MICROBIAL INOCULANTS ON WOODY LEGUMES TO RECOVER A MUNICIPAL LANDFILL SITE

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Abstract. Tree and shrubby legumes have great potential in degraded land rehabilitation because of their ability to form symbiotic associations with nitrogen fixing rhizobia and mycorrhizal fungi. Extensive soil disturbance reduces natural microbial propagules thus preventing the formation of beneficial plant-microbes symbiosis. Reintroduction of selected microbial symbionts may improve the recovery rate of disturbed ecosystems. We inoculated selected rhizobia and arbuscular mycorrhizal fungi on two woody legume species, the mediterranean shrub *Spartium junceum* L. and the exotic tree *Acacia cyanophylla* Lindl. in order to recover a sealed municipal landfill (Palermo, Sicily, Italy). Inoculated plants showed shoot growth parameters 2 to 12-fold higher than uninoculated plants. After transplanting on the municipal landfill site, inoculated plants showed no transplant shock and low mortality (6–15%). The chemical analysis of P and N plant content showed no differences between inoculated and uninoculated plants suggesting that a dilution effect occurred due to higher biomass production of the inoculated plants. The beneficial effects of mycorrhization and rhizobium inoculum on growth parameters were still detectable one year after transplanting in *S. junceum*.

Keywords: *Acacia cyanophylla*, Arbuscolar Mycorrhizal Fungi (AMF), inoculated wild woody legumes, landfill rehabilitation, nitrogen fixing shrubs, rhizobia, *Spartium junceum*

1. Introduction

Municipal landfill reclamation is a challenge due to the inhospitality of the environment (Tribis, 2000). Inadequate topsoil, physical and chemical characteristics, low organic matter content, structural instability, local toxicity, and other factors strongly limit the establishment of a natural plant cover, in particular on dry environments (Pastor *et al.*, 1993). Only ruderal vegetation is usually found on such sites and anthropic intervention is always necessary to re-establish a suitable plant cover in order to improve soil properties (La Marca *et al.*, 1998).

Legumes are the most appropriate candidates for revegetation of arid and/or disturbed ecosystems because of their ability to establish tripartite symbiotic associations with nitrogen fixing rhizobia and mycorrhizal fungi (Herrera *et al.*, 1993;

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Barea and Jeffries, 1995; Barea *et al.*, 1996; Zaharan, 1999). Rhizobia are widespread soil bacteria able to induce the formation of root nodules and fix nitrogen in cultivated and wild legumes. They reduce atmospheric nitrogen into ammonium that becomes available to legumes and associated non legume plants. Mycorrhizal fungi are soil fungi able to form mutualistic symbiosis with many plant species. Mycorrhizas enhance plant nutrition (P and other nutrients) and drought tolerance. In most cases, especially when nitrogen and phosphate are limiting in soil, AM fungi and rhizobia act synergistically. Combined inoculation leads to a higher degree of host colonization and enhances plant growth more than inoculation with either microsymbionts alone (Requena *et al.*, 1997; Birò *et al.*, 2000). This synergistic effect seems related not only to nutritional factors but also to molecular mechanisms involving the early stages of rhizobium-legume interaction that stimulate fungal colonization through Nod-factors stimulated flavonoids (Xie *et al.*, 1995).

Disturbed and degraded soils, such as those used to cover a sealed lanfill site, lack normal microbial activity and natural microbial propagules, thus preventing the formation of beneficial plant-microbe symbiosis (Scullion, 1992; Del Val *et al.*, 1999). Reintroduction of selected microbial symbionts with plants may improve the recovery rate of disturbed ecosystems (Prakash *et al.*, 1995).

We report on a medium term field experiment of landfill reclamation in a mediterranean environment with two woody legumes, the native shrub Spanish Broom (*Spartium junceum* L.) and the multipurpose exotic tree legume *Acacia cyanophylla* Lindl. (Kaplan, 1979) that were dually inoculated at the seeding stage with selected AM fungi and specific rhizobia. Mycorrhizal fungi were added either as a single *Glomus* species or, in order to verify the enhanced ecological features of the mixture, as a mixture of three different *Glomus* species.

2. Materials and Methods

Acacia cyanophylla and *Spartium junceum* seeds were collected from field growing plants in Sicily, scarified with H_2SO_4 (15 and 30 min, respectively), pre-germinated on water agar and seeded in 1,2 l plastic pots on steam sterilized soil (sand 47%, silt 17%, clay 36%, O.M. 1.51%, N 0.2%). This soil was sampled from a site of the same mounts where the Landfill of Bellolampo lies (Palermo Mountains, PA, Italy) and with similar pedologic characteristics. Both are typical lithosols with rock outcrop- chromic luvisols, diffused in occidental Sicily. These soils are considered poor and have reduced agronomic potentiality. Mycorrhizal inoculum (AM) was applied by replacing 1/3 of the pot volume with a crude inoculum (soil, infected roots and spores) from pot cultures of Sudan grass. Two different AM fungal treatments were applied: a single species, *Glomus constrictum* (LCST) and a mixture of three *Glomus* species, namely *G. clarum*, *G. intraradices* and *G. brasilianum* (INVAM).

All mycorrhized plants were also inoculated with the specific rhizobium strain. *A. cyanophylla* was inoculated with *Bradyrhizobium* sp. strain RFH 383 selected in Tunisia (Nasr *et al.*, 1999); *S. junceum* was inoculated with *Bradyrhizobium* sp. strain Sj9 isolated on *S. junceum* in Sicily (Quatrini *et al.*, 2002). Rhizobia were grown on Yeast Mannitol medium and inoculated (1 ml plant^{-1}) at a concentration of about 10^9 CFU ml^{-1} after germination. A second inoculum dose was added after 1 month. Plants were grown in a greenhouse from July 1999 to March 2000 and irrigated with tap water.

A sample of three plants for each treatment was analysed after 4 months' growth in the greenhouse and shoot, root length and weight were measured. Shoots were dried at 45 °C for 3–4 days (to constant weight) and weighed. AM colonisation was analysed on the entire root system by clearing and staining with Trypan blue (Koske and Gemma, 1989) using the gridline intersect method (Giovannetti and Mosse, 1980). Root nodules were counted and weighed.

P content in plant material was measured according to Jones and Case (1990) with slight modifications. A wet digestion under reflux was automatically performed. Finely ground dry plant matter (0.2 g) was placed at the bottom of 25 cm high glass digestion tubes, treated with 3 ml of acid digestion mixture (1 part $HClO_4$ 60% (w/w), 4 parts HNO_3 70% (w/w)), allowed to stand overnight and then digested for 30 min at 150 °C, 45 min at 200 °C, 140 min at 240 °C. After filtration through Whatman 42, phosphorus content was colorimetrically determined by the ammonium molybdate-ammonium metavanadate reagent (Olsen and Sommers, 1982).

Nitrogen content determination was based on the Kjeldahl method (Novozamsky *et al.*, 1983). Finely ground dry plant material (0.2 g) was placed at the bottom of 25 cm high glass digestion tubes, treated with 4 ml of H_2SO_4 (96%, w/w) and 1.0 g catalyst (10 parts K_2SO_4 and 1 part $CuSO_4$) and then automatically digested for 30 min at 150 °C, 1 h at 250 °C and 3 h at 360 °C. The digested content was then treated with an excess of NaOH (32% w/w), distilled and collected on an excess of boric acid (10 ml 2%, w/w). The formed borate anion was then automatically back-titrated with 0.01 N H_2SO_4.

After 35 weeks' growth in the greenhouse, plants were transferred to an eight yr-old sealed municipal landfill (Bellolampo, Palermo, Italy, 42°22′ North; 3°48′ East; average T 18,8 °C; rainfall 816 mm/year. Soil characteristics: Sand 54%, silt 18%, clay 28%, O.M. 0.85%, N 0.1%). One hundred sixty three plants were transplanted in a Completely Randomised Design in march 2000, with no addition of fresh substrate. Distances between plants were 1.5 m × 2 m within and among rows, respectively. Individual plants were supported by a wooden stake and protected from domesticated and wild grazing animals by a plastic fence. The entire area was fenced, summer irrigation and manual weed control were also performed.

Plant growth was followed every three weeks in the greenhouse and every seven weeks in the landfill site by measuring the following parameters: plant height, collar diameter, number of total and differentiated leaves (only in *A. cyanophylla*), branch number and branching height, plant survival. P and N measurements were repeated on randomly collected leaves and stems after 65 weeks from seeding (30 weeks from transplanting in the landfill). Leaves and stems of *S. junceum* were analysed together because leaves were too small and rare at the sampling time.

Data for each species were statistically analysed by 1-way analysis of variance using inoculation as treatment. The means were separated by the HSD Tukey test ($P = 0.05$).

3. Results and Discussion

3.1. SYMBIOTIC FEATURES

Inoculated plants were mycorrhizal and nodulated after 4 months' growth in the greenhouse. Uninoculated plants were not infected. Root colonisation by AMF ranged from 54 to 93% and was higher in plants of both species inoculated with the *Glomus* mixture compared with the *G. constrictum* alone (Table I). Nevertheless, plant height and stem diameter (Figure 1) were higher in plants inoculated with *G. constrictum*. Mycorrhizal infection degree is generally related to plant growth parameters and nutrient content. The percentage of root colonisation is considered a good approach to evaluate plant growth response to AM fungi (Ibijbijen *et al.*, 1996). Nevertheless, our results demonstrate that the extent of root colonisation is not necessarily an indication of the endophytes efficiency (Roldan-Fajardo, 1994). Nodule fresh weight, rather than nodule number, showed that the selected rhizobium inoculated on *A. cyanophylla* was, as expected, highly efficient with respect to the strain inoculated on *S. junceum* that was not selected (Table I). Nodulation was not significantly influenced by different AMF inocula (Table I).

3.2. PLANT SURVIVAL, DEVELOPMENT AND NUTRIENT CONTENT

Inoculated plants developed better than uninoculated controls as shown by heights, stem diameters (Figure 1), number of leaves and branches, differentiated/undifferentiated leaves rate in *A. cyanophylla* (data not shown) and higher aerial biomass for both species (Table I). Root biomass was not influenced by inocula (Table I). Inoculated plants had greater shoot/root ratio than uninoculated controls (Table I). This parameter means a greater biomass efficiency since less energy is directed to root formation. *S. junceum* was generally more stimulated by inoculation than *A. cyanophylla*.

Legumes established in the sealed landfill of Bellolampo without aftercare, apart from manual weed control and summer irrigation after transplanting. No fresh soil was added before transplanting. *S. junceum* survival in the landfill site was highly enhanced by inoculation (94% vs. 52%, Table III) when compared to controls and other landfill recovery experiments. Adversi and Monti (1996) in an experiment of evaluation of different plant species for the environmental recovery

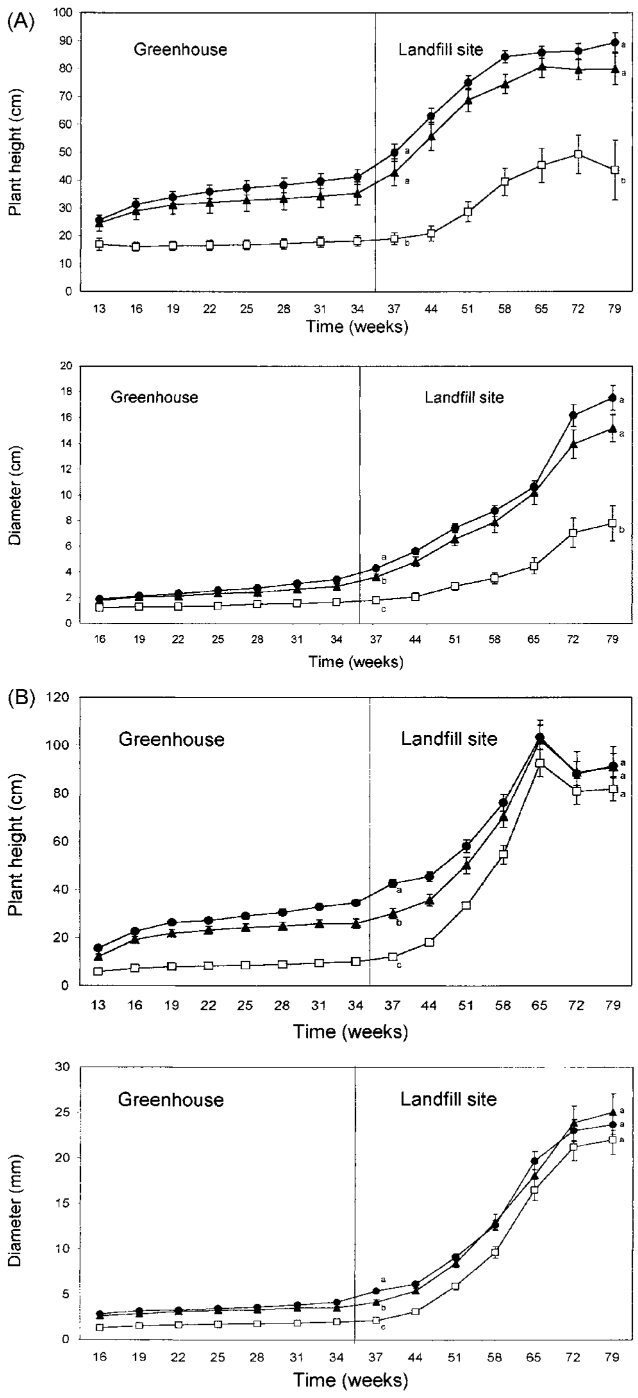

Figure 1. Heigths and collar stem diameter of (A) *Spartium Junceum* and (B) *Acacia cyanophylla* growing in the greenhouse and in the municipal landfill uninoculated (☐) and inoculated with specific rhizobia and two different AMF inocula: (●) *Glomus constrictum* and (▲) a *Glomus* mixture. Heights decreases in the landfill growth curves are due to accidental grazing. Bars indicate S.D.. Means at the transplanting time (37th week) and last field measurement (79th week) with different letters, are significantly different at P = 0.05 (Tukey's HSD range test).

TABLE I

Symbiotic and developmental parameters (means of 3 samples ± S.D.) of *Acacia cyanophylla* and *Spartium junceum* plants grown for 4 months in a greenhouse, double inoculated or not with rhizobia and AM fungi. Means with different letters, within each species, are significantly different at $P = 0.05$ (Tukey's HSD range test). Shoot/Root Ratio was calculated on means

Plant species	Treatment	Microbial inocula		AM colonization %	Nodule number plant^{-1}	Nodule f.wt. (mg)	Shoot f.wt. (g)	Shoot d.wt. (g)	Root f.wt. (g)	Shoot/ Root ratio
		AM fungi	Rhizobium strain							
S. junceum	Double inoculation	*G. constrictum*	Sj9	54.8 ± 2.4 b	12 ± 3.5 a	17.7 ± 8.6 a	2.01 ± 0.09 a	0.46 ± 0.02 a	0.64 ± 0.11 a	3.14
	Double inoculation	*Glomus* mix[a]	Sj9	76.7 ± 3.2 a	27 ± 17.0 a	21.3 ± 8.5 a	1.74 ± 0.5 a	0.39 ± 0.11 a	0.53 ± 0.20 a	3.28
	Uninoculated control	–	–	0 c	0 b	0 b	0.29 ± 0.07 b	0.09 ± 0.03 b	0.42 ± 0.15 a	0.69
A. cyanophylla	Double inoculation	*G. constrictum*	RFH383	71.0 ± 1.7 b	16.3 ± 3.2 a	235 ± 131 a	6.47 ± 3.6 a	1.25 ± 0.69 a	2.30 ± 0.93 a	2.81
	Double inoculation	*Glomus* mix[a]	RFH383	93.3 ± 2.5 a	22.7 ± 5.8 a	193 ± 109 a	5.25 ± 0.9 ab	0.92 ± 0.13 ab	1.78 ± 0.67 a	2.95
	Uninoculated control	–	–	0 c	0 b	0 b	0.51 ± 0.17 b	0.12 ± 0.02 b	0.62 ± 0.28 a	0.82

[a] *G. clarum, G. intraradices* and *G. brasilianum.*

of a landfill in Bologna (Italy) scored uninoculated *S. junceum* survival rate of 48% and 41% after one and two years from transplanting, respectively (Adversi and Monti, 1996).

Higher mortality of our inoculated *Acacia* plants was observed during the last period of growth in the greenhouse (Table III) as they developed so fast that the containers became too small to fit them.

In spite of fences, cows got into the experimental area during autumn 2000 and ate plants, *A. cyanophylla* being the preferred species (Figure 1). However, this inconvenient allowed us to evaluate the recovery ability of both species (Figure 1). Bellolampo soil contained AM fungi and rhizobia capable of nodulating both *A. cyanophylla* and *S. junceum* as shown in a greenhouse experiment on unsterilized Bellolampo soil using both species as trap plants (data not shown). It is thus feasible that new symbiotic relationships were established between our plants and native microrganisms after transplanting. Nevertheless, the beneficial effect of pre-inoculation with selected microrganisms was still evident after more than one year from transplanting for *S. junceum*.

N and P content analysis were performed to clarify the nutrient effect of the two symbiosis (Joshi and Maikhuri, 1996). Chemical analysis of plant material (Table II) showed that P concentration was higher in inoculated *A. cyanophylla* only after 16 weeks, while little or no effect on N content was detected. After 65 weeks N and P concentration was similar for inoculated and uninoculated plants or even higher in uninoculated ones (Table II). N and P concentration, however, is referred to mg^{-1} d.w. of plants and does not account for the higher biomass of the inoculated plants (Figure 1 and Table II). Total N and P content per plant were not measured but they were surely enhanced by double inoculation. This dilution effect has already been described in plants growing in infertile sites (Dela Cruz *et al.*, 1988) and is caused by the dilution of relatively unavailable nutrients within a larger biomass.

Other beneficial effects of AM inoculants other than P nutrition cannot be excluded, i.e. different hormone balance, better drought tolerance (Ruiz-Lozano *et al.*, 2001), enhanced mineral uptake (Joner and Leyval, 1997), sequestering of toxic elements (Kaldorf *et al.*, 1999) and modifications in root architecture (Citernesi *et al.*, 1998).

4. Conclusions

Dual inoculation with rhizobia and mycorrhizal fungi strongly benefited greenhouse and field growth and survival after transplanting of *S. junceum* and *A. cyanophylla* in the landfill of Bellolampo (Palermo, Sicily, Italy).

The beneficial effect of rhizobia and AM fungi largely depends on the specific fungus-bacterium combination used (Requena *et al.*, 1997). In our experiment *Glomus constrictum* appeared the best performing mycorrhizal fungus in spite

TABLE II

Phosphorus and nitrogen content (means of 3 samples±S.D.) of *Spartium junceum* and *Acacia cyanophylla* inoculated or not with rhizobia and AM fungi, 16 and 65 weeks after seeding (30 weeks after transplanting in the landfill at Bellolampo.)

Plant species	Treatment	Microbial inocula		Nitrogen % (d.w.)			Phosphous % (d.w.)		
		AM fungi	Rhizobium strain	16 weeks	65 weeks		16 weeks	65 weeks	
					Leaves	Stems		Leaves	Stems
S. junceum	Double inoculation	*G. constrictum*	Sj9	2.33 ± 0.20	1.65 ± 0.10		0.30 ± 0.01	n.d.	
	Double inoculation	*Glomus* mix [a]	Sj9	1.86 ± 0.12	1.65 ± 0.23		0.27 ± 0.03	n.d.	
	Uninoculated control	–	–	1.92 [b]	1.69 ± 0.34		n.d.	n.d.	
A. cyanophylla	Double inoculation	*G. constrictum*	RFH 383	2.73 ± 0.30	2.04 ± 0.07	0.75 ± 0.08	0.30 ± 0.01	0.27 ± 0.03	0.19 ± 0.06
	Double inoculation	*Glomus* mix [a]	RFH 383	2.92 ± 0.20	2.26 ± 0.21	0.92 ± 0.16	0.30 ± 0.01	0.30 ± 0.02	0.23 ± 0.06
	Uninoculated control	–	–	2.50 [b]	2.56 ± 0.30	0.96 ± 0.16	0.11 [b]	0.36 ± 0.08	0.24 ± 0.04

n.d. = not determined.

[a] *G. clarum, G. intraradices* and *G. brasilianum.*

[b] Uninoculated control plants after 16 weeks were analysed together because of their small size.

TABLE III

Greenhouse (8 months) and field survival (1.5 years) of *S. junceum* and *A. cyanophylla* inoculated or not with specific rhizobia and AM fungi

Plant species	Treatment	Microbial inocula		Survival %	
		AM fungi	Rhizobium strain	Greenhouse	Landfill
S. junceum	Double inoculation	*G. constrictum*	Sj9	77.5	94.2
	Double inoculation	*Glomus* mix[a]	Sj9	80	94.1
	Uninoculated	–	–	82.7	52.3
A. cyanophylla	Double inoculation	*G. constrictum*	RFH383	81	86.6
	Double inoculation	*Glomus* mix[a]	RFH383	65.6	83.3
	Uninoculated	–	–	88.2	70.3

[a] *G. clarum, G. intraradices* and *G. brasilianum.*

of its lower colonization rate in respect to the AMF mixture. Although the *Bradyrhizobium* strain Sj9 contributed positively to ameliorate growth of *Spartium junceum*, the rhizobium performances could still be enhanced by selecting the most efficient one among those belonging to our collection of rhizobia isolated from *S. junceum* root nodules in Sicily (Quatrini *et al.*, 2002).

Native shrubby legumes are particularly suitable for reclamation of arid and semiarid environments (Barea *et al.*, 1996). *Spartium junceum* strongly responded to dual inoculation more than *A. cyanophylla* and confirmed its suitability for arid landfill recovery programs. *A. cyanophylla*, although not native, has a short vital cycle and non-invasive behaviour in Sicily that make it suitable as a pioneer tree for disturbed soil restoration in dry mediterranean areas. The mycorrhizal legumes may act as 'fertile islands' serving as sources of inoculum for the surrounding area and improving nutrition for the non N-fixing vegetation that may follow (Barea and Jeffries, 1995).

We conclude that arid landfill reclamation using woody legumes and in particular native shrubby legumes, endowed with optimized rhizosphere symbiosis is feasible and worthwile. Mechanisms which determine the beneficial effect on plants of dual inoculation need to be further investigated.

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