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# *Vicia faba* aqueous extracts and plant material can suppress weeds and enhance crops

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## ABSTRACT

The phytotoxic potential of faba bean (*Vicia faba* L.) was evaluated by both soil-based and aqueous extract laboratory bioassays. A preliminary bioassay was conducted in Petri dishes filled with faba bean-amended soil. The results revealed strong inhibitory effects on the early growth of the model species lettuce. The phytotoxic potential of aqueous extracts of faba bean aerial biomass was then determined on lettuce, maize and soybean as model crops in a temperate forage-based system, and *Amaranthus retroflexus* (redroot pigweed), *Echinochloa crus-galli* (barnyard grass) and *Digitaria sanguinalis* (large crabgrass) as the most common associated weeds. Dose-response curves and $IC_{50}$ and $IC_{90}$ values for faba bean aqueous extracts were obtained. The results indicate that faba bean aqueous extracts significantly inhibited the germination and early growth of weeds. Except for the highest concentration, crops were not affected, or crops growth was enhanced by the aqueous extracts at concentrations that suppressed the weeds. Thus faba bean aerial biomass may be a promising material for weed control in sustainable-based crop systems.

**Keywords:** Allelopathy, *Amaranthus retroflexus,* aqueous extract, barnyard grass, crab grass, *Digitaria sanguinalis,* dose-response curve, *Echinochloa crus-galli,* early growth, faba bean, germination, *Glycine max*, hormesis, phytotoxicity, maize, redroot pigweed, selectivity index, Soybean, *Vicia faba*, *Zea mays*.

## INTRODUCTION

The current awareness of the problems from the widespread use of synthetic herbicides since the second half of the 20th century has resulted in increasing efforts to find sustainable and effective weed management tools (25). One of the possible strategies is the exploitation of allelopathic interactions for weed control. Allelochemicals may reach the receiver plants in various ways including leaching from plant foliage, exudation from the roots, and mostly decomposition of dead residues of the donor plants into the soil. In cropping systems, the occurrence of natural allelopathic activity in certain crops, or allelochemicals produced and released from crop plants that exert phytotoxic effects against weeds, can be exploited by introducing these species as cover crops or green manures (23,39).

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In temperate areas, the legume *Vicia faba* L. (faba bean, broad bean, horse bean, or field bean) is cultivated as winter cover crop for grain, for protein-rich forage and silage, and as green manure (12). Although the introduction of N-fertilizers gradually eliminated intercrops of grain legumes in the industrialized countries, modern sustainable agriculture is reassessing the potential role of legumes as a source of N for future sustainable cropping systems, especially in organic agriculture (21). When used as a cover crop, faba bean is frequently included in rotations with maize (*Zea mays* L.), one of the most important crops in Europe covering a production area of more than 18 million ha in 2012 (15). In the Atlantic and Cantabrian cultivation regions of Spain, maize is typically grown for silage in rotation with silage ryegrass, grass-clover pastures or other crops, and has notable importance in dairy livestock production. Our field observations on forage crop rotations in these areas suggest that weed proliferation after incorporation of faba bean as green manure is less in the subsequent maize crop.

The effectiveness of faba bean aqueous extracts and plant material for weed control has not been thoroughly studied to date. Allelopathy has been investigated in other leguminous crops, but only few citations provide some evidence of the presence of phytotoxic compounds in faba bean. Fujii (16) included it into a wide screening of crops showing significant inhibition of lettuce growth. Kaletha *et al.* (22) and Nava-Rodríguez *et al.* (30) tested the *in vitro* effects of faba bean aqueous leachates and obtained promising allelopathic effects on weeds and crops.

Phytochemistry of faba bean has received high attention regarding the nutrition value of grains and grain flour (38), as well as their nutraceutical profile. Pulses are rich in nutritional bioactive substances, such as antioxidants, anti-inflamatory, antiviral, or protection against UV radiation (33,40). Yao *et al.* (40) identified the phenolic acids caffeic, *p*-coumaric, ferulic and sinapic from faba bean grains, but not isoflavones. But tannins vicine and convicine are also known to be the main antinutritional factors reducing faba bean protein digestibility (38). Apart from grains, faba bean sprouts are known to be a rich source of levo dihydroxy phenylalanine (l-DOPA) the precursor of dopamine, which is used in the treatment of Parkinson's disease (33).

Laboratory assays done in controlled conditions are a key element to demonstrate the existence of allelopathy (20) as they provide information such as the sensitivity of different weed and crop species to a given allelopathic plant. *In vitro* bioassays also avoid the large number of factors interacting in the field, which make extremely complex to separate allelopathy from other phenomena such as competition. On the assessment of phytotoxicity, germination and seedling growth bioassays are considered the primary tool for assessing the phytotoxic activity of a given plant material in the laboratory (20).

This study aimed to explore the *in vitro* phytotoxicity of faba bean plant material and aqueous extracts on target model species and representative summer weeds and crops of a temperate forage system, as a first step to appraise its use in the field as a potential tool for weed control.

## MATERIALS AND METHODS

### I. Plant and soil material

Plants of faba bean cv. Prothabat 69 (Batlle S.A., Bell-lloc, Spain) were grown in 5.0 L plastic pots in a greenhouse, in Vigo University campus, Galicia, NW Spain (42.15º N, 8.43º W) from September 2010 to March 2011, providing homogeneous conditions for all plants (natural light, temperature ranging from 10 to 26 ºC). Pots were filled with commercial substrate Gramoflor® (GmbH & Co., Vechta, Germany). Substrate characteristics were pH ($CaCl_2$) 5.6, and concentrations of N, $P_2O_5$, $K_2O$ and MgO were 100-300, 200, 250 and 150 mg $L^{-1}$, respectively. The substrate was supplemented with Patent PK (K+S KALI GmbH, Kassel, Germany) ($P_2O_5$ 12%, $K_2O$ 15%, MgO 5%) at a dose of 800 kg $ha^{-1}$, and Lithothamne 400 (Timac Agro, Orcoyen, Spain) (MgO 2.5%, CaO 36%) at 3000 kg $ha^{-1}$. Tillers (shoots, leaves and flowers) were subsequently harvested at flowering throughout February and March. After each harvest, tillers were dried in dark at room temperature (18-20 ºC) until constant weight. Total collected aerial biomass was cut in 1 $cm^2$ pieces and stored in the dark at room temperature until used. The dry weight/fresh weight ratio was obtained by drying fresh material aliquots at 60 ºC for 72 h. Mean C and N contents on dry mass basis, determined in triplicate in a Fisons Instruments EA1108, were 38.25 and 3.52%, respectively. Mean $PO_4^-$, $K^+$, $Mg^{2+}$, $Mn^{2+}$ and $Ca^{2+}$ values determined by ICP-OES (Perkin Elmer Optima 4300DV) were 7.30, 49.43, 2.90, 0.03 and 11.95 mg $g^{-1}$, respectively.

A sandy-loam top-soil (A horizon) was collected from an agricultural field devoted to ryegrass-forage maize production for 30 years and then left fallow over the last three years. Soil physicochemical characteristics were pH (1:2.5 $H_2O$) 4.6, EC<0.13, organic matter 3.12 %, total N 0.17 %, and concentrations of $Ca^{2+}$, $K^+$, $Mg^{2+}$, $Na^+$ and $P^{3-}$ 234, 71, 23, <15 and 115 mg $kg^{-1}$, respectively, and a maximum water retention capacity (WRC) of 316 mL $kg^{-1}$ dw. For *in vitro* experiments, soil was air-dried in lab. and sieved through 2 mm mesh to remove debris and plant tissues.

## II. *In vitro* green manure preliminary bioassay

Bioassays of *in vitro* incorporation of plant material into the soil were done in 9 cm dia Petri dishes filled with the described soil mixed with faba bean aerial biomass, which for this experiment was powdered to avoid physical interferences and spatial heterogeneities in the dishes. Lettuce (*Lactuca sativa* L. cv. Great Lakes) from Fitó S.A. (Barcelona, Spain) was selected as standard target species. Plant material was incorporated at 2.75 g dw in 26 g dw of soil (10% dw/dw), this first-approach dose being representative of that in our field observations of weed control (approximately 4300 kg dw $ha^{-1}$). Plates were divided in four equal sectors, sowing two opposite sectors with 10 seeds and the other two with 10 pregerminated seeds (radicle length 1-3 mm). Plates were watered to soil capacity and incubated in the dark at 27 ºC for 48 h. The number of germinated seeds was counted every 12 h until no further seeds germinated. A seed was considered to be germinated when the seed coat was ruptured and the root emerged $\geq$ 1 mm. The total percentage of germinated seeds (Gt) was calculated from the cumulative germination data. For pregerminated seeds, root lengths were measured 48 h after incubation. Negative control consisted of dishes filled with 26 g dw of soil and watered to soil saturation. The synthetic pre-emergence herbicide S-metolachlor (Dual® Gold 960 EC, 960 g a.i. $L^{-1}$, Syngenta Agro, Spain), widely used in maize cultivation, was used as a positive control. Metolachlor was used at the label dose, i.e., dishes filled with 26 g dw of soil were watered with a solution of metolachlor prepared to attain 0.96 kg a.i. $ha^{-1}$ in each dish. Germination and root growth values were expressed as percentage of the negative control. Each treatment was replicated five times.

## III. Preparation of aqueous plant extracts

Extractions were done in 2 L Erlenmeyer flasks by immersing 100 g dried and cut plant material in 1.5 L distilled water (1:15 w/v), the maximum ratio to keep a volume of chopped plant material completely soaked. Flasks were left in the dark at room temperature for 24 h, and gently soaked every 6 h. A plant dry weight/distilled water volume ratio of 66.7 g $L^{-1}$ was obtained. The aqueous extract was vacuum filtered successively through a 0.45 µm cellulose membrane to clear impurities, and through a 0.2 µm millipore-filter to sterilize the extract. The extract was frozen at -20 ºC in sterile plastic bottles until bioassayed during the next two months. At the time of use, this crude extract served as the initial aqueous extract, being successively diluted in distilled water at 100, 80, 60, 40, 20, 15 and 0% v/v to get final concentrations corresponding to 66.7, 50.0, 25.0, 16.7, 12.5, 10.0, and 0.0 g $L^{-1}$ on a dry mass basis. Values for pH (Crison MicropH 2001), electrical conductivity (EC, Crison CDTM-523), and osmolarity (Gonotec OSMOMAT 030 cryoscopic osmometer) were measured for each solution. Values of pH for the increasing concentrations of faba bean extracts ranged between 5.7 and 5.9. Values of EC of the test solutions were 0.99, 1.25, 1.45, 2.18, 4.30 and 5.13 dS $m^{-1}$ at the concentration of 10.0, 12.5, 16.7, 25.0, 50.0 and 66.7 g dw $L^{-1}$, respectively. Osmolarity values of these solutions were 0.049, 0.060, 0.065, 0.109, 0.164 and 0.179 osmol $kg^{-1}$.

## IV. *In vitro* aqueous extracts bioassays

Dose-response assays for seed germination, root and shoot growth were done for lettuce as standard target species, for maize cv. Anjou 387 and soybean (*Glycine max* (L.) Merr.) cv. Isidor both ceded by Limagrain Ibérica S.A. (Elorz, Spain) as typical monocotyledon and dicotyledon forage summer crops in temperate areas, and *Amaranthus retroflexus* L. (redroot pigweed), *Echinochloa crus-galli* (L.) P. Beauv. (barnyard grass) and *Digitaria sanguinalis* (L.) Scop. (large crabgrass) from Herbiseed (Twyford, UK) as representative dicotyledon and monocotyledons summer weed species in European forage production (29). Seeds of *A. retroflexus* and *E. crus-galli* were pre-treated by soaking in distilled water at 4 ºC for 15 days, and seeds of *D. sanguinalis* by storing under continuous light at 4 ºC for 56 days.

For germination tests, concentrations between 12.5 and 66.7 g dw $L^{-1}$ were used. Maize and soybean seeds were incubated in 13 cm dia Petri dishes at 15 seeds per dish placed on a Whatman No. 1 filter paper layer (Whatman Ltd., Maidstone, UK) wetted with 10 mL of the corresponding solution, and incubated in the dark at 27 ºC. Lettuce and weed seeds were placed in 3.48 cm dia well in 6-well plates (Corning Inc., NY, USA) at a rate of 15 seeds per well placed on a Whatman No. 1 filter paper layer wetted with 750 µL of the corresponding solution, and incubated in the dark at 27 ºC for lettuce, in the dark at 35 ºC for *A. retroflexus*, at 26/16 ºC, 16/8 h light/dark for *E. crus-galli*, and in the dark at 25 ºC for *D. sanguinalis*. Gt index was calculated as described above. Additionally, other germination indices were derived from primary germination data to obtain extra information about the effects on the ontogeny of germination: speed of germination (S), speed of accumulated germination (AS), coefficient of rate of germination (CRG) and mean germination time (MGT), following Chiapusio *et al.* (5) and De Bertoldi *et al.* (9). For growth bioassays, concentrations between 10.0 and 50.0 g dw $L^{-1}$ were tested. Ten pregerminated seeds of each species were placed on Petri dishes or 6-well plates as described above. After 48 h of incubation, root and shoot lengths were measured on all seedlings in each dish or plate, and values were expressed as a percentage of the respective control. Control solutions were adjusted to pH 6.0, as this is considered the optimal for plant growth (27). Metolachlor was used as positive control, attaining the label dose in each plate or well. Each treatment was replicated five times.

The osmolarity values of increasing concentrations of faba bean extracts led us to carry out additional dose-response osmolarity assays, to assess the potential involvement of osmotic effects on the observed phytotoxicity. Lettuce and maize were used as representative small-seeded and large-seeded test species, respectively. They were bioassayed using polyethylene glycol (PEG) 6000 solutions at a range of osmotic potentials including those of the test solution, from 0 to 0.180 osmol kg$^{-1}$ (0 to 155 g L$^{-1}$ PEG 6000). Germination and growth bioassays were carried out by following the methodology explained above.

### V. Statistical analysis

Replicated experiments were conducted in a completely randomized design. Data were firstly tested for normality by Kolmogorov-Smirnov test and homogeneity of variances by Levene's test. Treatments from *in vitro* green manure bioassay were compared by Student's t test. For the osmolarity and aqueous extract bioassays, data were analyzed by one-way ANOVA and LSD test for post hoc multiple comparisons when variances were homogeneous. In the case of heteroscedasticity, variance was analyzed by Kruskall-Wallis H test and Tamhane's T2 for post hoc multiple comparisons. For each dose-response curve, the best-fit equation based on the coefficient of determination ($R^2$) was selected from linear, logarithmic, inverse, quadratic, cubic, potential, growth, and exponential models. The $IC_{50}$ and $IC_{90}$ values (concentrations required to obtain 50% and 90% inhibition of a given parameter, respectively) were calculated from the generated equations. Statistical analyses were performed using the IBM SPSS Statistics 19.0 software package (IBM SPSS Inc., Chicago, IL, USA).

## RESULTS

### *In vitro* green manure preliminary bioassay

A strong inhibition of the germination and root early growth of lettuce was observed (Table 1), with reductions of 94% and 82% with respect to the control, respectively ($P < 0.001$). The level of root growth suppression was higher than that achieved by metolachlor ($P < 0.05$), which had little effect on lettuce germination.

Table 1. Effects of incorporation of faba bean at 10% dw/dw into the soil on the germination and root growth of lettuce in a Petri dish bioassay

| | Faba bean | | Metolachlor | |
|---|---|---|---|---|
| Germination | 6.3 ± 1.84 | *** | 91.0 ± 7.64 | * |
| Root growth | 18.3 ± 2.32 | *** | 42.5 ± 2.93 | *** |

Values denote mean ± SD. Values given as percentage with respect to the control. For each treatment, asterisks denote significant differences with respect to the control * $P \leq 0.05$; *** $P \leq 0.001$ level (independent samples t-test).

## Osmotic effects of aqueous extracts

From the PEG 6000 solutions prepared to mimic the osmolarity of faba bean aqueous extracts, lettuce germination was significantly inhibited with respect to the control (distilled water, 0.00 dS $m^{-1}$) at osmolarity values higher than 0.064 osmol $kg^{-1}$ ($P < 0.05$, data not shown), whereas root and shoot lengths were significantly reduced only at osmolarities above 0.100 osmol $kg^{-1}$. Maize germination and growth was only affected at osmolarities above 0.125 osmol $kg^{-1}$.

## *In vitro* aqueous extracts bioassays

The aqueous extract of faba bean significantly affected the germination and early growth of all the tested species when assayed at different concentrations ($P < 0.05$). As shown in Fig. 1, lettuce germination was significantly reduced at extract concentrations higher than 16.6 g $L^{-1}$ ($P < 0.001$), and completely inhibited from 50 g $L^{-1}$ on. Root and shoot lengths were reduced at all concentrations ($P < 0.001$). Metolachlor reduced lettuce seedling growth. Values for maize and soybean germination ranged between 97-55%, and 100-28% respectively, but only at the highest concentration values (from 66.7 and 50 g dw $L^{-1}$ for maize and soybean, respectively) were significantly lower than the control ($P <$ 0.05 and $P < 0.001$ for maize and soybean). In both species, root growth was slightly stimulated at low concentrations of the aqueous extract (no significantly stimulated for maize, but highly significantly for soybean, $P < 0.001$). Shoot growth of maize increased up to two-fold and a half with respect to the control ($P < 0.001$), and was not affected in soybean ($P > 0.05$). As expected, no significant differences were found between control and metolachlor for maize since is one of the most used pre-emergence herbicides in maize cropping systems. However, metolachlor inhibited soybean seedling growth (Fig. 1).

Weeds were much more sensitive than forage crops, being inhibited in a dose-dependent manner by the faba bean aqueous extracts (Fig. 2). Total germination of the weed species was significantly inhibited at all concentrations ($P < 0.001$), and completely inhibited at concentrations higher than 50 g $L^{-1}$ for *A. retroflexus* and *E. crus-galli*, whereas *D. sanguinalis* germination was completely inhibited at all concentrations. Metolachlor did not affect germination of *A. retroflexus* and *D. sanguinalis*, and gave poor control of germination in *E. crus-galli*. Significant dose-response effects were observed for inhibition of root and shoot growth of weeds ($P < 0.001$), and in all cases excepting *D. sanguinalis* shoot growth, inhibitory effects were stronger than metolachlor. Root length inhibition ranged between 75-96% for *A. retroflexus*, 68-94% for *E. crus-galli*, and 64-95% for *D. sanguinalis*. Moreover, in lettuce and weeds abnormal root morphology was observed in seeds that germinated or seedlings that developed in aqueous extracts, being more evident at the higher concentrations. Root browning and restriction or absence of root hairs was typical at all concentrations, with root tip necrosis at the highest concentrations. Also, anomalous seedling development from pre-germinated seeds was observed for *E. crus-galli* and *D. sanguinalis* at all extract concentrations, as shoot growth occurred together with a retarded root extension. In *D. sanguinalis* germinating seeds, episodes of shoot emergence but complete inhibition of radical extrusion were observed, thus obtaining unviable seedlings. These effects were not observed in the osmolarity assays.

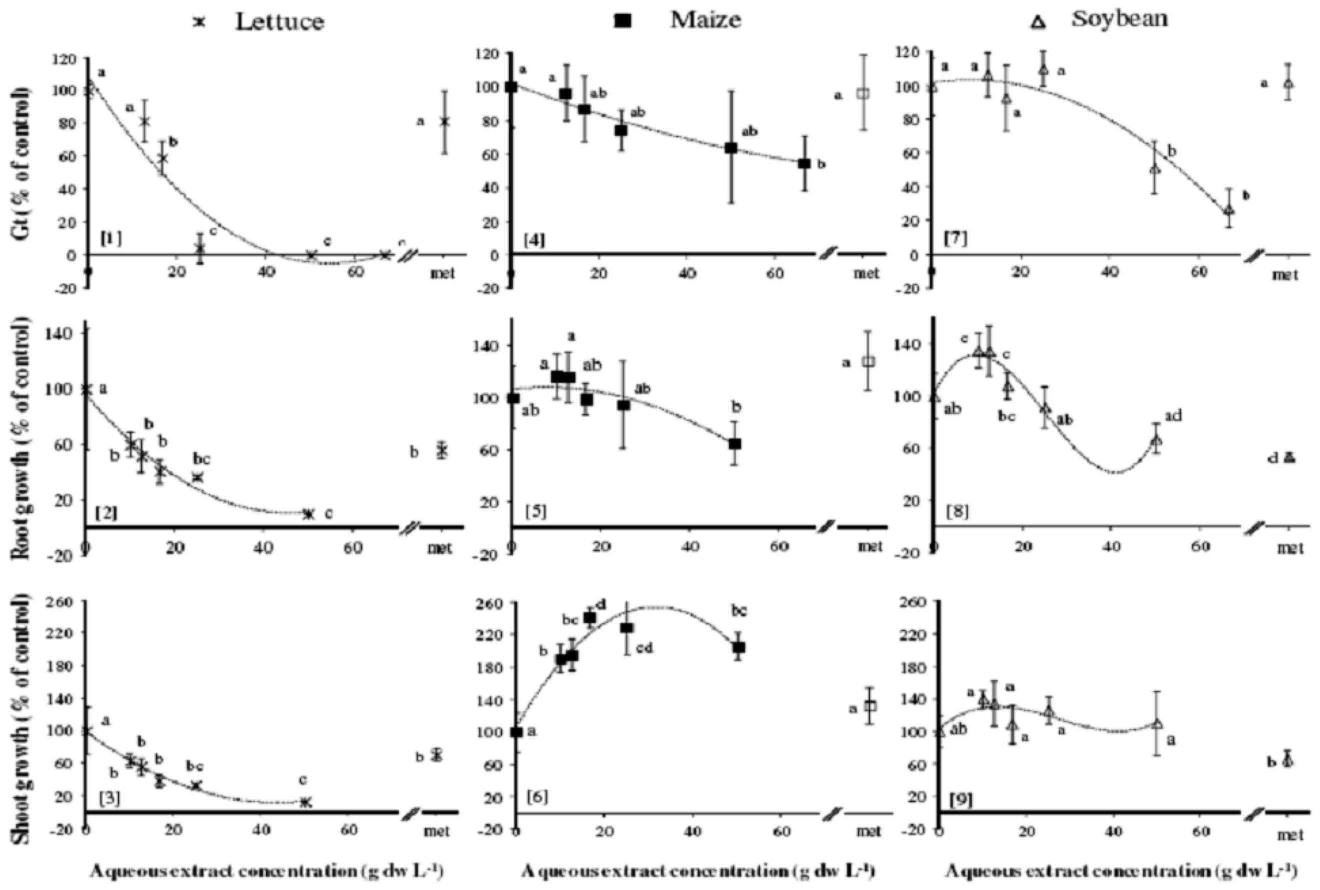

Figure 1. Dose-response curves for faba bean aqueous extracts on total germination and early growth of lettuce, maize and soybean. Within each species and parameter, mean values labeled with distinct letters are significantly different at $P \leq 0.05$ (LSD or Tamhane's T2 test). Symbols and vertical bars represent mean ± SD. Met, metolachlor 0.96 kg a.i.ha$^{-1}$; Gt, total germination.

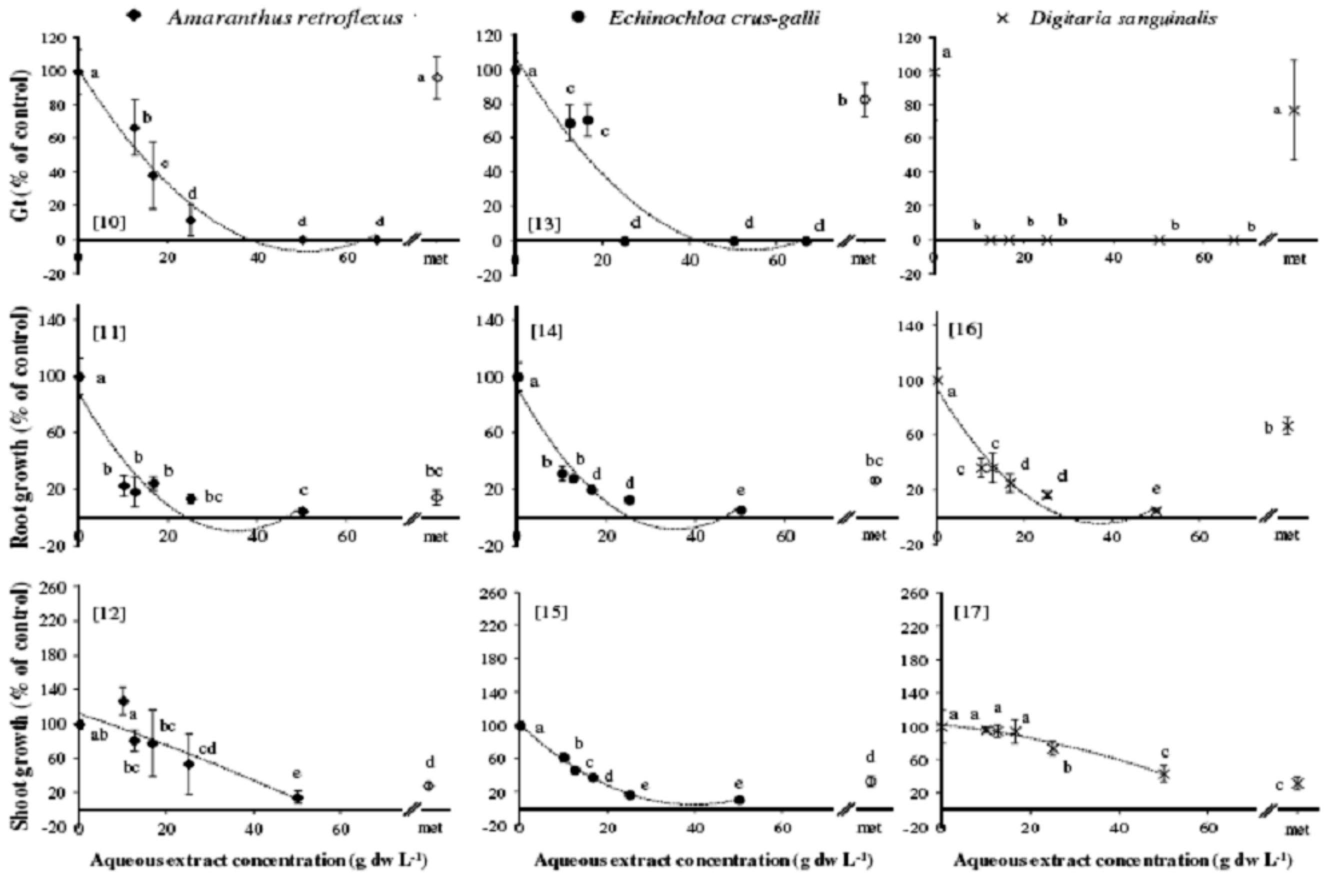

Figure 2. Dose-response curves for faba bean aqueous extracts on total germination and early growth of the weed species *Amaranthus retroflexus*, *Echinochloa crus-galli* and *Digitaria sanguinalis*. Within each species and parameter, mean values labeled with distinct letters are significantly different at $P \leq 0.05$ (LSD or Tamhane's T2 test). Symbols and vertical bars represent mean ± SD. Met, metolachlor 0.96 kg a.i. $ha^{-1}$; Gt, total germination.

**Table 2.** Regression analyses of dose-response curves for effects of faba bean aqueous extracts on the germination and growth parameters of crop and weed species expressed in percentage with respect to the control

| Species | | Regression equation | | $R^2$ | $IC_{50}$ (g dw $L^{-1}$) | $IC_{90}$ (g dw $L^{-1}$) |
|---|---|---|---|---|---|---|
| Lettuce | Germination | $y = 0.039x^2 - 4.232x + 109.12$ | [1] | 0.889 | 16.49 | 34.20 |
| | Root growth | $y = 0.041x^2 - 3.756x + 96.18$ | [2] | 0.970 | 14.63 | 45.81 |
| | Shoot growth | $y = 0.046x^2 - 3.994x + 98.62$ | [3] | 0.984 | 14.64 | o.r. |
| Maize | Germination | $y = 0.005x^2 - 1.016x + 102.33$ | [4] | 0.951 | o.r. | o.r. |
| | Root growth | $y = -0.025x^2 - 0.393x + 106.00$ | [5] | 0.803 | o.r. | o.r. |
| | Shoot growth | $y = -0.149x^2 + 9.412x + 105.80$ | [6] | 0.930 | o.r. | o.r. |
| Soybean | Germination | $y = -0.024x^2 + 0.402x + 101.92$ | [7] | 0.929 | 55.90 | o.r. |
| | Root growth | $y = 0.0058x^3 - 0.441x^2 + 6.861x + 101.26$ | [8] | 0.929 | 34.81 | o.r. |
| | Shoot growth | $y = 0.0028x^3 - 0.229x^2 + 4.648x + 102.57$ | [9] | 0.518 | o.r. | o.r. |
| *Amaranthus retroflexus* | Germination | $y = 0.042x^2 - 4.258x + 101.85$ | [10] | 0.963 | 14.13 | 31.13 |
| | Root growth | $y = 0.079x^2 - 5.551x + 88.17$ | [11] | 0.851 | 7.72 | 19.49 |
| | Shoot growth | $y = -0.005x^2 - 1.731x + 112.03$ | [12] | 0.803 | 32.63 | 51.33 |
| *Echinochloa crus-galli* | Germination | $y = 0.039x^2 - 4.203x + 107.53$ | [13] | 0.867 | 16.09 | 33.82 |
| | Root growth | $y = 0.079x^2 - 5.625x + 91.99$ | [14] | 0.932 | 8.47 | 20.45 |
| | Root growth | $y = 0.061x^2 - 4.859x + 100.71$ | [15] | 0.996 | 12.36 | 29.87 |
| *Digitaria sanguinalis* | Germination | – | | | – | – |
| | Root growth | $y = 0.070x^2 - 5.227x + 93.13$ | [16] | 0.946 | 9.44 | 22.97 |
| | Shoot growth | $y = -0.013x^2 - 0.561x + 102.14$ | [17] | 0.969 | 45.93 | o.r. |

$R^2$, coefficient of determination; $IC_{50}$, $IC_{90}$, concentrations required to obtain 50% or 90% inhibition of germination, root or shoot growth with respect to the control; o.r., out of range.

**Table 3.** Effects of faba bean aqueous extracts (g dw $L^{-1}$) on the germination indices (S, AS, CRG and MGT) of crop and weed species

| Species | Extract concentration | S | AS | CRG | MGT |
|---|---|---|---|---|---|
| Lettuce | 0 | 38.8 ±6.06 a | 108.1 ±20.67 a | 1.9 ±0.07 a | 34.8 ±6.31 a |
| | 12.5 | 12.9 ±6.29 b | 25.5 ±14.96 b | 1.6 ±0.09 c | 59.7 ±5.66 c |
| | 16.6 | 17.1 ±3.46 b | 33.0 ±10.41 b | 1.6 ±0.05 c | 59.5 ±4.81 c |
| | 25 | 0.7 ±1.52 c | 0.7 ±1.52 c | 1.4 ±0.00 | 72.0 ±0.00 |
| | 50 | 0. 0 ±0.00 c | 0.0 ±0.00 c | | |
| | 66.6 | 0.0 ±0.00 c | 0.0 ±0.00 c | | |
| | Metolachlor | 39.7 ±8.83 a | 114.5 ±25.14 a | 2.1 ±0.02 b | 25.3 ±1.82 b |

Contd.

Table 3. Contd.

| Species | Extract concentration | S | AS | CRG | MGT |
|---|---|---|---|---|---|
| Maize | 0 | 32.8 ±10.51 a | 113.9 ±35.62 a | 1.4 ±0.04 a | 42.3 ±2.73 a |
| | 12.5 | 28.8 ±3.43 ab | 97.7 ±14.30 ab | 1.3 ±0.03 ab | 48.4 ±3.04 ab |
| | 16.6 | 27.5 ±4.40 abc | 94.6 ±17.50 ab | 1.4 ±0.04 ab | 43.9 ±5.35 ab |
| | 25 | 21.0 ±3.78 bcd | 71.2 ±12.88 bc | 1.3 ±0.01 abc | 49.8 ±5.32 ab |
| | 50 | 15.8 ±9.77 cd | 52.3 ±34.50 c | 1.2 ±0.11 c | 56.3 ±14.57 b |
| | 66.6 | 13.6 ±4.32 d | 46.5 ±15.31 c | 1.3 ±0.02 bc | 53.0 ±8.72 ab |
| | Metolachlor | 29.9 ±7.31 ab | 103.4 ±24.83 ab | 1.3 ±0.04 ab | 43.8 ±3.09 ab |
| Soybean | 0 | 38.5 ±6.86 a | 132.2 ±23.04 a | 1.4 ±0.06 a | 36.9 ±6.60 a |
| | 12.5 | 35.2 ±3.66 a | 121.9 ±13.76 a | 1.4 ±0.04 ab | 41.4 ±2.84 ab |
| | 16.6 | 29.2 ±13.16 a | 98.9 ±15.16 ab | 1.3 ±0.05 bc | 47.4 ±7.59 ab |
| | 25 | 41.0 ±6.74 a | 139.9 ±24.59 a | 1.4 ±0.01 a | 40.0 ±2.12 ab |
| | 50 | 14.2 ±9.05 b | 49.5 ±32.63 bc | 1.3 ±0.06 a | 48.6 ±10.30 b |
| | 66.6 | 7.7 ±3.48 b | 27.3 ±12.39 c | 1.3 ±0.03 bc | 45.5 ±3.00 ab |
| | Metolachlor | 39.3 ±10.67 a | 134.2 ±37.35 a | 1.4 ±0.05 ab | 41.6 ±5.73 ab |
| *Amaranthus retroflexus* | 0 | 50.1 ±6.49 a | 127.8 ±17.39 a | 2.4 ±0.01 a | 24.4 ±0.98 a |
| | 12.5 | 25.3 ±5.61 b | 60.0 ±12.86 b | 2.2 ±0.06 bc | 33.9 ±2.37 bc |
| | 16.6 | 13.6 ±7.06 c | 31.4 ±16.61 c | 2.1 ±0.06 c | 36.4 ±3.58 cd |
| | 25 | 4.2 ±4.17 d | 9.4 ±10.31 d | 2.0 ±0.21 c | 41.0 ±8.25 d |
| | 50 | 0.0 ±0.00 d | 0.0 ±0.00 d | | |
| | 66.6 | 0.0 ±0.00 d | 0.0 ±0.00 d | | |
| | Metolachlor | 43.5 ±6.47 a | 109.3 ±17.37 a | 2.3 ±0.07 ab | 27.7 ±3.18 ab |
| *Echinochloa crus-galli* | 0 | 16.3 ±1.79 a | 39.9 ±5.45 a | 1.0 ±0.02 a | 82.5 ±4.00 a |
| | 12.5 | 9.8 ±1.53 c | 16.9 ±5.19 c | 1.0 ±0.07 b | 103.6 ±9.32 b |
| | 16.6 | 10.2 ±1.58 c | 18.4 ±4.14 c | 1.0 ±0.03 b | 100.6 ±3.49 b |
| | 25 | 0.0 ±0.00 d | 0.0 ±0.00 d | | |
| | 50 | 0.0 ±0.00 d | 0.0 ±0.00 d | | |
| | 66.6 | 0.0 ±0.00 d | 0.0 ±0.00 d | | |
| | Metolachlor | 12.5 ±1.49 b | 25.9 ±5.13 b | 1.0 ±0.03 ab | 94.5 ±7.75 b |
| *Digitaria sanguinalis* | 0 | 21.6 ±5.91 a | 56.5 ±14.97 a | 1.4 ±0.01 a | 61.1 ±4.81 a |
| | 12.5 | 0.0 ±0.00 b | 0.0 ±0.00 b | | |
| | 16.6 | 0.0 ±0.00 b | 0.0 ±0.00 b | | |
| | 25 | 0.0 ±0.00 b | 0.0 ±0.00 b | | |
| | 50 | 0.0 ±0.00 b | 0.0 ±0.00 b | | |
| | 66.6 | 0.0 ±0.00 b | 0.0 ±0.00 b | | |
| | Metolachlor | 17.3 ±6.60 a | 47.8 ±18.30 a | 1.4 ±0.04 a | 55.7 ±4.91 a |

S: Speed of germination; AS: Speed of accumulated germination; CRG: Coefficient of rate of germination; MGT: Mean germination time. Values represent means ± SD. For each species, mean values labeled with distinct letters are significantly different at $P \leq 0.05$ (LSD or Tamhane's T2 test).

Almost all parameters fitted to a quadratic model ($R^2$ values ≥ 0.803), according to the described dose-dependent inhibition caused by the faba bean extract (Table 2). Exceptions were soybean growth data, which better fitted to a cubic model due to the significant hormesis at the lower concentrations of the extract. No equation was calculated for *D. sanguinalis* germination, as no germination events were observed. Values of $IC_{50}$ and $IC_{90}$ for all parameters of maize, $IC_{90}$ for soybean and $IC_{50}$ for soybean shoot length were above the detectable range of the assay.

The complementary germination indices S, AS, CRG and MGT were calculated for germination rates above zero (Table 3), as their values are nulls when germination does not occur. The same information was obtained from S and AS indices, and CRG and MGT gave results consistent with those of S and AS. The little effect of the aqueous extracts on the germination of maize and soybean is confirmed. As most germination values for monocotyledon weeds were zero, CRG and MGT indices were poorly indicative in these cases. When considering S and AS, significant delays in the germination process were observed in lettuce and weeds at all the extract concentrations ($P < 0.001$), even those that had no effect on final germination. According to our results, the highest concentrations of the faba bean aqueous extract inhibited *A. retroflexus* and *E. crus-galli* germination completely, whereas lower concentrations significantly delayed the onset of germination.

## DISCUSSION

Only two studies of the effects of aqueous leachates of faba bean on several crops and weeds have been published to date (22,30). For *Vicia* species, the phytotoxic effects of aqueous extracts from hairy vetch (*V. villosa* Roth) have received attention. Hill *et al.* (19) reported slight reductions on maize germination and inhibition of root growth of *A. retroflexus* and *E. crus-galli*, but no effects on germination of these weeds. Ercoli *et al.* (14) found no effects of hairy vetch aqueous extracts on *A. retroflexus* germination, but a strong inhibition of root growth.

Plant material was selected from the evaluation of faba bean cultivars for mild-fresh winter areas (28), where cv. Prothabat 69 showed the smallest seed size besides the highest number of flowers per plant if compared to other cultivars with similar biomass production. These are important characters for the selection of a cultivar for green manuring purposes because i) the cost of seed is a major expense for faba bean growers (12) and the main deterrent to use it as green manure, and ii) faba bean plants are managed for mulch or green manure at flowering stage. Although allelochemicals can be found in any part of the plant, flowering plants are prone to contain higher quantities and wider variety of them, the flowers being especially rich in compounds that play important roles in biotic interactions like attraction of pollinating insects (17).

The *in vitro* green manure bioassay was carried out in order to check the reproducibility of our previous field observations under laboratory controlled conditions. Our hypothesis was that, if allelopathic interactions are responsible for the weed suppression observed in the field, then soluble allelochemicals released from the plant material into the soil solution would inhibit germination and early growth of a model species. Our results suggest that faba bean has a strong suppressive ability once incorporated into the soil. However, this experiment cannot exactly predict what would happen in the field (26,32) as the temperature and humidity conditions provided by the growth chamber were higher than those in the field, and favoured the rapid decomposition of plant material. Moreover, the lack of drainage from Petri dishes may favour the accumulation of allelochemicals, thus overestimating the suppressive activity of our plant material. As this experimental design does not support long-term assays, further experiments must be conducted under more realistic field conditions using rates based upon well established dose-response laboratory bioassays.

Prior to the bioassay of faba bean aqueous extracts, pH values of each solution were measured in order to discard any toxicity effects due to inadvertent acidification (3). According to Macías *et al.* (27), pH values between 5 and 7 do not affect lettuce development, so that pH was considered irrelevant in our case. On the contrary, the high EC and osmotic potential of the solutions may be exerting inhibitory effects on the germination and growth of test species, especially in the small-seeded ones. According to our results with PEG 6000, and as in Haugland and Brandsaeter (18) and Chon *et al.* (6), osmotic potential affected seed germination more than seedling growth. Our results are in agreement with literature data, that consider that values lower than 2 dS $m^{-1}$ and 0.07 osmol $kg^{-1}$ (corresponding to extract concentrations lower than 25 g $L^{-1}$) do not explain inhibitory effects *per se* (10,26). We cannot discard the concurrence of osmotic effects above the referred critical concentrations, but in all cases inhibitory effects were significant from extract concentrations lower than the minimum for the appearance of osmotic toxicity. Thus, our data strongly suggest that phytotoxins in the aqueous extracts are the main responsible for the observed inhibitory effects. Moreover, osmolarity seems irrelevant in forage crop species, as in studies involving crude extracts, the final effects are a result of complex interactions of several factors, and not solely due to the bioactivity of the compounds (11).

As expected, the type and magnitude of the effects observed for the dose-response bioassays were dependent on the extract concentration and the species. We performed germination and seedling growth bioassays individually, as different processes may have varying sensitivity to the effects of the active compounds present in the aqueous extract. Root growth was the most sensitive parameter measured, followed by germination and shoot growth. This observation is consistent with most other studies (31,35), probably because radicle is already emerged at the beginning of the experiment and therefore is the first organ that comes in contact with the toxic compounds present in the extract. The abnormalities observed in lettuce and weed seedlings are a typical response to the presence of allelochemicals (8). Visual observation also provides useful information related to the physiological effects and the mode of action of phytotoxic compounds, where inhibition of root hairs development are usually attributed to hormonal effects (37).

Some authors consider that Gt, which gave us an overall assessment of the inhibition or stimulation of germination, is not enough to evaluate phytotoxic effects on germination, and so have reported useful information provided by other indices (5,18). Indices S, AS, CRG and MGT allowed us comparing the dynamics and progress of germination between treatments, revealing delays in the germination process in cases where Gt did not show any effect. Some theoretical considerations can be extrapolated on these differences measured in short-time periods under field conditions, but these results should be compared with glasshouse or field data.

Dose-response curves together with the IC parameters are extremely useful to model phytotoxic effects, to effectively compare the results for all species, and to establish a reference to compare subsequent experiments (8). Lettuce was very sensitive to the global effects of the extracts. In fact, it is considered one of the most sensitive test species in allelopathy research due to its responsiveness to low quantities of phytotoxic compounds (8). This sensitivity is especially important when working with crude extracts, where the active compounds are typically present in low quantities (20). Nevertheless, lettuce alone would not detect all the variety and intensity of the phytotoxic effects. In fact, weeds proved to be more sensitive than lettuce for seed germination at a given concentration. Model forage crops, on the contrary, were not significantly affected or even sometimes stimulated by faba bean aqueous extracts. Although hormetic effects at the lower concentrations of an extract or a phytotoxin have been described previously (4,13), it is unusual to observe this phenomenon at high concentrations, as in the case of maize shoot growth. Nutrients released during the extraction of plant material may be partly responsible for the hormesis observed in forage crops, but we do not discard a bio-stimulative effect. The seed size is also an important factor when assessing the phytotoxicity of a given material, so the same concentration of an aqueous extract may seem more phytotoxic on small-seeded species rather than in the large-seeded ones (24). This is consistent with our results, and could partly explain the tolerance of maize and soybean and the sensitivity of weeds. This represents an additional advantage in systems based on “large-seeded” forage crops associated with “small-seeded” weeds.

The introduction of a commercial herbicide as an internal standard is rarely cited in literature (31), despite its usefulness to compare the biocidal activity with a synthetic herbicide model. The effectiveness of faba bean aqueous extracts for controlling weed germination at concentrations that do not affect crops was greater than that provided by metolachlor. As the primary site of absorption and action of metolachlor is the roots, the results for root growth inhibition highlight the biocidal potential of the aqueous extract of faba bean.

Reports of allelochemicals of faba bean vegetative biomass include the identification of phenolic compounds of slight phytotoxicity that are rapidly exuded from the emerging roots (1,2,34). Tannins are also known to be the main antinutritional factors reducing faba bean protein digestibility; nonetheless, Cubero and Duc (7) found no correlation among tannin and pyrimidine glucosides concentrations and resistance to pathogens. Our results for *in vitro* bioassays on the phytotoxicity of faba bean suggest the presence of phytotoxic substances that are almost partly released from the plant biomass in the soil solution as a result of its incorporation into the soil. So, identification of other potential allelopathic compounds from faba bean is required.

Interestingly, from our results faba bean aqueous extracts could enhance maize and soybean growth at concentrations that controlled their commonly associated weeds. When considering a potential herbicide for its use in the field, it is basic to ensure its capability of controlling most weeds without damaging the crop. This selectivity potential is typically estimated by means of the selectivity index (SI) calculated as the ratio between the doses that caused 10% of damage to the crop ($IC_{10}$) and 90% of damage to the weed ($IC_{90}$) (36): the larger selectivity index, the higher the sensitivity of weeds with respect to the crop. SI values above 1 are desirable for practical field application. When these selectivity indices are calculated on the basis of our dose-response curves, SI values for root growth range from 1.45 to 1.75 for maize, and from 1.13 to 1.33 for soybean. In the case of shoot growth, $IC_{90}$ could not be calculated for crops because stimulation was always observed. Then, faba bean represents a promising material for weed control. Glasshouse assays and field approaches are required for determining an optimal rate of plant material to achieve significant weed control and concomitantly promote crop growth.

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