Physiological and biochemical parameters: new tools to screen barley root exudates allelopathic potential (*Hordeum vulgare* L. subsp. *vulgare*)

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Abbreviations

Chl – chlorophyll; F_0 – initial fluorescence, F_v/F_m – maximum quantum yield of PS II photochemistry; G – genotype; S – substrate; AC – activated charcoal; SS – sandy substrate; SCSS, silty clay sand substrate

Abstract Morphological markers/traits are often used in the detection of allelopathic stress, but optical signals including chlorophyll *a* fluorescence emission could be useful in developing new screening techniques. In this context, the allelopathic effect of barley (*Hordeum vulgare* subsp. *vulgare*) root exudates (3 modern varieties and 3 landraces) were assessed on the morphological (root and shoot length, biomass accumulation), physiological (F_v/F_m and F_0) and biochemical (chlorophyll and protein contents) variables of great brome (*Bromus diandrus* Roth., syn. *Bromus rigidus* Roth. subsp. *gussonii* Parl.). All the measured traits were affected when great brome was grown in a soil substrate in which barley plants had previously developed for 30 days before being removed. The response of receiver plants was affected by treatment with activated charcoal, dependent on barley genotype and on the nature of the growing substrate. The inhibitory effect was lower with the addition of the activated charcoal suggesting the release of putative allelochemicals from barley roots into the soil. The barley landraces were more toxic than modern varieties and their effect was more pronounced in sandy substrate than in silty clay

sand substrate. In our investigation, the chlorophyll content and F_v/F_m were the most correlated variables with barley allelopathic potential. These two parameters might be considered as effective tools to quantify susceptibility to allelochemical inhibitors in higher plants.

Keywords Allelopathy, barley, root exudates, chlorophyll *a* fluorescence, total soluble protein content, soil

Introduction

Allelopathy is a kind of 'chemical warfare' between neighboring plants competing for nutrient resources through the production of molecules named allelochemicals (Rice 1984; Ding et al. 2007). Most allelochemicals are secondary metabolites and are emitted in the surrounding environment by leaching, residue decomposition, volatilization and root exudation (Koocheki et al. 2013). Currently, the crop allelopathic performance to suppress weeds receives increasing interest and could complement chemical and mechanical inputs for weed control in farming systems.

Barley (*Hordeum vulgare* L. ssp. *vulgare*) is considered to be a weed-competitive species (Christensen 1995; Didon and Hansson 2003; Bertholdsson 2005; Hansen et al. 2008; Dhima et al. 2010). It is also known to have allelopathic proprieties involved in plant-plant interactions against wild (e.g. *Lolium perenne*; Bertholdsoon 2004) or crop species (e.g. *Hordeum vulgare*, *Tritium durum* and *Triticum aestivum*; Ben-Hammouda et al. 2001; Bouhaouel et al. 2015; Ninkovic 2003). Compared with aboveground plant organs, the allelopathic potential of barley roots is still poorly studied. The assessment of this power and the identification of allelochemicals emitted by root tissues remains challenging, because of the belowground location of plant root systems (Delory et al. 2016) and of the involvement of resource competition which intermingles with allelopathic interference in the plant-to-plant interactions under field conditions (Qasem and Hill 1989). The establishment of an efficient, inexpensive, simple and reliable screening method is the first step in identifying crop genotypes with allelopathic potential (Courtois and Olofsdotter 1998). Several screening methods have been developed to assess the allelopathic interactions between donor-receiver species (Wu et al. 2001). Few bioassays have, however, adequately addressed to distinguish allelopathy from other interference mechanisms using living plants under controlled or field conditions (Nilsson 1994; Weidenhamer 1996; Ridenour and Callaway 2001; Li et al. 2015; Bouhaouel et al. 2015, 2016). In this context, recent investigations (Bouhaouel et al. 2015, 2016) reported that barley root exudates (donor species) have an inhibitory effect against the great brome (receiver species) using novel/modified bioassays in conditions reducing resource competition between both species. This species (*Bromus diandrus* Roth., syn. *Bromus rigidus* Roth. subsp. *gussonii* Parl.) is a troublesome grassy weed largely

distributed in Tunisian cereal crops and resulting in yield losses that can reach up to 80% in heavily infested cereal-growing areas (Souissi et al. 2000, 2001). The inhibitory effects of barley against this weed were only assessed at early stage of growth using laboratory bioassays (Petri dishes, beaker with agar medium) (Bouhaouel et al. 2015, 2016), but never in soil, a complex and living medium that might affect the allelopathic activity. Reliable screening bioassays should simulate the natural release of allelochemicals from the living donor plants into the growth medium and simulate field conditions as much as possible (Wu et al. 2001) to achieve meaningful results. The interspecific allelopathic potential of barley may be greatly influenced by both plant age and the environmental conditions including geo-edaphic characteristics and might explain the significant changes in the production (Gallet and Pellissier 2002), the sorption and the fate of allelochemicals in the soil.

Field and laboratory observations on morphological traits are usually the first step when demonstrating allelopathy, focusing on germination inhibition, reduction in the root and shoot growth or yield (Chiapusio et al. 2008). To understand the underlying mechanisms of this process, these observations should be completed at the physiological level by identifying cellular targets of allelochemicals. In fact, it has been shown that some compounds affect a wide range of physiological and biochemical processes including cell division, water status, phytohormones metabolism, respiration, photosynthesis, function of enzymes, absorption of nutrients, cell signaling and gene expression, etc. (Li et al. 2010).

In vivo measurements of chlorophyll content and chlorophyll *a* fluorescence were found as a sensitive, non-destructive and rapid method to estimate the photosynthetic performance of plants. Measuring the kinetics of chlorophyll *a* fluorescence emission by plant tissues allows to evaluate the functional integrity of photosystem II (Maxwell and Johnson 2000). These parameters have been extensively used in plant adaptation studies to different environmental stresses, including salinity, water stress, low and high temperatures, and nutritional deficiency (Artus et al. 1996; Jin et al. 2002; Faraloni et al. 2011; Kalaji et al. 2014; Zahra et al. 2014).

Protein content, in particular the soluble proteins in shoots or roots of several species, was also shown to be a useful biochemical parameter to quantify changes in plant performance against environmental stress (Singh and Rai 1982; El-Tayeb 2005). In the case where root exudates affect physiological and biochemical processes, these parameters could also serve as markers for the monitoring of the allelopathic stress and for screening purposes.

Most studies have emphasized the effect of aqueous extracts of residue or fresh material (Colton and Einhellig 1980; Yu et al. 2003; Kamal 2011; Elisante et al. 2013; Farhoudi and Lee 2013) or of specific, exogenously applied allelochemicals (i.e. cinnamic, *p*-coumaric, ferulic and vanillic acids, benzoxazolin-2(3H)-one,

flindersine and N-methyl-flindersine) (Mersie and Singh 1993; Barkosky et al. 2000; Hussain and Reigosa 2011; Hussain et al. 2011) on the photosynthetic activity or production of proteins. However, to the best of our knowledge, few researches were focused on the effect of root exudates on physiological and biochemical variables (Yu et al. 2003; Uddin et al. 2014; Zhang et al. 2016).

In this context, this paper reports on (i) the allelopathic potential of barley root exudates against the great brome in two growing substrates, (ii) chlorophyll *a* fluorescence and leaf contents in chlorophyll and protein in this context, and (iii) the usefulness of these physiological and biochemical traits as allelopathic stress markers, in a perspective of fast trait characterization and genotype screening.

Materials and methods

Plant materials

Six barley (*Hordeum vulgare* L. subsp. *vulgare*) genotypes were selected for this study, constituted by three Tunisian modern varieties (i.e. improved by conventional breeding) (‘Manel’, ‘Rihane’ and ‘Tej’) and two landraces (‘Ardhaoui’ and ‘Arbi’), and one Saudi Arabian barley landrace (‘Saudi’). The most cultivated modern varieties, ‘Rihane’ and ‘Manel’, were chosen in this study (El Felah 2011; El Gharbi and Felah 2013). In addition, the modern variety ‘Tej’ and barley landraces, ‘Ardhaoui, ‘Arbi’ and ‘Saudi’, better adapted to local environmental constraints, including water (El Faleh et al. 1985) and saline stress (Hammami et al. 2016), were used. Barley seeds were obtained from the National Agronomic Institute of Tunis. Seeds of great brome (*Bromus diandrus* Roth., syn. *Bromus rigidus* Roth. subsp. *gussonii* Parl.), however, were collected from infested sites in the Beja region in northern Tunisia (between 36°42'07.0"N, 9°12'46.3"E and 36°41'00.2"N, 9°13'09.8"E).

Sterilization and pre-germination

The barley and great brome seeds were surface-sterilized as previously described by Bouhaouel et al. (2015, 2016). After sterilization, the seeds were maintained on moist sterile filter paper and placed in darkness in a growth chamber at 22 °C and a relative humidity of 65%. Barley and great brome seeds were pre-germinated for 72 and 96 h, respectively.

Donor-receiver experiment

Thirty (30) pre-germinated seeds of the six barley genotypes (donor species) were sown in polypropylene square pots (13x13 cm) that had been disinfected with sodium hypochlorite. Each pot contained 800 g of sandy substrate (USDA classification system) or a mixture of soil (sand : soil; 50 : 50). The soil was taken from the surface layer of a field (0–20 cm) and the mixture was identified as silty clay sand substrate (USDA classification system). The physical and chemical proprieties of the two substrates were illustrated in Table S1. These substrates were autoclaved three times at 120 °C and at a pressure of 1 bar for 20 min. With the aim to study the release of organic molecules from barley roots and to assess their allelopathic role, a second treatment was applied with the addition of activated charcoal (RPL, Belgium) (20 g kg^{-1} soil) to each type of substrate (Batish et al. 2009). The activated charcoal has a great affinity for phenolic metabolites and does not adsorb inorganic molecules (Cheremisinoff and Ellerbusch 1978). Pots without barley seeds were used as controls. The experiment was conducted in a glasshouse at 26/22 °C day/night temperature, 16 h light/8 h dark photoperiod with a photon flux density of about 220 µmol m^{-2} s^{-1} and a relative humidity of 60%. The experimental design was arranged as a completely randomized block design with five replicates per treatment (i.e. combination of genotypes, types of substrates and treatment of activated charcoal). A total of 5 blocks were used. Each block contained one repetition per treatment (i.e. one pot). The pots were irrigated adequately every day with autoclaved tap water. After 30 days, the above- and below-ground parts of barley plants were removed. The substrates were then sieved using 2 mm mesh to remove, as much as possible, remaining barley roots that might be allelopathically active (Ben-Hammouda et al. 2002). Thereafter, ten (10) pre-germinated seeds of great brome (receiver species) were sown in the recovered substrate. After 30 days, the allelopathic effect of barley roots on the great brome growth was quantified using morphological and growth-related parameters: root length, shoot length, roots dry weight and shoot dry weight. Both the root and shoot parts of the plants were removed and placed in an oven at 70 °C for 72 h in order to determine their dry matter content.

Effect of activated charcoal on the growth of barley

To explore the effect (neutral, stimulatory or inhibitory) of activated charcoal on barley growth, barley landrace 'Ardhaoui' (high allelopathic potential), chosen with reference to present and previous study results (Bouhaouel et al. 2015, 2016), was used as donor genotype. The activated charcoal was mixed with both types of substrates (20 g kg^{-1} soil) and 30 pre-germinated seeds were sown / pot. Pots without activated charcoal were considered as

controls. The experimental conditions and design were maintained as described above. After 30 days, the four morphological parameters (root and shoot length, root and shoot dry weight) were determined.

Chlorophyll and chlorophyll *a* fluorescence parameters

A chlorophyll meter SPAD 502 Plus (Minolta, Japan) was used to estimate chlorophyll (Chl) content. After 28 days, the 'SPAD value' was determined on leaves of great brome, in particular on the new formed leaf of three randomly selected plants per pot. Four SPAD readings were taken per leaf and averaged to produce a single observation.

Chlorophyll *a* fluorescence measurements were also conducted after 28 days on young leaves of three great brome plants per pot, using a portable pulse-modulated fluorometer OSI 5P (modulating measure by ADC, BioScientific Ltd). Briefly, leaf samples were clipped into a leaf clip (dark-adaptation cuvettes) and kept in darkness for 20 min. The fluorometer automatically sets the following parameters: the initial minimum fluorescence (F_0), the maximum fluorescence (F_m) after a subsequent application of 0.8 s saturating pulse light at 8000 µmol m^{-2} s^{-1}, the variable fluorescence calculated as ($F_v = F_m - F_0$) and the maximum quantum yield of PS II photochemistry (F_v/F_m) in dark-adapted plants (Kalaji and Guo 2008). The F_0 was measured at the initial state following the unloading of electron carriers, which are found in the oxidized state, while F_m was measured at time of full operation of photosystems when the electron carriers were fully reduced. Therefore, F_0 should be low in optimal growth conditions and increases in cases of stress and *vice-versa* for F_m (Denden et al. 2005). In our study, F_0 and F_v/F_m were only considered for assessing the effect of allelopathic stress on the photosynthetic activity.

Total soluble protein content

Total soluble protein content was quantified in 28-days-old leaves of great brome plants using the Spectrophotometric Bradford assay (1976). A 200 mg of fresh leaves from five replicates per treatment were ground in liquid nitrogen to fine powder. To avoid protein denaturation, mortar, pestle and the Eppendorf tubes were previously frozen in liquid nitrogen. Then, 1.2 ml of extraction buffer (K-0.2 M phosphate at pH 7.8; 0.1 mM EDTA and 1% insoluble PVP) was added to the powder. Samples were vortexed and centrifuged at 4 °C and 15000 *g* for 30 min. A 5 µl-aliquot of the supernatant was carefully collected and mixed with 795 µl of distilled water and 200 µl of reagent Bradford Bio-Rad (Protein assay). Absorbance was recorded at a wavelength of 595 nm after 15 to 20 min of reaction using a spectrophotometer (Shimadzu). A calibration curve

(0, 2.5, 5, 7.5 and 10 mg l^{-1}) were made from a stock solution (20 mg ml^{-1}) of bovine serum albumin (BSA) used as a standard.

Statistical analysis

A three-way analysis of variance (ANOVA) was performed using PROC MIXED of SAS package (SAS V9.1) and the subroutine PDMIX 800.SAS to compare means according to Least Significant Difference (LSD) at a 0.05 level of probability. The rate of reduction of morphological and growth-related traits, F_v/F_m, Chl and total soluble protein contents, and the rate of increase of F_0 in great brome plants were calculated as [(Control – Treatment) / Control] x 100. Pots without barley seeds were considered as controls. A linear regression analysis ($y = mx + c$) was performed between the physiological (e.g. F_0 and F_v/F_m) or biochemical (e.g. Chl and protein) variables and the morphological variables in order to establish their mutual relationship. Figures were created using a Sigma-Plot 13.0 program for Windows (Systat Software Inc., Point Richmond, CA, USA).

Results

Effect of the barley root allelochemicals on great brome growth

Before using activated charcoal in the allelopathic interactions, the possible impact of adding this material to the growing substrate on barley growth was evaluated. The differences between the two treatments (i.e. in the presence or in the absence of activated charcoal) for the four morphological traits were not significant in sandy and silty clay sand substrates (Fig. 1).

Thereafter, the effect of the allelopathic potential of barley root exudates was assessed on the growth of great brome. The allelochemicals of barley roots did show a tendency to reduce the growth of great brome plants. The ANOVA showed highly significant variations of this effect between barley genotypes (G), growing substrates (S) and treatments with activated charcoal (AC) for the four morphological parameters of great brome (Table 1). However, a non-significant difference between the two types of growing substrate was recorded for the shoot length and root dry weight. Significant (G x S), (G x AC), (S x AC) and triple (G x S x AC) interactions were observed particularly for root and shoot length, and root dry weight.

In our conditions, the inhibitory action of barley roots affected, to a greater extent, the root and shoot length of great brome compared to the root and aerial biomass in the presence or in the absence of activated charcoal (Fig. 2). For example, in the absence of activated charcoal, the rate of inhibition of great brome growth by the six barley genotypes in sandy and silty clay sand substrates were respectively 27.8% and 20.7 % for the root length,

24.5% and 21.1% for the shoot length, 18.9% and 14.8% for the root dry weight, and 18.3% and 10.3% for shoot dry weight. In this study, barley roots affected in similar way the growth of the root and aerial parts of great brome.

In the presence of activated charcoal, the inhibitory activity of barley roots was significantly reduced (Table 1) for the four morphological traits (Fig. 2). For example, the rate of inhibition of root length of great brome plants by the six barley genotypes was decreased to 16.3% and 13.9% respectively in sandy and silty clay sand substrates in the presence of activated charcoal, while it reached 27.8% and 20.7% in its absence. Under these conditions, the rate of inhibition of the four determined morphological parameters was higher in the sandy substrate for all genotypes than in the silty clay sand substrate in the presence or in the absence of activated charcoal (Fig. 2).

The different barley genotypes affected the growth of the great brome to variable extents (Table 1). In sandy substrate, the rate of inhibition of root and shoot length of great brome ranged from 9-42% and 12-36%, respectively (Fig. 2). The inhibitory activity of the barley landraces 'Saudi', 'Arbi' and 'Ardhaoui' was higher than that of modern varieties, 'Manel' and 'Tej'.

Effect of the barley root allelochemicals on the photosynthetic activity of great brome plants

In order to determine the possible allelopathic effect of barley root exudates on the physiological and biochemical level of receiver plants, the Chl content and the chlorophyll florescence parameters (i.e. F_v/F_m and F_0) of the great brome were measured. Our data showed a reduction in the Chl content (SPAD value) of great brome plants subjected to barley root exudates. This effect was strongly dependent on the genotypes (G), types of substrate (S) and presence or not of activated charcoal (AC; Table 2). For example, the rates of reduction in the Chl content by the six barley genotypes in the presence of the two treatment of activated charcoal (i.e. in the presence or in the absence of activated charcoal) were more pronounced in sandy substrate (28.7%) as compared with silty clay sand substrate (22.7%). On the other hand, the rate of reduction was lower in the presence of activated charcoal (21.8%) than in its absence (29.6%). There was also a significant interaction between the two variables (G x S) and (G x AC), whereas interactions (S x AC) and (G x S x AC) were not significant.

The results showed also that the allelopathic activity of the six barley genotypes have decreased the maximum quantum yield of photosynthesis (F_v/F_m) and increased the initial fluorescence (F_0) (Table 2). The ANOVA showed that F_0 significantly varied between the tested genotypes (G) and treatments with/without activated charcoal (AC), but not with the type of substrate (S). For F_v/F_m, a highly significant difference was also obtained

for G, S and AC. A significant interaction between the two (G x S; G x AC; S x AC) or three variables (G x S x AC) were obtained for both F_0 and F_v/F_m, except a non-significant (S x AC) in F_v/F_m.

Considering the different treatments (i.e. activated charcoal or not, and type of substrate), the similar trend was also observed for F_0 and F_v/F_m (Table 2). The reduction of F_v/F_m or increase of F_0 by the six barley genotypes in the presence of the two treatments of activated charcoal were slightly greater in sandy substrate (8.1% and 8.5% for F_v/F_m and F_0, respectively) than in silty clay sand substrate (7.5% and 7.6% for F_v/F_m and F_0, respectively). The addition of activated charcoal reduced the inhibitory effect on F_v/F_m and the increase in F_0 compared to the control. Overall, 'Manel' showed the lowest reduction rate of F_v/F_m and increase rate of F_0, while 'Ardhaoui' and 'Saudi exhibited the highest values.

In order to test the suitability of the physiological and biochemical variables as markers of the allelopathic stress in receiver plants, correlations were studied between these variables and the barley allelopathic potential. Most of the positive correlations between the rate of reduction in Chl content (Fig. 3) or F_v/F_m (Fig. 4) and the inhibition rate of the four morphological parameters in great brome plants were significant. However, most of the positive correlations with F_0 were not significant (Fig. 5).

Effect of barley root allelochemicals on the total soluble protein content in great brome plants

In this study, the effect of allelopathic activity of barley roots on the protein homeostasis was tested. The results showed that barley roots reduced the total soluble protein content in great brome shoots and this effect was dependent on barley genotypes (G), types of substrate (S) and treatments with/without activated charcoal (AC). The interaction (G x S) was significant (Table 2). The reduction in the total soluble protein content by the six barley genotypes was higher in sandy (29.3%) than in silty clay sand substrate (25.2%) in the presence and absence of activated charcoal. The addition of activated charcoal reduced this inhibitory effect. No significant positive correlations between the rate of reduction in the total soluble protein content and the rate of inhibition of the four morphological traits in great brome plants were obtained for both types of substrates (Fig. 6). Indeed, the modern variety 'Tej', one of the least allelopathic genotypes allowed a high total soluble protein content in great brome leaves (e.g. 12.7 ng g^{-1} fresh leaves in silty clay sand substrate), close or higher to that of highly allelopathic genotypes (e.g. 13.0 ng g^{-1} fresh leaves for 'Arbi' or 11.6 ng g^{-1} for 'Ardhaoui' in silty clay sand substrate).

Discussion

Effect of the barley root allelochemicals on great brome growth

The growth of great brome plants in substrates containing the root exudates of six barley genotypes was significantly reduced after 30 days of culture (Fig. 2). The inhibitory effect was more pronounced on root and shoot length compared to root and shoot dry weight, suggesting that these two first traits are the best variables to assess the allelopathic potential of barley against great brome. Our previous investigations showed that great brome root is the primary target of barley allelochemicals at 5 and 10 days of growth (Bouhaouel et al. 2015, 2016). In this study, the rates of inhibition of root and shoot parts of the weed, however, were very similar. This result suggests that the aerial part is also sensitive to barley allelochemicals after 30 days of growth.

Until now, a few allelochemicals (~12 compounds) have been identified in barley root exudates (Kremer and Ben-Hammouda 2009), most of them alkaloids and phenolic acids. Liu and Lovett (1993) identified two species-specific alkaloids from root exudates, hordenine and gramine, the first allelochemicals proposed to explain the allelopathic effects of barley. Later, Baghestani et al. (1999) proposed two phenolic acids (*o*-coumaric acid, vanillic acid) and one phenylpropanoid derivative (scopoletin) as indicators of the allelopathic effectiveness of barley root exudates. These compounds might contribute to the observed effects, but further investigations are needed to support this hypothesis.

Great brome responded differentially to the barley genotypes (Table 1; Fig. 2) and this might be explained by variations in the profiles and quantities of produced allelochemicals. Variation in the allelopathic barley activity is in accordance with previous reports (Baghestani et al. 1999; Bertholdsson 2004; Bouhaouel et al. 2015, 2016; Oveisi et al. 2008). In general, barley landraces (‘Saudi’, ‘Arbi’ and ‘Ardhaoui’) showed a better capacity to inhibit growth of the weed species, as compared to modern varieties (‘Manel’, ‘Tej’ and ‘Rihane’) (Fig. 2). This finding support the view that barley or wheat landraces, although less productive, are better adapted to environmental stress than modern cultivars (El Felah et al. 1991). This performance may be due to their population genetic structure, buffering capacity, and a combination of morpho-physiological traits (Jaradat 2013). This result might also indicate a depressive effect of the allelopathic activity with the introduction of new varieties, but further work is needed to confirm this hypothesis, using a large number of genotypes. Interestingly, the newly introduced landrace ‘Saudi’ which is the most toxic genotype against great brome (Fig. 2) and is also salt-tolerant (Hammami et al. 2016) could be useful in future breeding programs of barley cultivated in Tunisia. This genotype might be also recommended for small farmers in Tunisian marginal environments (e.g. semi-arid and arid regions) that still cultivate landraces (El Felah 2011; El Gharbi and Felah 2013).

Effect of the activated charcoal on great brome growth

The allelopathic effect of the six barley genotypes depended on the presence of activated charcoal (Tables 1, 2). The activated charcoal seemed to decrease the allelopathic effect of barley against the great brome at the morphological (Fig. 1), physiological and biochemical (Table 2) levels. The activated charcoal is frequently used in the allelopathic interactions studies with the aim of altering the chemical composition of the rhizosphere of some plants and recommended as an effective approach in such studies. This material was assumed to adsorb organic molecules with low affinity for inorganic nutrients (Nilsson 1994; Ridenour and Callaway 2001; Hierro and Callaway 2003; Semchenko et al. 2007; Gómez-Aparicio and Canham 2008; Morvillo et al. 2011). However, its use has been recently criticized based on a few side effects, specially the availability of some nutrients (e.g. nitrogen, phosphate) (Lau et al. 2008; Weißhuhn and Prati 2009). Morvillo et al. (2011) demonstrated that the activated charcoal has no effect on soybean biomass and yield and sweet wormwood (*Artemisia annua* L.) biomass. Wurst and Van Beersum (2009) found, however, a negative impact of activated charcoal on the growth and flowering of some legumes. Meanwhile, Wurst et al. (2010) found that the addition of the activated charcoal had not improved the availability of nutrients for plants, but reduced the growth of *Lupinus polyphyllus* Lindl. and *Plantago lanceolata* L. and the mycorrhiza rate, regardless of the presence of competitive species. Therefore, the effect of the addition of activated charcoal seems to depend on its quantity and its quality in addition to environmental conditions and to the tested species. In our conditions, the addition of this substance produced a weak, non-significant stimulatory effect on barley growth, compared to the control for both types of substrates (Fig. 1). The decline of the inhibitory activity of barley can be explained by the adsorption of the growth inhibitory molecules.

Differences in allelopathic activity of barley according to soil type

The soil texture showed also a significant influence on the allelopathic activity of Tunisian barley. The inhibitory action of barley roots was more pronounced in the presence of sandy substrate. Similar finding was also reported by Shaukat et al. (2003) where the inhibitory activity of shoot aqueous extracts of *Conyza canadensis* L. was higher in sandy soils. In fact, clay or organic matter content allows phenolic acid adsorption (Cecchi et al. 2004; Tharayil et al. 2006). On the other hand, the nutrients deficiency that characterizes the sandy substrates has been proposed to increase the allelopathic activity of plants (Inderjit and Asakawa 2001). The results showed that expression of that potential may depend on the species or genotype, but could also be affected by several factors, including the physicochemical properties of the soil (pH, percentage of organic matter, availability of some

nutrients, etc.). Therefore, assessing the allelopathic potential of plant roots needs to be performed in several environmental contexts.

Chlorophyll content, chlorophyll *a* fluorescence or total soluble protein content: which is the best indicator of barley allelopathic activity?

The present research showed that the Chl content in great brome leaves was affected by the allelopathic activity of barley. As suggested by Yang et al. (2002), allelochemicals (e.g. *o*-hydroxyphenyl acetic, ferulic and *p*-coumaric acids) can reduce Chl accumulation in three ways: by inhibiting the biosynthesis of Chl, stimulating the degradation of Chl or by both processes. In fact, it has been reported that some allelochemicals can interfere with the synthesis of the porphyrin, a precursor for the Chl synthesis (Rice 1984). Later, Yang et al. (2004) showed that three allelochemicals (*o*-hydroxyphenyl acetic, ferulic and *p*-coumaric acids) have increased the activities of chlorophyllase and Mg-dechelatase, enzymes responsible for the Chl degradation pathway.

The reduction of Chl content is expected to decrease the photosynthesis efficiency (Hu et al. 2013). The maximum quantum yield (F_v/F_m) and the initial fluorescence (F_0) that reflect the photochemical efficiency of photosystem II (Maxwell and Johnson 2000), showed respectively a decrease and an increase as compared to the control. Declining values of F_v/F_m are usually associated with increases of F_0 values (Lindqvista and Bornman 2002), which often indicate a damage of the reaction centers embedded in the thylakoid membranes, especially those of PSII, and to the inhibition of resonance energy transfer from molecules antenna to the reaction center (Krause and Weis 1984).

The Chl content and F_v/F_m were significantly correlated with the inhibitory action of barley roots on the great brome growth for most of the treatments (i.e. type of substrate and activated charcoal; Figs. 3, 4). Similar patterns were also observed for F_0 (Fig. 5), but the number of correlations was much lower compared to Chl content and F_v/F_m. Previous reports showed that F_v/F_m was specifically highly correlated with several stresses including low temperatures (Artus et al. 1996; Baker and Rosenqvist 2004; Mishra et al. 2011), salt (Zahra et al. 2014) or water stress (Faraloni et al. 2011). Hussain et al. (2011) reported that F_0 was less affected by the exogenous application of benzoxazolin-2(3H)-one (BOA), as compared to F_v/F_m.

The allelopathic activity of barley roots seems to reduce protein biosynthesis in great brome leaves and / or to stimulate protein degradation (Table 2). Several studies showed the effect of allelochemicals (e.g. cinnamic acid and benzoxazolin-2 (3H) –one) on protein production in plant species (e.g. *Dactylis glomerata*, *Lactuca sativa*,

360 *Lolium perenne, Phaseolus vulgaris, Zea mays*) other than barley (Hussain and Reigosa 2011; Hussain et al.
361 2011; Romero-Romero et al. 2002; Singh et al. 2009). More specifically, Baziramakenga et al. (1997) reported
362 that the exogenous application of phenolic acids reduced the incorporation of some amino acids into proteins and
363 the rate of protein synthesis. For example, Mersie and Singh (1993) have shown that ferulic acid reduced by 50%
364 the incorporation of leucine [^{14}C] at a concentration of 1.0 µM after 60 min of incubation.
365 The total soluble protein concentration was not significantly correlated with the inhibitory action of barley root
366 exudates, whatever was the type of substrate (Fig. 6). Taken together, these results suggest that root exudates
367 have an effect on protein homeostasis and on growth traits of the receiver plant, but that different genotypes
368 seem to act on both sets of traits in a distinctive way. It would be interesting to compare the allelochemical
369 compounds produced by the different genotypes and to better understand their modes of action, on protein
370 synthesis and/or growth related-traits.
371 Overall, this study showed that non-destructive techniques of foliar diagnosis focusing on the determination of
372 the Chl content and chlorophyll *a* fluorescence, particularly F_v/F_m might be considered as promising tools for the
373 rapid assessment of plant response to the allelopathic stress.
374

375 **Conclusions**

376 The present investigation highlights the allelopathic effects of barley on great brome *via* root exudates. The
377 allelopathic relationships between plants are obviously complex since they depend on interacting factors,
378 including genotype, type of soil and their interaction. The barley roots seem to release allelochemicals that affect
379 the light-capturing processes of photosynthesis, and protein homeostasis of receiver plant. Such physiological
380 and biochemical disturbances result in reduced growth of leaves and roots with less plant biomass. The Chl
381 content and F_v/F_m seem to be useful criterions to assess the allelopathic stress in plants. Further field studies of
382 the interactions between barley root allelochemicals with soil microorganisms and minerals could provide
383 pertinent informations to understand the allelopathic phenomenon in natural environments and in its usefulness
384 in weed biological control.
385

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575

576 **Fig. 1** Effect of the addition of activated charcoal (AC) on root (**a**) and shoot (**b**) length, and root (**c**) and shoot
577 (**d**) dry weight of 'Ardhaoui' plants grown in two types of substrates. Graph bars (mean of five replicates ± SE)
578 with the same letter are not significantly different ($P < 0.05$; LSD test). SS, sandy substrate; SS+AC, sandy
579 substrate with activated charcoal; SCSS, silty clay sand substrate; SCSS+AC, silty clay sand substrate with
580 activated charcoal

581 **Fig. 2** Inhibition rate of root (**a**) and shoot (**b**) length, and root (**c**) and shoot (**d**) dry weight of great brome plants
582 after 30 days, grown in two types of substrates in the presence or absence of activated charcoal (AC) and
583 exposed to allelochemicals of six barley genotypes. Graph bars (mean of five replicates ± SE) with the same
584 letter are not significantly different ($P < 0.05$; LSD test) according to the three factors simultaneously. Since
585 interaction is not significant between these factors for shoot dry weight parameter, the LSD test was conducted
586 for each type of substrate showing difference between the six genotypes. SS, sandy substrate; SS+AC, sandy
587 substrate with activated charcoal; SCSS, silty clay sand substrate; SCSS+AC, silty clay sand substrate with
588 activated charcoal

589 **Fig. 3** Relationship between the reduction rate of Chl content (SPAD value) ($n = 15$) and the inhibition rate of
590 root (**a**) and shoot (**b**) length, and root (**c**) and shoot (**d**) dry weight of great brome plants ($n = 50$). Each point
591 represents the average value for one genotype grown in sandy substrate (SS; black filled symbols), sandy
592 substrate with activated charcoal (SS+AC; grey filled symbols), silty clay sand substrate (SCSS; black hollow
593 symbols) and silty clay sand substrate with activated charcoal (SCSS+AC; grey hollow symbols). The
594 coefficients of regression (R^2) are given and followed by the level of significance: $^{ns}P > 0.05$, $^{*}P < 0.05$, $^{**}P <$
595 0.01, $^{***}P < 0.001$

596 **Fig. 4** Relationship between the reduction rate of F_v/F_m ($n = 15$) and the reduction rate of root (**a**) and shoot (**b**)
597 length, and root (**c**) and shoot (**d**) dry weight of great brome plants ($n = 50$). Each point represents the average
598 value for one genotype grown in sandy substrate (SS; black filled symbols), sandy substrate with activated
599 charcoal (SS+AC; grey filled symbols), silty clay sand substrate (SCSS; black hollow symbols) and silty clay
600 sand substrate with activated charcoal (SCSS+AC; grey hollow symbols). The coefficients of regression (R^2) are
601 given and followed by the level of significance: $^{ns}P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

602 **Fig. 5** Relationship between the increase rate of F_0 ($n = 15$) and the reduction rate of root (**a**) and shoot (**b**)
603 length, and root (**c**) and shoot (**d**) dry weight of great brome plants ($n = 50$). Each point represents the average
604 value for one genotype grown in sandy substrate (SS; black filled symbols), sandy substrate with activated
605 charcoal (SS+AC; grey filled symbols), silty clay sand substrate (SCSS; black hollow symbols) and silty clay

606 sand substrate with activated charcoal (SCSS+AC; grey hollow symbols). The coefficients of regression (R^2) are
607 given and followed by the level of significance: $^{ns}P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$
608 **Fig. 6** Relationship between the reduction rate of total soluble protein concentration (n = 15) and the reduction
609 rate of root (**a**) and shoot (**b**) length, and root (**c**) and shoot (**d**) dry weight of great brome plants (n = 50). Each
610 point represents the average value for one genotype grown in sandy substrate (SS; black filled symbols), sandy
611 substrate with activated charcoal (SS+AC; grey filled symbols), silty clay sand substrate (SCSS; black hollow
612 symbols) and silty clay sand substrate with activated charcoal (SCSS+AC; grey hollow symbols). The
613 coefficients of regression (R^2) are given and followed by the level of significance: $^{ns}P > 0.05$, $^{*}P < 0.05$, $^{**}P <$
614 0.01, $^{***}P < 0.001$
615 **Table 1** Analysis of variance (ANOVA) for the inhibition rate of four morphological parameters in great brome
616 plants
617 **Table 2** Reduction rate of Chl content, F_v/F_m and total soluble protein content (%), and increase of F_0 (%) in
618 great brome plants after 30 days of exposure to allelochemicals of six barley genotypes. The associated
619 probabilities level calculated through the analysis of variance (ANOVA) is shown for genotype (G), substrate
620 (S), activated charcoal treatment (AC) and interaction (G x S), (G x AC) (S x AC) and (G x S x AC) effects