DOI:10.1021/jf9038106

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Phenolic and Short-Chained Aliphatic Organic Acid Constituents of Wild Oat (*Avena fatua* L.) Seeds

R. S. Gallagher,*,† R. Ananth,† K. Granger,† B. Bradley,† J.V. Anderson,‡ and E. P. Fuerst§

†Department of Crop and Soil Sciences, The Pennsylvania State University, University Park, Pennsylvania 16802, ‡Biosciences Research Laboratory, Fargo, North Dakota 58105, and §Department of Crop and Soil Sciences, Washington State University, Pullman, Washington 99164

The objective of this research was to identify and quantify the phenolic and short-chained aliphatic organic acids present in the seeds of three wild-type populations of wild oat and compare these results to the chemical composition of seeds from two commonly utilized wild oat isolines (M73 and SH430). Phenolic acids have been shown to serve as germination inhibitors, as well as protection for seeds from biotic and abiotic stress factors in other species, whereas aliphatic organic acids have been linked to germination traits and protection against pathogens. Wild oat populations were grown under a "common garden" environment to remove maternal variation, and the resulting seeds were extracted to remove the readily soluble and chemically bound phenolic and aliphatic organic acid components. Compounds were identified and quantified using gas chromatography−mass spectrometry. Ferulic and *p*-coumaric acid comprised 99% of the total phenolic acids present in the seeds, of which 91% were contained in the hulls and 98% were in the chemically bound forms. Smaller quantities of OH benzoic and vanillic acid were also detected. Soluble organic acids concentrations were higher in the M73 isoline compared to SH430, suggesting that these chemical constituents could be related to seed dormancy. Malic, succinic, fumaric and azelaic acid were the dominant aliphatic organic acids detected in all seed and chemical fractions.

KEYWORDS: Phenolic acids; seed chemistry; seed persistence; seed dormancy; seed vigor

INTRODUCTION

Wild oat (*Avena fatua* L.) is considered to be among the world's worst weeds, causing serious competitive yield losses in grain crops throughout North America, Eurasia and Australia (*1*). Management of this weed has become particularly problematic as it has developed resistance to many of the herbicides that were once available for its control (*2*). As a consequence, many scientists have advocated gaining a more comprehensive understanding of the mechanisms governing weed seed persistence in the soil seed bank to aid in the development of integrated weed control strategies that rely less on herbicides (*3*−*5*). The chemical constituents of wild oat seeds may contribute to the ecological success of this species by providing direct and indirect defense mechanisms against pathogens and seed predators, by regulating the timing and vigor of germination and seedling establishment, and/or by protecting against oxidative aging processes (reviewed in ref *6*). Specifically, phenolic compounds in seeds have been associated with several weed seed persistence mechanisms, such as inhibiting microbial colonization of the seed, promoting seed dormancy, and buffering oxidative seed aging processes. Relatively short-chained (< 10 C) aliphatic organic acids (organic acids hereafter) in seeds have been linked to potential modification of the spermosphere to reduce Al toxicity (*7*), the regulation of seed dormancy (*8*), fixed carbon storage possibly related with germination vigor (*9*, *10*), and endogenous protection against pathogens (*11*). Elucidating the phenolic and organic acid composition of weed seed and determining the relationships of these compounds with microbial colonization, dormancy, and seed longevity will advance our understanding of seed persistence in the soil and aid in developing more effective management strategies.

Free phenolic acids are readily extractable in polar solvents (e.g., water, methanol, acetone, etc.), whereas phenolics that are chemically bound to cellular constituents require hydrolysis to be liberated (*12*). Within the readily extractable fraction, "free" phenolic acids exist as chemical monomers in addition to the "soluble esters" that are present as chemical conjugates that can be hydrolyzed into the free forms. In cereal grains such as wheat (*Triticum aestivum* L.), rye (*Secale cereale* L.) and triticale (*Triticale hexaploide* Lart.), the most prominent free phenolic acids are ferulic (3-[4-hydroxy-3-methoxyphenyl]-2-propenoic acid), sinapic (3,5-dimethoxy-4-hydroxycinnamic acid) and *p*-coumaric (3-[4-hydroxyphenyl]-2-propenoic acid), and have been linked with the prevention of preharvest sprouting (*13*−*16*). In these crops, the composition of phenolic acids as soluble esters was similar to those in the free forms, but also included vanillic (4-hydroxy-3-methoxybenzoic acid) and caffeic acids (3-[3,4-dihydroxyphenyl]-2-propenoic acid). The concentration of soluble esters typically exceeded those in the free form in these studies, and has also been positively correlated with reduced preharvest sprouting in these crops (*16*).

*Author to whom correspondence should be addressed [tel (814) 865-1547, fax (814) 863-7043, e-mail rsg14@psu.edu].

 Published on Web 12/17/2009

In wheat, the bound phenolics constituted in excess of 90% of the total seed phenolics and have been positively correlated to midge resistance in numerous cultivars (*17*). The identification and quantification of the phenolic acids associated with seeds of common weed species have received considerably less attention, although some researchers have utilized more generalized spectrophotometric methods to estimate the concentration of the readily extractable suite of phenolic compounds in weed seeds (*18*, *19*). Hendry et al. (*18*) found a strong positive correlation between spectrophotometric estimates of *o*-dihydroxyphenol (1,2,3-trihydroxybenzene or pyrogallol) and published estimates of weed seed longevity. In contrast, Davis et al. (*19*) found that seeds from weed species with transient seed banks tended to have higher *o*-dihydroxyphenol concentrations than seeds from weeds with more persistent seed banks. Neither study quantified the esterified or bound phenolic constituents of the seeds.

Organic acids are typically soluble in aqueous seed extraction phases (*20*), although we are not aware of studies that have investigated chemically bound forms of these compounds. As is the case with phenolic acids, there has been limited characterization of the organic acids in seeds, particularly in weed species. In Eurasian catchfly (*Silene nutans* L.) seeds, pyruvic (2-oxopropanoic), malic, oxalic, citric, and aconitic (1-propene-1,2,3-tricarboxylic) acids were identified, and thought to play a possible role in chelating toxic Al ions in low pH soils (*7*). Malic and succinic (butanedioic) acids were found in wild oat, with the concentrations of malic acid inversely correlated with seed dormancy (*8*). Chickpea (*Cicer arietinum* L.) seeds contained high levels of succinic, malic and citric acids that were attributed to be fixed carbon stores associated with the tricarboxylic acid cycle (TCA) (*9*). *Arabidopsis* (*Arabidopsis thaliana* L.) seeds and other plant tissues contained fumaric acid, which they also hypothesized was an important form of transient fixed carbon in C_3 plants (*10*). Finally, azelaic acid was found in the grain of some cultivars of sorghum (*Sorghum bicolor*), although no physiological or ecological role for this compound was proposed (*21*). Azelaic acid, however, has known antimicrobial properties (*11*), and has been proposed to be part of the systemic acquired resistance mechanism in *Arabidopsis* leaf tissue in response to pathogen attack (*22*).

The objective of this research was to identify and quantify the specific phenolic and organic acids commonly found in wild-type populations of wild oat seeds and compare these findings to the phenolic composition of seeds from two wild oat isolines commonly utilized by researchers: M73 and SH430 (*23*). The SH430 isoline is nondormant and therefore ideal to study physiological seed aging and vigor processes without the interference of endogenous seed dormancy mechanisms. In contrast, M73 is highly dormant, making it well suited for dormancy and seed decay studies. Both isolines are integral parts of our research evaluating the impact of plant stress during seed maturation and development on seed chemistry and its regulation of seed longevity. Comparing wild-type populations with these isolines is necessary to ensure that the seed chemistry of the isolines is representative of typical field populations of wild oat. In addition, comparison of SH430 versus M73 offers the possibility of elucidating potential chemical regulators of seed dormancy. The data from this study will serve as the foundation for subsequent reports where the impact of plant stress during seed maturation on seed chemistry in the SH430 and M73 isolines will be characterized.

MATERIALS AND METHODS

Plant Materials. Seeds from the wild-type wild oat populations were initially collected from agricultural fields near Davis, CA, Pendleton, OR, and Powell, WY, in 2004 or 2005 and stored at −20 °C until February 2007. A "common garden" seed multiplication procedure was then initiated to remove any differences in seed quality associated with the initial maturation environment. To remove the primary dormancy, seeds were first subjected to 8 week accelerated after-ripening at 40 °C and 32% RH regulated by a saturated salt solution (*24*) during which seed moisture content was ∼10%. Following this after-ripening period, seeds were sown in Sun-Gro Sunshine Mix #1 (SunGro Horticulture, Vancouver, British Columbia, Canada) in pots measuring 10 × 10 × 35 cm (Stuewe & Sons, Inc., Tangent, OR). The mix for each pot contained ∼15 g of Osmocote (19:6:12) slow release fertilizer which was supplemented with biweekly additions of ∼100 mL of Miracle-Gro soluble fertilizer solution (1% w/v; 18:18:21 + micronutrients). Seedlings were thinned to 1 per pot after 2 weeks, and there were 12 pots per population. The M73 and SH430 isolines were also included in this common garden grow-out. The M73 seeds were subjected to the same after-ripening regimes as outlined for the wild-type populations, whereas the SH430 seeds were inherently nondormant and required no after-ripening. Wild oats are considered self-pollinating, but 5−10% cross pollination has been reported in the field (*25*). To prevent cross-pollination, plants were arranged in a glasshouse as population clusters that were separated by sheets of polyester row cover. Repeated measurements of the temperature and light conditions associated with each population cluster indicated that there were no significant differences in growth conditions among the clusters (data not shown). Greenhouse temperatures ranged from 20 to 38 °C for the duration of the grow-out period. Natural lighting was supplemented with two 400 W high pressure sodium lamps set to a 16 h photoperiod. Seeds were collected from each population as they matured, which began in July and ceased in September of 2007. The seeds from each population were pooled weekly and stored at −20 °C until the phenolic acid extraction procedure was initiated. Weekly harvest pools for each population were combined after all the seed had been collected.

Phenolic Acid Extraction. The phenolic acids were extracted using a method adapted from Krygier et al. (*12*). The awns from four replicates of 50 seeds from each seed population were removed and the whole seeds lyophilized for 4 days. Seeds were separated into two fractions: hull (palea and lemma) and caryopsis. Seed fractions were ground into a fine powder in liquid nitrogen, weighed, and stored at −20 °C until further processing. For the initial extraction, each sample was transferred to a 15 mL Corex tube and extracted with 5 mL of hexane (3 times for hulls and 6 times for caryopses) to remove the nonpolar chemical constituents. Samples were centrifuged at ∼10000*g* for 3 min between extractions, and the hexane fraction was discarded. After the final hexane extraction, the pellets were air-dried for ∼20 min to allow the remaining hexane to evaporate before proceeding with the next extraction step. Each sample was then homogenized using a Powergen 125 homogenizer with a 10 mm diameter generator (Fisher Scientific, Pittsburgh, PA) in 4 mL of methanol:acetone:water (1:1:1) for ∼15 s. The generator unit was rinsed with 3 mL of the extraction solvent mixture in a test tube, which was added back to the sample. Samples were centrifuged for 3 min (∼10000*g*), and the supernatant was removed from tube and saved. This step was repeated six times, resulting in an extracted pellet and a total supernatant volume ∼40 mL that was split among three 20 mL scintillation vials. The extract volume was reduced by ∼70% in a SpeedVac (Savant AES2020, Thermo Fisher Scientific, West Palm Beach, FL) set at full vacuum with the radiant cover activated and the vacuum set to the "high drying" setting for 2 h. The remaining volumes from the three vials for each sample were combined into one vial with an end volume of ∼12 mL to comprise the "soluble" chemical fraction. Our method deviated from Krygier et al. (*12*) at this point in that free and esterified components were not separated based on the assumption that both these chemical components would be mobile in the spermosphere of the seed and have a similar ecological role. The soluble fraction was hydrolyzed with 5 mL of 4 N NaOH in a 60 °C shaking water bath overnight to hydrolyze esterified phenolic constituents to the free forms. The headspace (∼5 cm^2) of the vial contained ambient air. Similarly, the extracted pellet of the samples was hydrolyzed by the same process to convert the "bound" chemical constituents into the free forms. The pellet was transferred to a 20 mL scintillation vial by rinsing the Corex tube with two ∼2 mL aliquots of the methanol:acetone:water solvent mixture and adding 5 mL of 4 N NaOH to the vial. After this overnight hydrolysis step, all samples were acidified to a pH of ∼2 with the

Table 1. Identification of the Target Phenolic and Organic Acids Based on Retention Time of the Quantification Ion and the Corresponding Ratio (In Parentheses) of the Qualifying Ions Relative to the Quantification Ion for Each Compound[a]

peak no.	retention time (min)	compound	quantification ion	qualifying ions	
1	4.9	succinic	147	247 (15)	172 (5)
2	5.1	fumaric	245	147 (40)	143 (10)
3	6.2	malic	147	233 (20)	245 (10)
4	7.3	OH benzoic	267	233 (70)	193 (40)
5	8.3	vanillic	297	267 (82)	253 (58)
6	8.4	azelaic	201	317 (60)	147 (65)
7	9.4 and 9.5	*p*-coumaric	293	219 (90)	308 (70)
8	10.4	ferulic acid	338	323 (65)	249 (60)

[a] Retention times (see **Figure 1**), quantification and qualifying ions based on the trimethylsilate (TMS) derivatives of the target compounds.

addition of 2 mL of concentrated HCl. Samples were again extracted with hexane as previously described to remove any nonpolar constituents liberated by the hydrolysis. Samples were subsequently extracted with 4 mL of 1:1 diethyl ether + ethyl acetate (DE/EA). A viscous emulsion layer was common, which was consolidated by centrifuging in the SpeedVac for 1 min. The DE/EA layer was removed and saved. This step was repeated six times resulting in a total DE/EA volume ~20 mL, and the aqueous phase was discarded. Residual water was removed from the DE/EA extract by passing it through ~2 g of anhydrous $MgSO_4$ (Mallinckrodt Chemicals, Phillipsburg, NJ) contained in a funnel with 12.5 cm diameter filter paper (VWR Qualitative 413, VWR Scientific, West Chester, PA) followed by an additional rinse with 5 mL of DE/EA. The DE/EA fraction was dried under nitrogen and reconstituted in 1.5 mL of HPLC grade acetone and stored at −20 °C. Two to three molecular sieves (8−12 mesh, EDM Chemical Inc., Gibbstown, NJ) were added to each vial to ensure anhydrous conditions.

GC/MS Analysis. Samples were derivatized with 45 μL of BSTFA + 1% TMCS (Supelco, Bellefonte, PA) just prior to the initiation of the gas chromatography−mass spectrometry (GC/MS) runs. Based on preliminary method development, we determined that 10 and 75 μL of the extracted bound hull and caryopsis samples, respectively, in a total GC sample volume of 500 μL resulted in optimum low and high detection limits using a splitless injection, whereas 250 and 455 μL was optimum for the soluble hull and caryopsis samples, respectively. Acetone was used to adjust total volumes in the GC vials to 500 μL. Molecular sieves were added to the GC vials as previously described to prevent water-induced degradation of the derivatives (*26*). Samples were analyzed on a Thermo Scientific Trace Ultra GC with a PTV inlet, DSQ MS and a TriPlus autosampler (Thermo Fisher Scientific, West Palm Beach, FL). The GC column was a 30 m by 0.25 mm i.d. Rxi-5 ms 30 (Restek, Bellefonte, PA). The injection volume was 1 μL sample + 1 μL internal standard (20 mg L^{-1} hexachlorobenzene in chloroform) injected simultaneously by the autosampler. Initial inlet temperature was held at 60 °C for 12 s, ramped to 280 at 10 °C s^{-1} and a postrun cleaning phase of 350 °C for 30 s. Initial oven temperature was held at 80 °C for 30 s and ramped to 150 at 30 °C min^{-1} and then to a final run temperature of 276 °C at a rate of 14 °C s^{-1}. A postrun oven temperature of 300 °C was held for 12 s before the next injection to clear the column. The transfer line temperature was held at 300 °C, and the ion source temperature was held at 250 °C. The carrier gas (He) flow was 1.5 mL min^{-1}. The MS detector was configured to the positive electronic ionization mode.

Xcalibur 1.4 (Thermo Fisher Scientific, West Palm Beach, FL) was used as the primary software interface with the GC/MS and for data processing. The initial identification of the target peaks was accomplished with the NIST MS Search Library (Version 2.0; National Institute of Standards and Technology, Gaithersburg, MD) on a subset of preliminary wild oat extraction samples. The likely NIST library matches were compared against derivatized analytical standards to verify the retention times, the dominant ions, and their respective ratios. The parameters from verified

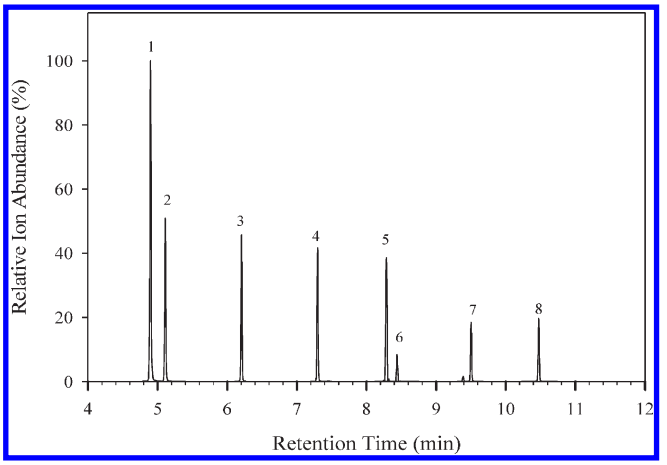

Figure 1. A typical selective ion chromatogram of the TMS derivatives of the standards of the prominent phenolic and short-chained aliphatic organic acids found in wild oat seeds (1 = succinic; 2 = fumaric; 3 = malic; 4 = OH benzoic; 5 = vanillic; 6 = azelaic; 7 = *p*-coumaric; 8 = ferulic). All standards were injected at 20 mg L^{-1} except ferulic acid, which was injected at 40 mg L^{-1}. Chromatograms based on the abundance and retention times of quantitative ion for each target compound (**Table 1**).

matches were used to configure the selective ion monitoring (SIM) scans for the subsequent runs (**Table 1**). For each compound, a dominant quantification ion was selected, as well as at least two qualification ions. Positive detection of the target compounds was based on a match for the retention time, quantification ion, and the appropriate ratio of the qualification ions relative to the quantification ion based on the spectra of the standards. Target compounds were quantified based on the integration of the extracted ion chromatograms of the quantifying ions relative to the integration of the extracted ion chromatogram for the internal standard. These values were referenced against standard curves generated from the calibration standards (6 levels), which ranged from 1 to 20 mg L^{-1} for all compounds except ferulic acid, where 1 to 40 mg L^{-1} was used. *p*-Coumaric acid tended to be represented by two peaks within 0.2 s of each other (**Figure 1**); the sum integration of both peaks was used to calculate the concentrations of this compound. The values for the calibration standards were also normalized relative to the internal standard. Quality control samples were also integrated into the GC/MS runs and consisted of the second, fourth and sixth level calibration mixtures. Quality control criteria were set to ±15% of the specified concentration for each target compound. In addition to the targeted phenolic and organic acids, various fatty acids (> 16 C long), including palmitic (*n*-hexadecanoic acid), linoleic (9,12-octadecadienoic acid), oleic acid and stearic (octadecanoic acid), were positively identified to be present in many of the samples. Analysis of the hexane fractions from a subset of extractions revealed that a large portion of these fatty acids were removed by the hexane (data not shown). As such, accurate quantification of the fatty acids in our seeds was not feasible with our extraction methodology. Analysis of variance of the data was conducted using a general linear model (GLM) and least squared means tests in SAS Version 9.1 (SAS Institute, Cary, NC).

RESULTS

The predominant phenolic acids that were positively identified in the various populations of wild oat seed included OH benzoic (benzenecarboxylic), vanillic, *p*-coumaric and ferulic (**Figures 1**, **2**). Trace amounts (< 1 μg L^{-1}) of cinnamic (3-phenyl-2-propenoic acid), sinapic and caffeic acid were intermittently detected in some of the seed extract samples; but since no discernible pattern of distribution was apparent, these compounds were not quantified. In the wild-type population, the total concentration of the phenolic acids in the seed (caryopsis + hull) was on average ~148,000 ng $seed^{-1}$ (**Table 2**). Ferulic and *p*-coumaric acid were present in the largest quantities, representing 68 and 31% of the total phenolics, respectively, with vanillic and OH benzoic acid representing < 1%

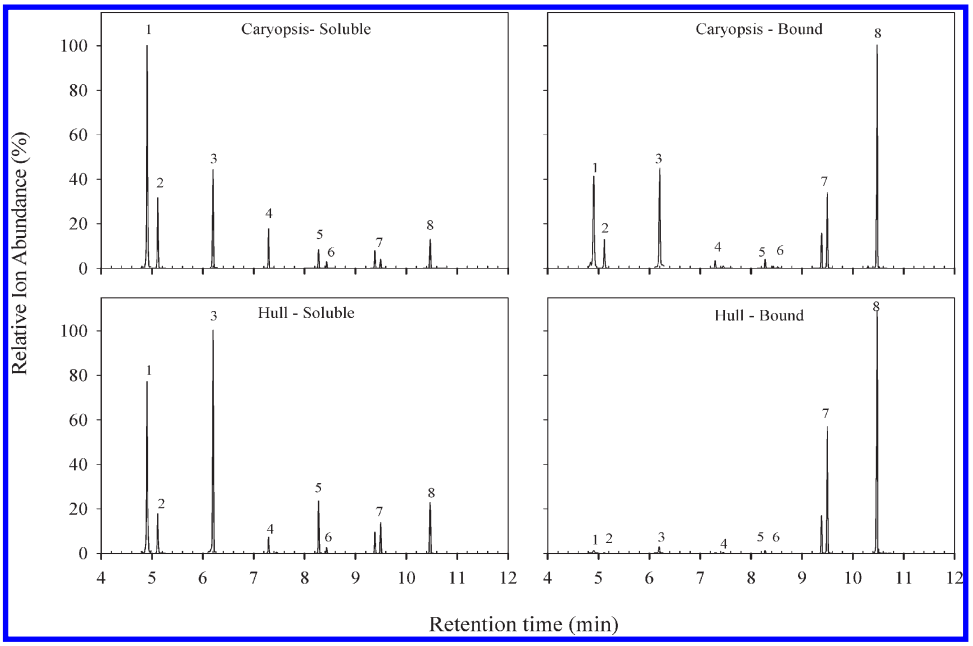

Figure 2. A typical selective ion (SIM) chromatogram of the TMS derivatives of the prominent four phenolic acids and aliphatic organic acids found in the soluble and chemically bound extraction fractions of wild oat caryopsis and hulls (California population shown). Peak designation and quantification are the same as in **Figure 1**.

Table 2. The Concentration of Individual Phenolic Acids per Seed in the Wild Oat Populations Grown under Common-Garden Greenhouse Conditions[a]

	wild-type		SH430		M73	
phenolic acid composition	mean, ng seed^{-1}	% total	mean, ng seed^{-1}	% total	mean, ng seed^{-1}	% total
OH benzoic	671 a	>1	749 a	>1	430 b	>1
vanillic	795 b	>1	901 b	>1	1471 a	>1
p-coumaric	41642 a	31	47063 a	29	25799 b	16
ferulic	92042 b	68	114887 ab	70	131579 a	83
total	147504 b		163600 a		159279 ab	
			Seed Fraction			
caryopsis	12086 a	9	12670 a	7	10958 a	7
hull	123064 a	91	150930 a	93	148321 a	93
			Chemical Fraction			
			OH Benzoic + Vanillic			
soluble	774 b	52	743 b	45	1178 a	62
bound	692 c	48	907 a	55	723 b	38
total	1466 c		1650 b		1901 a	
			p-Coumaric + Ferulic			
soluble	3768 a	3	2741 b	2	4527 a	3
bound	129916 a	97	159209 a	98	152851 a	97
total	133864 b		161950 a		157378 a	

[a] Means within a row followed by the same letter are not significantly different ($p > 0.05$) according to a least squared means test.

each. Of the total seed phenolics, 91% were in the hull and 9% in the caryopsis. For OH benzoic and vanillic acids collectively, 52% were in the soluble fraction and 48% in the bound fraction (caryopsis and hull combined). In contrast, 97% of the *p*-coumaric and ferulic acids were collectively in the bound chemical fraction and 3% in the soluble chemical fraction. In seed from the SH430 isoline population, total phenolic acids were 11% higher (~164,000 ng seed^{-1}) than in the wild-type populations, which was primarily attributed to a greater concentration of ferulic acid in the bound form. There was, however, a similar relative phenolic acid composition and the distribution in the seed and chemical fractions in the SH430 and wild-type seeds. The total phenolic acid concentration in the M73 isoline population was 15% higher than in the wild-type population, but similar to that of the SH430 isolines. Soluble phenolic acids, however, were 25 to 39% higher in the M73 seed compared to the wild-type or SH430 populations.

The organic acids that were positively identified in our wild-type wild oat seed populations included three intermediates of the TCA cycle: succinic, fumaric, and malic acids, as well as azelaic acid (**Figures 1**, **2**). Malic acid was the most abundant of these compounds, with a total seed concentration of ~3000 ng seed^{-1} (**Table 3**). Forty percent of the malic acid was found in the soluble

Table 3. The Amount of Aliphatic (C < 10) Organic Acids per Seed in Wild Oat from Wild Populations and Two Isolines (SH430 and M73)[a]

	organic acid constituent							
	malic		succinic		fumaric		azelaic	
seed and chemical fraction	mean, ng seed^{-1}	% total	mean, ng seed^{-1}	% total	mean, ng seed^{-1}	% total	mean, ng seed^{-1}	% total
				Wild Type				
caryopsis								
soluble	418 e	14	441 c	29	244 b	37	123 e	17
bound	629 d	20	297 d	20	125 c	19	72 g	10
hull								
soluble	1239 b	40	679 b	45	216 b	33	377 c	54
bound	796 c	26	83 e	6	74 c	11	132 de	19
total	3082		1500		659		704	
				SH430				
caryopsis								
soluble	1398 ab	33	231 d	15	91 c	12	98 f	15
bound	707 d	17	214 d	14	123 c	17	71 g	11
hull								
soluble	1619 a	39	817 a	54	234 b	32	345 c	52
bound	481 e	11	240 d	16	281 b	39	148 d	22
total	4205		1502		729		662	
				M73				
caryopsis								
soluble	624 d	27	738 ab	37	365 a	33	160 d	11
bound	393 e	17	354 d	18	179 bc	16	106 f	7
hull								
soluble	957 c	42	675 b	34	433 a	39	715 a	49
bound	308 e	13	236 d	12	122 c	11	490 b	33
total	2281		2002		1098		1471	

[a] Means within a column followed by the same letter are not significantly different ($p < 0.05$) according to a least squared mean test.

fraction of the hull, with 14 to 26% found in the other fractions. The second most abundant compound was succinic acid, with a total seed concentration of 1500 ng seed^{-1}. As was the case with malic acid, succinic acid was most abundant in the soluble fraction of the hull (45%), with most of the remaining portion of the succinic acid in the two fractions of the caryopsis (49%). Fumaric acid was the least abundant of the TCA intermediates, with a total seed concentration of ~660 ng seed^{-1}. Over 60% of the fumaric acid was in the soluble fractions, divided near equally between the caryopsis and hull. The total seed concentration of azelaic acid was ~700 ng seed^{-1}, of which over 70% was found in the hull. Seeds of the SH430 isoline population contained the same suite of organic acids in the same relative concentrations as in the wild-type seeds. The malic concentration of the SH430 seeds, however, was 36% higher than in the wild-type seeds, with 72% of this compound in the soluble fractions. The concentration and distribution of the other organic acids among the seed and chemical fractions of the SH430 seeds was similar to that of the wild-type seeds. Seeds of the M73 isoline population also contained the same suite of organic acids as in the wild-type and SH430 seeds. The total seed concentration of malic acid in the M73 seeds was 26% lower than in the wild type seeds, but the concentration of succinic and fumaric acids in the M73 seeds was 33 and 66% higher, respectively, than in the wild-type seeds. The distribution of the three TCA intermediates among the seed and chemical fractions in the M73 seeds was similar to that of the other seed populations. The total seed concentration of azelaic acid in the M73 seeds was ~1500 ng seed^{-1}, which was over 100% higher than in the other seed populations. As in the other seed populations, the majority (82%) of this compound was found in the hull.

Table 4. The Mean Mass of the Caryopsis and Hull Fractions from 50 Seeds Extracted for Each Test Population Extracted for Phenolic Acid Composition[a]

	mass of seed fraction, mg dry wt seed^{-1}	
population	caryopsis	hull
wild-type		
California	20.6 (3.2)	6.8 (0.4)
Oregon	17.2 (0.2)	6.6 (0.4)
Wyoming	14.4 (1.0)	7.2 (0.2)
isoline		
SH430	15.0 (1.0)	6.8 (0.2)
M73	13.0 (1.0)	5.7 (0.2)

[a] Numbers in parentheses represent one standard error of the mean.

DISCUSSION

Seeds from the wild-type and isoline wild oat populations tested in this study had a similar phenolic acid composition to that reported for cereal grain crops (*13*−*17*). When our data are converted to concentration of the phenolic acid per unit seed mass (**Table 4**), the total phenolic acid concentration in the caryopsis ranged from ~50 to 110 μg (g tissue)$^{-1}$ for the soluble fraction and ~600 to 800 μg (g tissue)$^{-1}$ for the bound fraction (**Table 5**), which is in the range of the concentrations reported for the above-mentioned cereal grain crops. In our wild oat hulls, total soluble phenolic acid concentration was much higher than in the wild oat caryopses, ranging from ~400 to 800 μg (g tissue)$^{-1}$, which is similar to that reported for free phenolic acids extracted from the glumes, palea and lemma of spring and winter wheat varieties (*27*). In this same wheat fraction, however, the bound fraction of ferulic plus *p*-coumaric was ~8000 μg (g tissue)$^{-1}$ compared to the ~17,000 to 25,000 μg (g tissue)$^{-1}$ for the bound

Table 5. The Concentration of Total Phenolics per Gram of Lyophilized Tissue for the Three Seed Populations[a]

	seed population, μg (g tissue)$^{-1}$		
	wild-type	SH430	M73
caryopsis			
soluble	106 a	54 b	103 a
bound	646 b	819 a	738 ab
hull			
soluble	508 b	393 b	766 a
bound	17648 b	22273 ab	25115 a
total	18908 b	23539 ab	26722 a

[a] Means within a row followed by the same letter are not significantly different ($p < 0.05$) according to a least squared mean test.

fraction of the extracted from the hulls of our wild oat populations. Modest differences in phenolic acid concentration per seed between M73 and the other seed populations (**Table 2**) were greatly augmented as a result of the low seed mass in M73 (**Tables 4-5**). Seeds from the M73 isoline also had notably higher soluble phenolic concentrations than the seeds from the SH430 isoline, suggesting that these chemical constituents may be related to the pronounced dormancy typically seen in M73 seeds.

In our study, we elected not to separately quantify the free and the esterified components of the soluble fraction, so therefore cannot conclusively comment on the relative concentrations of these two soluble components or their potential physiological role in wild oat. In cereal grain crops, however, total phenolic acids liberated from soluble esters were on average ~10 times higher than the free forms (*16*). Movement of the soluble phenolic acids and similar compounds in the seed zone (i.e., spermosphere) has not been well documented. We speculate that soluble compounds from the hull may be drawn into the caryopsis with the negative water potential gradient between the soil solution and the seed during seed imbibition. However, as the seed reaches a water potential equilibrium with the surrounding soil, compounds may diffuse from the seed to the spermosphere along a concentration gradient. Both of these phenomena, however, depend on the solubility of the seed compounds in the soil solution and the relative chemical attraction of the compounds to the seed and soil matrix. Since our extraction protocol included methanol and acetone in the aqueous extraction phase to improve extraction efficiency (*12*), the soluble phenolic fraction in our study may be higher than what would occur in a pure aqueous environment.

Ferulic and *p*-coumaric acid were the most prominent phenolic acids in the caryopsis and hull in both the soluble and the bound fractions, which is consistent with the data for the cereal grain crops (*13*−*17*, *27*). These two compounds are produced as part of the phenylpropanoid biosynthesis pathway and are commonly found in suberized plant tissues that help to protect against water loss and pathogen invasion (*28*). The metabolic pathway of OH benzoic acid is not well understood, but this compound has been found to be a precursor for glucosinolate biosynthesis in *Arabidopsis* (*Arabidopsis thaliana*) (*29*), as well as vanillic acid (*30*). Davis et al. (*19*) reported the presence of all of the phenolic acids we identified in wild oat in addition to the presence of numerous other phenolic compounds in unhydrolyzed methanol extracts of seeds from six common weed species, two of which were in the same family as wild oat. Since the GC/MS parameters and system capabilities were similar between the two studies, this result indicates that wild oat has a considerably less complex suite of phenolic compounds than reported in Davis et al. (*19*). Although the hydrolysis procedure utilized in our study to convert the esterified and bound phenolic acids into the free forms did not change the profile of phenolic acids present in our samples, it did liberate >95% of the total seed phenolics that would not have been accounted for with less comprehensive extraction protocols. Other compounds, such as cinnamic, sinapic and caffeic acid, that were intermittently detected at low concentrations in our samples could likely be more reliably quantified by tailoring the extraction and GC/MS methodology to low concentration compounds.

The exact role of phenolic acids in the ecology of wild oat seeds is speculative at this point; however, these compounds have been linked to the regulation of seed germination and the protection of seeds from abiotic and biotic stress factors in other plant species (*6*). Although there is considerable evidence that higher phenolic acid concentrations are correlated with reduced preharvest sprouting and subsequent seed germination in cereal grain crops (*13*−*16*, *31*), it is less clear if seed phenolic composition and concentration is a major regulatory factor for seed dormancy in wild oat. With the exception of the aforementioned differences in soluble phenolic acids between the M73 and SH430 isolines, the nondormant SH430 isoline generally had a similar suite and concentration of phenolic acids on a per seed basis to the other seed populations. Reigosa et al. (*32*) reported that exogenous addition of phenolic acids as individual compounds as well as in mixtures of these compounds had little effect on the germination of six common broadleaf weed species, suggesting that phenolic acids alone are probably not ubiquitous regulators of seed dormancy.

Compared to cereal grain crops, the allocation to phenolic acids in wild oat seeds is substantial, particularly in the hulls. There is evidence of a potential role of these compounds in protecting seeds against microbial infection and/or granivory. For example, Abdel-Aal et al. (*17*) found a strong correlation between bound ferulic acid concentration in the caryopsis of wheat cultivars and the resistance to orange blossom wheat midge. Likewise, higher concentrations of free phenolic acids in the secondary structures of wheat seeds corresponded with cultivar resistance to *Fusarium culmorum* (*27*). Wild oat seeds can persist in the soil seed bank for up to 9 years (*1*, *33*), and the protective role of phenolic acids may contribute to this longevity.

The concentration of organic acids was always greater in the soluble than in the bound fraction for both caryopsis and hull in all wild oat lines. Wild oat seed contained many of the organic acids common to the seeds of other plant species (i.e., malic acid, succinic acid, fumaric acid), but other organic acids, including citric, oxalic, and pyruvic, found in some other species were not detected in our study. Further study will be needed to determine whether this is due to variability among species or differences in protocols. We have not determined whether these other organic acids would be recovered by our protocol. There are very few reference points available in the published literature to compare the concentrations of these compounds in our wild oat populations to that of other wild oat populations or in other species. Hart and Berrie (*8*) reported malic and succinic acid concentrations in wild oat seed ranging from 3 to 11 μg seed^{-1}, which is somewhat higher than the concentration of these compounds in our study. In chickpea, malic acid concentrations were reported to be 268 to 804 μg seed^{-1} (*9*), much higher than we observed in wild oat. Although the mass of a typical chickpea seed is likely to be considerably larger than a wild oat seed, these data suggest that chickpea is particularly rich in these compounds. In contrast, the concentration of succinic plus malic acid in Eurasian catchfly was reported to be 3 to 6 ng (g tissue)$^{-1}$ (*7*), which is much lower than the mean value of 215 μg (g tissue)$^{-1}$ in our wild oat populations. Although we would expect differences among plant species, the extraction efficiency of organic acids can vary widely among compounds and extraction protocols (*20*). The extraction protocol used in our study was quite comprehensive, including multiple

aqueous/organic extractions to remove the organic acids from the plant tissue, hydrolysis with NaOH, reacidification, and extraction of the organic acids into a nonpolar organic phase (i.e., diethyl ether + ethyl acetate).

In our study, we evaluated the organic acid only in fully mature seeds with a moisture content of ~10%. There is evidence from other studies, however, that both the composition and concentrations of these compounds can depend on the development stage of the seeds and post dispersal environmental conditions. For example in *Arabidopsis*, the concentrations of succinate, fumarate and malate decreased as seed development approached physiological maturity, but increased substantially in the subsequent desiccation phase, suggesting that these compounds may be linked to germination and early seedling vigor (*34*). In chickpea, there was an initial increase in concentrations of malic and succinic acids during the first 24 h of seed imbibition that was attributed to dark CO_2 fixation (*9*). In wild oat, malic acid concentration increased with after-ripening as seeds became less dormant, and was considerably higher in nondormant cultivated "tame" oat than in wild oat (*8*). Similarly, exogenous additions of a number of organic acids, including malic, succinic and fumaric, stimulated the germination in conditionally dormant wild oat seeds at concentrations ranging from 50 to 100 mM (*35*), but this is far in excess of what we found endogenously in wild oat seeds. Monitoring endogenous organic acid concentrations throughout an after-ripening period is necessary to more precisely link the role of these compounds in regulating seed dormancy. The presence of azelaic acid in our wild oat seeds has interesting implications as a potential antimicrobial mechanism. Although azelaic acid has documented antimicrobial activity (*11*), its direct effect on *Pseudomonas syringae* infection of *Arabidopsis* leaf and stem tissue was minimal (*22*). Instead, it appeared to induce salicylic acid accumulation, which was the primary signal for defense against this pathogen. Similar mechanisms might occur in the seeds of wild oat and other weed species.

In summary, there is a common suite of phenolic acids in seeds of wild-type populations of wild oat and in the isoline populations evaluated in this study, dominated by ferulic and *p*-coumaric acid. These data suggest that the nondormant SH430 and the dormant M73 isolines should be suitable proxies for the wild populations with respect to phenolic acid composition and concentration to further evaluate the potential ecological and physiological role of these compounds in wild oat. Plants in this study were grown under greenhouse conditions, which would likely limit the exposure of the plants to ultraviolet (UV) radiation. It has been found that UV light can affect the production of phenolic compounds in some species (*36*), and this issue should be considered in future studies evaluating plant allocation to seed phenolics . The three TCA cycle intermediates (i.e., malic, succinic, and fumaric acids) appear to be commonly present in wild oat seeds, although variability in overall concentrations of these compounds did exist among the seed populations. The precise role of these compounds in wild oat seeds is unclear, although there is evidence from other studies linking them to seed vigor (*34*, *37*) and modification of the spermosphere/rhizosphere to improve seedling growth and development (*7*, *38*). The fact that these compounds were present at quite high concentrations in the soluble fraction of the hulls suggests that they could move with soil−water into the caryopsis as the seed imbibes as we hypothesized for the phenolic acids. The water solubility of these compounds ranges from ~6 g L^{-1} for fumaric acid to ~76 g L^{-1} for succinic acid, compared to 3 g L^{-1} for OH benzoic acid or 360 g L^{-1} for sodium chloride (at 25 °C) (*39*). Azelaic acid also appears to be commonly present in wild oat seeds, although we found it in notably higher concentrations in the M73 seeds compared to the seeds from the other population. There are no reports to our knowledge relating this compound to seed dormancy. If this compound has antimicrobial properties, however, M73 seeds may be less susceptible to pathogen infection than seeds from typical wild populations.

ABBREVIATIONS USED

GC/MS, gas chromatography−mass spectrometry; SIM, selective ion monitoring; TMS, trimethylsilate.

ACKNOWLEDGMENT

We thank A. Mesbah (University of Wyoming), L. Bodely and A. Fischer (University of California at Davis), and D. Thill (University of Idaho) for the wild oat seeds for the wild-type populations. We greatly appreciate the GC/MS support provided by Thermo Fisher Scientific (Dr. M. Bonilla, Dr. G. Harkey, B. Drakontaidis and M. Hendry), the Restek Corp, and Dr. E. Conklin. Special thanks to W. McCoy, J. Rossi, L. Kesser, L. Koller, C. Tien, J. Vincenty, and C. Ye for their technical support on this aspect of the project. We thank Dr. Richard Alldredge (Washington State University) for his guidance on the statistical analysis.

LITERATURE CITED

(1) Holm, L. G.; Plucknett, D. L.; Pancho, J. V; Herberger, J. P. *Avena fatua* L. and other members of the 'wild oat' group. In *The World's Worst Weeds: Distribution and Biology*; Hawaii Univ. Press: Honolulu, HI, 1977; pp 105−113.

(2) Heap, I. *The International Survey of Herbicide Resistant Weeds*. http://www.weedscience.com (accessed Aug 11, 2009).

(3) Buhler, D. D. Expanding the context of weed management. In *Expanding the Context of Weed Management*; Buhler, D. D., Ed.; Haworth Food Products Press: Binghamton, NY, 1999; pp 1−8.

(4) Benech-Arnold, R. L.; Sanchez, R. A.; Forcella, F.; Kruk, B. C.; Ghersa, C. M. Environmental control of dormancy in weed seed banks in soil. *Field Crops Res*. **2000**, *67*, 105–122.

(5) Forcella, F.; Benech-Arnold, R. L.; Sanchez, R.; Ghersa, C. M. Modeling seedling emergence. *Field Crops Res*. **2000**, *67*, 123–139.

(6) Gallagher, R. S.; Fuerst, E. P. The ecophysiology of seed longevity. In *Seed Science and Technology*; Basra, A. S., Ed.; Haworth Food Products Press: Binghamton, NY, 2006; pp 521−557.

(7) Bruun, H. H.; Van Rossum, F.; Ström, L. Exudation of low molecular weight organic acids by germinating seeds of two edaphic ecotypes of *Silene nutans* L. *Acta Ecol*. **2001**, *22*, 285–291.

(8) Hart, J. W.; Berrie, A. M. M. Relationship between endogenous levels of malic acid and dormancy in grains of *Avena fatua* L. *Phytochemistry* **1968**, *7*, 1257–1260.

(9) Aldasoro, J.; Nicolas, G. Fermentative products and dark CO_2 fixation during germination of seeds of *Cicer Arientinum*. *Phytochemistry* **1980**, *19*, 3–5.

(10) Chia, D. W.; Yoder, T. J.; Reiter, W. D.; Gibson, S. I. Fumaric acid: an overlooked form of fixed carbon in *Arabidopsis* and other plant species. *Planta* **2000**, *211*, 743–751.

(11) Charnock, C.; Brudeli, B.; Klaveness, J. Evaluation of the antibacterial efficacy of diesters of azelaic acid. *Eur. J. Pharm. Sci*. **2004**, *21*, 589–596.

(12) Krygier, K.; Sosulski, F.; Hogge, L. Free, esterified, and insoluble-bound phenolic acids. 1. Extraction and purification procedure. *J. Agric. Food Chem*. **1982**, *30*, 330–334.

(13) Weidner, S.; Amarowicz, R.; Karamac, M.; Frączeka, E. Changes in endogenous phenolic acids during development of *Secale cereale* caryopses and after dehydration treatment of unripe rye grains. *Plant Physiol. Biochem*. **2000**, *38*, 595–602.

(14) Weidner, S.; Frączeka, E.; Amarowicz, R.; Abe, S. Alternations in phenolic acids content in developing rye grains in normal environment and during enforced dehydration. *Acta Physiol. Plant*. **2001**, *23*, 475–482.

(15) Weidner, S.; Krupa, U.; Amarowicz, R.; Karamac, M.; Abe, S. Phenolic compounds in embryos of triticale caryopses at different stages of development and maturation in normal environment and after dehydration treatment. *Euphytica* **2002**, *126*, 115–122.
(16) Weidner, S.; Amarowicz, R.; Karamac, M.; Dąbrowski, G. Phenolic acids in caryopses of two cultivars of wheat, rye and triticale that display different resistance to pre-harvest sprouting. *Eur. Food. Res. Technol.* **1999**, *210*, 109–113.
(17) Abdel-Aal, E. S. M.; Hucl, P.; Sosulski, F. W.; Graf, R.; Gillott, C.; Pietrzak, L. Screening spring wheat for midge resistance in relation to ferulic acid content. *J. Agric. Food Chem.* **2001**, *49*, 3559–3566.
(18) Hendry, G. A.; Thompson, K.; Moss, C. J.; Edwards, E.; Thorpe, P. C. Seed persistence: A correlation between seed longevity in the soil and ortho-dihydroxyphenol concentration. *Funct. Ecol.* **1994**, *8*, 658–664.
(19) Davis, A. S.; Schutte, B. J.; Iannuzzi, J.; Renner, K. A. Chemical and physical defense of weed seeds in relation to soil seedbank persistence. *Weed Sci.* **2008**, *56*, 676–684.
(20) Pagel, H. A.; McLafferty, F. W. Use of tributyl phosphate for extracting organic acids from aqueous solution. *Anal. Chem.* **1948**, *20* (3), 272.
(21) Mehmood, S.; Orhan, I.; Ahsan, Z.; Aslan, S.; Gulfraz, M. Fatty acid composition of seed oil of different *Sorghum bicolor* varieties. *Food Chem.* **2008**, *109*, 855–859.
(22) Jung, H. W.; Tschaplinski, T. J.; Wang, L.; Glazebrook, J.; Greenberg, J. T. Priming in systemic plant immunities. *Science* **2009**, *324*, 89–91.
(23) Adkins, S. W.; Loewen, M.; Symons, S. Variation within pure lines of wild oats (*Avena fatua*) in relation to degree of primary dormancy. *Weed Sci.* **1986**, *34*, 859–864.
(24) Dhingra, O. D.; Sinclair, J. B. *Basic Plant Pathology Methods*, 2nd ed.; CRC Press: Boca Raton, FL, 1995.
(25) Imam, A. G.; Allard, R. W. Population studies in predominantly self-pollinated species. IV. Genetic variability between and within natural populations of wild oats from different habitats in California. *Genetics* **1965**, *51*, 49–62.
(26) Halket, J. M. Derivatives for gas chromatography-mass spectrometry. In *Handbook of Derivatives for Chromatography*; Blau, K., Halket, J. M., Eds.; John Wiley and Sons: New York, 1993; pp 297−325.
(27) Siranidou, E.; Kang, Z.; Buchenauer, H. Studies on symptom development, phenolic compounds and morphological defence responses in wheat cultivars differing in resistance to *Fusarium* head blight. *J. Phytopathol.* **2002**, *150*, 200–208.
(28) Croteau, R.; Kutchan, T. M.; Lewis, N. G. Natural products (secondary metabolites). In *Biochemistry & Molecular Biology of Plants*. Buchanan, B., Gruissem, W., Jones, R., Eds.; American Society of Plant Physiologists: Rockville, MD, 2000; pp 1250−1318.
(29) Ibdah, M.; Chen, Y. T.; Wilkerson, C. G.; Pichersky, E. An aldehyde oxidase in developing seeds of Arabidopsis converts benzaldehyde to benzoic acid. *Plant Physiol.* **2009**, *150*, 416–423.
(30) Kovacik, J.; Klejdus, B.; Backor, M.; Repcak, M. Phenylalanine ammonia-lyase activity and phenolic compounds accumulation in nitrogen-deficient *Matricaria chamomilla* leaf rosettes. *Plant Sci.* **2007**, *172*, 393–399.
(31) Kong, L.; Wang, F.; Si, J.; Feng, B.; Li, S. Water-soluble phenolic compounds in the coat control germination and peroxidase reactivation in *Triticum aestivum* seeds. *Plant Growth Regul.* **2008**, *56*, 275–283.
(32) Reigosa, M. J.; Souto, X. C.; Gonzale, L. Z. Effect of phenolic compounds on the germination of six weeds species. *Plant Growth Regul.* **1999**, *28*, 83–88.
(33) Chancellor, R. J. Seed behavior. In *Wild Oats in World Agriculture*; Jones, D. P., Ed.; Agricultural Research Council: London, U.K., 1976; pp 65−87.
(34) Fait, A.; Angelovic, R.; Less, H.; Ohad, I.; Urbanczyk-Wochniak, E.; Fernie, A. R.; Galili, G. Arabidopsis seed development and germination is associated with temporally distinct metabolic switches. *Plant Physiol.* **2006**, *142*, 839–854.
(35) Adkins, S. W.; Simpson, G. W.; Naylor, J. M. The physiological basis of seed dormancy in *Avena fatua*. VII. Action of organic acids and pH. *Physiol. Plant.* **1985**, *65*, 310–316.
(36) Kotilainen, T.; Haimi, J.; Tegelberg, R.; Julkunen-Tiitto, R.; Vapaavuori, E.; Aphalo, P. J. Solar ultraviolet radiation alters alder and birch litter chemistry that in turn affects decomposers and soil respiration. *Oecologia* **2009**, *161*, 719–728.
(37) Delhaize, E.; Ryan, P. R.; Randall, P. J. Aluminum tolerance in wheat (*Triticum aestivum* L.) II. Aluminum-stimulated excretion of malic acid from root apices. *Plant Physiol.* **1993**, *103*, 695–702.
(38) Jones, D. L. Organic acids in the rhizosphere—a critical review. *Plant Soil* **1998**, *205*, 25–44.
(39) *Merck Index*, 14th ed.; O'Neil, M., Ed.; Merck: Whitehouse Station, NJ, 2006; pp 905, 4285, 5707, 8869.

Received for review September 1, 2009. Revised manuscript received November 27, 2009. Accepted November 30, 2009. Funding for this research was provided in part by the USDA NRI Biology of Weedy and Invasive Species Program (Award No. 2005-35320-15375), The Pennsylvania State University, and Washington State University.