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Rock phosphate solubilization by *Aspergillus niger* on olive cake-based medium and its further application in a soil–plant system

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A citric acid-producing strain of *Aspergillus niger*, grown on olive cake-based medium, was able to solubilize rock phosphate. Solubilization of insoluble phosphate increased during the solid-state fermentation process, reaching a maximum of 164 μg/ml. Various combinations of olive cake and rock phosphate, previously treated or untreated by the fungus, were introduced into a calcareous, phosphorus (P)-deficient soil to improve the growth of *Trifolium repens* in a greenhouse experiment. Synergistic action of both the filamentous and arbuscular fungi caused considerable improvement of growth and plant P uptake. Greater growth and P uptake of mycorrhizal and non-mycorrhizal plants were achieved when microbe-treated olive cake and rock phosphate were applied to soil compared with all other treatments.

Key words: *Aspergillus niger*, *Glomus deserticola*, olive cake, rock phosphate, solubilization, plant growth.

The greater part of soil phosphorus (P), approximately 95–99%, is present in the form of insoluble phosphates which are considered as plant-unavailable P-sources. For this reason, the additional application in soil of cheap P-materials such as rock phosphate has received significant interest in recent years. However, rock phosphate is plant-available only in soils with pH lower than 5.5–6.0 and even then it needs up to 4 years of annual application to release sufficient amounts of P (Ghani *et al*. 1994). One very attractive approach for rock phosphate solubilization is the utilization by microorganisms able to excrete organic acids – the latter can strongly increase phosphorus solution concentration by mechanisms involving chelation and exchange reactions (Kpomblekou & Tabatabai 1994).

Filamentous fungi are widely used as producers of organic acids (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; Cerezine *et al*. 1988; Cunningham & Kuiack 1992; Vassilev *et al*. 1995, 1996a,b, 1997). A possible microbial treatment of rock phosphate in medium containing agricultural wastes can, in addition to avoiding soil contamination, enrich soil–plant systems with a soluble P-mineralized organic matter complex. This combination is of significant importance for Mediterranean soils which are poor in organic matter content and soluble P. Moreover, the utilization of such biosystems for plant growth improvement is believed to be an important part of sustainable agriculture.

The objective of the present study was to perform rock phosphate solubilization by *A. niger* on a medium based on olive cake (a waste material derived from olive oil production) and to analyse the plant response as affected by the application of the resulting system in soil.

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Materials and Methods

Fermentation Process

Microorganism, Culture Medium and Fermentation Conditions. The strain *A. niger* NB2, a producer of citric acid, used throughout this study, was maintained on potato–dextrose agar slants.

Olive cake (OC), a solid waste material from the olive-oil extraction process contained: cellulose 24%, hemicellulose 8%, lignin 30%, total carbon 54%, oxidizable carbon 5%, total nitrogen 1.7%. It was ground in an electrical grinder to 1 mm fragments and used at concentration of 10% (w/v) as a solid-phase substrate for static fermentation in 50 ml Czapek's solution.

After sterilization at 120 °C for 30 min, experiments were carried out in 250-ml Erlenmeyer flasks (in triplicate) inoculated with 1.2×10^7 spores per flask. Rock phosphate (fluorapatite from Morocco with 12.8% P, 1 mm mesh) at a concentration of 3.0 g/1 was added before the sterilization. Fermentation was performed at 30 °C for 17 days.

Soil–Plant Experiment

Four treatments were established for this experiment: control (C, without amendments), untreated olive cake (OC), untreated olive cake + rock phosphate (OC + RP), preincubated olive cake (OC + *A. niger*), preincubated olive cake + rock phosphate (OC + *A. niger* + RP). All variants were mixed directly with a steam-sterilized soil-sand mixture (1:1, v/v), and then left to equilibrate for 2 weeks at room temperature. The soil used was the top 0–20 cm of a Granada (southern Spain) province field soil with a pH of 7.5. The soil contained 8 μg P/g (Olsen & Dean 1965), organic carbon 0.46%, total N 0.046%. The waste material was added to soil–sand mixture at a rate of 5% (w/w) and rock phosphate at a rate of 0.75 g/pot, according to the recommendations of the FAO (Dahlzell 1987). Equal amount of rock phosphate was introduced into soil in OC + *A. niger* + RP treatment but 42% of the total rock P was in soluble form after the fermentation stage. It should be noted that fluorapatite contains traces of Na and Mg which do not affect plant growth.

Ten seeds of *Trifolium repens* were planted in each pot (12.2 cm; 500 g capacity) inoculated or not with the arbuscular mycorrhiza (AM) fungus *Glomus deserticola*. A 5 g sample of the AM inoculum (spores, mycelium and mycorrhizal root fragments) was applied to each of the corresponding pots in the bottom of a 5-cm deep hole. *Rhizobium trifoli*, 1 ml suspension, was introduced into all pots. The seedlings were thinned to three per pot 10 days after emergence. The plants were grown in a greenhouse under a day/night cycle of 16/8 h, 21/15 °C, 50% relative humidity. Water loss was compensated by watering every day after weighing.

Analytical Methods

Mycelium was carefully separated from the medium, washed several times with distilled water and dried in an oven at 90 °C to constant weight. Medium pH was measured with a glass electrode and titratable acidity was determined by titrating each sample with 0.1 M NaOH. Phosphorus content was determined by the molybdovanado method described by Lachica *et al.* (1973). The weight loss of lignocellulose during the fermentation process was calculated on the basis of ash content according to Kumar & Sign (1990) and presented as a percentage of mineralization.

The plants were harvested after 6 weeks. Shoot and root weight were recorded after drying at 70 °C. Shoot P content was determined by the molybdovanado method. 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 & Mosse (1980).

Results and Discussion

A. niger grew well on the medium containing olive cake (Table 1), with a rapid mycelial growth during the first 3 days when the average growth rate reached 0.23 g/fl.d compared with 0.07 g/fl.d after the day 9. Similar high fungal growth has been reported in other works when agro-wastes were applied in solid-state fermentation (Oriol *et al*. 1986; Nahas *et al*. 1990; Vassilev *et al*. 1995). Despite the low content of oxidizable carbon and the insoluble, crystalline nature of cellulose associated with a high amount of lignin in the olive cake, the weight loss of the waste material reached 21%. Such a process at low pH values is not surprising bearing in mind that even at pH 2.5–3.0 some filamentous fungi possess lignocellulolytic activity (Chahal *et al*. 1992). The initial pH value of 5.8–6.0 significantly decreased to about 3.6–3.7 at the end of the first half of the process with a slight increase thereafter. Similarly, the titratable acidity was higher at the end of the first week and reached its lowest value at the end of the fermentation. Under these conditions the data indicated a partial solubilization of the rock phosphate with a maximum of 165 μg soluble P/ml. This amount corresponded to about 42% solubilization of the total P present in the rock phosphate.

The fermentation material with or without rock phosphate was further mixed with soil and plant growth and P uptake were analysed (Table 2). These were negatively affected by the presence of olive cake alone or in combination with rock phosphate without microbial preincubation. This observation was more pronounced in non-mycorrhizal plants. Although the liquid and solid wastes, produced during the extraction of oil from the olive fruits are phenolic and toxic, it was reported that *A. niger* can partly neutralize them (Kieslich 1976). Thus, we can explain the plant growth and P content values, reaching 65 mg/pot and 0.7 mg/g plant dry weight, and

Table 1. Mycelial growth, titratable acidity and rock phosphate solubilization by *Aspergillus niger* grown on olive cake-based medium.

Time (day)	Biomass (g/fl)	pH	Titratable acidity (mmol/1)	Phosphate (μg/ml)	Soluble P/ total P (%)
3	0.70 ± 0.09	3.70	18.3 ± 0.3	89.0 ± 1.7	23.17
6	0.79 ± 0.11	3.56	25.8 ± 0.5	120.2 ± 0.9	31.25
9	0.83 ± 0.05	3.64	19.8 ± 0.7	164.6 ± 2.2	42.71
12	0.89 ± 0.02	4.00	13.1 ± 0.2	134.6 ± 2.8	34.89
17	1.10 ± 0.03	3.75	12.9 ± 0.3	159.8 ± 0.9	41.41

Table 2. Dry matter and phosphorus uptake for mycorrhizal and non-mycorrhizal *Trifolium repens* as affected by rock phosphate and *Aspergillus niger*.

	Treatments*			Shoot dry weight (mg/pot)	P content in shoot (mg/g)
	OC	RP	*A. niger*		
Mycorrhizal					
	+	+	+	141 ± 3.2	2.02 ± 0.09
	+	−	+	82 ± 3.1	1.38 ± 0.04
	+	+	−	62 ± 1.7	0.87 ± 0.03
	+	−	−	41 ± 1.1	0.46 ± 0.02
	−	−	−	44 ± 1.0	0.39 ± 0.01
Non-mycorrhizal					
	+	+	+	127 ± 2.9	1.78 ± 0.06
	+	−	+	65 ± 3.5	0.69 ± 0.03
	+	+	−	24 ± 0.3	0.46 ± 0.02
	+	−	−	27 ± 0.2	0.39 ± 0.01
	−	−	−	41 ± 1.1	0.53 ± 0.01

*OC, untreated olive cake; RP, rock phosphate. See text for details.

82 mg/pot and 1.38 mg/g dry weight, registered respectively in non-mycorrhizal and mycorrhizal plants grown in soil amended with the preincubated lignocellulosic material without rock phosphate. The growth response in these treatments were 59% and 86% higher than the respective controls. The presence of mycorrhizal fungus influenced positively the plant growth and P uptake but this effect was more significant in the treatment amended with untreated olive cake compared with the respective non-mycorrhizal variant. *G. deserticola* showed a higher percentage of root colonization in the treatment with untreated rock phosphate (59%) compared with other mycorrhizal plants (43.1% in OC + RP + *A. niger*, and 45% in OC + *A. niger* treatments). The synergistic action of both (filamentous and mycorrhizal) fungi (OC + *A. niger* + AM) doubled the plant growth and P plant uptake compared with the OC + AM treatment. However, the best combination was between the microbe-treated olive cake and rock phosphate, applied either alone or with the AM fungus. Under the conditions of these two treatments, three times greater plant growth was achieved compared with that in the control plants. Similarly, the P content was five and 3.4 times greater that in the controls, depending on the presence of the AM fungus. The role of *A. niger* was most pronounced when comparing the non-mycorrhizal treatments OC + RP and OC + A. *niger* + RP where the plant growth increase with the second treatment was 429% that of the first.

It appears that the preincubation of the waste material is the key factor in the effectiveness of this system. However, the possible role that both *A. niger* and *G. deserticola* play in the solubilization of organic forms of phosphate is unclear (Tarafdar, 1995).

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