https://doi.org/10.26651/allelo.j/2018-44-2-1166 0971-4693/94 Euro 20.00
Allelopathy Journal 44 (2): 233-244 (July, 2018) *International Allelopathy Foundation* 2018
Table: 2, Figs : 4, Diagram: 1.

Phytotoxic effects of Mediterranean plants extracts on lettuce, tomato and onion as possible additive in irrigation drips

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(Received in revised form: May 19, 2018)

ABSTRACT

The study aimed to identify the mixtures of natural products to be added to the drippers (used in drip irrigation) during their preparation, so that the slow release of phytotoxic substances with the passage of water reduces or eliminates the intrusion of roots in the drippers. In field studies, we selected five phytotoxic plant species [*Vetch villosa* Roth., *Brassica juncea* L., *Secale cereale* L., *Juncus effusus* L. and *Vallisneria natans* L.] and their hydroalcoholic extracts were tested on two dicotyledons [*Lactuca sativa* L. cv Cavolo di Napoli and *Lycopersicon esculentum* L.] and one monocotyledon, *Allium cepa* L. We determined the structure of the main components of each extract. The *Vetch villosa* extract was most inhibitory. Further studies are required to determine whether to use the extract as such or one or more of its individual components can be used as possible additive in subsurface irrigation drip.

Key words: *Allium cepa* L., *Brassica juncea* L., *Juncus effusus* L., *Lactuca sativa* L., *Lycopersicon esculentum* L., Mediterranean plants, phytotoxic extracts, phytotoxicity, secondary metabolites, *Secale cereale* L., *Vallisneria natans* L., *Vetch villosa* Roth.

INTRODUCTION

Plastic has many applications in agriculture (20), hence, at the end of crop production cycles, large quantities of plastic materials [PVC pipes for irrigation; polystyrene seedbeds; and empty containers of herbicides, pesticides and fertilizers] are disposed of improperly (21), which can pollute the groundwater. This has stimulated interest in other options e.g. the use of bioplastics, in subsurface drip irrigation (SDI) systems (4), where, drip lines are buried in soil at various depths depending on the soil type and the plants to be irrigated. SDI distributes precise amounts of water directly into the root area reducing the diseases and pests, and economising use of irrigation water. However, the main limitation is the intrusion of plant roots into the drippers, which may completely block water supply. The solution may be to add a phytotoxic (plant extract) compound directly in the plastic during the manufacture of drippers. Therefore there is need to identify phytotoxic plants, which slowly release phytotoxins in irrigation pipes to prevent the growth of plants in drippers, (which block the drippers) to ensure water supply tin the pipes. This study aimed (i). to identify easily accessible phytotoxic terrestrial or aquatic plants from the Mediterranean area to prevent the plants growth in dripper, (ii). to test their phytotoxicity in the field and then *in vitro* toxicity of their hydroalcoholic extracts against 3-recipients crops (lettuce, tomato, onion).

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MATERIALS AND METHODS

I. Donor Plants: We collected 5-common weeds plants [Hairy vetch (*Vetch villosa* Roth.) (23,24), brown mustard (*Brassica juncea* L.) (3,6,30), cereal rye (*Secale cereale* L.) (1,7,26), soft rush (*Juncus effusus* L.) (11,12) and vallisneria (*Vallisneria natans* L.)] at the flowering stage in spring (April-May) of 2015 near Naples (Italy). Voucher specimens (HERBSIDLS105) were deposited in Department of Functional Biology, University Federico II of Naples. These plants were easily available in large quantities, from geographically accessible locations and the extracts of which in some cases have been described as phytotoxic.

II. Recipient Plants

The recipient plant species were: two dicotyledons [*Lactuca sativa* L. cv Cavolo di Napoli and *Lycopersicon esculentum* L.] and one monocotyledon, *Allium cepa* L. The seeds of *L. sativa, L. esculentum* and *A. cepa* were purchased from Ingegnoli SpA. All undersized or damaged seeds were discarded, and the uniform seeds were selected.

III. Apparatus

Column chromatography (CC) was carried out on Merck Kieselgel 60 (230−400 mesh). Electronic Impact Mass Spectra (EI-MS) were obtained with a QP-5050A (Shimadzu) EI 70 eV spectrometer. Anal. TLC: Kieselgel 60 F254 plates (0.2-mm; Merck); visualization under UV light and by spraying with H_2SO_4:CH_3CH_2OH (4:96), followed by heating for 7 min at 120 °C. Liquid chromatography (LC) analysis was carried out using UFLC prominence series (Shimadzu Corp., Kyoto, Japan), equipped with a vacuum degasser, a quaternary pump, an autosampler, a column heater and PDA detector. Separation was accomplished using an Phenomenex Luna C18 column (Phenomenex, Italy) (5.0 µm, 2.0 × 30 mm).

IV. Extraction and Isolation

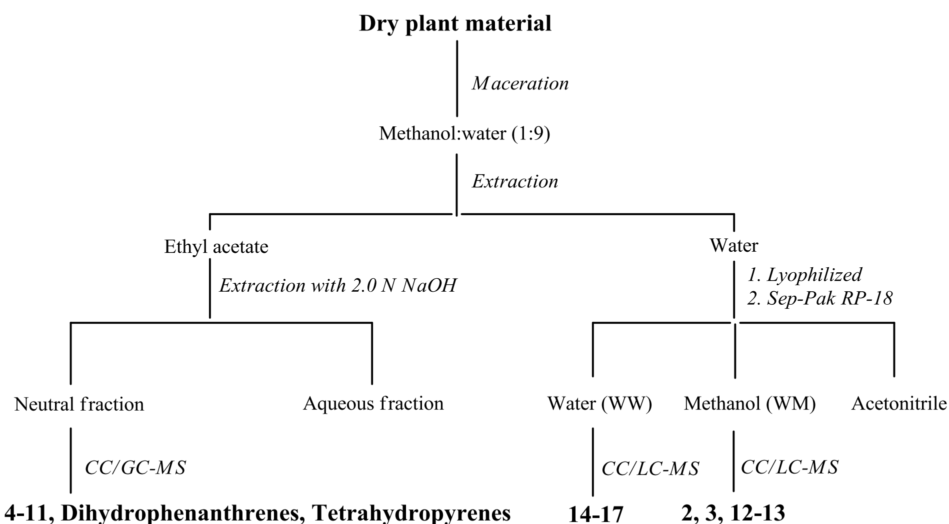
The branches and leaves of five donor plants were air-dried and pulverized. The powdered material was extracted with methanol water (1:9) for 72 h by maceration. The extract was filtered and concentrated to dryness *in vacuo* at 40 °C. The resulting extract was then suspended in water and partitioned with ethyl acetate and after drying, gave ethyl acetate-soluble residue. The organic fraction was fractionated into acidic and neutral fractions with aqueous 2N NaOH solution. The neutral solution was washed with water and concentrated *in vacuo*, while the aqueous solution was first acidified with aqueous 2N HCl to neutral pH and then extracted with ethyl acetate, according to the scheme below.

The neutral fraction was subjected to silica gel column chromatography using gradient solvent systems. The fractions cleaner were injected to GC-MS. For comparison with the data reported in the literature and in the database of the detector, compounds **1**, **4-11**, dihydrophenanthrenes and pyrenes were identified (10,11,15,16,27-29).

A volume equal to one tenth of water was lyophilized (320 mg) and filtered on Sep-Pak C18, eluting with water, methanol and acetonitrile. The first two fractions were analyzed by LC-MS and compounds **14-17** were identified from the water fraction and compounds **2, 3, 12** and **13** were identified from the methanol fraction (Table 1).

Table 1. Major components of selected plants.

		Hydroalcoholic extract		
V. villosa	*B. juncea*	*S. cereale*	*J. effusus*	*V. natans*
Cyanamide (**1**)	Allyl isothiocyanate (**4**)	2-(3H)-benzoxazolinone (BOA) (**12**)	Vanillic acid (**14**)	Vanillic acid (**14**)
cis-Cinnamic acid (**2**)	3-Butenyl isothiocyanate (**5**)	2,4-dihydroxy-1,4(2H)-benzoxazin-3-one (*DIBOA*) (**13**)	p-Coumaric acid (**15**)	Caffeic acid (**16**)
trans-Cinnamic acid (**3**)	Benzyl isothiocyanate (**6**)		Dihydrophenanthrenes (**18-20**) R = H, Glc R' = H, Glc R'' = H, Glc, CH_3	Ferulic acid (**17**)
	cis-3-Hexen-1-ol (**7**)		Tetrahydropyrenes (**21-22**) R = H, Glc, CH_3 R' = H, Glc, CH_3	
	trans-2-Hexenal (**8**)			
	Benzaldehyde (**9**)			
	β-Ocimene (**10**)			
	α-Farnesene (**11**)			



Scheme 1. Separation procedures of compounds **1-17.**

V. GC-MS/MS analyses

About 1 mg of neutral fractions were dissolved in 0.8 mL acetone and subjected to Triple Quadrupole Gas Chromatograph Mass Spectrometer (GC-MS). Qualitative analyses were obtained on the Shimadzu TQ-8030 (Milano, Italy) equipped with a RTXi capillary column (Sigma Aldrich; 30 m × 0.25 inside diameter x 0.25 film thickness); flow rate of 1.4 mL/min; injector temperature: 240 °C; splitting ratio: 1 : 20; detector temperature: 275 °C using helium as carrier gas and argon in collision cell. Analyses were performed with the following temperature program: 40 °C for 5 min, 220 °C at 5 °C/min, and 220 °C for 10 min. Injection volume was 1 µL. The acquisition parameters were as following: interface voltage 1.4 kV; interface temperature 250 °C; desolvation line temperature, 250 °C; desolvation gas, helium; desolvation gas flow rate, 3.5 L/min; drying gas, helium; drying gas flow rate, 20 L/min; collision gas, argon; dwell time, 20 ms; and collision gas pressure, 240 kPa.

VI. LC-MS/MS analyses

About 1 mg of fractions obtained from column chromatography of fractions WW and WM were dissolved in 0.8 mL MeOH filtered through a 0.45 mm filter and subjected to high performance liquid chromatography (HPLC). For qualitative analysis, the Shimadzu (Milano, Italy) LCMS-8040 Triple Quadrupole Liquid Chromatograph Mass Spectrometer (LC-MS/MS) was used. Separations were accomplished on Phenomenex Luna C18 column (100 mm × 2.1 mm, 1.6 µm) at a flow rate of 0.30 mL/min with water and acetonitrile gradients as mobile phase. The column temperature was maintained at 45 °C and the injection volume was 10 µL. Separation of compounds was monitored with DAD at 254 and 190 nm and with a mass spectrometry detector. Mass spectrometric analysis (ESI) was carried out on LCMS-8030 triple-quadrupole mass spectrometer (Shimadzu, Kyoto, Japan). Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was set in the negative and positive ionization mode with spectra acquired over a

mass range of 50-1000 *m/z*. The acquisition parameters were as following: interface voltage, 4.5 kV; interface temperature, 230 °C; desolvation line temperature, 230 °C; heat block temperature, 350 °C; desolvation gas, nitrogen; desolvation gas flow rate, 3.5 L/min; drying gas, nitrogen; drying gas flow rate, 20 L/min; collision gas, argon; dwell time, 20 ms; and collision gas pressure, 240 kPa. The most appropriate precursor ion, daughter ion, cone volt-age, collision energy (CE) were adjusted according to each analyte.

VII. Phytotoxicity bioassays

(i). Soil bioassay

From the aerial parts and roots, individually considered, and whole plants, aerial parts and roots mixed and pulverised together, of selected donor plants, 3-doses (x, 2x and 3x; where x is equal to 60, 52, 42, 47 and 0.53 g, quantity of each plant which in nature grows in a surface area of 400 cm^2, corresponding to the area of the containers used for the tests below) of *V. villosa*, *B. juncea*, *S. cereale*, *J. effusus*, *V. natans*, were applied, respectively. The experimental treatments consisted of three factors (i). Donor plants: 5 (*V. villosa*, *B. juncea*, *S. cereale*, *J. effusus*, *V. natans*), (ii). Recipient plants: 3 (lettuce, tomato, onion) and (iii). Biomass doses buried in soil: 3 (x, 2x, 3x) as described in Table 2.

Table 2. Details of donor crops biomass buried in soil for soil bioassay.

	Quantity (in g) buried in soil		
Donor plants	**x**	**2x**	**3x**
Brassica juncea	52	104	156
Juncus effusus	47	94	141
Secale cereale	42	84	126
Vetch villosa	60	120	180
Vallisneria natans	0.53	1.06	1.59

The phytotoxic effects were evaluated by burying in the soil the particular dose of each selected plant in rectangular containers (10 cm x 40 cm = 400 cm^2), at a depth of 5-7 cm, about 6 months before the sowing of lettuce, tomato, onion occurred at the beginning of summer. The phytotoxicity tests were done on the germination of recipient plants: *Lactuca sativa* L., *Lycopersicon esculentum* L. and *Allium cepa* L. All undersized or damaged seeds of recipient plants were discarded and the uniform test seeds were selected, sterilized with sodium hypochlorite solution (0.4 %, v/v) for 3 min and soaked in sterile distilled water for 30 min. The containers were irrigated with drip irrigation system to keep the soil moisture at field capacity. After 4-weeks, 3-seeds of lettuce, tomato and onion were planted at plant to plant distance of 3 cm, in rows 3 cm apart. The treatments were replicated thrice in completely randomized design. The data were transformed to percent of control for analysis. The germination (%) index (GI) was calculated as under:

$$GI\ (\%) = (RSG \times RRG)/100$$

Where, RSG: Relative seed germination (%) and RRG: Relative root growth.

(ii). *In-vitro* Bioassay

The phytotoxicity was tested from whole plant parts biomass in ratios corresponding to the relative amounts of donor plants that grow per 400 cm^2 of land (60, 52, 42, 47 and 0.53 g

of *V. villosa*, *B. juncea*, *S. cereale*, *J. effusus*, *V. natans*, respectively). The plant materials were frozen at -80 °C, powdered and macerated in dark at room temperature with 200 mL of methanol:water (1:9) for 5 days. The hydroalcoholic solutions were filtered using Whatman No. 1 filter paper, the methanol was removed in vacuum at 40 °C using a rotary evaporator. The obtained residues from *V. villosa*, *B. juncea*, *S. cereale*, *J. effusus*, *V. natans* extracts weighed 1.05, 0.98, 0.89, 0.85 and 0.015 g respectively and were stored at -80 °C until needed for further tests.

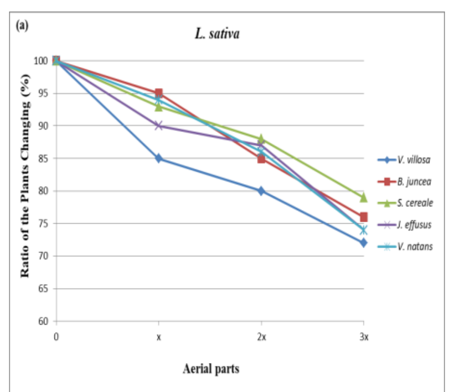
The target seeds (lettuce, onion and tomato) were surface sterilized with sodium hypochlorite solution (0.4 %, v/v) for 3 min and soaked in sterile distilled water for 30 min. The hydroalcoholic extracts were tested on the germination of target species at 33 %, 50 % and 100 % concentration, prepared using 10 mM MES buffer (2-(N-morpholino-ethanesulfonic acid, pH 6). Then, 25 sterilized seeds of recipient plants were separately placed on the filter paper (Whatman No. 1) in 5 cm Petri dishes. After adding the seeds and 4.5 mL of the test solution, the Petri dishes were sealed with Parafilm® to ensure closed-system models were tested and kept in a growth chamber (KBW Binder 240) at 25 °C, in the dark (5 days for lettuce and tomato, and 7 days for onion). After growth, the recipient plants were frozen at -20 °C to avoid subsequent growth until measurement. The crude extracts of donor plants were tested on germination of the recipient plants through measuring the percentages of the germination index (GI), which were estimated before. Treatments were arranged in a completely randomized design with three replications and data were transformed to percent of control for analysis.

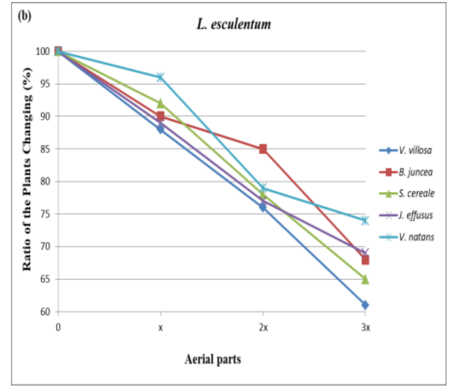
RESULTS AND DISCUSSION

We determined the phytotoxicity of five common weeds [viz., *V. villosa*, *B. juncea*, *S. cereale*, *J. effusus*, *V. natans*] of Mediterranean area on recipient plants (lettuce, tomato and onion) in soil bioassay. The biomass (equivalent to that present in field conditions) of these 5-donor plants was buried in soil in containers at the end of autumn for decomposition. Thereafter the recipient plants (lettuce, tomato and onion) were sown at the beginning of summer. The buried biomass of donor weeds reduced the number of recipient plants in each container, the reduction was dose-dependent on the amount of buried biomass i.e. the population of recipient plants depended on the donor plant and biomass dose. The lettuce and onion proved most sensitive crops, while among the donor plants, Vetch proved most inhibitory to donor plants (Fig. 1).

Plants produce a wide number of secondary metabolites that can be either actively released by plants (8,13,17) or passively produced during the decomposition process of both above- and below-ground plant residues (2,9,22). Natural products are the expression of the individuality of a species and they affect a wide range of biological activities. Many of these compounds can interfere with the germination and/or growth of other plants (14,25) and in this way they can affect structural and functional processes of coexisting plant species at community level (5,19). In particular, many studies evaluate the effects of natural substances on germination and plant growth, focusing mainly on their potential use in agroecosystem management (14,18,31-33).

Aerial parts: The extracts of aerial parts of donor plants inhibited the seed germination of recipient crops. In considering only the aerial parts of the donor plants studied in dose corresponding to the biomass quantity produced in the field (x), the germination was inhibited from 3 to 15 %, regardless of the donor plant (Fig. 1). The extract of *V. villosa* was most inhibitory and caused 15 % inhibition in *L. sativa* and *A. cepa*, and ~12 % in *L. esculentum* (Fig. 1). The amount of aerial biomass corresponding to twice the dose produced in the field per unit area (denoted as 2x) decreased the seedlings numbers between 12 and 20 % in *L. sativa*, 15 and 25 % in *L. esculentum* and 12 and 15 % in *A. cepa* (Fig. 1). The most inhibitory extract of *V. villosa*, caused 30 % inhibition in *A. cepa* (Fig. 1). Finally, an amount of aerial biomass corresponding to three times the dose produced in the field (denoted as 3x) decreased the seedling numbers between 20 and 28 % in *L. sativa* and *A. cepa*, and between 25 and 40 % in *L. esculentum* (Fig. 1). The most inhibitory extract was from *V. villosa*, with peak inhibitions of 30-40 % in all three donor crops (Fig. 1).





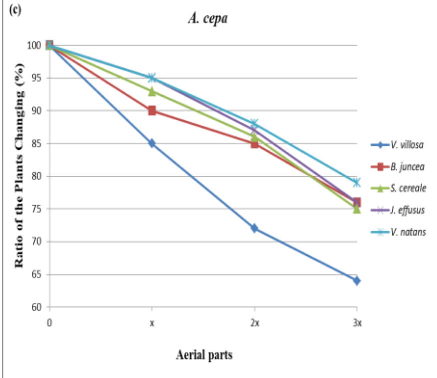
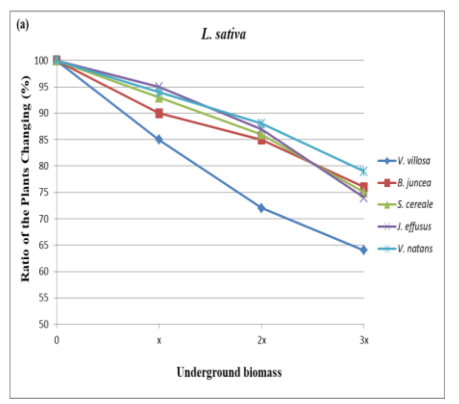
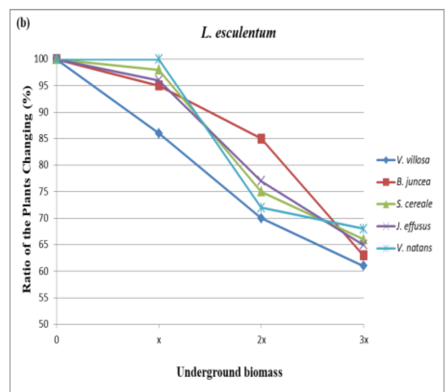


Figure 1. Number of lettuce, tomato and onion seedlings (% of control) germinated in relation to the buried amount of aerial biomass of vetch, mustard, rye, rush and vallisneria. Three different quantities were prepared from the aerial parts of selected plants at different doses, where x is equal to 60, 52, 42, 47 and 0.53 g of vetch, mustard, rye, rush and vallisneria, corresponding to the amount of each plant produced in a 400 cm^2 in the field.

Roots: This experiment determined the effects only on roots of treated recipient plants, using the same protocol. Considering only the roots of recipient plants using the dose corresponding to the quantity produced in the field (denoted as x) for the considered unit area, the inhibition ranged between 5 and 10 % for *L. sativa* and *A. cepa* and < 5 % in *L. esculentum* (Fig. 2). An exception was the extract of *V. villosa,* with 15 % inhibition in *L. sativa* and *L. esculentum* and 20 % inhibition in *A. cepa* (Fig. 2). An amount of root biomass corresponding to twice the dose produced in the field (2x) for the considered unit area caused decrease of 10 and 15 % in *L. sativa* and between 15 and 30 % in *L. esculentum* and *A. cepa* (Fig. 2). The most active extract was that of *V. villosa*, with peak inhibitions up to 30 % in all three recipient crops. Finally, an amount of root biomass corresponding to three times the dose produced in the field (3x) for the considered unit area caused 20-35 % inhibition in *L. sativa,* 30-40 % in *L. esculentum* and 25-40 % for *A. cepa*. The most inhibitory extract was that of *V. villosa*, with peak inhibitions up to 40 %.





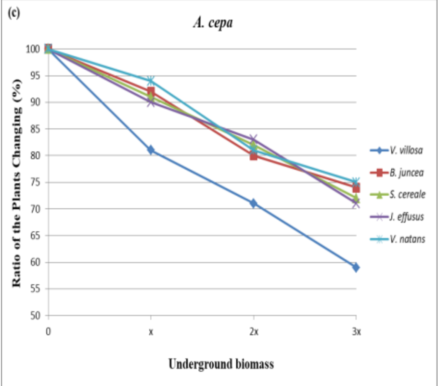
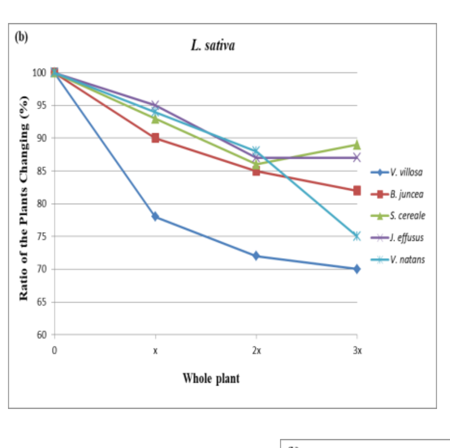
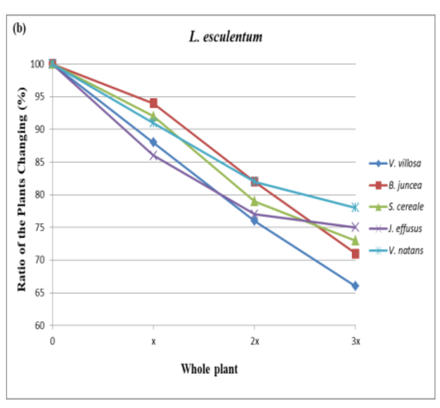


Figure 2. Number of lettuce, tomato and onion seedlings (% of control) germinated in relation to the buried amount of underground root biomass of vetch, mustard, rye, rush and vallisneria. Three different quantities were prepared from the roots (underground biomass) of selected plants at different doses, where x is equal to 60, 52, 42, 47 and 0.53 g of vetch, mustard, rye, rush and vallisneria, corresponding to the amount of each plant produced in a 400 cm^2 in the field.

Whole plant: The experiment was repeated with the biomass of whole plant (aerial parts + roots). Considering the entire plant (aerial parts and roots) in dose corresponding to the quantity produced in the field (x) for the considered unit area, there was 5-15 % inhibition in all recipient plant species, with the extract of *V. villosa* 5-10 % more active than others (Fig. 3). A dose of biomass corresponding to twice the quantity produced in the field (2x) for the considered unit area caused 10-20 % inhibition in *L. sativa* and *A. cepa* and 15-25 % in *L. esculentum* (Fig. 3). Once again, the most inhibitory extract was that of *V. villosa*, with peak inhibitions of 25-30 %. Finally, an amount of biomass dose corresponding to 3-times the quantity produced in the field (3x) for the considered unit area caused decrease of 10-30 % in *L. sativa*, 20-35 % in *L. esculentum* and 20-40 % in *A. cepa* (Fig. 3). The most inhibitory extract was that of *V. villosa*, with peak inhibitions up to 40 % in all three recipient crops.





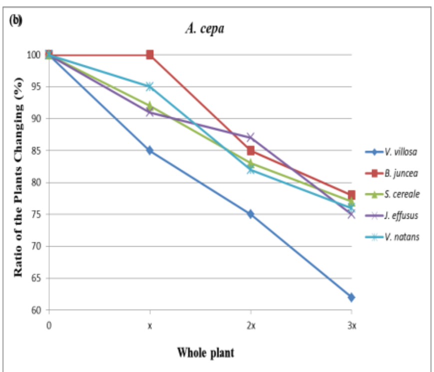


Figure 3. Number of lettuce seedlings, tomato and onion (% of control) germinated in relation to the buried amount of whole plant biomass of vetch, mustard, rye, rush and vallisneria. Three different quantities were prepared from the whole parts of selected plants at different doses, where x is equal to 60, 52, 42, 47 and 0.53 g of vetch, mustard, rye, rush and vallisneria, corresponding to the amount of each plant produced in a 400 cm^2 in the field.

Tested extracts and recipient crops.

Then we studied the *in-vitro* phytotoxicity of hydroalcoholic extracts of 5-selected donor plants to determine their inhibitory or stimulatory effects on the seeds germination and seedling growth (roots and seedling growth hypocotyls elongation) of recipient plants grown with and without hydroalcoholic extracts.

The five dry hydroalcoholic extracts, dissolved in 200 mL of 10 mM MES buffer showed similar inhibitory effects, with peak inhibitions up to 80 % for the extracts without dilution (CE), and up to 70 % and 60 % if diluted 1:2 and 1:3, respectively (Fig. 4). Generally, the most inhibitory hydroalcoholic extract was of *V. villosa*, causing 10 % more inhibition than extracts from other donor plants (Fig. 4).

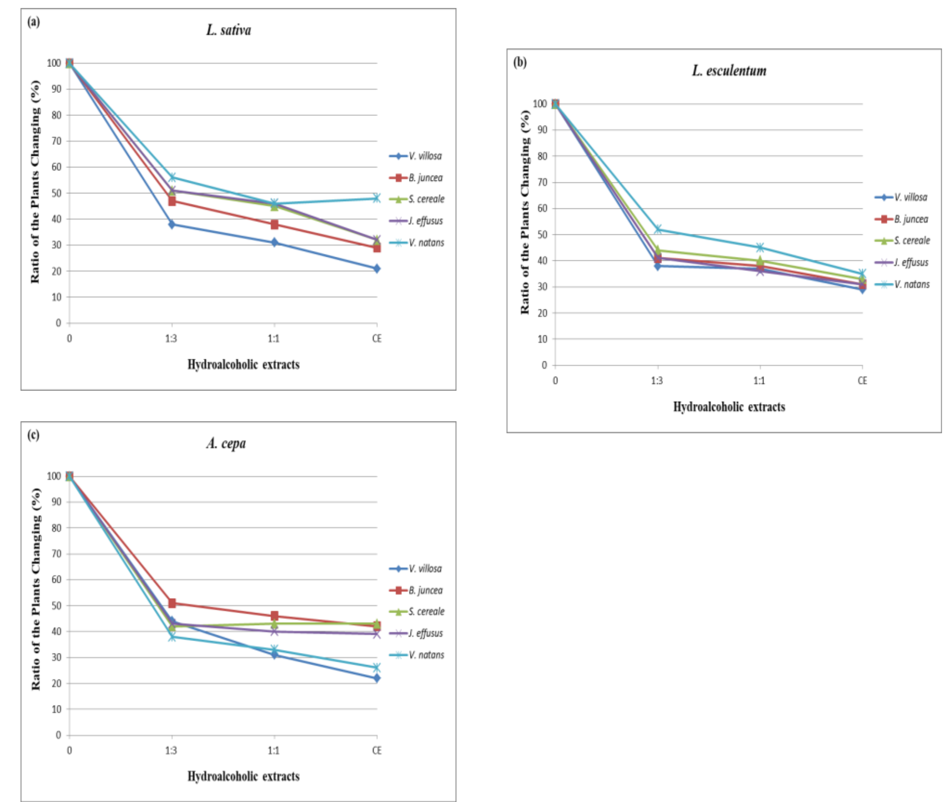


Figure 4. Effect (% of control) of the five hydroalcoholic extracts of vetch, mustard, rye, rush and vallisneria on seeds of lettuce, tomato and onion. The phytotoxicity of the crude extract (CE) and of the dilution 1:1 and 1:3 were reported.

ACKNOWLEDGMENTS

We would like to thank the AIPRAS Onlus (Associazione Italiana per la Promozione delle Ricerche sull'Ambiente e la Salute umana) for providing funds and Professor Antonino Pollio of the Department of Structural and Functional Biology of the University of Naples for the identification of the donor plants.

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