Timmons, 2005). Herbicide use therefore increased rapidly thereafter; 7,800 metric tonnes were applied over 23 million hectares of British fields in 2016 (FERA, 2018). In the European Union, over 350,000 tonnes of herbicide were sold each year between 2011 and 2017 (Eurostat, 2019). As weeds compete directly with crops for resources, they can reduce crop yield by up to 34% worldwide, compared to estimated 18% and 16% reductions from animal pests and pathogens respectively (Oerke, 2006). Weeds thus present a major challenge to global agriculture, so control is essential.

1.1.2. Black-grass (*Alopecurus myosuroides* Huds.)

In Europe, including the UK, black-grass (*Alopecurus myosuroides* Huds.) (Poaceae) (Figure 1.1), is a major agricultural weed and an increasing threat to winter cereal production (Cavan, Biss and Moss, 1998; Bertholdsson, 2012). Black-grass is a cross-fertilised weed with wind-pollinated flowers (Naylor, 1972a). Black-grass seeds mature in early summer, lose dormancy after several months, then emerge in early autumn. A minority of ungerminated seeds enter secondary dormancy during winter

to emerge in a minor second flush in the following spring (Naylor, 1972a; Menchari, Délye and Le Corre, 2007). Dormant seeds remain viable for four years in agricultural soil (Naylor, 1972a). Black-grass is abundant in autumn-sown cropland of winter wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), oilseed rape (*Brassica napus*), and field beans (*Vicia faba*) (Lutman *et al.*, 2013).

Black-grass is persistent despite annual herbicide use (Wilson and Brain, 1991). This is the result of a narrow susceptibility window, as the efficacy of many herbicides is reduced beyond the three-leaf stage (Naylor, 1972a; Pintar *et al.*, 2021). Heavy black-grass infestation can reduce crop yield by 35% in the UK (Naylor, 1972b), while a 45% reduction was possible in the warmer conditions of Greece (Vizantinopoulos and Katranis, 1998). A hundred plants per square metre can reduce crop yield by a ton per hectare through competition (Mennan and Işik, 2004; Bertholdsson, 2012). The species is responsible for an estimated annual wheat yield loss of 800,000 tonnes in England, translating to a loss of £400 million profit (Varah *et al.*, 2020). Black-grass control is further hindered by resistance to multiple classes of synthetic herbicide (Davies and Neve, 2017).

Cultural methods (non-chemical practices such as rotation or tillage) can contribute to black-grass control (Lutman *et al.*, 2013). While individual practices in isolation may not sufficiently abate black-grass, it may be achieved in combination, these 'many little hammers' together providing meaningful control (Liebman and Gallandt, 1997).

Figure 1.1: Heavy infestation of black-grass in a wheat crop near Banbury, Oxon, UK.
Photograph by D.T. Hickman, 17/05/2019.

1.2. The quandary of herbicide resistance

1.2.1. Herbicide resistance: crowning a Red Queen

Overuse of selective herbicides, particularly a single active in repeated applications, constitutes an intense selection pressure which can stimulate the development of resistant populations (Harper, 1957; Vila-Aiub, Neve and Powles, 2009). Repeated application of herbicides with the same mode of action leads to the accumulation of low residual doses in soil, which is particularly conducive to the development of resistance (Manalil *et al.*, 2011; Soltys *et al.*, 2013). Increasing resistance to synthetic herbicides, and the absence of a viable large-scale alternative, ensures that weeds remain detrimental to modern agricultural systems.

As of 2022, herbicide resistance was reported in 266 species, to 165 herbicides, in 71 countries (Heap, 2022). Such weeds were responsible for economic losses of US$33 billion annually in the United States alone in 2005 (Pimentel, Zuniga and Morrison, 2005), over £32 billion today. Actual global yield loss from weed interference was around 10% between 2001 to 2003, as it had been since the 1960s in spite of extensive developments in weed control (Oerke, 2006).

The persistence of weeds as an agricultural issue lends credibility to the 'Red Queen' Hypothesis, suggesting that plants are affected by an evolutionary arms-race (Figure 1.2). This hypothesis dictates that a species must constantly evolve adaptations to survive and thrive while faced with other species which are evolving similarly, effectively running as fast as it can to maintain its place, in the same manner as its namesake from *Through The Looking Glass* (Carroll, 1871; Benton, 2009). Natural selection is therefore dynamic, and all species are constantly evolving to counter the defences of competitors, hosts or prey, to the extent that fitness of these organisms will decline unless natural selection facilitates the evolution of counter-adaptations. It is thus ubiquitous across biological kingdoms, as it constitutes an element of maximising ecological fitness. By extension, anthropogenic endeavours in weed abatement also represent a necessary rapid advancement to counter the dynamic genetic landscape of the weed ecosystem, in an extension of the Red Queen hypothesis (Vigueira, Olsen and Caicedo, 2013).

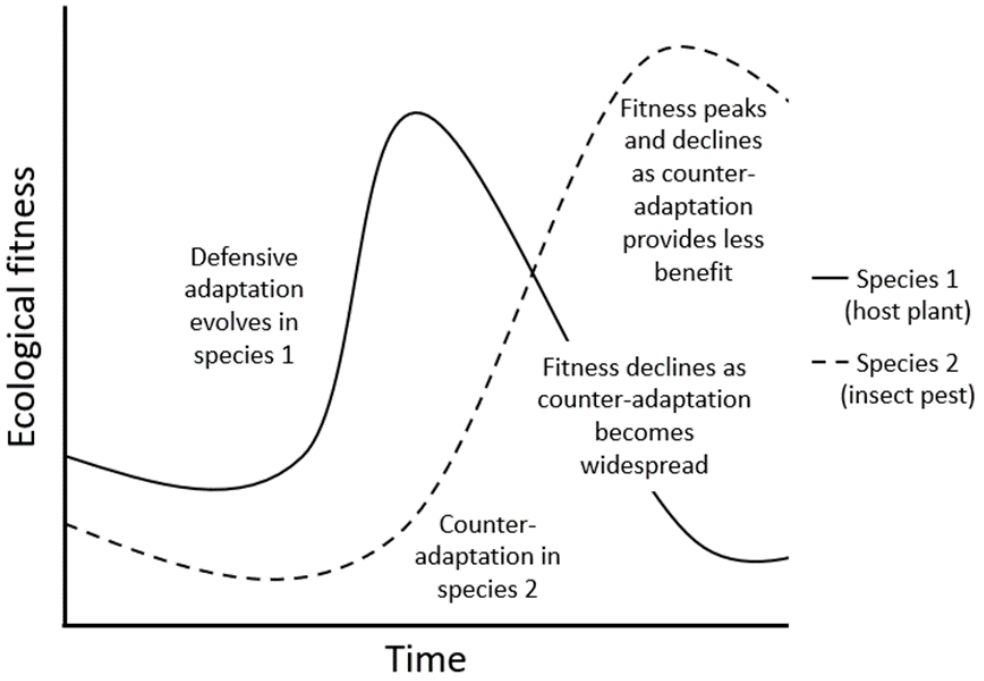

Figure 1.2: The red queen hypothesis depicted in relation to ecological fitness, provided in this example in the case of a host plant (Species 1) and an insect pest (Species 2). As depicted, when Species 1 evolves a defensive adaptation, such as the exudation of a defensive compound, Species 2 declines in fitness, which constitutes a selection pressure for the evolution of a counter-adaptation. Fitness increases in Species 2 and declines in Species 1 as this counter-adaptation becomes widespread. This will either force a counter adaptation in Species 1 to ensure survival.

The evolution of herbicide resistance is the predominant counter-defence of weeds against their attempted control. Herbicide resistance is defined as the ability of a plant biotype to survive and reproduce following application of a dose expected to be lethal (Yuan, Tranel and Stewart, 2007). Resistance is most common against compounds inhibiting Acetolactate synthase (ALS), Photosystem II, and Acetyl CoA carboxylase (ACCase) activity (Soltys *et al.*, 2013). Resistance has now even evolved to the notably broad-spectrum herbicide glyphosate, a 5-enolpyruvylshikimate-3-phosphate-synthase (EPSP-synthase) inhibitor. As of 2022, 55 weed species were resistant to glyphosate, catalysed by a large increase in application in recent years (Heap, 2022), and the development and boom in prevalence of glyphosate-resistant crops (Heap and Duke, 2018; Peters and Strek, 2018). In spite of this, there has been a de-emphasis in the study of weed biology or evolution with the widespread prevalence of selective herbicides (Neve *et al.*, 2009), as their potency rendered the understanding of target species less consequential in the eyes of the scientific community.

Herbicide resistance issues are exacerbated by the phenomenon of cross-resistance. Resistance to one active within a mode of action, may simultaneously confer resistance to other actives within that same mode of action, as they share a biological target within the plant (Manalil *et al.*, 2011). For example, some black-grass individuals were resistant to sulfonylurea herbicides prior to their application (Chauvel *et al.*, 2001).

1.2.2. Herbicide resistance in black-grass

Black-grass is currently resistant to seven modes of action (Davies and Neve, 2017; Figure 1.3). Partial herbicide resistance in black-grass was first discovered towards the photosynthesis inhibitor chlorotoluron in 1982. By 1984, herbicide effectiveness was drastically reduced in black-grass populations exhibiting cross-resistance with diclofop and pendimethalin, apparently due to non-target-site-resistance through enhanced metabolism (Moss, 1990). Most commonly, such enzymatic detoxification is conferred by cytochrome P450-monooxygenases (CYP-450s) or glutathione *S*-transferases (GSTs) (Cocker, Moss and Coleman, 1999; Délye, 2013). Indeed, much black-grass herbicide resistance is metabolic (Moss, 1990), accounting for around 80% of resistance in France (Menchari, Délye and Le Corre, 2007), with the remaining 20% due to target-site resistance (Cocker, Moss and Coleman, 1999). Sethoxydim, for example, cannot be metabolised, an insensitive ACCase gene instead indicating target-site-resistance (Cavan, Biss and Moss, 1998). Seven different point mutations were found in this gene, confirming this theory (Menchari, Délye and Le Corre, 2007). Thus, multiple resistance pathways exist between populations.

Black-grass resistance to ACCase inhibitors such as fenoxaprop-*p*-ethyl, intended to lethally disrupt fatty acid synthesis (Vila-Aiub, Neve and Powles, 2009) was also found in 1990 (Cocker, Moss and Coleman, 1999). This was followed by diclofop-methyl, fluazifop-*P*-butyl, sethoxydim, and tralkoxydim in the next decade (Cavan, Biss and Moss, 1998). Steady emergence of resistance to different herbicide classes has continued consistently in more recent years (Figure 1.3).

Compounding resistance issues is the imposition of European Union legislation limiting the development of new modes of action and banning effective

herbicides like isoproturon and trifluralin, leaving a greatly-reduced toolkit of herbicides to which black-grass remains sensitive (Lutman *et al.*, 2013; Davies and Neve, 2017). While glyphosate resistance has not currently been observed in the species, reduced sensitivity suggests that caution should be employed in its usage (Davies and Neve, 2017). As previously noted, some other weeds have developed glyphosate resistance, many of which are highly economically destructive (Powles, 2008; Heap and Duke, 2018).

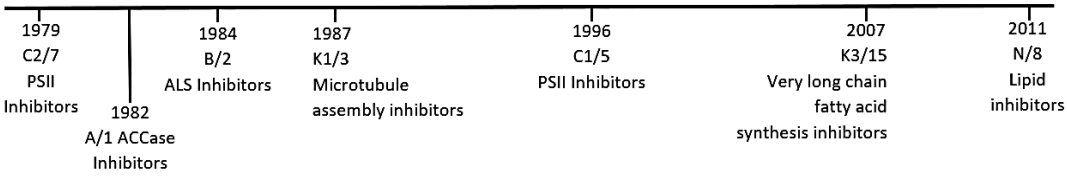

Figure 1.3: A timeline of herbicide-resistance discovery in black-grass by mode of action. Data from Heap (2022).

1.3. Allelopathy as a phenomenon

1.3.1. Allelopathy: definition and early history as a discipline

Given the prevalence of black-grass, its resistance to multiple synthetic herbicides, and the desire for alternative control methods, allelopathy may be of great interest for its control. Allelopathy is defined at its broadest as the biochemical interference between two organisms (Mallik and Inderjit, 2002). This usually describes plant-plant interactions, as its original description by Molisch (1937) suggests: 'The influence of one plant on another'.

Although basic observations of allelopathy had long been made, modern allelopathy studies were established with the first isolation of allelopathic compounds. Juglone was first identified in walnut trees (Hoy and Stickney, 1881), and many plant-derived compounds would later be isolated from soils by the US Department of Agriculture (*e.g.* Schreiner and Shorey, 1910). More physiologically orientated experiments by Pickering (1917) would lead to his acknowledgement of allelopathy as a widespread, if then-unnamed phenomenon. Exploration of this fledgling subject would continue over the following decades, reviewed by Loehwing (1937), and later Bonner (1950), but it was in the 1960s that experimental validation of allelopathy was extensively undertaken. Seminal works in this period began to explain agroecological trends by connecting in-field allelopathy to the characterisation of inhibitory compounds (Abdul-Wahab and Rice, 1967; Wilson and

Rice, 1968). Others successfully investigated allelopathic behaviours in natural ecosystems (Muller, Muller and Haines, 1964; Muller, 1966), placing allelopathy in a real-world context. Further review of allelopathy for weed control in agriculture, and its advocation as an alternative to synthetic herbicides would follow (Tukey, 1969; Rice, 1974). Modern works have been encouraged by advances in chemical analysis techniques making allelochemical identification cheaper and easier.

1.3.2. Allelopathy in the context of root exudation

Root exudates are a significant plant carbon sink, with around 10% of photosynthetically fixed carbon exuded *via* the root system (Farrar *et al.*, 2003). Root exudates primarily consist of carbohydrates and sugars involved in beneficial root-soil and root-microbe interactions, but there is often a great diversity of compounds exuded (Vranova *et al.*, 2013). *Arabidopsis thaliana* exudes over 100 compounds, including coumarins, hydroxycinnamic acids, nucleosides, aromatic amino acids, dipeptides, degradation products of salicylic and jasmonic acids, and glucosinolates (Strehmel *et al.*, 2014). These compounds are believed to have myriad ecological roles (Table 1.1; Uren, 2001). The presence of allelochemicals among this great diversity of root exudates does, however, present difficulties for isolation and elucidation given the amount of processing required (Tukey, 1969; Strehmel *et al.*, 2014). Phytotoxins thus remain poorly understood; around 10,000 are known, and an estimated 400,000 postulated to exist (Villagrasa *et al.*, 2006).

The degree of allelochemical exudation fluctuates throughout the life of a plant. In wheat, germination is believed to induce allelochemical synthesis, and concentrations peak in the first few days of growth (Copaja, Nicol and Wratten, 1999). Modelling indicates that allelochemical concentration *in planta* and exudation *ex planta* then declines with plant age in a stable environment (An *et al.*, 2003).

There are also multiple exudation pathways. Low molecular weight compounds are exuded from roots by direct diffusion through the lipid bilayer of root cells (Neumann and Römheld, 2001). Larger compounds are exuded *via* vesicular transport and exocytosis, preventing interaction between potentially bioactive substances and surrounding cells (Neumann and Römheld, 2001; Weston, Ryan and Watt, 2012). For sorgoleone, the primary allelochemical associated with sorghum

(*Sorghum bicolor*), exocytosis enables accumulation in the apoplast region of the roots (Weston, Ryan and Watt, 2012), forming into globules which exit the plant through root hair cells (Dayan and Duke, 2003).

Table 1.1: Possible roles of some types of root exudate, modified from Uren (2001).

Role	**Action**
Nutrient acquisition	Seek and fetch (*e.g.* phytosiderophores); modification of rhizosphere soil nutrition (*e.g.* protons, reductants)
Ectoenzymes	Conversion from unusable to usable organic forms (*e.g.* phosphatase)
Water acquisition	Modification of rhizosphere soil with mucilage
Protection against physical stress	Lubrication or amelioration of rhizosphere soil in response to high soil strength
Protection against pathogens	Defensive response to invasion (*e.g.* phytoalexins)
Protection against toxic elements	Response to toxic entity (*e.g.* complexation of Al^{3+})
Protection against competition	Modification of rhizosphere soil with phytotoxic allelochemicals
Establishment of symbiotic relationships	Chemotactic response to rhizobia, endo- and ectomycorrhizae

1.3.3. Stress, recognition, and induction of allelopathy

The factors influential to allelochemical exudation and composition remain nebulous. It is understood that they are altered by biotic stresses, and abiotic environmental cues such as temperature (Tang *et al.*, 1994) and drought (Tongma, Kobayashi and Usui, 2001; Gargallo-Garriga *et al.*, 2018). But allelopathy is also influenced by recognition, mediated by chemical signalling, and the fitness benefits conferred by allelochemical exudation are optimised through inducibility (Uesugi, Johnson and Kessler, 2019). Both volatile aboveground, and root-secreted belowground stress-related metabolites act as signalling compounds which indicate the relatedness of a neighbouring plant, and may therefore induce a heightened allelopathic response. Allelopathic induction has recently been linked to signalling compounds such as jasmonic acid and (-)-loliolide (Li, Zhao and Kong, 2020).

Recognition interactions with competitive neighbours may relate to phenotype matching, *i.e.* a plant's ability to identify related individuals through the use of chemical signatures (Penn and Frommen, 2010).

Allelochemical synthesis and exudation is elevated in response to recognition of neighbouring, competing plant species. This is an example of 'allelobiosis' (Li, Xia and Kong, 2016), essentially the priming of biochemical defence responses to the detection of a biotic stress (Ninkovic, Glinwood and Pettersson, 2006). Root exudates from velvetleaf (*Abutilon theophrasti*), Tausch's goatgrass (*Aegilops tauschii*), redroot pigweed (*Amaranthus retroflexus*), and hairy crabgrass (*Digitaria sanguinalis*), all stimulated allelochemical accumulation in wheat (Li, Xia and Kong, 2016). Wider bioassays determined that accumulation varies depending on the identity of the competing species (Kong *et al.*, 2018). In a striking example, the weed barnyard-grass (*Echinochloa crus-galli*) stimulated a six-fold increase in exudation of the known rice (*Oryza sativa*) allelochemical momilactone-B (Kato-Noguchi, 2011). Such interactions, and their connection with allelopathy, are poorly understood however, and require further elucidation.

1.3.4. Allelochemicals: novel weapons for intensifying challenges?

The ecological significance of allelopathy can be understood through the behaviour of invasive plants in natural ecosystems. Some invaders can inhibit development of local competitor plants through allelopathy, using unfamiliar compounds to dominate, as resistance or tolerance to these allelochemicals (discussed in Section 1.3.6) has not evolved in the invaded ecosystem. This is described as the 'novel weapons' hypothesis (Callaway and Ridenour, 2004) (Figure 1.4). Examples include garlic mustard (*Alliaria petiolata*) and common sow thistle (*Sonchus oleraceus*) (Prati and Bossdorf, 2004; Gomaa *et al.*, 2014). In the case of garlic mustard, the glucosinolate compounds allyl isothiocyanate and benzyl isothiocyanate may be the active compounds, while multiple potential allelochemicals have been theorised for common sow thistle. Allelopathy in an agro-ecological context, and potential applications for agricultural benefit, have been extensively reviewed elsewhere (Putnam and Duke, 1978; Qasem and Foy, 2001; Weston and Duke, 2003; Jabran *et al.*, 2015).

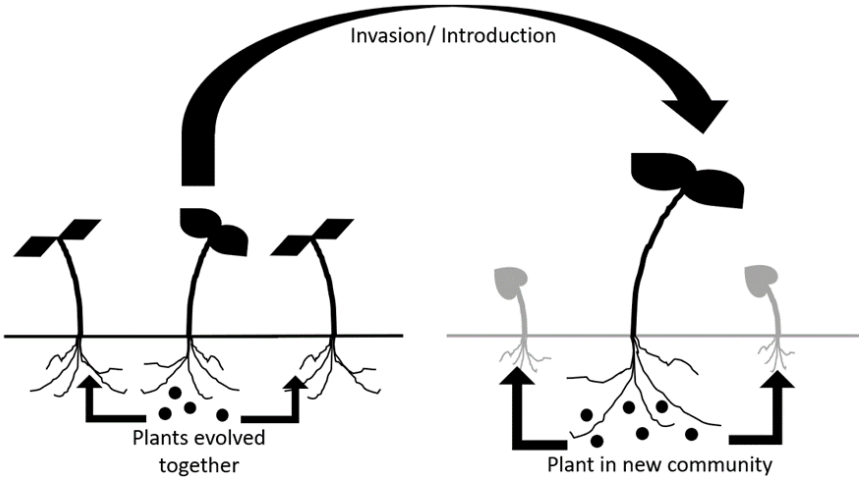

Figure 1.4: The Novel Weapons hypothesis with relation to allelochemical exudation and its inhibitory effects on unfamiliar competitors. Plants that have co-evolved with an allelopathic species have evolved a degree of tolerance; in a new community, as an invasive or introduced species, this allelochemical has stronger inhibitory effects against neighbours as they have not had opportunity for tolerance to evolve. From Hickman *et al.* (2021)

Sorghum species, and their allelochemical sorgoleone, are an extensively studied example of allelopathy at molecular, physiological, and agroecological scales (Weston, Alsaadawi and Baerson, 2013). The plant has weed-suppressive properties in the field, through the exudation of sorgoleone from root hairs (Czarnota *et al.*, 2001; Weston, Alsaadawi and Baerson, 2013). Sorgoleone is a potent allelochemical, reducing hairy crab-grass shoot growth by 50% at a dose of 10 µM, and reducing velvetleaf and barnyard-grass development by the same degree at 200 µM *in vitro* (Nimbal *et al.*, 1996). It has multiple modes of action, including inhibition of photosynthetic and mitochondrial electron transport, the photosynthesis-related enzyme *p*-hydroxyphenylpyruvate dioxygenase (HPPD), and root H^+-ATPase activity required for water uptake (Weston, Alsaadawi and Baerson, 2013).

Some plant secondary metabolites have indirect effects on resource competition or fitness to their habitat, *via* stimulation of beneficial donor plant-microbe interactions, increasing donor competitive ability, or through allelopathic effects, as reduced growth vigour in target plants reduces competitive ability. Nodding thistle (*Carduus nutans*) root exudates, possibly through the action of the alkatetraene aplotaxene (Silva *et al.*, 2014), are particularly inhibitory to legume species. Thistle has the effect of inhibiting acetyline reduction, thereby hindering nitrogen fixation and creating nitrogen-deficient conditions in the soil to which the thistle is comparatively tolerant (Wardle *et al.*, 1994; Inderjit and Callaway, 2003). Thus, allelopathy and resource competition are intrinsically interconnected (Inderjit

and Callaway, 2003); allelopathy may have evolved from intense resource competition to the detriment of the allelopathic species (Williamson, 1990). These interactions are therefore components of a complex web of rhizosphere-based interactions, involving nutrient availability (governing resource competition), secondary metabolism (including allelochemicals), and soil microbial communities (Maggi *et al.*, 2005).

1.3.5. Methodological considerations in allelopathy studies

Given the close relationship between allelopathy and other interspecific interactions (Duke, 2015), findings in the field have long been viewed with some suspicion, especially as providing indisputable proof of inhibition from an allelochemical is notoriously difficult. Williamson (1990) poetically likened this challenge to a 'neck riddle', the unsolvable nature of which a condemned prisoner could stake their life upon. Stowe (1979) was first to doubt the role of allelopathy experimentally, reporting that few apparently allelopathic species maintained their bioactivity in the transition from laboratory-based experiments to field trials. This may be related to the sensitive nature of allelopathic behaviour, influenced by the induction pressures mentioned in Section 1.3.3 (Tang *et al.*, 1994).

The dangers of false positives in allelopathy research are exacerbated by a prevalent approach described concisely but dismissively as '*grind and find*' (Romeo, 2000). This involves grinding tissues to release compounds which would not occur through root exudation or natural decomposition. Another limiting methodology described by Romeo (2000) is the '*thrill of the kill*', which does not consider ecological relevance or practicality for application in the identification of a phytotoxin. Further, many studies are criticised for investigating target species with limited ecological relevance, and a high sensitivity to allelochemicals (Romeo, 2000).

Attempts have consequently been made to provide an idealised framework for allelopathy studies. Willis (1985) offered six points to be satisfied (Figure 1.5), acknowledging that these points were rarely all achieved, with most studies achieving three or four at most. The six points, in simplified form, are as follows:

1. One plant species must demonstrate a pattern of inhibition on another.
2. The inhibitory plant must produce a toxin (an allelochemical).

3. The inhibitory plant must have a mode of toxin release into the environment.
4. The putative allelochemical must have a means of transport and/or accumulation which would facilitate an allelopathic effect.
5. The inhibited plant must imbibe said toxin.
6. The pattern of inhibition cannot be comprehensively explained by biotic factors such as resource competition or herbivory.

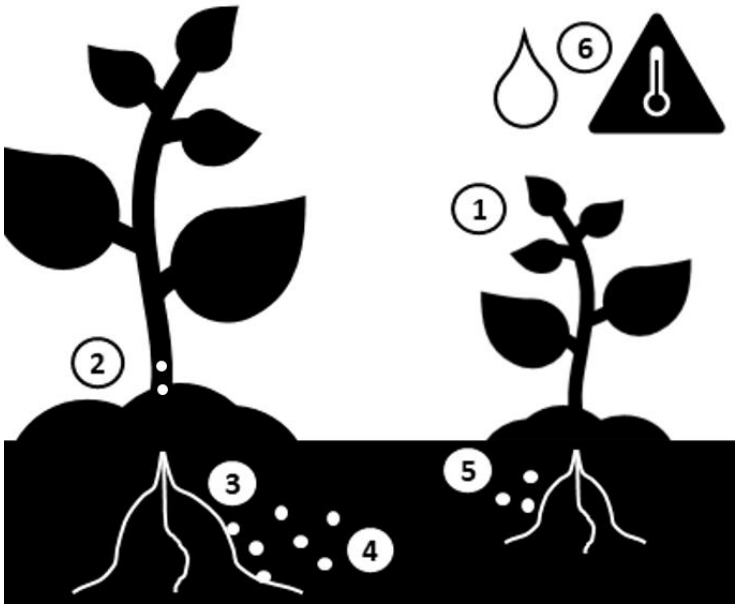

Figure 1.5: Willis' six points for proving allelopathic behaviour in diagram form.

This framework is not beyond criticism, as the last point in particular is subjective and impossible to prove comprehensively, but it remains useful (Blum, Shafer and Lehman, 1999). Nonetheless, other guidelines have been proposed. Both Fuerst and Putnam (1983), and Williamson (1990), for instance, suggested that Koch's postulates for disease causation could be adapted to apply to allelopathy studies as follows:

1. The putative allelochemical is present in media where inhibition occurs, and absent from those where it does not.
2. The putative allelochemical can be recovered from the donor plant.
3. The isolated putative allelochemical can inhibit growth of the target species.
4. The putative allelochemical can be recovered from affected plants in the same form as it exists in growth media.

While these frameworks emphasise isolation and identification of putative allelochemicals, there is debate over the necessity of their elucidation from a practical perspective. Multiple works describe multiple allelochemicals with multiple modes of action responsible for the allelopathy of a single species (Inderjit and Weston, 2000; Inderjit, Weston and Duke, 2005). An ideal alternative is to compare natural exudate bioactivity with the allelopathy of isolated compounds to examine synergy.

Another issue confounding understanding of allelopathy is the rarity of a single study covering *in vitro* bioassay of crude root exudates, chemical analyses of their composition, the maintenance of effects in biologically active media, and larger-scale field trials (Inderjit and Nilsen, 2003). This can be partially attributed to a lack of interdisciplinary science (such work covers synthetic and analytical chemistry, plant physiology, soil science, agroecology and agronomy at least). Romeo (2000) suggested that it is also a symptom of the modern need for frequent publication in science, noting that early works from times when publication pressure was less severe combined these approaches more effectively (*e.g.* Muller, 1966). A long-form research project such as a doctoral thesis presents an opportunity to attempt satisfaction of these tenets in a specific case of allelopathic potential.

1.3.6. Allelochemical resistance and autotoxicity avoidance

Utilising allelochemicals in agricultural weed control provides benefits through mode of action, potentially being multi-site inhibitors with novel mechanisms to which these weeds have not evolved resistance. There is some evidence that resistance can develop as in synthetic herbicides, however. This has been more extensively examined in arthropod pests (Section 1.6.5), but some work has investigated allelochemical resistance in plants, particularly as a component of the novel weapons hypothesis. Specifically, there is evidence suggesting that native plant communities have greater resistance to allelochemicals than invaded ones (*e.g.* Thorpe *et al.*, 2009). From an ecological perspective, the benefit of novel weapons will ultimately be overcome by counter-selection to negate their novelty in time, but this first requires the invader to become dominant and disrupt the ecosystem. The

large variation in sensitivity to allelochemicals between different target species is likely to be attributable to such resistance (Inderjit and Duke, 2003).

There is also varying sensitivity to allelochemicals in the plants that exude them. Exposure of multiple wheat varieties to their own crude root exudates correlated with reduced root growth by around 30% (Wu *et al.*, 2007). This is an example of autotoxicity, the intraspecific delay of germination or growth through allelochemical exudation (Friedman and Waller, 1985). The compounds present in these autotoxic wheat exudates were not examined but, given the importance of benzoxazinoids in the allelopathy of wheat (Section 1.4; Pérez, 1990), they are likely to be the autotoxic constituent.

Autotoxicity is avoided through storage of harmful compounds in the vacuole, isolating them from vulnerable tissues (Wink, 1993). The common incidence of allelopathy and autotoxicity in the same species suggests that the same compounds can be implicated in both interactions. In alfalfa (*Medicago sativa*) autotoxicity (Hall and Henderlong, 1989), the compounds of greatest effect were coumarin, *trans*-cinnamic acid and *o*-coumaric acid (Chon and Kim, 2002), compounds also associated with interspecific allelopathy (Abdul-Rahman and Habib, 1989; Siqueira, Hammerschmidt and Nair, 1991).

The reasons for the evolution of autotoxicity are unclear, although explanations have been posited which rationalise the phenomenon in spite of the existence of adaptations to prevent it. One hypothesis is that of biochemical recognition, postulating that intraspecific inhibition of germination provides selective advantages for population fitness in avoiding intense intraspecific competition, favouring later germination and establishment when conditions are more suitable (Renne *et al.*, 2014). This can be compared to phytoalexin-regulated hypersensitive cell death to contain pathogenic infection, such as the response to resveratrol in pathogen-infected grape plants (Chang *et al.*, 2011). Autotoxicity may alternatively be a trade-off for net benefit, as allelopathy may inhibit competitors to a greater extent simultaneously. Nevertheless, autotoxicity undermines the assumption that a plant species is resistant to its own phytotoxins, so further work is required on its nature as a constituent of allelopathic interactions.

1.3.7. Microbial degradation of allelochemicals

Many allelochemicals are degraded by microbial action, such as simple phenolic acids, benzoxazinoids, juglone, quercetin, rutin, and *meta*-tyrosine (Kaur *et al.*, 2009). As such, allelochemicals often lack persistence in soil. Benzylglucosinolate, common in crucifers, has a half-life of six hours (Gimsing *et al.*, 2006), while *p*-coumaric acid, although comparatively persistent, only remains intact for five days (Pue *et al.*, 1995). The bioactivity of putative allelochemicals is therefore hugely affected by microbial activity, and may explain Stowe's (1979) findings of limited allelopathy in biologically active field soils. Similarly, of nine weed species reported to have allelopathic root exudates by Li *et al.* (2015), only one, Billygoat-weed (*Ageratum conyzoides*), remained bioactive in unsterilised soil. Many bioassays investigating the allelochemical potency in artificial conditions therefore overestimate their effects (Kaur *et al.*, 2009), highlighting the necessity of supplementing fundamental laboratory with experimental treatments which mimic or utilise field environments (Inderjit, Kaur and Foy, 2001; Inderjit and Nilsen, 2003).

In other cases, however, rapid degradation of allelochemicals can produce more persistent compounds with greater bioactivity, so may sometimes be ecologically rational. One example is phytotoxic APO (2-amino-phenoxazin-3-one), a benzoxazinoid degradation compound which persists for up to 90 days in biologically active soil, hence its acknowledgment by some as an important allelochemical (Macías, Marín, *et al.*, 2005; Trezzi *et al.*, 2016).

1.4. Wheat, defence, and allelopathic potential

1.4.1. Defence capabilities of wheat

Wheat is the most important grain on Earth, with annual global production estimated at 750 million tonnes in 2018, and consumption comprising one-fifth of global human calorific intake (FAO, 2018). Improved yield has been the primary goal of wheat breeding since domestication (Shewry, 2009), but progress in this area has been achieved at the cost of genetic resources conferring defence capability against competitors, pests or other stressors (Haudry *et al.*, 2007; Bertholdsson, 2010). Ancestral wheat biotypes are therefore linked with potential for greater

allelochemical content than modern wheat lines (Escobar and Niemeyer, 1993; Stochmal *et al.*, 2006).

For instance, lines of the diploid wheat ancestor *Triticum monococcum* have elevated defence capability against take-all fungus (*Gaeumannomyces graminis* var. *Tritici*), and the English grain aphid (*Sitobion avenae*) (McMillan, Gutteridge and Hammond-Kosack, 2014; Simon *et al.*, 2017). Resistance to bird cherry-oat aphid (*Rhopalosiphum padi*) in the wheat ancestor *Aegilops speltoides* is also connected to allelochemical content in aboveground tissues (Elek *et al.*, 2013). Other ancestor lines have more limited defence capabilities (Batyrshina *et al.*, 2020), and therefore defence in wheat ancestors is better described as being more varied, cohering with Putnam and Duke's (1974) assertion that genetic bottlenecking has reduced the range of defensive potential in crops. Given that defence compounds accumulate dynamically according to biotic stresses (McCall and Fordyce, 2010), aphid or microbial resistance may also indicate potential for plant-plant allelopathy (Section 1.5 onwards), although this must be experimentally verified.

1.4.2. Benzoxazinoids, their synthesis, and degradation

Benzoxazinoids are cyclic hydroxamic acids synthesised by a range of plant and crop species, including wheat, barley, rye (*Secale cereale*) and maize (*Zea mays*) (Belz and Hurle, 2005; Niemeyer, 2009), long studied for biological activity. The benzoxazinoids most commonly attributed to cereal allelopathy are DIMBOA (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one), DIBOA (2,4-dihydroxy-1,4-benzoxazin-3-one), and their breakdown products, although their allelopathic potential was not discerned until the 1990s (Pérez, 1990). These compounds degrade to MBOA (6-methoxy-2-benzoxazolinone) and BOA (benzoxazin-2-one) respectively, then degrade further into AMPO (2-amino-7-methoxy-phenoxazin-3-one) and APO (2-amino-phenoxazin-3-one) respectively (Fomsgaard, Mortensen and Carlsen, 2004).

The benzoxazinoid biosynthesis pathway was first determined in maize by Frey *et al.* (1997), and first involves indole-3-glycerol phosphate. This is converted to indole by a specific gene product, *Bx1*, rather than the TSA (tryptophan synthase α) gene product. This begins a series of conversion reactions (Figure 1.6), induced by genes *Bx2* to *Bx5* encoding for cytochrome P450-monooxygenases (CYP-450s). The

hydroxylation and methylation reactions that convert DIBOA to DIMBOA occur through expression of genes *Bx6* and *Bx7*, a 2-oxoglutarate-dependent dioxygenase and an *O*-methyltransferase respectively (Jonczyk *et al.*, 2008). DIMBOA and DIBOA are then converted to apparently nontoxic glucosides (DIMBOA-Glc, 2-*β*-D-glucopyranosyloxy-4-hydroxy-7-methoxy-1,4-benzoxazin-3-one, and DIBOA-Glc, 2-*β*-D-glucopyranosyloxy-4-hydroxy-1,4-benzoxazin-3-one) for storage (Cambier, Hance and De Hoffmann, 1999). The genes involved are the homologous glucosyltransferase genes *Bx8* and *Bx9* (von Rad *et al.*, 2001). DIMBOA-Glc is commonly found in cereals, while only rye contains high levels of DIBOA-Glc (Hietala and Virtanen, 1960). These compounds are then believed to be enzymatically converted and deglucosylated into their aglucone allelochemical forms prior to exudation (Belz and Hurle, 2005), partially due to the activity of the genes *Bx10* to *Bx14* (Cotton *et al.*, 2019).

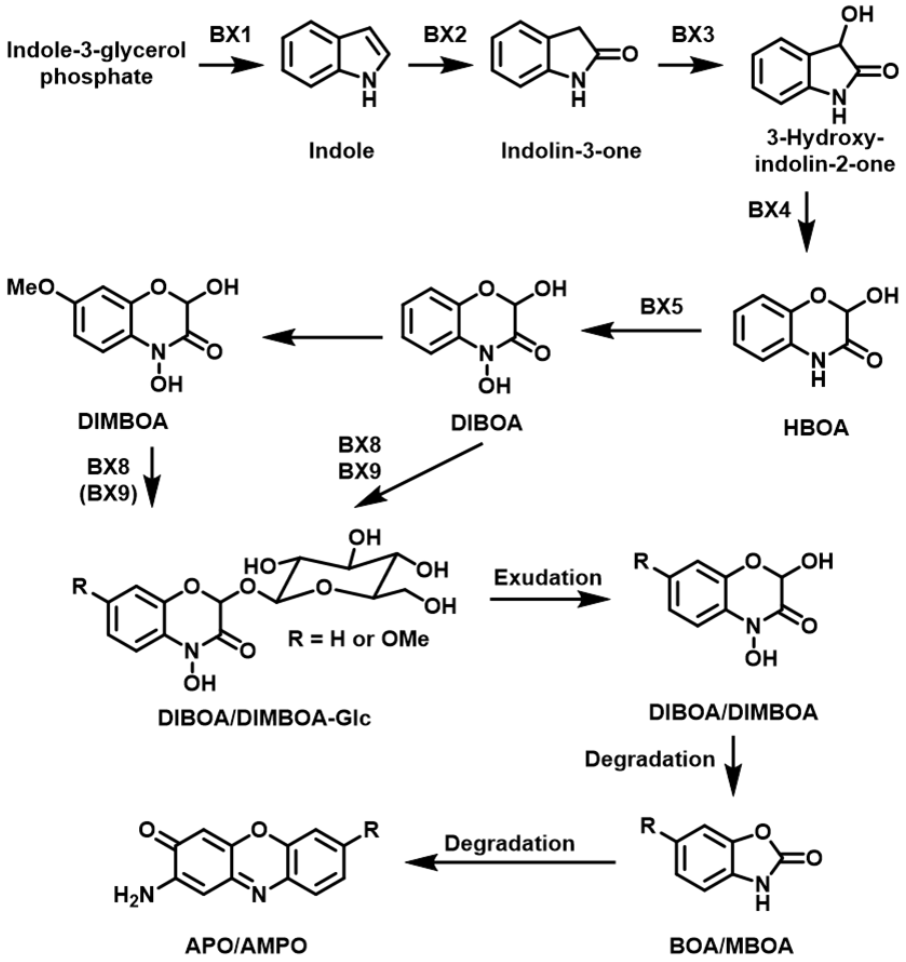

Figure 1.6: The biosynthesis and degradation pathways of benzoxazinoids in cereals, adapted from von Rad *et al.* (2001). Note that 'R' is a variable group indicative of the DIMBOA or DIBOA families; R= OMe indicates the presence of a methoxy group (degradation route DIMBOA- MBOA- AMPO) while R= H indicates the presence of a hydrogen atom (degradation route DIBOA- BOA- APO).

DIMBOA and DIBOA degrade quickly in unsterilised soil, their half-lives around 24 hours (Wu *et al.*, 2007), and 43 hours (Trezzi *et al.*, 2016), respectively. The persistence of these compounds may suggest their relative allelopathic potential, but no connection has been made between allelopathy and persistence. Degradation dynamics of benzoxazinoids are also unclear, although poor stability explains their limited persistence. In DIMBOA, greater electron density around the aromatic ring promotes degradation through the stability of the degradation state (Atkinson *et al.*, 1991).

Studies of benzoxazinoid degradation have focused on the BOA-APO step, representing the transition to a more potent and persistent allelochemical according to existing literature (Macías *et al.*, 2004). Microbial activity is required to hydrolyse BOA into *O*-aminophenol, which is then oxidised into APO upon contact with air (Gagliardo and Chilton, 1992). Not all microbes stimulate degradation, however. One study identified *Fusarium sambucinum* as the only one of four fungi capable of this interaction, development of the other three inhibited by BOA (Zikmundová *et al.* 2002). Other microbial species capable of BOA degradation include take-all and *Fusarium culmorum*, which did not degrade MBOA in the same study (Friebe *et al.*, 1998). These interactions are related to the resistance and tolerance discussed in Section 1.6.5. Degradation appears to be necessary for the evolution of resistance (Friebe *et al.*, 1998), comparable to the metabolism of herbicides by some herbicide-resistant crops (*e.g.* Cocker, Moss and Coleman, 1999), but beyond this, much remains unknown about benzoxazinoid degradation or its implications for allelopathic potential.

1.4.3. Benzoxazinoid allelopathy: a precedence of inhibition

Benzoxazinoids are highly potent allelochemicals. Just 200 ppm of DIBOA and BOA reduced cucumber (*Cucumis sativus*) germination by as much as 69%, while 370 µM of DIBOA reduced root growth in cress (*Lepidium sativum*) by over 50% (Burgos *et al.*, 2004). Around 69% of durum wheat (*T. durum*) allelopathy has been attributed to DIMBOA and DIBOA (Fragasso, Iannucci and Papa, 2013), although this varies between cereal species, cultivar, (Belz, 2007) and geographic origin (Wu *et al.*, 2001). Elevated benzoxazinoid exudation by multiple species correlates with suppression of

white mustard (*Sinapis alba*) development (Belz and Hurle, 2005), while a 500 µM dose of DIMBOA significantly inhibits root development in wild oat (*Avena fatua*) and rigid ryegrass (*Lolium rigidum*) (Macías *et al.*, 2006). Similarly, DIMBOA isolated from wheat root exudates reduced biomass of orange foxtail (*Alopecurus aequalis*), a close relative of black-grass, by around 20% (S.-Z. Zhang *et al.*, 2016).

Different target species are vulnerable to different benzoxazinoid compounds, however. In assays against onion (*Allium cepa*) and tomato (*Lycopersicum esculentum*), APO was the most potent inhibitor of plant development. This was also the case in the weed species wild oat and rigid ryegrass (Macías *et al.*, 2006), but not in barnyard-grass, where DIBOA was more potent (Macías, Chinchilla, *et al.*, 2005). It is therefore necessary to examine the effects of this family of compounds on black-grass at a variety of doses and conditions.

1.4.4. Potential and proven benzoxazinoid modes of action

There are many modes of action posited for benzoxazinoid allelochemicals, including reduced activity of enzymes like papain, *α*-chymotrypsin, and GSTs (Sicker *et al.*, 2000). When DIBOA was applied axenically to oat and broad bean plants, H^+ATPase activity in roots was reduced (Friebe *et al.*, 1997). This may be related to DIBOA's electrophilicity, its attraction to electrons and electron-dense molecules (Wouters, Gershenzon and Vassão, 2016). Benzoxazinoids may thus limit supply of adenosine triphosphate (ATP) by inhibiting electron transport, hindering energy release in cells.

The mode of action has not been conclusively identified for DIMBOA or DIBOA, or their respective degradation products, however (Venturelli *et al.*, 2016), and has only been elucidated for APO and AMPO (Venturelli *et al.*, 2015). These compounds bind to and inhibit highly conserved histone deacetylase (HDAC) enzymes, necessary for amino acid transcription and cell development (Venturelli *et al.*, 2015). This occurs at concentrations as low as 3.25 µM, sufficient for physiological relevance (Venturelli *et al.*, 2016), explaining the reported allelopathic potency of APO in particular (Macías, Marín, *et al.*, 2005).

Complicating understanding of benzoxazinoid allelopathy is evidence that some plants are capable of metabolic detoxification. BOA can be detoxified by GST

and CYP-450 activity in *Arabidopsis* (Baerson *et al.*, 2005), while barnyard-grass, and multiple biotypes of perennial ryegrass (*Lolium perenne*) demonstrate varying potential to metabolise MBOA (Oliveros-Bastidas *et al.*, 2021). It is therefore necessary to examine allelopathic potential of potent benzoxazinoids on multiple biotypes of an intended target.

1.4.5. Cereal defence and black-grass: an unknown quantity

There are thus a number of questions which should be investigated in this study, considering current understanding of black-grass, cereal allelopathy, and the methodologies discussed in Section 1.3.5. The potency of benzoxazinoids examined in other target species should be verified in black-grass, including herbicide-resistant varieties, with the intention of identifying resistance-related limitations to application of allelopathic potential. Wheat should also be bioassayed to determine its tolerance. These findings should be linked to agroecological effects through the bioassay and characterisation of crude cereal root exudates and their constituent compounds. The effects of potent allelochemicals and biotypes should be validated *in vitro*, then in solid axenic media and biologically active soil systems in order to identify shortcomings related to growth media or microbial presence.

1.5. Introduction to the concept of multi-kingdom allelopathy

Multi-kingdom effects of some allelopathic plant secondary metabolites have long been acknowledged in definitions and discussions of allelopathy (Whittaker and Feeny, 1971; Putnam and Duke, 1978), in spite of the original definition solely addressing plant-plant interactions (Molisch, 1937). The remainder of this chapter will expand on this aspect of plant defence by examining documented examples of allelochemicals with potential to control multiple kingdoms of antagonistic organisms.

Multiple examples of compounds exhibiting allelopathy and toxicity to other organisms were described in the 1980s (Wink, 1988), and the term ‘allelopathy’ was used in this context by the International Allelopathy Society in the 1990s (Mallik and Inderjit, 2002). Other works have documented multiple ecological roles and applications for individual plant-derived secondary metabolites (Siqueira, Hammerschmidt and Nair, 1991; Strugstad and Despotovski, 2012; Wouters,

Gershenzon and Vassão, 2016; Schandry and Becker, 2020). Such works nonetheless remain exceptions, with most literature focusing on the identification of inhibitory effects towards specific targets rather than potential multi-kingdom functions. This affects the scope of their applications for crop protection.

The case is therefore made that the existence of allelochemicals with multiple ecological functions necessitates the need for definitions that encompass both generic allelopathic interactions and more specific interactions with plants, animals and microbes. It is hereby suggested that 'allelopathy' should be used in its wider definition in affecting multiple kingdoms as previously described (Whittaker and Feeny, 1971; Mallik and Inderjit, 2002), and the terms 'phytoallelopathy', 'zooallelopathy' and 'microbial allelopathy' should be used to describe specific interactions with plants, animals, and microbes respectively in support of this. More detailed definitions of these terms as used throughout the remainder of this chapter are provided in Table 1.2. Having defined these interactions more clearly, it is possible to describe the roles they could play in pest management.

Table 1.2: Proposed definitions of allelopathy and associated terms regarding potential for multi-kingdom applications.

Allelopathy: The inhibition or stimulation of the growth or development of an organism through the biological action of secondary metabolites produced by plant species. These chemicals can be described as **allelochemicals** given this bioactivity, and will have effects on competition dynamics, and the stress tolerance of competitors.		
Phytoallelopathy: Allelopathy specifically towards another plant species, mediated by **phytoallelochemicals**.	**Zooallelopathy**: Allelopathy towards an animal species, typically an herbivore and most commonly observed in arthropods. This is mediated by **zooallelochemicals**.	**Microbial allelopathy**: Allelopathy towards a microbial species, such as a bacterium or fungus, mediated by **anti-microbials**, **phytoalexins** or **phytoanticipins**.

The burgeoning issue of herbicide resistance in weeds (Heap, 2022) drives a growing need to develop more diverse and integrated weed management systems, to which phytoallelochemicals could contribute (Section 1.1.1). Parallel to this, there is a growing cohort of insecticide-resistant invertebrate species, over 600 species resistant to at least one insecticide by 2020 (Whalon *et al.*, 2020), driving a similar desire for alternative approaches to their management. Fungicide resistance is also an issue, occurring in nine classes of fungicide by 2015 (Lucas, Hawkins and Fraaije, 2015). The recognition of multi-kingdom allelochemicals which could potentially

provide benefits against pesticide-resistant organisms, and the development of control strategies which utilise these allelochemicals, should therefore be considered. The incorporation of biologically derived herbicides or allelochemicals into integrated weed management plans has been advocated, albeit usually with few specifics as to the envisioned nature of their deployment (*e.g.* Bertholdsson, 2005; Cordeau *et al.*, 2016). Nonetheless, multi-kingdom functionality offers the chance to apply an additional 'little hammer' (Liebman and Gallandt, 1997) more broadly in integrated pest and disease management plans.

1.6. Multi-kingdom allelochemicals in an evolutionary context

1.6.1. Plant fitness and chemical defence

Plant productivity, and ultimately fitness, is not only impacted by resource competition with other plants but also by herbivory, disease and abiotic stresses. A key component of plant fitness is the ability to defend against these multiple stresses. Thus, it would be evolutionarily advantageous for plant species to select for generic defence mechanisms that maximise fitness when faced with multiple stressor organisms. Indeed, it has been posited that secondary metabolites provide general defence against multiple enemy organisms (Figure 1.7) (Wink, 1988). This assertion is connected to the optimal defence allocation theory, which suggests that allelochemicals are allocated to a greater extent where tissues are of greatest value, albeit encountering trade-offs between growth, fecundity and defence (McCall and Fordyce, 2010). Allelopathy would thus be linked to the ecological roles of these compounds through the vulnerability to different tissues of different antagonistic organisms.

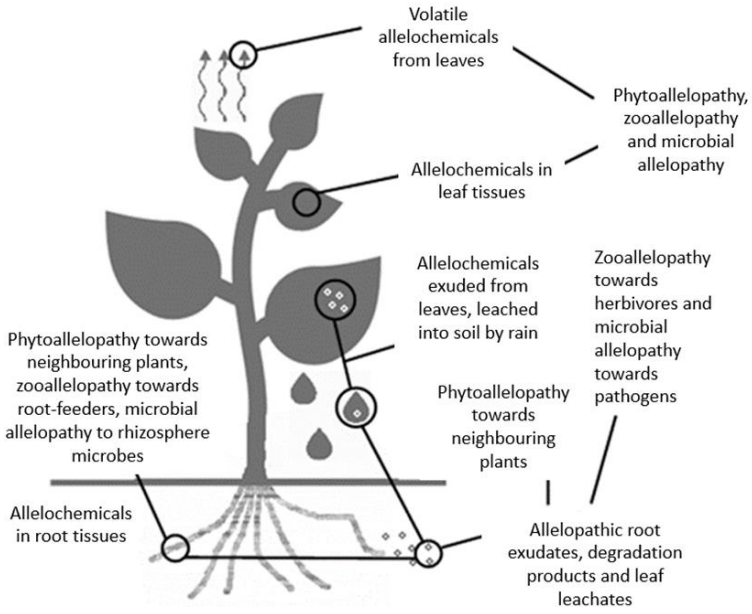

Figure 1.7: Antagonistic interactions between a plant and other species. The optimum defence allocation theory suggests a single compound can confer resistance to multiple species, potentially of multiple kingdoms.

1.6.2. Recognition of non-plant stresses and allelochemical induction

Beyond the recognition of plants (Section 1.3.3), evidence exists of multi-kingdom functionality in the induction of allelochemical synthesis; some allelochemicals accumulate *in planta* at atypically high levels under pressure from herbivores or pathogens. For example, tissue disruption or wounding by the bird cherry-oat aphid and the northern blight fungus (*Setosphaeria turtica*) stimulated benzoxazinoid accumulation in maize (Ahmad *et al.*, 2011). Similarly, feeding of the cabbage stem flea beetle (*Psylliodes chrysocephala*) on oilseed rape promotes the accumulation of multiple phytotoxic glucosinolates (Brown and Morra, 1995; Bartlet *et al.*, 1999). Plants therefore both recognise and react to multiple biotic stresses in a comparable manner, their inducible allelopathic behaviours displaying some consistency between multiple kingdoms of hostile organisms. It is therefore logical that the compounds involved in these behaviours have potential for multi-kingdom effects.

1.6.3. Allelochemical allocation and fitness consequences

The theory of multi-kingdom functionality in allelochemicals is dependent on ecologically rational allocation *in planta*. It is a reasonable extension of the optimal defence allocation theory that the distribution of a compound within a plant may be indicative of its fitness benefits (McCall and Fordyce, 2010).

For example, benzoxazinoids are found at greatest levels in the roots of wheat and rye (Niemeyer, 2009). Glucosinolates and their isothiocyanate breakdown products also accumulate at greater levels in crucifer roots (Tsunoda and van Dam, 2017). One could thus suggest that root exudate phytoallelopathy, or microbial allelopathy to the rhizospheric community, are the primary factors driving their selection. This can be disproven, at least in crop species such as wheat which have undergone selection under unnatural conditions, by variability in phytoallelochemical exudation. Benzoxazinoid exudation was only detectable in 11 of 57 wheat cultivars despite all containing high concentrations within root tissues (Huang *et al.*, 2003). It may instead be that allelochemical accumulation in root tissues provides the additional functional benefit of defence against root-feeding herbivores such as the nematode *Pratylenchus neglectus* (Potter *et al.*, 1999).

Alternatively, the presence of high concentrations of allelochemicals in roots may indicate sequestration in root vacuoles to prevent autotoxic interactions (Section 1.3.6). The apparent necessity of synthesising and sequestering these compounds constitutes a fitness cost, which must be overcome by a combination of benefits that confer a net competitive advantage. The compounds involved must therefore be conferring considerable fitness benefits, which may be explained by their multi-kingdom potential.

Some putative allelochemicals can also be found in high concentrations in aboveground tissues. This is particularly common in young tissues, of greater value to the plant due to their active growth, so allelochemical accumulation would appear to provide greater functional benefit as a feeding deterrent (McCall and Fordyce, 2010). This is the case in sweet wormwood (*Artemisia annua*), where artemisinin accumulates in flowers and buds, and is exuded from glandular trichomes on the surface of leaves and stems (Knudsmark Jessing, Duke and Cedergreen, 2014). Artemisinin is a potent phytoallelochemical, inhibiting the development of lettuce (*Lactuca sativa*), as well as the weeds redroot pigweed and common purslane (*Portulaca oleracea*), at a concentration of 33 µM (Duke *et al.*, 2008). There is evidence that artemisinin is also zooallelopathic, indicating additive functional benefit in relief of insect herbivory pressure. The beetle *Epilachna paenulata* and the armyworm *Spodoptera eridania* both suffered significant mortality when fed on

pumpkin leaves treated with a dose of 1.5mg cm^{-2} of artemisinin (Maggi *et al.*, 2005). Considering this, zooallelopathy may be the primary fitness benefit conferred by such allocation. Even then, artemisinin may provide additional phytoallelopathic benefits in nature through leaching from the leaf surface by rainwater. This effect would be enabled by its relatively long half-life, around 30 days, ensuring that it would persist sufficiently for uptake by surrounding plant competitors (Knudsmark Jessing, Duke and Cedergreen, 2014).

In summary, the major benefit of allelochemical synthesis is likely to be defence against multiple hostile organisms, as would be suggested from the phenomenon of multi-kingdom functionality. The resources required to produce such compounds and their tendency towards autotoxicity are major costs. Both of these costs appear to be minimised by the inducibility of compound synthesis in response to stress, tissue localisation of these compounds, or resistance or tolerance. Nonetheless, with these fitness costs in mind, multi-kingdom function would optimise net fitness benefit for a plant, especially when one considers the 'grow or defend' dilemma (Herms and Mattson 1992), suggesting that these two behaviours are mutually antagonistic. Thus, if defence is strongly selected in favour of growth, the benefits of defence must be substantial and potentially multiple.

1.6.4. Hormesis and the dose question

One potential explanation for the existence of autotoxicity (Section 1.3.6) is that it is an undesired fitness cost for the promotion of hormesis, *i.e.* the stimulation of growth at low concentrations by compounds that may be detrimental at higher concentrations (Friedman and Waller, 1985). Hormesis specifically occurs at around one-tenth of an effective inhibitory dose (Duke *et al.*, 2006). Several reasons for hormesis of autotoxins have been discussed, including the theory that exudation of these compounds is intended to stimulate, rather than inhibit, further growth of the species (Sinkkonen, 2007). In the case of hormesis, inhibitory effects would occur due to unnaturally high plant density, such as in a planted monoculture field. Alternatively, exudation may be over-stimulated to the detriment of the producing species by other stress factors, including the presence of competitors, underpinned by the recognition interactions described in Sections 1.3.3 and 1.6.2. In this case, the

occurrence of autotoxicity would be a consequence of the dose-dependency of phytoallelochemicals. Hormesis was reported in some wheat lines (Wu *et al.*, 2007), as well as in a number of cases where pure phytoallelochemicals were applied to target species (Duke *et al.*, 2006).

Hormesis also appears to occur in inhibition of arthropods by zooallelochemicals, as has been observed in azadirachtin derived from neem (*Azadirachta indica*), towards the bean weevil (*Zabrotes subfasciatus*) (Vilca Mallqui *et al.*, 2014). A trade-off occurs here, however, increasing fecundity but reducing longevity in an apparent case of *r*-selection (Vilca Mallqui *et al.*, 2014).

Hormesis and autotoxicity exemplify two extreme outcomes in the governance of the 'Paracelsus axiom' over allelochemical interactions. This is the theory that toxicity is only ever determined by dose, and by extension, all compounds can exhibit stimulatory and inhibitory interactions towards an organism at the correct dose (Duke *et al.*, 2006). In the case of hormesis, allelopathic behaviour is not detrimental; indeed, it would be of ecological and evolutionary benefit for a plant to evolve the synthesis of a compound stimulatory to growth of kin and inhibitory to competitors at low concentrations, allowing their benefit from plentiful resources in their environs while inhibiting competitors, but which became autotoxic at higher concentrations where seed germination is inhibited with intense intra-specific competition.

1.6.5. Resistance to allelochemicals by herbivores and microbes

There is propensity for resistance to allelochemical compounds to evolve in herbivorous animal species and microbes, in much the same manner as resistance to synthetic pesticides. This is why multi-kingdom effects are not universal at uniform concentrations. Indeed, evolution of tolerance or resistance by the intended target species in its natural setting explains limitations in the universality of such compounds.

In addition to several plant species (von Rad *et al.*, 2001), multiple fungal wheat pathogens, including several *Fusarium* species, have evolved the ability to detoxify benzoxazinoids (Niemeyer, 2009). Similarly, the presence of low concentrations of glucosinolates from garlic mustard in growth media through partial

degradation by the native rhizosphere community, is linked to eventual resistance of these microbes to these compounds (Lankau, 2011). Insect herbivores can similarly evolve tolerance to secondary plant metabolites, circumventing zooallelopathic defences through counter-resistance in the manner suggested by the Red Queen hypothesis (Section 1.2.1). This is particularly apparent where host resistance is only encoded by one gene, with selectively bred lettuce resistant to the lettuce aphid (*Pemphigus bursarius*) for just 10 years before the aphid evolved counter-resistance (Smith and Chuang, 2014). Similar dynamics are apparent in lepidoptera that evolved mechanisms to glucosylate DIMBOA back to its non-toxic storage form (Wouters *et al.*, 2014).

There also appears to be further association in the form of cross-resistance, as insect pests of allelopathic herbaceous species have a greater likelihood of evolving resistance to synthetic pesticides. A recent example of this can be found in cotton bollworm (*Helicoverpa armigera*) larvae, which were less sensitive to the synthetic insecticide methomyl when fed with allelochemicals like coumarin and DIMBOA. This metabolic cross-resistance was correlated with elevated activity of both GSTs and CYP-450s, common resistance enzymes (Chen *et al.*, 2019). Such a phenomenon is connected with the theory of pre-adaptation, that the mechanisms to detoxify zooallelochemicals of insect pests may incidentally provide a degree of resistance to synthetic insecticides prior to application (Hardy *et al.*, 2017), or *vice-versa*. The dynamics of pre-adaptation must be further explored to facilitate more effective application of allelochemical-derived biocides.

1.7. Examples of allelochemical multi-kingdom functionality

An integrated approach utilising the multi-kingdom behaviour of allelochemicals could optimise benefit to crop yield. It is important to consider individual compounds within this multi-kingdom framework. To this end, the examples of benzoxazinoids, *meta*-tyrosine and juglone, are presented as multi-kingdom allelochemicals that give credence to this recurring concept (Table 1.3). Such examples are not exhaustive, and also include momilactones in rice, which are both phytoallelochemicals (Kato-Noguchi and Peters, 2013), and phytoalexins (Cartwright *et al.*, 1981), and parthenin from Santa-Maria (*Parthenium*

hysterophorus), which is both phyto- (Batish *et al.*, 2002) and zooallelopathic (Datta and Saxena, 2001). The example of artemisinin from sweet wormwood, detailed in Section 1.6.3, is a further example of a defence compound with both phyto- and zooallelopathic potential (Maggi *et al.*, 2005; Duke *et al.*, 2008).

Table 1.3.: Summary of multi-kingdom effects in allelochemicals discussed in this section.

Allelochemical	Plant Producer	Phytoallelopathy	Zooallelopathy	Microbial Allelopathy
Benzoxazinoids	Various (Belz and Hurle, 2005)	*Sinapis alba, Lolium rigidum, Avena fatua* (Belz and Hurle, 2005; Macías *et al.*, 2006)	*Ostrinia nubilalis, Diuraphis noxia, Meloidogyne incognita* (Klun, Tipton and Brindley, 1967; Gianoli and Niemeyer, 1998; Meyer, Rice and Zasada, 2009)	Various (Virtanen, Hietala and Wahlroos, 1957; Bravo and Lazo, 1993)
***meta*-Tyrosine**	*Festuca rubra*	*Digitaria sanguinalis*, *Trifolium repens*, *Taraxacum officinale* (Bertin *et al.*, 2007)	*Coptotermes formosanus* (Gautam and Henderson, 2008)	*Bacillus* spp. (Aronson and Wermus, 1965)
Juglone	*Juglans nigra* (Strugstad and Despotovski, 2012)	Various (Rietveld, 1983)	*Callosamia promethea* (Thiboldeaux, Lindroth and Tracy, 1994)	Various (Clark, Jurgens and Hufford, 1990)

1.7.1. Benzoxazinoids

Some benzoxazinoids confer zooallelopathy against invertebrate herbivores, known long before their phytoallelopathic potential was discovered (Section 1.4). DIMBOA is inhibitory to larval development in the European corn borer (*Ostrinia nubialis*), translating to a 25% mortality rate at a concentration of around 1.5 mM kg^{-1} in no-choice diet assays (Klun, Tipton and Brindley, 1967). Similarly, DIBOA in wild barley species negatively impacted development of the Russian wheat aphid (*Diuraphis noxia*) (Gianoli and Niemeyer, 1998), and when exuded from rye, also inhibited egg development of the nematode *Meloidogyne incognita* (Meyer, Rice and Zasada, 2009). This suggests that both DIMBOA and DIBOA are broadly toxic to

invertebrate species, a reasonable assertion given that higher benzoxazinoid content in wheat leaves correlated with enhanced resistance to various aphid species at around 3 mM kg^{-1} fresh weight (Corcuera, Argandoña and Zúñiga, 1992).

Benzoxazinoids additionally have well-documented anti-microbial potential. BOA, for instance, was first discovered as an anti-fungal agent against pathogenic *Fusarium* species (Virtanen, Hietala and Wahlroos, 1957). Moreover, multiple bacteria and yeasts are sensitive to DIMBOA, DIBOA and BOA at concentrations typically below 3 mM (Bravo and Lazo, 1993), suggesting that this family of compounds has applications as broad-spectrum antimicrobials. Some benzoxazinoids have been suggested to inhibit ATP synthesis (Friebe *et al.*, 1997), central to all life excepting viruses, as is the case for the HDAC enzymes targeted by APO and AMPO. It is therefore logical that they would be toxic to multiple taxa of plants, animals and microbes (Venturelli *et al.*, 2015; Milazzo *et al.*, 2020).

These examples form a strong case for phytoallelochemicals having applications in other areas of plant defence, and strongly indicate that benzoxazinoids offer leads for potential development of pesticides with multiple applications. This is corroborated by research into the functions of these compounds, and the relationship that chemical structure has on these functions, which has already been reviewed in detail elsewhere (Schulz *et al.*, 2013; Wouters, Gershenzon and Vassão, 2016).

1.7.2. *meta*-Tyrosine

Grasses like red fescue (*Festuca rubra*) exude *meta*-tyrosine, a consistent finding in their phytoallelopathic crude root exudates. *meta*-Tyrosine inhibited weeds such as hairy crabgrass, white clover (*Trifolium repens*), and dandelion (*Taraxacum officinale*) (Bertin *et al.*, 2007). The compound also inhibited *Arabidopsis* root length by 50% at a concentration of 25 µM, a potent phytoallelopathic effect (Bertin *et al.*, 2007). *Arabidopsis* root tip browning was observed in the phytoallelopathic activity of *m*-tyrosine, indicative of cell necrosis (Movellan *et al.*, 2014). Leaf necrosis has also been reported in *m*-tyrosine treated *Arabidopsis* at concentrations over 40 µM (Zer *et al.*, 2020). Non-protein amino acids may have phytotoxic properties through their substitution of protein amino acids during

translation, modifying protein folding as a result (Bertin *et al.*, 2007). This mode of action has recently been verified for *m*-tyrosine, which is specifically incorporated in place of phenylalanine, an essential amino acid for protein production (Zer *et al.*, 2020).

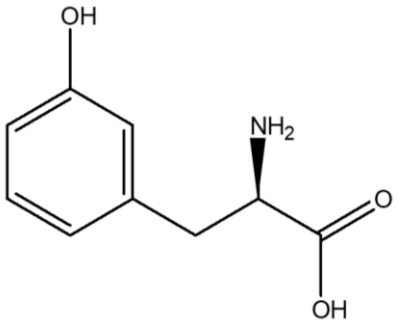

Figure 1.8: The chemical structure of *meta*-tyrosine.

There is evidence of allelopathy towards other organisms by *m*-tyrosine. Greater concentrations than those required to confer phytoallelopathy (over 50 mM) are antifeedant and toxic to the Formosan subterranean termite (*Coptotermes formosanus*) (Gautam and Henderson, 2008). The development and sporulation of multiple *Bacillus* bacterial species was inhibited by 500 µM of *m*-tyrosine (Aronson and Wermus, 1965). *m*-Tyrosine is therefore capable of multi-kingdom toxicity, in spite of an apparent specificity to plant proteins which would explain evidence that zooallelopathy may be an unrealistic expectation at natural concentrations. It may be that the observed wider allelopathic effects could instead be conferred by other, yet undiscovered mechanisms.

1.7.3. Juglone

The phytoallelopathy of juglone (Figure 1.9), a naphthoquinone produced by walnut trees, particularly black walnut (*Juglans nigra*), was discovered in the late 1800s (Hoy and Stickney, 1881; Strugstad and Despotovski, 2012). The inhibitory effects of juglone on other plant species have been widely explored and documented (Strugstad and Despotovski, 2012). For example, on both blotter paper and in soil, dry weight of five woody herbaceous plant species were significantly reduced by concentrations above 10 µM, while a further ten species were affected above 100 µM (Rietveld, 1983). Dry weight of duckweed (*Lemna minor*) was also significantly reduced at a 10 µM dose of juglone, caused by a reduction in net photosynthetic

activity seemingly related to mitochondrial disruption (Hejl, Einhellig and Rasmussen, 1993). While multiple modes of action have been theorised and none confirmed for juglone, inhibition of maize and soybean (*Glycine max*) development at similar concentrations were associated with mitochondrial inhibition in root cells through the reduction of H^+ATPase activity, and the disruption of plasma membrane function (Hejl and Koster, 2004). These results confirm that juglone is phytoallelopathic to a wide range of plant species (Willis, 2000).

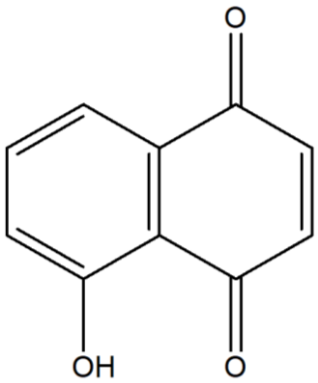

Figure 1.9: The chemical structure of juglone.

Juglone ostensibly has additional zooallelopathic potential. The growth rate of the silkmoth *Callosamia promethea* was reduced 3.6-fold when fed on paper birch (*Betula papyrifera*) leaves treated with 0.05% juglone (w/w), similar to the concentration in black walnut leaves (Thiboldeaux, Lindroth and Tracy, 1994). The compound also exhibits microbial allelopathy to a wide range of plant pathogens, which were significantly inhibited at concentrations above 75 µM (Clark, Jurgens and Hufford, 1990). Fungal species seemed particularly highly sensitive to the compound, to the extent that its effects are comparable to those of some commercial anti-fungal agents (Clark, Jurgens and Hufford, 1990). It would therefore appear that juglone has some multi-kingdom functionality, the full range of which is apparent from extensive review of its biological effects (Strugstad and Despotovski, 2012).

1.8. What does multi-kingdom functionality mean for crop protection?

1.8.1. Potential applications of multi-kingdom allelochemicals

As the examples provided indicate, a number of plant species are involved in multi-kingdom allelopathic interactions. Bringing such multi-kingdom effects to application for the benefit of agroecosystems first requires consideration of factors

influencing in-field crop allelopathy, and broader ecological impacts, both reviewed previously (Putnam and Duke, 1978; Inderjit and Duke, 2003; Weston and Duke, 2003). As outlined in Section 1.4.1, ancestral varieties of domesticated crops have potential to be more defensively capable, so there is interest in assessing and re-introducing this material into breeding programmes to augment natural defences (Quader *et al.*, 2001). Few breeding programmes prioritising plant defence have been explored, even solely for weed suppression. The only example in this case is rice, where weed suppression related to competitive and phytoallelopathic potential has been characterised and commercialised (Worthington and Reberg-Horton, 2013).

Such multi-kingdom allelochemicals could also provide leads for the development of future pesticides. These allelochemicals are often multi-target site inhibitors (Gniazdowska and Bogatek, 2005), which may rationalise their defence against multiple biotic threats. Prioritising the development of such multi-site inhibitors has recently been advocated given the greater difficulty of any organism evolving resistance against multiple targets (Gressel, 2020). It is hereby suggested by extension that multi-kingdom functionality may be an added, ecologically rational benefit, which would provide a broader-ranging basis for pesticide development and deployment in crop protection. From a practical perspective, developing naturally-inspired biocides protective against multiple biotic pressures is economically and agronomically rational.

Compared to the very limited applications of phytoallelochemicals as bioherbicides, multiple examples exist of insecticides developed from zooallelochemicals (Sparks, Hahn and Garizi, 2017). Examples include pyrethroids developed from the pyrethrins found in *Chrysanthemum* species, and insecticides derived from neem, which have been extensively reviewed (Regnault-Roger and Philogène, 2008). Even then, this is an underdeveloped tool in crop protection. More pertinently, there are no records of allelochemicals which have inspired the development of multi-kingdom pesticides, in spite of the examples of multi-kingdom functionality posited throughout.

1.8.2. Barriers to development of natural product-based pesticides

There are a number of contributory reasons for the underdevelopment of natural product-based pesticides, particularly herbicides. A major caveat of harnessing phytoallelochemicals is their potential for nontarget effects. *Poecilus cupreus* larvae and *Folsomia candida* springtails are beneficial soil organisms detrimentally affected by benzoxazinoid compounds (Fomsgaard *et al.*, 2006). The benzoxazinoid degradation product APO is also inhibitory to the growth and development of the water flea *Daphnia magna*, used as an indicator of aquatic pollution (Fritz and Braun, 2006). It is vital to fully determine the full environmental impact of a new crop protection compound, in spite of the perceived environmentally benign nature of allelochemicals or allelochemical-inspired formulations. High concentrations of allelochemicals may be required to elicit the desired inhibitory effects in the desired target, moreover, as a result of some degree of tolerance. This issue can be minimised by the identification of a maximum relevant dose, be it in terms of how much can be synthesised while remaining economically viable, or in terms of the concentrations of these compounds occurring in the allelopathic plant. The correct dose is further necessitated by hormesis, as there becomes a concern that the incorrect dose could stimulate, rather than inhibit, the growth of a detrimental species.

The development of a breeding programme for phytoallelopathic potential is dependent on a huge amount of knowledge (Putnam and Duke, 1978). The germplasm of a given species must be explored widely for phytotoxic potential, and this must be proven consistently on multiple relevant target species (Worthington and Reberg-Horton, 2013). Myriad (in some cases poorly understood) factors which can influence allelochemical synthesis and exudation, including the recognition interactions described in Sections 1.3.3 and 1.6.2, as well as the influences of pest insects, pathogens, and environmental factors. All of these factors must be understood for a breeding programme to succeed in providing agronomic benefit (Schulz *et al.*, 2013). Dynamics of allelochemical degradation in field soil must be characterised to ensure that there is no detriment to succeeding crops, but also that said compound persists sufficiently to have the intended effects (Sparks, Hahn and Garizi, 2017), which means that the active allelochemicals must therefore be isolated

and identified (Putnam and Duke, 1978; Schulz *et al.*, 2013). Given the modern necessity to sustain or even increase current food output, any allelopathic crops produced by breeding programmes must be extensively examined to confirm comparable yield to those currently commercialised (Worthington and Reberg-Horton, 2013). There is therefore a large amount of interdisciplinary work attached to the development of a viable agronomic outcome, and this is increased significantly when multi-kingdom effects are desired. It is for this reason that crop protection products inspired by allelopathy are rare, but not impossible to produce.

1.9. A perspective on multi-kingdom potential in allelochemicals

Given the number of existing examples of apparent phytoallelochemicals with anti-microbial or zooallelopathic properties, it is obvious that these compounds exhibit a degree of multi-kingdom functionality. This must be a result of these defences co-evolving to confer an overall net fitness benefit in natural habitats, likely to constitute tolerance to herbivores, plant competitors, and soil microbes. Therefore, it is acknowledged that phytoallelochemicals are a sub-class of multi-kingdom inhibitors, all of which are allelochemicals. It is unlikely that biosynthesis and release of currently recognised allelochemicals has evolved entirely due to the functional benefit of phytoallelopathy, given the distribution of a number of these compounds aboveground *in planta* and the dynamics associated with such allocation.

From a practical perspective, this means that allelochemical compounds, delivered as weed management tools either through enhanced production and delivery *in planta via* crop breeding or genetic engineering, or through the production of pesticide formulations using these chemicals as leads, may in fact have application in plant defence to multiple biotic stresses. Testing would be required, however, given that resistance, tolerance, or other factors may exist to limit the multi-kingdom functionality of some allelochemicals. It remains highly likely that other examples of previously researched phytoallelochemicals currently exist which have currently not been examined for multi-kingdom effects, but which do exhibit them.

Conversely, the area of phytoallelochemical discovery is currently hindered by its reliance on the demonstration of phytoallelopathy, a notoriously difficult

phenomenon to demonstrate in isolation (Section 1.3.5); it is hereby argued that it would benefit from greater consideration of compounds with proven allelopathic effects on herbivorous pests or microbial pathogens. Naturally-derived allelochemicals have potential to assist in relieving the selection pressure imposed by the overuse of existing synthetic actives. Identification and development of such multi-kingdom inhibiting, naturally-derived pesticides could delay the evolution of further resistance to existing synthetic chemistries while also providing effective new tools for weed, arthropod, and pathogen management.

The future outlined here would be realised by the testing of potent allelochemicals with little documented evidence of multi-kingdom functionality for inhibition of problematic target species. The adoption of such a multidisciplinary outlook in informing the discovery of potential crop protection compounds has the potential to reduce the considerable time and economic cost required to bring new natural product formulations to market (Lorsbach *et al.*, 2019) by reducing the likelihood of producing and testing ineffective compounds, thereby benefitting both consumers and industry.

Chapter 2

DIMBOA and DIBOA as promising cereal allelochemicals *versus* black-grass

2. DIMBOA and DIBOA as promising cereal allelochemicals *versus* black-grass

2.1. Introduction

Allelochemicals may be valuable in offering novel, or multiple, modes of action which could overcome growing challenges concerning resistance to synthetic herbicides (Gniazdowska and Bogatek, 2005; Gressel, 2020). Existing literature indicates that benzoxazinoids have broad allelopathic effects towards weed species (Chapter 1). However, there is also substantial evidence of the variation in efficacy of each of these compounds towards different target species (Macías, Chinchilla, *et al.*, 2005; Macías, Marín, *et al.*, 2005; Macías *et al.*, 2006), so it is important to determine the potency of these pure compounds towards black-grass to confirm that they, or the crop species that exude them, could be utilised as an alternative control for the species.

Little is known of the links between non-target-site herbicide resistance, effectively the metabolism of would-be phytotoxins (Yuan, Tranel and Stewart, 2007), and allelopathy. It has been suggested that non-target-site herbicide resistance may equate to allelochemical resistance in black-grass (Bertholdsson, 2012), but little experimental work has been undertaken to verify this hypothesis. Therefore, a wide range of germplasm must be screened in this species, including populations with verified resistance to herbicides, for consistency of effects of potent allelochemicals.

Considering the partially mutually exclusive hypotheses of novel weapons and autotoxicity (Sections 1.3.4 and 1.3.6 respectively), the insensitivity of wheat to benzoxazinoids (Macías, Marín, *et al.*, 2005), likely facilitated by its 'familiarity' through documented synthesis and exudation (*e.g.* Wu *et al.*, 2000), must be corroborated. Reports of autotoxicity of crude root exudates in the species (Wu *et al.*, 2007), are contrary to this supposition. Any allelopathic or bioherbicidal control for in-field black-grass must be ineffective to wheat development. Therefore, potent inhibitors of black-grass will also need to be screened against a variety of wheat cultivars.

The hypotheses to be tested in this chapter are as follows:

1. Benzoxazinoids will be inhibitory to early-stage black-grass root and shoot growth *in vitro*, with the level of inhibition increasing with dose.
2. The inhibitory potential of these benzoxazinoids will be uniform across a range of black-grass germplasm.
3. Wheat, as a producer of benzoxazinoid allelochemicals, will be relatively tolerant to these compounds compared to black-grass.

2.2. Methods

To address these hypotheses, the herbicide-susceptible standard black-grass population, Rothamsted-17 (Moss *et al.*, 2004), was initially examined in Petri dish dose-response assays against the six predominant benzoxazinoid allelochemicals, DIMBOA, DIBOA, MBOA, BOA, AMPO and APO, for inhibitory potential. Potent allelochemicals and discriminatory doses were identified, and assays expanded to include a range of black-grass field populations, including those with documented resistance to multiple classes of synthetic herbicide, to determine the extent to which cross-resistance to benzoxazinoids could be expected. Wheat was also assayed against promising compounds at potent concentrations to determine inhibitory potential towards crops grown in black-grass infested fields.

2.2.1. Seed surface sterilisation and pre-germination

Prior to attempts to develop allelopathy assays, two sterilisation methods were tested for their suitability in sterilising both wheat and black-grass seed. The first of these involved a ten-minute immersion in 70% ethanol, followed by a triple-rinse with autoclaved RO water. The second was adapted from Speakman and Krüger (1983): seeds were immersed in 10 ppm oxytetracycline hydrochloride for 20 hours, then 0.1% silver nitrate for ten minutes, rinsed in 0.5% sodium chloride, and then triple-rinsed with autoclaved RO water. Both methods were performed on wheat seed, plated onto standard 1% nutrient agar (Fluka Analytical, St. Gallen, Switzerland. Composition: 1g L^{-1} meat extract, 2g L^{-1} yeast extract, 5g L^{-1} peptone, 5g L^{-1} sodium chloride, 15g L^{-1} agar), and incubated at 28°C under light for five days. A control plate was also included with no seed, and a positive control containing unsterilised wheat seed. The results of this test (Figure 2.1) indicated the Speakman and Krüger method

to be most effective in surface-sterilising this seed and was therefore used throughout the following experimentation.

Figure 2.1: Testing of seed surface-sterilisation methods on nutrient agar plates, indicating the method derived from Speakman and Krüger (1983) to be preferable for axenic work.

For all assays, surface-sterilised black-grass seed was placed onto 0.8% RO water agar Petri dishes, sealed with Parafilm, and incubated at a light/dark regime of 17°C/11°C for 14/10 hours in a Sanyo MLR-351 versatile environment chamber for five days for pre-germination. Wheat was prepared similarly but incubated for only one day due to its faster pre-germination.

2.2.2. Determining allelopathic potential of benzoxazinoids towards black-grass *in vitro*

Petri dish root and shoot growth assays were undertaken for DIMBOA, DIBOA, MBOA, BOA, AMPO, and APO. While some of these compounds are commercially available in small quantities, all were synthesised by Dr. David Withall, BCP Department, Rothamsted Research. Brown 76# germination paper (Anchor Paper Company, St. Paul, MN, USA) was cut to fit the bottom half of a 12 x 12 cm square Petri dish, autoclaved, placed in such a dish, and wetted with 6 ml of benzoxazinoid solution. All compounds were tested individually at concentrations of 56.25 µM, 112.5 µM, 225 µM, 450 µM, 900 µM, 1800 µM, and a control without any benzoxazinoid compounds present. These concentrations were selected as they encompassed a range likely to be ecologically relevant, while additionally being practical for potential large-scale applications. Multiple concentrations were tested to determine the range of any biological activity found. All treatments were dissolved

in 0.25% DMSO in autoclaved RO water (v/v). This was the minimum concentration of the solvent required to dissolve all benzoxazinoids into solution, but not sufficient to affect plant growth (Schmitz and Skoog, 1970). Although RO water may have induced some stress in plants through its pH, ionic strength, or lack of nutrients, it was used over a nutrient medium to maintain simplicity of the treatments, ensuring that observed differences were attributable to benzoxazinoid content.

Eight Rothamsted-17 black-grass seeds were sown into each square dish (Figure 2.2). Rothamsted-17 is a herbicide susceptible standard originating from the Broadbalk field experiment (Rothamsted Research, UK, Grid Reference 51.809381, -0.370950), which has never been exposed to synthetic herbicides (Moss *et al.*, 2004). Four replicates were sown for each dose of each compound.

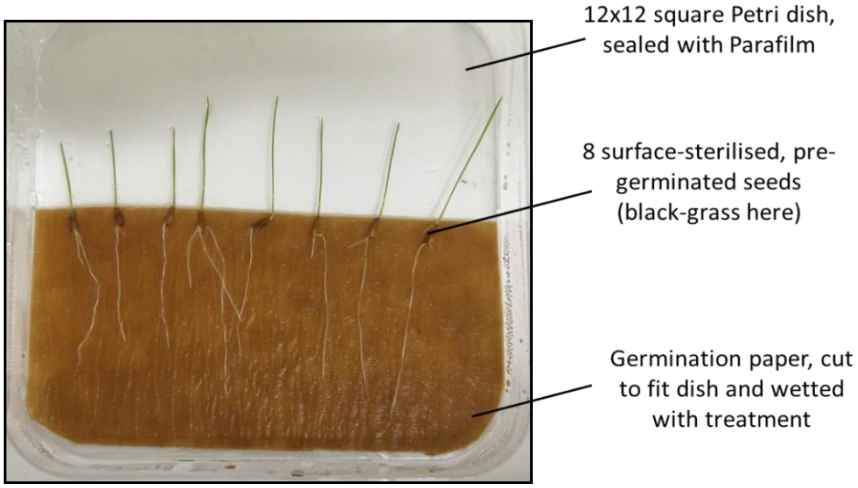

Figure 2.2: Diagram of Petri dish assay setup.

Plates were sealed with Parafilm and placed into plastic trays of seven plates each, at a shallow backward-sloping angle to aid seedling adherence and facilitate root growth along the germination paper. A brown paper envelope was placed at each end as a light control. Plates were specifically distributed to ensure that one of each dose was present in each tray, and that different compounds were as evenly distributed as possible between trays. They were then incubated in the conditions previously described for pre-germination (light/dark regime of 17°C/11°C for 14/10 hours) for nine days.

Following the growth period, all plates were photographed, and root and shoot length of all seedlings measured with the FIJI package of ImageJ (Schindelin *et*

al., 2012). As appropriate, two-parameter, log-logistic dose-response analyses and determination of ED_{50} (effective dose conferring a 50% reduction of a given metric) were undertaken in R Studio and the associated 'drc' package (Ritz *et al.*, 2015). One-way ANOVA analysis in Genstat (VSN-International, 2019) was also undertaken when suitable.

Promising results of black-grass inhibition by DIMBOA were further examined through bioassay of black-grass germination. Applications of 5 ml of the previously used set of doses were made to three sheets of autoclaved Whatman no.1 filter paper placed in 9 cm diameter circular Petri dishes (a more suitable system for germination analysis than the square dishes previously used), five replicates for each dose. Fifteen surface-sterilised black-grass seeds were placed onto each dish, evenly spaced, and incubated under pre-germination conditions for seven days. After this time, radicle emergence was counted. Differences were again analysed by one-way ANOVA in Genstat and depicted by scatterplot produced in R Studio.

2.2.3. Understanding DIBOA phytotoxicity to black-grass in greater detail

To better characterise the ED_{50} for the effect of DIBOA on black-grass development, and more accurately determine a discriminatory dose for further assays, a repeat dose-response assay was undertaken using adjusted doses to separate lower ranges. Specifically, this assay used doses of 12.5 µM, 25 µM, 50 µM, 100 µM, 200 µM, 400 µM, and 800 µM, all dissolved in 0.25% DMSO, and a 0.25% DMSO negative control. The black-grass populations included were Rothamsted-17, the herbicide-susceptible control, and Peldon-13, the herbicide-resistant control, as well as the field collected populations LoLa-44 and LoLa-59. Previous work has determined Peldon-13 is resistant to seven modes of herbicide action (Moss, 1990). This assay was intended to encompass the variability of black-grass sensitivity to DIBOA.

2.2.4. Assessing connections with DIMBOA and herbicide resistance in black-grass and wheat

In exploring the variation of benzoxazinoid sensitivity in black-grass, an initial assay was undertaken subjecting four black-grass populations to 250 µM, 500 µM, and 1000 µM all dissolved in 0.25% DMSO, and the 0.25% DMSO control, to confirm

dose-response relationships. Two wheat varieties, 'Gravity' and 'Cadenza', were also included to test the specificity of this compound to black-grass.

Black-grass populations were selected based on herbicide-resistance, with Rothamsted-17 and LoLa-81 used as herbicide-susceptible populations, while Peldon-13 and LoLa-91 were used as herbicide-resistant populations. LoLa-81 is susceptible to the Mesosulfuron/ Iodosulfuron mix and Fenoxaprop, two commonly used synthetic herbicide treatments with different modes of action in British agriculture, while LoLa-91 displayed a high rate of survivorship when treated with these actives (Table 2.1).

2.2.5. Determining variability in benzoxazinoid inhibition of black-grass populations

A larger assay was undertaken using 22 black-grass populations at the discriminating dose of 500 µM of DIMBOA, and 100 µM of DIBOA, both of which were determined from the results of initial compound screens against Rothamsted-17 black-grass. Specifically, the DIMBOA dose was decided as a convenient concentration above, but close to, the identified ED_{50} value for the compound. For DIBOA, a value great enough to induce consistent inhibitory effects, but low enough to be practical and convenient for application, was selected (as no consistent ED_{50} value was determined at this point). The populations selected consisted of the four populations tested in the DIMBOA variation assay (Section 2.2.4), and 18 further LoLa populations (Table 2.1), selected to optimise the range of herbicide resistance and geographic spread (assumed to be analogous to genetic variability) screened in this assay (Figure 2.3). Mean values of black-grass root and shoot length were compared in control and benzoxazinoid treatments using calculated confidence intervals and analysed by one-way ANOVA. Where a treatment significantly influenced black-grass development, relevant data were compared with herbicide survivorship for each population as depicted in Table 2.1, through linear regression analyses of data for each individual herbicide in Genstat.

Table 2.1: Black-grass populations used in this study and herbicide resistance, colour-coded by resistance rating (using data from Hicks *et al*. (2018)). Populations marked with an asterisk were used in the initial DIMBOA assay. Overall resistance determined through calculation of mean survivorship percentage; 'None': no known survivorship, 'Very Low': 0.1-39.9%, 'Low': 40-59.9%, 'Moderate': 60-79.9%, 'High': 80-89.9%, 'Very High': 90% +

	Mesosulfuron/ Iodosulfuron	Fenoxaprop	Cycloxydim	Overall Resistance
Population	Survival %	Survival %	Survival %	Rating
8	100.00	100.00	61.11	High
15	83.33	100.00	57.59	High
20	100.00	100.00	83.34	High
21	100.00	100.00	94.44	Very High
23	100.00	100.00	38.89	Moderate
30	88.89	100.00	44.44	Moderate
35	16.67	94.44	44.44	Low
43	100.00	100.00	66.67	High
44	100.00	100.00	77.78	Very High
55	100.00	100.00	94.44	Very High
59	94.44	100.00	38.89	Moderate
67	100.00	100.00	83.33	Very High
81*	5.56	61.11	0.00	Very Low
91*	77.78	100.00	10.53	Moderate
103	83.33	100.00	33.33	Moderate
108	94.44	100.00	55.56	High
113	94.44	100.00	83.33	Very High
120	94.44	100.00	83.33	Very High
122	88.89	100.00	33.33	Moderate
131	0.00	94.44	22.22	Very Low
Rothamsted-17*	N/A (Susceptible Standard)			None
Peldon-13*	N/A (Resistant Standard)			Very High

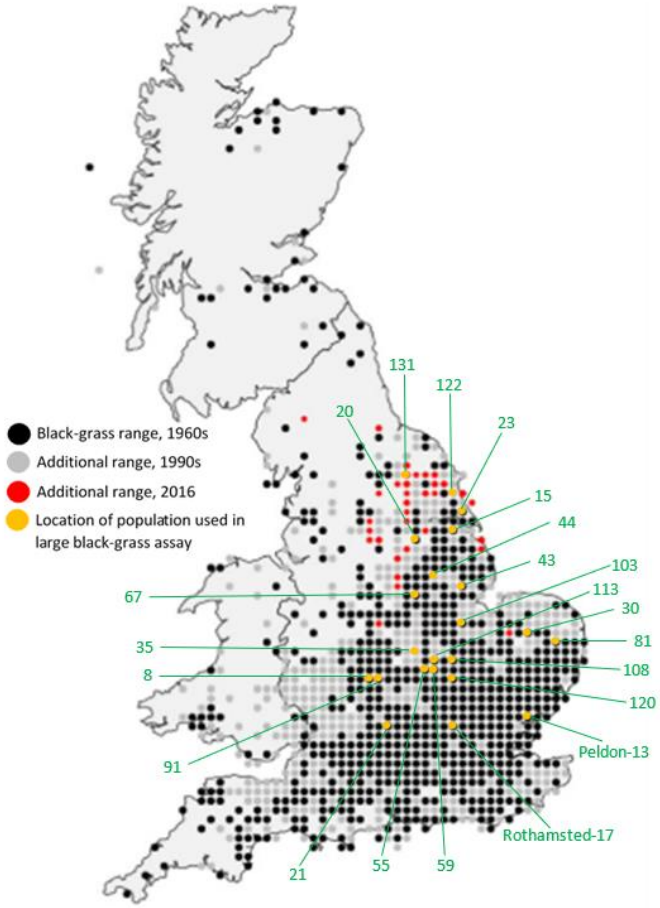

Figure 2.3: Geographic spread of black-grass, and annotated collection locations for populations used in this study within the UK, adapted from Hicks *et al.*, 2018. Original figure derived from black-grass range described in Botanical Society of Britain and Ireland atlases (Perring and Walters, 1962; Preston, Pearman and Dines, 2002).

2.2.6. Comparing benzoxazinoid inhibition of black-grass and wheat

The specificity of potent compounds must be understood in terms of their potential applicability to cereal agricultural systems. Therefore, the same discriminating doses of DIMBOA and DIBOA as used in black-grass assays (Section 2.2.5), were applied to a selection of 11 modern commercial wheat varieties (Table 2.2). 'Cadenza' was obtained from Rothamsted Research stock and included given its use in previous studies into its responses under stress, which are therefore better understood in this cultivar compared to others. The remaining ten varieties used were found in the AHDB winter wheat recommended list for 2019 (AHDB, 2019), and obtained from Dalton Seeds Ltd. (Eye, Suffolk, UK). Additionally, the four black-grass populations assayed in the initial sensitivity assay were included as controls (Rothamsted-17, Peldon-13, LoLa-81 and LoLa-91). ANOVAs were again conducted to determine statistical differences in root and shoot growth.

Table 2.2: Wheat cultivars used in the wheat sensitivity assay. Shaded cultivars were also used in the assay in Section 2.2.4.

Variety	**AHDB Grouping**
KWS Zyatt	Group 1
KWS Siskin	Group 2
KWS Firefly	Group 3
LG Skyscraper	Soft Group 4
LG Spotlight	Soft Group 4
RGT Gravity	Hard Group 4
Gleam	Hard Group 4
Shabras	Hard Group 4
Graham	Hard Group 4
Costello	Hard Group 4
Cadenza	Former Group 2

2.3. Results

2.3.1. Benzoxazinoid allelopathic potential to black-grass *in vitro*

AMPO was ineffective towards root growth of black-grass at any of the tested doses (Figure 2.4c) (p= 0.22), although a slight, statistically insignificant decline in root length did occur at the highest dose (1800 µM). BOA also had no significant growth effects on root length (Figure 2.4e) at the doses used in this investigation (p= 0.165).

MBOA was significantly inhibitory to black-grass root growth (Figure 2.4b) at the highest dose used in this investigation (1800 µM) (p< 0.001), but none of the lesser doses. Black-grass roots were also significantly inhibited by APO above 450 µM (Figure 2.4f) (p< 0.001). Even at the highest doses, however, root growth varied greatly between plants on one plate (Figure 2.5), suggesting some intra-population differences in sensitivity which were not apparent in Figure 2.4f. In corroboration, ED_{50} analysis determined a large value with an impractically large degree of error.

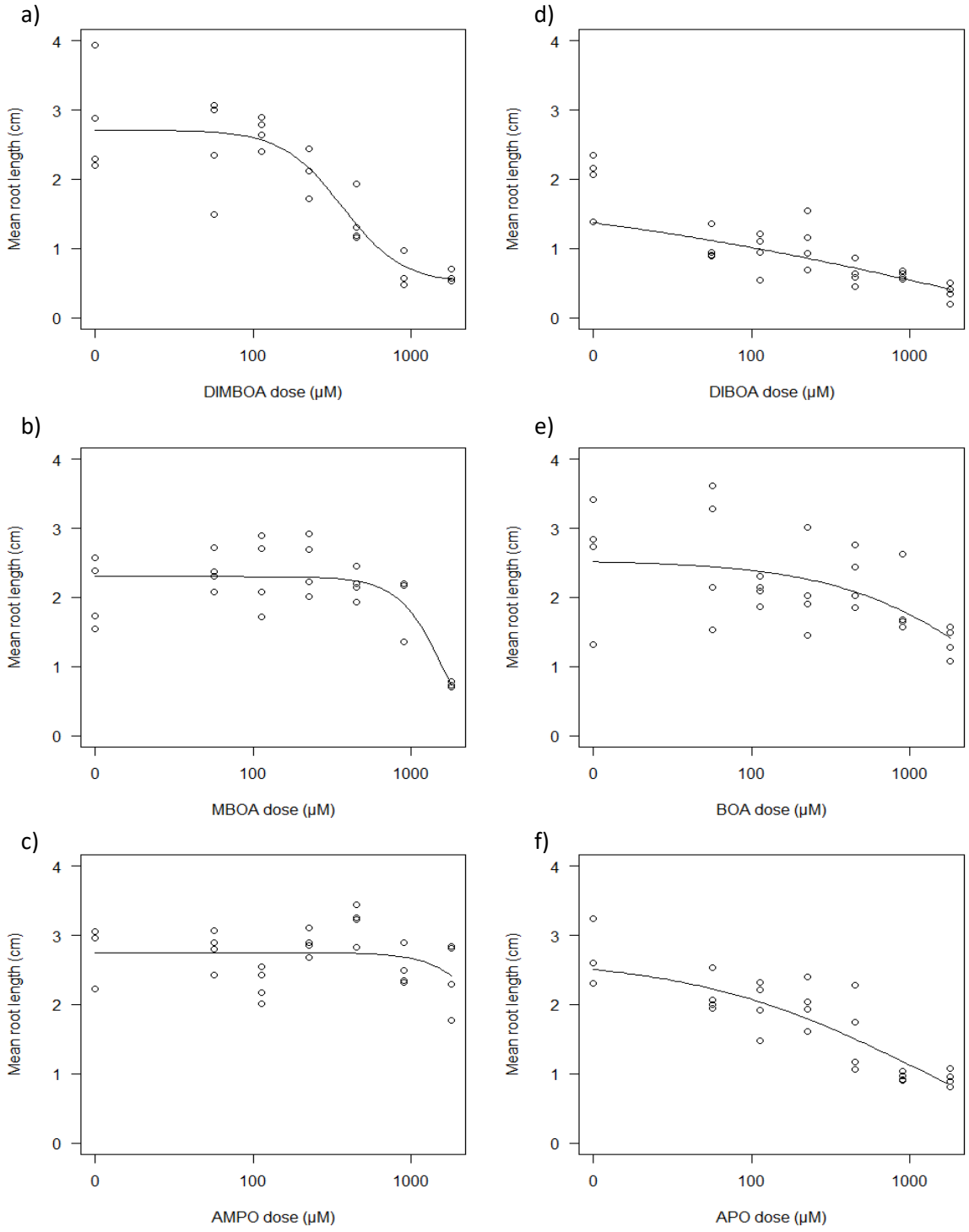

Figure 2.4: Dose-response curves of Rothamsted-17 black-grass root growth under treatment with: a) DIMBOA; b) MBOA; c) AMPO; d) DIBOA; e) BOA; f) APO; doses depicted on log scale. In the production pathways DIMBOA can be converted to MBOA and then to AMPO and parallel, DIBOA can be converted to BOA and then to APO; N= 168, plate replicates= 4, individual seeds per plate= 8, curves produced using the 'drc' package in R.

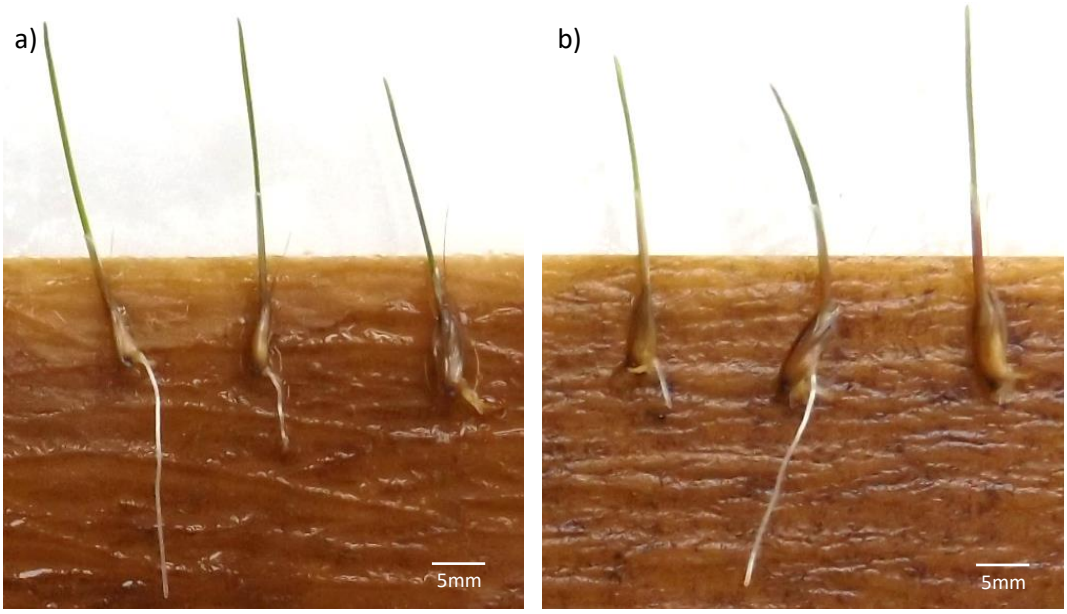

Figure 2.5: Variation of Rothamsted-17 black-grass growth between individual seedlings treated with 1800 µM of APO. a) and b) represent different replicate plates.

Under DIMBOA treatment, black-grass root growth was also significantly inhibited above 450 µM, in a sigmoidal curve (Figure 2.4a) (p< 0.001). The ED_{50} of DIMBOA had a low range of error compared to APO, and therefore the value of 371.60 µM is likely to be accurate. DIMBOA was also significantly inhibitory to black-grass germination at the highest 1800 µM dose (p< 0.001) (Figure 2.6), further indicating its allelopathic potential, but also confirming germination to be a less sensitive metric than root development.

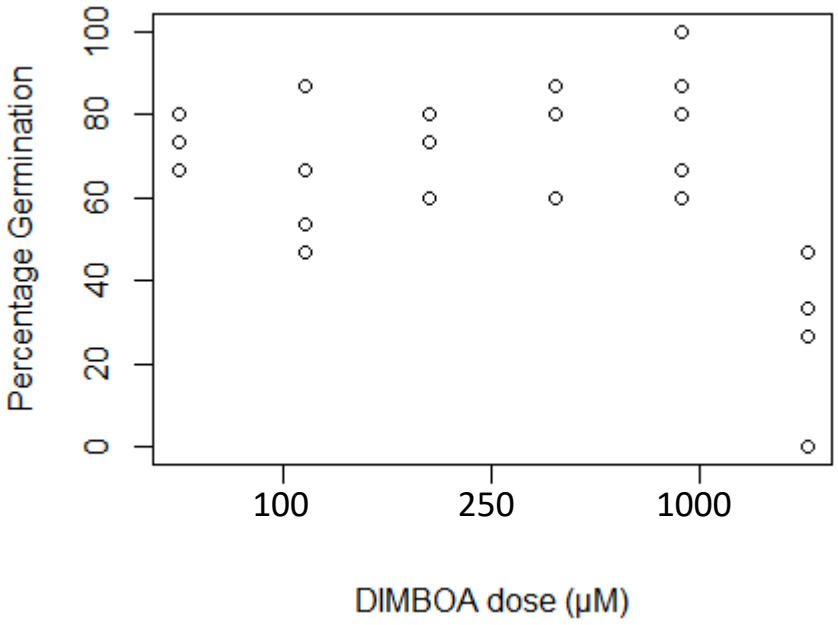

Figure 2.6: Black-grass germination percentages by replicate and dose of DIMBOA treatment on a log-logistic scale.

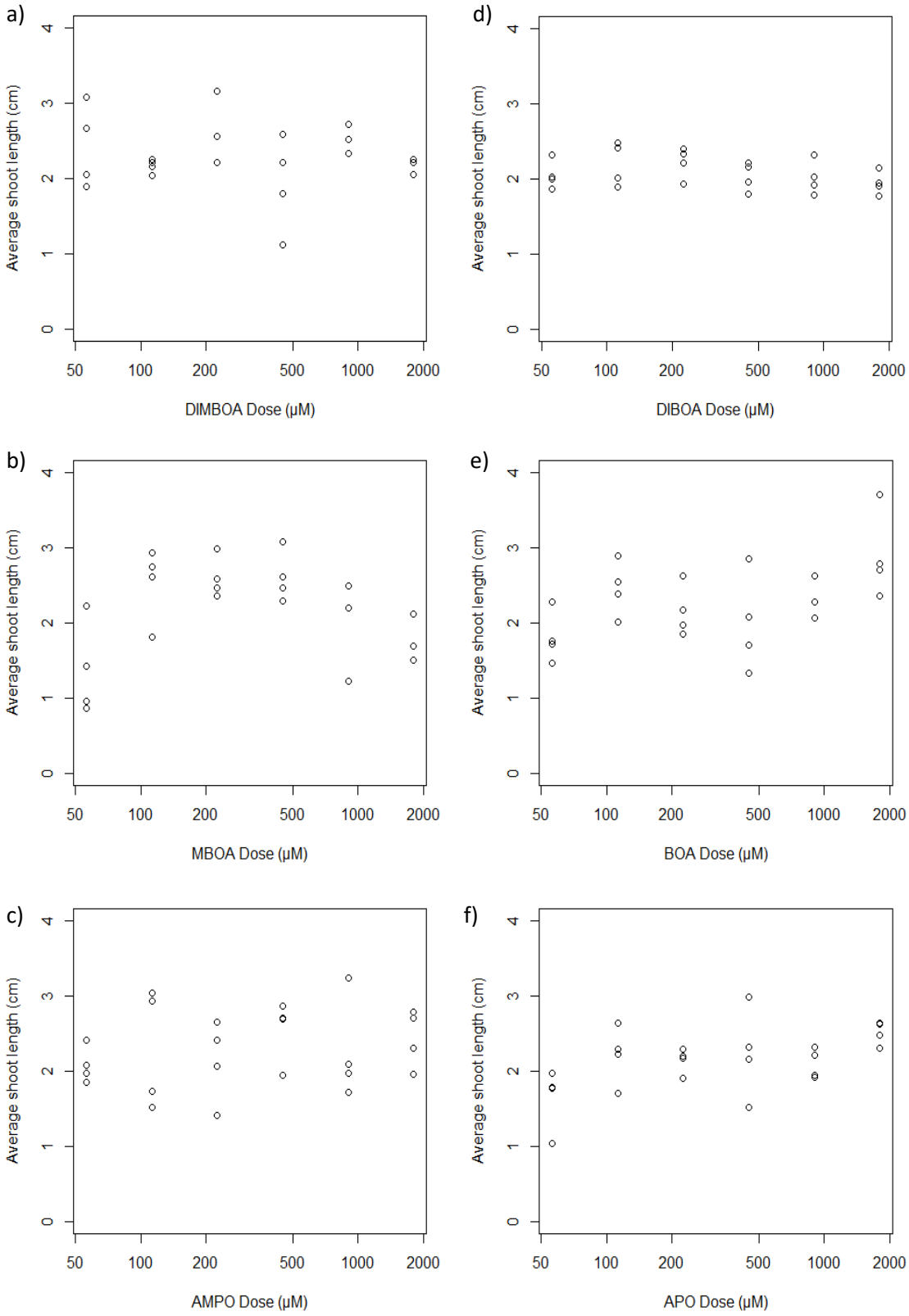

Figure 2.7: Variation in Rothamsted-17 black-grass shoot growth under treatment with: a) DIMBOA; b) MBOA; c) AMPO; d) DIBOA; e) BOA; f) APO; doses depicted on log scale. DIMBOA can be converted to MBOA and then to AMPO and parallel, DIBOA can be converted to BOA and then to APO; N= 168, plate replicates= 4, individual seeds per plate= 8, curves produced using R Studio.

DIBOA was also significantly inhibitory to black-grass root length at all doses compared to the control (Figure 2.4b) (p< 0.001). DIBOA was moreover the only screened compound to demonstrate meaningful inhibition to black-grass shoot length, only at the highest 1800 µM dose (p= 0.041) (Figure 2.7d). All other compounds except MBOA did not affect black-grass shoot length significantly (DIMBOA: p= 0.284, AMPO: p= 0.63, BOA: p= 0.054, APO: p= 0.073; Figures 2.7a, 2.7c, 2.7e, and 2.7f respectively). MBOA treatment correlated with significantly increased shoot growth at the 450 µM dose (p= 0.018) (Figure 2.7b), further indicating its unsuitability for black-grass management in field conditions.

2.3.2. A better understanding of DIBOA phytotoxicity to black-grass

In assay of multiple black-grass populations, DIBOA again significantly inhibited development at lower concentrations than DIMBOA. The root length of black-grass varied significantly by population (p= 0.002), dose of DIBOA (p< 0.001), and the interaction between DIBOA dose and population, with Peldon-13 significantly more sensitive than other populations (p= 0.036). This indicates that DIBOA is inhibitory to varying degrees on roots of different populations tested, thereby vindicating the need for a new discriminatory dose to be determined. All populations were significantly inhibited when treated with 100 µM of DIBOA or greater and described a similar dose-response curve (Figure 2.8). Natural variation in growth vigour between populations was apparent at low doses, but this was undone by the harsh stress of DIBOA doses above 200 µM. Calculation of ED_{50} values was possible for all populations (Table 2.3). All of these values have low error, and overlap, suggesting that all black-grass populations are similarly affected by DIBOA above 250 µM.

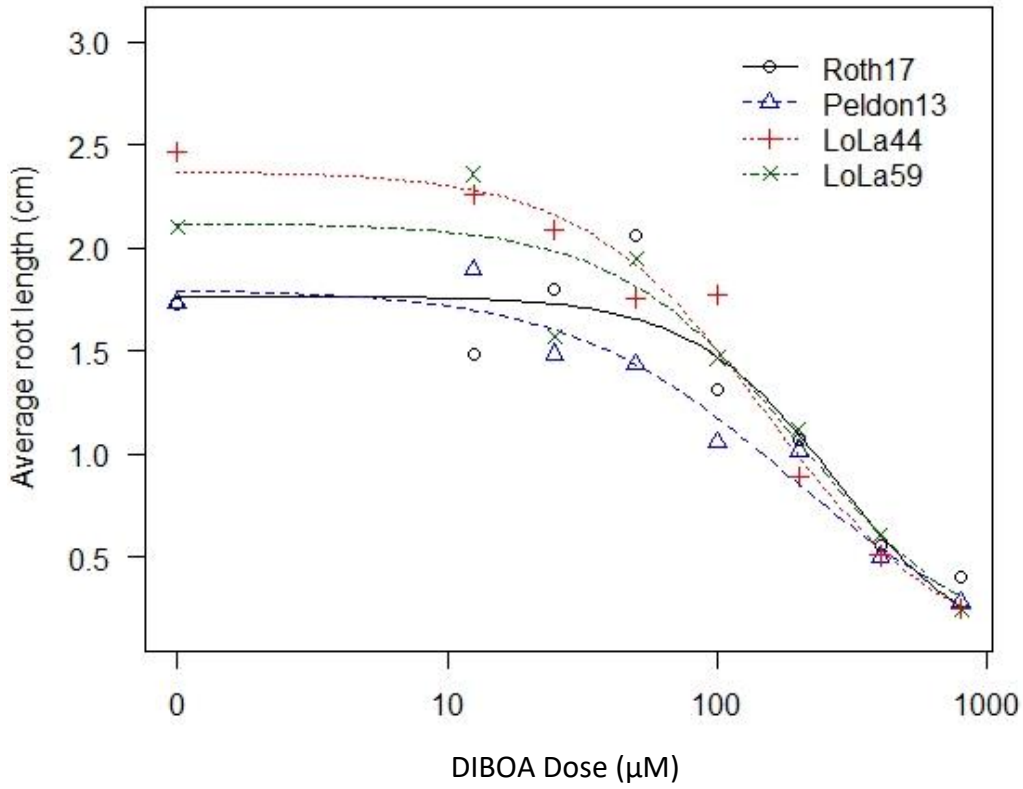

Figure 2.8: Dose-response curves of root growth in four black-grass populations treated with various doses of DIBOA on a log-logistic scale. Produced using the 'drc' package in R Studio.

Table 2.3: ED_{50} values and standard errors of black-grass populations present in Figure 2.8.

BLACK-GRASS POPULATION	ESTIMATED ED_{50}	STANDARD ERROR	LOWER	UPPER
LOLA-44	135.895	30.795	92.656	215.135
PELDON-13	182.250	52.843	77.165	287.335
LOLA-59	198.883	41.937	115.486	282.281
ROTHAMSTED-17	271.017	51.953	167.703	374.331

Significant differences in black-grass shoot length were identified for both DIBOA dose and population (both $p< 0.001$), but the effect of DIBOA dose did not differ significantly between populations ($p= 0.161$). Thus, intrinsic differences in shoot growth between black-grass populations did not affect their inhibition by DIBOA. Specifically, growth of Peldon-13 and LoLa-59 was significantly different to Rothamsted-17, while Peldon-13 was also significantly different to LoLa-44. Dose also had a significant effect, specifically doses 6 and 7 (above 400 µM), where shoot length was significantly reduced compared to lower doses (Figure 2.9) ($p <0.001$). These concentrations are therefore inhibitory to black-grass shoot growth, and this effect is generally not significantly different between populations.

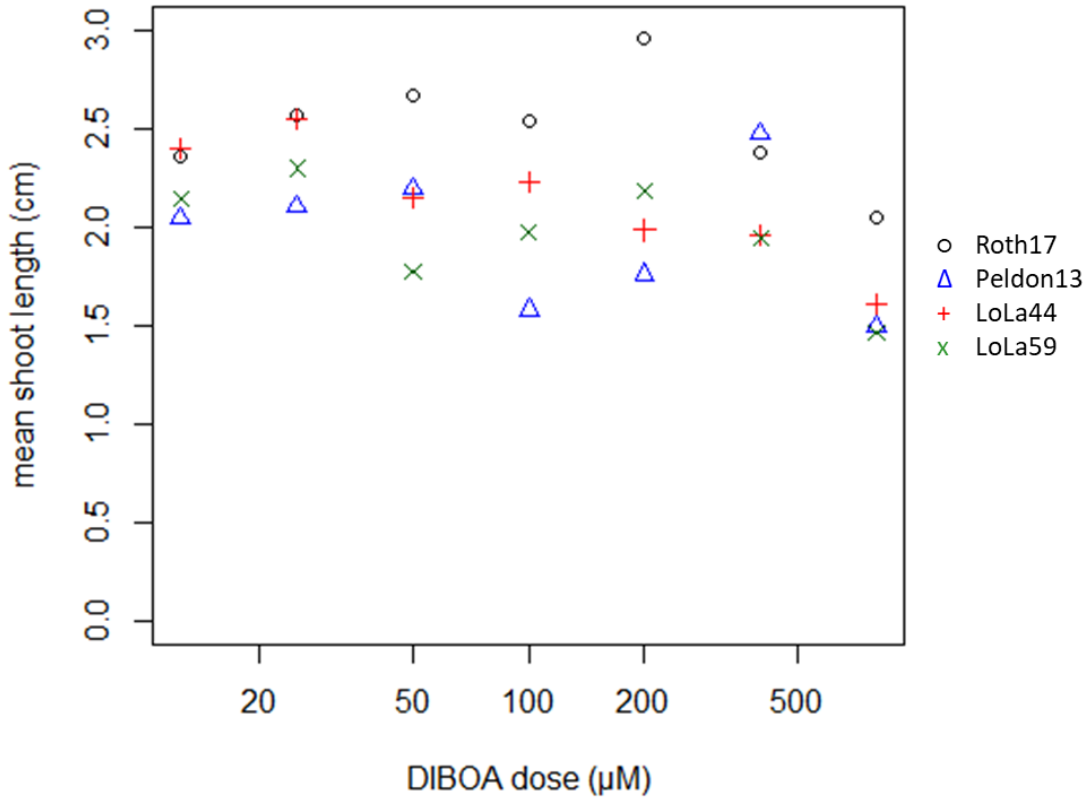

Figure 2.9: Shoot growth between DIBOA doses applied to four black-grass populations. Produced using R Studio.

2.3.3. Limited potential for DIMBOA resistance in black-grass

A consistent and significant reduction of black-grass root growth was found in all populations tested with increasing doses of DIMBOA (Figure 2.10a). ANOVA analyses found that the effect of variation in dose was strongly significantly different (p <0.001). In comparison, no significant difference between DIMBOA doses was found in terms of wheat root growth (Figure 2.10c) (p= 0.372), although a slight reduction in mean root length was observed at high doses (above 500 µM).

Shoot length of black-grass and wheat was not consistently affected by increasing doses of DIMBOA (Figures 2.10b and 2.10d). DIMBOA dose and black-grass population were both significant factors influencing this (p= 0.014 and 0.002 respectively). The greater significance of population further suggests that this is the major cause of variation in shoot growth, and thus there is no clear effect of DIMBOA on this metric, in corroboration of earlier findings.

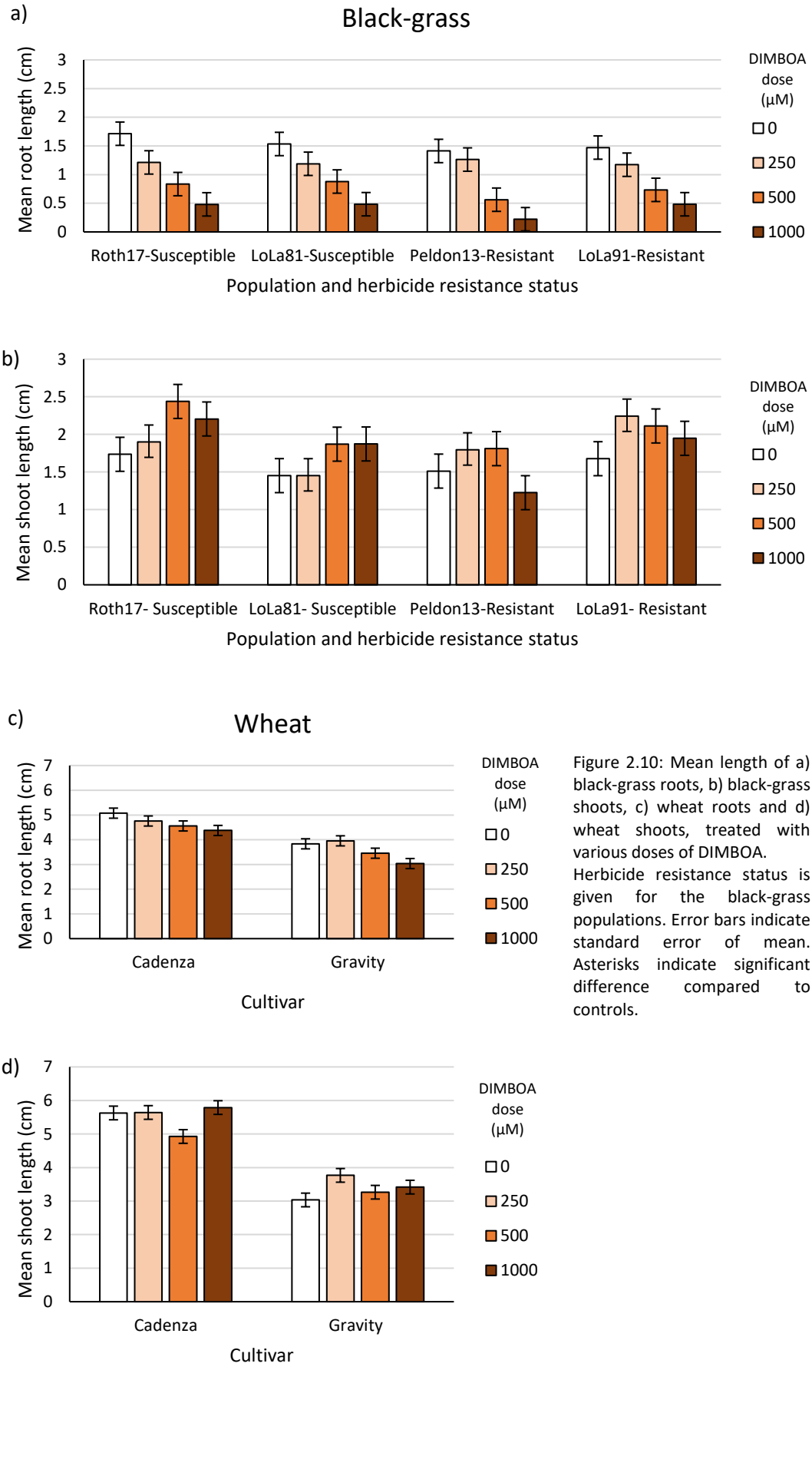

Figure 2.10: Mean length of a) black-grass roots, b) black-grass shoots, c) wheat roots and d) wheat shoots, treated with various doses of DIMBOA. Herbicide resistance status is given for the black-grass populations. Error bars indicate standard error of mean. Asterisks indicate significant difference compared to controls.

2.3.4. Benzoxazinoid inhibition across black-grass populations

Across the 22 black-grass populations assayed in this more extensive screening of population sensitivity, DIMBOA treatment (at a dose of 500 µM) inhibited root length in 5 populations (15, 67, 91, 108, and 120) by a maximum of 48% (Figure 2.11a). Root length of different black-grass populations therefore differed with statistical significance ($p< 0.001$) as a result of DIMBOA treatment. This treatment effect did not vary significantly between populations, however ($p= 0.274$), indicating statistically consistent inhibition across the populations tested. This corroborates previous results in proving the potency of DIMBOA in black-grass root inhibition, with differences between populations likely to be attributable to natural variation in growth vigour (this was verified by ANOVA which found significant differences between populations in terms of root length, $p< 0.001$). Shoot growth was not significantly inhibited in any population, although it was significantly greater in one population, 23, with DIMBOA treatment (Figure 2.11b). This was not sufficient to produce a significant difference across the test populations ($p= 0.424$), consistent with other results which do not indicate shoot inhibition occurring with DIMBOA treatment.

Under the 100 µM DIBOA treatment, root length of only one population (Lola-91) was inhibited, by 44% compared to controls (Figure 2.11c). This weaker effect than observed for DIBOA produced no statistical significance of DIBOA treatment on root length across the 22 populations tested ($p= 0.153$). Shoot length was not significantly different between any black-grass populations in this experiment with DIBOA treatment (Figure 2.11d). This was again not statistically significant inhibition across all populations ($p= 0.544$), further corroborating previous results suggesting that black-grass shoots are less sensitive to benzoxazinoid phytotoxicity than roots.

The significance of DIMBOA in inhibiting black-grass roots was compared by linear regression with survivorship of herbicide treatments by these populations. The results indicated no significant correlation between the effect of DIMBOA on roots and survivorship of treatment by Mesosulfuron/ Iodosulfuron mix ($p= 0.625$; Figure 2.12a), Cycloxydim ($p= 0.480$; Figure 2.12b), or Fenoxaprop ($p= 0.554$; Figure 2.12c), indicating no connection between sensitivity to these herbicides and degree of root inhibition by DIMBOA.

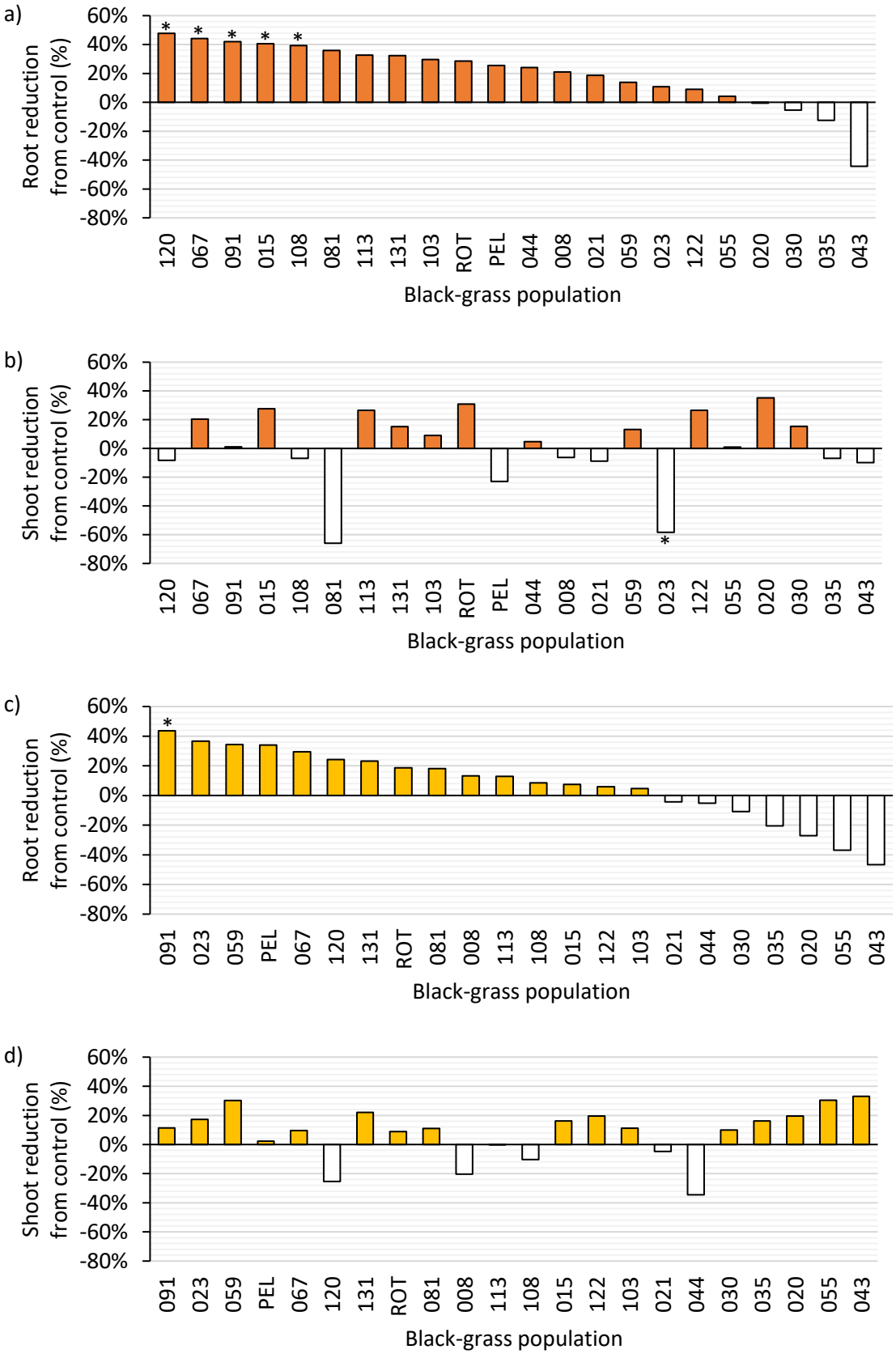

Figure 2.11: Effects of a 500 µM dose of DIMBOA on a) roots and b) shoots and a 100 µM dose of DIBOA on c) roots and d) shoots of 22 black-grass populations. Asterisks indicate significant differences according to confidence intervals and Tukey posthoc analyses. N=198, plate replicates= 3. 'ROT' is 'Rothamsted-17', 'PEL' is 'Peldon-13'.

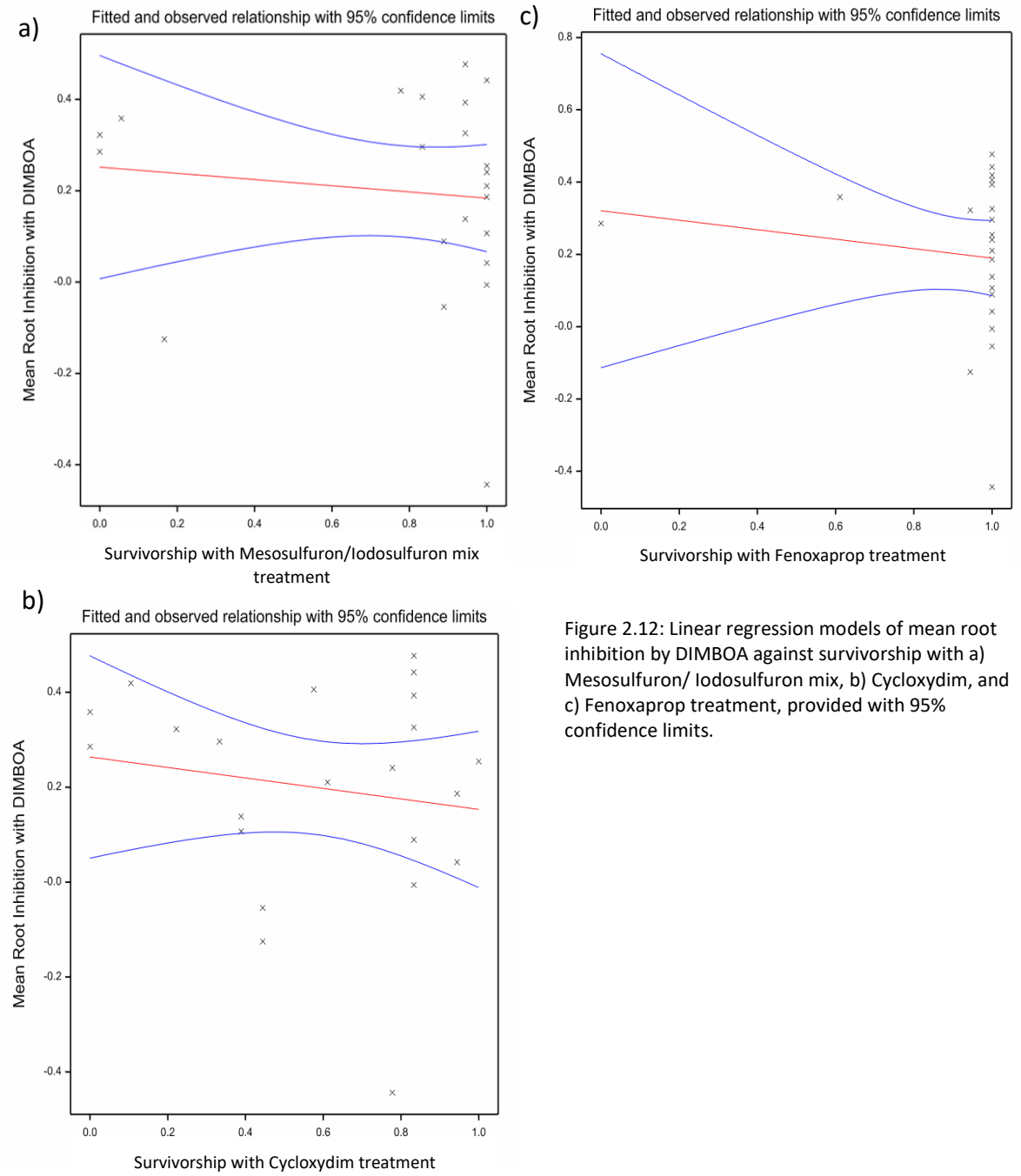

Figure 2.12: Linear regression models of mean root inhibition by DIMBOA against survivorship with a) Mesosulfuron/ Iodosulfuron mix, b) Cycloxydim, and c) Fenoxaprop treatment, provided with 95% confidence limits.

2.3.5. Comparing black-grass and wheat sensitivity to benzoxazinoids

To better understand potential applicability of benzoxazinoid treatments, effects of DIMBOA and DIBOA were assayed on multiple modern wheat cultivars. DIMBOA treatment did not result in significant influence on wheat root growth. It did correlate with significant inhibition of all four control black-grass populations included in this assay, however (Figure 2.13a). With DIBOA, effects were again less clearly observable on black-grass, with significant reduction of root growth in one black-grass population (LoLa-81). The root development of no wheat cultivar was significantly affected by DIBOA (Figure 2.13c).

Thus, the effect of benzoxazinoid treatment on root growth varied significantly by species ($p< 0.001$). Both compound treatments significantly reduced black-grass root growth, DIMBOA significantly more than DIBOA. In wheat cultivars, there was no significant difference between treatments ($p= 0.168$), corroborating the reported statistical difference in effects between test species.

Shoot growth demonstrated a less clear picture under benzoxazinoid treatment. Shoot growth of one black-grass population (LoLa-81) was significantly inhibited under DIMBOA treatment (Figure 2.13b), with no wheat cultivars displaying shoot inhibition. DIBOA treatment did not correlate with any significant reductions in shoot growth of either wheat or black-grass (Figure 2.13c). Thus, shoot length was not influenced by DIMBOA or DIBOA treatment in either species ($p= 0.455$).

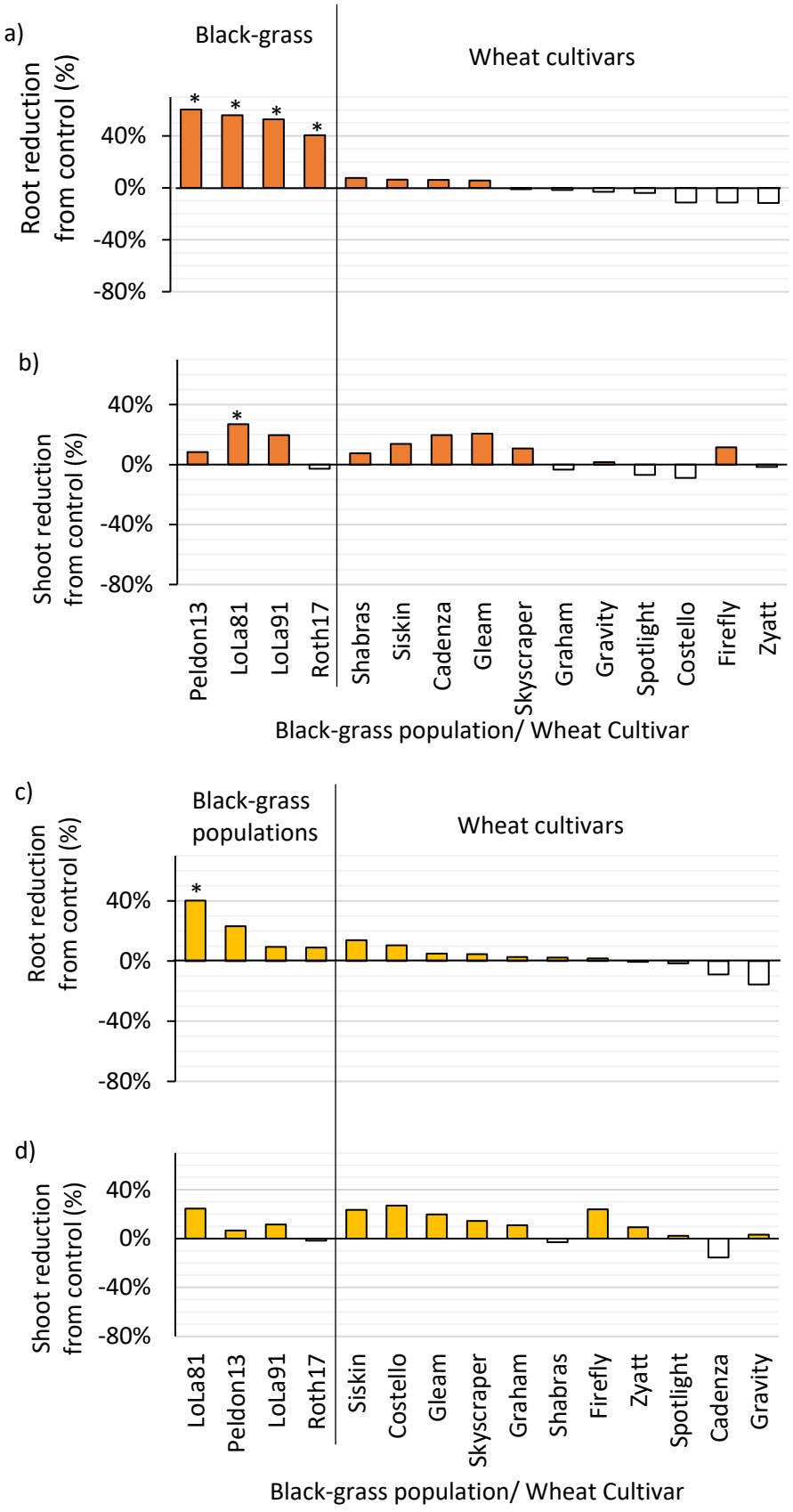

Figure 2.13: Effects of a 500 µM dose of DIMBOA on a) roots and b) shoots and a 100 µM dose of DIBOA on c) roots and d) shoots of four black-grass populations and 11 modern wheat cultivars. Asterisks indicate significant differences according to confidence intervals and Tukey posthoc analyses. N=198, plate replicates= 3.

2.4. Discussion

Considering the results of this chapter and existing knowledge of these compounds, DIMBOA and DIBOA are promising allelochemicals for control of black-grass, especially given their documented ineffectiveness towards wheat (Macías, Marín, *et al.*, 2005), corroborated by the results presented here. This study also contributes to the assertion of benzoxazinoids as black-grass inhibitors by confirming the phytotoxicity of these compounds at doses which may be practical for application. But the short half-lives of DIMBOA and DIBOA necessitate further work to elucidate their effectiveness in more biologically active conditions, as they may degrade quickly there. The other screened compounds are not especially promising, either due to a lack of effectiveness towards black-grass (MBOA, BOA, AMPO), or in the case of APO, a combination of inconsistency of results, previously documented inhibition of wheat, and problematically long half-life. AMPO is a particularly ineffective black-grass inhibitor, in line with previous findings in other species (Figure 2.14).

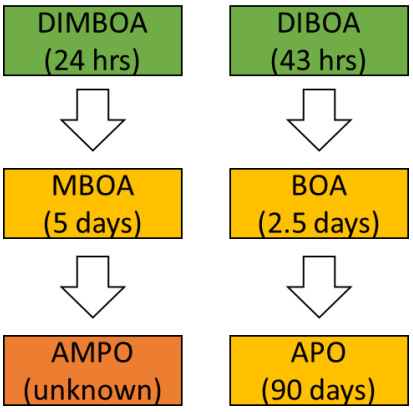

Figure 2.14: A simplified degradation diagram of the compounds tested in this chapter, with estimated half-lives in parentheses where known (Macías *et al.*, 2004; Macías, Marín, *et al.*, 2005). The half-life of AMPO is not known in detail but is noted to be greater than other benzoxazinoids (Etzerodt, Mortensen and Fomsgaard, 2008). Boxes are coloured by allelopathic potential to black-grass considering phytotoxicity found in this chapter; green indicates high potential, yellow indicates some potential, and orange indicates little potential.

2.4.1. Variation in benzoxazinoid phytotoxicity to black-grass, and persistence

The observation of DIMBOA and DIBOA as the most phytotoxic benzoxazinoids towards black-grass is at odds with some of the existing results emphasising the allelopathy of benzoxazinoid degradation products, particularly APO, towards other target species (Macías, Marín, *et al.*, 2005) (Table 2.4). It is

perhaps the most surprising result of this chapter that black-grass appears much less sensitive to APO than many previously tested target species, especially given that the HDAC enzymes that it is believed to inhibit are present in all eukaryotes (Venturelli *et al.*, 2015; Milazzo *et al.*, 2020). Still, inhibition of black-grass by APO was inconsistent even across a single Petri dish (containing one black-grass population) at 500 µM. This is a far greater dose than inhibits root growth in species such as cress or rigid ryegrass, although it is comparable to inhibitory doses for wheat or barnyard-grass (Macías, Chinchilla, *et al.*, 2005; Macías, Marín, *et al.*, 2005; Macías *et al.*, 2006). Inhibition of wheat is concerning for application, as it would mean that APO could not be applied to a planted field, and its persistence for three months or more in soil may lead to undesirable inhibitory effects in following crops (Macías, Chinchilla, *et al.*, 2005; Macías, Marín, *et al.*, 2005). Further, the level of variation in inhibition is concerning for applicability as it may leave tolerant outliers to thrive. Indeed, such variation may explain the reduced effectiveness of the compound towards black-grass. Thus, while APO may have some allelopathic potential, further examination of more species-specific and consistently inhibitory compounds is preferred.

Table 2.4: Inhibitory concentrations of pertinent benzoxazinoids towards root length of other test species (both standard tests species (STS) and weeds) from previous works, in micromolar dose. Data from Macías, Chinchilla, *et al.*, (2005); Macías, Marín, *et al.*, (2005); Macías *et al.*, (2006).

		INHIBITORY CONCENTRATIONS TO ROOT DEVELOPMENT (µm)						
	Species	**DIMBOA**	**DIBOA**	**MBOA**	**BOA**	**AMPO**	**APO**	**Reference**
STS	*Allium cepa*	500	100	1000	500	>1000	50	Macías, Marín *et al*., 2005
	Lepidium sativum	500	500	1000	500	>1000	10	Macías, Marín *et al*., 2005
	Lycopersicum esculentum	100	500	1000	500	>1000	100	Macías, Marín *et al*., 2005
	Triticum aestivum	>1000	1000	>1000	>1000	>1000	500	Macías, Marín *et al*., 2005
Weeds	*Lolium rigidum*	500	500	500	500	>1000	10	Macías, Marín *et al*., 2006
	Avena fatua	500	100	500	500	>1000	50	Macías, Marín *et al*., 2006
	Echinochloa crus-galli	>1000	500	>1000	>1000	>1000	500	Macías, Chinchilla *et al.*, 2005

By comparison, DIMBOA and DIBOA have promise as inhibitors of black-grass, and while not typically found to be as potent as APO, previous studies have identified allelopathic effects in these compounds in a variety of species at similar concentrations to those determined for black-grass in this study (around 500 and 250 µM respectively) (Table 2.4).

DIMBOA-mediated wheat allelopathy was significantly inhibitory to nine of 38 Chinese agricultural weeds, including a 21% reduction in orange foxtail (S.-Z. Zhang *et al.*, 2016), closely related to black-grass. On the other hand, Japanese foxtail

(*Alopecurus japonicus*) was not inhibited by DIMBOA, so it is not uniformly allelopathic towards *Alopecurus* species (S.-Z. Zhang *et al.*, 2016). The ED_{50} for DIMBOA towards barnyard-grass found in another study was 2590 µM (Mathiassen, Kudsk and Mogensen, 2006), indicating little inhibitory potential towards this species. This is an atypically high value, however, with those of annual meadow grass (*Poa annua*), loose silky-bent (*Apera spica-venti*), and redroot pigweed determined as 1370 µM, 600 µM and 510 µM respectively (Mathiassen, Kudsk and Mogensen, 2006). By comparison, then, the ED_{50} for black-grass, 371.60 µM, indicates that the species is relatively sensitive. The inhibition of black-grass germination by DIMBOA at the 1800 µM dose is also encouraging, although it corroborates previous findings that indicate germination to be a less sensitive metric for examining allelopathic effects compared to root growth (Haugland and Brandsaeter, 1996). For this reason, further investigations of benzoxazinoid effects on black-grass germination were not undertaken.

The insensitivity of wheat root or shoots to DIMBOA indicates its tolerance in comparison to black-grass. This is corroborated by Macías, Marín, *et al.*'s (2005) findings that no significant root inhibition occurred in wheat through DIMBOA treatment in doses below 1000 µM. It is also consistent with results from the assay of two wheat cultivars against DIMBOA up to this concentration in this study, and the lack of inhibition at 500 µM dose in the large-scale wheat bioassay.

The greater sensitivity of black-grass to DIBOA is somewhat unexpected, as results from other target species are broadly comparable to those of DIMBOA, and these two compounds have been similarly prominent allelochemicals since their phytotoxicity was determined (Pérez, 1990). Perhaps the greatest indication of the potential of DIBOA is in barnyard-grass, for which both root and shoot growth was significantly inhibited above 500 µM, a lower concentration than required by DIMBOA (Macías, Chinchilla, *et al.*, 2005). Moreover, there is precedent for DIBOA demonstrating greater phytotoxicity towards monocots (Barnes, Putnam and Burke, 1986). Przepiorkowski and Gorski (1994) correlated allelopathy against barnyard-grass specifically with DIBOA content of rye. DIBOA was the only compound to inhibit shoot development in the present study, at concentrations as low as 400 µM in the dose-response assay of multiple black-grass cultivars. This is an indication of the

anecdotal difficulty in controlling black-grass shoot growth through chemical means (R. Neale, Pers. Comm., 2019), as well as the lesser sensitivity of shoots to allelochemicals when compared with roots (Haugland and Brandsaeter, 1996).

On the other hand, there is some inconsistency to black-grass inhibition by DIBOA. At 100 µM, the initially identified discriminatory dose, there was still variation between populations, explaining the inconsistency of inhibition in the variation assay of 22 black-grass populations. In spite of this insufficient concentration of DIBOA used, root growth in one wheat cultivar, and shoot growth of multiple others, was inhibited. This is unlikely to be antagonistic to its applicability, but these results do indicate that wheat has a lower degree of tolerance to DIBOA than DIMBOA (Table 2.4; Macías, Marín, *et al.* 2005). Additionally, there is little evidence that modern wheat exudes noteworthy quantities of DIBOA through its roots, even though it is believed to synthesise the compound (Belz and Hurle, 2005). This supposition supports the theory that allelochemical tolerance is associated with its concentration in root exudates, and the resulting presence of sublethal doses in the surrounding rhizosphere.

Other cases of the limited allelopathic potential of AMPO, MBOA and BOA have also been reported in other target species. AMPO has little documented allelopathic potential towards any target species (Table 2.4). The explanation posited is that it is lipophobic, hampering diffusion through lipid cell membranes (Macías *et al.*, 2006). With both MBOA and BOA, black-grass is notably tolerant compared to other target species, considering the results in Table 2.4, and the slight, inconsistent root inhibition of black-grass noted at the 1800 µM dose. This may be attributable to the common occurrence of black-grass in arable soils which may contain low doses of these compounds. This contrasts to their parent compounds, which degrade too quickly to accumulate in this way. Tolerance was also observed in the herbicide-susceptible Rothamsted-17 population, which has only been exposed to naturally-occurring wheat root exudates (Moss *et al.*, 2004), indicating that it is unlikely to be associated with herbicide resistance, at least not entirely. Both BOA and MBOA persist in soil for several days (half-lives of around 2.5 and five days respectively) (Macías *et al.*, 2004), sufficient for black-grass to receive the low doses required for tolerance to evolve, as occurs with synthetic herbicides (Manalil *et al.*, 2011).

Incidentally, the much longer half-life of AMPO (Etzerodt, Mortensen and Fomsgaard, 2008) may also play a role in its lack of allelopathic potential, as most weed populations in arable cereal fields are likely to have experienced low doses and developed insensitivity.

There is precedence for resistance to BOA in other plants as noted in Chapter 1. Detoxification has been confirmed in velvetleaf (Schulz, Marocco and Tabaglio, 2012), while CYP-450 and UDP-glucosyltransferase activity in *Arabidopsis* facilitated *O*- and *N*-glucosylation of BOA when exogenously supplied, detoxifying it (Baerson *et al.*, 2005). Although not verified in black-grass, monocots are generally less sensitive to BOA than dicots (Barnes, Putnam and Burke, 1986), so it is unsurprising that black-grass is relatively insensitive. Additionally, BOA contains the active group in polycyclic urea herbicides (Moreland and Hill, 1963), inferring a shared mode of action (Barnes, Putnam and Burke, 1986). This is striking as herbicide-resistance in black-grass was initially discovered in phenyl-urea herbicides (Moss, 1990), which are similarly ineffective towards black-grass compared to BOA (Kemp, Moss and Thomas, 1990). Accompanying this theory, there are multiple examples of enzymatic upregulation of GSTs or CYP-450s in herbicide-treated black-grass with metabolic herbicide resistance (Yuan, Tranel and Stewart, 2007), so it may be that this same effect is occurring at a lesser scale with BOA treatment. There is therefore potential for both target-site (TSR) and non-target-site (NTSR) herbicide resistance to contribute to tolerance of BOA by black-grass.

2.4.2. Herbicide resistance does not guarantee benzoxazinoid resistance

A wide range of the black-grass populations used to test benzoxazinoid sensitivity are highly resistant to multiple herbicides, predominantly through TSR, as can be noted from the resistance data in Table 2.1 and associated findings by Hicks *et al.* (2018). Specifically, the formulations previously screened were the mesosulfuron/ Iodosulfuron mix, fenoxaprop-*P*-ethyl and cycloxydim. Mesosulfuron and Iodosulfuron are acetolactase synthase (ALS) inhibitors. As ALS is a catalyst of synthesis for the essential amino acids valine, leucine and isoleucine, the inhibition of this enzyme stunts the growth and development of sensitive plants (Burgos, Kuk and Talbert, 2001). Both of the other herbicides previously screened (Fenoxaprop

and cycloxydim) are acetyl-coenzyme A carboxylase (ACCase) target site inhibitors which affect the catalysis of fatty acid synthesis (Tal, Zarka and Rubin, 1996).

There is some precedent for the observed lack of connection between target-site herbicide resistance and allelochemical efficacy. For instance, Przepiorkowski and Gorski (1994) reported that triazine-resistant and susceptible barnyard-grass were similarly inhibited by crude rye root exudates. In extension to this, Yang *et al.* (2020) even suggest that resistance to synthetic herbicides (specifically mesosulfuron) in black-grass increases susceptibility to wheat-exuded DIMBOA. Heightened vulnerability to allelochemicals in herbicide-resistant biotypes has been reported in other species, for example the inhibition of penoxsulam-resistant barnyard-grass by allelopathic rice (Yang *et al.*, 2017).

NTSR mechanisms may also play a role in resistance, however, as noted in Section 1.2.2. While these can reduce absorption or enhance sequestration of phytotoxins, increased metabolism to detoxify inhibitory compounds is the most commonly occurring mechanism, predominantly conferred by CYP-450s and GSTs (Powles and Yu, 2010; Gaines *et al.*, 2020). Herbicide metabolism has been examined in black-grass, and it is noted that even herbicide-susceptible populations are capable of limited metabolism of chlortoluron, but the response is significantly greater in resistant lines (Hyde, Hallahan and Bowyer, 1996). A particular protein, *Am*GSTF1, is consistently upregulated with such resistant individuals, but different gene networks were upregulated in different populations. Some of these networks were linked variously with stress resistance, multidrug resistance in humans, cold shock, and resistance to fungal infection in cereals (Franco-Ortega *et al.*, 2021). Considering these various functions, it is reasonable to assume that one of these networks is associated with herbicide and, by extension, potentially benzoxazinoid resistance. Such vastly varying genetic resources for metabolic resistance further rationalise the examination of multiple black-grass populations for resistance to benzoxazinoids.

The allelopathic effect of DIMBOA did not correlate with herbicide resistance in black-grass, a highly encouraging finding for application. The absence of significant correlation between root inhibition by DIMBOA and survivorship of any of the three herbicides examined indicates that benzoxazinoids do not exclusively act through ALS or ACCase target-site inhibition. Interestingly, a recent work has set some precedent

for herbicide-breaking activity from plant-derived compounds, with multiple flavonoids noted for effectively binding with the multiple-herbicide-resistant gene *Am*GSTF1 in black-grass, thereby reducing its effectiveness to synthetic herbicides (Schwarz *et al.*, 2021). With the consistent allelopathy of benzoxazinoids to multiple-herbicide-resistant black-grass, it is possible that they have similar binding and resistance-breaking effects.

This result is furthermore predicated on the common incidence of resistance to the herbicides tested, which has the effect of reducing confidence in values towards the lower end of the range. With this profusion of highly resistant populations present in field populations, it is essential for application of DIMBOA and DIBOA that they remain effective against them. Moreover, this lack of correlation with common target-site herbicide resistance mechanisms indicates that the effectiveness of DIMBOA is novel by comparison. The findings presented here could therefore lead to the discovery of one, or multiple new target sites. Such a discovery is highly desired for the development of new herbicidal compounds or treatments (Gressel, 2020), and would represent a major advancement in the battle against black-grass.

2.5. Conclusion

This chapter successfully determines that benzoxazinoids display bioactivity against black-grass across a range of doses, albeit of great variability throughout the members of this family of compounds screened. Such results are of course caveated by a lack of results in the presence of competitor crops or biologically active media, which will be explored in later chapters.

The lack of bioactivity of AMPO compared with other benzoxazinoids is in accordance with previous results, while black-grass also demonstrates a lack of sensitivity to BOA and MBOA. This may be related to their persistence in soil which would constitute low-dose exposure and, consequently, the potential for evolution of tolerance or resistance in black-grass. Nonetheless, their lack of phytotoxicity in this study indicates that these three compounds should not be pursued further in attempts to control black-grass.

While APO demonstrates some allelopathy towards black-grass, this is to a lesser potency than in other target species. Additionally, the lack of consistency in its effects, and its problematically long half-life in soil are factors which limit its favourability for application. APO is therefore of less interest than other phytotoxic benzoxazinoids, namely DIMBOA and DIBOA. Long posited as the primary allelochemicals produced by cereals, these compounds are of interest for further study. DIMBOA is effective at doses comparable to APO, but displays a greater level of consistency in its control of black-grass. DIBOA, meanwhile, is particularly potent in its inhibition of black-grass.

These findings suggest potential for application. Broadly, black-grass displays consistent sensitivity to DIMBOA, and some consistency of sensitivity to DIBOA which would be increased by testing of a greater discriminating dose. Additionally, and crucially for application, neither compound is inhibitory to modern commercial wheats at comparable concentrations. This is to be expected to a degree given that benzoxazinoids are produced by wheat, so one would assume some tolerance. As a result, it can be said that both compounds could be applied to a mixed wheat and black-grass field with the intention of suppressing black-grass and allowing the tolerant wheat to outcompete it.

Considering the various discrepancies in sensitivity between different target species previously examined and black-grass, it is apparent that allelopathic effects vary greatly between target species (Macías, Chinchilla, *et al.*, 2005; Sturm, Peteinatos and Gerhards, 2018). Despite this, many such studies have instead traditionally employed only lettuce (*e.g.* Bremm *et al.*, 2021; Shi *et al.*, 2021), or other 'standard test species' which have been noted for their relative susceptibility to such allelochemicals (Kaur *et al.*, 2009), and which are likely to return false positive results on bioactivity towards more ecologically relevant target species. To this end, the species-specific, germplasm-wide examination of allelochemicals identified as potential bioherbicides is strongly advocated.

Chapter 3

Crude root exudate allelopathy towards black-grass

3. Crude root exudate allelopathy towards black-grass

3.1. Introduction

DIMBOA and DIBOA clearly hold considerable promise for allelopathic control of black-grass (Chapter 2), especially as these compounds have been documented in common cereal tissues (Barnes *et al.*, 1987; Pérez, 1990). It is important for application, however, to determine the extent to which these potentially benzoxazinoid-exuding cereals can inhibit black-grass through their crude root exudates at naturally-occurring concentrations. If so, such cereals could be used in an integrated weed management programme for black-grass control. There is, moreover, value to understanding the potential of black-grass to induce allelobiotic effects, whereby communication interactions between species can stimulate allelochemical exudation (Li, Xia and Kong, 2016), given that this phenomenon could affect allelopathic interactions.

It is also important to know if black-grass has potential to inhibit a crop through allelopathy, because this effect could negate the benefit of control measures. Agronomic observations have perpetuated the belief that black-grass is allelopathic to crops, given well-documented yield declines where it is prevalent (Naylor, 1972b; Vizantinopoulos and Katranis, 1998). Marczewska-Kolasa, Bortniak and Domaradzki (2010) noted a reduction of wheat root length between 64 and 77% in the presence of black-grass, and there is some evidence of allelopathy in other wild grasses (Bertin *et al.*, 2007), including in grass weeds (Viard-Crétat *et al.*, 2009). Still, the few studies of cereal inhibition by black-grass to date have not directly excluded the potential confounding effect of resource competition. Further study is therefore necessary to discern the allelopathic potential of black-grass towards cereals, a separate phenomenon to resource competition with different implications for the application of allelopathic cereals for black-grass control. Moreover, given evidence of autotoxicity in the grass weed rigid ryegrass (Canals, Emeterio and Peralta, 2005), there is rationale to examining black-grass for any such autotoxic effects which may assist in its control.

Therefore, the central hypotheses tested in this chapter are as follows:

1. Crude root exudates of cereals will inhibit black-grass development similarly to the synthetic compounds screened in Chapter 2.
2. Black-grass root exudates will not be inhibitory to wheat or black-grass.

3.2. Methods

Experiments detailed in this chapter were designed to assess the allelopathic potential of cereal crude root exudates believed to contain benzoxazinoid allelochemicals in axenic media. Initial assays were performed hydroponically in Magenta vessels, before moving into centrifuge tubes containing coarse sand. Successes in demonstrating allelopathic interactions in the sand centrifuge tube system led to further work examining the autotoxic and allelopathic potential of black-grass.

3.2.1. Hydroponic bioassays to unravel crude allelopathic interactions

The prototype hydroponic system consisted of a Caisson Brand MK-5 polycarbonate Magenta vessel (Gentaur UK, London), autoclaved for sterility, and filled with 100 ml of autoclaved RO water. Stainless steel mesh (aperture of 1.36 mm; Robinsons Wire Cloth, Stoke-on-Trent, UK), was fixed onto low-density foam using a glue gun. These floats were sterilised with 70% ethanol and dried in a laminar flow cabinet, then sowed with seed sterilised as described in Section 2.2.1. Floats sown with seeds were placed into vessels containing autoclaved RO water, and sealed with Parafilm (Figure 3.1).

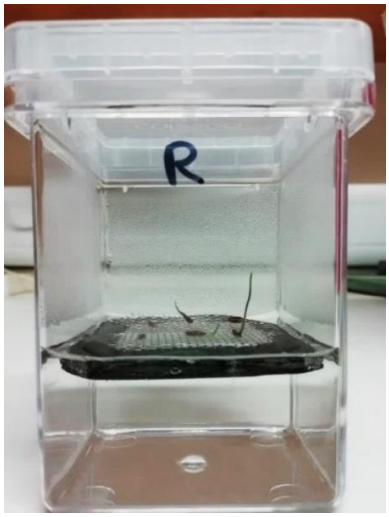

Figure 3.1: The Magenta hydroponic setup sown with six black-grass seedlings.

Water from these vessels was sampled, and sterility determined following assay setup through plating onto nutrient agar and incubating under constant 28°C light conditions for five days. A positive control using water from a system containing an unsterilised float was also produced, and found to contain extensive microbial growth, while water from the sterilised system did not support microbial growth (Figure 3.2). This confirmed the hydroponic system as axenic at the start of plant development.

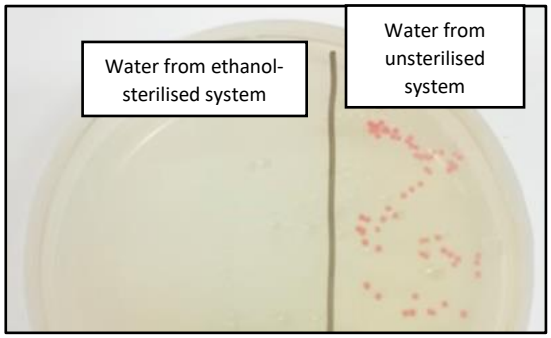

Figure 3.2: Nutrient agar plate containing water from unsterilised and ethanol-sterilised systems. Red patches are areas of microbial growth.

Black-grass, and modern and ancestor wheat, were sown into this system in various combinations (Table 3.1) to analyse the inhibitory potential of their root exudates towards black-grass. A mixed culture of modern wheat and black-grass was included to examine the effect of species recognition on root exudation, as well as being included in isolation. 'MDR049' ancestor wheat was additionally included given its aphid-resistant properties which were hypothesised to be associated with elevated benzoxazinoid content (Ahmad *et al.*, 2011; Simon *et al.*, 2017). A second assay omitted ancestor wheat exudates in favour of a 'combined' culture, essentially isolated wheat and black-grass exudate solutions which were combined after the growth period. This would indicate differences between additive effects of respective exudates, and the potential for greater allelopathic potential through allelobiosis (Li, Xia and Kong, 2016) (which would be observed in the mixed but not combined cultures).

Cultures were grown for fourteen days in the conditions used for assays in Chapter 2 (light/dark regime of 17°C/11°C for 14/10 hours). Following this growth period, plants were removed and photographed. Hydroponic media were freeze-dried to completion, and residue re-diluted in 20 ml of autoclaved H_2O DI for bioassay

of more concentrated material. These 5x concentrated exudates were applied to the Petri dish system (Section 2.2.2), with each dish testing 8 seeds of the Rothamsted-17 standard black-grass population. Dishes were incubated for 9 days in the same bioassay conditions as previous, before assessment of seedling root and shoot growth.

Table 3.1: Cultures produced from plant root exudates in Magenta vessel assays.

Culture name	Contents per culture replicate
Black-grass	16x Rothamsted-17 black-grass seeds in 100 ml of water
Modern wheat	16x 'Gravity' modern wheat seeds in 100 ml of water
Mixed	8x Rothamsted-17 black-grass seeds + 8x 'Gravity' modern wheat seeds in 100 ml of water
Ancestor wheat (first set only)	16x 'MDR049' ancestor wheat seeds in 100 ml of water
Combined (second set only)	16x Rothamsted-17 black-grass seeds in 50 ml of water + 16x 'Gravity' wheat seeds in 50 ml of water
Control	No plants, 100 ml of water

3.2.2. Centrifuge tube sand assays of cereal crude root exudate allelopathic potential against black-grass

Following unsatisfactory results from the hydroponic system, a different system using a porous matrix was developed, inspired by Pétriacq *et al.*'s (2017) exudate collection methods in soil. Sterile 50 ml Falcon centrifuge tubes were filled with 15 ml of autoclaved coarse sand, wetted with 5 ml of autoclaved H_2O DI, planted with the desired plant cultures at a depth of approximately 2mm, and sealed (Figure 3.3).

Cultures consisted of a no-plant negative control, black-grass, 'Gravity' modern wheat, and 'Edmondo' rye, a species believed to exude high levels of benzoxazinoids (*e.g.* Burgos and Talbert, 2000), and therefore representing a positive control (Table 3.2). Cultures were grown for seven days under the same environmental conditions used for hydroponic assays (Section 3.2.1). The length of the growth period was intended to maximise allelochemical concentration; benzoxazinoid concentrations in wheat peak around the seventh day of growth, decreasing thereafter and containing little by the eighteenth day (Argandoña, Niemeyer and Corcuera, 1981; Zúñiga and Massardo, 1991).

After this growth period, cultures were removed and photographed, and bioassay plants were sown in their place, again at a depth around 2mm. This consisted of four pre-germinated seedlings of Rothamsted-17 (herbicide-susceptible) or Peldon-13 (herbicide-resistant) black-grass in each tube. Rothamsted-17 was used as the standard population in previous assays, while Peldon-13 was also included to ensure that allelopathic effects were also observed within an herbicide resistant black-grass biotype. Eight replicates of each black-grass population were treated with each of these root exudates and grown for a further seven days prior to removal for photography and measurement.

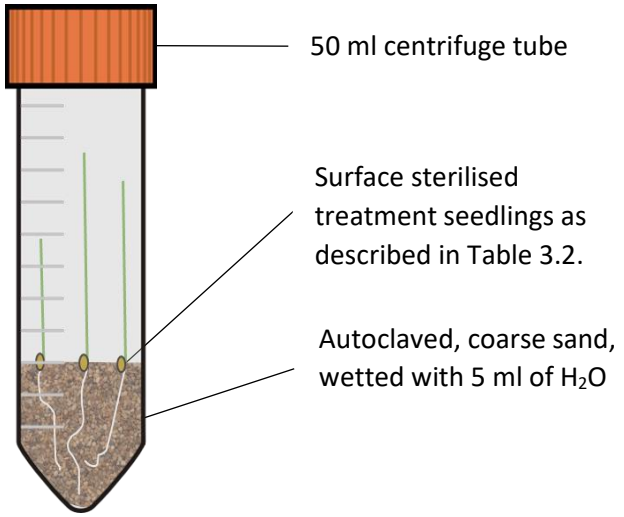

Figure 3.3: sand centrifuge tube assay used for growing cultures prior to elution with methanol for LCMS analyses.

Table 3.2: Cultures used for sand centrifuge tube assays

Culture name	**Contents**
Wheat	4x 'Gravity' wheat seeds
Rye	4x 'Edmondo' rye seeds
Black-grass	4x Rothamsted-17 black-grass seeds
Control	No plants

To investigate allelopathic potential in ancestor wheat varieties, treatments were expanded to include 'MDR' ancestor wheat (*Triticum monococcum*) lines with documented variation in defence capabilities, specifically 'MDR037', 'MDR043', and 'MDR049' (previously included in Section 3.2.1), prior to transplantation. 'MDR043'

(*T. monococcum* var. *monococcum*, spring wheat, collected from Greece, 1950) is detrimental to development of the soil-borne pathogenic fungus take-all (Jing *et al.*, 2007; McMillan, Gutteridge and Hammond-Kosack, 2014), while aboveground tissues of 'MDR049' (*T. monococcum* var. *monococcum*) were less supportive of aphid population growth compared to tissues of other cultivars tested (Elek *et al.*, 2009). 'MDR037' (*T. monococcum* var. *macedonicum*, spring wheat, collected from Armenia, 1934), does not show such detriment towards aphids or take-all (Jing *et al.*, 2007; Elek *et al.*, 2009; McMillan, Gutteridge and Hammond-Kosack, 2014).

Four surface-sterilised, pre-germinated seeds of each line were sown at 2mm depth for each culture. The other cultures were the no-plant controls, four 'Gravity' (modern) wheat seeds, and four 'Edmondo' rye seeds, as previously used (Table 3.3). Cultures were again grown for seven days under the pre-germination growth regime (light/dark at 17°C/11°C for 14/10 hours). Eight replicates of each culture were bioassayed, with pre-germinated, surface-sterilised Rothamsted-17 black-grass sown in at 2mm depth and grown for a further week for root and shoot length measurement and analysis.

Table 3.3: Cultures used in the ancestor wheat assay.

Culture name	**Contents**
Modern Wheat	4x 'Gravity' wheat seeds
Rye	4x 'Edmondo' rye seeds
MDR037	4x 'MDR037' ancestor wheat seeds
MDR043	4x 'MDR043' ancestor wheat seeds
MDR049	4x 'MDR049' ancestor wheat seeds
Control	No plants

3.2.3. Determination of potential for autotoxicity or allelopathy from black-grass

To further assay 3.2.2, a second set of treatments was set up, this time sowing black-grass or wheat into tubes which had previously contained black-grass seedlings. This assay allowed the investigation of whether black-grass itself has any allelopathy towards wheat, or autotoxicity. This assay contained a no-plant control, or a culture of four pregerminated, surface-sterilised Peldon-13 black-grass seedlings (selected as a more representative field population than Rothamsted-17). After seven days, black-grass seedlings were removed and photographed. In their place

was sown either another set of Peldon-13 black-grass seedlings for the evaluation of autotoxic potential, or four pregerminated, surface-sterilised 'Gravity' wheat seeds, with eight replicates of each combination of exudate and target species. All seedlings were again sown at a depth of approximately 2mm. After another seven days all plants were removed, photographed, and measured with ImageJ. Measurements were analysed by ANOVA and Tukey's posthoc tests as necessary.

3.2.4. Confirming benzoxazinoid uptake by black-grass

As a step to validating the findings of putative crude exudate allelopathy, the tracking of benzoxazinoid uptake was attempted. The centrifuge sand system was set up with three treatments, Control, DIBOA and DIMBOA. The control tubes were inoculated with 0.25% DMSO only. The DIBOA treatment consisted of 250 µM DIBOA in 0.25% DMSO, while the DIMBOA treatment was 500 µM DIMBOA in 0.25% DMSO. These are the discriminatory doses identified within Chapter 2, and in each case tubes (n= 18) were inoculated with 5 ml of the respective solution.

Into these tubes, four black-grass seeds were sown at 2mm depth and grown under the previously used environmental conditions for seven days. Both Rothamsted-17 and Peldon-13 were included to give some indication of variation in uptake or potentially even metabolism between populations.

Following this time, plants were removed and photographed for ImageJ analysis if necessary, allowing correlation between benzoxazinoid uptake and growth inhibition. They were then thoroughly washed with RO water to remove external droplets of benzoxazinoid solution, and cut off directly into liquid nitrogen, before freeze-drying. Frozen, freeze-dried root tissue was ground by pestle and mortar, and eluted with 500 µl of solvent (2% acetic acid v/v in methanol) for extraction of constituent compounds, and confirmation of benzoxazinoid presence in black-grass tissues. Specifically, this solvent was vortexed with ground root tissue for 30 seconds, then centrifuged for fifteen minutes at 3000 rpm to separate out eluate from solid material, and then siphoned into chromatography vials for LCMS analysis.

3.2.5. LCMS Analysis (Performed by Dr. J. Caulfield, BCP Department, Rothamsted Research)

LCMS analysis used an Acquity ultra-high-pressure liquid chromatography (UPLC) system coupled to a Synapt G2Si Q-Tof mass spectrometer with an electrospray ionisation source (Waters Ltd., Wilmslow, UK). The system was controlled through Masslynx 4.1 software (Waters). Chromatographic separation was undertaken at a flow rate of 0.21 mL min^{-1} using a UPLC BEH C18 column (2.1 x 150 mm, 1.7 µm, Waters) coupled to a C18 Vanguard pre-column (2.1 x 5 mm, 1.7 µm, Waters). The mobile phase consisted of solvent A (0.02% formic acid v/v, water) and solvent B (0.02% formic acid v/v in methanol) with the following gradient: initial conditions 95% A, 0 – 3 min 95% A, 3 – 7 min 85% A, 7 – 11 min 75% A, 11 – 13 min 70% A, 13 – 15 min 70% A, 15 – 18 min 50% A, 18 – 21 min 50% A, 21 – 25 min 25% A, 25 – 30 min 0% A, 30 – 39min 0% A, 39 – 39.1 min 95 % A, 39.1 to 43 min 95% A. The column was maintained at 50°C and the injection volume was 1 µl. Samples were run in positive and then negative modes with two consecutive injections of methanol between modes for stabilisation.

An Acquity photodiode array (PDA) detector monitored the UV trace (range 200-450 nm), sampling rate of 10 points s^{-1} with resolution set to 2.4 nm. The Synapt was operated in sensitivity mode and set to a mass range of 50 – 1200 Da and scan time = 0.1s in both ionisation modes. The system was operated in MS1 mode with the following conditions: capillary voltage – 2.5KV, sample cone voltage 30V, sample offset 80V, source temperature 100°C, desolvation temperature 300°C, desolvation gas flow 800L h^{-1}, cone gas flow 57L h^{-1}. The Synapt MS system was calibrated by infusing sodium formate solution and accurate mass detection was made by infusing the internal lockmass reference peptide leucine enkephalin during runs.

3.3. Results

3.3.1. Allelopathic interactions are inconsistent in hydroponic systems

Initial results from the Magenta vessel hydroponic system indicated that concentrated crude exudate treatments significantly reduced black-grass root length (p= 0.002), specifically from the mixed black-grass/wheat root exudate treatment. Additive effects of the modern wheat and black-grass exudates were assumed to

cause this effect, as neither treatment was significantly different to the control in isolation (Figure 3.4a). The 'MDR049' ancestor wheat treatment, in spite of its greater documented propensity for defence chemistry, did not significantly affect black-grass root growth in this system. In the repeat assay, no significant inhibition was found in root length (p= 0.066; Figure 3.5a) resulting from any exudate treatment. Although not significantly different, the combined treatment had less pronounced effects on black-grass growth than the mixed treatment.

No exudate treatments significantly affected black-grass shoot length in either of these assays (Assay 1: p= 0.861; Figure 3.4b; Assay 2: p= 0.305; Figure 3.5b). These findings together indicate that the hydroponic system could not consistently and effectively isolate allelopathic effects.

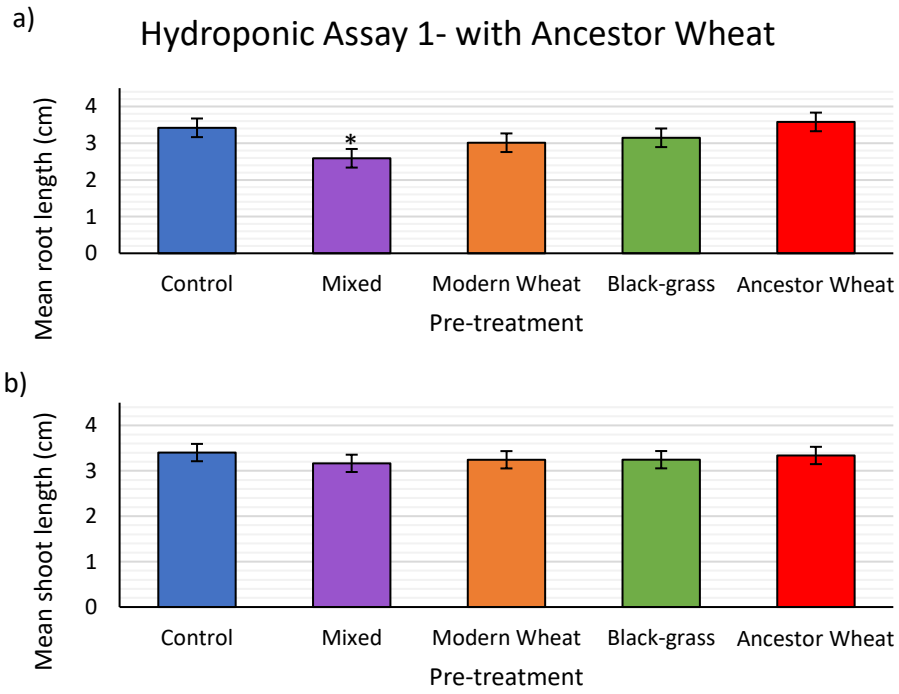

Figure 3.4: a) root growth and b) shoot growth of black-grass treated with 5x concentrated crude hydroponic exudates produced from pre-treatments with various plant species. Error bars indicate SEMs. Asterisks indicate treatments significantly different to the control.

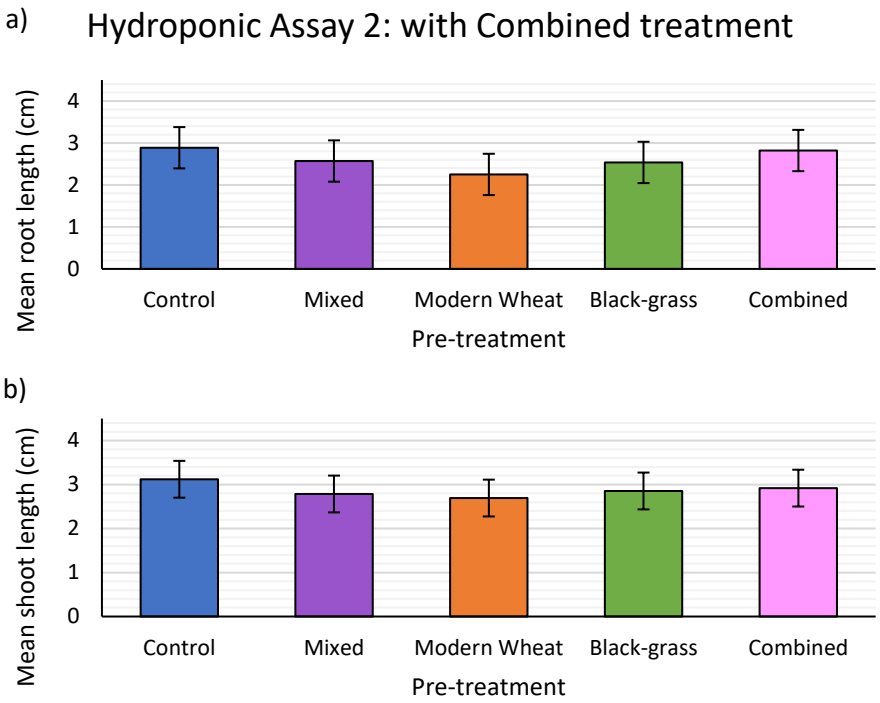

Figure 3.5: a) root growth and b) shoot growth of black-grass treated with 5x concentrated crude hydroponic exudates produced from pre-treatments with various plant species; a repeat assay including combined modern wheat and black-grass exudates produced in isolation. Error bars indicate SEMs.

3.3.2. Crude cereal exudates are allelopathic towards black-grass

Allelopathic effects of crude exudates were more clearly elucidated in the centrifuge tube sand system. While black-grass shoot length was again not significantly affected by root exudates of different species in this system (p= 0.400), such exudates did significantly affect root growth (p <0.001). These effects were not statistically different between the two black-grass populations tested (p= 0.135) (Figure 3.6). Specifically, root length of black-grass was significantly reduced by wheat root exudates compared to the no-plant control, and root inhibition was significantly greater from rye root exudates compared to either of the other treatments.

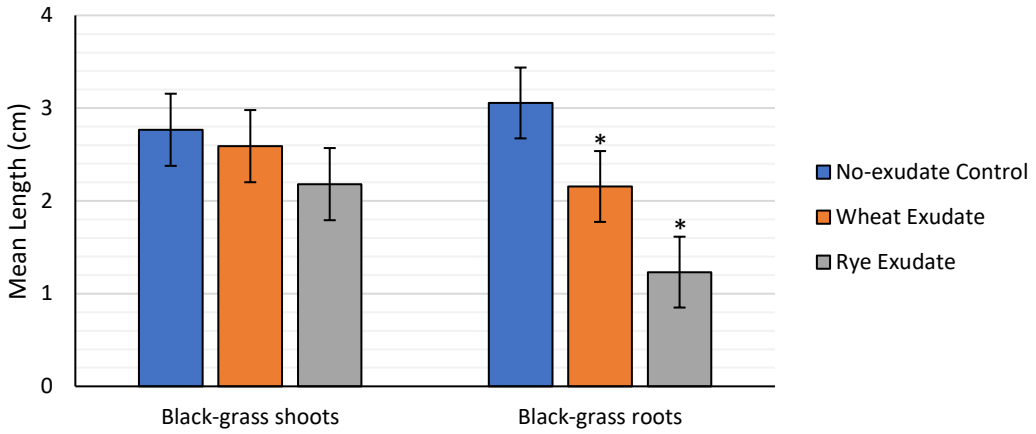

Figure 3.6: Mean root and shoot length of both Peldon-13 and Rothamsted-17 black-grass populations subjected to control, wheat and rye exudates. Error bars depict SEMs. Asterisks indicate treatments significantly different to controls.

3.3.3. Ancestor wheats vary in allelopathy towards black-grass

In the ancestor wheat variety screen, root exudates of 'Edmondo' rye and 'Gravity' wheat again significantly inhibited black-grass root length, as did the ancestor wheat line 'MDR037', compared with no-plant controls (p <0.001) (Figure 3.7). 'MDR043' and 'MDR049' exudates also tended to reduce black-grass root growth, but not significantly.

All non-control exudates were significantly inhibitory to black-grass shoot growth (p= 0.001). This was partially consistent with root inhibition, as the greatest growth reduction in shoots was found in exudates of the 'MDR037' ancestor wheat line, and 'Edmondo' rye.

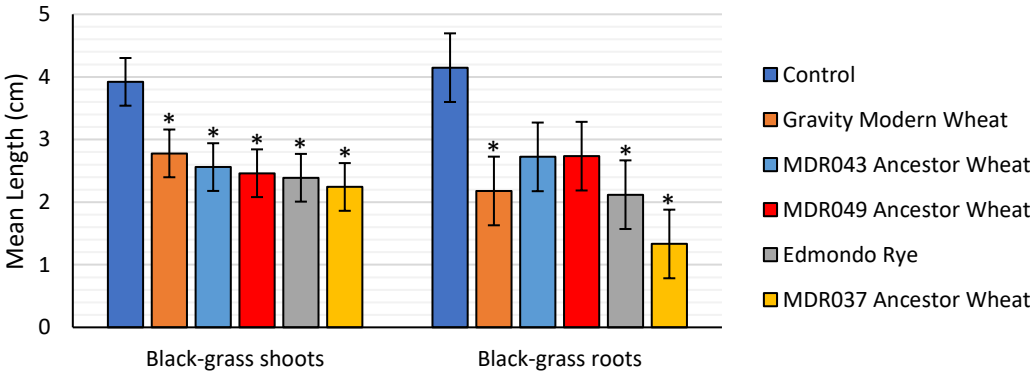

Figure 3.7: Black-grass root and shoot growth measurements treated with various cereal root exudates. Error bars represent SEMs. Asterisks indicate exudate treatments significantly different to controls.

3.3.4. No evidence of allelopathic potential in black-grass

In the centrifuge tube sand system, Peldon-13 black-grass root exudates had no significant influence on the length of other Peldon-13 black-grass roots (p= 0.646) or shoots (p= 0.359) (Figure 3.8a), or 'Gravity' wheat roots (p= 0.760) or shoots (p= 0.297) (Figure 3.8b), indicating no evidence of autotoxicity or allelopathy to wheat.

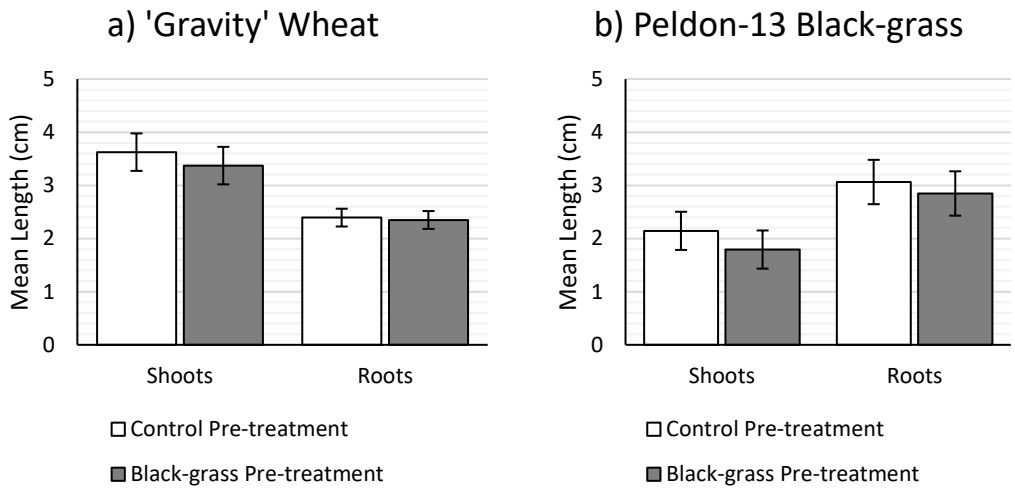

Figure 3.8: Mean root and shoot length of Peldon-13 black-grass (a) and 'Gravity' wheat (b) under no-plant control and Peldon-13 black-grass root exudates. Error bars depict SEMs.

3.3.5. Uptake of benzoxazinoid allelochemicals by black-grass

LCMS analyses of methanol-based root extracts of black-grass treated with DIMBOA tentatively identified trace levels of MBOA as a constituent. Both DIMBOA and DIBOA-treated root extracts also contained 2-β-D-glucopyranosyloxy-1,4-benzoxazin-3-one (HBOA-Glucoside, hereafter HBOA-Glc). The occurrence of these compounds was consistent between Rothamsted-17 and Peldon-13 populations. Roots grown in the control treatment contained no benzoxazinoid compounds. LCMS traces of these results in Rothamsted-17 roots are depicted in Figure 3.9.

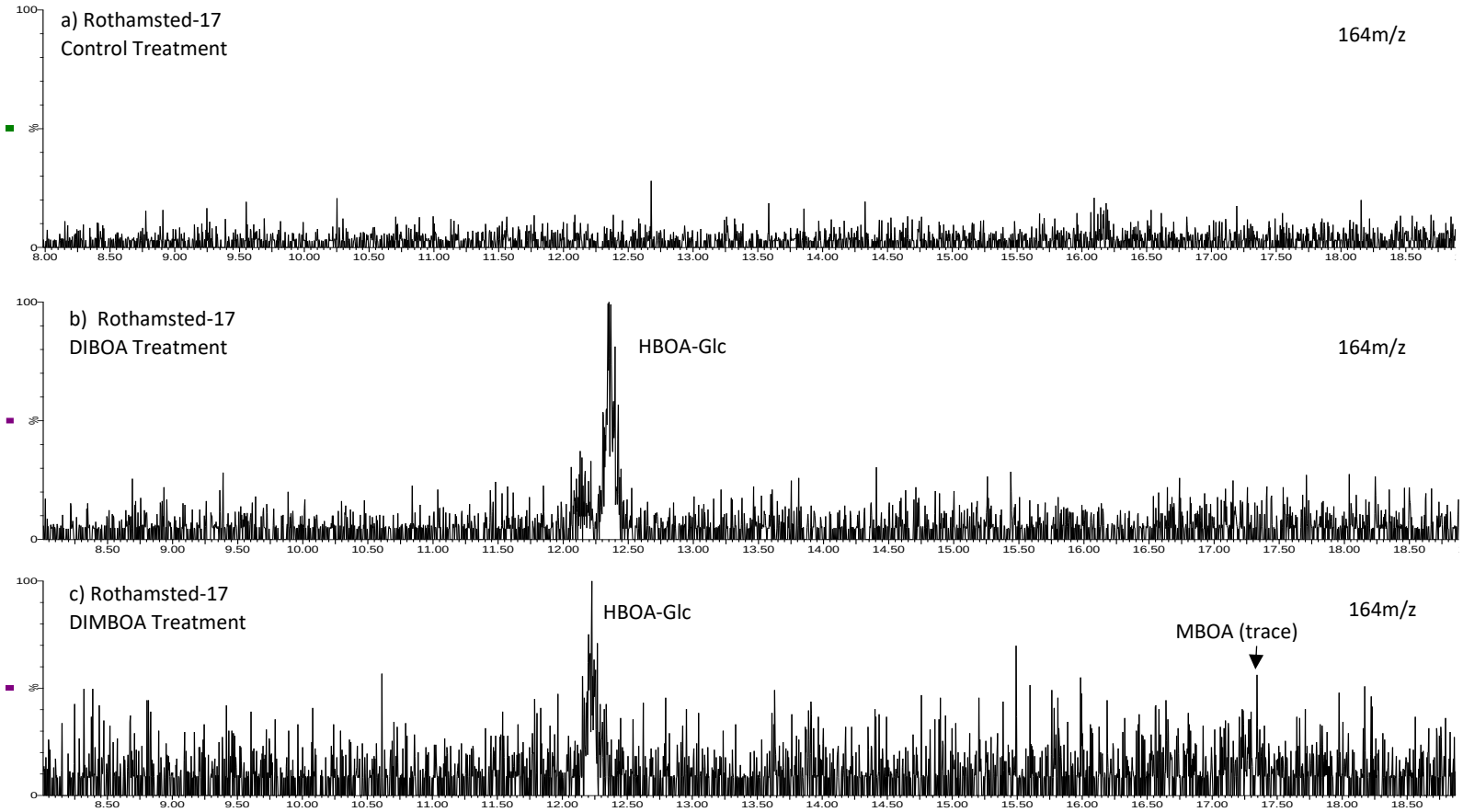

Figure 3.9: LCMS traces from Rothamsted-17 roots treated with a) the no-benzoxazinoid control DMSO treatment; b) DIBOA in DMSO treatment, and c) DIMBOA in DMSO treatment, indicative of their ability to imbibe benzoxazinoid compounds, at a frequency of 164 m/z.

3.4. Discussion

3.4.1. Crude cereal exudates: a precedent for allelopathy

Validation of benzoxazinoid allelopathy in real-world scenarios requires the demonstration of crude exudate allelopathy, comparable to the allelopathic effects of the synthesised compounds (Chapter 2) they have been suggested to contain (Pérez, 1990; Rice *et al.*, 2005), although their presence requires corroboration. The results in this chapter demonstrate that both wheat and rye root exudates are inhibitory to black-grass roots, supporting the hypothesis that allelopathy can occur in the field.

These results confirm previous, extensively documented allelopathic potential of wheat (Steinsiek, Oliver and Collins, 1982; Inderjit, Olofsdotter and Streibig, 2001; Wu *et al.*, 2001; Bertholdsson, 2010). Most pertinently to this study, Yang *et al.* (2020) outlined the potency of the Chinese winter wheat variety 'Jing411', selected for assay due to its high level of DIMBOA exudation, towards black-grass. On the other hand, the 'Gravity' cultivar was selected for these studies as a common commercial variety at time of writing, rather than any predication of allelopathic potency. It is therefore encouraging that this cultivar confers allelopathy towards black-grass, and suggests that allelopathic potential, at least to this species, is widespread across modern wheat germplasm.

Rye also has precedent as an allelopathic species. Although not consistent across all cultivars, agronomic bioassay of a range of wheat, rye, and *Triticosecale* hybrids against black-grass identified a rye cultivar, 'Dinaro' as the most allelopathic (Bertholdsson, 2012). More broadly, rye exudates are extensively reported to have broad allelopathic potential (Barnes, Putnam and Burke, 1986; Barnes *et al.*, 1987; Reberg-Horton *et al.*, 2005; Tabaglio *et al.*, 2008; Schulz *et al.*, 2013). Thus, rye is likely to be a more potent inhibitor of black-grass than wheat.

3.4.2. Ancestor wheats: range of allelopathic potential indicative of genetic bottlenecking with modern breeding

Greater defensive potential in ancestor and landrace wheats has been substantiated by works on their resistance to both aphids and fungal pathogens (Elek *et al.*, 2013; McMillan, Gutteridge and Hammond-Kosack, 2014). In holistic terms this

probably relates to greater genetic variability of these ancestors (Quader *et al.*, 2001). It is thus unsurprising that some ancestor lines have elevated allelopathic potential towards black-grass compared to commercial varieties (*e.g.* 'MDR037'), while others have more limited allelopathic capabilities (*e.g.* 'MDR043' and 'MDR049').

'MDR037' is a particularly potent ancestor line, reducing mean black-grass root length by around 70% in spite of its lack of potential for resistance to take-all or aphid herbivory (Elek *et al.*, 2013; McMillan, Gutteridge and Hammond-Kosack, 2014). Contextualising this finding, Huang *et al.* (2003) reported that fewer than 20% of the wheat varieties assayed exuded benzoxazinoids from roots despite high concentrations present in root tissues. Thus, while individual compounds may have effects on multiple biotic kingdoms, their allocation, concentration, and dynamics *in planta* vary to facilitate different defensive capabilities, which may explain the lack of correlation between allelopathic lines and capability of defence against other biotic stressors.

3.4.3. Benzoxazinoid uptake by black-grass confirmed: a vital tenet of allelopathy studies satisfied

Proof of allelochemical uptake is a typically underappreciated facet of allelopathy studies, with emphasis usually resting instead on the proof of a potent inhibitory effect. Still, Willis (1985) correctly outlines the importance of proving the presence of an inhibitory compound in the tissues of an inhibited target species as one of the six points to be satisfied in an allelopathy study. In terms of benzoxazinoids, uptake of compounds exuded from rye were recently elucidated in root tissues of hairy vetch (*Vicia villosa*), with their translocation to shoots also observed (Hazrati, Fomsgaard and Kudsk, 2020). There is thus reassuring precedent for the observation of benzoxazinoid compounds in DIMBOA and DIBOA-treated black-grass roots, which contributes to the picture of benzoxazinoid allelopathy inhibiting black-grass root development.

On the other hand, neither of these compounds were directly taken up by the target. The presence of MBOA in DIMBOA-treated plants coheres with the understood degradation pathway (Macías *et al.*, 2004), but the presence of HBOA-

Glc in both DIMBOA and DIBOA-treated roots is a curiosity given that no glucoside sugars were applied therein. The most likely explanation is that this indicates some degree of metabolism of benzoxazinoid compounds by black-grass, given that glucosides are typically explained as less toxic molecules produced for storage in the vacuole (Zhou, Richter and Jander, 2018). It is at least noted that plants which do not exude benzoxazinoids are capable of their metabolism into glucoside forms, although the recorded example of this phenomenon, in common purslane, did not involve the synthesis of HBOA-Glc (Hofmann *et al.*, 2006).

3.4.4. The limitations of a hydroponic system for elucidating allelopathy

The inconsistency of inhibitory effects of wheat root exudates on black-grass in the hydroponic Magenta vessels contrasts strongly with the consistent elucidation of significant effects in the sand system. Hazrati, Fomsgaard and Kudsk (2020) reported discrepancies in rye root exudate composition between hydroponic and sterilised soil media, indicating that potent allelochemicals may not be exuded by cereals in the hydroponic system.

Hydroponic systems can stimulate atypical root development and responses (Tavakkoli, Rengasamy and McDonald, 2010), which may be the cause of altered allelochemical exudation dynamics. A possible additional confounding factor may have been the exposure of roots to light in this system, potentially modifying their growth given that light has been identified as a stress factor in root tissues (Yokawa *et al.*, 2014). Discrepancies between systems may also be related to the dilution effects of a hydroponic system in reducing allelochemical efficacy. Such findings highlight the influence of the assay system on resident plants, in terms of allelochemical exudation, persistence and efficacy (*e.g.* Inderjit and Callaway, 2003). Moreover, it is possible that the use of RO water, a medium with no pH buffer and atypical ionic strength, may have had a detrimental influence on allelopathic interactions compared to more sophisticated growth media. Both pH and ionic strength have documented effects on root exudation and allelochemical stability (Anderson and Reilly, 1968; Scavo, Abbate and Mauromicale, 2019).

Additionally, cloudiness of hydroponic media indicated the presence of microbes, ostensibly from the endogenous community within the wheat germ, which

may have degraded the potent allelochemicals preserved in the sand system. The excision of wheat embryos has been advocated to ensure axenic conditions in such cultures (Robinson *et al.*, 2016), but this was not attempted given its intricacy and the likelihood of tissue damage altering root exudate composition. The centrifuge tube sand assay is therefore a more useful system for examining allelopathic potential under controlled, axenic conditions.

3.4.5. Black-grass: weediness is not the result of allelopathy

It is important for application of allelopathic black-grass control that the weed itself is not allelopathic. The results reported here suggest that, under the experimental conditions tested, black-grass does not display an allelopathic trait towards cereal plants (Marczewska-Kolasa, Bortniak and Domaradzki, 2010), given the absence of autotoxicity in black-grass, or allelopathy towards wheat. Although the geographic origins of black-grass are not known in detail, the species is not a recent invader to the range in which it is problematic (Naylor, 1972a), at least not in the timescales of many problematic weeds. Any putative allelochemical constituents of black-grass root exudates are therefore unlikely to be 'novel weapons' inhibitory to modern European wheat varieties, which may explain its limited allelopathic potential.

Agronomic observations and the findings of Marczewska-Kolasa, Bortniak and Domaradzki (2010) are more likely to be caused by intense resource competition. This previous study did not attempt to disentangle allelopathy from resource competition experimentally or identify putative allelochemicals associated with black-grass. This indicates the importance of using a detailed framework in scientifically determining allelopathic effects (Section 1.3.5), rather than merely reporting an unexpected inhibitory effect.

3.5. Conclusion

The findings of this chapter crucially confirm that crude wheat and rye exudates are inhibitory to black-grass root growth, while ancestor wheats demonstrate a wider range of allelopathic capability. Some lines appear more highly allelopathic than the modern variety screened, but others are ostensibly less capable. This is indicative of the greater genetic variability that these ancestor varieties have

411 in comparison to genetically bottlenecked modern varieties. It is moreover highly
412 encouraging that the uptake of benzoxazinoid allelochemicals is noted in black-grass
413 roots, as this strengthens the case for their allelopathy being the cause of observed
414 patterns in inhibition. While these results indicate that potent cereal species may be
415 useful in integrated weed management strategies against black-grass, their
416 application would be better understood through isolation and characterisation of
417 benzoxazinoid compounds in these crude root exudates.

Chapter 4

Isolation, characterisation, and germplasm differences in benzoxazinoid compounds from cereal root exudates

4. Isolation, characterisation, and germplasm differences in benzoxazinoid compounds from cereal root exudates

4.1. Introduction

Earlier chapters of this thesis indicate that benzoxazinoids, documented as allelochemicals exuded from cereal roots, are inhibitory to black-grass development (Chapter 2), and that crude wheat and rye root exudates are similarly inhibitory to the weed (Chapter 3). A vital step in understanding these effects, however, is to examine and confirm the presence of these phytotoxic compounds in the root exudates of the cereals believed to contain them. This would greatly benefit application of allelopathic crops by explaining variation in their inhibition of black-grass. To this end, it is a necessary step to analyse root exudates of a wide range of cereal germplasm (both modern and ancestor lines), and to characterise constituent compounds.

As noted in Chapter 3, there is precedent for elevated potential for secondary metabolite defences in landraces and ancestor cereal lines (Jing *et al.*, 2007; McMillan, Gutteridge and Hammond-Kosack, 2014; Simon *et al.*, 2017), as well as the developing hypothesis that these defence compounds have multi-kingdom effects against multiple biotic stresses. Such secondary metabolite defences may thus extend to allelopathy, and benzoxazinoids with documented multi-kingdom inhibitory potential represent prime candidates as causative compounds of these multiple interactions in cereals (Hickman *et al.*, 2021). As plant-plant allelopathy is a comparatively little-studied concept compared to plant defence against other biotic kingdoms, examination of germplasm with inhibitory potential towards biota outside of the plant kingdom may provide hints towards lines with allelopathic potential. Greater understanding of benzoxazinoid exudation by cereal roots would inform their application for black-grass control, given variations in potency of these compounds towards this species documented in Chapter 2.

To this end, the hypotheses of this chapter are as follows:

1. Tentatively identified benzoxazinoid allelochemicals and their degradation products will be recoverable from root exudates of wheat and rye.
2. The constituent compounds of cereal root exudates will vary by species, while ancestor and landrace wheats will vary in root exudate composition compared to modern commercial relatives, explaining variation in inhibitory effects towards black-grass in Chapter 3.

4.2. Methods

The development of axenic systems documented in Chapter 3 was intended to facilitate collection of crude root exudates for LCMS analyses, as well as the bioassay of their allelopathy detailed previously. The hydroponic system was initially used to collect root exudates (Section 3.2.1), but difficulty in consistently identifying benzoxazinoids present in root exudates necessitated efforts to innovate a more suitable system. To this end, later screening of root exudates utilised the centrifuge tube system (Section 3.2.3), which facilitated more effective elucidation of allelopathic compounds present in root exudates.

Following initial assays of wheat, rye and black-grass plants in isolation to confirm root exudate composition, mixed cultures were grown and root exudates collected to determine potential for heterospecific cultures to stimulate allelochemical exudation through allelobiosis (Li, Xia and Kong, 2016). A wide range of both modern and ancestor wheat germplasm was thereafter analysed for differences in exudation profile, which could suggest the manner in which allelochemical exudation has altered with domestication. Informed by the consistent tentative identification of its glucoside form in allelopathic cereal root exudates, HBOA (2-hydroxy-1,4-benzoxazin-3-one) was also analysed in the Petri dish system detailed in Chapter 2.

4.2.1. Magenta vessel system for root exudate collection

Treatments in the Magenta vessel hydroponic system were produced for exudate collection by growing black-grass and wheat seedlings, as had previously been set up for bioassay of crude exudates (Section 3.2.1), but without the transplant

of a second set of black-grass seedlings for bioassay. Rather, hydroponic media were retained and processed for LCMS analysis.

The treatments initially grown and analysed in this system were a no-plant control group, and groups containing 16 seedlings of 'Gravity' wheat, 16 seedlings of Rothamsted-17 black-grass, and a mixed treatment of eight seedlings of each of these species ('Gravity' wheat and Rothamsted-17 black-grass). Black-grass was included as a non-cereal control, and as an interspecific competitor in the examination of the induction of cereal benzoxazinoid exudation through allelobiosis in this hydroponic system. Seedlings were grown for fourteen days in the same conditions used for pre-germination and Petri dish assays (light/dark regime of 17°C/11°C for 14/10 hours). At the endpoint of this growth period, hydroponic media were filtered through a 0.22 µm pore-size sterile vacuum filter (Merck Millipore, Nottingham, UK), then freeze-dried to completion over five days at -50°C. Dried samples were resuspended in 500 µL of 99% HPLC-grade methanol for LCMS analyses as described in Section 3.2.5.

4.2.2. NMR analyses of DIMBOA degradation in hydroponic media (performed by Dr D. Withall, BCP Department, Rothamsted Research)

To track the degradation of DIMBOA into MBOA in hydroponic media throughout the growth period, NMR (Nuclear Magnetic Resonance) spectrometry was used. A 1 mg aliquot of synthetic DIMBOA per 100 ml of deuterium oxide was spiked into hydroponic media containing exudates from the following cultures: one each of the modern wheat, black-grass, mixed modern wheat/black-grass, and the no-plant control. Standards were also produced using the same DIMBOA solution spiked into sterile, autoclaved H_2O DI, and an equivalent MBOA solution for comparison. Insolubility of AMPO in D_2O prevented further examination of MBOA degradation into that compound.

Samples were run by AVANCE Bruker DRX-500 MHz Nuclear Magnetic Resonance spectrometer with a 5 mm BBO BB-1H probe set at 500 MHz for ^1H spectra. Bruker data were analysed using Topspin 4.0.7. A double-peak at the retention time between 6.91- 6.97 ppm was consistently found to indicate MBOA, while another between 7.26 and 7.33 ppm was indicative of DIMBOA. Thus, areas of

these two peaks were compared to determine the ratio between these compounds and track degradation. All media described were tested on days 0, 4, 7, 11, and 14, allowing comparison with the 14-day growth period of the Magenta vessel assays. The results for each of these media were assessed as the change in DIMBOA: MBOA ratio over this time course.

4.2.3. Centrifuge tube sand system for root exudate collection

Given that LCMS results from hydroponic media were inconsistent in terms of compounds identified, the centrifuge assay using axenic sand media was used to collect exudates and characterise compounds present. The initial assay of root exudate composition in this system used the same plant treatments as the Magenta assay described in Section 4.2.1, but using four seedlings for single-species treatments, two seedlings of each species for heterospecific treatments, and again no plants for the negative control. After one week of growth under the same conditions as the hydroponic assay, root exudates were collected. To prepare root exudates for LCMS analysis, tube bases were pierced with an acetone-washed scissor blade to drain. Contents were eluted with 5 ml of 70% methanol, dried completely with a Büchi R-300 rotary evaporator and resuspended in 0.5 ml of 99% methanol. Exudates were then analysed by LCMS (Section 3.2.5).

4.2.4. Further examining the influence of allelobiosis on cereal root exudate profile

Having tentatively elucidated compounds of interest in cereal root exudates, it was of interest to further examine the inducibility of allelochemical exudation in wheat, rye and black-grass. This was done by planting a more extensive set of mixed cultures of these species in the centrifuge tube system, compared to the previous examination of wheat and black-grass co-culture in the hydroponic system. Therein, eluates were collected from the cultures described above (4x 'Gravity' wheat, 4x 'Edmondo' rye, 4x Rothamsted-17 black-grass, and a no-plant control), and additionally the cultures of two wheat and two rye seedlings respectively, both grown along with two black-grass seedlings. The chromatograms produced through LCMS analysis (Section 3.2.5) were compared with those of each of these species in isolation.

4.2.5. Wheat variety screening

To build on indications of variation in benzoxazinoid exudation between modern and ancestor wheat germplasm (Section 3.3.3), a larger screen of wheat ancestor root exudates was devised. This consisted of five 'MDR'-line ancestor wheats, three of which were included in crude root exudate assays ('MDR037', 'MDR043', and 'MDR049', Section 3.2.2). Two other lines, 'MDR031' (*Triticum monococcum* var. *monococcum* x *macedonicum*, spring wheat collected from Turkey, 1931), which demonstrates some detriment to take-all establishment, and 'MDR045' (*T. monococcum* var. *vulgare*, spring wheat collected from Denmark, 1970), which is partially detrimental to aphid feeding (Jing *et al.*, 2007; McMillan, Gutteridge and Hammond-Kosack, 2014; Simon *et al.*, 2017), were also included. The variation in vulnerability of cereals to such biotic stresses has previously been related to elevated levels of benzoxazinoids (Gianoli and Niemeyer, 1998; Wilkes, Marshall and Copeland, 1999). Additionally, five Watkins lines, hexaploid landraces originally collected in the 1920s and 30s, were included; '258', '546', '624', '777', and '821'. 'Gravity' was also included as a modern control to better examine differences between ancestor and modern germplasm (Table 4.1).

Table 4.1: Wheat lines included in ancestor wheat screening for benzoxazinoid content. Lines included in previous assays are shaded in grey. A no-plant control was also included in this assay. Notes on defence capabilities provided by McMillan, Gutteridge and Hammond-Kosack, 2014; Simon *et al.*, 2017, V. McMillan (Pers. Comm.), and G. Aradottir (unpublished data).

Variety/Line	**Classification**	**Notes**
'RGT Gravity'	Modern	Modern control
'Watkins 258'	Landrace	Poor aphid resistance
'Watkins 546'	Landrace	
'Watkins 624'	Landrace	Aphid resistant
'Watkins 777'	Landrace	Take-all resistant
'Watkins 821'	Landrace	
'MDR031'	Ancestor	Take-all resistant
'MDR037'	Ancestor	Ancestor control; poor defence
'MDR043'	Ancestor	Take-all resistant
'MDR045'	Ancestor	Aphid resistant
'MDR049'	Ancestor	Aphid resistant

Ten modern wheat varieties were separately assayed in the same manner, all of which were previously tested in the screen of wheat sensitivity to DIMBOA and DIBOA (detailed in Section 2.2.5). These varieties were 'Skyscraper', 'Spotlight',

'Firefly', 'Siskin', 'Zyatt', 'Cadenza', 'Gleam', 'Graham', 'Costello', and 'Gravity' as a control culture included in all screens. Additionally, 'MDR037' was included as an ancestor control to confirm consistency of results. All exudates were collected and refined using the system and methods detailed in Section 3.2.5.

4.2.6. HBOA Petri dish assay

Following the consistent but tentative identification of HBOA-Glc (Figure 4.1a) in LCMS analyses, it was desirable to gain some understanding of its phytotoxicity. HBOA aglucone (Figure 4.1b) was screened against Rothamsted-17 black-grass in the Petri dish system described in Section 2.2.2 at doses of 12.5 µM, 25 µM, 50 µM, 100 µM, 200 µM, 400 µM, 800 µM, and a control containing no compound. This compound was again synthesised by Dr. David Withall of the BCP department, Rothamsted Research. It was originally envisioned that HBOA-Glc and DIBOA-Glucoside (2-*β*-D-glucopyranosyloxy-4-hydroxy-1,4-benzoxazin-3-one, DIBOA-Glc) would be tested, but difficulties in synthesis related to restrictions as a result of the Covid-19 pandemic meant that these compounds could not be examined. In rationalisation of the examination of HBOA specifically, it is believed that glucosides degrade too quickly *ex planta* to be major allelochemicals in their own right (Macías, Oliveros-Bastidas, *et al.*, 2005), and are typically similarly allelopathic to their corresponding aglucones (Macías, Marín, *et al.*, 2005). As previously in Petri dish assays, this compound was dissolved in 0.25% DMSO.

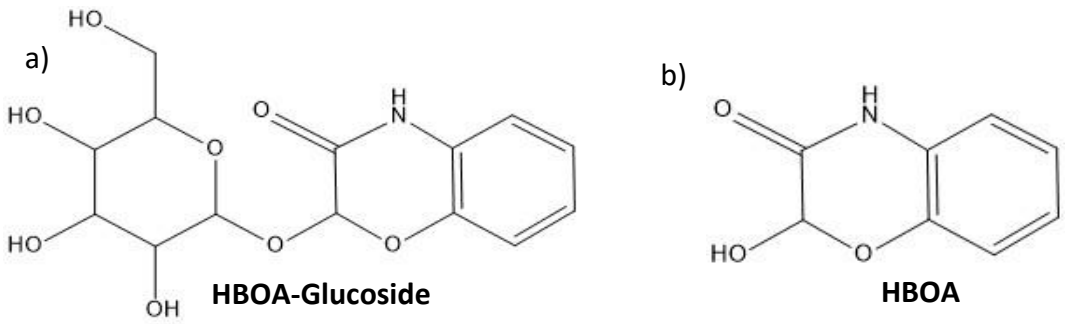

Figure 4.1: Structural formulae of a) HBOA-Glucoside and b) HBOA aglucone.

4.3. Results

4.3.1. Benzoxazinoids are present in cereal root exudates

Root exudates from the Magenta hydroponic system were processed and initially analysed for comparison between different species. Multiple chromatographic peaks relating to benzoxazinoid compounds were identified in root exudates of wheat and rye (Figure 4.2). Predominant amongst those in rye were tentatively identified as DIBOA-Glc and HBOA-Glc (retention time for both 11.60 - 12.00 minutes). Rye therefore appeared to exude mostly DIBOA-related compounds, although HMBOA-Glucoside (2- *β*-D-glucopyranosyloxy-7-methoxy-1,4-benzoxazin-3-one, HMBOA-Glc) was also tentatively identified.

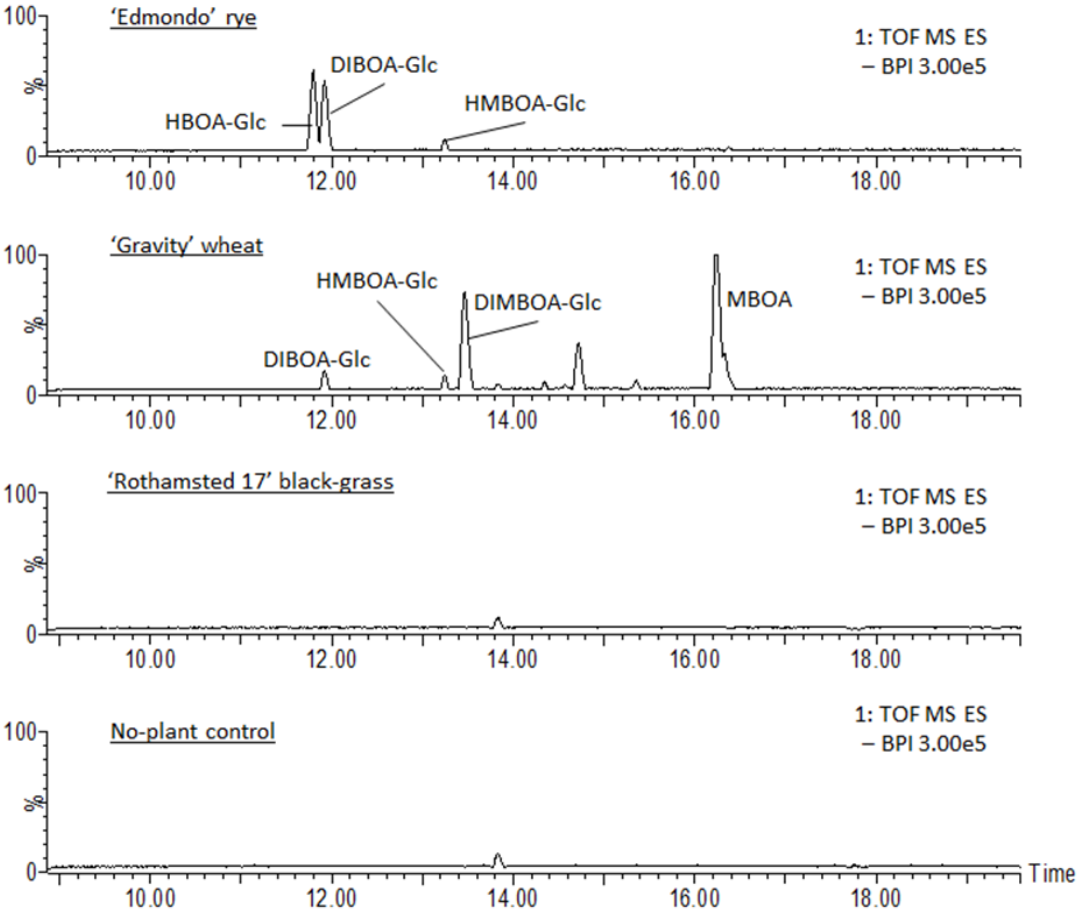

Figure 4.2: LCMS traces of rye, wheat, and black-grass exudates and no-plant controls, with annotations of tentative identifications. Of note, the predominant benzoxazinoid compounds exuded from rye were related to DIBOA, while those exuded by wheat were predominantly related to DIMBOA. Benzoxazinoids were not identified in black-grass or control treatments.

The composition of modern wheat root exudates differed markedly. Although HMBOA-Glc and DIBOA-Glc were again tentatively identified, provisional detection

of DIMBOA-Glucoside (2-*β*-D-glucopyranosyloxy-4-hydroxy-7-methoxy-1,4-benzoxazin-3-one) and MBOA (retention times of 13.40 – 13.60 minutes and 16.10 – 16.60 minutes respectively) was also noted, suggesting DIMBOA-related compounds as the primary benzoxazinoids exuded. Benzoxazinoids were not detected in either black-grass or the control culture, as hypothesised.

4.3.2. The presence of MBOA is an indicator of DIMBOA exudation

NMR analyses were used to track the degradation of DIMBOA to MBOA in the hydroponic media used to produce the chromatographic traces in Figure 4.2. The relative presences of the MBOA peak at 6.91- 6.97 ppm, and the DIMBOA peak at 7.26- 7.33 seconds (visible in Figure 4.3) indicate that degradation of DIMBOA to MBOA over the time period was likely in the hydroponic system. In the DIMBOA standard, no change was apparent between Day 0 and Day 7, and no MBOA was formed, evident by the lack of an MBOA peak. In the hydroponics system, over the same time period, a peak corresponding to MBOA had developed (the doublet at 6.91 ppm). All hydroponic media stimulated degradation in comparison to the RO water standard (Figure 4.4). Strikingly, the ratio of MBOA to DIMBOA increased in the wheat medium, then decreased again, before increasing substantially to the final time point. This may be the result of the MBOA to AMPO conversion step occurring at this late stage, which would effectively decrease the MBOA: DIMBOA ratio.

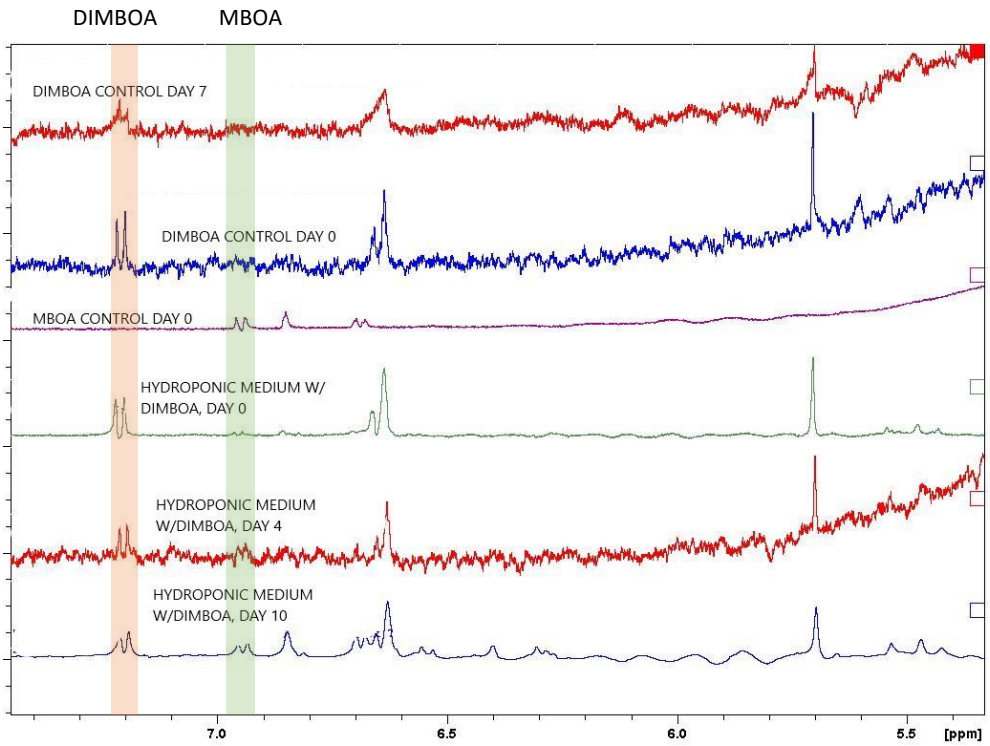

Figure 4.3: NMR traces from the preliminary assay containing peaks for both DIMBOA and MBOA. Note DIMBOA peak at 7.26-7.33 and MBOA double-peak at 6.91-6.97 ppm (denoted by the labelled orange and green lines of highlighting) which were used as indicators; the 'hydroponic medium w/DIMBOA' traces indicate a degree of conversion from DIMBOA to MBOA over time. Traces are coloured to distinguish between treatments, and peaks are depicted in terms of magnitude on a one-dimentional scale.

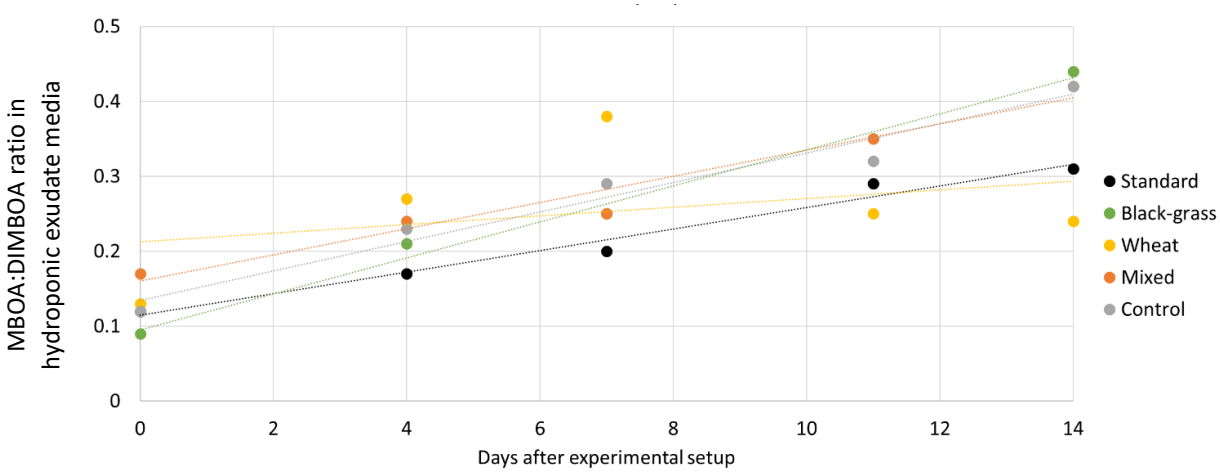

Figure 4.4: MBOA: DIMBOA ratio following spiking of DIMBOA into Magenta vessel hydroponic exudates, and also in the sterile standard solution. The 'standard' treatment consisted of DIMBOA in autoclaved RO water, while all others denote the hydroponic root exudate treatment, with 'mixed' indicating a wheat/ black-grass co-culture.

4.3.3. Black-grass presence does not alter benzoxazinoid exudation

Initial LCMS analyses of root exudates collected from the sand centrifuge tube system tentatively identified compounds consistent with those found in the hydroponic system, but the greater consistency of exudate profiles between replicates suggested the centrifuge tube system to be preferable for further exudate

collection. Further LCMS analysis of root exudates from mixed cereal/black-grass cultures in the sand centrifuge system suggested that the presence of black-grass as a competing weed has no clear effect on benzoxazinoid exudation from cereals. There was no variation in LCMS profile where plants were grown in combination compared to in isolation.

For rye exudates, both in isolation and in co-culture with black-grass, HBOA-Glc, DIBOA-Glc, HMBOA-Glc and DIMBOA-Glc were tentatively identified (Figure 4.5). Non-benzoxazinoid compounds were found but not identified, as these compounds were consistent with the exudate traces of these species in isolation and therefore unlikely to be related to interspecific signalling interactions.

Modern wheat root exudates similarly did not alter in composition with the presence of black-grass (Figure 4.6). The compounds provisionally identified in wheat root exudates were HBOA-Glc, DIBOA-Glc, HMBOA-Glc, DIMBOA-Glc, although again concentrations of these compounds were not discerned. All peaks found in mixed culture corresponded to either wheat or black-grass root exudates in isolation, again indicating that interspecific biochemical communication is limited between black-grass and allelopathic cereals.

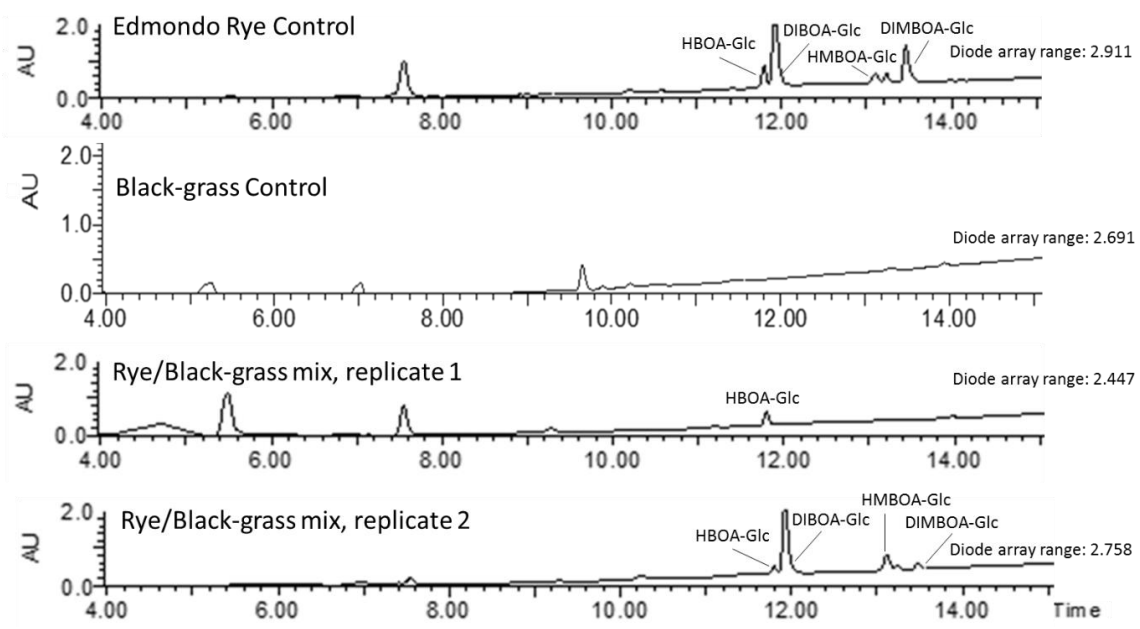

Figure 4.5: Annotated LCMS chromatograms of 'Edmondo' rye, black-grass, and two replicates of a mixed rye/black-grass culture as indication of the variation in compound exudation that can occur.

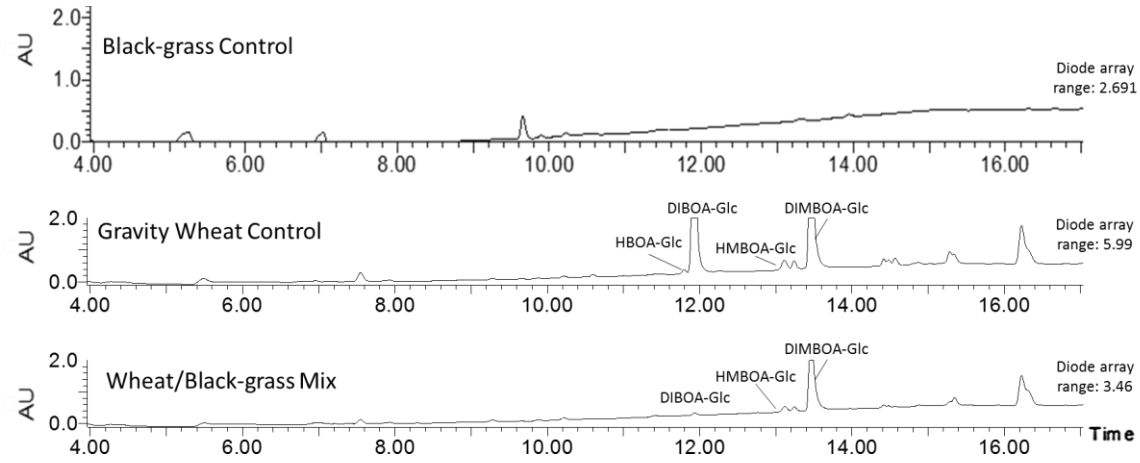

Figure 4.6: Annotated LCMS chromatograms of 'Gravity' wheat, black-grass, and a mixed culture of the two species.

4.3.4. Wheat ancestor screening

Further LCMS analyses (Figure 4.7) found consistency in 'Gravity' wheat root exudate composition with previous results (Figure 4.1), provisionally identifying DIBOA-Glc, HMBOA-Glc, DIMBOA-Glc and MBOA. Given the enhanced clarity of exudates collected from the sand centrifuge system, however, this repeat assay also tentatively identified HBOA-Glc and BOA. By contrast, the only benzoxazinoid compounds tentatively identified in 'MDR037' root exudates were DIBOA-Glc and HBOA-Glc. Although not presented in the figure for brevity, the other ancestor wheats assayed, 'MDR043' and 'MDR049' produced exudates with similar profiles, suggesting that they exuded the same benzoxazinoid compounds as 'MDR037'. This indicates a variation in root exudate composition between modern and ancestor wheats.

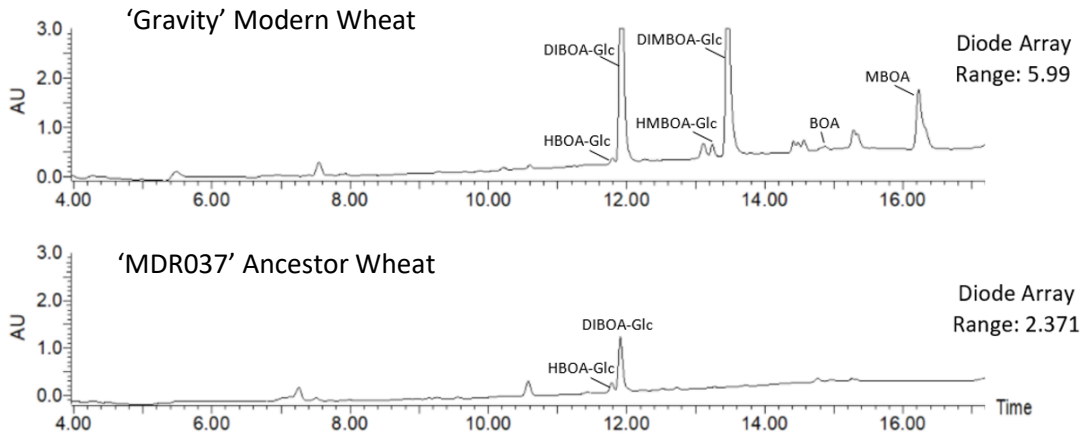

Figure 4.7: A comparison of LCMS chromatographs from root exudates of 'Gravity' modern wheat (top) and the potent allelopathic ancestor wheat 'MDR037' (above), annotated with tentative identifications of peaks.

Wider screening of ancestor wheats and landraces further indicated variations in root exudation within wheat germplasm as suggested by data from Chapter 3, with differences between 'MDR'-line ancestor lines, and more modern germplasm, including landraces.

Exudates of 'MDR'-line ancestors generally contained only four tentatively identified benzoxazinoid compounds: DIBOA-Glc, DIBOA, BOA, and HBOA-Glc (Table 4.2). Thus, DIMBOA-related compounds were not detected in ancestor wheat root exudates. Watkins landraces (denoted by 'W' in Table 4.2) were believed to exude a comparable diversity of benzoxazinoids to the modern cultivar 'Gravity' (specifically the tentatively identified MBOA, HMBOA-Glc and DIMBOA-Glc in addition to the compounds provisionally identified in 'MDR'-line root exudates). Exceptions occurred, however, such as failure to detect DIBOA in 'MDR031' exudates, or DIBOA and DIBOA-Glc from some Watkins line exudates. 'Gravity' appeared to exude the most diverse range of benzoxazinoids of those tested here. For further detail, comparative chromatograms of 'Gravity' (modern), 'MDR045' (ancestor), and 'Watkins 258' (landrace), are depicted in Figures 4.8, 4.9, and 4.10. respectively.

Table 4.2: Presence/ absence of tentatively identified benzoxazinoid compounds in root exudates of wheat lines and cultivars used in wheat ancestor screening. A question mark indicates an inconclusive result.

	Gravity	MDR031	MDR037	MDR043	MDR045	MDR049	W258	W546	W624	W777	W821
MBOA	Y	N	N	N	N	N	Y	Y	Y	Y	Y
BOA	Y	Trace	Trace	Trace	Y	Trace	Y	Trace	Y	Trace	N
DIBOA	Y	N	Trace	Y	Y	Y	Y	N	Y	N	N
DIMBOA	?	N	N	N	N	N	N	N	N	N	N
DIBOA-Glc	Y	Y	Y	Y	Y	Y	Y	N	Y	N	N
HBOA-Glc	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
HMBOA-Glc	Y	N	N	N	N	N	Y	Y	Y	Y	Y
DIMBOA-Glc	Y	N	N	N	N	N	Y	N	Y	Y	Y

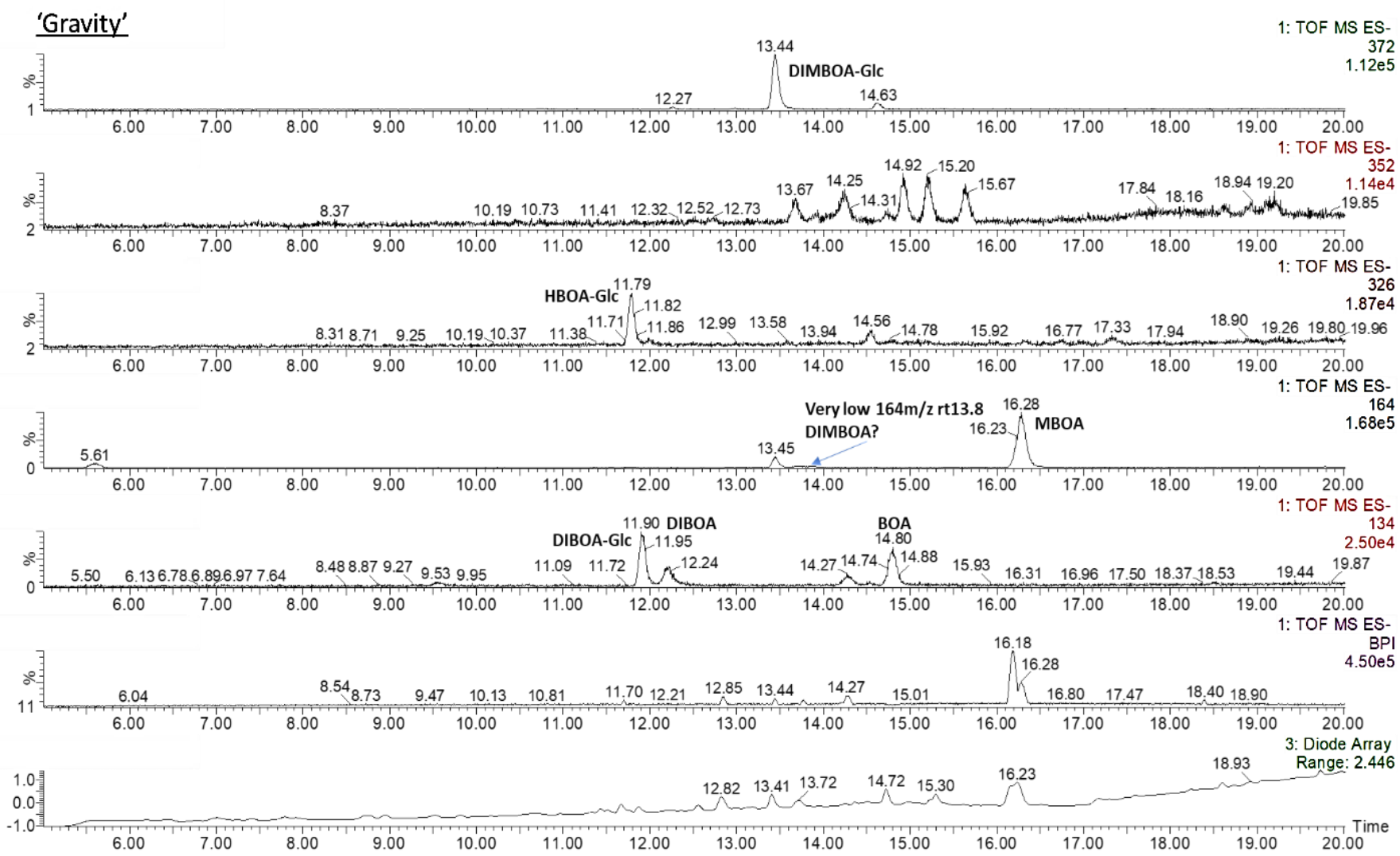

Figure 4.8: LCMS chromatograms of 'Gravity' wheat root exudate under a variety of intensities, annotated with tentative identifications of all compounds of interest found.

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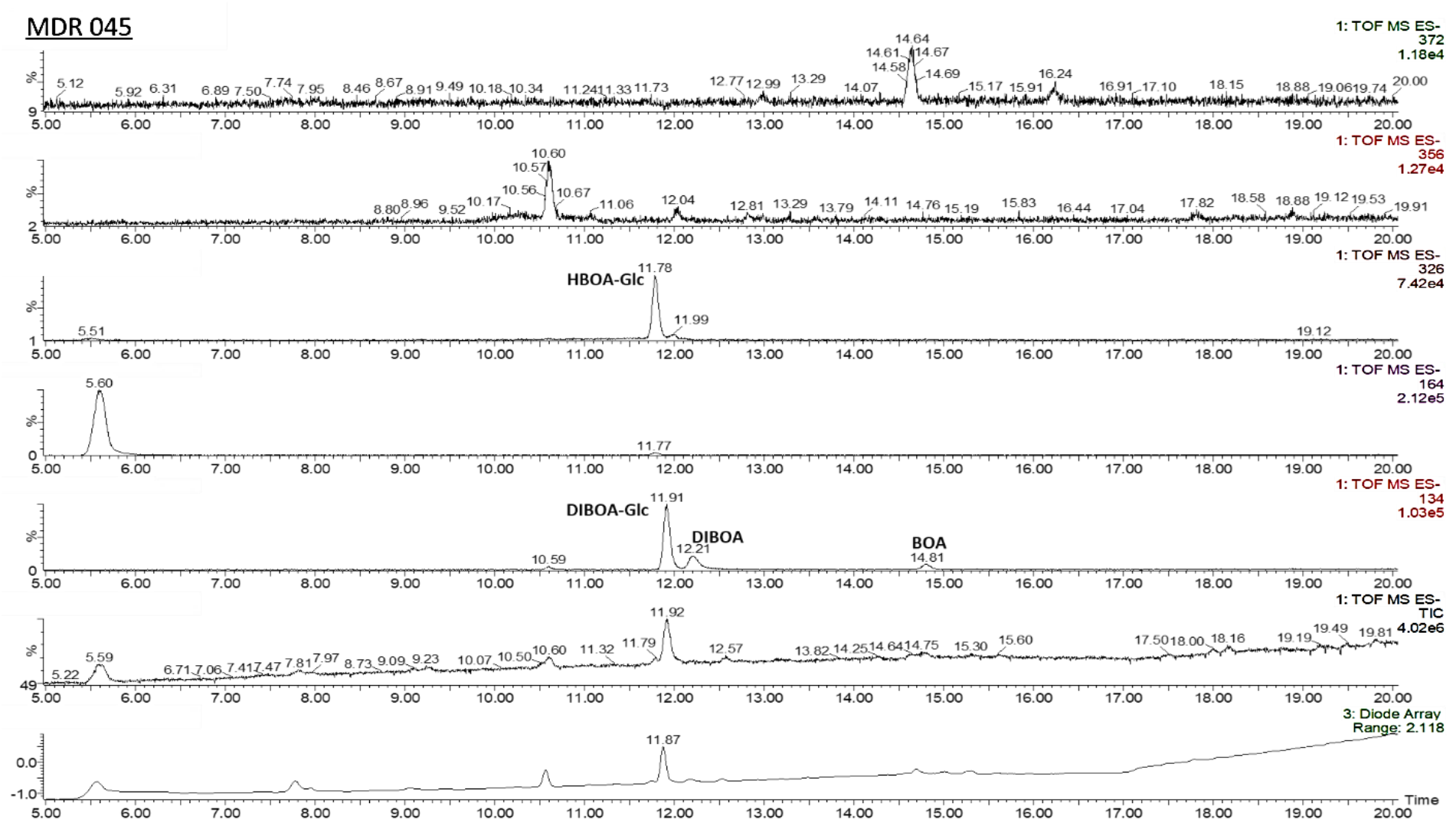

Figure 4.9: LCMS chromatograms of 'MDR045' ancestor wheat root exudate under a variety of intensities, annotated with tentative identifications of all compounds of interest found.

277

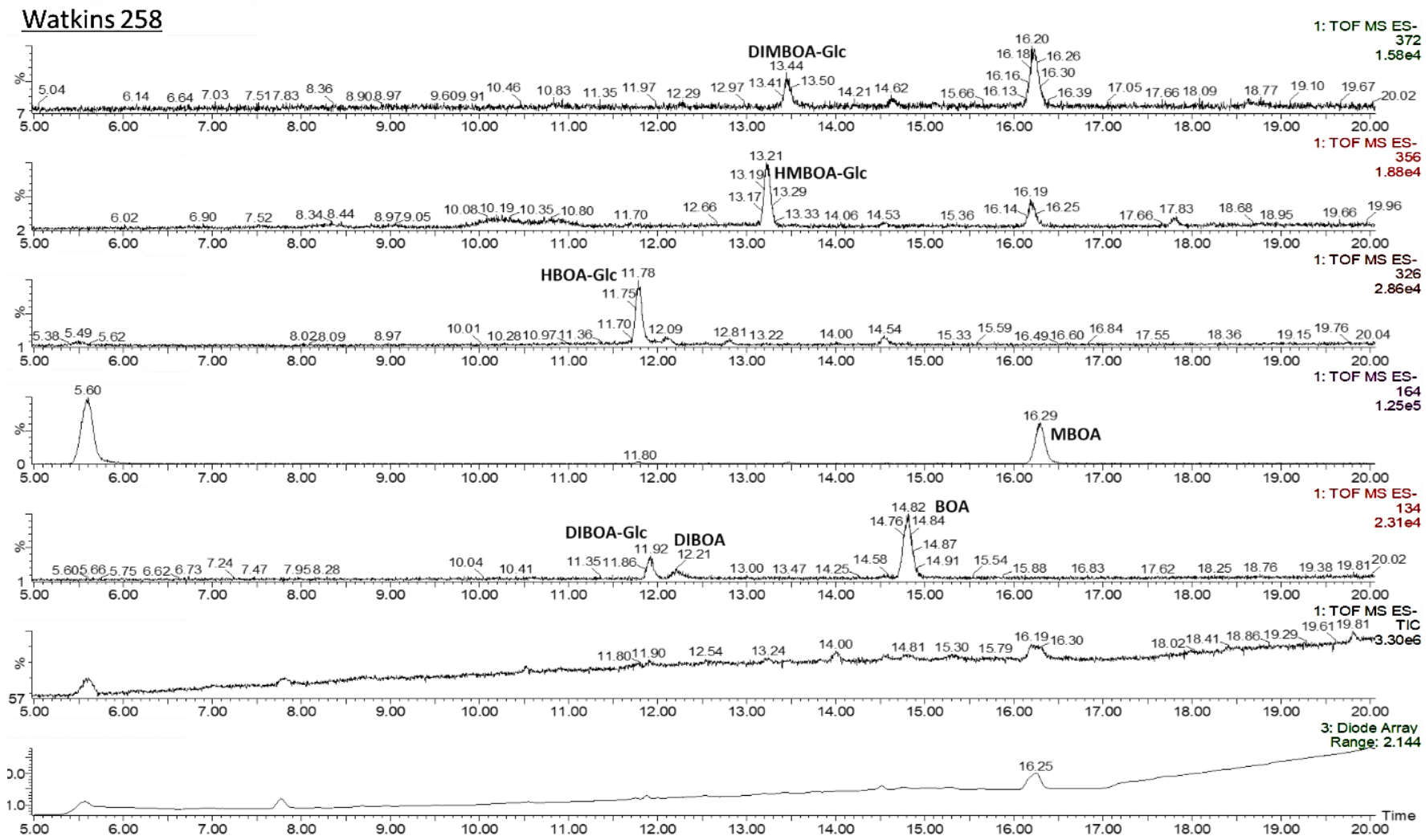

Figure 4.10: LCMS chromatograms of 'Watkins 258' landrace wheat root exudate under a variety of intensities, annotated with tentative identifications of all compounds of interest found.

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4.3.5. Commercial wheat screening

Less variation in root exudate profile was observed from commercial wheats compared to ancestors (Table 4.3). All varieties exuded compounds provisionally identified as MBOA, HBOA-Glc, HMBOA-Glc, HDMBOA-Glucoside (2-(2-hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one)-*β*-D-glucopyranose, HDMBOA-Glc), and trace quantities of BOA. The only noted differences were the absence of the tentatively identified DIBOA-Glc and DIMBOA-Glc from exudates of 'Gleam', and the additional absence of the latter compound from 'Gravity' and 'Siskin'. As with previous results, DIMBOA-Glc was not detected in root exudates of the ancestor line 'MDR037', which was also the only tested biotype to produce exudates where the provisionally identified HDMBOA-Glc was not detected. No DIBOA or DIMBOA aglucones were detected in any wheat root exudates screened in this experiment.

Table 4.3: Presence/ absence of tentatively identified benzoxazinoid compounds in root exudates of wheat lines and cultivars used in commercial wheat screening.

	MDR037	Cadenza	Costello	Gleam	Graham	Gravity	Siskin	Skyscraper	Spotlight	Zyatt
MBOA	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
BOA	TRACE	TRACE	TRACE	TRACE	TRACE	TRACE	TRACE	TRACE	TRACE	TRACE
DIBOA	N	N	N	N	N	N	N	N	N	N
DIMBOA	N	N	N	N	N	N	N	N	N	N
DIBOA-Glc	Y	Y	Y	N	Y	Y	Y	Y	Y	Y
HBOA-Glc	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
HMBOA-Glc	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
DIMBOA-Glc	N	Y	Y	N	Y	N	N	Y	Y	Y
HDMBOA-Glc	N	Y	Y	Y	Y	Y	Y	Y	Y	Y

4.3.6. HBOA Petri dish assay

HBOA treatment of black-grass in Petri dishes resulted in dose-dependent inhibition of root growth (Figure 4.11). Specifically, the reduction in root length between 50 and 100 µM was relatively great, and greater doses were similarly inhibitory. For this reason, the ED_{50} value for root inhibition from this compound was calculated as 71.074 µM. Consistent with previously screened benzoxazinoid compounds, HBOA had no obvious inhibitory effects on black-grass shoot length within the dose range screened (Figure 4.12).

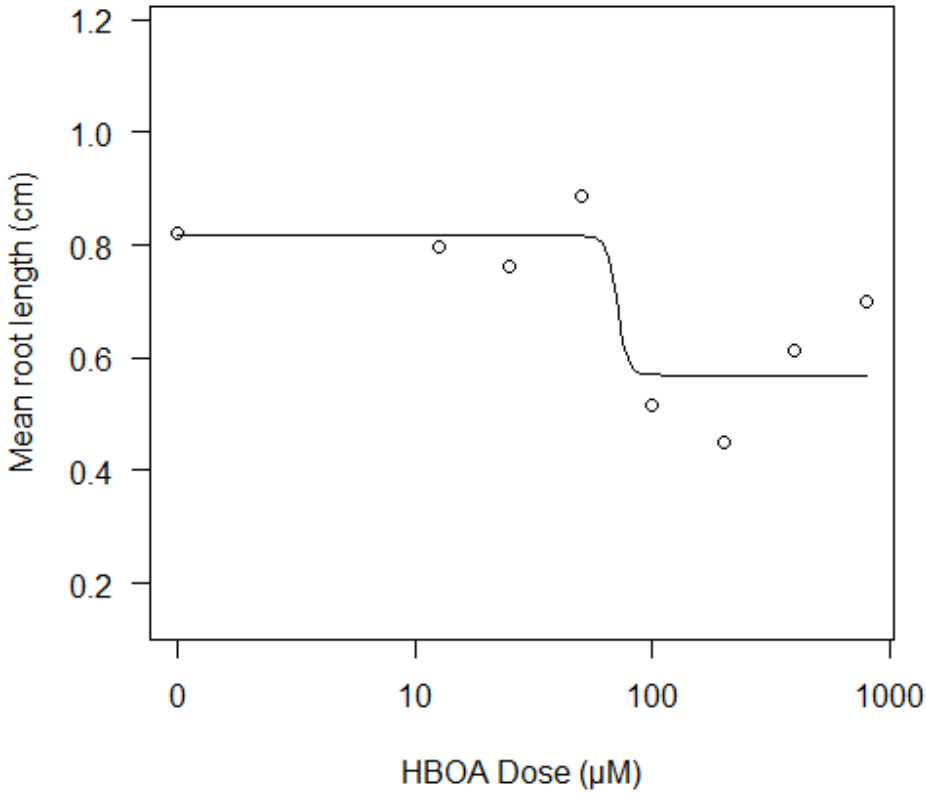

Figure 4.11: Dose-response curves of Rothamsted-17 black-grass root growth under treatment with HBOA doses depicted on log scale. Plate replicates= 6, individual seeds per plate= 8, curve produced using the 'drc' package in R.

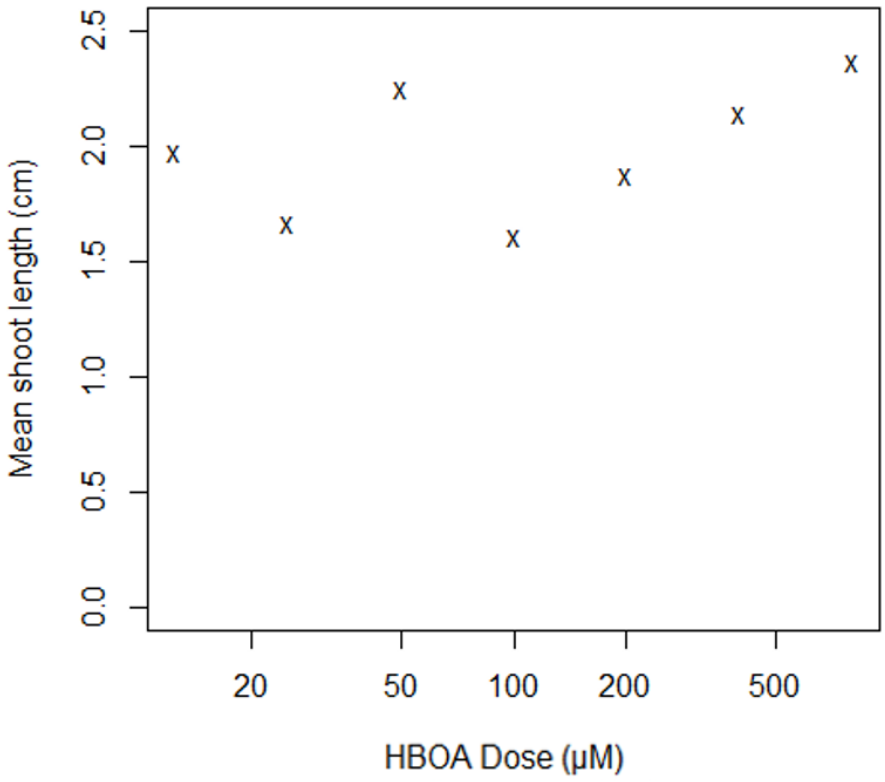

Figure 4.12: Variation in Rothamsted-17 black-grass shoot growth under treatment with HBOA doses depicted on log scale. Plate replicates= 6, individual seeds per plate= 8, produced using R Studio.

4.4. Discussion

The results in this chapter contribute several key findings to understanding black-grass inhibition through benzoxazinoid allelopathy. Wheat and rye do indeed exude compounds tentatively identified as benzoxazinoids as constituents of their root exudates. This suggests promise for weed suppression through application as companion or cover crops, corroborated by the allelopathy of these crude root exudates against black-grass (Chapter 3). The tentative identification of benzoxazinoid glucosides within these cereal root exudates is contradictory to traditional understanding of cereal root exudation, so these compounds may be of greater importance to allelopathy than previously believed. The variation in compounds exuded by rye and wheat germplasm also offers some indication of the variation of benzoxazinoid synthesis with domestication and polyploidy. The presence of black-grass does not appear to stimulate the exudation of additional allelochemical compounds from wheat, indicating a limited allelobiotic effect which may partially explain the prevalence of black-grass in cereal fields.

4.4.1. Benzoxazinoid exudation by wheat and rye, and the question of glucosides

Both wheat and rye appear to exude benzoxazinoid compounds examined in Chapter 2, as well as related compounds. The most consistently found of these in wheat exudates was tentatively identified as MBOA. NMR analyses of hydroponic media in Section 4.3.2 confirm that DIMBOA degrades into MBOA throughout the growth period in this system, suggesting that DIMBOA, or possibly DIMBOA-Glc, is in fact the exuded compound. The recovery of MBOA from wheat growth media has been documented previously (Belz and Hurle, 2005; Chen *et al.*, 2010; Lu *et al.*, 2012), but has rarely been explicitly related to extensively reported DIMBOA exudation (Wu *et al.*, 2001; Macías, Marín, *et al.*, 2005; S.-Z. Zhang *et al.*, 2016). NMR analyses therefore contribute to understanding of these compounds by confirming that degradation of this compound is the most logical source of what is believed to be MBOA in these extracts.

Rye exudates collected from both the hydroponic and centrifuge tube systems included the tentatively identified DIBOA. This corroborates previous

findings that DIBOA and derivatives are the primary benzoxazinoid allelochemicals in rye, which is therefore distinct from DIMBOA-exuding cereals (Pérez and Ormeño-Nuñez, 1991; Burgos *et al.*, 2004). Moreover, DIBOA and its derivatives are commonly reported as the primary cause of the documented greater allelopathy of rye compared to other cereals (Barnes, Putnam and Burke, 1986; Barnes *et al.*, 1987; Burgos and Talbert, 2000; Burgos *et al.*, 2004).

Exudates also contained compounds tentatively identified as DIBOA-Glc, HBOA-Glc, and HMBOA-Glc in rye, and DIBOA-Glc, HBOA-Glc and DIMBOA-Glc in wheat. Existing literature usually describes these glucosides as storage compounds, preventing autotoxic effects of their constituent benzoxazinoids, and deglucosylation is presented as a precursor to exudation (von Rad *et al.*, 2001; Macías *et al.*, 2009). As such, the exudation of glucosides would not be expected and thus, their apparent observation in root exudates throughout this chapter is an unexpected result. A recent study, however, similarly found HMBOA-Glc and HBOA-Glc in crude rye exudates collected from soil, and DIMBOA-Glc and DIBOA-Glc at greater concentrations than their aglucone forms (Hazrati, Fomsgaard and Kudsk, 2020). Timelier sample collection was offered as a potential explanation in this study, although the methodology used in the present study was less rapid. It is also unlikely that tissue damage by 70% methanol during elution could have elicited leakage of glucosides from vacuoles, as Pétriacq *et al.* (2017) reported no increase in electrolyte leakage from *Arabidopsis* roots between elution with 0, 50, and 95% methanol solutions. The present study also eluted exudates prior to disturbance of plants, preventing any stimulation of glucoside exudation through tissue injury (Pentzold *et al.*, 2014). The findings presented in this chapter therefore corroborate the identification of glucosides from cereal root exudates by Hazrati, Fomsgaard and Kudsk (2020).

The fact that the provisionally identified compounds DIBOA-Glc and HBOA-Glc were noted in phytotoxic root exudates vindicates the need to examine these compounds specifically. Little information exists concerning the bioactivity or degradation of HBOA-Glc, or HMBOA-Glc, another compound found in cereal root exudates, but DIBOA-Glc has been more extensively studied. Its effects appear comparable to DIBOA aglucone in tomato inhibition (both at a dose of 500 µM), and

slightly more potent towards onion (inhibitory at 10 µM compared to 100 µM for the aglucone) (Macías, Marín, *et al.*, 2005). Yenish *et al.* (1995) determined that in-field weed suppression by rye residue was closely associated with DIBOA-Glc content, although this is contextualised by its rapid degradation into DIBOA aglucone (*e.g.* Macías, Oliveros-Bastidas, *et al.*, 2005). In lettuce, glucosylation was detrimental to DIBOA phytotoxicity, with DIBOA-Glc not allelopathic, even mildly stimulatory at 1000 µM, while DIBOA aglucone was allelopathic at 500 µM (Macías, Marín, *et al.*, 2005). Moreover, the apparently short half-life of DIBOA-Glc (around 24 hours) suggests that it cannot be a major factor in cereal allelopathy (Macías, Oliveros-Bastidas, *et al.*, 2005). On the other hand, degradation could provide additional benefits, potentially contributing glucosyl sugars to stimulate the development of a benign rhizospheric microbial community, in addition to the DIBOA aglucone allelochemical.

That HBOA aglucone is similarly allelopathic to DIBOA is corroborative of the inhibitory effects of crude cereal root exudates which were believed to contain glucosides of these compounds. Their additive, or even synergistic effects are indeed the most likely cause of the observed root inhibition of black-grass. The observed allelopathy of HBOA towards black-grass is striking given that none of the species screened by Macías, Chinchilla, *et al.* (2005) or Macías *et al.* (2006) (wheat, onion, tomato, cress, rigid ryegrass, or wild oat) were significantly inhibited by the compound below 1000 µM. It therefore appears that black-grass is atypically sensitive to this compound, a finding which could be exploited for its control.

4.4.2. The long-standing quandary of benzoxazinoid concentration in cereal root exudates

It was not possible to quantify the benzoxazinoid concentrations within cereal root exudates due to time lost resulting from Covid-19 restrictions. This is a limitation of the work undertaken in this chapter, especially as naturally-occurring concentrations of these compounds have only been examined within plant tissues to date. Concentrations of benzoxazinoids *in planta* are expected to vary greatly. Niemeyer (1988) initially presented benzoxazinoid concentrations between 0.21 and 16.0 mM Kg^{-1}, while a narrower range between 1.4 and 10.9 mM Kg^{-1} was later

suggested (Copaja, Niemeyer and Wratten, 1991). In Polish wheat germplasm, DIMBOA similarly varied in concentration *in planta* between 0.19 and 9.46 mM Kg^{-1} (Stochmal *et al.*, 2006). The same study reported low concentrations of MBOA, BOA and DIBOA (Stochmal *et al.*, 2006), in partial corroboration of findings in this chapter. A narrower range of benzoxazinoid concentrations, between 1.14 and 2.37 mM Kg^{-1}, has also been reported since (Escobar and Niemeyer 1993).

Crucially, though, such works have only examined benzoxazinoid concentrations *in planta*, of limited applicability to the present study given the obvious difference between accumulation and exudation (*e.g.* Li *et al.*, 2015). Inconsistencies in root tissue concentrations from existing literature ensure that this remains a curious aspect of cereal allelopathy. Where DIMBOA content within wheat roots has been examined, concentrations between 3 µM and 7 µM per gram of dry weight were documented, depending on growing conditions (S.-Z. Zhang *et al.*, 2016). These values require further validation, but if correct they are much lower than the ED_{50} values for DIMBOA determined in Chapter 2. This apparently low concentration of exuded benzoxazinoids is inconsistent with crude exudate phytotoxicity, however (Section 3.3.2). Specifically, the effects of crude wheat root exudates in this section are comparable to the ED_{50} of DIMBOA (Section 2.3.1), the prime allelochemical in the species (*e.g.* Zhang *et al.*, 2016). Moreover, crude rye exudate follows the effects observed in DIBOA around the ED_{50} concentration (Section 2.3.2), again salient with the presence of this compound therein (Belz and Hurle, 2005). An alternative, and currently unutilised approach to quantifying benzoxazinoid concentrations may be to use stable isotope tracers such as ^{13}C to track their exudation and activity in the rhizosphere, as has been undertaken in examining plant-microbe interactions and the fate of minerals in soil (Prosser, Rangel-Castro and Killham, 2006; Pett-Ridge and Firestone, 2017; Pausch and Kuzyakov, 2018). In any case, breeding, genetic upregulation, or treatment of allelopathic plants would promote exudation of these compounds at greater concentrations.

4.4.3. Allelobiosis and inducibility in crop-weed interactions: a cryptic influence

The work presented here suggests that wheat does not alter its exudation profile in response to black-grass presence, at least in terms of the compounds present. This is contradictory to Li, Xia and Kong's (2016) findings that wheat is capable of detecting competing weeds, and modifying exudation dependent on their density and identity. Further work identified (-)-loliolide and jasmonic acid, known stress indicators, in the exudates of such weed competitors, which indicates their role in inducing benzoxazinoid exudation in wheat (Kong *et al.*, 2018). No such indicator compounds were identified in the present study, although their short-lived nature impedes their elucidation. Elsewhere, the presence of competitor root exudates in growth media have been proven to alter the growth habit of a plant as it acts to stifle or evade its neighbour (Semchenko, Saar and Lepik, 2014). Wheat response to black-grass may therefore be limited due to the sparse exudation profile of the target species (Figure 4.4), which also rationalises the lack of crude exudate allelopathic effects from black-grass specifically (Section 3.3.4). Muted signalling to the rhizosphere may have even evolved as a counter-defence against allelopathy, preventing detection and therefore an elevated allelopathic response. The inconsistency of cereal root exudate profiles indicates that allelochemical exudation is highly variable (*e.g.* Quader *et al.*, 2001), the minutiae of which remain unexamined.

4.4.4. Allelopathic potential in ancestor wheat germplasm: what has changed with selection?

The compounds in ancestor wheat root exudates vary in comparison to modern wheats (Table 4.2). The apparently fewer benzoxazinoid compounds exuded by ancestral lines is likely to relate to their lower ploidy, with *T. monococcum* being diploid rather than hexaploid like modern *T. aestivum* (Jing *et al.*, 2007). It is crucially DIBOA-Glc and HBOA-Glc that were tentatively identified in root exudates of ancestors, rather than DIMBOA, so it can be theorised that the absence of DIMBOA-related compounds in *T. monococcum* results from the inability to convert DIBOA to DIMBOA *in planta*. The genes conferring this conversion, either the *Bx6* or *Bx7* gene

(Jonczyk *et al.*, 2008), or at least their expression, may thus have evolved in wheat with domestication. Transcriptome analysis of benzoxazinoid synthesis genes through RNA-seq performed on actively growing ancestor wheats, similar to the work of Tzin *et al.* (2015) on aphid-infested maize plants, would facilitate the drawing of more definitive conclusions. A larger range of exuded benzoxazinoid compounds, and therefore more general defences, may thus be conferred by modern wheat. Given documented stimulation of benzoxazinoid synthesis in modern wheat with colchicine treatment to induce polyploidy (Oliveros-Bastidas *et al.*, 2018), it is logical that this factor affects defence in the species. There appears to be a trade-off between the diversity of compounds, and the quantity of the potent black-grass inhibitor DIBOA synthesised, which has consequently reduced the potential for modern wheat to inhibit black-grass. The suite of benzoxazinoids exuded by modern wheats may inhibit a wider range of target species, which could even have evolved to circumvent the tolerance of some species to a specific benzoxazinoid compound, given the species-specific sensitivity of different target species towards these compounds (examined in detail in Chapter 2). The exudation of DIBOA by some ancestor wheats could make them more effective inhibitors of black-grass specifically.

As hypothesised, the root exudate composition of modern commercial wheat cultivars was relatively consistent given the limited genetic variability and regular use of common ancestors in modern breeding programmes (Haudry *et al.*, 2007). That said, there appears to be some variation, indicative of there at least being varieties better than others for benzoxazinoid-induced suppression of a weed. There was, interestingly, no detection of DIMBOA-Glc in exudates from 'Gleam', 'Gravity', or 'Siskin', but past work has noted the presence of HDMBOA-Glc as an indicator of DIMBOA-Glc presence *in planta* (Oikawa *et al.*, 2001). Therefore, failure to detect DIMBOA-Glc or HDMBOA-Glc in 'MDR037' would cohere with previous conclusions on the inability of ancestor wheats to exude DIMBOA-related compounds. The provisional presence of MBOA in the trace for the ancestor line 'MDR037' does not follow this narrative, however, and would suggest that further exploration is needed.

Such variation in both root exudation dynamics between ancestor and modern crops has been documented more generally, in terms of both concentration and profile, with consequences for defensive capability (Ku *et al.*, 2020; Preece and

Peñuelas, 2020). For example, the maize ancestor teosinte (*Zea mays parviglumis*) is unique to modern North American cultivars for its exudation of (*E)*-β-caryophyllene, a metabolite involved in indirect defence against root-feeding herbivores (Rasmann *et al.*, 2005; Köllner *et al.*, 2008). More specifically to allelopathy, glucosinolate allelochemicals in cabbage (*Brassica oleracea*) are found in much lesser concentrations in modern domesticated varieties compared with their wild ancestor (Moreira *et al.*, 2018). There is precedent, therefore, for ancestor crops to exude greater concentrations of defence compounds, or even novel compounds for this purpose. It is interesting, then, that modern wheat breeding has increased the diversity of benzoxazinoid root exudates, as the trend is typically for artificial selection to reduce metabolite diversity (Ku *et al.*, 2020). Still, given that *T. monococcum* has rarely been integrated into breeding programmes in the modern era (Jing *et al.*, 2007), integration of potent DIBOA-exuding ancestral lines for elevated allelopathic effect may have potential for black-grass suppression.

4.5. Conclusion

These findings contribute an important aspect to the story of benzoxazinoid-mediated crop-weed interactions. The tentative confirmation of benzoxazinoid presence in wheat and rye root exudates here provides a vital link between the dose-response testing of these compounds in Chapter 2 and the agroecological interactions of putatively benzoxazinoid-exuding species with competing black-grass in Chapter 3. Moreover, the tentatively identified common presence of DIBOA-Glc and HBOA-Glc in bioactive crude root exudates of rye and ancestor wheat indicates that these compounds are ecologically important inhibitors of black-grass, corroborated by the potent phytotoxicity of DIBOA as examined in Chapter 2 and HBOA as reported in Section 4.3.6. As noted, there is little consistency between studies examining benzoxazinoid concentrations in aboveground tissues, and limited knowledge of concentrations in roots, so efforts should be made to better understand this quandary, as well as the question of concentrations in root exudates. Such work would facilitate the further exploration of black-grass allelobiosis and concentration of exuded benzoxazinoids.

The most novel finding of this chapter is the distinction in wheat root exudate benzoxazinoid diversity between ancestor and modern wheats. A wider and more consistent range of compounds exuded in landraces and modern cultivars providing evidence of a generalist suite of defences, potentially to overcome a high level of tolerance to a specific compound. By contrast, diploid lines of *T. monococcum* with more limited genetic resources appear to lack the means to synthesise DIMBOA. This may be the result of a lack of expression in the relevant genes, or the absence of the genes altogether. In the case of black-grass, its sensitivity to DIBOA (Chapter 2) rationalises the potency of crude ancestor wheat and rye root exudates towards black-grass seedlings (Chapter 3) given the identification of related compounds in such exudates in this chapter.

These results also provide some understanding of the degradation rate of DIMBOA, which remains the largest concern in its potential application for black-grass control, and a common stumbling block in the development of crop protection chemistry from natural defence compounds. Given the degradation of this compound within the timeframes of these basic experiments, it is important that this aspect is explored further in more natural, microbially active media, such as soils.

Chapter 5

Does cereal-mediated benzoxazinoid allelopathy to black-grass persist in soil?

5. Does benzoxazinoid allelopathy to black-grass persist in soil?

5.1. Introduction

Resistance and metabolism of allelochemical compounds by soil microbes has been extensively documented (Kaur *et al.*, 2009; Cipollini, Rigsby and Barto, 2012; Li *et al.*, 2015). This interaction is the likely cause of the often-observed reduction in allelopathic effects between *in vitro* systems and soil (*e.g.* Stowe, 1979). Additionally, it is consistent with the 'novel weapons' hypothesis (Section 1.3.4) that microbes resident in wheat soils would be more likely to have evolved to metabolise benzoxazinoids, so these limitations should be characterised and compared between soils of different land uses. Such interactions may therefore present a limitation of these benzoxazinoids as black-grass inhibitors, so it is vital for successful field application to examine how inhibitory allelopathic interactions are affected by a soil medium and the microbial community resident within, as outlined in Section 1.3.6.

Even if benzoxazinoids maintain a degree of effectiveness as allelochemicals in some field soils, optimising persistence of these compounds would still benefit their applications for weed control. Specifically, the modification of DIBOA into D-DIBOA (4-hydroxy-1,4-benzoxazin-3-one) was noted to have reduced its phytotoxicity slightly, but increased the half-life of the compound from less than two days to around 3.6 days (Chinchilla *et al.*, 2015; Trezzi *et al.*, 2016). To this end, the examination of this compound as a black-grass inhibitor, and comparison to DIBOA, may identify a more suitable compound for inhibition of black-grass.

Having elucidated a consistent allelopathic effect of both allelopathic cereals and applications of the pure synthetic compounds they contain in soil, it is vital to examine the effects of these inhibitors outside of *in vitro* conditions. To this end, a final step of this investigation is to apply these two different treatments, both individually and in combination, to black-grass grown in glasshouse conditions. The intention of this step is to confirm that black-grass can be inhibited by benzoxazinoid treatments in less controlled conditions (true, biologically active soil) than those used previously.

To this end, the hypotheses tested in this chapter are:

1. The allelopathic effects of DIMBOA and DIBOA will be maintained in soil, including those with diverse and active rhizospheric microbial communities.
2. D-DIBOA, proposed as a more environmentally persistent compound related to DIBOA, will have similar inhibitory potential towards black-grass.
3. Growth of black-grass in co-culture with benzoxazinoid-exuding cereals, and applications of discriminatory doses of pure DIMBOA and DIBOA in combination, will culminate in the cereal gaining a greater competitive advantage in glasshouse assays.

5.2. Methods

The centrifuge assays previously undertaken (Section 3.2.2) were modified to include unsterilised soil, providing the possibility of microbial degradation altering allelopathic effects. Preliminary work was required to determine water holding capacity of these experimental soils, to allow the maintenance of a consistent moisture level for the establishment of a microbial community and conditions comparable to the field. Soils of varying land uses were then examined for their mediation of DIMBOA and DIBOA phytotoxicity. D-DIBOA was also examined for phytotoxic potential in the Petri dish system used in Chapter 2, given its potential as a more environmentally persistent inhibitor than previously screened compounds. This work culminated in glasshouse competition assays to determine the potential of these crop and chemical treatments both in isolation and combination to suppress black-grass across environmental conditions more comparable to the field.

5.2.1. Soil selection and collection

An initial assay to examine crude exudate efficacy in biologically active soil specifically used a Kettering loam hereafter described as ‘Weed Mix’, a standard field soil used for glasshouse trials of black-grass inhibition at Rothamsted Research (*e.g.* Comont *et al.*, 2019). This material was sieved through a 1 mm aperture sieve for use in these centrifuge tube assays.

Additional soils for a follow-up assay were collected from the Highfield experiment (Rothamsted Research, UK, grid reference 51.804393, -0.362667), specifically from three varying land uses; bare fallow, grassland, and wheat field. These plots were established in 1959, providing adequate time for a consistent and representative microbial community to develop for each land use (Hirsch *et al.*, 2017). According to supplemental material provided by Kavamura *et al.* (2019), the microbial communities present in these different land uses contain a similar species richness but vary in relative abundance. Approximately 150 g of topsoil was taken from each plot, three plots for each land use (Figure 5.1). The rationale for testing soil from these three land uses originates from the likelihood of their variability in metabolising allelochemicals through their microbial communities. Soil was dried to friability, again sieved through a 1 mm aperture sieve, and bulked by land use.

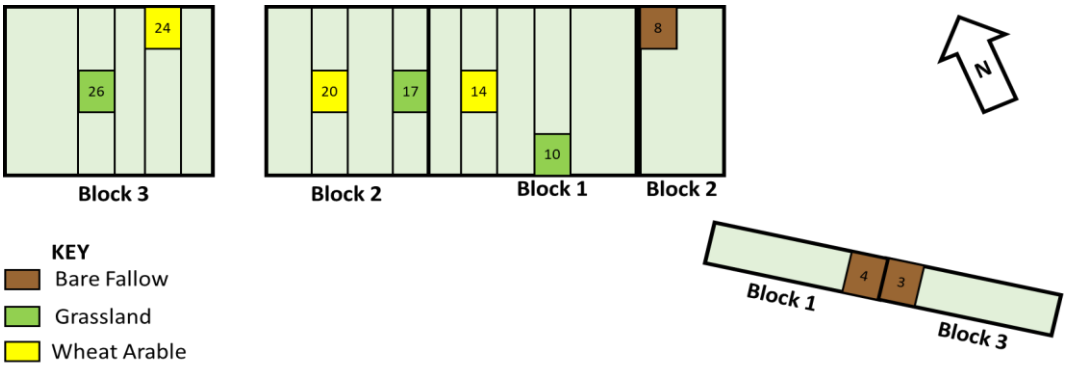

Figure 5.1: Schematic of numbered plots from which soil was collected for Highfield soils assay, coloured by land use treatment.

5.2.2. Calculation of soil water holding capacity and wetting

Water holding capacity (WHC) is the maximum volume of moisture which the soil can hold prior to saturation. WHC was calculated initially through wetting 10 g of soil with 10 ml of RO water and draining overnight. This soil was then oven dried to completion at 105°C, along with an unwetted sample to determine initial moisture. Water holding capacity values for each soil used in centrifuge tube assays throughout this chapter can be found in Table 5.1.

Table 5.1: Water holding capacities of soils used for allelopathy assays in this chapter.

SOIL TREATMENT	WHC (ml g^{-1})
WEED MIX	0.3365
HIGHFIELD BARE FALLOW	0.4868
HIGHFIELD GRASSLAND	0.8894
HIGHFIELD WHEAT FIELD	0.5646

These soils were initially wetted to 30% of WHC and incubated under the previously used regime (light/dark regime of 17°C/11°C for 14/10 hours) for 14 days. The 30% WHC level was used for this initial incubation period as this is considered sufficient for the establishment of a stable microbial community (*e.g.* Xue, Shen and Marschner, 2017). Soils were further wetted to 60% of WHC upon sowing of biological treatment plant species, allelochemical treatments and target plants depending on the structure of each experiment (Section 5.2.3). This final WHC value was selected based on an average calculated from ongoing soil WHC measurements of the Highfield field experiment from which soils were collected (M. Khandoker, Unpublished Data). Moreover, this value was believed to be sufficient not to deprive plants of moisture while also minimising quantity of chemical treatments required for experiments involving allelochemical applications.

5.2.3. Centrifuge system assay setup for soil assays

Initial assay of crude exudate allelopathy in biologically active soil used weed mix soil added to 50 ml centrifuge tubes, 20 g each, accurate to two decimal places. The bioassay system and plant species selected for exudate treatment were otherwise similar to the axenic centrifuge tube assays in sand undertaken in Section 3.2.2 (Table 5.2). Soil was wetted according to Section 5.2.2.

Table 5.2: Exudate treatments tested against black-grass in initial soil centrifuge tube assays.

EXUDATE TREATMENT	CONTENTS
CONTROL	No plants
BLACK-GRASS	4x Rothamsted-17 black-grass seedlings
'GRAVITY' WHEAT	4x 'Gravity' modern wheat seedlings
'EDMONDO' RYE	4x 'Edmondo' rye seedlings
'MDR037' WHEAT	4x 'MDR037' ancestor wheat seedlings

Following seven days of growth, plants conferring exudate treatments were removed and photographed for future reference. Soil was mixed and aerated to reduce bulk density and facilitate planting. Four surface-sterilised black-grass seeds were then sown into each tube at 2mm depth, grown for a further seven days, then removed for photography, measurement and ANOVA analyses (Chapter 2).

Follow-up work used the three Highfield soils previously described in addition to the weed mix standard, and the pure synthetic benzoxazinoid chemical treatments

previously described in Chapter 2, at the discriminatory doses discerned in those experiments. As previously, each replicate consisted of 20 g of a chosen soil added to 50 ml centrifuge tube (although the lower density of the 'grassland' soil necessitated that only 15 g of soil was used in this treatment to allow for sufficient 'aboveground' growth space). Wetting and incubation was initially undertaken as described in Section 5.2.2, with the post-incubation wetting applied using the chosen chemical treatment rather than water. Specifically, these treatments were a control of 0.5% DMSO, and discriminatory doses of DIBOA and DIMBOA (500 µM and 1000 µM respectively, double-doses of those determined in Chapter 2 to allow for dilution effects), again dissolved in 0.5% DMSO. Four replicates were produced of each combination of soil and biological treatments (summarised in Table 5.3). Four surface-sterilised, pre-germinated Rothamsted-17 black-grass seeds were sown into each tube at approximate depth of 2mm, and grown for seven days prior to removal, photography, measurement and ANOVA analyses as previously undertaken.

Table 5.3: A summary of soil and chemical treatments included in the Highfield soils assay: all three chemical treatments were assayed in each soil.

SOIL TREATMENTS	CHEMICAL TREATMENTS
Weed Mix (Control)	0.5% DMSO (Control)
Highfield Bare Fallow	500 µM DIBOA in 0.5% DMSO
Highfield Grassland	1000 µM DIMBOA in 0.5% DMSO
Highfield Wheat Field	

5.2.4. DIMBOA and DIBOA degradation in soil

To accompany results of benzoxazinoid allelopathy to black-grass in soils, it was useful to track degradation of DIMBOA and DIBOA in a range of media (Table 5.4), over the course of seven days, to give an indication of allelopathic potency to black-grass across this timeframe. The hypothesis of this experiment was that degradation of these compounds would be limited in the sand system, but would be quicker in all soils, particularly in grassland and wheat arable soils where the microbial community may have co-evolved with benzoxazinoid compounds.

This was done by placing 0.5 g of the soils or sand into a 2 ml Eppendorf tube, wetting to 25% of water holding capacity, and incubating in the previously used conditions for 14 days before adding DIBOA and DIMBOA treatments respectively.

Three replicates for each treatment combination were tested (*n*= 30). Wetting calculations for 0.5 g of each medium are presented in Table 5.5.

Table 5.4: List of media and chemical treatments used in assay of DIMBOA and DIBOA microbial degradation across a seven-day period.

MEDIUM	CHEMICAL TREATMENTS
Autoclaved Coarse Sand (ACS)	500 µM DIBOA in 0.5% DMSO
Weed Mix Soil (WMX)	1000 µM DIMBOA in 0.5% DMSO
Highfield Bare Fallow Soil (HBF)	
Highfield Grassland Soil (HGS)	
Highfield Wheat Arable Soil (HWA)	

The tubes were then sealed and incubated for a further 7 days. Each day, to track benzoxazinoid degradation, 500 µL of MeOH was added to the soil sample, vortexed for 30 seconds, then centrifuged for 15 minutes at 3,000 rpm. Excess methanol eluate was then siphoned off and analysed by LCMS according to the protocols described in Section 3.2.5.

Table 5.5: WHC calculations for requirements in 0.5g of each medium for benzoxazinoid degradation testing.

MEDIUM	WHC (µl g^{-1})	25% OF WHC IN 0.5g (µl)	STARTING % OF WHC (µl)	REQUIREMENT FOR 25% WHC (µl)
Autoclaved Coarse Sand (ACS)	?	50 (assumed)	0	50 (assumed)
Weed Mix (WMX)	336.5	42	0	42
Highfield Bare Fallow Soil (HBF)	486.8	61	21.19	9
Highfield Grassland Soil (HGS)	889.4	110	22.42	11
Highfield Wheat Arable Soil (HWA)	564.6	71	17.29	22

5.2.5. Glasshouse competition assay setup

Glasshouse assays were designed to combine the knowledge of inhibitory effects in synthetic benzoxazinoids and crude cereal exudates, to compare their inhibition of black-grass in conditions comparable to those in the field.

Pots of 15 cm diameter were considered an appropriate size to support the desired number of plants (maximum of eight per pot), while preventing them from becoming pot-bound throughout the growth period. These were filled with 1.875 kg of weed mix soil, sown with four biological treatments at approximately 2mm depth,

and treated with three chemical treatments (Table 5.6). Each biological treatment contained four black-grass seeds and four cereal seeds in all replicates excepting the control, which only contained four black-grass seeds. Each pot was placed into an individual saucer to retain runoff. Soil was wetted as uniformly as possible with autoclaved RO water to around 25% water holding capacity using a cement mixer prior to potting and sowing (equating to 168.25ml per pot). The consistency of moisture level between multiple pots was confirmed using a W.E.T. soil probe (Delta-T devices, Cambridge, UK). Unplanted pots were left overnight under experimental conditions prior to sowing, which led to some degree of drying as was indicated by the soil moisture probe. Treatments of 100 ml of the chemical treatments described in Table 5.6 were applied to each pot after sowing of biological treatments, and runoff was captured and re-applied to the pot after approximately an hour to minimise loss of chemical treatments. All pots were watered uniformly with tap water thereafter to maintain soil wetness, especially on the surface of the pots to prevent capping. Again, excess water collected in any runoff was re-applied to the pots. Given available quantities of compounds used in chemical treatments, five replicates of DIMBOA treatment were set up for each treatment except for the 'MDR037' treatment, where four replicates were produced. Eight replicates of all other treatment combinations were produced. Pot placement was randomised on a bench in a glasshouse, their positions blocked by replicate.

Table 5.6: Chemical and biological treatments used in the first glasshouse competition assay.

CHEMICAL TREATMENTS (BEFORE DILUTION WITH WETTED SOIL)	**BIOLOGICAL TREATMENTS**
Control (0.5% DMSO)	**CTL** (4x Rothamsted-17 Black-grass seeds)
DIBOA (500 µm in 0.5% DMSO)	**GRW** (4x Rothamsted-17 Black-grass seeds + 4x 'Gravity' modern wheat seeds)
DIMBOA (1000 µm in 0.5% DMSO)	**EDR** (4x Rothamsted-17 Black-grass seeds + 4x 'Edmondo' rye seeds)
	037 (4x Rothamsted-17 Black-grass seeds + 4x 'MDR037' ancestor wheat seeds)

Unfortunately, poor seedling establishment across all treatments (including controls) meant that seedling numbers were insufficient in many pots. Therefore, after three weeks growing in a cycle of 17°C/11°C for 14/10 hours, established plants

from each treatment were counted and aggregated to ensure three black-grass and three crop plants (as appropriate) in each pot. Effort was made to minimise transplantation and therefore disturbance. This resulted in the production of two replicates of all treatment combinations involving DIMBOA, and four replicates of all treatment combinations containing DIBOA and the control chemical treatment. Positions were re-randomised following the removal of obsolete pots. Counts of black-grass plant survival were analysed against treatment and blocking factors using one-way ANOVAs in Genstat to determine contributing factors to variation in establishment.

After another five weeks, plants of each species were removed and separated. Root and shoot material was separated, dried overnight at 80°C, and total dry weight of all roots and shoots of each species per pot recorded. The sum of these values was also used for the metric of total biomass. One replicate of each treatment combination was processed in this manner each day until completion. ANOVA was then used to analyse the growth of both black-grass and cereal treatments under different treatments, and black-grass root and shoot biomass compared between biological treatments.

To overcome limitations in the previously described experiment, a second assay was established with modifications. The weed mix pots used were of similar diameter but lesser capacity (approximately 800 g). To optimise establishment, black-grass and wheat were first sown ungerminated into compost seed trays and grown for nine days for black-grass, and five days for wheat prior to transplantation into the pots of weed mix soil. Pots were thoroughly wetted one day prior to plant sowing. Three biological treatments were then sown in at 2mm depth, specifically Rothamsted-17 black-grass, 'Gravity' wheat (included to determine detriment of black-grass and chemical treatments on crop establishment), and a mixed treatment of the two species, with six seedlings grown on in each pot and three of each species in the mixed treatment. These treatments were left for another day to allow for plant establishment before the chemical treatments described in Table 5.7 were applied as in the previous assay.

A standard glasshouse was used for this second assay as the airflow of the cooling system was theorised to have contributed to soil capping in the first assay,

antagonising establishment. Six replicates of each treatment combination were set up in consideration of statistical power estimations undertaken in Genstat. Plant heights were measured at seven and 14 days after treatment (DAT), and removed for root washing, drying, and biomass measurement at 17-18 DAT according to replicate.

Table 5.7: Chemical and biological treatments used in the modified glasshouse competition assay.

CHEMICAL TREATMENTS (BEFORE DILUTION WITH WETTED SOIL)	**BIOLOGICAL TREATMENTS**
Control (0.5% DMSO)	**BLG** (6x Rothamsted-17 black-grass)
DIBOA (500 µm in 0.5% DMSO)	**GRW** (6x 'Gravity' wheat)
DIMBOA (1000 µm in 0.5% DMSO)	**MIX** (3x Rothamsted-17 black-grass + 3x 'Gravity' wheat)

5.2.6. D-DIBOA Petri dish assay

To examine the phytotoxic potential of D-DIBOA towards black-grass, both this compound (Figure 5.2a) and DIBOA (Figure 5.2b) were applied to Petri dishes and sown with eight surface-sterilised, pre-germinated Rothamsted-17 black-grass seedlings each, in the manner undertaken in Section 2.2. Both of these compounds were synthesised, as previously, by Dr. David Withall of BCP Department, Rothamsted Research. The concentrations of both compounds applied were consistent with the HBOA assay undertaken in Section 4.2.6, specifically 12.5 µM, 25 µM, 50 µM, 100 µM, 200 µM, 400 µM, 800 µM, and a control containing no compound. After eight days, treated plants were again photographed, shoot and root lengths measured using ImageJ, and analysed using the 'drc' plugin of R Studio (Ritz *et al.*, 2015).

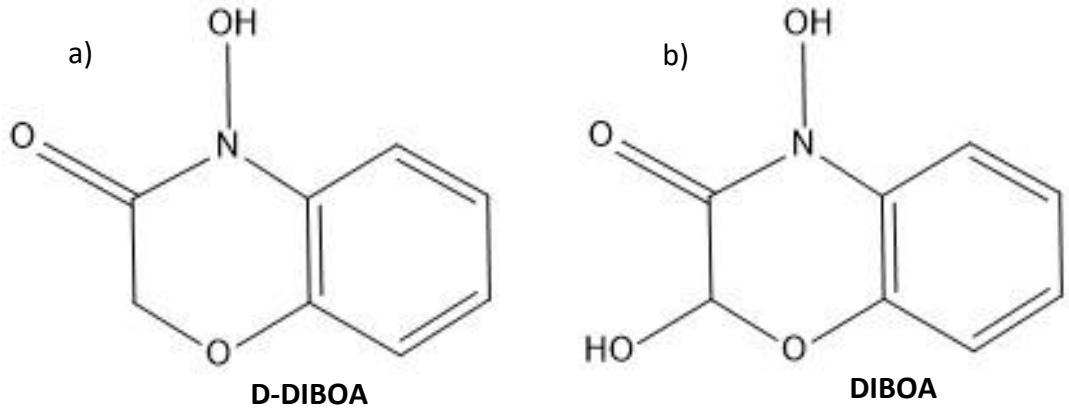

Figure 5.2: Chemical structures of: a) D-DIBOA and b) DIBOA.

5.3. Results

5.3.1. Crude cereal root exudate allelopathy to black-grass remains in a standard soil

Black-grass inhibition *via* crude exudates in centrifuge tubes containing biologically active weed mix soil broadly followed results from axenic sand (Section 3.3.2). Shoot length was not significantly different as a result of any plant treatment (p= 0.126) (Figure 5.3). Roots, however, were significantly different between the control treatment and those pre-treated with crude exudates of 'Edmondo' rye (p <0.001), while some reduction in root growth was also apparent in other non-control exudate treatments. This corroborates the inhibitory potential of these crude root exudates, while also indicating that biological activity in soil is not sufficient to degrade allelochemical constituents prior to the induction of a phytotoxic effect.

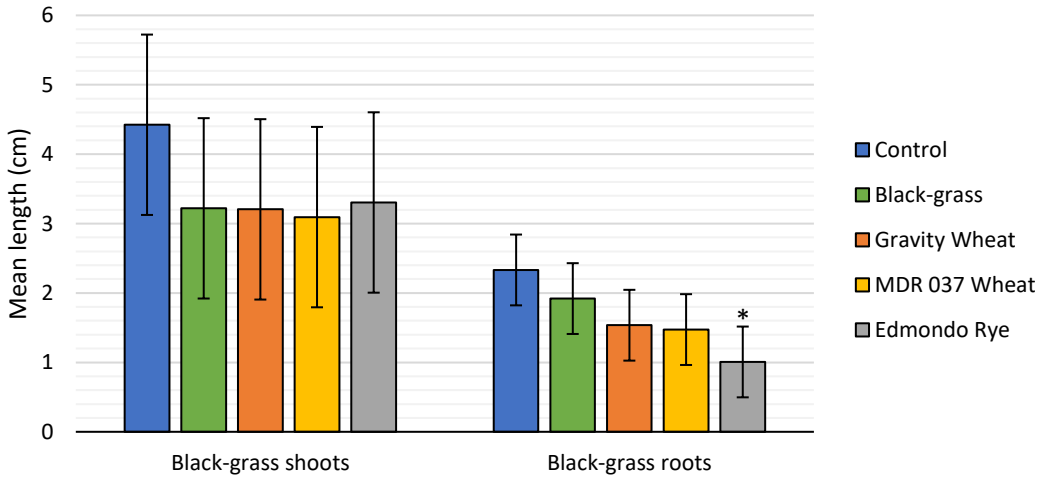

Figure 5.3: Mean length of Rothamsted-17 black-grass shoots and roots in weed mix soil centrifuge tube assay pre-treated with various root exudates. Error bars depict 95% confidence intervals. Asterisks indicate significant differences of crude exudate treatments compare to controls.

5.3.2. Benzoxazinoid allelopathic potential to black-grass varies in different soils

In the assay of soils from different land uses, the influence of different soils, assumed to contain different microbial communities, had no effect on the potency of chemical treatments towards black-grass shoots (p= 0.694) (Figure 5.4). Chemical treatments also did not significantly affect black-grass shoots when the effect of different soil media was removed (p= 0.810). The one significant factor for black-grass shoot development was that of soil without consideration of chemical

treatments (p= 0.044). Herein, black-grass grown in bare fallow soil had significantly shorter shoots than the grassland soil, a trend unrelated to allelopathic effects. Black-grass root length was also similar between different benzoxazinoid treatments (p= 0.063) (also Figure 5.4), as was the effect of these various benzoxazinoid treatments in varying soils (p= 0.105). Again, soils alone had significant effects on black-grass root growth, where the weed mix specifically correlated with shorter roots across the growth period (p< 0.001). While not significant, the greatest reduction in black-grass root length compared to the control chemical treatment occurred with DIMBOA treatment in the bare fallow soil (Figure 5.4b). No such reduction was observed in grassland or wheat arable soils (Figures 5.4c and 5.4d). This indicates some degree of variation in allelopathic potential of the treatment compounds according to soil type.

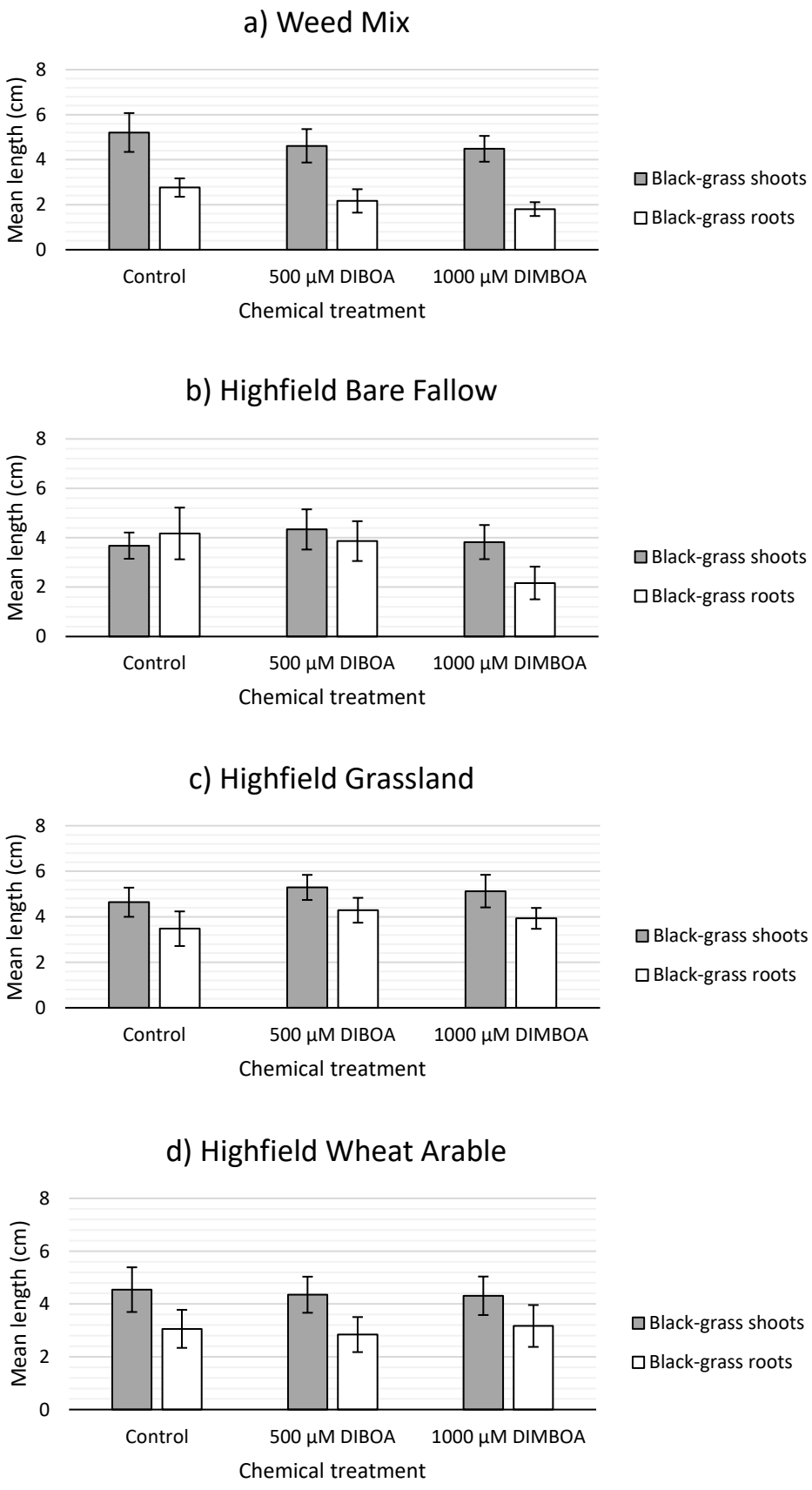

Figure 5.4: Mean black-grass root and shoot length with differing benzoxazinoid treatments in a) weed mix control; b) Highfield bare fallow; c) Highfield grassland; and d) Highfield wheat arable soils. Error bars represent confidence intervals.

5.3.3. DIMBOA and DIBOA degradation in soil

Degradation analyses of DIMBOA and DIBOA in the four soils examined in the Highfield assay, and the additional negative control of autoclaved coarse sand, identified correlations between allelopathic potential of these compounds and their rate of degradation.

DIMBOA degradation was similar between the five media examined. In autoclaved coarse sand, a faint trace peak of DIMBOA was still apparent on Day 3 (Figure 5.5a), but in all other media, the DIMBOA peak had diminished by this time, regardless of medium (Figure 5.5b). MBOA was identified in all media at all time points.

The degradation rate of DIBOA was more variable between media. In autoclaved coarse sand, the compound was still recovered after three days (Figure 5.6a), although a peak was also observed for its degradation product BOA on all days. By comparison, DIBOA was not detected in Highfield soils after two days, and in the case of the bare fallow soil, after one day (Figure 5.6b). These data indicate that varying soil media have more pronounced effects on DIBOA degradation than DIMBOA degradation.

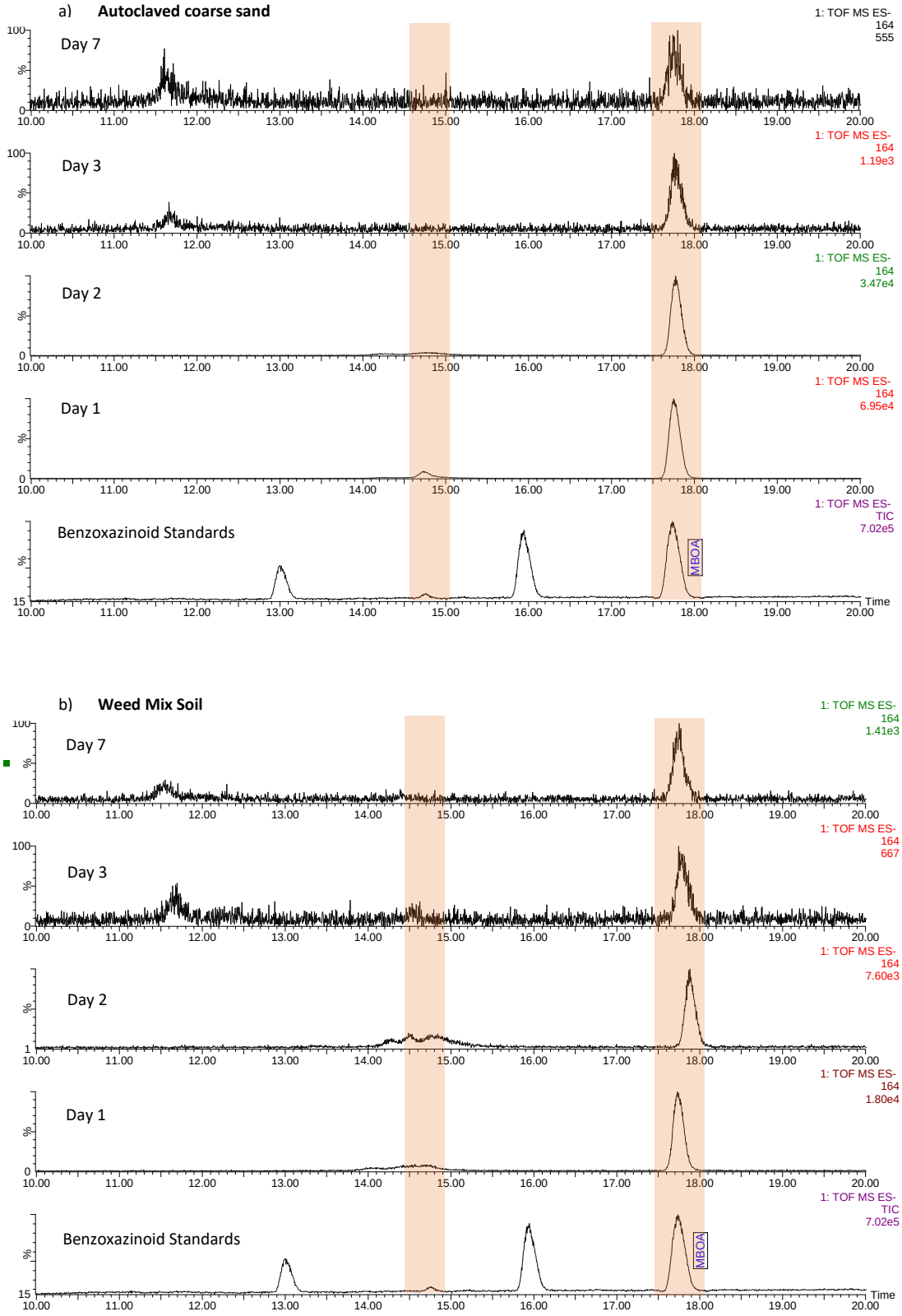

Figure 5.5: LCMS Chromatograms of a) autoclaved coarse sand, and b) Weed mix soil media treated with DIMBOA, across days 1 to 3 and day 7, both compared with benzoxazinoid standards.

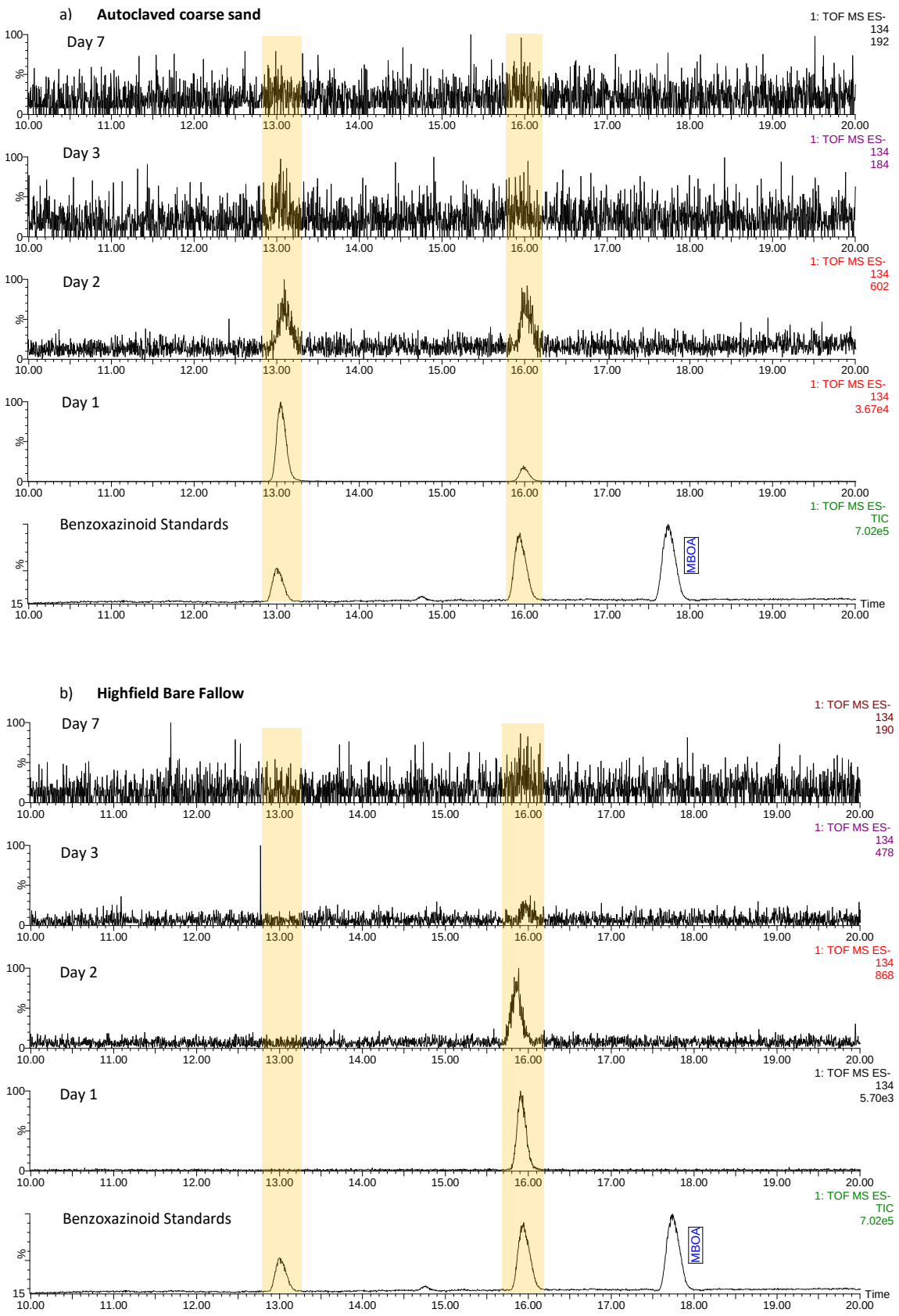

Figure 5.6: LCMS Chromatograms of a) autoclaved coarse sand, and b) Highfield bare fallow soil media treated with DIBOA, across days 1 to 3 and day 7, both compared with benzoxazinoid standards.

5.3.4. Glasshouse competition assays indicate efficacy of biological treatments

When black-grass was grown in conjunction with crop species in the glasshouse competition assay, and benzoxazinoid treatments added, total dry biomass of black-grass per pot was significantly reduced with the presence of non-control biological treatments ($p< 0.001$). The presence of each crop species correlated with a significant reduction in black-grass biomass when compared to the control (Figure 5.7a). Black-grass root biomass was significantly inhibited by 'Edmondo' rye and 'Gravity' wheat ($p <0.001$), while shoot biomass was significantly reduced by these crop species as well as the ancestor wheat line 'MDR037'.

DIMBOA treatment significantly reduced black-grass dry weight in comparison to DIBOA treatment ($p< 0.001$), but neither differed significantly from the control treatment (Figure 5.7b). This pattern was also apparent in black-grass shoot biomass ($p= 0.002$), while root biomass significantly increased in growth with DIBOA treatment compared to others ($p< 0.001$). In all cases, DIMBOA reduced black-grass biomass compared to the control treatment, but not significantly as a result of a large degree of error.

In terms of biomass of crops growing in co-culture with black-grass, 'MDR037' had significantly lower biomass than the modern crop cultivars used ($p< 0.001$) (Figure 5.7c), a pattern which was consistent in crop roots ($p< 0.001$). Shoot biomass was significantly different between crop treatments, with 'Edmondo' rye having the greatest and 'MDR037' again having the least biomass ($p< 0.001$).

Total crop biomass also varied by chemical treatment (Figure 5.7d), with DIMBOA significantly reducing mean total crop biomass ($p< 0.001$) and mean root biomass ($p= 0.002$) compared to other treatments. Although shoot biomass differed between treatments ($p< 0.001$), neither benzoxazinoid treatment was significantly different to the control. Again, DIBOA produced significantly increased shoot biomass compared to DIMBOA, with the control value being between these two extremes.

Neither biological treatment ($p= 0.995$), nor chemical treatment ($p= 0.566$), were significant factors in numbers of surviving black-grass plants prior to transplantation, but replicate ($p= 0.003$) was, indicating that blocking by replicate had led to confounding effects. This was used to inform setup of the following assay,

as it indicates that some replicates were more conducive to plant survival than others, thereby partially explaining these unexpected results.

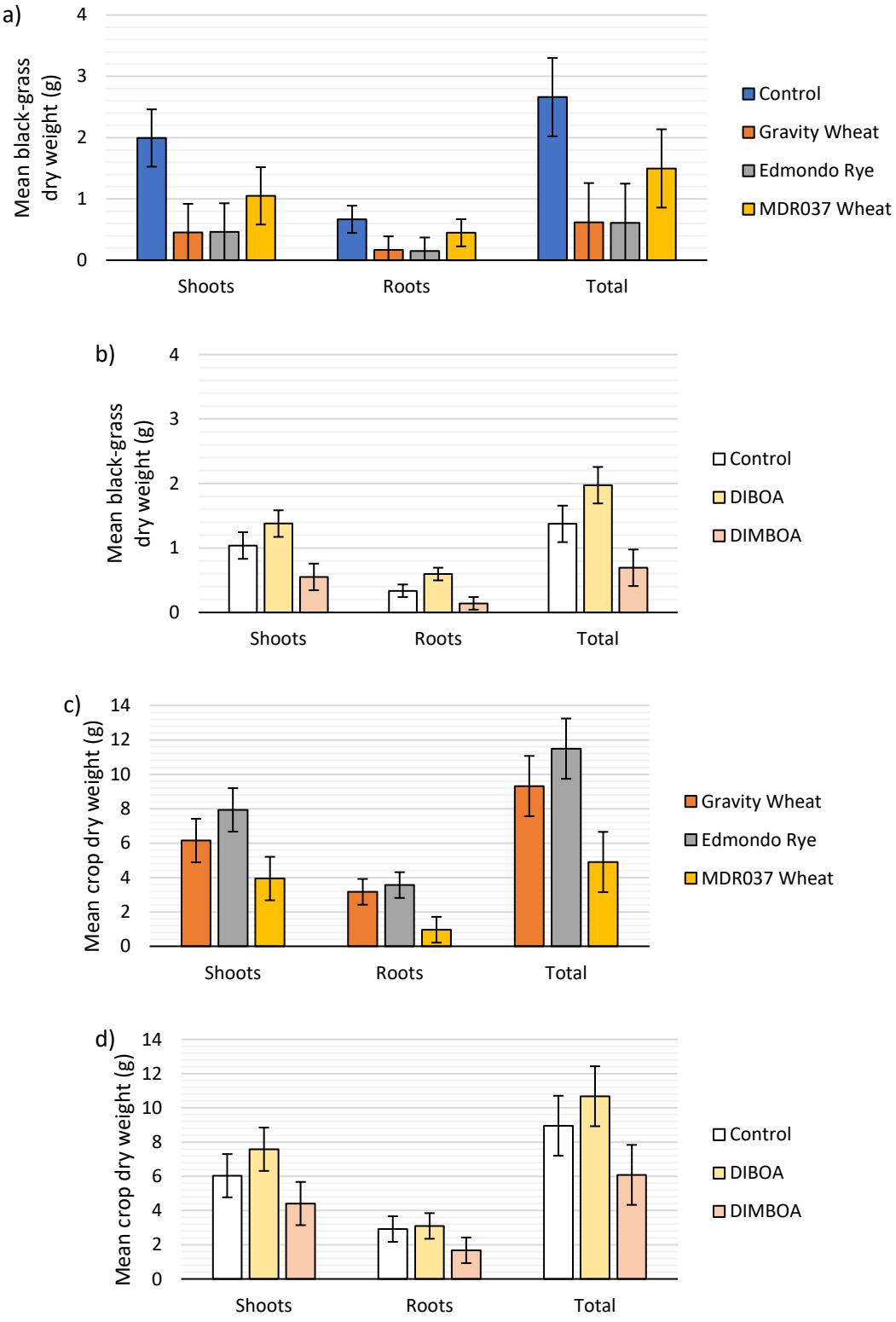

Figure 5.7: Effects of a) biological and b) chemical treatments on mean black-grass shoot, root and total dry weight. Mean crop shoot, root, and total dry weights are also presented for each different biological treatment crop (c), and the mean dry weight of crop shoot, root, and total biomass according to chemical treatment (d). Error bars depict confidence intervals.

In the repeat assay, aboveground measurement of wheat and black-grass at seven DAT indicated that the presence of wheat had a detrimental effect on black-grass shoot length across the three chemical treatments applied (p= 0.009, Figure 5.8a). By contrast, the presence of black-grass was not detrimental to wheat shoot length (p= 0.228), and may even have been partially stimulatory, although this result was not significant. Neither black-grass (p= 0.809), nor wheat shoots (p= 0.218), were significantly affected by chemical treatment, in coherence with previous results (Figure 5.8b).

Wheat was similarly unaffected by the presence of black-grass at 14 DAT (p= 0.960) (Figure 5.8c), but the previously significant detriment of wheat towards black-grass shoot length was no longer significant at this later time point (p= 0.210). As previously, neither benzoxazinoid chemical treatment had significant inhibitory effect on black-grass (p= 0.463) or wheat (p= 0.054) at 14 DAT (Figure 5.8d).

Dry weights of plants measured after three weeks of growth corroborated findings from shoot length measurements at seven DAT. Total black-grass biomass was significantly affected by biological treatment (p= 0.049); specifically with the mixed treatment reducing black-grass biomass (Figure 5.9a). Neither the constituent root or shoot biomass was significantly reduced in the mixed biological treatment however (p= 0.143 and p= 0.058 respectively, Figures 5.9b and 5.9c). Benzoxazinoid chemical treatments did not significantly alter the development of black-grass in terms of total, root, or shoot biomass (p= 0.760, p= 0.963, and p= 0.703 respectively, Figures 5.9d to 5.9f). Chemical treatment also had no effects on black-grass across isolated and mixed biological treatments (total biomass: p= 0.931, roots: p= 0.784, shoots: p= 0.772).

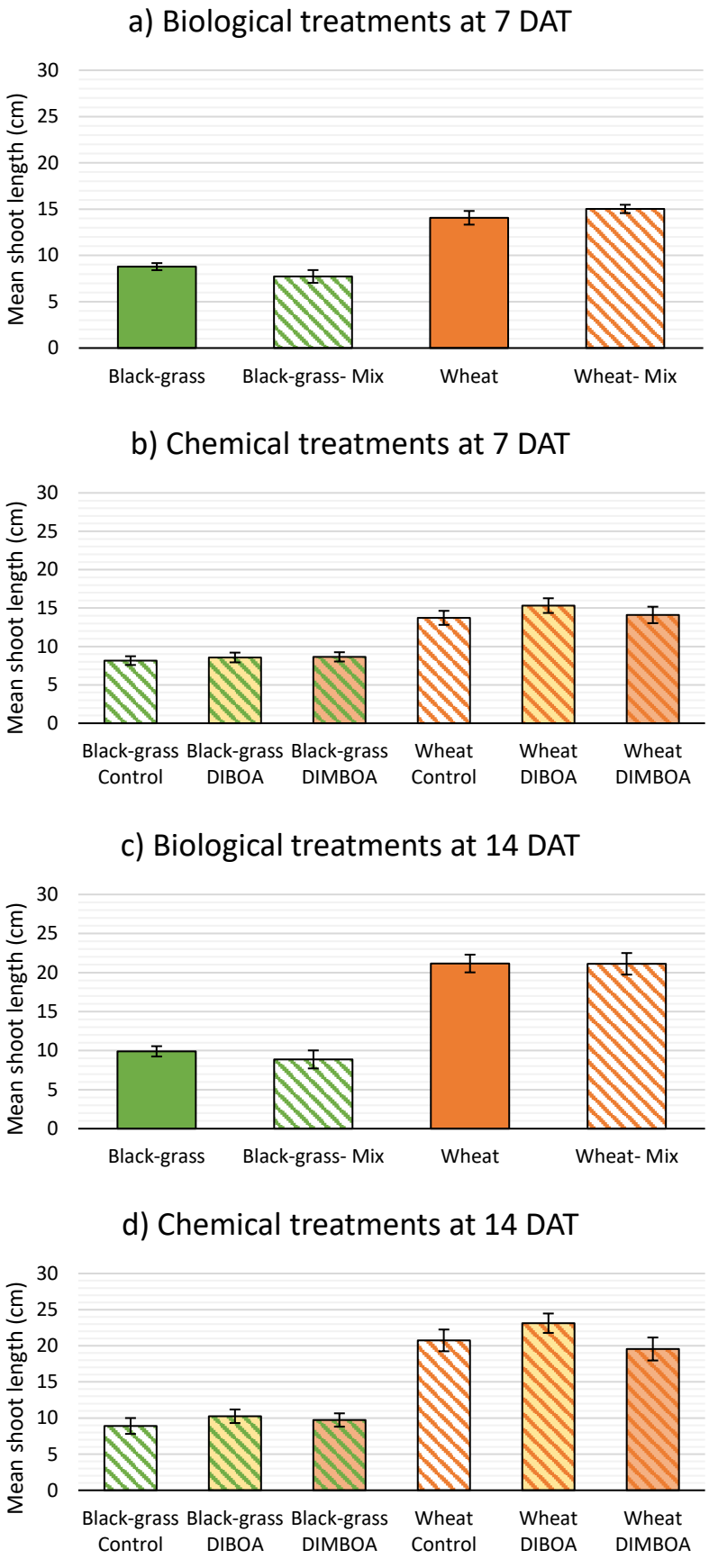

Figure 5.8: Mean shoot length of black-grass and wheat under a) biological and b) benzoxazinoid chemical treatments at 7 DAT, and 14 DAT (c) and d) respectively) under glasshouse conditions. Error bars represent 95% confidence intervals.

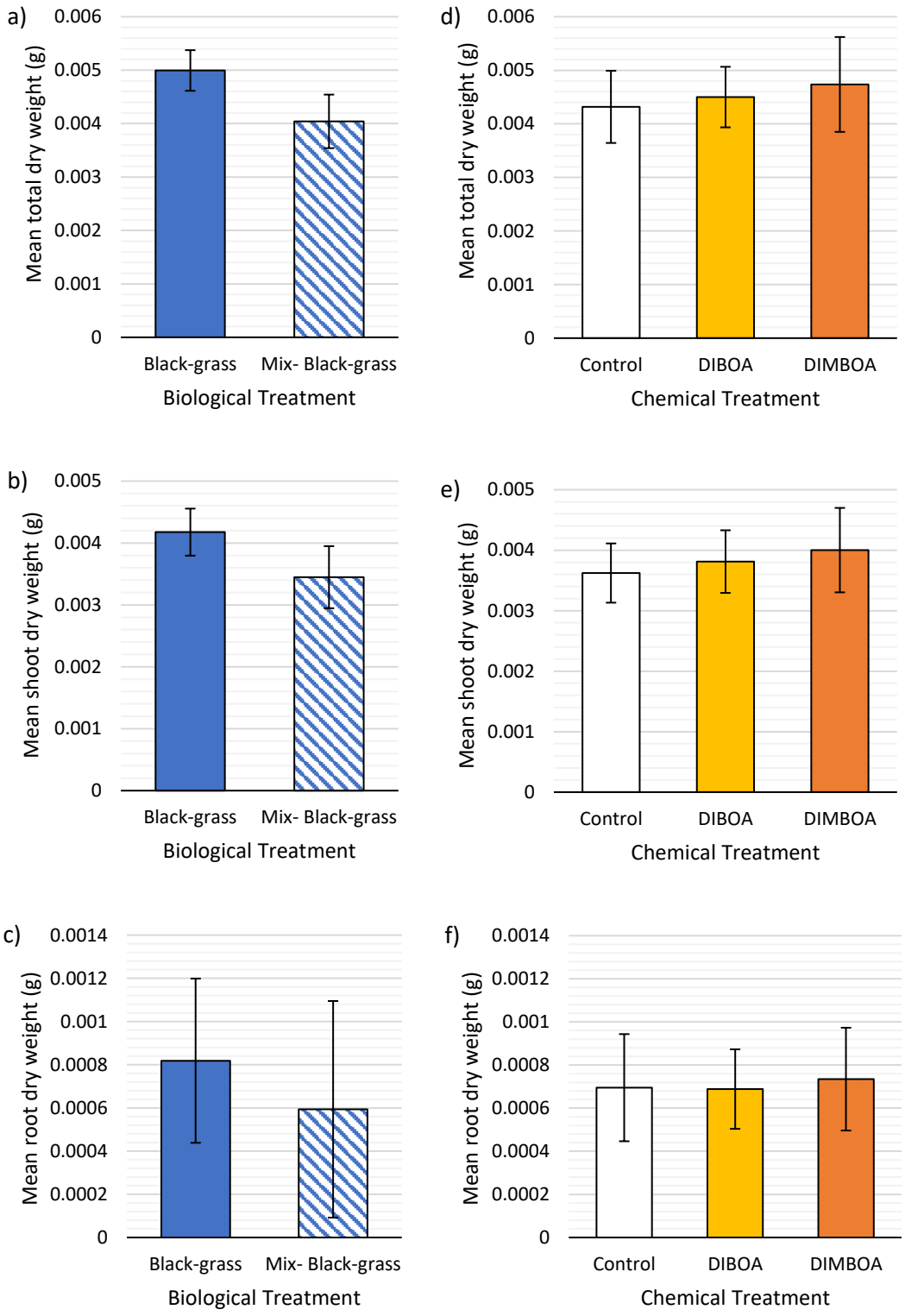

Figure 5.9: Mean dry weight of black-grass after three weeks growth under glasshouse conditions under various treatments: a) total dry weight, b) shoot dry weight, and c) root dry weight by biological treatment; d) total dry weight, e) shoot dry weight and f) root dry weight by benzoxazinoid chemical treatment. Error bars represent 95% confidence intervals.

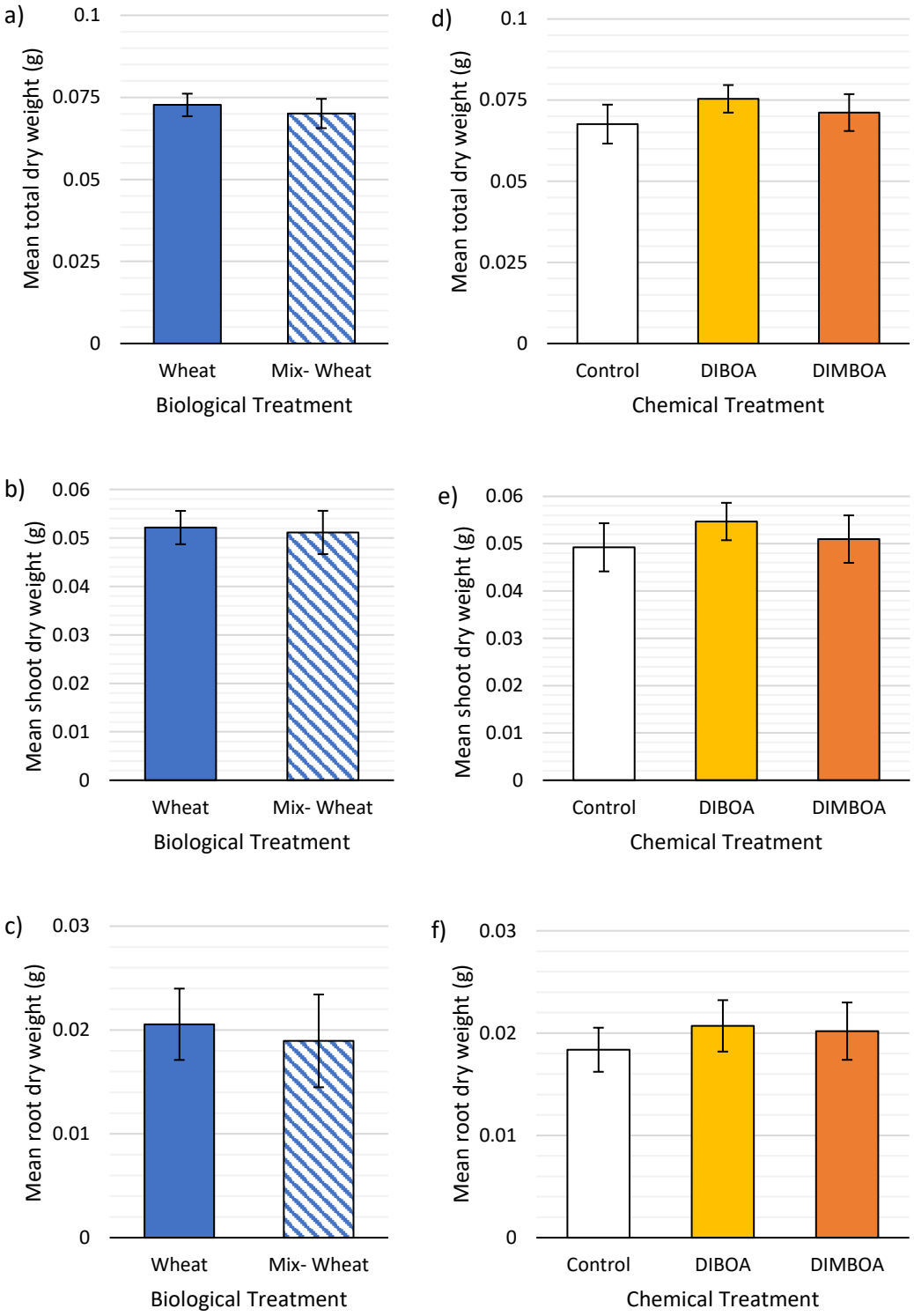

Figure 5.10: Mean dry weight of wheat after three weeks growth under glasshouse conditions under various treatments: a) total dry weight, b) shoot dry weight, and c) root dry weight by biological treatment; d) total dry weight, e) shoot dry weight and f) root dry weight by benzoxazinoid chemical treatment. Error bars represent 95% confidence intervals.

In comparison to black-grass dry weights, wheat biomass was not significantly inhibited by biological treatment (total biomass: p= 0.441, roots: p= 0.199, shoots: p= 0.707, Figures 5.10a to 5.10c), or chemical treatment (total biomass: p= 0.182, roots: p= 0.275, shoots: p= 0.251, Figures 5.10d to 5.10f). There was also no significant interaction between these two factors in any metric (total biomass: p= 0.468, roots: p= 0.726, shoots: p= 0.375), indicating that chemical treatments did not differentially affect wheat in isolation compared to in the presence of black-grass.

5.3.5. D-DIBOA is an ineffective black-grass inhibitor

Petri dish analysis of D-DIBOA and DIBOA identified differences in bioactivity on black-grass. As with previous assays (Sections 2.3.1 and 2.3.2), DIBOA was highly inhibitory to black-grass root development (Figure 5.11), with a large degree of reduction apparent at doses above 100 µM. An ED_{50} value calculated as 68.806 µM, with a very low range of error, indicated a high likelihood of this value being accurate. D-DIBOA, meanwhile, was not clearly inhibitory at any of the doses applied, with inconsistent inhibition even at the highest 800 µM dose. Due to this inconsistency, the ED_{50} value calculated had a high degree of error, and so cannot be described as meaningful. Neither compound was significantly inhibitory to black-grass shoot growth across the dose range tested (Figure 5.12), in corroboration of previous findings of benzoxazinoid inhibition.

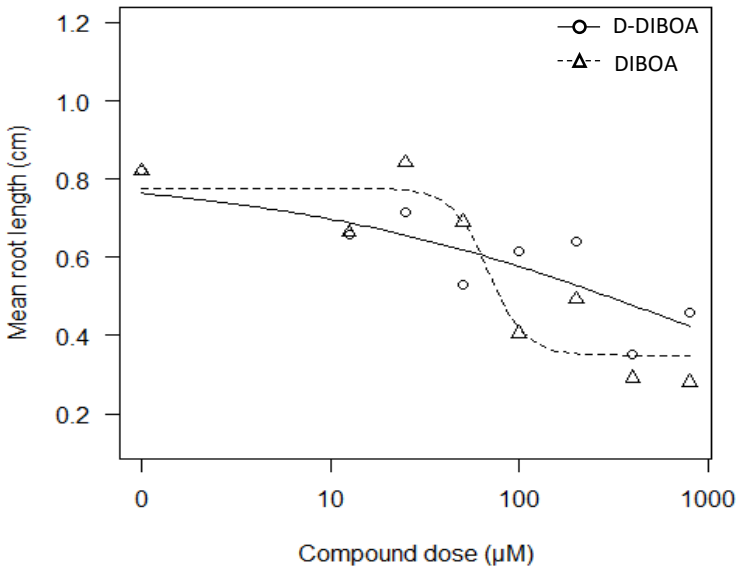

Figure 5.11: Dose-response curves of Rothamsted-17 black-grass root growth under treatment with D-DIBOA (solid line with circles) and DIBOA (dashed line with triangles) doses depicted on log scale. Plate replicates= 6, individual seeds per plate= 8, curves produced using the 'drc' package in R.

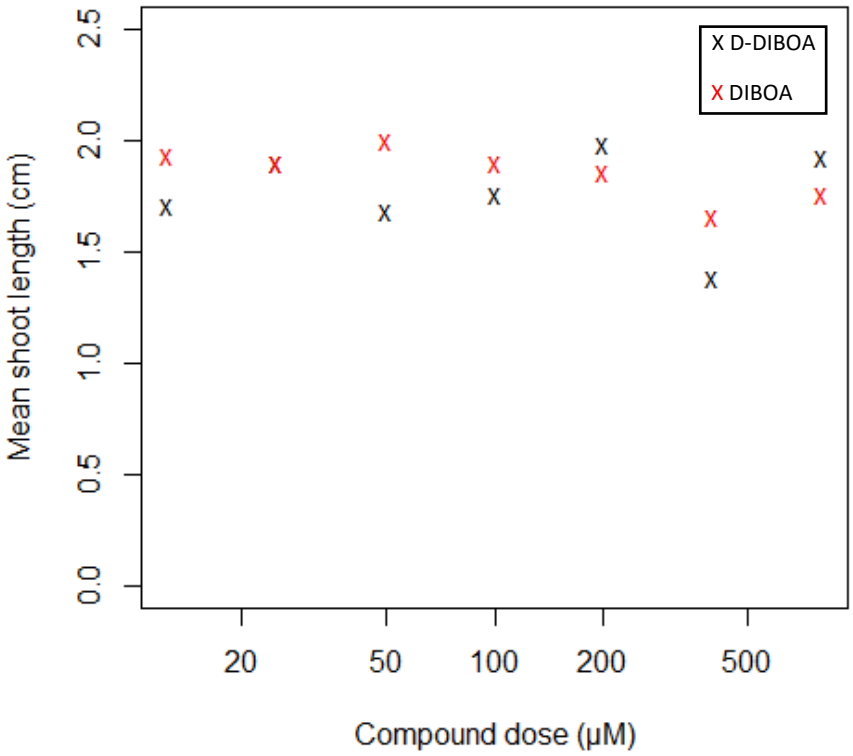

Figure 5.12: Variation in Rothamsted-17 black-grass shoot growth under treatment with D-DIBOA (black) and DIBOA (red) doses depicted on log scale. Plate replicates= 6, individual seeds per plate= 8. Produced using R Studio.

5.4. Discussion

5.4.1. Crude cereal root exudate allelopathy in soils: microbes have a role

There is a growing body of literature confirming that benzoxazinoid degradation is stimulated by microbial action. This is likely to be related to metabolic resistance and tolerance by these microorganisms (Section 1.3.6), and in more general terms the diminished allelopathic effect often observed in the transition from axenic to bioactive conditions (Stowe, 1979; Li *et al.*, 2015). Concerning benzoxazinoids, a degree of degradation from DIBOA to BOA in soil microbial cultures is apparent within 24 hours, through the action of rhizobacteria such as *Bacillus cereus*, *B. megaterium*, *B. methylotrophicus, and Mycobacterium fortuitum* (Schütz *et al.*, 2019). There does appear to be a trade-off between benzoxazinoid metabolism potential and development in these microbes, as resources are committed to detoxification of these compounds in preference to their fecundity (Schütz *et al.*, 2019).

Little effort has been made to date to examine the connection between phytotoxicity of putative allelochemicals and their rapid degradation. Results in this

chapter indicate that crude wheat and rye root exudates maintain allelopathic activity towards black-grass between axenic coarse sand and biologically active Kettering loam weed mix soil. Phytotoxic effects are thus still possible from constituent allelochemicals in spite of the ostensible effects of biological action by soil microorganisms.

There are a number of works reporting allelopathy from cereals which could not be explained by DIMBOA or DIBOA content. Incorporation of whole organic wheat plants of six cultivars into field soils inhibited growth of annual meadow grass, loose silky-bent, chickweed (*Stellaria media*), false mayweed (*Tripleurospermum inodorum*), fat-hen (*Chenopodium album*), and poppy (*Papaver rhoeas*), although such inhibition was greater than any comparable dose of isolated benzoxazinoid (Mathiassen, Kudsk and Mogensen, 2006). Similar findings have been reported in rye, which was still significantly inhibitory to lettuce and smooth pigweed (*Amaranthus hybridus*) in field soils (Rice, Cai and Teasdale, 2012). This again did not correlate with the content of any specific benzoxazinoid compounds, including breakdown products or glucosides (Rice, Cai and Teasdale, 2012). This same study found that benzoxazinoid concentrations varied greatly at different time points, which may indicate that target species are vulnerable to different compounds at different stages of development in a currently unknown manner. To this end, it is apparent that further experimentation is required to reconcile the allelopathy of crude cereal exudates with the diminished effects of their identified phytotoxic benzoxazinoid constituents in isolation.

5.4.2. Land use, microbial community, and benzoxazinoid phytotoxicity to black-grass

It was established in Chapter 1 that there may be an ecological connection between microbial degradation of allelochemicals and the resistance of these microbes to the compounds. The novel weapons hypothesis (explained in Section 1.3.4) dictates that microbial resistance (and presumably degradation through metabolism) is dependent on the historical 'familiarity' of the community with such compounds. In this case, microbial resistance would evolve gradually through exposure to sublethal doses, as occurs in antibiotic resistance (Baym *et al.*, 2016).

The results in Section 5.3.2 suggest that this may be the case. Phytotoxic effects of benzoxazinoids, particularly in wheat field soil, but also grassland soil to a lesser degree, were diminished compared to other soils, although this was not to the point of statistical significance. In the case of both DIMBOA and DIBOA, degradation assays in a variety of porous media highlighted rapid degradation, which is likely to be the cause of their diminished allelopathic effects in both the assays in Highfield soils and glasshouse experiments (*i.e.* Weed mix soil). Studies of microbial benzoxazinoid degradation have previously prioritised the elucidation of degradation dynamics over the effect that it may have on allelopathic interactions (Gents *et al.*, 2005; Understrup *et al.*, 2005), so further work would be required to inform these findings further. An understanding of how the degradation of these compounds affects their allelopathy would be essential for their real-world application.

Further work is also required to explore the influence of physicochemical factors, which differed between soil treatments and may affect allelopathic potential. Kaur *et al.* (2009) estimated that only around 20% of allelopathic potential can be expected in biologically active soil media, and while the predominant mitigating factor is microbial degradation, it is noted that sorption onto soil colloids will also play a role (Inderjit and Bhowmik, 2004). One method that could be used in further research to remove the influence of edaphic properties on allelopathy would be to produce an inoculum from this variety of soils for inoculation into a standard, sterilised soil (Robinson *et al.*, 2021). Such methods could be adapted to explore particularly potent microbial species in the degradation of specific compounds if desired.

5.4.3. Cereal allelopathy and black-grass control: the road to application

It is clear from glasshouse assay results (Section 5.3.4) that the growth of an allelopathic cereal crop in close proximity to black-grass leads to a reduction in its biomass and therefore development. Additionally, it is striking that the degree of inhibition correlated to some degree with the biomass of the crop, with the ancestor wheat 'MDR037' standing out as being inhibitory to a lesser degree than other crop treatments while also producing significantly less biomass. This is a useful finding from an application perspective, although it does not indicate the relative

importance of allelopathy or resource competition given that both of these interactions would be more pronounced in individuals with greater root biomass.

The potency of crop treatments in inhibiting black-grass is notable throughout the glasshouse assays conducted in this chapter, contrasting with the synthetic benzoxazinoid chemical treatments which were ineffective towards black-grass in this system. It is easy to attribute this effect to resource competition given that, again, no measures were taken to exclude the phenomenon. On the other hand, it is important to consider both the proven allelopathy of crude cereal exudates towards black-grass (Chapter 3), and the loss of a significant effect in black-grass shoot growth inhibition between seven and 14 DAT in the second glasshouse assay. This may indicate allelopathy as the cause because younger plants are believed to be more sensitive to allelochemicals (Zhang *et al.*, 2021). Moreover, benzoxazinoid concentrations in wheat (Copaja, Nicol and Wratten, 1999), and exudation of other allelochemicals from roots of other species, peak a short time after the germination of the donor plant (An *et al.*, 2003). The interval between seven and 14 DAT could therefore coincide with sufficiently reduced benzoxazinoid exudation to permit black-grass to grow relatively uninhibited. By comparison, resource competition would logically intensify as space and nutrients became more limited with plant growth, at odds with the observed nullification of inhibitory effects towards black-grass as plants develop. Any resource competition that may have occurred would therefore only have provided additional benefit. To this end, it is fitting to invoke the assertion of Inderjit and Del Moral (1997) that separating these two interactions is prohibitively difficult, unrealistic, and to some extent unnecessary from the perspective of ecological application.

The absence of consistent results from synthetic benzoxazinoid applications in glasshouse and other biologically active systems may be the result of dilution or rapid microbial degradation (Section 5.4.1). Regardless, this outcome indicates that, for in-field application, benzoxazinoid allelopathy is more likely to be effective through the presence of an allelopathic crop providing a constant, steady supply of allelochemicals, rather than the application of a one-time dose of synthesised pure allelochemical. The effect of repeated doses of such an allelochemical, or multiple compounds in combination, have not been examined but would be undesirable given

the cost and effort required to synthesise the necessary quantities of benzoxazinoids. An alternative method of delaying allelochemical release for a more consistent supply could be the application of mulches, stubble and debris. Such an approach has effectively inhibited weed development in field in both benzoxazinoid-exuding cereal species (*e.g.* Steinsiek, Oliver and Collins, 1982; Al Hamdi *et al.*, 2001), and other allelopathic plant species (*e.g.* White, Worsham and Blum, 1989).

The preference of allelopathic crops over pure synthetic benzoxazinoids is further founded upon the much-reduced inhibitory effect of D-DIBOA to black-grass, when compared to DIBOA (Section 5.3.5). Previous work has offered D-DIBOA as a similarly allelopathic compound but with more persistence through testing with a number of standard test species and weeds (Macías, Chinchilla, *et al.*, 2005; Macías *et al.*, 2006; Chinchilla *et al.*, 2015; Trezzi *et al.*, 2016), and therefore a more promising compound for application. On the other hand, this is not the case in black-grass, where it appears to have little allelopathic potential. To this end, any attempts to improve the persistence of DIBOA would require the identification of a more allelopathic alternative to D-DIBOA.

Dispensing with the debate of interlinkages between allelopathy and resource competition, results consistently prove that black-grass development is detrimentally affected by the presence of a cereal crop, but that the cereal crop is not affected by black-grass. In other words, cereals can outcompete black-grass when in co-culture. This would appear contrary to the problematic status of black-grass in cereal fields, but this is likely to be due to the greater simplicity of the systems, the low number of plants, and the early growth stages used throughout this work. It is noted by Naylor (1972) that the inhibition of cereals by black-grass is unlikely to be the result of shading given the diminutive size of the weed. Rather, the prevailing theory from early pot experiments with black-grass is that it specifically competes for nitrogen (Welbank, 1963), which was not monitored or controlled during these experiments. Nonetheless, the results here indicate that, in the right (controlled) conditions, wheat can outcompete black-grass with limited detriment, even without any chemical treatment. It therefore appears that the informed application of allelopathic crops represents an often-overlooked additional 'little

hammer' in the toolbox of integrated weed management for the benefit of modern agriculture (Liebman and Gallandt, 1997).

5.5. Conclusion

The results of this chapter corroborate previous findings that microbial activity is detrimental to the stability of benzoxazinoids. While it is interesting to note the maintenance of allelopathic effects in some soils (*e.g.* Section 5.3.1), it is likely that black-grass and wheat are predominantly found in agricultural soils with a microbial community that is tolerant, and therefore effectively degradative, of benzoxazinoid allelochemicals. To this end, future applications must prioritise the examination of approaches to prolong allelochemical delivery into soil. In terms of pure synthetic benzoxazinoids, the poor performance of D-DIBOA as a black-grass inhibitor is a disappointing complication in the development of an environmentally persistent alternative to DIBOA, and therefore other alternatives would be required if this approach was pursued further.

Encouragingly, however, results from biological treatments in this chapter indicate that a potent, benzoxazinoid-exuding cereal crop is capable of significant inhibition of black-grass without suffering its own detriment in co-culture. Therefore, although both this approach and the treatment of a pure synthetic benzoxazinoid are valid for future application with further work, it is the use of an allelopathic crop, or at least its tissues, which appears to be a more suitable candidate for black-grass suppression at present.

Chapter 6

General Discussion:
Can allelopathy tip the balance against weeds?

6. General Discussion: Can allelopathy tip the balance against weeds?

6.1. Benzoxazinoid allelopathy and black-grass: not a 'silver bullet', but an underutilised approach with potential

The previous chapters outline DIBOA and DIMBOA as cereal-derived natural products with great potential for selective black-grass control. These inhibitory effects are consistent with those of crude cereal exudates in both axenic sand and biologically active soil, and are comparable in a range of British black-grass field populations (findings detailed in greater detail in Figure 6.1). Results from glasshouse experiments show less consistency, but there remains evidence of black-grass inhibition from the competition and allelopathy of benzoxazinoid-exuding cereal species.

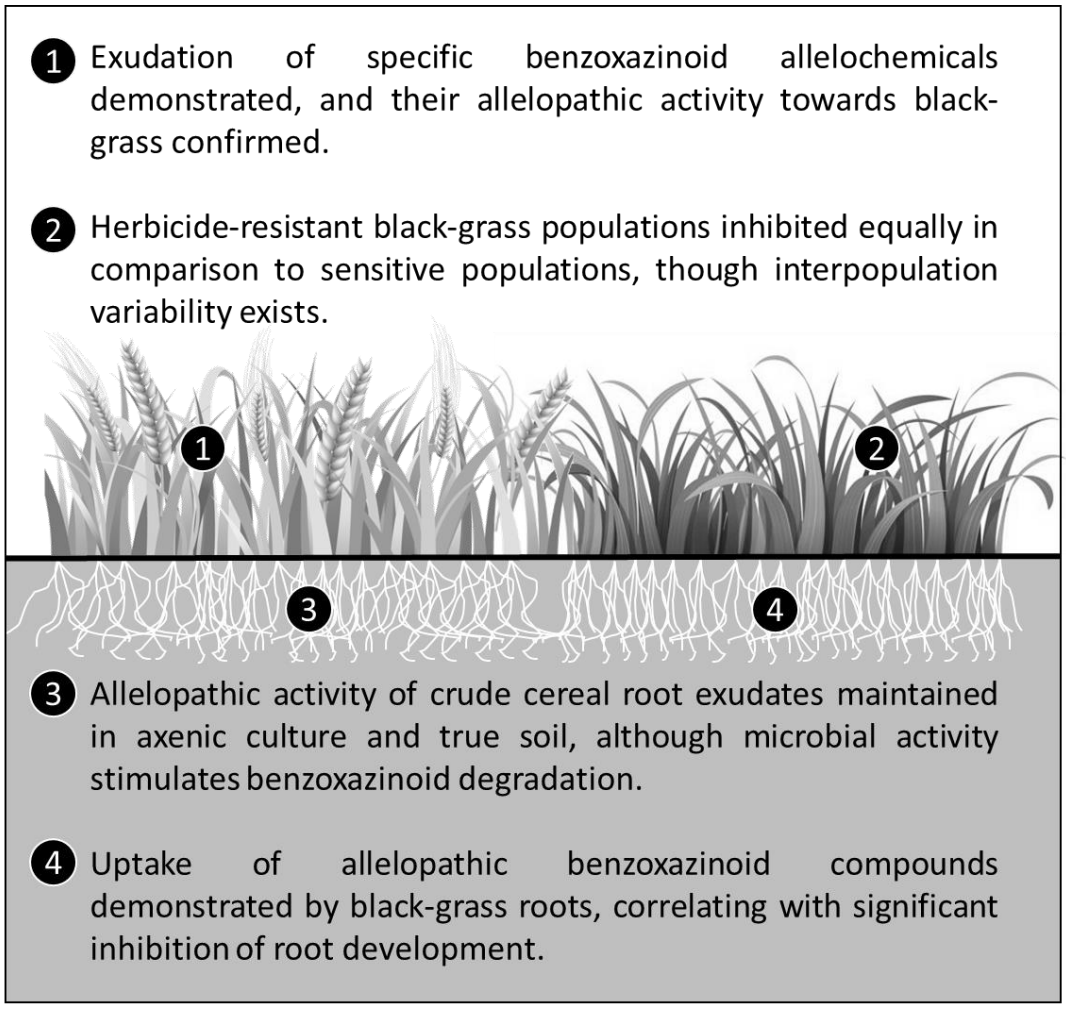

Figure 6.1: A summary schematic of key contributions of this project to understanding of potential application of benzoxazinoid inhibitors in control of black-grass.

Even with these findings in mind, it is unreasonable to assume that any control measure could be a 'silver bullet' against black-grass, or any other herbicide-resistant weed. This situation is only exacerbated by increasingly stringent regulation of existing synthetic herbicides (Cordeau *et al.*, 2016). Rather, the use of benzoxazinoid allelopathy, through cultivation of an allelopathic crop, or direct application of a pure naturally-derived product, may contribute another of the 'many little hammers' required for control of weeds in place of synthetic herbicide (Liebman and Gallandt, 1997). This combinatorial approach forms the basis of ecologically orientated integrated weed management strategies, currently advocated as best practice for weed control (Storkey *et al.*, 2021). As such, the application of benzoxazinoid allelopathy is envisioned in conjunction with currently advocated cultural controls (Lutman *et al.*, 2013). Throughout this work in controlled conditions, black-grass showed no evidence of allelopathic inhibition towards wheat, and small numbers of black-grass seedlings did not effectively outcompete a similar number of crop seedlings. It therefore seems that the black-grass problem in agriculture is exacerbated by in-field factors, and it is envisioned that benzoxazinoid activity would combine with cultural controls to at least partially contribute to overcoming the large populations in the field. Together with the findings of this thesis, these approaches could combine to tip the balance against black-grass and in favour of the crop.

6.2. Returning to Willis' Framework

As acknowledged in Section 1.3.5, the framework set by Willis (1985) represents a useful basis for investigating a case of allelopathy, but one which is rarely fully satisfied. Returning to this framework, it is useful in appraisal of the current case of cereal benzoxazinoid allelopathy to black-grass;

1. Both wheat and rye demonstrate inhibitory effects towards black-grass (Chapter 3).
2. Wheat and rye produce allelochemicals (benzoxazinoids which vary by donor species) (Chapter 4; phytotoxicity of benzoxazinoids examined in Chapter 2).
3. Benzoxazinoid compounds and their degradation products have been found in wheat and rye root exudates (Chapter 4), indicating that a mode of release does exist even though it has not been specifically elucidated.

4. The means of accumulation or transport has not been elucidated for these compounds but their occurrence in growth media indicates that such means exist.
5. The imbibition of benzoxazinoids by black-grass was examined and confirmed in degraded or partially metabolised forms (Section 3.3.5)
6. Bioassays of black-grass throughout this thesis have prioritised the isolation of allelopathic effect, and therefore the documented inhibition cannot be explained by environmental factors, herbivory, or resource competition, all of which were eliminated, or at least controlled, in the assays undertaken.

Therefore, this thesis has satisfied all of Willis' tenets at least to some extent, and together form a compelling case for an allelopathic effect, albeit with caveats. Points 3 and 4 are somewhat subjective in terms of how they can be satisfied in a modern allelopathy study but, as noted, there is evidence in this case of allelochemical release and environmental accumulation through their consistent recovery from growth media.

6.3. Outstanding questions on cereal allelopathy towards black-grass

6.3.1. The quandary of quantity: can wheat exude enough allelochemical for in-field weed inhibition?

Willis' framework, although useful, has limitations, and thus there are other outstanding questions that remain within the scope of this investigation. Perhaps the largest quandary in benzoxazinoid allelopathy is the discrepancy between concentrations required to elicit allelopathic responses and the much more limited (around 100 times lesser) concentrations identified in wheat roots (S.-Z. Zhang *et al.*, 2016). This quandary was not examined further throughout this thesis, but was discussed in detail in Chapter 4. Even assuming some degree of accumulation, it remains surprising that crude wheat and rye root exudates are consistently inhibitory to black-grass in porous media, especially as short-lived benzoxazinoids were the only putative allelochemicals identified therein. One potential explanation may be a potent synergistic effect between benzoxazinoids (as no crude cereal root exudate samples contained a single compound), which could be explored by further dose-response analyses as undertaken in Chapter 2. Other, non-benzoxazinoid compounds

may also synergise with these putative allelochemicals, or contribute to their environmental persistence by altering degradation dynamics in the rhizosphere. The contribution of these compounds could be examined through bioassay-guided fractionation of crude root exudates (Duke, 2015). The use of an allelochemical crop will always carry a risk of variability in potential dose, which could be inhibitory to the crop if too high, and ineffective towards target species if too low. Such unpredictability was offered as an explanation by Masiunas, Weston and Weller (1995) for the inconsistent effects of rye mulch applied to control weeds in tomato fields, and may be explanatory of the limited effects observed in-field by Stowe (1979).

There is precedent in existing literature for the detrimental influence of microbial activity on benzoxazinoids (Macías *et al.*, 2004; 2005), and by extension their allelopathy to black-grass, which further contributes to the sensitive nature of in-field allelopathy (examined in detail in Chapter 5). The next step would be a competition assay such as those undertaken under glasshouse conditions, but transplanted to a field setting. Without this step, it cannot be confirmed that such interactions would occur in agricultural soils under unregulated conditions, hence the advocation of field assays in literature on allelopathy (Inderjit and Weston, 2000; Inderjit, Kaur and Foy, 2001; Inderjit and Nilsen, 2003).

6.3.2. Stacking the deck: approaches to stimulate benzoxazinoid exudation

For application, alternative approaches could be used to optimise benzoxazinoid release. Previous works have noted the allelopathic potential of wheat straw (Steinsiek, Oliver and Collins, 1982; Al Hamdi *et al.*, 2001), and rye mulch (Tabaglio *et al.*, 2008), on weeds. Given that benzoxazinoid content within plant tissues is likely to be greater than levels in root exudate (Escobar and Niemeyer, 1993; Stochmal *et al.*, 2006), the application of cereal straw would increase allelochemical concentration and also delay its release as tissues decompose, potentially delaying degradation of these short-lived compounds.

As a more sophisticated alternative, the anthropogenic application of a stress signalling compound like *cis*-jasmone has stimulated benzoxazinoid accumulation in wheat tissues (Moraes *et al.*, 2008), and comparable defence behaviours in other

crop species (Sobhy *et al.*, 2017). This may present a method for augmenting allelopathic exudation where weed infestation is great. The induction of polyploidy in wheat, rye and maize with colchicine *in vitro* has been correlated with increased benzoxazinoid exudation given the existence of more genetic material conferring it (Oliveros-Bastidas *et al.*, 2018). The application of this finding in-field would require a great deal of further work to ensure that other developmental metrics are not detrimentally affected. At the least, however, it vindicates the examination of inducibility interactions and, more broadly, approaches to stimulate benzoxazinoid exudation for allelopathic benefit.

6.3.3. Seeing wood for the trees: the importance of understanding the limitations of an assay system

Throughout the experiments undertaken in this thesis, repeats of the same experiment have, on occasion, produced differing results which are a source of curiosity. Such results were particularly notable in the hydroponic experiments undertaken in Chapter 3, which can stimulate atypical root development and responses (Tavakkoli, Rengasamy and McDonald, 2010). These conditions could alter allelochemical exudation dynamics and should therefore be avoided for studies of this nature. Moreover, some results from glasshouse competition assays disagreed with previous *in vitro* assays. Allelopathy is a sensitive phenomenon, and variability in these systems highlights the importance of designing an effective elucidating system and controlling confounding factors (*e.g.* Haugland and Brandsaeter, 1996).

Even without the limitations inherent in optimising a system in a limited timeframe, it is essential to understand the caveats inherent in lab-based examination of allelopathic interactions. It is typically the roots of target plants which are particularly sensitive in traditional allelopathy studies (Haugland and Brandsaeter, 1996), which are confirmed by results throughout this thesis. Neglected in this statement, however, is its consequence for application, as it is important to prove that this control of roots culminates in an overall inhibition of development, or at the very least a reduction in competitive ability. Otherwise, such root inhibition is of little application for the purpose of weed control. Glasshouse assays undertaken in Chapter 5 were intended to confirm the connection between allelopathic root

inhibition and altered competitive ability. This was examined through the utilisation of a longer growing period than *in vitro* assays, and measurement of whole-plant and shoot biomass as well as root development. While this step is necessary, however, it is complicated by the reduced sensitivity of older plants or aboveground tissues to the effects of allelopathy (Zhang *et al.*, 2021).

In effect, this is a fine balance to achieve in assay design; donor plants must be large enough to exude compounds into soil sufficiently widely to elicit effects in neighbours, and target plants must be large enough to reach and uptake such allelochemicals, but young enough for such effects to be clearly elucidated. The fact that this balance is likely to depend on the specific conditions of each assay and the plant material therein only further complicates this conundrum. Even individual plants of a single line or accession (particularly in ancestor material) can differ genetically, which may influence allelopathic potential on an individual level (Quader *et al.*, 2001). The assays developed here were valuable in evaluating responses in the appropriate target plant species and facilitating the identification and quantification of individual exudate components. Nevertheless, the limitations of such systems mean that evaluation under true field conditions would assist the elucidation of clear effects, but such assays were not possible within the timeframe or resources of this project.

6.4. Solving the neck riddle of allelopathy

6.4.1. The same flawed approaches, the same complicated conundrum

While this study has attempted to follow the guidance of literature on best practice in investigations into allelopathic behaviour, it is still common for contemporary studies to prioritise '*grind and find*' or '*thrill of the kill*' approaches (Romeo, 2000; Duke, 2015). It is at least partially for this reason that the reputation and adoption of allelopathy as a potential alternative weed control method remains limited. The adherence to flawed approaches to address an incredibly complicated challenge only proliferates the reputation of allelopathy as a neck riddle (Williamson, 1990); as Harper (1975) summarised, a phenomenon near impossible to prove, but logically impossible not to exist. Even the pioneering work of Muller (1966) followed frustrated reports of limited allelopathic interference in some desert shrubs. In one

of these preceding works, it was concluded that even in the relative simplicity of a desert environment, ecological interactions were too complex for allelopathy to be successfully untangled at the time (Muller, 1953). Throughout this thesis, inter-replicate variability in allelopathic potential indicates that such complexity still cannot be entirely unpicked *in vitro* almost 70 years later. Such factors, and the range of inhibitory potential that they confer, must therefore be better understood.

6.4.2. Novel approaches for elucidating allelopathy

There do however exist novel, underutilised methods which may circumvent traditional challenges related to allelopathy studies. One such method, pioneered in the early days of root exudation studies, and still applicable today, uses activated carbon in experimental soil to absorb bioactive molecules and thereby prevent allelopathy in a system without altering other variables (Schreiner and Reed, 1907; Inderjit and Callaway, 2003). In this way, allelopathic crops could be grown with and without activated carbon in order to examine the specific effect of allelopathy on development while not removing effects of resource competition. Although it is important to check that the presence of activated carbon does not alter plant growth through increasing nutrient availability (Lau *et al.*, 2008), this approach remains a valuable option (*e.g.* Abhilasha *et al.*, 2008).

A more sophisticated approach to limiting other factors is to genetically modify allelopathic species. In sorghum, recent effort has been made to identify genetic regions involved in sorgoleone synthesis and allelopathy (Shehzad and Okuno, 2020), paving the way for future efforts to attempt upregulation of these genes for greater allelopathic effect. A related option is to knock out the genes coding for synthesis of the identified allelochemical (*sensu* Yoshida *et al.*, 2017), which would theoretically allow for the comparison with a wild type for allelopathic effects. Promising advances in CRISPR/Cas9 gene editing technology indicate its great value in modifying wheat genes to alter desirable traits (Y. Zhang *et al.*, 2016), for example the recent development of wheat plants with reduced content of free asparagine through knockout of a gene conferring its synthesis (Raffan *et al.*, 2021). Such an approach could therefore be used to alter benzoxazinoid synthesis genes in cereals. Specifically, examination of the role of the *Bx1* gene in maize indicates that it is likely

to be the predominant determinant of its potential to synthesise and exude DIMBOA (Butrón *et al.*, 2010). It is possible that its knockout could therefore allow the examination of the influence of allelopathy as described, although the involvement of multiple genes in benzoxazinoid synthesis (Frey *et al.*, 1997) may complicate this approach. Given the findings of Chapter 4, it is likely that the *Bx6* and *Bx7* genes facilitating conversion from DIBOA to DIMBOA *in planta* have evolved with modern wheat breeding (Jonczyk *et al.*, 2008). Knockout of these genes could therefore act as a switch between these compounds and should thus be examined as a potential avenue to provide further understanding of benzoxazinoid allelopathy.

On the other hand, plant growth (and therefore ability to compete for resources) may be significantly affected by the removal of a gene conferring allelochemical synthesis. Züst *et al.* (2011) reported that the removal of allelochemical synthesis genes confirmed the fitness cost of defence metabolite synthesis, as the knockout of glucosinolate synthesis genes in *Arabidopsis* had the effect of stimulating early growth compared to the more defensively capable wild type. Nonetheless, with further understanding of such effects, the knockout of allelochemical synthesis genes may be a useful tool for the examination of the effects of allelopathy.

6.4.3. Enduring doubts lead to limited adoption

The need for novel approaches in elucidating allelopathy is great, as the enduring reputation of allelopathy as an almost unknowable quantity has translated to scepticism of end users. The result is a lack of adoption, or even knowledge of potential allelopathic benefits in agriculture (Trezzi *et al.*, 2016). As noted in Section 1.3.4, sorghum (through its allelochemical metabolite sorgoleone) represents one of the best understood examples of an allelopathic crop species at time of writing. The allelopathic potential of sorghum may be of great value given its predominant cultivation in low-input, smallholder systems in arid and semi-arid (and therefore stressful) environments. In spite of this, however, knowledge of sorghum allelopathy among farmers in these systems is limited, a recent study from Zimbabwe reporting that only 29% of farmers were aware that the species has potential to inhibit weeds (Tibugari, Chiduza and Mashingaidze, 2020). More broadly, only around 10% of rice

farmers interviewed in Côte D'Ivoire were knowledgeable of any potentially beneficial allelopathic species (Yao *et al.*, 2019). Such statistics are concerning as knowledge of these benefits is required for their application to reach a meaningful scale. The widespread uptake of allelopathy-inspired weed control solutions will only be achieved by the adoption of reliable approaches to prove their potential for benefit, which would proliferate education about the applications of these approaches for agriculture.

6.5. Vision: what can allelopathy contribute to weed control?

As outlined throughout this work, there are multiple novel applications possible for allelopathic crops or compounds, as a main crop, a cover or companion crop within an integrated weed management strategy, as a trait for breeding or genetic engineering into modern crop cultivars, or as a naturally-derived herbicide.

6.5.1. Allelopathy-inspired bioherbicides and modes of action

The potential for allelochemicals to contribute to the development of naturally-derived herbicides is greatly undervalued. Not one of the bioherbicides detailed by Cordeau *et al.* (2016) were of phytic origin, and only few such compounds are known to exist (Dayan, Owens and Duke, 2012). With benzoxazinoids, the compound closest to application is D-DIBOA (Chinchilla *et al.*, 2015), but examination of this compound in Section 5.3.5 outlines, somewhat surprisingly, its ineffectiveness towards black-grass. Even if an effective and persistent black-grass inhibitor was identified, there would remain a great deal of outstanding work, examining such factors as nontarget toxicity and efficacy towards a wide range of agricultural weeds, which would be required prior to commercialisation. It is therefore clear that allelochemical-derived benzoxazinoid herbicides are still far from application.

The missed opportunity of allelochemical-derived bioherbicides is especially important given the potential for novel or multiple modes of action to be discovered with potential to overcome herbicide resistance (Gressel, 2020; Hachisu, 2021). The discovery of novel herbicide modes of action has been very slow since the 1980s, due to widespread reliance on glyphosate (Duke, 2012; Peters and Strek, 2018; Dayan, 2019). Only one herbicide containing a novel mode of action has been commercialised since this time, specifically the dihydroorotate dehydrogenase

inhibitor tetflupyrolimet (Dayan *et al.*, 2019). Other novel modes of action have been retrospectively identified in old actives (*e.g.* cinmethylin and aclonifen) (Campe *et al.*, 2018; Kahlau *et al.*, 2020), while other recently discovered molecules exhibit novel modes of action which may be used to develop herbicides in the future (*e.g.* Shino *et al.*, 2018). While this recent progress is encouraging, it is unlikely to be sufficient to outpace the development of herbicide resistance without a change in approach (Gaines, Busi and Küpper, 2021).

6.5.2. Focusing the search: clues to phytotoxic potential in nature

The identification of allelochemicals with potential to break herbicide resistance may provide a shortcut in the development of bioherbicides. The traditional approach of herbicide discovery involves ideation and modelling based on known effective synthetic molecules (Peters and Strek, 2018). A comparable, but more informed, approach has recently been advocated in anti-malarials, some of which also have phytotoxic properties (Corral *et al.*, 2017). Indeed, a modelling approach to identify potential herbicidal compounds recently produced a promising candidate from a pool of anti-malarials (Sukhoverkov *et al.*, 2021). On a related note, this study coheres with the wider theory of multi-kingdom potential in plant allelochemicals, the extent to which will only be determined by further investigation (Hickman *et al.*, 2021). More generally, the great potential of a modelling approach to identify novel compounds with herbicidal properties is well-recognised (Sparks, Hahn and Garizi, 2017; Oršolić *et al.*, 2021). To this end, a high-tech modelling approach to identify compounds related to allelochemicals or phytotoxic molecules could streamline and assist the development of effective plant-derived bioherbicides and improve their chances of reaching application or commercialisation.

It is, by extension, striking that a number of plant species with documented allelopathic potential have uses in traditional medicines, such as olive (*Olea europaea*), squill (*Drimia maritima*), rue (*Ruta graveolens*), lavender (*Lavandula angustifolia*) (Aliotta, Mallik and Pollio, 2008), and sage (*Salvia officinalis*) (Bouajaj *et al.*, 2013). Specific allelochemical compounds are also linked, for example artemisinin (or '*Qinghaosu*') from sweet wormwood (*e.g.* Knudsmark Jessing, Duke and Cedergreen, 2014), a traditional treatment for fever in China for over 2,000 years

(Klayman, 1985). A recent review even examined derivatives of the benzoxazinoids APO and AMPO as potentially effective anti-cancer drugs (Zorrilla *et al.*, 2021), further drawing the connection between potentially pharmacological and allelopathic compounds. The trend between medicinal plants and allelopathy has been explored more widely in some studies (Islam, Yeasmin, *et al.*, 2018), but has primarily been approached in broad screens of a large range of species, followed by recommendations of suitable candidates for further work (Fujii *et al.*, 2003; Islam, Hasan, *et al.*, 2018; Sothearith *et al.*, 2021). As such, this link is far from utilisation for bioherbicide production to date.

Many of the medicinal plants examined for allelopathic potential are of tropical origin, and there is some belief that this link is not coincidental. Ooka and Owens (2018) hypothesised that tropical conditions may be particularly conducive to the evolution of allelopathic behaviour given otherwise favourable growing conditions and great plant diversity. Thus, exotic plants wherein 'novel weapons' to widespread agricultural weeds are more likely to exist may have greater potential for discovery of compounds with allelopathic properties (Zhang *et al.*, 2021). Such an approach would constitute a form of bioprospecting, the search for novel compounds in biodiverse ecosystems, typically for pharmaceutical applications (Mateo, Nader and Tamayo, 2001). Bioprospecting for agrochemical compounds, although not specifically herbicides, has also been advocated (Strobel and Daisy, 2003). Souza *et al.* (2008) exemplify this approach in their testing of tropical species from Brazil for inhibitory activity towards agricultural pests. These methods could easily be fitted into an allelopathy study given the simple and versatile methods developed throughout this thesis, meaning that bioprospecting for allelopathic compounds is a future possibility.

6.5.3. Allelopathic crops for integrated weed management

The deployment of allelopathic crops may also contribute to the control of herbicide-resistant weeds. Such application would benefit from contributions to the emergent understanding of plant-plant communication and recognition, as well as greater understanding of potential non-target effects of putative allelochemicals (*e.g.* Fritz and Braun, 2006). This greater understanding would elevate allelopathic

plants from blunt objects for weed control into intelligent devices to fit into an integrated weed management programme. The widespread herbicide resistance of black-grass, so detrimental to cereal agriculture, requires novel approaches to overcome. The existence of potent benzoxazinoid allelochemicals therefore suggest one such novel approach which may tip the balance against the weed in certain conditions. As an aspect of integrative weed management, there is great importance in inhibiting emerging weed seedlings given the effect this has on crop-weed competition at later stages (Storkey *et al.*, 2021), and thus benzoxazinoid allelopathy may be an important component of IWM programmes at this stage. This provides an example of the potential for allelopathy to be used in weed control, and as such it is envisioned that similar cases exist where these opportunities are currently undervalued.

6.6. Novel aspects, findings, and contributions to understanding

This investigation has contributed significantly to understanding of cereal defence and plant-plant interactions, which can be used to inform the potential applications detailed in Section 6.5. The most novel contributions to this area of science were those involving the importance of screening promising compounds against an ecologically relevant target species across multiple genetically variable populations to confirm inhibitory potential, the understanding of intraspecific diversity in root exudate composition, the use of cultivars of interest from defence against other biotic stresses, and the inclusion of biologically active media in laboratory assays to gain understanding of their degradative effect on allelopathy.

6.6.1. Know your target: the effect of target species and population on allelopathic potential

An important contribution of this work is the use and analysis of multiple biotypes of an ecologically relevant target species for differences in phytotoxicity with herbicide resistance. The results presented are striking as they improve the reliability of findings of DIMBOA and DIBOA allelopathy towards black-grass but, more crucially, confirm that herbicide resistance status does not have a clear influence on their inhibitory potential. While the mode of action of these compounds

has not been examined, these results indicate promise that it may be novel in comparison to existing synthetic black-grass herbicides.

While phytotoxicity of benzoxazinoids was not associated with known herbicide resistance biotypes, there was some variation in magnitude of allelopathy by black-grass population. This highlights the importance of assaying multiple populations to negate the misleading effects of atypically sensitive or resistant biotypes. Such findings build on previous works detailing the necessity of testing specific target species against an allelopathic entity (*e.g.* Macías *et al.*, 2006), rather than a standard test species which has limited ecological relevance and, in the case of a species like lettuce, is atypically sensitive to phytotoxins (*e.g.* Romeo, 2000). Parallels can be drawn with a recent study of the more extreme effects of benzoxazinoids on nematodes, of which some species suffer toxicity or repulsion, while others are tolerant and ostensibly attracted to the roots of the exuding species (Sikder *et al.*, 2021). Both plants and other biota therefore appear to have species-specific (and indeed biotype-specific) vulnerability to benzoxazinoids (and by extension other plant defence compounds), the genetic causes of which must be understood for the sake of application.

6.6.2. Understanding cereal root exudates: allelopathic potential in wheat germplasm

The variable nature of root exudates is known to be related to nutrient availability, plant age, both biotic and abiotic stress, and the interpretation of signalling compounds (Tang *et al.*, 1994). In spite of this great variability on an individual scale, it is rare for root exudates of a wide range of both modern and ancestor germplasm within a single species to be screened for variability in terms of either inhibitory potential or composition. Quader *et al.* (2001) provide a good example of this approach (although only using one species) in their screening of DIMBOA content in 26 lines of *Triticum spelta*, correlating this with allelopathic potential. Macías *et al.* (2004) also examined variations in benzoxazinoid content in six modern wheat varieties, but did not correlate such variation with allelopathic potential. In-field screening of wheat, rye, and *Triticosecale* hybrids for allelopathic potential towards black-grass was undertaken by Bertholdsson, Andersson and

Merker (2012), but this did not attempt to identify the causative allelochemicals exuded. To this end, results relating crude root exudates and benzoxazinoid content in both modern and ancestor cereal biotypes are a vital validation of findings of allelopathy elsewhere in this thesis.

Moreover, there is novelty to the finding that wheat ancestors do not exude the same benzoxazinoid allelochemicals as modern commercial cultivars, suggesting variability in genes relating to their synthesis. Previous works have noted that ancestors can exude different compounds, but wheat appears to be an outlier given more limited diversity of root exudates in ancestors, rather than the greater diversity of the maize ancestor teosinte (Köllner *et al.*, 2008). This may relate to the greater ploidy of modern wheat, but the precedent that this work has set for germplasm-wide analysis of root exudate variability must be pursued further to facilitate better understanding.

6.6.3. The Guidance of other Kingdoms: shortcut to allelopathy or a false summit?

As extensively detailed in Section 1.7.1 of this thesis and its associated literature review (Hickman *et al.*, 2021), individual benzoxazinoid compounds have inhibitory potential towards multiple kingdoms of biotic stress, discovered long before their allelopathy towards plants (*e.g.* Virtanen, Hietala and Wahlroos, 1957; Klun, Tipton and Brindley, 1967). The rationale of examining ancestor wheats for allelopathic potential was that these biotypes had varying potential for defence against take-all fungus and aphids, which may logically be associated with variations in benzoxazinoid synthesis given their defensive roles. Such knowledge on plant defence towards other biological kingdoms has potential to guide identification of potent allelopathic material.

In fact, 'MDR037' was a particularly potent allelopathic treatment against black-grass, an unexpected result given its lack of defensive potential in previous assays (McMillan, Gutteridge and Hammond-Kosack, 2014; Simon *et al.*, 2017). The likely explanation of this result is the influence of allocation in plant defence (discussed in Section 1.6.3); logically, defence against aphids would require accumulation in aboveground tissues, while take-all may be inhibited by accumulation within root

material. In both cases, accumulation elsewhere is not a guarantee of exudation from roots. Assuming a comparable degree of benzoxazinoid synthesis in different varieties, it may instead be that such a requirement to accumulate these compounds *in planta* to deter biotic stressors is detrimental to their exudation from roots and therefore allelopathic potential. Of course, this theory would need to be explored through quantification of compounds in different tissues of biotypes with varying defensive capabilities. At present, however, there may be value in examining crop lines which synthesise known allelochemicals, but which do not have noteworthy defensive capability against other biological kingdoms. For application, the relative defensive potential of different crop biotypes to all enemies they may encounter in-field would be required, as well as the examination of such defensively capable lines for yield penalties.

6.6.4. Bridging gaps: an attempted holistic approach

While partially limited by equipment availability, expertise, Covid-19-related restrictions and other unforeseen challenges, it is significant that this investigation attempted to balance the fields of chemical ecology, plant physiology, soil microbiology, and agronomy, guided by the framework of Willis (1985) (Sections 1.3.5 and 6.2). It is common for one or more of these fields to be neglected in allelopathy studies, and disciplinary gaps are part of the reason for the poor reputation of allelopathy as a subject. Indeed, most studies focus on experiments comparable to those detailed in a single chapter of this study. This disciplinary focus opens such works to obvious criticism, as *in vitro* assays rarely extend to proof of effect in biologically active media (*e.g.* Stowe, 1979). Conversely, results from in-field or glasshouse assays are often hindered by the absence of an isolated effect (removing the effect of resource competition or other potentially confounding factors which may induce an allelopathic effect), or the identification of a putatively allelopathic compound.

The result of this multi-disciplinary approach is a clearer picture for application: there is inter- and intra-specific variation in crude cereal root exudate allelopathy, associated with the benzoxazinoid group of allelochemicals. For application, it is highlighted that microbial action is likely to be detrimental to

benzoxazinoid persistence in soil, and, crucially, this limits their inhibitory potential towards black-grass, as identified in small-scale axenic systems. It is for this reason that biological treatments in the shape of allelopathic crops, likely exuding lesser concentrations of these compounds but over a much longer timescale, are advocated as more promising inhibitors. This conclusion can be linked to both crude exudate allelopathy of these treatments, and chemical analysis of their composition, confirming the presence of compounds previously noted as inhibitory. This range of disciplines and approaches have therefore combined to produce an examination of a case of putative allelopathy which is more holistic in scope than most existent literature on the subject.

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Appendices

Appendices

Appendix 1: Benzoxazinoid Synthesis (Undertaken and provided by Dr. David Withall, BCP Department, Rothamsted Research)

A1.1. Synthesis of benzo[d]oxazol-2(3H)-one (BOA)

To a solution of 2-aminophenol (250 mg, 2.29 mmol) in DMF (20 ml), under N_2, was added N,N'-carbonyldiimidazole (446 mg, 2.75 mmol) and the reaction heated to 85 ^oC for 24 hours. The reaction mixture was cooled to RT, poured into water and extracted with EtOAc. The combined organics were dried ($MgSO_4$) and concentrated under vacuum. The crude product was purified on silica gel (30% EtOAc in petroleum ether) to give BOA (217 mg, 70% yield) as a white solid.

^{1}H-NMR ($CDCl_3$, 500 MHz): 9.64 (bs, 1H), 7.25 (d, 1H, *J = 7.7 Hz*), 7.20 (m, 1H), 7.15 (m, 2H). **^{13}C-NMR ($CDCl_3$, 125 MHz):** 156.13, 143.91, 129.33, 124.23, 122.80, 110.23, 110.16.

A1.2. Synthesis of 2-amino-5-methoxyphenol

To a solution of 5-methoxy-2-nitrophenol (500 mg, 2.96 mmol) in 1:1 MeOH:EtOAc (10 ml) was added 10% Pd/C (40 mg). The reaction vessel was flushed with hydrogen gas ten times before being allowed to stir for 5 hours. The reaction flask was flushed with N_2 ten times before removal of the catalyst via celite filtration. Concentration of the filtrate gave 2-amino-5-methoxyphenol (374 mg, 91% yield) as a red solid.

^{1}H-NMR ($CDCl_3$, 500 MHz): 6.81 (d, 1H, *J = 8.5 Hz*), 6.46 (d, 1H, *J = 2.7 Hz*), 6.37 (dd, 1H, *J = 8.5, 2.7 Hz*), 3.76 (s, 3H). **^{13}C-NMR ($CDCl_3$, 125 MHz):** 155.34, 148.21, 125.54, 121.08, 105.58, 101.74, 55.62

A1.3. Synthesis of 6-methoxybenzo[d]oxazol-2(3H)-one (MBOA)

To a solution of 2-amino-5-methoxyphenol (374 mg, 2.69 mmol) in DMF (20 ml), under N_2, was added N,N'-carbonyldiimidazole (656 mg, 4.04 mmol) and the reaction heated to 85 ^oC for 36 hours. The mixture was cooled to RT, poured into water and extracted with EtOAc. The combined organics were dried ($MgSO_4$) and concentrated under vacuum. The crude product was purified on silica gel (40% EtOAc in petroleum ether) to give MBOA (353 mg, 80% yield) as a pink solid.

^{1}H-NMR ($CDCl_3$, 500 MHz): 9.06 (bs, 1H), 7.00 (d, 1H, *J = 8.5 Hz*), 6.87 (d, 1H, *J = 2.3 Hz*), 6.74 (dd, 1H, *J = 8.5, 2.4 Hz*), 3.83 (s, 3H).**^{13}C-NMR ($CDCl_3$, 125 MHz):** 156.21, 156.07, 144.62, 122.69, 110.05, 109.70, 97.55, 56.01

A1.4. Synthesis of potassium 2-nitrophenolate

To a solution of 2-nitrophenol (5 g, 35.94 mmol) in DCM (15 ml) was added potassium hydroxide (2.02 g, 35.94 mmol) in water (10 ml) and the solution vigorously stirred for 16 hours. The solvent was removed under vacuum before drying further in a vacuum desiccator for 3 days to give potassium 2-nitrophenolate (6.32 g, 99% yield) as an orange solid.

^{1}H-NMR (d_6-DMSO, 500 MHz): 7.66 (d, 1H, *J = 8.4 Hz*), 7.04 (t, 1H, *J = 7.5 Hz*), 6.52 (d, 1H, *J = 8.7 Hz*), 6.08 (t, 1H, *J = 7.5 Hz*).**^{13}C-NMR (d_6-DMSO, 125 MHz):** 166.54, 136.70, 134.22, 126.81, 126.73, 109.55

A1.5. Synthesis of potassium 5-methoxy-2-nitrophenolate

To a solution of 5-methoxy-2-nitrophenol (5 g, 29.56 mmol) in DCM (15 ml) was added potassium hydroxide (1.66 g, 29.56 mmol) in water (10 ml) and the solution vigorously stirred for 16 hours. The solvent was removed under vacuum

before drying further in a vacuum desiccator for 3 days to give potassium 5-methoxy-2-nitrophenolate (5.91 g, 97% yield) as an orange solid.

^{1}H-NMR (d_6-DMSO, 500 MHz): 7.63 (d, 1H, *J = 9.5* Hz), 5.78 (d, 1H, *J = 2.3* Hz), 5.61 (dd, 1H, *J = 9.5, 2.3* Hz). **^{13}C-NMR (d_6-DMSO, 125 MHz):** 170.57, 164.55, 130.93, 128.36, 106.35, 101.19, 55.06

A1.6. Synthesis of methyl 2-methoxy-2-(2-nitrophenoxy)acetate

To a solution of methylmethoxyacetate (4.56 g, 43.86 mmol) and N-bromosuccinimide (7.81 g, 43.86 mmol) in carbon tetrachloride (30 ml) was added dibenzoyl peroxide (104 mg, 0.43 mmol) and the mixture heated to 80 ^{o}C for 2 hours. The reaction mixture was cooled to 0 ^{o}C for 15 mins before being filtered through cotton wool directly into a suspension of potassium 2-nitrophenolate (5 g, 29.24 mmol) in THF (60 ml) and stirred for a further 16 hours. The reaction mixture was diluted with DCM before being washed with water, dried ($MgSO_4$) and concentrated under vacuum. The crude product was purified on silica gel (25% EtOAc in petroleum ether) to give methyl 2-methoxy-2-(2-nitrophenoxy)acetate (5.56 g, 79% yield) as a pale yellow solid.

^{1}H-NMR ($CDCl_3$, 500 MHz): 7.87 (dd, 1H, *J = 8.2, 1.7 Hz*), 7.55 (td, 1H, *J = 8.1, 1.8 Hz*), 7.32 (dd, 1H, *J = 8.5, 0.9 Hz*), 7.20 (td, 1H, *J = 8.5, 0.9 Hz*), 5.60 (s, 1H), 3.86 (s, 3H), 3.62 (s, 3H). **^{13}C-NMR ($CDCl_3$, 125 MHz):** 165.91, 148.81, 133.93, 125.52, 123.09, 118.97, 98.64, 55.19, 53.04

A1.7. Synthesis of methyl 2-methoxy-2-(5-methoxy-2-nitrophenoxy)acetate

To a solution of methylmethoxyacetate (1 g, 9.60 mmol) and N-bromosuccinimide (1.71 g, 9.60 mmol) in carbon tetrachloride (20 ml) was added dibenzoyl peroxide (20 mg, 0.08 mmol) and the mixture heated to 80 ^{o}C for 2 hours. The reaction mixture was cooled to 0 ^{o}C for 15 mins before being filtered through cotton wool directly into a suspension of potassium 5-methoxy-2-nitrophenolate (2.19 g, 10.56 mmol) in THF (12 ml) and stirred for a further 2 hours. The reaction mixture was diluted with DCM before being washed with water, dried ($MgSO_4$) and concentrated under vacuum. The crude product was purified on silica gel (25% EtOAc in petroleum ether) to give methyl 2-methoxy-2-(5-methoxy-2-nitrophenoxy)acetate (1.89 g, 73% yield) as a pale yellow solid.

^{1}H-NMR ($CDCl_3$, 500 MHz): 7.99 (d, 1H, *J = 9.3 Hz*), 6.78 (d, 1H, *J = 2.5 Hz*), 6.67 (dd, 1H, *J = 9.1, 2.5 Hz*), 5.59 (s, 1H), 3.88 (s, 3H), 3.86 (s, 3H), 3.62 (s, 3H).**^{13}C-NMR ($CDCl_3$, 125 MHz):** 165.94, 164.24, 151.41, 134.29, 128.06, 107.96, 104.73, 98.72, 56.03, 55.18, 53.03

A1.8. Synthesis of 4-hydroxy-2-methoxy-2H-benzo[1,4]oxazin-3(4H)-one

To a vigorously stirred suspension of 10% Pd/C (200 mg) in 1:1 1,4-dioxane:water (180 ml) was added sodium borohydride (1.05 g, 27.67 mmol). Methyl 2-methoxy-2-(2-nitrohenoxy)acetate (5.56 g, 23.06 mmol) in 1,4-dioxane (10 ml) was added dropwise and the reaction mixture stirred for a further 30 mins after complete addition. The reaction mixture was filtered through celite and the filtrate pH adjusted to 3 with 2M HCl before being extracted with EtOAc. The combined organics

were dried ($MgSO_4$) and concentrated under vacuum. The crude product was purified on silica gel (35% EtOAc in petroleum ether) to give 4-hydroxy-2-methoxy-2H-benzo[1,4]oxazin-3(4H)-one (3.22 g, 72% yield) as a pink solid.

^{1}H-NMR (d_6-DMSO, 500 MHz): 11.07 (bs, 1H), 7.29 (m, 1H), 7.14 (m, 2H), 7.09 (m, 1H), 5.57 (s, 1H), 3.45 (s, 3H).**^{13}C-NMR (d_6-DMSO, 125 MHz):** 156.31, 140.42, 129.19, 124.60, 123.64, 117.64, 113.61, 98.25, 56.32.

A1.9. Synthesis of 4-hydroxy-2,7-dimethoxy-2H-benzo[1,4]oxazin-3(4H)-one

To a vigorously stirred suspension of 10% Pd/C (32 mg) in 1:1 1,4-dioxane:water (32 ml) was added sodium borohydride (167 g, 4.42 mmol). Methyl 2-methoxy-2-(5-methoxy-2-nitrophenoxy)acetate (1 g, 3.68 mmol) in 1,4-dioxane (3 ml) was added dropwise and the reaction mixture stirred for a further 30 mins after complete addition. The reaction mixture was filtered through celite and the filtrate pH adjusted to 3 with 2M HCl before being extracted with EtOAc. The combined organics were dried ($MgSO_4$) and concentrated under vacuum. The crude product was purified on silica gel (40% EtOAc in petroleum ether) to give 4-hydroxy-2,7-dimethoxy-2H-benzo[1,4]oxazin-3(4H)-one (501 mg, 61% yield) as a pink solid.

^{1}H-NMR (d_6-DMSO, 500 MHz): 10.99 (bs, 1H), 7.17 (d, 1H, *J = 8.8 Hz*), 6.77 (d, 1H, *J = 2.4 Hz*), 6.71 (dd, 1H, *J = 8.8, 2,4 Hz*), 5.54 (s, 1H), 3.74 (s, 3H), 3.45 (s, 3H) .**^{13}C-NMR (d_6-DMSO, 125 MHz):** 156.69, 155.57, 141.46, 122.82, 114.34, 108.68, 103.98, 98.65, 56.30, 56.00

A1.10. Synthesis of 2,4-dihydroxy-2H-benzo[1,4]oxazin-3(4H)-one (DIBOA)

To a solution of 4-hydroxy-2-methoxy-2H-benzo[d][1,4]oxazin-3(4H)-one (3 g, 15.38 mmol) in DCM (100 ml), cooled to -50 ^{o}C under N_2, was added precooled 1M boron trichloride solution in DCM (46.1 ml, 46.14 mmol). The reaction mixture was allowed to warm to RT over 3 hours before THF (30 ml) was added, poured into water and extracted with EtOAc. The combined organic layers were concentrated to ~20 ml under vacuum and diluted with THF (20 ml). This solution was added to a vigorously stirred suspension of silver carbonate (8.48 g, 30.76 mmol) in 2:1 water:THF (20 ml) and stirred for 30 mins. The mixture was filtered and extracted with EtOAc. The combined organics were dried ($MgSO_4$) and concentrated to ~25 ml under vacuum. Hexane was added dropwise to initiate crystallisation, after which the mixture was placed in a freezer for 3 days. Collection of the precipitate gave DIBOA (2.10 g, 76% yield) as an off-white solid. Spectroscopic data in accordance with previously reported literature.

A1.11. Synthesis of 2,4-dihydroxy-7-methoxy-2H-benzo[1,4]oxazin-3(4H)-one (DIMBOA)

To a solution of 4-hydroxy-2,7-dimethoxy-2H-benzo[1,4]oxazin-3(4H)-one (501 mg, 2.23 mmol) in DCM (20 ml), cooled to -50 ^{o}C under N_2, was added precooled 1M boron trichloride solution in DCM (6.7 ml, 6.69 mmol). The reaction mixture was allowed to warm to RT over 3 hours before THF (30 ml) was added, poured into water and extracted with EtOAc. The combined organic layers were concentrated to ~5 ml under vacuum and diluted with THF (4 ml). This solution was added to a vigorously stirred suspension of silver carbonate (1.23 g, 4.46 mmol) in 2:1 water:THF (6 ml) and

stirred for 30 minutes. The mixture was filtered and extracted with EtOAc. The combined organics were dried ($MgSO_4$) and concentrated to ~2 ml under vacuum. Hexane was added dropwise to initiate crystallisation, after which the mixture was placed in a freezer for 3 days. Collection of the precipitate gave DIBOA (189 mg, 40% yield) as a brown solid. Spectroscopic data in accordance with previously reported literature.