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Allelopathy of plants from Deliblato Sands-Serbia I. Allelopathic influence of *Festuca vallesiaca*

L. DJURDJEVIĆ*, G. GAJIĆ, S. JARIĆ, O. KOSTIĆ, M. MITROVIĆ, LJ. OBERAN and P. PAVLOVIĆ

Department of Ecology, Institute for Biological Research 'Siniša Stanković', University of Belgrade, Bulevar Despota Stefana 142, 11060 Belgrade, Serbia
E. Mail: kalac948@gmail.com

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ABSTRACT

The *Festuceto-Potentilletum arenariae* steppe community is dominated by *Festuca vallesiaca* Schleich. We investigated its allelopathic influences on seed germination and seedling growth of neighbouring plants (*Cynodon dactylon* and *Lolium perenne*), by determining the amount of total phenolics and phenolic acids in the aboveground parts of *F. vallesiaca*, litter and soil. An aqueous extract of the aboveground parts of *F. vallesiaca*, hydroponic growth solution in which *F. vallesiaca* grew, root exudates, its litter, soil and the phenolic-containing fraction from the soil inhibited the seed germination and seedling growth of neighbouring plants. Total phenolic compounds contents followed the order: vegetative *F. vallesiaca* parts > litter > soil. The same phenolic acids detected in the aboveground parts of the dominant *F. vallesiaca* were also found in plant litter and soil, but in significantly lower amounts. Phenolics produced in its tissue accumulated in the litter and soil, reaching toxic concentrations, thus inhibiting the seed germination and seedling growth. In addition to other ecological factors, the dominance of *F. vallesiaca* mainly resulted from its negative allelopathic effects on other species due to the synthesis and secretion of phenolics, which accumulate in the litter and soil.

Keywords: Allelopathy, degraded habitats, *Festuca vallesiaca*, growth and germination inhibition, phenolics, phenolic fraction, root exudates, sandy soil, steppe community, water extract.

INTRODUCTION

The Eurasian Steppes is grassland [4,000 to 5,000 mile-long, running from central Europe (Western border of Hungary) to Asia (Eastern border of Mongolia). At its western edge, there are Steppes in Pannonian region of Austria, dominated by members of Poaceae, the fourth largest plant family [about 10,000 species under 660-700 genera (24)].

The dominance of trees, bushes and species from the herbaceous layer arises due to their negative allelopathic effects on other species in the phytocoenosis (7,15,18,35). Allelochemicals are present in all types of plants and tissue, (leaves, flowers, fruits, stems, roots, rhizomes, seeds, and pollen). They are released into the soil rhizosphere through aboveground leaching, microbial decomposition of plant residues, volatilization and root exudation (15,18,28,35,38). Most allelochemicals are secondary metabolites, of which phenolic

*Correspondence author

compounds are the most intensively investigated group of compounds. When susceptible acceptor plants are exposed to allelochemicals, seed germination, growth and development may be affected, owing to the negative impacts on many physiological processes [plant-water relationships (3,4), net P, K, and water uptake (31), transpiration, water utilisation, leaf area expansion (11), stomatal aperture, photosynthesis, protein synthesis, respiration (19,26), and chlorophyll accumulation (43)].

The dominance of certain grass species and their phytotoxicity are consequences of their allelopathic effect on other species in grasslands (7,8,13,28,29,35,39,40). Many grasses produce secondary metabolites which are toxic to seed germination and growth of other plants in plant communities (13,35). The steppe element *Festuca vallesiaca* Schleich is distributed in southern and eastern Europe, temperate Asia and Asia Minor, this densely-tufted perennial plant is frequently found in Serbia in xerothermic meadows, pasture land and rocky areas (sub-Mediterranean and steppe in character). It forms numerous phytocoenoses,creating dense cover and conditioning the physiognomy of the whole community (36). In general, transitions from more arid grasslands to montane steppe communities have a predictable pattern in terms of vegetation structure and are associated with the scarcity or absence of C4 grasses and short-lived taxa, the increasing importance of perennial forbs and perennial C3 grasses and the emergence of *Festuca vallesiaca* as the dominant species (37). According to data from Wagner, similarities between the dominant species in the montane grassland group of this study and the dominant or diagnostic species from the north-western Tien Shan include the widespread *Festuca vallesiaca, Iris sogdiana, Koeleria cristata, Poa angustifolia,* and *Bromopsis inermis* (41). Furthermore, in the semi-arid steppe rangelands of Central Turkey, *F. vallesiaca* and *Thymus sipyleus* ssp *rosulans* have become the dominant species on degraded pastures. It is believed that decreases in species richness and abundance correlate with the increasing prevalence of these two species (23).

In the subalpine meadows of the Alps and other mountains of southern Europe, similar to the domination of *F. vallesiaca* in arid grasslands and montane steppe communities, the meadows are often dominated by *Festuca paniculata*, a slow growing caespitose grass that becomes overdominant and reduces the biodiversity when mowing is abandoned. Then its abundance increases from 38 to 70 % of the biomass. Allelopathy could be one of the mechanisms determining the abundance of *F. paniculata* (8,9,39,40).

The *F. vallesiaca* grows in tussocks, surrounded by growth inhibition zones with few other meadow-steppe plant species in the Deliblato Sands *Festuceto-Potentilletum arenariae* steppe community may be a result of an allelopathic influence on associated steppe plants. To investigate this phenomenon, chemical analysis was done to identify the phenolic allelopathic compounds released by fescue, i.e. the total phenols and phenolic acids (free and bound forms). This analysis included the aboveground parts of *F. vallesiaca*, its partially decomposed litter and the sandy soil of its rhizosphere. We tested the allelopathic potential of *F. vallesiaca* a steppe community on germination of seeds and the growth of seedlings of two grass species : *Cynodon dactylon* and *Lolium perenne*, which grow between its tussocks, but are sparse and suppressed. To monitor the effects of natural concentrations of phenolic acids (free forms) detected in soil under *F. vallesiaca* plants on seed germination and seedling growth, we made a series of their concentrations. We also studied the effects of *F. vallesiaca* root exudates and hydroponic growth solution in which *F. vallesiaca* grew on the seed germination and seedling growth of *C. dactylon*, *L. perenne* and *F. vallesiaca.* This is the first study, which, investigated the allelopathic influences of *F. vallesiaca* on other herbs.

MATERIALS AND METHODS

I. Description of Deliblato Sands and plant community

Deliblato Sands is located in the southern part of Serbia, between the River Danube and the western Carpathian slopes, 40 km northeast of Belgrade. It is a 35.4 km long and approximately 20 km wide region, and it extends from south-east to north-west. Once called the European Sahara, Deliblato Sands represents a unique landscape in Europe due to its genesis, orography, climate, and specific flora and fauna. In section 331/1 of the Rošijana locality (N 45^0 55′ 03″ E 21^0 06′ 55″), a study area of 100 x 100 m was selected. The habitat lies on a gentle slope, north-east facing sand dune. The short, well-developed tufts of dominant grass *F. vallesiaca* gives the entire community a specific appearance, with other meadow-steppe species present in relatively low numbers (Table 1). A control area without vegetation (Control sandy soil), is situated several hundred metres away on a bare sand dune.

II. Sampling of soil, litter and plant material for phenolics analyses

Plants were collected during the flowering on June 18, 2011. The aboveground parts of *F. vallesiaca* were uniformly collected along a transect from five 10 x 10 m plots (5 x 500 g) in the study area. The material was air-dried, ground and passed through a 2 mm sieve prior to phenolic compounds analysis. Litter mainly consisted of the partially decomposed leaves and stems of *F. vallesiaca* and was uniformly collected along a transect from five 10 x 10 m plots (5 x 500 g) in the study area. The material was air-dried, ground and passed through a 2 mm sieve prior to phenolic compounds analysis. The soil beneath the dominant *F. vallesiaca* was sampled simultaneously with the litter. After the removal of the partially decomposed litter, soil samples of the control sandy soil (without plants) and of soil beneath *F. vallesiaca* plants were taken from the surface layer (0-10 cm). Each sample was of equal weight (1 kg) and five replicates were gathered per plot. After the removal of visible plant remains, the soil was air-dried, ground and passed through a 2 mm sieve.

III. Preparation of phenolic-containing extracts from *F. vallesiaca* vegetative parts and plant litter

Both phenolic acids and total phenolic were extracted from plant material (5x2 g) with distilled water for 24 h. Water extracts were evaporated, the residues were dissolved in water (pH 2.0 adjusted with 2N HCl) and transferred to ethylacetate. The mixture was evaporated to dryness and the residue was dissolved in 4 mL of 80% (v/v) MeOH (phenolics free) and used for HPLC analysis or stored at -20 °C until use. Bound phenolics were prepared by boiling the residue in 2N HCl for 60 min and transferred to ethylacetate. For a detailed procedure, see Djurdjević *et al.* 2007 (17).

IV. Extraction of phenolics from soil samples

Free forms of phenolics were extracted from 5x30 g of dried sandy soil with distilled water for 24 h. The extracts were evaporated, adjusting pH to 2.0 with 2N HCl and transferred to ethylacetate. The mixture was evaporated to dryness and the residue dissolved in 4 mL of 80% (v/v) MeOH (phenolics free). To obtain the bound phenolics

residual soil was treated with 15 ml of 2N NaOH and after boiling for 24 h the mixture was acidified with concentrated HCl to pH 2.0, phenolic compounds were transferred into ethyl acetate (3x50ml) and were evaporated to dryness. Dry residue was dissolved in 4 ml 80% (v/v) MeOH(17).

V. Preparation of phenolic-containing extracts from soil for biotesting

The phenolic fraction from the surface layer of soil beneath *F. vallesiaca* plants and from the control soil without plants was extracted for biotesting from 5x20 g of dried sandy soil with 30 ml of 2 N NaOH and after boiling for 24 h, the mixture was acidified with concentrated HCl to pH 2.0. Phenolic compounds were transferred to ethyl acetate (3x50 ml) and evaporated to dryness. The dry residue was dissolved in 4 ml 80 % MeOH.

VI. Determination of total phenolics

Total phenolics content (free and bound forms) were determined in plant or soil using the Folin-Ciocalteu reagent and calculated with ferulic acid (Serva, Germany) as standard, with the absorbance measured at 660 nm (a Shimadzu UV 160 spectrophotometer). Take a 0.02 ml aliquot of the sample solution from MeOH extracts for free phenolics and 0.005 ml for bound phenolics (see above: III. Preparation of phenolic-containing extracts from *F. vallesiaca* vegetative parts and plant litter and IV. Extraction of phenolics from soil samples) and add 7 ml distilled water plus 0.1 ml Folin-Ciocalteu's phenol reagent, and after 3 min add 0.2 ml of 20% Na_2CO_3. After boiling at 90 ^{0}C (exactly 5 min) cool the samples at room temperature and dilute with H_2O to 10 ml volume. Use the distilled water and reagents as a blank. Units of total phenolics were expressed in micrograms of ferulic acid equivalent per g dry weight (17).

VII. Determination of phenolic acids by HPLC analysis

Phenolic aicds were detected between 210 and 360 nm using Hewlett Packard diode array detector (HP 1100 HPLC system). Separation was achieved with a Nucleosil 100-5 C_{18} column; 5 µm; 4.0x250 mm (Agilent Technologies, USA). A step-gradient of acetonitrile in water was used: 15% acetonitrile (5 min, gradient), 30% acetonitrile (20 min, gradient), 40% acetonitrile (25 min, gradient), 60% acetonitrile (30 min, gradient), 60% acetonitrile (35 min, gradient), and 100% acetonitrile (45 min, isocratic). In order to avoid tailing of the phenolic acids, 0.05% orto-phosphoric acid was added to the solvents.The flow rate was 1 ml/min, the injection volume was 5 µl. The identity of phenolic acids was determined by comparing of the retention times and maximum of absorption of known peaks with pure standards. *p*-Hydroxybenzoic and syringic acids (Acros organics, USA), ferulic, vanillic and *p*-coumaric acids (Serva, Germany), were used as phenolic standards. Units of phenolic acids were microgram per g dry weight. All analyses were performed in triplicates (17).

VIII. Plant growth tests with soil, seedling growth tests and seed germination tests

Test plants were grown in 300 mL plastic dishes. Surface samples (0-10 cm) of sandy soil under *F. vallesiaca* as dominant plants were used. Two-day-old etiolated seedling of *Cynodon dactylon* and *Lolium perenne* were planted into experimental soil-containing pots and grown in a green-house for 15 days (14 h light, white fluorescent lamp

$3.6x10^3$ erg/cm^2/sec, 25 °C and 10 h dark, 20 °C). The controls were grown under the same conditions in the pots containing sandy soil taken from the spots without plants. Measurements were performed using 500 individuals of each test plant species.

Test plant seeds sterilized with 5% sodium hypochlorite for 10 min and thoroughly rinsed with distilled water germinated at 25 °C in the dark for 36 h. Uniform seedlings approximately 5 mm long were used. They were grown in Petri dishes (10 cm dia) containing 5 mL of aqueous extracts: (i). Litter (dead plant parts of *F. vallesiaca*) and (ii). Vegetative plant parts (0.25 g/ml respectively) or (iii). Distilled water (control). After 96 h growth at 25 °C the radicle or coleoptiles length was measured. In each experiment 500 seedlings of test plants were used.

Test plant seeds (5x100 seeds of each) germinated at 25 °C in Petri dishes (10 cm dia) containing 5 ml of aqueous extracts of : (i). Litter (dead plant parts of *F. vallesiaca*) and (ii). Vegetative plant parts (0.25 g/ml respectively), (iii). Distilled water (controls). In order to examine the soil impact to seed germination test plant seeds (5x100 seeds of each) germinated at 25 °C in Petri dishes (10 cm dia) containing 50 g of surface sandy soil (0-10 cm) under *F. vallesiaca*. Sandy soil without of plants served as control. After 6 days, the number of germinated seeds was counted. For a detailed procedure, see Djurdjević et al. 2007(17).

IX. Biotests with natural concentrations of five phenolic acids detected in soil under *F. vallesiaca*

To monitor the effects of natural concentrations of phenolic acids (Free forms) detected in soil under *F. vallesiaca* plnts on seed germination and seedling growth, we made a series of their concentrations (Table 5). Test plant seeds (5x100 seeds of each) were germinated at 25 °C in Petri dishes (10 cm dia) containing 5 ml of aqueous solutions of : *p*-Coumaric acid (4.5 µg/g), Ferulic acid (3.5 µg/g), *p*-Hydroxy benzoic acid (1.5 µg/g), Vanillic acid (2.5 µg/g), Syringic acid (1.7 µg/g), Mix of five acids (13.7 µg/g), distilled water (controls). After 6 days, the number of germinated seeds was counted.

Test plant seeds sterilized with 5% sodium hypochlorite for 10 min and thoroughly rinsed with distilled water were germinated at 25 °C in dark for 36 h. Uniform seedlings approximately 5 mm long were used. They were grown in Petri dishes (10 cm dia) containing 5 mL of aqueous solutions of: *p*-Coumaric acid (4.5 µg/g), Ferulic acid (3.5 µg/g), *p*-Hydroxy benzoic acid (1.5 µg/g), Vanillic acid (2.5 µg/g), Syringic acid (1.7 µg/g), Mix of five acids (13.7 µg/g), distilled water (controls). After 96 h growth at 25 °C the length of the radicle or coleoptiles was measured. In each experiment 500 seedlings of test plants were used. For a detailed procedure, see Djurdjević *et al.* 2007 (17).

X. Establishment of grass culture, collection of root exudates and bioassay

Tufts of *F. vallesiaca* were collected from Deliblato Sands. They were washed and treated with 5 % sodium hypochlorite for 10 min and rinsed thoroughly with distilled water prior to planting. The containers used were made from 3 L brown glass solvent bottles with the bottoms removed. The five containers were filled with a 4-cm layer of crushed rock (about 2-cm in size), followed by a 2:1 (v/v) sand : rock mixture up to 3 cm from the edge. One tuft of grass was planted in each container (in five replicates, n=5) in a greenhouse (14 h light, white fluorescent lamp 3.6×10^3 erg/cm^2/sec, 25 °C and 10 h dark,

20 °C) and irrigated with 0.1-strength Hoagland solution at 100 ml/day. Additional distilled water was supplied as needed. Two weeks after planting the grass was well established and had a full-grown root system. Hydrophobic or partially hydrophobic exudates were selectively retained by the XAD-4 resin column. Methanol eluates from 5 columns were pooled and methanol was evaporated under reduced pressure at 60°C. The concentrate was diluted with water to 50 ml acidifying the remaining aqueous fraction to pH 2 with 1NHC1 and extracting three times with 100 ml CH_2Cl_2. The extracts (designated as the acidic fraction) were combined, dried over anhydrous $MgSO_4$ and concentrated to 20 ml in a rotary evaporator at room temperature. To monitor the effects of root exudates of *F. vallesiaca* on seed germination and seedling growth, aliquots (0.2 ml) of acidic fractions were applied separately on 3.5-cm^2 discs of Whatman No. 3 MM filter paper. To monitor the effects of hydroponic growth solution, 2 ml solution was added into each Petri dish. Distilled water served as control. For a detailed procedure, see Tang and Young (38).

Statistical analyses

The statistical analysis was done with ANOVA. Data was processed using Statistica 6.0 for Windows statistical package.

RESULTS AND DISCUSSION

Phytocoenological investigations

The *Festuceto-Potentilletum arenariae* steppe community at Deliblato Sands, earlier known as the European Sahara, extends over 565 ha and occupies 1.91 % surface area. It is dominated by the steppe element *F. vallesiaca*, which forms short, well-developed tufts, creating a dense cover and conditioning the physiognomy of the whole community. Between its tussocks (surrounded by growth inhibition zones) 20-other meadow-steppe plant species grow, but their abundance and cover is extremely low (1-2 %) (Table 1, Figure 1). The sands are characterised by large day-night and summer-winter variations in air and soil temperature, early autumn and late spring frosts, soil moisture deficit during the summer and intense solar radiation, and drought during the summer and autumn (End of May to end of September). Most of the plants (67 %), of this community are hemicryptophytes i.e. plants whose aboveground parts die during winter, but their underground buds at the base of the stems remain alive and sprout during the summer. Most of the plants (90 %) are xerophytes (plants adapted to extremely dry conditions) and subxerophytes (plants which are found in both extremely dry and mesophilic phytocoenoses). The *Festuceto-Potentilletum arenariae* steppe community is an open habitat, so in relation to light, the majority of plants (76 %) belong to a transitional group between semi-sciophytes and heliophytes well adapted to daily insolation.

Phenolic compounds in the aboveground parts of *F. vallesiaca* and its litter

In the tissues of vegetative parts, high amounts of both free (9,242.67 µg/g) and bound forms (12,344.30 µg/g) of total phenolics were found. The amount of total free and bound phenolics in the litter [partially decomposed aboveground remains of *F. vallesiaca*]

Figure 1. The short, well-developed tufts of the dominant grass *Festuca vallesiaca* in the *Festuceto-Potentilletum arenariae* steppe community (Deliblato Sands-Serbia).

Table 1. Phytocoenotic related data of *Festuceto-Potentilletum arenariae* plant community with *Festuca vallesiaca* domination

Plant species	Abundance
Festuca vallesiaca	8
Andropogon ischaemum	2
Chrysopogon gryllus	2
Carex humilis	1
Potentilla arenaria	1
Poa bulbosa	1
Euphorbia segueriana	1
Thymus glabrascens	1
Asperula cynanchica	1
Festuca sulcata	1
Achilea millefolium ssp.collina	1
Teucrium chamaedrys	1
Cynodon dactylon	1
Lolium perenne	1
Adonis vernalis	1
Paeonia tenuifolia	1
Plantago lanceolata	1
Poa pratensis	1
Veronica spicata	1
Koeleria gracilis	1
Filipendula hexapetala	1

For each plant species, the numbers represent an estimation of its abundance and cover in the examined community, expressed in numbers on a scale of 1-9, equivalent to abundance of 1-100%: 1 (1%), 2 (2%), 3 (5%), 5 (25%), 7 (50%), 8 (75%), and 9 (100%), according to Westhoff and van der Marrel (42).

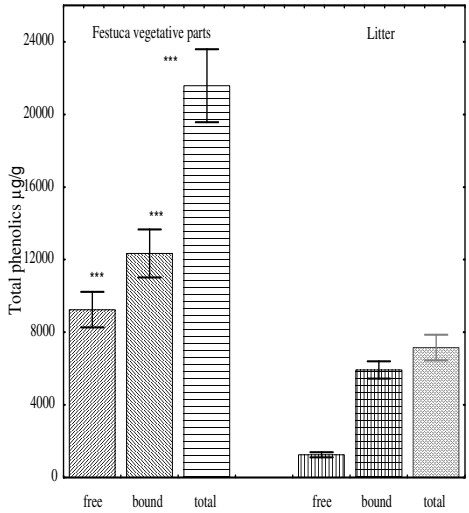

Figure 2. Content of total phenolics (free and bound forms) in *Festuca vallesiaca* aboveground parts and litter. (ANOVA; *** p <0.001; n=5).

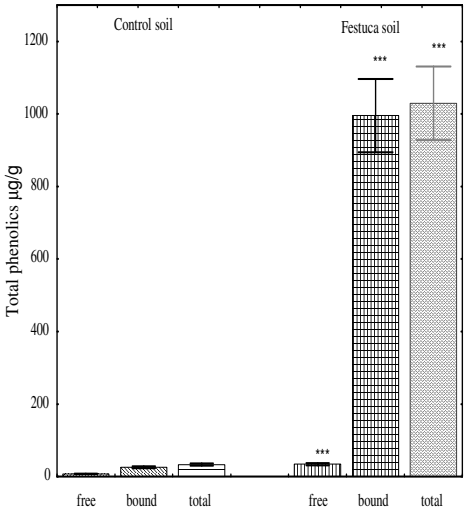

Figure 3. Content of total phenolics (free and bound forms) in control and *Festuca vallesiaca* sandy soil. (ANOVA; *** p <0.001; n=5).

was several times lower than in the aboveground parts (p <0.001). The level of free phenolics as a percentage of total phenolics in the aboveground parts of *F. vallesiaca* and the litter was 42.81 and 17.36 %, respectively, while bound forms accounted for 57.18 % and 82.64 %, respectively (Figure 2).

The tissue of aboveground part of *F. vallesiaca*is the main source of allelochemicals contained higher contents of all five free phenolic acids than the partially decomposed litter (Table 2). It also contained higher amounts of bound forms of ferulic, *p*-hydroxybenzoic and syringic acid than the litter (p<0.001). There was no statistically significant difference between the tissue of *F. vallesiaca* and the litter in terms of the amount of bound *p*-coumaric and vanillic acids. In grasses (Gramineae), derivatives of cinnamic acid (*p*-coumaric and ferulic acid) predominate over benzoic acid derivatives (*p*-hydroxybenzoic, vanillic and syringic acid) in the tissue and litter. In the vegetative tissues, free phenolic acids comprise only 3.40 % of the total free phenolics, while in the litter, their level was 13.63 %. The ratio of cinnamic/benzoic acid derivatives (free forms) in the aboveground parts of *F. vallesiaca* is 3.42, while it was significantly higher 6.32 in litter.

In aboveground vegetative plant organs in grasslands, leaves are the main source of allelopathic substances and contain a large amount of allelochemicals with broad range of activity (14,18,29,35). Many allelopathic compounds present in aboveground plant parts (leaves, stems, flowers) are soluble in water and can therefore be leached by rain, fog or mist (29). In the tissue of vegetative parts of *F. vallesiaca*, (dominant species in the meadow-steppe community), large amounts of free and bound forms of total phenols accumulated (9,242.67 µg/g and 12,344.30 µg/g, respectively). The content of individual free phenolic acids in the aboveground parts ranged from 15.11 to 123.93 µg/g, and from

Table 2. Phenolic acids contents in *Festuca vallesiaca* plant parts, litter and sandy soil

Phenolic acid		*Festuca vallesiaca* parts		Sandy soil	
(µg/g)	Forms	Litter	Vegetative	Control soil	*Festuca vallesiaca* soil
p-	Free	91.01±8.9	117.81±12.43**	1.15±0.20	4.25±0.43***
Coumaric	Bound	1060.86±111.01	1180.44±120.00^{NS}	6.28±0.71	61.21±6.31***
Ferulic	55.36±6.01	123.93±6.01***	1.21±0.20	3.24±0.37***	55.36±6.01
	1,294.86±130.00	2079.88±208.00***	6.16±0.52	63.73±6.40***	1294.86±130.00
p-Hy.	Free	5.68±0.65	27.60±10.08***	0.26±0.13	1.24±3.11***
benz	Bound	41.37±5.11	97.42±10.08***	1.24±0.13	27.44±3.11***
Vanillic	Free	8.11±0.90	27.91±3.00***	0.49±0.03	2.16***±0.25
	Bound	406.32±40.10	395.17±40.10^{NS}	4.01±0.40	36.75±3.71***
Syringic	Free	9.36±0.10	15.11±1.53***	0.63±0.05	1.69±0.17***
	Bound	139.22±14.10	239.01±24.03***	1.27±0.13	40.42±4.2***

Compared are: (a) litter/vegetative *Festuca vallesiaca* parts; (b) control soil/soil under *Festuca vallesiaca*; (ANOVA, NS-not significant; ** $p<0.01$; *** $p<0.001$; n = 5). Means ± S.D.

97.42 to 2,079.88 µg/g for bound forms. The leaves of three *Sorghum* hybrids have the most total phenolics compared to other plant organs. In different plant parts, five phenolic acids are found: *p*-hydroxybenzoic, vanillic, syringic, *p*-coumaric and ferulic (0.9-613 mg/kg) (7). Ferulic and *p*-coumaric acids are the main phenolic acids present in the cell wall of monocotyledons and especially of Gramineae. Moreover, 19-species of dry-season tropical grasses contain large amounts of *p*-coumaric, ferulic and caffeic acid, and trace amounts of sinapinic acid. The total amount of acids is approximately 100 mg/g d.w. in majority of grass species. The acids are present in the lignin of cell walls (30).

Litter mainly composed of partially decomposed aboveground remains of *F. vallesiaca*, contains significantly lower amounts of total free phenolics (17.36 %) than the aboveground parts (42.81 %). This decrease in total free phenolics during litter decomposition is caused by the leaching of water-soluble phenolics and by the microbial decomposition of dead plant remains. Due to the microbial degradation of lignin, free phenolic acids in litter were 13.63 %, while only 3.40 % in the vegetative parts of *F. vallesiaca*. In addition, the ratio of cinnamic/benzoic acid derivatives (Free forms) in the aboveground part of *F. vallesiaca* is 3.42, but is considerably higher (6.32) in litter, which indicates the occurrence of microbial degradation of lignin in the grass, an increase in the amount of free phenolic acids in it, and the transformation of cinnamic acid derivatives into derivatives of benzoic acid (27,32).

Sandy soil phenolics

The amount of total free phenols in the soil was 4.78 times higher than in the control soil (sand prone to wind erosion), while that of bound forms was 38.73 times higher ($p<0.001$). The proportion of free phenolics in the total phenolics in the soil beneath *F. vallesiaca* was 3.31 %, while bound forms was 96.67 % (Figure 3). The same phenolic acids were found in the soil beneath *F. vallesiaca* as were detected in its tissue and partially decomposed litter (Table 2). The content of free and bound phenolic acids was several times higher than in control soil without vegetation ($p<0.001$). Free phenolic acids in the soil beneath *F. vallesiaca* was much as 36.92 % of the total free phenolics, while the bound forms were less (23.06 % of the total bound phenols). Thus the sandy soil beneath

the dominant grass *F. vallesiaca* contains more of total phenols (free and bound forms) and the five phenolic acids than the control soil without plants.

In the sandy soil beneath the dominant grass *F. vallesiaca*, the amount of total phenolics is 1029.54 µg/g, of which bound forms were 96.67 %, and is several times greater than in control soil without vegetation. According to our earlier findings, the highest amount of total phenolics (1589.61 µg/g to 6561.30 µg/g) were found in forest soil, beneath various species of dominant trees . The phenolics were less (598.81 µg/g) in the sandy soil of forest-steppe vegetation at Deliblato Sands and the lowest level (271.39 µg/g) was in the young sandy soil dominated by the introduced species *C. canadensis* (15,16). In the soil beneath sorghum, there is 0.07-0.20 mg/g of water-soluble total phenolics, and in terms of phenolic acids, there is *p*-hydroxybenzoic, vanillic, and syringic (7). Studying the phenolic compounds in 12 subtropical grasses, Chou and Young identified the phytotoxins [ferulic, syringic, *p*-coumaric, vanillic, *p*-hydroxybenzoic and *o*-hydroxyphenylacetic acids]. Most of these compounds were also found in the associated soils; the toxic compounds were in lower amounts in control (non herb-growth) soil than in the grass soils (14). This is in accordance with our finding of 5 phenolic acids, in both the aboveground part of the dominant grass *F. vallesiaca* and the sandy soil beneath it, and their levels being significantly higher than at the control soil without plants.

Seed germination and seedling growth

The aqueous leachates from the aboveground part of *F. vallesiaca* and litter inhibited the seed germination of both test species (*C. dactylon* and *L. perenne*). However, leachate from the vegetative plant parts were more inhibitory than the partially decomposed litter. Allelopathically active substances from the vegetative parts of the dominant species *F. vallesiaca* and its partially decomposed litter proved more inhibitory to the germination of *C. dactylon* seeds than those of *L. perenne*.

The aqueous leachates from the aboveground parts of *F. vallesiaca* and its litter were also inhibitory to the growth of aboveground parts and roots of both test plants, although the leachates of vegetative plant parts were more inhibitory to the elongation of roots and aboveground parts than plant litter. The roots of both test species were more sensitive than the aboveground parts to the inhibitory matter from *F. vallesiaca* aboveground parts and litter. However, the process of root elongation in *C. dactylon* was more sensitive to inhibitory matter from the litter and aboveground parts of *F. vallesiaca* than in *L. perenne* (Table 3).

The aqueous extracts from living vegetative plant parts and litter of dominant *F. vallesiaca* had inhibitory effects on seed germination and seedling growth of both test plants, *C. dactylon* and *L. perenne*. According to the results of Viard-Cretat et al. (39), *Festuca paniculata* leachates inhibited the seedling growth of *Dactylis glomerata* and *Bromus erectus*. Inhibition was correlated with polyphenol concentrations. The results suggest that allelopathy may be at least partially responsible for *F. paniculata* dominance in subalpine meadows by inhibiting colonization by neighbouring species (39). The inhibition of wheat radicle growth was positively associated with concentrations of total phenolics contained in sorghum hybrid parts (7). The fresh material extracts were more phytotoxic than litter extracts. The chemical composition of litter and fresh material indicated that the later contained comparatively more phenolic substances than litter

Table 3. Effects of water extracts from litter and vegetative plant parts of *Festuca vallesiaca* (0.25g/ml) on seed germination and seedling growth of *Cynodon dactylon* and *Lolium perenne*

Treatment	Germination (%)	Aerial part length (cm)	Root length (cm)
		Cynodon dactylon	
Control	79.10[a]	100	100
Litter	43.63(-44.84)[b]	43.17(-56.83)[b]	39.58(-60.42)[b]
Vegetative	19.01(-75.97)[b]	38.71(-61.29)[b]	20.16(-79,84)[b]
		Lolium perenne	
Control	71.01 [a]	100	100
Litter	51.98(-26.80)[b]	49.79(-50,21)[b]	41.02(-58.98)[b]
Vegetative	17.88(-74.82)[b]	37.75(-62.25)[b]	35.91(-64.09)[b]

[a] Numbers in parentheses are % of inhibition. Control=distilled water.
[b] Statistically significant difference, $p<0.001$ (t-test), n=500.

materials. This is confirmed by our results, as the aqueous leachates from the vegetative parts of *F. vallesiaca* were more inhibitory than the partially decomposed remains (litter). It was due to the higher content of total phenols and phenolic acids in the vegetative parts of *F. vallesiaca* than in litter. This is in accordance with earlier findings in which aqueous extracts from the living and decaying shoots of *C. canadensis*, 12 subtropical grasses, three *Sorghum bicolor* hybrids, *Echinochloa crus-galli*, and *Allium ursinum* inhibited the germination and root and shoot growth of different test species. The phytotoxins ferulic, syringic, *p*-coumaric, vanillic, *p*-hydroxibenzoic and o-hydroxyphenylacetic acids were identified. Additionally, most of these compounds were also found in the associated soils. The control non herb-growth soil contained significantly lower amounts of toxic compounds than the grass soils (7,9,15,16).

The humic surface layer of soil beneath *F. vallesiaca* had an inhibitory effect on the seed germination and seedling growth (elongation of aboveground parts and roots) of both test plants. The allelopathically active matter accumulated in the soil had a more inhibitory effect on the elongation of the roots and aboveground parts of *L. perenne* than of *C. dactylon* (Table 4). The effects of phenolic fraction from *F. vallesiaca* soil (0-10 cm) on the seed germination and seedling growth of *C. dactylon* and *L. perenne* are shown in Table 5. The phenolic fraction from soil obtained by alkaline hydrolysis inhibited the germination of seeds of both species, but was more inhibitory to the germination of *C. dactylon* seeds than those of *L. perenne*. The elongation of aerial parts and roots of both test species was inhibited due to the effects of the allelochemicals contained in the phenolic fraction from the soil. According to the degree of inhibition of seed germination and seedling growth (elongation of aerial parts and roots), followed the order: vegetative parts of *F. vallesiaca* > phenolic fraction of soil > litter > soil.

Although the humic surface layer of soil beneath *F. vallesiaca* contains several times lower amounts of phenols than the aboveground part and the partially decomposed litter, it was inhibitory to seed germination and the growth of seedlings (elongation of aboveground parts and roots) of both test plants. The allelopathically active matter accumulated in the soil was more inhibitory for the elongation of roots and aboveground parts of *L. perenne* than of *C. dactylon.* Our results also support the research of Chou and

Table 4. Effects of sandy soil (0-10 cm) beneath *Festuca vallesiaca* on seed germination and seedling growth of *Cynodon dactylon* and *Lolium perenne* test plants

Treatment	Germination (%)	Aerial part length (cm)	Root length (cm)
		Cynodon dactylon	
Control soil	81.84[a]	100	100
F. vallesiaca soil	63.65(-22.23)[b]	69.13(-30.87)[b]	71.96(-28.04)[b]
		Lolium perenne	
Control soil	73.17[b]	100	100
F. vallesiaca soil	62.71(-14.30)[b]	61.03(-38.97)[b]	53.58(-46.42)[b]

[a] Numbers in parentheses are % of inhibition. Control soil=sandy soil without plants.
[b] Statistically significant difference, $p<0.001$ (t-test), n=500.

Table 5. Effects of phenolic fraction from *Festuca vallesiaca* soil (0-10 cm) (0.25g/ml) on seed germination and seedling growth of *Cynodon dactylon* and *Lolium perenne*

Treatment	Germination (%)	Aerial part length (cm)	Root length (cm)
		Cynodon dactylon	
Control	78.14[a]	100	100
Phenolic fraction	33.98(-56.51)[b]	49.01(-50.99)[b]	57.21(-42.79)[b]
		Lolium perenne	
Control	71.98	100	100
Phenolic fraction	35.25 (-51.03)[b]	53.23 (-46.77)[b]	47.11 (-52.89)[b]

[a] Numbers in parentheses are % of inhibition. Control=distilled water.
[b] Statistically significant difference, $p<0.001$, (t-test), n=500.

Lee (13), who established that the rhizosphere soils under *Miscanthus transmorrisonensis* exhibited significant phytotoxicity on the seed germination and radicle growth of the test plants tested, indicating that allelopathic interaction was involved. Some responsible phytotoxic phenolics were identified. The phenolic fraction from soil beneath *F. vallesiaca* obtained by alkaline hydrolysis was inhibitory to seed germination and seedling growth of both test plants. These results resemble the data of Li *et al.* (28), who reported that the phenolic fraction extracted with 1 M NaOH from the rhizosphere soil of *Sasa cernua* caused significant inhibition to the seed germination and seedling growth of lettuce, timothy, green amaranth and barnyard grass.

Seed germination and seedling growth of *C. dactylon* and *L. perenne*

Of the 5- phenolic acids in natural concentrations, four (Except *p*-hydroxibenzoic acid) were inhibitory (-0.66 % to -4.80 %) to the seed germination of *C. dactylon* and *L. perenne* (Figure 4). The combination of 5-phenolic acids (as total concentrations of individual acids) inhibited the seed germination of the test plants considerably more than each individual acid in natural concentrations (up to -14.06 %). The elongation of aerial parts of the test plants were also inhibited (-2.13 % to -4.89 %) by the phenolic acids, detected in soil under *F. vallesiaca*. The combination of 5-phenolic acids was more inhibitory (-25.12 %) to the elongation of aerial parts of *L. perenne* than of *C. dactylon* (-20.96 %). All 5-acids in natural concentrations were inhibitory (-1.22 % to -4.06 %) to root elongation. Their combination was more inhibitory to the root elongation of *L. perenne* (-23.43 %) than of *C. dactylon* (-15.61 %).

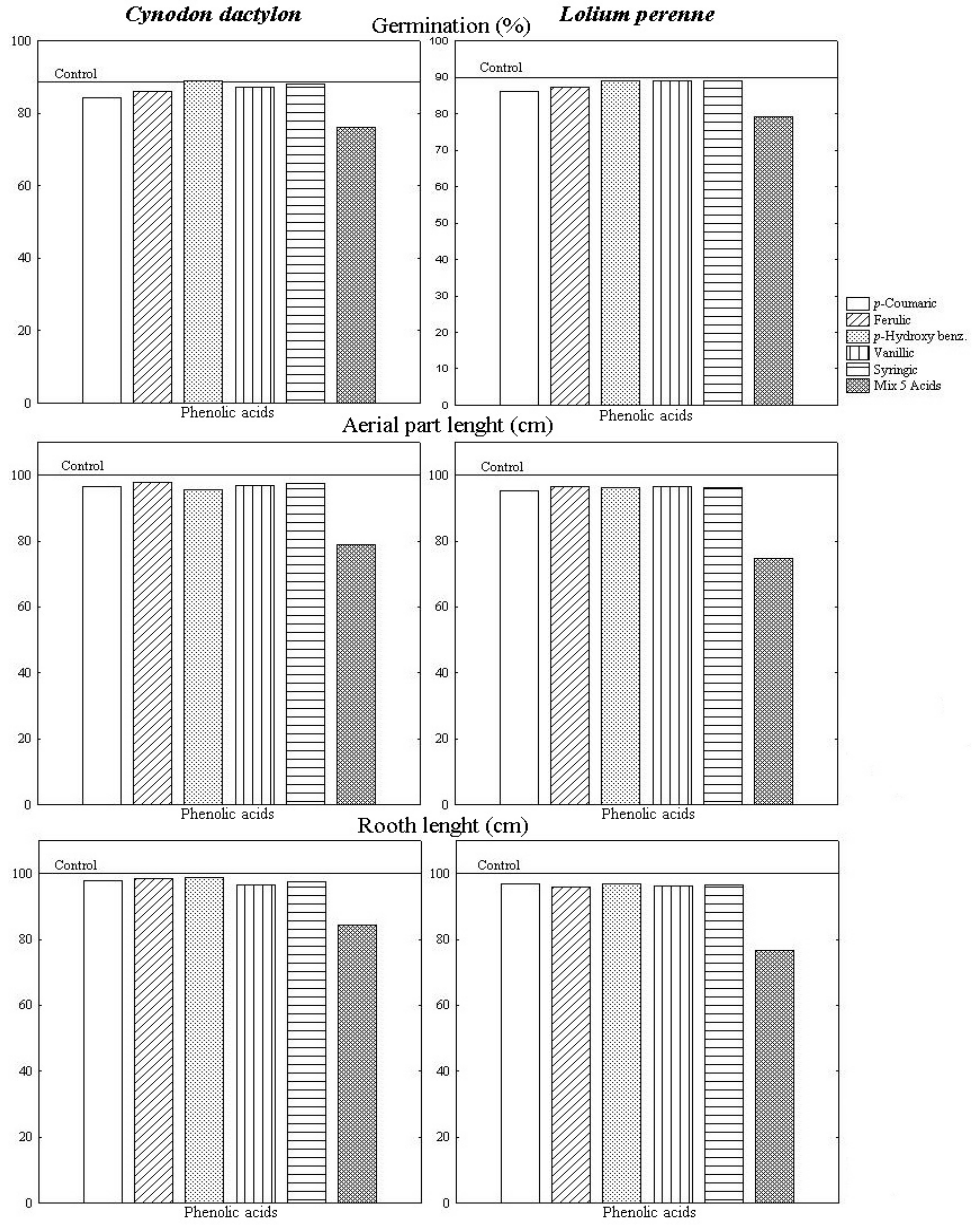

Figure 4. The effects of five phenolic acids in natural concentrations (*p*-Coumaric 4.5 µg/g; Ferulic 3.5 µg/g; *p*-Hydroxy benzoic 1.5 µg/g; Vanillic 2.5 µg/g; Syringic 1.7 µg/g; Mix of five acids 13.7 µg/g) detected in soil under *Festuca. vallesiaca* on seed germination and seedling growth of *Cynodon dactylon* and *Lolium perenne.*

Table 6. Effects of *Festuca vallesiaca* root exudates (0.2 ml of acidic fraction) and hydroponic growth solution (2 ml) in which *Festuca vallesiaca* grew on seed germination and seedling growth of *Cynodon dactylon*, *Lolium perenne* and *Festuca vallesiaca*

Treatment	Germination (%)	Aerial part length (cm)	Root length (cm)
		Cynodon dactylon	
Control	88.67	100	100
Root exudates	79.11[a] (-10.78)	90.21[a] (-9.79)	89.14[a] (-10.86)
Hydroponic solution	78.94[a] (-10.97)	87.44[a] (-12.56)	88.97[a] (-11.03)
		Lolium perenne	
Control	88.32	100	100
Root exudates	80.45[a] (-8.91)	88.85[a] (-11.15)	87.98[a] (-12.02)
Hydroponic solution	77.75[a] (-11.97)	87.98[a] (-12.02)	88.12[a] (-11.88)
		F. vallesiaca	
Control	87.89	100	100
Root exudates	88.79[a] (+1.02)	101.87[a] (+1.87)	103.06[a] (+3.06)
Hydroponic solution	89.66[a] (+2.01)	102.66[a] (+2.66)	104.97[a] (+4.97)

Numbers in parentheses are % of inhibition or stimulation in relation to control. Control=distilled water. ANOVA, levels of significance: [a] $p<0.001$, ns = not significant, n=500.

Natural concentrations of free phenolic acids in soil under *F. vallesiaca* range from $1.1x10^{-5}$M, to $8.56x10^{-5}$M and, according to our findings, they are inhibitory for the seed germination and seedling growth of test plants. According to data from literature (Table 7), inhibitory concentrations for seed germination and seedling growth ranges from 10^{-6} to 10^{-3}M (1,10,22,26,33). Much literature is available about stimulatory effects of allelochemicals. Water soluble and volatile compounds from decaying leaves of some *Acer* species were tested against growth of *Lepidium sativum* and both inhibitory and stimulatory compounds were found (35). The consumption-orientation of Chl was significantly stimulated by the exogenously applied phenolics. The order of stimulation effect on chlorophyllase a activity is: o-hydroxyphenylacetic acid > ferulic acid > p-coumaric acid (43). Net P and K uptake of roots in contact with ferulic acid increased in a linear and curvilinear (convex) manner, respectively (31).

Root exudates, seed germination and seedling growth of *C. dactylon*, *L. perenne* and *F. vallesiaca*

Root exudates and hydroponic growth solution in which *F. vallesiaca* grew were inhibitory for the seed germination of both test plants, although the hydroponic solution was somewhat more inhibitory for the germination of *L. perenne* seeds (Table 6). On the other hand, root exudates and hydroponic solution was stimulatory to germination of *F. vallesiaca* seeds. The growth of aerial part of test plants was inhibited by the effect of the root exudates and hydroponic solution (up to -12.56 % for *C. dactylon*). The growth of the aerial part of *F. vallesiaca* was stimulated by its own root exudates and hydroponic solution (+1.87 and +2.66 % respectively). The elongation of roots of *C. dactylon* and *L. perenne* was inhibited by the effect of root exudates and hydroponic solution in which *F. vallesiaca* grew (from -10.86 % to -12.02 %). *F. vallesiaca* root exudates and hydroponic solution were stimulatory to the root elongation of its own species (+3.06 % and +4.97 % respectively).

Table 7. The inhibitory influence of phenolic compounds on some physiological process in plants

Phenolics	Physiological process	Inhibitory concentrations	Reference
p-Hydroxybenzoic, salycilic, and caffeic acid	Plant-water relationships, chlorophyll fluorescence, stomatal conductance, growth	0.25 mM, 0.5 mM, and 1.0 mM	5
Sinapinic, syringic, vanillic, ferulic, *p*-coumaric, chlorogenic, gallic, gentisic, protocatechuic, *p*-hydroxybenzoic, *trans*-cinnamic acids, eucalyptol, quercetin, vanillin, syringaldehyde, rutin, 2-benzoxazolinone, protocatechualdehyde, tyrosol, juglone, and L-mimosine	Seed germination, root growth, hypocotyls length, root morphology of alfalfa, barnyardgras and weed species, leaf expansion, seedling growth	20 -700µM, 10^{-2}M, 10^{-3} M, 10^{-4}M 10^{-5}M, 10^{-6}M 0.05-0.25 µM/g soil	10,12,34
Scopoletin	Growth, CO_2 exchange rates	10^{-4}M, 10^{-3}M	21
Vanillic and *p*-hydroxybenzoic acids	Radish and grain sorghum germination and sorghum root and shoot elongation	2.5 x10^{-3}M, 5x10^{-3}M	20
Benzoxazolin-2(3*H*)-one, *p*-hydroxybenzoic acid and cinnamic acid	Growth, leaf water relations, PSII photochemistry, non-photochemical fluorescence quenching, and heat energy dissipation	1.5 mM	26
Ferulic acid	Net P, K, and water uptake	0.5 mM	31
p-Coumaric, ferulic, *p*-hydroxybenzoic, vanillic, salicylic and chlorogenic acids	Seed germination and growth of grain sorghum and birch	10^{-3} M and 5×10^{-4} M	33
o-Hydroxyphenyl acetic, ferulic and *p*-coumaric acids	Chlorophyll, porphyrin and chlorophyllide contents, and chlorophyllase a and b activities.	20, 50, 100, 200 ppm	43
Coumarin and phenylpropanoids	Inhibition of radish seed germination and root growth	2×10^{-4}M	2
Ferulic, *p*-coumaric, vanillic, cinnamic *p*-Hydroxybenzoic acid and coumarin	Seed germination, root and shoot length, fresh and dry weight, chlorophyll, carotenoids and anthocyanin content, guaicol peroxidase activity	10^{-3} M, 10^{-4}, 10^{-5} M	1,22

The roots exudes a variety of low-molecular weight organic compounds (sugars and simple polysaccharides, amino acids, organic acids and phenolic compounds. Some of these compounds), especially the phenolics, influence the growth and development of surrounding plants and soil microorganisms (6,9). Our results on the inhibitory effects of root exudates and their rhizochemicals on seed germination and seedling growth are supported by data from many authors (8,9,25,38).

CONCLUSIONS

The steppe element *F. vallesiaca* dominates the *Festuceto-Potentilletum arenariae* steppe community at Deliblato Sands. Between its well-developed tussocks, there are few other meadow-steppe plants. Phenolic compounds produced in its tissue accumulated in litter and soil, reaching toxic concentrations and thus inhibited the seed germination and seedling growth of surrounding plants. Natural concentrations of free phenolic acids in soil under *F. vallesiaca* ranged from $1.1x10^{-5}$M to $8.56x10^{-5}$M and, according to our findings, they are inhibitory for the seed germination and seedling growth of test plants. The elongation of roots of *C. dactylon* and *L. perenne* was inhibited by the root exudates and hydroponic solution in which *F. vallesiaca* grew. Phenolics as secondary metabolites of this dominant grass may have a key influence on the composition and structure of *Festuceto-Potentilletum arenariae* steppe community, and on the formation and characteristics of the soil beneath it.

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