Auto-Toxicity of Barley Residues in Direct Sowing

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Keywords: Barley, Allelopathy, Auto-toxicity, Total-phenolics, Phenolic acids.

Abstract

Barley is known as an allelopathic crop and a good understanding of the eco-physiology of a cover crop is required in a conservation agriculture based on mulch management. The role of phenolic acids in barley (Hordeum vulgare L.) auto-toxicity was investigated by seedling growth bioassays, spectrophotometry and HPLC. Total-phenolics (TP) of barley plant components (BPC) was determined, whereas 5 phenolic [p-hydroxybenzoic (POH), vanilic (VAN), syringic (SYR), p-coumaric (PCO), ferulic (FER)] acids were quantified in barley water-extracts (BWE).

Four local barley varieties ('Manel', 'Martin', 'Esperance', 'Rihane') were studied. TP in BPC varied within and among varieties across 3 (99/00, 00/01, 01/02) growing seasons (GS). For the 00/01 GS, inhibition of barley radicle growth (BRG) was found positively correlated ($r = 0.42$, $p < 0.10$) with TP of BPC. A multiple regression analysis showed that among BPC, only stems TP contributed significantly ($\beta stems = 11.1$, $p < 0.06$) to barley auto-toxicity. The coleoptile-length/radicle-length ratio (ratio: CL/RL) was significantly correlated with TP content of only 2 barley varieties ('Esperance', 'Rihane'). But contrary to expectation, the correlation was negative ($r = -0.53$*) for 'Esperance' and positive ($r = 0.51$*) for 'Rihane'. So, auto-toxicity in both varieties is of different nature.

Concentrations of the 5 targeted phenolic acids differed in all BPC within and among GS. FER and VAN are the least and the most frequent in BPC tissues, respectively. POH, SYR and PCO were positively correlated with barley auto-toxicity, with POH as the most highly ($r = 0.31$*) correlated one. FER was the unique phenolic acid significantly correlated ($r = 0.41$*) with CL/RL ratio.

Anytime the correlation, between inhibition and TP was found significant with a relatively high level of probability, is taken as a sign of other allelochemicals implication in the chemical basis of barley auto-toxicity. Variations in TP and individual phenolic acids across GS is an indication of a weak heredity control on phenolic accumulation in BPC tissues. However, barley auto-toxicity in a direct sowing could be attenuated by an appropriate eco-physiological approach (grow a species/variety with mild phyto-toxic potential prior to a highly tolerant one) in order to grow barley/barley as long as an economic return still possible.

Introduction

Several crop residues are recognized for allelopathic properties. Barley (*Hordeum vulgare* L.) residues expressed a phytoxic effect in the form of auto-toxicity (Ben-Hammouda et al 2002) and hetero-toxicity on durum wheat (*Triticum durum* L.) and bread wheat (*Triticum aestivum* L.) (Ben-Hammouda et al 2001). Wheat shoot residues inhibited significantly the growth of annual ryegrass (*Loium rigidum* Gaud.) (Wu et al 2001-b). Wheat grain yield is depressed when cropped after grain sorghum [*Sorghum bicolor* (L.) Moench] (Ben-Hammouda et al 1995). In a recent work, tilled grain sorghum residues delayed development of wheat crop but did not affect its grain yield, whereas no-till stover did not affect wheat stand establishment but reduced frequently its grain yields (Roth et al 2000). In a rye (*Secale cereale* L.)/corn (*Zea mays* L.) double cropping sequence, corn development was delayed and its biomass yield was reduced due to rye residues , with a more pronounced effect with no-till when compared with tilled soil (Raimbault et al 1990). Leachates from dead giant foxtail (*Setaria faberii* Herrm.) reduced corn (*Zea mays* L.) growth (Bell and Koeppe 1972). Under controlled conditions, a soluble active compound determined as L-tryptophan in water-extracts of oat (*Avena sativa* L.) shoots inhibited the germination and growth of radicle and hypocotyl of lettuce (*Lactuca sativa* L.) (Kato-Noguchi et al 1994). The benzoxazolinone, an allelochemical found in maize seedlings exhibited an inhibitory effect on roots and shoots of oat and ryegrass (*Lolium multiflorum*) suggesting that this compound partially contributes to the allelopathic potential of maize (Kato-Noguchi et al 1998).

Phenolic acids are known to play a role in the allelopathy of a large number of crop residues, including sorghum (Ben-Hammouda et al 1995), bread wheat (Baghestani et al 1999; Wu et al 2000; Wu et al 2001-b), oat (Baghestani et al 1999) and rice (*Oryza saliva* L.) (Rimando et al 2001). Wheat and barley residues were found to release ferulic acid (Sancho et al 2001). Seven phenolic acids (p-hydroxybenzoic, vanillic, cis-p-coumaric, syringic, cis-ferulic, trans-p-coumaric, trans-ferulic) were exuded by 17 days old wheat seedlings into agar growth medium (Wu et al 2001-a). Wheat accessions differed significantly in the production of phenolic acids. Those with high level of total phenolic acids in shoot and root tissues are generally the most allelopathic (Wu et al 2000; Wu et al 2001-b). Sorghum root exudes p-hydroxybenzoic, vanilic and syringic acids that may enhance the overall allelopathic potential in the field when residues are abandoned on soil surface or are incorporated (Ben-Hammouda et al 1995).

An increasing availability of nutrients to two winter wheat cultivars lowered the concentration of phenolic compounds in the plants (Harder et al 1998). Allelopathy is strongly coupled with a variety of crop environment stresses such as high temperature, irradiance, nutrient limitation and insect damage, that enhance allelochemical production and increase the potential for allelopathic interference (Einhellig 1996). Availability of nitrates and environment stress such as high temperature influenced barley (*Hordeum* spp.) defense mechanism by increasing the content of gramine in leaves which is a compound that enhances resistance to aphids (Hanson et al 1983; Corcuera 1993; Liu and Lovett 1993-b). Secondary metabolites such as gramine and hordenin play a role in barley allelopathic potential and its defense against fungus (*Drechslera teres*) and armyworm (*Mythimna convecta*) larvae. Gramine which is an indole protoalkaloïd, was identified in leaves of two sub-species (*vulgare*, *spontaneum*) of barley (Yoshida et al 1993). For barley, phenolics content was more influenced by growth conditions than variety (Jacobsen and Lie 1974).

To better understand the contribution of phenolics in the auto-toxic potential of barley, the present work was conceived and conducted to investigate the presence and the role of total-phenolics and 5 phenolic acids [p-hydroxybenzoic (POH), vanillic (VAN), syringic (SYR), p-coumaric (PCO), ferulic (FER)] in the differential auto-toxic potential among four barley varieties during 3 growing seasons.

Methods and Materials

Collection of barley plant components

Four local barley varieties (‘Manel’, ‘Martin’, ‘Esperance’, ‘Rihane’) were cropped in an RCBD with 4 replications over 3 (99/00, 00/01, 01/02) growing seasons (GS) at the Experimental Station of Ecole Supérieure d’Agriculture du Kef (ES/ESAK) located in the semi-arid zone of Tunisia on a clay soil with a pH of 8.1 and 2 % of organic matter.

Mature plants of barley, free of diseases and insect infestation, were collected randomly over 6 rows plots at 12 m2 (10 m × 1.2 m). Roots were washed with tap water to remove the soil and whole plants were stored at less than 5 °C until extraction.

Extraction of plant tissues and growth medium

Barley plants were gently washed with distilled water, blotted between 2 paper towels, and then separated into roots, leaves, stems, and grains. Except grains, all barley plant components (BPC) were chopped into 1-cm long pieces and dried at 50 °C for 24 h. The extraction was done following the procedure described by Ben-Hammouda et al (1995).

Extraction and growth medium preparation were done following the procedure described by Ben-Hammouda et al (1995).

Bioassays

After an incubation period of 60 h at 25°C, lengths of both coleoptile and radicle of seedling of a barley test-variety (‘Manel’) were measured and coleoptile length/radicle length ratio (Ratio: CL/RL) was calculated. Measurments were done for 3 PC (roots, leaves, stems) of the four (‘Manel’, ‘Martin’, ‘Esperance’, ‘Rihane’) tested barley varieties, during 3 (99/00, 00/01, 01/02) GS.

To evaluate the inhibitory potential of the 4 barley varieties mentioned earlier, ‘Manel’ was chosen as the test-variety, due to its high sensitivity to barley water-extracts (BWE) (Ben-Hammouda at al 2002). Water-extracts (WE) of the four varieties were tested by radicle growth bioassay following the procedure described by Ben-Hammouda et al (2001). Barley radicle growth (BRG) inhibition of the test-variety was calculated as follow: [(Control – Treatment)/Control × 100].

Determination of total-phenolics

The Folin-Denis method was used for total-phenolic (TP) analysis (A. O. A. C 1990) with tannic acid as a standard. Folin-Denis reagent is a mixture of 10 g of sodium thungstate, 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.

To use the tannic acid as a standard, the procedure described by Makkar (2000) was applied: A sodium carbonate saturated-solution was obtained by adding 40 g of sodium carbonate to 150 ml of distilled water, then dissolved for 1h at dark and adjusted to 200 ml. A standard-solution of tannic acid was obtained by dissolving 50 mg of tannic acid in 100 ml of distilled water. Aliquots of 0, 20, 40, 60, 80 and 100 µl of the standard solution were dispensed into tubes containing 0.5 ml of Folin-Denis reagent and 2.5 ml of sodium carbonate saturated-solution. Finally, standards were diluted to 4 ml with distilled water and quickly shaken. Their absorbances were determined after 35 min in dark at 750 nm (A.O.A.C 1990).

Determination of TP was prepared by adding 0.5 ml of Folin-Denis and 2.5 ml sodium carbonate reagents to 1 ml of each BWE. Using absorbance standard curve, TP content was estimated. Units of TP were expressed in µg of tannic acid equivalents per ml of WE. For BWE, tannic acid equivalents were multiplied by 20, based on an extraction ratio of 1:20 (w/w).

Qualitative and quantitative analysis of phenolic acids

WE of BPC used for TP estimation were analyzed to trace 5 individual phenolic acids [p-hydroxybenzoic (POH), vanillic (VAN), syringic (SYR), p-coumaric (PCO), ferulic (FER)] known to be implicated in wheat (Wu et al 2000; Wu et al 2001-b) and sorghum (Ben-Hammouda et al 1995) allelopathy. Prior to analysis, WE were filtered through a 0.45 µm sterile membrane.

Crude WE were analyzed for phenolic acids using an HPLC system with two pumps operating at a 0.7 ml/min flow rate and a wavelength UV detector set at 280 nm. Separation was done by a reversed-phase column while the mobile phase consisted of a WE. Quantification of individual phenolic acids was managed by a calibrated computerized package.

Data analysis

Related data to WE effects on CL/RL ratio were subjected to analysis of variance. Treatments with significant main effects based on the protected Fisher-test were separated, using an LSD-test at the 0.05 probability level (Steel and Torrie 1980).

A multiple regression analysis of radicle growth (RG) inhibition on TP and individual phenolics (as a quantitative parameter) and the source (roots, leaves, stems) of WE (as a qualitative parameter) was carried out. Also, CL/RL ratio was subjected to a multiple regression analysis on the parameters mentioned earlier. Both regressions were executed depending on PC, variety and GS. Occasionally, a data transformation upon an independent variable was conducted to reach an acceptable level of probability. For both analysis of variance and regression analysis, SAS package was used was used (SAS institute 1985).

Results

When compared to control, WE of all PC of the four varieties (‘Manel’, ‘Martin’, ‘Esperance’, ‘Rihane’) reduced substantially the RG of the test-variety (‘Manel’) over experiments of 3 (99/00, 00/01, 01/02) GS, with respectively stems and ‘Martin’ having the most across inhibitory activities (Table 1). All WE of BPC have a significant effect on CL/RL ratio during the 3 GS. For the first (99/00) GS, all BPC expressed an allelopathic potential that was stimulatory to CL/RL ratio, except root-WE of ‘Rihane’. Stem-WE expressed the most stimulatory effect in 75% of the cases. In the second (00/01) GS, all WE of BPC were stimulatory with stem-WE having the highest activity in 50 % of the cases. During the third (01/02) GS, all BPC expressed a stimulatory effect on CL/RL ratio, except grain-WE of ‘Manel’ and root-WE of ‘Esperance’. Stem-WE expressed the most stimulatory effect in 75 % of the cases (Table 2).

For the second (00/01) GS, a correlation ($r = 0.42$) was found significant at 0.10 level of probability between radicle inhibition (Y) and TP as $(TP + 0.25)0.01‡$, independently of variety and PC. When studied within variety and independently of PC and GS, a correlation ($r = 0.56$) between Y and $(TP + 0.7)0.1‡$ was found significant only for ‘Rihane’, at 0.06 level of probability. Regression of BRG inhibition on TP (X1 = TP) as quantitative variable and the source of phenolics (X2 = roots, X3 = leaves, X4 = stems, X5 = grains) as a qualitative variable, showed that stem was the sole significant variable. Parameters in the best fitting

equation were: $\beta 0 = 56.6$ ($p < 0.06$) and $\beta stems = 11.1$ ($p < 0.06$). TP content of stems expressed a significant inhibitory activity on RG, whereas leaves were characterized by the highest TP content (Figure 1-a). This result suggests that inhibition may be associated with qualitative rather than quantitative trait of phenolics.

Barley varieties seemed to accumulate differently phenolics depending on conditions of the GS (Figure 1-b).

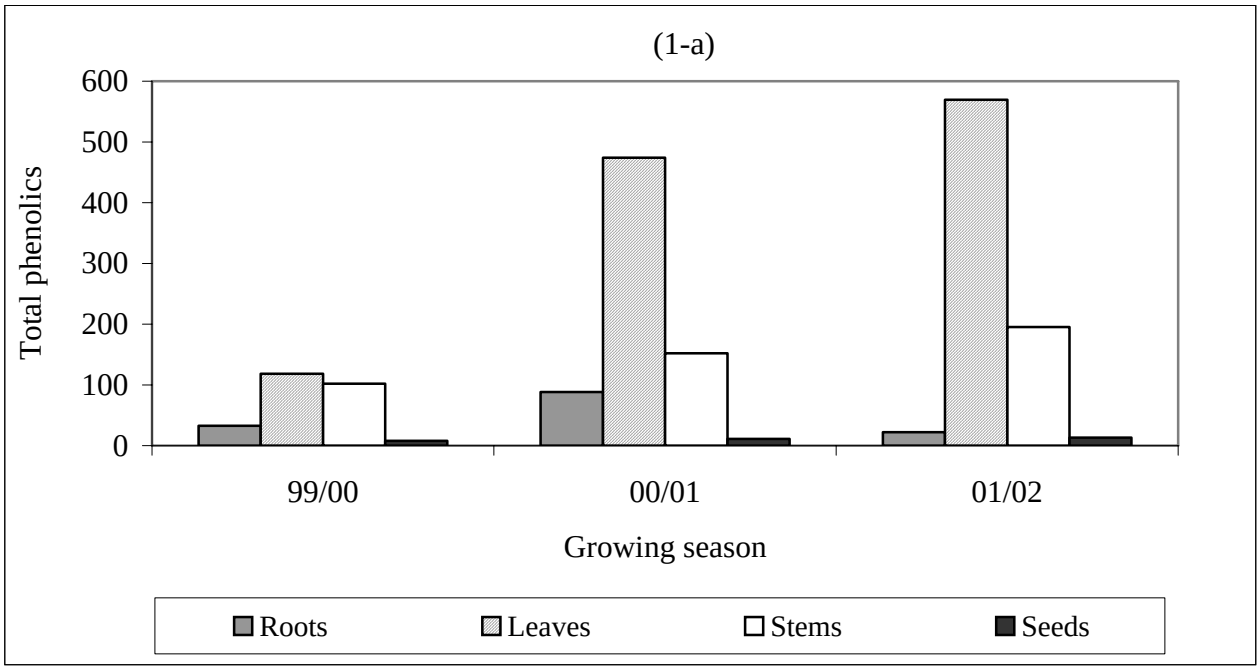

Figure 1. Means of total phenolics content (µg tannic acid equivalent/g) of plant components across 4 varieties (1-a) and 4 varieties across plant components (1-b) during 3 (99/00, 00/01, 01/02) growing seasons.

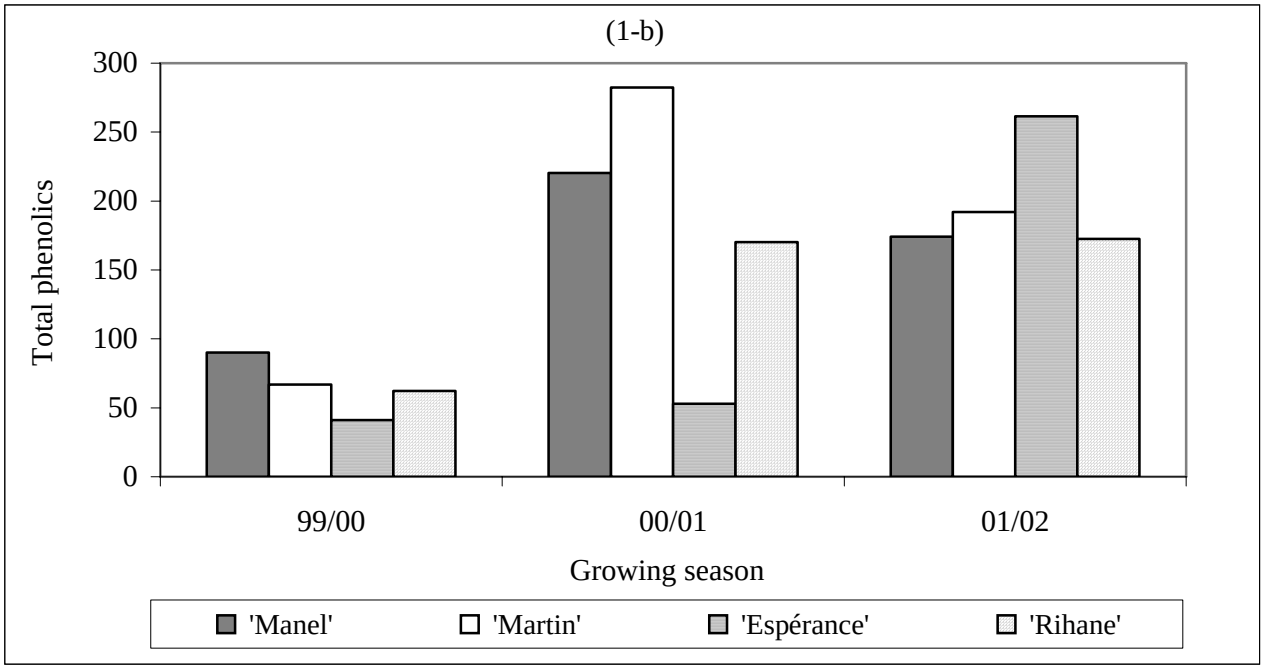

Figure 1. Continues.

‡ Transformation for TP data.

As for RG inhibition during the second (00/01) GS, a correlation (r = 0.46) was found significant at 0.08 level of probability between CL/RL ratio (Y') and TP as Y' = (PT + 0.25)0.01‡ independently of variety and PC. Correlation analysis within variety and independently of PC and GS, showed a negative correlation (r = -0.53), significant at 0.08 level of probability between Y' and TP, only for the case of 'Esperance'. However, a correlation (r = 0.51) was positive and significant at 0.09 level of probability for 'Rihane' [between Y' and (TP + 8)0.01‡]. There were no significant correlation between CL/RL ratio and TP even at 0.10 level of probability for any BPC, independently of variety and GS.

Three phenolic acids (POH, VAN, SYR) out of 5 (POH, VAN, SYR, PCO, FER) were always present but in different amounts in stems of the 4 tested barley varieties over 3 (99/00, 00/01, 01/02) GS. Concentrations of individual phenolic acids varied among PC within and among both barley varieties and GS (Table 3). FER and VAN were absent in 54.2 % and 12.5 % of the cases, respectively. Generally, concentrations of the 5 phenolic acids where higher in the second (00/01) GS than in the first (99/00) or the third (001/02) GS. This seemed to be coupled with a relatively dry 00/01 GS with 283 mm of rain over the growing cycle of barley (November-May) and a high monthly variability (CV = 45.8 %) of rain (Data not shown). Three phenolic acids (POH, SYR, PCO) were found to be positively correlated with RG inhibition ($r = 0.31$, $p < 0.05$), ($r = 0.25$, $p < 0.09$) and ($r = 0.30$, $p < 0.05$), respectively. Only FER was significantly correlated ($r = 0.41$, $p < 0.05$) with CL/RL ratio.

Discussion

Results suggest that total inhibitory effect of barley residues may involve partially other allelochemicals than phenolics. Hordenine and gramine were reported as two alkaloids, playing a role on the allelopathic potential of barley (Liu and Lovett 1993-a; Hoult and Lovett 1993), a kind of potential that was not tested in this present work. Stem TP content was significantly correlated to BRG inhibition, indicating its contribution into barley allelopathy.

Auto-toxic potential of barley seemed to be associated to the amount of selective phenolic acids rather to TP content. This is very similar to the foundings obtained by Ben-Hammouda et al (1995), when working on the allelopathy of grain-sorghum on wheat.

Out of 5 tested phenolic acids (POH, VAN, SYR, PCO, FER), only 3 (POH, SYR, PCO) were significantly associated to barley auto-toxicity. Other phenolic (vanilic, o-coumaric) acids were mentioned as possible allelochemicals of barley (Baghestani et al 1999). Identified phenolic acids as barley allelochemicals, exhibited the same activity in other cereal species, for example PCO in rice (Rimando et al 2001) and (POH, SYR, PCO) in grain-sorghum (Ben-Hammouda et al 1995) and wheat (Wu et al 2000; Wu et al 2001-b).

Generally, WE of BPC manifested a stimulatory allelopathic effect on CL/RL ratio of the test-variety 'Manel', which makes the coleoptile grow better than radicle. Essentially, CL/RL ratio was significantly associated to TP content but also to FER as an individual phenolic acid, despite the fact that FER acid was the less frequent across PC. This result indicates that FER impaired growth of shoot and root of barley at the seedling stage, with an advantage to shoot growth. Similar results were reported about lantana (Lantana camara L.) phenolic effects on rey-grass seedling growth (Singh et al 1989).

When contrasting a negative correlation ($r = -0.53$, $p < 0.09$) between LC/LR ratio and TP content for 'Esperance' with a positive one ($r = 0.51$, $p < 0.10$) for 'Rihane', allelopathic activity appeared to be essentially associated to the nature or to the proportionality of individual phenolic acids or to both within TP that a barley plant could synthesize. These results are in agreement with those obtained with wheat (Wu et al 2000; 2001-a; 2001-b) and rice (Oryza sativa L.) cultivars (Caassi-Lit et al 1997).

There were great differences in phenolic acid contents among BPC within and between varieties across GS. Such differences were similar to those obtained with grain-sorghum hybrids (Ben-Hammouda et al 1995). Variations in phenolic acid contents among GS for the same variety may be due partially to change in climatic conditions over seasons, at the experimental site where tested barley varieties were grown. Working with sahelian sorghum genotypes similar variations in phenolics content were thought to be coupled with climatic conditions rather than cropping and soil factors (Sène et al 2001). This is in agreement with results reported by Einhellig (1996), stating that the production of allelochemicals depends on the degree of an environmental stress. What could happen to phenolics, was true for hordenine production by barley leaves, determined more by environmental conditions than genetics (Lovett et al 1994).

‡ Transformation for TP data.

Conclusion

TP content could explain partially the auto-toxic potential of barley, leaving the eventuality of other allelochemicals to be implicated in. Auto-toxic potential was not stable over time, indicating that is not highly genetically controlled. In fact, a relatively dry 00/01 GS, characterized by the highest concentrations of phenolic acids in BP tissues, was the sole GS that showed a significant relationship ($r = 0.42$, $p < 0.10$) between RG inhibition and TP and at the same time ($r = 0.46$, $p < 0.8$) between CL/RL ratio and TP, though the probability level in both cases was relatively high.

Three phenolic acids (POH, SYR, PCO) were found to be involved significantly in the expression of barley auto-toxicity. The most present phenolic acid (VAN) in barley plant tissue did not show an individual significant role in barley auto-toxicity, suggesting rather a synergetic effect with one or more implicated phenolic acids. Also, the less frequent phenolic acid (FER) was the sole involved with CL/RL ratio, suggesting that its effect is a differential allocation of dry matter in favor of barley shoot growth.

'Rihane' was the sole barley variety of which TP content was significantly correlated to barley auto-toxicity.

Since phenolic production appeared to be controlled largely by environmental changes, varieties characterized by a relatively stable and low auto-toxic potential can be used in a barley/barley cropping sequence, specially when practicing direct sowing as long as an economic return still of reach.

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Table 1. Inhibition mean of the test-variety radicle growth by water-extracts from plant components of 4 varieties over 3 (99/00, 00/01, 01/02) growing seasons.

	Inhibition of radicle growth (%)				
Plant component	'Manel'	'Martin'	'Esperance'	'Rihane'	Across mean
Roots	62.43	64.63	48.57	48.50	56.03††
Leaves	58.60	63.40	55.17	58.57	58.94
Stems	72.33	67.57	68.83	62.20	67.73
Seeds	52.83	60.47	67.70	38.23	54.81
Across mean	61.55†	64.02	60.07	51.88	

†, †† Mean of each variety over plant components and plant component over varieties across 3 growing seasons.

Table 2. Effects of water extracts from plant components of 4 barley varieties ('Manel', 'Martin', 'Esperance', 'Rihane') on CL/RL ratio of 'Manel' during 3 (99/00, 00/01, 01/02) growing seasons.

	Ratio: CL/RL											
	'Manel'			'Martin'			'Esperance'			'Rihane'		
Treatment	99/00	00/01	01/02	99/00	00/01	01/02	99/00	00/01	01/02	99/00	00/01	01/02
Control	1.05 b†	0.98 c	0.77 c	1.19 b	1.12 d	0.81 d	1.25 b	1.02 c	0.94 d	1.38 c	1.01 c	0.90 d
Root extract	2.86 a	4.27 a	1.56 ab	4.69 a	5.59 a	1.43 bc	3.36 a	2.65 b	1.38 cd	1.53 bc	5.48 a	2.20 b
Leaf extract	2.93 a	4.12 a	1.82 ab	4.72 a	3.86 c	1.64 b	3.25 a	3.09 b	1.68 c	1.86 ab	4.87 a	2.75 a
Stem extract	3.84 a	4.76 a	2.31 a	4.37 a	4.55 bc	2.16 a	3.96 a	4.47 a	2.38 b	2.06 a	4.94 a	3.11 a
Seed extract	3.23 a	2.56 b	1.27 bc	4.25 a	4.80 ab	1.20 c	3.64 a	3.63 ab	3.35 a	1.81 ab	2.38 b	1.44 c
LSD(P < 0.05)	1.27	0.96	0.78	1.23	0.90	0.29	1.48	1.03	0.61	0.36	0.73	0.44

† Means within a column followed by different letters are significantly different at 0.05 level of probability.

Table 3. Five phenolic acid contents in plant components of 4 barley varieties during 3 [99/00 (GS-1), 00/01(GS-2), 01/02 (GS-3)] growing seasons.

		Phenolic acid content (μg/g)											
		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'	POH	0.64	0.00	0.00	4.04	18.16	1.82	5.34	14.14	2.32	0.00	1.48	0.00
	VAN	0.10	0.46	0.00	7.02	17.42	3.26	5.30	19.28	5.24	0.00	1.62	0.22
	SYR	0.02	0.00	0.00	0.02	6.70	3.08	3.20	1.80	14.94	0.00	0.38	0.04
	PCO	0.02	0.00	0.00	0.08	2.26	0.42	0.66	1.18	5.36	1.34	0.70	0.00
	FER	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'	POH	0.40	11.90	0.00	2.28	24.92	0.72	0.62	14.22	1.68	0.00	1.00	0.00
	VAN	0.44	9.88	0.02	3.04	49.02	6.38	0.20	6.30	0.88	0.00	1.00	0.46
	SYR	0.00	5.78	0.04	0.10	4.52	0.88	0.36	0.64	0.46	0.00	0.30	0.14
	PCO	0.00	1.36	0.00	0.06	2.92	0.64	0.08	2.74	1.08	0.00	0.38	0.00
	FER	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'	POH	0.20	3.48	0.02	0.60	0.26	3.50	6.54	6.90	1.68	0.00	0.02	0.00
	VAN	0.00	0.90	0.02	0.10	0.86	5.36	3.52	6.64	0.76	0.00	0.02	0.02
	SYR	0.00	3.28	0.00	0.00	1.18	2.28	3.62	8.86	1.62	0.00	0.00	0.56
	PCO	0.00	0.64	0.02	0.04	0.06	0.48	0.90	1.28	0.50	0.00	0.00	0.00
	FER	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'	POH	1.40	0.00	0.06	7.08	0.00	3.18	0.16	19.88	0.76	0.00	1.58	0.00
	VAN	1.38	0.14	0.04	1.92	0.00	1.92	0.20	8.96	1.78	0.26	1.16	0.10
	SYR	0.46	0.00	0.02	0.38	0.00	0.76	0.06	1.26	1.26	0.00	0.52	0.02
	PCO	0.00	0.02	0.00	0.10	0.00	0.34	0.00	2.74	0.36	0.00	0.06	0.00
	FER	0.00	0.02	0.00	0.00	0.00	2.66	0.00	1.06	28.76	0.00	0.18	0.00