



A laboratory approach to investigate auto-toxicity of soils cultivated with barley

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ABSTRACT

Monoculture of barley, where direct drilling is applied as mean of conservation agriculture, may encounter allelopathic risk. Both tissue leachates and root exudates of a crop could persist in the soil with a high depressive potential for the subsequent one. Most studies on barley allelopathy were on above-ground residues and little is known about the allelochemicals released in to the soil by living residues. Auto-toxicity of extracts from soils cultivated with 4 barley varieties ('Manel', 'Martin', 'Esperance', 'Rihane') was studied using seedling growth bioassays, with 'Manel' as the test-variety. To investigate the role of phenolics in barley auto-toxicity, inhibition of radicle growth by soil extracts was regressed on total-phenolics and 5 phenolic acids (p-hydroxybenzoic, ferulic, vanillic, syringic, p-coumaric) contents of all soil extracts. Only radicle growth bioassays permitted the detection of barley auto-toxicity expressed by soil extracts. Radicle growth inhibition by soil extracts from a non-cultivated soil (soil-Control) was not significant, which was not the case for extracts of cultivated ones. Such result suggests that, allelochemicals were essentially released by plant tissues (leachates) and root exudates into the rhizosphere. No significant correlation was found between radicle growth inhibition by soil extracts and total-phenolic contents, though this content varied considerably among the 4 tested varieties. Three phenolic acids (vanillic, syringic, p-coumaric) were identified in soil extracts. As for total-phenolic, no significant correlation was found between radicle growth inhibition by soil extracts and phenolic acids contents. This is an indication that other allelochemicals highly determine barley auto-toxicity.

Keywords: barley varieties, soil extracts, bioassays, phenolic acids, total-phenolics.

Introduction

Understanding soil-residues interaction in the expression of an allelopathic potential could be an aid to manage allelopathic risk in case of Direct Drilling (DD). The DD is one of the conservation agriculture three principles: i) no soil disturbance and DD, ii) permanent mulching, and iii) adapted crop rotation that should be applied simultaneously. Soil extracts from DD wheat plots, revealed a significant production of allelochemicals after harvest and during the following cropping season when compared to soil extracts from conventional drilled wheat plots (Cast *et al.*, 1990). Two allelopathic rice (*Oryza sativa* L.) varieties released momilactone B into the soil, at phyto-toxic levels (Kong *et al.*, 2006). Four phenolic acids (p-hydroxybenzoic, vanillic, p-coumaric, ferulic) were identified at various levels in four different soils inducing differential plant growth (Whitehead, 1964). Working with alfalfa (*Medicago sativa* L.), auto-toxic factors were identified in sandy and clay soils, with higher inhibitory activity to seedling growth in sandy soils (Jennings

and Nelson, 1978). Coumarin and cis-cinnamoyl glucosides are two allelochemicals found in three different (volcanic ash, alluvial, calcareous) soils (Hiradate *et al.*, 2010). Decaying rice residues released five phenolic acids (p-hydroxybenzoic, p-coumaric, ferulic, vanillic, syringic) into the soil, that inhibited successive growing plants of a paddy weed, barnyard grass [*Echinochloa crus-galli* (L.) P. Beauv.] (Kong *et al.*, 2006).

Exuded allelopathic compounds from living roots helps wheat seedlings to compete against annual ryegrass (*Lolium rigidum* Gaud.) growth (Wu *et al.*, 2001b). At different growth stages, wild oat (*Avena fatua* L.) exuded phenolic compounds into the rhizosphere, which are potentially allelopathic substances (Iannucci *et al.*, 2012). Wheat seedlings, from accessions with low allelopathic potential, did not exude detectable amounts of allelochemicals into the growth medium in comparison with strongly allelopathic ones (Wu *et al.*, 2001a, b). Jasmonic acid is an allelochemical identified in root exudates of bread wheat (Dathe *et al.*, 1994). Grain-sorghum roots [*Sorghum bicolor* (L.) Moench.], exuded 3 phenolic

acids (p-hydroxybenzoic, vanillic, syringic) that may enhance the overall allelopathic potential of sorghum in the field (Ben-Hammouda *et al.*, 1995b).

Since root exudates and residue leachates may have synergic effect in the expression of barley auto-toxicity, in a continuous cropping system, the study of this potential risk of soil extracts from soil cultivated with barley could be of great interest to cereal producers. Previous work on above-ground tissues of the same barley varieties was conducted and showed that phenolics play a role in barley allelopathy/auto-toxicity (Oueslati *et al.*, 2009). So, investigating the contribution of phenolics in barley auto-toxicity, expressed by leachates/root-exudates, would be an integrative approach to better understand barley auto-toxicity and improve cereal residues management in Tunisia under conservation agriculture cropping systems.

The present work aims to: i) investigate auto-toxic potential of soils cultivated with barley, ii) characterize the role of total-phenolics (TP) content and 5 individual phenolic acids (PA) [p-hydroxybenzoic (POH), vanillic (VAN), syringic (SYR), p-coumaric (PCO), ferulic (FER)] in the expression of such potential.

Materials and Methods

Preparation of soil extracts

Immediately after harvest, at late June 2002 soil samples were randomly collected from plots cultivated with 4 barley varieties ('Manel', 'Martin', 'Espérance', 'Rihane'), grown at the Experimental Station of Ecole Supérieure d'Agriculture du Kef. The station was located in the semi-arid zone of North-West of Tunisia on an alkaline (pH = 8.1) clay (59% clay, 23% silt, 18% sand) soil according to (Donahue *et al.*, 1983) with 2 % organic matter and classified as calcisol (Deckers *et al.*, 1983). Samples were collected across 30 cm depth; air dried during 1 day, and then passed through a sieve of 0.3 mm stitches. Soil extraction was done following the procedure described by (Read and Jensen, 1989). A sample of 250 g equivalent of dry soil was extracted in 250 ml distilled water on a horizontal shaker for 24 h at 200 rpmn. Soil suspensions were filtered across Whatman N° 2 filter paper under vacuum then stored at 5°C until bioassay. Prior to TP analysis, soil extracts were centrifuged at 12500 rpmn for 20 mn at 8 °C.

Seedlings bioassay

A 1.2 % growth medium of agar, amended with 20 ml extracts of soil cultivated with 4 barley varieties

('Manel', 'Martin', 'Espérance', 'Rihane'), was used to be tested on seedlings (coleoptile, radicle) growth of 'Manel', chosen to be a test-variety. In addition, to a control of distilled water-agar (1.2 g agar/100 ml of distilled water), a second water-agar control of non-cultivated soil (fallow and hand weeded) was considered. The second control was a mix of 1.2 g agar in 80-ml of distilled water and 20 ml water-extract from the non-cultivated soil.

Surface sterile seeds were pre-germinated in Petri dish [(180x10) mm] between 2 filter papers moistened with 1.2 ml of distilled water. Seedling bioassays were conducted, using a test tube technique. Test tubes [(180x10) mm] were covered with cotton, slanted at 45° allowing 15 ml growth medium to solidify. Seedlings with radicle 3 mm long were transplanted into tubes. After 60-h of incubation, in dark at 25°C, lengths of coleoptile and central radicle of each barley seedling were measured and recorded, a procedure reported by (Ben-Hammouda *et al.*, 2001, 2002). Radicle and coleoptile's growth inhibition [Inhibition (%) = (Control – Treatment)/control × 100] of 'Manel' by soil extracts were calculated for all bioassays.

Determination of total-phenolics

For TP analysis, Folin-Denis method was applied (1), with tannic acid (TA) as a standard. Folin-Denis reagent is a mix of 10 g of sodium tungstate, 2 g of phosphomolybdic acid and 5 ml of phosphoric acid added to 75 ml of distilled water. The mixture was refluxed for 2 h, cooled, and then diluted to 100 ml with distilled water. A saturated sodium carbonate solution was prepared by dissolving 40 g of sodium carbonate to 150 ml of distilled water for 1 h in dark, and then adjusted to 200 ml. TP content determination was made following (Makkar, 2000) procedure: Saturated TA solution was prepared by dissolving 50 mg of TA in 100 ml distilled water. Aliquots [(0, 20, 40, 60, 80, 100) µl] of standard TA solutions were dispensed into tubes containing each 0.5 ml of Folin-Dennis reagent and 2.5 ml of saturated sodium carbonate solution. Standards were diluted to 4 ml with distilled water and quickly shaken. Absorbance was determined after 35 min in dark at 750 nm by a spectrophotometer (A.O.A.C., 1990), and then a standard curve was plotted.

For soil extracts, determination of TP was realized by adding 0.5 ml of Folin-Denis reagent and 2.5 ml of a saturated sodium carbonate solution to 1 ml of extract. Absorbance was registered and TP content of each sample was estimated based on a standard curve. TP were directly expressed in µg TA equivalents/g-soil, based on an extraction ratio of 1:1 (w/w).

Determination of phenolic acids

Soil extracts of barley were also, analyzed for 5 PA (POH, VAN, SYR, PCO, FER), which are known to be associated to barley (Oueslati *et al.*, 2009) and wheat allelopathy (Wu *et al.*, 2001c). First, crude soil extracts were filtered through a 0.45 µm sterile membrane, than analyzed for PA using a Shin-pack CLC (M-ODS) HPLC system. The system consists of: i) 2 pumps operating at 0.7 ml/min flow rate, and ii) a wavelength UV-detector, set at 280 nm. Separation of PA was done on a C18 reverse-phase. The mobile phase consisted of 0.1% phosphoric acid in 70% acetonitrile. Quantification of the individual PA was managed by a computerized package.

Data analysis

The experimental design for seedlings (radicle, coleoptile) growth bioassays was a CRD with 4 repetitions. Bioassays data were submitted to an analysis of variance, using SAS package (SAS Institute, 1985) and Fisher's protected test for least significant difference (LSD) at the 0.05 level of probability (Steel and Torrie, 1980), to make mean separation. A correlation analysis between the inhibitory effects of soil extracts on barley radicle growth and TP contents was conducted using SAS package. A second correlation between the inhibitory effects of the same extracts on barley radicle growth and PA contents was also conducted.

Results and Discussion

Extracts from soils cultivated with 4 barley varieties ('Manel', 'Martin', 'Esperance', 'Rihane')

and from a non-cultivated soil (soil-control) had no significant effects on 'Manel' coleoptile's growth. Though, all soil-extracts had a significant effect on radicle growth, excepting soil-control. As expected, radicle growth bioassays were the most reliable test to detect auto-toxic potential of soil extracts cultivated with barley. Similar results were obtained by (10) and (Ben-Hammouda *et al.*, 2001, 2002), working on oat (*Avena sativa* L.) and barley allelopathy, respectively. Soil-control expressed a slight but not significant (15 %) inhibition when compared to water-control. This shows that a non-cultivated soil could release phytotoxic substances in addition to those potentially leached by above-ground biomass and exuded by roots. A kind of activity that could be attributed to soil microbial effect (Waniska *et al.*, 1988; Sturz and Christie, 1996; Ito *et al.*, 1998; Kong *et al.*, 2007).

Soil extracts that are relevant to 'Esperance' were the most inhibitory, reducing radicle growth by 29 % and 39 % when compared to soil-control and water-control, respectively. 'Manel' soil extracts were the least inhibitory, reducing radicle growth only by 17 % and 29 % when compared to soil-control and water-control, respectively. The later and the former results indicate that the varietal auto-toxicity is less pronounced than the varietal hetero-toxicity. Using an increasing order of inhibition, tested varieties were classified as follows: 'Espérance' > 'Martin' > 'Rihane' > 'Manel' (Table 1). The differential inhibitory effect of soil extracts, among barley varieties, is always higher when compared to soil-control. Such differences could be due largely to leachates/root-exudates of barley. These results are in agreement with those found with grain-sorghum (Ben-Hammouda *et al.*, 1995a) and rice allelopathy (Om *et al.*, 2002).

Table 1.
Effects of extracts from cultivated soils with 4 barley varieties ('Manel', 'Martin', 'Esperance', 'Rihane') on radicle growth (cm) of 'Manel', during the 01/02 growing season.

Treatment	Radicle growth
Water-control	5.30 a*
Soil-control	4.52 ab
Soil extract/'Manel'	3.75 bc
Soil extract/'Rihane'	3.28 c
Soil extract/'Martin'	3.25 c
Soil extract/'Espérance'	3.23 c
LSD (5%)	0.97

* Means followed by the same letter are not significantly different at 5% level of probability.

There were great differences in TP content among soils cultivated with the four barley varieties. Phenolics were not detected in soil extracts relevant to 2 varieties ('Manel', 'Martin'), while those of 'Rihane' had the highest TP content. Similar results were found about differences of phenolics in soil cultivated with three sorghum hybrids (Ben-Hammouda *et al.*, 1995b). The relatively high amount of TP (1.27 μg /g-soil), in soil-control (Table 2) could be explained by decaying old residues (more than one growing season) or by microbial activity products (Whitehead *et al.*, 1981).

Correlation analysis between inhibition of 'Manel' radicle growth by barley soil extracts and their relative TP contents showed no significant association between the two parameters. Such result, suggests that barley auto-toxicity is not highly determined by phenolics. The fact that 'Martin' with no detected phenolics had a higher inhibitory activity than

'Rihane' with 3.89 μg /g-soil as a TP content explains clearly the absence of a cause-effect relationship.

Soil extracts analysis revealed that 3 (VAN, SYR, PCO) out of 5 (POH, VAN, SYR, PCO, FER) targeted PA were present with low concentrations, varying slightly among varieties. While in a precedent work realized on tissues of the same barley varieties the five targeted PA, were generally present (Oueslati *et al.*, 2009). PCO was detected only in extracts of soils cultivated with 'Manel' and 'Esperance', but SYR was the only PA found in all extracts. Its concentration was higher in soil extracts when compared to its concentration in soil-control, by about 2, 3, 6, and 10 times, respectively for soil extracts of 'Rihane', 'Manel', 'Esperance' and 'Martin'. This suggests that SYR could be produced by leachates/root-exudates and soil microbial activity, but with less intensity by soils (Table 2).

Correlation analysis, between inhibition of radicle growth of barley by soil extracts and TP

Table 2.
Total phenolic (TP) and phenolic acid (PA) contents ($\mu\text{g/g}$ -soil) of extracts from cultivated soils with 4 barley varieties ('Manel', 'Martin', 'Esperance', 'Rihane'), during the 01/02 rowing season.

Source of extracts	TP Content	PA*	PA Content
Soil-control	1.27	VAN	0.002
		SYR	0.001
		PCO	0.000
Soil extract/'Manel'	0.00	VAN	0.001
		SYR	0.003
		PCO	0.001
Soil extract/'Martin'	0.00	VAN	0.002
		SYR	0.010
		PCO	0.000
Soil extract/'Esperance'	0.20	VAN	0.000
		SYR	0.006
		PCO	0.001
Soil extract/'Rihane'	3.89	VAN	0.001
		SYR	0.002
		PCO	0.000

*PA Vanillic (VAN), syringic (SYR) and p-coumaric (PCO).

content of these extracts, revealed no significant association. Also no association, between inhibition of radicle growth and PA contents, was found. Three of the targeted PA (POH, SYR, PCO) were identified responsible of the auto-toxicity of tissues from the same barley varieties (Oueslati *et al.*, 2009). Such result suggests that barley auto-toxicity appeared to be more determined by growing season and other types of allelochemicals, such the 2 protoalkaloids (hordenine, gramine) found in tissues extracts and root exudates of barley (Liu and Lovett, 1993a, 1993b). The probability of indirect effect of phenolic compounds should not be omitted. A future work with an experiment separating leachates from root exudates will better express their relative intensity.

Conclusion

Auto-toxic potential of barley was identified in soil extracts, using the technique of seedlings growth bioassays. Such potential varied among soils where barley varieties were cultivated. Neither TP content nor individual PA concentrations of soil extracts were able to explain barley auto-toxicity. The possibility of implication of other allelochemicals than phenolics should be taken in consideration. Leachates/root-exudates must be considered in any case study of barley allelopathy to assess its over-all potential, before making decision at the farm level to go for a barley/barley sequence especially in a conservation agriculture cropping system based on a permanent mulching and a direct drilling . A kind of decision that becomes more delicate when direct drilling is practiced, as a mean to rebuilt soil fertility and control erosion.

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