THE ECOLOGICAL ROLE OF THE ROOT ENZYME POLYPHENOL OXIDASE IN THE INVASIVE PLANT GENUS BROMUS

by

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ABSTRACT OF THE DISSERTATION

The ecological role of the root enzyme polyphenol oxidase in the invasive plant genus *Bromus*

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Biological invasions adversely affect and disrupt natural ecosystems at great economic costs. The vast body of theory and research focus on which factors advance these invasions and is geared toward understanding, prevention, and management of non-native species. Roots of grasses in the genus *Bromus* constitutively possess high levels of the enzyme polyphenol oxidase (PPO), a catalyst for the oxidation of phenolics into visible melanin-like compounds. Phenolic substrates for PPO are plant-produced secondary metabolites with phytotoxic allelopathic properties. Through the conversion of these harmful phenolics by PPO, we hypothesized PPO may be used as a defense mechanism against phenolic-allelopathic plants and thereby contribute to the competitive success of *Bromus* species, many of which are non-native invaders.

To test these hypotheses, we first assayed a wide range of Poaceae (grass) species for root PPO activity with a focus on bromes. Results showed significantly higher PPO levels in invasives than non-invasives, suggesting the ability to produce high root PPO concentrations is a trait contributing to invasion potential of non-native species, an important corollary that may be a useful tool for identifying future invasives. Second, through phylogenetic reconstructions, phenetic PPO was phylogenetically tractable and was only present in two taxonomically distinct genera, hinting at a high-PPO ancestral condition, later lost by some genera. Third, we examined effects of allelopathic competitor species on PPO and non-PPO-producing grasses in direct competition and exposed to leachate and litter; experiments supported our hypothesis as (a) PPO-producer *Bromus* tolerated allelopathic phenolic *Centaurea*, (b) but non-PPO *Festuca* was suppressed, and (c) non-phenolic allelopathic *Artemisia* suppressed both PPO-*Bromus* and non-PPO-*Festuca*. Fourth, field surveys showed allelopathic plants further distances from *Bromus* than non-allelopathic plants. Finally, we exposed a range of grass species of variable PPO activity to the phenolic-allelochemical caffeic acid (CA). PPO was constitutively expressed, but the utility was weakly observed, possibly due to sub-toxic doses. Overall, we illustrate PPO as a novel defense against phenolic-allelochemicals and as a trait correlated to invasiveness, and highlight ongoing taxonomic classifications that may shed light on evolutionary understanding of selection benefits of PPO and grass evolution, which are agriculturally, economically, and environmentally important.

For Mum, Dad, Jennifer, and Nicholas

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TABLE OF CONTENTS

TABLE OF CONTENTS
(Continued)

LIST OF TABLES

LIST OF FIGURES

1.1. Polyphenol oxidase (PPO)

Introduction to the chemistry of PPO

Enzymes are protein catalysts which propagate biochemical processes without themselves being consumed or destroyed. Polyphenol oxidase (PPO) is a common, naturally-occurring oxidizing enzyme that is stable for long periods, first characterized in 1896 (Bertrand 1896, Mayer 2006). The PPO cDNA sequence, located on the p arm of chromosome 2, is a GC-rich sequence of 2,055 bp (He et al. 2007, Massa et al. 2007). There are different PPO genes and structures (Tran et al. 2012). The amino acid sequence varies by species and even within species, but the active site is always conserved (Mayer 2006, Nokthai et al. 2010). As a result of this variation, molecular weight of PPO isozymes is variable; purified plant-produced PPO varies in from 35, 40, to 67 kDa (Mayer 2006).

Accordingly, PPO enzyme nomenclature varies (Valero et al. 1991, Mayer 2006). PPO is in the oxidoreductase class of enzymes (first E.C. integer of 1). The International Union of Biochemistry classifies PPO as number E.C. 1.21.3.6. Polyphenol oxidase searches reveal the most common to be E.C. 1.14.18.1 where E.C. 1 represents oxidoreductases, E.C. 1.14 as acting on paired donors with incorporation or reduction of molecular oxygen, and E.C. 1.14.18 as with another compound as one donor, and incorporation of one atom of oxygen. The accepted name of polyphenol oxidase is monophenol monoxygenase (IUPAC, Queen Mary, University of London, access date 1 June 2012). Other names for PPO include:

N-acetyl-6-hydroxytryptophan oxidase
o-diphenol oxidase
o-diphenol oxidoreductase
o-diphenol:O2 oxidoreductase
o-diphenolase
catecholase or catechol oxidase
chlorogenic acid oxidase
chlorogenic oxidase

cresolase
diphenol oxidase
dopa oxidase
monophenol dihydroxyphenylalanine:oxygen oxidoreductase (E.C. 1.10.3.1, E.C. 1.10.3.2)
monophenol monooxidase
monophenol oxidase
monophenolase
phenol oxidase
phenolase
polyaromatic oxidase
polyphenol oxidase
polyphenolase
pyrocatechol oxidase
tyrosinase (E.C. 1.14.18.1; MW 128kDa)
tyrosine-dopa oxidase

Ubiquity of PPO genes in nature

PPO has been the subject of several review papers and hundreds of papers regarding both fungus and plants, as it is a ubiquitous enzyme, common in the environment and edibles, such as apples, bananas, and potatoes (Mayer 2006). PPO genes of various copy numbers have been found in animals, fungi, and green plants (Tran et al. 2012). PPO is present in many familiar species, including *Prunus armeniaca* (apricot), *Ipomoea batatas* (sweet potato), *Saccharum spp.* (sugar cane), *Nicotiana tobacum* (tobacco), and poplar (Bucheli et al. 1996, Shi 2002, Aniszewski et al. 2008, Tran and Constabel 2011). Bryophytes contain a PPO gene named PP_ppo1, of 2402 bp length with a 94 bp intron (Richter et al. 2005). *Malus spp.* (apples), have a multiplicity of genes; *Musa spp.* (bananas) have four PPO genes; *populus spp.* (aspen, cottonwood, poplar) have nine PPO genes; *Trifolium spp.* (clover) have at least three PPO genes; *Vitis spp.* (grape vine) have one single PPO gene; hexaploid *Triticum spp.* (wheat) kernels have six PPO genes; *Solanum lycopersicum* (tomatoes) have seven PPO genes; and *Solanum tuberosum* (potatoes) have six PPO genes (Mayer 2006, Tran and Constabel 2011). Poplars have nine PPO genes that are 36-98% identical at the amino acid level, arisen through diversions and duplications (Tran and Constabel 2011).

Browning reactions from secondary polymerization which yields coloration are the focal point of much of the research regarding PPO (melanogenesis) (Mayer 2006, Massa et al. 2007, Aniszewski et al. 2008). Studies have shown that increasing

expression of PPO yields an increase in browning (Mayer 2006). Tyrosinase, an example PPO, is common in produce (Rawel et al. 2001). Tyrosinase activity results in a browning process, first of "yellowish-pink coloration, then reddish, then brown, and finally black. This reddish black oxidation or condensation product is called melanin and is closely related to the natural animal pigments in dark hair, etc., and also in the so called melanotic tumors" (Clark 1911). Oxidation, if continued, can also form cross-linked phenol-protein complexes resistant to proteolytic activity (Rawel et al. 2001).

Substrates for and mechanism of PPO

The mechanism PPO catalyzes is two steps: first, there is the oxidation of phenolic compounds to o-quinones which second, polymerize spontaneously into high molecular weight melanin-like pigments (Valero et al. 1991, Apel and Hirt 2004, Massa et al. 2007, Aniszewski et al. 2008). PPO has remarkably high specificity (Keilin & Mann 1938), and requires two substrates: oxygen and a mono-, di-, or polyphenolic (Mayer 2006). Substrates found so far have all been ortho-diphenolics (Queiroz et al. 2008, Kafkewitz 2012). In general, substrates for PPO are substituted aromatics, C6-C1 aromatic rings with hydroxylations and methoxylations (Leicach et al. 2009). Examples of phenolic substrates include catechin, chlorogenic acid, and 4-methylcatechol (Table 1; (Queiroz et al. 2008)).

Table 1. Not all phenolic compounds are substrates for PPO (Kafkewitz, personal comm.). Note: this is not a comprehensive list.

Substrates for PPO:	***Not PPO substrates:***
Catechin	2-hydroxybenzoic acid
L-DOPA (L-3,4-dihydroxyphenylalanine)	3-hydroxybenzoic acid
Caffeic acid	4-hydroxybenzoic acid
Catechol (1,2-dihydroxybenzene)	2, 3, or 4-courmaric acids
Chlorogenic acid	2, 3, or 4- hydroxycinnamic acids
4-methylcatechol (Queiroz *et al.* 2008)	Vanillin
	Pyrogallol
	L-tyrosine
	Protocatechuic acid

Clarification

PPOs can be catecholases (plant-produced, versus their animal-produced counterpart, tyrosinase (E.C. 1.14.18.1), cresolases, or laccases (E.C. 1.10.3.2) (Aniszewski et al. 2008). These are all the same enzyme, with the same structure, all containing CuA and CuB at N and C termini, however, they have different activity per organism and species specificity (Aniszewski et al. 2008). Tyrosinases are melanin-forming enzymes present in animals and some higher plants including *Hordeum vulgare* (barley), *Beta vulgaris* (beets), *Papaver orientale* (Oriental poppy), *Solanum tuberosum* (potatoes), *Rhus spp.* (sumac), and *Triticum spp.* (wheat) (Clark 1911). Some bacteria have tyrosinase (Clark 1911). Tyrosinases are not as widespread as laccases (Clark 1911). Laccase, an oxidase like tyrosinase, cannot produce browning effects because during electrophilic aromatic substation, PPO is an oxidizer ortho-polyphenolics, versus laccase, an oxidizer of para-polyphenolics (Clark 1911).

PPO: problems in the food industry

PPO is considered to be a significant economic detriment in the food processing industry due to browning reactions which reduce food quality. Consumer acceptance is a major consideration of the food industry in addition to sustainability concerns and cost control. Accordingly, research focuses on enzyme inhibition and deactivation in order to prevent discolorations. Browning from PPO is a direct cause of decreased appetitiveness, appearance, nutritional quality and value of produce, but is not to be confused with rotting from bacterial or fungal contamination. These PPO-catalyzed discolorations result in moderate to severe loss of harvested fruits and vegetables of up to 50% or more

(Thipyapong and Steffens 1997, Queiroz et al. 2008). Notable products of great loss include discolorations in french fries from potatoes (*Solanum tuberosum*), and coconut water from coconuts.

Inhibitors of PPO have been studied with the aim of understanding deactivation of the enzyme, but inactivation processes can be costly in terms of both equipment required and operation. As with all enzymes, PPO requires specific environmental conditions, e.g., temperature and pH, in order to maintain their active states; this knowledge is used in the inactivation of PPO to minimize detrimental browning. PPO can also be inhibited by copper chelators, such as tropolone (Valero et al. 1991). Use of cysteine (an amino acid) and ascorbic acid (the antioxidant vitamin C) remain usable chemical antibrowning effectors (Thipyapong and Steffens 1997). Thermal deactivation of enzymes (PPO is thermosensitive at temperatures of 70-90C) is common practice but blanching will result in the loss of carbohydrates, color, flavor, texture, vitamins, and other water-soluble components (Thipyapong and Steffens 1997). Heating and other methods are not sustainable, require a lot of energy, and produce a lot of waste. Several other possibilities to inactivate PPO have similarly been eliminated from use because in the food industry, such as sulfur dioxide, which is dangerous to human health (Thipyapong and Steffens 1997). High hydrostatic pressure can also deactivate the enzyme and works best in conjunction with increased ambient temperatures. Pulsed electric field has very little supporting data and is also only about 70% effective (Thipyapong and Steffens 1997). Gamma irradiation is not as effective as heating deactivation of the enzyme (Thipyapong and Steffens 1997). Other less effective and more destructive technologies include supercritical carbon dioxide, which explodes cells from within, Ohmic heating via

electric currents, and microwave heating used in liquid food processing (Thipyapong and Steffens 1997).

Broader impacts of PPO

There are a variety of functions of phenolic oxidases from organismal to environmental scales (Sinsabaugh 2010). This class of enzyme is expressed for acquiring carbon and nitrogen, defense, and ontogeny (Sinsabaugh 2010). PPO functions are varied, and include degradation, mineralization, and transformation of soil organic matter. PPO has a role in the detoxification of phenolics via oxidation to less harmful forms (Dorantes and Zúñiga 2012). Phenolics, substrates for PPO, are notorious as being allelopathic chemicals, which are plant-produced secondary metabolites with phytotoxic effects (Rice 1984, Blum 1996, Estabrook and Yoder 1998). Phenolic compounds inhibit nutrient absorption and regulate phytohormones (Leicach et al. 2009). It is important to note that limited nutrients limits plant growth even more than limited photosynthesis (Leicach et al. 2009).

PPO has great potential for use in global issues including as a cleanup tool in bioremediation and in creating biofuel alternatives to fossil fuels, such as those generated from the degradation of lignocelluloses (Sinsabaugh 2010). Peroxidases in this family of enzymes are localized in primary plant cell walls and vacuoles and are exuded and lysed in great amounts into the rhizosphere, serving a multitude of functions including metabolism of auxin, plant defense, and lignin and suberin formation (Dorantes and Zúñiga 2012).

Enzymatic degradation of xenobiotic pollutants by PPO is well documented (Ling et al. 2012). Enzymes such as peroxidases and catalases scavenge for reactive oxygen species (ROS), and thereby detoxify the environment via these oxido-reductive enzymes (Dorantes and Zúñiga 2012). PPO is multifunctional and can also serve in the reduction of H_2O_2 (Dorantes and Zúñiga 2012). Structural modifications through the enzyme-catalyzed oxidation, particularly of phenols, transform organic pollutants. Dorantes *et al.* (2012) suggests the use of various "swamp" species (*Phragmites australis*, common reed, *Iris pseudacorus*, yellow flag, and *Typha latifolia*, broadleaf cattail) for use as potential oil spill cleanup species, as there has been evidence of enzymatic up-regulation in response to substrate availability in *Cyperus elegans*, *Cyperus hermaphroditus*, and *Rhynchospora* species.

At high levels of the environmental contaminants polycyclic aromatic hydrocarbons (PAH's), plants respond by increasing levels of PPO, which metabolizes aromatics, as seen in *Festuca arundinacea* (tall fescue) in exposure to phenanthrene (Ling et al. 2012). PPO increased 153 to 359% higher root PPO activity. Uptake of persistent organic pollutants (POPs) by plants is caused by humans and also a danger to humans, notably from mobility in the food chain (Ling et al. 2012).

1.2. Background on the plant family Poaceae (grass) and the genus *Bromus*

The importance of the Poaceae (grass) family

Poaceae (grass) include over 10,000 species of immense environmental and ecological significance used fundamentally for biofuel, food, and materials. Agriculturally, grass is the most important plant family as it includes species of high global consumption, such as *Oryza sativa* (rice), *Triticum* (wheat), and *Zea mays* (maize), is used in myriad other ways, including prevention of erosion through action as a soil stabilizer (Salse et al. 2008, United States Department of Agriculture 2012). Grasslands are also an important biome type, covering about one-third of the earth's land surface (Shantz 1954).

Poaceae evolution

Fossilized pollen, easily distinguished by its spherical morphology with a single surface pore, gives an idea of when grass originated. Estimates range from the Paleocene onwards, at least 60-55 million years ago (MYA) but no more than 70 MYA (Jacobs et al. 1999, Kellogg 2001). Grass genomes are highly variable in chromosome number, ploidy levels, and size (Salse et al. 2008). Comparative studies in genomics indicate genome conservation of rice (*Oryza sativa*), wheat (*Triticum sp*.), and other grasses, have evolved from a common ancestor. *Aegilops* is the progenitor of wheat (Belyayev and Raskina 2013), and wheat is believed to be 8,000 years old (Huang et al. 2002). As a result of advances in genetic techniques, it is now believed that grass likely evolved from one 90 million year old ancestor with just five chromosomes (Salse et al. 2008). Over

time, there were likely several evolutionary events of whole genome or chromosomal duplications, fusions, and translocations from which the grasses of today speciated (Salse et al. 2008).

Poaceae morphology

The monophyletic group Poaceae (grass) from the (former) Gramineae has family has several unique morphological characteristics which define it as such, including a pollen wall sans scrobiculi, highly differentiated leaves, shoot and root mersistems and vascular system, and lateral embryo (Grass Phylogeny Working Group 2001). Prior to molecular data, this unique morphology was used inform cladograms (Kellogg 2001). One example of differences in grass morphology includes channels that do not penetrate the inner wall of the embryo (Kellogg 2001). Grass spikelet origination has been surprisingly gradual in multiple steps (Kellogg 2001). Glumes (bracts at the base of a spikelet), lemma (external bract of a floret), lodicules (possibly petal modifications), and palea (internal bract of a floret) "ancestry and origins" are still under debate (Kellogg 2001).

Among the grasses, morphology has been used to key out and categorize species. Grass, a monocotyledonous, wind-pollinated angiosperm, have stems called culms along which are nodes from which leaves grow in various patterns. Morphological characteristics of Poaceae also include the rhizome and stolon, sheath, ligule, and blade (Ibrahim and Peterson 2014). The growth morphology of grass is beneficial to their survival and persistence from grazing and trampling of animals as well as mowing. This

allows aboveground growth to continue at the base, rather than at apical meristems, which are snipped off by grazers.

Floral anatomy (inflorescence and spikelet) is the easiest way to identify grass species (Ibrahim and Peterson 2014). Grasses generally have characteristic spikelets, which are the flowers, with one or more florets. Historically, other initial defining characteristics of grass classification included embryo anatomy, leaf anatomy, lodicules, and starch content (Grass Phylogeny Working Group 2001).

Poaceae phlylogenetic trees and divergences

The source of the accepted grass family is a consortium formed in 1996 known as the Grass Phylogeny Working Group (GPWP). They combined data from chloroplast restriction sites, rbcL, ndhF, rpoC2, phytochrome B, ITS, GBSSI, and morphology and sought to rectify cladistic methods with phenetic analysis to reconstruct the most parsimonious Poacease tree (Grass Phylogeny Working Group 2001).

There are 3 known lineages of Poaceae: Anomochlooideae, Pharoideae, and Puelioideae, which are all C3 early-diverging taxa that subtend the BEP (Bambusoideae, Ehrhartoideae [formerly Oryzoideae and Pooideae]) and PACMAD (Panicoideae, Arundinoideae, Chloridoideae, Micrairoideae, Aristidoideae, Danthonioideae) clades (Grass Phylogeny Working Group II 2012). After the split of Poaceae into the now extinct Joinvilleaceae and other Poales, arm cells evolved, seed coats fused to ovary walls, perianths were reduced, and rpoC2 was inserted into chloroplast DNA (cpDNA), which changes at a reputably slow rate, and there was a 6.4 kb trnT inversion in cpDNA (Grass Phylogeny Working Group 2001). Spikelets and lodicules evolved, stigma number

was reduced to two, and ndhF was inserted into cpDNA (Grass Phylogeny Working Group 2001). Poaceae diverged to one large clade and Anommochlooideae, Pharoideae, and Puelioideae in others (Grass Phylogeny Working Group 2001). One new subfamily, Danthonioideae, has recently been proposed (Grass Phylogeny Working Group 2001).

Pooidaeae clade and the genus *Bromus*

The original *Bromus* species from Eurasia became extinct during the Pliocene and Pliestocene, but differentiated into sections during the Pliocene, spreading from North America to South America (Stebbins 1981). Bromes are quite speciose and are found worldwide. The number of *Bromus* species, also known as bromes, listed on The Plant List (http://www.theplantlist.org/browse/A/Poaceae/Bromus/ Access date 22 August 2012) includes 788, of which 165 are accepted species names. The USDA taxonomy recognizes 71 species and 95 accepted taxa overall (http://plants.usda.gov Access date 22 August 2012). Pooideae (cool season) grasses are believed to be a result of diversification in cooler climates (Kellogg 2001). Within this subfamily are the genera *Avena* (oats), *Bromus* (bromes), and *Triticum* (wheat) (Pavlick et al. 1995). The name *Bromus* means broma, food, from the Greek bromos, or oat. Morphological characteristics of *Bromus* species include connate leaf sheath margins, subapically inserted awns, simple starch grains (rounded, like that of Triticeae), and ovaries with hairy apical bilabiate appendages (Smith 1970).

The tribes Bromeae and Triticeae shared a common ancestor, and the genus *Littledalea* is the sister group of this Bromus-Triticeae lineage; thus, the closest non-*Triticeae* relative of *Bromus* is *Littledalea* from Asia (Schneider *et al.* 2009). Previously,

the genus *Littledalea* Hemsl. of three species from eastern Asia was believed to be *Bromus*' closest relative, but does not form a clade with *Bromus*, and morphologically similar *Megalachen*, *Metcalfia*, *Pseudodanthonia*, and *Sinochasea* are also now found to be distant relatives (Saarela et al. 2007). New molecular techniques led to the belief that Triciceae are the most genetically close to Bromeae (Saarela et al. 2007).

Bromus **species phylogeny:** ***Bromus*** **"sections"**

Within the genus *Bromus*, there have been several studies regarding the phylogenetic history and structuring of extant species and *Bromus* species within the genus *Bromus* have been classified as one of seven "sections": Boissiera, Bromus, Ceratochloa, Festucaria, Neobromus, Neuskiella, and Stenobromus (e.g., (Smith 1970, Smith 1985, Scholz 1998, Saarela et al. 2007, Williams et al. 2011). The sections of *Bromus* have morphological differences in their spikelets (Saarela et al. 2007). Based on the 136 kbp chloroplast DNA changes, the tree indicates the existence of two major clades, one within Neobromus and one within Ceratocholoa, as a result of a single synaptomorphy (Pillay & Hilu 1995). Festucaria (60 species) is the newest to have evolved, with the largest chromosome (2n = 70) of the *Bromus* subgenera (Pillay and Hilu 1995). Bromus and Stenobromus diverged recently, whereas the oldest are Ceratochloa (2n = 56) and Neobromus (2n = 42), which are both hypothesized to have arisen from a now extinct ancestor. No information is available on either of the sections Neuskiella or Boissiera (Pillay and Hilu 1995).

1.3. Allelochemicals and allelopathy

Introduction to allelopathy

Allelopathy can be described as the phytotoxic effect produced by one plant on another (Rice 1984). Allelochemicals are those compounds which impose an allelopathic effect. Allelochemicals are naturally-produced plant secondary compounds which are non-essential for physiological functions for plant growth and development, but can impact neighboring plants in various ways. Allelopathy is a competition type of interaction between organisms and is therefore not a beneficial relationship, even for the winner of the competition, who must expend resources to produce phytotoxins. The loser of the competition may express decreased fecundity or face mortality. Each competitive plant is better off without the presence of the other, because in competition there are fewer nutrients, resources, and space. Allelopathy has also been implicated as a mechanism for dominant vegetation, invasion, and plant succession (Muller 1953).

Historical notions of allelopathy

Allelopathy has long been known in the agricultural realm to play a detrimental role in patterning and spacing in plant vegetation, as vegetation can be allelopathically determined as a result of toxins produced by plants. Plants distribute randomly, spaced, or clumped; when plants are found clumped together, it is often indicative of shared resource use, but when found spaced, allelopathy is often implicated (Muller 1953). Although this concept has notoriety as controversial and difficult to prove, the International Allelopathy Society (formed in 1998) provides guidelines and strict criteria

for a plant to be considered allelopathic and articles have been published to give credence to the subject (Fitter 2003).

Chick peas were used to eliminate weeds as early as 300 BC, and Cato the Elder described walnut *(Juglans* species) to be toxic. The word allelopathy itself is built from the two Greek words *allelon*, which means of each other, and *pathos*, which means to suffer. German plant physicist Hans Molisch coined the term in 1937 to describe the harm on one plant as caused by another, but the phenomenon was known since much earlier. For example, "replant syndrome" avoidance has been known to be beneficial for crop success in the agricultural realm for quite some time, and even Charles Darwin mentioned the importance of crop rotations in his *On the Origin of Species* (Darwin 1859). Historically, "soil sickness" and "soil tiredness" can be cured by rotating crop species not from nutrient depletion, rather, accumulation of exudates in soil (Leicach et al. 2009).

Now the term allelopathy encompasses both negative and positive effects from a variety of fungi, microorganisms, and plants (de Albuquerque *et al.* 2011). Allelopathy also has a long history of academic debate (Muller 1964, Bartholomew 1970, Fitter 2003, Duke 2010), but here we focus on important ecological applications of interactions among plants mediated by complex chemistry.

Allelochemical compound structure diversity

The biochemistry of allelopathy has been extensively reviewed (Bertin et al. 2003). Enumeration of all known allelochemicals is not feasible because of the diversity of compounds in structure; even describing group classification is challenging. Generally

allelochemicals are secondary metabolites, and although the distinction is sometimes blurred, secondary metabolites are simply those that are considered not primary. Over 100,000 naturally-produced phytochemicals have been described (Dixon 2001). The list of allelochemicals is long and complex, with no single compound or even a single category of compounds represented; types of allelopathic compounds can include amino compounds, fatty acids and sterols, growth factors, organic acids, sugars, nucleotides, flavonones and enzymes (Curl and Truelove 1986). Over 400 alkaloids, 100 amines, 50 cyanogenic glycosides, 1200 flavanoids, 100 glucosinolates, 400 non-protein amino acids, 750 polyacetylenes, 700 polyketides, and 4500 terpenoids have been identified as playing a role in deterring insects, plants, or microorganisms (Wink and Twardowski 1992).

In classification of compounds, there have been a few notable types or groups of compounds cited that elicit responses by other plant; categories of allelochemicals include carbolines, cinnamic acid and derivatives, coumarins, fatty acids (long chain) and polyacetylenes, flavanoids, hydroxamic acids, lactones (simple, unsaturated), phenolics, polyacetylenes, quinines, quinones, quinalones, steroids, tannins, terpenes and terpenoids, and water-soluble organic acids, straight-chain alcohols, including aliphatic aldehydes and ketones (Bais et al. 2001, Bais et al. 2006, Li et al. 2010). Three main groups tend to emerge: n-containing compounds, phenolics, and terpenoids, from the parent compounds acetyl coenzymeA, deoxyxylulose phosphate, meualonic acid, and shikimic acid (De Albuquerque et al. 2011). The allelochemicals compounds themselves vary widely among these organized groups, and formed chemical compounds readily change in the environment, and may be more or less stable, changing from harmful to harmless or inert

to allelopathic. Chemical conversions can be caused by chemical interactions or microorganisms (Bais et al. 2001). These instances of indirect effects also require disentanglement from direct cause-and-effect allelopathic interactions.

Methods for allelochemical identification

Methods for allelochemical extraction, isolation, purification, analysis, and identification have been reviewed (Eljarrat and Barcelo 2001). Allelochemicals can now be subjected to a slew of modern scientific techniques for compound extraction, purification, quantification, and identification: ecological or analytical chemistry: gas chromatography coupled with mass spectrometry (GC-MS) to identify volatile compounds, high performance liquid chromatography (HPLC) for polar and non-volatiles, and structures of compounds can be determined by infared spectroscopy (IR), nuclear magnetic resonance (NMR), and ultraviolet-visible spectroscopy (UV-V) (Leicach et al. 2009). For instance, HPLC using copper oxidation can be used to analyze phenolics in soil (Mitrovic et al. 2012). Various other analytical techniques include near infrared reflectance spectroscopy (NIRS), cyclic voltammetry analyzing flavonoids, and ESR spectrometry can be used for chemical identification (Mitrovic et al. 2012). Methods for allelochemical analysis can also include liquid chromatography (LC) (Eljarrat and Barcelo 2001). Typical steps toward any of these methods include extraction of compounds followed by separation via chromatography, followed by characterization of the properties (MP, BP, solubility, etc.), and finally spectroscopy (Leicach et al. 2009).

Allelochemical production: constitutive or induced

Allelochemicals are produced both constitutively throughout the duration of the life of the plant and *de novo*, as induced post-attack (Leicach et al. 2009). Production of allelochemicals is highly variable, not just between species, but also among species, and during different stages of plant development, and also between tissue types. Plant defense theory suggests that plants produce (constitutive or induced) allelochemical compounds as a way to defend themselves against herbivory, but both biotic and abiotic stressors can elicit increased allelopathic responses. Insect or other herbivorous damage increases allelochemical release, as does post-biotic attack via phytopathogens or phytophagy or other mechanical wounding, resulting in allelochemical release (Leicach et al. 2009). One constitutively expressed allelochemical defense is the legume genus *Lupinus*, which produces bitter-tasting quinolizidine alkaloids, repelling herbivores (Leicach et al. 2009).

The environment itself or interactions therein can trigger the biosynthesis of allelopathic phenolic compounds, particularly the phenylalanine ammonia-lysase (PAL) genes (De Albuquerque et al. 2011). Environmental factors that impact the allelochemical release into the environment include soil moisture, temperature (higher temperatures allow the release of a greater number of toxins), humidity, oxygen, and minerals (Leicach et al. 2009).

When allelopathy is induced, biotically in response to pathogens or insects or other plants or abiotically from chemicals, including herbicides, allelochemical production increases are possible via gene upregulation (Belz 2007). For example, rice genes encoding allelopathic phenolics, analyzed by qRT-PCR and HPLC, were found to be upregulated upon stress induction by the presence of *Echinochloa crus-galli* L.

(barnyard grass), resulting in increased allelochemical biosynthesis (He et al. 2012). Alternatively, induction of allelopathy is perhaps not promoted by interaction, rather, it may be that allelochemicals are produced at certain developmental stages (Belz 2007).

Allelochemical release into the environment

Allelochemicals can be volatilized, exuded, or simply present in plant biomass, which later expresses toxic effects from ordinary processes of decomposing litter, referred to as foliar leaching (Rice 1984, Inderjit and Callaway 2003). Leaves, stems, roots, rhizomes, seeds, flowers, and pollen have all been found to be allelopathic (De Albuquerque et al. 2011). Allelochemicals enter the environment by various means: a) decay of litter or roots and/or b) by exudation, deposition, volatilization (such as the pungent scent of leaves), or release from roots (De Albuquerque et al. 2011). The decomposition of plant tissues is the greatest source of phytotoxic chemicals (Leicach et al. 2009). Decomposition involves membrane disruption, thereby hydrolyzing allelochemicals, and these biologically active and physical allelochemicals are free to leach into soils, and are taken up by the roots of other plants.

Allelopathic effects on a cellular level

Allelochemical effects are diverse, and scale from the cellular level up to the whole-plant and even community-level effects. Production, release, or activity of an allelochemical may influence plant dominance, important in an ecological context, the influence of allelochemicals on plant productivity (such as a reduced crop yield) (Inderjit et al. 2011b).

Allelopathy elicits a great number of variable effects on the plant cellular level, including cellular division, cellular enlargement, and cellular maturation and specialization (Leicach et al. 2009). Allelochemicals change soil pH, decrease chlorophyll biosynthesis, decrease chlorophyll content, inhibit of biosynthesis of primary metabolites, and cause free radical formation which in turn attack double bonds of unsaturated acyl groups in membranes of lipids (Leicach et al. 2009). Allelochemicals cause increases in membrane permeability resulting in increased lipid peroxidation, and cellular contents can spill, fewer nutrients are absorbed, and ultimately cellular growth slows, slowing overall plant growth; or, cell death may occur, leading to overall death of the plant. Cell division decreases in roots, also interfering with overall plant growth and development (Li et al. 2010). Stomatal conductance decreases photosynthetic products, similarly yielding lesser growth and development in comparison to uninhibited plants (Li et al. 2010). The electron transport chain of photosystem II is inhibited by secondary metabolite-induced oxidative stress (Shunmugam et al. 2014).

Allelochemicals have to been shown to function to inhibit a multitude of ordinary plant activities, including ion uptake, nutrient uptake, stomatal conductance, photosynthesis, respiration, enzyme activity, protein synthesis, water balance, and cell division, thereby inhibiting seed germination and growth (Rice 1984, Wu et al. 2000, Bais et al. 2001, Walker et al. 2003, Perry et al. 2006). These negative effects may take some time to be observed on the whole-plant scale, but if veritable, nonetheless effect the community composition. Upon entering the complex environment of organisms and soil, these cellular-level impacts may have grave consequential indirect effects on the whole-plant scale (Leicach et al. 2009).

Effects of allelopathy are observed macroscopically via various methodologies, such as inhibition of seed germination in bioassays. Allelopathic effects can also be measured and observed as deficiencies in plant growth and development. Scaled up, allelopathic effects from cellular to whole plant levels show correlations between the photosynthetic distress (measurable as Fv/Fm ratio of variable fluorescence over maximum fluorescence), which will manifest as decreased plant productivity and growth (Mitrovic et al. 2012).

Allelopathy on an ecosystem-size scale

Plant community organization may be in part determined by the chemistry of that community (Inderjit et al. 2011a). Allelopathy can have an ecosystem-scale role in effects of plant-plant interactions (Inderjit et al. 2011b). Allelopathy affects community development on a broad scale through biochemical interactions between plants (Wardle et al. 1998). Plant community composition is at least in part determined by both positive (faciliatative) and negative (such as allelopathic) interactions between and among plants (Holzapfel and Mahall 1999). Plants compete in a number of ways for nutrients, resources, water, and light (Holt and Lawton 1994, Fitter 2003). Interference through chemical competition is of biological interest because there is evidence to suggest that plants produce phytotoxic allelochemical compounds, thereby precluding other plants from existing by use of this chemical arsenal (Callaway and Aschehoug 2000, Callaway and Ridenour 2004, Callaway et al. 2004). Detection of allelopathy by plant community composition may be possible if the hypothesis that allelopathy negatively effects plant species richness and therefore community composition is correct. Thus, allelopathy

influences communities in several ways: determining dominant vegetation, species succession patterns, and population dynamics (Leicach et al. 2009).

Allelochemicals are ecologically relevant, and ecologists studying plant communities must take a multidisciplinary approach when deciphering allelopathy; allelopathy encompasses plant physiology, molecular biology, and chemistry in addition to ecology. For example, Allelopathic *Solidago* species (goldenrod) have been shown to influence succession in communities, such as during the turnover from farmland to wildland (Pisula and Meiners 2010). Also important is the evolution of this type of chemistry, and why plants have invested energy in producing secondary compounds. Plant survival and fitness may be contingent upon the compounds in the environment produced by surrounding plants and also the compounds they produce. Primary effects on a molecular level manifest themselves as secondary consequences, observed in growth and development of target plants.

Allelopathic autotoxicity

Autotoxicity from allelopathy, that is, chemical inhibition of and by the same species, may have evolved as a way to reduce intraspecific competition, as inhibiting other plants make scarce nutrients more available (Mitrovic *et al.* 2012). Plants use several strategies to protect themselves against their own toxins (autotoxicity). Ways around the toxic effects are varied, from harboring enzymes that quickly break down the toxins to sequestration compartmentalization of toxic allelochemicals, to storage as non-toxic precursor chemicals that can be formed into allelochemicals as needed.

An example of a species that is autotoxic is *Juglans nigra* (black walnut), which produces the allelochemical juglone (5-hydroxy-1,4-napthoquinone) (Rietveld 1983). Juglone inhibits photosynthesis and respiration (Rietveld 1983, Leicach et al. 2009). Juglone can inhibit other species in a low concentration as 1 μm, and prevents seedlings of its own species from germinating, which is useful for controlling the population density, as a sort of self- or natural selection (Rietveld 1983). By preventing germination, the plant thus avoids competition for limited light, nutrients, and water.

Juglone also exists within walnut tree cells in a non-toxic precursor form called hydrojuglone and thereby avoids localized autoxicity. Hydrojuglone is colorless and is generally non-toxic, but is immediately converted to juglone by oxidation. Upon continual contact with oxidative conditions or tissue drying, juglone is tied up and decomposed.

Allelopathy and biological invasions: novel weapons hypothesis

In plant interactions, phytotoxic secondary plant products may have a role in enabling successful biological invasions. Allelopathy may be a mechanism that allows introduced non-native species to become problematic, dominating, invasive species. The novel weapons hypothesis (NWH) suggests that plants invading a previously-established plant community may be able to do so by use of their offensive chemical arsenal (Callaway and Aschehoug 2000, Callaway and Ridenour 2004). Coming equipped with their allelochemicals weapons that are unknown by the native species, the allelopathic species may be successful invasive species and cause problems for the native community

by displacing native species, which may be integral members of the community (whether as sources of food with a role in the food web, or as habitat).

Examples of allelopathic plant species

Just as there are countless allelochemical compounds, there are similarly numerous documented instances of allelopathy across the globe. Chemically important allelochemicals of notoriety include the alkaloids atropine from the plant *Atropine belladonna*, cocaine from *Eritroxilum coca*, strychnine from *Strychnos nux-vomica*, and the anti-auxin nicotine compound from *Nicotiana* species, which includes tobacco. The stimulant caffeine is a purine alkaloid, the alkaloids notorious class of compounds for their bitter taste, which is important for plant self-defense, from coffee (*Coffee Arabica*).

Allelopathic cultivars include many important agricultural and crop species, such as *Avena sativa* L. (oats; scopoletin), *Helianthus annus* L. (sunflower), *Hordeum vulgare* L. (barley; gramine and hordenine), *Oryza sativa* (rice; benzoxazinoids, momilactone B, 3-isopropyl-5-acetoxycyclohex-2-enone, and 5,7,4'-trihydroxy-3',5'-dimethoxyflavone), *Secale cereale* (rye; phenolic acids PLA and HBA, hdroxamic acids AZOB, DIBOA, and BOA) *Sorghum* Moench spp. (sorghum; sorgoleone), and *Triticum aestivum* L. (wheat; numerous benzoxazinoids; phenolic acids including p-coumaric, p-hydroxybenzoic, ferulic, syringic, and vanillic acids; short-chain fatty acids) (Narwal 2004, Belz 2007, De Albuquerque et al. 2011). Among the curcurbitae family, cucumber, watermelon, and melon roots produce benzoic and cinnamic acid allelochemicals. Sugarcane straw phenolic allelochemicals include the compounds ferulic, syringic, and vanillic acids, which cause increased root cell leakage, decreased chlorophyll content, and decreased

dehydrogenase activity in *Lactuca sativa* (lettuce). Phenolic allelochemicals in *Aulonemia aristulata* (bamboo) include ferulic acid, rutin, and quercetin, which causes inhibition of seed germination and seedling growth of *Lactuca sativa* and *Sesbania virgata.* Allelopathic tree species also leach allelochemicals into the environment; species include but are not limited to *Ficus spp., Rhododendron spp., Phyllostachys spp.*, and *Leucaena leucocephala*, each negatively affect *Acacia Ainus casuarinas*.

Allelopathic invasive species

Sometimes, allelopathic plants are problematic invasives. Allelochemical compounds may confer a benefit when those plants are in competition with non-allelopathic plants, and may thus be phytotoxic to and outcompete native plants. Allelopathic weed species include but are not limited to *Alliaria petiolata* (garlic mustard; exudes flavonoid glycosides and glucosinates), *Agropyron repens*, *Parthenium hysterophorus*, *Phragmites australis* (common reed, gallic acid, Rudrappa *et al.* 2007), *Sorghum halepense*, *Chenopodium album* (lambsquarters, produces phenolics), and *Elytrigia repens* L.. *Leonurus sibiricus* L., a roadside weed in India, produces caffeic acid, and *Sambucus nigra* L., an Italian shrub that invades crops, produces 24 aromatics, cyanogenins, lignans, flavonoids, and phenolic glycosides. Root exudates of *Trifolium pratense* (red clover) including the isoflavanoid compounds (6aR,11aR)-maackiain and (6aR,11aR)-trifolirhizin were allelopathic to *Arabidopsis thaliana* and *Poa annua* (Liu et al. 2013). In the United States, the weed barnyard grass (*E. crus galli*) is an allelopathic plant that invades rice fields and adversely affects crop yields by exuding 18 allelochemical compounds (terpenes, steroids, long-chain fatty acids, and derivatives of

cinnamic and ferulic acids). Black locust (*Robinia pseudoacacia*), native to the southeastern United States but elsewhere an invasive species, produces a list of allelochemicals that includes the phytotoxic compounds robinetin, myricetin, and quercetin, whose presence was determined by nuclear magnetic resonance and mass spectroscopy (Nasir et al. 2005).

Allelopathy can be an anti-predator defense

In addition to anti-plant weapons, allelopathy can also manifest as anti-herbivore defense in large mammals. Deer herbivory at high levels can have severe detrimental effects on plant species richness (Kimball et al. 2012). Monoterpenes are unpalatable, and deer preferentially browse foliage with lower concentrations at higher incidences (Kimball et al. 2012). Phenolic compounds can also be useful to plants to combat plant pathogens. For example, in the presence of condensed tannins, there was a decreased prevalence of the rust *Melampspora amygdalina* Kleb. (Leicach et al. 2009).

Phenolic allelochemicals

One important class of allelochemical compounds is phenolics (Mitrovic et al. 2012). The biomass of plants is 50% or more primary metabolic pathways synthesized via the Shikimate Pathway, which yields phenolic compounds, which can make up 25% of a plants dry green leaves (Vitousek et al. 1997, Shetty 2004, Leicach et al. 2009). The six-carbon aromatic ring with hydroxyl functional group(s) has a geometry similar to that of water, and is water-soluble, will diffuse through soil, and is soluble in plant membranes (Leicach et al. 2009). Phenolics are weak bases and weak acids, like water.

Also like water, phenolics form hydrogen bonds in liquid state. To vaporize phenolics, intermolecular attractions must be broken; this can be achieved through boiling, but is a state not usually observed in nature, so it is easy to see the stability of these molecules and their persistence in the environment. Ferulic acids, para-hydroxybenzoic, para-coumaric, and vanillic acid are ubiquitous phenolics in weedy plant species, such as *Digitaria decumbens* (pangola grass, slenderstem, a pasture grass) and *Leucaena leucocephala* (white leadtree, an invasive mimosoid tree) (Leicach et al. 2009). Phenolic allelochemical compounds include flavanoids, p-hydroxy acids, and quinones (Estabrook and Yoder 1998). These phenolics enter the environment one of two ways: leachate or litter, where upon they may be adsorbed into clay, degraded and mineralized by microorganisms as a source of carbon, transformed, or simply remain in their dissolved form and exit the ecosystem (Haettenschwiler and Vitousek 2000).

Allelochemical resistance to degradation

Although fates of allelochemicals remain largely unknown, rapid degradation of allelochemicals in the environment via hydrolysis and acetylation can still yield surviving bioactive phytotoxic compounds (Belz 2007). Phenolic allelochemicals are protected from degradation by microbes by chemical binding to soil (Leicach et al. 2009).

Novel weapons hypothesis

Allelochemicals have great significance in ecological interactions. To answer the questions of plant community composition determinants, allelopathy is studied as a mechanism that structures these plant communities. The novel weapons hypothesis

(NWH) proposes that allelochemicals produced by non-native plant species make the plants successful because the allelochemical compounds are foreign to the communities they invade, and the natives cannot defend themselves (Callaway & Aschehoug 2000, Callaway & Ridenour 2004).

Applications of allelopathy

Allelochemicals have the potential to be sustainable, natural herbicides (Singh et al. 2003). Environmentally friendly for their biodegradability, allelochemicals have the potential to be used as an alternative to synthetic herbicides or using allelopathic crop species could a viable strategy for weed management. Unfortunately, weed-fighting crops have received little research and lacked development attention due to the yield-cost (such as biomass reduction) of those crops producing chemicals for defense (Belz 2007).

Reduction in use of man-made organocarbonates and organophosphates would decrease environmental contaminants, especially as weeds evolve and become resistant to synthetic herbicides, which are expensive and can be persistent environmental pollutants (de Albuquerque *et al.* 2011). Pollution worldwide has spurned a need for alternatives to synthetic herbicides, which can be toxic and have long-lasting effects. Natural chemicals are more safe than synthetics for the environment and human health. Organic gardening movements desire for solutions to global problems of food crises and resource use. Allelochemicals are biodegradable natural plant products with potential to be an eco-friendly alternative to synthetic herbicides and pesticides, perhaps of interest to the chemical industry, as synthetic, man-made herbicides can be toxic to human consumption.

One well-studied allelopathic species is *Oryza sativa* (rice), which has recently been modified to withstand invasive barnyard grass (Gealy et al. 2014). Rice and other species grow in flooded areas and so the spraying of liquid herbicide is illogical and the physical difficulties and cost of labour of hand-weeding are not always viable options. Additionally, increased use of herbicide leads to evolution of herbicide resistance. There also exists a social demand for and need to develop and implement use of natural herbicides. The future is bright for allelochemical defense for use as natural pesticides, for control of insect species which feed on crops, and as natural herbicides, as deterrents for invading weedy species.

Future research in allelopathy

Future directions toward reaching goals of environmental sustainability include genetic enhancement of allelopathic traits for use in weed suppression, particularly crop species, even by using traditional plant breeding methods of artificial selection, could be beneficial, and a reversal of the decreasing trend in allelopathic activity (de Albuquerque *et al.* 2011). There is a present focus on the genetic manipulation of allelopathic traits for crop development. This is achieved through phenotypic screening and selection for those high on the allelopathic spectrum (Belz 2007). As agriculture shifted from collection to cultivation toward uniformity (high yield, same phenology, same seed germination) results in decreased genetic variation and increased susceptibility to pathology. Techniques to integrate a selected allelopathic trait include chemical profiling followed by molecular breeding and transgenics (Belz 2007). Genetically, targets of domestication of crop species tend to be part of developmental pathways (Olsen & Wendel 2013). Many

modern tools serve for genetic modifications and plant breeding and seed banking (e.g., Olsen & Wendel 2013). Weed management programs utilizing germplasm selection, molecular breeding, and transgenics, whereby genes incorporated with suppress weeds, reduce herbicidal applications (de Albuquerque *et al.* 2011). Morphological manifestations of genetic and developmental biology improvements to crops can feed the worlds increasing population either directly or indirectly by feeding livestock (Olsen & Wendel 2013). Studying allelopathy is important for agroecosystems, as weeds cause great crop losses, whether due to their own autotoxicological and soil sickness or changed microbial populations. Increased food production is a major goal for farmers and consumers alike, especially in regards to the projected continuation of population growth and global food demands.

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Phylogenetic distribution of polyphenol oxidase (PPO) within the plant family Poaceae (grass) and the genus *Bromus*: a link to invasiveness

ABSTRACT

Grasses (Family: Poaceae) include >11,000 species of agricultural, economic, and environmental importance, but their evolution is not fully understood despite numerous attempts to reconstruct the phylogeny with both morphological and genetic data. Plant classification in phylogenies is a reflection of that species' evolutionary history and the biological traits they possess. In contrast to whole genome sequencing, researchers sometimes just look at a few target sequences or traits to understand phylogenetic relationships. Previous work has shown that the enzyme polyphenol oxidase (PPO) is present in the roots of some Poaceae species but not others. Of particular interest is the genus *Bromus*, represented by 71 species and 95 accepted taxa, which includes a number of notoriously problematic highly invasive species. Here, we (1) conducted PPO enzyme assays of roots of 40 Poaceae species of bromes and non-bromes (combined with 24 published PPO values of previously assayed species for a total n=64) to test the hypothesis that invasive *Bromus* species have high levels of PPO, whereas non-invasive grasses have little or no PPO, (2) reconstructed a phylogeny through numerous methods using previously published data and modern methodology, and (3) mapped PPO and invasiveness onto our tree to investigate the phylogenetic history and evolution of PPO. Results showed that (1) roots of invasive grasses (n=27) do indeed have significantly higher PPO levels than roots of non-invasive (n=37) grasses ($P<0.01$), in both *Bromus* and non-*Bromus*, and (2 & 3) phylogenetic distribution of phenetic polyphenol oxidase

(PPO) of selected species in Poaceae is somewhat tractable, offering two interpretations: (a) reliance on PPO or PPO expression was much lower in the ancestor, or (b) moderate PPO is the ancestral condition and some genera lost the activity. These new findings point to PPO as a trait correlated to invasiveness, and highlight ongoing taxonomic classifications that may shed light on evolutionary understanding of selection benefits of the PPO enzyme and grass evolution.

INTRODUCTION

Polyphenol oxidases (PPOs, or monophenol monooxygenase (E.C. 1.14.18.1, International Union of Biochemistry (IUPAC)), have been the subject of several reviews and hundreds of papers regarding its presence and variability in animals, bacteria, fungi, green plants, and even bryophytes (e.g., (He et al. 2007; Massa et al. 2007; Mayer 2006; Nokthai et al. 2010; Richter et al. 2005; Tran et al. 2012). PPOs are common, naturally-occurring, highly stable oxidoreductase enzyme catalysts for ubiquitous natural browning reactions, first characterized over a century ago (Bertrand 1896) and more recently (Anderson and Morris 2003). The genes for PPO enzymes exhibit different sequence variation, gene copy numbers, gene families, as well as variety in PPO enzyme products, but maintain a conserved active site containing CuA and CuB at N and C termini (Aniszewski et al. 2008; Mayer 2006; Tran et al. 2012). PPO enzyme structures vary, thus, reported molecular weight of PPO isozymes are variable (Mayer 2006).

The PPO enzyme has remarkably high substrate specificity, though PPO enzyme substrates can be generalized as ortho-diphenols, six-carbon aromatic rings with

hydroxylations and methoxylations (Keilin and Mann 1938; Leicach et al. 2009; Queiroz et al. 2008). Ultimately, PPO enzymes catalyzes the oxidation of various mono-, di-, or polyphenolic compounds to o-quinones, generating melanin-like compounds that are visible as pigments (Valero et al. 1991). Following the first PPO-catalyzed reaction, quinone products react with amino acids, phenols, or proteins and polymerize spontaneously into melanin-like compounds; this produces visible browning through the creation of high molecular weight brown pigments (Aniszewski et al. 2008; Apel and Hirt 2004; Massa et al. 2007; Valero et al. 1991). Self-assembly of quinones into the reddish-black melanin-like condensation product is measured in colorimetric bioassay, as increased expression of PPO yields an increase in browning (Anderson and Morris 2003; Clark 1911; Mayer 2006). Here, to quantitatively measure enzymatic reaction, we determined levels of PPO by oxidation of added L-DOPA substrate increase in spectrophotometric absorbance at 475 nm due to the formation of melanin-like compounds (Holzapfel et al. 2010).

PPO enzymes are localized in primary plant cell walls, chloroplasts, and vacuoles and are exuded in great amounts into the rhizosphere through excretion or lysis (Sinsabaugh 2010). Research from our lab shows localized browning of root tissues, accordingly, PPO does not need to be released to be active, as the PPO in roots responds to phenolics in assay (Holzapfel et al. 2010). Fractionation and histochemical experiments suggest the PPO enzyme may solely be an enzyme of the plastid, including presence in root plastids, and is absent in plastid mutants (Vaughn and Duke 1984).

Understanding the evolution and classification of the plant family Poaceae (grass) and the genus *Bromus* is of great importance because Poaceae species are

economically and environmentally vital in several roles. As crops, grass species are a major human dietary food component (e.g., *Avena sterilis* (oats), *Hordeum vulgare* (barley), *Oryza sativa* (rice), *Secale cereale* (rye), *Sorghum bicolor* (sorghum), *Triticum aestivum* (wheat), and *Zea mays* (maize)), and are an important animal feed as it is both harvested as fodder for and directly foraged by livestock) USDA Agricultural Statistics 2012). In addition, grasses are sources of biofuel, and grass products are used as building materials (e.g., bamboo). Poaceae provide myriad ecosystem services (e.g., grasses prevent erosion through action as a soil stabilizer, they are medicines, they produce oxygen and store carbon, and they absorb superfluous nutrients and water (United States Department of Agriculture 2012). Finally, grasslands are an important biome type, covering about one-third of the earth's land surface, and are unique refuges for biodiversity (Shantz 1954).

The evolution and diversification of the more than 11,000 species in the monophyletic group Poaceae (grass) has been well-studied, with dates of origin ranging from 70 – 55 MYA (Grass Phylogeny Working Group 2001; Kellogg 2001; Salse et al. 2008). Domestication of plants started less than 10,000 years ago, thus, ancestral species are mostly still extant and their evolution is therefore tractable. For that reason, as suggested as early as 1859 by Charles Darwin (Olsen and Wendel 2103), crop plant species in particular pose as excellent models for evolution in action.

Phylogenetic tree reconstructions are ongoing, dynamic, and constantly changing and rearranging when new and additional data are available due to usage of new techniques or increased taxon sampling. Next generation sequencing technology and genomics are furthering these advances at an increasingly fast rate. Comparative studies

in genomics indicate grass genome conservation, and grass likely evolved from one 90 million year old ancestor with just five chromosomes (Salse et al. 2008). After several likely events of whole genome or chromosomal duplications, fusions, and translocations, grass genomes today are highly variable in chromosome number, ploidy levels, and size (Salse et al. 2008).

The source of the most widely cited and accepted Poaceae family tree is the Grass Phylogeny Working Group (GPWP), a consortium of scientists formed in 1996 to establish a phylogeny of grasses assessing current datasets and seeking to rectify cladistic methods with phenetic analysis. For instance, classic papers (Hamby and Zimmer 1988) and (Doebley et al. 1990) published a ribosomal RNA molecular phylogeny and rbcL (ribulose 1,5-bisphosphate carboxylase/oxygenase, large subunit) molecular sequence phylogenies, respectively. The resulting GPWG phylogenetic tree is a combination of these and other trees into the most parsimonious tree of grasses showing evolutionary history and speciation events (Grass Phylogeny Working Group 2001). This work was later updated in 2012 by the Grass Phylogeny Working Group II (Grass Phylogeny Working Group II 2012), constructing a more robust grass phylogeny by using 3 chloroplast markers (rbcl, ndhf, and matK/trnK introns), screening GenBank, and supplemented with genomic DNA as needed but were only able to include 5% of all grass species to ultimately create a Bayesian consensus tree of 545 accessions.

We chose to concentrate here on bromes (genus: *Bromus*), because of their high levels of root PPO enzyme (Holzapfel et al. 2010) and notoriety as invasive species. Bromes are quite speciose, and the genus is represented by at least 71 species and 95 accepted taxa in the United States (USDA Plants Database, http://plants.usda.gov Access

date 22 August 2012). The global number of *Bromus* species (bromes) listed on The Plant List, (http://www.theplantlist.org/browse/A/Poaceae/Bromus/ Access date 22 August 2012) includes 788 species, of which 165 are accepted species names.

Bromus is a genus in the subfamily Pooideae (cool season) grasses, and is thought to be derived from diversification events in cooler climates (Kellogg 2001). The original *Bromus* species from Eurasia may have become extinct during the Pliocene and Pliestocene, but differentiated into sections during the Pliocene, spreading from North America to South America (Stebbins 1981).

The grass family has numerous morphologically unique, defining characteristics that were used to inform cladograms prior to molecular data (Kellogg 2001). Grasses are monocotyledonous, wind-pollinated angiosperms. Historically, initial defining characteristics of grass classification included embryo anatomy, leaf anatomy, lodicules, and starch content, a pollen wall sans scrobiculi (GPWG 2001). Grasses have stems are called culms along which are nodes from which leaves grow in various patterns. Another difference in grass morphology includes channels that do not penetrate the inner wall of the embryo (Kellogg 2001). Floral anatomy characteristics (inflorescence and spikelet) are the easiest way to identify grass species (Ibrahim and Peterson 2014). The growth morphology of grass is beneficial to their survival and persistence amongst grazing and trampling of animals and mowing, allowing growth to continue, regardless of grazers.

Some aspects of grass morphological evolution are not yet resolved. Though it is known that grass spikelet origination has been surprisingly gradual, in multiple stages, glumes (bracts at the base of a spikelet), lemma (external bract of a floret), lodicules (possibly petal modifications), and palea (internal bract of a floret) “ancestry and origins”

are still under debate (Kellogg 2001). In bromes, distinguishing morphological characteristics include connate leaf sheath margins, subapically inserted awns, simple starch grains, and ovaries with hairy apical bilabiate appendages (Smith 1969).

Biological invasions by *Bromus* species cause severe economic and environmental detriment and have been increasingly studied, as traits of *Bromus* species contribute to their invasive success and characteristic problematic spread (Del Tredici 2010; Knapp 1996). Bromes have been introduced around the world through numerous intentional and accidental means, for aesthetics, fibers, and livestock feed (Pimentel et al. 1997), and have subsequently established successfully for myriad reasons (e.g., climate match, empty niche, and other theories of biological invasion success).

There are numerous negative effects of invasive grass species, and strands of *Bromus* species in particular have dramatically changed ecosystems in many ways. The major problem with aggressive non-natives is that they can overtake ecosystems, reducing native plant species presence (Knapp 1996; Pimentel et al. 1997). This is a product not just of the fires spread by bromes, but other alterations as well. Invasive grasses change resource supply e.g., nitrogen availability, as nitrogen (in addition to carbon) is volatilized by fire, yielding a nutrient loss to the atmosphere (D'Antonio and Vitousek 1992). *Bromus tectorum* is a soil engineer, increasing nutrient availability, which is beneficial to itself (Blank and Morgan 2013; Blank et al. 2013). Decreased capability of binding soil particles by invasive species can cause erosion pattern changes different from that of the native species, resulting in larger or smaller dunes, etc. (D'Antonio and Vitousek 1992). Invasives can reduce biomass of native plant and animal species, and decrease survival and growth of natives (Davis et al. 2005). Invasive grasses

can even change trophic structures as keystone species may be lost (D'Antonio and Vitousek 1992). Invasive grasses outcompete natives for water and nutrients by growing dense, shallow root systems (D'Antonio and Vitousek 1992). For example, *Bromus madritensis* subsp. *rubens* from the Mediterranean has invaded the United States and now dominates parts of the Mojave desert, as it is competitively better at resource use through quicker water acquisition, creation of larger surface area of roots and canopy, and production of higher biomass than the native desert annuals *Vulpia octoflora* and *Descurainia pinnata* (DeFalco et al. 2003).

B. tectorum has variable phenology, but generally the inflorescence forms in early spring and lasts until late summer, producing seeds that can germinate in spring or fall, providing an advantage over later-germinating species (Del Tredici 2010; Klemmedson and Smith 1964). *Bromus tectorum* has an atypically short lag phase time from initial introduction and subsequent invasion (Blank and Morgan 2013). The reproductive traits (high propagule pressure from prolific seeds with high germination rates) and competitive abilities such as rapid root growth, which enables *Bromus* to tolerate droughts and use up water and nutrient resources first as *Bromus* species roots are characteristically dense and fibrous, unlike those of native annuals, which mostly have taproots, are traits that allow grasses to invade and establish monotypic stands, altering ecosystem processes, tolerate or enhancing fires, and spreading better because of said fires (D'Antonio and Vitousek 1992; Klemmedson and Smith 1964; Knapp 1996; Pavlick et al. 1995). Occupation of a previously un-occupied niche, due to previous absence of a dominant native annual grass, in addition to excess grazing of species other than bromes

in a place matched for climate with where it originates in Eurasia and North Africa, of rainy winters and dry summers.

One such problematic invasive species is *Bromus tectorum* in North America. *Bromus tectorum*, also known as cheatgrass, downy brome, drooping brome, early chess, gas station grass, or thatch grass, is an annual cool season grass native to Eurasia and North Africa. It is believed to have arrived in North America via contaminated grain in 1889, then likely took hold on abandoned farm lands in the 19th century, as the species easily colonizes bare ground, disturbed, and degraded land, growing well in open areas with high levels of sunlight (Klemmedson and Smith 1964; Pavlick et al. 1995). *B. tectorum* seeds may have affixed themselves to animal hides or furs in transit westward, are now dominant in up to 20% of the shadscale (*Atriplex confertifolia*) and sagebrush steppe habitats of the Great Basin Desert in Western North America (Knapp 1996). Overgrazing of native perennial species by newly introduced domestic cattle decreased numbers of natives and resulted in a further increase in *B. tectorum* (Klemmedson and Smith 1964).

The problems caused by *B. tectorum* are numerous. *Bromus tectorum* has invaded over 98 million acres of US rangeland (DiTomaso 2000), and invades fields of *Medicago sativa* (alfalfa), an important forage crop species (Klemmedson and Smith 1964). *B. tectorum* precludes native plant establishment, which is particularly of critical importance in areas that burn, as post-fire native reestablishment is important for maintenance of biodiversity (Knapp 1996).

Among these concerns, a major problem attributed to *Bromus tectorum* is increased size and number of fires in the western United States, causing *B. tectorum* to be

cited as the most significant of plant invasive species (D'Antonio and Vitousek 1992). Traits of *B. tectorum* certainly contribute to the species flammable notoriety, a major disturbance, as the hazard of fire is likely due to the species low moisture content and early maturation (Klemmedson and Smith 1964). The biomass of grasses is low-moisture, coupled with *Bromus* species ability to grow well in dry environments, and post-senescence persistence as standing dead material all contribute to the flammability of the species, creating a vicious feedback cycle (D'Antonio and Vitousek 1992).

In the western U.S., fire frequency has increased from 60-100 year occurrences to every 3-5 years (Pimentel et al. 1997). Average annual costs for total fire management from 1980-1992 in the Great Basin was approximately twenty million USD, about $48.63/acre (Knapp 1996). These extreme environmental fluctuations (non-equilibrium, stochasticity) make the system extinction-prone (Pimm et al. 1988), and after fires, there is colonization by non-native species (Knapp 1996).

The phylogenetic history of PPO is understudied in grasses, which comprise a large, diverse, and important plant family. Classification in phylogenetic trees is a reflection of that species' evolutionary history and the biological traits it possesses; molecular data lends foundation and support of these trees. In contrast to whole genome sequencing, researchers sometimes just look at a few target sequences to understand phylogenetic relationships (Kellogg 2001). The phylogenetic distribution of PPO among plants shows inconclusive evidence for convergent evolution. There is likely genetic variability. A multigene family for PPO was found within cultivated and wild *Triticum* (wheat) species as well as within *Aegilops* species, a diploid wild relative (Massa et al. 2007; Wichers et al. 2003).

Previous work demonstrated that seedlings of 23 accessions of the grass genus *Bromus* representing 11 species consistently possessed high levels of polyphenol oxidase (PPO) enzyme activity in their roots while grasses of other genera with various taxonomic affiliation to the genus *Bromus* did not (Holzapfel et al. 2010). Both the function and exact phylogenetic distribution of root PPO enzyme among grasses remains unresolved. Therefore we expanded the scope of our PPO enzyme assays beyond the genus *Bromus* to a number of other genera in the grass family to investigate the presence of high PPO enzyme activity in Poaceae in general. By creating several phylogenetic reconstructions and mapping PPO activity, invasiveness, and growth strategy (plant life duration: annual or perennial) onto them we explored the potential evolutionary history of PPO activity in grasses.

METHODS

Plant material preparation. Seeds were surface-sterilized with 10% bleach for thirty minutes, washed twice in sterile DI water, and plated with flamed forceps on sterile moist filter paper in 10 mm glass petri dishes. If microbial contamination was observed during germination or growth, specimens were excluded from study. Seeds remained in these sterile dishes and were germinated until growth of a few millimeters of root was observed; this root was used in assay described below.

Enzyme assay protocol. A 2 mg/mL L-DOPA (L-3,4-dihydroxyphenylalanine) in MOPS solution was prepared in 50 mM MOPS (3-(N-Morpholino)propanesulfonic acid sodium salt, 4-Morpholinepropanesulfonic acid sodium salt) buffered to pH 6.5 with 1N NaOH. The enzyme assays were performed at room temperature in 13x100 mm culture tubes and

total reaction volume was 5 mL per tube. 2.5 mL water followed by a pre-measured piece of root (about 1cm) were added to the each of five tubes. At t = 0, 2.5 mL DOPA-MOPS was pipetted into each tube and vortexed and the first measurement was recorded. An additional reaction tube was set up with no root as a control for spontaneous, nonenzymatic color development as well as a water blank control.

Calculations and data analysis. To avoid pseudoreplication, one piece of root from five different individuals per each species was assayed separately. Absorbance (A_{475}) values of the incubation using a UV-VIS spectrophotometer (Spectronic 20D+, Milton Roy) were averaged for each species. The total activity ΔA_{475} was divided by the root length, giving the enzyme activity per unit root, thus, one unit of PPO was defined as the absorbance at 475 nm per cm root length. Data was analyzed by ANOVA in SPSS version 21 with 95% confidence intervals. (independent variable: species treatment, dependent variable: PPO), and when a treatment effect was detected, the data were then run through Tukey's *post hoc* test).

PPO, life history traits, and phylogenetic relationships. We collated published data on species' life history traits: life cycle duration (annual or perennial), invasiveness (invasive or non-invasive), and plant origin. We added to this referenced data base the PPO values taking into account time of assay and root length, seed source, any known information on PPO genes for each species. We then synthesized phylogenetic hypotheses from previous publications by manually combining extant phylogenetic trees to form a consensus tree using Mesquite evolutionary analysis (Version 3.02, (Maddison and Maddison 2015) for a single character (Catalán and Olmstead 2000; Fortune et al. 2008; Grass Phylogeny Working Group II 2012; Kellogg 2001; Saarela et al. 2007; Saarela et al. 2010; Salamin

et al. 2002). In the event of a disagreement among studies we chose the most congruent topology or settled on polytomous relationships.

Preliminarily we mapped continuous PPO values on this tree using Mesquite's parsimony driven ancestral state reconstruction function. Ancestral states calculated for species with multiple replicate individuals we used as tip data in subsequent analyses where replicate taxa were eliminated from the analysis. Branches of the tree were colored as a heat map of PPO. 56 different species were included in the phylogenetic analysis, including species that were previously assayed by (Holzapfel et al. 2010).

We compiled sequence data from Genbank to ascertain a more robust phylogenetic hypothesis, and to have a tree with meaningful branch lengths on which likelihood estimations of ancestral states could be made (Table 1). We eliminated taxa for which sequence data was not available. We took sequence data from a close relative for genera represented by only one taxon in the analysis but lacking appropriate genetic information in GenBank (Table 1).

Table 1. Accession numbers for genes by taxa.

Species	Accession numbers			
	ITS	NDHF	RBCL	TRNL
Aegilops longissima	AF149196	DQ247912	LN626635 (*A. vavilovii*)	EU013622
Avena sterilis	DQ995458	JX438124	HE963347	GU367247
Boissiera squarrosa	KM077288	-	HE575813	-
Bouteloua gracilis	GU359285	HE575771	JX848489	HM590247
Brachypodium distachyon	JX665601	BDU71043	LN626640	AF478500
Brachypodium phoenicoides	JN187620	AF051847	-	JN187670
Brachypodium pinnatum	AF019782	BPU71041	AM849347	KJ746414
Brachypodium rupestre	-	-	FR865135	JN187673
Brachypodium sylvaticum	GQ373321	BSU71040	AJ746258	EF137593
Briza maxima	KJ598893	HE575736	FN870384	KJ599344
Bromus carinatus	HQ600553	-	HQ600457	AB732921
Bromus coloratus	AY367943	-	-	AY367992
Bromus diandrus	KF713201	-	KF712968	AB732924
Bromus inermis	KF713194	DQ786821	JX848491	AY829228
Bromus japonicus	KF713199	-	KF712963	EU036181
Bromus kalmii	AY367916	-	-	EU119360
Bromus madritensis	EU036205	-	HM849827	EU036170
Bromus marginatus	AY367921	-	-	AY367971
Bromus pectinatus	AY367939	-	-	AY367988
Bromus riparius	AY367931	-	-	AY367980
Bromus scoparius	AY367932	-	-	EU036176
Bromus squarrosus	KM077303	-	-	EU036173
Bromus sterilis	KM077296	DQ247874	AY836155	EU036167

Bromus tectorum	KF713207	HF558459	KF712974	KF600709
Dactylis glomerata	-	-	HM849945	-
Elymus glaucus	JN009815	-	JX848496	AF519138
Elymus virginicus	FJ040171	-	KC237165	AF519143
Festuca idahoensis	AF147177	-	KJ756344	AF533064
Festuca rubra	KJ598996	JX438169	AJ746261	AY118099
Hordeum vulgare	KC193780	DQ290657	LN626641	KF600708
Oryza sativa	EF141824	-	KF731225	DQ131552
Panicum virgatum	AY129730	PVU21986	FR821347	AY142750
Phalaris arundinacea	KF713258	AY589121	AJ784827	EU639579
Secale cereale	AF303400	EU012710	LN626639	AF478501
Stipa capensis	KF850613	GU254773	HE573441 (*S. juncea*)	JF698046
Triticum aestivum	AY346121	DQ247921	LN626619	AF148757

These were first aligned with MUSCLE (Edgar 2004) and then imported into Mesquite and revised manually. Hard to align regions were omitted to come to a conservative assumption of homology among characters. We used the K-means method in PartitionFinder (Frandsen et al. 2015; Lanfear et al. 2012) to find the optimal partitioning scheme and partition specific evolutionary models. As an alternative, we also partitioned using PartionFinder's "all function" and defined subsets by gene and then codon position for protein coding genes (Table 2). Using these partitioning schemes and alignments, we computed the phylogenetic tree using Bayesian inference. In the program BEAST (Drummond et al. 2012), we ran a chain of 100,000,000 generations with 10,000,000 being burn-in. The tree prior was specified as a Yule Process (Gernhard 2008) and all others were set to uniform priors. The effective sample size was well above 2000 for each statistic calculated.

Table 2. Partitioning scheme.

Scheme	Subset	Model	Nuc. Length	Subset Type
K-Means	1	F81	2939	Site specific
	2	K80	76	
	3	K80	281	
	4	K80	110	
	5	K80	140	
	6	TrNef	181	
All	1	HKY + G	1201	TRNL
	2	GTR + G	1214	NDHF + codon pos. 3 of RBCL 1
	3	TrNef + G	392	ITS
	4	HKY + I + G	460	Codon pos. 1 of RBCL 1
	5	K80 + I	460	Codon pos. 2 of RBCL 1

Concurrently with these methods, we reconstructed a phylogenetic tree using maximum likelihood with the same alignments and partitioning schemes. In the program Garli (Zwickl 2006) we ran 100 search replicates to obtain the best possible tree and then ran 100 bootstrap replicates to obtain support values for each node. A significant improvement in topology was defined as .01 lnL.

We used the Bayesian tree resulting from the K-means partitioning scheme to retrodict ancestral states for three characters: PPO (continuous), lifecycle duration (2 state - categorical), invasiveness (2 state - categorical). We chose the tree from the K-means partitioning scheme because we believe this to be the more biologically meaningful scheme. In the R-package "ape" (Paradis et al. 2004) we used the function "ace" to calculate PPO ancestral states using the maximum likelihood method (Felsenstein 1973) and a Brownian motion model. We estimated ancestral states for categorical traits in Mesquite using the maximum likelihood criteria.

RESULTS

1. PPO enzyme assays. Results of enzyme assays of the roots of individual plants show that PPO level varies by species (Table 3).

Table 3. Species assayed, seed source, corresponding PPO values, and other meaningful traits used in analyses.

Genus	Species	Seed source	PPO	Invasive	Plant origin	Duration
Aegilops	*longissima*	Isreal	0.26	No	Mediterranean Europe and Western Asia	Annual
Aegilops	*speltdoides var speltoides*	Turkey	0.40	No	Mediterranean Europe and Western Asia	Annual
Aegilops	*tauschii*	Azerbaijan	3.37	No	Mediterranean Europe and Western Asia	Annual
Andropogon	*gerarrdii*	Commercial, USA	1.29	No	Native to Great Plains and prairie regions of central North America	Perennial
Avena	*sterilis*	Collected by CH	0.45	Yes	Native to Mediterranean	Annual
Boissiera	*squarrosa*	Siirt, Turkey	0.71	No	Native to Western Asia	Annual
Boissiera	*squarrosa*	Uzbekistan	2.08	No	Uzbekistan	Annual
Boissiera	*squarrosa*	Van, Turkey	1.80	No	Turkey	Annual
Bouteloua	*gracilis*	Commercial, USA	2.82	No	Native to United States	Perennial

Brachypodium	*distachyon*	USDA	8.93	Yes	Native to southern Europe, Northern Africa, and southwestern Asia east to India	Annual
Brachypodium	*phoenicoides*	Spain	8.33	Yes	Native to Mediterranean	Perennial
Brachypodium	*pinnatum*	Former USSR	6.86	Yes	Eurosibera	Perennial
Brachypodium	*pinnatum*	Turkey	14.94	Yes	Eurosibera	Perennial
Brachypodium	*rupestre*	Russian Federation	10.05	Yes	Europe, northern Turkey	Perennial
Brachypodium	*sylvaticum*	China	8.08	Yes	North Africa, Europe and Asia	Perennial
Brachypodium	*sylvaticum*	Iran	10.88	Yes	North Africa, Europe and Asia	Perennial
Briza	*maxima*	Commercial, USA	1.32	No	Northern Africa, the Azores, Western Asia, Southern Europe	Annual
Bromus	*biebersteinii*	Canada	9.70	No	Turkey; (Middle East, western and central Europe and China); seeds from Canada	Perennial
Bromus	*biebersteinii*	Turkey	7.42	No	Turkey; (Middle East, western and central Europe and China)	Perennial
Bromus	*carinatus*	Commercial, USA	6.35	No	North America, Western	Perennial
Bromus	*coloratus*	USDA	6.72	Yes	South America	Perennial
Bromus	*diandrus subsp. rigidus*	Belgium	4.20	Yes	Mediterranean Europe	Annual
Bromus	*fasciculatus*	Israel	4.30	No	Africa, temperate Asia, Southern Europe (Greece, Italy, France, Spain)	Annual
Bromus	*hordaceaus*	Afghanistan	2.18	No	Eurasia	Annual
Bromus	*hordaceaus*	Commercial, USA	5.90	No	Eurasia	Annual
Bromus	*inermis inermis subsp.*	Commercial, USA	4.52	Yes	North America & Introduced	Perennial
Bromus	*inermis*	Russia	10.61	Yes	North America & Introduced	Perennial
Bromus	*japonicus*	Belgium	5.66	Yes	Europe	Annual
Bromus	*japonicus*	New Jersey	6.38	Yes	Europe	Annual
Bromus	*kalmii*	Commercial, USA	2.32	No	North America	Perennial
Bromus	*kalmii*	USDA	1.76	No	North America	Perennial
Bromus	*lanceolatus madritensis subsp.*	USDA	3.35	No	Mediterranean Europe	Annual
Bromus	*dlilelea madritensis*	Isreal	5.56	Yes	Europe	Annual
Bromus	*subsp. rubens madritensis*	California, USA	6.72	Yes	Europe	Annual
Bromus	*subsp. rubens*	California, USA	10.70	Yes	Europe	Annual
Bromus	*marginatus*	USDA	2.54	No	Western North America	Perennial
Bromus	*oxyodon*	USDA	7.74	Yes	Western Asia, China, Mongolia and India	Annual
Bromus	*pectinatus*	USDA	3.56	No	Africa, Asia	Annual
Bromus	*riparius*	USDA	6.70	No	From Russia; present in SW Asia, Europe	Perennial
Bromus	*scoparius*	USDA	3.98	No	Eurasia	Annual
Bromus	*secalinus*	USDA	8.12	Yes	Eurasia	Annual
Bromus	*squarrosus*	USDA	2.01	No	Europe, western Asia, northern Africa	Annual
Bromus	*sterilis*	(none listed)	9.76	Yes	Eurasia	Annual
Bromus	*sterilis*	USDA	3.39	Yes	Eurasia	Annual
Bromus	*tectorum*	Iowa, USA	4.08	Yes	Mediterranean Europe	Annual
Bromus	*tectorum*	New Jersey, USA	1.83	Yes	Mediterranean Europe	Annual
Bromus	*tectorum*	Turkey	5.37	Yes	Mediterranean Europe	Annual
Bromus	*tomentosus*	USDA	2.50	No	Distribution: Asia-temperate: Caucasus and western Asia. Asia-tropical: India	Perennial
Bromus	*vulgaris*	USDA	2.15	No	Western North America	Perennial
Dactylis	*glomerata*	Commercial, USA	1.84	Yes	North Africa, temperate Asia, Europe	Perennial
Elymus	*glaucus*	Commercial, USA	2.67	No	North America	Perennial
Elymus	*virginicus*	Commercial, USA	3.00	No	North America	Perennial
Festuca	*idahoensis*	Commercial, USA	4.18	No	North America	Perennial
Festuca	*rubra*	Commercial, USA, Stover Seed Company	1.40	Yes	North America & Introduced	Perennial
Festuca	*rubra subsp.*	Commercial, USA	2.22	Yes	North America & Introduced	Perennial

	communtata					
Hordeum	*vulgare*	USDA	0.62	No	Africa, Eurasia	Annual
Nasella	*pulchra*	California, USA	0.65	No	North America	Perennial
Oryza	*sativa*	USDA	1.53	No	Africa, Asia	Annual
Oryzopsis	*hymenoides*	Commercial, USA	1.51	No	North America	Perennial
Panicum	*virgatum*	Commercial, USA	0.00	No	North America	Perennial
Phalaris	*arundinacea*	Commercial, USA	1.95	No	North America	Perennial
Secale	*cereale*	USDA	1.18	No	Western Asia and India	Annual
Stipa	*capensis*	Isreal	0.74	No	Mediterranean	Annual
Triticum	*aestivum*	USDA	1.67	No	Mediterranean, southwest Asia	Annual

2a. PPO activity and invasiveness and 2b. PPO activity and plant life duration. Roots from invasive plants had significantly higher root PPO activity than roots from non-invasives (Fig. 1). Perennial plants roots had on average more PPO activity than annuals, but the difference was not significant (Fig. 1).

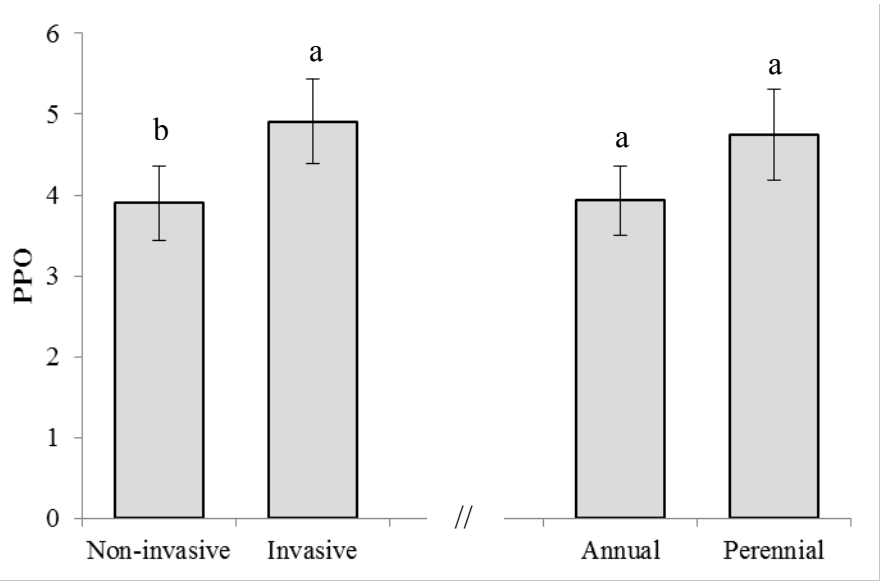


Fig. 1. Mean PPO levels for groups by invasiveness and life cycle duration (n = 38 non-invasive, 40 invasive; n = 41 annual, 37 perennial) +/- 1 SE, different lowercase letters indicate significant differences, ANOVA, Tukey's *post hoc* test, $P < 0.05$.

Significantly more PPO activity was found in the both invasive annuals and invasive perennials than non-invasive annuals and perennials (Fig. 2). There was not a significant difference in PPO levels among invasive annuals or perennials, except that annual non-invasives showed significantly less root PPO activity than perennial non-invasives .

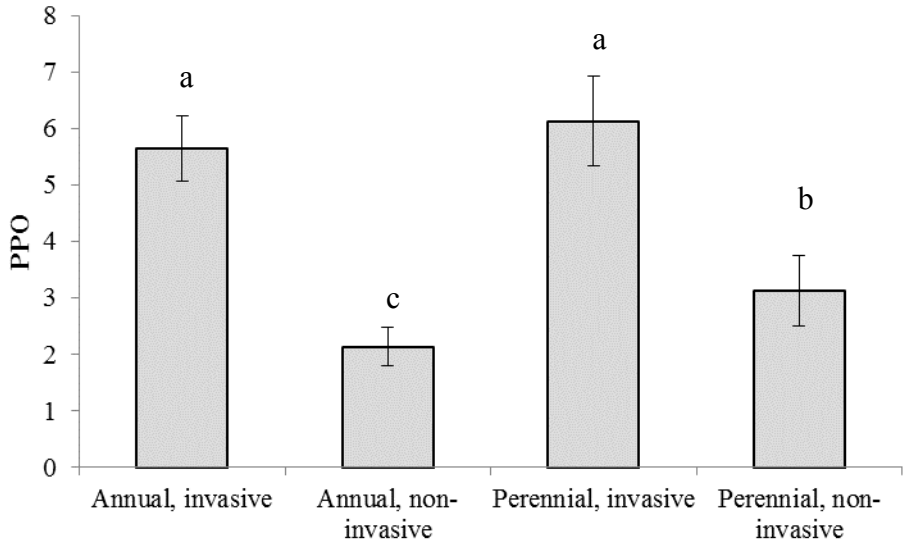


Fig. 2. Mean PPO levels for groups by life cycle duration and invasiveness (n = 21 annual invasive, n = 20 annual non-invasive, n = 20 perennial invasive, n = 17 perennial non-invasive), +/- 1 SE; different lowercase letters indicate significant differences, ANOVA, Tukey's *post hoc* test, $P < 0.05$.

We then grouped our species into bromes and non-bromes, and found that the mean amount of root PPO activity in the genus *Bromus* was significantly greater than non-bromes (Fig. 3). To determine if this was attributable to the tendency of bromes to be invasive, we further divided those groups in invasive or non-invasive bromes and non-bromes (Fig. 4). Here, we saw that indeed, invasive *Bromus* species have significantly higher root PPO activity than non-invasive *Bromus* as well as non-invasive non-*Bromus*, but that these invasive bromes have amounts of root PPO levels similar to that of non-*Bromus* invasives (Fig. 4). That is, both invasive bromes and invasive non-bromes had significantly higher PPO activity than both non-invasive groups (brome and non-brome; Fig. 1).

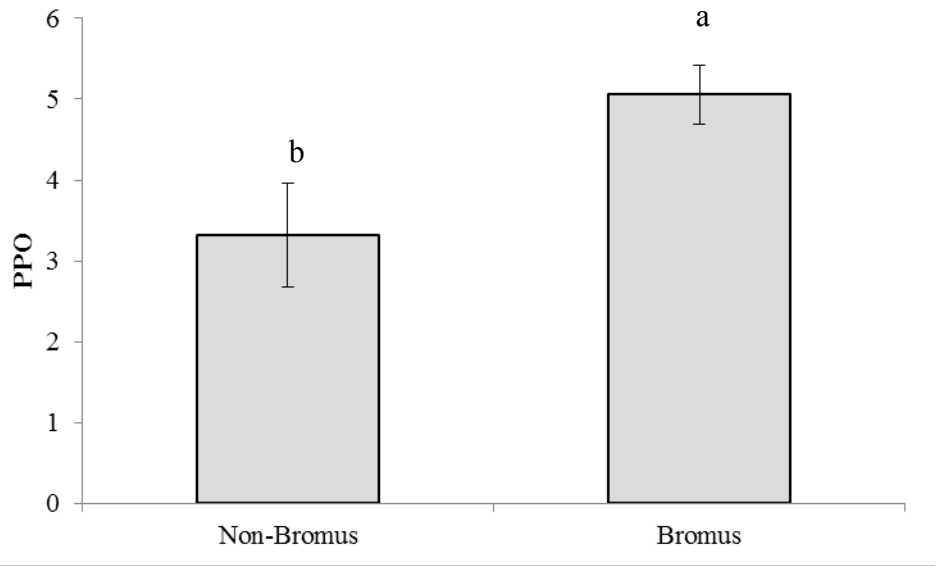


Fig. 3. Mean PPO levels by genus (n = 33 non-*Bromus*, 45 *Bromus*), +/- 1 SE; different lowercase letters indicate significant differences, ANOVA, Tukey's *post hoc* test, $P < 0.05$.

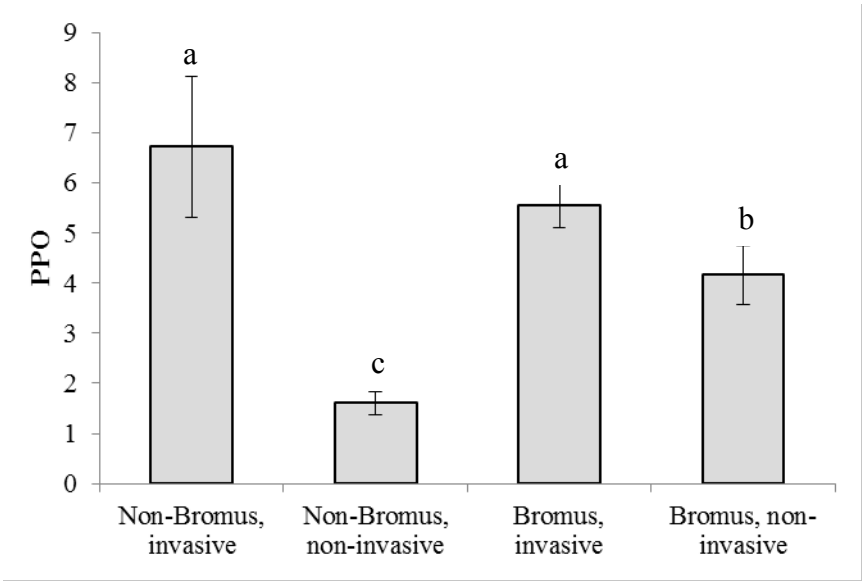


Fig. 4. Mean PPO levels by genus and invasiveness (n = 11 non-*Bromus* invasive, n = 22 non-*Bromus* non-invasive, n = 29 *Bromus* invasive, n = 16 *Bromus* non-invasive, +/- 1 SE; different lowercase letters indicate significant differences, ANOVA, Tukey's *post hoc* test, $P < 0.05$).

We tested for any trends of PPO levels when species were divided into groups of bromes and non-bromes by plant life cycle duration (Fig. 5). There was significantly lower PPO activity in the roots of non-*Bromus* annuals than any other perennial or annuals tested ($P < 0.05$), but no other statistical differences were observed.

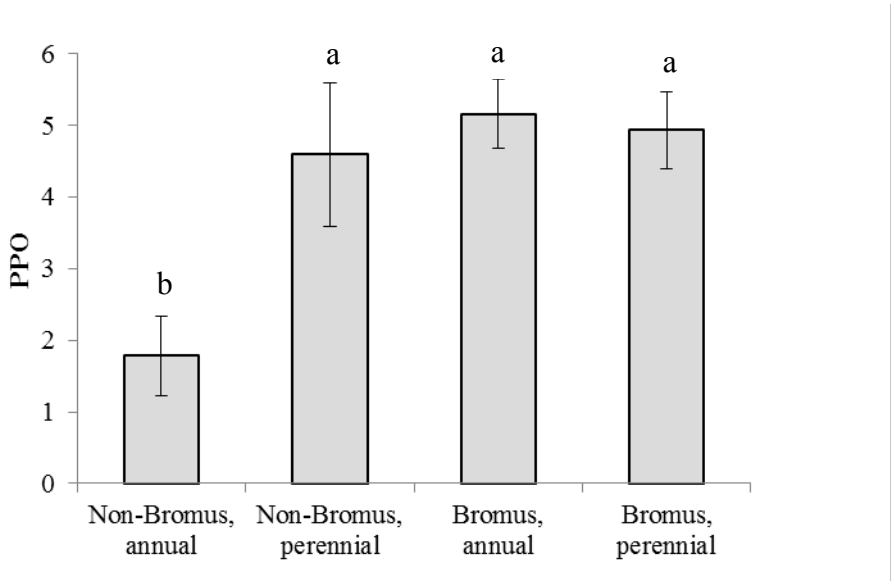


Fig. 5. Mean PPO levels by genus and life cycle duration (n = 15 non-*Bromus* annual, n = 18 non-*Bromus* perennial, n = 24 *Bromus* annual, n = 21 *Bromus* perennial, +/- 1 SE; different lowercase letters indicate significant differences, ANOVA, Tukey's *post hoc* test, $P < 0.05$).

3. Phylogenetic tree reconstructions. All Bayesian analyses converged quickly, within 10,000,000 generations but were allowed to run for 100,000,000 total generations. ML trees run with the full data and different random start seeds set all had identical topologies. The final trees chosen from each analysis had minor differences in young relationships when compared to the tree constructed from the literature, but the backbone had the same topology.

Table 4. Phylogenetic reconstruction methods and probabilities.

Method	Partitioning and model selection scheme	Number of partitions	Posterior Prob. (mean)	ln Likelihood (best)
Bayesian	All	5	-15691.20	-
Bayesian	K-Means	6	-15698.85	-
Max. Likelihood	All	5	-	-12192.60
Max. Likelihood	K-Means	6	-	-10849.05

We chose the ML-Kmeans (Fig. 6) tree as the best overall tree because its topology agreed best with the others (see below) and it showed a great improvement in likelihood over the ML-All tree. Figure 6 shows the ML-Kmeans tree with clade

bootstrap support values (100 replicates) and posterior probabilities from the Bayes-Kmeans tree.

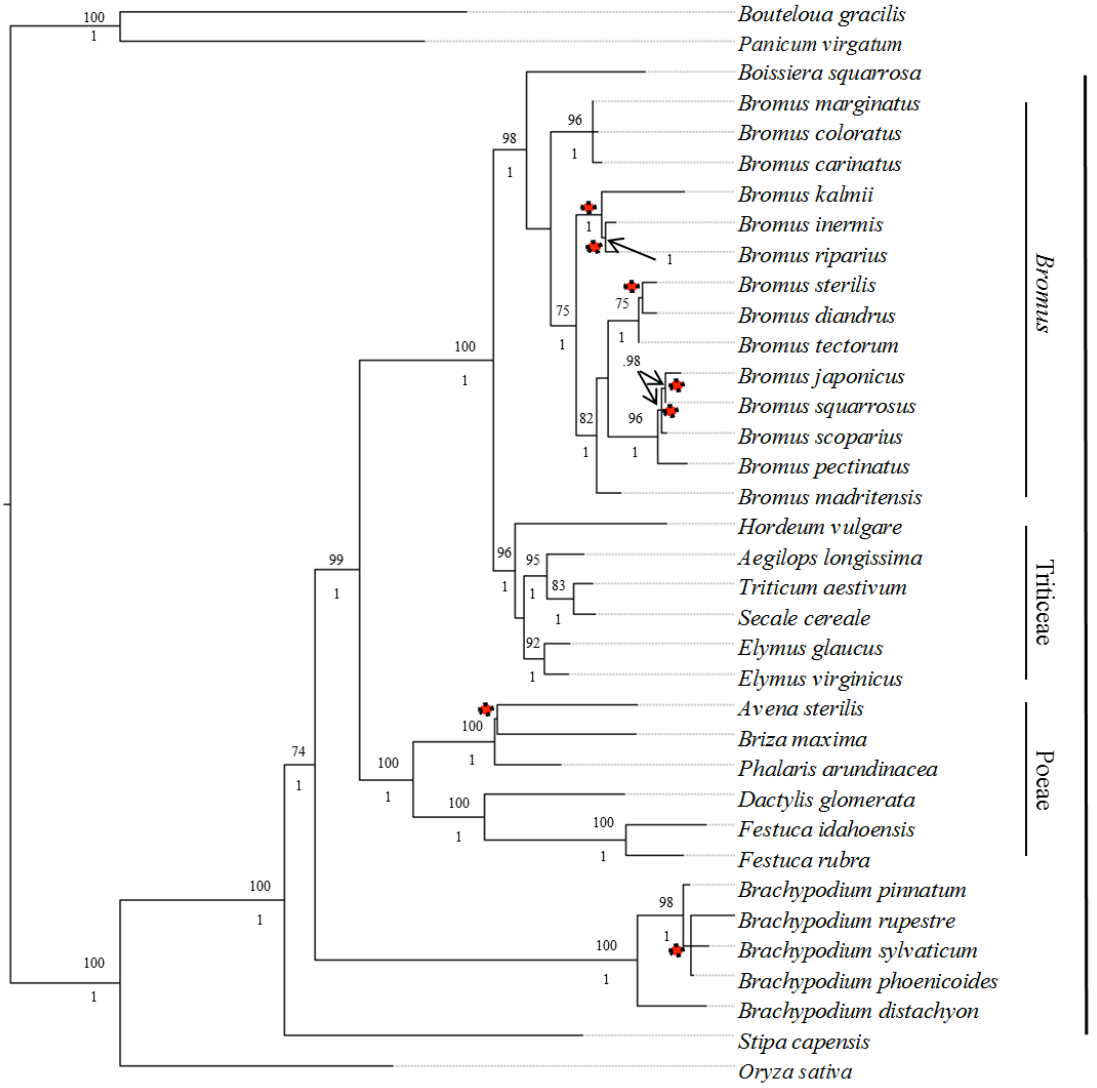


Fig. 6. The ML-Kmeans was chosen as the best tree. Numbers along branches are bootstrap support values from parsimony analyses and posterior probabilities from Bayesian analyses, respectively.

Our four estimated trees had similar topologies with the exception of a few relationships that were volatile across analyses. Within *Brachypodium*, *B. distachyon* was always sister to the remaining species but the relationship among those remaining species was unsettled. *B. pinnatum* was sister to *B. sylvaticum* + *B. phoenicoides* + *B. rupestre* in

all but the Bayes-All tree. In every case, the topologies were in disagreement with the previously accepted topology (Fig. 7). No relationship among *B. sylvaticum*, *B. phoenicoides* and *B. rupestre* was highly supported (bootstrap values or posterior probability). The *Phalaris* + (*Briza* + *Avena*) was supported by all trees except the Bayes-Kmeans tree. But the (*Briza* + *Avena*) clade always had a very short stem and was supported by low posterior probability. This overturns the *Briza* + (*Avena* + *Phalaris*) relationship from previous studies (Fig. 7). The *Hordeum* + (*Elymus* + (*Aegilops* + (*Secale* + *Triticum*))) clade was highly supported in all trees, although the basal relationship was not well supported by ML bootstrapping (<70%). Again, these relationships differ from the previously accepted topology (Fig. 7). *Boissiera* was placed as sister to *Bromus* (the previously accepted relationship; Fig. 7) in all but the Bayes-Kmeans tree. In the aberrant tree, *Boissiera* was nested within *Bromus*, but with low posterior probability. Within *Bromus*, a few relationships were not well supported but all supported relationships were largely different from the previous phylogeny. Compare among Fig. 6, Fig. 7, and (Saarela et al. 2007) for details of these differing relationships. *B. kalmii* + (*B. inermis* + *B. riparius*) was supported across all trees, however the relationship collapsed in ML bootstrapping. *Bromus madritensis* was placed as sister to the clades containing *B. diandrus*, and *B. pectinatus* in the ML trees but placed as sister to the clade containing *B. pectinatus* in the Bayesian trees. No relationship (except for their monophyly) was supported among the *B. tectorum*, *B. diandrus*, *B. sterilis* clade. *B. pectinatus* + (*B.scoparius* + (*B. japonicas* + *B. squarrosus*) was recovered in all trees but was not supported with ML bootstrapping.

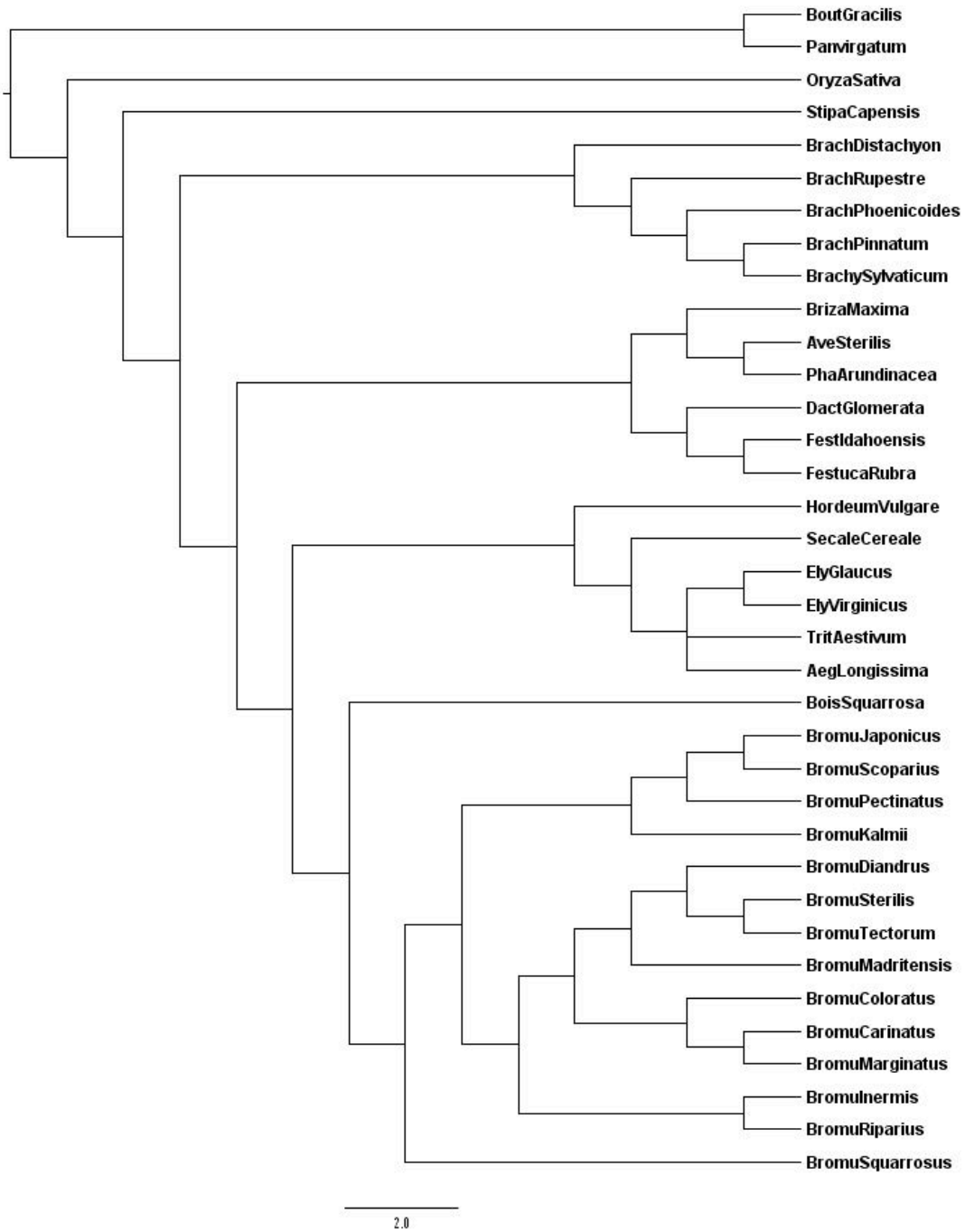


Fig. 7. Phylogenetic reconstruction from manually combined extant phylogenetic hypotheses (Catalan & Olmstead 2000, Kellogg 2001, Fortune *et al.* 2008, GPWG II 2011, Saarela *et al.* 2007).

Analysis of trait evolution. We chose the Bayes-Kmeans tree for probabilistic ancestral state reconstruction. The ML-Kmeans tree was not entirely dichotomous and therefore could not be analyzed by the "ape" package. Figure 8 shows the character

reconstruction for three traits on the Bayesian Tree with the trait values reconstructed by Maximum Likelihood mapped onto the tree.

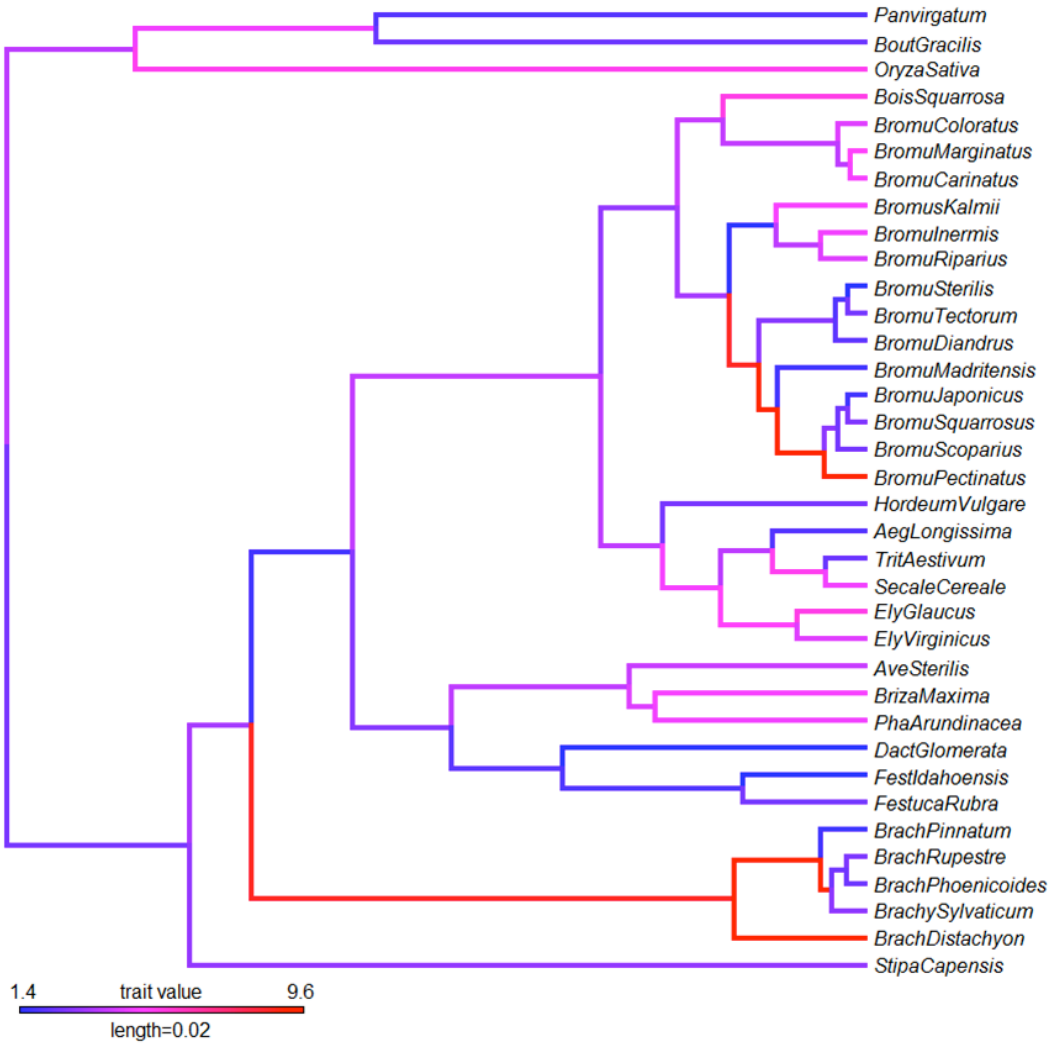


Fig. 8. Bayes-KMeans tree, ML reconstruct Brownian Motion.

PPO activity and taxonomy. All *Brachyodium* accessions show strong activity that are comparable to activity expressed by *Bromus inermis*, while both available *Boissieria* accessions of the monotypic genus clearly lacked the activity, as did several other genera (Fig. 9). The PPO reconstruction gives the ancestor of *Stipa* + remaining taxa to have high PPO values considering the very low PPO of *Stipa*.

The ancestors along the backbone of the tree all show overall moderate PPO values (~5). In a few cases, reduction in PPO expression was retained among daughter lineages (Fig. 6). The greatest expressers of PPO, *Brachypodium* and *Bromus* display a convergent increase in PPO expression, possibly indicating a convergence in functionally related biology (thus far unexplored).

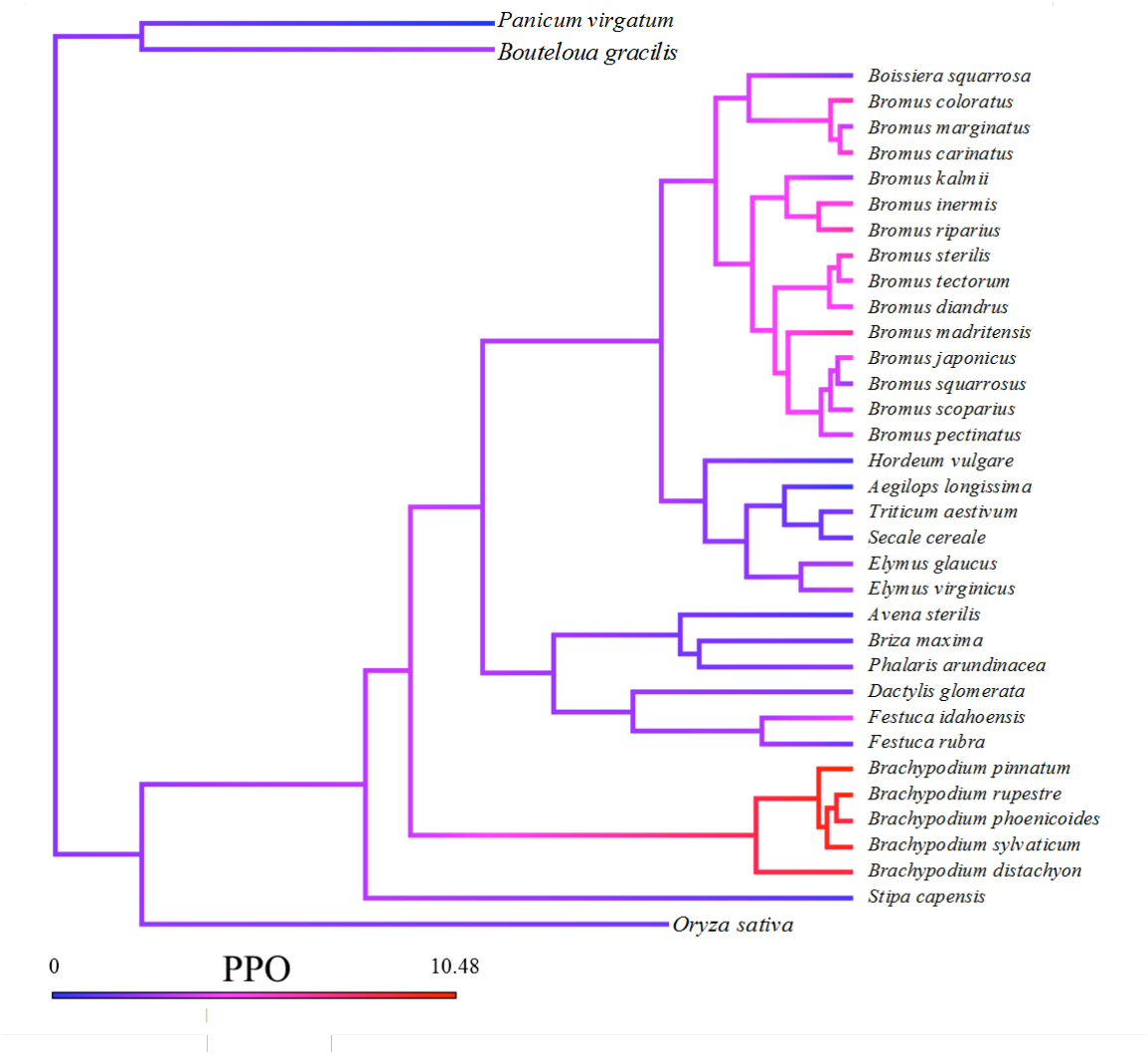


Fig. 9. PPO activity levels projected as a heat map of colored branches using ancestral state reconstruction.

DISCUSSION

Through investigating the relationships between PPO activity and life history traits (invasiveness, life cycle duration: annual vs. perennial), and the use of phylogenetic comparative methods mapping these traits onto a new phylogenetic reconstruction based on existing hypothesized phylogenies of Poaceae, we identified several key findings regarding PPO level, invasiveness, and the phylogeny of Poaceae species.

1. PPO enzyme activity. We related known life history traits with values from our assays for PPO activity, predicting that PPO activity would be found more prevalently in more invasive species, especially invasive *Bromus* species. All bromes tested positively for PPO activity. Most grass genera previously assayed for the enzyme do not express levels of root PPO activity as high as that found in *Bromus* species (Holzapfel et al. 2010). We found that non-*Bromus* species within the Poaceae (grass) family including accessions of *Aegilops longissima, Aegilops speltoides var. speltoides*, (progenitor of wheat), *Avena sterilis* (oats), *Briza maxima* (quaking grass), *Hordeum vulgare* (barley), *Oryza sativa* (rice), *Secale cereale* (rye), and *Triticum aestivum* (wheat) had little or no PPO activity.

Genetics of PPO and PPO expression. Gene expression is not necessarily directly correlated to gene copy numbers. Within the grass family, *Oryza sativa* (rice) has two PPO genes and hexaploid *Triticum spp.* (wheat) has six PPO genes (Tran and Constabel 2011; Tran et al. 2012). We found that *O. sativa* and *T. aestivum* have extremely low levels of PPO activity. Simply finding PPO genes does not necessitate that the genes are expressed and the PPO enzyme product is produced.

2a. PPO activity and invasiveness. Invasive *Bromus* species displayed significantly higher root PPO activity than non-invasive *Bromus* as well as non-invasive non-*Bromus* grass species, but these invasive bromes produce amounts of root PPO levels similar to that of non-*Bromus* invasives (Fig. 4). This suggests that invasiveness is more clearly associated with higher PPO production than taxonomic affiliation alone (e.g., *Bromus* vs. other grasses). This important finding presents a possible future test for predicting invasiveness: by assaying plant roots for PPO activity, high levels suggest the plant may become an invasive. Case in point, the Eurasian invasive grass *Brachypodium sylvaticum* (slender false brome) has been recently reported as invading Ontario, Canada (Miller *et al.* 2012). Predictably, this invasive grass has high levels of PPO - we found mean PPO values of 8.08 (China) and 10.88 (Iran), which are significantly higher than non-invasive PPO levels.

2b. PPO activity and plant life duration. Annual and perennial species of the genus *Bromus* were not found to be different in their PPO activity. Non-*Bromus* annuals produced significantly lower PPO activity than *Bromus* annuals, and lower than either genera (bromes or non-bromes) of perennials. We cannot conclusively relate plant life duration and the PPO trait because PPO seems instead related to genus and invasiveness. There was not a significant difference in PPO levels among invasive annuals or perennials so the trait seems unique to invasiveness rather than life cycle duration, except that annual non-invasives showed significantly less root PPO activity than perennial non-invasives. To determine if this was attributable to the tendency of bromes to be invasive, we further divided those groups in invasive or non-invasive bromes and non-bromes, and still found strong PPO activity among invasives, both brome and non-brome.

3. PPO activity and Poaceae taxonomy.

The taxonomy of grasses is important for understanding when the PPO root enzyme first arose and its activity increased, how plants might have evolved and even coevolved with one another, and how this has been a factor in determining plant community composition. The grasses are derived from a common ancestor, an ancient progenitor, thus they have a shared history, and we can study the selection for or against PPO expression this way (e.g., Darwin 1872).

PPO expression was studied to gain understanding of the evolutionary history of trait. By understanding the grass family, the grass family evolution, and the enzyme PPO, we may be closer to understanding its function as it relates to the distribution of the enzyme in nature. Among grass family relatives of *Bromus*, the PPO gene or expressed trait is sometimes absent, which remains an unsolved evolutionary mystery. Within the genus *Bromus*, there have been several studies regarding the phylogenetic history and structuring of extant species and species within the genus *Bromus* have been classified as one of seven "sections": Boissiera, Bromus, Ceratochloa, Festucaria, Neobromus, Neuskiella, and Stenobromus (e.g., Saarela *et al.* 2008; Smith 1970, 1985; Williams *et al.* 2011). We did not delve into this area of the phylogeny because most bromes have high levels of PPO and can group them into a single clade. The tribes Bromeae and Triticeae shared a common ancestor (Schneider et al. 2009). Triciceae are now believed to be the most genetically close to Bromeae (Saarela et al. 2007), but PPO expression varies greatly between these two tribes, with little or no expression in *Triticum aestivum*.

Brachypodium species stand out as high PPO-producers among branches of little-to-no PPO-producing close relatives, and of all of the Pooideae we assayed. These results

could aid in the understanding the evolutionary history of the grasses. *Brachypodium* is the earliest pooid to diverge among Bromeae, Triticeae, and Poeae according to RFLPs and RAPD nuclear genomic analysis (Catalan et al. 1995). *Brachypodium distachyon* has six copies of the PPO gene (Tran et al. 2012). The high amount of root PPO produced by *Brachypodium* could mean that the ancestral grasses had it originally, a conserved early trait that originated prior to the grasses themselves; alternatively, the trait may have evolved multiple times by convergent evolution. It is possible the PPO gene was lost in *Festuca* and *Triticum* species, as we saw little or no PPO enzyme in assay. Perhaps the PPO activity in *Brachypodium* and *Bromus* roots reflects some common environmental condition experienced early in their evolutionary history. All seven assayed accessions of five species of *Brachypodium* were found to have root PPO levels comparable to those of *Bromus*. *Brachypodium* is the only genus we have found thus far to produce PPO levels similar to bromes. Incidentally, *Brachypodium distachyon* has the common name "purple false brome." In addition, our data are potentially of interest because *Brachypodium* has been proposed as the monocot equivalent of *Arabidopsis* as a model organism for genomic studies whose small genome has recently been sequenced ((Vogel et al. 2010), http://brachypodium.pw.usda.gov/).

One of the genera lacking root PPO activity is *Boissierra*, which has been the subject of some taxonomic uncertainty. *Boissiera* has been considered by some taxonomists, including by the USDA (http://www.ars-grin.gov/ Accessed 2012), to be a member of the genus *Bromus* L. (Species Plantarum, ed. 1, 1753), but the inclusion of *Boissiera* within the genus *Bromus* or as a separate genus has been a matter of contention and remains unresolved. (Smith 1969) suggests abandoning the *Boissiera* genus

altogether as antisera of seed albumin and globulin of *Bromus* and *Boissiera* show serological nearness, whereas Stebbins (Stebbins 1981) named *Boissiera* a subgenus of *Bromus*, which was later called a section (Scholz 1998; Smith 1985). Our PPO activity data argue against the inclusion of *Boissiera* in the genus *Bromus*. Every species of *Bromus* we have assayed has had significant levels of PPO activity in its seedling roots, whereas the *Boissiera* seedlings have little or no activity. While a single trait cannot establish taxonomic position, our data at least support the idea that *Boissiera* is a distinct genus. These results may offer two interpretations: (a) PPO is not the ancestral condition, and *Boissiera* diverged prior to PPO acquisition (b) PPO is the ancestral condition and *Boissiera* lost the activity. The misclassification of *Boissiera* in the genus *Bromus* implies that the lack of PPO activity is an ancestral condition and that *Boissiera* diverged from the brome lineage before constitutive PPO activity was acquired. The molecular phylogeny suggests the alternate possibility that *Boissiera* is derived from ancestors that had high levels of PPO activity, but that it has lost that activity, perhaps due to a change in the soil microorganisms or other selective factors. If the latter hypothesis is true, then we would predict that *Boissiera* still has the gene for the enzyme but that it is regulated differently or has been somehow inactivated.

These new findings highlight ongoing taxonomic classifications and may shed light on evolutionary understanding of the PPO enzyme and grass evolution. From the consensus phylogenetic tree with PPO level mapped as a heat map, it is evident that PPO is somewhat tractable, offering two interpretations: (a) reliance on PPO or PPO expression was much lower in the ancestor or (b) PPO is the ancestral condition and some genera lost the activity, as is suggested by the phylogeny. These new findings point

to PPO as a trait correlated to invasiveness, and highlight ongoing taxonomic classifications that may shed light on evolutionary understanding of selection benefits of the PPO enzyme and grass evolution.

Role of PPO. Functions of PPO are unresolved, and the role of PPO is debated (Aniszewski et al. 2008). There are a multitude of functions of phenolic oxidases, not just defense (e.g., anti-predator, anti-competitor) and ontogeny (e.g., metabolism of auxin, plant defense, and lignin and suberin formation), but also some of great ecological importance: from detoxifying phenolics, acquiring carbon and nitrogen, and alleviating oxidative stress (e.g., reduction of H_2O_2), to rehabilitating environmental degradation (e.g., of lignin through depolymerization), mineralization, and transformation of soil organic matter (Dorantes and Zúñiga 2012; Sinsabaugh 2010; Tran et al. 2012). PPO as a plant defense may be a reason for evolutionary persistence of the enzyme, and may explain the high activity in some species.

Here, we saw invasive *Bromus* species have significantly higher root PPO than non-invasive *Bromus* species. Furthermore, non-invasive *Bromus*, have significantly lower root PPO than invasive non-bromes. The invasive bromes have amounts of root PPO similar to that of non-*Bromus* invasives; clearly, the PPO trait is linked to invasiveness.

Caveats of phylogenetic studies. Convergent evolution can confound phylogenetic studies. For example, C4 photosynthesis, characterized by Kranz leaf anatomy, has evolved multiple times. Members of the grass family can be either C3 (cool season) or C4 (warm season), therefore we cannot establish phylogeny based on these photosynthetic distinctions. However, this example of parallel adaptation among others

can be helpful in studying mechanisms and processes involved in evolution (Olsen and Wendel 2103). Furthermore, dynamic environments cause continuing genomic changes, further confounding evolutionary studies. Further still, the evolutionary trees hold one assumed rate of evolution for all taxa in the tree. Chromosomal rearrangements will become fixed as a result of positive selection from changing environments, if adaptive. Thus, in general, discovering origins of the more than 4,500 C4 plants through phylogenetics has been difficult because of numerous evolutions (4 evolutions, (Kellogg 2001); (17-20 evolutions, (Edwards et al. 2010).

Future research. There are several areas needing further research regarding PPO. Although the distribution, location, properties, and structure are well-understood, the exact biological function of PPO remains puzzling (Mayer 2006). A possible function for PPO may be for immunity and protection in animal organisms, but in plants, the activity seems undirected, unlike in fungi where PPOs purpose is likely (1) defense, and (2) offense or attack (Aniszewski et al. 2008). Browning itself may play a role in deferring herbivory as unappetitive, yet this remains to be definitively shown (Aniszewski et al. 2008). Other enigmatic characteristics of PPO involve the PPO reaction sequence, from PPO gene activation, PPO formation, where PPO is synthesized, to defining the target site of PPO (Mayer 2006). How PPO present in its latent form is activated is unclear (Mayer 2006). Studying the substrates for PPO may aid in understanding the enzyme activation.

CONCLUSION

Generally, root PPO was found more prevalently in invasive species and in bromes. Non-invasive grasses (both brome and non-brome) had little or no PPO. This suggests that the ability to produce high root PPO concentrations may be a trait that contributes to invasion potential of non-native species. Screening species for such a trait therefore can provide the opportunity to identify future invaders. This important corollary may serve as a useful tool for identifying future invasive species.

ACKNOWLEDGEMENTS

Thank you to the USDA GRIN for providing germplasm and Dominic Evangelista for help with phylogenetic reconstructions.

APPENDIX

Figures A 1 – A 49 display the time-dependent activity of PPO as determined by spectrophotometric assay using L-DOPA (5 mM) as a substrate and a seedling root of approximately 1 cm as the enzyme source. Figures A 50 and A 51 represent phylogenetic reconstructions and likelihoods for single character traits (plant life duration and PPO).

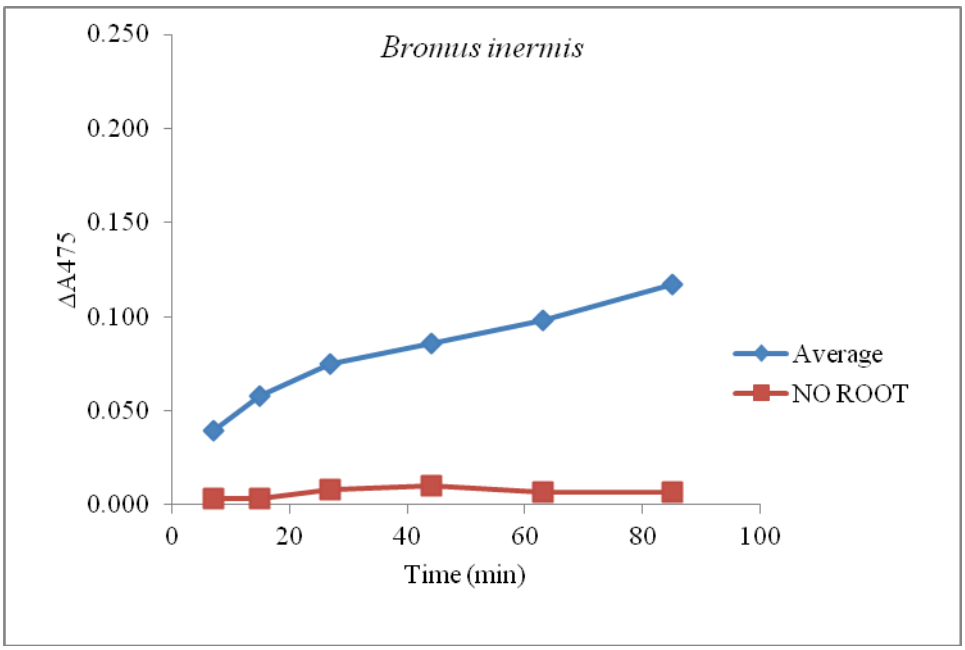


Fig. A 1. PPO enzyme assay results of root and blank control (for each data point, n = 5).

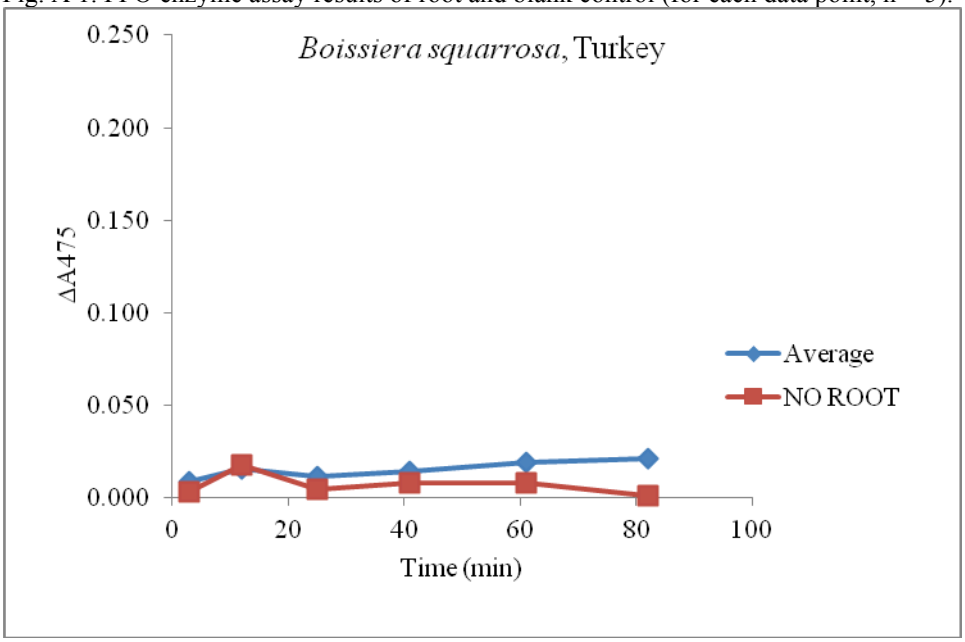


Fig. A 2. PPO enzyme assay results of root and blank control (for each data point, n = 5).

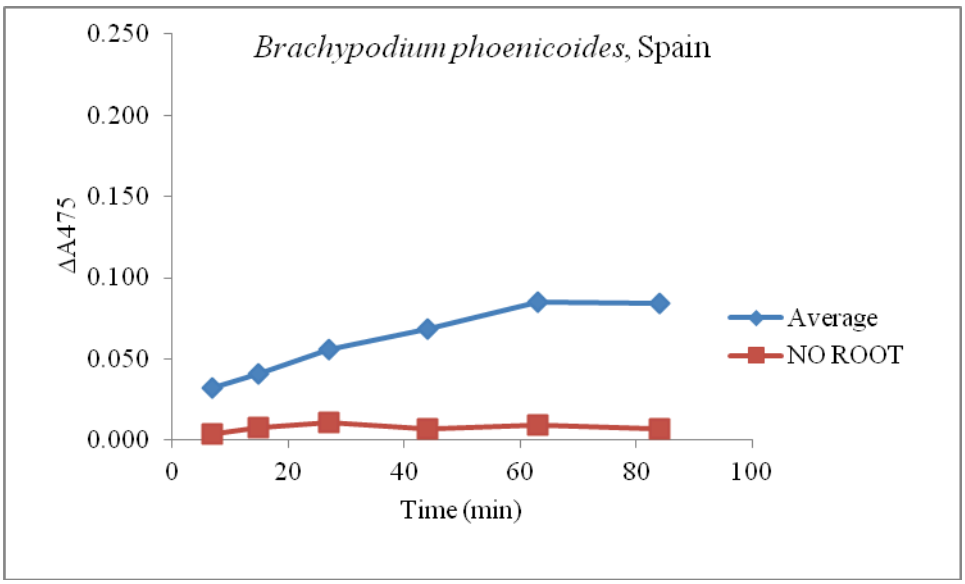
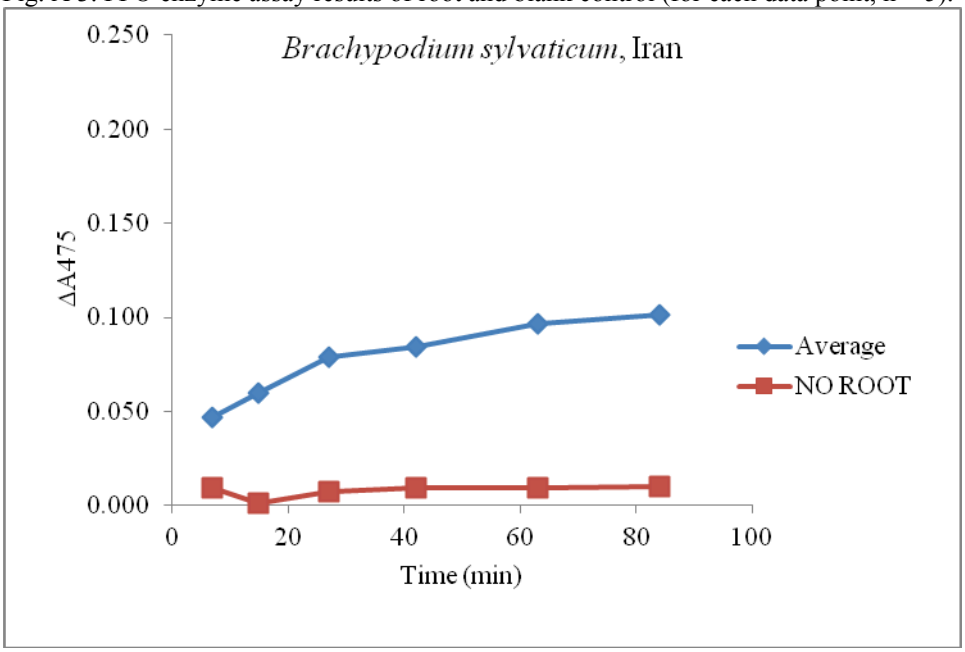


Fig. A 3. PPO enzyme assay results of root and blank control (for each data point, n = 5).



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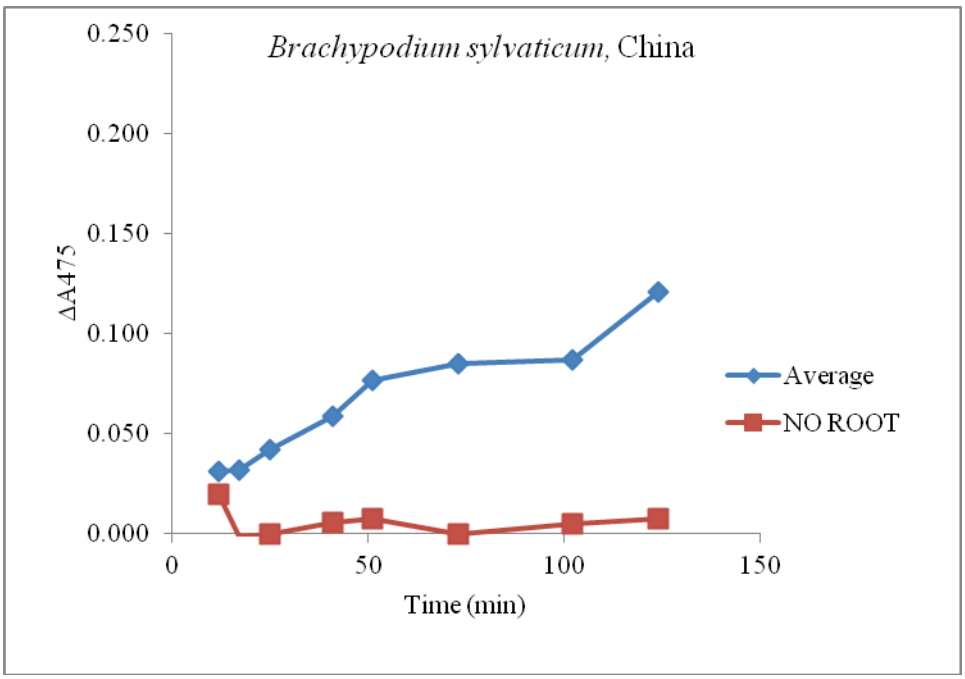

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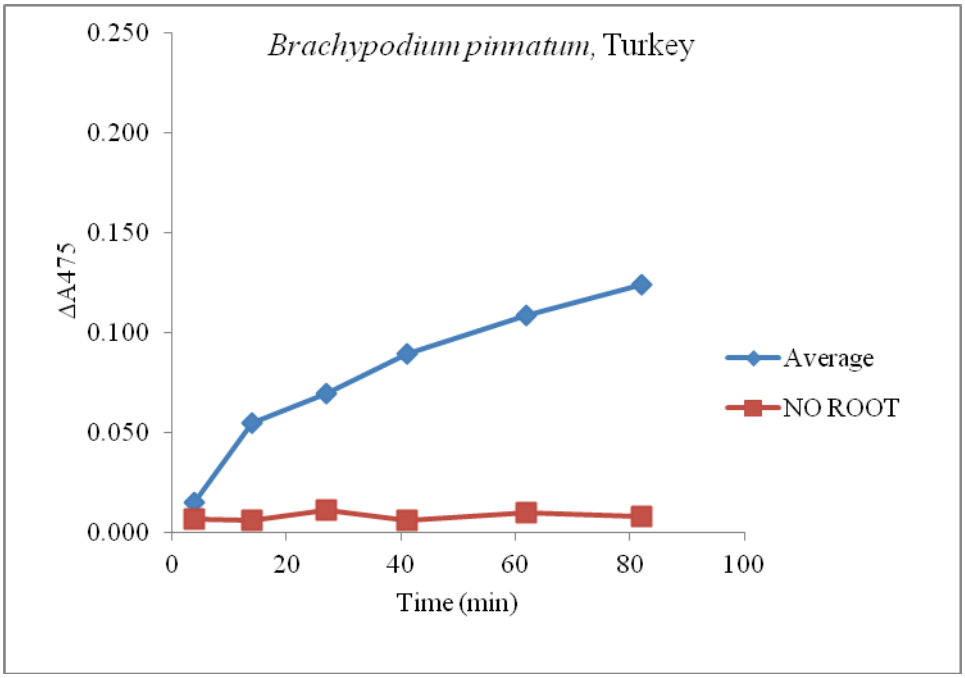

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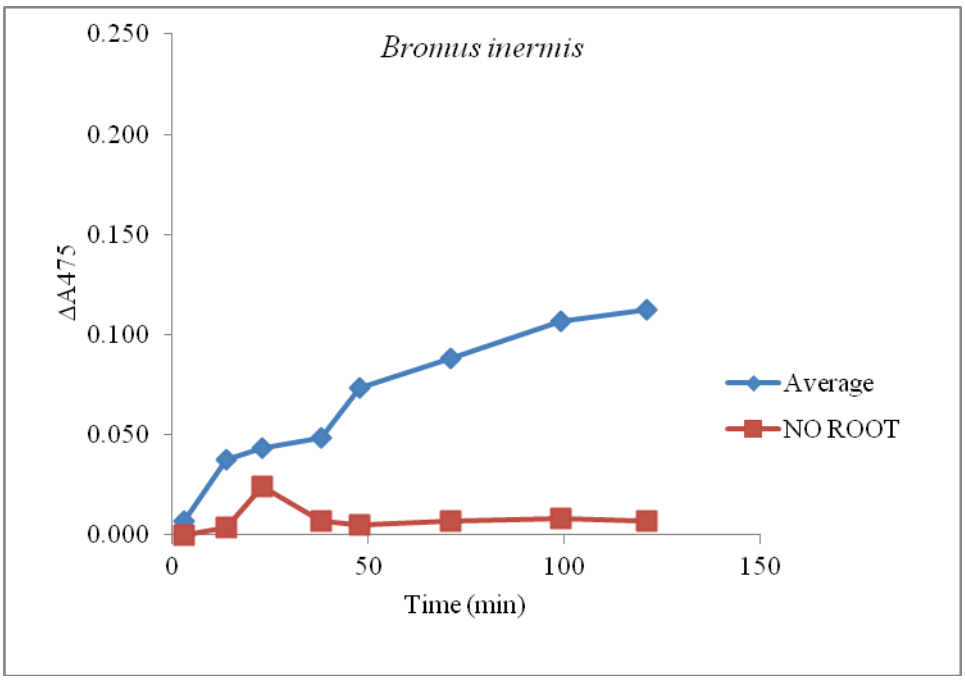
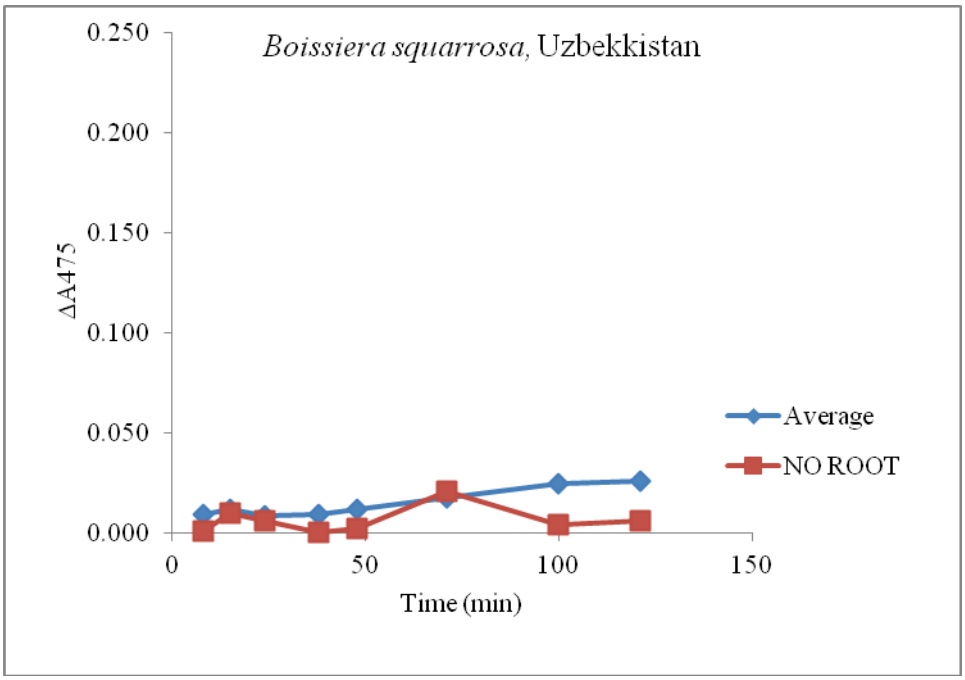


Fig. A 7. PPO enzyme assay results of root and blank control (for each data point, n = 5).



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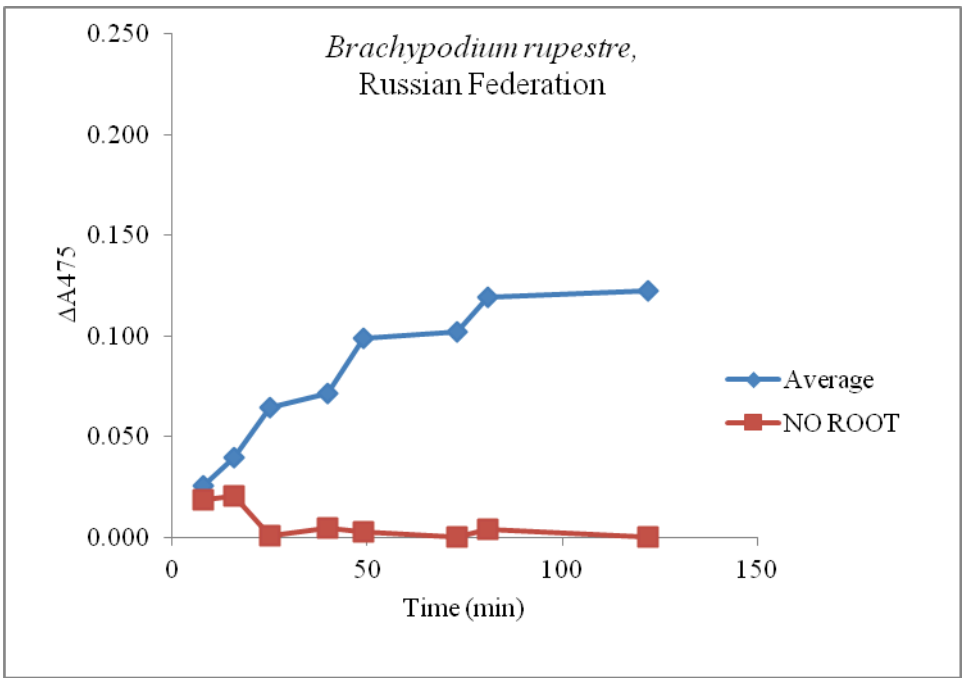

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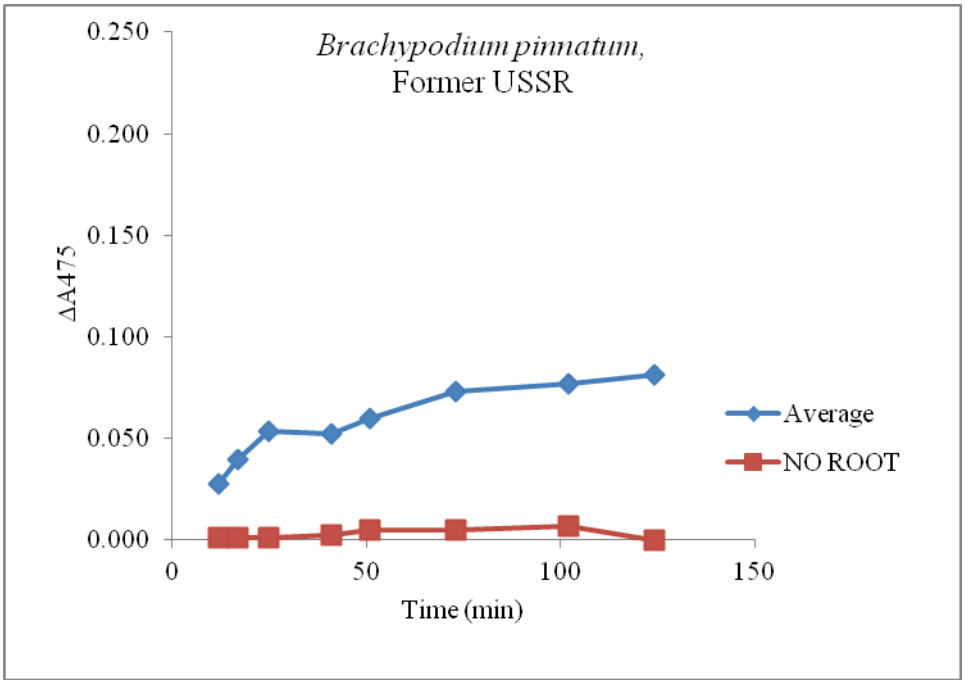

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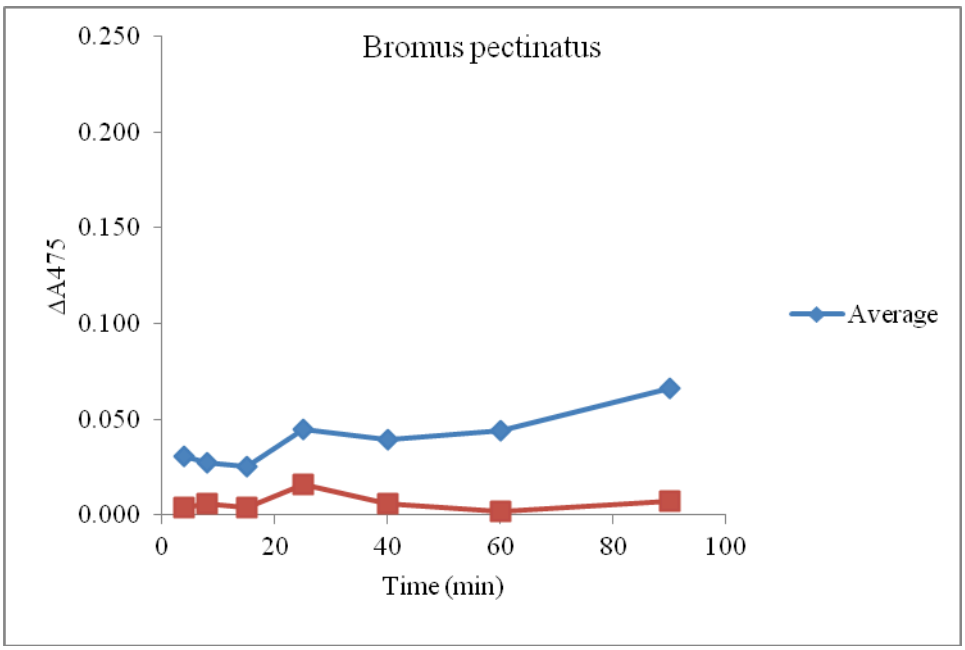

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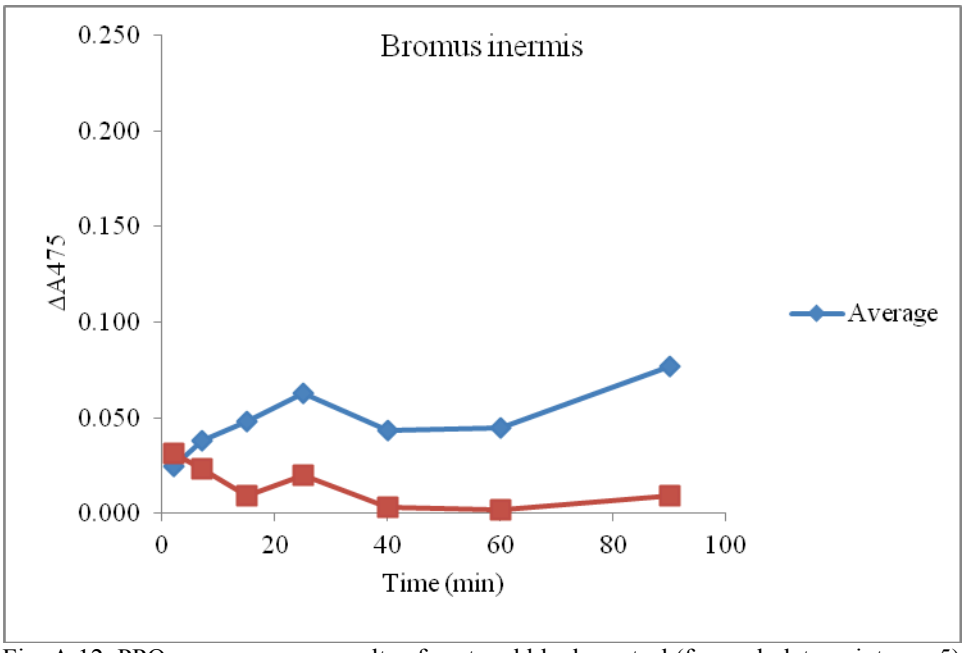

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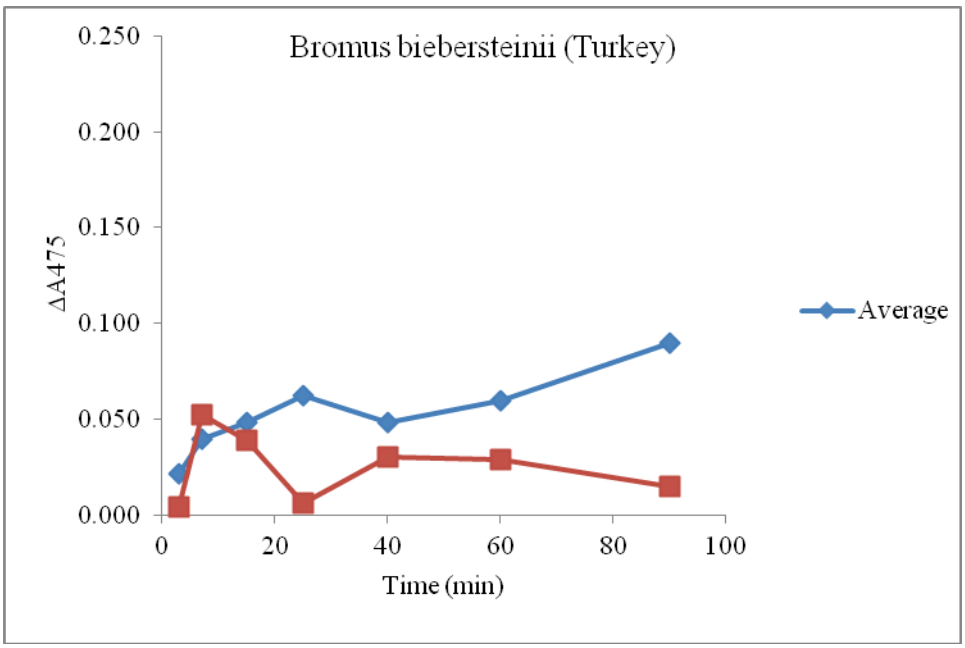


Fig. A 13. PPO enzyme assay results of root and blank control (for each data point, n = 5).

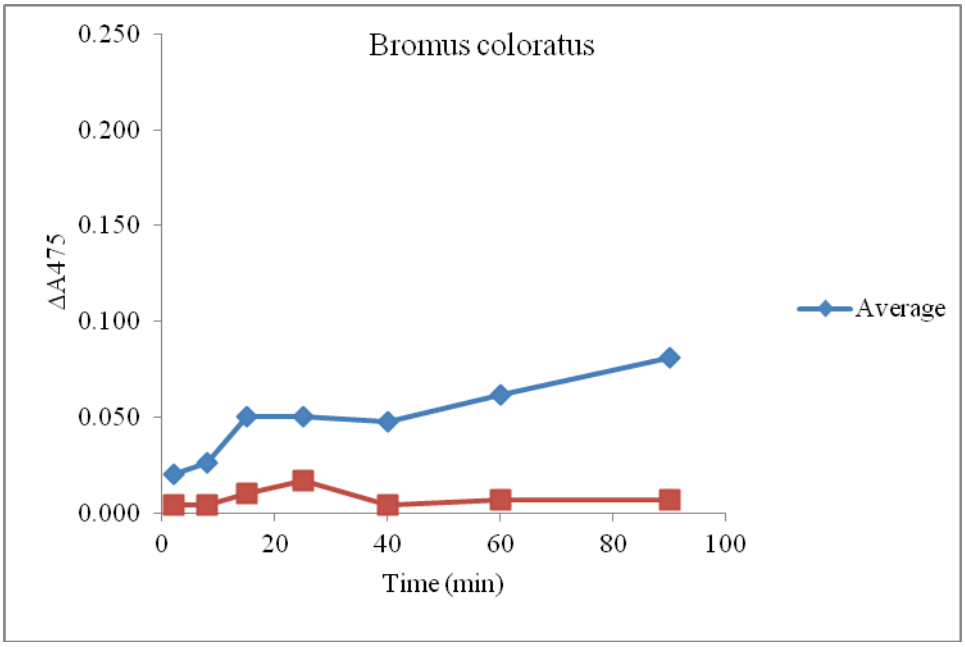
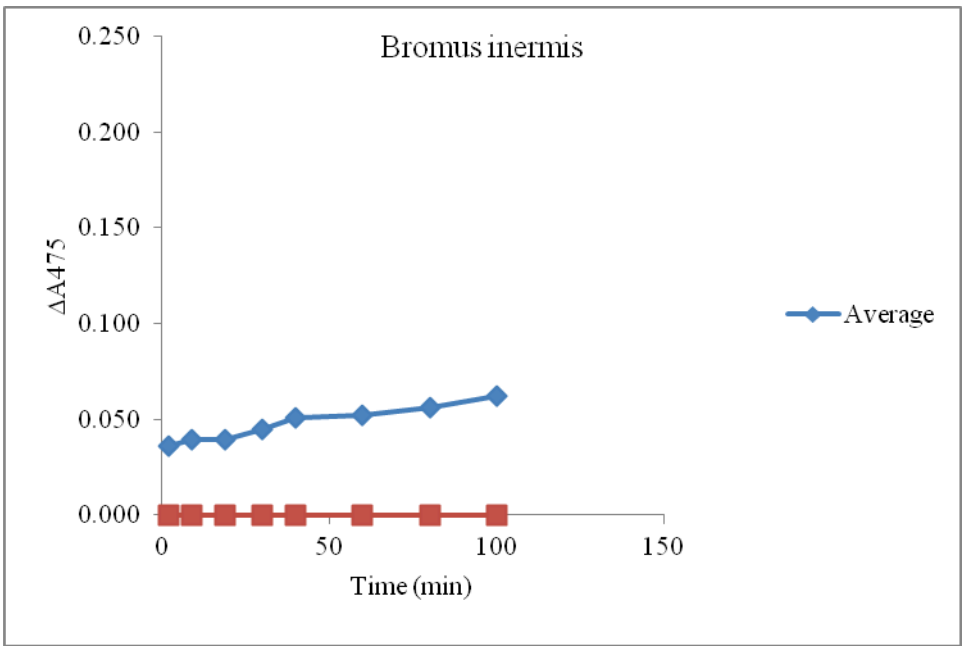


Fig. A 14. PPO enzyme assay results of root and blank control (for each data point, n = 5).



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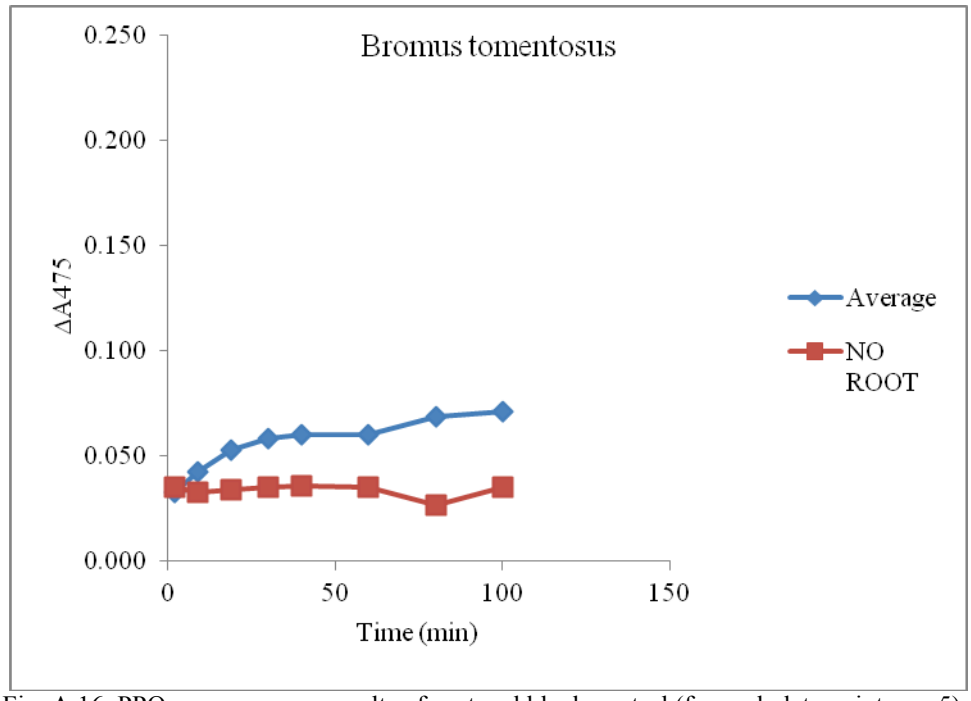

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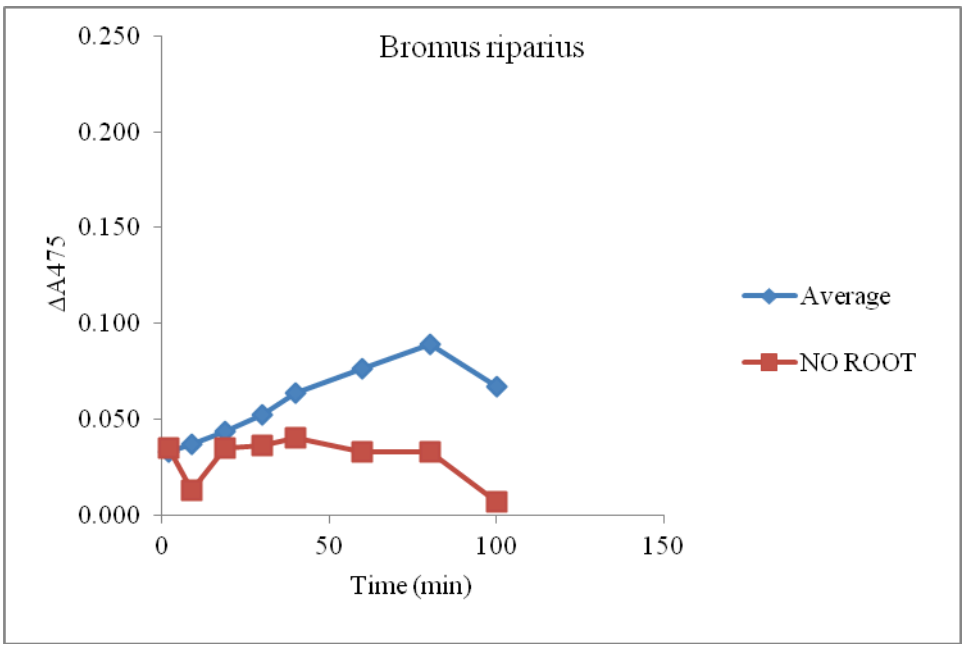

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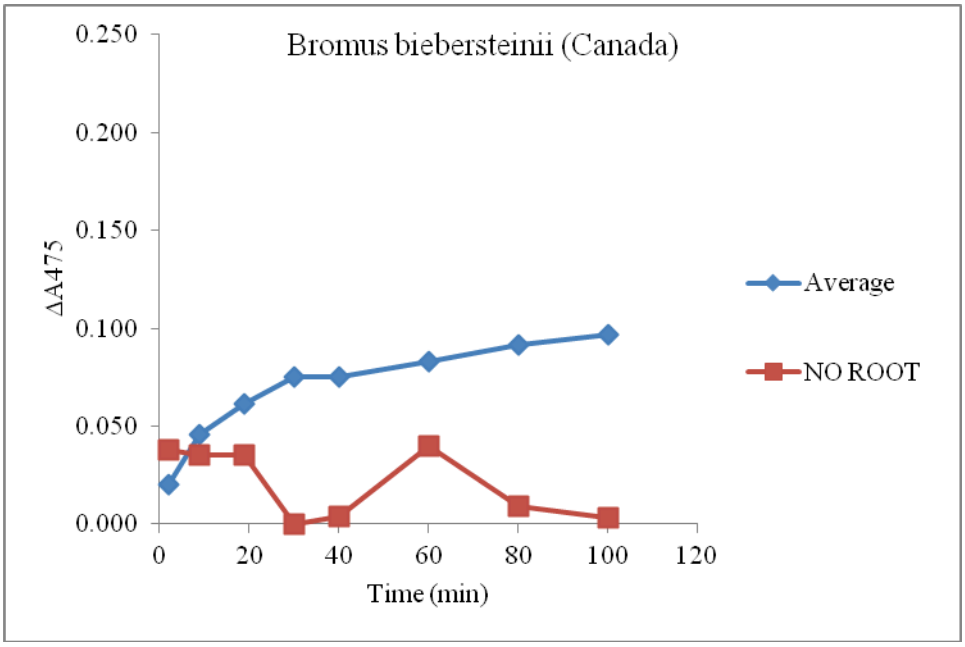

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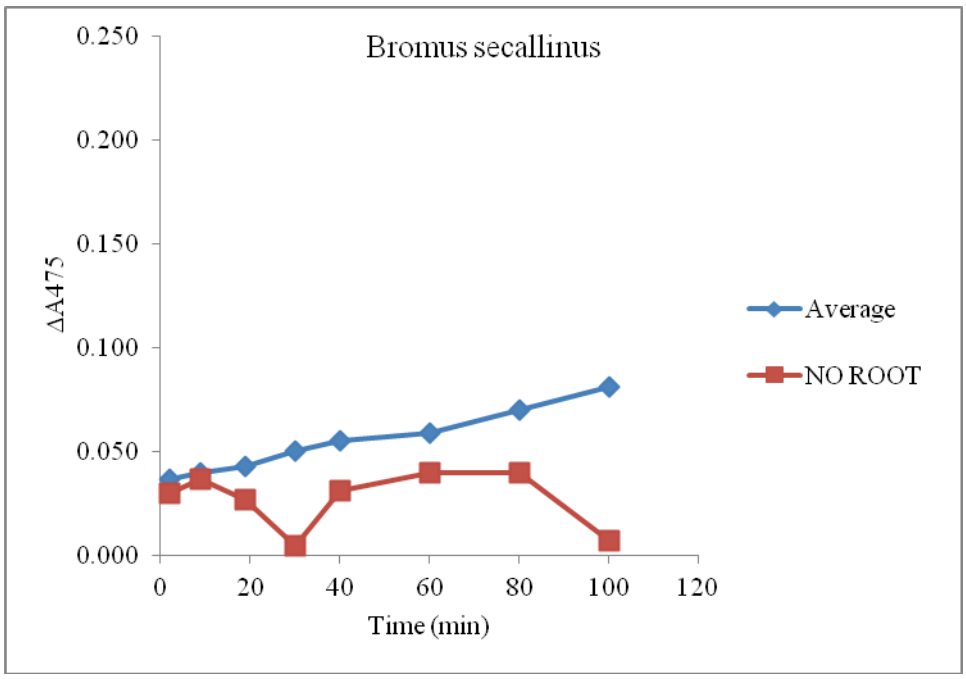

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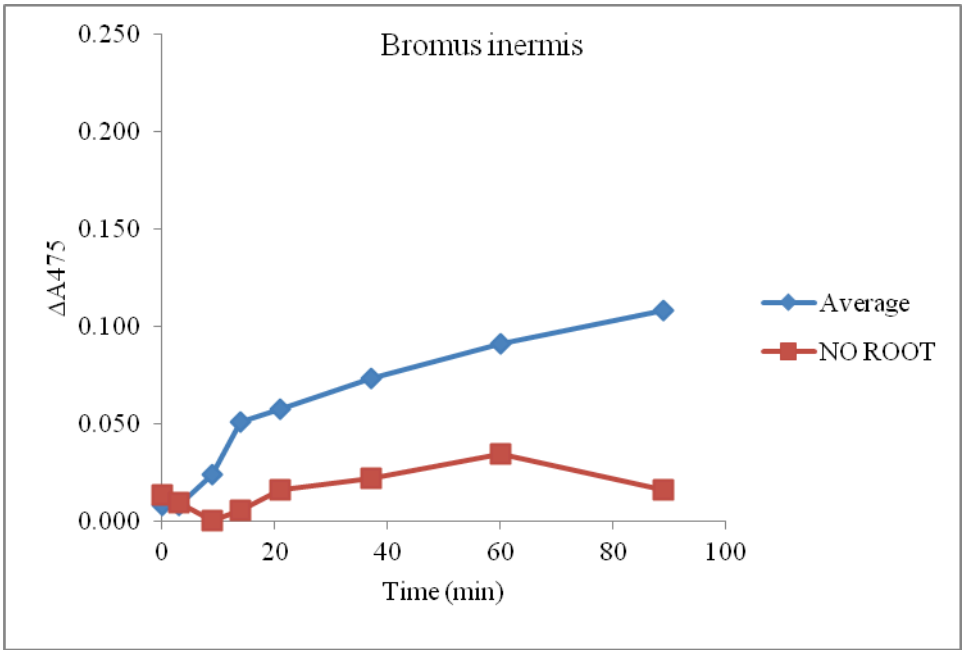

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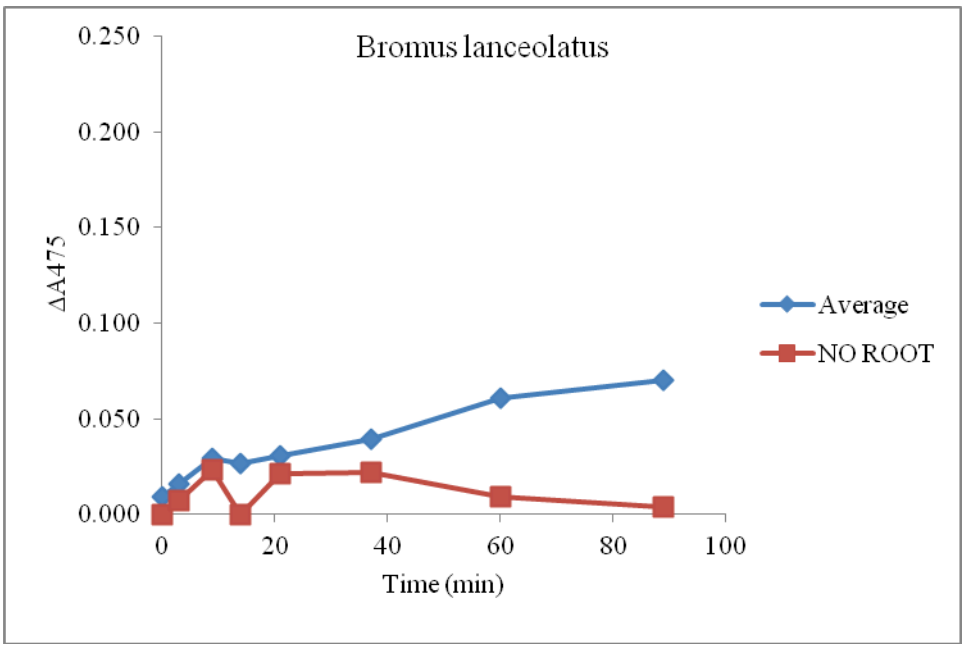

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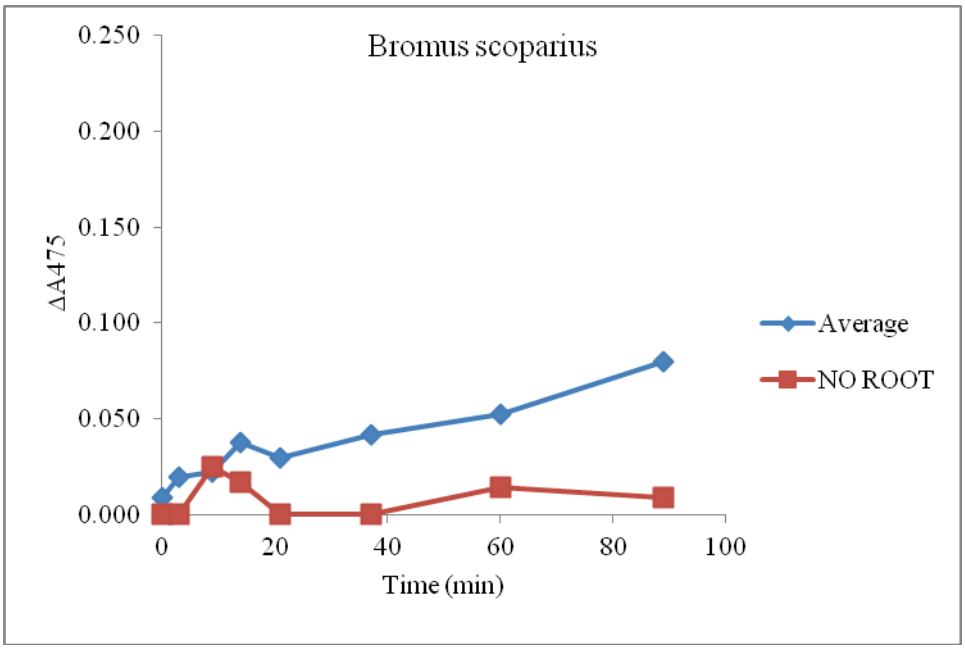

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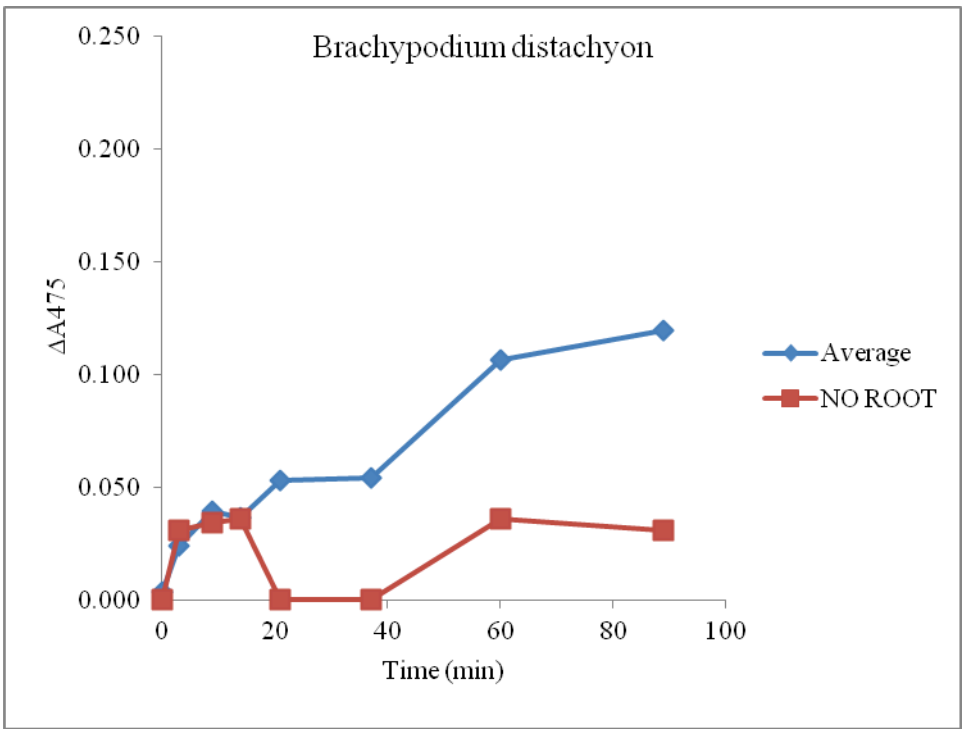

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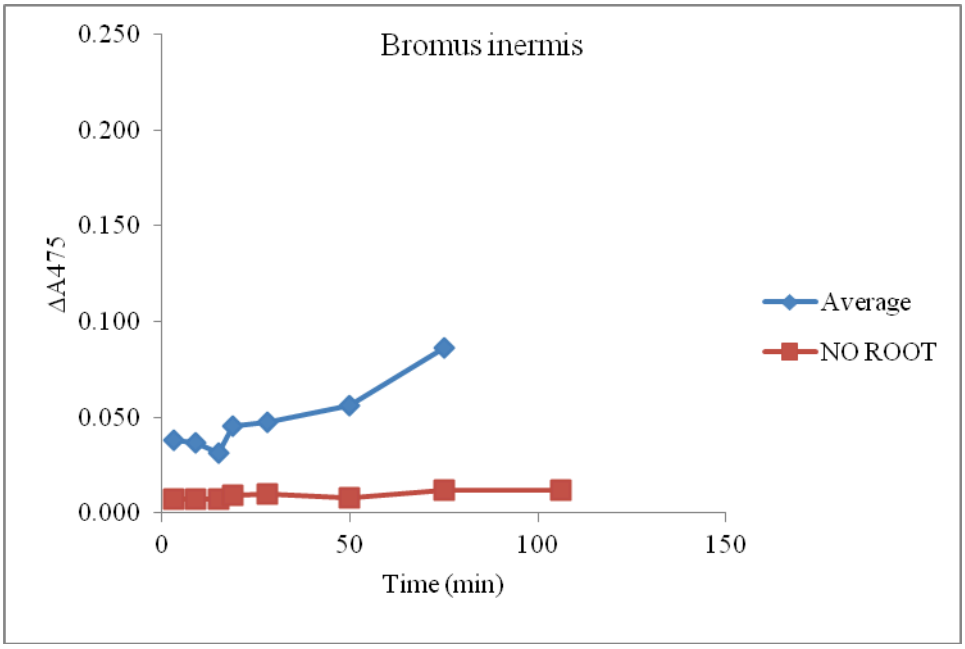

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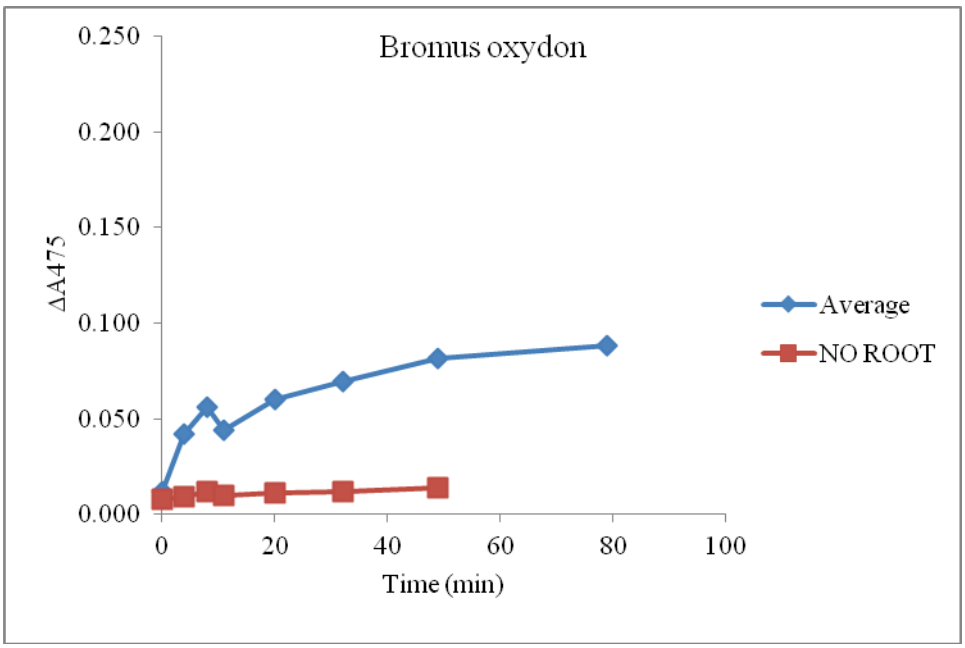

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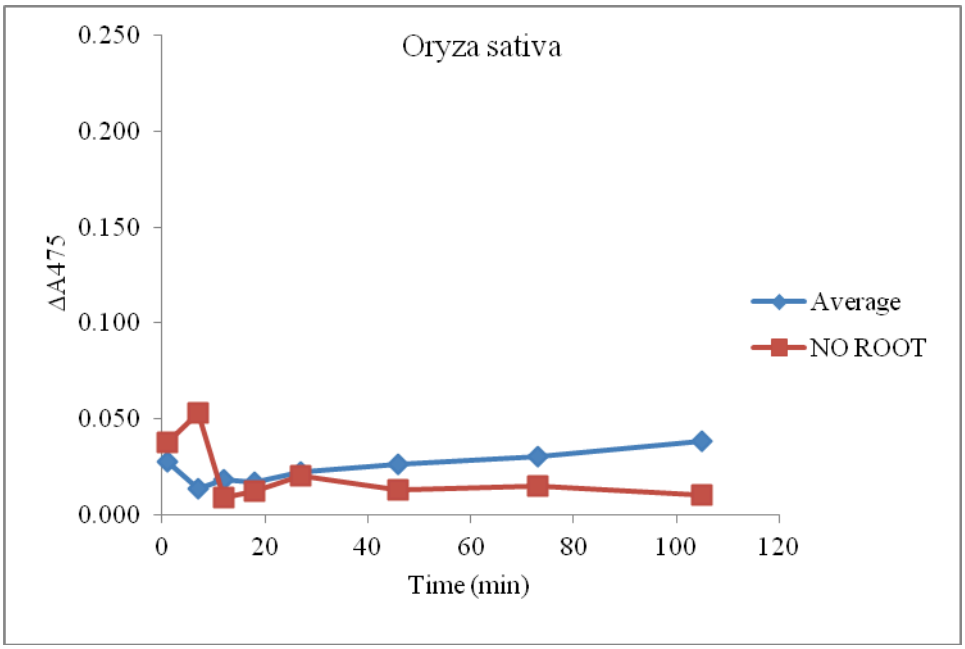

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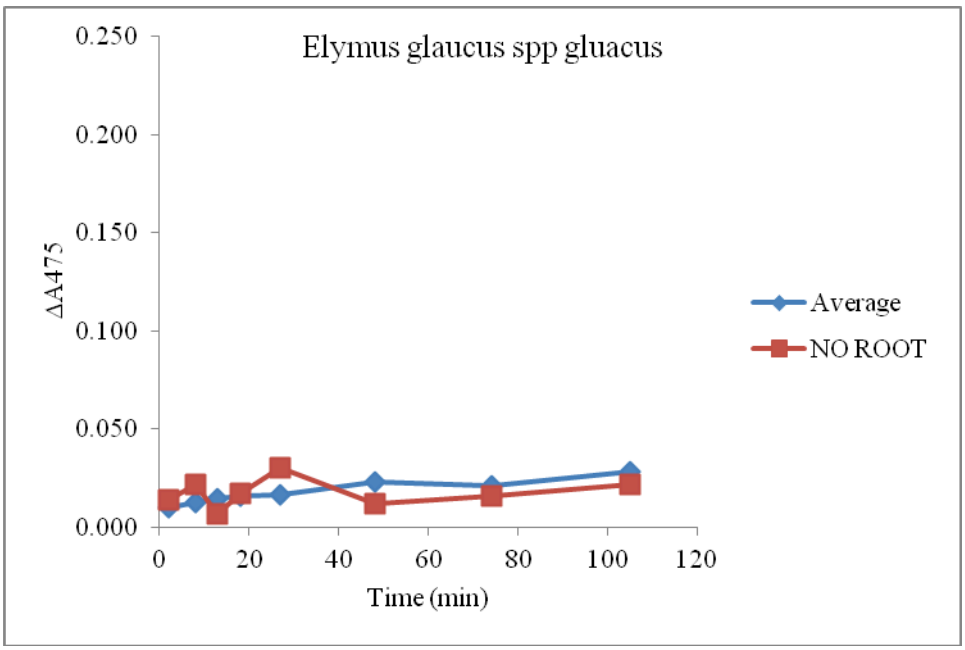

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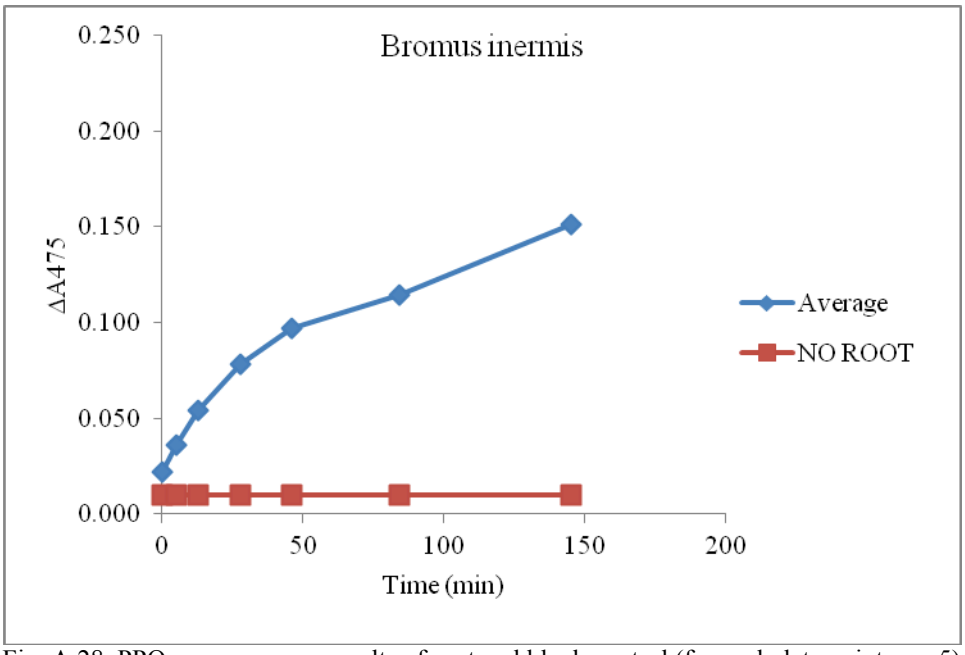

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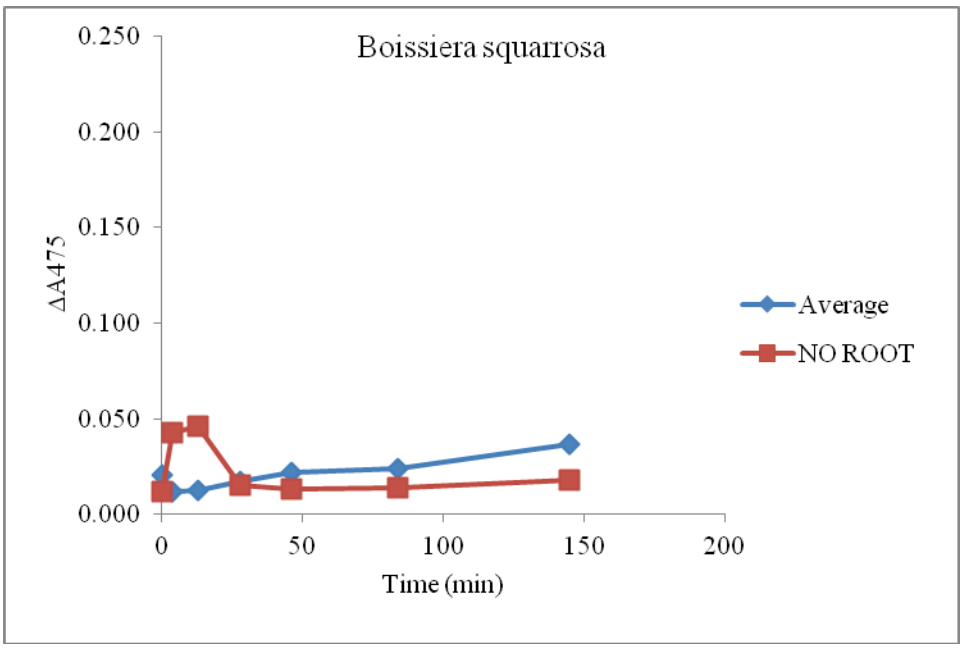

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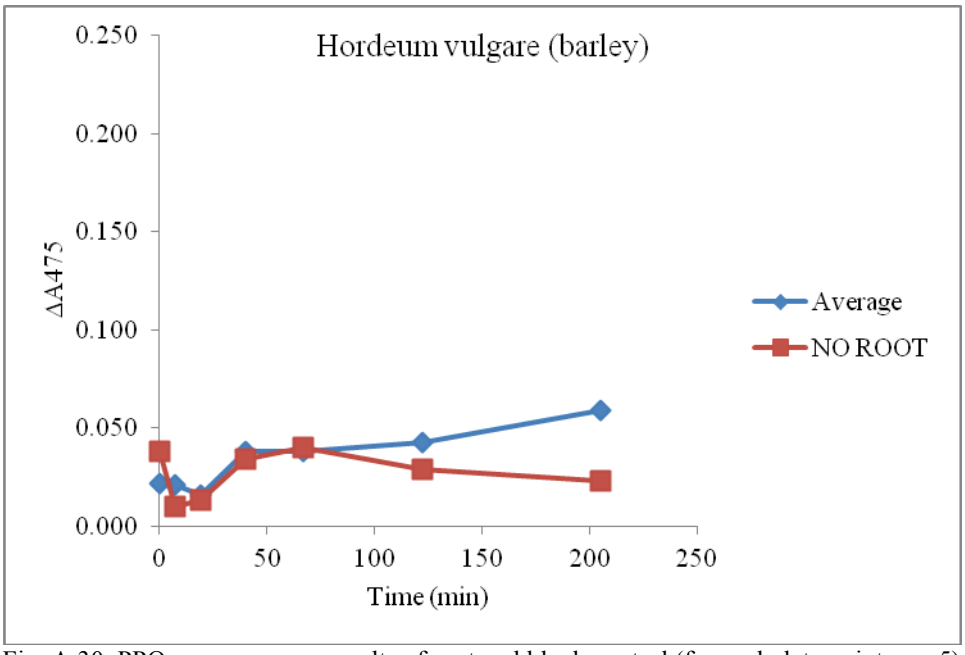

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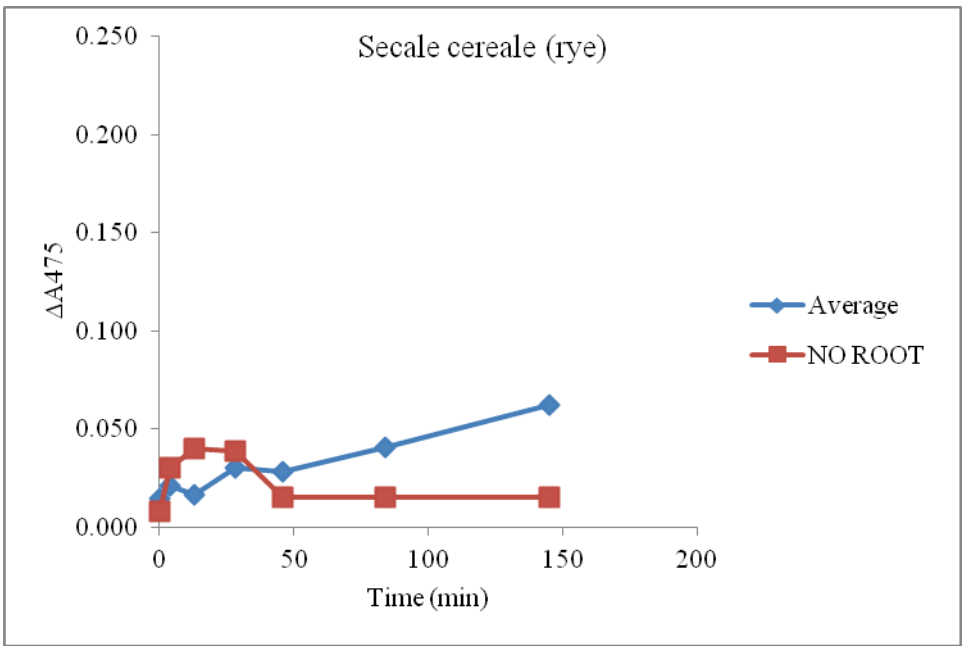

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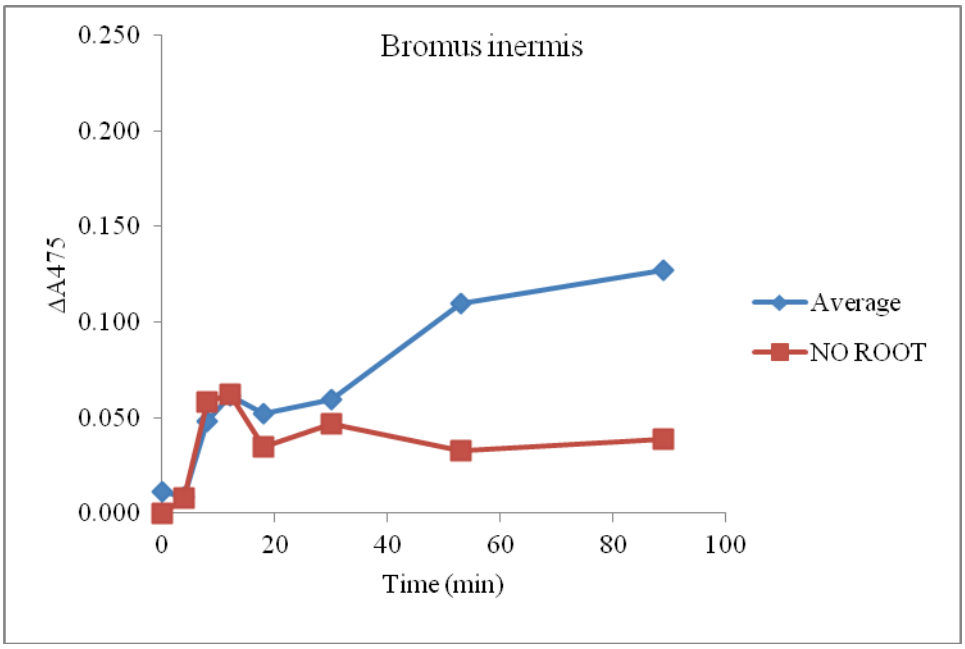


Fig. A 32. PPO enzyme assay results of root and blank control (for each data point, n = 5).

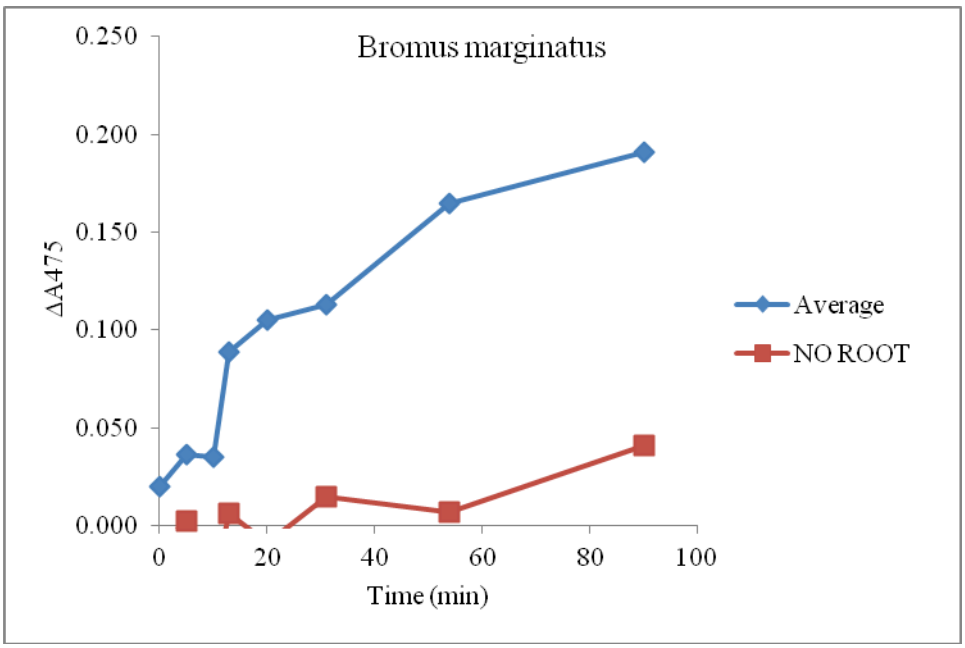


Fig. A 33. PPO enzyme assay results of root and blank control (for each data point, n = 5).

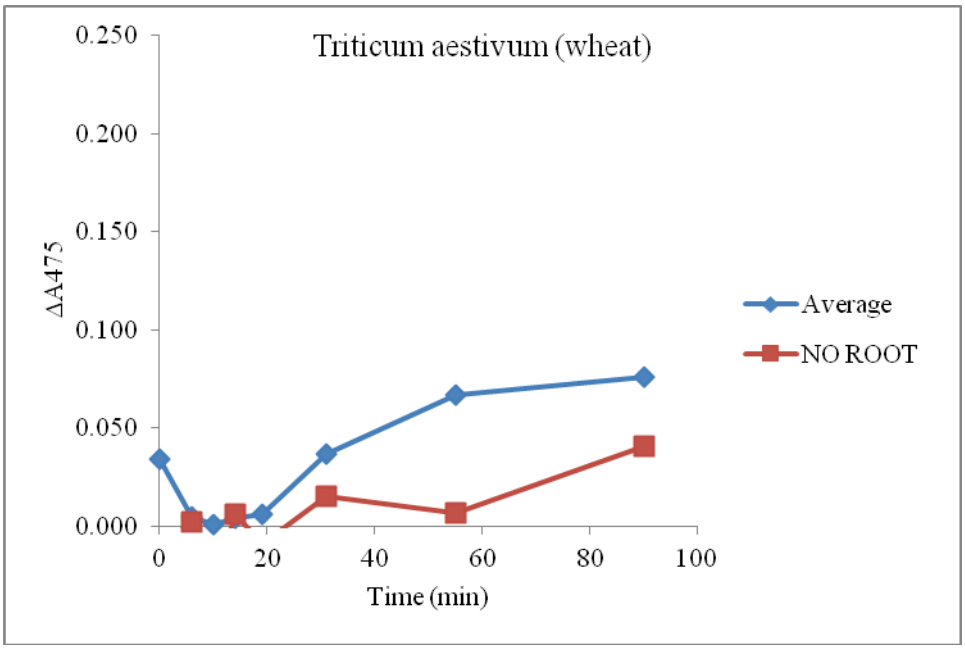


Fig. A 34. PPO enzyme assay results of root and blank control (for each data point, n = 5).

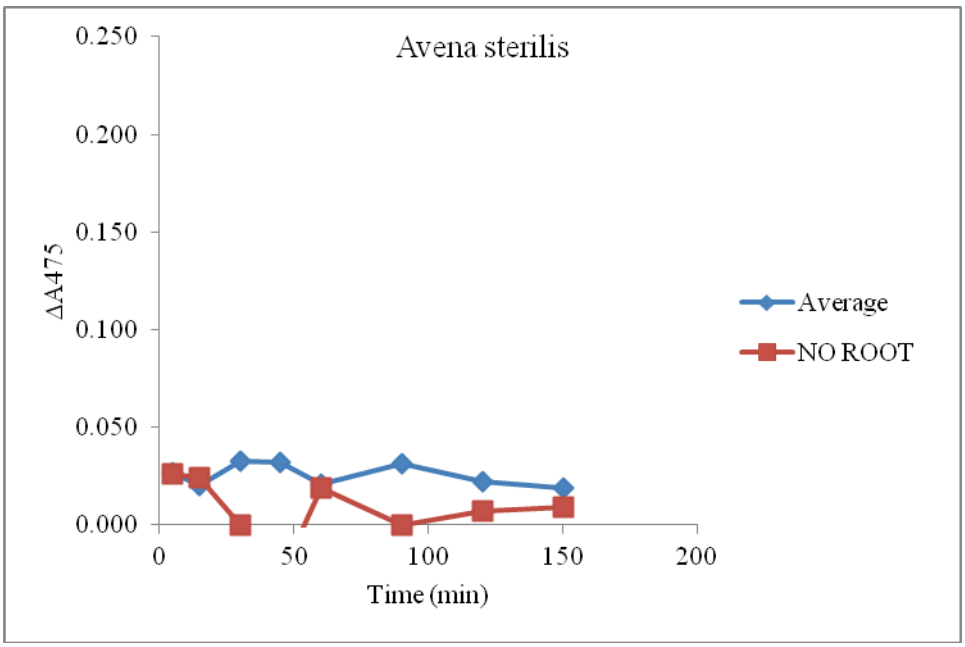
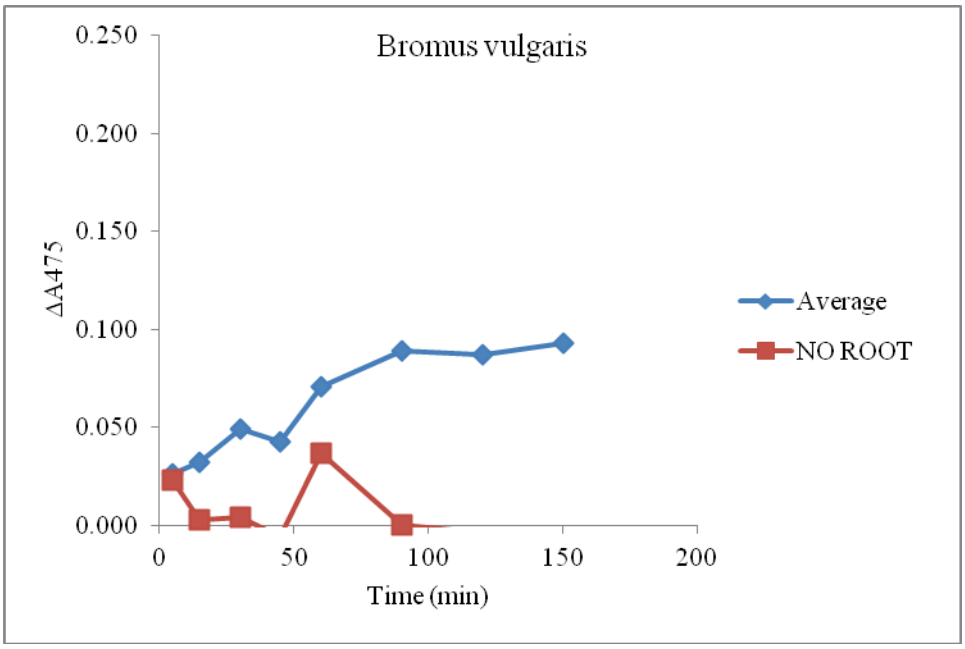


Fig. A 35. PPO enzyme assay results of root and blank control (for each data point, n = 5).



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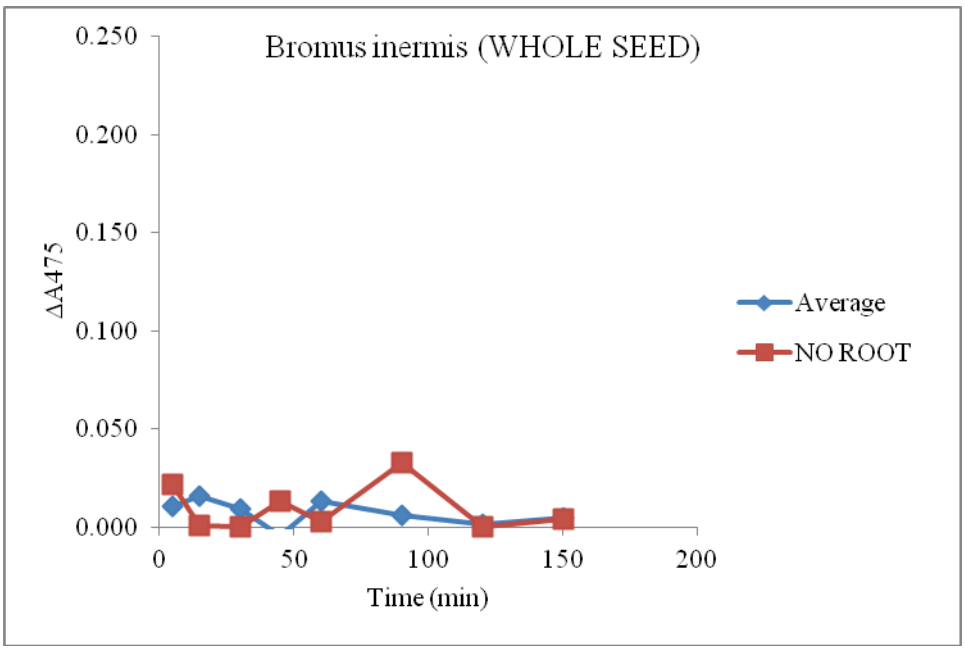

Fig. A 37. PPO enzyme assay results of root and blank control (for each data point, n = 5).

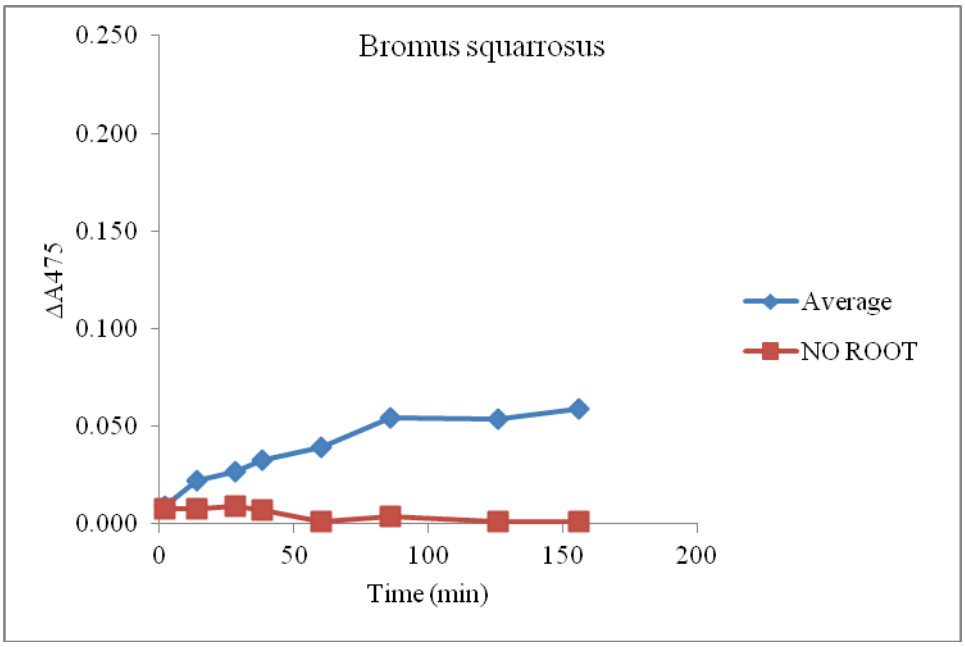

Fig. A 38. PPO enzyme assay results of root and blank control (for each data point, n = 5).

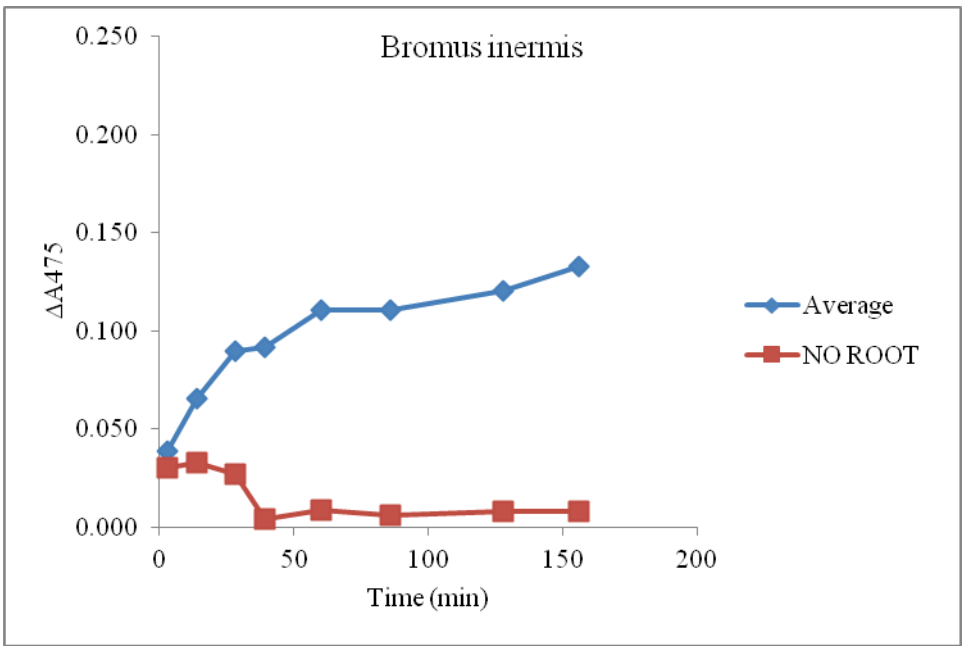

Fig. A 39. PPO enzyme assay results of root and blank control (for each data point, n = 5).

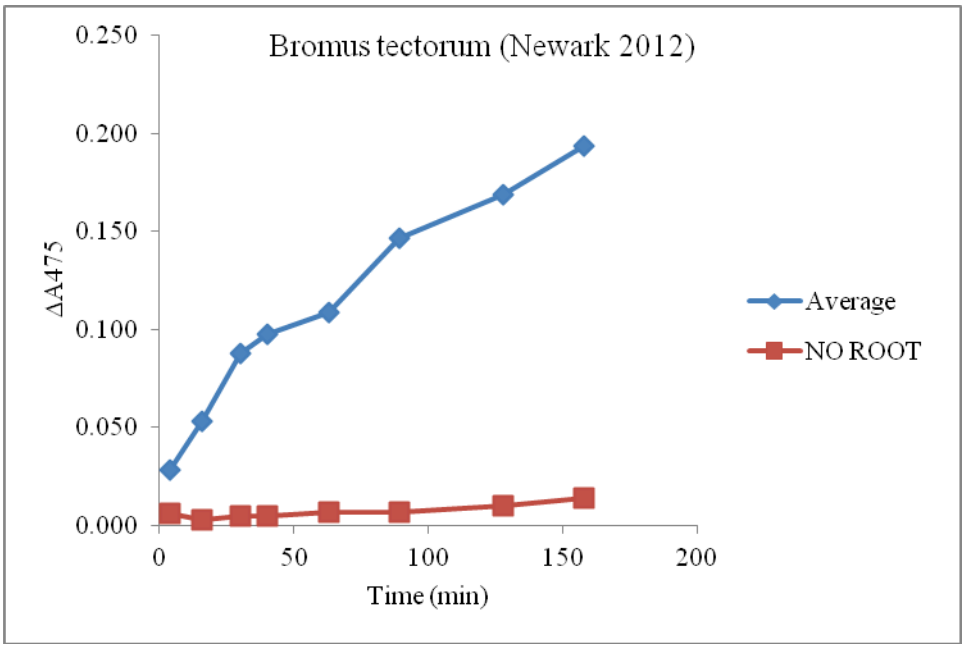

Fig. A 40. PPO enzyme assay results of root and blank control (for each data point, n = 5).

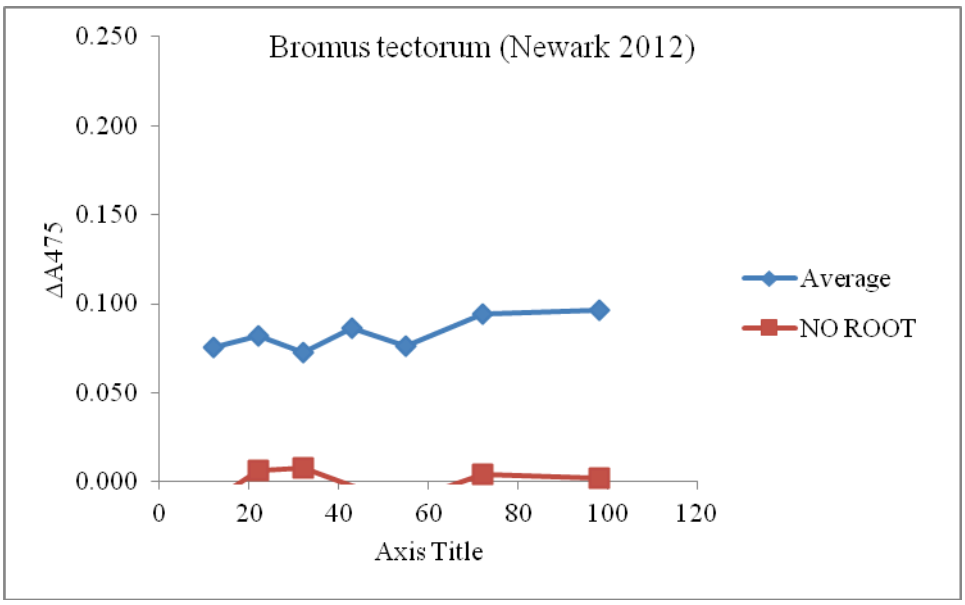

Fig. A 41. PPO enzyme assay results of root and blank control (for each data point, n = 5).

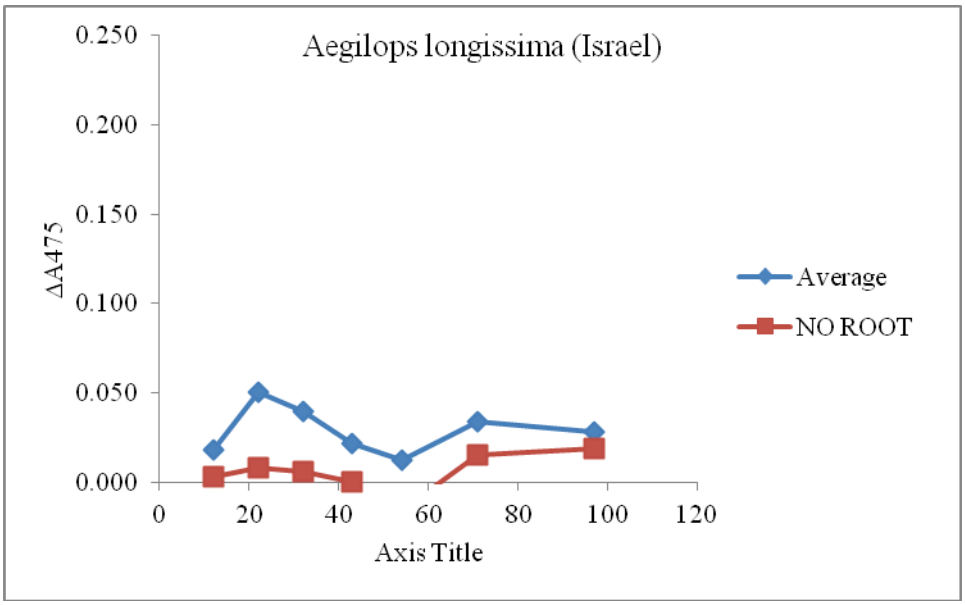

Fig. A 42. PPO enzyme assay results of root and blank control (for each data point, n = 5).

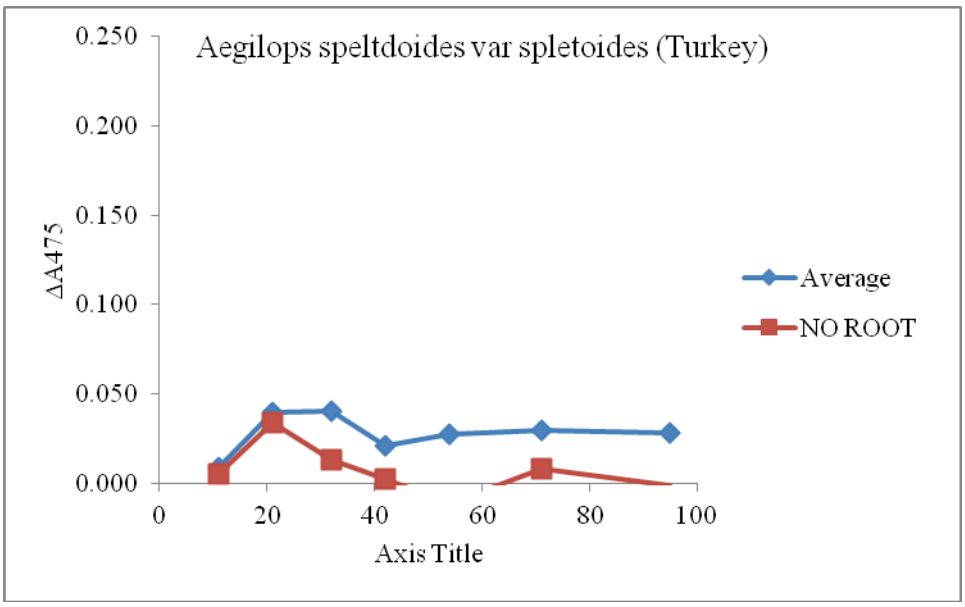

Fig. A 43. PPO enzyme assay results of root and blank control (for each data point, n = 5).

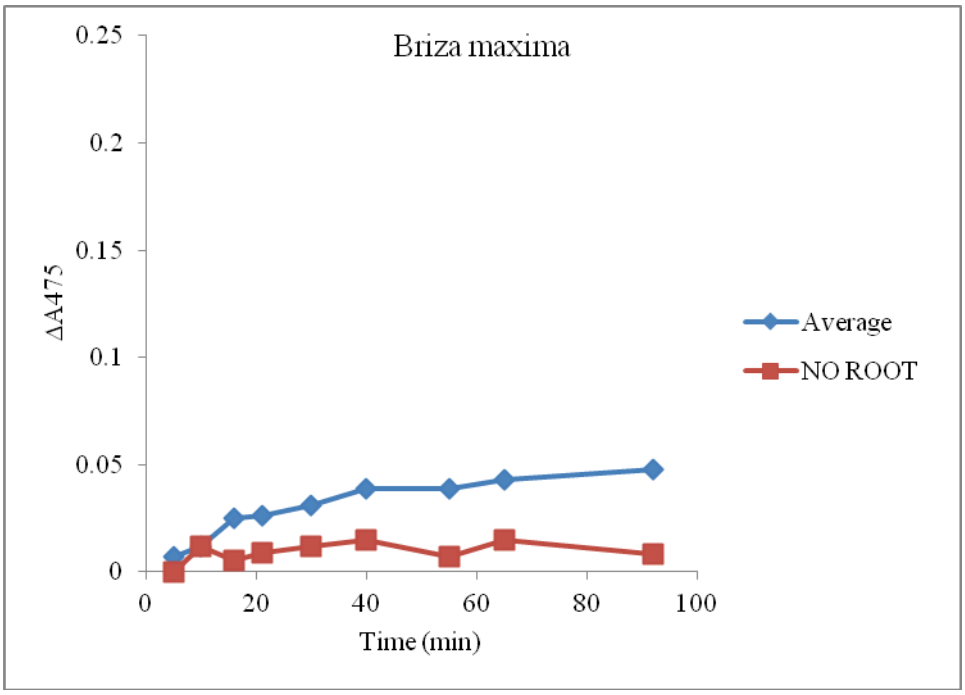

Fig. A 44. PPO enzyme assay results of root and blank control (for each data point, n = 5).

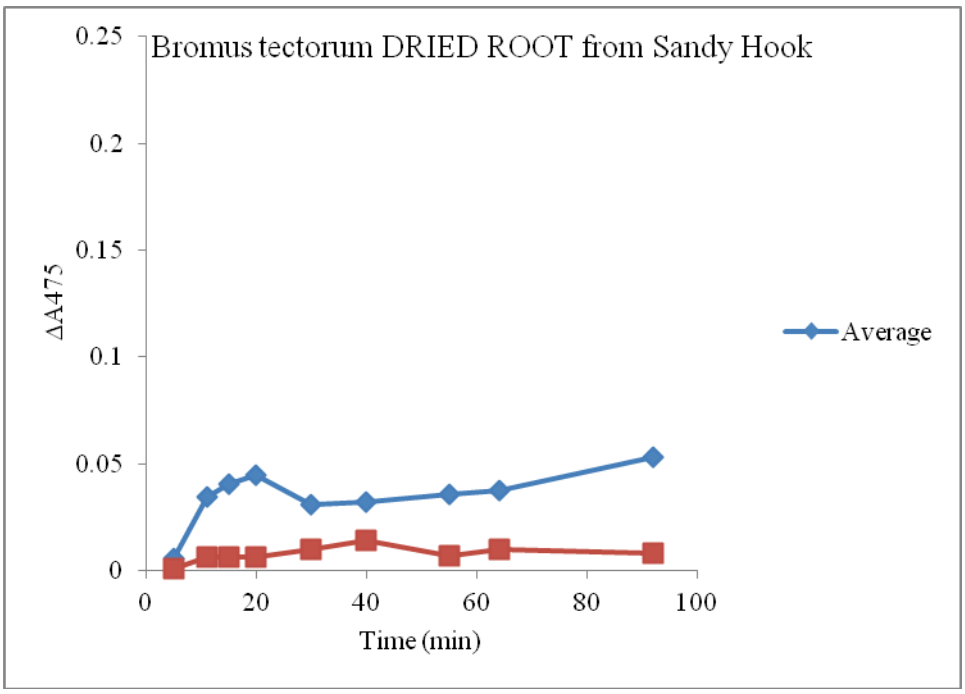

Fig. A 45. PPO enzyme assay results of root and blank control (for each data point, n = 5).

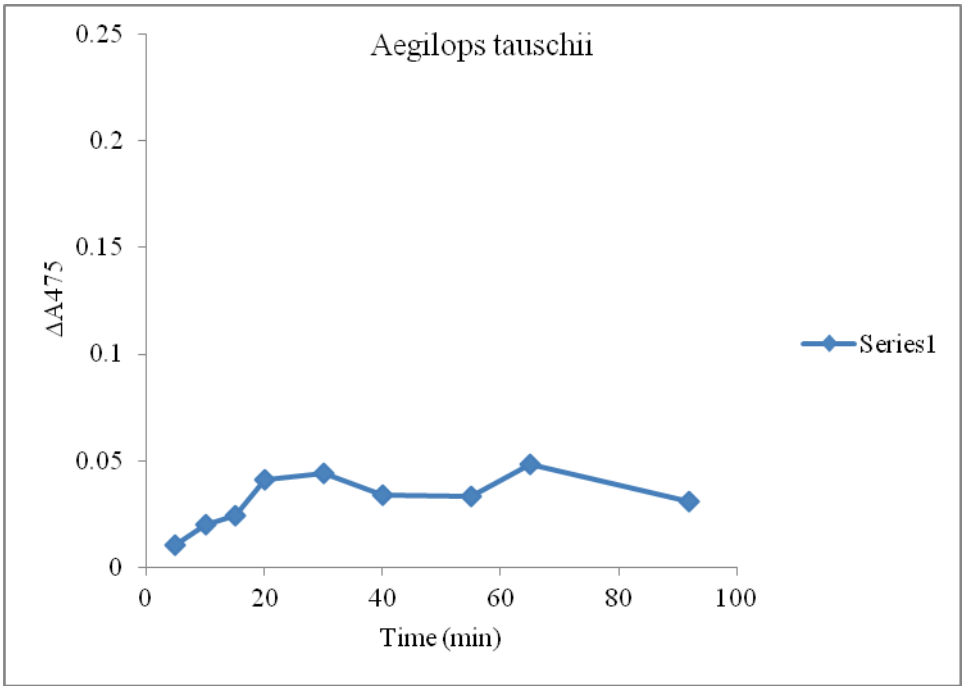

Fig. A 46. PPO enzyme assay results of root (for each data point, n = 5).

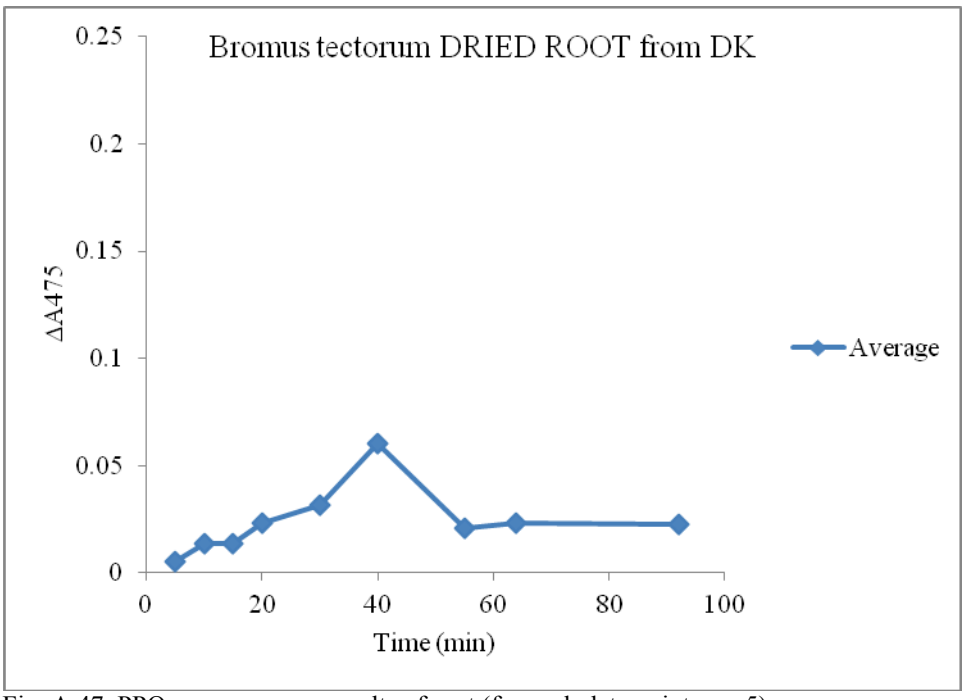

Fig. A 47. PPO enzyme assay results of root (for each data point, n = 5).

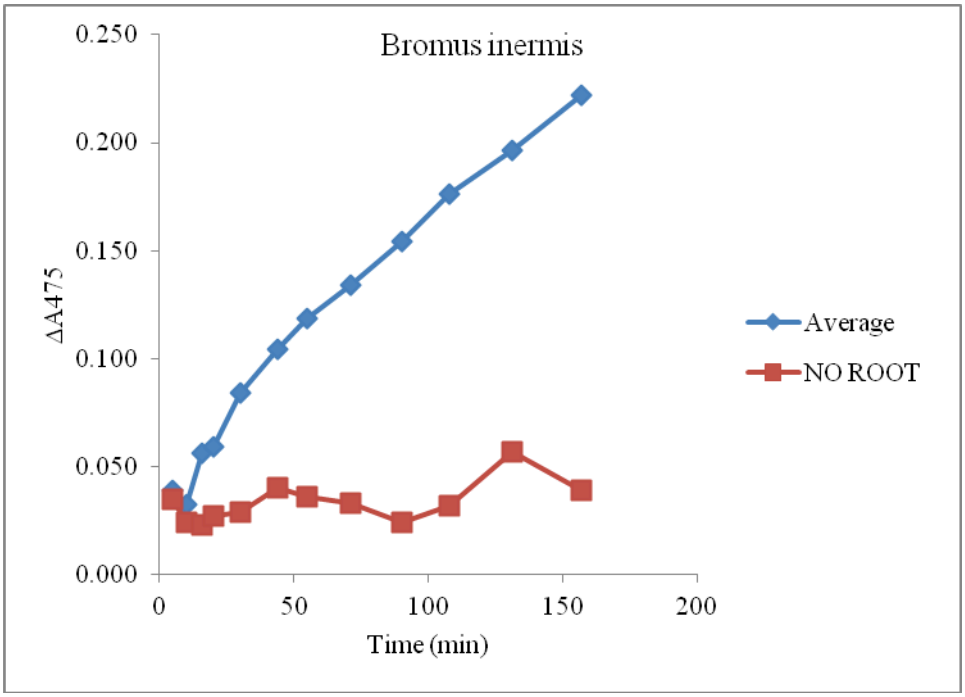

Fig. A 48. PPO enzyme assay results of root and blank control (for each data point, n = 5).

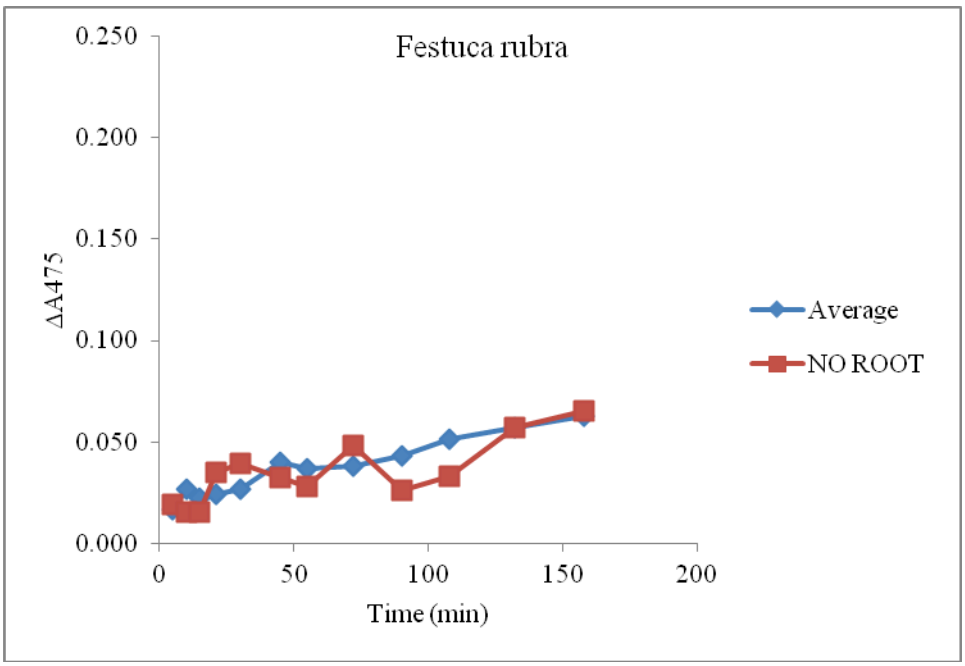

Fig. A 49. PPO enzyme assay results of root and blank control (for each data point, n = 5).

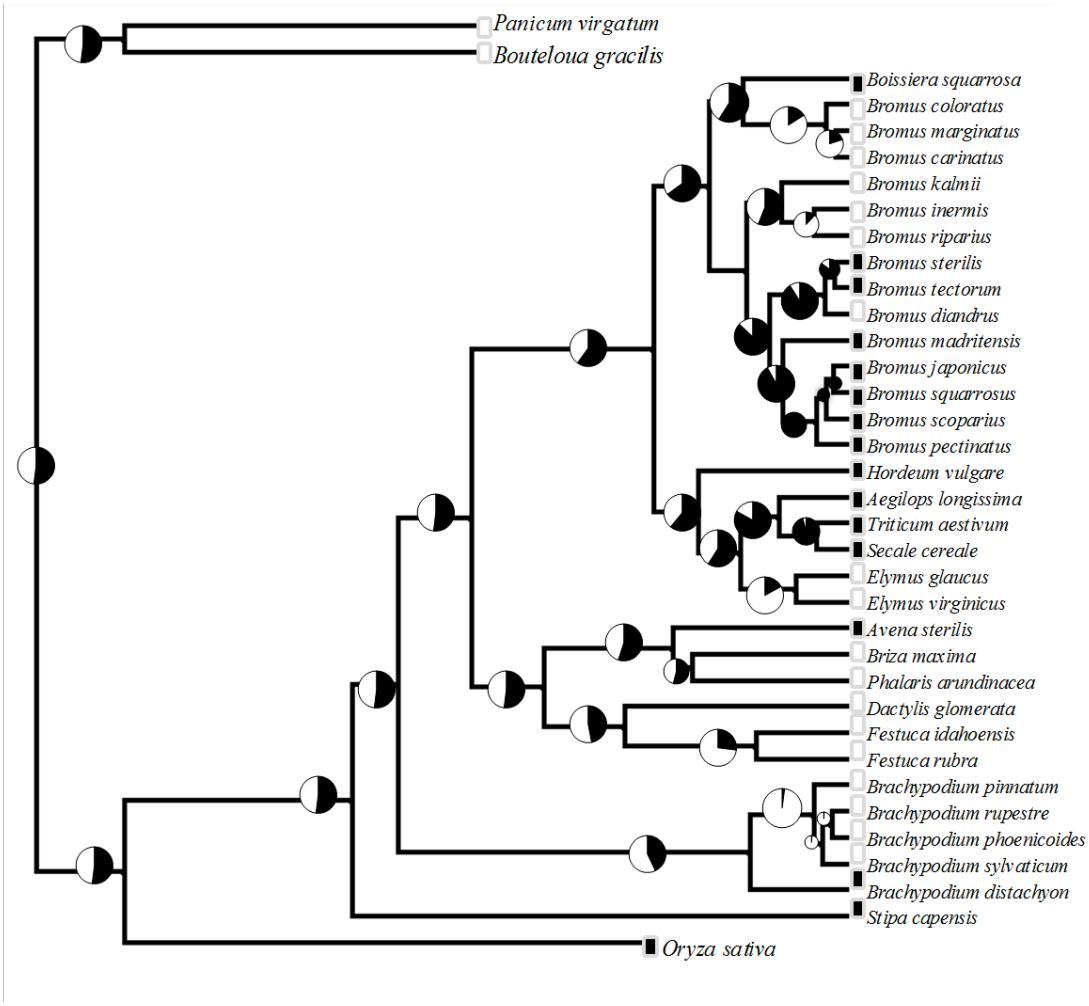

Fig. A 50 Plant life duration ancestral state reconstruction (ASR). Pie charts represent likelihood (0-100%).

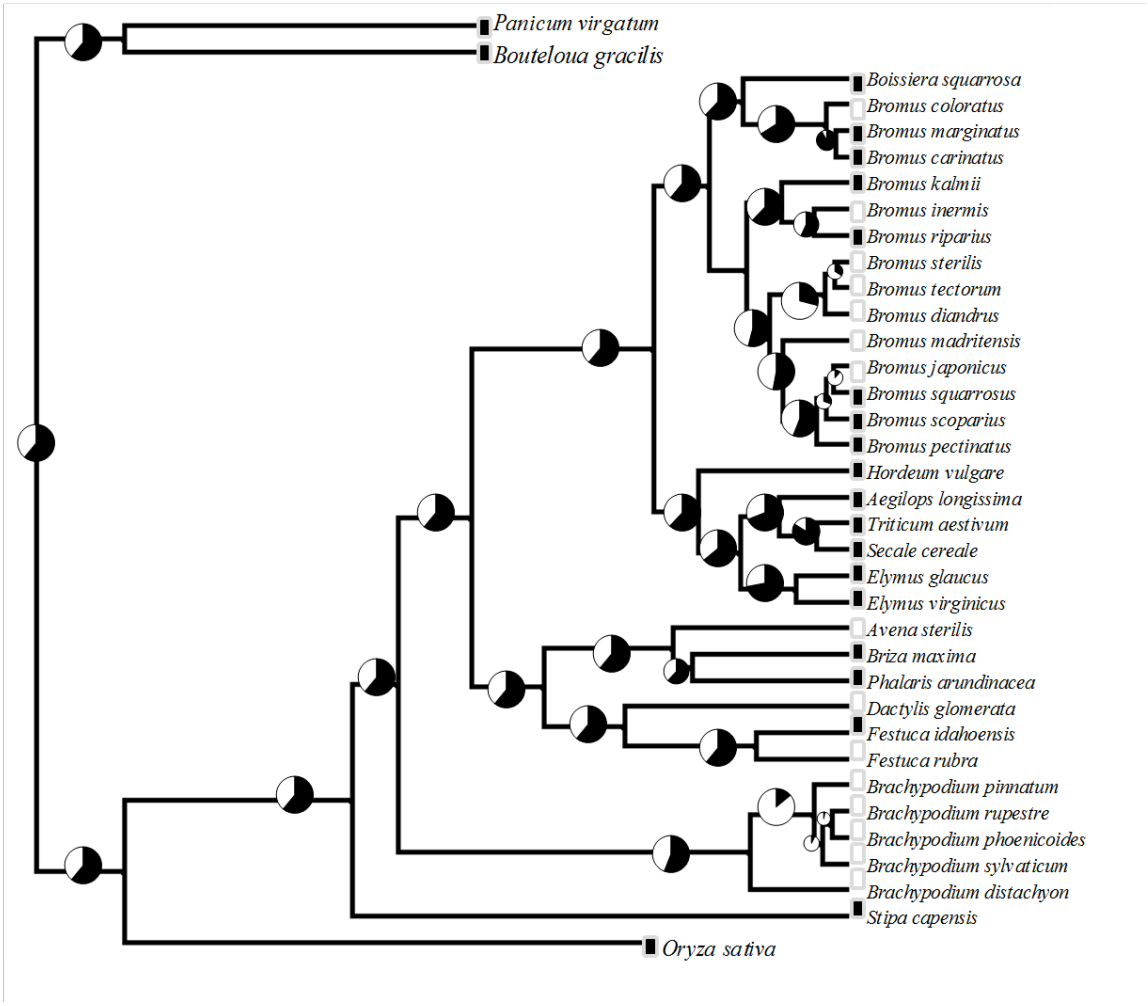

Fig. A 51. PPO activity ancestral state reconstruction (ASR). Pie charts represent likelihood (0-100%).

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Oxidation detoxification: Polyphenol oxidase (PPO) in roots of the grass genus *Bromus* as a novel defense, a new hypothesis for invasion biology

ABSTRACT

In plant community ecology, one principal question is what enables species to be invasive. The novel weapons hypothesis states that some plant species produce phytotoxic allelochemicals unknown to the invaded environment. In phenolic conformation, allelochemical compounds can serve as substrates for the enzyme polyphenol oxidase (PPO), which is known to be exuded by roots of the grass genus *Bromus* but not by other grass genera. This highly invasive genus constitutively produces high levels of PPO, thus, we hypothesized *Bromus*-PPO may be able to defend against phenolic-allelopathic plants which otherwise suppress plants that do not produce root PPO. We analyzed responses of PPO- and non-PPO-producing grasses to phenolic and non-phenolic-allelopathic plants in greenhouse competition, litter, and leachate experiments. Both competition and leachate greenhouse experiment results supported the hypothesis as the PPO-producer *Bromus inermis* was not suppressed by allelopathic-phenolic *Centaurea stoebe w*hile the non-PPO producing grass *Festuca rubra* was. Both PPO/non-PPO grasses were suppressed by allelopathic-non-phenolic *Artemisia vulgaris*. Field studies were conducted to assess the relationship between PPO-producer *Bromus tectorum* and nearby plants. The majority of plants near *Bromus* were allelopathic, and were found at distances further than non-allelopathic plants; phenolic-allelopathic plants were found closer than non-phenolic allelopathic plants, but these results were not significant. Experiments with added allelopathic plant litter showed an unexpected

overall net benefit of litter which probably indicates presence of nutrients and carbon in the litter that possibly overwhelmed potentially allelopathic effects. Overall, these results lend support to the hypothesis that the unique presence of root PPO in the genus *Bromus* enables these grasses to cope with phenolic-allelopathic competitors. This PPO enzyme with previously unresolved functions therefore might act as a novel defense in invaded ranges.

INTRODUCTION

By definition, invasive species typically are able to increase in population size after introduction and in that process displace native species (e.g., (Inderjit et al. 2011; Maron et al. 2014; Pimm et al. 1988; Pyšek et al. 2012). One major focus of invasion ecology is exploring the mechanisms of interactions of invasive species to can explain their success in overtaking communities. Proposed explanations of biological invasions fall into several, well described categories including empty niche, enemy release, invasional meltdown, evolution of increased competitive ability (EICA), dense growth, biodiversity as a barrier, high propagule pressure, fire tolerance, and more recently the evolutionary imbalance hypothesis (EIH), which hypothesizes that invasives are successful because they evolved that way through previous histories of diverse competition (e.g., (Blossey and Nötzold 1995; D'Antonio and Vitousek 1992; Elton 1958; Fridley and Sax 2014; Kennedy et al. 2002; Simberloff and Von Holle 1999). Allelopathy is increasingly reported as a major factor influencing plant communities by determining invasion and establishment (Hierro and Callaway 2003; Ridenour and Callaway 2001). The “novel weapons” hypothesis suggests invasive plants exude allelochemicals that are

unknown to the native populations and therefore cannot be readily defended against (Callaway and Aschehoug 2000; Callaway and Ridenour 2004). In allelopathic interactions among plants, plants naturally produce and release phytotoxins into the environment (Rice 1984). Allelochemical compounds are secondary metabolites, natural phytochemical compounds that are not used for any of the primary processes of the plant: nutrient assimilation, photosynthesis, protein synthesis, respiration, or solute transport, and have no generalized direct role in the veracity of the plant, but which impose an allelopathic effect of phytoxicity. Allelochemicals can be volatilized, exuded, or simply present in plant biomass, which later expresses toxic effects in decomposing litter, referred to as foliar leaching (Inderjit and Callaway 2003; Rice 1984).

Allelochemical effects are diverse, and can be scaled up from cellular level to whole-plant and even community-level effects. Production, release, or activity of an allelochemical may influence plant dominance or yield, which can have importance in an ecological context, or be detected as a reduced crop yield (Inderjit et al. 2011). In plant interactions in the interface of roots and soil, known as the rhizosphere, allelochemicals have to been shown to inhibit a multitude of ordinary plant activities, including membrane permeability (spilling cellular contents), ion uptake, nutrient uptake, stomatal conductance, photosynthesis, respiration, enzyme activity, protein synthesis, water balance, and cell division, thereby inhibiting seed germination and growth (Bais et al. 2001; Perry et al. 2006; Rice 1984; Walker et al. 2003; Wu et al. 2000). *Juglans nigra* (black walnut), producing juglone, a nathoquinone, and *Sorghum bicolor*, producing sorgoleone, a benzoquinone, are examples of classic allelopathic species (Bertin et al. 2003).

Phenolic compounds are an example class or type of allelochemical, and negatively affect plants in various ways: phenolics lower the soil pH, making it more acidic, and are capable of changing the electric potential of cellular membranes, often having a depolarizing effect; ion losses may also occur from permeability of membranes; they negatively influence the concentration of auxin, a photosynthetic hormone responsible for cell growth and development in plant tissues (Leicach et al. 2009). Phenolic compounds are also phytotoxic when they are absorbed by plant roots and taken up via xylem and introduced to the chloroplast, where phenolics from soil reduce chlorophyll biosynthesis and thus the foliar chlorophyll content (Mitrovic et al. 2012).

Phenolic compounds are common, and have been frequently implicated as the source of allelopathy, as they are notorious allelopathic chemicals, negatively effecting plants in various ways (e.g., (Blum 1996; Estabrook and Yoder 1998; John and Sarada 2012; Keilin and Mann 1938; Mitrovic et al. 2012; Rice 1984). Phenolics make up 1-25% of total biomass of dry green leaves (Haettenschwiler and Vitousek 2000). One of five photosynthetically-fixed carbon atoms become phenolics, derived from the amino acid phenylalanine via the phenyl propanoid biosynthesis pathway to become one of more than 3,000 possible compounds including the phenolics: favanols, flavones, and isoflavonoids (Moore et al. 2014).

A plant species that may tolerate allelochemicals is *Bromus inermis*. Polyphenol oxidase (PPO), characterized by (Anderson and Morris 2003), has been found in roots of all assayed *Bromus* species, but little or no PPO has been found in roots of other grass genera (Holzapfel et al. 2010). The characteristics of PPO and other phenolic oxidizing enzymes such as peroxidases, laccases, and catecholases, have been well described and

reviewed (e.g., (Aniszewski et al. 2008; Keilin and Mann 1938; Mayer 2006; Valero et al. 1991)). PPO is localized in plant cell walls and vacuoles and exuded in great amounts into the rhizosphere through lysis and excretion, and PPO in soil is 1^{-50} μmol/hour/gram (Dorantes and Zúñiga 2012; Sinsabaugh 2010). The general mechanism of PPO is catalysis of the oxidation of phenolic compounds to o-quinones which react with phenolics, generating melanin-like compounds visible as brown pigments. PPO catalyzes two reactions: (1) cresolase activity, a hydroxylation of monophenols to o-diphenols and (2) catecholase activity, where there is oxidation of o-diphenols to o-quinones (Valero et al. 1991). These semiquinones and quinones (2,5-cyclohexadiene-1,4-diones) produced in the second reaction react with amino acids, phenols, or proteins and produce visible browning from the production of melanin-like compounds, which notoriously reduce food quality, as in the case of pre-cut apples or potatoes for french fries (Aniszewski et al. 2008). PPO has remarkably high specificity (Keilin and Mann 1938). The reaction will not proceed without both substrates: molecular oxygen and a mono-, di-, or polyphenolic (Mayer 2006). PPO can oxidize various substituted aromatics, a group of arene compounds with an hydroxyl group, including L-DOPA, caffeic acid, and catechol (Kafkewitz, pers. comm.). Other possible phenolic substrates for PPO are cinnamic acid derivatives and benzoic acid derivatives, and catechin, chlorogenic acid, and 4-methylcatechol (Leicach et al. 2009; Queiroz et al. 2008).

Although the mechanism of enzymatic action is well understood, the function and rationale for production of the enzyme by some plants and not others has yet to be determined (Mayer 2006). A physiological role for PPO in *Bromus* species is not apparent (Holzapfel et al. 2010). The roots of a number of other grass genera have been

similarly assayed and shown to have little or no PPO activity (Holzapfel et al. 2010). The *Bromus* species enzymatic activity exhibits substrate specificity (Keilin and Mann 1938), suggesting the plant makes use of various phenolic compounds; *Bromus* species may use PPO as a defense against phenolic allelochemicals through the enzymatic destruction of allelochemicals before they can exert their toxic effects. PPO has previously been suggested as a "putative defensive oxidative enzyme" (Constabel and Ryan 1998). The natural presence of *Bromus* PPO is hypothesized to be advantageous in plant interactions if demonstrated by resilience of *Bromus* species in the presence of allelopathic phenolics. PPO comes in contact with the substrates as the enzyme is found in *Bromus* roots, phenolics are released from leaching and in litter. We hypothesized that by using PPO, *Bromus* species are able to "disarm" allelopathic plants whose allelochemicals are phenolic compounds and can be used by *Bromus* species as substrates.

We tested the general hypothesis that PPO-acitivity in *Bromus* acts as a novel defense against phenolic allelochemicals. The proposed novel defense mechanism invoked by plants involves preemptive enzymatic destruction of the allelochemical compounds, thereby averting toxic effects of the allelochemicals. As it has been shown that roots of seedlings of species within the grass genus *Bromus* constitutively possess high levels of PPO but the function of the enzyme remains unknown (Holzapfel et al. 2010), we hypothesized the enzyme polyphenol oxidase (PPO) is used as a "novel defense" against allelopathic species since many of the allelochemical compounds serve as substrates for PPO (e.g., (Blum 1996; Keilin and Mann 1938; Mitrovic et al. 2012)).

Here we investigated the ecological role of PPO as a hypothesized novel defense. For this, we conducted a series of experiments: (1) greenhouse competition experiments

comparing the effects a phenolic-allelopathic herbaceous plant (*Centaurea stoebe,* spotted knapweed) and non-phenolic-allelopathic plant (*Artemisia vulgaris*, mugwort) on a PPO-producing grass (*Bromus inermis,* here *Bromus)* and a non-PPO-producing grass (*Festuca rubra)*; (2) litter and (3) leachate experiments that expose grasses varying in PPO-acitivity to litter and extracted leachate of the same two allelopathic forbs; and (4) we also conducted spatially explicit field surveys of plant communities that contain *Bromus tectorum* in order to test whether this PPO-active grass is able to occur closer to plants that are phenolic-allelopathic than to plants that are not.

Specifically, we anticipated decreased growth of non-PPO grasses and tolerance of PPO grasses to the phenolic-allelochemical producers; both species were anticipated to be suppressed by the non-phenolic allelochemical producers. In testing plant-produced PPO as a novel defense against plant-produced phytotoxic allelochemicals, we predicted that the PPO-producing plants would be able to cope with the phenolic-allelopathic environment by detoxification, as phenolic-allelochemical compounds were predicted to be converted to melanin-like compounds by the PPO enzyme.

METHODS

Study species. Experiments were conducted using two aggressor plants, non-phenolic allelopathic *Artemisia vulgaris* (mugwort) and phenolic allelopathic *Centaurea stoebe* (spotted knapweed) against *Bromus inermis* (smooth brome), an invasive perennial grass which produces root PPO, and *Festuca rubra* (red fescue), an indigenous perennial grass from the west coast of the United States that produces little or no root PPO. *Artemisia vulgaris,* a perennial forb native to Asia, Europe, and northern Africa, naturalized in the

United States since arriving with Jesuit clergy in the 1600's but remains a high risk invasive to remnant wild lands (Fernald 1900). *A. vulgaris* displays significant allelopathic effects, and the chemistry of species within the genus *Artemisia* has been well-studied, with many studies focused on terpenes (Barney and DiTommaso 2003; Duke et al. 2002; Weston et al. 2005), *A. vulgaris* is allelopathic in both roots and leaves, producing the compounds alpha- and beta-pinene, camphor, eucalyptol, and thujone, a ketone monoterpene (Barney et al. 2005). This plant was selected as an allelopathic-non-phenolic competitor. *Centaurea stoebe* (spotted knapweed) is a perennial forb native to Eastern Europe, brought to the United States in contaminated seed (USDA Plants Database, https://plants.usda.gov). In its native habitat, *Centaurea* is not a problem species, but in the United States it colonizes disturbed land, prairies and ranges, often forming dense monotypic stands and crowding out and competitively excluding native species, and is a serious threat to millions of acres. Invasive success of *Centaurea* has been attributed to the production of the flavn-3-ol phenolic allelochemical +/-catechin, 0.65 +/-45 mg/g in invaded soils (Bais et al. 2001; Bais et al. 2003; Bertin et al. 2003; Perry et al. 2005; Perry et al. 2007). These forbs were chosen because they are naturalized, allelopathic, common, and readily available in the northeastern United States. These plants were also chosen because of their reproducibility and ease of manipulation.

1. Competition experiment.

Seed germination. Seeds of perennial grasses *Bromus inermis* and *Festuca rubra* (Seed Trust, Littleton, CO, USA) were surface-sterilized with 10% bleach for thirty minutes, washed twice in sterile, autoclaved water, and plated with flamed forceps on sterile, autoclaved moist filter paper in glass petri dishes.

Plant collection. Thirty small *Artemisia vulgaris* rhizome fragments were collected early in their development from a semi-natural urban habitat on the campus of Rutgers University in Newark, New Jersey, USA on 16 March 2012. Thirty small *Centaurea stoebe* specimens were collected from a former brownfield at Liberty State Park in Jersey City, New Jersey, USA on 22 March 2012 and transported in plastic bags to prevent desiccation.

Planting. Growing media was created by mixing Scott's topsoil with natural play sand (1:1) to approximate the same field density. Allelopathic plants *Artemisia* and *Centaurea* were potted singly in the center of square pots (10 cm x 10 cm x 13 cm height) with drainage holes lined with paper towels and placed in the greenhouse at Rutgers University in Newark, NJ for acclimation on natural photoperiod and auto-watered on schedule for three weeks to establish roots (22 March – 12 April 2012). One teaspoon of slow-release Osmocote dry plant fertilizer was added to each pot (18-6-12, Osmocote Smart-Release Outdoor and Indoor Plant Food, Scotts Company LLC, Model # 272101).

Experimental design. For each treatment, four freshly germinated grass seedlings (*Bromus inermis* or *Festuca rubra*) were planted surrounding the allelopathic center plant while conspecific controls consisted of five seedlings in one pot. With ten replicates the full experiment included 60 pots (10 x 3 (*Artemisia, Centaurea*, Control) x 2 (*Bromus*, *Festuca*).

Data collection. Plants were measured initially and after that, weekly. In the grasses leaf length, leaf number, and survival were measured. Allelopathic plants *Artemisia vulgaris* and *Centaurea stoebe* were measured for plant height, from soil to the top of the longest leaf was measured as by (Thorpe et al. 2009), number of leaves, and number of floral

parts. After 12 weeks of growth together, final measurements were taken and plants were harvested on 24 July 2012.

Harvest. Individual plants were carefully removed from pots and washed, separated into aboveground and belowground parts, bagged separately, and dried at 60^{o}C for at least 48 hours and weighed after weight constancy was reached. Aboveground and belowground biomass was measured to the nearest hundredth milligram and recorded after drying. Roots from all species were imaged using a root scanner (Epson Expression 16801.D) and diameter and length was measured to the nearest tenth of a millimeter using the WinRHIZO Pro program (Regent Instruments, Inc.).

2. Decomposition/litter experiment.

Experimental design. Live *Artemisia vulgaris* and *Centaurea stoebe* were collected from Northern New Jersey, rinsed, and dried for 48 hours at 60 C. Dried plants were chopped into 1 cm^2 pieces, and added in the following amounts to 150 g of the growing media: 1.0 g roots, 3.5 g shoots, or both 1.0 g roots and 3.5 g shoots of either allelopathic species mixed in to decompose similar to (Singh et al. 2005). Controls containing no plant material were subjected to the same conditions until plants were added. Growing media and litter mixtures were set up on 29 October 2012. These were kept moist and periodically mixed until 11 March 2013, when mixtures were split into 150 mL tubes with drainage holes of ten replicates for each treatment. On this same day *Bromus inermis* and *Festuca rubra* seedlings were planted, initially measured, and grown until 15 April 2013, for a total of 35 days (ten replicates for each species, four treatments, n = 80). Harvest, root scans, and analyses were conducted as above in competition experiments.

3. Leachate experiment.

Planting. Ten replicates of germinated seedlings of each grass, *Bromus inermis* and *Festuca rubra*, were planted in conical tubes with 150 mL pure silica sand in the greenhouse (n = 60). Initial height was recorded for all plants.

Leachate preparation. Leachate was prepared fresh every other day. Fresh plants of *Artemisia vulgaris*, a non-phenolic-allelopathic plant, and *Centaurea stoebe*, a phenolic-allelopathic plant, were collected in Newark, New Jersey, USA every other day from 25 October to 25 November 2013. 25g of fresh biomass and 10 g of dry biomass of each species of allelopathic plants were coarsely chopped and added to a flask containing 500 mL DI water to aqueously extract allelochemicals, as by (MC et al. 2010; Uddin et al. 2012). The flask was sealed with parafilm, inverted three times until all plant material was wet, and then placed on the rotating shaker (Fisher Scientific) at a speed of 80 RPM. After 24 hours, leachate was filtered through commercially available coffee filters to water both species of grass.

Leachate watering. Ten replicates of each *Festuca rubra* and *Bromus inermis* seedlings were watered with 20 mL for three treatments: with water, leachate from *Artemisia vulgaris*, and leachate from *Centaurea stoebe* (n = 60). Leachate was applied avoiding leaves, until leachate began to drip from the bottom of the pots. Controls of both grass species were watered with DI water at these times. This procedure was repeated every other day for the duration of the experiment. Miracle Gro (24-8-16) was added twice during the experiment to all plants on non-leachate watering days, and the auto-water system was used for normal watering on non-leachate watering days, so that all plants were watered daily.

Leachate experiment harvest. The experiment ran for four weeks (25 October - 25 November 2013). Final height and number of leaves was recorded immediately prior to harvest, which was conducted as above in competition experiments.

4. Field surveys.

Field survey methods. In the spring of 2013, we conducted field surveys in Northern New Jersey, USA to identify plant species in the vicinity of *Bromus tectorum* to assess the ecological system and plant community, as effects of PPO may manifest as changes on the physical environment and thus community composition (e.g., (Kennedy et al. 2002; Mack and Harper 1977; Meiners et al. 2012; Pacala and Silander 1987)), and allelopathic plants directly affect neighboring species (e.g., (Kong 2010; Thorpe et al. 2009)). In 29 field sites mostly within the uncultivated urbanized landscape of New Jersey, *Bromus tectorum* populations were located in patches of vegetation, and one individual stem was randomly selected and marked with flagging tape. Radiating outward up to 50 cm, species and distance of these nearest neighbors were measured and recorded. Plant family, life form (annual or perennial), and origin (native, invasive, endemic) were also referenced and recorded. After surveyed species were identified, we conducted literature searches to determine allelopathic properties of the species present, specific traits like life-cycle duration and plant origin were assigned as designated by the United States Department of Agriculture (USDA, NRCS. 2015. The PLANTS Database [http://plants.usda.gov, 5 March 2015] National Plant Data Team, Greensboro, NC 27401-4901 USA). 5 species could not be identified and were categorized as unknown.

Data analysis. In general, statistical analyses tested for significant differences within and among species using general linear model, univariate ANOVA (independent variable:

treatment, dependent variable: growth parameter – height, number of leaves, shoot mass, root mass, root to shoot ratio, distance to target plant), and Tukey's *post hoc* tests when a treatment effect was detected were conducted in SPSS (Version 21.1) with 95% confidence intervals. In competition, litter, and leachate experiments we tested for significant differences in growth and survival, using changes in height and number of leaves (calculated as final minus initial), root, shoot, and total dry biomass, and root diameter and root length.

RESULTS

1. Competition experiment results.

1. a. *Artemisia vulgaris* significantly suppressed both PPO- and non-PPO grasses both above and belowground. Results of competition experiments of the non-phenolic-allelopathic *Artemisia vulgaris* with each of the grasses indicate significantly negative allelopathic effects on some of the measured variables (Table 1, Fig. 1). *A. vulgaris* significantly suppressed the number of leaves ($P = 0.00$), root mass ($P = 0.05$), change in height ($P = 0.02$); the following were suppressed, but not significantly: shoot ($P = 0.10$) and total biomass ($P = 0.20$) of *B. inermis* in comparison to *B. inermis* grown conspecifically (Fig. 1a). Similarly, when the non-PPO grass *Festuca rubra* was grown with allelopathic *A. vulgaris*, number of leaves ($P < 0.001$), change in height ($P < 0.001$), total biomass ($P = 0.01$), and both shoot ($P < 0.05$) and root ($P < 0.05$) biomass (Fig. 1b) were all significantly suppressed in comparison to *F. rubra* conspecifics.

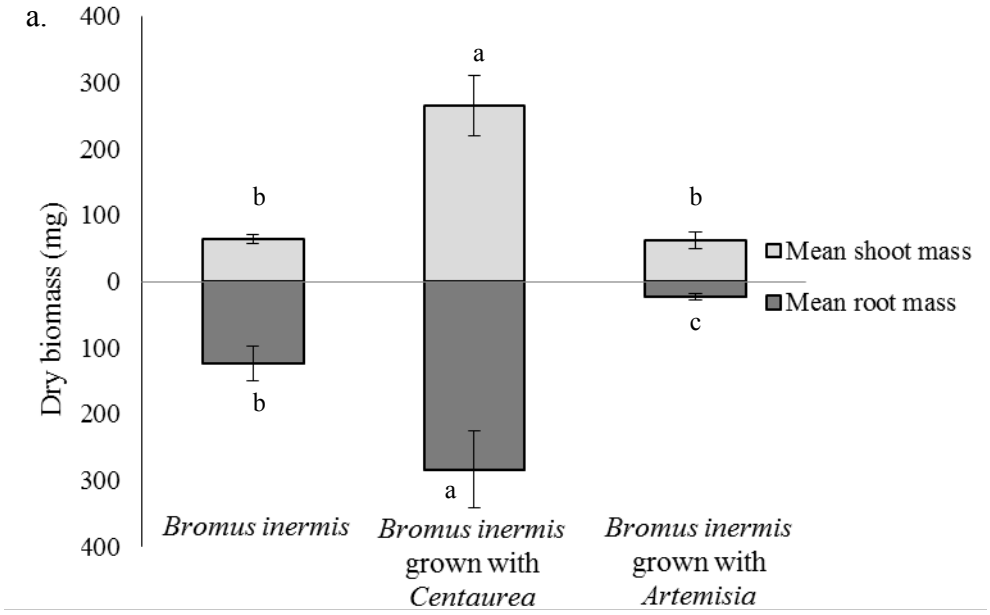
1.b. *Centaurea stoebe* suppressed roots of non-PPO *Festuca rubra*, but not PPO *Bromus inermis*. Competition experiment results showed that the phenolic-allelopathic

plant *Centaurea stoebe* was not capable of suppressing PPO-producer *Bromus inermis* (Table 1, Fig. 1). When grown with *C. stoebe*, *B. inermis* produced significantly both more shoots and roots than when *B. inermis* was grown conspecifically (Fig. 1a) as shown by root mass that was significantly promoted ($P = 0.01$), shoot mass that was significantly promoted ($P < 0.001$), and total mass that was significantly promoted ($P < 0.001$). Mean change in height was not significantly different from the control ($P = 0.16$), nor was number of leaves significant from control means ($P = 0.97$). By comparison, competition of the phenolic-allelopathic *Centaurea stoebe* with non-PPO producer *Festuca rubra* results show insignificant increases in number of leaves ($P = 0.10$), change in height ($P > 0.05$), and shoot mass ($P > 0.05$) produced by *F. rubra*, but root biomass was significantly lower in competition than conspecifics, showing evidence of competitive supression (Fig. 1b, $P < 0.05$). Total mass was not significantly different than controls ($P > 0.05$).

1.c. Neither grass affected the growth either allelopathic plant. Lastly, competition experiment results showed that grasses did not affect forb growth: neither of the allelopathic forb species *Centaurea stoebe* or *Artemisia vulgaris* were affected (neither suppressed nor promoted) by the addition of either PPO/non-PPO grasses *Bromus inermis* or *Festuca rubra* for any of the response variables measured: root mass, shoot mass, total mass, change in height, or number of leaves ($P > 0.05$ for all, Tukey's *post hoc* test).

Table 1. Results of greenhouse competition experiments, grouped by competitor and target plant species.

Target:		Competitor:											
		Artemisia				*Centaurea*				None (control)			
		Mean	N	Std. Dev.	Std. Error of Mean	Mean	N	Std. Dev.	Std. Error of Mean	Mean	N	Std. Dev.	Std. Error of Mean
Bromus inermis	Shoot mass	0.06	40	0.08	0.01	0.26	40	0.29	0.05	0.06	50	0.05	0.01
	Root mass	0.02	40	0.03	0.00	0.28	40	0.37	0.06	0.12	50	0.18	0.03
	Total biomass	0.09	40	0.10	0.02	0.55	40	0.62	0.10	0.19	50	0.21	0.03
	Height change	10.87	40	11.63	1.84	21.44	40	13.30	2.10	17.19	50	7.26	1.03
	Leaf change	2.63	40	2.68	0.42	5.70	40	4.38	0.69	5.86	50	3.22	0.46
Festuca rubra	Shoot mass	0.01	40	0.02	0.00	0.09	40	0.08	0.01	0.02	50	0.02	0.00
	Root mass	0.00	40	0.01	0.00	0.02	40	0.02	0.00	0.07	50	0.22	0.03
	Total biomass	0.01	40	0.02	0.00	0.11	40	0.09	0.01	0.09	50	0.22	0.03
	Height change	-0.24	40	6.21	0.98	14.36	40	9.43	1.49	6.15	50	4.45	0.63
	Leaf change	1.48	40	3.44	0.54	15.70	40	15.37	2.43	11.50	50	6.04	0.85



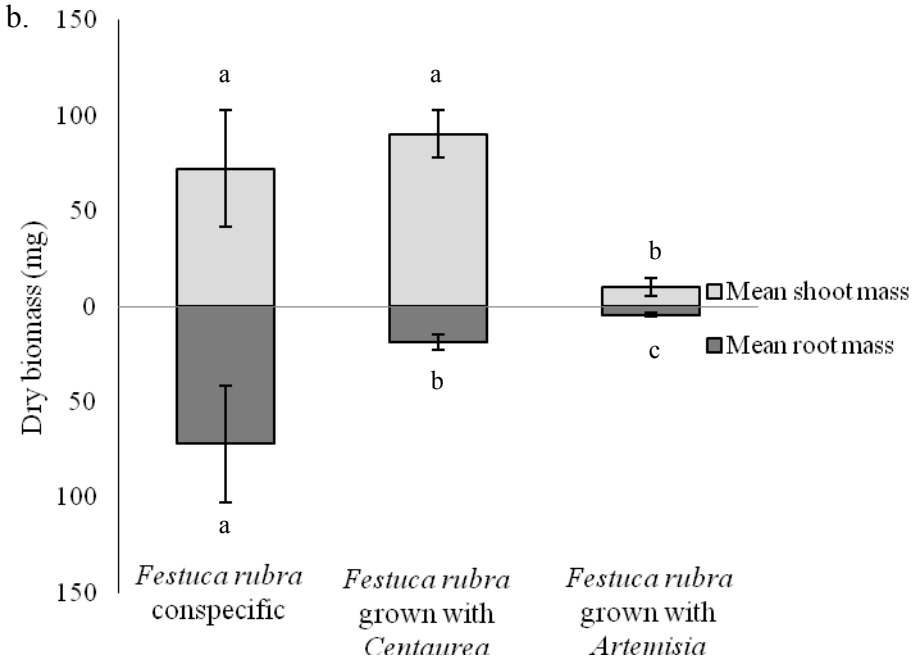


Fig. 1. Results of mean above and belowground biomass of grasses in greenhouse competition experiments. (a) Shoot and root biomass of *Bromus inermis* from greenhouse competition experiments with *Artemisia vulgaris* and *Centaurea stoebe*. (b) Shoot and root biomass of *Festuca rubra*; *F. rubra* shoots were significantly suppressed by *Artemisia vulgaris* as were roots significantly suppressed by both *A. vulgaris* and *Centaurea stoebe*. Different lowercase letters indicate significant differences (ANOVA, Tukey's *post hoc* test, $P < 0.05$); error bars +/- 1 SE; n = 10.

Root to shoot ratio does not vary by grass species ($P = 0.30$), but varies significantly by treatment (competitor identity) ($P < 0.001$), but is there is no significant interaction of treatment and species, that is, not significant by treatment*species ($P = 0.09$). A high root to shoot ratio means that there are more roots than shoots, thus, here, when in competition with either *Artemisia* or *Centaurea*, the significantly lower root to shoot ratio of *Festuca* indicates that root growth was suppressed by each of the competitor plants (Fig. 2, $P < 0.001$ with competitors). *Bromus* was grown in competition with *Centaurea*, the root to shoot ratio was also significantly supressed ($P < 0.05$). The root to shoot ratio of *Bromus* grown with non-phenolic-allelopathic *Artemisia* was significantly decreased ($P < 0.001$).

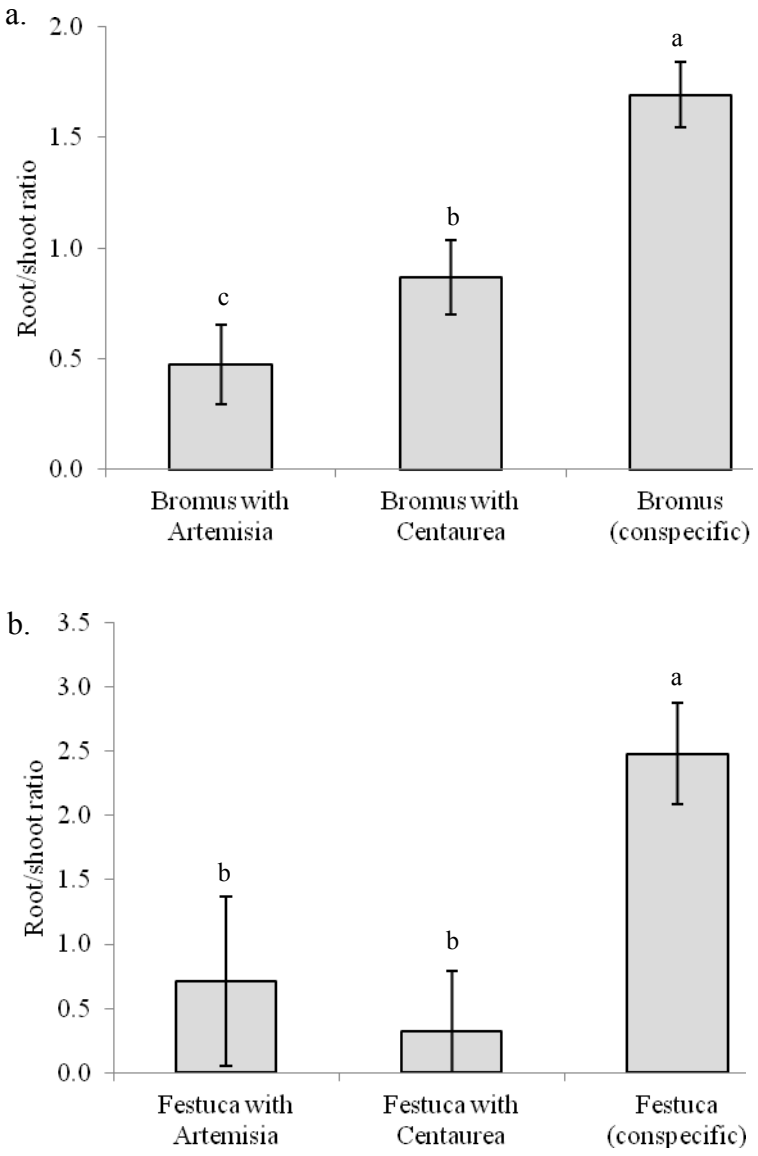


Fig. 2. Results of competition experiments. Mean root/shoot ratios for both (a) *Bromus inermis* and (b) *Festuca rubra*. Different lowercase letters indicate significant differences (ANOVA, Tukey's *post hoc* test, $P < 0.05$); error bars +/- 1 SE; n = 10.

2. Litter experiment results. All experiments of added litter show nominal increases in both above and belowground biomass (Fig. 3).

2.a. *Artemisia vulgaris* litter experiment results. Neither grass species was adversely affected by litter of allelopathic forb *Artemisia vulgaris* (Fig. 3). *Bromus inermis* showed a nominal increase when the litter (whether roots, shoots, or both roots and shoots) of *A.*

vulgaris were added to the sand and soil, but are treated as having no effect because the increase is not significant (Fig. 3a, $P > 0.05$). *Festuca rubra* showed a similar trend of a nominal increase (in number of leaves, height, and both shoot and root biomass, Fig. 3b) when grown in the litter biomass, but results were not significant ($P < 0.05$) with the exception of aboveground responses when shoots or both roots and shoots were added ($P < 0.05$).

2.b. *Centaurea stoebe* litter experiment results. Neither grass species was adversely affected by litter of phenolic-allelopathic forb *Centaurea stoebe* (Fig. 4). The PPO-producing grass *B. inermis* was not suppressed by litter of *C. stoebe* roots ($P < 0.05$) or shoots ($P < 0.05$), and produced significantly greater biomass above ($P < 0.05$) and belowground ($P < 0.05$) in comparison to controls when the combination treatment of both roots and shoots were added, supporting our hypothesis that *B. inermis* tolerates *C. stoebe* and is suggestive of a benefit of growing with *C. stoebe* litter (Fig. 4a). When grown with litter (whether roots, shoots, or both roots and shoots) of *C. stoebe,* number of leaves, height, and both shoot and root biomass of *F. rubra* were all significantly promoted, likely due to a compost effect that swamped out possible allelopathic suppression (Fig. 4b, $P < 0.05$).

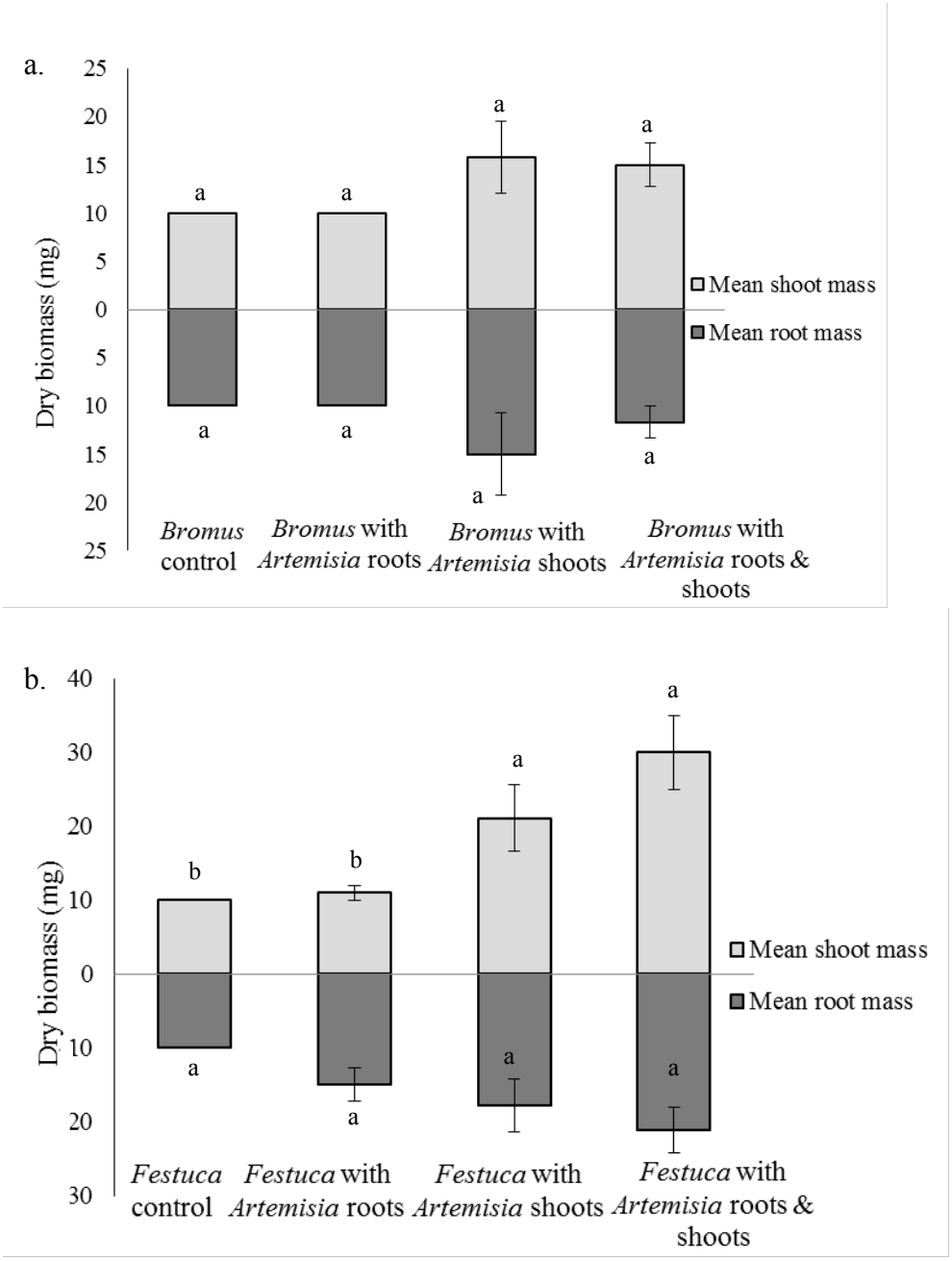


Fig. 3 Results of litter experiments. Mean dry biomass of (a) *Bromus inermis* and (b) *Festuca rubra* controls and with added roots, shoots, or both roots and shoots of *Artemisia vulgaris*. Different lowercase letters indicate significant differences (ANOVA, Tukey's *post hoc* test, $P < 0.05$); error bars +/- 1 SE; n = 10.

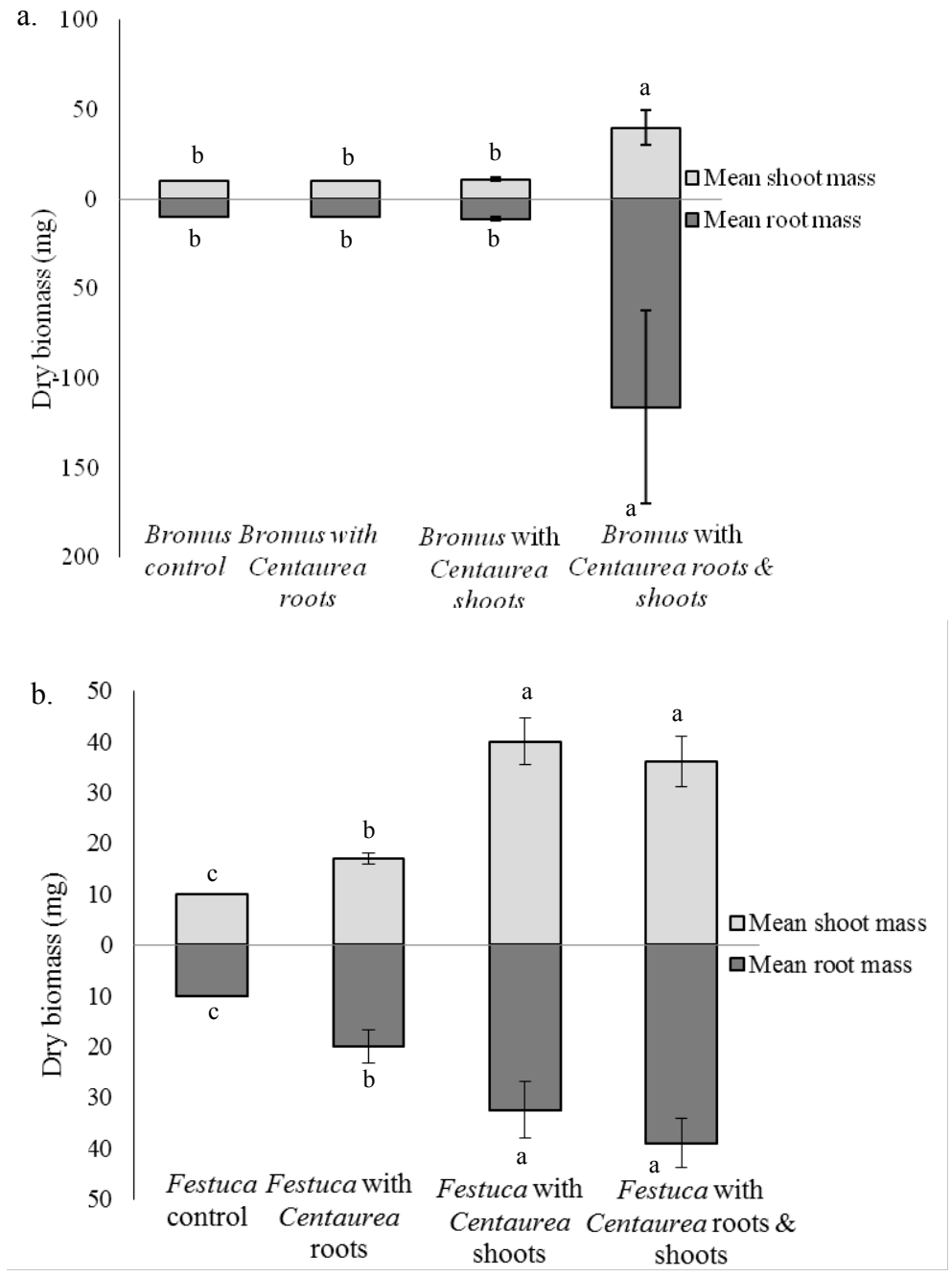


Fig. 4. Results of litter experiments. Mean dry biomass of (a) *Bromus inermis* and (b) *Festuca rubra* controls and with added roots, shoots, or both roots and shoots of *Centaurea stoebe*. Different lowercase letters indicate significant differences (ANOVA, Tukey's *post hoc* test, $P < 0.05$); error bars +/- 1 SE; n = 10.

The trends seen in the final root and shoot mass of *Bromus inermis* and *Festuca rubra* are representative of all measured variables: height, number of leaves, longest leaf

lengths, and root scans of root length and root diameter confirm/corroborate the other metrics (root mass, shoot mass) for the litter experiment. The length of the scanned roots of *B. inermis* were not significantly impacted by the addition of roots ($P = 0.38$), shoots ($P = 0.08$), or both roots and shoots ($P = 0.81$) of the allelopathic species *C. stoebe*. Similarly, *A. vulgaris* did not significantly suppress or promote root elongation of *B. inermis* upon addition of *Artemisia vulgaris* roots ($P = 0.34$), shoots ($P = 0.34$), or both roots and shoots ($P = 0.81$). The root diameter of *B. inermis* was significantly increased with the addition of roots and shoots of *C. stoebe*, added *A. vulgaris* roots, and added *A. vulgaris* shoots, in comparison to the controls ($P < 0.05$). Again, this is likely due to a compost effect, a positive plant response to added carbon, nitrogen, etc. Root length of the other grass, *F. rubra*, significantly increased only when *C. stoebe* roots and shoots (both) were added ($P < 0.05$), again indicative of a positive effect of added carbon, nitrogen, etc.; no other addition of separate roots or shoots of either allelopathic species was significantly positive or negative to the root length of *F. rubra* ($P > 0.05$).

3. Leachate experiment results. Results of the leachate watering greenhouse experiments confirmed our hypothesis and corroborated results of the competition experiments; specifically, that *Bromus* tolerates *Centaurea*, but is suppressed by *Artemisia*; further, that *Artemisia* and *Centaurea* suppress *Festuca*.

3.a. *Artemisia vulgaris* leachate experiments. Both *Bromus inermis* and *Festuca rubra* watered with leachate of *Artemisia vulgaris* were suppressed by several metrics (Fig. 5). *B. inermis* control plants grew an average of 9.0 cm during the experiment ($n = 10$; $SD = 5.3$; min = 0.5 cm, max = 16.0 cm) and 2.7 leaves (min = 1, max = 4), for an average leaf area ([cm-cm*#leaves]) of 29.2. By comparison, *B. inermis* watered with leachate from

Artemisia vulgaris grew an average height of 4.7 cm (n = 10; SD = 1.2; min = 3.0 cm, max = 7.0 cm) and 3.2 leaves (min = 2, max = 4), for an average leaf area of 15.1. This is significantly (P= 0.02) less growth in height than the control plants. Although *B. inermis* watered with leachate from *A. vulgaris* grew more leaves than the control, the acquired height during the experiment was significantly (P = 0.02) suppressed, as was root mass (P = 0.02). Root scans of *B. inermis* watered with leachate of *A. vulgaris* revealed similar significant suppression of both root length (P < 0.001) and diameter (P < 0.001). The shoot mass (P = 0.07) was not significantly suppressed, perhaps because the suppression impact was realized belowground, but total mass was overall significantly lower than that of controls (P = 0.03).

Similar to *Bromus, Festuca rubra* watered with leachate from *Artemisia vulgaris* was also significantly suppressed in several measures (Fig. 5b). The non-PPO producing grass *Festuca rubra* controls grew an average height of 4.6 cm (n = 10; SD = 1.9; min = 0.5 cm, max = 7.0 cm) and produced an average of 4.8 leaves (min = 3, max = 8). When *F. rubra* was watered with leachate of *A. vulgaris*, we expected to see less growth than that of the control; results confirmed our hypothesis: *F. rubra* watered with leachate from *Artemisia vulgaris* was indeed suppressed, and grew an average height of only 1.6 cm (n = 10; SD = 1.5; min = 0 cm, max = 5 cm; P < 0.001 different than control) and produced an average of 3.7 leaves (min = 2, max = 5). Root scans of *F. rubra* watered with leachate of *A. vulgaris* revealed that both root length (P < 0.001) and diameter (P < 0.001) were significantly less than those of controls. None of the following variables were significantly different from controls: root mass (P = 0.07), shoot mass (P = 0.61), or total mass (P = 0.10).

3.b. Results of *Centaurea stoebe* leachate experiments. Results of *Centaurea stoebe* leachate watering experiments showed and overall suppression of *Festuca rubra* and resilience by *Bromus inermis* (Fig. 5). We observed suppression of *Festuca rubra* by *Centaurea stoebe* leachate waterings as *F. rubra* grew a mean height of 2.1 cm, compared to the 4.6 cm control ($P < 0.001$). Results further show that the leachate of *Centaurea stoebe* was allelopathic to *Festuca rubra* because of significant suppression seen in root length ($P < 0.001$), root diameter ($P < 0.001$), and root mass ($P = 0.05$). Shoot mass was not significantly different from controls ($P = 0.27$), nor was total mass ($P = 0.06$).

As predicted, *Bromus inermis* was not significantly suppressed by *C. stoebe* by several measures. When leachate from *C. stoebe* were used to water *B. inermis*, we observed a mean growth in height of 6.5 cm that was not significantly different from controls ($P = 0.24$). Similarly, no other variable showed significant difference from controls as results showed no suppression of *B. inermis* by the leachate of *C. stoebe* including root mass ($P = 0.92$), shoot mass ($P = 0.28$), total mass ($P = 0.53$). The exception to this trend of resilience is root length, which was significantly lower than controls ($P < 0.001$), perhaps balanced by root diameter, which was significantly greater than controls ($P < 0.001$).

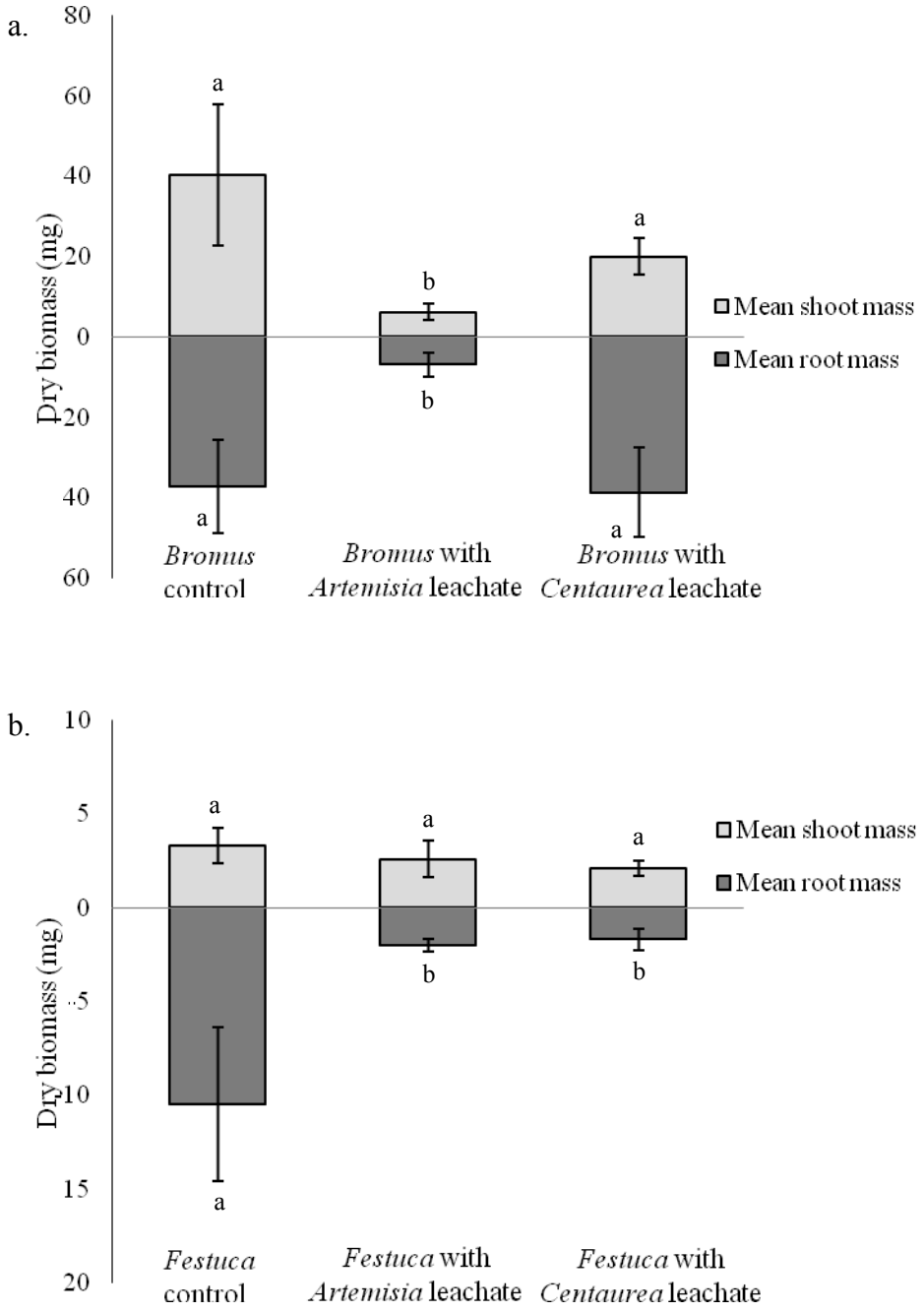


Fig. 5. Results of mean dry biomass of (a) *Bromus inermis* and (b) *Festuca rubra* when watered with DI, leachate of *Artemisia vulgaris,* or leachate of *Centaurea stoebe*. Different lowercase letters indicate significant differences (ANOVA, Tukey's *post hoc* test, $P < 0.05$); error bars +/- 1 SE; each bar n = 10.

Root to shoot ratio was not significantly different from controls when watered with either *A. vulgaris* ($P > 0.05$) or *C. stoebe* ($P > 0.05$).

4. Field survey results. 432 individual plants belonging to 59 species of 50 genera in 23 plant families were recorded areas among the 10 nearest neighbors of individual target of *Bromus tectorum*. Among these, 56 individual plants were annual, 29 were annual/biennial, 10 were annual/perennial, 14 were biennial, 15 were biennial/perennial, and 303 were perennial (Table 2).

Table 2. Results of nearest neighbor field surveys, count of plants by life cycle duration.

Duration	Count
Annuals	56
Annual, Biennial	29
Annual, Perennial	10
Biennial	14
Biennial, Perennial	15
Perennial	303
Unidentified	5
Total	432

When plants were grouped by family, the most common plant family found occurring with *Bromus* was by far Asteraceae (Fig. 6a). We also calculated mean distances from *Bromus* by plant family (Fig. 6b). The average distance of any plants that occurred with *Bromus* was 33.03 cm. The closest distance of all species that occurred with *Bromus* was *Plantago aristata* at 1.0 cm.

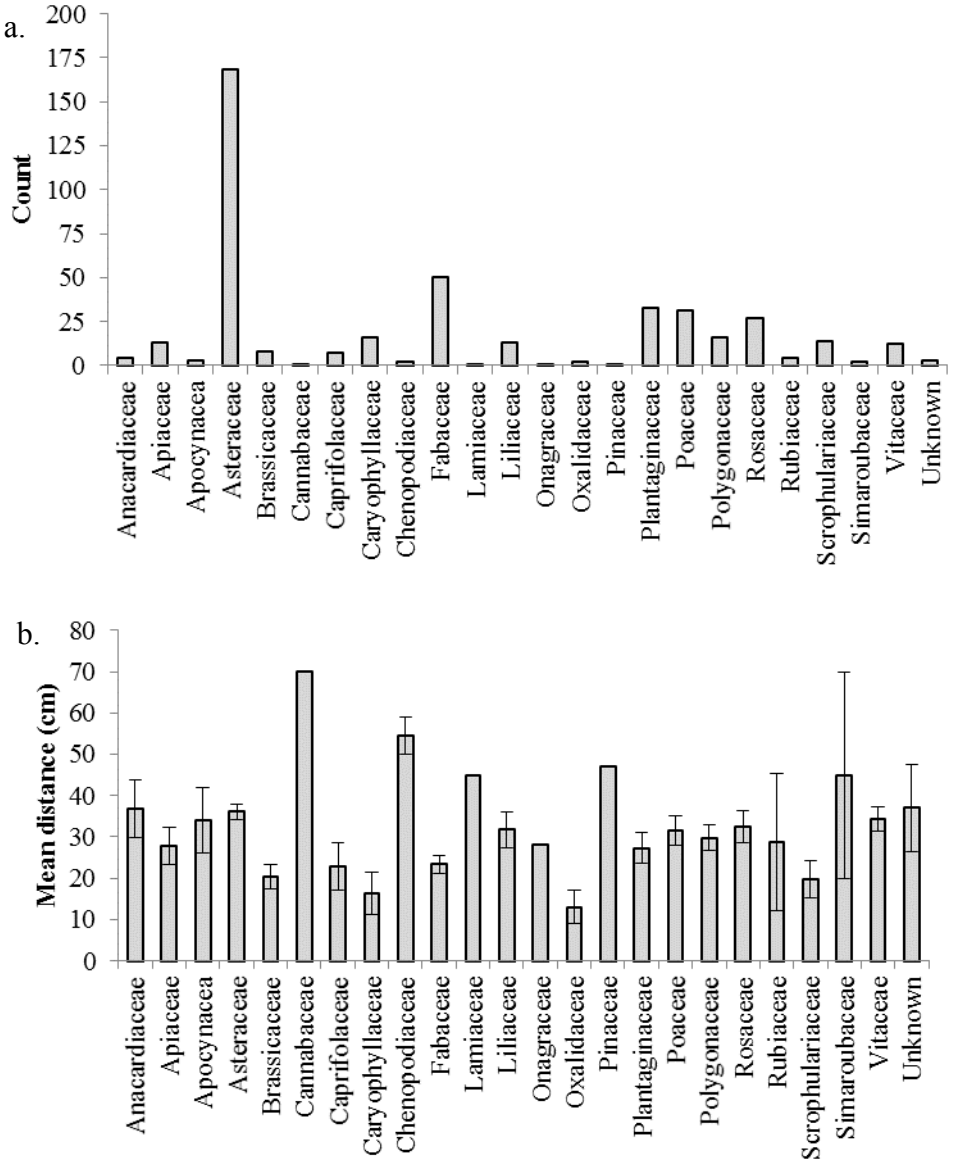


Fig. 6. Results of field surveys. (a) distribution of count (number) of individual plants near *Bromus* by plant family, and (b) mean distance (cm) from *Bromus* by plant family. Error bars +/- 1 SE.

The majority (73%) of plants in surveys near *Bromus* were allelopathic, 17% were non-allelopathic, and the remaining 10% were unknown to be allelopathic or not. The average distance of the alleopathic plants nearby *Bromus* was 32.1 cm (SD = +/- 20.8, n = 349). The average distance of non-alleopathic plants within the 50 cm diameter of *Bromus* was 30.6 cm (SD = +/- 19.5, n = 79). The allelopathic plants in our surveys were found significantly at larger distances from the target *Bromus* than the non-allelopathic

plants (P = 0.041, ANOVA, Tukey's *post hoc* test). The unidentifiable unknown species were a mean distance of 38.5+/-15.2 cm from *Bromus* (n = 4).

The mean distance of allelopathic plants (32.1, n = 349) was significantly greater than the mean distance of non-allelopathic plants (25.8, n = 79) from *Bromus* (P = 0. 041; +/- 1 SE). 4 unknown plants were found a mean distance of 32.0 cm from *Bromus*. Different lowercase letters indicate significant differences (ANOVA, Tukey's *post hoc* test, P < 0.05); error bars +/- 1 SE.

When dividing the allelopathic plants into groups of plants either exuding phenolic or non-phenolic compounds, no significant difference in average distance was found between the two groups (30.7 cm [n=150] vs. 33.9 cm [n=172], Tukey's *post hoc* test after ANOVA p > 0.05), even though the phenolic exuding plants on average were nominally closer.

We analyzed by plant origin (Table 3, Fig. 7). The majority (72%) of species in the surveyed areas were introduced species not native the to the United States. Introduced species were found closer (29.1 cm, +/- 20.1 cm, n = 311) to bromes than the native species (34.3 cm, +/- 21.5, n = 83) were to bromes, but this was not significant (P > 0.05).

Table 3. Results of field surveys, mean distance from *Bromus* by plant origin.

Origin	n	Mean distance from *Bromus* (cm)	SD	SE
Introduced	311 (72%)	29.1	20.1	1.1
I,N	33 (8%)	39.0	22.8	4.0
Native	83 (19%)	34.3	21.5	2.4
Unidentified	5 (1%)	40.2	13.7	6.1

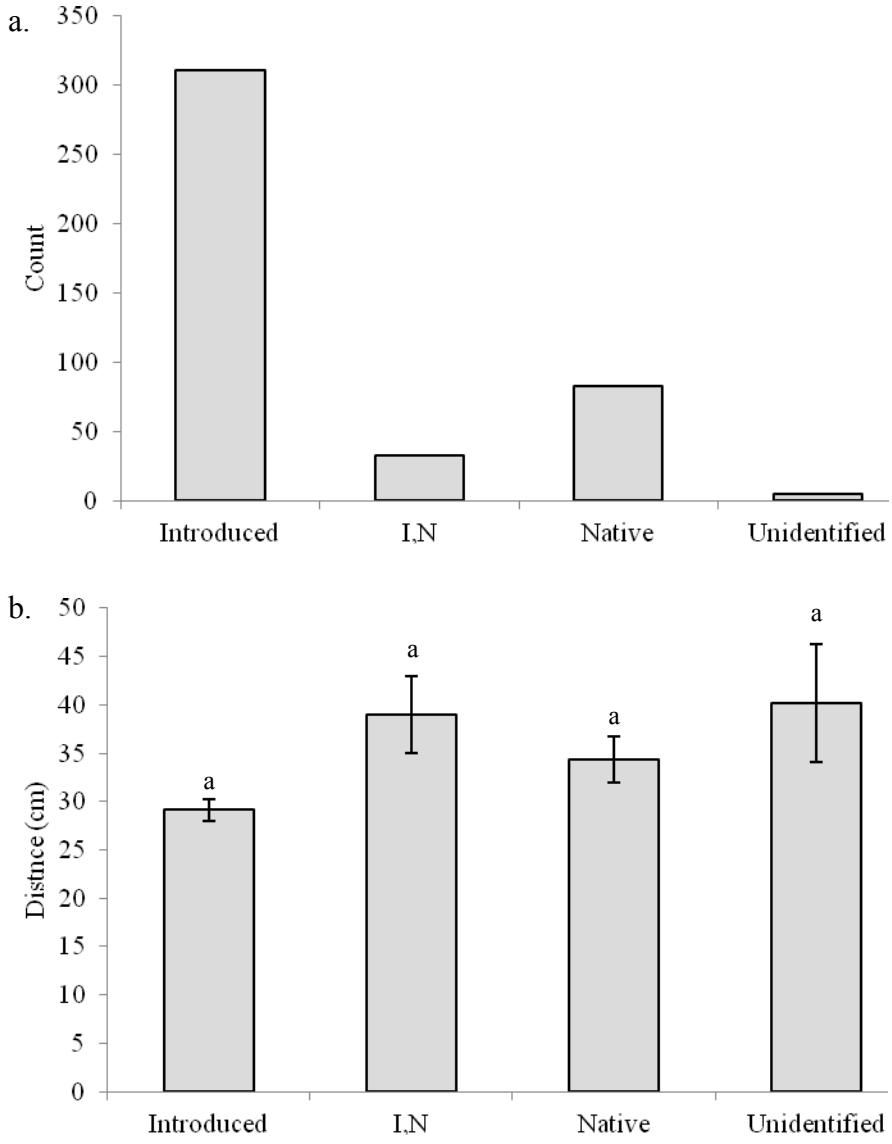


Fig. 7. Results of field surveys. (a) Count and (b) mean distance from *Bromus* by USDA Plants Database Native Status within L48 (Lower 48 States) jurisdiction (I,N = both native and introduced). Different lowercase letters indicate significant differences (ANOVA, Tukey's *post hoc* test, $P < 0.05$). Error bars +/- 1 SE.

The non-phenolic allelopathic Asteraceae *Artemisia vulgaris* which commonly co-occurs in the same habitats as *Bromus tectorum* does occur at distances slightly further (33.4 +/- 21.2 cm, n = 109) than all other species (30.2 +/- 20.5 cm, n = 323), however this difference was not significant ($P = 0.159$). It is worth noting that there were serveral native and introduced phenolic allelopathic plants that were found particularly

close to *Bromus*, including *Trifolium repens* (1, 2, and 4 cm), *Lonicera japonica (*3 cm), *Plantago lanceolata* (5 cm), *Prunus serotina* (6 cm), and *Centaurea stoebe* (7 cm).

DISCUSSION

This project was motivated in attempts to understand the biochemical traits that may aid in the success of biological invasions in plant communities. Inherent difficulties make effects of allelopathy are difficult to detect, thus, our evaluation was three-fold to tease apart a true chemical interaction from resource competition, and accompanied by field surveys for validation of laboratory findings. Here, we have shown the potential for a defense mechanism that exists through the enzymatic destruction of allelochemicals before they can exert their toxic effects. We call this a novel defense, a reference to the novel weapons hypothesis, a defensive response to the offensive chemicals (Callaway (Callaway and Aschehoug 2000; Callaway and Ridenour 2004). As roots of species within the plant genus *Bromus* constitutively possess high levels of the enzyme polyphenol oxidase (PPO), whose function is unknown (Holzapfel et al. 2010), PPO may be used as a "novel defense" against allelopathic plants because many of the allelochemical compounds produced by these plants serve as substrates for PPO (Blum 1996; Keilin and Mann 1938; Mitrovic et al. 2012). Overall, our data suggest that PPO is a potential defense against allelopathy. Specifically, in both competition and leachate experiments, PPO-producing *Bromus* species tolerated phenolic allelochemical-producer *Centaurea* whereas non-PPO-producing *Festuca* did not; these results support our hypothesis and suggest a benefit conferred by PPO when growing with phenolic-

allelopathic *Centaurea stoebe.* The non-phenolic-allelochemical producing species *A. vulgaris* suppressed the growth of both PPO and non-PPO producing grasses.

1. Competition experiments. Our greenhouse competition and leachate watering experiments add to an emerging body of work demonstrating that phenolic allelopathic invaders can be withstood by PPO-producers, thus we could conclude the presence of PPO may be a mechanism for detoxification of allelopathic plants. Although we did not investigate subcellular mechanisms, and as a caveat, this is one factor among many in the complex environment, we did quantify the biological response in growth over time and final harvest biomass, and our quantitative population-level impact data indicates a response of increased root growth by *B. inermis* when in competition with *C. stoebe*.

Our data of larger competition effect on non-PPO producing plants are similar to published results from the competition of *Festuca idahoensis* (native bunchgrass), which was suppressed 50% by *Centaurea* which was attributed to allelopathy by *Centaurea*, as the effects were not seen when activated carbon was added to absorb the allelochemicals (Ridenour and Callaway 2001). In our competition experiment, resource competition was minimized as a variable by fertilization and watering (Inderjit and Callaway 2003).

A possible mechanistic explanation for the increase in both above and belowground biomass production by *B. inermis* in competition with *C. stoebe* may be attributed to possible benefits conferred by melanogenesis. In competition results, not only did we see lack of suppression, but a benefit to bromes growing with allelopathic plants; we believe the PPO first detoxifies *Centaurea* by oxidation, explaining the lack of suppression, and second, posit that the increased biomass production is due to melanin. Biochemically, the quinone products generated by the PPO enzyme activity polymerize,

producing the indolic polymer melanin via the melanogenic synthetic pathway, called melanogenesis. The definite molecular structure of the melanin product remains ambiguous and resistant to enzymatic lysis and degradation (Prota and Thomson 1976; Riley 1997). Three possibilities may explain our results: (1) melanin provides chemoprotective properties for the organism, possibly acting as a "sink" for free radicals by binding and avoiding toxic effects through both one- and two-electron redox and cation chelation properties; (2) allomelanins have antibiotic properties as they react toward nucleophilic groups, including amino groups ($-NH_2$) and thiols (-SH); or (3) melanin provides increased structural strength through rigidity (Riley 1997).

2. Litter experiments. As a follow up to the hypothesis-confirming competition experiments, we designed allelopathic litter experiments similar to tease apart competitive effects from enzymatic ones by eliminating variables of nutrient competition, or competition for light when plants are grown concurrently. The allelopathic plant litter did not suppress growth of PPO/non-PPO grasses as in similar allelopathic decomposition experiments by (Singh et al. 2005) and (Batish et al. 2006) used 40g biomass per kg of soil and 10g per kg soil, respectively; both saw allelopathic suppression in these amounts.

Indeed, litter experiments did not support our hypothesis. Despite these published litter experiments demonstrating allelopathy in this manner of amended soil using plant material, when grown with litter (roots, shoots, and roots and shoots) of allelopathic forb *Centaurea stoebe*, both the PPO-producing grass *Bromus inermis* and non-PPO producer *Festuca rubra* produced longer leaves, more shoots, and more roots, suggestive of a benefit of growing with *C. stoebe*, which may be due to a compost effect, a result of the

carbon, nitrogen, and other possible nutrients and micronutrients contained in the added plant litter in comparison to controls, which contained no added litter. Similarly, when both PPO-producer *B. inermis* and non-PPO producer *F. rubra* were grown with litter (roots, shoots, and roots and shoots) of the allelopathic forb *Artemisia vulgaris*, number of leaves, height, and both shoot and root growth increased, also likely due to a compost affect. These results were not predicted by our hypothesis, as *A. vulgaris* produces allelochemicals that do not serve as PPO substrates.

It seems reasonable to believe that the allelopathic plants may need to be alive to actively produce allelochemical compounds, and so the added dried litter in experimentation did not produce a phytotoxic effect, rather, we attribute the slight increase in grass biomass may be due to a fertilizer or "compost" effect, a benefit of growing plants with added litter, which may have provided carbon, nitrogen, and other nutrients to the plants that was not available in controls. Production of allelochemicals is highly variable, not just between species, but also among species, during different stages of plant development, and also between different tissue types (Belz 2007).

Our litter experiment could be improved by first growing allelopathic plant species then removing them and finally planting in PPO/non-PPO grasses and measuring effects as by (Friedman et al. 1977). In Israel, *Artemisia* species suppress native neighboring plants through use of chemical inhibition, a phenomenon observed even after *Artemisia* is removed, suggesting allelopathic compounds remain in the soil (Friedman et al. 1977). Laboratory experiments showed a similar pattern of suppression of germination of natives by aqueous shoot extracts of *Artemisia* (Friedman et al. 1977).

3. Leachate experiments. Greenhouse leachate watering experiments supported our hypothesis. Similar to (MC et al. 2010), freshly-made aqueously-extracted leachate from allelopathic plants *Artemisia* and *Centaurea* were used to water both PPO-*Bromus* and non-PPO-*Festuca*. This system was ideal to tease apart competitive effects from enzymatic ones, (for example, versus nutrient competition, or competition for light when plants are grown concurrently), and eliminated confounding factors of nutrient additions from added biomass, as in the litter experiment.

First, we saw allelopathy where anticipated. Leachate watering experimental results demonstrate the allelopathy phenomenon, as both change in height and root mass were significantly (P = 0.02 and 0.02, respectively) suppressed, confirming our hypothesis that despite having the PPO enzyme, *B. inermis* would not be able to tolerate non-phenolic allelochemicals of *A. vulgaris*. *Festuca rubra* roots were significantly suppressed by both *Centaurea stoebe* and *Artemisia vulgaris* leachate. Secondly, *Centaurea* produces catechin, a phenolic compound that may be a substrate for PPO (Bais et al. 2001; Bais et al. 2003; Bertin et al. 2003; Perry et al. 2005; Perry et al. 2007). Catechin causes death of the root meristem tissue from the accumulation of reactive oxygen species (ROS) (Bais et al. 2003). Catechin targets roots, but here, were saw *Bromus* roots were defended, possibly by PPO in that *Bromus inermis* watered with *Centaurea stoebe* leachate show tolerance of *C. stoebe* by *B. inermis*. The documented allelopathic ability of *Centaurea stoebe* predicts that leachate from the plant used to water other species would result in suppression of the target plant (Bais et al. 2001; Bais et al. 2003; Bertin et al. 2003; Perry et al. 2005; Perry et al. 2007).

4. Field surveys. Plant community composition can inform ecologists of successful plant strategies and mechanisms of competition and coexistence (Meiners et al. 2012). Field surveys were conducted to assess close relationships between the grass *Bromus tectorum* and nearby plants in nature and assess the nature of such relationships, as ecosystems are governed by species, which vote with their presence. Both competition and facilitation are common and important in close proximities (Wright et al. 2014). Allelochemistry may influence plant communities (e.g., (Kong 2010; Thorpe et al. 2009)). Accordingly, information about what grows nearby *Bromus* species in nature and what does *not* grow nearby *Bromus* species was assessed via field observation data of the nearest neighbors. The effects of PPO, such as the predicted defense against allelochemical compounds, may manifest as changes on the physical environment thus, using known methods and analyses neighborhood analyses, we assessed the extant plant communities comprising the ecological system, a reflection of the effects of PPO (e.g., (Kennedy et al. 2002; Mack and Harper 1977; Meiners et al. 2012; Pacala and Silander 1987)).

We statistically determined species mean distances, and grouped by allelopathic properties. Results indicate that on average, the non-phenolic allelopathic forb *Artemisia vulgaris* occurs at larger distances from *Bromus* than do other species; this is predicted by (1) the allelopathic activity of *A. vulgaris* and (2) our greenhouse competition results wherein *Bromus* was suppressed by *A. vulgaris*, thus, we expected the species to occur at far distances. The larger distances of *Bromus* to *A. vulgaris* versus *Bromus* to all other plants, albeit insignificantly, makes sense ecologically and biochemically because previous experiments involving the *Bromus* and *A. vulgaris* species showed that *Bromus*

was suppressed by *A. vulgaris* in the greenhouse competition experiments, particularly belowground.

Our study helps further understand of ecological theories by testing plant competition and community determinants. Plants are in a constant competition and attack and defense arms race, not only with other plants both inter- and intra-specifically, but also with parasites, parasitoids, pests, and pathogens through physical and chemical means; our study adds to this complex body of knowledge by suggesting a population, community, or ecosystem, may be resilient to an invasion through the insight on the PPO trait. These results corroborate results from other studies, antagonistic effects of competitor plants.

Evidence of the "novel defense" hypothesis from the literature. In the field of ecology, observed phenomena are sometimes reported without explanation for said phenomena; we found several such published incidences which may be explained by our novel defense hypothesis. Likely many factors contribute to interactions in the rhizosphere, but our findings suggest PPO may have a vital role in the defense against phenolic allelochemicals. The results of several published studies might be understood in the light of an active defense against phenolic allelopathic agents. (Lindquist et al. 1996) noted that *Centaurea* rarely invades *Bromus inermis* dominated areas; upon experimental competition, *Bromus* showed low relative competition index (RCI) values and suppressed growth of *Centaurea*. In comparison *Festuca* had no impact and was not capable of suppressing weedy growth of *Centaurea* (Lindquist et al. 1996). The invasive species *Centaurea stoebe* and *Bromus tectorum* were both inhibited by litter and leachate from *Pinus ponderosa*, but *Bromus* was able to persist and was less inhibited by *Pinus* than

Centaurea (Metlen et al. 2013). *Pinus ponderosa* produces caffeic acid, chlorogenic acid, quercitin (all of which are phenolics), and large amounts of condensed tannins, released into the soil (Lodhi & Killingbeck 1980). The competitive interaction mediated by the allelopathy of *P. ponderosa* allowing for the success of *Bromus* may be due to the use of PPO to detoxify the allelochemicals in phenolic conformation. Various plant candidates were screened for restoration potential post-*Centaurea* invasion and *Bromus marginatus* (mountain brome) was found to be an ideal candidate; this was attributed to high resistance to catechin produced by *Centaurea* (Perry et al. 2005). A gradient of invasive success of 3 *Bromus* species was due to: (1) phenotypic plasticity; (2) high competitive response (*B. tectorum* was most unaffected by competition); and (3) ability to be more generalist; but the authors pointed out that they do not know the trait that enhances the invasiveness (Fenesi et al. 2011). This is where our research is of particular importance and we point out the following regarding their 3 test species:

a) *Bromus squarrosus* (corn brome, not invasive) – Low PPO (2.01; Plank 2012, unpublished).
b) *Bromus sterilis* (poverty brome, invasive) – High PPO (3.4-9.8; Holzapfel et al. 2010; Plank 2012, unpublished).
c) *Bromus tecoturm* (cheatgrass, highly invasive) – High PPO (3.6-6.5; Holzapfel et al. 2010; Plank 2012, unpublished).

Bromus sterilis can become the dominant species even in stands of the highly allelopathic and invasive *Robinia pseudoacacia* (black locust) which produces robinetin, myricetin and quercetin (all of which are phenolics); the mechanism by which *Bromus* is successful may be the utility of PPO (Fenesi et al. 2011; Nasir et al. 2005). *B. tectorum* is cited

anecdotally as not invading *Eurotia lanata* (winterfat) or *Atriplex nuttallii* (Nuttall's saltbush), but prevents the establishment of *Agropyron desertorum* and *A. smithii* (Klemmedson and Smith 1964).

The mechanism that could possibly allow these results and other field observations may be the disarmament of phenolic-allelochemicals by PPO, the novel defense hypothesis we propose. Geared toward an understanding for conservation and management purposes and tested by a host of experiments, we accurately predicted that *Bromus* species, with high root PPO, would be less suppressed than other grass species which do not have high levels of PPO, in competing against phenolic-allelopathic plants.

CONCLUSIONS

A large body of previous work on allelopathy has emphasized the importance of plant biochemistry in community-defining interactions. We investigated the effects of allelochemicals on plant ecosystems to contribute to a larger focus on plants suitable for restoration as understanding how to successfully restore an invaded ecosystem is important in effective environmental planning. The strong evidence for a novel defense by PPO presented here demonstrates a probable negative impact of *Bromus* cover on the species in the plant communities of urban wildlands. From our finding that the invasive *Bromus* can tolerate phenolic allelochemicals, we advise planting native non-phenolic allelopathic plants may be an optimal management recommendation. Additionally, allelochemicals are a natural alternative to synthetic herbicides, which can cause many undesirable effects. Future actions for complete control of invasive *Bromus* species or even restoration of invaded ecosystems is unrealistic, however, species control is

necessary for the conservation of natural populations of native and rare species. Just as in epidemiology, one size does not fit all for management strategies to meet objectives, and there is an ongoing need for continuation of empirical studies for better conservation and ecosystem restoration strategies through improved understanding of these processes.

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The utility of high root polyphenol oxidase (PPO) in Poaceae (grass) species in protection from the phytotoxic phenolic caffeic acid

ABSTRACT

The number of species in the Poaceae (grass) family exceeds 11,000, and these species exhibit a continuum of levels of root polyphenol oxidase (PPO), an enzyme of unknown utility. Within this family, one genus in particular, *Bromus*, exhibits particularly high levels of PPO. Here, we tested the hypothesis of PPO as a "novel defense" against the naturally-occurring phenolic allelochemical caffeic acid (CA). By using a known concentration of CA as a substrate for different Poaceae species having variable levels of PPO, we measured the response of a spectrum of five individuals of eight low-to-high PPO-producing *Bromus* and non-*Bromus* species. The same eight species were used as controls and watered without the CA treatment. Roots of all plants used in the experiment were assayed for PPO levels using established enzyme assay methods. We then tested for a relationships among PPO levels and performance (measured by parameters of change in height, number of leaves, root biomass, shoot biomass, root length, and root diameter) at the whole-plant level. Results show that (1) root PPO is constitutively expressed, and was not induced by the presence of CA, that is, higher levels of PPO were not observed when CA was added; (2) (a) *Bromus* species root biomass was not significantly reduced in CA treatments compared to controls whereas non-bromes were, (b) roots of both genera groups had significantly smaller diameter when treated with CA ($P = 0.025$), and (c) roots were also marginally longer, although not significant ($P > 0.05$); (3) when grouped by genera, we found that *Bromus* root biomass was not significantly reduced in CA

treatment ($P > 0.05$) whereas non-bromes were ($P < 0.05$); (4) there was not a significant treatment effect on shoot mass, total mass, change in height, or root:shoot ratio ($P > 0.05$); (5) performance (metrics: root mass, shoot mass, total mass) weakly correlated with PPO level. In spite of these findings of significant belowground suppression of low-PPO non-bromes by CA and tolerance of CA by bromes, the 0.25 mM dose of CA may not have been sufficiently antagonistic. 0.25 mM CA likely represents a low, sub-toxic dose wherein treatments did not significantly decrease growth metrics relative to controls. Because the 0.25 mM CA did not significantly suppress the low-PPO species by all metrics in the experiment, we cannot conclude whether PPO was beneficial to plant growth and survival when faced with CA. The use of this environmentally realistic dose may have precluded us from observing significantly antagonistic effects by CA.

INTRODUCTION

The numerous environmentally detrimental effects of invasive species and their associated economic costs are well-described in the literature, but the mechanisms are not completely understood (Callaway et al. 2011; Maron et al. 2014; Pimentel et al. 1997; Pimentel et al. 2005; Pimm et al. 1988; Pyšek et al. 2012) Allelopathy is one such mechanism, increasingly reported as a major factor influencing plant communities by determining invasion and establishment (Ridenour and Callaway 2001). The “novel weapons” hypothesis suggests invasive plants exude allelochemicals that are unknown to the native populations and therefore cannot be readily defended against (Callaway and Aschehoug 2000; Callaway and Ridenour 2004). In allelopathic interactions, plants naturally produce and release secondary metabolites, natural phytoxins not used for any

of the primary processes of the plant (e.g., photosynthesis) but which impose an allelopathic effect of phytoxicity (Rice 1984). Allelochemicals can be volatilized, exuded, or later leach from biomass into the environment (Inderjit and Callaway 2003; Rice 1984).

The documented effects of allelochemicals are diverse. These effects scale from the cellular to whole-plant and even community-level effects. In plant interactions in the rhizosphere, allelochemicals inhibit a number of plant activities, including membrane permeability (spilling cellular contents), ion uptake, nutrient uptake, stomatal conductance, photosynthesis, respiration, enzyme activity, protein synthesis, water balance, and cell division, thereby inhibiting seed germination and growth (Bais et al. 2001; Perry et al. 2006; Rice 1984; Walker et al. 2003; Wu et al. 2000). Production, release, or activity, and effects of allelochemicals thus ultimately have important ecological effects, as they influence plant dominance and yield (Inderjit et al. 2011).

One class or type of allelochemical compound is phenolics. Phenolics are common, naturally-occurring compounds that have been frequently implicated as the source of allelopathy, negatively effecting plants in various ways (Blum 1996; Estabrook and Yoder 1998; John and Sarada 2012; Keilin and Mann 1938; Mitrovic et al. 2012; Rice 1984). Phenolics, both dietary and environmental are ubiquitous. Phenolics make up 1-25% of total biomass of dry green leaves (Haettenschwiler and Vitousek 2000). One of five photosynthetically-fixed carbon atoms become phenolics, derived from the amino acid phenylalanine via the phenyl propanoid biosynthesis pathway to become one of more than 3,000 possible compounds including the phenolics: favanols, flavones, and isoflavonoids (Moore et al. 2014).

These phenolics negatively affect plants in numerous ways. First, phenolics lower the soil pH, making it more acidic, and second, are capable of changing the electric potential of cellular membranes, often having a depolarizing effect; ion losses may also occur from permeability of membranes (Leicach et al. 2009). Third, they negatively influence the concentration of auxin, a photosynthetic hormone responsible for cell growth and development in plant tissues (Leicach *et al.* 2009). Fourth, phenolic compounds absorbed by plant roots and taken up via xylem and introduced to the chloroplast are phytotoxic in that they reduce chlorophyll biosynthesis and thus the foliar chlorophyll content (Mitrovic et al. 2012). Finally, phenolic compounds inhibit nutrient absorption and regulate phytohormones; it is important to note that limited nutrients limits plant growth even more than limited photosynthesis (Leicach et al. 2009).

Phenolics are both antioxidants and proxidants. Phenolics act oxidatively especially where ions of the transition metals iron and copper are present (Bhat et al. 2007). Phenolic compounds are frequently referenced for their antioxidant benefits when included in the diet, are also antioxidant, antiviral, anti-tumor, antifibrotic, antihypertensive, antithrombotic bioactive compounds, but are also known to cause oxidative DNA damage (Prasad et al. 2011).

One enzyme that binds these phenolic substrates is polyphenol oxidase (PPO). PPO, characterized in 2003 (Anderson and Morris 2003), has been found in roots of all assayed *Bromus* species, but little or no PPO activity has been found in roots of other grass genera (Holzapfel *et al.* 2010). The characteristics of PPO and other phenolic oxidizing enzymes have been well described and reviewed (e.g., (Aniszewski et al. 2008; Keilin and Mann 1938; Mayer 2006; Valero et al. 1991). PPO is localized in plant cell

walls and vacuoles and exuded in great amounts into the rhizosphere through lysis and excretion at a rate of 1^{-50} μmol/hour/gram in soil (Dorantes and Zúñiga 2012; Sinsabaugh 2010). PPO only proceeds when provided with both molecular oxygen and a mono-, di-, or polyphenolic (Keilin and Mann 1938; Mayer 2006). The general mechanism of PPO is catalysis of the oxidation of phenolic compounds to o-quinones (semiquinones and quinones [2,5-cyclohexadiene-1,4-diones]) which react with amino acids, phenols, or proteins, generating melanin-like compounds visible as brown pigments, which notoriously reduce food quality, as in the case of pre-cut apples or potatoes for french fries (Aniszewski et al. 2008; Valero et al. 1991).

Oxidizable phenolic substrates include cinnamic acid derivatives and benzoic acid derivatives, catechin, chlorogenic acid, and 4-methylcatechol (Leicach et al. 2009; López-Molina et al. 2003; Queiroz et al. 2008). Thus, the general structure of the substrate for PPO is C6-C1, aromatic ring with hydroxylations and methoxylations. Data from our lab indicates that all substrates for the PPO enzyme are ortho-phenols, such as L-DOPA, caffeic acid, and catechol, but not para-phenols (Kafkewitz 2012).

Caffeic acid (3,4-dihydroxy cinnamic acid) (CA) is one such structure: caffeic acid has an ortho-diphenolic conformation, and has been shown to be a substrate for the enzyme PPO (Queiroz et al. 2008). Caffeic acid is found naturally in the diet in fruits, vegetables, olive oil, and coffee, but is not to be confused with caffeine, an unrelated compound. Caffeic acid is a cinnamic acid derivative and has wide-ranging physiologically toxic allelopathic effects, including increased cell membrane permeability and the reduction of hydraulic conductance as well as decreased nutrient uptake (Blum 1996; Li et al. 2010). CA has anti-cancer properties at the 30, 40, and 50

ug/mL level, exhibiting inhibition of *in vitro* cellular growth, likely via significant DNA damage (Prasad et al. 2011).

Although the mechanism and substrates required for enzymatic action of PPO are well understood, the function and rationale for production of the enzyme by some plants (and not others) has yet to be determined (Mayer 2006). A physiological role for PPO in such high ambient levels in the roots of *Bromus* species is not apparent (Holzapfel et al. 2010).

Bromus species may use PPO as a defense against phenolic allelochemicals through the enzymatic destruction of said allelochemicals before they can exert their toxic effects by oxidation of the phenolic allelochemicals before they reach their targets within the invaded plant. PPO has been previously suggested as a "putative defensive oxidative enzyme" (Constabel and Ryan 1998). The PPO enzyme, found in root plastids, comes in contact with phenolic substrates as they are released from leaching and in litter (Vaughn and Duke 1984).

PPO, present in some Poaceae species, oxidizes certain phenolic compounds, thus we hypothesize PPO is an advantageous defense for plants in interactions with phytotoxic phenolic allelochemicals through oxidation detoxification.

We tested the general hypothesis that PPO activity is a novel defense against phytotoxic allelochemicals, demonstrated by response of PPO-producing species in the presence of such phenolics, whereby the conversion into non-toxic forms of these harmful compounds by PPO may confer benefits to the plant, such as defense.

Here, we measured the response of a spectrum of five individuals of each of four high-low PPO-producing *Bromus* species and four little to no PPO-producing non-

Bromus species in response to treatment of watering with caffeic acid and compared to water controls. Roots of all individual plants used in the experiment were assayed for PPO levels using established enzyme assay methods. We tested the relationship among PPO level and performance (we measured several parameters: change in height, number of leaves, total, root, and shoot biomass, as well as root length and root diameter) to detect inhibitory or stimulatory responses, if there were any, by correlation and regression.

METHODS

Seed sterilization and germination. Seeds of each of the following eight species: *Bromus inermis*, *Bromus kalmii, Bromus tectorum*, *Bromus sterilis*, *Festuca rubra, Hordeum vulgare, Seceale cereal,* and *Triticum aestivum* were surface-sterilized with 10% bleach for 30 minutes, rinsed twice in sterile water, then plated into sterile 9 cm petri dishes with moist Fisher P8 filter paper.

Planting. 125 mL of pure silica sand was aliquoted into 150 mL capacity conical tube-pots with drainage holes. Ten replicates of germinated seedlings of each grass were planted in the conical tube-pots. These tube-pots were placed in racks designed specifically for them, which provided ample space between pots to avoid any interactions between plants. These racks were placed in the greenhouse at Rutgers University in Newark, New Jersey, USA. Racks were rotated periodically to avoid any block effects. All plants were watered with MiracleGro (24-8-16). Initial height was measured.

Caffeic acid preparation and watering. 0.25 mM (250 μM) caffeic acid (3,4-dihydroxy cinnamic acid) (CA) solution was prepared fresh for each watering as by Barkosky *et al.* (2000). Each plant was watered with 20 mL of either treatment, water or caffeic acid, which were applied avoiding leaves, until solution began to drip from the bottom of the pots. This procedure was repeated every other day for the duration of the experiment. The auto-water system was used for normal watering on non-leachate watering days so that all plants were watered daily. The experiment ran for four weeks (3 February – 10 March 2014). Final height was recorded immediately prior to harvest.

Plant harvest. Individual plants were carefully removed from pots and washed. After 1 cm of root was cut (this root was used in assay described below), plants were separated into aboveground and belowground parts and bagged separately in labeled coin envelopes. Plants were dried in the drying oven (Fisher Scientific Isotemp oven) at 60C until constant weight to the nearest hundredth milligram. Dry roots were then imaged using the root scanner described below.

Enzyme assay protocol. A 2 mg/mL L-DOPA (L-3,4-dihydroxyphenylalanine) in MOPS solution was prepared in 50 mM MOPS (3-(N-Morpholino)propanesulfonic acid sodium salt, 4-Morpholinepropanesulfonic acid sodium salt) buffered to pH 6.5 with 1N NaOH. The enzyme assays were performed at room temperature in 13x100 mm culture tubes and total reaction volume was 5 mL per tube. 2.5 mL water followed by the 1 cm piece of root were added to the each of five tubes. At t = 0, 2.5 mL DOPA-MOPS was pipetted into each tube and vortexed and the first measurement was recorded. An additional reaction tube was set up with no root as a control for spontaneous, nonenzymatic color development as well as a water blank control. To avoid pseudoreplication, one piece of

root from five different plants per species were assayed. Absorbance (A_{475}) values of the incubation using a UV-VIS spectrophotometer (Spectronic 20D+, Milton Roy) were averaged for each species. The total activity ΔA_{475} was divided by the root length, giving the enzyme activity per unit root, thus, one unit of PPO was defined as the absorbance at 475 nm per cm root length.

WinRHIZO program and root scanner. The WinRhizo Pro (Regent Instruments, Inc.) program for washed root measurement was used in conjunction with TWAIN compatible top scanner Epson Expression 16801.D, which captures 8 bits per pixel per color or 1 bit per pixel for black and white images. Roots were scanned for both diameter and length to the nearest tenth of a millimeter.

Data analysis. In general, statistical analyses tested for significant differences within and among species using general linear model, univariate ANOVA were conducted in SPSS (Version 21.1) with 95% confidence intervals. We tested for significant differences in growth and survival, using changes in height (calculated as final minus initial), root, shoot, and total dry biomass, and root diameter and root length. We then tested for relationship between PPO levels and individual performance in those variables, comparing also caffeic acid treatments to controls. We also log transformed root mass and PPO for linear regression, and PPO was used as the explanatory variable to understand the relationship with the response variables.

RESULTS

PPO enzyme assays. Results of enzyme assays of the roots of individual plants show that PPO activity varies significantly by species (Table 1, Fig. 1, $P < 0.05$). PPO activity

was not induced by the caffeic acid (CA) treatment in comparison to controls (Table 1, Fig. 1).

Table 1. Enzyme assay results by species and treatment, caffeic acid or control.

Genus	Species	Treatment	Seed source	Invasive	Plant origin	Duration	N	PPO	PPO SD	PPO SE
Bromus	*inermis*	Control	Commercial, USA	Yes	North America & Introduced	Perennial	5	3.15	0.87	0.44
Bromus	*inermis*	Caffeic acid	Commercial, USA	Yes	North America & Introduced	Perennial	5	2.99	0.84	0.37
Bromus	*kalmii*	Control	USDA	No	North America	Perennial	5	0.89	0.28	0.16
Bromus	*kalmii*	Caffeic acid	USDA	No	North America	Perennial	5	1.00	1.20	0.69
Bromus	*sterilis*	Control		Yes	Eurasia	Annual	5	3.98	2.41	1.08
Bromus	*sterilis*	Caffeic acid	USDA	Yes	Eurasia	Annual	5	4.59	2.10	0.94
Bromus	*tectorum*	Control	Iowa, USA	Yes	Mediterranean Europe	Annual	5	4.56	5.07	2.54
Bromus	*tectorum*	Caffeic acid	Iowa, USA	Yes	Mediterranean Europe	Annual	5	3.70	0.96	0.56
Festuca	*rubra*	Control	Commercial, USA	Yes	North America & Introduced	Perennial	5	0.98	0.69	0.31
Festuca	*rubra*	Caffeic acid	Commercial, USA	Yes	North America & Introduced	Perennial	5	1.80	1.21	0.54
Hordeum	*vulgare*	Control	USDA	No	Africa, Eurasia	Annual	5	0.10	0.11	0.05
Hordeum	*vulgare*	Caffeic acid	USDA	No	Africa, Eurasia	Annual	5	0.15	0.15	0.07
Secale	*cereale*	Control	USDA	No	Western Asia and India	Annual	5	0.19	0.26	0.12
Secale	*cereale*	Caffeic acid	USDA	No	Western Asia and India	Annual	5	0.17	0.21	0.09
Triticum	*aestivum*	Control	USDA	No	Mediterranean, southwest Asia	Annual	5	0.47	0.27	0.12
Triticum	*aestivum*	Caffeic acid	USDA	No	Mediterranean, southwest Asia	Annual	5	0.40	0.41	0.18

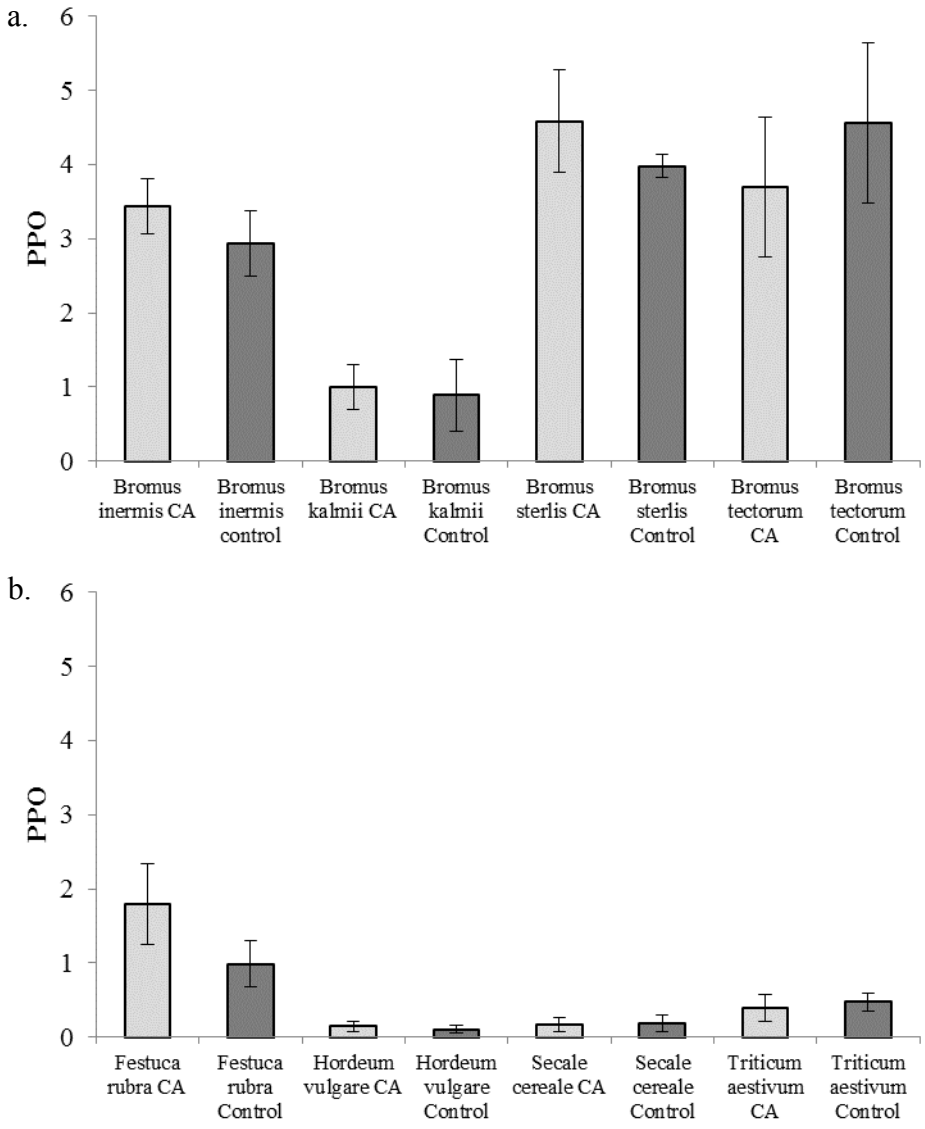


Fig. 1. Mean PPO for control and caffeic acid (CA) watering treatments of both (a) *Bromus* species and (b) non-*Bromus* species (each bar n = 5, error bars +/- 1 SE).

Root and shoot biomass. There was a trend toward lower root mass in caffeic acid treatments compared to controls of the non-brome genera group (Table 2, Figs. 2 & 3, $P < 0.05$). An overall species effect was detected on root mass ($P < 0.001$), additionally, root mass was significantly different by treatment ($P = 0.037$). Root mass was not significant by treatment*species ($P = 0.215$), or by PPO amount ($P = 0.612$).

Aboveground we saw fewer trends. Although shoot mass was significantly different between species ($P < 0.05$), shoot mass results of CA treated species were not significantly different from controls (Figs. 2 & 3, $P > 0.05$).

Table 2. Above and belowground effects of caffeic acid experiments by species and treatment (each species n = 5).

Genus	Species	Treatment	Root mass (mg)	SE Root mass	Shoot mass (mg)	SE Shoot mass	Root/ shoot	Height change (cm)	SE Height change	Root length	SE Root length	Root diam.	SE root diam.
Bromus	*inermis*	Control	8.92	2.56	3.90	0.90	2.29	0.20	0.77	32.00	12.28	0.29	0.02
Bromus	*inermis*	Caffeic acid	6.32	1.66	3.78	0.99	2.09	1.00	0.20	45.31	13.33	0.28	0.03
Bromus	*kalmii*	Control	6.57	2.79	4.67	1.74	1.41	0.50	1.36	60.35	37.81	0.40	0.10
Bromus	*kalmii*	Caffeic acid	8.85	4.86	6.93	3.08	1.70	1.40	0.61	47.17	30.86	0.19	0.01
Bromus	*sterilis*	Control	42.84	8.82	13.90	2.05	3.08	1.00	0.63	60.35	37.81	0.40	0.10
Bromus	*sterilis*	Caffeic acid	41.74	8.86	17.00	3.53	2.46	1.90	0.73	155.59	22.14	0.27	0.01
Bromus	*tectorum*	Control	11.12	3.72	2.20	0.76	5.05	10.30	1.17	17.33	8.35	0.30	0.04
Bromus	*tectorum*	Caffeic acid	3.80	1.43	2.00	0.51	3.80	10.50	0.50	53.04	20.61	0.30	0.04
Festuca	*rubra*	Control	11.12	3.36	3.96	0.71	2.81	0.20	0.60	35.05	8.95	0.24	0.01
Festuca	*rubra*	Caffeic acid	8.44	2.71	3.68	0.69	2.37	1.80	0.20	35.05	8.95	0.24	0.01
Hordeum	*vulgare*	Control	77.72	13.69	43.90	8.74	1.77	1.70	1.11	127.23	30.02	0.31	0.02
Hordeum	*vulgare*	Caffeic acid	35.14	7.72	23.68	5.39	1.48	2.30	1.45	299.88	33.09	0.30	0.01
Secale	*cereale*	Control	82.68	40.80	22.33	8.26	3.70	3.10	1.20	30.88	10.90	0.31	0.01
Secale	*cereale*	Caffeic acid	33.18	66.14	21.58	17.86	1.54	2.40	0.84	46.05	20.14	0.29	0.02
Triticum	*aestivum*	Control	16.10	9.17	11.62	3.75	1.39	1.40	1.38	157.44	31.89	0.29	0.01
Triticum	*aestivum*	Caffeic acid	14.94	5.18	25.72	12.64	0.58	2.20	0.98	147.22	96.12	0.28	0.03

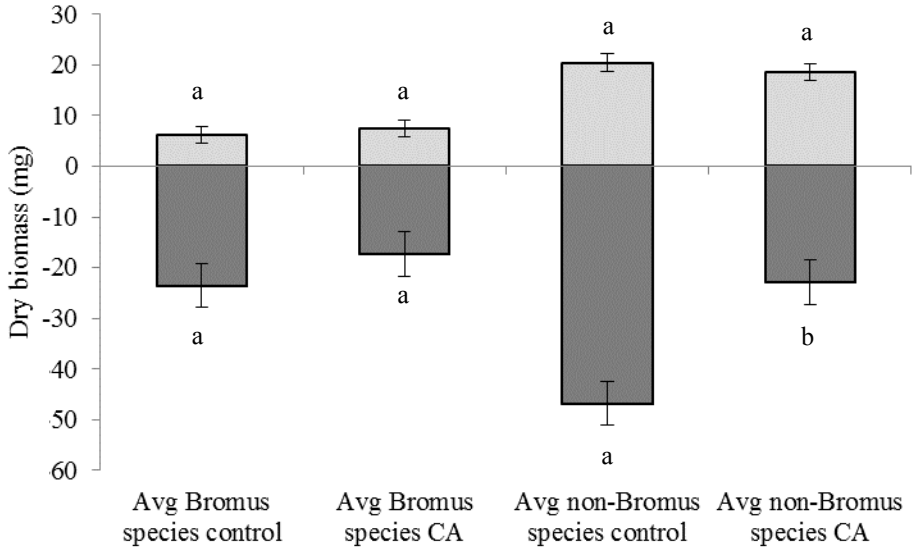


Fig. 2. Effects of caffeic acid (CA) and control treatments on both root and shoot biomass grouped by brome or non-brome and treatment. Error bars +/- 1 SE, each bar n = 20. Different lowercase letters indicate significant differences (ANOVA, Tukey's *post hoc* test, $P < 0.05$).

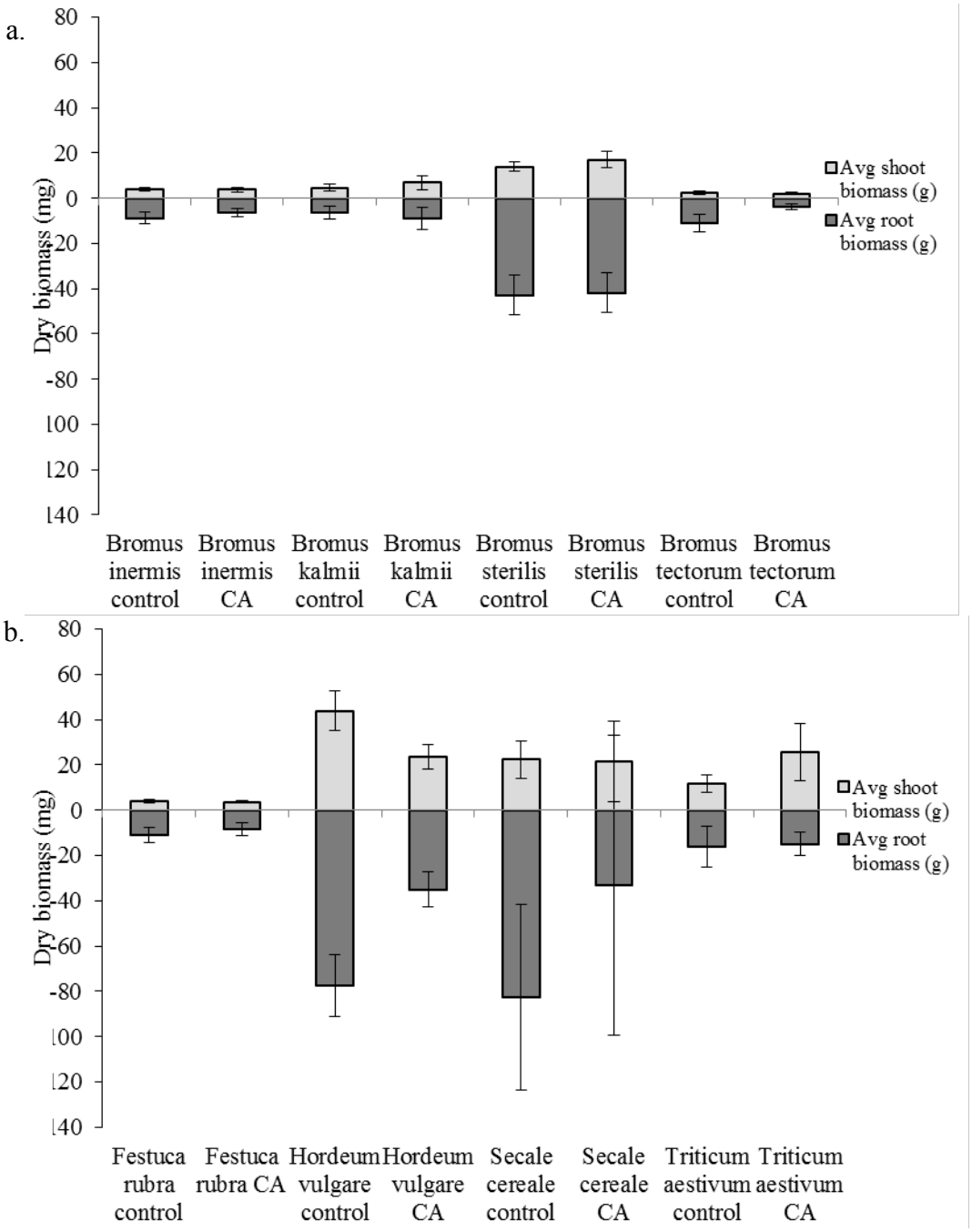


Fig. 3. Effects of caffeic acid (CA) watering experiments and controls on mean shoot and root biomass of both (a) *Bromus* species and (b) non-*Bromus* species (error bars +/- 1 SE, each bar n = 5).

Change in height. Mean change in height for all species and treatments show that none of the individual comparisons were significantly different when treated with CA (Figs. 4 & 5, $P > 0.05$). Change in height was significantly different between species ($P < 0.001$),

but was not significant different between treatments (P = 0.232) nor was treatment*species interaction effect detectable (P = 0.934).

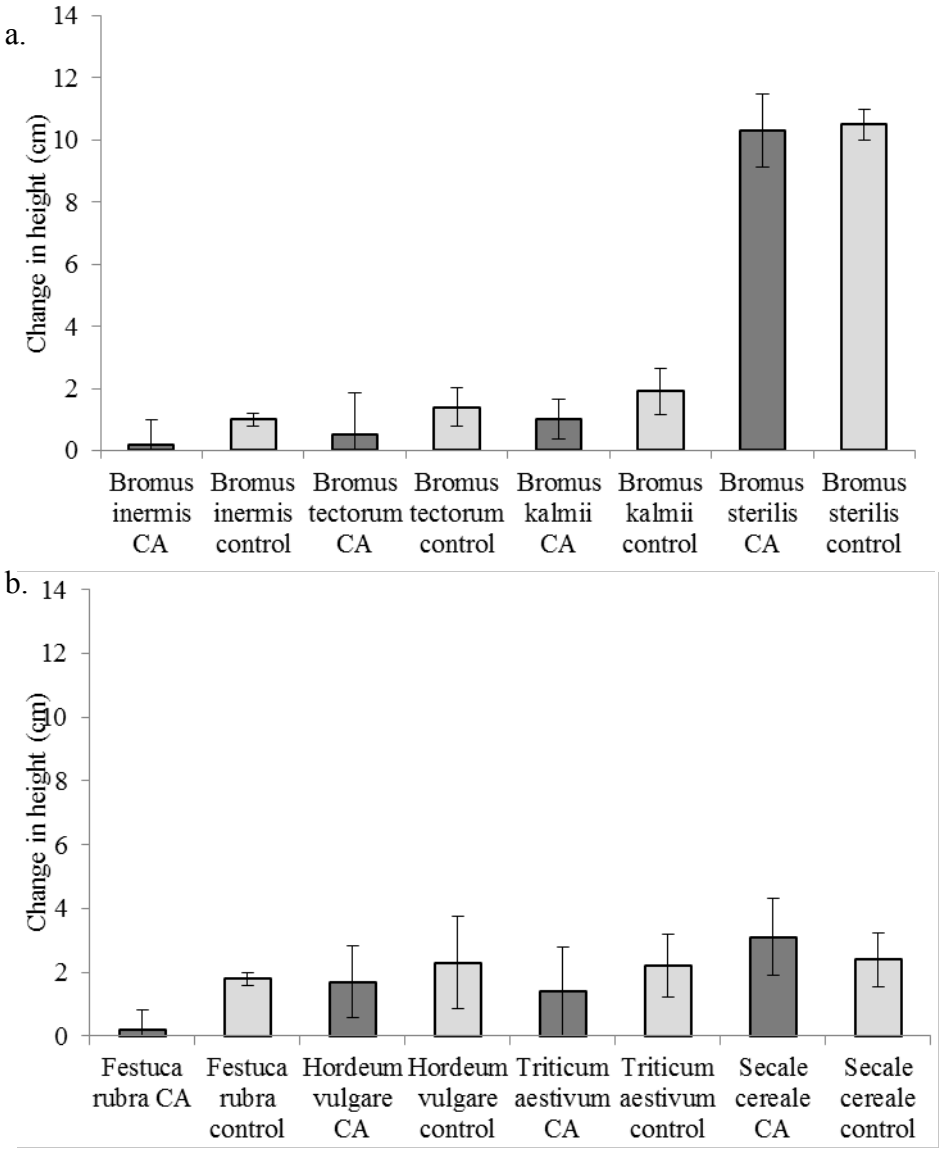


Fig. 4. Effects of caffeic acid (CA) watering experiments and controls on mean change in height of both (a) *Bromus* and (b) non-*Bromus* species (error bars +/- 1 SE, each bar n = 5).

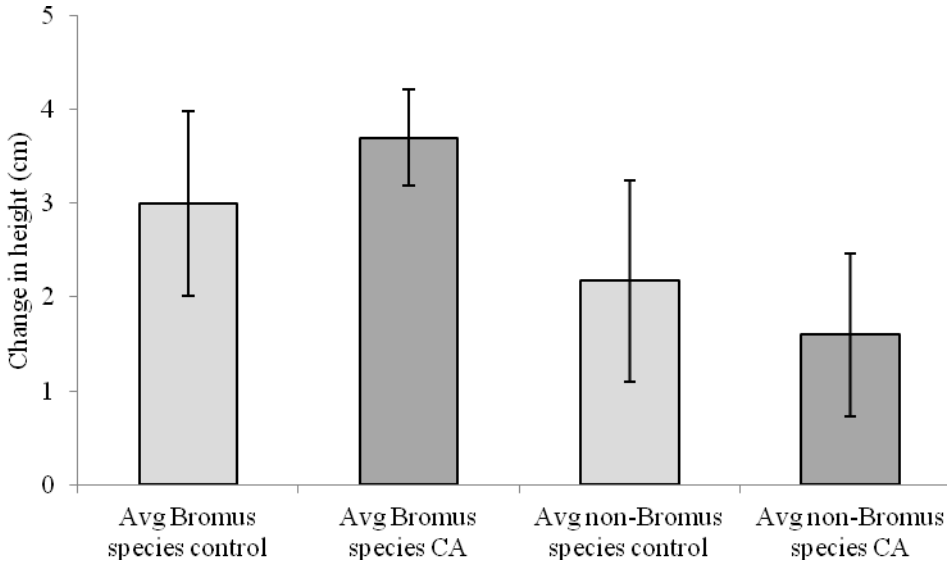
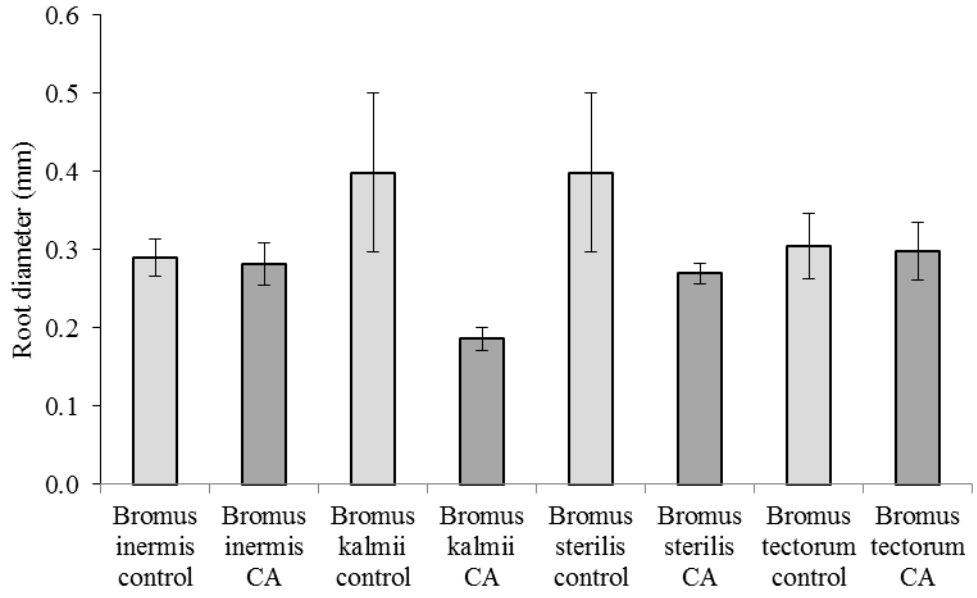


Fig. 5. Effects of caffeic acid (CA) watering experiments and controls on mean change in height of both (a) *Bromus* and (b) non-*Bromus* species (error bars +/- 1 SE, each bar n = 5).

Root diameter and length. Roots had significantly smaller diameter when treated with CA versus controls (Figs. 6 & 8, P = 0.025). Root diameter was not significantly different between species (P = 0.562), but was significantly different between treatments and was effected by treatment*species interaction (P = 0.045 and 0.025, respectively). Roots were also marginally longer, although not significantly different between treatment (Figs. 7 & 9, P = 0.114). Root length was significantly different between species (P < 0.001), but no significant treatment*species interaction effect was detected (P = 0.126). Among all species, *Bromus tectorum* was an exception in that it produced longer roots when treated with CA in comparison to controls (P < 0.05).

a.



b.

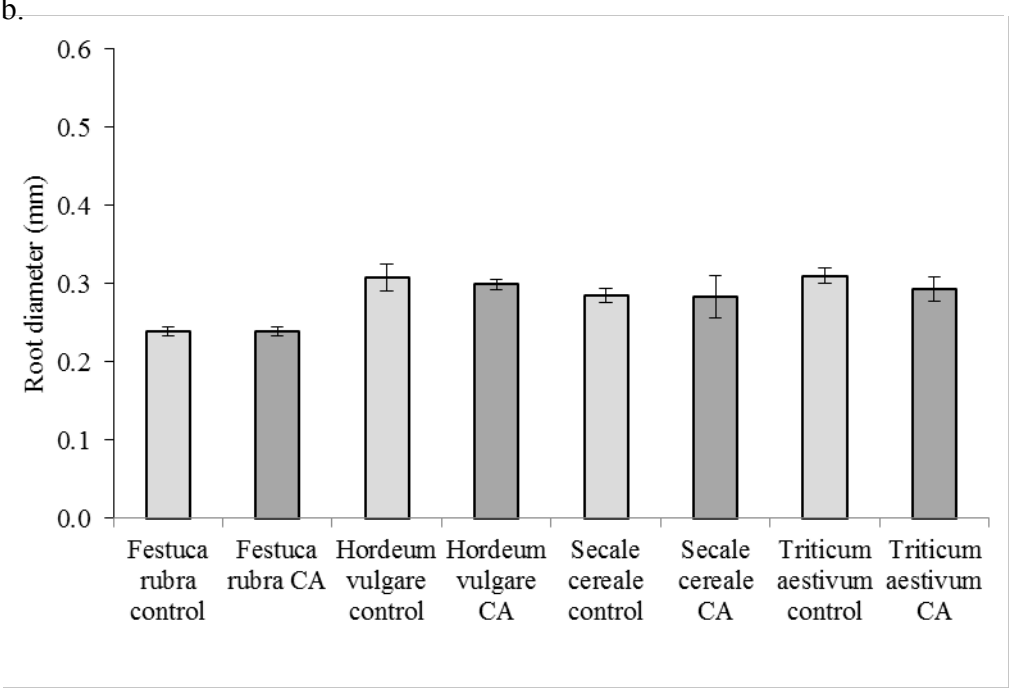


Fig. 6. Effects of caffeic acid (CA) watering experiments and controls on mean root diameter of both (a) *Bromus* and (b) non-*Bromus* species (error bars +/- 1 SE, each bar n = 5).

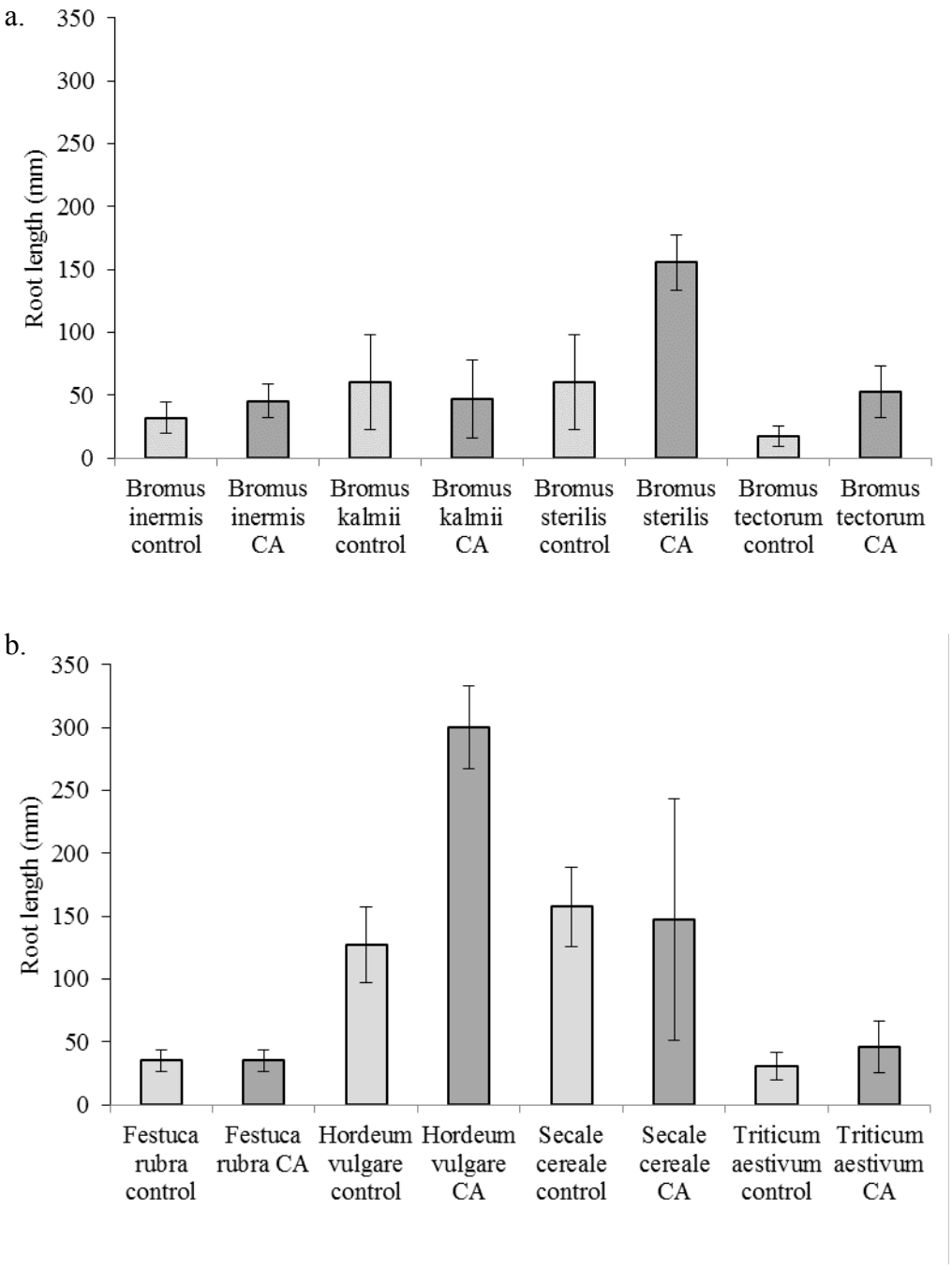


Fig. 7. Effects of caffeic acid (CA) watering experiments and controls on mean root length of both (a) *Bromus* and (b) non-*Bromus* species (error bars +/- 1 SE, each bar n = 5).

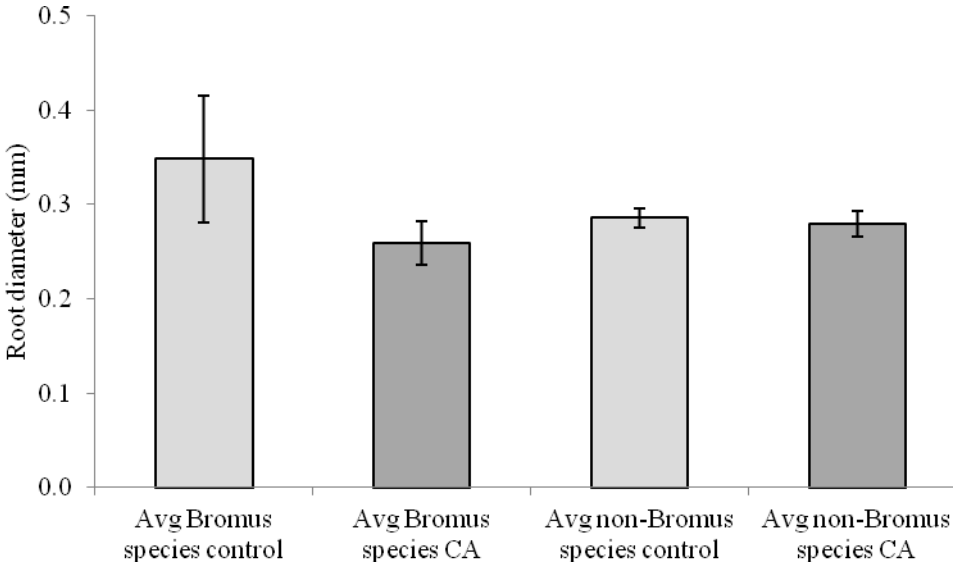


Fig. 8. Effects of caffeic acid (CA) watering experiments and controls on mean root diameter of both *Bromus* and non-*Bromus* species (error bars +/- 1 SE, each bar n = 5).

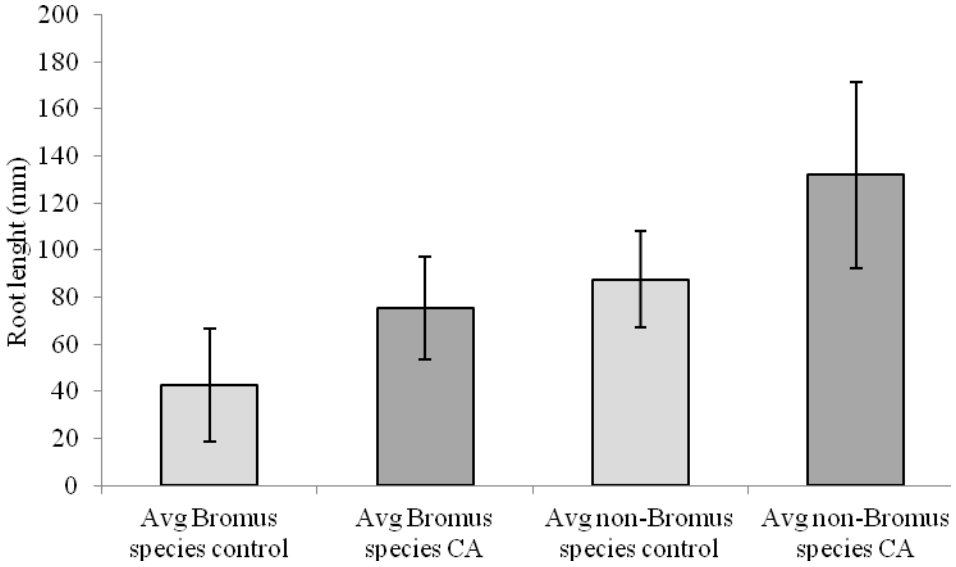


Fig. 9. Effects of caffeic acid (CA) watering experiments and controls on mean root length of both *Bromus* and non-*Bromus* species (error bars +/- 1 SE, each bar n = 5).

Root/shoot ratio. Overall mean root to shoot ratio was lower in the caffeic acid treatment than the control, a trend that was prominent in the non-*Bromus* species (Figs. 10 & 11). Root to shoot ratio varies significantly by species ($P < 0.001$) and treatment ($P = 0.026$), but there was no significant treatment by species interaction effect ($P = 0.507$).

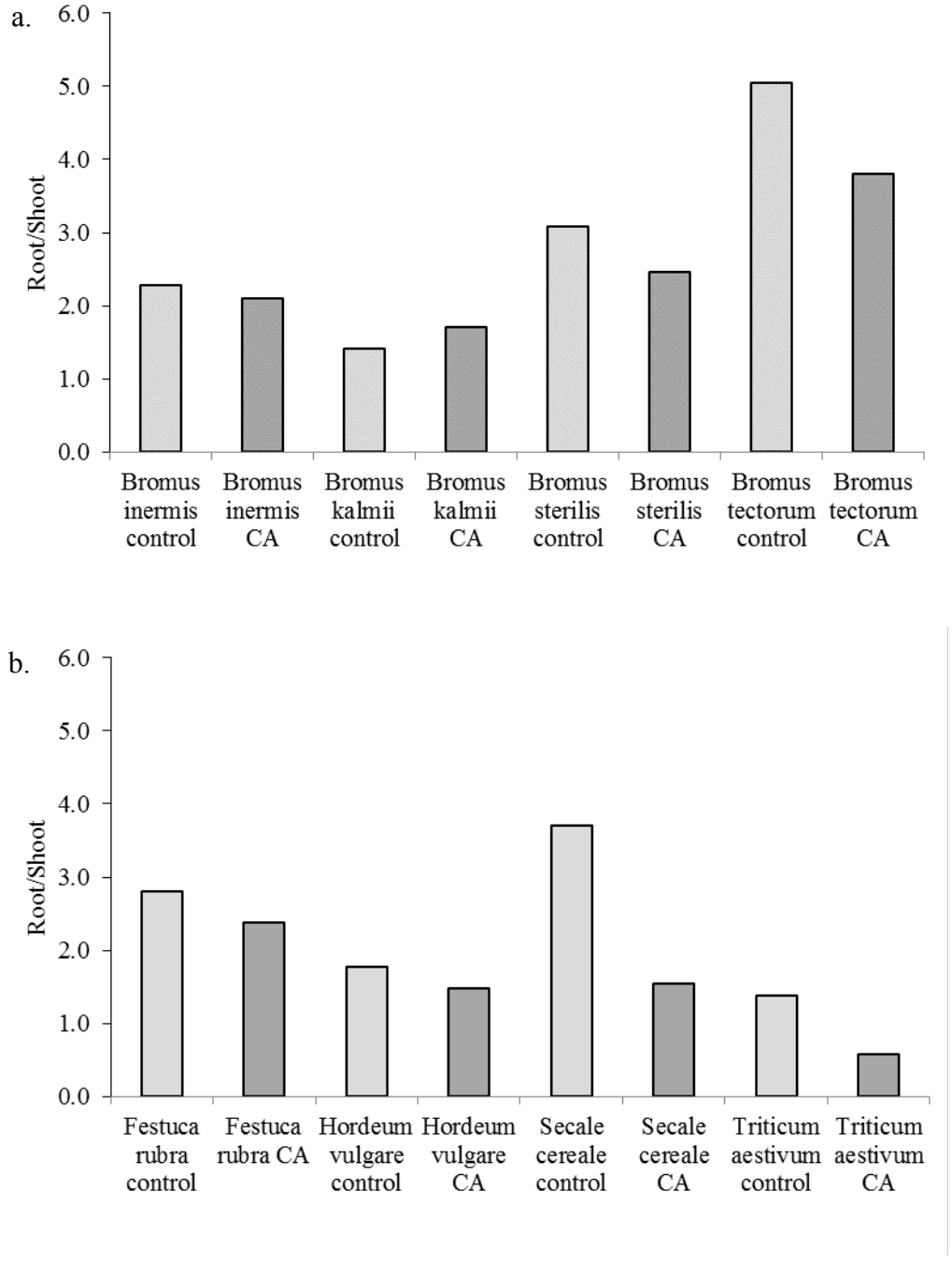


Fig. 10. Effects of caffeic acid (CA) watering experiments and controls on mean root/shoot ratio of both (a) *Bromus* and (b) non-*Bromus* species (each bar n = 5).

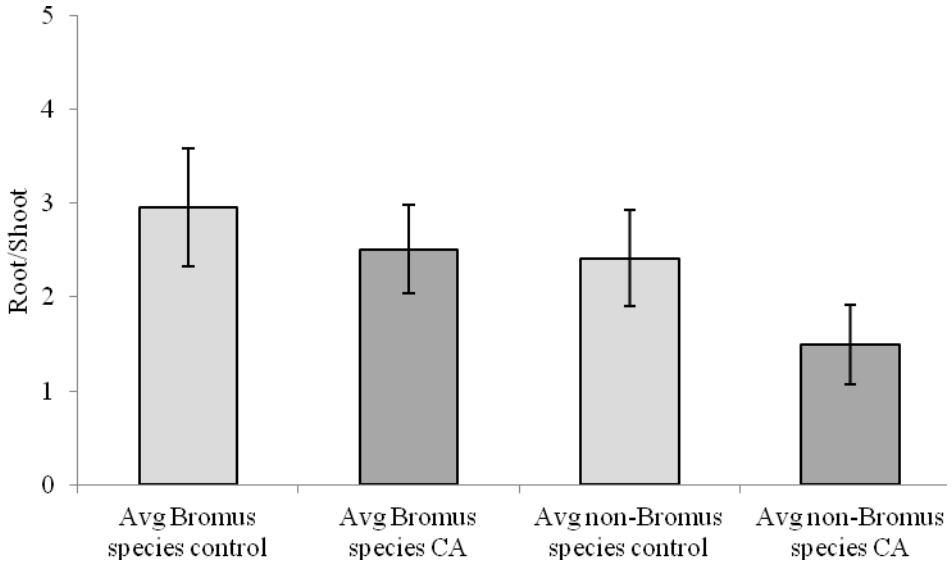


Fig. 11. Effects of caffeic acid (CA) watering experiments and controls on mean root/shoot ratio of both *Bromus* and non-*Bromus* species (each bar n = 5).

Correlation of PPO with response variables. GLM (dependent variable of total biomass and independent variable of PPO) results showed that total biomass was not significantly correlated with PPO amount ($P = 0.618$) although mass production was significantly different by species ($P < 0.001$). In addition, there was no significant treatment effect on total mass ($P > 0.05$). Root mass was not significantly predicted by PPO amount ($P = 0.20$), although root mass was significantly different by species ($P < 0.001$). Shoot mass was not significantly predicted by PPO amount ($P = 0.618$), nor was total mass dependent on PPO amount ($P = 0.637$). Total biomass was not significant by treatment ($P = 0.237$), and was not significant by treatment*species ($P = 0.315$). The correlation of root, shoot, or total mass and PPO was not significant (Table 3).

Table 3. Regression of various dependent factors with PPO concentration.

Regression with PPO	R^2	n
Root mass, all species	0.0324	80
Root mass, *Bromus* species	0.0211	40
Root mass, non-*Bromus*	0.0098	40
Log root mass, all species*	0.0395	80

Log root mass, *Bromus* species*	0.0056	40
Log root mass, non-*Bromus**	0.0063	40
Shoot mass, all species	0.0075	80
Shoot mass, *Bromus* species	0.0706	40
Shoot mass, non-*Bromus*	0.0038	40
Total mass, all species	0.0036	80
Total mass, *Bromus* species	0.0005	40
Total mass, non-*Bromus*	0.0053	40

*regressed with log PPO

DISCUSSION

Results show that (1) PPO is constitutively expressed, and was not induced by the presence of caffeic acid since higher levels of PPO were not observed when caffeic acid was added; (2) *Bromus* species root biomass was not significantly reduced in CA treatments compared to controls ($P > 0.05$) whereas non-bromes were ($P < 0.05$), but these effects were lost when we considered biomass by individual species; (3) roots of both genera groups (brome and non-brome) had significantly smaller diameters when treated with CA ($P = 0.025$), and (c) roots were also marginally longer, although this was not significant ($P > 0.05$); (4) there was not a significant treatment effect on shoot mass, total mass, change in height, or root:shoot ratio ($P > 0.05$); (5) performance (metrics: root mass, shoot mass, total mass) weakly correlated with PPO level.

Root PPO was constitutively expressed. PPO did not seem to be induced by the caffeic acid treatment. Just as allelochemical production can be both constitutive and induced via gene upregulation, biotically induced in response to pathogens, insects, or other plants, or abiotically induced via chemicals, including herbicides (Belz 2007), so too can PPO expression (Constabel and Barbehenn 2008). Induction of PPO production occurs in the plant families Betulaceae (Black alder), Fabaceae (beans), Solanaceae (potato, tomato), Salicaceae (poplar), and Theaceae (tea), and *Populus* species (aspen, cottonwood, poplar), but not Poaceae (grass) (Constabel and Barbehenn 2008;

Thipyapong and Steffens 1997; Tran and Constabel 2011). Tomatoes (*Lycopersicon esculentum* Mill.) in particular exhibit a complexity of differential expression patterns during growth and development and in response to wounding (Thipyapong and Steffens 1997; Tran and Constabel 2011). Upregulation of PPO was observed in response to stresses from mechanical wounding (abiotic injury, physical stress), pathogenicity via biotic injury (*A. solani*, *P. pyringae*), and exposure to vapors of salicylic acid, jasmonate, and ethylene (Thipyapong and Steffens 1997). The inducability of PPO upregulation in such scenarios suggests a role for PPO as a defensive mechanism and an evolutionary rationale for maintaining the PPO enzyme. However, similar to previous studies on grasses, our results showed PPO does not seem to be induced, evidenced by the insignificant variation within species (control versus CA treatment). The lack of induction of PPO activity by our eight assayed grasses is interesting, as PPO has been implicated in aiding plant persistence in survival in the face of attack, namely insect herbivores and pathogens, but we have seen constitutive root expression of PPO.

Non-brome root mass suppression by CA. Toxicity of CA varied between target species. *Bromus* species root biomass showed tolerance to the CA in that the bromes were not significantly reduced in CA treatments compared to controls, whereas non-bromes were significantly suppressed by CA treatment (allelopathic effect). This would be perhaps our most significant finding, supportive of our hypothesis in that bromes tolerated the CA treatment, possibly through the use of PPO activity to convert CA to a less harmful form, however, these effects were lost when we considered biomass by individual species. In addition to demonstrating how CA is tolerated by the roots of *Bromus* species, in this instance, this study illustrates a direct connection between an

organism's response and biochemical properties of the PPO it employs by three out of four brome species.

Shoot mass, total mass, change in height. Aboveground metrics were not significantly negatively affected by CA. There was no significant treatment effect (CA versus control) on shoot mass, total mass, or change in height. Because aboveground our non-brome group tolerated CA, we cannot draw any conclusions about the similarly demonstrated brome tolerance of CA aboveground. We avoided leaves when watering plants, so this is not surprising, rather, we expected to see a major inhibition belowground, so we also compared root scans and root/shoot ratios for the two treatments.

Root length and diameter. Through root scan analysis, we further show that CA suppressed roots, but here, both *Bromus* and non-*Bromus* species were suppressed, independently of their PPO activity. We anticipated resilience by *Bromus* roots in response to CA treatments because of root mass results, however, root scans revealed that both *Bromus* and non-*Bromus* genera were significantly thinner than controls when treated with CA. This demonstrates the allelopathic affect of CA. Both groups of *Bromus* and non-*Bromus* roots were also marginally longer than controls when treated with caffeic acid, though not significantly so. This finding is interesting but does not support our hypothesis because *Bromus* root diameter does not show resilience to the CA treatment as we would expect.

Root/shoot ratio. A high root to shoot ratio means that there are more roots than shoots, thus, here, when watered with caffeic acid, the low root to shoot ratios indicate that root growth was suppressed by the caffeic acid. Here, we found that neither group of

genera (brome or non-brome) significantly shifted their root to shoot allocation ratios when treated with CA in comparison to controls.

Correlation of performance with PPO. We saw no correlations of target species performance with PPO activity levels. The individual species (both *Bromus* and non-*Bromus*) performance (metrics: root mass, shoot mass, total mass) did not correlate with PPO level. Despite the observed significant suppression of root mass among non-*Bromus* species, this metric varied insignificantly with PPO activity.

PPO and mass trade-offs. Resource allocation is one of the fundamental underlying principles of plant growth and development. This is particularly important as plants respond to dynamic environments. PPO production and root biomass may have had a trade-off in plant expenditures for production of each in response to CA treatment. Specifically, *Bromus* species overwhelmingly produce higher PPO activity than non-*Bromus* species, and may rely upon the enzyme for defense. There may be fitness costs associated with allocating resources to the production of PPO, and the diversity of PPO expression may reflect trade-offs between a plant's energetic cost for PPO and benefit conferred by defense against environmental phenolics. The "trade-off" hypothesis, though often applied to host-parasite interactions, generally implies that adaptive responses are at some cost to the organism; alternatively, the "relaxed selection" hypothesis implies these adaptive responses are lost when not in use (Arbiv et al. 2012). Organisms exhibit trade-offs as alternative strategies between costs and yields, particularly in metabolic pathways (Agrawal 2007; Flamholz et al. 2013; Futuyma and Moreno 1988; Mole 1994; Wright et al. 2004). The proximate costs of plant chemical defenses may later be offset by ultimate growth and development rewards (Neilson et al.

2013). Here, there may have been a trade-off in plants that produced high levels of PPO thereby produced less biomass; this would have been evidenced by lower root mass - that is, high PPO activity may have been correlated with low root mass, as biosynthesis is expensive.

Caffeic acid. The overall lack or weak response of all grasses to caffeic acid treatment is surprising. It is possible that the caffeic acid treatment was not sufficiently strong enough (0.25 mM (Barkosky et al. 2000), which is a realistic concentration occurring in nature) and that we did not witness a strong negative effect from the weakness of the treatment. However, the concentration used did allow the plants to grow, striking a balance between total plant death and slight suppression (as seen in non-brome roots) or tolerance of the caffeic acid. It is also possible that a mixture of phenolic acids in the treatment was necessary to elicit a response (Blum 1996). Although field conditions are impossible to replicate in the laboratory, a phytotoxic mixture of compounds would have been more realistic to replicate the myriad compounds found in nature, and may have elicited a stronger suppression of growth parameters (Blum 1996). More research is needed to disentangle PPO and plant performance in response to CA. A follow up experiment would involve growth of these eight species with the CA-producing perennial roadside weed in India, *Leonurus sibiricus* (Mandal 2001).

CONCLUSION

Although we saw tolerance of CA by *Bromus* species (possibly due to the utility of PPO) and some significant belowground suppression of non-bromes, which have low PPO activity, 0.25 mM CA likely represents a low, sub-toxic dose wherein treatments did

not significantly decrease growth metrics relative to controls. Because the 0.25 mM CA did not significantly suppress any of the eight species in the experiment in metrics other than root mass, we cannot conclude whether PPO was beneficial to plant growth and survival when faced with CA. The use of this environmentally realistic dose may have precluded us from observing highly significant antagonistic effects by CA.

APPENDIX

Figures display the time-dependent activity of PPO as determined by spectrophotometric assay using L-DOPA (5 mM) as a substrate and a seedling root of approximately 1 cm as the enzyme source.

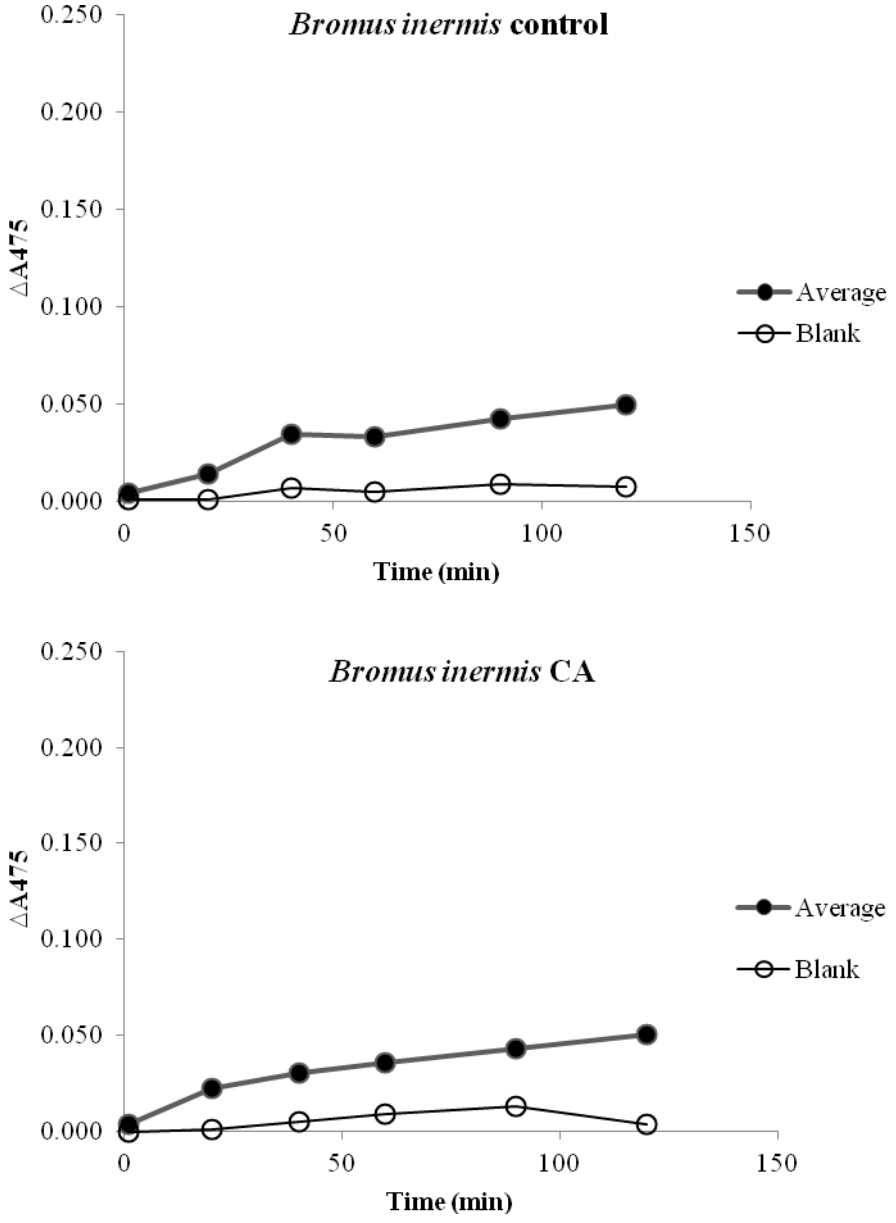


Fig. A 1. PPO enzyme assay results of both (a) control and (b) caffeic acid (CA) treatments (for each data point, n = 5).

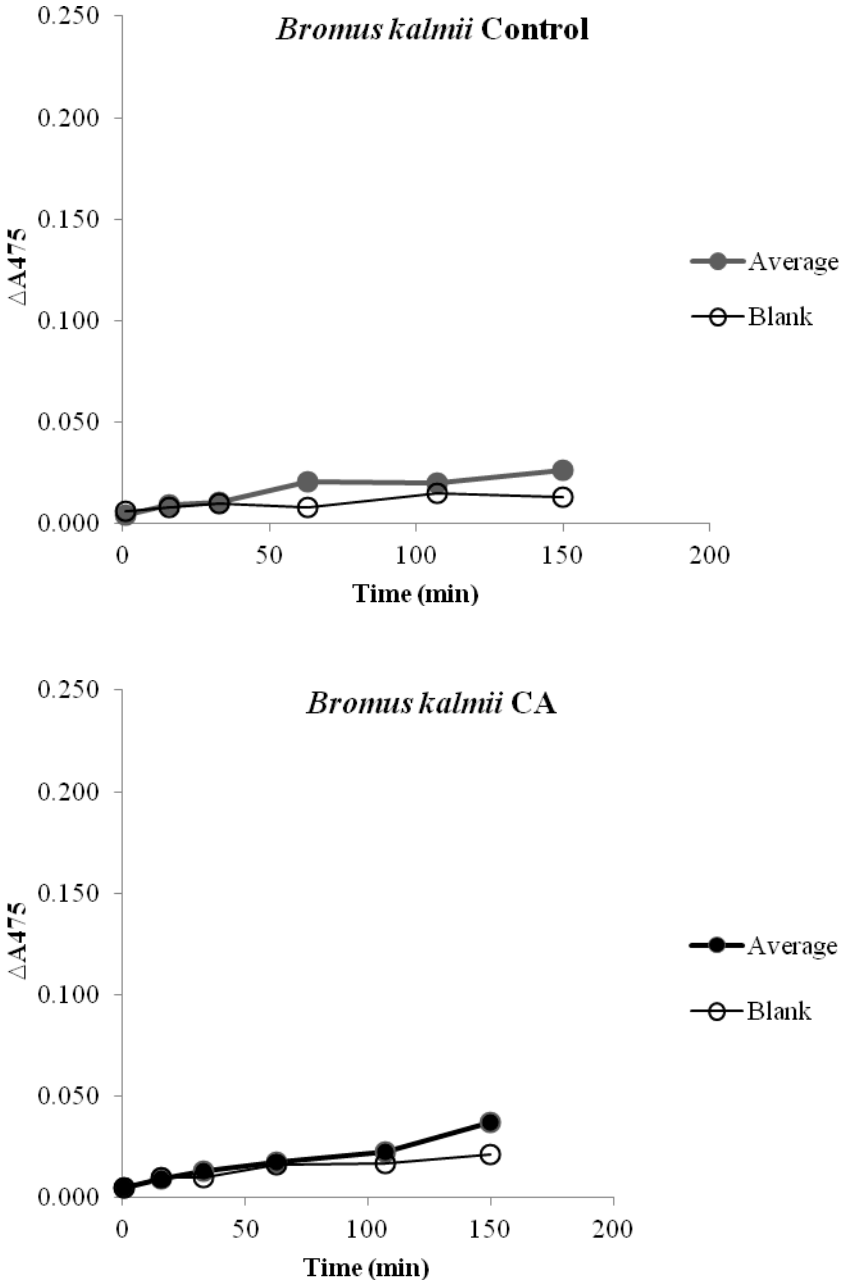


Fig. A 2. PPO enzyme assay results of both (a) control and (b) caffeic acid (CA) treatments (for each data point, n = 5).

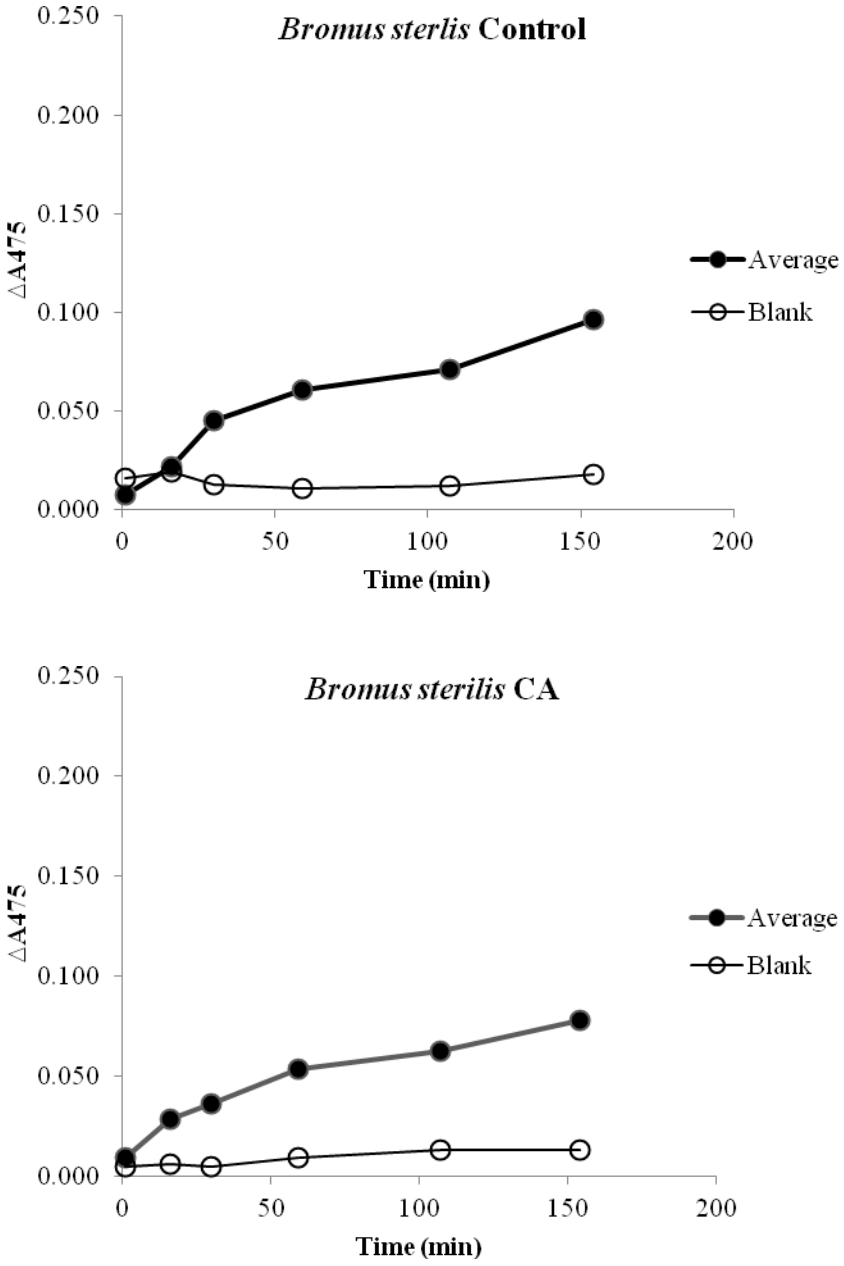


Fig. A 3. PPO enzyme assay results of both (a) control and (b) caffeic acid (CA) treatments (for each data point, n = 5).

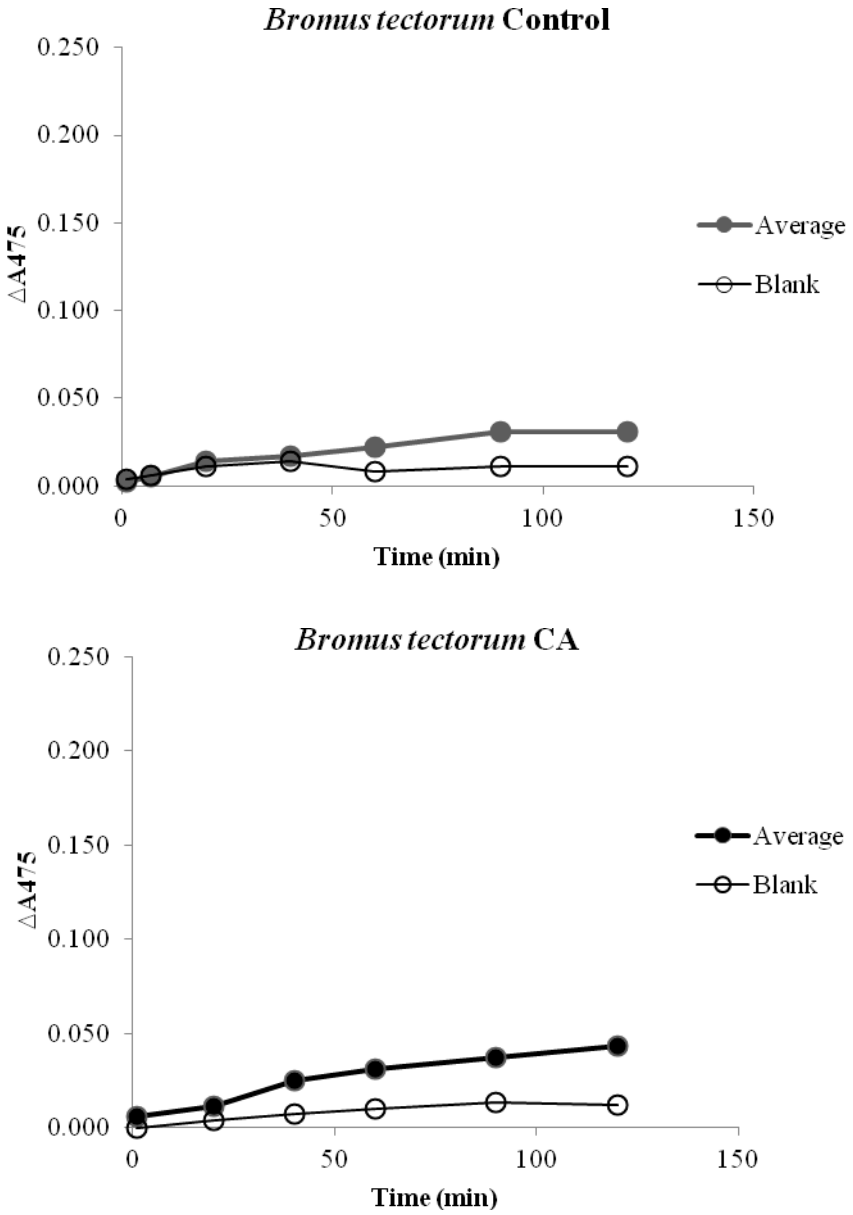


Fig. A 4. PPO enzyme assay results of both (a) control and (b) caffeic acid (CA) treatments (for each data point, n = 5).

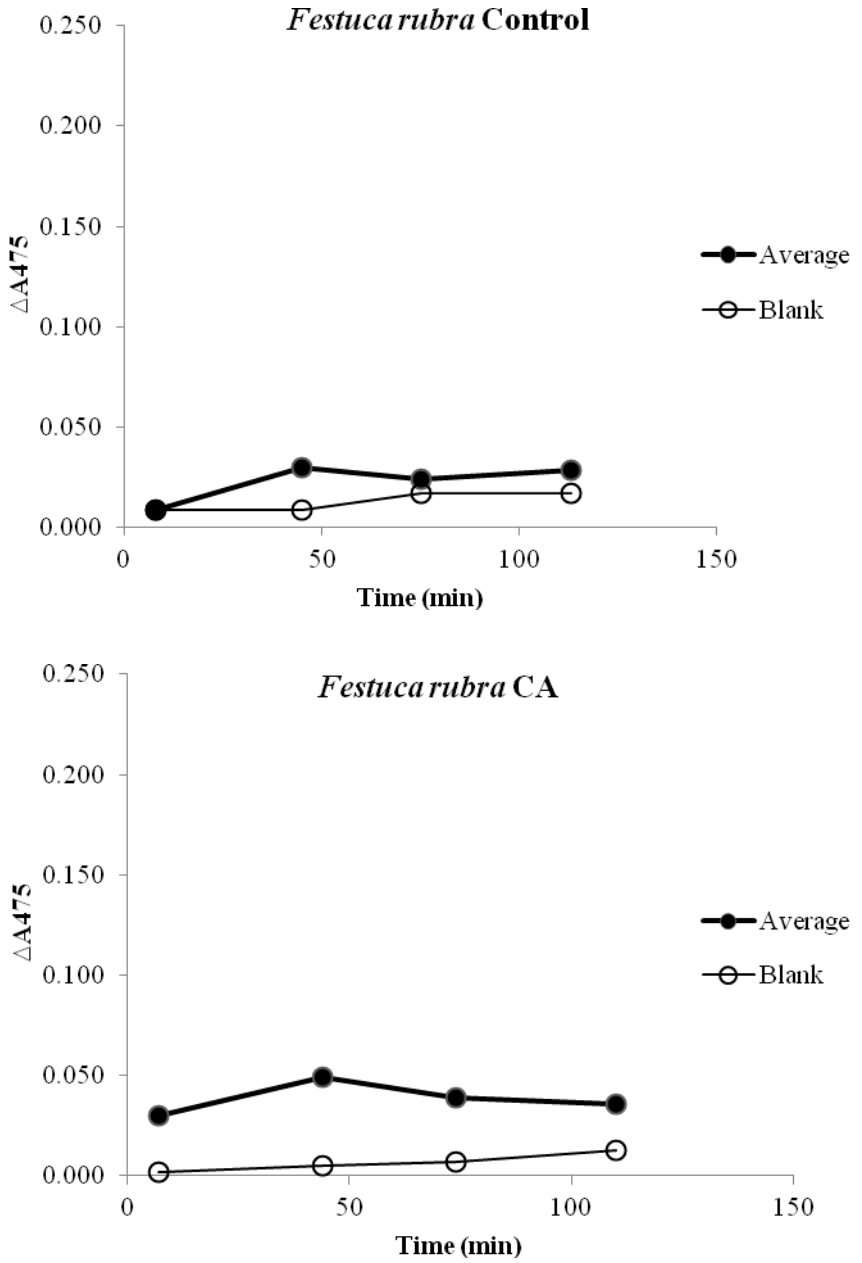


Fig. A 5. PPO enzyme assay results of both (a) control and (b) caffeic acid (CA) treatments (for each data point, n = 5).

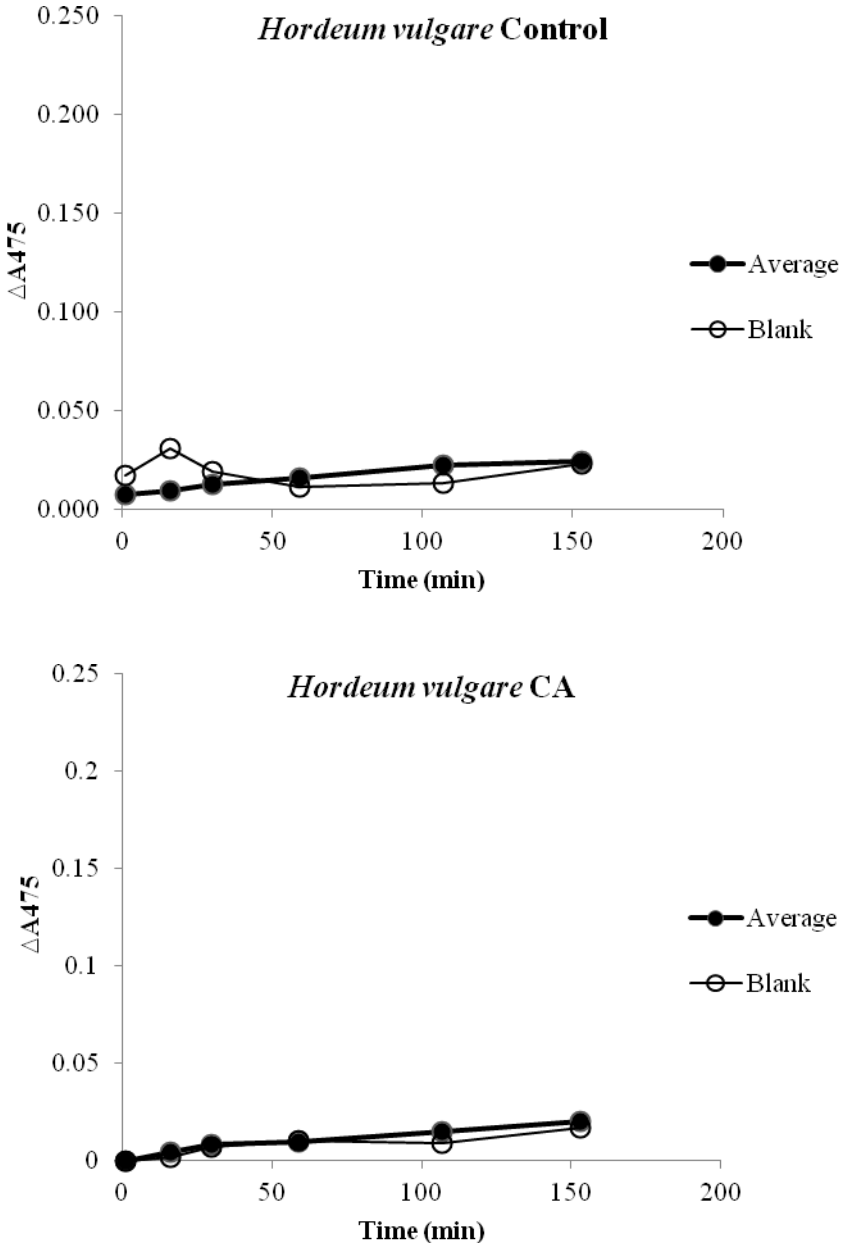


Fig. A 6. PPO enzyme assay results of both (a) control and (b) caffeic acid (CA) treatments (for each data point, n = 5).

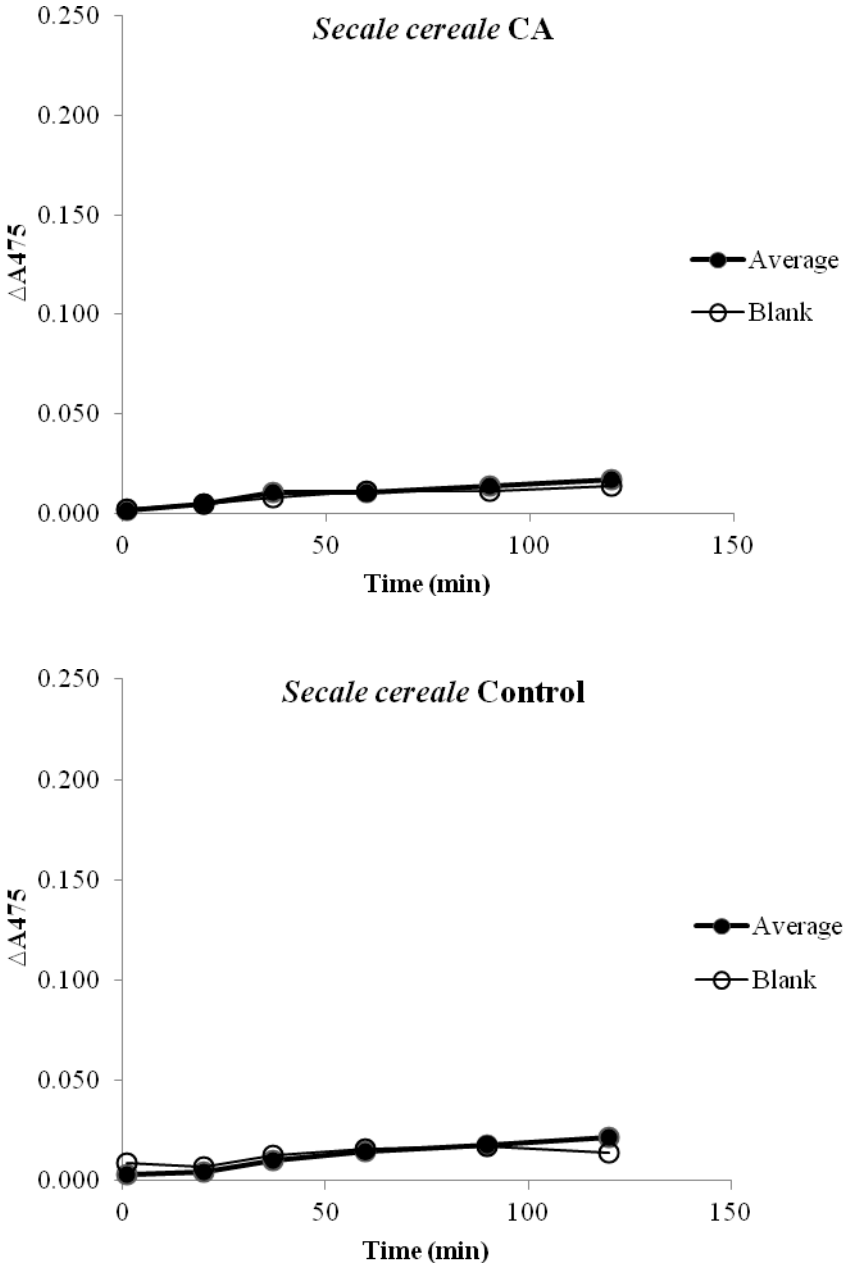


Fig. A 7. PPO enzyme assay results of both (a) control and (b) caffeic acid (CA) treatments (for each data point, n = 5).

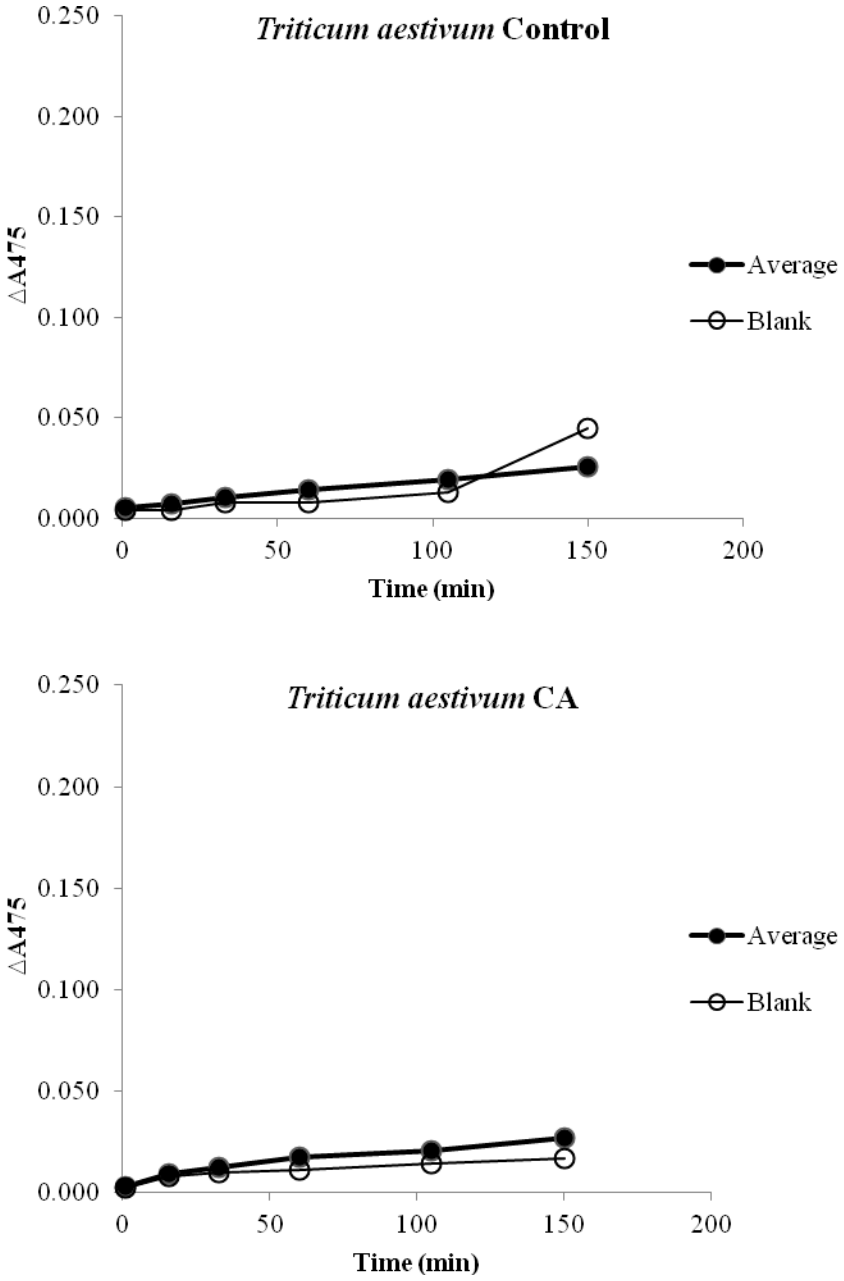


Fig. A 8. PPO enzyme assay results of both (a) control and (b) caffeic acid (CA) treatments (for each data point, n = 5).

Table 4. ANOVA table for species and response variable interactions.

ANOVA Table

Factors		Sum of Squares	df	Mean Square	F	Sig.
Root mass * Species	Between Groups	0.03	7	0.01	6.01	<0.001
	Within Groups	0.05	67	0.00		
	Total	0.08	74			
Shoot mass * Species	Between Groups	0.01	7	0.00	8.14	<0.001
	Within Groups	0.01	63	0.00		
	Total	0.02	70			
Total biomass * Species	Between Groups	0.07	7	0.01	8.05	<0.001
	Within Groups	0.09	72	0.00		
	Total	0.16	79			
RootmassRelativeMeanControl * Species	Between Groups	0.66	7	0.10	0.24	0.97
	Within Groups	28.67	73	0.39		
	Total	29.33	80			
ShootmassRelativeMeanControl * Species	Between Groups	5.52	7	0.79	0.91	0.51
	Within Groups	62.51	72	0.87		
	Total	68.03	79			
TotalmassRelativeMeanControl * Species	Between Groups	3.94	7	0.56	0.73	0.65
	Within Groups	55.61	72	0.77		
	Total	59.55	79			

Table 5. Regression of mass (dependent: total, root, shoot) and PPO (predictor).

ANOVAa

Model	Sum of Squares	df	Mean Square	F	Sig.
Regression	0.22	1.00	0.22	0.25	0.618b
Residual	61.73	70.00	0.88		
Total	61.95	71.00			

a. Dependent Variable: TotalmassRelativeMeanControl

b. Predictors: (Constant), PPO_Abs_MinusBlank_DivByRootLength_DivByTime_*10000

ANOVAa

Model	Sum of Squares	df	Mean Square	F	Sig.
Regression	0.88	1.00	0.88	1.67	0.200b
Residual	37.00	70.00	0.53		
Total	37.89	71.00			

a. Dependent Variable: RootmassRelativeMeanControl

b. Predictors: (Constant), PPO_Abs_MinusBlank_DivByRootLength_DivByTime_*10000

ANOVAa

Model	Sum of Squares	df	Mean Square	F	Sig.
Regression	0.20	1.00	0.20	0.25	0.618b
Residual	54.98	70.00	0.79		
Total	55.18	71.00			

a. Dependent Variable: ShootmassRelativeMeanControl

b. Predictors: (Constant), PPO_Abs_MinusBlank_DivByRootLength_DivByTime_*10000

Tests of Between-Subjects Effects
Dependent Variable: Height change

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	330.838a	7.00	47.26	11.37	0.00
Intercept	147.23	1.00	147.23	35.42	0.00
Treatment	0.00	0.00	.	.	.
BiSpecies	330.84	7.00	47.26	11.37	0.00
Treatment * BiSpecies	0.00	0.00	.	.	.
Error	87.30	21.00	4.16		
Total	710.00	29.00			
Corrected Total	418.14	28.00			

a. R Squared = .791 (Adjusted R Squared = .722)

Tests of Between-Subjects Effects
Dependent Variable: Height change

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	618.104a	15.00	41.21	8.11	0.00
Intercept	460.19	1.00	460.19	90.54	0.00
Treatment	7.48	1.00	7.48	1.47	0.23
BiSpecies	596.49	7.00	85.21	16.77	0.00
Treatment * BiSpecies	11.91	7.00	1.70	0.34	0.93
Error	223.64	44.00	5.08		
Total	1573.25	60.00			
Corrected Total	841.75	59.00			

a. R Squared = .734 (Adjusted R Squared = .644)

Tests of Between-Subjects Effects
Dependent Variable: Root length

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	430882.698a	15.00	28725.51	5.25	0.00
Intercept	643158.40	1.00	643158.40	117.59	0.00
Treatment	14038.67	1.00	14038.67	2.57	0.11
BiSpecies	351826.34	7.00	50260.91	9.19	0.00
Treatment * BiSpecies	65130.33	7.00	9304.33	1.70	0.13
Error	328165.18	60.00	5469.42		
Total	1452179.24	76.00			
Corrected Total	759047.88	75.00			

a. R Squared = .568 (Adjusted R Squared = .460)

Tests of Between-Subjects Effects
Dependent Variable: Root diameter

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.106a	15.00	0.01	1.77	0.06
Intercept	6.12	1.00	6.12	1533.70	0.00
Treatment	0.02	1.00	0.02	4.20	0.05
BiSpecies	0.02	7.00	0.00	0.84	0.56
Treatment * BiSpecies	0.07	7.00	0.01	2.50	0.03
Error	0.24	60.00	0.00		
Total	6.62	76.00			
Corrected Total	0.35	75.00			

a. R Squared = .306 (Adjusted R Squared = .133)

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CONCLUSION OF THE DISSERTATION

The novel weapons hypothesis posits that phytotoxic allelochemicals produced and released by invasive species allow for successful biological invasion into new territories. Here, we have shown the potential for a new hypothesis about a possible mechanism promoting invasion: a defense mechanism that exists through the enzymatic destruction of phenolic allelochemicals before they can exert their toxic effects. We call this a novel defense, since we postulate that this response to the offensive allelochemicals is novel in the environment due to new arrival of these invasive species. This defense manifests through roots of species within the plant genus *Bromus* which constitutively produce high levels of the enzyme polyphenol oxidase (PPO), whose original function is unknown. We tested the utility of PPO as a "novel defense" against allelopathic plants because many allelochemical compounds produced by plants serve as substrates for PPO.

The possible role of the extreme PPO activity in the roots of *Bromus* species was the focus of this study because many *Bromus* species are invasive in North America. In particular, the species *B. tectorum* is considered one of the most destructive invasive grasses in western North America because it displaces native plants. Generally, root PPO was found more prevalently in invasive species. Non-invasive grasses (both brome and non-brome) had little or no PPO. This suggests that the ability to produce high root PPO activity may be a trait that contributes to invasion potential of non-native species. Screening species for such a trait therefore can provide the opportunity to identify future invaders. This important corollary may serve as a useful tool for identifying future

invasive species. Phylogenetic reconstructions demonstrated the tractability of phenetic PPO, and suggested high-PPO may be the ancestral condition, later lost by some genera.

In greenhouse experiments we also found evidence that *Bromus* species may rely upon the enzyme for defense in direct competition, when exposed to plant leachates, and subjected to caffeic acid, adding to an emerging body of work emphasizing the importance of plant biochemistry in community-defining interactions, demonstrating that phenolic-allelopathic invaders can be withstood by PPO-producers. Specifically, PPO-producing *Bromus* species tolerated phenolic-allelochemical-producer *Centaurea* whereas non-PPO-producing *Festuca* did not. The non-phenolic-allelochemical producing species *Artemisia* predictably suppressed the growth of both PPO and non-PPO producing grasses. We conclude the presence of PPO may be a mechanism for detoxification of phenolic-allelopathic plants but not non-phenolic-allelopathic plants. Field surveys showed validation of laboratory findings in that allelopathic plants were found significantly further from *Bromus* than non-allelopathic plants.

The strong evidence for a novel defense by PPO presented here demonstrates a probable negative impact of *Bromus* cover on the species in plant communities. From our finding that the invasive *Bromus* can tolerate phenolic allelochemicals, we strongly advise planting native non-phenolic-allelopathic plants as an optimal defense in *Bromus*-invasion prone systems, as we saw significant suppression of *Bromus* by *Artemisia* in both competition and leachate experiments.

We have illustrated the strong potential of PPO as a novel defense, a trait correlated to invasiveness, and highlighted ongoing taxonomic classifications that may

shed light on evolutionary understanding of selection benefits of PPO and grass evolution, which are agriculturally, economically, and environmentally important.

Several areas are in need of further research regarding PPO. Although the distribution, location, properties, and structure are well-understood, the exact biological function, gene activation trigger, synthesis location, reaction sequence, and target site of PPO remains puzzling. A possible function for PPO may be for immunity and protection in animal organisms, but in plants, the activity seems undirected. Browning itself may play a role in deferring herbivory as unappetitive, yet this remains to be definitively shown. Studying the substrates for PPO may aid in understanding the enzyme activation. More research is needed to disentangle PPO and plant performance in response to phenolics.

Curriculum vitae **Kimberly L. Plank** **kimplank@rutgers.edu**

EDUCATION

- **PhD in Biology**, Rutgers, the State University of New Jersey, Newark, 2009 – 2015
- **Bachelor of Science in Biology** from Temple University, Philadelphia, PA, 2003 – 2007

EXPERIENCE

- **Graduate Student at Rutgers University** Newark, New Jersey 2009 – 2015
  - Research: Planned, developed, implemented, managed, and modified all scientific experiments and protocols for dissertation in the laboratory (e.g., seed germination, polyphenol oxidase enzyme assays), field (e.g., plant identification and association patterns), and greenhouse (e.g., competition, leachate experiments); used statistical methods such as analysis of variance (ANOVA), regression, and correlation to analyze, evaluate, and interpret experimental data for accuracy, validity, and significance; interpreted and discussed data, results, and summarized conclusions; prepared data in final form for publication and presentation at laboratory & scientific meetings/conferences.
  - Botanist (Summer 2014). Conducted Whittaker Plots, identified plant species, determined diversity.
  - Lab rotation with Dr. Daniel Bunker (Spring 2011). I used R to review, analyze, and synthesize existing information found from literature searches to conduct a metaanalysis on allelopathy, a mechanism of plant chemical competition.
  - Lab rotation with Dr. Claus Holzapfel (Fall 2010). Through collaboration with Rutgers Chemistry Department, I used GC-MS to analyze root exudates of *Spartina*, a native wetland plant, grown in my own hydroponic greenhouse design.
- Teaching Assistant for various undergraduate and graduate-level labs including:
  - General Biology I & II (Fall 2009, Spring 2010, Summer 2010), Comparative Vertebrate Anatomy (Fall 2010), Developmental Plant Physiology (Spring 2014), Field Ecology (Fall 2010, Fall 2011, Spring 2012, Spring 2013, Spring 2014), Field Studies in Plant Ecology (Fall 2013), Mammalian Physiology (Summer 2012, Spring 2013, Summer 2013, Summer 2014), Plant Growth & Development (Spring 2011, Spring 2012), Plant Physiology (Fall 2011, Fall 2012, Fall 2013), Aim High Academy Summer Earth Ecology Program (July 2012, July 2014).

- **Teaching Fellow, Citizen Schools/New York Academy of Sciences**, 2011 – 2013
  • Designed and delivered afterschool curriculum including both lessons and lab activities over ten weeks each semester in role of both instructor and mentor to 6-8th grade students, oversaw final public presentations. Curriculum taught: Genetics (Fall 2011), Earth Science (Spring 2012), Climate Conscious Urban Gardening (Fall 2012, Spring 2013).

- **Research Technician, Monell Chemical Senses Center**, Philadelphia, PA, 2007 – 2009
  • Planned, executed, and summarized high-quality experiments in experiments involving the breakdown of starch by salivary amylase. The aim of this project involved correlation of perceptual testing results with actual molecular structures and gene copy number variants.
  • Involved with numerous experiments occurring simultaneously, assisted writing manuscripts and graphed collected data for publication in scientific journals and conference/meeting presentations.
  • Standardized two rheometers and created protocols for their use.
  • Maintained proper standard operating procedures as outlined by NIH and local IRB committees as to the treatment of humans in testing involving compounds of chemical, agricultural, and pharmaceutical nature. Included maintaining and presenting proper informed consent to ensure safety and protection of participants, as well as adhering to rules concerning confidentiality of records.
  • Mentored student (Estee Rubien Thomas) in summer 2008 Apprenticeship Program; assisted with basic lab training, supervised human testing and data analysis techniques; oversaw final presentation.
  • Responsible for lab upkeep including general inventory and ordering as well as choosing appropriate equipment for the lab based on need and budget. Stayed educated about equipment, use of, and training other members of the lab as needed.
  • Responsible for TRPA1 knockout *Mus musculus* colony of approximately 300; arranged and carried out weaning and mating schedules, tagging, and cutting tail sections for DNA extraction and genotyping. Created and maintained logs and submitted monthly census. Conducted two-bottle choice tests, analyzed and compared data collected of transgenics and controls. Adhered to IACUC guidelines.

PEER-REVIEWED PUBLICATION

Mandel AL, Peyrot des Gachons C, **Plank KL**, Alarcon S, Breslin PAS (2010) Individual Differences in AMY1 Gene Copy Number, Salivary a-Amylase Levels, and the Perception of Oral Starch. PLoS ONE 5(10): e13352. doi:10.1371/journal.pone.

ORAL PRESENTATIONS

The possible ecological role of the root enzyme polyphenol oxidase in the invasive plant genus Bromus. Kimberly Plank, David Kafkewitz, and Claus Holzapfel, Department of Biological Sciences, Rutgers University, Newark, NJ, United States. Ecological Society of America, Annual Meeting. Sacramento, California. 10 – 15 August 2014.

A possible ecological role of polyphenol oxidase in roots of Bromus, an invasive plant genus. Kimberly Plank, David Kafkewitz, and Claus Holzapfel, Department of Biological Sciences, Rutgers University, Newark, NJ, United States. Ecological Society of America, Mid-Atlantic Chapter Annual Meeting. University of Maryland, College Park, Maryland. March 29, 2014.

A novel defense? Understanding the role of the enzyme polyphenol oxidase in the invasive genus Bromus in plant competition. Kimberly Plank, David Kafkewitz, and Claus Holzapfel, Department of Biological Sciences, Rutgers University, Newark, NJ, United States. Rutgers University Department of Biological Sciences Colloquium, 10 September 2013.

A novel defense? Understanding the role of the enzyme polyphenol oxidase in the invasive genus Bromus in plant competition. Kimberly Plank, David Kafkewitz, and Claus Holzapfel, Department of Biological Sciences, Rutgers University, Newark, NJ, United States. Ecological Society of America, Annual Meeting August 4 – 9, 2013, Minneapolis, Minnesota.

Clonal diversity and resistance to invasion in remnant salt marsh patches. Kimberly Plank, TingMin Wu, Sahil Wadhwa, Edward Kirby, and Claus Holzapfel. Biology, Rutgers University Newark, Newark, NJ, United States. Ecological Society of America, Annual Meeting August 7 – 12, 2011, Austin, Texas.

POSTER PRESENTATIONS

A phylogenetic study of polyphenol oxidase (PPO) enzyme distribution within the plant family Poaceae (grass) and the invasive genus Bromus. Kimberly Plank, Dominic Evangelista, David Kafkewitz, and Claus Holzapfel Department of Biological Sciences, Rutgers University, Newark, NJ, United States. Ecological Society of America, Annual Meeting. Baltimore, Maryland. August 9 – 14, 2015.

Patterns of diversity of plant communities in an urban tidal marshland. Claus Holzapfel, Marjolein Schat, Hadas A. Parag, Anthony Cullen, Kimberly Plank, Sahil Wadwha,Megan E. Litwhiler, and Rajan Tripathee, Department of Biological Sciences, Rutgers University, Newark, NJ, United States. Ecological Society of America, Annual Meeting. Baltimore, Maryland. August 9 – 14, 2015.

The root of the matter: A phylogenetic study of polyphenol oxidase (PPO) enzyme distribution within the plant family Poaceae (grass) and the highly invasive genus Bromus. Kimberly Plank, Dominic Evangelista, David Kafkewitz, and Claus Holzapfel Department of Biological Sciences, Rutgers University, Newark, NJ, United States. Ecological Society of America, Mid-Atlantic Chapter Annual Meeting. Elizabethtown, Pennsylvania. April 17 – 19, 2015.

The enzyme polyphenol oxidase in the invasive plant genus Bromus. Kimberly Plank, David Kafkewitz, and Claus Holzapfel, Department of Biological Sciences, Rutgers University, Newark, NJ, United States. New Jersey Institute of Technology X Annual Graduate Student Research Day. Newark, New Jersey. October 30, 2014.

The function of enzyme polyphenol oxidase in the invasive plant genus Bromus. Kimberly Plank, David Kafkewitz, and Claus Holzapfel, Department of Biological Sciences, Rutgers University, Newark, NJ, United States. New Jersey Institute of Technology IX Annual Graduate Student Research Day. Newark, New Jersey. October 31, 2013.

A novel defense? Understanding the plant competition role of the enzyme polyphenol oxidase in the invasive genus Bromus. Kimberly Plank, David Kafkewitz, and Claus Holzapfel, Department of Biological Sciences, Rutgers University, Newark, NJ, United States. Smithsonian Botanical Symposium, National Museum of Natural History and the U.S. Botanic Garden, Washington, DC April 19 – 20, 2013.

A novel defense? Understanding the role of the enzyme polyphenol oxidase in the invasive genus Bromus in plant competition
Kimberly Plank, David Kafkewitz, and Claus Holzapfel, Department of Biological Sciences, Rutgers University, Newark, NJ, United States. Ecological Society of America, Mid-Atlantic Chapter Annual Meeting. Delaware State University, Dover, Delaware. April 13 – 14, 2013.

Clonal diversity and resistance to invasion in remnant salt marsh patches. Kimberly Plank, TingMin Wu, Sahil Wadhwa, Edward Kirby, and Claus Holzapfel. Biology, Rutgers University, Newark, NJ, United States. Ecological Society of America, Mid-Atlantic Meeting. Montclair State University, Montclair, New Jersey. April 8 – 9, 2011.

The Nutritional Significance of Oral Starch Digestion. Abigail L. Mandel, Kimberly L. Plank, Paul A.S. Breslin. Monell Chemical Senses Center, Philadelphia, PA, United States. Association for Chemoreception Sciences 2009 annual conference, Sarasota, Florida. April 22 – 26, 2009.