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# Rock phosphate solubilization by *Aspergilli* species grown on olive-cake waste and its application in plant growth improvement

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**ABSTRACT**

Organic acids producing strains of *Aspergillus niger* and *Aspergillus fumigatus* grown on olive cake-based media were studied for ability to solubilize rock phosphate. The final fermented mixture was, acidified and contained mineralized organic matter, solubilized rock phosphate and fungal mycelium. Solubilization increased during the fermentation process, reaching a maximum of 400, 330 µg/ml at pH 3.6, 4.0 and titratable acidity 25, 20 m mol/L after 9 and 12 days of incubation time with *A. niger* and *A. fumigatus* respectively. Various combination of olive cake and rock phosphate, previously treated or untreated by the fungi were introduced into a phosphorus (P) deficient soil (0.016 mg/g soil) to improve the growth of broad beans in a pot experiment. Compared to other treatments, synergistic action of both the filamentous and arbuscular fungi caused considerable improvement of growth and P uptake. The greater growth rate and P concentration of mycorrhizal and non-mycorrhizal plant were achieved when microbes-treated olive cake and rock phosphate were applied to soil compared with all other treatments. Also, inoculation with the mycorrhizal fungus significantly increased plant growth than in the equivalent non-mycorrhizal treatment.

**KEYWORDS:** *Aspergillus niger*, *Aspergillus fumigatus*, vesicular-arbuscular mycorrhizal fungus, rock phosphate, solubilization, olive cake, broad bean and plant growth.

## INTRODUCTION

Phosphorus plays a vital role in plant nutrition (Hayman 1975), but its concentration in soil solution is very low. The greater part of soil phosphorus (P) is present in the form of insoluble phosphates mostly unavailable to plants. Recently, the possibility of the practical use of rock phosphate as a fertilizer has received significant interest.

Rock phosphate is not available to plants in soils with a pH greater than 5.5-6.0 (Khasawneh & Doll 1978). One very attractive approach for rock P solubilization is the application of microorganisms able to excrete organic acids, which can strongly increase phosphorous concentrations 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 some species have been tested in fermentation systems.

In recent years olive cake became an important agro-industrial waste because of its high organic content (Dimitris 1988). A possible microbial treatment of rock phosphate in a medium containing such waste could avoid soil pollution, and at the same time enrich soil-plant systems with soluble P and a mineralized organic matter complex. This combination is of significant importance for Egyptian soils that are poor in organic matter and soluble phosphate. The objective of the present work was to test rock phosphate solubilization by two *Aspergillus* species, *A. niger* and *A. fumigatus* on an olive-cake medium, and to analyze plant responses to the application of the resulting system in soil.

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## MATERIALS AND METHODS

**Microorganisms and fertilizers used:** Strains of *Aspergillus niger* and *Aspergillus fumigatus* used throughout this study were isolated from Egyptian soil and identified according to Fisher & Cook (1998). Identification was confirmed by Regional Centre for Micology and Bio-technology, Al-Azhar University, Cairo, Egypt. The pure cultures were maintained on glucose peptone agar medium and subcultured at monthly intervals. The vesicular-arbuscular mycorrhizal (VAM) fungus used in this study was *Glomus intraradices*. It was obtained from the Institute of Plant Pathology, University of Hannover, Germany. It was propagated on maize grown in green houses for three months.

The fertilizers used were:

(a) Phosphate: Egyptian raw rock phosphate (fluorapatite) was supplied from the Abu-Zabal Company for Chemical Fertilizers, Cairo, Egypt.

(b) Agro-industrial waste: Olive cake was obtained from the Olive-oil Extraction Factory, Ismailia, Egypt.

**Culture media and fermentation condition:** Olive cake is a solid waste material; it was ground in an electrical grinder to 1.0 mm fragments and used at a concentration of 10% (w/v) as a solid-phase substrate for static fermentation in 250 ml Erlenmyer flasks containing 50 ml Czapek's solution. Rock phosphate (1 mm mesh) at concentration of 3.0 g/L was added. After sterilization the flasks were inoculated with a spore suspension of either *A. niger* or *A. fumigatus* ($1.2\times10^6$ spores/flask) and incubated at 30°C for 15 days (Vassilev *et al.* 1996). This medium was used for the establishment of six treatments: pre incubated olive cake+rock phosphate+*A. niger* or *A. fumigatus*, pre-incubated olive cake+*A. niger* or *A. fumigatus*, untreated olive cake+ Rock phosphate, and rock phosphate with sterilized or non-sterilized control. Three replicates from each treatment were used.

**Analytical study for fermentation media:** At the end of incubation period, mycelial growth was determined by weighing the mycelium, it was carefully separated from the medium, washed with distilled $H_2O$ and dried in an oven at 100°C. Medium pH was measured with a glass electrode, and titratable acidity determined by titrating each sample with 0.1 N NaOH. The phosphorus content of the the medium was determined according to Rouser *et al.* (1970) The weight loss of lignocellulose during the fermentation was calculated on the basis of ash content according to Kumar & Sign (1990) and presented as a percentage of mineralization.

**Soil-plant experiment:** The previously fermented media were mixed directly at 1:1 (v/v) with sterilized clay-sand soil 2:1 (w/w) with pH of 8.0 and phosphorus content of 0.016 mg/g soil, in pots containing 750 g. The rock phosphate (containing 29% $P_2O_5$) and waste material were added to the soil at a rate of 2.25 and 75 g/pot respectively. All treatments were left to equilibrate for two weeks at room temperature before planting.

Seeds of broad bean (*Vicia faba* cv. Giza, 40) were surface-sterilized with 7% calcium hypochlorite for 20 min. and subsequently washed with distilled water and germinated for 2 to 4 days in a sterile water. Four uniform seedlings were then planted in each pot containing sterilized or nun-sterilized treated soil. The pots were inoculated or not with the mycorrhizal inoculum. A 10 g sample (spores, mycelium and mycorrhizal root fragments) of the vesicular-arbuscular mycorrhiza (VAM) fungus *Glomus intraradices* was placed 2 cm depth below the broad bean seeds at planting. All pots received 1.0 ml of *Rhizobium japonicum* (local strain). Pots were randomized in a glass house under natural day length and light intensity (winter) and watered regularly to near field capacity with tap water. The seedlings were thinned to three per pot after two weeks and the plants were harvested after nine weeks. Three pots from each treatment were used.

**Analytical study for plants:** At the end of growth period the fresh weight heights of shoots and roots and the number of leaves and nodules were recorded. Shoot and root dry weights

were estimated after drying at 70°C.

Total phosphorous in each of dried shoot material, rock phosphate and soil was determined by the Soil and Water Environment Research Institute, Unit of Analysis and Studies, Agriculture Research Centre, Egypt. The pH value of soil was determined according to Jackson (1958) by immersing the glass pH meter electrode in a soil suspension with a soil water ratio of 1:5. The percentage of mycorrhizal root length was estimated by microscopic examination of stained samples using trypan blue (Trouvelot *et al.* 1986). All results were treated statistically using analysis of variance.

## RESULTS AND DISCUSSION

**Fermentation study:** *A. niger* or *A. fumigatus* grown on media supplemented with 10% olive cake and 3.0 g/L rock phosphate. (Table 1) showed rapid mycelial growth over the fermentation time. Similar results have been reported by other authors applying sugar cane bagasse (Lakshminarayana *et al.* 1975 & Oriol *et al.* 1988), vinasse (Nahas *et al.* 1990) & sugar beet waste (Vassilev *et al.* 1996). Despite the insoluble crystalline nature of cellulose associated with the high amount of lignin in olive cake, the weight loss of this waste material was more than 20% at the end of incubation time. Such a process at low pH is not surprising since most filamentous fungi possess lignocellulytic activity (Czajkowska *et al.* 1988; Chahal *et al.* 1992).

Initial pH value of 6.0 decreased substantially with both species, reaching 3-4 at the end of the incubation. Titratable acidity, pH value and phosphate solubilization all increased throughout the fermentation time. An increase of the titratable acidity to 25 and 20 m mol/L was observed after 9 and 12 days of fermentation which resulted in P solubilization of 74 and 61% with *A. niger* and *A. fumigatus* respectively. Thereafter the fungi started to sporulate which was a sign of adverse conditions for acid production (Vassilev *et al.* 1996).

**Plant growth:** Growth response after mixing the various treatments with soil and after 9 weeks of growth are presented in Tables 2 and 3. There was a negative effect of the presence of rock phosphate alone or in combination with olive cake without microbial pre-incubation, more pronounced in non-mycorrhizal plants. The pre-incubated (OC+RP+*A. niger* or *A. fumigatus*) treatments had a positive effect on both mycorrhizal and non-mycorrhizal plants. The highest significant values of all parameters were obtained with *A. niger* in the presence or absence of mycorrhizal fungus. Also, inoculation with the mycorrhizal fungus resulted in a significantly higher ($P<0.05$) plant growth than that of the equivalent non-mycorrhizal treatments (Kucey *et al.* 1989).

In Table 2, the pre-incubated (OC+RP+*A. niger*) treatment had a shoot and root dry weights about 1.40, 0.90 g/pot and 1.00, 0.60 g/pot respectively in mycorrhizal and non-mycorrhizal plants. The growth response in these treatments were 87, 80% and 54, 58% higher than the respective controls.

**Nodule formation:** Mycorrhizal plants possessed significantly greater ($p<0.05$) nodule numbers than in non-mycorrhizal plants (Table, 3). Mycorrhizal status is a precondition for effective growth and nodulation of legumes and as a result of infection with both *Rhizobium sp.* 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 (Nahas *et al.* 1990).

**Root colonization:** Microscopic observation of *Vicia faba* roots stained by trypan blue showed that only AM-inoculated plants had their roots colonized (Table 4). The higher percentage of mycorrhization (100%) was found in the treatment with previously unsolubilized RP, in comparison with the other mycorrhizal plants. Similarly Barea *et al.* (1980), reported that RP did not reduce the level of mycorrhizal infection compared to soluble phosphate.

Table (1): Effect of fermentation time on mycelial growth, pH values, titratable acidity and rock phosphate solubilization by *A. niger* and *A. fumigatus* grown on olive-cake waste

| Time day | Biomass (g/L) | | | pH value | | | Titratable acidity (m mol/L) | | | Phosphate concentration (µg/ | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | *A. niger* | *A. fumig-atus* | Means | *A. niger* | *A. fumig-atus* | Means | *A. niger* | *A. fumig-atus* | Means | *A. niger* | *A. fumig-atus* |
| 3 | 0.21 | 0.08 | 0.145(c)* | 5.68 | 6.00 | 5.84(a)* | 18 | 13 | 15.5(d)* | 180 | 120 |
| 6 | 0.99 | 0.32 | 0.655(c) | 5.03 | 5.43 | 5.23(b) | 20 | 17 | 18.5(c) | 290 | 230 |
| 9 | 3.40 | 1.33 | 2.365(b) | 3.60 | 5.00 | 4.30(c) | 25 | 18 | 21.5(b) | 400 | 270 |
| 12 | 5.23 | 2.61 | 3.921(a) | 3.30 | 4.00 | 3.65(d) | 25 | 20 | 22.516(a) | 380 | 330 |
| 15 | 6.03 | 4.11 | 5.071(a) | 3.10 | 4.00 | 3.55(e) | 24 | 19 | 21.516(b) | 390 | 330 |
| Mean | 3.172 (a) | 1.69 (b) | | 4.142 (b) | 4.886 (a) | | 22.4 (a) | 17.4 (b) | | 328 (a) | 256 (b) |
| | F test for time = 14.525*** | | | = 179.864*** | | | = 84.392*** | | | = 142.774*** | |
| | L.S.D. for time at 0.05 = 1.328 | | | = 0.0022 | | | = 0.0295 | | | = 23.916 | |
| | F test for fungi = 29.063*** | | | = 124.322*** | | | = 31.472*** | | | = 120.991*** | |
| | L.S.D. for fungi at 0.05 = 1.840 | | | = 0.0014 | | | = 0.0186 | | | = 15.125 | |

*** = Highly significant. (df = 18).

* Values in each column followed by the same letter are not significantly different at P≤ 0.05 (Anova test).

Values are the means of three replicates.

**Phosphorus content:** Phosphorous concentration increased in shoots for most treatments compared with control (Fig. 1). However, although more pronounced in combination with the AM fungus, this effect was significantly greater ($p<0.05$) in the treatments where *A. niger* participated in the system. Synergistic action of both filamentous and mycorrhizal fungi (OC+RP+*A. niger*+AM) caused a significant plant P uptake (5.9 mg/g dry wt.)compared to other treatments. Gerke (1992) reported that the addition of citric acid increased phosphate concentration in solutions of alkaline soil, and Bolan *et al.* (1994) found that organic acids added to soils increased the P uptake from a water-soluble phosphate. Therefore it was clear that RP previously solubilized by OC+*A. niger* and /or *A. fumigatus* fermentation favoured the growth of *Vicia faba* and this process was significantly enhanced by infection with AM fungus. It appears that pre-incubation of the waste material is the key factor in the effectiveness of this system. Kieslich (1976) reported that *A. niger* can partly neutralize the phenolic effect of the olive cake. Finally, *A. niger* is more effective than *A. fumigatus* for rock phosphate solubilization which stimulated the growth of *Vicia faba*.

Inoculation of plants with mycorrhizal fungus significantly increased all growth parameters and phosphorus uptake relative to equivalent non-mycorrhizal plants in all treatments. These results agree with other researches (Kucey & Paul 1982; Fredeen & Terry 1988; Beniwal *et al.* 1992 & Daniels-Hylton & Ahmed 1994).

Finally, we can conclude that, rock phosphate previously solubilized by OC+*A. niger* fermentation favoured the growth of *Vicia faba* and this process was significantly enhanced by infection with AM-fungus.

Table 2: Influence of rock phosphate and *A. niger* or *A. fumigatus* on shoots and roots fresh and dry weight of mycorrhizal and non-mycorrhizal broad bean plants grown 9 weeks in sterilized soil.

| No. | Treatments | | | | Parameters | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | Shoot Fresh wt. (g/pot.) | Root Fresh wt. (g/pot.) | Shoot Dry wt. (g/pot.) | Root Dry wt. (g/pot.) |
| | | | Mycorrhizal | | | | | |
| | OC | RP | *A. niger* | *A. fumigatus* | | | | |
| A | + | + | + | - | 12.20 a* | 4.90 a* | 1.40 a* | 0.90 a* |
| B | + | + | - | + | 11.21 b | 4.22 b | 1.22 b | 0.81 a |
| C | + | - | + | - | 9.32 c | 3.95 c | 0.93 c | 0.80 a |
| D | + | - | - | + | 9.11 d | 3.00 d | 0.93 c | 0.72 b |
| E | + | + | - | - | 7.83 e | 2.87 e | 0.84 d | 0.61 c |
| F | - | + | - | - | 7.90 e | 2.62 f | 0.79 e | 0.53 d |
| C (st.) | - | - | - | - | 6.80 g | 2.62 f | 0.75 f | 0.50 d |
| C (unst.) | - | - | - | - | 7.70 f | 2.80 e | 0.81 de | 0.54 d |
| SE | | | | | 0.0019 | 0.0014 | 4.95 | 9.60 |
| | | | Non-Mycorrhizal | | | | | |
| A | + | + | + | - | 9.80 i* | 3.40 i* | 1.00 i* | 0.60 i* |
| B | + | + | - | + | 9.80 i | 3.03 j | 0.91 j | 0.54 j |
| C | + | - | + | - | 8.26 j | 2.92 k | 0.91 j | 0.50 j |
| D | + | - | - | + | 7.80 k | 2.70 i | 0.85 k | 0.50 j |
| E | + | + | - | - | 7.22 i | 2.35 n | 0.70 i | 0.40 k |
| F | - | + | - | - | 6.42 m | 2.30 n | 0.68 lm | 0.39 k |
| C (st.) | - | - | - | - | 6.20 o | 2.10 o | 0.65 m | 0.38 k |
| C (unst.) | - | - | - | - | 6.33 n | 2.42 m | 0.63 m | 0.39 k |
| SE | | | | | 0.0012 | 9.21 | 6.59 | 5.76 |

OC = Untreated olive cake; RP =Rock Phosphate; += Presence; - = Absence; Control (sterile, unsterile) = Soil without amendments
* Values in each column followed by the same letter are not significantly different (P = 0.05) for each treatment (Anova test). Values are the means of eight plants. df = 14; SE= Standard Error.

Table 3: Influence of rock phosphate and *A. niger* or *A. fumigatus* on shoots, roots height and number of leaves and nodules of mycorrhizal and non-mycorrhizal broad bean plants grown 9 weeks in sterilized soil.

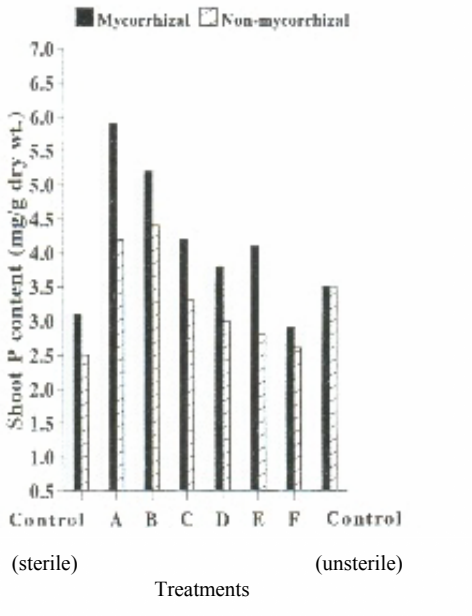
| No. | Treatments | | | | Parameters | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | Shoot height (cm) | Root height (cm) | No. of leaves/ plant | No. of nodules/ plant |
| | | | Mycorrhizal | | | | | |
| | OC | RP | *A. niger* | *A. fumigatus* | | | | |
| A | + | + | + | - | 57 a* | 20 a* | 19 a* | 84 a* |
| B | + | + | - | + | 51 b | 18 b | 17 b | 81 b |
| C | + | - | + | - | 51 b | 16 c | 14 d | 70 d |
| D | + | - | - | + | 45 c | 16 c | 15 c | 73 c |
| E | + | + | - | - | 43 d | 12 d | 14 d | 55 e |
| F | - | + | - | - | 42 e | 11 e | 12 e | 49 g |
| C (st.) | - | - | - | - | 41 f | 11 e | 11 f | 45 h |
| C (unst.) | - | - | - | - | 42 e | 12 d | 11.f | 50 f |
| SE | | | | | 4.17 | 1.30 | 2.60 | 4.17 |
| | | | Non-Mycorrhizal | | | | | |
| A | + | + | + | - | 42 i* | 22 i* | 16 i* | 53 i* |
| B | + | + | - | + | 39 j | 20 j | 14 j | 50 j |
| C | + | - | + | - | 35 k | 18 k | 13 k | 48 k |
| D | + | - | - | + | 32 i | 17 i | 13 k | 44 i |
| E | + | + | - | - | 30 m | 17 i | 12 i | 38 m |
| F | - | + | - | - | 29 n | 16 m | 11 m | 38 m |
| C (st.) | - | - | - | - | 26 p | 14 o | 11 m | 35 o |
| C (unst.) | - | - | - | - | 27 o | 15 n | 10 n | 37 n |
| SE | | | | | 0.0013 | 0.0013 | 0.0013 | 0.0013 |

OC = Untreated olive cake; RP = Rock Phosphate; += Presence; - = Absence; Control (sterile, unsterile) = Soil without amendments. * Values in each column followed by the same letter are not significantly different ($P \leq 0.05$) for each treatment (Anova test). Values are the means of eight plants. df= 14; SE= Standard Error.

Table 4: Effect of rock phosphate and *A. niger* or *A. fumigatus* on mycorrhizal infection percentage of mycorrhizal and non-mycorrhizal broad bean plants grown in sterilized soil.

| No. | Treatments | | | | Mycorrhizal infections (%) | |
|---|---|---|---|---|---|---|
| | | | | | Mycorrhizal | Non-mycorrhizal |
| | OC | RP | *A. niger* | *A. fumigatus* | | |
| A | + | + | + | - | 82 | -- |
| B | + | + | - | + | 80 | -- |
| C | + | - | + | - | 75 | -- |
| D | + | - | - | + | 70 | -- |
| E | + | + | - | - | 90 | -- |
| F | - | + | - | - | 100 | -- |
| C(st.) | - | - | - | - | 75 | -- |
| C(unst.) | - | - | - | - | 100 | 25 |

OC = Untreated olive cake; RP = Rock Phosphate; += Presence; - = Absence; Control (sterile, unsterile) = Soil without amendments. Values are the means of 50 root samples.

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## الملخص العربى

## ذوبان الفوسفات الصخرى بواسطة أنواع من *الأسبرجلس* نميت على مخلفات قشرة ثمار الزيتون – وتطبيقه فى تحسين نمو النبات

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قسم النبات – كلية البنات – جامعة عين شمس – القاهرة – مصر

يهدف هذا البحث إلى دراسة قدرة أنواع فطرية منتجة للأحماض العضوية وهما *أسبرجلس نيجر، أسبرجلس فيوماجاتس* على تحليل الفوسفات الصخرى، تم تنميتهما على بيئة أساسية من مخلفات قشرة ثمار الزيتون. أوضحت النتائج أن المزيج النهائى للتخمر حامضى ويحتوى على مادة عضوية ترسبت معدنياً وفوسفات صخرى ذائب وخيوط فطرية. لوحظ إزدياد الذوبان أثناء عملية التخمر حتى وصل إلى أقصى قيمة وهى 400، 330 ميكروجرام لكل مللى عند الأس الهيدروجينى 3,6، 4 وعند حموضة (معايره بالتحليل الحجمى) 25، 20 مللىمول لكل لتر بعد فترة تحضين 9، 12 يوماً بواسطة *أسبرجلس نيجر وأسبرجلس فيوماجاتس* على التوالى. وضح أيضا أن التركيبات المختلفة من قشرة الزيتون والفوسفات الصخرى التى تم معاملتهما أو لم تعامل بواسطة الفطريات قد إدخلت فى تربه فقيرة بالفوسفور (016, مليجرام لكل جرام تربه) لتحسين نمو نبات الفول البلدى.

عند المقارنه بالمعاملات الأخرى تبين وجود تأثير تعاونى لكل من الفطريات الخيطية والشجيرية تسبب عنه تحسن كبير فى النمو ودخول الفسفور للنبات. وجد أن أعلى معدل للنمو وأعلى تركيز للفوسفور للنباتات المصابة وغير المصابة بالفطريات الميكوروزية تم الحصول عليهما عندما إستعمل فى التربة مخلفات قشرة الزيتون المعالج بالميكروبات والفوسفات الصخرى وذلك بالمقارنة بكل المعاملات السابقة الأخرى. وجد أيضاً أن الحقن بفطر *ميكوريزا* أدى إلى زياده معنوية فى نمو النبات مقارنه بالنباتات غير المعاملة بالـ *ميكوريزا*.