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FERTILIZING EFFECT OF MICROBIALLY TREATED OLIVE MILL WASTEWATER ON *TRIFOLIUM* PLANTS

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Abstract
Olive mill wastewater (OMW) with added N and Mg was used as a medium in a shake-flask, repeated-batch fermentation process with a passively immobilized, acid-producing, strain of Aspergillus niger. *The latter was able to reduce the phenolic content of the waste material to 59% of its initial amount and to lower the pH of the medium. Rock phosphate, added to the OMW medium was solubilized to a maximum amount of 0·5 g/l during the fourth batch cycle with a corresponding productivity of 10·6 mg P/l.h. Five types of OMW±RP, microbially treated or not, were tested in a soil–*Trifolium *system for their fertilizing ability. The beneficial effect of microbially treated OMW was more evident during the first crop cycle. The best plant growth response and P uptake were found in mycorrhizal plants grown in soil amended with fungal treated OMW+RP. © 1998 Elsevier Science Ltd. All rights reserved.*

Key words: Aspergillus niger, immobilized cells, olive mill wastewater, rock phosphate, solubilization, mycorrhiza, plant response.

INTRODUCTION

During the extraction of oil from the olive fruits, large amounts of olive mill wastewater (OMW) are produced. Its uncontrolled disposal pollutes the environment, thus causing unforeseen effects on the soil–plant system in olive-producing areas. During the last two decades many processes have been described to reduce the phytotoxic and antimicrobial effects of the OMW (attributed mainly to its phenolic content) (Perez *et al.*, 1986; Peredes *et al.*, 1987). Treated OMW can be utilized as animal food, or raw material in various biotechnological processes (Hamdi, 1993). Another attractive approach is to use OMW as a fertilizer, since it contains some organic and inorganic compounds, beneficial to plants. This alternative is, however, limited due to the toxic characteristics of the waste. It is believed that pretreatment of OMW by microorganisms, particularly fungi, positively affects its composition (Makasinova and Martakov, 1982).

Recently, we have proved possible the application of immobilized, acid-producing filamentous fungi in rock phosphate solubilization (Vassilev *et al.*, 1996aVassilev *et al.*, 1997). The aim of the present work was to evaluate the effect of immobilized *A. niger* on decreasing the phytotoxicity of olive mill wastewater in a process with simultaneous solubilization of rock phosphate. Growth and P uptake of mycorrhizal or non-mycorrhizal *Trifolium repens* were used as criteria to verify the potential of the treated liquid for agricultural purposes.

METHODS

Microorganism, and fermentation media

An acid-producing filamentous fungus, *Aspergillus niger* NB2, was used throughout this study, maintained on potato–dextrose agar slants.

The olive mill wastewater (pH 5·7; total sugars, 29·8 g/l; total carbon, 30·7 g/l; total phenol content, 6·7 g/l; soluble P, 0·189 g/l) was obtained from the local (Granada, Spain) factory. The medium for cell immobilization was described in a previous work (Vassilev *et al.*, 1997). The medium for OMW treatment contained (g/l OMW): $(NH_4)_2SO_4$, 0·25; $MgSO_4.7H_2O$, 0·1. Rock phosphate (Morocco fluorapatite, 12·8% P; 1 mm mesh) was added at a concentration of 3·0 g/l when necessary.

Immobilization procedure and fermentation coditions

Polyurethane sponge cubes (0·3 cm^3) used for the immobilization of *A. niger* had an average pore size of 0·6–0·8 mm and were obtained from the local market. The immobilization procedure was performed as described earlier (Vassilev *et al.*, 1997). Immobilized cells were transferred to 100 ml of OMW-based medium in 250 ml Erlenmeyer flasks. The immobilization procedure and fermentation were carried out (five flasks per treatment) at 30°C in shaken culture at 200 rev/min. The OMW-based medium was changed every 48 h. The immobilized culture was separated from the liquid after the fifth batch which was further used for the soil–plant experiment.

Soil–plant stage

The experiment consisted of five treatments according to the material added to soil: control (soil alone); untreated OMW; untreated OMW+rock phosphate; OMW, preincubated with *A. niger*; OMW+rock phosphate, preincubated with *A. niger*. Treated or untreated OMW was applied at a rate of 50 ml per pot, the latter containing 300 g steam-sterilized soil–sand mixture (1:1, w/w), and left for equilibration for 30 days. 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 (Olsen test), organic carbon 0·46%, total N 0·046%.

Ten seeds of *Trifolium repens* were planted in each pot (9·5 cm; 300 g capacity) inoculated or not with the AM fungus *Glomus deserticola*. Introduction of AM inoculum and growth conditions were as described earlier (Vassilev *et al.*, 1996b).

Analytical methods

The dry weight of immobilized cells, medium pH and P content were analyzed as described previously (Vassilev *et al.*, 1997). The amount of total phenols was determined by the method of Swain and Hillis (1959).

The plants were harvested twice with a harvest period of 6 weeks. Shoot and root dry weight, shoot P content and the percentage of mycorrhizal root length were analyzed by methods described previously (Vassilev *et al.*, 1996b). Results are the average of five replicates, and standard errors were smaller than the dimensions of the symbols.

RESULTS AND DISCUSSION

Fermentation stage

The immobilized mycelium grew well inside the polyurethane sponge in supplemented olive mill wastewater (OMW)-based medium, forming a stable carrier–cell system without leaking of free cells into the medium. However, the biomass growth was restricted and reached only 4·1 g/g carrier (OMW+RP) and 2·9 g/g carrier (OMW−RP), as compared with 8·8 g/g carrier and 6·5 g/g carrier obtained in a glucose-based medium with twice the amount of nitrogen in a previous study (Vassilev *et al.*, 1997). The lowest pH values of 2·9 and 3·7 were registered after the third batch cycle for OMW and OMW+RP treatments, respectively, but the buffering effect of rock phosphate should be noted when assessing the acidifying capacity of immobilized fungal mycelium (Fig. 1(a)). Nevertheless, the level of pH in all treatments was lower by about 1·0 unit than in the case with higher amounts of nitrogen Vassilev *et al.*, 1997). These observations are in good accordance with results obtained by Vassilev *et al.* (1992) when decreased nitrogen concentration resulted in low biomass growth and high acid productivity by immobilized *A. terreus*. It is now well established that solubilizing microorganisms may bring about the solubilization of inorganic phosphates by acid production (Sperber, 1958). Under the conditions of this study, the immobilized fungus solubilized rock phosphate and the maximum concentration of total soluble P reached 510 mg/l during the fourth batch cycle with a corresponding productivity of 10·6 mg P/l.h (Fig. 1(b)). A part of the available soluble P was obviously consumed by the fungus, which resulted in

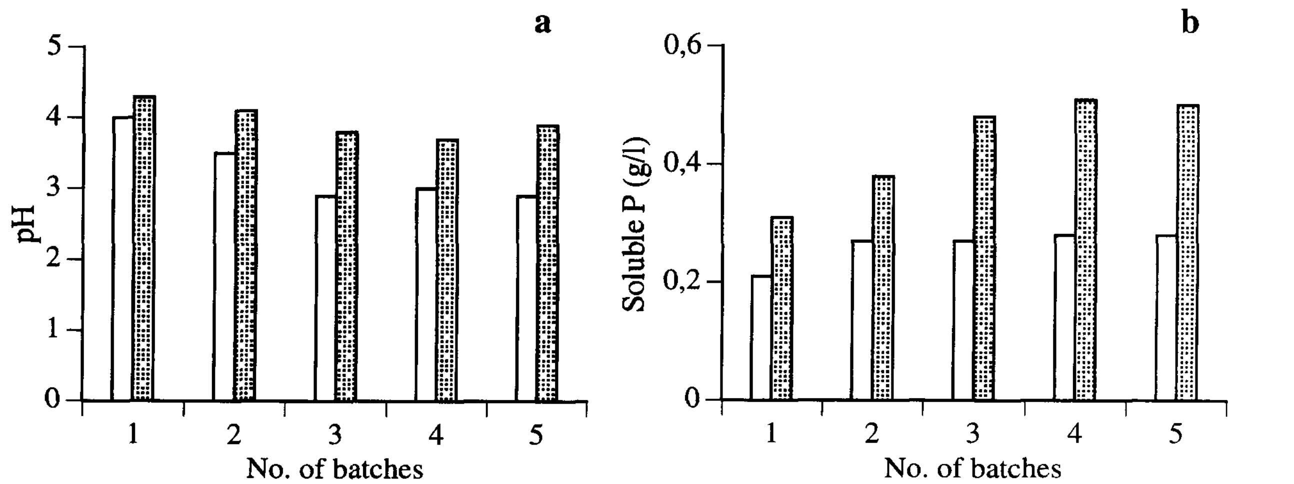

Fig. 1. pH values (a) and soluble phosphate accumulation (b) in repeated-batch process by immobilized *A. niger* on OMW-based media. Open symbols, OMW; shaded symbols, OMW+RP.

a greater immobilized biomass weight (0·82 g/flask) than in the rock-free treatment (0·58 g/flask). Similar enhanced biomass growth, but much more pronounced, was observed when immobilized *A. niger* was cultivated in glucose-based medium supplemented with different amounts of rock phosphate (Vassilev *et al.*, 1997).

The immobilized *A. niger* strongly affected the phenolic content of the OMW-based fermentation medium. The concentration of total phenols present in the OMW at an initial concentration of 6·7 g/l was reduced to values of 2·8 g/l (59% of the initial amount) and 4·3 g/l (36% of the initial amount) registered after the third batch cycle of the OMW+RP and OMW−RP treatments, respectively (Fig. 2). A similar reduction of phenolic compounds, attributed to the metabolic activity or adsorbtive capacity of freely suspended mycelium of *A. niger* was reported by Hamdi *et al.* (1991). However, they mentioned the abundant fungal biomass growth which caused problems during the attempts to scale-up the process. Therefore, the method of OMW treatment by immobilized fungal cells seems to be more advantageous than the free-cell OMW processing.

As a result of the immobilized-cell repeated-batch fermentation, two types of OMW (enriched or not with soluble P) were obtained and further introduced into a P-deficient soil poor in organic matter.

Soil–plant experiment

A soil–plant experiment was carried out analyzing the growth and P uptake of non-mycorrhizal and mycorrhizal *Trifolium* plants (Table 1 and Table 2). The results clearly demonstrated the phytotoxic nature of untreated OMW which was, however, partly neutralized in the case of mycorrhizal plants. It has been found that OMW negatively affected germination and the early growth stage of plants (Perez *et al.*, 1980, 1986). On the other hand, it was considered to promote plant growth in some field experiments (Torres Martin *et al.*, 1980; Potenz *et al.*, 1980). These findings were confirmed in this study by the retarded growth during the first crop cycle with OMW and an increase during the second crop cycle, particularly in the non-mycorrhizal treatment. In both treatments the growth of *Trifolium* during the second crop was higher than that of the control. Similarly, the introduction of untreated OMW in soil resulted in the lowest value of plant P uptake during the first crop and the greatest further enhancement during the second crop.

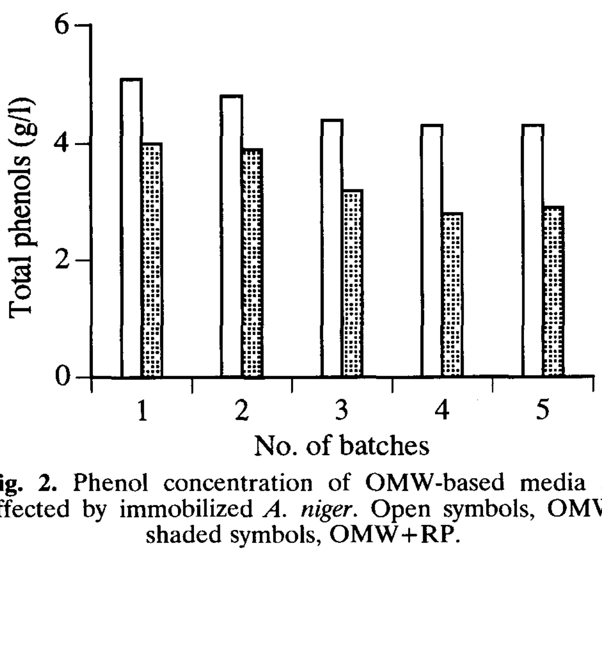

Fig. 2. Phenol concentration of OMW-based media as affected by immobilized *A. niger*. Open symbols, OMW; shaded symbols, OMW+RP.

Table 1. Dry weight of non-mycorrhizal and mycorrhizal plants as affected by OMW, rock phosphate and *Aspergillus niger*

Treatments			Crop period (mg/pot)		Total (mg/pot)
OMW	RP	*A. niger*	I	II	
Non-mycorrhizal					
+	−	−	60±4	130±6	190
+	+	−	80±2	150±5	230
+	−	+	130±4	140±3	270
+	+	+	170±7	200±6	370
−[a]	−	−	90±3	120±4	210
Mycorrhizal					
+	−	−	110±5	190±7	300
+	+	−	150±4	220±10	370
+	−	+	180±5	240±9	420
+	+	+	250±10	370±11	620
−[a]	−	−	120±5	160±6	280

Values are ± standard deviation for five replicate cultures. Results are significant at $P = 0{\cdot}001$.
[a]Control.

The enrichment of untreated OMW with rock phosphate resulted in a slight plant growth increase which was more pronounced in the mycorrhizal treatment. The higher growth and P uptake obtained by mycorrhizal plants could be explained by the findings that fungal hyphae are able to exploit forms of P which are not considered ‘available’ by the Olsen test (George *et al.*, 1995).

The beneficial effect of microbially treated OMW was verified with both RP-supplemented and non-supplemented variants and was more evident during the first crop cycle. Taken individually, the growth of non-mycorrhizal and mycorrhizal *Trifolium* plants in soil amended with *A. niger*-treated OMW during the first crop was 116% and 64% higher, respectively, than the variant amended with untreated OMW. This result could be explained by the reduced level of toxic OMW-compounds. On the other hand, a significant plant growth increase was observed in the treatments receiving OMW treated in the presence of rock phosphate. Particularly in the case of mycorrhizal plants, plant growth increased more than two-fold over the unamended control. Evidently, with an enhanced exploitation of soil, owing to the action of *G. deserticola*, plants

Table 2. Phosphorus content in shoots of non-mycorrhizal and mycorrhizal plants as affected by OMW, rock phosphate (RP) and *Aspergillus niger* (*A.n.*)

Treatments			Crop period				Total	
			(mg/pot)		(mg/g[a])		(mg/pot)	(mg/g[a])
OMW	RP	*A.n.*	I	II	I	II		
Non-mycorrhizal								
+	–	–	0·08	0·20	1·00	1·54	0·28	2·54
+	+	–	0·16	0·23	2·00	1·53	0·39	3·53
+	–	+	0·29	0·21	2·23	1·50	0·50	3·73
+	+	+	0·58	0·71	3·41	3·55	1·29	6·96
–	–	–	0·11	0·16	1·20	1·33	0·27	2·53
Mycorrhizal								
+	–	–	0·18	0·42	1·64	2·30	0·60	3·94
+	+	–	0·27	0·55	1·80	2·50	0·82	4·30
+	–	+	0·38	0·60	2·10	2·50	0·98	4·60
+	+	+	0·92	1·37	3·68	3·71	2·30	7·40
–	–	–	0·21	0·28	1·75	1·75	0·49	3·50

Values are means from five replicate cultures and the standard errors were smaller than 5%.
[a]1 g shoot dry weight.

could adsorb phosphate ions chemically liberated from rock phosphate during the fermentation stage. All mycorrhizal plants took up more P than the respective non-mycorrhizal treatments (Table 2) but this process was strongly pronounced in soil amended with treated OMW+RP. While the enhanced P uptake in mycorrhizal plants is not surprising, the high P concentration should be noted in non-mycorrhizal plants grown in soil enriched with treated OMW+RP.

Only AM-inoculated plants were root colonized (OMW, 48·0%; OMW+RP, 46·0%; treated OMW, 45·4%; treated OMW+RP, 42·6%; control, 50·0%). Introduction of treated or untreated OMW did not affect mycorrhizal root colonization. The percentage of mycorrhization depended on the level of solubilization of rock phosphate, as reported earlier by Barea *et al.* (1980).

The high buffering capacity of the calcareous soil should be mentioned but recently Gerke (1992) reported detectable soluble P concentration after 140 days in alkaline soil treated with citric acid. Similar observations were reported when sugar beet waste, treated by acid-producing fungus in the presence of rock phosphate, was introduced into calcareous soil (Vassilev *et al.*, 1996b). The synergistic effect of microbially treated OMW+RP and AM fungus was found to be decisive in obtaining the highest plant growth and P uptake, as compared with all other treatments. The recorded total plant growth in this case was almost 300% greater than the non-mycorrhizal control. The same percentage was registered for the plant tissue–P concentration during the first crop.

It can be concluded that the process of OMW treatment by immobilized *A. niger* might serve for the production of a fertilizer which, enriched or not with solubilized rock phosphate, is able to improve the growth of plants.

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