

Phenolics-oxidizing enzymes of invasive and native plant roots

David Kafkewitz & Claus Holzapfel
Dept. of Biological Sciences, Rutgers Newark
[kafkwitz@andromeda.rutgers.edu, holzapfe@andromeda.rutgers.edu]

Abstract

Phenolic compounds are allelopathic signal molecules exuded by the roots of many plants and detected by the roots of other plants. Invasive plants – plants that often were introduced from far-away regions - may not respond appropriately to these coevolved interspecies signals. Root enzymes that destroy phenolic compounds may be involved in the process of permitting an invasive plant to (a) ignore the signal molecules exuded by plants in the native community and (b) to render such allelopathic compounds ineffective. We screened and assayed roots of a wide range of invasive and native grasses for phenolic oxidase and peroxidase enzymes. An analysis of our assembled grass data base suggests that only members of the genus *Bromus* display elevated levels of phenolics-oxidizing enzymes. Species from that genus are known to be problematic invaders in many parts of North America; there are several non-native *Bromus* species in the Hackensack Meadowlands. Other grass genera, regardless of their life form and tendency to invasion, typically have lower levels of these enzymes. Current research is aiming at identifying the ecological function of elevated enzyme activities. Phenolic degrading enzymes have broad specificity. Phenolase and laccase, enzymes widely distributed in the plant kingdom, have been shown capable of degrading chlorophenols and other xenobiotic phenolics. Phytoremediation of xenobiotic chlorophenolics has been demonstrated. Thus knowledge of which plants in the ecosystem possess these enzymes in their roots should be useful in remediating contaminated sites.

Introduction

We had proposed to assay the roots of both terrestrial and aquatic plants of the Meadowlands ecosystem for monophenolic, diphenolic and polyphenolic oxidase and peroxidase enzymes. For the first stage of the research we concentrated on grass species since we indentified elevated enzyme levels in the roots of one invasive, annual grass species. We anticipated three reasons why the data obtained should be of interest.

(1) Phenolic compounds are allelopathic signal molecules exuded by the roots of many plants and detected by the roots of other plants (Ridenour and Callaway 2001, Bais et al. 2004, Callaway and Ridenour 2004). Invasive plants may not to respond appropriately to these interspecies signals. Root enzymes

that destroy phenolic compounds may be involved in the process of permitting an invasive plant to ignore the signal molecules exude by the native community.

(2) Phenolics degrading enzymes have broad specificity (Sherman et al. 1991). Phenolase and laccase, enzymes widely distributed in the plant kingdom, have been shown capable of degrading chlorophenols and other xenobiotic phenolics. Phytoremediation with genetically engineered plants has been demonstrated. Thus knowledge of which plants in the ecosystem possess these enzymes in their roots may be useful in remediating contaminated sites.

(3) Both aquatic and upland habitats of the Hackensack Meadowlands are dominated by invasive plants (e.g., *Phragmites australis*, *Bromus species*) while native plant communities still have some small footholds. The mechanism by which invasive plants control ecosystems are little understood. Screening for root enzymes can shed light on such mechanisms that are decisive in the interaction of invasive and native plants. Such knowledge is needed to develop management strategies that will favor native vegetation.

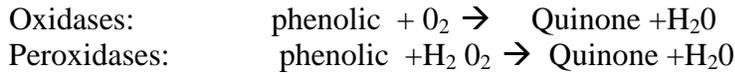
Hypothesis/Research questions: The first stage of the research program that has been funded by this MERI Fellows Grant concentrated on the survey of different grass taxa in order to establish whether high phenol-oxidase activity that have been found in one brome species (*Bromus madritensis*) is typical for the grass family as a whole (*Poaceae*).

Our goal was (a) to answer the question on how widespread this particular enzyme activity is across grass species, (b) to test whether it depending on the life history of species and (c) whether we can detect correlative pattern that might help to explain invasiveness of some grass species. Since we detected the enzyme activity in germinating seedlings we predicted in regard to life history that annual species might have higher activity than perennial species. The rationale is that annual plant species have to ensure recruitment from seed to a higher degree than perennial life forms. In regard to invasiveness we expected to find higher activity in grass species that were collected in invaded ranges compared to species that were collected in the native range. If so, this could be an indication of "Evolution of Increased Competitive Ability" (EICA, Blossey and Notzold 1995).

Methodology

Seedling preparation: Seeds were surface sterilized by for 30 minutes in a 1.2% sodium hypochlorite solution. The seeds were then rinsed with two changes of 200 ml sterile distilled water and then aseptically transferred to sterile glass Petri dishes containing Whatman 40 filter paper. The dishes were incubated on the lab bench at room temperature. Germination and root outgrowth times varied from 3-10 days. Fungal contamination was rare and contaminated plates were discarded.

L-DOPA-assay: The enzymes we used in the assay are:



A widely used substrate for the assay of enzymes is 2,3, dihydroxyphenylalanine (L-DOPA). This colorless compound is oxidized to a deeply colored product that can be visually detected and spectrophotometrically quantified (see photo 1).



Photo 1 : DOPA-stained root hairs and root surface indicate strong phenolase activity in a Smooth brome (*Bromus inermis*) seedling (scale line 1 cm). *B. inermis* a European invader that can form a dense sod which displaces and prevents seed recruitment of native species (photo: D. Kafkewitz).

The distal portion of the seedling root was assayed for polyphenol oxidase activity (PPO). Root segment length varied from about 0.5 -2.0 cm. Activity was determined by measuring the increase in absorbance at 475nm in a solution containing 5 micromoles/ml L- DOPA in 25 mM MOPS buffer, pH 6.5. The assay was performed in a total volume of 5.0 ml in 13x100 mm tubes. A Spectronic 20D spectrophotometer was used for absorbency measurements. The presence of the root in the culture tube did not significantly affect the absorbance readings. Readings were recorded at intervals, over a 60 to 120 minute period. The assays were performed at room temperature.

Data analysis: The accumulation of reaction products (tannins) in roots that possessed PPO activity caused the roots to darken during the first few minutes of the assay. For this reason the rate of change in absorbency as a function of time did not become linear until 10 -15 minutes after the reaction was initiated. The total amount of activity (DOPA oxidized/minute) was determined

by the slope of a linear regression plot. Specific activity (DOPA oxidized/minute/ length of root) was calculated by dividing the total activity by the length of the root as determined by measuring the length of a scanned image of the root performed at the completion of the enzyme assay. The scanning analysis did not have sufficient resolving power to measure root hairs; consequently the reported measurements are of the length of the root segment and not of its total surface area.

Root structure and the limitations of the assay: Roots of Bromus seeds that developed in contact with the filter paper in the Petri dishes had many root hairs. Roots of seeds that developed in water without significant contact with the paper were almost hairless. Microscopic observation of roots after the assay indicates that PPO activity is present in the root hairs, as well as the body of the root (see photo 1). Because the scanner used to measure the root could not resolve the root hairs and calculate total surface area, the specific activity measurements are at best, estimates.

“Seedbank” and Meta-Analysis of data: We assembled a large collection of viable grass seeds from a wide range of taxa. To date we have 77 specimen collections stemming from over 50 species available. These seeds originated from various commercial sources: own collection material (New Jersey, California, Israel), the USDA germplasm bank, and from collections acquired by our colleagues. The samples were entered in a data base that included phylogenetic/taxonomical (Gould and Shaw 1983, Salamin et al. 2002) and ecological information (e.g., life form, invasive/native status). To date 34 assays (see above) gained clear data and these have been entered in the data base. For data analysis in regard to the leading questions (see above) univariate linear models have been employed that have been conducted within SPSS (Windows version 11.5).

Results

Phylogeny and root enzymes: How wide-spread are phenolic-oxidizing enzymes within the family of Poaceae?

The assay screening across a wide range of grass taxa resulted in a surprising and clear finding: Species of the genus *Bromus* have clearly higher activity than any other tested grass taxa ($p= 0.002$ for the difference between *Bromus* and other taxa). The variation within the genus *Bromus* was clearly smaller than the variation among other genera.

Taxonomic distance from *Bromus*, as shown in Fig. 1 did not affect activity. Genera closely related to *Bromus* did not differ in their activity from phylogenetically distant taxa. For instance even the closely related genus *Festuca* did have significant lower activity than *Bromus*.

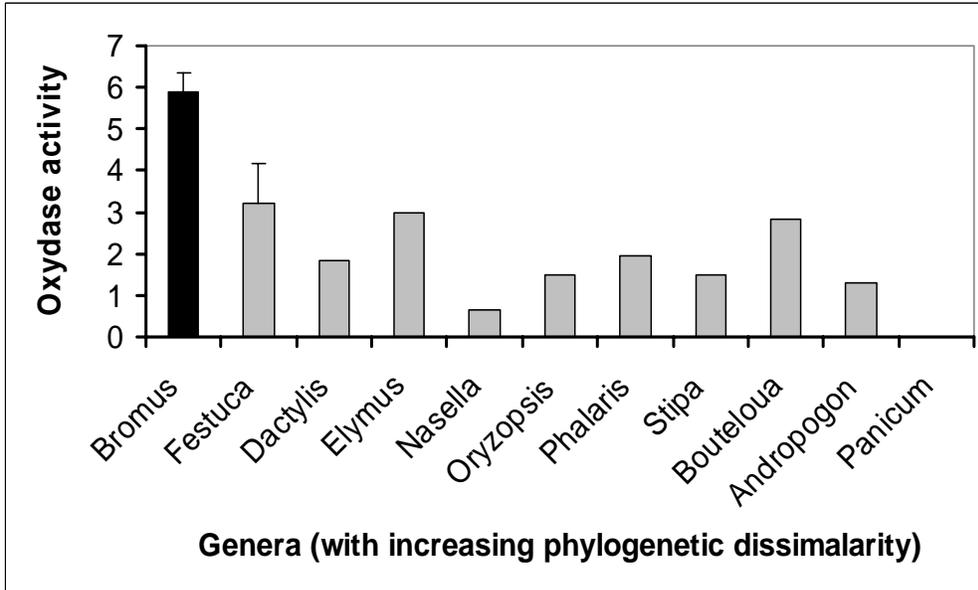


Fig. 1: Result of the phylogentic assay screening of oxydase activity (specific activity- S/L). Significance of genera is $p=0.0219$ (MS 12.88, $df=10$). Shown are means with 1 SE.

Life forms (annual versus perennial): Do annual grasses have higher oxidase activity than perennial grasses?

In contrast to our expectation we did not find any differences in oxydase activity between annual and perennial different life forms. Fig. 2 clearly shows that the variation of within our data set is clearly explained by phylogeny alone, neither brome species nor other genera differ in respect to life form. The effect of taxonomy was highly significant ($p= 0.00068$, $MS=57.4$, $df=1$), life form ($p=0.685$, $MS=1.5$, $df=1$) and the interaction between taxonomy and life form ($p=0.899$, $MS=0.063$, $df=2$) both were not.

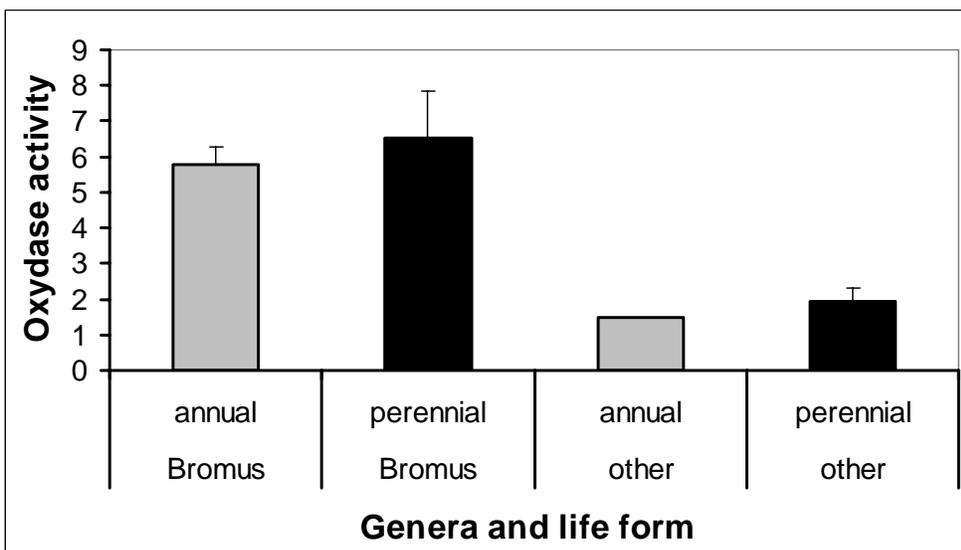


Fig. 2: Result of assay screening of oxydase activity in regard to life form (specific activity- S/L). Shown are means with 1 SE.

Origin (native and non-native): Do plants in invaded ranges display higher activity compared to plants in the native range?

Again, in contrast to prediction we did not detect a relationship between the degree of phenolic oxydase activity and invasiveness of species. Both *Bromus* species and other species did not differ between species in their home range and in their invaded range. As in the analysis for life form, the effect of taxonomy was highly significant ($p=0.00063$, $MS=83.67$, $df=1$), however origin ($p=0.6678$, $MS=0.1882$, $df=1$) and the interaction between taxonomy and life form ($p=0.7862$, $MS=0.655$, $df=2$) were not. An additional analysis (data not shown) that compared enzyme activity between species that are known to be invasive to species that are not, did not detect a difference.

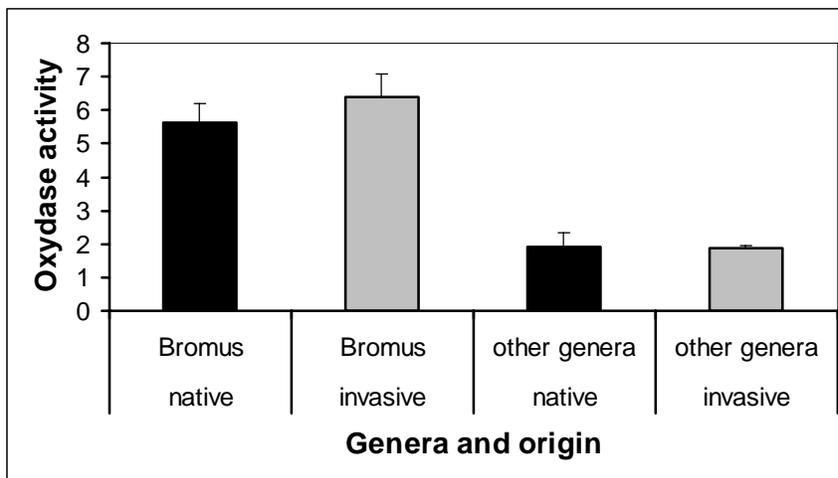


Fig. 3: Result of assay screening of oxydase activity (specific activity- S/L) in regard to invasion status (origin). Shown are means with 1 SE.

Discussion and Future Directions

The result of the first phase indicate a previously unknown physiological fact: grasses of the genus *Bromus* have significantly higher phenolic oxydase activity compared to any other tested grass taxa. This difference is not related to phylogenetic relatedness, even genera closely related to *Bromus* do not possess such elevated activity. Therefore we assume and predict that bromes are unique among grasses. We do not know what the ecological consequence of these elevated enzyme activity are, but expect that it may be related to the success of bromes in many environments. Several brome species are successful invaders in North America, in fact all of the annual species that are now dominant in the SW are annual, non-native species (Beatley 1966, Roy et al. 1988, MacDougall and Turkington 2004).

The next steps in our anticipated research program will address these topics. Gregg Burdulis, a master student in the Biology Graduate Program, who was partly supported by the MERI fellowship, started to investigate the ecological

consequences of high phenolic oxydase activity. For this he is using a two-pronged approach. Firstly, he is testing in a green house experiment the success of high activity species (chiefly bromes) and low activity ones in the interaction with a desert shrub (*Ambrosia dumosa*) that is known to have allelopathic root exudates (sesquiterpenes and phenolics). This desert shrub is also impacted negatively by invasive bromes (Holzapfel and Mahall 1999) and we are hypothesizing that bromes are “immune” to the phenolic exudates of these shrubs. Secondly, he will be testing whether different grasses will perform in experimentally created gradients of phenolic concentrations according to their oxidase activity. For this, he will be growing seedlings in agar cultures and will measure root growth and shoot extension.

A further step will address the potential use of bromes for bioremediation of contaminated sites. Testing the effectiveness of these grasses in the degradation of toxic phenolics in soils will involve collaboration with the Chemistry Department at Rutgers Newark. This step in the research program has strong relevance for polluted sites in the Hackensack Meadowlands. Several non-native brome species are found in disturbed sites in the Meadowlands (*Bromus inermis*, *B. japonicus*, *B. tectorum*, etc.) and the prospect to put these “dreaded” invaders to good use is promising to say the least.

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