0971-4693/94 US $ 5.00
Allelopathy Journal 23 (1): 157-166 (2009) *International Allelopathy Foundation* 2009
Tables: 3, Fig : 1

Role of phenolic acids in expression of barley (*Hordeum vulgare*) autotoxicity

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(Received in revised form: October 25, 2008)

ABSTRACT

The role of phenolic acids in autotoxicity of four barley (*Hordeum vulgare* L.) varieties was investigated using radicle growth bioassays and analytical techniques. Total phenolic content of barley plant components varied within and between varieties during the 1999-2002 growing seasons. Inhibition of barley radicle growth was positively correlated with total phenolics depending on growing season and variety. Only total phenolic content of barley stems contributed significantly to barley autotoxicity. Concentrations of five phenolic acids differed in all plant components, among barley varieties and growing seasons. Ferulic acid and vanillic acid occurred least and most frequently in barley plant tissues, respectively; *p*-hydroxybenzoic acid, syringic acid, and *p*-coumaric acids were positively associated with barley autotoxicity ($r \geq 0.31$). Inhibition was significantly correlated with total phenolics, although other allelochemicals could also contribute to barley autotoxicity. Variations in total phenolics and phenolic acid composition over growing seasons may indicate a strong impact of climatic conditions on phenolic accumulation in barley plants.

Key words: Autotoxicity, barley, phenolic acids, total phenolics.

INTRODUCTION

Phenolic compounds form a large group of naturally occurring and chemically diverse substances widely distributed among plants. Characterized by the presence of an aromatic ring with one or more hydroxyl groups, phenolics include alkaloids, flavonoids, terpenoids, and glycosides (1). The most important groups of phenolics are flavonoids, phenolic acids and polyphenols, commonly known as tannins (18). Phenolics are secondary metabolites, primarily synthesized through the shikimate metabolic pathway (27). Phenolic acids contribute to the allelopathic expression in numerous crops, including sorghum (*Sorghum bicolor* L. Moench) (4), wheat (*Triticum aestivum* L.) (3,28,31), oat (*Avena sativa* L.) (3), and rice (*Oryza sativa* L.) (24).

Phenolic acids as potential allelochemicals have been identified in numerous crop and weed species. For example, wheat and barley residues typically release ferulic acid (25). Eight phenolic acids (*trans*-cinnamic, salicylic, ferulic, chlorogenic, *p*-hydroxybenzoic, protocatechuic, *p*-coumaric, vanillic) were identified in spring barley

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and oat tissues (13). Potential allelochemicals including benzoic acids (*p*-hydroxybenzoic, vanillic, protocatechuic) and cinnamic acids (coumaric, caffeic, ferulic, chlorogenic) were detected in aqueous extracts of 30 barley varieties (33). Seven phenolic acids (*p*-hydroxybenzoic, vanillic, *cis*-*p*-coumaric, syringic, *cis*-ferulic, *trans*-*p*-coumaric, *trans*-ferulic) were exuded by 17 day-old wheat seedlings into agar growth medium (29). Other allelochemicals including hydroxamic acid 2, 4-dihydroxy-7-methoxy-1,4-benzoxazin-3-1 were also detected (30). Wheat accessions differ significantly in the production of phenolic acids; those with high levels of total phenolic acids in shoot and root tissues are generally the most allelopathic (28, 31). Highly allelopathic accessions exuded high concentrations of allelochemicals into the growth medium (29). Sorghum root exudates containing *p*-hydroxybenzoic, vanillic and syringic acids may enhance overall allelopathic potential in the field when residues on the soil surface or are incorporated by tillage (4).

Increased nutrient availability to two winter wheat cultivars lowered the concentration of phenolic compounds in the plants (11). However, water stress increased phenolic accumulation in maize plant tissues (23). Allelopathy is strongly coupled with a variety of crop environmental stresses such as high temperature and insect damage that enhance the potential for crop interference through increased production of allelochemicals (8). In barley, phenolic-based allelochemical content was influenced more by growth conditions than by variety (14). Temperature and nitrate availability increase gramine content in leaves, which contributes to self-defence mechanisms in barley varieties that exhibit resistance to aphid (*Aphis* spp.) attack (10, 7, 20). Secondary metabolites such as gramine and hordenin play a role in barley allelopathic potential and its defence against fungal pathogens (*Drechslera teres*) and armyworm (*Mythimna convecta*) larvae. Gramine, an indole protoalkaloid was identified in leaves of two sub-species (*H. vulgare* ssp. *vulgare*; *H. vulgare* ssp. *spontaneum*) of barley (32). Gramine is also released through roots and may aid barley in overcoming competitive effects of weeds such as white mustard (*Brassica hirta* Moench) (20).

The present work investigated the potential role of phenolics in the expression of barley auto-toxicity for two reasons: (i) phenolics as allelochemicals occur in several cereal crops and (ii) the concentration and effectiveness of phenolic compounds in barley tissues may be influenced by changes of environmental factors. Therefore, we focussed on the contribution of phenolic acid content to the allelopathic potential of barley, and investigated the presence and role of five phenolic acids for assessing differential allelopathic potential among plant components of four barley varieties during three growing seasons in Tunisia.

MATERIALS AND METHODS

Four local varieties (‘Manel’, ‘Martin’, ‘Espérance’, ‘Rihane’) of barley were cropped in a randomized complete block design (RCBD) with 4 replications over 3 growing seasons (1999/00, 2000/01, 2001/02) at the Experimental Station of Ecole Supérieure d’Agriculture du Kef (ESAK) located in the semi-arid zone of Tunisia, on a clay soil with a pH of 8.1 and 2% organic matter. Mature plants of barley, free of disease symptoms and insect infestation, were randomly collected from plots. Roots were washed

with tap water to remove the soil and whole plants were stored for one week in the dark at ≤5°C until extraction.

Extraction of Plant Tissues

Barley plants were gently washed with distilled water, blotted between two paper towels, and then separated into roots, leaves, stems, and grain. Except for grain, all plant components were chopped into 1-cm long pieces and dried at 50°C for 24 h, then extracted following the procedure described by Ben-Hammouda *et al*. (4).

Bioassays

To evaluate the inhibitory potential of 4 barley varieties, 'Manel' was chosen as the assay specie due to its high sensitivity to allelochemicals (6). Water-extracts of the 4 varieties were tested by radicle growth bioassay following the procedure described by Ben-Hammouda *et al.* (5). Radicle growth inhibition was calculated as: [(Control − Treatment)/Control × 100].

Determination of Total Phenolics

The Folin-Denis method was used for total phenol analysis (2), with tannic acid as the standard. Folin-Denis reagent is a mixture of 10 g of sodium tungstate, 2 g of phosphomolybdic acid and 5 ml of phosphoric acid in 75 ml of distilled water that was refluxed for 2 h, cooled, and diluted to 100 ml with distilled water. The assay for total phenolic content followed the procedure of Makkar (22): saturated sodium carbonate was prepared by adding 40 g of sodium carbonate to 150 ml of distilled water, dissolving for 1 h in the dark and adjusting to 200 ml; tannic acid standard solution was prepared by dissolving 50 mg of tannic acid in 100 ml of distilled water. Aliquots of 0, 20, 40, 60, 80 and 100 µl of standard tannic acid solution were dispensed into tubes containing 0.5 ml Folin-Denis reagent and 2.5 ml saturated sodium carbonate solution. Standards were diluted to 4 ml with distilled water and quickly shaken, incubated in the dark at room temperature for 35 min, and absorbance determined spectrophotometrically at 750 nm (2). For barley plant extracts, 0.5 ml Folin-Denis reagent and 2.5 ml saturated sodium carbonate were combined with 1 ml of the water extract. Absorbance was determined and the total phenolic content was estimated by using the standard curve derived with the tannic acid standards. Total phenolic content was expressed as µg of tannic acid equivalents. For barley water extracts, tannic acid equivalents were multiplied by 20, based on an extraction ratio of 1:20 (w/w), to yield units in µg of tannic acid equivalents per g of barley tissue.

Qualitative and Quantitative Analysis of Phenolic Acids

Water extracts of barley plants used to estimate total phenolics were analyzed for *p*-hydroxybenzoic (POH), vanillic (VAN), syringic (SYR), *p*-coumaric (PCO), and ferulic (FER), which are associated with allelochemical contents of several cereal crop plants including wheat (28, 31) and sorghum (4). Prior to analysis, water extracts were filtered through a 0.45-µm sterile membrane. The filtered crude water extracts were analyzed for phenolic acids using a Shin-pack CLC (M-ODS) HPLC system, with two pumps operating at a 0.7 ml/min flow rate and a UV detector set at 280 nm. Separation of phenolic acids was performed on a C18 reversed-phase column (4.6 × 250 mm). The mobile phase was

0.1% phosphoric acid in 70% acetonitrile. Quantification of the individual phenolic acids was managed by a calibrated computerized package.

Data Analysis

Analyses of variance were conducted on bioassay results and means were separated using Fisher's protected LSD at 0.05 level of probability (α). Regression of radicle growth inhibition on total phenolic content and individual phenolic acids was carried out with plant component, variety and growing season as qualitative variables. When necessary, data transformation of the independent variable was conducted to reach an acceptable level of probability.

RESULTS AND DISCUSSION

Extracts of all plant components of four tested varieties substantially reduced the seedling radicle growth compared to water control (without plant extract). Across all three growing seasons, the stem component (across varieties) and the 'Martin' variety (across sources of water-extracts) showed highest inhibitory activity than other barley varieties (Table 1). Total phenolic content was significantly ($\alpha = 0.05$) higher in all components of the 'Martin' variety and was highest in leaf and stem (Table 1).

Table 1. Growth inhibition (%) of seedling radicle of 'Manel' barley (assay variety) by water-extracts prepared from plant components (5 g tissue/100 ml) of four barley varieties. The respective total-phenolic contents of each plant component averaged over three growing seasons are presented for comparison with respect to growth inhibition

Extract	Parameter	Barley variety				
		'Manel'	'Martin'	'Espérance'	'Rihane'	Means
Roots	RGI [a]	62.43 a [c]	64.63 a	48.57 b	48.50 b	56.03
	TPC[b]	31.57 c	75.25 a	59.68 b	23.84 c	47.59
Leaves	RGI	58.60 a	63.40 a	55.17 a	58.57 a	58.94
	TPC	441.80 a	443.47 a	279.95 c	384.11 b	387.33
Stems	RGI	72.33 a	67.57 b	68.83 b	62.20 c	67.73
	TPC	159.73 b	182.88 a	130.61 c	126.13 c	149.84
Seeds	RGI	52.83 b	60.47 a	67.70 a	38.23 c	54.81
	TPC	12.91 b	20.11 a	3.84 c	5.81 c	10.67
Means	RGI	61.55	64.02	60.07	51.88	
	TPC	161.50	180.43	118.52	134.97	

[a] RGI, Radicle growth inhibition (%); [b] TPC, Total phenolic contents (µg of tannic acid equivalent/g of tissues); [C] Values within rows followed by the same letter do not significantly differ at $\alpha = 0.05$ based on Fisher's least significance test.

During the second growing season (2000/01) radicle inhibition (Y) correlated with total phenolics (TP) ($r=0.42$; $p<0.10$) as $(TP + 0.25)^{0.01}$, independent of variety and plant component as source of the water-extract. Within variety and independently of plant component and growing season, Y correlated ($r = 0.56$; $p<0.06$) with TP only for 'Rihane'.

Allelochemicals other than phenolic acids may also be involved in growth suppression. Two alkaloids (hordenine, gramine) responsible for the allelopathic potential

of barley (12, 19) may have caused the growth inhibition. Sorgholeone (*p*-benzoquinone), *l*-tryptophan, DIMBOA (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one) and DIBOA (2,4-dihydroxy-1,4-benzoxazin-3-one) play roles in allelopathy of sorghum (9), oat (15, 16), wheat (30) and maize (*Zea mays* L.) (17). The autotoxic potential of barley is apparently associated with concentrations of specific phenolic acids rather than with total phenolic content. This is very similar to the findings of Ben-Hammouda *et al.* (4), in which sorghum allelopathy to wheat was related to specific phenolic acids.

Multiple regression of Y on TP (X_1 = TP) as the quantitative variable and the source of phenolics (X_2 = roots, X_3 = leaves, X_4 = stems, X_5 = seeds) as qualitative variables showed that stems were the sole significant variable in the best fitting model. Significant parameters in the corresponding equation were: $\beta_0 = 56.6$ and $\beta_4 = 11.1$ ($p < 0.05$). Although leaves contained the highest total phenolic content, stem extracts were most inhibitory to radicle growth (Table 1). These results suggest that inhibition was associated with phenolic composition rather than the concentration of total phenolic compounds. Barley varieties also varied in total phenolic content across years, indicating the influence of variable growing conditions experienced during each growing season (Table 2).

Phenolic acid contents of plant components in all test varieties across 3-growing seasons (1999-2002) are given in Table 2. Three phenolic acids, POH, VAN, SYR were always present and were detected in different amounts in stem. Concentrations of individual phenolic acids varied widely in plant components within and among barley varieties and growing seasons (Table 2), which was problematic for statistical analyses. However, the data helped in deriving correlations between the seedling growth inhibition and phenolic content of plant component extracts (Table 3). Generally, concentrations of phenolic acids were higher in 2000/01 than 1999/2000 and 20001/02 seasons (Fig. 1a). This seemed to be related to the relatively dry season of 2000/01, as only 283 mm rain was received during the barley growing season (November-May). Independent of plant component, variety and growing season, the phenolic acids, POH, SYR, PCO, were positively correlated to radicle growth inhibition (Table 3). Vanillic and *o*-coumaric acids were also implicated by Baghestani *et al.* (3) as possible allelochemicals of barley. Inhibitory properties of phenolic acids originating from barley are similar to those from other cereal species, such as PCO in rice (24), POH, SYR, and PCO in sorghum (4) and in wheat (28,31).

Phenolic acid content differed greatly among plant components within and between barley varieties across growing seasons (Fig. 1b). Sorghum hybrids exhibit a similar pattern (4). Fluctuations in phenolic acid contents across growing seasons for the same variety are partially due to variations in climatic conditions experienced at the experimental sites where the test barley varieties were grown. This is in complete agreement with the results of Einhellig (8) who reported that the production of allelochemicals was dependent on the degree of environmental stress. The large variability in seasonal phenolic content among a set of sahelian sorghum genotypes was thought to be more due to climatic conditions than cropping and soil factors (26). The exudation of DIBOA by maize roots was influenced by the duration of light irradiation (17). Similarly, hordenine production by barley leaves was determined more by environmental conditions than genetic factors (21).

Table 2. Phenolic acid contents in plant components of four barley varieties during three growing seasons [1999/00 (GS-1), 2000/01 (GS-2), 2001/02 (GS-3)]

		Phenolic acid content[a]											
		Roots			Leaves			Stems			Seeds		
Variety	Phenolic acid	GS-1	GS-2	GS-3	GS-1	GS-2	GS-3	GS-1	GS-2	GS-3	GS-1	GS-2	GS-3
Manel	*p*-Hydroxybenzoic acid	0.64	0.00	0.00	4.04	18.16	1.82	5.34	14.14	2.32	0.00	1.48	0.00
	Vanillic acid	0.10	0.46	0.00	7.02	17.42	3.26	5.30	19.28	5.24	0.00	1.62	0.22
	Syringic acid	0.02	0.00	0.00	0.02	6.70	3.08	3.20	1.80	14.94	0.00	0.38	0.04
	p-coumaric acid	0.02	0.00	0.00	0.08	2.26	0.42	0.66	1.18	5.36	1.34	0.70	0.00
	Ferulic acid	0.02	0.00	0.00	0.00	0.68	0.00	0.08	0.96	18.20	0.00	0.82	0.00
Martin	*p*-Hydroxybenzoic acid	0.40	11.90	0.00	2.28	24.92	0.72	0.62	14.22	1.68	0.00	1.00	0.00
	Vanillic acid	0.44	9.88	0.02	3.04	49.02	6.38	0.20	6.30	0.88	0.00	1.00	0.46
	Syringic acid	0.00	5.78	0.04	0.10	4.52	0.88	0.36	0.64	0.46	0.00	0.30	0.14
	p-coumaric acid	0.00	1.36	0.00	0.06	2.92	0.64	0.08	2.74	1.08	0.00	0.38	0.00
	Ferulic acid	0.00	0.50	0.00	0.00	11.08	0.00	0.08	1.96	20.40	0.00	0.04	0.00
Espérance	*p*-Hydroxybenzoic acid	0.20	3.48	0.02	0.60	0.26	3.50	6.54	6.90	1.68	0.00	0.02	0.00
	Vanillic acid	0.00	0.90	0.02	0.10	0.86	5.36	3.52	6.64	0.76	0.00	0.02	0.02
	Syringic acid	0.00	3.28	0.00	0.00	1.18	2.28	3.62	8.86	1.62	0.00	0.00	0.56
	p-coumaric acid	0.00	0.64	0.02	0.04	0.06	0.48	0.90	1.28	0.50	0.00	0.00	0.00
	Ferulic acid	0.00	0.22	0.00	0.00	0.00	0.00	0.54	0.16	26.60	0.00	0.00	0.18
Rihane	*p*-Hydroxybenzoic acid	1.40	0.00	0.06	7.08	0.00	3.18	0.16	19.88	0.76	0.00	1.58	0.00
	Vanillic acid	1.38	0.14	0.04	1.92	0.00	1.92	0.20	8.96	1.78	0.26	1.16	0.10
	Syringic acid	0.46	0.00	0.02	0.38	0.00	0.76	0.06	1.26	1.26	0.00	0.52	0.02
	p-coumaric acid	0.00	0.02	0.00	0.10	0.00	0.34	0.00	2.74	0.36	0.00	0.06	0.00
	Ferulic acid	0.00	0.02	0.00	0.00	0.00	2.66	0.00	1.06	28.76	0.00	0.18	0.00

[a] µg of tannic acid equivalent/g of dry weight tissue.

Table 3. Simple correlation coefficients and regression equations between barley radicle growth inhibition and individual phenolic acids extracted from plant components of four barley varieties, grown over three seasons

Phenolic acid	Correlation coefficient [a]	Regression equation
p-Hydroxybenzoic acid	0.31*	Y = 0.1041X – 2.7881
Vanillic acid	0.21NS	Y = 0.0989X – 2.2238
Syringic acid	0.25†	Y = 0.0392X – 0.8848
p-coumaric acid	0.30*	Y = 0.0179X – 0.4611
Ferulic acid	0.12NS	Y = 0.0482X – 0.4628

[a] df = 46; * Significant at the 0.05 probability level; † Significant at the 0.09 probability level; NS Not significant at the 0.05 probability level.

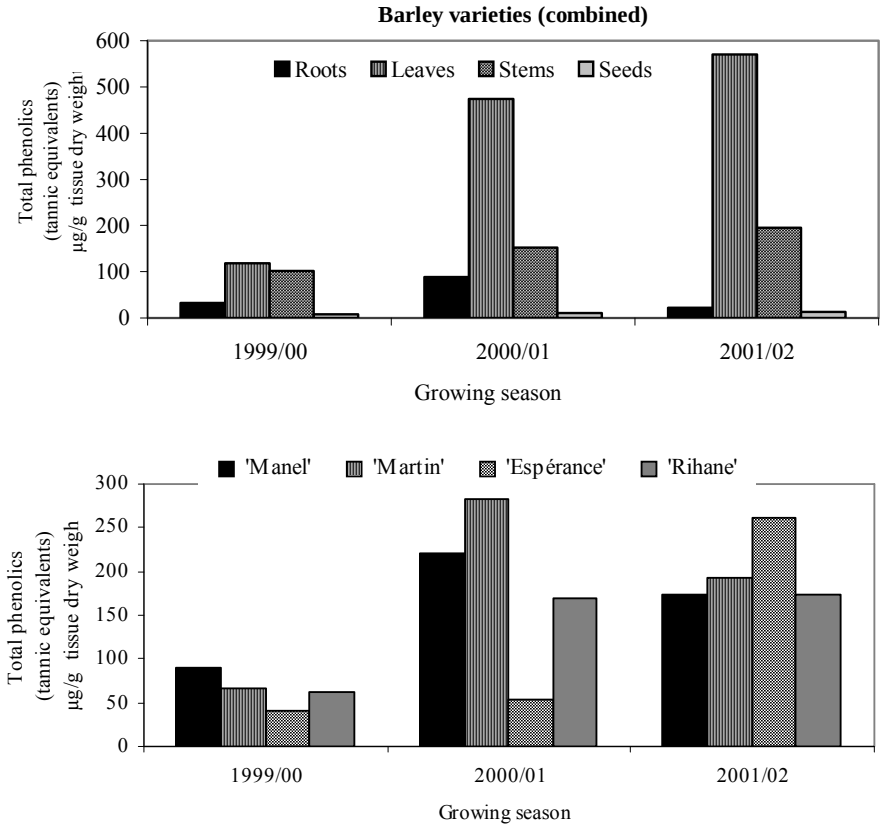

Figure 1. Total phenolics contents in plant components (a) in 4 barley varieties and (b) 3 growing seasons.

Autotoxic potential was not stable over time, indicating low genetic control of this trait. In fact a relatively dry growing season (2000/01) that contributed to the highest concentrations of phenolics in barley tissues, was the only season in which a significant relationship occurred between radicle growth inhibition and total phenolic content.

'Rihane' was the only variety to show barley autotoxicity and was significantly correlated with total phenolic content, suggesting that phenolic compounds play an essential role in the allelopathy of individual varieties. Since production of phenolic compounds appeared to be controlled largely by environmental changes, varieties characterized by relatively low autotoxic potential can be used in barley/barley cropping sequences, especially in conservation agriculture in which barley residues remain on the soil surface as mulch, which assures a net economic gain for farmers using this cereal grain production system.

CONCLUSIONS

Phenolic contents (primarily POH, SYR, PCO) partially explains the expression of barley autotoxicity. The most common phenolic acid (VAN) in barley tissues did not individually cause barley autotoxicity but may contribute synergistically with other phenolic acids.

ACKNOWLEDGMENTS

We wish to thank Agence Française de Développement and the Tunisian Research-Development Program for co-sponsoring the project "Direct Sowing" which supported the present work on phenolics and phytotoxic potential of barley residues. Thanks are extended to Pr. Sami SAYADI and Mr. Hedi ISSAOUI from the C.B.S (Centre de Biotechnologie, Sfax) for assistance with HPLC analysis, and to Dr. Hichem BEN SALEM from INRAT (Institut National de Recherche Agronomique de Tunis) for assistance with the total phenolic analyses.

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