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Soilborne microorganisms of *Euphorbia* are potential biological control agents of the invasive weed leafy spurge

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Abstract

Leafy spurge (*Euphorbia esula-virgata*), a native of Eurasia, is a serious invasive weed of grasslands of the northern Great Plains of the U.S. and prairie provinces of Canada. Leafy spurge is very difficult to control with herbicides, insect biological control agents, and other cultural practices. Previous field investigations revealed pathogen–insect interactions on the roots of leafy spurge leading to mortality. In order to exploit this synergistic relationship as an effective biological control strategy, we undertook an exploration of Europe for soilborne fungi and rhizosphere bacteria on *Euphorbia* spp. growing in a wide variety of soils in different landscapes. All microbial cultures were screened for growth suppressive or disease potential on leafy spurge plants or callus tissue. Study objectives were to determine relationships of some edaphic factors and host plant conditions with biological control activity, and to screen rhizobacteria isolated from *Euphorbia* spp. for traits that might contribute to suppression of leafy spurge growth. The most virulent soilborne fungal strains of *Fusarium* and *Rhizoctonia* species, based on greenhouse pathogenicity tests, were isolated from roots of *Euphorbia* spp. with insect feeding damage. High proportions (>50%) of rhizobacteria were classified as deleterious rhizobacteria (DRB) using a callus tissue bioassay. *Euphorbia* spp. at sites with high DRB numbers displayed severe fungal disease symptoms and supported insect infestations. Selected soil properties were not correlated with potential biocontrol activity of microbes on leafy spurge; however, insect presence and disease ratings were associated with incidence of growth-suppressive microbes. Certain physiological traits (i.e., exopolysaccharides and hydrogen cyanide production) were good indicators of deleterious activity of rhizobacteria. Our study illustrates that the most effective condition for inducing disease and subsequent mortality of leafy spurge includes a synergism between plant-associated microorganisms and root-damaging insects. Furthermore, the results are valuable for identifying sites for collecting soilborne microorganisms on weeds in their native range for evaluation as biocontrol agents in their invasive range.
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1. Introduction

Leafy spurge (*Euphorbia esula-virgata* L.) is an invasive, deep-rooted perennial weed that reproduces both by seed production and by vegetative propagation from apical buds located on the underground portion of the stem (crown) and lateral roots. Anderson et al. (2000) reported that leafy spurge infests approximately 2 million ha of land in the northern Great Plains of the U.S. and the prairie provinces of Canada. Leafy spurge is a highly successful competitor for resources and space, a result of its adaptation to a broad range of environmental conditions and its aggressive, extensive

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root system, the primary characteristics leading to its invasive nature. Although relationships between soil microorganisms and invasive plant species have received little attention, large changes in landscape vegetation suggest simultaneous alteration in the structure and activity of the soil microbial community (Klironomos, 2002). Vegetation composition changes under dense leafy spurge infestations may affect microbial community functioning by providing large amounts of organic residues different from native plant composition, and by altering the rhizosphere environment. Soil microbial inhabitants that benefit the proliferation of the invasive plant community may also be selected. Growth of desirable plant species is likely reduced by competition although leafy spurge may also limit neighboring plant growth through allelopathy (Lajeunesse et al., 1999). Beef production is indirectly decreased through impaired grazing of adjacent forage caused by latex-based chemicals present in the leafy spurge plant, which is toxic to cattle (Lajeunesse et al., 1999). Because leafy spurge is a major threat to vegetation in pastures, rangelands, and native habitats, it is listed as 1 of the 12 least desirable invasive species in U.S. ecosystems (Stein and Flack, 1996).

The vegetative reproduction trait of leafy spurge resists many weed control tactics because crown or root buds can regenerate new plants after foliage is treated with herbicides, mowing, biological control agents, burning, or grazing. Long-term control with herbicides is difficult to achieve (Markle and Lym, 2001); thus, it is imperative to develop effective biological control for use in leafy spurge management programs. Leafy spurge is considered an ideal target for biological control because the habitats it invades are generally incompatible with herbicide control and its perennial nature can provide a consistent food source for biological control organisms (Kirby et al., 2000).

Biological control of leafy spurge has centered on introduction and release of a suite of insect natural enemies. Insects for biological control have been comprised mainly of flea beetles (*Aphthona* spp.), the larvae of which feed specifically on leafy spurge roots. Examination of the impact of flea beetles released for control of leafy spurge in North America indicated successful insect establishment at numerous sites but with little or no measurable impact on the weed stand (Caesar, 2000). However, successful biological control observed as rapid declines in leafy spurge stands at release sites most frequently coincided with the presence of soilborne pathogens associated with root-feeding *Aphthona* larvae (Caesar, 2000). These observations strongly suggested that soilborne plant pathogens including *Rhizoctonia* and *Fusarium* associated with damage caused by *Aphthona* and other insects should be collected to supplement insect releases for more rapid and consistent suppression of leafy spurge infestations.

Biological control strategies based on the use of deleterious rhizobacteria (DRB) take advantage of selected, non-parasitic bacteria that colonize plant roots and suppress plant growth (Kremer et al., 1990; Kremer and Kennedy, 1996; Nehl et al., 1997). The biological control potential of DRB toward leafy spurge has been demonstrated using suspended cell and callus tissue culture bioassays (Kremer et al., 1998; Souissi and Kremer, 1994, 1998). DRB applied to soil in leafy spurge-infested field plots in South Dakota suppressed growth to a limited extent by decreasing root weight and root carbohydrate content (Brinkman et al., 1999).

Mechanisms by which rhizobacteria suppress plant growth are not fully understood but may include overproduction of indoleacetic acid (Sarwar and Kremer, 1995; Xie et al., 1996), production of siderophores (Loper and Buyer, 1991), extracellular polysaccharides (Fett et al., 1989; Kelman, 1954), and hydrogen cyanide (Kremer and Souissi, 2001; Owen and Zdor, 2001; Schippers et al., 1987). The presence of any or all of these traits may indicate potential growth-suppressive activity and may be useful in selecting DRB from rhizosphere bacterial isolates.

More information is needed on how soilborne fungi and rhizosphere bacteria interact with environmental conditions (soil properties, climate, etc.) to successfully colonize the rhizosphere and roots and express deleterious traits so that biological control efficacy is more predictable (Horwath et al., 1998). Little is known about the influence of edaphic factors on leafy spurge biocontrol activity by soil microorganisms. Because the synergism between insects and pathogens has been largely overlooked, a survey was conducted for soilborne pathogenic fungi and DRB associated with damage caused by root-feeding insects attacking *Euphorbia* spp. in its native range in Europe. Collection of organisms with biological control potential from various sites in the native range is a necessary step in the development of biological management of invasive weed species (Harley and Forno, 1992). Our objectives were to determine relationships of selected edaphic factors and host plant conditions at collection sites with biological control activity of soilborne microorganisms, and to screen rhizobacteria from *Euphorbia* rhizospheres for putative DRB traits that might contribute to suppression of leafy spurge growth.

2. Materials and methods

2.1. Site selection and characteristics

Euphorbia species were collected in their native range at sites located between 43° and 50°N from southeastern France to eastern Hungary in May–June 1995. This region has landscapes, soils, elevation, rainfall patterns, and latitude similar to the U.S. northern Great Plains. Collection sites, landscape and ecosystem features, soil characteristics, *Euphorbia* host species, and plant growth status including disease symptoms, presence of insect species, and plant damage incurred by insects were described and recorded (Tables 1 and 2). Soils at each site were classified to soil order based on characteristics described in the *World Reference Base* (Dekkers et al., 1998) and then correlated to soil orders described in *Soil Taxonomy* (Soil Survey Staff, 1999). Selected physical and chemical soil properties were determined by the Soil Characterization Laboratory at the University of Missouri.

2.2. Soilborne fungi isolation and pathogenicity testing

Euphorbia plants exhibiting stem rot at the soil line, water-soaked lesions on foliage, necrotic cankers, or dead root buds on crowns and roots were selected from

Table 1
Descriptions of the ecosystems and classifications and characteristics of soils at *Euphorbia* collection sites established in Europe during 1995

Site	Code	Ecosystem	Soil classification		Soil texture	SOC[a] (%)	pH
			WRB[b]	USDA[c]			
Austria							
Alland-1	AU-A1	Floodplain meadow; 0–2% slope	Fluvisol	Entisol	Silty clay	5.6	7.3
Alland-2	AU-A2	Upland shoulder-summit; near barley field; 5–10% slope	Luvisol	Alfisol	Loam	4.4	7.3
Krems	AU-K	Terrace; near vineyard; 0% slope	Fluvisol	Entisol	Silt loam	0.8	7.5
Stockerau	AU-S	Floodplain; riparian forest; 0% slope	Fluvisol	Entisol	Sandy loam	3.2	7.4
Guntersdorf	AU-G	Upland shoulder-summit; near vineyard; 5-10% slope	Cambisol	Inceptisol	Loam	2.5	7.4
Hungary							
Balmazujavanos	HU-B	Hungarian plain (“puszta”); railroad right-of-way; 0% slope	Chernozem	Mollisol	Silt loam	2.1	7.4
Puspokladany	HU-P	Hungarian plain (“puszta”); near maize field; 0% slope	Chernozem	Mollisol	Silty clay	3.6	7.4
Fuzesabony	HU-F	Hungarian plain (“puszta”); near wheat field; 0% slope	Chernozem	Mollisol	Clay loam	3.5	7.3
Veszprem	HU-V	Upland summit; disturbed area (topsoil removed); 0% slope	Cambisol	Inceptisol	Clay loam	3.9	7.4
Switzerland							
Sonceboz	SZ-S	Montane backslope; forest; 45–65% slope	Podzol	Spodosol	Silt loam	13.6	7.3
Brig-1	SZ-B1	Montane backslope; meadow; 30–45% slope	Luvisol	Alfisol	Fine sandy loam	4.1	7.2
Brig-2	SZ-B2	Floodplain; grass-forb vegetation; 0%	Fluvisol	Entisol	Sandy loam	3.8	7.3
France							
The Camargue	FR-C	Floodplain; peach orchard; 0% slope	Fluvisol	Entisol	Loam	1.7	7.4
Czech Republic							
Zelatova	CR-Z	Upland footslope; near wheat field; 2–5% slope	Cambisol	Inceptisol	Sandy loam	2.8	5.9
Slovakia							
Izsa	SL-I	Floodplain; grass, shrub riparian area; 0–2% slope	Fluvisol	Entisol	Loamy sand	3.6	7.4

[a] SOC, soil organic carbon.
[b] WRB, *World Reference Base* (Dekkers et al., 1998).
[c] USDA, *Soil Taxonomy* (Soil Survey Staff, 1999).

Table 2
Species classification, plant growth status, and occurrence of crown and root fungi on *Euphorbia* plants collected at sites in Europe in 1995

Site	Code	*Euphorbia* species	Plant growth status[a]	Crown and root fungi[b]
Austria				
Alland-1	AU-A1	*E. esula-virgata*	++	+
Alland-2	AU-A2	*E. esula-virgata*	+; leaf rust symptoms	+
	AU-A2	*E. lucida*	0	–
Krems	AU-K	*E. esula-virgata*	+++; leaf rust, stem lesions; insect-damaged roots	+
Stockerau	AU-S	*E. esula-virgata*	+++; insect-damaged roots; adult *Aphthona* spp. present	+
Guntersdorf	AU-G	*E. esula-virgata*	++; leaf rust symptoms	+
Hungary				
Balmazujavanos	HU-B	*E. esula-virgata*	++; leaf rust symptoms; adult *Aphthona* spp. present	+
Puspokladany	HU-P	*E. esula-virgata*	++++; severe insect feeding damage on roots	+
	HU-P	*E. cyparissias*	++; insect-damaged roots	+
Fuzesabony	HU-F	*E. esula-virgata*	+; adult *Aphthona* spp. present	+
Veszprem	HU-V	*E. stepposa*	0	–
Switzerland				
Sonceboz	SZ-S	*Euphorbia* sp. (“wood spurge”)	0	–
Brig-1	SZ-B1	*E. seguieriana*	++++; root and foliage damage by *Oberea* spp. and *Aphthona* spp.	+
	SZ-B-1	*E. cyparissias*	+++; root and foliage damage by *Oberea* spp. and *Aphthona* spp.	+
Brig-2	SZ-B2	*E. esula-virgata*	0	–
France				
The Camargue	FR-C	*E. seguieriana*	0	–
Czech Republic				
Zelatova	CR-Z	*E. esula-virgata*	+++; leaf rust symptoms; insect-damaged roots; adult *Aphthona* spp.	+
Slovakia				
Izsa	SL-I	*E. esula-virgata*	++; adult *Aphthona* spp. present	+

[a] 0, healthy plants; +, foliar symptoms; ++, foliar symptoms and dead root buds; +++, foliar symptoms, dead root buds and stunted plants; ++++, dead plants.
[b] +, highly virulent strains of *Fusarium* and *Rhizoctonia* spp. isolated from plant material; –, no virulent fungi isolated.

each site for isolation of fungal pathogens. Plant samples were collected, transported and stored under refrigeration (5 °C), and processed within 48–72 h of collection. Diseased tissues of collected plants were surface-sterilized, and cultured for isolation and characterization of *Rhizoctonia* and *Fusarium* spp. as described elsewhere (Caesar et al., 1993, 1998).

For pathogenicity tests, inocula of *Fusarium* and *Rhizoctonia* isolates were prepared as previously described (Caesar et al., 1993, 1998). A greenhouse soil mix was infested with the inocula and dispensed into pots. Rooted stem cuttings were planted into infested soil and grown at 20–28 °C. Each treatment comprised one cutting/pot, five pots/strain; all treatments were arranged in a randomized complete block design. Plants were harvested after 14 weeks and assessed on a 0–6 rating scale (0, no disease; 1, evident stunting and root discoloration; 2, as 1 with root lesions and/or necroses; 3, root lesions, necrosis and stunting, moderate overall stunting and evident chlorosis; 4, root lesions, root necrosis, overall severe stunting with chlorosis, crown rot; 5, death of plant after 8 weeks; 6, death of plant within 8 weeks). The tests were performed twice.

2.3. Isolation of rhizobacteria

A minimum of four replicate *Euphorbia* plant specimens categorized as either "healthy" or "damaged" were collected at each site for rhizobacteria isolation. Plants, including roots, were carefully removed from surface soil using a small shovel. Special care was taken to retain enough soil on the roots to avoid desiccation and maintain intact root systems before further processing in the laboratory. Plant samples were placed in a sterile plastic bag, transported in a cooler, and stored refrigerated at 8 °C before processing.

Bacteria associated with the root surface (rhizoplane) and with soil tightly adhering to roots were considered rhizobacteria. Rhizobacteria on roots were enumerated by serial dilution on media described elsewhere (Li and Kremer, 2000). Briefly, total bacteria were determined on modified King's B agar (KBA) medium (Sands and Rovira, 1970) supplemented with cycloheximide. Although King's medium was developed to selectively culture fluorescent pseudomonads, species of other culturable bacteria generally readily grow using this medium (Kremer et al., 1990). Bacterial colony forming units (CFU) were enumerated and representative colonies were selected and subcultured by streaking growth onto KBA and tryptic soy agars to obtain pure, single-colony isolates. Selection was based on distinct bacterial types according to morphological characteristics including pigment, colony form, elevation, margin, texture, and opacity (Smibert and Krieg, 1994). Fluorescent pigment production was detected by exposing bacterial colonies to ultraviolet light (<260 nm wavelength) for 1–2 s. Representative isolates were identified by gas chromatography–fatty acid methyl ester analysis (Sasser, 1990).

2.4. Characterization of rhizobacteria

Siderophore (SID) production was determined using the chrome azurol S (CAS) agar plate technique (Ames-Gottfred et al., 1989) as modified by Alexander and Zuberer (1991). Siderophore-producing bacteria that strongly chelate iron induce a change in the blue color of CAS agar to bright orange. CAS agar plates were inoculated by spotting small amounts of 48-h-old rhizobacterial cultures with an inoculating needle. After incubation at 27 °C for 48 h, plates were examined for growth and production of orange halos around the colonies developing from each inoculum spot. Rhizobacteria were scored for SID production as excreting high and low amounts of SID by measuring orange halos >1.0 or <1.0 cm surrounding the colonies, respectively.

Indoleacetic acid (IAA) production was determined using the in situ nitrocellulose membrane assay on agar plates (Bric et al., 1991). Each rhizobacterial isolate was cultured on KBA for the assay. Rhizobacteria showing positive reactions (development of pink color on membrane) for IAA production were scored based on intensity of color development.

Exopolysaccarides (EPS) were detected using the tetrazolium chloride (TTC) agar of Kelman (1954). TTC agar plates were inoculated in a manner similar to that used in siderophore assay. After incubation at 27 °C for 48 h, plates were evaluated for bacterial growth and production of EPS from colonies developing from each inoculum spot. Rhizobacteria developing large, fluidal (mucoid) colonies with light pink coloration were scored positive (rating = 1) for EPS production; dry, butyrous colonies with red coloration were scored negative (rating = 3) for EPS production.

Cyanide production was determined qualitatively as described by Kremer and Souissi (2001). Briefly, picrate/Na_2CO_3 saturated filter papers were affixed to the underside of Petri dish lids of rhizobacteria KBA cultures, which were sealed with parafilm before incubation at 28 °C. Color change of filter papers from yellow to light brown, brown, or reddish brown was recorded at 48 h as indication of weak, moderate, or strong cyanogenic potential, respectively.

Biological control potential was assessed for DRB using a microplate callus tissue bioassay (Souissi and Kremer, 1998). Viable pieces of leafy spurge callus tissue were placed on Gamborg's B5 medium in 24-well microtiter plates. Each callus piece was inoculated with 30 μl of bacterial suspension. After incubation in the dark for 48 h at 27 °C, callus pieces were visually rated for the appearance of injury due to inoculation on a 0–4 scale (0, no damage; 1, slight discoloration; 2, tissue color change, slight growth reduction; 3, tissue color change, obvious growth reduction and tissue shrinkage; 4, tissue color change, cellular leakage, callus disintegration, severe growth reduction).

2.5. Statistical analysis

All bioassays were conducted using a complete random design with four replications and were repeated at least once. Data were subjected to analysis of variance and regression procedures. Where F-values were significant at the $P < 0.05$ level, the means were compared using Fisher's protected least significant difference (LSD) test.

3. Results

3.1. Site characteristics

Collection sites represented diverse landscapes ranging from floodplain to mountainous regions (Table 1). The soils were classified across five different soil orders based on the *World Reference Base* (Dekkers et al., 1998) and correlated with USDA *Soil Taxonomy* (Soil Survey Staff, 1999). Surface soils (0–10 cm) collected with plant samples had textures ranging from clay loam to loamy sand. All surface soils had pH values between 7.2 and 7.5, except Zelatova (Czech Republic) with 5.9; soil organic carbon (SOC) ranged from 0.8 to 13.6%. Density of *Euphorbia* spp. stands at collection sites was generally light ranging from 1 to 5 plants m^{-2}.

3.2. Soilborne fungi

Soilborne fungi including *Fusarium* and *Rhizoctonia* species were consistently isolated from diseased *Euphorbia* spp. and from plants damaged by root-attacking insects including *Aphthona* spp. (Table 2). Populations of *Fusarium* spp. in soils associated with insect-damaged plants usually exceeded levels for plants free of insect damage (Caesar, 2003). Likewise, the most virulent strains, based on greenhouse pathogenicity tests, of *Fusarium* and *Rhizoctonia* species were isolated from roots of *Euphorbia* spp. with insect feeding damage (Table 2). The fungal isolates either severely reduced root density or caused mortality of leafy spurge plants. Disease progressed from chlorosis and necrosis of lower leaves to defoliation, stem chlorosis, and apical (i.e., crown buds) necrosis. Additionally, *Rhizoctonia* isolates often caused damping off disease symptoms.

3.3. Rhizobacteria characteristics

Culturable populations of rhizobacteria were always greater on roots of diseased or insect-damaged than on healthy individuals of *Euphorbia* spp. (Fig. 1). This observation was consistent at all sites where it was possible to compare damaged plants with healthy plants. Approximately 53% of the 2000 rhizobacteria isolated from *Euphorbia* roots were classified as fluorescent pseudomonads. Of the 2000 isolates, 1144 (57.2%) were positive for one or more of the physiological properties tested. The frequency of positive tests for the physiological properties varied based on origin of rhizobacteria (collection site) and condition of the plant (healthy versus damaged) (Table 3). The most common trait was IAA production, expressed by 55% of the isolates. Other traits were less common, including EPS production, which was detected for only 8.5% of the isolates. A high proportion (>50%) of rhizobacterial isolates were classified as DRB based on the leafy spurge callus bioassay (Souissi and Kremer, 1998).

No distinct patterns of potential "growth inhibitory traits" were evident for selected DRB isolates (Table 4). Several fluorescent pseudomonads and other

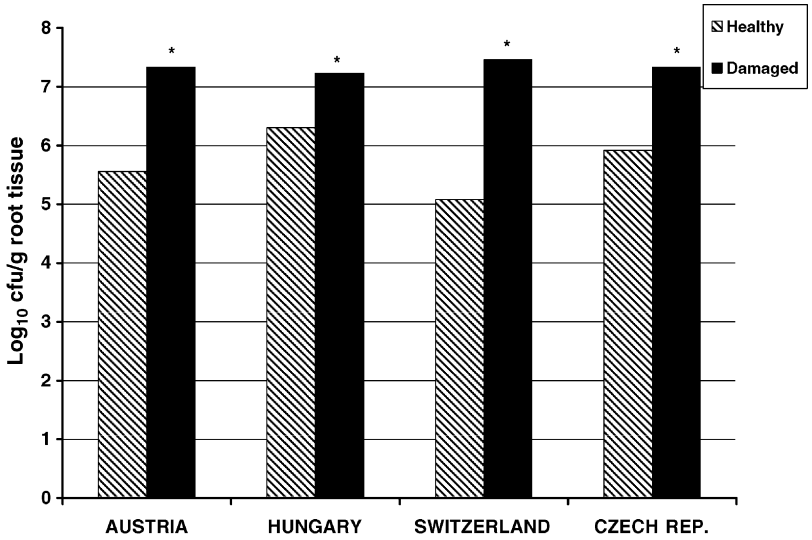

Fig. 1. Populations of rhizobacteria (*Y*-axis, number of colony forming units) from rhizospheres of *Euphorbia* spp. collected from stands at selected sites in Europe (*X*-axis), either healthy in appearance or damaged due to insect feeding and/or fungal disease. Each value represents the mean population detected for four different plants sampled within healthy and damaged *Euphorbia* plant stands at each site. Mean represented by a column designated with (*) is significantly ($P = 0.05$) different from its paired mean within sites based on Fisher's protected LSD.

Table 3
Frequency (%) of selected physiological properties among rhizobacteria isolated from *Euphorbia* spp. at European sites

Site code	*Euphorbia* species	Plant condition	No. of isolates tested	EPS[a]	SID[b]	IAA[c]	HCN[d]	DRB[e]
AU-A1	*E. esula-virgata*	Healthy	122	4.1	8.2	41.0	41.0	18.0
AU-A2	*E. esula-virgata*	Healthy	102	6.8	6.8	36.3	13.8	18.6
AU-K	*E. esula-virgata*	Healthy	98	2.6	10.2	35.7	28.6	25.5
AU-S	*E. esula-virgata*	Damaged	112	6.8	5.4	43.8	35.7	46.4
AU-G	*E. esula-virgata*	Healthy	124	8.8	10.5	43.5	21.7	45.2
AU-G	*E. esula-virgata*	Damaged	125	23.2	15.2	30.4	15.2	64.0
HU-B	*E. esula-virgata*	Healthy	131	0.8	6.1	48.8	26.0	26.0
HU-P	*E. esula-virgata*	Damaged	130	4.6	18.4	65.4	27.8	68.4
HU-F	*E. esula-virgata*	Healthy	135	7.4	22.2	66.6	14.8	40.0
HU-F	*E. esula-virgata*	Damaged	134	5.2	10.4	83.5	26.1	74.6
HU-V	*E. stepposa*	Healthy	83	8.5	33.7	61.4	14.4	33.8
SZ-S	*Euphorbia* sp.	Healthy	55	23.6	21.8	80.0	3.6	47.2
SZ-B1	*E. seguieriana*	Damaged	132	4.1	46.9	76.5	30.3	35.6
SZ-B2	*E. esula-virgata*	Healthy	134	12.0	48.0	76.1	18.0	29.1
FR-C	*E. seguieriana*	Healthy	53	3.8	45.2	62.2	28.3	41.5
CR-Z	*E. esula-virgata*	Healthy	90	0	5.6	66.6	22.2	56.6
CR-Z	*E. esula-virgata*	Damaged	122	4.1	4.1	61.5	21.3	63.1
SL-I	*E. esula-virgata*	Healthy	118	21.1	50.0	56.8	16.1	41.5
LSD (0.05)				1.5	2.1	6.5	3.0	7.8

[a] EPS, exopolysaccharide.
[b] SID, siderophore.
[c] IAA, indoleacetic acid.
[d] HCN, hydrogen cyanide.
[e] DRB, deleterious rhizobacteria [as determined by leafy spurge callus bioassay (Souissi and Kremer, 1998)].

Table 4
Physiological traits of selected rhizobacteria isolated from *Euphorbia* spp. at European sites[a]

Isolate	Origin	EPS	SID	IAA	HCN	Callus rating[b]
Pseudomonas aureofaciens 5-1	AU-A1; healthy	–	–	+	++	3.5
Pseudomonas syringae 8-1	HU-P; damaged	+	+	–	+	4.0
Chryseomonas luteola 19-2	HU-B; healthy	+	–	–	–	3.5
Burkholderia cepacia 29-7	AU-G; damaged	+	–	–	+++	4.0
Pseudomonas sp. 45-6	AU-S; damaged	–	++	+	++	3.0
Pseudomonas sp. 47-3	AU-S; damaged	++	++	–	–	3.0
Pseudomonas fluorescesns 67-2	CR-Z; damaged	++	++	–	++	3.8
Xanthomonas sp. 71-5	HU-B; healthy	++	–	+	–	3.5
Stenotrophomonas maltophilia 71-9	HU-B; healthy	–	–	–	–	0.5
Stenotrophomonas maltophilia 74-1	HU-P; damaged	–	–	++	–	4.0
Flavomonas oryzihabitans 75-11	HU-P; damaged	+	+	–	+++	3.5
Pseudomonas fluorescens 83-2	HU-P; damaged	–	–	++	–	4.0
Pseudomonas fluorescens 93-2	HU-F; healthy	–	–	–	–	3.0
Pseudomonas fluorescens 96-8	HU-F; damaged	–	++	+	+	4.0
Pseudomonas fluorescens 103-5	HU-V; healthy	–	–	+	+++	1.5
Pseudomonas fluorescens 130-6	FR-C; healthy	–	–	–	–	1.5
Pseudomonas sp. 177-4	SZ-S; healthy	–	–	–	–	3.5
Pseudomonas fluorescens 188-2	SZ-B1; damaged	–	–	++	+	3.5
Pseudomonas fluorescens 194-2	SZ-S; healthy	+	+	++	+	3.5

[a] See Table 3 for description of physiological trait codes.
[b] Determined by leafy spurge callus bioassay: 0, no damage; 1, slight discoloration; 2, tissue color change, slight growth reduction; 3, tissue color change, obvious growth reduction and tissue shrinkage; 4, tissue color change, cellular leakage, callus disintegration, severe growth reduction (Souissi and Kremer, 1998).

‘‘pseudomonad-like’’ bacteria were inhibitory to leafy spurge callus growth and produced one or more or none of the test metabolites (IAA, HCN, EPS, and SID). Several isolates that produced both EPS and HCN also significantly reduced leafy spurge callus growth. The DRB for which physiological characteristics are described in Tables 3 and 4 were detected at all sites with diverse landscape features including floodplain, steppe, and alpine slopes with soil textures ranging from clay loam to sandy loam (Table 1). Although soil properties did not correlate with potential biocontrol activity, a high incidence of DRB was associated with insect presence and plant disease ratings.

4. Discussion

Fusarium and *Rhizoctonia* isolates associated with *Euphorbia* plants wounded by insect biocontrol agents and characterized for comparative virulence and host range are important in advancing biological control of this invasive species. Higher rhizosphere fungal populations were apparently due to recurring cycles of insect attack on roots of *Euphorbia* spp., infection of damaged roots by the fungus, followed by death of plants and release of inoculum into soil. The most virulent strains of *Fusarium* and *Rhizoctonia* identified in our study were isolated from insect-damaged roots and crowns. The close relationship among *Euphorbia*, root-attacking insects, and the soilborne fungal pathogens *Rhizoctonia* and *Fusarium* supports the contention that plant pathogens in nature are necessary to synergize with root-attacking insects for growth suppression and limiting the spread of the host plants (Caesar, 2000).

Microbial invasion through injuries created by feeding-wounding insects is a common means of insect-mediated transmission of many fungal (Agrios, 1980) and bacterial (Harrison et al., 1980) plant pathogens. In the native range of *Euphorbia* spp., insects feeding on roots may transmit microorganisms including DRB and soilborne fungi into plants resulting in higher numbers on damaged plants. Insect damage likely provided readily available infection courts for entry of DRB into plants leading to greater rhizosphere bacterial populations in roots of *Euphorbia* plants with insect damage than in roots from healthy plants (Fig. 1). The presence of rhizobacteria with deleterious properties as well as fungal pathogens on damaged leafy spurge roots suggests that bacteria should be considered in the total synergistic relationship.

Based on previous research that demonstrated a strong relationship between weed-suppressive rhizobacteria and soil organic matter (Li and Kremer, 2000), we hypothesized that detrimental activity of DRB toward leafy spurge would be linked to such soil quality factors. Further, it has been recommended to include soil quality factors in studies designed to predict performance of resident microorganisms in soil as biological control agents (Horwath et al., 1998). However, the soil quality factors of soil texture and soil organic matter were not consistently related to the performance of DRB, indicated by the callus tissue bioassay (Tables 3 and 4). The clay composition of soils may support (Stutz et al., 1989) or suppress (Ownley et al., 2003) colonization and biological activity of pseudomonads. Likewise, soil organic matter may enhance weed suppression (Li and Kremer, 2000) or repress effects of rhizobacterial metabolites involved in biological control (Ownley et al., 2003). Soil and crop management influence rhizobacterial activity (Horwath et al., 1998; Li and Kremer, 2000); however, we were unable to collect information on these practices during the survey. These factors, in addition to soils, and landscape features need consideration in surveys of collection sites during explorations for potential soilborne microbial agents.

Examination of DRB characteristics revealed that no physiological factor was clearly responsible for growth-suppressive effects of rhizobacterial isolates. Previous research also showed individual metabolites of DRB could not explain deleterious effects on seedling growth in bioassays (Kremer et al., 1998). Each factor likely plays a specific role in suppressing plant growth. The production of EPS has been correlated with virulence of plant-associated bacteria. Indeed, a diverse array of EPS is often elaborated by *Pseudomonas* species that are considered opportunistic plant pathogens (Fett et al., 1989). The EPS aid bacteria in systemic colonization of plant tissue and causes wilting in affected plants, interfering with movement of water through xylem vessels (Husain and Kelman, 1958). Production of abundant EPS by rhizosphere bacteria may be a desirable trait that would allow potential biological control DRB to survive stress conditions in the field (Fett et al., 1989).

Siderophores are produced by bacterial phytopathogens, yet their importance as virulence factors or ecological determinants in bacterial–plant interactions is not fully understood (Loper and Buyer, 1991). Certain plant deleterious fluorescent pseudomonads produce SID, often in concentrations surpassing those of plant growth-promoting bacteria (Yang and Leong, 1984). Cyanide production is considered a trait of DRB because large amounts of HCN depress root respiration and indirectly impair nutrient uptake (Schippers et al.,

1987). The concentration of HCN produced by DRB determines the ability of specific isolates to be deleterious (Kremer and Souissi, 2001). Owen and Zdor (2001) reported that cyanide produced by inoculum DRB selectively inhibited velvetleaf but not maize seedling growth. Several researchers reported that production of excessive amounts of auxin-like compounds (IAA) by DRB suppressed seedling growth of plants including weed species (Loper and Schroth, 1986; Sarwar and Kremer, 1995; Xie et al., 1996). Growth-suppressive effects appear to be related to root colonization because the density of bacteria on root surfaces is often proportional to IAA production (Suckstorff and Berg, 2003).

It is likely that bacteria able to produce several growth-suppressive compounds co-exist in rhizospheres under natural conditions and interact to contribute to inhibition of plant growth. Some traits such as SID and EPS production may be highly associated with biological control activity, as indicated by callus ratings, and deserve more in-depth investigation to establish characteristics definitively associated with DRB. For example, reductions in leafy spurge callus growth (ratings ≥ 3.0) were frequently associated with rhizobacterial isolates expressing both EPS and HCN activity (Tables 3 and 4). Regression analysis indicated a good linear relationship ($R^2 = 0.586$) between EPS and HCN production. Detection of these traits using simple bioassays could be used with confidence in screening rhizobacteria with deleterious activity.

The presence of any physiological trait tested here does not assure that a particular rhizobacterial isolate is a DRB nor does its absence suggest it is not. However, given that a high proportion of rhizobacteria positive for one or more traits (Table 3) led to decreased plant growth suggests that these traits are worthwhile for consideration in screening for leafy spurge growth reduction. Few isolates, i.e., *Pseudomonas fluorescens* 93-2 and *Pseudomonas* sp. 177-4 (Table 4), that expressed none of the traits tested, suppressed callus growth. Why these acted as DRB is not clear although other traits not tested (i.e., cytokinin or gibberellic acid production, or pectinolysis) may be responsible for growth-suppressive effects. Also, some isolates may produce very complex phytotoxins such as the D7 toxin produced by *P. fluorescens* D7 upon colonization of downy brome (*Bromus tectorum* L.) seedlings (Gurusidadaiah et al., 1994). Other traits that may be associated with DRB and not screened in the present study might be considered in future testing.

5. Conclusions

Our study illustrates the most effective condition for inducing disease and subsequent mortality of leafy spurge includes plant-associated microorganisms contributing to a synergism with root-damaging insects. The synergism of soilborne fungi with *Aphthona* spp. has been documented (Caesar, 2003) and is verified here. The unique finding of our study is that enhanced disease of leafy spurge also involved DRB interacting with soilborne fungi and insects. Our study confirms that multiple-agent biological control strategies for effectively suppressing leafy spurge growth may be an effective tool for the management of invasive weed species. Certain soil properties (i.e., soil organic matter) may be important in affecting all agents involved in attacking leafy spurge roots. Thus, optimization of a multiple biological control agent approach requires consideration of the soils and landscapes of specific leafy spurge infestations. This has been demonstrated for effectiveness of individual *Aphthona* species as insect biological control agents at different North American sites (Gassmann and Schroeder, 1995). A better understanding of the interactions of biological control agents, leafy spurge host plant, and environment interactions described in this study will enhance development and effectiveness of the multiple agent approach.

The observations collected in this investigation are useful in planning collection strategies for soilborne microorganisms on *Euphorbia* spp. in its native habitats for evaluation as potential biological control agents. In addition to standard selection criteria for biological control agents (Harley and Forno, 1992), the tendency for insect–microorganism synergism to occur in nature is strongly suggested as a principal criterion in selecting biological control agents. Furthermore, the methods described in this study should be easily adapted for screening soilborne fungi and rhizosphere bacteria associated with other invasive weed species targeted for biological control.

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