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# IMPROVED PLANT GROWTH WITH ROCK PHOSPHATE SOLUBILIZED BY *ASPERGILLUS NIGER* GROWN ON SUGAR-BEET WASTE

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**Abstract**

Aspergillus niger *was successfully cultivated on sugar-beet-waste material (SB) supplemented with 3.0 g/l rock phosphate (RP) acidifying the medium and thus decreasing the pH to 3.0–3.5. The fermented mixture finally contained mineralized organic matter, rock phosphate solubilized to 224 μg P per mililiter, and fungal mycelium. Various combinations of SB and RP, previously treated or untreated by the fungus, were introduced into soil to improve the growth of* Trifolium repens. *Compared to other treatments, the results showed a higher growth rate and shoot phosphorus concentration when microbially treated SB and RP were applied to both mycorrhizal and non-mycorrhizal plants. However, combined introduction of both the filamentous and arbuscular fungi led to improved plant growth when degraded organic matter supplemented or unsupplemented with RP was used.* 

*Key words:* Rock phosphate, agro-waste, *Aspergillus niger*, solubilization, plant growth, arbuscular fungi.

## INTRODUCTION

Phosphorus plays a vital role in plant nutrition (Hayman, 1975) but its concentration in soil solution is only approximately 0.05 mg/l. For this reason, the possibility of the practical use of rock phosphate as a fertilizer has received significant interest in recent years. Unfortunately, rock phosphate is not plant-available in soils with a pH greater than 5.5–6.0 and, even when conditions are optimal, yields are as a rule lower than those obtained with soluble phosphate (Khasawneh & Doll, 1978). One very attractive approach for rock phosphate solubilization is the application of microorganisms able to excrete organic acids. It has been repeatedly shown that low-molecular-weight organic acids can strongly increase phosphorus solution concentration by mechanisms involving chelation and exchange reactions (Earl *et al.*, 1979; Fox & Comerford, 1990; Gerke, 1992).

Filamentous fungi are widely used as producers of organic acids (Mattey, 1992; Vassilev & Vassileva, 1992) and, in particular, *Aspergillus niger* and some *Penicillium* species have been tested in fermentation systems or inoculated directly into soil, in order to solubilize rock phosphate (Kucey, 1987; Asea *et al.*, 1988; Cerezine *et al.*, 1988; Cunningham & Kuiack, 1992).

Conversely, waste agroproducts can be degraded by biological processes, avoiding soil contamination. The utilization of such biosystems involving organic materials and microorganisms for rock phosphate solubilization and improvement of plant growth is believed to be an important part of a sustainable-agriculture concept. In a previous study, we examined three agroindustrial wastes, rice hulls, alperujo and sugar-beet waste, in order to test the level of biodegradability and titratable acidity during the course of *A. niger* fermentation. Sugar-beet waste at 10% concentration proved to be the best substrate, with a percentage of mineralization of 69 and 53 mmole/l titratable acidity.

The objective of the present work was to perform rock phosphate solubilization by *A. niger* on sugar-beet-waste medium and to study the effect of the resulting system applied in soil on the growth of *Trifolium* plants. Arbuscular mycorrhizal (AM) fungi occur naturally in most soils and often greatly improve growth of many agronomic crops, especially under stress situations. Thus, the effect of AM symbiosis was also studied in a legume plant associated with *Rhizobium*.

## METHODS

### Fermentation process

*Microorganism*

The strain of *Aspergillus niger* NB2 used throughout this study was selected from 20 acid-producing cul-

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tures of *Aspergillus* and shown to produce only citric acid on complex substrates (Vassilev *et al.*, 1986). It was maintained on potato-dextrose agar slants.

*Culture media and fermentation conditions*
Sugar-beet-waste material (SB), ground in an electrical grinder to 1 mm fragments, was used at concentrations of 10 and 20% as a substrate for static fermentation in 50 ml Czapek's solution. After sterilization at 120°C/30 min, experiments were carried out in 250 ml Erlenmeyer flasks (in triplicate) inoculated with $1.2 \times 10^7$ spores/flask. Rock phosphate (RP) at a concentration of 3.0 g/l was added when necessary. Fermentation was performed at 30°C for 20 days.

**Soil–plant experiment**
The experiment consisted of three treatments: untreated sugar-beet waste+RP; preincubated SB waste+*A. niger*; preincubated sugar-beet waste-+RP+*A. niger*, all mixed with a steam-sterilized soil–sand mixture (1:1, v/v) and left for equilibration for 5 weeks at room temperature. The soil used was the top 0–20 cm of a Granada (Spain) province field soil with a pH of 7.5 containing 8 μg P/g (Olsen test), organic carbon 0.46%, total N 0.046% and E.C. 1.89 (the latter changed before planting to 4.73 in the SB+RP treatment, 6.69 in SB+RP+*A. niger* treatment and 6.0 after the addition of SB+*A. niger*). The lignocellulosic material was added to soil at a rate of 5% SB and 0.75 g RP per pot (the latter was applied in an equivalent amount in SB+RP +*A. niger* treatment but 58% of its total P was solubilized during the fermentation process).

Ten seeds of *Trifolium repens* were planted in each pot (12.2 cm; 500 g capacity) inoculated or not with the VAM fungus *Glomus deserticola* and all pots received 1 ml of *Rhizobium trifoli* suspension. The AM inoculum (5 g) consisted of spores, mycelium and mycorrhizal root fragments and was applied to each one of the corresponding pots in the bottom of a 5-cm-deep hole. The seedlings were thinned to three per pot 2 weeks after emergence. The plants were grown in a greenhouse under a day/night cycle of 16/8 h, 21/15°C, 50% relative humidity. Throughout the experiment, the pots were weighed every day and water loss from field capacity was replaced by top watering.

**Analytical methods**
Mycelial growth was determined by weighing the mycelium, carefully separated from the fermentation medium and washed and dried in an oven at 100°C. Medium pH was measured with a glass electrode and titratable acidity was determined by titrating each sample to pH 7.0 with 0.1 N NaOH. Citric acid content was determined by the microcolorimetric method of Taussky (1949). Phosphorus content was determined by the molybdovanado method described by Lachica *et al.* (1973).

The plants were harvested three times with a single harvest period of 7 weeks. Shoot and root dry weight were recorded after drying at 70°C. Shoot P content was determined by a molybdovanado method described by Lachica *et al.* (1973). The roots were carefully washed and the number of nodules was determined visually. The percentage of mycorrhizal root length was estimated by microscopic examination of stained samples (Phillips & Hayman, 1970) using the grid-line intersect method of Giovannetti and Mosse (1980).

## RESULTS AND DISCUSSION

**Fermentation study**
In a preliminary study SB at 10% concentration was selected as a substrate for further work among three agroindustrial wastes, including rice hulls and alperujo (data are not shown). In this work a separate experiment was carried out with 10% SB supplemented with 3.0 g/l rock phosphate (RP), analyzing the fungal growth, titratable acidity and soluble phosphate (Table 1). The data indicated a rapid mycelial growth at the beginning of the fermentation process, followed by a slow growth phase. The growth was higher in the first days, with an average growth rate of 0.19 g/fl/day compared to that until day 15 when the average growth rate was about 0.03 g/fl/day. It increased thereafter again and the total mass of mycelium produced on the medium supplemented with RP was higher by 20% than that on the medium without RP. Biomass growth on the lig-

**Table 1. Mycelial growth, titratable acidity and rock phosphate solubilization by *Aspergillus niger* cultivated on sugar-beet-waste**

| Time (day) | Biomass (g/fl) | Titratable acidity (mmol/l) | Citric acid (% of total acidity) | Phosphate concentration (μg/ml) | Soluble P/total P (%) |
|---|---|---|---|---|---|
| 3 | 0.57±0.12 | 38.2±1.4 | 100 | 47.4±1.6 | 12 |
| 6 | 0.68±0.08 | 60.6±0.7 | 98 | 172.6±0.8 | 44 |
| 10 | 0.77±0.05 | 72.2±0.6 | 92 | 292.2±2.3 | 76 |
| 15 | 0.89±0.05 | 58.8±1.1 | 87 | 276.8±3.1 | 71 |
| 20 | 1.20±0.10 | 48.1±0.2 | 71 | 224.0±0.9 | 58 |

Values are mean ± standard deviation for three replicate cultures.

nocellulosic substrate tested in this study was higher than that under liquid culture conditions using the same strain of *A. niger* (Vassilev *et al.*, 1986). Similar results have been reported by other authors applying sugarcane bagasse (Lakshminarayana *et al.*, 1975; Oriol *et al.*, 1988) and vinasse (Nahas *et al.*, 1990). The most likely explanation of *A. niger* growth on SB is the presence of sufficient amounts of nutrients in the initial period of the fermentation and some lignocellulolytic activity, bearing in mind the ability of *A. niger* to degrade such kinds of substrates (Czajkowska *et al.*, 1988).

Mycelial growth and titratable acidity were parallel in the first half of the process. An increase of the titratable acidity to 72 mmol/l was observed during this period, which resulted in a solubilization of 76% of the insoluble rock phosphate. However, the fungus started to sporulate thereafter, which was a sign of adverse conditions for acid production and this caused a slow decrease in titratable acidity to a level of 48 mmol/l at the end of the experiment. Although the process was directed towards the biomass growth in this study, the level of acidity achieved by *A. niger* was sufficient to overcome the neutralizing effect of rock phosphate. The results also showed that the process of solubilization increased after the active growth phase. However, as the amount of fungal biomass continued to increase slowly, the determined phosphate in the solution probably corresponded to that amount which was not consumed by the mycelium. This speculation should not be surprising, bearing in mind that when trace metals are not limiting, the additional phosphate results in prolongation of mycelial growth and changes in the fungal metabolism (Martin & Steel, 1955). It was evident that the presence of RP added directly to the fermentation medium affected the behaviour of *A. niger*, particularly its growth and citric acid production. The latter accounted for 100% of the titratable acidity during the first days of the fermentation but started to decrease thereafter and further work should be performed to study the nature of acidic metabolites which are different from the main acid released by the mycelium.

**Soil–plant experiment**

Plant dry matter responses and P content of shoots after the addition of untreated SB+RP and SB+RP previously fermented by *A. niger* are presented in Table 2 and Table 3, respectively. A significantly high growth and P content, reaching 330 mg/pot and 4 mg/g plant dry weight, were observed during the first crop period in mycorrhizal plants grown in soil amended with the preincubated lignocellulosic substrate+RP+*A. niger* combination. In this case the plant growth response was 58 and 82% higher than

**Table 2. Dry weight of mycorrhizal and non-mycorrhizal plants as affected by rock phosphate and *Aspergillus niger***

| Treatments | | Crops period (mg/pot) | | | Total (mg/pot) |
|---|---|---|---|---|---|
| Mycorrhizal | | | | | |
| RP | *A. niger* | I | II | III | |
| + | + | 330±30 | 1830±90 | 1540±87 | 3700 |
| − | + | 140±7 | 1600±81 | 1460±84 | 3200 |
| + | − | 60±8 | 450±68 | 580±60 | 1100 |
| Non-mycorrhizal | | | | | |
| RP | *A. niger* | | | | |
| + | + | 34±2 | 450±68 | 720±67 | 1200 |
| − | + | 10±1 | 100±14 | 190±33 | 300 |
| + | − | 10±1 | 10±1 | 60±8 | 80 |

Values are ± standard deviation for five replicate cultures. Results are significant at $P = 0.0001$.

**Table 3. Phosphorus content in shoots of mycorrizal and non-mycorrhizal plants as affected by rock phosphate and *Aspergillus niger***

| Treatments | | Crops period (mg/g) | | | Total (mg/g) |
|---|---|---|---|---|---|
| Mycorrhizal | | | | | |
| RP | *A. niger* | I | II | III | |
| + | + | 4.08±0.12 | 4.58±0.09 | 4.98±0.22 | 13.64 |
| − | + | 3.02±0.07 | 4.06±0.14 | 4.72±0.12 | 11.80 |
| + | − | 0.50±0.01 | 3.00±0.27 | 3.12±0.16 | 6.62 |
| Non-mycorrhizal | | | | | |
| RP | *A. niger* | | | | |
| + | + | 1.46±0.05 | 3.68±0.13 | 2.82±0.18 | 7.96 |
| − | + | 0.53±0.01 | 2.20±0.04 | 2.54±0.14 | 5.27 |
| + | − | 0.46±0.01 | 0.54±0.03 | 0.58±0.01 | 1.58 |

Values are ± standard deviation for five replicate cultures. Results are significant at $P = 0.0001$.

those of treatments where preincubated SB-RP and untreated SB+RP were applied alone to the mycorrhizal plants. A low plant growth and P concentration in the shoot were found in the treatments without mycorrhizal infection, although there was some increase in soil amended with the preincubated SB+RB+*A. niger* combination. A possible negative influence of SB, especially applied at this concentration, during the initial period of the experiment should be noted. An increase of dry matter was observed in all treatments during the second crop period when the plant growth reached its maximum of 1830 mg/pot and 1600 mg/pot, respectively, for the mycorrhizal plants grown in soil supplemented with SB preincubated by *A. niger* with and without RP. The tendency of improved P uptake and plant growth was observed again during the third crop period in all treatments, but with a slight decrease in the best experimental mycorrhizal sets with preincubated SB with and without RP addition. Similarly, lower plant P-uptake was found in SB+RP+*A. niger*, but only during the third crop cycle.

The preincubated SB+RP+*A. niger* treatment had a positive effect on both mycorrhizal and non-mycorrhizal plants where a total shoot dry matter of 3700 and 1200 mg/pot, and 13.6 and 8 mg/g of P were found, respectively. The same combination without RP caused an increase in dry matter production only in the mycorrhizal plants. For the *Trifolium* plant weight and plant P uptake, the SB+RP treatment without preincubation with *A. niger* was significantly less effective than all other treatments in both mycorrhizal and non-mycorrhizal plants.

In general, inoculation with the mycorrhizal fungi resulted in a higher plant growth than that of the non-mycorrhizal treatments. Microscopic observation of *Trifolium* roots showed that only AM-inoculated plants were root colonized. The higher percentage of mycorrhization was found in the treatment with previously unsolubilized RP (49.5%), in comparison with the other mycorrhizal plants (45.1% in the SB+RP+*A. niger* and 46.0% in the SB+*A. niger* treatments) corroborating the findings of Barea *et al.* (1980) that RP does not reduce the level of mycorrhizal infection as does soluble P. Again, mycorrhizal plants possessed higher nodule numbers than did non-mycorrhizal plants (data are not shown). Mycorrhizal status is a precondition for effective growth and nodulation of legumes and as a result of infection with both *Rhizobium* spp. and AM fungi plants can receive benefits of improved N and P nutrition, respectively. However, the advantageous action of the AM combined with *A. niger* should be noted, even independently of RP addition. The assumption that *A. niger* can provide additional nutrient amounts derived from the mycelium (Nahas *et al.*, 1990) cannot explain the lower plant growth and P uptake in the SB-RP+*A. niger* treatment without AM fungal infection, in comparison to the same plants but colonized by *G. deserticola*. In contrast, apart from the positive symbiotic effect of the AM fungus, it can also play an important role in protecting plants from high E.C. and SB toxicity, although this does not explain the comparatively low growth in the SB+RP+*A. niger* treatment.

Although AM increases plant growth and nodulation of legumes, even when they grow on alkaline soils, the high phosphate-fixing capacity of soils in southern Spain should be noted. However, Gerke (1992) recently reported that the addition of citric acid increased phosphate concentrations in solutions of alkaline soil and this effect was detectable even after 140 days. In our experiment the fermented SB+*A. niger* system with and without RP was kept for equilibration for 5 weeks. Therefore, we could expect some reaction between titratable acid and insoluble organic and inorganic P during the first days of this period and this assumption should be considered when assessing the positive effect of SB preincubated with *A. niger* in comparison to the other treatments. An increase in P concentration was found in shoots, particularly during the first and second crops, for all treatments. However, although more pronounced in combination with the AM fungus, this effect was significantly greater in treatments where *A. niger* participated in the system.

Despite the possibility of rapid degradation of acid by the soil components, citrate ions can be adsorbed at the same sites as phosphate and, consequently, may desorb phosphate ions directly (Nagarajah *et al.*, 1970). It was also found that organic acids added to soils increased the plant uptake of P from a water-soluble P (Bolan *et al.*, 1994). In addition, organic acids can be considered as a source of available carbon or serve as plant growth stimulators (Kucey *et al.*, 1989). Therefore, the experimental results presented here could not evaluate the effect of all interacting factors that took place, but it was clear that RP previously solubilized by SB+*A. niger* fermentation favoured the growth of white clover and this process was significantly enhanced by infection with AM fungus.

Of significance, however, is which mode of application of *A. niger* will be used in practice: inoculation directly into soil+SB+RP mixture, in order to ensure a sequential release of soluble P; or provision of preliminary solubilization by a fermentation process, as described in this work.

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