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ORIGINAL PAPER

Nodulation and growth of black locust (*Robinia pseudoacacia*) on a desurfaced soil inoculated with a local *Rhizobium* isolate

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Abstract Nodulation and nitrogen fixation of black locust (*Robinia pseudoacacia* L.), a legume tree broadly used in Argentina for urban and agricultural afforestation, was studied in hydroponic culture. The development of seedlings inoculated with a local strain of *Rhizobium*, highly specific for *R. pseudoacacia*, was also compared with respect to non-inoculated but N-fertilised seedlings. This strain produced fast nodulation and high crop yield and leaf N content. Already nodulated plants with the local *Rhizobium* strain were assayed for growth in a greenhouse pot experiment with soil from a field where topsoil has been removed for industrial purposes, whilst pots with non-desurfaced soil from the same field were used as control. Non-inoculated plants were also grown in either control or desurfaced soil. Inoculated plants developed better than non-inoculated plants in desurfaced soil, and in control soil as well, suggesting that the symbiosis was able to overcome the nutrient limitation of the desurfaced soil. Non-inoculated plants were nodulated by native soil born *Rhizobium*, either in control or desurfaced soil, but they showed low final nitrogen leaf content and low nitrogen fixation activity, suggesting that native rhizobia were ineffective.

Keywords Nitrogen-fixing trees · Desurfaced soil · *Rhizobium* · Soil reclamation · Legume symbiosis

Abbreviations
DM Dry matter

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Introduction

Lands of very good agricultural quality in Buenos Aires Province (Argentina) have been traditionally mined to extract topsoil for the manufacture of bricks and other building materials. The extraction produces a loss of organic matter that severely limits or prevents the future agricultural production. Forestry and agroforestry have been proposed for rehabilitation of degraded lands (Lal 1996; Montagnini 1992). Gimenez et al. (2002) have tested *Eucalyptus* species in a desurfaced land in La Plata city (Buenos Aires Province), but a poor tree growth was observed due to bad physical and chemical properties of soil, mainly low available N. Thus, nitrogen-fixing trees would be a better option for these poor soils than non-nitrogen fixing trees. Many species of legumes (Franco and De Faria 1997) and actinorhizal plants (Dawson 1986; Diem and Dommergues 1990; Wheeler and Miller 1990) have been used for soil reclamation, and their growth can be increased in N-limited soils by proper inoculation with an effective microsymbiont (Gutteridge 1998).

Robinia pseudoacacia L. (subfamily *Papilionoideae*), known as black locust in the world and 'Acacia Blanca' or 'Falsa Acacia' in Argentina, is a legume tree native of North America, generally adapted to temperate regions (Hanover and Mebrahtu 1996). It is currently used for production of timber, fodder and honey, and also in pulp industry (Hanover and Mebrahtu 1996; Keresztesi 1980). In addition, it can colonise disturbed sites due to its fast growth and its capacity to fix atmospheric nitrogen by symbiotic association with *Rhizobium* spp. For this reason it has been used in rehabilitation of mining land and heavily industrialised areas and in erosion control and soil stabilisation (Dagar 1998; Keresztesi 1980; Hanover and Mebrahtu 1996; Panagopoulos and Hatzistathis 1995;

Zeleznik and Skousen 1996). *R. pseudoacacia* has also shown high growth and N_2 fixation under elevated CO_2 concentrations (Feng et al. 2004; Norby 1987; Olesniewicz and Thomas 1999), suggesting an important role of this legume for carbon sequestration.

The objective of this work was to characterise a local *Rhizobium* isolate and to study its symbiotic efficiency on the performance of *R. pseudoacacia*, from the point of view of rehabilitation of mined and degraded land. We used soil from a desurfaced land, which represents an extreme situation of degraded soils of Buenos Aires province in Argentina. To achieve this point, we started with the evaluation of nodulation parameters and plant growth under controlled conditions before running a field experiment.

Materials and methods

Plant growth, bacterial growth and inoculum

Seeds of *R. pseudoacacia*, collected in a forest located near the city of 25 de Mayo (Buenos Aires, Argentina), were manually scarified with abrasive paper, surface-sterilised in 10% *p/v* H_2O_2 for 5 min, washed three times with sterile water and germinated in sterile flasks on wet perlite. Seeds were germinated in a greenhouse with a temperature between 16 and 28°C, a relative humidity between 40 and 90%, and photoperiod of 16 h light and 8 h darkness, using 400 W wolfram lamps (Osram). Plant height was measured weekly. At harvest, shoot, roots and nodules were separated for biomass estimation as dry matter (DM) after 48 h at 60°C. The dried leaves were milled and analysed for their organic N content by the Kjeldahl method (Forster 1995).

A local strain of *Rhizobium* named RpI1 was isolated from a field nodule of *R. pseudoacacia* collected at the campus of Universidad Nacional de Luján, Buenos Aires Province. The nodule was surface-sterilised with 70% ethanol for 1 min, washed three times with sterile water and cut in two halves. The red inner tissue was extracted with a sterile pipette and was cultivated in YEM agar (Vincent 1980) at 28°C. A typical single pink colony was further sub-cultured and tested for good growth in liquid medium and maintained as the new isolate *Rhizobium* RpI1. The broad host range *Rhizobium* sp. strain NGR 234 was used in some experiments as a positive nodulation control (Pueppke and Broughton 1990).

For inoculum preparation, *Rhizobium* strains were cultivated 48 h in 50 ml of YEM at 28°C and 200 rpm. Bacteria were separated by centrifugation and re-suspended in 50 ml of a modified 1/10 strength Hoagland mineral solution (Hoagland and Arnon 1950) without N. Bacterial suspensions were diluted to have an optical density of 0.15 (λ=500 nm), which corresponded to a concentration of 3.10^8 bacteria/ml or a total protein content of 8.5 μg/ml, measured by the Bradford method (Bradford 1976) against an albumin standard.

Soil substrate

The soil of Alfisol order and Argiacuol great group was sampled from a field in the south west of Buenos Aires city, which had formerly been a brick manufacture industry for many years and now is abandoned. One part of the field had been desurfaced, with the exposure of the B2t horizon, having a silty clay loam texture (29.89% clay, 51.63% silt and 18.48% sand). The high content of silt gives the soil a slow water infiltration rate, which produces frequent water logging. As a control, we used the surface horizon of a nearby soil located in the same field, which had been not desurfaced and is actually used for soybean production. The chemical properties of the desurfaced and the control non-desurfaced soils are listed in Table 1. About 1 m^3 of a 5-cm deep layer of each soil was collected using a bulldozer. This material was transported to the university and stored outdoors. Soil substrates were gently milled to crash big fragments, sieved (8×10 mm) and used without any sterilisation.

Nitrogen fixation estimation

Symbiotic efficiency in terms of N fixation was estimated by measuring leaf N concentration and total leaf N content at time of harvest, and comparing data from inoculated plants vs non-inoculated controls.

Experiment 1: host specificity of the *Rhizobium* strain RpI1

To assess the host range of the *Rhizobium* isolate, seeds of some herbaceous or woody legumes representative of the

Table 1 Chemical properties of the soils used in this study

Soil properties	Control soil	Desurfaced soil
Organic C[a] (%)	2.7	0.7
Organic N[b] (%)	0.30	0.083
Available P[c] (mg kg^{-1})	19.9	3.85
pH[d]	6.50	6.95
Exchangeable calcium[e] (cmol kg^{-1})	13.9	13.0
Exchangeable magnesium[e] (cmol kg^{-1})	2.1	5.1
Exchangeable potassium[e] (cmol kg^{-1})	2.7	1.2

[a] Wet oxidation (Walkley and Black 1934)
[b] Kjeldahl (Forster 1995)
[c] Bray-Kurtz (Kuo 1996)
[d] Water 1:2.5
[e] 1 N ammonium acetate pH 7 (USDA 1972)

three subfamilies were germinated and inoculated in the same way as black locust. Some seeds were from commercial sources whilst others were collected in forests of Buenos Aires Province. The species tested were *Acacia visco* Lorentz ex Griseb., *Albizia julibrissin* Durazz., *Arachis hypogaea* L., *Centrosema macrocarpum* Benth., *Cicer arietinum* L., *Enterolobium contortisiliquum* (Vell.) Morong (ear pod tree), *Glycine max* (L.) Merr. (soybean), *Lens culinaris* Medik. (lentil), *Leucaena leucocephala* (Lam.) De Wit., *Lotus* glaber Mill., *Medicago sativa* L. (alfalfa), *Phaseolus vulgaris* L. (common bean), *Pisium sativum* L., *Prosopis chilensis* (Mol.) Stuntz, *Trifolium repens* L. (white clover), *Vicia faba* L. (broad bean) and *Vigna unguiculata* (L.) Walp. (cowpea); *R. pseudoacacia* was included as a positive control.

Three-day-old seedlings were transferred to pots with sterile perlite and inoculated by pouring 2 ml of the inoculum of RpI1. Seedlings were watered with 1/4 strength Hoagland with a concentration 0.036 mM for NH_4NO_3. Ten plants per species were inoculated whilst five plants were not and were used as negative controls. Roots were examined for nodulation 60 days after inoculation.

Experiment 2: nodulation kinetics in pouches

To monitor nodulation rate of *R. pseudoacacia* inoculated with RpI1 in our greenhouse conditions, a nodulation experiment was performed using plants growing in pouches (Mega International, Mineapolis, MN, USA). Two-day-old seedlings were placed in sterilised pouches watered with 1/4 Hoagland solution without N. The nutrient solution was removed and changed once a week. Seedlings were inoculated, 3 days after transfer, by pouring 500 μl of RpI1 suspension directly on the root. The appearance of nodules was recorded weekly. Non-inoculated plants remained as negative controls. The number of plants per treatment was 12.

Experiment 3: growth, nodulation and N_2 fixation in a drip–drop system

We conducted a third experiment to compare plant growth under N fixation vs plant growth with N mineral nutrition, and to assay the symbiotic efficiency of our local *Rhizobium* isolate. Three-day-old plants (one plant per pot) were settled in 330 cm^3 pots filled with perlite sterilised as inert substrate, and watered in a continuous drip–drop watering system (Valverde and Wall 2002). Briefly, four plastic pots with plants were accommodated hanging in a bigger pot (3,000 cm^3) which served as a reservoir of nutrient solution, a 1/4 strength Hoagland solution with or without N as described below. Mineral solution was forced to recycle from the reservoir into the pots, through an airlift system. Nutrient solution (800 ml) was renewed weekly during the experiment. Plants were inoculated at day 7 after germination by pouring 2 ml of the suspension of rhizobia, RpI1 or NGR 234 strains, directly on the base of the root. A group of non-inoculated plants were fertilised during the whole experiment by using the same Hoagland solution but with a 10-fold increase in N concentration (0.36 mM NH_4NO_3). A last group of plants was neither inoculated nor fertilised with N. The number of plants per treatment was eight. Plants were harvested at day 59 after inoculation, and root, nodules, stem and leaves were separated for biomass estimation as DM and for chemical analysis.

Experiment 4: growth and N_2 fixation in pots with soil substrate

We conducted a fourth experiment to assess the performance of nodulated plants with our local *Rhizobium* isolate and to compare to non-inoculated plants, on a substrate from a degraded soil. One-week-old seedlings of *R. pseudoacacia* were transferred to pouches, inoculated with 1 ml of RpI1 suspension, and watered with 1/4 Hoagland solution without N. Non-inoculated plants were kept as controls. Twenty-five days after inoculation, plants were examined for nodulation and then either the nodulated or the control plants were individually transferred to 330 cm^3 pots and filled with the desurfaced or the non-desurfaced-soil, as describe before. Four treatments were done (seven replicates per each treatment): plants inoculated with RpI1 in pots with non-desurfaced soil; plants inoculated with RpI1 in pots with desurfaced soil; non-inoculated plants in pots with non-desurfaced soil; and non-inoculated plants in pots with desurfaced soil. From the moment of transplantation, all pots were watered three times a week only with distilled water. Shoot height was weekly registered. Three months after inoculation, plants were harvested for nodule recording and biomass estimation. The comparison between inoculated and non-inoculated plants growing on desurfaced soil was repeated for confirmation three times in different seasons along three consecutive years. The first experiment was performed from October 2001 to January 2002 (Spring–Summer in Argentina), the second one was run between June 2002 and August 2002 (Winter in Argentina) and the third one between April 2003 and August 2003 (Autumn–Winter in Argentina). The soil substrate stored as a pile in an open place at the university campus was characterised by a decrease in the organic C and N contents, especially in the control non-desurfaced soil, as a result of mineralisation and leaching of C and N compounds during the storage. Consequently, in the second and third repetition, the plant growth in the control non-desurfaced soil was equal to that of the plants growing on

desurfaced soil (data not shown). For this reason, only the plant growth in the desurfaced soil substrate was compared between inoculated to non-inoculated plants. The statistical analysis was done comparing the mean values of different parameters by the Student *t* test.

Results and discussion

Symbiosis between *R. pseudoacacia* and *Rhizobium* RpI1 in hydroponic culture

The *Rhizobium* sp. strain RpI1 appeared to be very specific for its original host. Except for *R. pseudoacacia*, none of the 17 species of herbaceous and woody legumes tested developed nodules after root inoculation with RpI1. Otherwise *R. pseudoacacia* was nodulated with *Rhizobium* sp. NGR234 and with soil born rhizobia present in the soil samples used in this work (see below).

The nodules in *R. pseudoacacia* growing in pouches started to be visible at about 10 days after inoculation, and all plants had nodules at day 16 after inoculation. Once the nodulation process started, the nodulation rate was about 0.5 nodules per plant per day until day 25 after inoculation. After that day, the nodulation rate decreased and remained constant at about 0.15 nodules per plant per day (data not shown). At day 25, each plant had an average of 9.0±1.3 nodules. At time of harvest, 55 days after inoculation, the value reached 15.00±1.34 nodules per plant with an average of nodule biomass per plant of 19.3±2.0 mg. No nodules were found in any non-inoculated control plant.

To assay the efficiency of nodulation and the biomass allocation in symbiotic plants, inoculated, control and fertilised plants were grown in a drip–drop hydroponic system (see "Materials and methods"). The growth rate of plants inoculated with RpI1 show a bimodal curve with an initial growth rate of about 0.1 cm per day, between days 7 and 32, and a second fast phase of 0.3 cm per day (Fig. 1). The non-inoculated but N-fertilised plants show a constant growth rate of about 0.3 cm per day from the beginning of the experiment (Fig. 1). Plants inoculated with NGR234 show a growth pattern similar in shape to RpI1 inoculated plants but with a lower growth rate in the second phase. Control unfertilised plants watered without N did not grow well and died at day 45 as a consequence of N starvation; thus, plant biomass determinations could not be done. No nodules were found in any non-inoculated plant. *R. pseudoacacia* seeds had a mean N content of 3.3% *w*/*w*, with a mean value of 842 μg of N per individual seed. The growth rate of non-inoculated unfertilised seedlings (Fig. 1) suggests that the N content of the seed could sustain the initial growth of either inoculated or non-inoculated plants for about 18 days (grey arrow in Fig. 1). The change in the shape of the growth curve suggests that about 30 days after the inoculation (black arrow in Fig. 1) the plants would start to grow supported by the N fixed symbiotically. At time of harvest, 59 days after germination, non-inoculated but N-fertilised plants reached a higher shoot height ($P<0.05$) than inoculated or control plants (Fig. 1).

Each growth parameter of plants inoculated with RpI1 showed significantly ($P<0.05$) higher DM values than those of plants inoculated with NGR 234 (Fig. 2). Plants

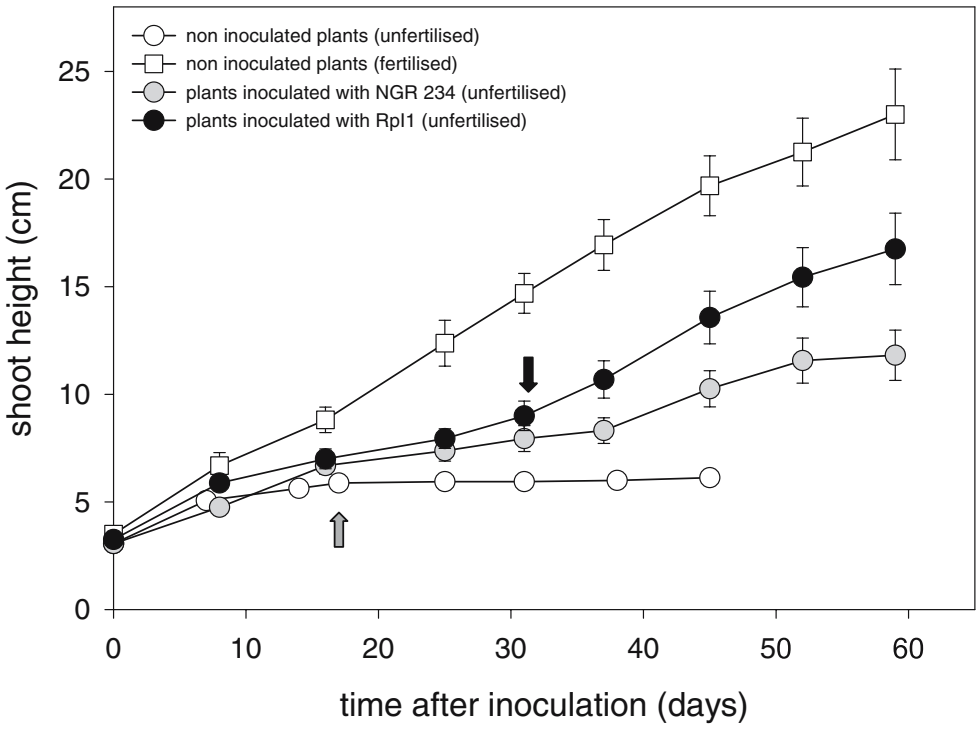

Fig. 1 Kinetics of shoot growth of seedlings in pots with perlite in a drip–drop system (*black circle* plants inoculated with RpI1; *grey circle* plants inoculated with NGR 234; *white circle* non-inoculated controls; *white square* non-inoculated and fertilised plants)

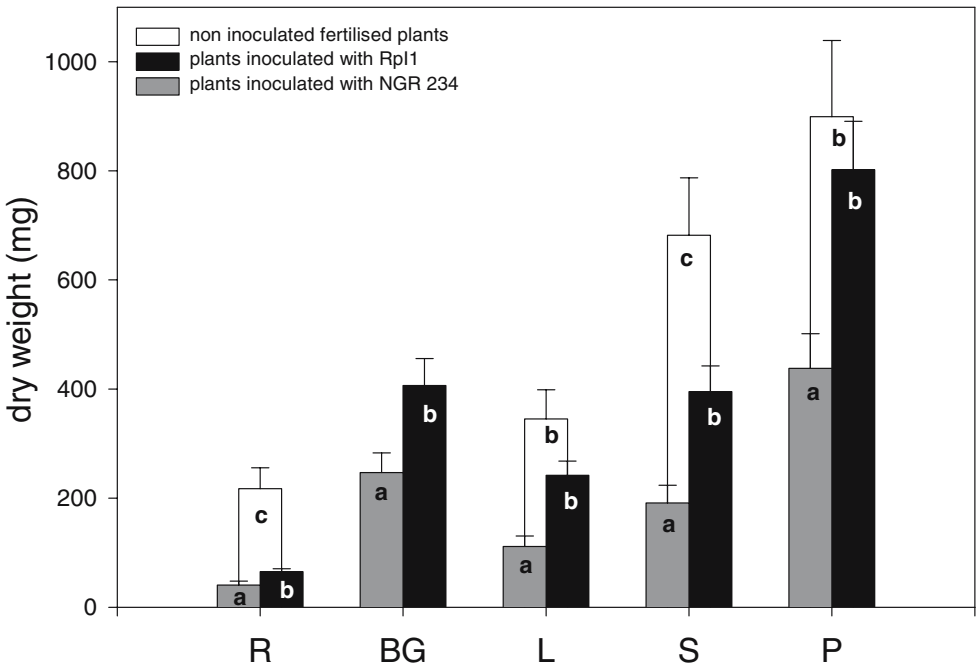

Fig. 2 Final dry weight of root (*R*), belowground (root + nodule) (*BG*), leaf (*L*), shoot (*S*) and whole plant (*P*) of seedlings grown in pots with perlite in a drip–drop system (*black* plants inoculated with RpI1; *grey* plants inoculated with NGR 234; *white* non-inoculated fertilised plants). *Different letters* indicate significant different means for $P<0.05$

inoculated with RpI1 showed (Table 2) significantly more nodules ($P<0.034$) and higher nodule biomass per plant ($P<0.053$) than those inoculated with NGR 234 although the individual nodule size was similar ($P<0.30$). The higher leaf N concentration in inoculated plants than in fertilised plants (Table 2) would suggest a better assimilation of fixed N than mineral N, also evidenced by the intense green colour of leaves of inoculated plants. This difference could also be important when considering N input to soil by leaf fall.

Regarding plant biomass allocation, plants inoculated with RpI1 show higher shoot/root ratio than fertilised plants (Table 2), apparently showing a lower need of root exploration due to the atmospheric N supply. The lower root dry weight in inoculated than N-fertilised plants support this hypothesis (Fig. 2).

The lower shoot/plant ratio in plants inoculated with RpI1 compared to fertilised plants (Table 2) suggests that the nodulated root system functions as a more important sink of photosynthate than the roots of fertilised plants; consequently, nodulated plants are more important for soil reclamation than fertilised plants due to the higher input of belowground organic matter. It should be noted that the nodule biomass in RpI1-inoculated plants represents 83% of the total belowground biomass.

The differences in the final plant growth between fertilised and RpI1-inoculated plants could depend on the fact that it took 25 days for inoculated plants to reach a growth rate similar to that of N-fertilised plants. Indeed, inoculated and fertilised plants showed a similar growth rate after 25 days (Fig. 1) ($P=0.60$). Both slopes were also significantly higher ($P<0.05$) than the slope of plants

Table 2 Nodulation and nitrogen analysis of plants inoculated with RpI1 and NGR 234 strains and non-inoculated fertilised plants grown in pots with perlite in a drip–drop system at day 67 after inoculation

	Fertilised plants	RpI1 plants	NGR 234 plants
Number of nodules per plant	0	19.8 (2.4) b	10.4 (2.6) a
Nodule biomass per plant (mg)	–	341.5 (46.8) b	206.1 (29.3) a
Single nodule biomass (mg/nodule)	–	19.1 (3.3) a	31.3 (9.3) a
Nodule/plant ratio (mg/mg)	–	0.42 (0.02) a	0.48 (0.02) b
Shoot/root ratio (mg/mg)	3.4 (0.3) a	6.4 (0.8) b	5.2 (0.9) ab
Shoot/plant ratio (mg/mg)	0.76 (0.02) b	0.49 (0.02) a	0.43 (0.02) a
Leaf N concentration (% *w*/*w*)	2.3 (0.2) a	4.7 (0.1) c	3.8 (0.2) b
Leaf N content (mg)	7.7 (1.2) ab	11.4 (1.4) b	4.2 (0.9) a

Standard errors are presented inside parentheses. Different letters indicate significant differences for $P<0.05$.

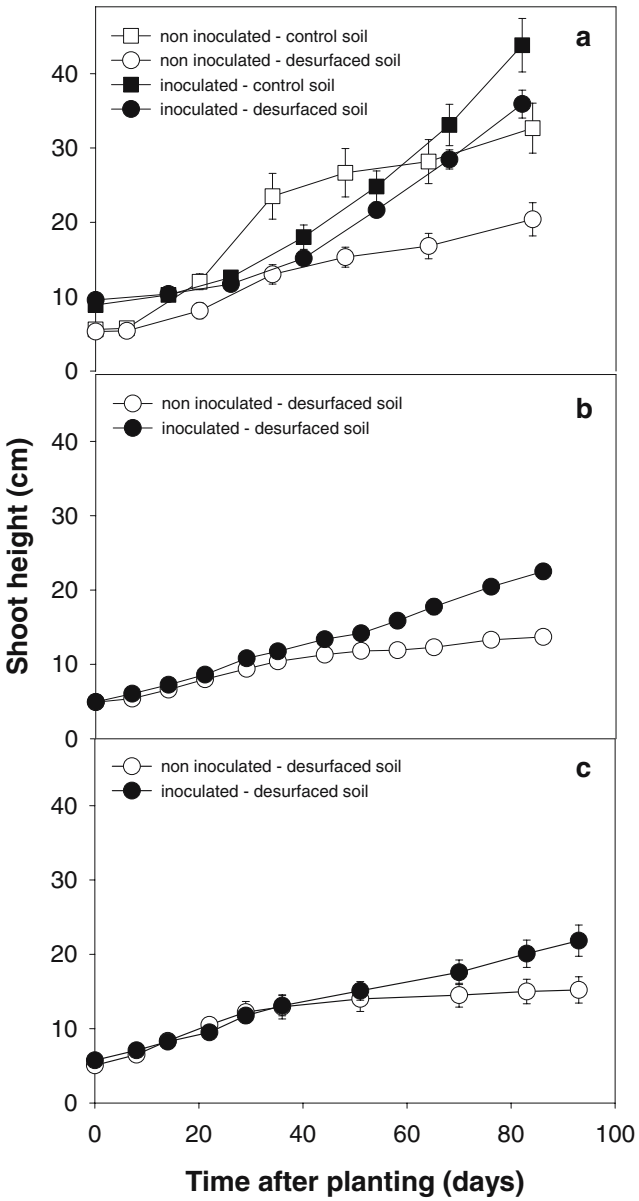

Fig. 3 Growth kinetic of *R. pseudoacacia* seedlings in pots filled with both soils during Spring–Summer (**a**), and on desurfaced soil during Winter (**b**) and Autumn–Winter (**c**) (*white circle* non-inoculated seedlings on desurfaced soil; *black circle* RpI1-inoculated seedlings on desurfaced soil; *white square* non-inoculated seedlings on control soil, *black square* RpI1-inoculated seedlings on control soil). *Different letters* indicate significant different means for $P<0.05$

inoculated with *Rhizobium* NGR 234, which being a broad host range *Rhizobium* it may be not the best one for black locust.

Symbiotic performance on desurfaced soil

During the first experiment (year 2001), the growth of previously inoculated and nodulated plants was compared to the growth of non-inoculated plants in pots with either desurfaced or control non-desurfaced soil (Fig. 3a). Non-inoculated plants growing on desurfaced soil showed the worst performance. Previously inoculated and nodulated plants showed the highest growth rate (Fig. 3a).

Repetition of the experiment in the year 2002 (Fig. 3b) and year 2003 (Fig. 3c) showed the growth rate, and consequently, the final shoot growth of inoculated and previously nodulated *R. pseudoacacia* seedlings being always higher than those of non-inoculated seedlings in pots filled with a desurfaced soil substrate (Fig. 3a–c), irrespective of different years and different seasons.

At the end of each experiment, most of the non-inoculated control plants were nodulated. By excluding the possibility of cross-contamination during the experiments, and after the negative nodulation of non-inoculated plants growing in autoclaved soils, the presence of nodules in non-inoculated control plants can be attributed to the existence of infective and quite inefficient strains of *Rhizobium* for *R. pseudoacacia* in the desurfaced soil. At the end of the experiment in year 2003, inoculated plants had about three times more nodules than at the time of the transfer to pots with soil substrate (Table 3) showing continuous nodulation after transplantation.

Conclusions

Rhizobium sp. RpI1 appears to be a very specific strain for *R. pseudoacacia* with good symbiotic performance and efficiency for N fixation. The growth of seedlings previously nodulated by *Rhizobium* RpI1 showed on desurfaced soil a constant growth rate during the whole experimental period, whilst non-inoculated plants reduced their growth rate about 40 days since the transfer to pots (Fig. 3a). These observations suggest that N would be a major limiting growth factor in this type of soil, and inoculated plants could overcome this problem by carrying out N_2 fixation. In agreement with Hanover and Mebrahtu (1996), N_2 fixation in black locust would be more effective when inoculated with local rhizobia isolates. However, these data

Table 3 Number of nodules per plant before and after transferred to soil, for plants previously nodulated with *Rhizobium* RpI1 and non-inoculated control plants grown in pots with desurfaced soil for 125 days

	Control plants	RpI1 plants
Number of nodules per plant at planting	0	6.8 (1.9) a
Number of nodules per plant at harvest	7.0 (1.6) a	20.8 (3.0) b

Standard errors are presented inside parentheses. Different letters indicate significant difference for $P<0.05$.

need to be confirmed under field conditions. For this reason, a field experiment on a degraded land with desurfaced soil had been started in 2005 by planting seedlings of *R. pseudoacacia* previously nodulated with *Rhizobium* RpI1 as described before. The plant performance under field conditions has been compared to non-inoculated *R. pseudooacacia* and to *Fraxinus americana* as a non-nitrogen fixing reference tree. The preliminary results after 1 year showed that previously nodulated *Robinia* have a shoot height 23% higher than non-inoculated *Robinia*, thus supporting the conclusion of this paper.

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