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Microbial solubilization of rock phosphate on media containing agro-industrial wastes and effect of the resulting products on plant growth and P uptake

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Abstract

Four agro-industrial wastes were assayed as substrates for microbial solubilization of rock phosphate (RP). Sugar beet wastes (SB), olive cake (OC) and olive mill wastewaters (OMWW) were treated by *Aspergillus niger*, and dry olive cake (DOC) was treated by *Phanerochaete chrysosporium*. In conditions of solid-state fermentation 46% of SB and 21% of OC were mineralized by *A. niger* while 16% of DOC was mineralized by *P. chrysosporium*. Repeated-batch mode of fermentation was employed for treatment of OMWW by immobilized *A. niger*, which resulted in conversion of 80% of the fermentable sugars. Acidification of all media treated by *A. niger* was registered with a simultaneous solubilization of 59.7% (SB), 42.6% (OC), and 36.4% (OMWW) of the total P present in the RP. The same mechanism of RP solubilization was observed in DOC-based medium inoculated with *P. chrysosporium* but other mechanisms were probably involved during the process. A series of microcosm experiments were then performed in the greenhouse to evaluate the effectiveness of the resulting fermented products. All amendments improved plant growth and P acquisition, which were further enhanced by mycorrhizal inoculation. The level of all studied parameters including the root mycorrhizal colonization depended on the substrate characteristics. The reported biotechnological schemes offer a potential application particularly for degraded soils.

Introduction

Although phosphorus (P) is quite abundant in many soils, it is one of the major plant nutrients limiting plant growth. P is added to soil in the form of phosphate fertilizers, part of, which is utilized by plants but another part rapidly forms insoluble complexes with soil constituents, thus lowering the overall P use efficiency. Therefore, frequent application of soluble forms of inorganic P is needed. However, in practice, as the capacity of soil to bind P is limited, many soils receive P in excess of crop requirements which results in its leaching to the ground water. The runoff from P-loaded soil is accepted as the main factor in eutrophication of natural water reservoirs (Del Campillo et al., 1999). In view of environmental concerns and current developments in sustainability, research efforts are concentrated on elaboration of agro-techniques that involve the use of less expensive, though less bioavailable, sources of plant nutrients such as rock phosphate (RP).

It is accepted that there is no substitute of RP as a source of P. However, and particularly for non-acidic soils, a minimum processing is required before application. Even when the soil

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acidity is below 5.5–6.0 it has been shown that only after 4 years of annual direct application, does RP become as effective as superphosphate (Ghani et al., 1994). It is well established that RP application is not economically feasible, particularly at soil conditions characterized by a high P sorption capacity, low cation exchange capacity, high pH, low rainfall, low organic matter content, and low microbial activity (Simpson et al., 1997). For these reasons, various strategies for RP solubilization have been recently proposed with an increasing emphasis on application of P-solubilizing microorganisms (Rodriguez and Fraga, 1999; Vassilev et al., 2001; Whitelaw, 2000).

A number of *in vitro* studies have shown that bacteria, fungi and actinomycetes are able to liberate phosphate ions from sparingly soluble inorganic P-bearing compounds (Kucey et al., 1989). The P-solubilizing activity is determined by the ability of the microbes to release metabolites such as organic acids, which through their hydroxyl and carboxyl groups chelate the cations bound to phosphate, the latter being converted to soluble forms (Sagoe et al., 1998). In any case, metabolizable C compounds must be applied to the microbes to ensure their growth, organic acid production, and, simultaneously, RP solubilization. On the other hand, the choice of C sources determines the mode of the fermentation processes and the form of application of the microbial cultures. A wide range of carbohydrates, have been tested such as glucose, sucrose, fructose, xylose, starch, etc. (Cerezine et al., 1988). Studies, which include alternative carbohydrate sources such as agro-industrial wastes should also be taken into consideration, bearing in mind the importance of utilizing renewable resources and the simplicity of the cultivation equipment. Processes for RP microbial solubilization based on inexpensive and abundant organic substrates seem to be economically attractive for soils, which are characterized by low contents of organic matter and of soluble P.

The aim of this work was to evaluate solubilization of rock phosphate by fungi utilizing agro-industrial wastes, typical for Southern Spain, such as sugar beet wastes (SB), olive cake (OC), dry olive cake (DOC), and olive mill waste waters (OMWW).

Materials and methods

Microorganisms

The strain of *Aspergillus niger* NB2 used throughout this study was obtained from the Culture Collection of the Institute of Microbiology, Bulgarian Academy of Sciences, and was maintained on potato-dextrose agar slants at 4 °C. It was proved to produce only citric acid on complex substrates (Vassilev et al., 1986) and mineralize lignocellulosic materials (Vassilev et al., 1998). For inoculum preparation, *A. niger* was grown on a slant at 30 °C for 7 days and spores were scraped in sterile distilled water. The spore amount was measured by optical density measurement at 750 nm following calibration between this data and direct haemocytometer counting.

Phanerochaete chrysosporium was obtained from the Culture Collection of the Faculty of Pharmacy, University of Granada, Spain. It was maintained on malt extract plates at 4 °C. *P. chrysosporium* was incubated at 26 °C for 7 days before use as inoculum in fermentation experiments. A spore suspension for inoculation was prepared by dislodging spores from the plate surface in sterile distilled water. The spore number was measured as described for *A. niger*.

Culture media

Sugar beet wastes (SB), olive cake (OC), dry olive cake (DOC), and olive mill wastewaters (OMWW) were used as substrates in the fermentation trials. The characteristics of wastes were determined before fermentation (Table 1). The solid residues were dried in a 60° oven and then ground to pass a 2-mm pore screen. Portions of 15 g of each solid substrate were placed in 250-ml Erlenmeyer flasks. Preliminary studies (data not reported) were carried out to determine the media composition for each waste–microorganism combination using as criteria microbial growth, pH, and acid production. Czapek-Dox mineral salt solution, 40 mL, was added to the treatments with SB and OC, while DOC was mixed with 40 mL distilled water. OMWW was supplemented with $(NH_4)_2SO_4$, 2.5 g L^{-1} and $MgSO_4 \cdot 7H_2O$, 0.2 g L^{-1} and 100 ml of this OMWW-based medium was then

Table 1. Characteristics of sugar beet waste (SB), olive cake (OC[a]), dry olive cake (DOC[a]), and olive mill wastewaters (OMWW[a])

Component	SB	OC	DOC	OMWW
Cellulose (%)	29	24	18	ND
Hemicellulose (%)	23	8	16	ND
Lignin (%)	5	30	26	ND
C_{total} (g kg^{-1} dw)	520	532	464	29[b]
N_{total} (g kg^{-1} dw)	7	9	11	2[b]
P_{total} (g kg^{-1} dw)	0.7	0.8	0.6	0.4[b]

[a]Polyphenol content: 3.3 g kg^{-1}, OC; 2.1 g kg^{-1}, DOC; 6 g L^{-1}, OMWW.
[b](g L^{-1}).

poured into 250-ml Erlenmeyer flasks. Rock phosphate (Morocco fluorapatite, 12.8% P, 1 mm mesh) was added when necessary to all treatments at a rate of 0.75 g per flask. Media were sterilized by autoclaving at 120 °C for 30 min. Spore suspension of *A. niger* (1.2×10^7) and *P. chrysosporium* (2.3×10^6) were carefully spread over the surface of the respective media. OMWW was treated with *A. niger* that had been passively immobilized in polyurethane 0.5-cm^3 cubes. The immobilization system was prepared as described previously (Vassilev et al., 1993) on a medium containing g L^{-1}: glucose, 60; NH_4NO_3, 2.0; KH_2PO_4, 1.0; $MgSO_4 \cdot 7H_2O$, 0.5; yeast extract, 1.0.

Culture conditions

All experiments were carried out in 250-ml Erlenmeyer flasks (in triplicate) with or without RP. Solid-state fermentations were performed with SB, OC (inoculated with *A. niger*), and DOC (inoculated with *P. chrysosporium*) at 30 °C for 20 days. Repeated-batch mode of fermentation was employed in experiments with OMWW treated with immobilized *A. niger* at 30 °C in shaken culture at 200 rpm. In this case the medium was changed every 48 h during five fermentation cycles. Mixtures of waste materials and RP, microbially treated or not, were further used in soil–plant experiments.

Soil–plant experiment

The treatments used in this experiment were as follows: (i) SB/RP: control; SB/RP treated with *A. niger*; (ii) OC/RP: control; OC/RP treated with *A. niger*; (iii) DOC/RP: control; DOC/RP treated with *P. chrysosporium*; (iv) OMWW/RP: control; OMWW/RP treated with immobilized *A. niger*. The fermentation products from treatments (i)–(iv), prepared as described before, were mixed with a steam-sterilized soil–sand mixture (1:1, v/v) and left for equilibration for 4 weeks at room temperature. Topsoil (0–20 cm) from a field of Granada (Spain) province was used. The main soil characteristics were pH 7.5; 8 μg P g^{-1} (Olsen test); organic carbon 0.46%; total N 0.046%. Waste materials and RP mixtures microbially treated or not, were added to the soil at a rate supplying 5 g solid waste in 100 g soil or 5 ml liquid waste in 100 g soil with a corresponding amount of 0.15 g RP in 100 g soil. Three seedlings of *Trifolium repens* were transplanted in each pot (d = 12.2 cm; 500 g capacity; five pots per treatment) inoculated or not with the arbuscular mycorrhizal fungus *Glomus deserticola*. In treatments with DOC treated or not with *P. chrysosporium*, the experimental plant was *Dorycnium pentaphyllum*. All pots received 1 mL (10^8 cells per ml) of *Rhizobium trifoli* suspension. The AM inoculum consisted of 5 g spores, mycelium and mycorrhizal root fragments and was applied to each of the corresponding pots in the bottom of a 5-cm deep hole. The plants were grown in a greenhouse under a day/night cycle of 16/8 h, 21/15 °C, and 50% relative humidity. Water loss was compensated by watering every day after weighing.

Analytical methods

Analyses of fermentation products were carried out by homogenising 4-g sample in 96 mL distilled water. After centrifugation the supernatant was analyzed for citric acid content by the method of Taussky (1949). Medium pH was measured with a glass electrode and titratable acidity (TA) was determined by titrating each sample to pH 7.0 with NaOH. Percentage of citric acid concentration of the total acidity was calculated as described by Tsay and To (1987). Phosphorus content was determined by the molybdo-vanado method described by Lachica et al. (1973). Solubilization productivity was expressed as mg soluble P per kg of waste per hour. Weight loss of lignocellulose during the fermentation

processes was calculated on ash content basis according to Kumar and Sign (1990) and presented as a percent of mineralization. Lignin, cellulose, and hemicellulose contents were measured according to the method of Goering and Van Soest (1970). Total carbon was determined by the method of Jackson (1960) and total nitrogen was measured as described by Baethgen and Alley (1989). Total phenolic content in OC, DOC, and OMWW was determined as described by Ribereau-Gayon (1968).

The plants were harvested after 2 months. Shoot dry weight was recorded after drying at 70 °C. Shoot P content was determined by the molybdo-vanado method described by Lachica et al. (1973). The percentage of mycorrhizal root length was determined by microscopic examination of stained root samples (Phillips and Hayman, 1970) using the gridline intersect method of Giovanetti and Mosse (1980).

Results

Fermentation experiments

The results obtained after 20 days of solid-state fermentation with or without RP showed that SB, OC, and DOC proved to be excellent substrates as rapid growth of *A. niger* and *P. chrysosporium* was observed, particularly at the beginning (the first 3–4 days) of the cultivation process (data not shown). The highest final dry biomass was measured in treatments with SB while the lowest mycelia production was registered in flasks with OMWW-based medium treated with immobilized *A. niger*. The slight neutralizing effect should be mentioned when RP was added to the fermentation media without changing significantly the values measured for fungal biomass, pH and titratable acidity. There was a drop in the initial pH of 6.8–7.0, which at the end of the studied period reached its lowest value in treatments with SB (Table 2). The final pH was higher when DOC was used as substrate treated with *P. chrysosporium*. The production of citric acid, independently of the presence of RP, was well pronounced in all treatments with *A. niger* thus accounting for 91, 75, and 68% of the titratable acidity measured at the end of the fermentation processes based on OMWW, SB, and OC, respectively. The percent of substrate mineralization ranged from 16% in experiments with DOC to 80% in OMWW treatments. The concentrations of polyphenols dropped three times in treatments with OC, DOC, and OMWW and reached 1.2 g kg^{-1}, 0.7 g kg^{-1}, and 2 g L^{-1}, respectively.

Under these conditions, *A. niger* and *P. chrysosporium* were able to solubilize the phosphate rock supplemented in the fermentation media. The highest concentration of soluble P was found in SB-based treatments with *A. niger*. However, the solubilization productivity of 1.24 mg P kg^{-1} h^{-1} obtained in this case was lower as compared to 10.6 mg P L^{-1} h^{-1} reached in OMWW-based repeated-batch process with polyurethane foam immobilized *A. niger*. On the other hand, the percentage of soluble P of total P in the RP, 59.7% and 42.6%, was higher in the treatments with SB and OC treated with *A. niger* in conditions of solid-state fermentations as compared to 32.6% and 36.4% in the case of DOC and OMWW treated by *P. chrysosporium* and immobilized *A. niger*, respectively.

The soluble P concentrations measured in the absence of RP in the fermentation media were low ranging from 21 mg kg^{-1} in the solid-state fermentation with DOC to 156 mg L^{-1} in OMWW-based repeated-batch fermentations. These values of soluble P could not be explained by the microbial action and most likely corresponded to the soluble P fraction of each crude waste. The highest total soluble P concentration of 635 mg kg^{-1} was obtained in SB/RP-based solid-state fermentation and the lowest one was 347 mg kg^{-1} in the treatment with DOC/RP mixture.

Soil–plant experiments

Dry matter and P concentration of shoots of non-mycorrhizal and mycorrhizal plants are presented in Table 3. Overall, plant responses to combinations of agroindustrial wastes and RP depended on whether the wastes were previously treated by *A. niger* and *P. chrysosporium*. Shoot dry weight of plants grown in soil amended with pre-treated SB waste and RP was increased more than five times and reached 330 mg per pot, compared with treatment supplemented with untreated SB/RP. Similarly, two-fold and

Table 2. pH, titratable acidity (TA), soluble P, and solubilization productivity of fermentation processes by *A. niger* and *P. chrysosporium* on media containing sugar beet waste (SB), olive cake (OC), dry olive cake (DOC), and olive mill wastewaters (OMWW) supplemented or not with rock phosphate (RP)

Substrate	Microorganism	PH/TA (mmol kg^{-1})	P_{sol}[d] (mg kg^{-1})	Productivity (mg P kg^{-1} h^{-1})
SB	*A. niger*	2.6/64 ± 3	38 ± 0.7	0.08
SB + RP	*A. niger*	2.9/53 ± 1	597 ± 6	1.24
OC	*A. niger*	3.1/20 ± 0.3	79 ± 1.1	0.16
OC + RP	*A. niger*	3.7/13 ± 0.4	426 ± 4.2	0.89
DOC	*P. chrysosporium*	5.6/8 ± 0.1	21 ± 0.2	0.04
DOC + RP	*P. chrysosporium*	5.7/6 ± 0.2	326 ± 2.9	0.68
OMWW[c]	*A. niger*	3.8/91 ± 3.0	156 ± 3.1[a]	3.25[b]
OMWW[c] + RP	*A. niger*	4.1/72 ± 1.8	364 ± 0.8[a]	10.6[b]

[a]mg L^{-1}.
[b]mg P L^{-1} h^{-1}.
[c]Average result after five repeated-batch cycles.
[d]Soluble P concentrations obtained in experiments with and without RP must be sum to calculate the total soluble P.

three-fold plant growth increase was registered in treatments amended with microbially treated OMWW/RP, DOC/RP, and OC/RP, respectively. In treatments supplemented with untreated wastes and RP, the highest shoot P concentrations of 1.75 and 1.2 mg g^{-1} shoot dry weight were found in mycorrhizal and non-mycorrhizal *T. repens*, respectively, grown in presence of OMWW. Increased P plant acquisition was demonstrated in all soil–plant systems amended with previously treated wastes. The highest, eight-fold increase of P concentration, was measured in mycorrhizal plants grown in microbially treated SB/RP-amended soil.

Microscopic observations of plant roots showed that only AM-inoculated plants were root colonized. Taken individually, the presence of *G. deserticola* influenced positively the plant weights and P uptake in shoots in each treatment. The percentage of AM root length colonization was 58% in the treatments with plants grown in soil amended with untreated SB/RP but decreased in presence of untreated OC, DOC, and OMWW. However, this parameter increased

Table 3. Shoot dry weight (DW) and P content of *Trifolium repens* and *Dorycnium pentaphyllum* as affected by fermentation-resulting products and mycorrhizal inoculation

Substrate/ plant	AM	**Mycorrhiz. (%)		*Shoot DW (mg per pot)		*P concen. in shoots (mg g^{-1} shoot DW)	
		C	+TW	C	+TW	C	+TW
SB/	+	58a	42b	60	330	0.50	4.1
T. repens	−			10	34	0.41	1.5
OC/	+	41ab	51ab	44	141	0.53	2.02
T. repens	−			41	110	0.30	1.78
DOC/	+	38b	71a	280	520	0.20	0.8
D. pentaph.	−			70	290	0.07	0.6
OMWW/	+	32b	48b	110	250	1.75	3.68
T. repens	−			80	170	1.20	3.41

C: soil enriched with untreated waste and RP.
+TW: C + microbially treated RP-enriched waste.
*LSD < 0.5.
**Within each column, means followed by the same letters are not significantly different ($P < 0.05$) using Duncan's multiple range tests.

in all treatments supplemented with microbially treated olive oil wastes and RP except in SB/RP-amended soil where an 18% decrease was measured.

Discussion

This work has proved possible RP solubilization in conditions of solid-state fermentation, on media containing solid agro-industrial wastes. In general, lignocellulosic materials do not give good yields of organic acids without some pre-treatment because of the slow rate of hydrolysis and low level of available sugars. However, the microorganisms used in these experiments are known to have lignocellulolytic activity and particularly *P. chrysosporium* is characterized by its high lignolytic enzyme activity (Bastawde, 1992; Kerem and Hadar, 1993). Therefore, SB, OC, and DOC were mineralized successfully in fermentation systems by *A. niger* and *P. chrysosporium*. It is important to note that in all treatments with *A. niger*, a high value of acidity (mainly citric acid) was measured, while the titratable acidity in the treatment with *P. chrysosporium* was only 6 mmol kg^{-1}. Recently, *P. chrysosporium* was reported to produce low-molecular weight organic acids thus lowering pH outside of the fungal hyphae (Makela et al., 2002). In addition, some metal chelating compounds released by white-rot fungi, including *P. chrysosporium*, have been reported to take part in wood mineralization (Milagres et al., 2002).

The overall effect of the extracellular compounds with their properties of chelators is the most likely reason for some RP solubilization by *P. chrysosporium* although other mechanisms could be also involved bearing in mind that the above mentioned processes occur in the early stage of fungal development and the amounts of the released compounds is low. However, this work demonstrated that, although *P. chrysosporium* is not a typical organic acid producer, it can be used efficiently in RP solubilization providing a percentage of soluble P at least comparable to that obtained by *A. niger*.

The microbial solubilization of RP based on the liquid agro-industrial waste, OMWW, demonstrated the highest system productivity of 10.6 mg P L^{-1} h^{-1}. The most likely explanation is the application of immobilized living cells during the repeated-batch fermentation. Immobilization is known to prevent shear stresses, typical for submerged operations, thus ensuring higher metabolic activity, catalytic longevity, and stability (Vassilev and Vassileva, 1992). The main advantage of solubilizing RP by immobilized microbial cells is that for a period of one batch cycle (48 h), the immobilized system provides an amount of soluble P equal or higher that that obtained by free fungal cultures in conditions of solid-state and even conventional submerged process.

Both *A. niger* and *P. chrysosporium* were able to decrease the concentration of total phenols. Filamentous fungi and white-rot fungi are known to significantly lower the concentration of phenolic compounds in residues of olive oil extraction processes (Blanquez et al., 2002; D'Annibale et al., 2003), which was confirmed in this work.

Our results from microcosm experiments confirmed the important role of mycorrhizal fungi in P uptake, particularly in combination with P-solubilizing microorganisms. By the use of isotopic ^{32}P dilution technique, we have recently reported that mycorrhizal plants benefited from P solubilized from RP by *A. niger* (Vassilev et al., 2002).

The mycorrhizal development in this work depended on the nature of the waste material. Root mycorrhizal colonization in control plants, amended with untreated mixtures of OC, DOC, OMWW, and RP was lower than that measured in soil amended with microbially treated substrate/RP mixtures. The addition of *A. niger*-treated SB/RP resulted in lower percentage of AM root length colonization compared with the treatment amended with untreated SB/RP. It is now well established that cellulose stimulates mycorrhizal development in certain conditions (Gryndler et al., 2002). SB (as a cellulose carrier) stimulated the mycorrhizal colonization of the experimental plants when added untreated. However, lowering of this parameter was observed in case of treated SB/RP amendment, which could be caused partly by the substrate mineralization and/or by the presence of soluble P in the fermented product.

A different trend was observed in all experiments based on OC, DOC, and OMWW. Here, the lower root mycorrhization in the control soil–plant systems could be explained by the presence of polyphenol compounds which are well known antimicrobial and phytotoxic agents

(Rodriguez et al., 1988). Information regarding the effect of residues of olive oil extraction processes on AM development is scarce. Recently, Martin et al. (2002) have reported that the application of untreated DOC decreased the percentage of AM colonization of plants. The obtained data showed that AM fungi increased the phytotoxicity of DOC in soybean and lettuce. Our results with olive oil wastes confirm these statements which were more pronounced particularly in the case of plants amended with untreated OMWW where the highest concentration of polyphenols of 6 g L^{-1} was the most likely reason for the lowest value of AM colonization compared with all other treatments amended with untreated wastes. Under these conditions, however, *T. repens* was able to accept high concentrations of P, which was due to the high concentration of plant available soluble P (156 mg L^{-1}) in the crude OMWW. The results reported here indicated that the detoxification of olive oil wastes by *A. niger* and *P. chrysosporium* increased the level of mycorrhiza formation along with all effects related to plant growth and P uptake. The experiment with DOC/RP, previously treated with *P. chrysosporium* should be mentioned as it provided the lowest both total polyphenol and total soluble P concentrations which resulted in a high percentage of root length AM colonization compared with the control treatments. In this case the P concentration of 0.8 mg g^{-1} shoot dry weight was the lowest one among all treatments amended with treated wastes which is, however, most likely caused by the dilution (high plant growth) effect.

Because of the different physical and chemical waste characteristics it would be difficult to strictly compare their effect on RP solubilization and further on plant growth and P uptake. However, some conclusions can be drawn:

1. *Aspergillus niger* and *Phanerochaete chrysosporium* were able to grow and simultaneously solubilize the phosphate rock independently of the mode of fermentation.
2. The process of RP solubilization was related to the release of organic acids, mainly citric acid, particularly in the treatments with *A. niger*. In cultures with *P. chrysosporium* the process of RP dissolution depended on the same mechanism during the first stage of fermentation but other mechanisms could be later involved.
3. The concentration of phenolic compounds in residues of olive oil extraction process decreased in microbially treated OC, DOC, and OMWW.
4. Microbially treated wastes and RP enhanced plant growth and P uptake. Inoculation with *G. deserticola* appeared to have an important role in these processes by increasing the overall positive effect of the introduced amendments.
5. The level of root mycorrhization depended on the substrate characteristics and its microbial treatment.

Further studies should be carried out to determine the effect of untreated and microbially treated wastes and the corresponding microorganisms solubilizers on the soil physical, chemical, microbiological, and biochemical quality in greenhouse and natural conditions.

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