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Rock Phosphate Solubilization using Oxalic Acid - secreting *Laccaria fraterna*

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Abstract

Laccaria fraterna is an ectomycorrhizal fungus commonly associated with conifers and angiosperms. To check whether *L. fraterna* possess mechanism to solubilize rock phosphate by secreting organic acids, it was grown in Pikovskaya's medium with rock phosphate as the main 'P' source in the presence of ammonium sulphate. The fungus grew profusely in the medium by secreting oxalic acid which caused a reduction in the pH of the medium. To understand the mechanism behind the rock phosphate solubilization, an attempt was made to clone the fungal genomic DNA. In the Pikovskaya's medium amended with rock phosphate, the clone MAP 22 released 80 µg/mL of P at the end of 24 h while, *L. fraterna* released 65 µg/mL of P at the end of 30 days incubation. We used HPTLC to confirm that oxalic acid is the main mineral-transforming agent that was secreted. The wild and the sub-clone MAP 22 released 1,490 and 2,950 µg/mL of oxalic acid, respectively. Therefore, it is possible to use the clone MAP 22 for more efficient inoculum production *in vivo*.

Keywords: Ectomycorrhizal Fungus, *Laccaria fraterna*, Phosphate Solubilization, Rock Phosphate.

1. Introduction

Next to nitrogen, Phosphorus (P) is the second most essential macronutrient, and is required for plant growth, photosynthesis and development[1]. However, in many types of soil, approximately 95–99%, throughout the world larger parts of P is rapidly converted into less available forms and are not directly available to plants[2]. To overcome P deficiency, synthetic fertilizers are applied. However, a large proportion of synthetic fertilizer P is quickly transformed into an insoluble form[3]. Thus, a very small percentage of the applied P is available to plants making the continuous application of fertilizer necessary[4]. P deficiency is wide spread throughout the world which makes P fertilizers a major expenditure in agricultural production because of the cost of application and its rapid depletion[5]. Recently Rock Phosphate (RP) the natural phosphate bearing material was found to be less costly thus forms an alternative for the more expensive commercial phosphate fertilizer. However, the naturally available RP is not available because of its poor solubility and lesser P content. It is known that phosphate solubilizing microorganisms convert these insoluble phosphates into soluble forms through a series of process such as acidification, chelation and exchange reactions[6], thus have become an integral component of the soil 'P' cycle.

To overcome the inorganic orthophosphate (Pi) limitation and increase P acquisition, plants form a symbiotic relationship with a group of specialized soil fungi to form mycorrhizal roots[7]. If this mycorrhizal association exists between herbaceous plants and Arbuscular Mycorrhizal (AM) fungi it is termed as AM symbiosis, and those with woody gymnosperms and angiosperms are termed Ectomycorrhizal (ECM) associations[8]. Ectomycorrhizal fungal hyphae can take up, store and translocate P within the host plant. These fungi increase the availability of P

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as a result of flexible mycelial growth, and by their ability to produce and exude organic acids, protons and other metabolites[9].

Mycorrhizal fungi release P from RP[10] or apatite[11] by reducing the pH through organic acid secretion[12]. These organic acids released by microorganisms and plants have the ability to dissolve RP or insoluble hydroxyapatite via acidification such as citric, oxalic, malic, lactic, succinic and gluconic acids. Among 30 species of ECM tested so far, oxalic acid is the organic acid most abundantly released by ECM[14].

The ectomycorrhizal fungus *Laccaria fraterna* is found in all the warm regions and is probably widespread throughout the world, where *Eucalyptus* and other ectomycorrhizal associated plants are grown. *L. fraterna* is abundant in natural forests where the litter layer is sparse. Despite impressive data on the phosphate solubilization by other ectomycorrhizal fungi[14], little or nothing is known about *L. fraterna* and therefore, an attempt has been made in the present study to examine (1) the RP solubilization by *L. fraterna,* (2) mechanism underlying RP solubilization, (3) acids secreted during RP solubilization.

2. Materials and Methods

2.1 Strains, Plasmids, and Culture Conditions

The ectomycorrhizal fungus, *L. fraterna,* was isolated from the basidiomata associated with *Eucalyptus globulus* plantations in Nilgiri hills, Tamilnadu, South India obtained from the culture collection of the Centre for Advanced Studies in Botany, Chennai, India. Fungal cultures were maintained on Modified Melin-Norkrans (MMN) medium[16] at 30 ± 2°C in the dark. Mycelial discs of 0.9 mm were grown on Pikovskaya's (PKS) medium[17] with RP as sole P source. Growth of the fungus was measured after 30 days at 30 ± 2°C in the dark. Selection of clones was based on the solubilization of RP in PKS agar containing ampicillin (100 µg/mL) plates. The vector *pANUMV2* (Gift from CCMB, Hyderabad) and *DH 5α* were cultured in Luria Broth (LB) broth at 37°C.

2.2 Construction of Genomic Library and Subcloning

Total genomic DNA was isolated according to Zolan and Pukkila[18]. Restriction enzymes, calf intestinal phosphatase, T4 DNA ligase, and RNase A (Genei, Bangalore, India) were used according to Sambrook et al.[19]. In order to obtain fragments of size 5–10 kb, the genomic DNA was partially digested with *Hind III*. Fragments sizes ranging from 5–10 kb were isolated from low melting agarose. Cosmid DNA from the vector *pANUMV2* was isolated using QIAprep spin miniprep kit (Qiagen Inc, CA, USA) and linearized with *Hind III*. This fragment was ligated to the dephosphorylated *Hind III* site of the cosmid vector *pANUMV2*. The ligation in vitro packaged using Sternberg et al.[20] and gives an pfu of 2.2×10^8/µg of cosmid DNA. This packaged cosmid was diluted and added to 10 µL of *DH 5α* for infection. Transformants were selected and replica plated onto PKS medium amended with RP as sole P source. Plasmids were isolated using minipreps and restriction analysis was conducted to confirm the insert[19]. Of the 726 colonies plated on the selective medium, only 2 colonies that were positive for mineral phosphate solubilizing (mps+) and these were designated as MLA 46 and MLA 50. Since MLA 50 grew faster than MLA 46, further studies were conducted using MLA 50 (data not shown).

To reduce the size of the insert which involved in RP utilization, the clone MLA 50 and vector DNA were digested with *Bam HI*. Out of 228 colonies, only one sub-clone grew on PKS medium with RP as sole P source and was designated as MAP 22. The plasmid DNA from the sub-clone MAP 22 was isolated and digested with *Bam HI* to release the insert. Using *λ Hind III DNA* digest marker the size of the released insert was estimated to be 3 kb. This 3 kb insert was sequenced and the mineral phosphate solubilizing gene was given an accession number of *AF333771*. Overnight grown cultures (10 µL) of the vector *pANUMV2* and the sub-clone MAP 22 that grew on PKS medium with RP as sole P source were used to confirm the RP solubilization by the sub-clone but not by the vector. Phosphate solubilization was accompanied by a decrease in pH of the medium in both the wild type and the clone.

2.3 Identification of Organic Acids Released

The sub clone MAP 22 was grown on PKS medium supplemented with RP as sole P source and its growth was monitored at 24 h interval for 3 days. The release of phosphorous was measured at 24 h intervals for 3 days using Murphy and Riley's procedure[21].

To determine the release of oxalic acid upon utilization of RP by both wild and clone, a quantitative and qualitative analyses was performed using High

Performance Thin Layer Chromatography (HPTLC). Samples were prepared from cultures of the wild fungus as well as sub-clone MAP 22 grown in PKS broth with RP, and 100 mL culture filtrate from the fungus was withdrawn at intervals of 3 days for 30 days and routinely extracted with chloroform. The same amount of culture filtrate from the sub-clone was extracted with chloroform at intervals of 24 h for 3 days. The chloroform fractions were pooled and evaporated and the residue was dissolved in 1 mL of 80% ethanol and TLC was performed on fluorescent dye coated silica TLC plates[22]. The residue was used for analysis in a Linomat IV automated TLC sampler and the chromatogram was developed in a solvent system of ethanol: water: and ammonium hydroxide (78: 9.5: 12.5 v/v)[23]. It was scanned in a CAMAG TLC densitometric scanner at 366 nm. Standard oxalic acid was co-chromatographed and scanned. The Rf values of the samples and the standard were compared. Based on samples and standard peak heights, the amount of oxalic acid in each spot was estimated. Densitometric measurements were made by absorbance of fluorescence in a spectral range of 190–800 nm.

2.4 Statistical Analysis

Statistical interpretation of the various measurements made was carried out using Analysis Of Variance (ANOVA) and $P < 0.05$ was used.

3. Results and Discussion

To confirm the phospholytic activity of *L. fraterna*, the fungus was cultured on PKS medium amended with glucose as carbon source, ammonium sulphate as nitrogen source and RP as sole P source. The results revealed that the fungus grew profusely on PKS medium confirming the utilization of rock phosphate by this fungus. Recent results with other fungi have confirmed the efficacy of P solubilization of PKS medium as most cost effective without compromising the solubilization[24]. In *Aspergillus aculeatus* and *Aspergillus sp,* glucose supported maximum phosphate solubilizing activity[24, 25]. Other nitrogen sources such as ammonium nitrogen or nitrate nitrogen significantly influenced phosphate solubilization by ectomycorrhizal fungi[14]. It has also been reported that in the presence of ammonium nitrogen, calcium phytate and calcium phosphate were easily solubilized, due to acidification[26]. In the present study it was also observed that ammonium ions increased RP solubilization by decreasing the pH from 7.0 to 3.8, thus confirming that solubilization was indeed due to acidification. Previous results[24, 25], also shown that ammonium sulphate is the best nitrogen source for reduction of pH and phosphate solubilization from RP.

Research advances have shown that the mineral phosphate solubilizing (mps+) phenotypes formed a worthwhile basis for a renewable phosphate fertilizer technology for crop growth[27]. Martin et al.[28] suggested that crop productivity can be increased due to the use of superior strains derived by breeding or engineering a fungus with desirable characteristic(s) as fungi have been reported to possess greater ability to solubilize insoluble phosphate than bacteria[13]. Despite intensive research on ectomycorrhizal colonization, there is very limited research on mps+ of ectomycorrhizal fungi. In the present study we have aimed to obtain the mps+ of *L. fraterna* as follows: The genomic library of *L. fraterna* was constructed in *E. coli DH 5α* using the vector *pANUMV2*. Of the two clones MLA 46 and MLA 50, MLA 50 was chosen for further studies because of its faster growth and utilization of RP. The recombinant plasmid that released 5 kb insert was not associated with mineral phosphate solubilization. Therefore, by sub-cloning a 3 kb sub-clone designated as MAP 22 which grew efficiently on PKS medium amended with RP as sole P source was identified.

L. fraterna solubilized RP and released 65 µg/mL of P at the end of 30 days (Table 1). The sub-clone MAP 22 released 80 µg/mL of P at the end of 24 h, while releasing 70 µg/mL and 52 µg/mL of P at the end of 48 and 72 h, respectively (Table 2). In comparison, an *Aspergillus sp*

Table 1. Phosphorus released from the rock phosphate by *L. fraterna* at different day intervals

Incubation time (days)	Release of phosphorus (µg/mL)
3	22
6	28
9	32
12	37
15	40
18	44
21	50
24	53
27	59
30	65

isolated from rice field soils of Orissa released 58 µg/mL of P after 7 days of incubation in PKS medium with RP as sole P source[24]. In this case, the release of P was lower or less efficient when compared to tricalcium phosphate due to the complexity of RP.

Mycorrhizal fungi are known to release P from RP by reducing the pH through organic acids secreted by them[12]. Oxalic acid was mostly secreted by ECM in mineral weathering[14]. Oxalic acid is both an acid and a complexing agent of calcium, iron and aluminium and this indicates that it could be an active solubilizing agent by complexing the cations as well as by acidification[29]. It has been reported that *L. laccata* secreted oxalic acid when grown on mineral phosphates[14]. Also when ECM fungi such as *Amanita muscaria*, *H. crustuliniforme*, *Piloderma byssinum*, *P. fallax*, *Paxillus involutus, Mycena galopus* and *Suillus bovines* grew on MMN medium with Tri-Calcium Phosphate (TCP), apatite, potassium feldspar, pink calcite and quartz as their P source, *P. fallax* and *M. galopus* showed dense growth on TCP. The researchers reported that *M. galopus* secreted nearly 7.5 µM of oxalic acid, while *P. involutus* secreted nearly 6 µM of oxalic acid followed by *C. glaucopus* (5.2 µM), *S. bovinus* (5 µM) of oxalic acid, respectively[30]. To confirm the secretion of organic acids by *L. fraterna* and by the sub-clone MAP 22, HPTLC analysis was performed in the present study.

Table 2. Phosphorus released from the rock phosphate by sub-clone MAP 22 at different time intervals

Incubation time (h)	Release of phosphorus (µg/mL)
24	80
48	70
72	32

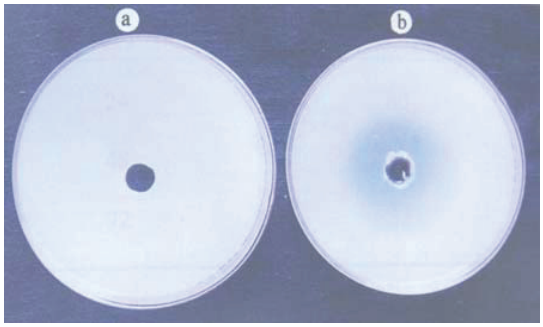

Figure 1. Growth of vector and MAP 22 on Pikovskaya's medium.(a) Vector *pANUMV2* (b) Sub-clone MAP 22.

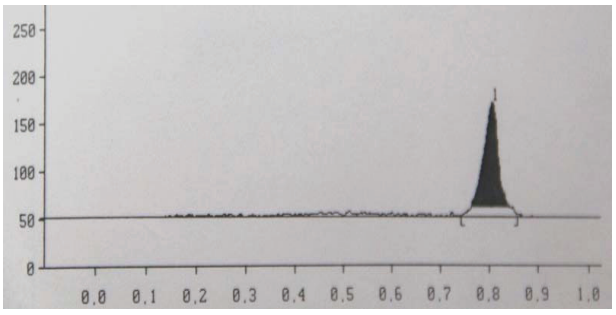

Figure 2. HPTLC analysis of standard oxalic acid.

Densitometric scanning using the CATS software provided quantitative estimation of the intermediate. Using HPTLC, oxalic acid secretion was identified in both wild *L. fraterna* as well as the sub-clone MAP 22.

When 10 µL overnight grown cultures of the sub-clone and vector grew on PKS medium with RP, the sub-clone MAP 22 produced a clearing zone confirming the utilization of RP (Figure 1). The intermediate identified from the fungus and the sub-clone at intervals of 3 days for 30 days and 24 h for 3 days was oxalic acid. The wild and the sub-clone MAP 22 released 1,490 and 2,950 µg/mL of oxalic acid when compared with authentic oxalic acid (Figures 2–4) and reduced the pH of the medium

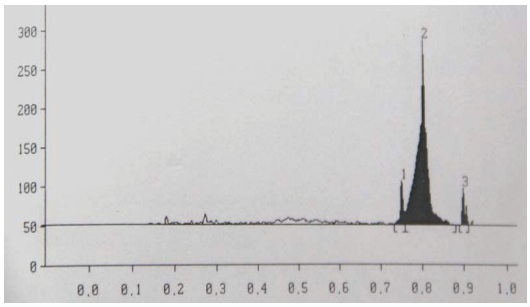

Figure 3. HPTLC analysis of extracts from wild *L. fraterna.*

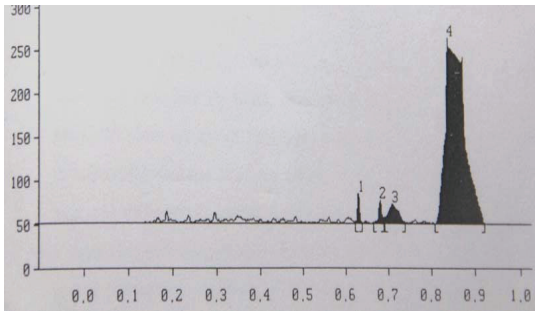

Figure 4. HPTLC analysis of extracts from sub-clone MAP22.

(data not shown). However, no significant relationship could be established between the quantity of phosphate solubilization and drop in pH. These findings are in line with the previous reports[25].

Oxalic acid is toxic, and is known to function as a nonspecific phytotoxin[31] which has played an important role in pathogenesis, fungal development and in the degradation of cell walls of pathogens[32]. The secretion of oxalic acid by the wild fungus *L. fraterna* and the clone most possibly helps its host plants to protect against pathogens besides supplying the Pi to the plants. This is the first report of RP solubilization by *L. fraterna* and it has been shown that this fungus may serve as an excellent rock phosphate solubilizer when inoculated into soils where RP is used as P fertilizer.

4. Conclusion

In the present study, a genetic clone MAP 22 of *L. fraterna* is identified as one of the most efficient strains for solubilization of RP. Thus its application can lead to combating the P deficiency in soils and also reducing the cost of application of P fertilizers. The secretion of oxalic acid by both wild and clone is believed to protect against the pathogens. Further research needs to be carried out using this efficient strain in inoculum production on soil or plant application to monitor its effect on plant growth *in vivo*.

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6. Conflict of Interest

The authors declare no conflict of interest.

7. References

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