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Increases in growth and nutrient uptake of alfalfa grown in soil amended with microbially-treated sugar beet waste

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Abstract

Sugar beet waste (SB), treated by *Aspergillus niger* under the conditions of 10-, 20-, and 30-day-solid state fermentation, supplemented or not with rock phosphate (RP), was added to a soil-plant system. Plant growth responses depended on the time period of preincubation of the agrowaste characterized by different lignocellulosic composition and N and P contents before introduction into soil. Maximum growth and nutrient uptake of alfalfa during three crop cycles were recorded in a soil amended with microbially-treated SB waste+RP. This effect was more pronounced in treatments with arbuscular mycorrhizal (AM) fungus grown in soil enriched with 10- and 20-day-microbially-treated SB+RP, when the respective average total plant growth increased 233% and 343% over the non-mycorrhizal control containing untreated SB. Compared to other treatments, plant mycorrhization was ineffective when 30-day-treated agrowaste was used. Similarly, plant nodule numbers and uptake of metal ions depended on both the time period of waste preincubation and mycorrhization. © 1999 Elsevier Science B.V.

Keywords: SB agrowaste; *Aspergillus niger*; Ligno-cellulolytic micro-organism; Rock-phosphate solubilization; *Glomus deserticola*; Plant growth

1. Introduction

Rock phosphates (RPS) are natural inexpensive sources of phosphorus but their solubilization rarely occurs in non-acidic soils. However, microbially-mediated processes involving chelation and exchange reactions are able to solubilize inorganic P forms. In previous studies (Vassilev et al., 1995, 1996) used a very attractive approach to RP solubilization by a selected strain of *Aspergillus niger* grown on sugar beet (SB) waste material. The agronomic use of agrowastes as substrates cause changes in the soil affecting its physico–chemical characteristics and microbial activity in the rhizosphere (Iyamnremya and Dick, 1996). The breakdown of such materials to simple sugars provide energy sources for heterotrophic micro-organisms such as P-solubilizing and N_2-fixing bacteria. Normally, the growth and metabolic activity of soil micro-organisms are limited by the availability of nutrients. However, in a preliminary study we have reported (Vassilev et al., 1995, 1996) different levels of mineralization of SB waste treated by *A. niger*, as well as different pH, N and P concentrations in the fermented material. All these may induce positive or negative effects on rhizosphere micro-organisms and/or plant growth. So far, the experimental work has been limited to an assessment

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of the effect of 3-week-treated SB waste on plant growth. The aim of the present study was to evaluate the influence of the same material treated for different periods (10, 20 and 30 days) on plant growth and nutrient uptake. A further objective of the study was to investigate the behaviour of plant-beneficial microorganisms such as arbuscular mycorrhizal (AM) fungi and *Rhizobium* as affected by the addition of microbially-treated SB waste in the presence of RP.

2. Materials and methods

2.1. Fermentation process

A strain of *Aspergillus niger* NB2 was used in this study. It had previously been selected as producing citric acid on complex substrates (Vassilev et al., 1986).

SB waste, a lignocellulosic material was ground in an electrical grinder to 1 mm fragments. It was mixed at a concentration of 10% with 50 ml Czapeck solution for static fermentation in 250 ml Erlenmeyer flasks. The latter were inoculated with or without 3 ml of *A. niger* spore suspension (1.2×10^6 spores). RP at a concentration of 3 gl^{-1} was added when appropriate. Static fermentation was performed at 28°C for 10, 20 and 30 days. Four treatments were studied as follows: control (SB untreated), control+RP (SB supplemented with RP), control+*A. niger* (SB preinoculated with *A. niger*), control+RP+*A. niger* (SB supplemented with RP and preinoculated with *A. niger*).

2.2. Soil, plant and soil inoculation

The SB (control), SB+RP, *A. niger*-treated SB and *A. niger* treated SB+RP treatments preinoculated for 10, 20 or 30 days as described above, were evaluated as soil amendments with or without AM colonization. The soil used was from the Granada province (Spain) with a pH of 7.5, contained 15 μg P g^{-1} (Olsen test), 0.8% organic C; 2600 mg l^{-1} total N and texturally contained 58.7% sand, 26.4% silt and 14.9% clay. The soil was deficient in soluble Ca.

The steam-sterilized soil (soil:sand mixture, 1:1) was mixed with SB, at a rate of 5%, treated according to the fermentation scheme design.

Seeds of *Medicago sativa* L. cv Aragon, were planted in pots of 500 cc capacity and were inoculated with or without the AM fungus *Glomus deserticola*. All pots, arranged in a randomized block design (one plant per pot), received 1 ml of *Rhizobium meliloti* culture containing 10^8 CFU ml^{-1}. The AM inoculum of 5 g/pot was thoroughly mixed with the soil. It was obtained from a stock pot culture where *Lactuca sativa* L. was the host plant and consisted of spores, mycelia and mycorrhizal roots fragments.

The plants were grown in a greenhouse under a day–night cycle of 8–16 h, 21–15°C, and 50% relative humidity. Water loss was replaced by top watering (tap water).

2.3. Measurements

Total N was estimated by the Kjeldal method and total P content was determined by the Molibdo–Vonado method (Lachica et al., 1973).

Plants grown on soil–sand mixtures amended with the product fermented for 10, 20 or 30 days, were harvested three times at intervals of 8 weeks. Shoot dry weight was recorded after drying at 70°C for 20 h. Concentrations of N and P were colorimetrically measured using an autoanalyzer (Technicon). K was determined by flame photometry and Ca and Mg by atomic absorption spectrophotometry using a Perkin-Elmer 5000 spectrophotometer. Shoot metal concentrations were determined only after the first crop (which was selected as being the most representative).

AM infection was assessed microscopically using the grid-line intersect method of Giovannetti and Mosse (1980) after staining by the procedure of Phillips and Hayman (1970).

Number of nodules was determined visually on carefully washed roots. Data for each parameter from the five replicates were analyzed statistically for differences using analysis of variance and the Duncan new multiple range test at the significance level of $p\leq0.05$.

3. Results and discussion

Fig. 1 shows that the concentration of N in the incubated product was highest after 10-day incubation with *A. niger*. Highest P concentrations were found in

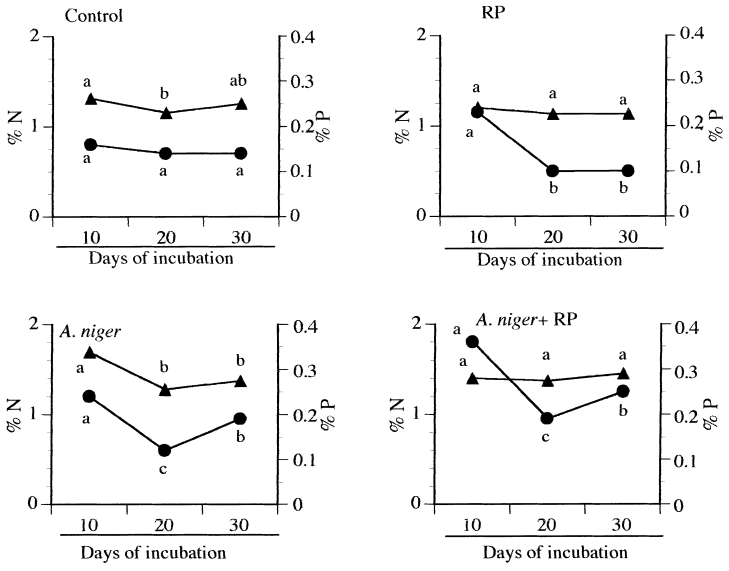

Fig. 1. Total % N (▲) and % P (●) of sugarbeet waste (control) as affected by rock-phosphate (RP) and/or *A. niger* after three incubation periods (10, 20 and 30 days). For each response variable, values not sharing a letter in common differ significantly ($p \leq 0.05$) from each other.

the *A. niger*+RP treatment with the maximum P content value being obtained after 10 days (219% higher than in the microbially untreated control).

Plant growth responses were strongly affected by both chemical (RP addition) and biological (*A. niger* and/or AM fungus) treatments. The results also showed significant relationships between plant growth, time of microbial preincubation of the lignocellulosic substrate (±RP), and crop (Table 1).

The maximum growth and nutrient uptake of mycorrhizal and non-mycorrhizal alfalfa plants was reached when SB waste, precultured with *A. niger* in the presence of RP, was applied to the soil-plant system (Table 1). These results agree well with the N and P contents of the precultured material before its introduction into the soil. On the other hand, the length of the fermentation period influenced the mycorrhizal effect on plant nutrient uptake and growth according to the treatment.

Higher growth of mycorrhizal and non-mycorrhizal plant was achieved in soil amended with *A. niger-treated* SB+RP (Table 1) in comparison to other 10-day preincubation treatments. Alfalfa growth was at a maximum with the combined effects of *A. niger*, RP applied during the preincubation stage, and AM fungus, applied in the soil-plant stage. This effect was maintained over successive crops, with the exception of mycorrhizal plants in the third crop.

Similar trends were observed when SB, preincubated for 20 days, was introduced into the experimental soil. In this case, higher plant growth was also registered during the three crop periods in nearly all mycorrhizal treatments in comparison to the non-mycorrhizal control. Mycorrhizal colonization of plants grown in SB+RP+ *A.niger*-amended soil resulted in 219% (first crop), 432% (second crop) and 387% (third crop) plant growth increase over the non-mycorrhizal control containing untreated agrowaste.

Mycorrhizal symbiosis was effective in all, except the SB+ *A. niger*+RP-amended treatment, when the soil contained 30-day-treated agrowaste. It appears that the material microbially-treated with *A. niger* for 30 days in the presence of RP was not compatible with the AM fungus in any of the three crop cycles (Table 1). However, RP- and *A. niger*-amended treatments proved highly efficient in association with the AM fungus. The differentiation in the AM fungal response in relation to the length of preincubation is an important characteristic of this system. Perhaps, the large amount of *A. niger* biomass formed on the medium enriched with RP, decreased the total level of

Table 1
Dry alfalfa biomass (mg) of mycorrhizal (M) and non-mycorrhizal (−M) plants as affected by the period of substrate preincubation (10, 20 or 30 days) with and without rock-phosphate (RP) and/or *A. niger* for three successive crops

First crop treatments	Days of incubation					
	10		20		30	
	−M	+M	−M	+M	−M	+M
Control	155d	165cd	142d	205cd	196c	197c
RP	145d	190cd	187c	247b	187cd	280ab
A. niger	172cd	225bca	185cd	270b	172cd	267b
A. niger+RP	300a	335a	277ab	312ab	315a	280ab
Second crop						
Control	75d	97d	52d	100cd	93d	120cd
RP	85d	140cd	125cd	127cd	106cd	155c
A. niger	80d	160bc	110cd	193ab	122cd	190ab
A. niger+RP	200b	255a	183bc	225ab	172b	165ab
Third crop						
Control	111bc	127bc	80c	252a	102bc	130bc
RP	95bc	245ab	87c	292ab	223a	330a
A. niger	110bc	247ab	153bc	273ab	145bc	253a
A. niger+RP	222a	205a	260a	310a	270a	137b

Values within crop not sharing a letter in common differ significantly ($p \leq 0.05$) from each other.

available nutrients derived from the SB or antagonized the development of the AM fungus. The AM colonization evaluated in this treatment was lower than where preinoculated for 10 or 20 days (Table 2). Another possible explanation could be the adverse effect of lignin, released as a result of mineralization, on the functional activity of the AM fungus.

Plant nutrient acquisition was also stimulated by the chemical (RP), biological (*A. niger* or/and AM fungus) treatments and particularly by their combination (Figs. 2–4). Plant uptake of N and P was significantly increased by the addition of *A. niger*-treated agrowaste when preincubated for 10 or 20 days and AM colonization. The supplementation of biological treatments with RP seemed to be more effective after an incubation time of 10 days for N (increasing by 215% over control) and 20 days for P (increasing by 290% over control) despite the decrease in the P content in the fermented product after this period of incubation (Fig. 1). After 30 days preincubation the mycorrhizal fungus did not enhance N and P uptake in the most efficient A+RP treatment. In fact, N and P uptake were affected mainly by the length of the preincubation period with a decrease in N and P content with increasing preincubation time. In the presence of RP the synergistic interactive effect of AM fungus and

Table 2
AM colonization and nodule numbers in mycorrhizal (M) and non-mycorrhizal (−M) plants as affected by the period of sugar beet waste (control) with and without rock phosphate (RP) and/or *A. niger* after the third crop

	Days of incubation											
	10				20				30			
	% AM		No. nodules		% AM		No. nodules		% AM		No. nodules	
	−M	+M	−M	+M	−M	+M	−M	+M	−M	+M	−M	+M
Control	—	88±15	45±9	29±10	—	44±6	14±4	5±3	—	73±7	20±2	17±2
RP	—	64±12	8±6	34±6	—	81±17	40±7	19±6	—	58±7	49±9	36±6
A. niger	—	56±82	28±7	37±11	—	34±10	23±3	50±14	—	68±4	41±8	81±12
A. niger+RP	—	64±10	50±12	51±12	—	66±13	82±12	61±18	—	45±5	42±7	30±4

Standard errors of the mean are given ($p \leq 0.05$).

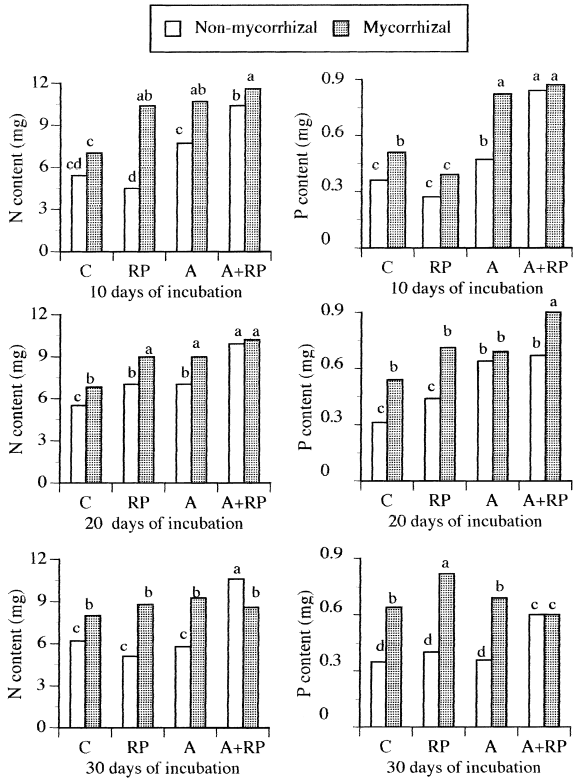

Fig. 2. Shoot N and P content of mycorrhizal (M) and non-mycorrhizal plants treated with sugar beet waste (C) as affected by RP and/or *A. niger* (A) after three incubation periods (10, 20 or 30 days). Values not sharing a letter in common differ significantly ($p \leq 0.05$) from each other.

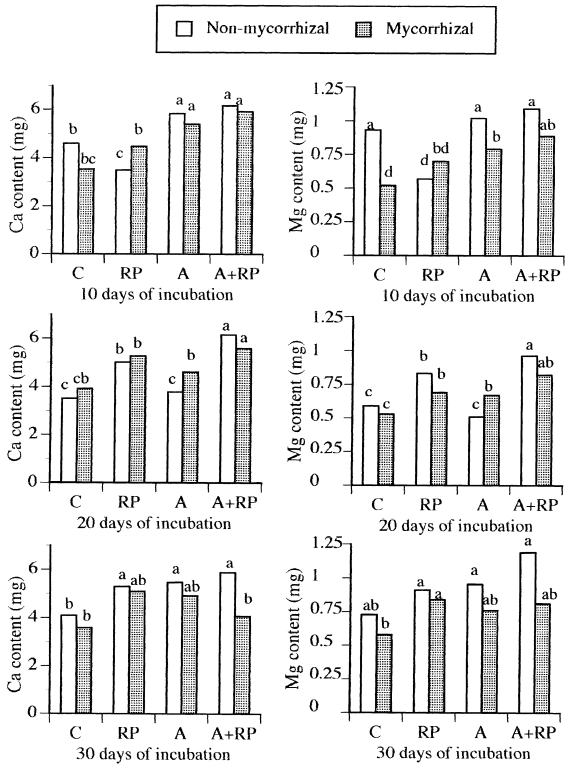

Fig. 3. Shoot Ca and Mg content of mycorrhizal (M) and non-mycorrhizal plants treated with sugar beet waste (C) as affected by RP and/or *A. niger* (A) after three incubation periods (10, 20 or 30 days). Values not sharing a letter in common differ significantly ($p \leq 0.05$) from each other.

A. niger on N and P uptake was absent particularly when the fermentation period lasted 30 days.

As Fig. 3 shows, the uptake of Ca and Mg decreased when 30-day-treated SB was added to the mycorrhizal plants. The highest amounts of Ca and Mg were found in the experimental plants grown in soil supplemented with microbially-treated SB+RP. The period of pre-incubation affected the uptake of Ca and Mg mainly in plants colonized by AM fungus. The latter obviously caused a decrease of Ca and Mg in the plant tissue thus preventing an excessive cation uptake, particularly in treatments containing Ca derived from the RP (Fig. 3). Similar response of mycorrhizal plants was not surprising bearing in mind the ability of AM fungi to balance the cation–anion equilibrium in plants (Azcón and Barea, 1992b). Major ions can influence each other's absorbtion by competitive interactions or by affecting ion selectivity of membranes (Ramalho et al., 1995).

Regarding acquisition of K by the plant, similar trends were observed as with P plant uptake (Fig. 4).

Mycorrhizal colonization positively affected Na plant acquisition of Na in soil amended with 20-day-treated agrowaste. The addition of 30-day-*A. niger*-treated with SB+RP to mycorrhizal plants decreased the plant Na uptake as compared to the non-mycorrhizal treatments as occurred for all the elements in this treatment (Fig. 4).

General trends can be drawn regarding the symbiotic development parameters (Table 2). For 10 and 30 days preincubated treatments the highest AM colonization and the lowest were observed in the control and

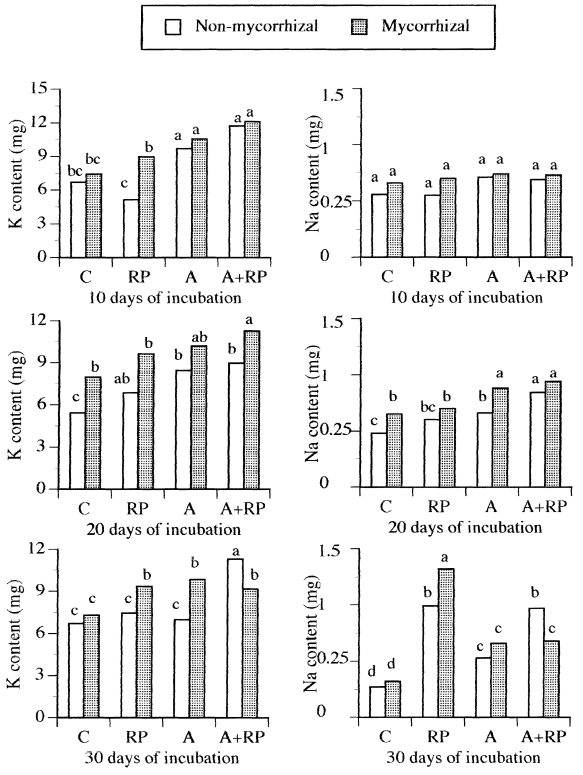

Fig. 4. Shoot K and Na content of mycorrhizal (M) and non-mycorrhizal plants treated with sugar beet waste (C) as affected by RP and/or *A. niger* (A) after three incubation periods (10, 20 or 30 days). Values not sharing a letter in common differ significantly ($p \leq 0.05$) from each other.

microbially-treated SB+RP treatments, respectively. By contrast, the nodule number in mycorrhizal plants was higher in the treatments containing *A. niger*-treated SB than in the control plants. In general, the nodule number was higher in non-mycorrhizal than in mycorrhizal plants. The maximum level of nodulation after 10 and 20 days of incubation was found in SB+*A. niger*+RP-amended treatments. There was no significant relationship between the nodule number and preincubation time-length.

It is known that chemical characteristics of the crop residue may influence some soil biological groups and processes. Any effect on rhizosphere micro-organisms may reflect on the plant growth and nutrition. In this study, the high level of nodulation and AM colonization (Table 2) are strong indicators of microbial-substrate compatibility. In fact, the dual *A. niger*–AM fungus synergistic effect was demonstrated using as criteria the high amounts of N and P in plants infected with both fungi. In addition, these micro-organisms positively affected the *Rhizobium*-plant symbiosis while such an effect was not observed when *A. niger* and the AM fungus were applied separately. This work indicates that the P demand of plants can be satisfied by micro-organisms able to solubilize RP through organic acid production on agrowaste materials. Similar results have been obtained by using agrowastes such as olive mill waste water (Vassilev et al., 1997) and olive cake (Vassileva et al., in press). Further application of AM fungi additionally increases the positive effect of mineralized organic material and solubilized RP. The combined microbial activity resulted in higher plant growth and P uptake rates (Fig. 2). Increased P uptake strongly facilitates biological N_2 fixation by alfalfa–*Rhizobium* symbiosis (Azcón and Barea, 1992a). On the other hand, the external AM mycelium also supports plant N acquisition (Tobar et al., 1994a, b) and this fact should be taken into account when assessing the amount of biologically fixed nitrogen. Both mechanisms may account for the increased N plant content found in the mycorrhizal plants (Fig. 2).

4. Conclusions

In conclusion, the most effective treatment was that containing material pre treated for 10 days with *A. niger* in the presence of RP and mycorrhizal plants. Another advantageous effect of this system was that the polysacharidic component of the agrowaste stabilized the soil structure (data not shown). Degraded soils are convenient model-soils for such studies because their organic matter content is very low and there is no inherent soil structure. Therefore, the application of microbially-treated agrowastes may significantly improve the above-mentioned soil properties.

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