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Potential Allelopathic Effect of Six *Phaseolus Vulgaris* Recombinant Inbred Lines for Weed Control

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Abstract: The phytotoxicity of aqueous foliar extracts and ground dried residues of six common bean (*Phaseolus vulgaris*) Recombinant Inbred Lines (RILs 147, 83, 104, 115, 34 and 75), recently introduced from France for possible cultivation and improvement under Egyptian conditions, against certain weed and crop species was investigated as a possible source for natural herbicides. Aqueous extracts at 10% were prepared and evaluated on seedling root and shoot growth of perennial ryegrass (*Lolium perenne*), slender amaranth (*Amaranthus viridis*), wheat (*Triticum aestivum*) and lettuce (*Lactuca sativa*) as bioassay. Root and shoot growth were significantly inhibited by the different extracts, with a superior activity against root growth rather than on shoot growth. No great differences were observed between the extracts obtained at flowering stage and those obtained at harvesting stage in affecting assayed species, and crop seedlings were generally more sensitive than weed seedlings. The suppressions by the RILs 34 and 147 were comparatively the greatest, especially with the RIL-34 where it displayed remarkable inhibitory effect against weeds growth (in particular ryegrass). The stability of the RIL-34 extract (along eight days of lab experimentation) against soil conditions was measured on the same test species. The recovered extracts showed good activity with decline in toxicity with time. Ryegrass and lettuce were the most sensitive (root growth in particular). Wheat was, to some extent, the most tolerant followed by slender amaranth. No clear toxicity was observed on shoot growth after the day two of incubation in most cases. Analysis (using paper chromatography) for the existence of the phenolics that might have a role in the observed allopathic effect in the RIL-34 (at flowering stage), revealed two phenolics (e.g., caffeic acid and *p*-coumaric acid) could be involved in this. This study demonstrates that the potential exists for *P. vulgaris* plants to be used as natural source for herbicides, but this shackled by many considerations that should be taken into account.

Key words: allelopathy, common bean, herbicides, natural products, phytotoxicity, Recombinant Inbred Lines (RILs), weeds

INTRODUCTION

The chemical interactions between plants (including microorganisms) have taken these days a new meaning in weed management strategies. Allelopathy, the term which expresses the phenomenon, could be used either directly or indirectly in controlling weeds (Einhellig and Leather, 1988; Khalid *et al*., 2002; Bhowmik and Inderjit, 2003; Xuan *et al*., 2005; Dhima *et al*., 2009; Bhadoria, 2011). Due to human concerns by environment and public health, there was an urgent need for searching for new herbicides that could be more safely and environmentally friendly than current used ones. With natural products-based herbicides many of these advantages could be achieved, starting from safety to all forms life to reduce herbicide-resistant pests (Duke *et al*., 2000). To date, a number of allelochemicals have been isolated and investigated to develop new natural herbicides (Kelton *et al*., 2012).

Herbicides producers look at allelopathy and its arms from the allelopathic agents (allelochemicals) as a new hope in producing and developing new herbicides. Plant-derived compounds can be used either directly as crude preparations or as pure compounds in controlling weeds (Duke, 1992; Vaughn and Spencer, 1996; Duke *et al*., 2000; Duke *et al*., 2002; Vyvyan, 2002). Rather than being used directly, they have been used more often as structural leads for the discovery and development of natural products-based herbicides (Dayan *et al*., 2009).

Allelopathy in legumes has recently been received considerable attention. As cover crops, they have been thought to play a role in the biological control of weeds. Cover crops can suppress weeds in cropping systems by competing on available resources and by promoting conditions that are unfavorable for weed germination and establishment (Adler and Chase, 2007). Allelopathy is involved in this (Farooq *et al*., 2011). Recently, there has been increased interest in using leguminous cover crops in sustainable and organic cropping systems worldwide (Collins, 2004; Abdul-Baki *et al*., 2005; Scholberg *et al*., 2006). Species for example like cowpea, sunn hemp, and velvet bean can be used to suppress weeds through resource competition however, it is likely that weed suppression by these crops may also be in part the result of allelopathy (Collins, 2004). Aqueous leachates from

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fresh and dry cultivated legumes (1%, at vegetative and flowering stages) were found to inhibit weeds growth in ranges from 20% to 43% for fresh material, and from 26% to 84% for dry material (Veronica *et al*., 2005).

The objective of this work is to determine and characterize potentially allelopathic effect among six common bean Recombinant Inbred Lines (RILs) recently introduced from France for possible cultivation and improvement under Egyptian conditions. Isolation and identification of the main responsible phytotoxic components (phenolic compounds in the main standing) was also carried out.

MATERIALS AND METHODS

Experimental Procedures:

Six Recombinant Inbred Lines (RILs) of common bean (*P. vulgaris*, 147, 83, 104, 115, 34 and 75) were introduced from France (Institut National de la Recherche Agronomique, UMR1222 Ecologie Fonctionnelle & Biogéochimie des Sols, INRA-IRD-SupAgro, 2 Place Viala, 34060 Montpellier Cedex) via Dr. Jean-Jacques Drevon for possible cultivation and improvement under Egyptian conditions. The allelopathic effect of the RILs was studied *in vitro* at two stages of growth (flowering and harvesting stages).

Healthy common bean (RILs 147, 83, 104, 115, 34 and 75) seeds were sown at the experimental station of the National Research Centre, Nubaria District, Egypt, 30.73 N, 30.55 E on 25th February 2009 and harvested on 14th June 2009. Recommended amount of common bean seeds of 95 kg/ha was used to achieve the required plant density.

Common bean seeds were selected for uniformity by choosing those of equal size and with the same colour. The selected seeds were washed with distilled water, sterilized with 1% sodium hypochlorite solution for about 2 min and thoroughly washed again with distilled water and, left to dry at room temperature (25 °C) for about 1h. Uniform air dried common bean seeds were sown in rows with 60 cm spacing and hills were spaced 10 - 15 cm apart within plots (3 x 3.5m). Thinning was done before first irrigation to secure two plants/hill. During soil preparation and plant growth, soil was supplemented with full dose of NPK according to recommendations of the Ministry of Agriculture and Land Reclamation, Egypt under the studied area. The recommendations are 475 kg/ha of calcium super phosphate (15.5 % P_2O_5), 120 kg/ha of ammonium sulphate (20.5 % N) and 60 kg/ ha of potassium sulphate (48 % K_2O) and applied with seedbed preparation. Additional 120 kg/ha of ammonium sulphate and 60 kg/ha of potassium sulphate were added at the first irrigation (two weeks after sowing). Irrigation water was supplied as a ratio from ETo (Fayoum Meteo Station) at 100%. Number of irrigation times was 7 with total calculated amount of 2829.6 m^3/ha. All other recommendations were also followed.

Plant Processing:

One sample was collected at flowering and another one at harvesting (56 and 110 days after sowing, respectively). The plant samples were dried at room temperature and then ground to a fine powder using a sharp grinder. The ground materials were kept in paper bags until use in the extraction.

Extraction and Bioassay Test:

A 25-g aliquot of the dried materials was extracted overnight (17 h) with distilled water (150 ml/each) in dark-screw bottles. After good shaking several times, the homogenate was filtered through six layers of cheesecloth (to remove the majority of the cellulose material) and one layer of filter paper Whatman no. 1 (to remove particulate matter). The filtrate was then made up to a 250-ml volume to yield a 10% w/v solution at a dry weight basis. The extracts were stored at 4 °C until use within 24 h.

The effect of the extracts on seedling root and shoot growth was evaluated using two weed (perennial ryegrass, *Lolium perenne* L.; slender amaranth, *Amaranthus viridis* L.) and crop (lettuce, *Lactuca sativa* L.; wheat, *Triticum aestivum* L., cv. Sakha 61) species. Root and shoot growth were particularly chosen for their high sensitivity in comparison with the seed germination percentage (Lydon and Duke, 1989; Moosavi *et al*., 2011). A 7-cm circle of Whatman no. 1 (Whatman International Ltd. Maidstone, England) filter paper was placed in the base of each 7-cm Petri dish and moistened with 2 - 3 ml extract based on seeds size. Ten uniform seedlings with root lengths of about 2 mm were transferred to the dishes. (The test species were germinated beforehand in 9-cm diameter glass Petri dishes for 2-3 days until reach the required growth stage). The lid of the dish was used to close the dishes to reduce moisture loss. Controls were made from distilled water. Dishes were held in darkness (to reduce competition as much as possible) at room temperature (18 – 25 °C), and checked after 5 days where root and shoot growth (cm) were determined.

P. Vulgaris Extract and its Recovery from The Soil:

Since RIL-34 (i.e., at flowering stage) exhibited the highest inhibitory effect against tested species, especially against weeds growth, it was decided to continue with such source in a soil incubation trail. The experiment was conducted under lab conditions (at 18 – 25 °C) using a silty clay soil. Two kg of the soil was collected from the field for this purpose. The soil was purified from the agricultural wastes/plant debris, left to dry at room temperature, and ground to a suitable size. An equal amount of sand and gravels was washed very carefully with 2 M HCl to remove adsorbed organic materials that might interfere with the process. The sand and gravels were rinsed several times with distilled water to remove any traces from the HCl. An amount equal 100 g from each of the soil and sand were thoroughly mixed and added to glass columns (44 × 3.5 cm), involved glass filter wool from the bottom and 3.5 cm of the washed sand and gravels (2 + 1 cm, respectively) over glass wool. The columns were then covered with another 3 cm of the sand. Nine columns were prepared following this procedure. As a standard one additional column was prepared with sand only. *P. vulgaris* RIL-34 extract (obtained at flowering stage) was applied onto the columns (25 ml/each). Two zero time samples were collected by elution with distilled water (2 times × 50 ml); one from the sand column and the other from one of the soil-sand columns. The remainder columns (eight columns) were eluted during a definite time course depending on leaching one column every 24 h. The leachates (for each column) were bulked, and stored at (-21°C) until use in bioassay-plants reaction experiments. The resultant solution was approximately 50 ml for each column, and that equal the same concentration that we used before (10%). Bioassay test was conducted following the same technique, using the same test organisms and under the same circumstances.

Isolation and Identification of the Main Responsible Phytotoxic Components:

The aqueous extract (obtained at flowering) of the RIL-34 was partitioned with an equal volume of diethyl ether (3 times × 150 ml) in a 250-ml separating funnel. The ether extracts were bulked, and evaporated under vacuum (at 29 °C) to dryness. The residues were re-dissolved in 5 ml 95% ethanol, and stored at (-21°C) until use in chromatography analysis.

The ethanol extract was chromatographed one-dimensionally on paper chromatography [(PC), (Chrom.-Paper, Sartorius AG, 37070 Gottingen, Germany)] with *n*-BuOH-HOAc-H_2O (4:1:5, top layer). Marker solutions of coumarin, caffeic acid, cinnamic acid, ferulic acid, *p*-coumaric acid, gallic acid, 2,5-dihydroxybenzoic acid, *o*-coumaric acid, *p*-hydroxybenzoic acid, *p*-hydroxybenzaldhyde and benzoic acid were applied in parallel with the extracts as controls. The dried chromatograms were sprayed with 1% aqueous ferric chloride after exposure to UV light (at 254 nm) Typical R_f values, and colours under UV and with ferric chloride were recorded as a general procedure.

Statistical Analysis:

The randomized complete design was employed in all experiments with four replications each treatment. The data were analyzed using ANOVA table and LSD test at 0.05 and 0.01 *probabilities* for comparing means (Snedecor and Cochran, 1989). The inhibition (%) was also determined using the equation (data not reported):

(%) of inhibition = 100 – (growth in treatment/growth in control×100)

Results:

The aqueous extracts of the different RILs of *P. vulgaris* significantly suppressed the seedling growth in all tested species (Table 1). Root and shoot growth were markedly inhibited in wheat and lettuce as well as in slender amaranth and perennial ryegrass weeds. Overall, crop seedlings were more sensitive than weed seedlings, and the effect was more pronounced on root growth rather than on shoot growth. From 55 to 82% growth inhibition was recorded on root growth of wheat compared to 5 - 75% for shoot growth. Lettuce showed relatively high response, where a range of 78 - 85% growth inhibition was estimated on root growth, but less on shoot growth (13 - 48%). Weeds were, to some extent, more tolerant than crops. The results showed an inhibition range from 31 to 67% for root growth and from 3 to 53% for shoot growth in ryegrass and in slender amaranth from 57 to 73% for root growth and from 4 - 45% for shoot growth in comparison with the control (data not reported).

Comparing to the other treatments and the control the RIL-34 was, to some extent, the most effective especially against weeds growth. No big differences were observed in this regard between the activity of the extracts obtained at flowering stage and those obtained at harvesting stage, except with the RIL-34 where a highly influence was recorded at flowering stage rather than at harvesting stage.

As *P. vulgaris* RIL-34 (especially at flowering) was relatively the most effective, it was decided to continue with such source to measure the stability under soil conditions along eight days of the experimentation. The data revealed an activity for the whole leachates, especially against root growth (Table 2). A remarkable inhibition was obtained on reducing ryegrass and lettuce roots (20 - 73% and 49 - 74%, respectively) comparing to 28 - 70% and 9 - 69% for slender amaranth and wheat, respectively (data not reported). Shoot growth slightly affected in this regard if compared with the root growth and the control. No clear toxicity was observed after the day two of incubation in most cases.

Two phenolic acids (e.g., caffeic acid and *p*-coumaric acid) were identified in *P. vulgaris* RIL-34 extract obtained at flowering stage (Table 3).

Table 1: Effect of water extract of the different RILs of *P. vulgaris* obtained at two stages (flowering and harvesting stages) on the growth and development of certain assayed weed and crop species. The experiment was conducted *In vitro* (under Petri dishes conditions), with five days old seedlings and 22°C temperature in average.

Physiological stage	*Phaseolus vulgaris* RILs	Assayed species							
		Perennial ryegrass (*Lolium perenne* L.)		Slender amaranth (*Amaranthus viridis* L.)		Wheat (*Triticum aestivum* L., cv. Sakha 61)		Lettuce (*Lactuca sativa* L.)	
		Root growth	Shoot growth	Root growth	Shoot growth	Root growth	Shoot growth	Root growth	Shoot growth
		- cm -		- cm -		- cm -		- cm -	
Flowering	147	1.78	3.63	0.28	1.12	1.60	1.17	0.49	1.12
	104	2.06	3.78	0.26	1.17	2.71	3.20	0.56	1.25
	34	1.25	2.02	0.21	0.78	3.03	3.60	0.50	1.15
	83	1.63	3.18	0.30	0.88	2.25	2.66	0.56	1.14
	115	1.77	3.36	0.23	1.10	2.18	3.11	0.55	1.24
	75	1.52	2.62	0.22	1.03	2.95	3.60	0.58	1.18
Harvesting	147	2.19	4.18	0.23	1.72	3.43	3.64	0.67	1.54
	104	2.41	3.58	0.28	1.99	3.97	3.97	0.67	1.59
	34	1.92	3.46	0.19	1.53	3.73	4.11	0.60	1.57
	83	2.31	3.63	0.22	1.84	3.45	3.77	0.70	1.58
	115	2.63	4.04	0.21	1.69	3.56	4.45	0.65	1.85
	75	2.09	3.22	0.27	1.55	3.71	4.29	0.71	1.86
control		3.82	4.28	0.68	1.61	8.83	4.69	3.21	2.14
LSD $_{0.05}$		0.44	0.62	0.07	0.26	0.36	0.42	0.10	0.34
$_{0.01}$		0.59	0.83	0.09	0.35	0.49	0.56	0.14	0.46

Table 2: Effect of soil recovered extracts of *P. vulgaris* RIL-34 on the growth and development of certain assayed weed and crop species. The experiment was conducted *in vitro* under the same circumstances of time, temperature and test organisms.

Period of incubation (Day)	Assayed species							
	Perennial ryegrass (*Lolium perenne* L.)		Slender amaranth (*Amaranthus viridis* L.)		Wheat (*Triticum aestivum* L., cv. Sakha 61)		Lettuce (*Lactuca sativa* L.)	
	Root growth	Shoot growth	Root growth	Shoot growth	Root growth	Shoot growth	Root growth	Shoot growth
	- cm -		- cm -		- cm -		- cm -	
1	1.36	4.62	0.46	1.34	3.77	8.29	1.05	2.24
2	2.63	6.63	0.47	1.44	4.91	8.64	1.16	2.63
3	3.43	6.56	0.64	2.06	7.76	8.65	1.43	2.91
4	3.70	7.00	0.72	2.26	9.02	9.20	1.59	2.91
5	3.89	7.27	0.71	2.39	9.58	9.22	1.75	3.12
6	3.91	7.42	0.84	2.46	10.87	9.74	1.92	3.25
7	3.94	7.36	0.80	2.44	12.23	9.56	2.12	3.44
8	4.13	7.36	0.83	2.49	13.45	9.78	2.09	3.57
Zero time (sand)	2.39	6.12	0.51	1.58	4.52	7.24	1.18	2.62
Zero time (soil)	1.40	4.93	0.44	1.66	2.70	6.41	1.06	2.40
control	5.13	6.58	1.17	2.16	11.98	9.07	4.05	2.57
LSD $_{0.05}$	0.57	0.62	0.12	0.32	2.06	0.95	0.34	0.33
$_{0.01}$	0.77	0.85	0.16	0.43	2.79	1.28	0.46	0.45

Table 3: Phenolics in *P. vulgaris* RIL-34 extract as phytotoxic agents.

Phenolics	R_f (x 100) *in* BAW	*Fluorescence in* UV light at 245 nm	*Colour with* 1% ferric chloride	*Presence in P. vulgaris* RIL 34
Coumarin	-	none	None	-
Caffeic acid	78.67	bright blue	green	+
Cinnamic acid	96.00	blue	yellow	-
Ferulic acid	89.33	bright blue	reddish- brown	-
o-coumaric acid	88.00	bright yellow	orange	-
p-coumaric acid	92.00	blue	brick red	+
Gallic acid	58.00	blue	gray	
2,5-dihydroxybenzoic acid	87.33	strong bright blue	clear blue	-
p-hydroxybenzoic acid	93.33	blue	light yellow	-
p-hydroxybenzaldhyde	94.66	blue	mauve	-
Benzoic acid	-	none	none	-

Solvent key: BAW = *n*-BuOH-HOAc-H_2O (4:1:5, top layer)

Discussion:

The data showed good inhibitory effect for the whole extracts against test species, with a special activity against crops growth rather than on weeds growth, and on root growth rather than shoot growth. The allelopathic effects in legumes have been discussed in several locations with a confirmation of stunting growth (Martin *et al*., 1990; Ohno *et al*., 2000; Farooq *et al.*, 2011; Asaduzzaman and Asao, 2012). As cover crops (competing aggressively on light, water, and nutrients), they studied more extensively. Lately, Adler and Chase (2007) reported that the allelopathic activity within such plants play a substantial role besides resource competition in affecting associated plants (Collins, 2004). The allelopathic effect has been demonstrated in leguminous cover crops as many as *P. vulgaris*, *P. coccineus*, alfalfa, faba bean, and vetch where an inhibition range from 20 to 84% were estimated on different plant species including leguminous cover crops themselves (Veronica *et al*., 2005). These all might provide evidence in supporting our data in this regard.

Bioassays using soil leachates exhibited moderate stability of *P. vulgaris* RIL-34 extract against soil conditions. Although there was a diminution in toxicity with time, the influence still in the active extent, especially against root growth. There are evidences that the allelopathic chemicals interact in the soil environment similarly as herbicides and are subject to processes of degradation such as microbial degradation, oxidation, and photolysis, and processes of removal or transfer, such as volatilization and adsorption (Vidal and Bauman, 1997). Researchers also reported that the activity of allelochemicals in nature are limited in time (because of slow release from the donor material) and in space (because of the interaction with the environment). That might give an explanation regarding our reported results.

The chromatography analysis using paper chromatography showed two predominant phenolic acids (namely, caffeic acid and *p*-coumaric acid) in *P. vulgaris* RIL-34 extract (obtained at flowering). Phenolic compounds are a class of the most important and common plant allelochemicals (Li *et al*., 2010; Kelton *et al*., 2012). To date, a number of phenolics have been isolated and tested as a proof of the allelopathic action. Species which have been noted to produce phenolic acids are many including leguminous cover crops themselves (Wu *et al*., 2001; El-Shahawy *et al*., 2006; Cai-xia *et al.*, 2007; Uddin *et al*., 2012). Phenolics were detected as allelopathic agents in dry shoot material of vegetative alfalfa, pinto bean, and vetch, and flowering faba bean (Veronica *et al*., 2005). Benzoic, salicylic, and malonic phenolic acids have been found to play a role in *P. vulgaris* allelopathic activity (Asaduzzaman and Asao, 2012). Such evidences, anyway, come in agreement with our finding.

This study demonstrates that the potential exists for *P. vulgaris* cultivars to be used as a source for natural herbicides and that very important at both the environmental and the public health levels. But the matter needs more advanced research to solve many of the problems connected with such source (natural products generally) before we announce the feasibility of using these chemicals in field situations.

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