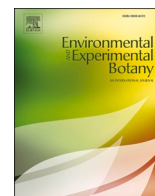




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Environmental and Experimental Botany

journal homepage: www.elsevier.com/locate/envexpbot

Benzoxazinoids in wheat allelopathy – From discovery to application for sustainable weed management

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ARTICLE INFO

Keywords:

Allelopathy
Benzoxazinoids
Detoxification
Phenoxazinones
Organic farming
Soil persistence
Weed control

ABSTRACT

Allelopathic activity of wheat (*Triticum aestivum* L.) has previously been associated with the production of phenolic acids and flavonoids (PAF), benzoxazinones (BXZs) and phenoxazinones (PXZs). The biosynthesis of BXZs is closely regulated during cereal growth, with accumulation highest in young tissues with variation associated with genotype and environmental conditions. This review is focused on BXZ metabolites and their impact on germination, seedling growth and physiological, biochemical, transcriptional and proteome traits of surrounding plants and weeds. The major pathways employed by plants for benzoxazinoid detoxification involve hydroxylation and glucosylation and polymerisation of intermediates in these pathways. Allelochemicals from various wheat genotypes have been shown to inhibit the growth of selected weed species, including *Bromus japonicus*, *Chenopodium album*, *Portulaca oleracea*, *Avena fatua* and *Lolium rigidum*. Wheat allelopathy is potentially exploited from the standpoint of crop mulches, incorporation of crop residues, tissue disruption, intercropping with allelopathic cultivars and application of aqueous wheat extracts. BXZs have been shown to suppress the growth and development of certain agricultural pests, including insects, fungal pathogens, and weeds. Many native plants, fungi and insect herbivores inherently possess varying tolerance levels towards BXZs. However, other BXZ- susceptible species are adversely impacted by elevated BXZ levels in crop plants. Thus, considerations for the selection and breeding of wheat genotypes possessing enhanced defensive ability via elevated BXZ contents are discussed. Here, these objectives are reconsidered with a focus on co-evolutionary aspects and their potential impacts on biodiversity in the agroecosystems under study. For future breeding efforts to be successful, it is important to take such potential adverse environmental impacts into account, in combination with an increased focus on enhancing beneficial allelopathic effects within agricultural systems.

1. Introduction

Plants have evolved over millions of years, with a diverse microbiome, plant symbionts and pests and other competitors, including a plethora of plant species. Co-evolution of diverse ecosystems and communities has resulted in developing individual species-based chemical profiles that reflect the highly specific defense metabolism undertaken

by plants to enhance their competitiveness with other organisms, including plants (Speed et al., 2015). It is well documented that plants release diverse primary and secondary metabolites through volatilisation, exudation, or decomposition of residues (Birkett et al., 2001; Latif et al., 2017). Plant-produced metabolites can directly or indirectly impact other organisms, depending on the metabolite mixture and mode of release. In this way, plants can attract, repel or 'advise' other

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<https://doi.org/10.1016/j.envexpbot.2022.104997>

Received 18 March 2022; Received in revised form 11 July 2022; Accepted 13 July 2022

Available online 19 July 2022

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organisms in close proximity or occasionally located more distantly. The capacity of plants to impact the growth and development of other plants and organisms through the production and release of chemical signals is referred to as allelopathy and the compounds involved are called allelochemicals (Rice, 1984).

Current research has shown that plant metabolites are present ubiquitously in higher plants and algae and concentrations and composition vary with respect to tissues, organs, and species (Latif et al., 2017). The production of such specialised defense metabolites is spatially and temporally regulated (Sweetlove and Fernie, 2013). Some are produced constitutively but in specific tissues or cell types, whereas others are present at low concentrations unless the plant is exposed to specific stress (Garagounis et al., 2021). This chemical network arising in natural ecosystems also exists in crop plants and other associated organisms within managed agroecosystems. Phenolic acids, terpenoids and nitrogen-containing compounds can serve as chemical messages that allow plants' rapid and direct communication with their surrounding environment. The principal biosynthetic pathways responsible for the cellular synthesis of these metabolites include the shikimic acid pathway (benzoic and cinnamic acids and their derivatives, coumarins, glycosides, alkaloids, etc.), and pathways derived from acetic and mevalonic acids (terpenoids, steroids, complex quinones, etc.) (Waste-mack and Strnad, 2019).

The presence of secondary metabolites is dependent on plant family, species and the chemotype (Russo et al., 1998). For example, the Labiatae family is particularly rich in terpenoids and phenolic acids (Verdeguer et al., 2020; De Mastro et al., 2021; Hamed et al., 2021), representing the main allelochemicals driving plant-plant interaction, whereas, in the Apiaceae family, the most prevalent secondary metabolites are represented by coumarins, furanocoumarins and terpenoids (Sousa et al., 2021) and the Poaceae family contains a plethora of hydroxamic acids and benzoxazinoids (Sánchez-Moreiras et al., 2004).

2. Occurrence and function of benzoxazinoids (BXZs)

Benzoxazinoids (usually called hydroxamic acids) are specialised metabolites commonly occurring in cultivated and wild graminaceous species. In maize, wheat and rye, the key hydroxamic acids identified included the benzoxazinones 2,4-dihydroxy-7-methoxy-(2H)-1,4-benzoxazin-3(4H)-one (DIMBOA) and 2,4-dihydroxy-(2H)-1,4-benzoxazin-3(4H)-one (DIBOA) (Macías et al., 2005a, 2005b). DIBOA has been documented also in other cereal species, including wheat, rye, maize, and various wild relatives (Macías et al., 2005a, 2005b). DIBOA catabolism in two wheat cultivars (Astron and Ritmo) was further evaluated. DIBOA was metabolised to the more active compound 2-benzoxazinone (BOA), which was further transformed microbially into several phenoxazinones, including the highly active APO and AAPO (Macías et al., 2005a, 2005b). The microbes responsible for transformation include both soil bacteria and fungi. BOA and APO degradation in soil is further dependent on soil moisture availability, microbial presence and environmental factors (Fomsgaard et al., 2004). However, phenoxazinones are generally less labile in the rhizosphere than their BXZ precursors (Mwendwa et al., 2021).

The phytotoxicity of BOA and DIBOA was initially characterised by several researchers (Barnes and Putnam, 1987; Sánchez-Moreiras and Reigosa, 2005; Macías et al., 2014). More recently, related microbial transformed metabolites phenoxazinones have been characterised and quantified in the rhizosphere of field-grown wheat and rye, and their biological activity determined in weed and crop spp. (Venturelli et al., 2015; Mwendwa et al., 2021). From the roots of *Acanthus mollis* L., two acetylated glycosylated BXs compounds, 2-(6-acetyl- β -D-glucopyranoside)-2,4-dihydroxy-2H-1,4-benzoxazin-3(4H)-one and (-2-(6-acetyl- β -D-glucopyranoside)-2-hydroxy-2H-1,4-benzoxazin-3(4H)-one) and a BXZ-derived glucoside carbamate olide, (2-hydroxy-6-(2-deoxy- β -D-glucopyranosyloxy)-phenyl) carbamic acid 2'-olide, were isolated and identified. Such previously unknown BXs may

contribute to the reported biological activities of *A. mollis* L. (Bhattarai et al., 2022).

Various benzoxazinoids and hydroxamic acids (BXZs), including BOA, DIMBOA, HMBOA, HBOA, MBOA and DIBOA, are synthesised in cereals via slightly different pathways, but are released from the plant tissues and residues by decomposition and through root exudation (from root hairs or secondary roots) into the surrounding soil environment (Macías et al., 2005a; Mwendwa et al., 2021).

Once produced and released, plant allelochemicals experience physico-chemical and microbial transformations resulting in alterations in phytotoxicity (Inderjit, 2005; Jilani et al., 2008; Mwendwa et al., 2021), resulting in production of novel allelochemicals by associated microorganisms. Allelochemical transforming non-pathogenic bacteria can directly impact crop growth by inducing toxicity in the crop plant or indirectly by suppressing a necessary symbiont (Barazani and Friedman, 2001).

Several climatic factors also contribute to the transformation of BXZ metabolites to phenoxazinones, including soil moisture, soil temperature, and the microbial community present in local soils (Mwendwa et al., 2021). In other studies, Krogh et al. (2006) and Mwendwa et al. (2021) both found MBOA, HMBOA and HBOA in the wheat rhizosphere, with MBOA present in relatively high concentrations in the rhizosphere and rhizoplane. In laboratory bioassays, MBOA, DIBOA, HBOA, HMBOA, and BOA were detected in wheat seedlings as early as 2–3 days of age (Krogh et al., 2006). Under field conditions, 17 related BXZ metabolites and phenoxazinones were detected in the rhizosphere and in root extracts of various wheat genotypes. Rye typically produced the highest concentrations of APO (up to ten times greater than those detected in wheat). Meanwhile, the wheat cultivar Federation and commercial cultivars (Condo, Espada, Federation, Gregory, Janz CL, Livingston, Wedgetail, Grazer) expressed greater early vigour and also produced higher levels of phenoxazinone metabolites than less vigorous wheat cultivars. Greatest phenoxazinone concentrations were recovered from soils experiencing dry production years or lower soil moisture content and also in soils collected from early season wheat stands in contrast to stands collected at crop maturity (Mwendwa et al., 2021).

While numerous crops have been reported to release allelochemicals able to suppress weeds, the graminaceous species, including sorghum, wheat, oats, rye, and maize, have been employed most successfully in weed suppression (Sánchez-Moreiras et al., 2003). The presence of benzoxazinones, hydroxamic acids, simple phenolics and flavonoids in these species makes them especially interesting in terms of their allelopathic properties. Certain competitive cultivars of wheat and barley have recently been shown to exhibit differential potential to suppress weeds both in the laboratory and in the field (Mwendwa et al., 2020, 2022; Hendriks et al., 2022). In a recent review Schandry and Becker (2020) evaluated potential benzoxazinoidphytotoxicity to surrounding weeds/plants, bacteria and fungi. While other cereals have shown strong allelopathic potential to suppress weeds, wheat has also been noted to express allelopathic potential associated with the presence of hydroxamic acids, benzoxazinones, phenoxazinones and flavonoids that could possibly be exploited for environmentally friendly weed management (Wu et al., 2003; Mwendwa et al., 2021).

3. Chemical stability of benzoxazinoids

Most natural products can be rapidly degraded in agricultural soils or the environment, and many are considered environmentally friendly. However, rapid degradation may affect their half-life or efficiency as agrochemicals as rapid degradation may limit their persistence and subsequent bioactivity (Dayan and Duke 2009).

Since the isolation of 2,4-dihydroxy-7-methoxy-(2 H)-1,4-benzoxazin-3(4 H)-one (DIMBOA) (Hamilton et al., 1962) and 2,4-dihydroxy-(2 H)-1,4-benzoxazin-3(4 H)-one (DIBOA) (Hietala et al., 1960) was reported in maize and rye respectively, benzoxazinoids with a (2 H)-1,4-benzoxazin-3(4 H)-one skeleton have attracted the attention of

researchers. These compounds are found in plants in their glycosylated forms and, by enzymatic activity, are released as aglycones (Cambier et al., 1999; Baumeler et al., 2000). As aglycones, they are generally unstable in aqueous solutions and in soil since they spontaneously transform into corresponding BXZs and other degradation products (Fomsgaard et al., 2004) through the loss of formic acid (Fig. 1), with the transformation products showing higher activity levels than their precursors (Friebe et al., 1998).

The characterisation of the degradation products from DIBOA-Glc and DIMBOA-Glc has been reported previously (Nair et al., 1990; Chase et al., 1991; Gagliardo and Chilton, 1992; Friebe et al., 1998; Zikmundová et al., 2002a, 2002b). More recently, some of the kinetic aspects of the degradation process have also been described, including metabolite half-life and the influence that certain microorganisms have on the degradation process itself (Macías et al., 2004; 2005a,b). Two principal factors, temperature and pH, impact degradation kinetics. Thus, the half-life of DIBOA varies from 16h at pH 4.0 at 75 °C to 4.5 min at pH 8.0 (Bredenberg et al., 1962). Similarly, the degradation process of DIMBOA varies from 20 h at pH 6.0 and 25 °C to just 7 h at a pH of 7.5 (Woodward et al., 1978). This suggests the influence of pH on the rate of degradation which is linked to deprotonation of the hydroxamic acid (a precursor in the degradation process) (Bravo and Niemeyer, 1985).

BOA, 2-benzoxazolinone, and MBOA, 6-methoxy-2-benzoxazolinone, are the first compounds to appear from spontaneous degradation in aqueous solutions from DIBOA and DIMBOA (Fig. 2) (Woodward et al., 1978; Macías et al., 2004, 2005b), as well as from transformations mediated by isolated microorganisms (Fomsgaard et al., 2004b) and the microflora found in cultivated soils (Gagliardo and Chilton, 1992; Kumar et al., 1993; Macías et al., 2005b). Therefore, the resulting products are significantly more stable than their precursors and are also easier to synthesise. As several are now commercially available, this has facilitated the completion of some interesting work on their bioactivity and possible modes of action (Sahi et al., 1990; Bravo and Lazo, 1996; Kato-Noguchi and Macías, 2006; Oliveros-Bastidas et al., 2021).

Fig. 3 presents the most widely accepted pathway associated with the benzoxazinone degradation process. Phenylacetamides, 2-hydroxy-N-(2-hydroxyphenyl)acetamide (HHPAA) and N-(2-hydroxyphenyl)acetamide (HPAA), are most likely derived from benzoxazinoids and are also described as conversion products from endophytic fungi isolated from the roots of *P. tabacinum*, *G. cibotii*, and *Chaetosphaeria* sp. (Zikmundová et al., 2002a; 2002b).

N-[2-hydroxyphenyl]malonic acid (HPMA) and N-[2-hydroxy-5-methoxyphenyl]malonic acid (HMPMA) are the detoxified metabolites of the benzoxazolinones (BOA and MBOA). Their biotransformation is carried out by the endophytic fungus *F. sambucinum*, and it is normally associated with BOA and MBOA-producing plants (Zikmundová et al., 2002a). These metabolites are also the only metabolites produced from HBOA and HMBOA since no further transformation occurs, suggesting that both metabolites are stable terminal products.

Through ¹³C labelling experiments, it has been demonstrated that these transformations also include *o*-aminophenol (*o*-AP), which is further transformed by acylation, nitration, and oxidation reactions into other compounds (DIBOA, DIMBOA) (Fig. 2). Thus, *o*-aminophenol seems to be the key intermediate compound in these biotransformations.

Aminophenoxacins constitute an interesting group of compounds related to benzoxazinones and benzoxazolinones. These tricyclic structures originate from the decomposition of benzoxazolinones into their

corresponding *o*-AP followed by dimerisation. Aminophenoxacins appear in certain cultivated soils after decomposition and dimerisation or due to the action of microorganisms (Gagliardo and Chilton, 1992; Kumar et al., 1993; Macías et al., 2004, 2005b). 2-(*N*-hydroxy)acetylamino-phenoxazine-3-one (NHAAPO) and 2-(2-hydroxyacetyl)amino-3-H-phenoxazine-3-one (HAAPO) appear after subsequent *N*-oxidation (Zikmundová et al., 2002a).

The dimerisation of aminophenols into aminophenoxazin-3-one results from the oxidation of aminophenols with oxygen in the air to produce aminophenoxazinone (APO). This compound was reported as 2,2'-oxo-1,1'-azonezene (AZOB) (Chase et al., 1991) and later, in 1992, its structure was corrected by Gagliardo and Chilton to 2-amino-3-H-phenoxazin-3-one. In fact, APO is most commonly synthesised in the laboratory through the oxidation of aminophenol (Macías et al., 2006b).

The accepted metabolic pathway begins with the hydrolysis of the hemiacetal ring of HBOA/HMBOA or the oxazolone ring of BOA/MBOA. *o*-aminophenol is formed in both cases and transformed by acylation into *N*-(2-hydroxyphenyl)acetamide or *N*-(2-hydroxyphenyl)malonic acid. *N*-acetyl- or *N*-malonyl-transferases are the enzymes reported to be associated with the biotransformation of these compounds, e.g. cytochrome P-450 monooxygenases.

4. Structure-activity relationship

The structure-activity relationship (SAR) in benzoxazinoids, (both natural and synthetic derivatives) was further elucidated in studies conducted by Macías et al. (2005a, 2006a). Weeds and cultivated species were tested for phytotoxicity. DIBOA and DIMBOA, together with the synthetic benzoxazinones 4-hydroxy-(2 H)-1,4-benzoxazin-3(4 H)-one (D-DIBOA) and 4-acetoxy-(2 H)-1,4-benzoxazin-3(4 H)-one (ABOA) were found to exhibit the highest phytotoxicity levels. Except for the highly toxic APO, none of the other compounds tested presented significant phytotoxicity. Wheat and lettuce registered the highest sensitivity of all the species tested and root formation was most affected in contrast to shoot growth.

Structural modifications of phytotoxic benzoxazinones were evaluated by Macías et al. (2006b) using D-DIBOA as the starting product. The objective was to implement several structural/conformational changes that might improve the phytotoxicity and selectivity of these compounds. One such modification was the esterification of D-DIBOA at the N-4 position, resulting in a new generation of compounds with enhanced lipophilicity proving superior as bioherbicides with greater selectivity than natural benzoxazinones (Macías et al., 2006b). Similar modifications were also implemented at the C-2 position, (Macías et al., 2008), which resulted in the modification of the steric and electronic characteristics of the base skeleton of the benzoxazinones proposed by Macías et al. (2006a), (2009). Halogenated models 6-chloro-(2 H)-1,4-benzoxazin-3(4 H)-one, 6-fluoro-(2 H)-1,4-benzoxazin-3(4 H)-one, and 7-fluoro-(2 H)-1,4-benzoxazin-3(4 H)-one (Fig. 4) were chosen based on their excellent results, where the last compound showed a very high *A. fatua*-wheat selectivity. The combination of the above-mentioned strategies has produced a more potent chemical that is more phytotoxic than the previous ones (Macías et al., 2010a, 2010b). The compounds with the highest selectivity levels in the system for *Avena fatua*-wheat were: 8-chloro-4-valeryl-(2 H)-1,4-benzoxazin-3(4 H)-one (8-Cl-4-Pr-D-DIBOA) and 6-fluoro-4-propyl-(2 H)-1,4-benzoxazin-3(4 H)-one (6-F-4-Pr-D-DIBOA). The results reported for

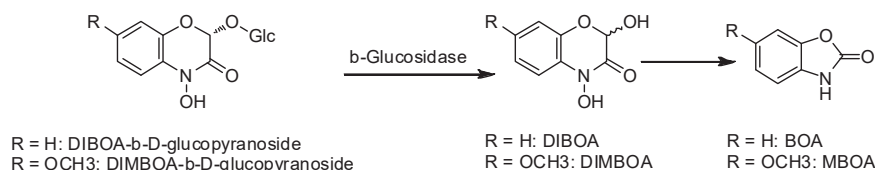


Fig. 1. Spontaneous degradation of DIBOA-Glc and DIMBOA-Glc to the corresponding benzoxazolinones BOA and MBOA.

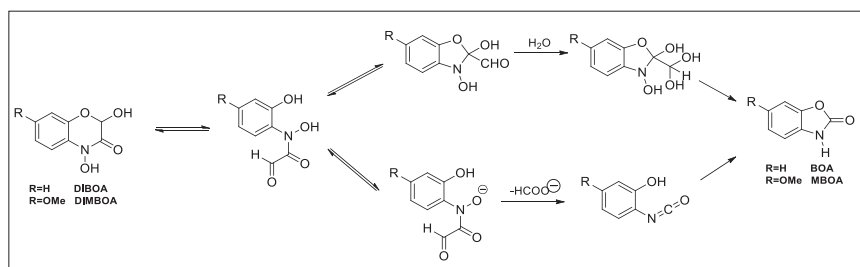


Fig. 2. Mechanisms for the transformation of DIBOA/DIMBOA to BOA/MBOA.

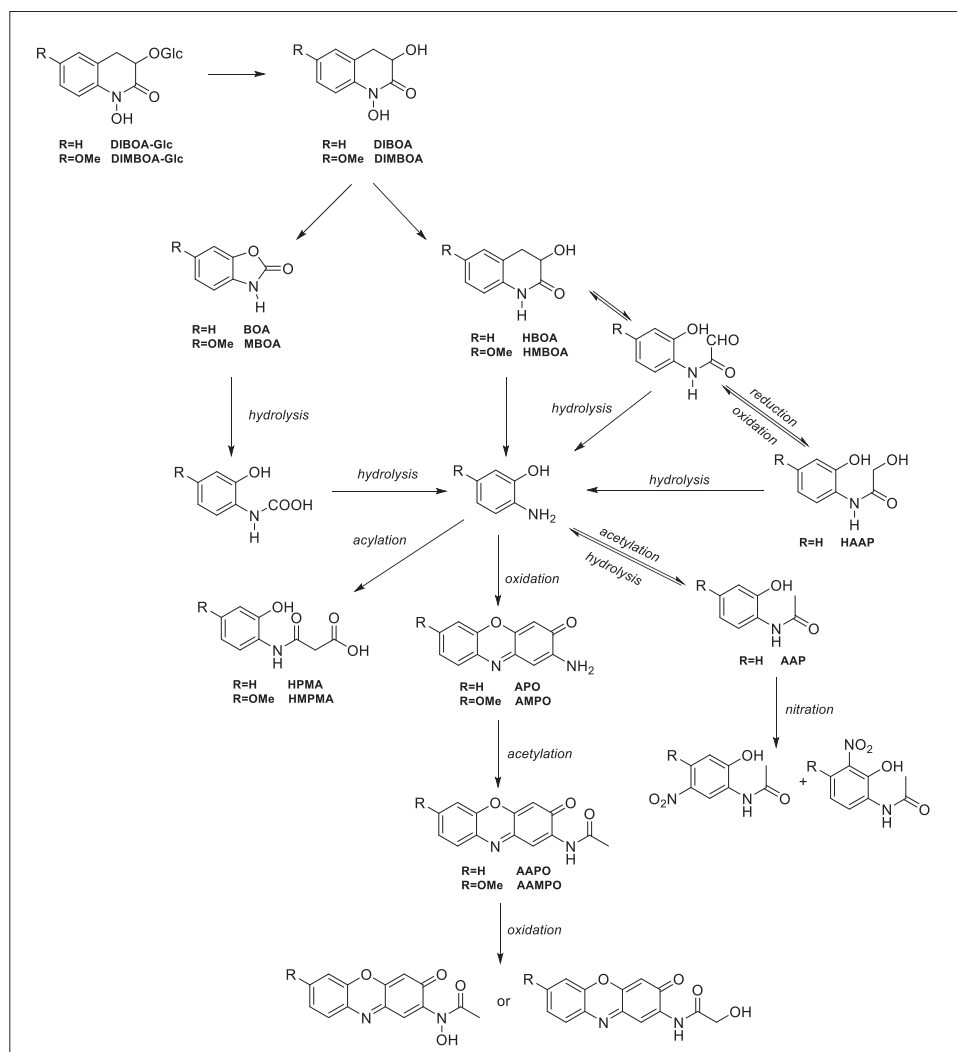


Fig. 3. Proposed scheme for biotransformation of natural Benzoxazinoids.

6-F-4Pr-D-DIBOA showed excellent phytotoxicity to *A. fatua* (IC₅₀ = 6 μM) (Fig. 4).

The effect of benzoxazinones and their degradation compounds, has also been measured on cress seedlings (Kato-Noguchi and Macias, 2008) with inhibition of root growth and reduced α-amylase activity. Although α-amylase is an enzyme directly related to seed germination, it also supports the growth of the seedling via the breakdown of starch reserves in the endosperm prior to the start of seedling photosynthesis. The structure-activity assays of these compounds show that benzoxazinone family compounds are the most active. Other aspects regarding the correlation between structure and activity suggest that, in the presence of a hydroxyl group at the C-2 position of the benzoxazinone skeleton,

activity remains unchanged. In contrast, a hydroxyl group at the N-4 position appears essential for significant inhibitory activity. Kato-Noguchi and Macías (2006) investigated the mode of action of MBOA as a bioherbicide. Secondary metabolites, MBOA inhibited the mycelial growth (*G. graminis* var. *tritici*, *C. gramineum*, and *F. culmorum*) by 50% (EC₅₀) following exposure to 77, 134, and 271 μg/mL concentration in a corn meal agar medium, respectively, and the corresponding BOA EC₅₀ values for the fungi were 11, 189, and 456 μg/mL. The BOA degradation products 2-amino-3H-phenoxazin-3-one (APO), 2-acetylamino-3H-phenoxazin-3-one (AAPO), and *o*-aminophenol (*o*-AP) were much more phytotoxic that exhibit inhibitory EC₅₀, < 0.58, 4.57, and 1.4 μg/mL, respectively, for *C. gramineum* and 0.78, 2.18, and 0.80 μg/mL for

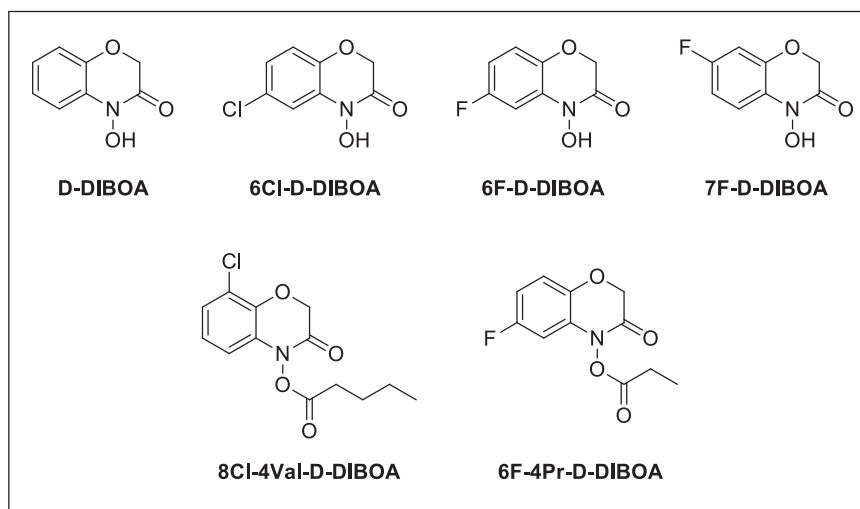


Fig. 4. Structures of optimised synthetic BHx.

G. graminis var. *tritici* (Martyniuk et al., 2006).

5. Exudation and release of benzoxazinoids in the rhizosphere

Root exudation is for a major means for plants to release allelochemicals involved in plant-plant and plant-microorganism interactions into the rhizosphere (Vives-Peris et al., 2020). Among root exudates, benzoxazinoids have been extensively studied and once in the rhizosphere, they can play several roles in plant defence, plant competition and the modulation of root-associated fungal and bacterial communities (Hickman et al., 2021; Schandry et al., 2021; Sikder et al., 2021).

It is commonly assumed that benzoquinones are stored in a glucosidic form in vacuoles. Once released into the cytoplasm, they are hydrolysed by the β -glucosidases increasing their reactivity and biological activity (Frey et al., 2009; Niemeyer, 2009). It has been demonstrated that hydrolysing glucosidases are present in the cytoplasm the cell wall and several organelles and that they are spatially separated from their glucoside substrates (Nikus et al., 2001). As a consequence of cell damage, the contact between the enzymes and their substrates catalyses the hydrolysis of benzoxazinone glucosides. The resulting unstable benzoxazinone aglucones and their benzoxazolinone degradation products are considered the main biologically active compounds (Sicker and Schulz, 2002; Niemeyer, 2009).

Root exudates are released from root hairs and epidermal cells in roots by transport proteins or via passive diffusion (Weston et al., 2012). Despite the presence of benzoxazinoids and their transport into the root apoplast (Ahmad et al., 2011), the mechanisms involved in root exudation are unclear. Others have reported that benzoxazinoid exudation occurs through passive transport (Niculaes et al., 2018) but this is unlikely due to the charged nature of the hydroxamic acids and BXZ metabolites.

Despite evidence of qualitative differences in benzoxazinoid biosynthesis among *Triticeae* species, the reasons for these differences at the species level remain unknown.

The coding sequence and chromosomal location of almost all the genes involved in the biosynthesis of benzoxazinoids (Makowska et al., 2015) have been characterised, suggesting that biosynthesis is similar in all the species belonging to the *Triticeae* family. In particular, it has been observed that seven genes drive the enzymatic steps necessary to produce DIMBOA from its demethoxylated precursor DIBOA (Frey et al., 1997, 2003; Gierl and Frey, 2001; Makowska et al., 2015). Therefore, it has been postulated that such differences could be due to a species-specific regulation of gene expression as well as to a partial loss

of their function, which could induce differential biosynthesis, accumulation and release of benzoxazinoids in the rhizosphere (Pérez and Ormeno-Nuñez, 1991; Glawischnig et al., 1999; Nomura et al., 2002; Frey et al., 2003; Xu et al., 2021).

Wu et al. (2001), screening differential benzoxazinoid production by different tissues and exudates of fifty-eight wheat accessions, demonstrated that DIMBOA production varied among tissues/exudates and accessions. In particular, DIMBOA content was higher in roots than in corresponding shoots and the concentration of DIMBOA exuded in the rhizosphere was positively correlated with the concentration observed in root tissues. Therefore, it has been speculated that the amount of benzoxazinoids exuded by roots is dependent on the corresponding accumulation of their glycosidic precursors in root tissues, a hypothesis supported by several reports (Argandoña et al., 1981; Nakagawa et al., 1995; Copaja et al., 1999; Sue et al., 2000a, 2000b).

In wheat, the benzoxazinoid aglucones DIBOA [2,4-dihydroxy-2H-1,4-benzoxazin-3(4H)-one] and its 7-methoxy derivative DIMBOA [2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one] are considered to be the most widely occurring and play several key roles in plant defense. Although DIBOA has been detected in several plant tissues, the main benzoxazinoid exuded by *Triticum aestivum* L. was thought to be DIMBOA (Niemeyer et al., 1992; Copaja et al., 1999; Sue et al., 2000a; Wu et al., 2000, 2001, 2002; Köhler et al., 2015). In contrast, DIBOA and DIMBOA were mainly identified in the root exudates of several *Triticum durum* cultivars, and the concentration observed was cultivar dependent (Pérez and Ormeno-Nuñez, 1991; Wu et al., 2000, 2001, 2002; Huang et al., 2003;).

Belz and Hurlé (2005) screened the root exudates collected from 146 cultivars of four species belonging to the *Triticeae* family (*Triticum aestivum* L., *Triticum durum* Desf., *Triticum spelta* L., and *Secale cereale* L.) observing that in all tested cultivars, DIBOA and DIMBOA were also observed in root exudates. The total amount of DIBOA and DIMBOA exuded was positively correlated with growth inhibition and their contribution to the overall allelopathic effect was modelled and estimated at 48 – 72%.

In a bioassay with pure phytotoxins, Bx concentrations consistent with the amounts quantified in the screening bioassay caused detrimental effects on *Sinapis alba* and (Belz and Hurlé, 2005). Chemical characterisation of the hexaploid species *T. spelta* and *T. aestivum* highlighted that DIMBOA was the only benzoxazinoid detectable, whereas DIMBOA (predominantly) and DIBOA were observable in the root exudates of the tetraploid species *T. durum* (Huang et al., 2003; Belz and Hurlé, 2005;). Those results confirmed that benzoxazinoid variation is dependent on the wheat ploidy level (Niemeyer et al., 1992; Sicker

et al., 2000; Nomura et al., 2002; Belz and Hurle, 2005).

Oliveros-Bastidas et al. (2018) carried out related studies to induce polyploidy in the wheat, maize, and rye via colchicine treatment and observed its impact on the production and root exudate content of 2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3-one (DIMBOA) and 2,4-dihydroxy-2H-benzoxazin-3-one (DIBOA). Caryopses treated with colchicine at concentrations of 0.1–10 mg/mL (at inoculation period of 8 and 48 h) enhanced allelochemical concentration in plant roots and stems.

More recently, Mwendwa et al., (2016, 2021), through liquid chromatography coupled to mass spectrometry, performed advanced metabolic profiling and detected in *Triticum aestivum* field-grown cultivars up to 14 different benzoxazinoids (including benzoxazinoid glycosides) and phenoxazinones, demonstrating that both qualitative and quantitative differences in wheat were dependent on year, cultivar, plant tissue/organ evaluated, and distance from the site of exudation.

6. Microbial activities, benzoxazinoid transformation products and their fate in the soil

Among benzoxazinoids, the most abundant metabolites in wheat extracts were found to be MBOA and DIMBOA (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one) (Mwendwa et al., 2021; Nakagawa et al., 1995). Weed suppressive wheat varieties release both metabolites into the rhizosphere through exudation or degradation of plant material, as well as a range of other BXZ metabolites (Mwendwa et al., 2016, 2021). DIBOA (2,4-dihydroxy-1,4-benzoxazin-3-one) and DIMBOA has been extensively studied in maize and wheat for involvement in the allelopathic response (Macías et al., 2004; 2005b). It is well established that the degradation of benzoxazinoids in the soil occurs mainly by microbial activity and, to a lesser extent, by other abiotic factors (Schütz et al., 2019). After root exudation or tissue degradation, DIBOA and DIMBOA are the precursors of several derivatives (directly produced by biotic degradation), which are quickly converted into BOA and MBOA (6-methoxy-benzoxazolin-2-one) through a heterocyclic ring contraction, which are more stable and readily detectable in the soil (Fomsgaard et al., 2004; Reiss et al., 2018; Macías et al., 2019, 2020; Soto-Cruz et al., 2021).

It has been demonstrated that, depending on the pH, the half-life of DIMBOA in water is between 7 h and 20 h (Macías et al., 2004). Degradation studies carried out by Macías et al. (2004) reported that the half-life of BOA and its biotransformation to 2-aminophenoxazin-3-one (APO) is around 2.5 days. In contrast, under sterile conditions, the derivatives BOA and MBOA are pretty stable but are converted in a few days by microorganisms via aminophenol, producing chemicals belonging to the malonic acids, acetamides and aminophenoxazinone catabolite classes (Fomsgaard et al., 2003, 2006; Schulz et al., 2013). After ring-opening, DIBOA generates an unstable and highly reactive intermediate AP (2-aminophenol) that, through oxidative dimerisation, produces the aminophenoxazinones APO (2-amino-phenoxazin-3-one) and AMPO (2-amino-7-methoxy-phenoxazin-3-one) which can have a half-life in the soil of several months (Fomsgaard et al., 2004; Macías et al., 2004). Therefore, the allelopathic potential of several species producing benzoxazinoids could be mainly due to these two aminophenoxazinones rather than their precursors. A relatively recent publication reported the detection of DIBOA and DIMBOA degradation products in the roots and shoots of the target weed (*Avena fatua*) (Fig. 2) (Chinchilla et al. 2015).

In the soil, bacteria and fungi can further transform BOA, MBOA and APO through acetylation, hydroxylation, nitration and other reactions. Soil degradation starts with the opening of the heterocycle ring mediated by a γ -lactamase, which several soil-borne fungi can produce (Friebe et al., 1998; Kettle et al., 2015; Glenn et al., 2016; Schulz et al., 2018b). Chase et al. (1991) also reported that *Acinetobacter calcoaceticus* could open the BOA heterocyclic ring allowing transformation to AZOB (2,2'-oxo-1,1'-azobenzene). After heterocyclic breakage, catalysed by

enzymes with domain similarity to the metallo-lactamase super-family, an extremely reactive molecule (AP) is produced. AP (2-aminophenol) is acetylated to AAP (2-acetamidophenol) by *Fusarium* species and also bacteria, which is subsequently detoxified by adding a malonyl group (mediated by a member of the arylamine N-acetyltransferase family) to generate HPMA [N-(2-hydroxyphenyl) malonic acid] (Friebe et al., 1998; Glenn et al., 2001, 2016, 2002; Glenn and Bacon, 2009; Kettle et al., 2015). Moreover, Chirino et al. (2013) reported that the proteobacteria *Burkholderia xenovorans* possess a functional AP catabolic central pathway, leading to picolinic acid production, which several microorganisms can further degrade (Qiu et al., 2017).

Another widespread natural reaction of AP is its auto-oxidation to 2-amino-phenoxazin-3-one (AMPO), which occurs very slowly in air-saturated solutions and has an estimated half-life of about 23 days under normal conditions, i.e. neutral pH and 25–30°C (Oancea and Puiu, 2003). Finally, Zikmundová et al., (2002a; 2002b) reported AP conversion to several derivatives mediated by different soil-borne fungi.

7. Influence of environmental stress on benzoxazinoids

As with most of the specialised metabolites produced by plants, benzoxazinoid production fluctuates depending on several biotic and abiotic factors and crop conditions. Kowalska and Jędrejek (2020) reported that differences in environmental conditions significantly changed the benzoxazinoid profile in *T. aestivum* spring and winter cultivars cropped under conventional and organic systems, concluding that organic management significantly increased their content.

Besides the availability of nutrients inter-specific competition has been shown to affect benzoxazinoid composition significantly. Lu et al. (2012) reported that wheat plants growing close to two competitors, i.e. wild oat and flixweed, altered their DIMBOA/MBOA production. In particular, they observed that the metabolite MBOA exerted a significant response to weed association, and the authors concluded that weed proximity induced biotic stress responses in wheat, triggering defence mechanisms that led to an overproduction DIMBOA/MBOA, suggesting that the synthesis and exudation of these metabolites was influenced by the environment.

Abiotic stress, which upregulates the jasmonate pathway, could be responsible for changes in benzoxazinoid content. Moraes et al. (2008) described the effects of cis-jasmone on *T. aestivum* metabolism, showing increasing DIMBOA content in shoots and roots in response to the hormonal stimuli, suggesting that jasmonate could selectively affect several pathways connected to benzoxazinoid metabolism.

The influence of jasmonates on benzoxazinoid metabolism has also been described by Oikawa et al. (2002), who observed that jasmonic acid treatment induced an increase of HDMBOA-Glc[2-(2-hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one)- β -D-glucopyranose] and a concomitant decrease in DIMBOA-Glc [2-(2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one)- β -D-glucopyranose] content. Further, they proved that such alteration was mainly induced by increased activity of S-adenosyl-L-methionine through DIMBOA-Glc 4-O-methyltransferase, which catalyzes the conversion of DIMBOA-Glc to HDMBOA-Glc inducing HDMBOA-Glc accumulation.

The induction of benzoxazinoid production in response to jasmonate is not surprising. It has been largely reported that the jasmonate pathway plays a pivotal role in protecting plants from herbivory, stimulating the production of several specialised metabolites with phytoalexin functions (Chen and Mao, 2020; Ghorbel et al., 2021).

8. Benzoxazinoids for weed suppression and the potential for sustainable weed management

Weed suppression by BXZs may be obtained during germination and early vegetative growth. During vegetative growth, allelopathy occurs through the exudation by the roots of phytotoxins which affect the

germination and particularly the reduction of seedling growth, while following crop harvest BXZs in crop residues (straw, chaff etc.) can leach into the soil and suppress the germination and seedling growth of weed species (Niemeyer, 2009). Roots exude BXZ glucosides into the rhizosphere which are converted by hydrolysis to aglucones, characterised by a higher biological activity than glucosides (Hazrati et al., 2020). Bioactive BX aglucones, such as 2,4-Dihydroxy-1,4-benzoxazin-3-one (DIBOA) and 2,4-Dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) and their breakdown products benzoxazolin-2-one (BOA) and 6-methoxy-benzoxazolin-2-one (MBOA) are the most effective allelochemicals in wheat (Makowska et al., 2015). DIMBOA is the most abundant BX aglucone, with variation observed between wheat varieties (Mathiassen et al., 2006). Benzoxazinoid aglucones have shown inhibitory activity (Mikić and Ahmad, 2018) against a wide range of weed species such as wild oat (*Avena fatua*), white mustard (*Sinapis alba*) and rigid ryegrass (*Lolium rigidum*).

The release of allelochemicals from seeds, leaves, stems, flowers, pollen, and roots of living or decomposing plant material with the capacity to influence the germination, growth and development of adventitious plant species in the agroecosystem can sometimes be a useful strategy to manage weeds, without the need for the use of herbicides (Farooq et al., 2011). Sustainable agriculture can take advantage of allelopathy in various ways, for example, for use in weed management through intercropping, mulching, residue or litter decomposition and incorporation into the soil, leaching by rainfall, and the establishment of weed suppressive living crops (Nawaz et al., 2012; Latif et al., 2017; Sathishkumar et al., 2020).

The specific effects of allelochemicals in intercropping with diverse crops, including for example legumes and cereals, have been previously studied, and crop rotation with suppressive crops can present a sustainable tool for the control of weeds, pests and diseases (Makoi and Ndakidemi, 2012; Gurusinghe et al., 2022), resulting in the potential to reduce the application of pesticides or synthetic fertilisers. Recent reports have shown that the use of allelopathic mulches or crop residues either left on the soil surface or lightly incorporated into the soil can significantly reduce the weed seedbank (Gurusinghe et al., 2022) and associated dependence on the use of synthetic herbicides (Abbas et al., 2018; Alsaadawi et al., 2020). Cheema et al. (2004) demonstrated the capacity of soil surface-applied sorghum mulch to reduce weeds in maize (26–37%) and cotton (23–65%). Tabaglio et al. (2008) demonstrated that mulching with rye cover crops was able to suppress weeds by more than 40% in the case of redroot pigweed and common purslane.

Crop residues of many other species, including wheat, sunflower, sorghum, clover, rice, and canola (Bilalis et al., 2003; Abbas et al., 2016; Rawat et al., 2017; Hussain et al., 2019, 2021), have also shown suppressive effects on germinating weed seedlings. Alsaadawi et al., (2011, 2013) found that sunflower and sorghum residues combined with reduced doses of pre- and post-emergence herbicides were highly effective in reducing the emergence of weeds with suppression values similar, or even higher, than those obtained from the recommended dose of herbicides. Similar suppressive results were obtained when allelopathic extracts of these crops were evaluated. In particular, in wheat, the combined use of sorghum, brassica, rice, mulberry and sunflower water extracts (Elahi et al., 2011; Razzaq et al., 2012; Mahmood et al., 2013), and sorgaab (sorghum water extract) application (Jamil et al., 2005; Sharif et al., 2005) supported the use of reduced rates of herbicides by providing significant weed suppression while increasing wheat yield.

The incorporation of living allelopathic crops in a rotation, either as primary field or cover crops, also appears to be a cost-effective and sustainable alternative for controlling diverse weed species in horticulture and field crops. Currently, plant breeders are incorporating key plant growth and developmental traits when breeding locally adapted cereal cultivars. Such traits contribute to crop competition and weed suppression early in the growing season, including early vigour, of cereal crops with broader leaves and faster canopy closure, along with drought tolerance and dwarf characteristics (Mwendwa et al., 2021).

The use of biotechnology to further enhance gene expression for allelochemical production and targeting sensitive weed species has also been recently examined; however has not yet been employed commercially (Nawaz et al., 2014). Mapping of key sections of the wheat genome has also been accomplished, and the use of consensus mapping combining genetic information from multiple populations significantly improves genome coverage and marker density when developing selection strategies (Jensen et al., 2008; Qu et al., 2021).

Therefore, the development of allelopathic strategies cannot be designed independently from knowledge of the soil biota. Deep knowledge of adventitious flora, rhizosphere and soil type is necessary for a complete understanding of the organisms that constitute the agroecosystem. Following a co-culture of weeds with crops, weeds will experience modifications in their biological and ecological patterns, as well as their ability to interfere with crops (Ramesh et al., 2017). Factors such as high CO₂ concentration or extreme temperatures can modify the solubility, uptake and transport of herbicides and allelopathic molecules by weeds, not only due to morpho-physiological and anatomical modifications induced by these conditions but also to the physical-chemical characteristics of the molecules and to the impact on the plant rhizosphere-microbe relationships (Ziska et al., 2004; Manea et al., 2011). In conclusion, climate change could alter the relationship of plants with other organisms in the ecosystem and the practices and cost associated with effective weed management.

Therefore, a holistic approach with multi-dimensional components including donor plants, soil biota, target plant and climate conditions should be considered before developing an effective alternative strategy to chemical control for weed management. In this scenario, allelopathy could play an essential role in crop productivity and weed management.

For many years, allelopathy research focused on allelochemical release from cover crops, intercropping and plant extracts to reduce the growth of weeds and thereby increase grain yield under field conditions. However, the majority of effects on weed growth, chlorosis, physiological, morphological and biochemical changes are caused by interactions between the allelochemicals released and the cellular/molecular systems of weed species (Chiapusio et al., 1997; Sánchez-Moreiras et al., 2003, 2008, 2004; Baerson et al., 2005; Sánchez-Moreiras and Reigosa, 2005; Reigosa and Sánchez-Moreiras, 2006). The herbicidal mode of action of BXZs is unknown but most allelochemicals appear to have multi-site rather than a single site mode of action affecting a range of growth and metabolic processes in the target weed (Dayan and Duke, 2009).

9. Benzoxazinoids and wheat allelopathy

Wheat allelopathy has been well documented by past researchers. While allelochemicals inhibit the germination and radicle length of herbicide-resistant weeds such as *Lolium rigidum* Gaud. In a replicated laboratory wheat accession trial, Wu et al. (2003a, 2003b) reported that wheat allelopathy significantly decreased germination in herbicide-resistant and herbicide-susceptible ecotypes of annual ryegrass and inhibition ranged from 3% to 100%. Among the parameters evaluated, root growth was more sensitive to wheat seedlings and exudates than seedling germination. Both *p*-coumaric acid and propionic acid observed in wheat accessions were associated with a reduction of germination in both resistant and susceptible ecotypes of annual ryegrass (Wu et al., 2000a,b,c,d and 2003b).

Allelopathic activity of different wheat tissues or organs, including roots, shoots and the whole plant, has been demonstrated (Zuo et al., 2005; Zuo et al. 2007), and roots and their extracts were found to be more phytotoxic than other plant tissues. Mathiassen et al. (2006) quantified BOA, DIMBOA and MBOA in wheat tissues and determined that all possess some allelopathic potential. In another study, Krogh et al. (2006) documented that the highest concentration of BXZ metabolites present in wheat leachates was MBOA, followed by HMBOA and HBOA. This was further confirmed by Mwendwa et al. (2021) in

field experiments with advanced metabolomics from the rhizosphere and rhizoplane together with wheat root extracts. The allelopathic properties reported from various wheat varieties from different world regions are reported in Table 1.

Root exudates of the wheat cultivar Ursita decreased the seedling growth (29–60%) and photosynthetic pigments of *Lolium rigidum* under co-culture conditions (Hussain et al., 2022). Several phenolic acids (vanillic, ferulic, syringic, and p-coumaric acids) and root exudates of BXZ (benzoxazolin-2-one (BOA), 2-hydroxy-7-methoxy-1,4-benzoxazin-3-one (HMBOA), 2-hydroxy-1,4-benzoxazin-3-one (HBOA), and 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA) were increased in wheat tissues (shoots, roots) and exudates in root rhizosphere agar medium following co-growth with either *L. rigidum* or *P. oleracea* (Hussain et al., 2022). Macías et al. (2006b) characterised the biological activity of BXZ metabolites. Some unique benzoxazinones such as 2,4-dihydroxy-(2H)–

1,4-benzoxazin-3(4H)-one and 2,4-dihydroxy-7-methoxy-(2H)– 1,4-benzoxazin-3(4H)-one, and the phenoxazinone APO showed significant inhibition of both wild oat and rigid ryegrass.

Wheat mulch and soil under wheat cultivation have revealed several phenolic compounds, particularly ferulic acid, p-coumaric acid, and related cinnamic or vanillic acids (Lodhi et al., 1987), which have shown a significant reduction in the growth and development of radish in laboratory experiments. The incorporation of wheat straw or its use as a surface mulch also proved to be toxic for the surrounding maize seedlings, and numerous phenolic compounds were reported in the soil where wheat straw was used as suppressive treatment (Opoku et al., 1997). Competitive traits in cereal crops also vary among years (Coleman et al., 2001; Vandeleur and Gill, 2004), locations and climatic conditions (Andrew et al., 2015). These variations may cause challenges in selecting the best cultivars. However, Wu et al. (2000c) found that the

Table 1
Wheat cultivars with allelopathic properties reported from different regions of the world.

Wheat cultivars/genotypes	Allelochemicals	Weed/test species	Country	References
Tasman, Triller, Wilgoyne, Meering, 3-J 27, Nabawa, Sunstar, 3-J 67, CH 31, AUS#375	DIMBOA, p-hydroxybenzoic acid, cis-ferulic acid, vanillic acid, cis-p-coumaric acid, trans-ferulic acid, syringic acid, trans-p-coumaric acid	<i>Lolium rigidum</i>	Australia	Wu et al. (2000a)
Karcagi 21	Syringic acid, trans-ferulic acid, p-hydroxybenzoic acid, cis-p-coumaric acid, cis-ferulic acid, trans-p-coumaric acid, vanillic acid	<i>L. rigidum</i>	Australia	Wu et al. (2000b)
Ursita	p-Hydroxybenzoic, trans-p-coumaric, cis-p-coumaric, syringic, vanillic, trans-ferulic, and cis-ferulic acids and 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA)	<i>L. rigidum</i> , <i>Portulaca oleracea</i>	Spain	Hussain et al. (2022)
Perquenco, Metrenco, Aztec and Baroudeur (total evaluated = 50)	NO INFO	<i>Spergula arvensis</i> (+10% and 88%), <i>Rumex acetocella</i> (+8% and 70%), <i>Avena fatua</i> (+36% and 74%) and <i>Vulpia bromoides</i> (+11% and 68%).	Chile	Bensch et al. (2009)
(Shafaq-06 and Faisalabad-08	Total phenolics (Folllen-Ciocaltue)	<i>P. minor</i>	Pakistan	Kashif et al. (2015)
Lumai168, Nongda211, Duokang	DIMBOA, MBOA	<i>Avena fatua</i> , <i>D. sophia</i>	China	Lu et al. (2012)
Azar2 (Azar2, Falat, Shahriar, Son 60, Zagross, Gohar, Maroon, Sardari and Nicknezhad) Total evaluated 9	NO INFO	<i>S. cereale</i>	Iran	Mardani et al. (2014)
WH533, WH542		<i>P. minor</i>	India	Om et al. (2002)
22 Xiaoyan, 6 Lankao		<i>L. rigidum</i>	China	Zuo et al. (2002)
Yecora Rojo		Lettuce	USA	Schuerger and Laible (1994)
22 Xiaoyan, No 131 Chanowu		Potato	China	Zuo et al. (2008)
Stakado	MBOA, HMBOA, HBOA		Denmark	Krogh et al. (2006)
Triller, Currawong		Wheat (autotoxicity)	Australia	Wu et al. (2007)
Ritmo, Astron	DIBOA, BOA, 2-aminophenoxazin-3-one (APO)		Spain	Macias et al. (2005b)
Wheat cultivars 15		Canola	USA	Wynne et al. (2019)
<i>Triticum vulgare</i> (Punjab 81)	Vanillic acid, ferulic acid, p-hydroxybenzoic acid, p-coumaric acid, syringic acid	Wheat (autotoxicity); Cotton	Pakistan	Lodhi et al. (1987)
	p-Coumaric acid		USA	Blum et al. (1993)
	MBOA		USA	Blum et al. (1992)
	scopoletin (7-hydroxy-6-methoxy-2 H-1-benzopyran-2-one); DIBOA (2,4-dihydroxy-2 H-1,4-benzoxazin3 (4 H)-one); BOA (benzoxazolin-2(3 H)-one); MBOA (6-methoxy-benzoxazolin-2(3 H)-one		Germany	Belz and Hurler (2005)
	Syringoylglycerol 9-O-β-D-glucopyranoside, L-tryptophan		Japan	Nakano et al. (2006)
	Ferulic acid, L-tryptophan		Japan	Nakano et al. (2007)
V6007, AS 2000, V6111, V6034, V4611, V7189, Uqab 2000, Chanab 2000, Bhakkar 2002, Pak 81 and Rohtas 90 (Total 35 accession evaluated)	DIMBOA	Wild Oat (<i>Avena fatua</i>)	China	Zhang et al. (2016)
wheat stubble and in the soil	p-Hydroxybenzoic, vanillic, p-coumaric, syringic, and ferulic acids		Pakistan	Mahmmud et al. (2005)
				(Guenzi and McCalla (1966); Lodhi et al. (1987)

best cultivars were generally consistent across years and locations irrespective of meteorological factors and soil profile. However, location, agronomic practices and environmental factors may certainly influence the weed suppression by some crop genotypes (Worthington et al., 2015).

Determination of the mechanism(s) associated with weed suppression is essential to determine if selected wheat cultivars possess allelopathic properties or competitive abilities to interfere with weed growth. Allelopathy and competition most likely act both separately and also interactively and such dual action might be important in the selection and breeding of highly competitive cultivars (Hendriks et al., 2022).

The activity observed, whether stimulatory or inhibitory, also depends upon the concentration and stability of allelochemicals in the rhizosphere, weed species, microbial transformants, climatic conditions and other abiotic/biotic stresses (Hussain et al., 2020; Hussain and Reigosa, 2021).

10. Benzoxazinoids (BXZs) as natural herbicides; insight into their mode of action

Identifying the mode of action of bioactive natural products can prove to be a challenge, requiring biochemical and physiological knowledge as well as the choice of effective concentration and the right time of exposure to the chemical (Duke et al., 2020). Moreover, one of the main problems generally encountered during the study of a secondary metabolite is the time of exposure to a given molecule. In fact, lengthy exposure to the chemical does not allow the observation of immediate effects on plant metabolism but only the side effects, mainly due to a cascade of biochemical and physiological responses. Therefore, depending on the experimental design and its complexity, the same molecule could exert several effects on different parameters, misleading interpretation about its actual mechanism of action. Concerning BXZs several manuscripts have reported their interaction with plants and microorganisms and several potential modes of action have been suggested, but it cannot be excluded that only a few of them are the main targets of the molecules, whereas the others are just potential side effects.

Plants generate reactive oxygen species (ROS) when exposed to biotic and abiotic stresses but also in response to the application of BXZs. In early studies, ROS were considered toxic by-products of aerobic metabolism, but in recent years have been shown to be key signalling molecules, regulators of growth, development and a role in defence pathways (Ding et al., 2007; Singh et al., 2016). ROS play an important role in activation of the cellular antioxidant system, involving enhanced activity of antioxidant enzymes and increased synthesis of molecular antioxidants such as glutathione, ascorbate and tocopherols (Gniazdowska and Bogatek, 2005). Following increased peroxidase activity and hydrogen peroxide (H_2O_2) production in plants treated with benzoxazinoids, lignin accumulation occurs with increased rigidity and mechanical strength of stems, which often leads to a reduction of growth (González and Rojas, 1999). Furthermore, the rate of NADH oxidation of cell wall peroxidases with the associated increased production of H_2O_2 following application of DIMBOA resulted in growth inhibition of common oat (*Avena sativa*) coleoptiles. The phototropism response of illuminated coleoptiles was attributed to an increase of H_2O_2 concentration following DIMBOA release, followed by lignin accumulation, cell-wall strengthening and growth suppression concomitant with disruption of lipid metabolism, protein synthesis and transport/secretory capabilities (Burgos et al., 2004).

Inhibition and/or delay of seed germination and reductions in radicle growth appear to occur in response to a disruption of cellular metabolism (Schulz et al., 2013). MBOA was shown to reduce α -amylase activity in cress (*Lepidium sativum*) and lettuce (*Lactuca sativa*) seeds (Kato-Noguchi and Macías, 2005; Kato-Noguchi and Macías, 2008), α -amylase is essential for germination because it converts starch to soluble sugars for seedling utilisation and growth.

It has been reported that DIMBOA and MBOA inhibited wheat germination through an inhibition of α -amylase activity (Kato-Noguchi and Macías, 2005, 2008; Ozaki and Kato-Noguchi, 2015). Furthermore, DIMBOA and MBOA have been shown to inhibit the seed germination of *Avena fatua* (Perez, 1990), while MBOA was more effective with the inhibition of germination and reductions in radicle and hypocotyl length of crimson clover (*Trifolium incarnatum*) and ivy-leaved morning glory (*Ipomoea hereracea*) (Blum et al., 1992). In contrast, DIMBOA was recently found to be more phytotoxic than MBOA and BOA in wheat (*Triticum aestivum*) (Macías et al., 2005a,b).

Disruption of photosynthesis is an important and frequently observed physiological effect of BXZs. Benzoxazolin-2-one (BOA) has been shown to induce changes in leaf water relations, photosynthesis and carbon isotope discrimination in lettuce (*Lactuca sativa*) (Sánchez-Moreiras et al., 2010; Hussain et al., 2011) and in thale cress (*Arabidopsis thaliana*) when exposed to high BOA concentrations for up to 8 days resulting in senescence and ultimately death (Sánchez-Moreiras et al., 2011). DIMBOA has been shown to inhibit photosynthesis by affecting chloroplast ATPase activity in spinach leaves, probably due to a reaction with the sulfhydryl groups of the enzyme (Queirolo et al., 1983). DIMBOA was also found to influence chloroplast metabolism by inhibiting the chloroplast ATPase but with no effects of the respective glucoside.

The effects of BXZs on respiration and photosynthesis, resulting in decreased ATP production, are likely to influence a whole host of metabolic processes, thereby influencing plant growth. BOA and DIBOA isolated from rye (*S. cereale*) were reported to lower the activity of plasma membrane H^+ -ATPase in the roots of both common oat (*Avena sativa*) and vetch (*Vicia sativa*) (Friebe et al., 1997). It is also likely that the action of BOA and DIBOA on plasma membrane H^+ -ATPase is closely linked to their effects on root and shoot growth. This supports the theory that the allelochemical activity of these compounds is linked to auxin-inhibition (Hasegawa et al., 1992).

The reduction in plant growth induced by BXZs is linked to reduced mitosis activity and/or a disruption of the structure of key cellular organelles, i.e. nuclei, mitochondria and chloroplasts (Gniazdowska and Bogatek, 2005). BOA and DIBOA reduced the regeneration of cucumber (*Cucumis sativus*) root cap cells and increased the width of cortical cells resulting in increased root diameter. Additionally, BOA and DIBOA increased the cytoplasmic vacuolisation, reduced ribosome and dictyosomes density and decreased the number of mitochondria. BOA and DIBOA have also been shown to cause changes in cellular ultrastructure and reduce root growth by disrupting lipid metabolism, protein synthesis and cellular transport (Burgos et al., 2004).

BXZs have also been shown to affect plant growth. Both DIMBOA and MBOA have been identified as auxin inhibitors binding to both membrane fractions and isolated membrane proteins (Hoshi-Sakoda et al., 1994; Burgos et al., 2004), with DMBOA being more effective than MBOA (Schulz et al., 2013). Exogenous addition of DIMBOA and MBOA also affected the auxin-induced growth of oat (*Avena sativa*), timothy grass (*Phleum pratense*), amaranth (*amaranth* spp.) and pea (*Pisum sativum*) (Zhou et al., 2018).

Recently Venturelli et al. (2015) reported that the BXZs APO and AMPO directly affect the chromatin-modifying machinery in *Arabidopsis thaliana*, inhibiting, in-vitro and in-vivo, the histone deacetylases and exerting their activity through locus-specific alterations of histone acetylation, targeting an evolutionarily highly conserved class of enzymes, and associated gene expression.

11. Benzoxazinoids and wheat breeding

Genes involved in benzoxazinone biosynthesis (*Bx1- Bx5*) have been characterized in wild diploid and hexaploid wheat (*T. aestivum*, 2n 6x42, genomes AABBDD; TaBx1A-TaBx5A, TaBx1B-TaBx5B, TaBx1D-TaBx5D) (Nomura et al., 2005, 2007). In *Triticum aestivum*, genome A is from *Triticum urartu* (AA), genome B from *Aegilops* species,

and genome D from *Aegilops tauschii* (Sue et al., 2011). *Bx1-Bx5* have been found in the A, B and D genomes. *TaBx1* and *TaBx2* are located on chromosomes 4A, 4B and 4D, *TaBx3–TaBx5* on chromosomes 5A, 5B and 5D, and *TaBx3* and *TaBx5* have duplicate loci on chromosome 5B (Nomura et al., 2008). The number of exons and introns in phase 0 is the same in genomes A, B and D; the coding sequences are also identical (Bakera et al., 2015). For maize *Bx7* and *Bx10–Bx14*, orthologous genes have not yet been identified from *Triticum aestivum* (Shavit et al., 2018).

Based on the relative transcript levels and benzoxazinone-associated enzyme activities in *T. aestivum*, genome B contributes more than genome A and D to benzoxazinone synthesis and benzoxazinone-related transcription varies with respect to tissue and wheat development (Sue et al., 2011). Preliminary evidence has also pointed to specific *cis*-acting promoter elements which direct the developmental stage-specific transcription of *TaBx3* and *TaBx4*. Thus, based on Sue et al. (2011), genome B might be of special importance for breeding strategies aimed at enhancing BXZ content in specific plant organs. Imbalance within the wheat genome by favouring genome B could have unpredictable consequences; for example interactions with arbuscular mycorrhizal fungi may be affected as loci associated with this function reside within genome D (Džafić et al., 2013). Gene orthologues of the elicitor peptide Pep1 could also represent important breeding targets. In *Zea mays* Pep1 (*ZmPep1*) was shown to activate BXZ biosynthesis (Huffaker et al., 2011) and although not directly examined, Pep1-related enhancement of BXZ biosynthesis may also occur in wheat.

12. Screening, selection and evaluation of allelopathic wheat cultivars for weed management

Previous research findings have generally indicated that allelopathic effects are typically due to the interaction of multiple compounds such as phenolics, aldehydes, hydroxamic acids, ketones and flavonoids, which may work together in synergistic or additive means (Kong et al., 2019). Biological weed management involves screening, selection and evaluation of crop cultivars with increased competitive ability against weeds and allelopathic activity (Andrew et al., 2015). Several wheat varieties have demonstrated significant weed suppressive ability (Andrew et al., 2015; Worthington et al., 2015; Mwendwa et al., 2016).

Screening, selection and cultivating wheat varieties with significant allelopathic potential will be a good choice for eco-friendly weed management (Jabran et al., 2015). Organic farmers are particularly interested in cultivars that possess the potential to inhibit weed growth (Schalchli et al., 2012). Biological screening of seven wheat genotypes, including five ancient emmer wheat genotypes (Zarneh, Singerd, Shahrekord, Khoysan, and Joneghan), one modern hexaploid wheat genotype (*Triticum aestivum* var. Roushan), and one modern tetraploid wheat genotype (*Triticum turgidum* var. Yavaroos), revealed that all genotypes significantly inhibited the germination and seedling growth of weeds (Fatholahi et al., 2020). Both modern hexaploid and ancient tetraploid emmer genotypes exhibit strong allelopathic potential because they possess a range of phenolic acids and flavonoids in plant tissues as revealed via HPLC diode array detector. The presence of allelochemicals in aqueous extract decreased *R. sativus* growth. Fatholahi et al. (2020), concluded that syringic acid, isolated and quantified from ancient tetraploid and modern hexaploid wheat genotypes, played a particular role in the allelopathic effects. Simultaneously, findings revealed that ancient tetraploid emmer wheat looks to contain a range of allelochemicals, indicating their potential for sustainable crop production systems. Several research teams are working on this theme to screen and evaluate the current allelopathic potential of wheat varieties and short-term genetic approaches to enhance the competitive or allelopathic activity (Bertholdsson, 2005, 2010; Hendriks et al., 2022 a and b; Wu et al., 2000d; Mwendwa et al., 2021).

Mardani et al. (2014) evaluated wheat genotypes and documented that the cultivar Azar2 showed significant allelopathic inhibition on the germination and seedling growth of *Secale cereale* L. Another study (Om

et al., 2002) from India documented that the wheat varieties WH533 and WH542 were highly allelopathic against *P. minor*. The germination and seedling growth traits were decreased by up to 30% in *P. minor* after treatment with these two wheat varieties, out of 11 wheat varieties evaluated. In this context, Kashif et al. (2015) reported that the growth and development of *P. minor* were reduced following exposure to the wheat variety, Shafaq-06. They found that the highest polyphenols were obtained from Shafaq-06 among all the ten genotypes (Faisalabad-08, Lasani-08, Shafaq-06, Sehar-06, Miraj-08, Farid-06, Chakwal-50, V-04178, V-05066 and V-05082) evaluated. As a general consequence, phenolic compounds in all the tested wheat varieties were increased following co-cultivation with *P. minor* weed species. A similar pattern was found by Lu et al. (2012) in their work i.e. exudation of hydroxamic acids, especially DIMBOA and MBOA, from the wheat roots when grown in the proximity of (*Descurainia sophia* (L.) and *Avena fatua*). Weed infestation of *Digitaria sanguinalis* (L.) and *A. retroflexus* caused an increased synthesis and release of DIMBOA from wheat tissues (Zheng et al., 2010). The allelopathic properties reported from various wheat varieties belonging to different world regions are reported in Table 1.

For sustainable weed management and eco-friendly agriculture, wheat cultivars can be employed for weed management (Jabran et al., 2015). This information is important for organic farmers who have to control weeds without the use of herbicides. Wu et al. (2000d) evaluated more than 800 genotypes, and found that some of them possess significant allelopathic activity against the monocot weed (*Lolium rigidum*). Several researchers have contributed to the identification of wheat crop allelopathy that can be exploited for biological weed management to reduce production costs and help maintain crop yield for in an eco-friendly environment (Bertholdsson, 2005, 2010). Mwendwa (2021) and Hendriks (2022 a,b) evaluated both commercial and early vigour genotypes and found that early vigour associated with canopy closure was also associated with production of high levels of associated BXZ metabolites and phenoxazinones, clearly suggesting that wheat breeders may select cultivars with superior competitive ability both above and below ground (Mwendwa et al., 2021; Hendriks et al., 2022 a, b).

The presence of allelopathic potential, early seedling vigour, and weed suppression ability are important traits that should be taken into account by plant breeders and plant ecophysiologicalists to select a competitive crop genotypes (Wu et al., 2003a). Recent research findings in both the field and under laboratory conditions have found that competition above ground for light and space drives weed suppression by competitive cultivars. Such factors as early vigour, canopy closure, leaf width and photosynthetic efficiency are important in driving weed suppression due to competitive above-ground traits. Below ground, we have limited information on the impact of root systems and root traits on crop growth and weed suppression. However, there are marked differences between cultivars and genotypes with respect to root growth rates in wheat, root branching, root hair numbers and density and root exudation. This also has included the finding that certain wheat genotypes with enhanced early vigour can produce and release higher concentrations of BXZ metabolites and phenoxazinones than others (Wu et al., 2003a). Additional research on pathway regulation, elicitation of BXZ metabolite production and the impact of seasonal and climatic factors on the regulation of biosynthesis is needed when considering breeding for enhanced weed competitiveness both above and below-ground (Mwendwa et al., 2020, 2021; Hendriks, 2022a, 2022b).

13. Benzoxazinoid content and the co-evolution of pest resistance

Several laboratories have also explored the potential for elevated expression of BXZ genes leading to increased benzoxazinone content in wheat as a tool to increase plant resistance to pests. For example, benzoxazinones have been evaluated for a possible role in repelling aphids. Hansen (2006) concluded from a study with MBOA that higher BXZ

contents in wheat could decrease aphid populations. However, in a more recent study involving eight hexaploid Brazilian wheat genotypes and the cereal aphids *Rhopalosiphum padi* and *Sitobion avenae* (Pereira et al., 2017), correlations between DIMBOA levels and their antagonistic effects were not found. In contrast, in the tetraploid *Triticum turgidum* ssp. *durum*, DIMBOA contributes to resistance against the grain aphid (*Sitobion avenae*) and to some extent resistance against bird cherry-oat aphid (*Rhopalosiphum padi* L.) (Shavit et al., 2018). Benzoxazinoids have also been reported to negatively affect the aphid *Diuraphis noxia* (Hickman et al., 2021). These contradictory findings could suggest different levels of advancement of co-evolutionary processes occurring between various wheat accessions and aphid species. DIMBOA was the sole benzoxazinoid detected in the root exudates of the hexaploid species *T. spelta* and *T. aestivum*, whereas DIMBOA (predominantly) and DIBOA were detected in the root exudates of the tetraploid *T. durum* (Huang et al., 2003; Belz and Hurlle, 2005). These findings revealed that benzoxazinoid variation is likely influenced by wheat polyploidy levels (Niemeyer et al., 1992; Sicker et al., 2000; Nomura et al., 2002; Belz and Hurlle, 2005). Copaja et al. (1999) found up to 10-fold differences in the levels of DIMBOA among a large number of Chilean and British wheat cultivar leaves, mostly *T. aestivum*.

Roots of allelopathic wheat cultivars exude specialized metabolites into the surrounding soil environment that have been shown to interfere with *Pseudomonas* secondary metabolism synthesis. In the presence of wheat root extracts, the synthesis of *Pseudomonas* bioactive metabolites such as phloroglucinol, phenazines, pyrrolnitrin, or acyl-homoserine lactones was altered (Rieusset et al., 2022). Metabolomic profiling techniques were utilised to determine if wheat metabolism in association with *Pseudomonas* has significant implications for the broader wheat metabolome. Wheat root extracts from two genotypes were used to condition two distinct *Pseudomonas* strains, resulting in bacterial specialised metabolite production changes. The wheat genotypes were subsequently injected with bacterial cells and the metabolomes of wheat roots studied. Non-targeted metabolomic analysis was performed and metabolites from the Adular genotype were identified via molecular network technique (Rieusset et al., 2022). Application of this technique will elucidate if wheat detects bacterial cells co-cultured with the plant previously and highlights the importance of the specialised metabolites that characterise the wheat—*Pseudomonas* relationship.

The potential benefits of increased benzoxazinone levels seem less clear for other insect pests. For example, Wouters et al. (2014) reported that fall armyworm (*Spodoptera frugiperda*) larvae can re-glucosylate DIMBOA, thus escaping its toxic effects. The glucosyltransferase gene involved has also been identified (Israni et al., 2020).

The benzoxazinoid contents in numerous wheat cultivars were too low for the effective suppression of *Fusarium* head blight (Baldwin et al., 2019). This study suggested that higher levels of benzoxazinoids would likely suppress *Fusarium* species and proposed the utilisation of wheat landraces and wild relatives as resources for breeding cultivars with higher benzoxazinoid content. Numerous wheat cultivars and landraces have previously been evaluated for benzoxazinoid content (Mogensen et al., 2006; Stochmal et al., 2006). Wu et al. (2000a, 2000b) screened the exudates of 58 wheat cultivars for benzoxazinoid content and found only 11 exuded detectable amounts of DIMBOA.

Fusarium species can detoxify BOA efficiently via lactamase-catalyzed heterocycle cleavage and subsequent conjugation with propanedioic acid to N-(2-hydroxyphenyl) malonamic acid (oHPMA) (Glenn et al., 2016). When *Fusarium verticillioides* are provided with BOA under culture conditions, the fungus will immediately initiate accumulation of fusaric acid, a picolinic acid derivative and known phytotoxin capable of inhibiting cell proliferation and DNA synthesis (Schulz et al., unpublished). Moreover, root rot in cereals caused by *Pythium lutarium* and *P. oopapillum* was more severe in the presence of MBOA (Acharya et al., 2021). There are numerous fungal pathogens which are tolerant to or are even stimulated by the presence of benzoxazinoids. Highlighting the potential differences in organismal co-evolutionary processes,

reports of benzoxazinoid-related suppression of the fungal pathogens *Helminthosporium turcicum*, *Cephalosporium maydis*, *Fusarium moniliforme*, *F. subglutinans*, *F. culmorum*, *Gaeumannomyces graminis*, *Microdochium nivale* and *Puccinia graminis* have been published (Kudjordjie et al., 2019).

Considerable BOA contents in weeds have also been correlated with reduced BOA detoxification capacity in both sensitive and tolerant species. Higher benzoxazinoid contents in wheat and other BXZ-producing cereals are thought to be advantageous for weed suppression. However, researchers have also documented that certain weeds have acquired the ability to detoxify and degrade benzoxazinoids, especially with the assistance of microbial consortia. It is problematic that many agronomically significant pests have acquired the ability to tolerate high BXZ concentrations.

Therefore, we suggest that it may be questionable whether higher BXZ content would be a desirable crop breeding aim with regards to the suppression of pests. Aside from the fact that many agricultural pests, including insects, *Fusarium* species and weeds, are already resistant to pesticides/herbicides, higher contents of benzoxazinoids may not potentially be regarded as an eco-friendly strategy for several reasons: (1) BOA, as found for many other plants specialised metabolites, dramatically influences the soil microbiome by altering species composition. While certain microbial species are diminished, disappear, or are otherwise inhibited, others may increase in abundance. (2) While it is not known whether the diversion of energy for the biosynthesis of higher amounts of benzoxazinoids would occur at the expense of reduced crop biomass, microbial colonisation and/or crop yields, the disruption of various plant defense mechanisms, or reduced symbiotic associations, cannot be discounted (Schütz et al., 2021). DIMBOA, for instance, reduces the colonisation of maize roots by arbuscular mycorrhizal fungi (Džafić et al., 2013). (3) Unfortunately, no studies are currently available examining the off-target effects of benzoxazinoids on harmless or so-called beneficial insects or soil macrobiota. It is also likely that high benzoxazinoid levels in crops would impair native plant species, which are not troublesome weeds, and even lead to their extinction in a worst-case scenario. Undoubtedly the so-called Sixth Mass Extinction embraces vertebrates, invertebrates and plants and all types of organisms (Wagner et al., 2021). Raven and Wagner (2021) stated that it is presently impossible to estimate the rate of extinction for bacteria, archaea, fungi and protists, as most are at present unidentified. Therefore, nothing is known about their ecological functions or interactions in nature. For example, the types of chemical interference that microorganisms may direct toward each other and susceptible plants under competitive or other stressful conditions are almost unknown. These are all reasonable precautions to consider before developing cultivars possessing higher levels of benzoxazinones or other related compounds that could significantly impact biodiversity.

14. Plant microbiomes and viromes – future targets for new breeding strategies?

It has long been known that plants exude chemo-attractants to recruit microorganisms. The abundance of some potentially beneficial rhizobacteria (Rhizobiaceae and Burkholderiaceae) increased after S-metolachlor (S-ME) treatment, indicating that plants recruited potential beneficial microorganisms to resist S-ME-induced stress. After S-ME treatment, organic acids secreted by plants were positively correlated with Rhizobiaceae and Burkholderiaceae, implying that potential beneficial microorganisms may be attracted primarily by organic acids (Qu et al., 2021).

Current research has also revealed that benzoxazinoids can play a similar role (Neal et al., 2012). Ultimately, microorganisms used for the biocontrol of certain plant diseases could be engineered for benzoxazinone tolerance, thus allowing higher BXZ production during pathogen attacks or when exposed to allelochemicals. Although plant disease suppression by rhizosphere colonising microorganisms is highly

complex (Whipps, 2001), future attempts toward breeding more resistant wheat cultivars must consider the ecological aspects. Plant-microbe interactions are known to play an important role in weed establishment and control (Trogitz et al., 2016) and improve plant vigour (Berendsen et al., 2012; Hacquard et al., 2017; Trivedi et al., 2020). Recently the systemically-induced root exudation of metabolites (SIREM), which includes the release of secondary metabolic products, was shown to be important for mediating plant-microbe signaling processes (Korenblum et al., 2020) as well as for priming plant immune responses (Köhl et al., 2019).

Plant-derived metabolites also play critical roles in plant-microbe recognition, association and biofilm formation (Fig. 5) (Tkacz and Poole, 2021). Interestingly, modern wheat cultivars show greater associations with different microbiota compared to landraces and ancestral wheat varieties (Mauger et al., 2021). *Acidobacteria* and *Actinobacteria* were more abundant in older wheat accessions (Kinnunen-Grubb et al., 2020), whereas *Candidatus saccharibacteria*, *Verrucomicrobia* and *Firmicutes* were present in modern accessions, along with other *Fusarium* species pathogens. Kinnunen-Grubb et al. (2020) concluded that a dramatic change occurred within the wheat root microbiome concomitant with breeding history. It appears that modern wheat varieties have lost the ability to establish associations with a variety of beneficial microorganisms, and this phenomenon is not restricted to wheat but is also evident for other crop species (Cordova et al., 2019; Mauger et al., 2021; Gholizadeh et al., 2022). In fact, plant breeding typically involves genotype-specific adaptations of microbiomes (Tkacz and Poole, 2021), which should be considered in future breeding programs.

While plant metabolites can shape the species composition of colonising microorganisms, those microorganisms can, in turn, modulate the plants' metabolome and alter the biosynthesis of plant specialised metabolites (Etalo et al., 2018). Regarding BXZ production, certain microorganisms influence benzoxazinoid biosynthesis and accumulation differentially in maize (Song et al., 2011; Walker et al., 2012; Planchamp et al., 2014). In cases where an increase was observed, it is questionable whether the BX pools produced are stable, exuded and high enough to effectively suppress weeds. In laboratory studies, damage to receiver plants is typically observed with benzoxazinoid concentrations higher than 500 μ M (Planchamp et al., 2014).

Viruses have also been recognised for playing an important role in the cycling of carbon and nutrients and the modulation of soil bacterial populations within ecological communities (Dolja et al., 2020). Given

the currently estimated levels of approximately 10^{10} virions/g occurring in typical soils, viruses are more abundant than soil bacteria (Roesch et al., 2007; Kuzyakov and Mason-Jones, 2018). Modern plant viromes, dominated by RNA viruses, co-evolved with plants and reflect evolutionary changes occurring within plant communities (Dolja et al., 2020). Viromes exist within the cells of all organisms and within all ecosystems. Like microbial communities, viral communities are composed of members that can be either beneficial or deleterious to their host. They can modulate host functions, play roles in gene transfer, and introduce new metabolic capabilities to symbiotic relationships (Dolja et al., 2020; Santos-Medellin et al., 2021). The DNA intercalating activity of some benzoxazinoids could also confer a role to these compounds in shaping the composition of viromes, as intercalating agents are generally inhibitory to double-stranded DNA viruses. This property would primarily affect soil viral communities rather than plant viromes, as the latter are comprised primarily of single-stranded RNA viruses. However, at present it is generally not possible to accurately predict the specific effects of secondary plant metabolites, or their often short-lived breakdown products, on soil viromes and microbiome-virome interactions (Fig. 5). Moreover, viromes cannot meaningfully be considered in plant breeding programs involving enhanced benzoxazinoid production at the present time, given the paucity of information that exists concerning the potential influence of benzoxazinoids on virome-microbiome interactions. Despite the fact that elucidating the effects of plant specialised metabolites on plant and soil microbiome community structures and their dynamics has recently become a major focus for plant research, additional studies will be required before breeding strategies can be devised that efficiently target microbiome structure.

For breeding efforts in the near future, it would therefore seem reasonable to emphasise the use of positive allelopathic interactions, for example, the optimization of plant-microbe interactions. Unfortunately, our current understanding of these complex positive allelopathic interactions is far from complete.

15. Weed defense against benzoxazinoids – degradation and detoxification strategies

Driven by coevolution, coexisting plants come to terms with one another mostly through their ability to cope with harmful compounds exuded by their neighbours. Maintaining a well-balanced community involves extensive chemical communication between plants and microorganisms, which also promotes biodiversity within a given habitat. Consequently, widespread infestations by generalist pathogens are relatively infrequent in natural ecosystems compared to agricultural systems (Goss and Timilsina, 2018). For example, agricultural systems are typically composed of monocultures, which can negatively impact following crops by diminishing, or even eliminating beneficial soil microorganisms that are potentially critical for the viability of subsequent crops (Schütz et al., 2021). Indeed, these issues have increasingly become the focus of current agricultural studies. The maintenance of plant and microbial biodiversity, and consequently, insect and animal diversity, is unfortunately in direct conflict with modern agricultural practices and this conflict is exacerbated by ever-increasing human populations.

Weed infestation in crop cultures can lead to lower yields and reduced product quality and represents a problem of great economic significance worldwide. The increasing frequency of weed resistance to common herbicides has suggested that natural compounds referred to as allelochemicals may represent suitable alternatives for weed control or provide the potential for the design of new synthetic herbicides (Duke et al., 2002). In the case of benzoxazinoid allelochemicals, however, many weeds have already evolved strategies to cope with these compounds, although the levels of actual tolerance vary widely from species to species (Hickman et al., 2021). However, it is clear, in the case of benzoxazinoids and other allelochemicals, that a more holistic understanding involving not only the knowledge of plant detoxification

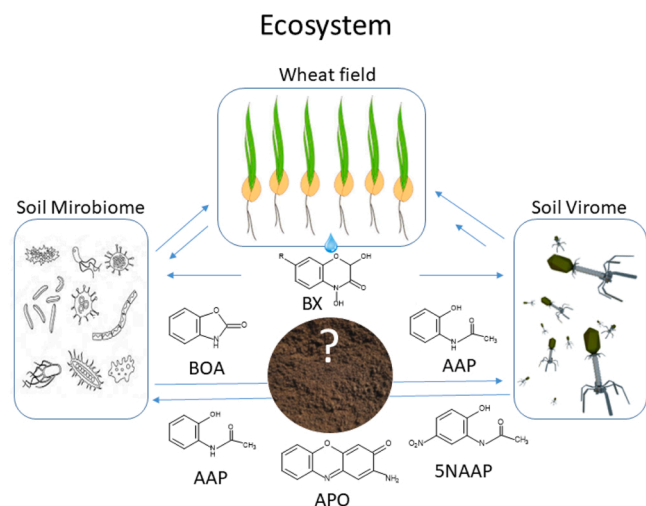


Fig. 5. Hypothetical interactions potentially occurring between wheat, the soil microbiome and the soil virome, possibly triggered by benzoxazinoids and their derivatives. Shown are the compounds BOA: benzoxazolinone, AAP: acetamidophenol, 5-nitroacetamidophenol: SNAAP, and APO: phenoxazinone. BX: benzoxazinoids.

mechanisms but also the impact of these compounds on associated microbial communities is essential to our understanding of their role in natural and agricultural systems (Fig. 6).

16. Uptake of benzoxazinoids by weeds

The concentrations of DIBOA, DIMBOA, HMBOA, HBOA, DIMBOA, BOA, and MBOA in roots and shoots of wheat varieties differ from cultivar to cultivar depending on developmental stage, culture conditions and the presence or absence of weed species (Hussain et al., 2022). Neighbouring weeds can trigger the content of DIMBOA in wheat tissues (shoots and roots), as was concluded from recent co-cultivation studies performed with wheat (Zhang et al., 2016; Hazrati et al., 2020; Hussain et al., 2022). These findings provide an important perspective on the complexity of benzoxazinone biosynthesis in maize, rye and wheat in response to intrinsic ecosystem factors. Due to the short half-lives of DIBOA and DIMBOA, their benzoxazinone derivatives BOA and MBOA are the compounds primarily absorbed by plants. However, Hazrati et al. (2020) recently identified DIBOA-glc and DIMBOA-glc in roots and shoots of hairy vetch (*Vicia villosa*) plants co-cultivated with (benzoxazinoid producing) rye plants, representing the only report to date of benzoxazinones detected in a target weed species exposed to exudates containing the parent compounds.

17. Detoxification via BOA-O-glucosylation

With few exceptions, laboratory studies aimed at investigating the effects of benzoxazinoids on plant growth are performed with seedlings or juvenile plants, primarily cultivated from surface-sterilised seeds; thus, the situation occurring in nature is quite different. Many weeds take up allelochemicals from their surroundings and mitigate their

toxicity via metabolic sequestration. In these cases, compounds may be transported and stored temporarily within the central vacuole or bound to cell wall constituents. For transport within the cytosolic compartment, it may be necessary to increase the hydrophilicity of the allelochemical. For example, in most dicotyledonous weeds, and to a lower extent in monocots such as cereals, BOA is hydroxylated at position 6 or 5 and subsequently glucosylated (Schulz et al., 2013). The success of this detoxification mechanism depends very much on the avoidance of BOA-6/5-OH accumulation, as these intermediates possess higher toxicity than BOA. Whereas BOA-insensitive species tend to utilise hydroxylation and glucosylation, sensitive species often accumulate considerable levels of the intermediates BOA-6/5-OH, and sometimes also unmodified BOA. Currently, available evidence indicates that BOA-O-glucosides are not maintained stably within receiver plants. For example, extractable levels appeared to drop rapidly following termination of incubation experiments performed with coriander (*Coriandrum sativum*) and shaggy soldier (*Galinsoga ciliata*) (Sicker et al., 2001).

The glucosylation of benzoxazinoids has been catalysed by UDP-glucosyltransferase enzymes (Zhang et al., 2022). Presently only one weed, *Arabidopsis thaliana*, has been investigated in more detail for glucosyltransferase activity, which catalyses the glucosylation of BOA-6-OH. The mutant SALK_04959c carrying a T-DNA insertion within the At4g01070 coding sequence encoding glucosyltransferase UGT72B1, is highly sensitive to BOA and produces only small amounts of BOA-6-O-glucoside in glucosyltransferase assays performed with crude soluble protein extracts prepared from seedlings, relative to wild-type Col-0 (Baerson et al., 2017). UGT72B1 is an enzyme important for the detoxification of xenobiotics (Brazier-Hicks and Edwards, 2005). Since the SALK_04959c mutant still produced low amounts of BOA-6-O-glucoside, additional UGTs likely exist with some activity towards BOA-6-OH, which would be in accordance with the observed

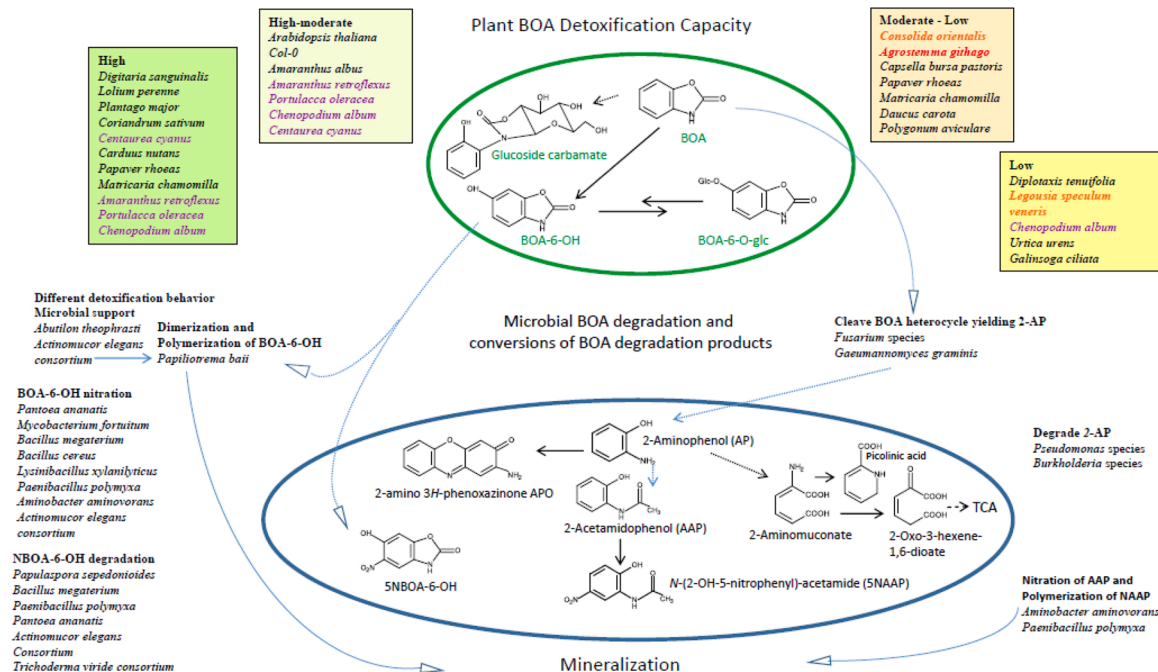


Fig. 6. Benzoxazinoids: Weed degradation and detoxification strategies are shown with their relationships with microbial conversion and degradation pathways. Plants with similar detoxification capacities are listed within four boxes (high, high-moderate, moderate-low, low). Extinct species are shown in red, endangered species are shown in orange. Predicted chemotypes are shown in mauve and fall under 'High', 'High-moderate' or 'Low' detoxification capacities. Structures of the most common plant detoxification products are shown circled in green; structures of microbial degradation and conversion products are shown circled in blue. The fungal-generated 2-aminophenol, a key compound for subsequent microbial conversions, is also highlighted (circled in white). Blue dashed lines indicate cross-kingdom metabolic interactions. Solid blue lines point to likely interactions with other soil microorganisms leading to complete degradation and mineralisation. Black dashed lines indicate multiple enzymatic steps involved in compound conversion and degradation. Headings indicate specific microbial activities (BOA-6-OH nitration, NBOA-6-OH degradation, Degradation of 2-AP, nitration of AAP), and the microorganisms capable of performing these activities are listed below each heading. *Abutilon theophrasti* utilises an atypical detoxification strategy for BOA(OH)s and is therefore separately grouped. TCA: citric acid cycle.

upregulation of several UGT genes in *Arabidopsis* after BOA exposure (Baerson et al., 2005). Considering the ubiquitous occurrence of BOA-OH glucosylation in weeds, plants apparently gain advantage from the acceptor substrate promiscuity of some of their UGT enzymes. The UGTs involved are likely co-opted from other metabolic pathways and may be involved in other detoxification reactions as well.

18. Detoxification via glucoside carbamate in combination with BOA-O-glucosylation

Species possessing higher tolerance levels to BOA, including many *Poaceae* members, replace BOA-O-glucosylation with glucoside carbamate formation during the detoxification process. Glucoside carbamate becomes the major detoxification product in uncultivated *Poaceae* members such as perennial ryegrass (*Lolium perenne*) and hairy crabgrass (*Digitaria sanguinalis*), (Sicker et al., 2004). Glucoside carbamate is also found in more sensitive species and accumulates in those plants as a minor detoxification product, for instance, in wild oat (*Avena fatua*) and many moderately sensitive dicot species (Schulz and Wieland, 1999; Sicker et al., 2004). The latter group encompasses species belonging to plant communities characterized by BXZ containing *Secale cereale* and *Triticum aestivum*.

Seedlings of the warm season weeds velvetleaf (*Abutilon theophrasti*), redroot pigweed (*Amaranthus retroflexus*), fat-hen (*Chenopodium album*) and common purslane (*Portulacca oleracea*) exhibit a dynamic, organ-specific pattern of BOA detoxification dependent on external BOA concentration (Schulz et al., 2012). Aside from BOA-6-O-glucoside, considerable amounts of glucoside carbamate were also found in *A. retroflexus*, *C. album* and *P. oleracea*. Additionally, *P. oleracea* has been shown to accumulate malonylglucoside carbamate as another major detoxification product and low amounts of gentiobioside carbamate. Glucoside carbamate derivatives were first identified in maize (Hofmann et al., 2006). However, the detoxification activities of all these warm-season weeds significantly decreased when incubations were performed with 200 μ M BOA/g_{FW}, a concentration that is certainly not relevant to conditions typically found in nature. Interestingly, velvetleaf (*Abutilon theophrasti*) accumulated none of the aforementioned detoxification products significantly. In fact, studies utilising channel blocking agents strongly suggest that this species exudes much of the absorbed BOA through efflux transporters (Schulz et al., 2012).

19. Elimination of benzoxazinoids at the root surface

Bacteria and other microorganisms attached to the outer surface of plant roots, and endophytic microorganisms within plant tissues, profoundly influence numerous plant processes, such as the extracellular release of enzymes such as catalases, laccases and peroxidases (Schmidt et al., 2018). Established assemblies of root zone-associated bacteria typically increase from the root tip through the mature part with lateral roots (Schmidt et al., 2018; Wheatley and Poole, 2018; Ruger et al., 2021). Roots of weeds incubated with BOA are often coated with phenoxazinone (Schulz and Wieland, 1999; Sanchez-Moreiras and Reigosa, 2005), which results from microbial degradation of BOA, yielding 2-aminophenol (2AP). This degradation product is dimerized to the phytotoxic 2-amino-3H-phenoxazin-3-one (APO), most probably by peroxidases and laccases (Voloshchuk et al., 2020).

Microorganisms capable of cleaving the BOA heterocycle include *Fusarium* species, strains of the take-all pathogen (*Gaeumannomyces graminis*), and several bacterial species. However, 2-aminophenol is not always converted to APO but can also be degraded by root colonising and soil bacteria such as *Pseudomonas* or *Burkholderia* species into fragments capable of entering the TCA cycle (Chirino et al., 2013, see 6. this review).

Thus, these microorganisms reduce the inhibitory effects of otherwise increasing amounts of APO on plant growth. In particular, older root zones have been shown to gain an advantage when well-colonised

by beneficial microorganisms, as they can effectively shield the host plant from the negative effects of certain phytotoxins (Wheatley and Poole, 2018). Furthermore, the extent of phenoxazinone precipitation seems to be heavily influenced by the establishment of bacterial biofilms on root surfaces (Voloshchuk et al., 2020).

20. Plant-Microbe cooperation in compound conversion

At present, no microorganism has been identified possessing the ability to hydroxylate BOA. Although BOA-6-OH can be spontaneously generated via Fenton reactions known to occur within the cell wall, the introduction of the OH group is seemingly catalysed exclusively by plant enzymes (Baerson et al., 2005). Candidates for catalysing this reaction are cytochrome P450 monooxygenase(s), which represent enzymes playing pivotal roles in plant defense. In *Arabidopsis*, several genes within the P450 superfamily are induced during BOA incubation, although the catalytic activities of the corresponding enzymes have not yet been determined.

BOA-OH isomers are known to be metabolised by specific microorganisms. Depending on the cultivation site, *Abutilon theophrasti* can be colonised by a microbial consortium composed of the zygomycete *Actinomyces elegans*, bacteria including *Stenotrophomonas maltophilia*, *Pantoea ananatis* and others, and the yeast *Papiliotrema baii*. These organisms act in concert when producing BOA-OH polymers at the root surface. Interestingly, *Abutilon theophrasti* plants inoculated with this consortium produced higher amounts of BOA-5-O-glucoside than BOA-6-O-glucoside when protein extracts were assayed for glucosyltransferase activity using the corresponding BOA-OHs as acceptor substrates (Haghi Kia et al., 2014; Schulz et al., 2017b). Microorganisms can subsequently hydrolyse glucosylated BOA-6-OH, and the released BOA-6-OH can be further converted. Thus, cooperation between plant and microbial pathways is responsible for the environmental fate of BOA.

In a nitrate-rich environment, certain bacteria, for instance, *Pantoea ananatis* isolated from the *Actinomyces elegans* consortium, or strains of the soil bacteria *Lysinibacillus xylanilyticus* and *Bacillus cereus* add a nitro group to BOA-6-OH in position 5, yielding 6-hydroxy-5-nitrobenzo[d]oxazol-2(3H)-one (NBOA-6-OH) (Schutz et al., 2017a; Schutz et al., 2019). The nitroaromatic compound is inhibitory to other bacteria, as was found for strains of *Mycobacterium fortuitum*, *Bacillus megaterium* and *Bacillus aryabhatti*. Other microorganisms are stimulated by NBOA-6-OH. For example, *Bacillus cereus*, and the fungus *Papulaspora sepedonioides* showed no effect. Thus, this compound has the potential to shape the composition of microbial communities. NBOA-6-OH can also be inhibitory to some plants, as it inhibits photosynthesis. The nitro-compound was also found in *Abutilon theophrasti* incubation media supplemented with BOA-6-OH, and on root surfaces. However, in contrast to BOA-6-OH, NBOA-6-OH has a short half-life and can be degraded by many microorganisms. Negative effects on plant growth are therefore not long-lasting. BOA-6-OH, which possesses a moderate inhibitory effect on photosynthesis, can be eliminated by polymerisation within an H₂O₂-enriched chemical environment, as was found with *Papiliotrema baii*.

Interestingly, the microbial 2-AP detoxification product 2-acetamidophenol (2-AAP) can also be nitrated by specialised bacteria (Schutz et al., 2019). Nitrated 2-AAPs were previously observed in fungal cultures incubated with benzoxazinoids (Zikmundova et al., 2002a; 2002b). The resulting nitro compounds did not inhibit plant growth up to 3 mM; thus, they seem less phytotoxic than NBOA-6-OH. These findings demonstrate the importance of microorganisms in protecting plants against the phytotoxic effects of exposure to allelopathic benzoxazinoids. Nitrated 2-AAPs can also be polymerised on the surfaces of *Aminobacter* and *Paenibacillus* species and perhaps other microorganisms (personal communication). Polymerisation of benzoxazinone derived metabolites presents one of the possible final fates. These polymers could also potentially undergo mineralisation and contribute to soil

fertility.

21. Conclusions and future perspectives

Wheat exhibits allelopathic potential due to the presence of phenolic acids, flavonoids (PAF), benzoxazinones (BXZ) and phenoxazinones (PXZ) that are released into the surrounding environment and affect the growth, development and physiological characteristics of neighbouring plants. The biosynthesis of BXZs is strongly regulated during plant growth, with accumulation being greatest in young tissues and dependent on wheat genotype and environmental conditions. The major pathways employed by plants for benzoxazinoid detoxication involve hydroxylation and glucosylation reactions and the polymerisation of intermediates within these pathways.

Allelochemicals from different wheat genotypes have been shown to inhibit the growth of various weed species, including *Bromus japonicus*, *Chenopodium album*, *Portulaca oleraceae*, *Avena fatua* and *Lolium rigidum*. Soil microflora is responsible for transforming BXZs into more potent bioherbicidal metabolites that can be exploited as weed control agents. Screening, selection and breeding of BXZ rich wheat cultivars could serve as bioherbicides models and avoid increasing levels of herbicide resistance for more sustainable weed management. BXZs have been shown to suppress the growth and development of certain agricultural pests, including insects, fungal pathogens, and weeds; thus, considerations for the selection and breeding of novel wheat genotypes possessing enhanced defensive ability via elevated BXZ contents.

Wheat BXZs may be useful as weed control agents due to their phytotoxicity, specific activity and limited soil persistence. Up to now, limited information is available regarding the ability of plant breeders to select for crop genotypic diversity that results in enhanced pest and disease management, but this form of management through competitive crops is cost-effective and technically easy to deploy. However, before seed companies and plant breeders invest in such long-term breeding and selection programs, information about the key metabolites released over time, and how they are transformed or detoxified in the rhizosphere by soil microbiota, including mycorrhizal fungi, is a new frontier that remains to be explored. An increasing focus on healthy foods and the sustainability of agroecosystems is now driving the activity of agricultural researchers.

The selection and breeding of BXZ rich wheat genotypes, and a better understanding of their soil persistence and transformation in the rhizosphere, along with the potential for weeds to develop resistance, will be required to generate improved wheat genotypes for use in more sustainable weed management programs. It will be important for future breeding efforts to take such potential adverse environmental impacts into account, in combination with an increased focus on enhancing beneficial allelopathic interactions between species within agricultural systems.

CRedit authorship contribution statement

M.I.H., and A.M.S.M., conceived and designed the study; F.A., M.S., S.B., Y.V.A., L.R., P.B., N.C.S., F.A.M., L.A.W., M.J.R., drafted the Ms and critically revised the manuscript; All authors review the literature, synthesize the data/material and draft the review; F.A., M.S., S.B., P.B., L.A.W., M.J.R., A.M.S.M., critically edited and revised the manuscript; V.A.Y., helped in correction, revision of tables, reference formatting, and correction of typo mistakes. All authors have read and agreed to the published version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

Acknowledgements

This work was supported by the European Commission, grant number 77136-2, for the project, 'Increasing the efficiency and competitiveness of organic crop breeding 'ECOBREED'. We highly appreciate the support and funding received for open access from University of Vigo / CISUG.

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