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Plant responses to wide-range polarity extracts from invasive *Acacia dealbata* Link

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ABSTRACT

We studied the *A. dealbata* extracts exert allelopathic effects on the germination and growth of *Lactuca sativa* L., *Dactylis glomerata* L. and the native shrub *Cytisus scoparius* (L.) Link. using realistic phytochemical concentrations. Oxidative stress parameters on *C. scoparius* were measured. Chemical compounds from flowers, roots and soils were sequentially extracted using solvents with increasing polarity: dichloromethane (DCM), acetone, methanol and water. DCM and acetone fractions affected germination and radicle length of the tested species. Non polar fractions affected the H_2O_2 levels, protein and malondialdehyde content but not the enzymatic activities from the oxidative metabolism. Soil and root extracts were highly bioactive than extracts from flowers (commonly considered as most bioactive material). This study showed that direct toxicity under natural conditions, may have minor relevance because of the main role of phytochemicals concentration. Our findings also suggested that responses were species-dependent and oxidative stress did not affect the early growth of *C. scoparius*.

Keywords: Allelopathic effects, antioxidant enzymes, *Cytisus scoparius, Dactylis glomerata, Lactuca sativa,* lettuce, orchard grass, common broom oxidative stress, plant invasion, soil extracts.

INTRODUCTION

In new habitat, non-native plants can become invasive by altering the ecosystem functions with substantial economic and ecological costs (43,44,56). The release of chemical compounds by plants, which influence the surrounding organisms (allelopathy), is used by non-native plants to be successful in competition with native plants (7).

Acacia dealbata Link is problematic invasive specie in Mediterranean Europe (1,21,23,30,34, 37,45,46,51,52). Allelopathy, mainly during the flowering period, has been suggested as powerful tool for its invasiveness (13,35,36,37). We hypothesized that the invaded soil, roots and flowers of *A. dealbata* possess allelopathic potential, influencing the germination, growth and oxidative metabolism of native species To evaluate these hypotheses, our experimental work was divided in 3-parts: (i) Evaluation of the Bioactivity of *A. dealbata* flowers, roots and soil extracts of different polarities using bioassay-guided procedure, (ii) Bioactivity of extracts at various concentrations were tested on *Lactuca sativa* L. and *Dactylis glomerata* L. were used as test species and (iii) Determining the biochemical mechanisms of extracts to damage the native shrub *Cytisus scoparius* (L.) Link.

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MATERIAL AND METHODS

Soil and plant material sampling

In March 2012, soil and plant material were collected from three patches (10 x 10 m) (Fig. 1) from invaded shrubland in O Ribeiro region [42°18′18″N, 8°10′18″ W 300 msl; Mediterranean sub-humid type climate, mean annual temperature 6.7 to 18°C (12)] Galicia, NW Spain. Soils were acidic (pH 4-6), rich in organic matter (8-16%) and classified as umbrisols (50). The shrublands were dominated by *Ulex europaeus* L, *Pterospartum tridentatum* (L.) Willk, *Arbutus unedo* L., *Cytisus scoparius* (L.) Link, *C. multiflorus* (L´Hér.) Sweet genus *Cistus*, *Erica* and *Genista* spp.

Figure 1. On the left, Agroforestry path completely occupied by *Acacia dealbata* due to plants growth, a common view in invaded areas across the NW Iberian Peninsula. On the right, detail of *A. dealbata* composed leave and flowers that produce large number of pods during the spring (photos by Souza-Alonso, P.)

Sampling was done during the flowering of *A. dealbata*, as the allelochemicals are mainly produced in flowers (13,36). At each invaded patch, 3-materials (soil, roots and flowers) were collected. The soil underneath 10 mature plants and within 10 cms around the live roots (up to 20 cm deep) was collected using hand shovel. Living roots (0.2-0.5 cm dia) were collected within 30 cms radius around the 10 mature *A. dealbata* plants. Flowers from apical inflorescences of 10 mature plants were also collected. Soil (500 g), roots (250 g) and flowers (250 g) were separately stored in plastic bags and transported to laboratory for further processing. Total amounts of collected soil and plant material were in same proportions as present under field conditions, e.g. the 500 g soil quantity represents approximately 1 dm^3, volume of soil that contains several seeds.

Extraction

In laboratory, soil was sieved through a 2-mm mesh to eliminate coarse roots, debris and small stones. Soil adhered to the roots was gently removed and roots were cleaned and cut into small pieces (< 1 cm). Extraction was done as per slightly modified protocol of Ens *et al*. (16). Soil, roots and flowers were separately kept in 2 L Erlenmeyer flasks and solvents of increasing polarity were sequentially added. Hence, we made four sequences of extraction in 30 h-cycles with 2 independent factors (Solvents and Natural materials). There were 12 treatments: 4-Solvents [Dichloromethane (DCM), acetone, methanol and water] and 3-Materials (soil, roots and flowers). Initially, we added 1 L of DCM (HPLC grade) in each Erlenmeyer flask, after 30 h DCM was collected and stored. Residual DCM, in the plant material, was allowed to evaporate until acetone (1 L) was added. After 30 h acetone was collected and stored. Residual acetone was allowed to evaporate until methanol was added. After 30 h methanol was collected and stored. Residual methanol was allowed to evaporate until water, the last solvent, was added, collected and stored. Solutions were filtered twice through filter papers using the vacuum pump (RV12, Edwards) until no particles remained and the liquid was totally clear. Filtered solutions were then concentrated using rotary evaporator (temperature < 40°C; Büchi RE 121, Rotary Evaporator). The dry residue, hereafter considered as the *Extract*, was then weighed and results were referred to the weight of original material kept in the Erlenmeyer (w/w; weight of dry residue/weight of original material used, in %) as described in Ens *et al*. (16).

A previous extraction with DCM and water of *A. dealbata* soils was identically done to determine the soil chemical concentration under natural conditions. Ten soil samples from invaded patches were collected, processed and extracted as described above. The weight of the final crude extract varied between 0.25-0.3% (w/w, weight of dry residue/weight of original soil used). Therefore, we assumed that this range includes the maximum chemical concentration in the invaded soil and then it was selected as the maximum reference value of concentration. To distinguish between the allelopathic or phytotoxic effects, we followed the criteria of Ens *et al*. (16). Evidence for allelopathy needs significant effects of soil extracts accompanied with significant effects produced by roots or flowers from the same fraction. In case of significant effects produced by roots or flowers extracts, but without significance in their soil equivalents, these results were considered as phytotoxicity. If the effects were exclusively produced by soil extracts, the effects will be classified as indirect soil effects.

A. dealbata extracts Bioassays

Preliminary assays

(i). *L. sativa*: We did preliminary Petri plate assay to know the presence of phytotoxic effects at the maximum concentration obtained. Two independent factors were used: Solvents (Dichloromethane (DCM), acetone, methanol and water) and Natural materials (soil, roots and flowers). The extracts pH was measured using pH meter (MicropH 2000, Crisom). We used plastic dishes (3.5 cm dia) lined with 1 filter paper Whatman 3 MM. Dichloromethane and acetone extracts (both dissolved in DCM) were applied, allowing the organic solvent to evaporate for 1 h (16), then 0.7 ml distilled water was added. Methanol and water extracts (both dissolved in water) were applied in similar manner. Ten sterilised (1% sodium hypochlorite, 5 min) seeds of *L. sativa var.* Trocadero were kept equidistant in each plastic dish. Adequate controls using distilled water (0.7 ml) and *L. sativa* were also included.

Additionally, six replicates with distilled water, in which DCM (previously applied and evaporated), were also included to test whether DCM had masking effects on the germination and seedling growth (16). Petri plates were then sealed with parafilm to prevent evaporation and kept at 27 ºC (± 1°C) in dark. After 3-days, germination (G_t) was recorded by counting all seeds that had radicle > 1mm and radicle length (R_L) was measured using a Ruler (mm).

(ii). *D. glomerata*: As per the results of preliminary assay, we included the decreasing range of concentrations that were applied to *Dactylis glomerata*. There were 30 treatments including 3 factors: solvents (DCM, acetone), concentrations (0, 0.01, 0.05, 0.15 and 0.3) and natural materials (soil, roots and flowers). The experimental procedure with *D. glomerata* was same as described before for *L. sativa*. Here, the petri dishes were kept at 20 ºC (± 1°C) in dark. After 10 days, germination (G_t) was recorded counting all those seeds that had a radicle greater than 1mm and radicle length (R_L) was measured using a Ruler (mm).

(iii). *C. scoparius* : We investigated the potential allelopathic impact of *A. dealbata* in Petri plates (3.5 dia) bioassay on the germination, growth and oxidative stress of pioneer and competitive shrub *C. scoparius*. Due to the identified bioactivity, only non-polar extracts from each material were selected. There were 6 treatments: 2 solvents (DCM, acetone) and 3 natural materials (soil, roots and flowers) replicated five times. The plastic dishes were lined with 1 filter paper). Extracts were applied at maximum concentration (0.3%). Ten sterilized seeds of *C. scoparius* (5 min in 5% hypochlorite solution and rinsed abundantly with distilled water) were kept equidistant in each plastic dish and 0.7 ml distilled water was added. Plates were sealed with parafilm and placed in growth chamber at 20 (±1 °C) in dark. In control plates, only distilled water was used.

Germination and biometric measurements:

(i) Germination: Germination was recorded daily. After 15 days, total germination (G_t) was recorded counting all seeds with radicle > 1 mm. Three germination indices: (Speed of germination (S), Speed of accumulated germination (AS) and Coefficient of rate of germination (CRG) were calculated (14, 25). At the end of experiment hypocotyl and radicle length were measured and fresh (FW) and dry weight (DW) were recorded (For dry weight the seedlings were dried in oven at 70 °C for 72 h).

(ii) Biochemical analyses: Four replicates were used for each biochemical parameter. Five days after incubation, hydrogen peroxide (H_2O_2) levels, lipid peroxidation, soluble protein content and antioxidant enzymatic activities were measured in germinated seeds. Hydrogen peroxide (H_2O_2) content was measured as per Aroca *et al*. (4). Lipid peroxidation was determined by estimating the malondialdehyde (MDA) content using the method of Hodges *et al*. (24). Total protein content was determined according to Bradford (5) and superoxide dismutase (SOD) and peroxidase (POX) activities were measured following the methods of Upadhyaya *et al*. (54) and Beauchamp and Fridovich (10), respectively.

Statistical analyses

Data normality and homoscedasticity were checked using the Kolmogorov-Smirnov test (K-S test) and Levene´s test, respectively, prior to statistical analyses. Pairwise comparisons using Student t-test were carried out to check for differences in pH, G_t and R_L between control and *A. dealbata* extracts on *L. sativa* and *D. glomerata*. In case of normality assumptions were not satisfied, pairwise comparisons were carried out using non-parametric test (Whitney's U test) and significant differences were inferred from the 0.05% probability level. Biometrical and biochemical responses of *C. scoparius* were analysed using the Student t-test (significant differences also inferred from the 0.05% probability level). Additionally, Pearson's correlation was carried out to assess the linear relationship between measured variables. All statistical analyses were done using the IBM SPSS Statistics 20.0 software package (IBM SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

L. sativa and *D. glomerata*

Our results indicated that the pH of *A. dealbata* extracts was lower than water controls ($p \leq 0.001$), especially the DCM fraction (Table 1). The influence of pH in bioassays is critical because inadequate pH causes abiotic stress in plants (42). In our assay, the reduction in pH was according to previous reports (36). Nevertheless, the pH is unlikely to be responsible for the phytotoxic effects due to the absence of significant correlation with biometrical and biochemical parameters. Moreover, pH was consistently reduced in DCM and acetone fractions, but physiological and biochemical effects were highly variable.

Table 1. Mean (±SD) of pH values (n=3) of the *A. dealbata* extracts from different natural material and extractant solvent.

		DCM	**Acetone**	**Methanol**	**Water**
Extract	Soil	5.12 (±0.04)***	5.44 (±0.04)***	5.20 (±0.03)***	5.35 (±0.02)***
	Root	4.95 (±0.03)***	5.34 (±0.03)***	5.04 (±0.02)***	5.26 (±0.02)***
	Flowers	5.15 (±0.04)***	5.92 (±0.06)***	5.80 (±0.03)***	5.54 (±0.01)***

Asterisks: Statistically significant with water control (pH=6.16±0.03) at $p \leq 0.05$ (*****), $p \leq 0.01$ (******) and $p \leq 0.001$ (*******) significance level**.**

In preliminary assay, DCM and acetone fractions significantly decreased the germination of *L. sativa* (Fig. 2). DCM extracts reduced G_t by 30 %, whereas the reduction with acetone fraction was also noticeable. On the contrary, methanol extracts had no significant effects, while water extract of roots were slightly stimulatory to G_t. Roots and flower extracts in DCM also inhibited R_L (Fig. 2). Despite the reduction trend, the acetone and methanol extracts of *A. dealbata* had no significant effects on R_L. The polar fraction of *A. dealbata* aerial parts was previously tested on *L. sativa* (36,37,38). Recently, non-volatile organic compounds possibly responsible for the phytotoxic activity of *A. dealbata* have been identified (1). These polar extracts from the aerial parts, did not affect the G_t of *L. sativa*, but had negative effects on the seedling growth (1). We found significant effects of soil and root extracts on G_t, which suggest that, hypothetically, molecules released from roots into the soil affects the seed germination and seedling development. The inclusion of soil extracts proved useful tool to distinguish between the allelopathic and phytotoxic effects caused by molecules that are not naturally leached or released.

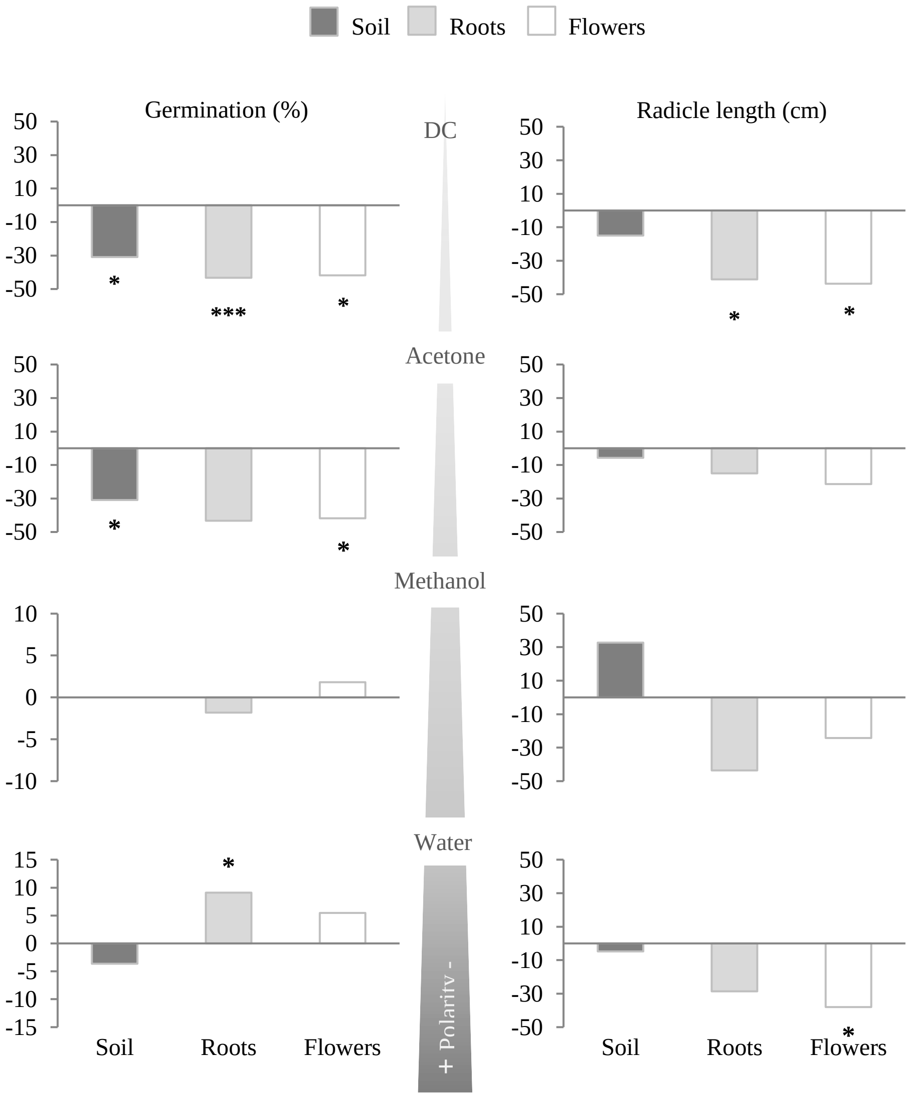


Figure 2. Effects of *Acacia dealbata* extracts from different natural material (soils, roots and flowers) and solvent extractant (DCM, acetone Methanol and water) on germination and radicle length of *L. sativa* seeds and seedlings. Values are in comparison to control (%).
Negative values: *y* axis indicate physiological reduction and Positive values: *y* axis indicate physiological stimulation than control. Asterisks: Statistical significance at $p \leq 0.05$ (*****), $p \leq 0.01$ (******) and $p \leq 0.001$ (*******) level.

Based on the protocol developed by Ens *et al*. (16), roots and flowers extracts in DCM and flowers extracts in acetone, exhibited allelopathic effects on the G_t of *L. sativa*, as suggested by the inhibition of comparable soil extracts (Fig. 2). The effects of root aqueous extract, was classified as phytoactive (stimulatory effect), since a comparable effect was not evident in soil extracts. The effects of flowers on the R_L of *L. sativa* were classified as phytotoxic, as the comparable soil extract did not exert significant influence. In R_L, the absence of related soil effects suggests that compounds affecting the root elongation might be present in the plant material, but probably they are released in small quantities or rapidly degraded in the surrounding soil -usually by microbial communities in the rhizosphere (28). In our study, the Non-polar extracts were inhibitory than polar fractions (Fig. 2). These results also showed that the inhibitory potential of *A. dealbata* extracts is only in non-polar fractions (16,38). Non-polar extracts (DCM) contains several bioactive compounds inhibitory to growth of neighbouring plant species (6,8), affecting R_L rather than G_t (57). Following this protocol, the presence of allelopathic molecules can be related to non-polar extracts, mainly to DCM, but also to the acetone extract.

Table 2. Effects of *Acacia dealbata* non-polar extracts concentrations on germination and early growth of *Dactylis glomerata*.

Solvent	**Material**	***Acacia dealbata* non-polar extracts concs (%)**			
		0.3%	**0.15%**	**0.05%**	**0.01%**
Gt					
DCM	Soil				
	Roots				
	Flowers				
	Soil				
Acetone	Roots				
	Flowers				
	Soil				
DCM	Roots			+	
Root length					
Acetone	Flowers	+	+		
	Soil	+			
	Roots	+	+	+	+
	Flowers				
DCM	Soil		+	+	
	Roots	+	+		
	Flowers	+	+		+
Shoot length					
Acetone	Roots	+	+	+	
	Flowers	+			
	Soil				
DCM	Roots			+	
	Flowers				
Biomass					
Acetone	Soil				
	Roots			+	
	Flowers				

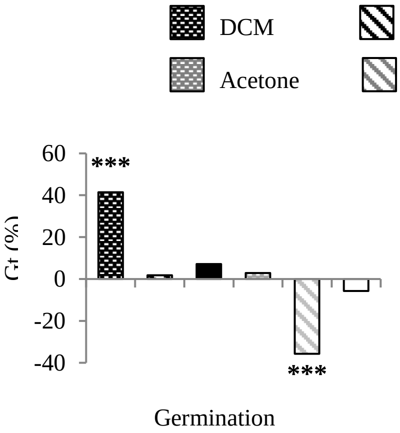
(+): Significant increase in specific parameter with respect to control, (-): Significant decrease at $p \leq 0.05$ significance level.

Based on the effects observed on *L. sativa* seeds, a whole range of concentrations (0, 0.01, 0.05, 0.15 and 0.3) were tested on *D. glomerata.* The application of non-polar extracts enhanced the biometrical parameters of *D. glomerata* (Table 2). The application of *Acacia* extracts did not affect the Total biomass and Gt, however, enhanced the biometrical parameters (root and shoot length) than control. These results are in agreement with the general observation that biological processes are stimulated at low allelochemical concentrations and inhibited as the concentration increases (33). At the same time, it reinforces the methodology used showing that the use of realistic concentrations in bioassays does not usually lead in toxic results. Furthermore, enhanced shoot and root growth were dependent on the concentration, material and solvent used. Previously, irregular responses to *A. dealbata* extracts were reported in comparing the different bioassay methodologies (38) or when the effects under natural conditions were evaluated (39). Regardless of the allelochemical concentration, the polarity was found responsible for the observed effects. However, the effects produced by the non-polar fraction of *A. dealbata* remained less studied. Structurally, the DCM extract of *A. dealbata* bark contains cinnamic acids esters, mainly aliphatic long-chain n-alkyl caffeates, together with other p-hydroxycinnamic acid esters (17), but their possible phytochemical effects are still unexplored.

Finally, different responses observed in biometrical results can be related to the different species tested. The use of *L. sativa,* alone or in combination with other species, is very common to develop bioassay-guided fractionation methods (3,29,38,40). Nevertheless, the use of sensitive species can overestimate the phytotoxic effects of extracts. Therefore, the inclusion of the allelopathic *C. scoparius* a strong competitor of *A. dealbata*, which occupies similar ecological niches (22), could provide realistic information regarding the effects of plant-plant chemical interactions.

C. scoparius

After 15 days, contrasting effects were observed in G_t of *C. scoparius* (Fig. 3). Soil DCM extract significantly increased the G_t (41 %; $p \leq 0.001$), whereas, acetone root extract was inhibitory (35 %; $p \leq 0.001$). Soil DCM extracts were very stimulatory to S (129 %; $p \leq 0.01$) and AS indices (304 %; $p \leq 0.001$), was the acetone soil extract also stimulatory to S (80 %; $p \leq 0.01$) and AS (164 %; $p \leq 0.001$). Despite the recent evidenced negative effects on Gt and physiological parameters after the exposure to *A. dealbata* leachates (36), we applied extracts at concentrations similar to those found in invaded soils, produced opposite effects. The increase in Gt, S and AS was evident in soil DCM extracts (Fig. 3), suggesting hastening in seed emergence. The S index is sensitive indicator of allelopathic effects (2), providing complementary information to G_t. In our case, S and AS provided parallel information and due to the significant increase in both indices, mainly in soil extracts, a probable effect of indirect allelopathy can be suggested (Table 3). Therefore, soil extracts from DCM increased the number of germinated seeds and shortened the germination period. The establishment of plant species is dependent on the timing of competitive interactions but the ecophysiological consequences of the altered germination can be largely unpredictable. Asymmetric competition resulting from the early or late germination may benefit the plant recruitment. However, early or late germination comes at a cost, increasing the exposure risk to unfavourable conditions (20,32).



	DCM			Acetone		
	Soil	Root	Flower	Soil	Root	Flower
S	+ +	n.s.	n.s.	+	n.s.	n.s.
AS	+ + +	n.s.	+ +	+ + +	n.s.	n.s.
CRG	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

Germination

Figure 3. Effects of *Acacia dealbata* extracts from different natural material (soils, roots and flowers) and solvent extractant (DCM, acetone Methanol and water) on germination on Total germination (left) and germination indices (right) of *Cytisus scoparius* seeds. Values are in comparison to control (%). Negative values: *y* axis indicate significant reduction and Positive values: *y* axis indicate significant stimulation than control. Positive symbols (+): Statistical significance at $p \leq 0.05$ (+), $p \leq 0.01$ (++) and $p \leq 0.001$ (+++) level. Asterisks: Statistical significance at $p \leq 0.05$ (*****), $p \leq 0.01$ (******) and $p \leq 0.001$ (*******) level.

The DCM and acetone extracts invariably increased the root and hypocotyl length (Fig. 4). Radicle length of *C. scoparius* were generally enhanced (25-90 %); however, the significant increase was only in soil DCM extracts. Hypocotyl length was increased (10-60 %), the soil and root DCM fractions were also stimulatory ($p \leq 0.001$ and $p \leq 0.05$, respectively). Seedling biomass and the FW/DW ratio were not affected (data not shown).

As far as we know, the oxidative damage of *A. dealbata* extracts on seedlings has never been tested. In this sense, the measurement of oxidative stress parameters (15,41,48) combined with physiological processes (26,36) offers a better understanding of the phytotoxic process. At the end of the assay, soluble protein content in *C. scoparius* seedlings was considerably enhanced (Fig. 5). The DCM and acetone fractions increased the protein content > 40%, almost 60% in soils (57 %, $p \leq 0.001$) and roots (56 %, $p \leq 0.001$). In general, protein metabolism is negatively affected by the exposure to allelochemicals (9,49); however, at certain levels, some allelochemicals stimulates the protein synthesis (27,47,53). In this case, the increase in protein content suggests an enhancement in metabolic pathways leading to protein synthesis; apparently, *C. scoparius* seedlings are mobilizing resources for radicle and hypocotyl elongation instead of reducing oxidative stress. Malondialdehyde content was reduced in acetone soil extracts (70%, $p \leq 0.05$). Low levels of MDA than controls suggest the absence of membrane damage. The impermeable coat layer of *C. scoparius*, together with the antioxidant capacity due to high content of phenolic compounds (19), may play an important role in seed protection against oxidative damage.

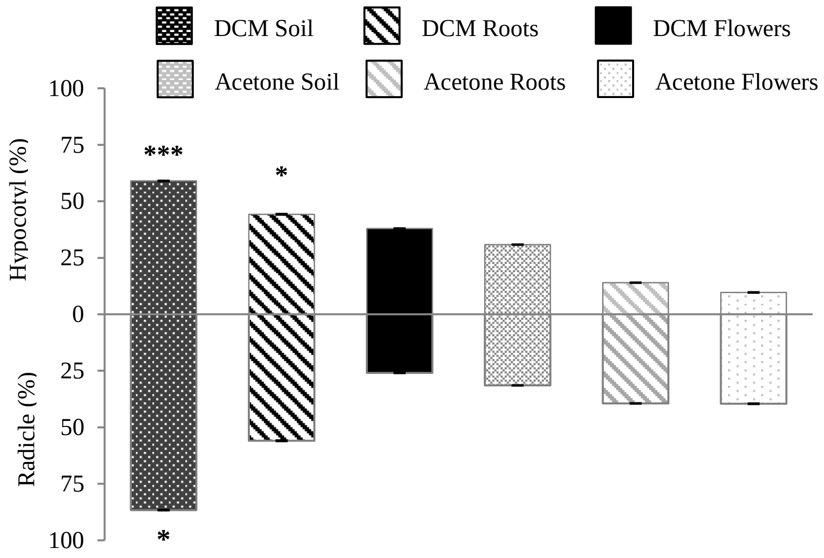


Figure 4. Effects of *Acacia dealbata* extracts from different natural material (soils, roots and flowers) and solvent extractant (DCM, acetone Methanol and water) on radicle and hypocotyl length of *Cytisus scoparius* seedlings.
Values are in comparison to control (%). Asterisks: Statistical significance at $p \leq 0.05$ (*), $p \leq 0.01$ (**) and $p \leq 0.001$ (***) level.

Hydrogen peroxide content was significantly increased after the exposure to soil and roots extracts from the DCM fraction, whereas acetone extracts showed no significant effects. Antioxidant enzymatic activities showed variable results after the seedling exposure to *A. dealbata* extracts (Fig. 6). Acetone soil extracts enhanced the POX values than control (91%; $p \leq 0.05$) and SOD activity was generally enhanced (26%; $p \leq 0.05$) by the DCM extracts of flowers. A general increase in SOD activity is the initial defence against O_2^- and its transformation enhances the H_2O_2 content. Therefore, we expected higher levels of SOD, particularly in the DCM fraction. Afterward, POX metabolizes H_2O_2, into H_2O to avoid the cell damage; however, POX and SOD levels were not correlated with H_2O_2. Regardless of the increase in H_2O_2 produced by DCM extracts, the general absence of differences in POX level was an indicator of allelopathic stress (15,48), suggests that seedlings were not suffering oxidative stress.

The heterogeneous trend of results observed (Table 3) might be related to the different extract composition, which depends on the nature of plant material and solvent polarity. With different solvents and polarities, the chemical nature of the extracts is highly variable (16). Soil, roots and flowers, either dissolved in DCM or in acetone, differed qualitatively and quantitatively in their chemical composition since the observed effects were highly variable. These differences were clearly reflected in the inconsistent effects observed on *C. scoparius* seedlings after the exposure to *A. dealbata* extracts.

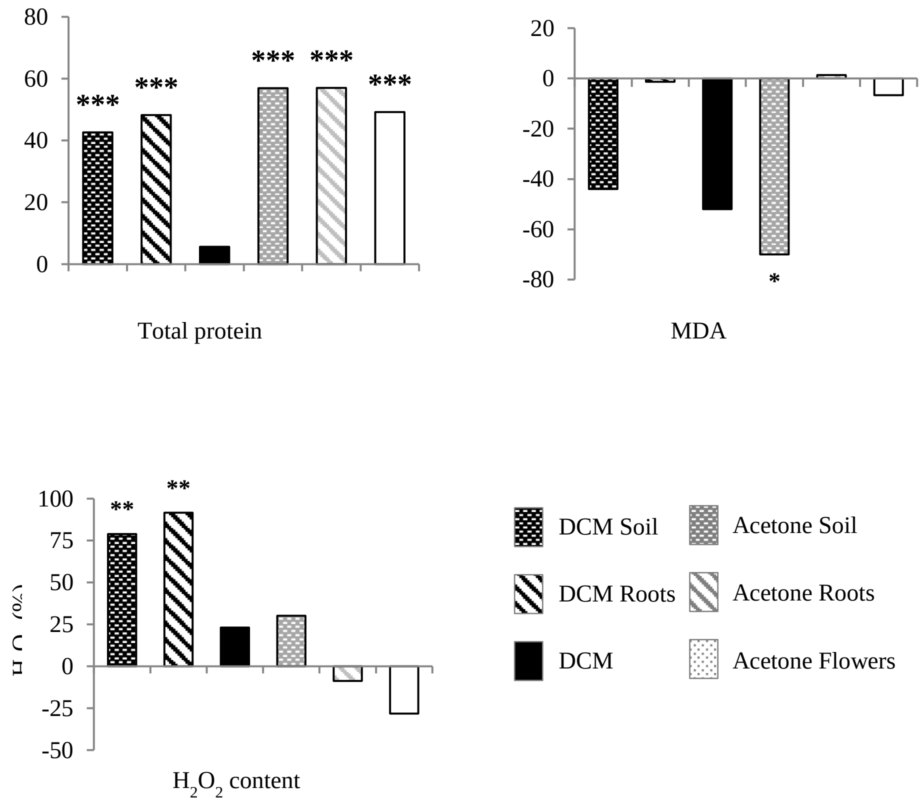


Figure 5. Effects of *Acacia dealbata* extracts from different natural material (soils, roots and flowers) and solvent extractant (DCM, acetone Methanol and water) on total protein content (left upper chart), H_2O_2 content (left bottom chart), MDA equivalents (right upper chart above) of *Cytisus scoparius*. Values are in comparison to control (%). Negative values: the *y* axis indicate reduction over control. Asterisks: Statistical significance at $p \leq 0.05$ (*), $p \leq 0.01$ (**) and $p \leq 0.001$ (***) level.

The observed stimulation, mainly related to non-polar fractions, could be explained by a hormetic response, described as a biphasic dose-response relationship exhibiting low-dose stimulation and a high-dose inhibition (11,55). Biological activity is guided by chemical concentrations and the allelochemicals can also produce positive responses at lower doses. The range of stimulation found in our study is within an acceptable range, with maximum responses 30-60% higher than controls (11). Contrasting effects with previous studies (1,35,36,37) could be due to the uncertain concentration of bioactive compounds in DCM and acetone extracts.

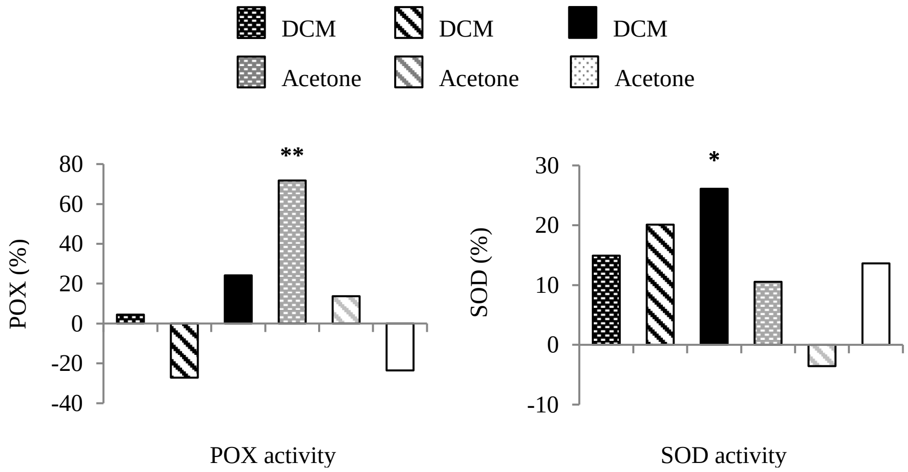


Figure 6. Effects of *Acacia dealbata* extracts from different natural material (soils, roots and flowers) and solvent extractant (DCM and acetone) on Peroxidase (POX) and superoxide dismutase (SOD) activity of *Cytisus scoparius* seedlings.
Values are in comparison to control (%). Negative values: *y* axis indicate reduction over control. Asterisks: Statistical significance at $p \leq 0.05$ (*), $p \leq 0.01$ (**) and $p \leq 0.001$ (***) level.

Table 3. Combined effects of *Acacia dealbata* extracts on measured variables. Asterisks represent significant differences between *A. dealbata* extracts and control at $p \leq 0.05$ (*), $p \leq 0.005$ (**) and $p \leq 0.001$ (**) significance level.

	DCM				Acetone			
	Soil	*Roots*	*Flowers*	**Effect**	*Soil*	*Roots*	*Flowers*	**Effect**
G_t (%)	0.037*	0.949	0.717	**I.A**	0.855	0.041*	0.839	**P**
S (speed of germination)	0.009**	0.630	0.109	**I.A**	0.048*	0.884	0.388	**I.A**
AS (accumulated speed of germination)	<0.001***	0.292	0.001**	**A**	<0.001***	0.174	0.192	**I.A**
CRG (coefficient of the rate of germination)	0.328	0.329	0.772	-	0.550	0.982	0.284	-
Radicle length (mm)	0.012*	0.017*	0.210	**A**	0.139	0.112	0.110	-
Hypocotyle length (mm)	0.046*	0.181	0.221	**I.A**	0.385	0.563	0.686	-
FW/DW	0.845	0.826	0.312	-	0.366	0.435	0.272	-
Proteins (mg·g^{-1} dry weight)	<0.001***	<0.001***	0.091	**A**	<0.001***	<0.001***	<0.001***	**A**
H_2O_2 (µmol·g^{-1} dry weight)	0.002**	0.003**	0.310	**A**	0.144	0.598	0.105	
Lip. Perox. (µmol· g^{-1} dry weight)	0.204	0.972	0.089	-	0.023*	0.971	0.862	**I.A**
POX (U·g^{-1} dry weight)	0.486	0.396	0.108	-	0.001**	0.194	0.598	**I.A**
SOD (U·g^{-1} dry weight)	0.446	0.071	0.035*	**P**	0.408	0.861	0.191	-

Abbreviations: G_t total germination; S, speed of germination; AS, speed of accumulated germination; CRG coefficient of the rate of germination; Rad. L, radicles length; Hyp. L., hypocotyl length; FW/DW, fresh weight/dry weight; Lipid P., lipid peroxidation; POX, peroxidase; SOD, superoxide dismutase. A= allelopathy; I.A=indirect allelopathy; P=phytotoxic effect, - = no effect.

Notwithstanding, it is important to remark that the observed physiological and biochemical changes (phytotoxic, allelopathic, or indirectly allelopathic) were mainly related to soil extracts. In the field, the extensive root system of *A. dealbata* completely occupies the first 30 cm of surface soil layer; massive root growth, both superficial but also in depth, has been suggested as an inhibitor of the plant establishment (18,21). Soil beneath *A. dealbata* acts as a final store house receiving chemical compounds from different sources, as litter and root system decomposition (21), together with the natural release (35,36,37), accumulation and modification of root and plant exudates. In addition, it has been suggested that molecules released from the *A. dealbata* litter contributes to the persistent allelopathic effects along the phenological cycle (1).

CONCLUSIONS

Our experimental approach showed that direct toxicity, under natural conditions, may have minor relevance because of the main role played by phytochemicals concentrations. We obtained two different plant responses, suggesting that effects were species-dependent and showing contrasting effects on susceptible or resistant species. After the exposure to *A. dealbata* extracts, germination and radicle length of *L. sativa* were reduced but the biometrical and biochemical parameters of *D. glomerata* and *C. scoparius* remained unchanged. Low concentrations of phytochemicals released into soil by *A. dealbata* have showed stimulatory effects on different physiological parameters. A change in the timing of germination or early growth may increase the risk for native species competing in unfavourable conditions. Soil and root extracts, but mainly soil extracts, showed higher bioactivity, contrasting the general idea that flowers are the most bioactive plant material. Finally, our results suggested that the non-polar fractions presents higher biological activity than the polar fraction, a fact that can be further explored in the search for useful novel compounds.

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