Seed Science Research

cambridge.org/ssr

Review Paper

Cite this article: Pollard AT (2018). Seeds *vs* fungi: an enzymatic battle in the soil seedbank. *Seed Science Research* **28**, 197–214. https://doi.org/10.1017/S0960258518000181

Received: 7 December 2017
Accepted: 18 April 2018
First published online: 5 June 2018

Keywords:
defence enzyme; dormancy; fungal enzyme; integrated weed management; seed; soil seedbank

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Seeds *vs* fungi: an enzymatic battle in the soil seedbank

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Abstract

Depleting the soil weed seedbank is an important integrated weed management strategy that has the potential to foster lasting weed control. Long-term dormancy and decay resistance of weed seeds pose a challenge to weed eradication efforts. Select soil fungi have been shown to cause significant decay of weed seeds. The physical and chemical mechanisms by which seeds in the seedbank defend themselves against pathogens have been well researched. However, very few studies have purposefully investigated the biochemical defence response of seeds. Enzyme-based biochemical seed defences have been detected in dormant and non-dormant seeds, and research supports their function in pathogen defence. This review summarizes current knowledge of the seed defence enzymes polyphenol oxidase, peroxidase, chitinase and oxalate oxidase. The fungal enzymes chitinase, protease and xylanase that function in pathogenesis of seeds in the soil seedbank are also reviewed. Progress in the development and standardization of *in situ* enzyme analyses fosters our understanding of actual enzyme activity present in soils, while high-throughput microplate techniques promote efficiency and enable greater scope. Application of genomic, proteomic and transcriptomic techniques to glean a deeper and more holistic understanding of the enzymatic interactions of weed seeds and soil fungi in the soil seedbank will support the development of improved integrated weed management strategies.

Introduction

Management of annual weeds is a major problem in agriculture, impacting crop quality (Akbar et al., 2011; Gibson *et al.*, 2017), crop yield (Oerke, 2006; Soltani *et al.*, 2016, 2017), and ultimately economic return on investment (Pimentel *et al.*, 2005; de Lange and van Wilgen, 2010). Many factors influence the effect of weeds on crop yields including weed density, weed species, crop species and soil conditions (Wagner *et al.*, 2007). A density of ten wild oat (*Avena fatua* L.) plants per m^2 can result in as much as 35% yield loss of wheat (*Triticum aestivum* L.) (Martin and Field, 1988). Weeds are responsible for 12% of annual yield loss to US agriculture, resulting in $33 billion annually in lost crop production (Pimentel *et al.*, 2005). Moreover, roughly $7 billion is spent annually for herbicidal weed control for US crops (Gianessi and Reigner, 2007).

A central tenet of weed management is to control weeds at the early stages of crop development when weed competition most impacts crop yield (Zimdahl, 1988; Swanton and Weise, 1991). However, weeds present in the field later in the season also produce viable seeds, which contribute to weed problems in subsequent years. Without high levels of control, weed seed density will not diminish over time, and may actually increase, unless weed management strategies aim to deplete this pool of weed seeds in the seedbank (Gallandt, 2006).

Weed seeds exist in high densities in soils, frequently surpassing 20,000 m^{-2} worldwide and more than 100,000 m^{-2} in the United States (Baskin and Baskin, 2006). Up to nearly 1 million seeds m^{-2} have been reported for seedbanks of agricultural lands (Baskin and Baskin, 2006). Adaptations that prolong the persistence of weed seeds in soils, including decay resistance and long-term dormancy, are especially problematic (Baskin and Baskin, 1985; Kremer, 1993; Dalling *et al.*, 2011). Wild oat has been the subject of several recent reports on seed defence mechanisms (Anderson *et al.*, 2010; Gallagher *et al.*, 2010; de Luna *et al.*, 2011; Fuerst *et al.*, 2011, 2014). In addition to increasing incidence of herbicide resistance, the seed characteristics of wild oat contribute to its status as one of the ten worst global weeds of temperate regions (Beckie *et al.*, 2012). Wild oat seeds are large, produced in abundance, have a tough outer hull, exhibit staggered germination, and can remain dormant in the soil for up to 9 years (Beckie *et al.*, 2012).

Interest in sustainable and organic agriculture is increasing steadily as a result of consumer demand, environmental concerns, trade regulations and herbicide resistance, necessitating the adoption of effective alternatives to chemical weed management strategies (Liebman and Davis, 2000; Scialabba, 2000; Wilson and Otsuki, 2004; Hughner *et al.*, 2007). Integrated weed management (IWM) systems focus on diversifying management approaches while

minimizing inputs, including reducing reliance on herbicides, without sacrificing crop productivity or economic returns (Swanton and Weise, 1991; Bastiaans *et al*., 2008; Chikowo *et al*., 2009). Coordinating a thorough understanding of weed ecology and demographics into cropping systems is key to successful IWM strategies (Buhler, 2002). Depleting the weed seedbank in the soil is an important component of IWM that is often overlooked in favour of targeting the aboveground tissue (Bastiaans *et al*., 2008). However, studies show that the diversity and abundance of the underground weed seedbank can drastically decrease over time in certain low-input and organic systems (Menalled *et al*., 2001). The major causes of seed loss from the soil are germination, predation and microbial decay (Buhler *et al*., 1997). Understanding the mechanisms underlying the relationships between persistent weed seeds, or those seeds that survive in the soil for more than 1 year (Thompson and Grime, 1979), and soil microbes will foster development of IWM strategies that utilize the inherent capacity of soil microorganisms to cause weed seed mortality. For example, organisms that can overcome weed seeds' enzymatic defences may be identified as candidates for biological control applications.

While the potential of soil microbes to cause decay is commonly associated with crop disease, it can also be utilized as a promising IWM strategy (Kremer, 1993; Kennedy and Kremer, 1996). Soil microbes can hinder weed proliferation and deplete the weed seedbank via indirect means, such as by influencing seed germination and development, and directly by eliciting seed decay through hyphal penetration and cell wall degrading enzymes (Kremer, 1993). For example, Kennedy *et al*. (1991) found the bacterium *Pseudomonas fluorescens* isolate D7 capable of reducing populations of downy brome (*Bromus tectorum* L.), a common weed of small grains, by up to 30% while increasing yields of winter wheat by up to 35% due to inhibition of seed germination and suppression of root elongation. Downy brome suppression eventually resulted in greater crop competitiveness and increased native plant biodiversity (Kennedy *et al*., 1991). Many microbial taxa are known to cause mortality of buried seeds in the soil, and fungi-induced decay of seeds in the seedbank has perhaps been the most studied (Wagner and Mitschunas, 2008; Baskin and Baskin, 2014). Decay of dormant seeds of velvetleaf (*Abutilon theophrasti* Medik.), an invasive weed of corn and soybeans, has been strongly correlated to fungal associations (Chee-Sanford, 2008). Ninety-nine per cent of velvetleaf seeds that had been incubated for 3 months on soil-inoculated agar plates showed decay symptoms, which the authors attributed to surface colonization by various *Ascomycota* fungal species. In contrast, the uninoculated control seeds, from which no 18S rRNA fungal genes amplified, exhibited no obvious decay (Chee-Sanford, 2008). Seed fatality of the annual grass weed downy brome due to the fungal seed pathogen *Pyrenophora semeniperda* has been extensively researched (Beckstead *et al*., 2007; Meyer *et al*., 2007, 2008, 2010; Finch *et al*., 2013). De Luna *et al*. (2011) isolated hundreds of soil fungi from dormant wild oat seeds incubated in the field for 6 months and 15% of the isolates were clearly linked with seed decay *in vitro*. This subset of isolates was tested further and *Fusarium avenaceum* isolate *F.a*.1 elicited the most rapid and pronounced decay of wild oat seeds.

Despite evidence of prevalent microbe-induced seed decay, seeds in the soil seedbank are innately equipped to resist pathogen attack via physical, chemical and biochemical means (Davis *et al*., 2008; Dalling *et al*., 2011; Fuerst *et al*., 2011, 2014). Physical mechanisms including dense pubescence and thick seed coats are important persistence mechanisms across many seed types, yet especially critical for seeds with long-term persistence (Pfeiffer *et al*., 2003; Davis *et al*., 2008, 2016). Chemical defences include those endogenous to the seed such as low molecular weight specialized metabolites (e.g. tannins, alkaloids, phenols), and those that arise from beneficial associations with soil microbes (Chee-Sanford and Fu, 2010; Dalling *et al*., 2011). Cereal grains are known to produce chemicals that appear to contribute to defence from pathogen attack. For example, flavonoids present in the testa of developing barley (*Hordeum vulgare* L.) caryopses are potent inhibitors of *Fusarium* spp. (Skadhauge *et al*., 1997). In contrast to chemical defences, biochemical seed defences refer to high molecular weight protein and enzyme-based mechanisms (Anderson *et al*., 2010; Jerkovic *et al*., 2010; Fuerst *et al*., 2011, 2014; Raviv *et al*., 2017a, b).

Under natural environmental fluctuations present in the soil seedbank, seeds with long-term physiological dormancy experience countless biotic and abiotic stresses, such as cycles of hydration and dehydration, while still maintaining dormancy and the ability to germinate at a later opportune time (Bolingue *et al*., 2010). Research suggests that these highly persistent seeds, especially weed seeds that have been shown to remain viable in the soil seedbank for 50–100 years, may employ complex biochemical mechanisms to guard against pathogen attack in the soil (Baskin and Baskin, 1985).

An analysis of the redox-sensitive proteome of dormant and non-dormant wheat seeds showed that 79 proteins responded differentially in dormant *versus* non-dormant seeds (Bykova *et al*., 2011). Dormant wheat seeds exhibited higher levels of proteins involved in the anti-oxidative defence response, including the thiol-dependent peroxidase, peroxiredoxin. They also expressed greater levels of proteins involved in the hypersensitive response, such as chitinases and other pathogenesis-related proteins, and serine protease inhibitors that counteract degradative fungal proteases (Bykova *et al*., 2011). In another study comparing gene expression patterns in dormant *versus* after-ripened *Arabidopsis thaliana* seeds, 442 genes had higher expression in dormant seeds and within this set, genes associated with stress response were two times more abundant (Cadman *et al*., 2006). Surprisingly, chitinase activity has even been detected in the seed coats of 37-year-old radish (*Raphanus sativus* L.) seeds (Raviv *et al*., 2017a). These studies support the hypothesis that seeds with physiological dormancy are capable of actively mounting complex biochemical defence responses (Fuerst *et al*., 2014).

Research into the physical and chemical defence mechanisms of seeds dates back decades (Rosenthal, 1977; Chrispeels and Raikhel, 1991; Siemens *et al*., 1992; Broekaert *et al*., 1995; Peumans and Van Damme, 1995; Davis *et al*., 2008), but biochemical seed defence mechanisms have only recently been the target of scientific investigation (Jerkovic *et al*., 2010; Fuerst *et al*., 2011, 2014; Raviv *et al*., 2017a,b). Moreover, research into the biochemical seed defence responses of dormant weed seeds is extremely sparse (Anderson *et al*., 2010; Fuerst *et al*., 2011, 2014). In this review, I summarize biochemical seed defences and the complementary degradative enzymes employed by soil fungi in their attack of seeds in the soil. Whenever possible, I highlight these interactions as they occur in weed seeds and dormant seeds.

Seed defence enzymes

Numerous enzymes and proteins implicated in defending seeds against microbial pathogens are active in seeds (Sultan *et al*.,

https://doi.org/10.1017/S0960258518000181 Published online by Cambridge University Press

Table 1. Characteristics of plant defence enzymes and fungal degradative enzymes discussed in this review

Enzyme	Reaction catalysed	Significant functions	Structural characteristics
Plant			
Polyphenol oxidase	Hydroxylation of monophenols to *o*-diphenols, and oxidition of phenolic compounds to quinones	Toxic quinone production; cell wall lignification; involvement in ROS generation; reduction of nutrient bioavailability by cross-linking molecules	Metalloenzyme with binuclear type-3 copper centre
Peroxidase	Single-electron oxidation of various hydrogen donors (e.g. phenolics, lignin precursors, auxin), thereby reducing H_2O_2 in the process	ROS production; synthesis of diverse antimicrobial phytoalexins; cell wall lignification	Common heme group formed from protoporphyrin IX and Fe(III)
Oxalate oxidase	Two-electron oxidative decarboxylation of oxalate to hydrogen peroxide and carbon dioxide	Production of toxic H_2O_2; cell wall strengthening; calcium regulation; stress response	Glycoprotein with jellyroll β-barrel structure and Mn(II) cofactor
Chitinase	Hydrolytic cleavage of β-1,4-glycoside bonds within chitin	Chitin degradation in fungal cell walls and insect exoskeletons; participation as PR proteins in systemic acquired resistance	Highly diverse; traits vary according to complex classification system
Fungal			
Chitinase	Hydrolytic cleavage of β-1,4-glycoside bonds within chitin	Mycoparasitism; entomopathogenesis; possible competitive advantage in seed pathogenesis	Belong exclusively to glycosyl hydrolase superfamily 18
Protease	Hydrolytic cleavage of peptide bonds in proteins to yield peptides and free amino acids	Plant defence enzyme degradation; plant host tissue degradation; signalling; nutrition; sporulation; morphogenesis; septum formation	Highly diverse; complex classification system; division into 9 families based on functional group at catalytic domain
Xylanase	Endohydrolysis of β-1,4-xylosidic bonds in xylan polysaccharide backbone	Plant cell wall degradation; may be required for fungal virulence	Highly diverse; complex classification system based on similarities of amino acid sequence of the catalytic domain

2016). For example, microdissection of wheat bran into outer (epidermis and hypodermis), intermediate (cross cells, tubes cells, testa and nucellar tissue), and inner (aleurone) layers, as illustrated by Lásztity (1999), followed by proteomic analysis revealed a complex wheat bran proteome in which enzymes are organized to provide distinct seed defence functions (Jerkovic *et al.*, 2010). The outer fraction was dominated by oxidative stress- and defence-related enzymes, including oxalate oxidase, polyphenol oxidase and peroxidase. Proteins of the intermediate fraction served similar oxidative stress- and defence-related functions, but the proteome in this layer was far more diverse than in the outer fraction. As this is the last line of pathogen defence before fungal hyphae enter the living aleurone tissue, this intermediate bran fraction contained not only oxalate oxidase, but also chitinases and numerous inhibitors of fungal enzymes. The majority of proteins in the living inner aleurone layer functioned in metabolism, but defence enzymes, including chitinase, and fungal enzyme inhibitors were also present. Herein I explore the classification, structure, function and mechanism of four common enzyme families believed to operate in seed defence: polyphenol oxidase, peroxidase, oxalate oxidase and chitinase (Fuerst *et al.*, 2011, 2014) (Table 1).

Polyphenol oxidase

Polyphenol oxidase (PPO) is often portrayed in scientific literature as a single enzyme, and one whose name is frequently used interchangeably with the enzyme names tyrosinase, polyphenolase, phenolase, catechol oxidase, cresolase and catecholase (Yoruk and Marshall, 2003). In reality, PPO is a broad term encompassing three distinct enzymes: catecholase, laccase and cresolase or tyrosinase. Cresolase and tyrosinase are the same enzyme, but they have sometimes been assigned the name cresolase in the case of plants, and tyrosinase in the case of animals and microbes (Aniszewski *et al.*, 2008; Kaintz *et al.*, 2014). However, the first tyrosinase reported of plant origin was recently isolated from walnut (*Juglans regia*) leaves (Zekiri *et al.*, 2014). Laccases are a diverse group of enzymes with broad substrate specificity that catalyse the oxidation of a wide range of phenolic substrates in plants, fungi, bacteria and insects (Giardina *et al.*, 2010).

PPO enzymes oxidize phenolic compounds to generate quinones. Quinones are highly reactive compounds that will further react non-enzymatically through self-polymerizing or covalently bonding and cross-linking with amino acids or proteins to form high molecular weight melanin pigments (Walker and Ferrar, 1998). PPOs catalyse distinct hydroxylation and oxidation reactions: (1) the hydroxylation of monophenols to *o*-diphenols, and (2) the oxidation of diphenols (and other substrates) to the associated quinones in the presence of molecular oxygen. The first reaction is catalysed by cresolase or tyrosinase (also known as monophenolase or monophenol monooxygenase). The second reaction is catalysed by catecholase (also known as diphenolase or diphenol oxidase) or laccase (Marusek *et al.*, 2006; Aniszewski *et al.*, 2008). Catecholases oxidize *o*-diphenols to *o*-diquinones, whereas laccases oxidize *o*- and *p*-diphenols, and other substrates including triphenols, to *p*-diquinones, other quinones, and semiquinones (Aniszewski *et al.*, 2008). The enzymes that catalyse only the latter reaction are often referred to broadly as catechol oxidases and their activity in a diverse range of living organisms has been extensively studied (Gerdemann *et al.*, 2002b). In

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contrast, cresolase activity has been far less investigated. Whether cresolase is always present in organisms that produce CO is debated in the literature (Marusek *et al.*, 2006).

PPOs are a highly diverse group of enzymes that differ widely in structure, amino acid sequence, function, temporal and spatial expression, and substrate specificity (Mayer, 2006; Cai *et al.*, 2013). Given the latter characteristic, measured activity may differ if one substrate is used *versus* another. A highly conserved trait shared by all PPOs is that they are metalloenzymes with an active site consisting of a binuclear type-3 copper centre containing two copper ions (CuA and CuB), each bound to three histidine side chains (Marusek *et al.*, 2006; Aniszewski *et al.*, 2008). Additional common structures shared by PPOs include a signal peptide, which directs the enzyme to the thylakoid lumen of chloroplasts or to the vacuolar lumen; a highly conserved N-terminal containing the features for copper binding, substrate catalysis, and structural maintenance; a variable C-terminal domain; and a linker region between the C- and N-termini that is highly variable in size and structure (Marusek *et al.*, 2006; Tran and Constabel, 2011; Cai *et al.*, 2013).

The described structure of the mature, latent PPO enzyme ranges in size from 39 to 73 kDa depending on plant species (Aniszewski *et al.*, 2008). Evidence suggests that proteolytic cleavage of the 15–20 kDa C-terminal is required for enzyme activation because the C-terminal physically shields the activation site (Gerdemann *et al.*, 2002a; Marusek *et al.*, 2006; Flurkey and Inlow, 2008). The mature, inactive form of PPOs is highly stable, but it shows much greater thermal lability following activation via proteolytic cleavage (van Gelder *et al.*, 1997). Endogenous and exogenous proteases, such as those from pathogenic fungi, are known to activate PPOs; anionic detergents, acids and lipids have been shown to activate PPOs *in vitro* (van Gelder *et al.*, 1997; Fuerst *et al.*, 2011, 2014).

The mechanism by which PPOs catalyse reactions in plants relies on the interaction of the copper ions in the enzyme with molecular oxygen and substrates (Aniszewski *et al.*, 2008). Possible ways which PPO imparts anti-fungal defences to seeds may include (1) producing toxic quinones, (2) reducing nutrient bioavailability by cross-linking molecules, (3) cell wall lignification, and (4) involvement in reactive oxygen species (ROS) generation (Constabel and Barbehenn, 2008; Fuerst *et al.*, 2014).

PPO is induced in seeds challenged with fungal pathogens and its activity exhibits spatial and temporal variability. Following inoculation of developing wheat heads with *Fusarium graminearum*, maximum PPO activity, as assessed using pyrocatechol (1,2-dihydroxybenzene) as the substrate, occurred during the milk stage for resistant and susceptible cultivars, and steadily declined thereafter (Mohammadi and Kazemi, 2002). The level of PPO activity was three times greater in the resistant cultivars compared with the non-inoculated controls, and twice as great as the susceptible cultivars, suggesting that PPO induction in developing wheat heads could be a defensive response to pathogen attack. Moreover, seven isoforms of the PPO enzyme were detected in the extracts of wheat heads and they were differentially expressed among the cultivars and throughout grain development.

PPO was induced in dormant caryopses of wild oat isoline M73 following incubation on *Fusarium avenaceum* isolate *F.a.*1, as detected using L-DOPA (L-3,4-dihydroxyphenylalanine) as the substrate (Anderson *et al.*, 2010; Fuerst *et al.*, 2011, 2018). Fractionation of the extracted proteins from caryopsis leachates showed predominantly 57 kDa proteins, and to a lesser extent 36 kDa, from untreated caryopses; several lower molecular weight proteins, including 24, 25 and 36 kDa, were present in the *F.a.*1-treated caryopses. The 36 and 57 kDa protein sequences were highly similar to wheat PPO, but the 24 and 25 kDa proteins were most similar to oxalate oxidase and chitinase, respectively. The authors hypothesized that *F.a.*1 induction of latent wild oat PPO involves proteolytic cleavage, thereby yielding a lower molecular weight activated form of water-soluble PPO that readily diffuses into the seed environment (Anderson *et al.*, 2010; Fuerst *et al.*, 2011). Detection of PPO activity in the leachates of wild oat caryopses following incubation on *F.a.*1 using L-DOPA as substrate (Fuerst *et al.*, 2011, 2018), as well as in the supernatant of imbibed wheat bran and using hydroquinone monomethyl ether and 3-methylbenzothiazolin-2-one hydrazone as substrates (Jerkovic *et al.*, 2010), suggests that PPO seed defence function is amplified by its mobility in the external seed environment. For example, PPO activity in leachates from dormant wild oat caryopses incubated on *F.a.*1 increased 7.5-fold compared with untreated caryopses, whereas PPO activity from whole caryopses increased only 3.4-fold after incubation on *F.a.*1 compared with the untreated controls (Fuerst *et al.*, 2018).

After wild oat caryopses were incubated *in vitro* on three *Fusarium* spp. isolates (*F.a.*1, *F. culmorum*-2, and *F. culmorum*-4), PPO activity, as measured with L-DOPA substrate, increased 106, 47 and 24%, respectively, compared with the untreated controls. PPO detection in the controls, however, indicated that PPO was also expressed constitutively. In contrast, incubation on a *Pythium* isolate decreased PPO activity by 26% (Fuerst *et al.*, 2011), illustrating a species-specific response. PPO induction from the three *Fusarium* isolates correlated with their virulence, as demonstrated by the rate at which they caused visible symptoms of seed decay, *F.a.*1 > *F.c.*2 > *F.c.*4 (de Luna *et al.*, 2011). The hull fraction (lemma and palea) of wild oat seeds also showed induction of PPO activity following incubation on *F.a.*1, and at a greater level than in the caryopses, suggesting that these non-living tissues are also capable of actively mounting a biochemical defence response (Fuerst *et al.*, 2011).

The mechanism by which dead maternal seed tissue can respond in defence has not been conclusively determined. Studies have shown that genes encoding enzyme expression are active at various developmental stages of the pericarp. For example, a gene encoding a protein similar to polyphenol oxidase is expressed in seed coats of developing *Arabidopsis thaliana* (Pourcel *et al.*, 2005). Barley seed transcriptomics indicates that numerous genes regulate expression of protease enzymes in the pericarp and that seed developmental stages are characterized by different expression patterns. For example, distinct proteases are involved at specific phases of programmed cell death in the pericarp (Sreenivasulu *et al.*, 2006). Programmed cell death of maternal seed tissues results in the degradation and redistribution of nutritional cellular materials to the developing filial tissues (Domínguez and Cejudo, 2014). Research suggests that enzymes that serve defence roles remain stored in the non-living outer seed layers following programmed cell death and that these active enzymes are released from the tissues upon hydration (Godwin *et al.*, 2017; Raviv *et al.*, 2017a,b). Additional hypotheses propose that fungal proteases activate latent PPO through proteolytic cleavage of its C-terminal peptide and that this can occur in non-living seed hulls (Fuerst *et al.*, 2014). Other seed defence enzymes may be activated in a similar manner, but the hypothesis has not been tested (Fuerst *et al.*, 2018). Given the presence of proteases in seed pericarps, it is also possible that endogenous seed proteases proteolytically activate PPO.

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Research indicates that PPO is generally localized in the outer layers of seeds. For example, microdissection followed by proteomic analysis using two-dimensional gel electrophoresis (2DGE) showed that PPO activity was only present in the water-extractable protein fraction from the pericarp of wheat bran (Jerkovic *et al*., 2010). Fuerst *et al.* (2010) likewise concluded that PPO activity is predominantly present in the bran fraction of wheat. Investigation of enzyme activity in the germ aleurone layer of mature barley kernels indicated that phenol oxidase was present in the cytoplasm of germ aleurone cells (Cochrane, 1994). This publication was also one of the earliest studies to suggest a possible anti-microbial defence function of phenol oxidases in seeds (Cochrane, 1994). PPOs most often are initially translocated into the chloroplast thylakoid lumen (Golbeck and Cammarata, 1981), but a vacuolar form has also been reported (Tran and Constabel, 2011), and other possible subcellular locations have been proposed (Mayer, 2006). Of course, such subcellular localization becomes less relevant when considering the outer tissues of seeds, some of which are non-living and thus lack cellular compartmentalization.

Peroxidase

The peroxidase (POD) enzyme category encompasses a vast diversity of enzymes that are found throughout living systems. Heme PODs are the most well-known PODs, appearing in the scientific literature since at least 1908 (Kastle and Porch, 1908). More recently, in 1996, heme-free and thiol-dependent PODs, termed *peroxiredoxins*, were discovered in plants (Stacy *et al*., 1996). While limited studies suggest peroxiredoxins may be involved in pathogen defence (Rouhier *et al*., 2004; Haddad and Japelaghi, 2015), relatively little is known about the function of these thiol PODs in seeds (Bhatt and Tripathi, 2011). Therefore, this review will address only heme-containing plant PODs.

Heme PODs are classified into two superfamilies: the ‘animal’ PODs (found only in animals), and the ‘plant’ PODs (found in plants, fungi and prokaryotes) (Dunford, 1999). The ‘plant’ PODs all have a common heme group, formed from protoporphyrin IX and an Fe(III) (Banci, 1997), and share a similar three-dimensional protein structure (Cosio and Dunand, 2008), but are further divided into three separate classes based on amino acid sequence (Welinder, 1985, 1992).

Class III PODs are the plant-specific secretory PODs. Unlike class I and II PODs, class III PODs are glycoproteins, contain two Ca^{2+} ions for enhanced structural stability (Banci, 1997), and they are secreted into plant cell walls and vacuoles (Barceló *et al*., 2003; Passardi *et al*., 2004). Found in all land plants and throughout the plant lifecycle, class III PODs are members of large multigenic families (Passardi *et al*., 2004; Cosio and Dunand, 2008). They catalyse the single-electron oxidation of various hydrogen donors, such as phenolics, lignin precursors, auxin or specialized metabolites, thereby reducing H_2O_2 in the process (Barceló *et al*., 2003; Passardi *et al*., 2004). PODs generally have broad substrate specificity, with a moderate specificity for phenols (Hiraga *et al*., 2001), and they demonstrate an unusually high degree of thermal stability (Vámos-Vigyázó and Haard, 1981; Fujita *et al*., 1995; Barceló *et al*., 2003). Numerous isoforms exist within the class III PODs, each exhibiting a distinct amino acid sequence and subject to heterogeneous regulation of gene expression (Hiraga *et al*., 2001). This vast isozyme diversity is likely to be responsible for the diverse physiological processes that PODs catalyse (Hiraga *et al*., 2001; Passardi *et al*., 2005).

PODs have been detected in seeds since as early as 1973 when 14 POD isozymes were extracted from ground mature barley grains (LaBerge *et al*., 1973). At that time, however, the functional role of PODs in seeds was relatively unknown, and they were viewed rather as possible genetic markers for breeding since the isozyme profiles varied widely by cultivar. PODs have since been implicated in numerous active and passive defence-related activities. Passive, or constitutive, defences include cell wall lignification and suberization via cross-linking cell wall compounds, whereas active defences include production of ROS and synthesis of diverse antimicrobial phytoalexins (Passardi *et al*., 2005; Almagro *et al*., 2009). Lignification of plant cell walls by POD inhibits penetration by fungal hyphae (Cochrane *et al*., 2000). Class III POD defence activity occurs in response to both abiotic and biotic stressors. These can include heavy metal exposure, physical wounding, or pathogen and herbivore attack (Passardi *et al*., 2005). Research suggests that class III PODs are constitutively produced, but that levels modulate in response to abiotic and biotic stressors (Barcelo *et al*., 2003; Almagro *et al*., 2009).

POD activity has often been shown to increase when seeds are challenged with pathogens, although the expression varies with fungal species and plant cultivar. POD was isolated from mature seeds of French bean (*Phaseolus vulgaris* cv. Kentucky Wonder) and *in vitro* tests showed it strongly inhibited the fungi *Coprinus comatus* and *Botrytis cinerea*, but weakly inhibited *Mycosphaerella arachidicola* and *Fusarium oxysporum* (Ye and Ng, 2002). This study demonstrated not only the active pathogen defence function of POD in seeds, but also the variable specificity of the enzyme activity towards different fungal species. Some pathogen-specific differences in the defence enzyme gene expression profiles of soybean [*Glycine max* (L.) Merr.] seeds 35 days post-anthesis (DPA) were also apparent; 48 h after inoculation with *Diaporthe phaseolorum* var. *meridionalis*, ~2-fold greater magnitude of upregulated POD gene expression was observed compared with inoculation with *Cercospora kikuchii* (Upchurch and Ramirez, 2010). Compared with the control, *D. phaseolorum* and *C. kikuchii* induced ~6 and ~12 times greater POD expression, respectively, in soybean seeds. Induction of POD activity in response to a fungal pathogen is also seen in dormant caryopses, although the degree of enzyme response differs by plant species. Following incubation of dormant wild oat and wheat caryopses on the seed-decay fungal isolate *F.a.*1, POD activity from the whole caryopses increased 2.4- and 3.4-fold in the wild oat and wheat, respectively, compared with the POD activity from untreated caryopses (Fuerst *et al*., 2018).

The POD isozyme profiles from different barley and wheat cultivars change as seeds develop and according to cultivar (Kruger and LaBerge, 1974; LaBerge, 1975). Mature barley kernels (24–72 DPA) exhibited a greater number of POD isozymes than immature kernels (10–19 DPA) and the overall activity was higher at 24–72 DPA than at 10–19 DPA (LaBerge, 1975). The opposite trend was seen in a recent study in which hull-less barley was inoculated during anthesis with *Fusarium graminearum*, the major fungal pathogen causing Fusarium head blight, and the albumin and globulin soluble protein fractions from the grain were assessed at five phenological stages post-inoculation. While albumin and globulin seed proteins function primarily in nutrient storage, increasing evidence suggests they also function in defence against fungi, bacteria and insects (Terras *et al*., 1992, 1993; Marcus *et al*., 1999; Sales *et al*., 2000; Freire *et al*., 2015). Using 2DGE and mass spectrometry, POD was detected at increasing abundance during the milk stages from 7 to 14 days after

inoculation (DAI), but decreased during the proceeding dough stages (21 to 54 DAI) to non-detectable levels (Trümper *et al.*, 2016). Comparable results were seen in the POD activity of resistant and susceptible wheat heads inoculated with *F. graminearum*, which increased significantly during the milk stage compared with the non-inoculated controls (Mohammadi and Kazemi, 2002). Gel staining indicated the presence of three basic and six acidic POD isozymes and that they were differentially distributed and expressed among the four cultivars. Proteomic analysis of resistant and susceptible peanut (*Arachis hypogaea* L). seeds subjected to variable watering conditions and *Aspergillus flavus* treatments showed differential responses between the two cultivars (Wang *et al.*, 2010). POD expression increased in the resistant and susceptible cultivars under drought conditions + *A. flavus* inoculation compared with drought without inoculation, but POD was upregulated ~1.5 times more in the resistant cultivar when pathogen challenged compared with the susceptible cultivar. Using similar methods and also quantitative real-time polymerase chain reaction, POD expression in non-inoculated field-grown wheat grain was increasingly up-regulated 12 to 16 DPA, but by 21 DPA its expression drastically declined (Dong *et al.*, 2012). The contrasting correlation between POD expression and developmental stage seen in LaBerge (1975) compared with the latter studies may be due to several factors, including innate differences between plant cultivars, or use of more precise methods and advanced technology in the more recent studies.

In one of the earliest studies to suggest a possible antimicrobial seed defence function of POD, POD activity was observed in the cytoplasm and cell walls of germ aleurone cells of mature barley (cv. Triumph) kernels following overnight incubation of tissue in substrate solution (Cochrane, 1994). In a study of 14 POD isozymes in barley (cv. Centennial) kernels, there was great variability in the distribution pattern of the individual isozymes among the grain layers; some isozymes were confined to particular layers, while others would be present throughout the grain (LaBerge, 1975). Studies also show that the distribution of individual POD enzymes changes as wheat and barley grains develop and this varies by cultivar (Kruger and LaBerge, 1974; LaBerge, 1975). For example, 10 days after flowering, 75% of total seed POD was located in the wheat grain pericarp, but by 40 days after flowering, this percentage had dropped to ~10% in cv. Hercules but to only ~30% in cv. Manitou (Kruger and LaBerge, 1974). Differences in proteomic profiles were also seen between cultivated wheat and wild emmer wheat (*Triticum turgidum* var. *dicoccoides*), with significantly greater expression of detoxifying and oxidative enzymes, including POD, in the wild emmer wheat (Raviv *et al.*, 2017b). In their analysis of the wheat bran proteome, Jerkovic *et al.* (2010) identified POD activity solely in the water-extractable protein fraction from the pericarp, and studies indicate that POD is located in the outer surface layers of wild oat (Fuerst *et al.*, 2014). Significant POD activity was detected in the glumes of wild emmer wheat (Raviv *et al.*, 2017b) and in the seed coat of *Sinapis alba* and *Anastatica hierochuntica* (Raviv *et al.*, 2017a).

Oxalate oxidase

The nomenclature surrounding oxalate oxidases (OxOs) needs to be clarified due to research developments and inconsistencies in nomenclature that occurred as biochemical advances in the enzymology of OxOs were refined. OxO enzymes are one of two main subgroups within the germin protein family, which itself belongs to the cupin protein superfamily (Dunwell, 1998; Dunwell *et al.*, 2004). Given that the other subgroup contains 'germin-like proteins', OxOs are sometimes referred to as 'true germins' (Davidson *et al.*, 2009). Germins comprise a group of homologous proteins that are found solely in true cereals (Lane, 2002). They were first identified as a marker associated with germination onset in wheat embryos, hence the name *germin* (Thompson and Lane, 1980; Lane, 2002). In older literature before it was widely determined that some cereal germins were in fact oxalate oxidases, OxOs were sometimes referred to as germin-like proteins, although that misnomer has been avoided in more recent literature. Thorough histories of germin and OxO characterization are presented in the literature (Dunwell, 1998; Lane, 2002; Dunwell *et al.*, 2008). OxOs are encoded by a large homogenous group of genes found exclusively in true cereals (Poaceae family) (Davidson *et al.*, 2009). Proteins with OxO activity have been discovered in non-cereal crops including banana, sorghum and beet, but they appear to be proteins distinct from germin-OxOs (Lane, 2000; Davidson *et al.*, 2009) and are instead considered germin-like proteins. Germin-like proteins and OxOs share an average of 50% sequence identity, including the conserved germin motif, and have similar biochemical properties (Dunwell *et al.*, 2000; Davidson *et al.*, 2009). However, germin-like proteins differ from OxOs in numerous ways. In contrast to OxOs, germin-like proteins are encoded by a heterogeneous group of genes found in diverse plant species (Davidson *et al.*, 2009). Germin-like proteins are also more functionally diverse, as they may exhibit activity from various enzymes, including OxO, superoxide dismutase, PPO and ADP glucose pyrophosphatase (Barman and Banerjee, 2015).

All OxOs are glycoproteins consisting of six β-jellyroll monomers, each a barrel consisting of four pairs of anti-parallel beta sheets, locked in a homohexamer (Woo *et al.*, 1998, 2000). This structure is responsible for the high stability and broad resistance of all OxOs to proteolysis, dehydration, heat, SDS and pH extremes (Woo *et al.*, 2000; Dunwell *et al.*, 2008). Aside from the conserved jellyroll β-barrel structure of all OxOs, specific OxO enzymes display a range of physical and chemical characteristics. For example, analysis of four purified OxOs from rice (*Oryza sativa* L.) leaves showed that they differed in molecular mass, optimum pH, stability, and responses to inhibitors and activators (Li *et al.*, 2015). A highly purified and crystallized germin-OxO from barley showed both OxO and extracellular superoxide dismutase activity, although superoxide dismutase activity is typically not detected in the germin-OxOs (Woo *et al.*, 2000).

OxOs are a group of water-soluble enzymes that catalyse the two-electron oxidative decarboxylation of endogenous oxalate to hydrogen peroxide and carbon dioxide. Insoluble crystallized calcium oxalate present in plant vacuoles and cell walls is an additional OxO substrate, whose oxidation also yields free Ca^{2+} (Lane, 1994; Dunwell *et al.*, 2008). OxO-catalysed reactions rely only on the presence of manganese (II) and unlike other apoplastic oxidase enzymes, such as the amine oxidases, OxO is independent of other external cofactors (Requena and Bornemann, 1999; Woo *et al.*, 2000). OxO preproteins usually contain apoplastic secretory signal peptides at the N-terminal that direct the protein from the site of synthesis in the cytosol to the endoplasmic reticulum membrane for secretion, consistent with their role in cell wall function and pathogen defence (Zimmermann *et al.*, 2006; Imai and Nakai, 2010; Fuerst *et al.*, 2014).

OxOs are a functionally diverse enzyme group, involved in calcium regulation, oxalate metabolism, cell wall strengthening,

https://doi.org/10.1017/S0960258518000181 Published online by Cambridge University Press

stress response, and pathogen defence (Dunwell *et al.*, 2008; Davidson *et al.*, 2009). Moreover, OxO may in fact function by several mechanisms. Cell wall strengthening may result from peroxidative cross-linking involving the generated hydrogen peroxide, and also via lignification and papillae formation (Davidson *et al.*, 2009; Kanauchi *et al.*, 2009). Fortified cell walls may impede fungal hyphal penetration and be more resistant to degradation by fungal enzymes (Davidson *et al.*, 2009). Hydrogen peroxide is also directly toxic to pathogens and plays a role in plant immune signalling cascades (Alvarez *et al.*, 1998). Fungal-derived oxalic acid may be degraded by plant OxOs (Kanauchi *et al.*, 2009). The defence role of oxalate oxidase is illustrated in the numerous studies in which the barley or wheat oxalate oxidase gene transformed into numerous crops such as soybean, oilseed rape, sunflower and peanut confers resistance to the fungal pathogen *Sclerotinia* spp. (Donaldson *et al.*, 2001; Hu *et al.*, 2003; Livingstone *et al.*, 2005; Dong *et al.*, 2008). In germinating barley seedlings, OxO and POD genes were induced in response to the seed-borne fungus *Pyrenophora graminea* (Haegi *et al.*, 2008). However, there are few studies documenting OxO induction in non-germinating seeds. Indeed, Fuerst *et al.* (2018) demonstrated inhibition of OxO activity in dormant wild oat seeds in response to a pathogen.

OxOs are generally constitutively expressed in multiple types of plant tissue (Davidson *et al.*, 2009), but OxO activity also displays temporal and spatial variability in cereal grains (Lane, 2000). For example, four rice OxO genes with >90% amino acid identity exhibit widely variable expression patterns: *OsOXO1* is expressed in the panicles and during flowering and pollination, *OsOXO3* is expressed in the roots and seeds, and *OsOXO4* is expressed in healthy roots, shoots, leaves and seeds as well as during drought, cold stress and $CuSO_4$ stress (Carrillo *et al.*, 2009). OxOs are extracted from soluble and cell wall plant protein fractions, suggesting that they are secreted into the apoplast (Davidson *et al.*, 2009). They concentrate in epidermal tissues of mature grains and developing embryos of cereals, and are also detected in mesophyll tissues (Lane, 2000; Wu *et al.*, 2000). OxO was detected in the aleurone and embryo of ungerminated malting barley (Kanauchi *et al.*, 2009). In contrast, OxO activity was detected in the pericarp and the intermediate fractions (testa and nucellar tissue) of wheat bran, but was not detected in the aleurone cells (Jerkovic *et al.*, 2010).

Chitinase

Chitinases (CHIs) are glycosyl hydrolase enzymes that catalyse the hydrolytic cleavage of β-1,4-glycoside bonds within chitin, a linear homopolymer of β-1,4-linked *N*-acetyl-D-glucosamine that is abundant in fungal cell walls, as well as in algae, bacteria and invertebrate exoskeletons (Grover, 2012). CHIs are ubiquitous in nature as they are produced by microbes, insects, plants and animals. CHI nomenclature and classification have changed over the decades and consistency is not apparent in the literature (Patil *et al.*, 2000; Dahiya *et al.*, 2006; Duo-Chuan, 2006). *N*-acetylglucosaminidases, which have different cleavage patterns from CHI, are sometimes referred to as chitinolytic enzymes, although they are considered CHIs in this review (Dahiya *et al.*, 2006; Seidl, 2008).

According to similarities in amino acid sequences of the catalytic domain, CHIs have been classified predominantly as members of families 18 and 19 within the glycosyl hydrolase (GH) superfamily, and the *N*-acetylglucosaminidases are found in GH20 (Seidl, 2008). Recent, yet very limited, research indicates that CHIs are also present in GH families 23 (Arimori *et al.*, 2013) and 48 (Fujita *et al.*, 2006). GH18 and GH19 are distinguished by their amino acid sequence, three-dimensional structure, signal peptide, isoelectric pH, enzyme localization and catalytic mechanism, suggesting that they have unique evolutionary origins (Duo-Chuan, 2006; Karlsson and Stenlid, 2008; Hamid *et al.*, 2013). GH18 CHIs have ancient evolutionary origins and are widely present in archaea, bacteria, fungi, viruses, plants and mammals (Funkhouser and Aronson, 2007), whereas GH19 CHIs comprise almost exclusively plant CHIs, as well as limited bacterial CHIs (Adrangi and Faramarzi, 2013). CHIs were traditionally categorized into as many as eleven classes (Gomez *et al.*, 2002), but modern molecular genetic techniques have resulted in redistribution of CHIs into seven distinct classes (Kasprzewska, 2003; Duo-Chuan, 2006; Hamid *et al.*, 2013). According to the CAZy classification [Carbohydrate Active Enzymes database (http://www.cazy.org)], GH18 contains classes III and V, and GH19 contains classes I, II, IV, VI and VII (Adrangi and Faramarzi, 2013; Lombard *et al.*, 2013). CHIs within each family are further divided into the major categories of endo- and exochitinases, depending on a cleavage pattern of either randomly within the polymer or from a single terminus (Horn *et al.*, 2006). Plant CHIs are most frequently endochitinases (Hamid *et al.*, 2013).

Given the huge diversity of CHI enzymes, the specific molecular structure and size, organismal location, substrate specificity and catalytic mechanism vary widely (Kasprzewska, 2003). CHIs found in seeds typically range in size from ∼20 to ∼40 kDa (Yeboah *et al.*, 1998; Santos *et al.*, 2004; Chang *et al.*, 2014; Raviv *et al.*, 2017a, b). CHIs contain a peptide signal sequence at the N-terminal that directs them into the lumen of the endoplasmic reticulum (Chrispeels, 1991).

Plant CHIs, and especially those containing a carbohydrate-binding module, function primarily in pathogen defence, notably against pathogenic fungi (Jashni *et al.*, 2015b). Induction of CHI activity in plants by several microbial pathogens has been reported (Metraux and Boller, 1986; Schlumbaum *et al.*, 1986; Zhu *et al.*, 1994; Robert *et al.*, 2002). CHIs are pathogenesis-related (PR) proteins, present in four (PR-3, -4, -8 and -11) of the 17 PR families (Ebrahim *et al.*, 2011; Sultan *et al.*, 2016). As such, they are produced by plants in response to pathogen attack and participate in systemic acquired resistance, a defence system that enables plants to respond to diverse pathogens (Adrangi and Faramarzi, 2013). PR-3 CHIs, which are specific fungal growth inhibitors, concentrate in vacuoles and are abundant in the intermediate bran layers of wheat grains where they may prevent fungal hyphae from attacking the living aleurone layer (Jerkovic *et al.*, 2010). A CHI isolated from seeds of the perennial legume *Adenanthera pavonina* was likewise localized to vacuoles within cotyledon cells (Santos *et al.*, 2004). Evidence shows that soluble CHI is secreted into the environment of both germinating and non-germinated seeds, possibly to aid in seed defence (Santos *et al.*, 2004; Jerkovic *et al.*, 2010).

CHI has long been suggested as a seed defence enzyme (Leah *et al.*, 1991; Huynh *et al.*, 1992; Gomes *et al.*, 1996). A 26 kDa CHI purified from mature barley seeds inhibited fungal growth alone and when combined with other seed proteins. For example, alone it inhibited growth of *Trichoderma reesei* 50%, but when paired with ribosome-inactivating protein, *T. reesei* was more than 95% inhibited (Leah *et al.*, 1991). *Fusarium sporotrichioides*, a barley seed rot pathogen, *Rhizoctonia solani*, and *Botrytis cinerea* were similarly inhibited by this barley seed CHI acting synergistically with other seed enzymes (Leah *et al.*, 1991).

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CHIs are expressed constitutively in plant stems, seeds, flowers and tubers (Hamid *et al*., 2013). They are also induced locally in response to stress, including heavy metal exposure, osmotic stress and low temperature, with expression exhibiting spatial and temporal variability (Gomez *et al*., 2002; Hong and Hwang, 2006; Rodríguez-Serrano *et al*., 2009; Su *et al*., 2014). Seed CHIs are present in several seed tissues, such as endosperm, aleurone, embryo or husk (Gomez *et al*., 2002). Proteomic analysis of wheat bran found CHI activity expressed in the intermediate bran layer, which includes the testa and nucellar tissue, and in the aleurone layer (Jerkovic *et al*., 2010). CHI expression in mature barley seeds was detected only in aleurone cells and not in the endosperm (Leah *et al*., 1991). Baek *et al*. (2001) discovered that four distinct CHIs extracted from rice seed were differentially located among the polished rice (endosperm), rice bran and rice hull fractions, with the greatest activity present in the hulls. Activity from a 22 kDa CHI was detected in the caryopses, glumes, lemmas and paleas of wild emmer wheat, with the strongest activity present in the lemma fraction (Raviv *et al*., 2017b). An additional 40 kDa CHI was also detected in the lemmas, illustrating the diversity of CHI found in seeds and their tissue specificity. CHI activity has also been detected in non-living seed coats of *Sinapis alba*, *Anastatica hierochuntica* and 37-year-old radish (Raviv *et al*., 2017a). Expression of a CHI transcript was strongly evident in developing soybean seeds, but virtually no expression of the transcript was detectable in the soybean leaves or stems, highlighting the tissue specificity of CHI enzymes. Two kidney bean (*Phaseolus vulgaris* L.) cultivars exhibited differential CHI distribution between the cotyledon, axis and seed coat, with the greatest activity present in the seed coat, although a significant difference was seen between cultivars (Ramos *et al*., 1998). For example, the seed coat and cotyledon of kidney bean cv. Maisugata had 597 and 27 units mg^{-1} CHI, respectively, whereas the levels in cv. Surattowonder were 1238 and 25 units mg^{-1}, respectively. These studies illustrate the diversity of CHIs present in seeds and the wide inter- and intraspecific variability in localization patterns between different plant species and cultivars. Of two CHIs present in rye (*Secale cereal* L.) seed, RSC-a localized only to aleurone cells, while RSC-c was predominantly located in the starchy endosperm; neither was present in the testa (Taira *et al*., 2001). Both rye CHIs significantly inhibited hyphal growth of soil-isolated *Trichoderma* sp. *in vitro* within 24 h, suggesting that seeds contain multiple layers of anti-fungal CHI defences.

Temporal variability of CHI expression may serve to protect developing seeds when they are most vulnerable, as seen in the increasing expression of a CHI transcript in developing soybean seeds from 13 to 38 DPA, followed by a drastic decline in expression by 48 DPA (Yeboah *et al*., 1998). A CHI enzyme isolated from dehulled cowpea (*Vigna unguiculata* L. Walp) seeds was shown to inhibit growth of the phytopathogenic fungi *Colletotrichum lindemuthianum in vitro* (Gomes *et al*., 1996), yet no CHI activity was detected in the exudates of germinating cowpea seeds, suggesting possible regulation of CHI expression according to developmental stage (Rose *et al*., 2006). A CHI isolated from maize seeds strongly inhibited mycelial growth of the plant pathogenic fungi *Fusarium oxysporum* and *Alternaria solani*, and the non-pathogenic *Trichoderma reesei* (Huynh *et al*., 1992). However, this CHI could not inhibit the pathogens *Sclerotinia sclerotiorum* or *Gaeumannomyces graminis*, illustrating the pathogen specificity of CHI.

Plant CHIs also fulfil some roles in plant growth and development, abiotic stress response, and beneficial microbe associations that may be indirectly associated with defence (Gomez *et al*., 2002; Grover, 2012). For example, enhancing relationships with beneficial microbes may enhance the protective capacity of the microbial consortium at the seed surface. CHIs perform these functions via hydrolysis of chitin-containing compounds including arabinogalactan and glycoproteins in plant cell walls, peptidoglycan in bacteria, and lipochitooligosaccharides, which are Nod factors produced by rhizobia (van Hengel *et al*., 2001, 2002; Dyachok *et al*., 2002; Grover, 2012). Plant CHIs also function in calcium storage (Masuda *et al*., 2015), which may increase CHI stability, or provide a calcium reservoir to utilize for seed protection mechanisms (Franceschi and Nakata, 2005).

Fungal pathogenic enzymes

Soil fungi employ various mechanisms in pathogenesis, including emission of volatile organic compounds (Fiers *et al*., 2013; Peñuelas *et al*., 2014) and production of diffusible compounds (Christensen, 1996), but direct hyphal penetration of plant tissues via cell wall degrading enzymes appears to be the most commonly used method of initiating pathogen attack. Examination of 103 proteomes from fungi representative of four fungal phyla concluded that fungal nutritional modes and infection mechanisms directly correlated with their carbohydrate activity enzymes (CAZymes) (Zhao *et al*., 2013). Plant pathogenic fungi were found to contain the largest number of specific CAZymes and pathogens of monocots tended to have fewer CAZymes than those that attack dicots. For example, the necrotrophic fungus of monocots and dicots, *Fusarium oxysporum*, contained the most CAZymes at ~875. Moreover, gene expression analysis of *Fusarium graminearum* determined that most cell wall degrading enzymes were upregulated during plant infection.

Scanning electron microscopy (SEM) allows visualization of fungal hyphae during seed infection. SEM of the seeds of the parasitic plant, broomrape (*Orobanche*), infected with the fungus *Aspergillus alliaceus* showed that within 24 h of inoculation, fungal hyphae covered the outer seed surface. Mycelia directly penetrated the seed testa without appressoria formation, and degraded the hilum, embryo and endosperm (Aybeke *et al*., 2014). The fungal hyphae of *Penicillium chrysogenum*, *Phoma* sp. and *Trichoderma koningii* penetrated the funiculus of dormant *Opuntia streptacantha* seeds, resulting in greater germination than in non-infected seeds (Delgado-Sánchez *et al*., 2011). Hyphae of *Fusarium nygamai* penetrate the testa of non-germinated *Striga hermonthica* seeds, a parasitic weed of maize, between adjoining cell walls and ultimately degrade the seed embryo and endosperm (Sauerborn *et al*., 1996).

Fusarium spp. also physically penetrate cereal caryopses via cell wall degrading enzymes and the genus does not form specialized penetration structures such as appressoria or haustoria (Kikot *et al*., 2009). After incubation on *Fusarium culmorum* plates for 1 week, histological investigations of non-germinated immature barley caryopses showed that fungal mycelia had infested the outer grain layers and the pericarp only minimally, but after 2 weeks, hyphae had penetrated and invaded the pericarp, testa and aleurone layers, and had completely degraded the cell walls of the endosperm (Skadhauge *et al*., 1997). In mature spring wheat kernels infected with *F. culmorum*, SEM showed that fungal hyphae enveloped the outer surface of caryopses, and was also present in all internal tissues (Jackowiak *et al*., 2005). The highest concentrations of hyphae were in the testa, with much less present in the endosperm, illustrating how the

https://doi.org/10.1017/S0960258518000181 Published online by Cambridge University Press

high concentrations of seed defence enzymes in the outer seed layers protected cereal endosperm from infection. Fungal infection resulted in degradation of cell walls, starch granules and the protein matrix. Similar results have been reported from the infection of barley, winter wheat, triticale and rye infected with various *Fusarium* sp. (Jackowiak *et al*., 2005), highlighting the capacity of fungi to produce hydrolytic enzymes to effectively attack cereal caryopses.

Soil fungi produce hundreds, if not thousands, of unique enzymes for functions including morphogenesis, growth and development, nutrient acquisition, stress defence, and plant–pathogen association (Rao *et al*., 1998; Baldrian, 2006; Hofrichter *et al*., 2010). Herein I explore the classification, structure, function and mechanism of three prevalent fungal enzyme families implicated in seed decay: chitinase, protease and xylanase (Table 1).

Chitinase

Fungal CHIs have not been classified as thoroughly as plant CHIs, but analysis of 25 fungal genomes shows they belong exclusively to GH18 (Seidl, 2008; Hamid *et al*., 2013) and are contained within classes III and V. Phylogenetic analyses resulted in the division of GH18 fungal CHIs into subgroups A, B and C (Seidl *et al*., 2005), based on differences in the structure of their substrate-binding site and their carbohydrate-binding modules. CHI class V (exochitinase) and class III (endochitinase) are contained within subgroups A and B, respectively. Subgroup C contains a newly discovered group of CHIs not previously identified in fungi, but predicted to be related to class V exochitinases (Seidl, 2008; Hartl *et al*., 2012). Carbohydrate-binding modules, found in CHI B and C subgroups and other glycosidases, allow enzymes to bind substrates tighter and as such, enhance enzyme efficiency (Eijsink *et al*., 2008; Hartl *et al*., 2012; Paës *et al*., 2012). Fungal CHIs are typically 30–60 kDa in size, but subgroup C variants range from 120 to 200 kDa (Hartl *et al*., 2012).

Fungal CHIs catalyse the same basic reaction as plant CHIs, namely the hydrolysis of chitin, a polysaccharide of β-1,4 linked *N*-acetylglucosamine units (Hartl *et al*., 2012). Unlike plants, fungal cell walls are composed of chitin; consequently, they utilize CHI not only for degradation of exogenous chitin, but also for endogenous roles including growth, development and morphogenesis (Hartl *et al*., 2012; Hamid *et al*., 2013). Fungal CHIs function in nutrient acquisition, defence, mycoparasitism and entomopathogenesis (Seidl, 2008; Hamid *et al*., 2013; Langner *et al*., 2015). All fungi contain CHI, but the number of different CHI genes varies widely, as it depends partially on the fungal growth form (Latgé, 2007; Hartl *et al*., 2012) and is correlated to the chitin content of fungal cell walls. For example, cell walls of yeast-like species have only 0.5 to 5% chitin and contain few CHI genes, as seen in the model yeast *Schizosaccharomyces pombe* which contains a single CHI gene (Karlsson and Stenlid, 2008; Kubicek *et al*., 2011). In contrast, filamentous fungi cell walls consist of ± 20% chitin and contain an average of 15 unique CHI-encoding genes, such as the 27 in *Fusarium oxysporum* (Seidl, 2008). Mycoparasitic or entomopathogenic fungi tend to have exceedingly high numbers of CHI genes, needing this diverse arsenal to degrade their chitin-containing hosts (Gao *et al*., 2011; Kubicek *et al*., 2011). For example, the mycoparasitic fungi *Trichoderma virens* and *T. atroviride* contain the greatest numbers of chitinolytic enzymes of any fungi, with 36 and 29, respectively (Kubicek *et al*., 2011).

Specific knowledge about the genetic regulation and biochemical mechanisms of fungal CHIs, especially as they pertain to plant pathogenesis, remains sparse (Langner and Göhre, 2016), although recent fungal genome sequencing has improved our understanding (Seidl, 2008; Hartl *et al*., 2012). The high number of unique fungal CHIs reflects the diversity of roles they fulfil in fungi, but characterizing the function of individual CHI enzymes proves challenging because the CHI genes exhibit pronounced redundancy (Langner *et al*., 2015). The physiological function of individual CHIs influences how they are regulated. Housekeeping CHIs involved in cell wall maintenance are constitutively expressed, whereas those involved in pathogenesis or specific morphogenic processes are spatially and temporally regulated (Langner and Göhre, 2016). Studies that characterize specific fungal CHIs or analyse CHI gene expression and regulation do so in relation to mycoparasitism, entomopathogenesis, or transgenic plants expressing fungal CHI genes. Very little research exists on fungal CHIs in relation to plant pathogenesis, and virtually no research has been conducted on assessing fungal CHI as it pertains to seed decay. Preliminary studies suggest that when dormant wild oat or wheat caryopses are incubated on *Fusarium* isolate *F.a.*1, the fungal mycelia produce approximately 2.6 times greater activity levels of β-*N*-acetylglucosaminidase, compared with mycelia from the control *F.a.*1 plates that do not contain caryopses (Fuerst *et al*., 2018). While fungi do not theoretically require CHI to degrade chitin-free seed tissue, fungal CHIs may impart a competitive advantage to fungi by aiding their attack of beneficial fungi associated with seeds or other competing fungal pathogens in the seed microbiome (Dalling *et al*., 2011). Moreover, upregulation of specific fungal CHIs may enhance fungal cell wall plasticity, thereby hastening filamentous growth which represents the first phase of pathogenic development.

Protease

Proteases catalyse the hydrolytic cleavage of peptide bonds in proteins, yielding peptides and free amino acids. Fungi produce an extensive variety of proteases, also known as peptidases, proteolytic enzymes and peptide hydrolases. Given the enormous structural and functional diversity of proteases, they are classified at several levels and into numerous groups. Proteases are divided into nine families based on the functional group present at the catalytic domain and each is denoted by a capital letter: aspartic (A), cysteine (C), glutamic (G), metallo (M), asparagine (N), mixed (P), serine (S), threonine (T), or unknown (U) (Monod *et al*., 2002; Rawlings *et al*., 2016). Proteases are additionally classified by the hydrolytic cleavage site (endo- or exo-), their pH optima (acid, neutral or alkaline), and clans based on phylogenetic associations (Rao *et al*., 1998). Serine proteases constitute the most well-studied protease family, comprising over 33% of all identified proteases (Yike, 2011). Extracellular fungal proteases concentrate in the serine family (Chandrasekaran *et al*., 2016), which includes the common subtilisin and trypsin types (Kudryavtseva *et al*., 2013). Alkaline proteases contain either a serine or metallo- centre (Sharma *et al*., 2017). Subtilisin proteases are also sometimes referred to as alkaline proteases (Kumar and Takagi, 1999).

Optimum pH, temperature, ionic strength and substrate to achieve peak activity vary within and between classification groups (Sharma *et al*., 2017). For example, an alkaline thiol protease from *Botrytis cinerea* shows maximum activity at pH 10–11 and ~60°C (Abidi *et al*., 2007), whereas peak activity for an alkaline serine protease identified in *Fusarium culmorum* is at pH

8.3–9.6 and 50°C (Pekkarinen *et al.*, 2002). Moreover, Pekkarinen *et al.* (2002) assayed a serine protease using five different substrates and the activity levels ranged from 0.2 to 1360 nkat (mg protein)$^{-1}$. Research on the model fungus *Aspergillus nidulans* shows that extracellular fungal protease production is regulated by carbon, nitrogen, sulphur, pH, and in certain circumstances, exogenous proteins (Yike, 2011).

Proteases are important enzymes secreted by plant pathogenic fungi that function in signalling, nutrition, plant host tissue degradation, sporulation, morphogenesis, septum formation, and plant defence enzyme degradation (Abidi *et al.*, 2007; Yike, 2011). Fungal proteases appear to play significant roles at certain phases of plant infection, such as host cell adhesion and initial cell wall penetration and colonization (Olivieri *et al.*, 2004; Soberanes-Gutiérrez *et al.*, 2015; Chandrasekaran *et al.*, 2016). Fungal proteases function in plant pathogenesis by activating or inactivating plant proteins such as plant defence enzymes, influencing autolysis, and by increasing plant plasma membrane permeability (Chandrasekaran *et al.*, 2016). Fungal metalloproteases, notably Zn-metalloproteases, are known to function in plant pathogenicity and virulence by directly degrading the plant cell wall (Staats *et al.*, 2013). Recent studies indicate that the significance of proteases in plant pathogenesis is influenced by the specific plant–pathogen association (Dong *et al.*, 2014; Figueiredo *et al.*, 2014; Jashni *et al.*, 2015a). For example, *Fusarium graminearum* and *F. culmorum*, the fungal pathogens responsible for Fusarium head blight disease, produce alkaline serine proteases in the endosperm of infected wheat and barley grains, with the highest enzyme activity produced by the most virulent fungal strains, suggesting their role in pathogenicity (Nightingale *et al.*, 1999; Pekkarinen *et al.*, 2003).

Numerous examples exist of fungal pathogens that escape plant defence CHIs by secreting proteases to degrade or otherwise modify plant CHI enzymes, especially those containing a carbohydrate-binding module, thereby nullifying their ability to serve a defensive role (Jashni *et al.*, 2015b). This behaviour has been widely demonstrated by numerous *Fusarium* spp., including *Fusarium verticillioides*, *F. oxysporum*, *F. graminearum*, *F. proliferatum* and *F. subglutinans* that secrete Zn-metalloproteases capable of degrading three unique class IV carbohydrate-binding module-CHIs in maize (Naumann *et al.*, 2011; Slavokhotova *et al.*, 2014). *Fusarium* proteases with anti-CHI activity are also seen in *F. solani* f. sp. *phaseoli* that modifies CHI in bean to promote fungal colonization (Lange *et al.*, 1996); *F. oxysporum* f. sp. *lycopersici* that modifies a carbohydrate-binding module-CHI in tomato (Jashni *et al.*, 2015a); and a subtilisin serine protease from *F. solani* f. sp. *eumartii* that modifies CHI present in potato tubers (Olivieri *et al.*, 2002). Interestingly, cleavage efficiency and specificity for different CHI enzymes vary widely among different *Fusarium* proteases (Naumann *et al.*, 2011; Jashni *et al.*, 2015a). Moreover, different proteases cooperate to enhance enzyme degradation. For example, synergistic effects were evident when metallo and serine proteases acted together to degrade CHI in tomato (Jashni *et al.*, 2015a) and it is hypothesized that fungi simultaneously secrete exo- and endoproteases for synergistic functioning (Girard *et al.*, 2013). Additional fungi display protease activity against plant CHI which ultimately leads to increased fungal virulence, including *Bipolaris zeicola*, *Cochliobolus carbonum*, *Stenocarpella maydis*, *Botrytis cinerea* and *Verticillium dahliae* (Naumann *et al.*, 2009; Naumann and Wicklow, 2010; Jashni *et al.*, 2015a).

Seeds also produce inhibitors to protect against fungal proteases and these are abundant in cereal grains (Pekkarinen and Jones, 2003). For example, wheat and barley seeds produce multiple inhibitors in response to the subtilisin- and trypsin-like proteases produced by *Fusarium graminearum* and *F. culmorum* during Fusarium head blight infection (Pekkarinen *et al.*, 2003). Three inhibitors were likewise isolated from dormant buckwheat (*Fagopyrum esculentum*) seeds that inhibited trypsin-like proteases from *Fusarium oxysporum* and *Alternaria alternata*, common phytopathogenic fungi, and suppressed their mycelial growth *in vitro* (Dunaevskii *et al.*, 1995). High concentrations of protease inhibitors were identified in the surface proteome of mature barley seeds (Sultan *et al.*, 2016).

Xylanase

Xylanases are hydrolytic enzymes that degrade xylan, which is a general term to describe a variety of hemicellulosic polysaccharides of D-xylose linked by β-1,4-bridges which is abundant in cell walls of commelinid monocots (Collins *et al.*, 2005; Polizeli *et al.*, 2005; Hatsch *et al.*, 2006). Xylans can be further classified according to the substitutions present along the main xylose backbone. The predominant hemicellulose of endosperm in cereal grains is arabinoxylan (McCleary *et al.*, 2015). Endo-1,4-β-D-xylanase (endoxylanase) is one of multiple enzymes required for the complete degradation of xylan. It catalyses the hydrolytic cleavage of β-xylosidic bonds in xylose (Collins *et al.*, 2005). Xylanases are produced by various organisms, but predominantly by microorganisms, and fungal-derived xylanases show especially high activity (Polizeli *et al.*, 2005).

Xylanases are a diverse group of enzymes, exhibiting differences in catalytic domain, optimum pH and temperature, substrate specificity, and catalytic efficiency (Paës *et al.*, 2012). Fungal xylanases are integral to plant pathogenesis and most fungi produce numerous different xylanase enzymes (Paës *et al.*, 2012). Fungal xylanases have inter- and intraspecific variability, and are regulated both spatially and temporally. The large number of unique xylanases stems from factors including genetic redundancy (Wong *et al.*, 1988), a strategy that allows fungi to adapt to diverse plant substrates at different growth stages and in different environments. Each enzyme is probably fine-tuned to optimally degrade a specific substrate under certain environmental parameters (Paës *et al.*, 2012).

The majority of plant pathogenic fungi contain numerous genes encoding endoxylanases (Sella *et al.*, 2013). Fungal endoxylanases are present primarily in families 10 and 11 of the glycosyl hydrolase superfamily (Lombard *et al.*, 2013), but they are also distributed in GH families 5, 8, 16, 26, 30, 43 and 62, albeit to a far lower extent (Collins *et al.*, 2005; Paës *et al.*, 2012; Lombard *et al.*, 2013). They were traditionally divided into GH10 and GH11 according to high and low molecular weight, respectively (Sella *et al.*, 2013), but with increasing discovery and characterization of xylanases, this classification system gave way to one based on similarities of the amino acid sequence of the catalytic domain (Lombard *et al.*, 2013). Additional distinctions between GH10 and GH11 include substrate specificity and structure. GH10 xylanases have broad substrate specificity, are preferentially active on soluble substrates, can degrade linear chain xylans and those with multiple substitutions, and have a TIM-barrel fold at the active site (Beaugrand *et al.*, 2004). In contrast, GH11 have high substrate specificity, can degrade insoluble substrates, cannot degrade xylan backbones with a high degree of substitution, and have a highly conserved β-jelly roll structure at the active site (Pollet *et al.*, 2010; van den Brink and de Vries, 2011; Paës *et al.*, 2012;

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Sultan *et al.*, 2016). The narrow substrate specificity of GH11 xylanases may be supported by their carbohydrate-binding module, which is a distinct region in xylanases and other glycosidases (Paës *et al.*, 2012) that enhances substrate binding and hastens cell wall disruption (Pollet *et al.*, 2010). Carbohydrate-binding modules also significantly increase the thermostability of the xylanase enzyme (Paës *et al.*, 2012). In a comparative study of a GH10 and a GH11 xylanase, the GH11 xylanase was more efficient in hydrolysing wheat bran than GH10 and it had a 2-fold greater affinity for wheat bran than the GH10 xylanase (Beaugrand *et al.*, 2004). Interestingly, in a proteomic analysis of the barley grain surface, only GH11 fungal xylanases were detected (Sultan *et al.*, 2016).

While cereal grains produce endogenous xylanases, research suggests that over 90% of the xylanase activity detected on cereal grains is of microbial origin (Dornez *et al.*, 2006b). Several studies show that microbial xylanases concentrate in the outer bran layers of cereal grains rather than in the endosperm, as seen in a comprehensive survey of common wheat, durum wheat, spelt, einkorn, emmer, barley, rye and oat (Gys *et al.*, 2004; Dornez *et al.*, 2006a; Gebruers *et al.*, 2010). Moreover, cereal grains with hulls, such as oat, generally contain higher levels of fungal xylanases compared with hull-less varieties because the space between the hull and caryopsis provides a niche for microbial colonization (Noots *et al.*, 1999). For example, fungal mycelia covered an average of 59.2% of the lemma and 70.2% of the palea of barley grain (Warnock, 1971).

Proteomic analysis of the barley grain surface identified numerous fungal enzymes that function in plant cell wall degradation and are also required for virulence, including β-1,4-xylanase (Sultan *et al.*, 2016). Numerous xylanases specifically identified in the grain washing liquids came from the following fungi: (1) the soil-borne pathogen *V. dahlia* (contributed two different xylanases), (2) the highly virulent cereal grain pathogen *Cochliobolus sativus*, and (3) the broadly specific grass pathogen *Pyrenophora tritici-repentis* (Sultan *et al.*, 2016). Genomic analysis of *F. graminearum*, the causative agent of Fusarium head blight, indicates that it contains 10 xylanase-encoding genes, six of which are expressed during infection of hop and barley, and five during wheat infection (Güldener *et al.*, 2006; Hatsch *et al.*, 2006; Sella *et al.*, 2013).

Knowledge of the mechanisms underlying the interaction between fungal xylanases and plant cells remains sparse, yet it is known that plant cells have defences to fungal xylanases by means of protein inhibitors. For example, three classes of inhibitors (XIP, TAXI and TLXI) have been identified in several cereal grains such as barley, wheat and rye (Elliott *et al.*, 2003; Goesaert *et al.*, 2003; Dornez *et al.*, 2010) that differ in their structure and xylanase specificity (Paës *et al.*, 2012). XIP-I is concentrated in the xylan-rich nucellar tissue within the intermediate layer of wheat bran, illustrating its importance in protecting the inner seed fraction from fungal attack (Jerkovic *et al.*, 2010). The endosperm of cereal grains has also been identified as a region of concentrated xylanase inhibitors. For example, the flour produced from debranned wheat kernels showed no significant reduction in xylanase inhibitor proteins compared with wholemeal flour (Gys *et al.*, 2004), and strong inhibition of the xylanases produced by *Aspergillus niger* and *Bacillus subtilis* was seen in the flour fraction of various dormant European wheat varieties (Gebruers *et al.*, 2002).

Secreted during the early infection stages of cereal crops including wheat, barley and rye, endoxylanases degrade cell walls in seeds and leaves; however, the exact role they play in virulence or pathogenicity is not well known. A xylanase from *Botrytis cinerea* is required for virulence in grape berries, but its contribution to infection resides in its ability to induce tissue necrosis, and not in its catalytic hydrolysing activity (Brito *et al.*, 2006; Noda *et al.*, 2010). Mutated xylanases from *Trichoderma reesei* with enzymatic activity decreased 100- or 1000-fold elicited a defence response of hypersensitive necrosis in tomato and tobacco leaves equivalent to the wild-type xylanase, indicating that the enzymatic function of xylanases is not necessarily required for fungal virulence (Enkerli *et al.*, 1999). Numerous *Fusarium graminearum* isolates with a mutated xylanase gene displayed only 40% xylanase activity 4 and 7 DAI compared with the wild type (Sella *et al.*, 2013). However, 21 DAI of wheat spikes with these mutants, there was no significant reduction in necrosis symptoms compared with the wild type, indicating that reduction in this level of xylanase activity was not required for virulence. *Fusarium graminearum* xylanase in the wheat lemma tissues, which are high in arabinoxylans, resulted in accumulated hydrogen peroxide deposits under the epidermal tissues and localized tissue necrosis, suggesting that this fungal xylanase not only degraded plant cell walls, but also triggered acute cell death (Sella *et al.*, 2013).

The activities of microbial xylanases are strongly influenced by environmental conditions and to a lesser extent, by plant genotype; in contrast, environment minimally influences the activity of xylanase inhibitors in seeds (Dornez *et al.*, 2008; Gebruers *et al.*, 2010; Sultan *et al.*, 2016). In an assessment of xylanase and xylanase inhibitor activity among more than 200 cereal varieties that included winter wheat, spring wheat, durum, einkorn, emmer, spelt, barley, rye and oat, variability in xylanase activity expressed in the grain was attributed to environmental conditions (50%), genotype (11–14%), and environment–genotype interactions (34–39%) (Gebruers *et al.*, 2010). Similarly, in a comparison of surface proteomes of barley cultivars grown in different years, Sultan *et al.* (2016) concluded that microbial xylanases associated with barley grains are strongly affected by environmental conditions, due to the significant influence of environment on the grain microbial consortia; meanwhile, expression of xylanase inhibitors was more stable and not strongly affected by environment.

Research challenges and future directions

Research interest into utilizing soil microbes to deplete the soil weed seedbank has existed for decades (Charudattan, 1991; Kremer, 1993; Wagner and Mitschunas, 2008), and while technological advances have enhanced the scientific understanding of the interaction between soil fungi and weed seeds in the seedbank, much remains to be learned to enable utilization of soil microbes as part of an IWM strategy.

Understanding biochemical interactions occurring in the soil weed seedbank is challenging not only because every seedbank is unique, but also because it is tedious and time-consuming to analyse the influence of soil fungi on seeds buried in soil. Moreover, it is challenging to parse the complex weed seed–soil fungi seedbank interaction into the contributing factors of environmental variables (Pake and Venable, 1996; Benech-Arnold *et al.*, 2000), soil microbial communities (Kremer, 1993; Wagner and Mitschunas, 2008) and agronomic practices (Clements *et al.*, 1996; Gallandt *et al.*, 2004).

For decades, enzyme research methodology has garnered significant scientific investigation and debate (Burns, 1982; Schinner and von Mersi, 1990; Deng *et al.*, 2013). A fundamental

https://doi.org/10.1017/S0960258518000181 Published online by Cambridge University Press

limitation in enzyme research progress is the lack of universally accepted methods, which makes it challenging to compare study results. There are two general approaches to enzyme analysis, the classical method and the *in situ* method. Classical enzymology methods measure potential activity of an enzyme, which could be a poor indicator of the actual enzyme activity in the environment (Wallenstein and Weintraub, 2008). Classical methods measure activity in a homogenous soil slurry at the optimal temperature and pH for that particular enzyme (Steinweg *et al*., 2012). However, this fabricated scenario does not replicate natural soil conditions. The natural soil temperature and pH, as well as heterogeneity of soil physical properties including soil texture, will influence enzyme efficiency, diffusion rates and substrate binding; therefore, potential enzyme activity measured under controlled laboratory conditions may significantly differ from actualized enzyme activity (Steinweg *et al*., 2012). However, as assays for the vast majority of enzymes have been developed using the classical approach, it remains the most widely accepted. The *in situ* approach, which attempts to mimic realistic soil conditions in the laboratory, likewise has limitations as exact field conditions cannot be replicated in the laboratory. For example, the simple process of collecting and storing soil for analysis disturbs enzyme balance (Burns *et al*., 2013).

Experimental design, assay protocols and the reagents used can significantly impact results of enzyme studies. Studies show that the media on which fungi are cultured directly impact the measured optimum temperature and pH, substrate specificity and inhibitor sensitivity of protease enzymes (Yike, 2011). A proteomic analysis using 2DGE and mass spectrometry was conducted on the pathogenic fungi *Fusarium oxysporum* after culturing the fungus for 96 h in media with pH ranging from 5 to 8 (da Rosa-Garzon *et al*., 2017). Enzymes involved in pathogenesis exhibited differential profiles at each pH; proteases were more active at neutral–alkaline pH, whereas xylanases favoured neutral–acid pH. These studies illustrate how the classical enzyme assay approach may yield drastically different results than what actually occurs in nature.

An additional problematic aspect of enzyme assays is the variable results achieved from different standard substrates and across different soils and pH values. Bach *et al*. (2013) measured phenol oxidase and POD activity in three different soils across a pH gradient (3 to 10) and using their three commonly used substrates: pyrogallol (PYGL: 1,2,3-trihydroxybenzene), L-DOPA (L-3,4-dihydroxyphenylalanine) and ABTS (2,20-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid). Each substrate produced a unique trend according to soil type and pH. For example, PYGL POD activity was significantly negatively correlated to pH in Alaskan and Costa Rican soils, but significantly positively correlated to pH in Californian soils. In contrast, ABTS POD activity in Alaskan soils showed no significant correlation to pH, and ABTS activity was nearly undetectable in Californian and Costa Rican soils. This study underscores the importance of determining the best assay conditions for acquiring reliable data prior to conducting assays (German *et al*., 2011). Enzyme protocols are rooted in bench-scale colorimetric assays that are time and resource intensive. High-throughput microplate-based fluorometric methods have improved on time efficiency and reagent usage, but lack of procedural consistency among researchers continues to hinder the direct comparison of results and the ability to draw firm conclusions (Deng *et al*., 2017).

Technological advancements have enhanced our ability to analyse enzyme activity via different approaches, such as molecular-based methods to measure enzyme gene expression (Damon *et al*., 2012) and proteomics to characterize functional interactions of soil microbes and seeds in the soil (Sultan *et al*., 2016). Genomic studies that assess microbial community composition and functional gene concentrations enable us to predict the enzymatic capacity and ecosystem services of a soil (Fierer *et al*., 2012), including its potential to elicit decay of weed seeds. More fine-tuned analysis of *in situ* enzyme activity can be ascertained via reverse transcription PCR, though soil properties can seriously challenge the effective implementation of this method (Saleh-Lakha *et al*., 2011). This promising technique is currently limited by the minimal number of functional genes which have been sequenced (Wallenstein and Weintraub, 2008).

A discussion of the enzymatic interactions between seeds and fungi in the soil seedbank must take into consideration the seed microbiome. The microbiome is defined as the collective communities of microorganisms associated with an ecosystem (Lederberg and McCray, 2001). Crop rhizosphere microbiomes are known to provide critical ecosystem services such as enhancing disease suppressive soils, assisting plant nutrient uptake, and promoting plant growth (Berendsen *et al*., 2012). However, our understanding of weed rhizosphere microbiomes is in its infancy (Samad *et al*., 2017) and weed seed microbiome research nearly non-existent (Müller-Stöver *et al*., 2016). Increased metagenomics, transcriptomic, and proteomic research focused on the microbiome of weed seeds in the soil will foster new opportunities for weed management.

Research into plant defence enzymes has predominantly focused on aboveground plant tissue or plant roots, and when seed defence enzymes are studied, it is generally in relation to germination. Relatively few studies focus on defence enzymes secreted by dormant or quiescent seeds, and yet their activity in weed seeds greatly enhances their ability to persist in the seedbank and hinder long-term weed management efforts. Additional aspects of biochemical seed defence enzymes whose investigation would promote ecological weed management strategies include: the spatial and temporal expression of enzyme activity during fungal attack; differential response of monocot, dicot, annual and perennial weed species to different common soil pathogens; the specific influence of diverse soil properties on seed enzyme activity; and the synergistic and/or antagonistic interactions between different seed defence enzymes and between different fungal enzymes.

Acknowledgements. I am grateful for the feedback, suggestions, advice and support of Dr E. Patrick Fuerst and Dr Patricia Okubara during the creation of this manuscript.

Financial support. This work was supported by the United States Department of Agriculture, National Institute of Food and Agriculture, Agriculture and Food Research Initiative Foundational Program award number 2014-67013-21575.

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