Effects of Root Exudates of Two Mangrove Species on *in vitro* Spore Germination and Hyphal Growth of *Glomus mosseae*

T. Kumar, M. Ghose and R.L. Brahmachary
Agricultural and Ecological Research Unit, Indian Statistical Institute,
203 B.T. Road, Kolkata-700 108, India

Abstract: The present study deals with the effects of root exudates of two mangrove species viz. *Bruguiera gymnorrhiza* (L.) and *Excoecaria agallocha* L. on *in vitro* spore germination and hyphal growth of *Glomus mosseae* (Nicol. & Gerd.) Gerd. & Trappe. Root exudates of both the mangrove species showed significant inhibitory action on hyphal growth, but the inhibitory effect of *E. agallocha* root exudate was more than that of *B. gymnorrhiza* root exudate. Root exudates of these two mangrove species exerted no significant effect, stimulatory or inhibitory, on spore germination. Putative active compounds were detected in the root exudates of the plants. The two mangrove species are unlikely to be the hosts for colonization by *G. mosseae*.

Key words: *Bruguiera gymnorrhiza*, *Excoecaria agallocha*, root exudates, putative active compounds, *Glomus mosseae*, hyphal growth, inhibition

INTRODUCTION

Mangroves constitute a thrust area and the Sundarban Biosphere Reserve is a hot-spot. We have been interested in the morphological, ecological and ecophysiological aspects of mangroves in the Sundarbans.

At present we are studying the mycorrhizal status of the mangroves and the Arbuscular Mycorrhizal Fungi (AMF) present in the soils of the Sundarbans. One aspect of this is to study the allelopathic effect, if any, of Root Exudates (RE) on AMF. Allelopathy has been described by Molisch (1937) as biochemical interactions (both stimulatory and inhibitory) between all types of plants including microorganisms. RE have been studied in many plants (Mukerji *et al*., 2002; Rice, 1984), but mangroves have not been touched. There are several works regarding the effects of RE of mesophytic plants on the growth of specific AMF (Elias and Safir, 1987; Hirosuke *et al*., 2002; Gianinazzi-Pearson *et al*., 1989; Nagahashi and David, 2000; Ratanayake *et al*., 1978; Scervino *et al*., 2005; Tawaraya *et al*., 1996; Tsai and Phillips, 1991), but such work appears lacking in the case of mangroves. Thus, on the one hand the effect of mangrove RE on AMF growth is worth studying and on the other, the chemistry of mangrove RE is of interest *per se*.

Here we report such findings on two mycorrhizal mangrove species, namely, *Bruguiera gymnorrhiza* (L.) and *Excoecaria agallocha* L. and their effects on *Glomus mosseae* (Nicol. & Gerd.) Gerd. & Trappe spore germination and hyphal growth. Since *G. mosseae* occurs as one of the most dominant species of AMF in the soils of Sundarbans (Sengupta and Chowdhuri, 2002; Kumar and Ghose, 2006).

Corresponding Author: T. Kumar, Agricultural and Ecological Research Unit, Indian Statistical Institute, 203 B.T. Road, Kolkata-700 108, India Tel: +(91) (033) 2575 3225 Fax: +(91) (33) 2577 3049

MATERIALS AND METHODS

Collection of Root Exudates (RE)

Six seedlings each of *B. gymnorrhiza* and *E. agallocha* of approximately a height (shoot system) of 45 cm were uprooted together with soil adhering to the root systems from Sajnekhali island of the Sundarbans Biosphere Reserve, India. After removing the soil the root systems of the seedlings were washed thoroughly with distilled water. Six seedlings were kept in a conical flask containing 1000 mL of distilled water for 48 h. The solutions (root exudates) were evaporated to 20 mL. The resulting solutions were stored at 4°C until use.

Fungal Growth in RE

Spores of *G. mosseae* were collected by wet sieving (Gerdemann and Nicolson, 1963), followed by sucrose centrifugation method of Daniels and Skipper (1982) from a starter inoculum (Tata Energy Research Unit, New Delhi, India). Spores were washed in distilled water for 5 min, surface sterilized with a solution containing 20 mg L^{-1} chloramine T, 200 mg L^{-1} streptomycin and 3 mL L^{-1} Tween 80 for 15 min (Mosse, 1962) and then rinsed with distilled water. Root exudates were passed through membrane filter (0.22 µm, AAWGO4700, Millipore) and 2 mL aliquots of the filtrates were added to pads on the bottom of 50 mm Petri dishes. Distilled water was used as control. Mixed cellulose ester gridded membrane filter (0.8 µm, GSWPO4700, Millipore) was placed on the pad and surface-sterilized spores were transferred onto the membrane filter, 5-10 spores per Petri dish. The spores were incubated in the dark at 27°C. Germination was assessed by scoring the emergence of a germ tube at 7, 14, 21, 28, 35, 42, 49 and 56 days after incubation. Hyphal lengths were determined by the grid-line intersect method (Giovannetti and Mosse, 1980).

The three treatments have been depicted as control, *B. gymnorrhiza* RE (B RE) and *E. agallocha* RE (E RE).

Statistical Analysis

The data on germination percentages was subjected to the test of proportions to check whether the percentages were significantly different or not between the treatments at each day of observation. The data on hyphal lengths was subjected to Analysis of Variance (ANOVA). When a significant ($p<0.05$) effect was found, the mean values were compared using the Tukey test.

Preliminary Detection of the Active Compounds Present in the RE

Detection of the putative compounds present in the RE were attempted with the help of paper chromatography. Initially the presence/absence of the compound(s) was detected by spot tests. Phenolic compounds were stained using silver nitrate reagent (1 mL saturated aqueous silver nitrate, with stirring, to 20 mL acetone; the solution was then treated dropwise with water just until the precipitated silver nitrate has just dissolved) (Stahl, 1969). For staining fatty acids, a solution of 0.03% methyl red in 0.05 (N) borate buffer (pH 8) was used; for staining amino acids, 0.2% ninhydrin solution in butanol was used. After spot tests Whatman paper No. 3 was used to develop chromatograms with n-butanol:acetic acid:water (4:1:1) solvent system (for phenolic compounds).

RESULTS

Fungal Growth

Germination

The germination of spores was less than 25% till 56 days of incubation and the percentages remained constant from 28 to 56 days in all the three cases (Fig. 1). Upto 21 days of incubation the

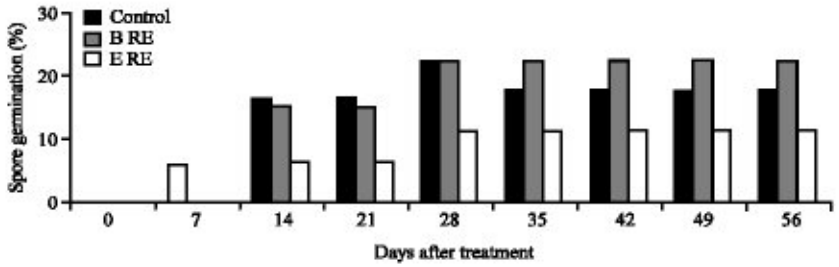

Fig. 1: Effect of root exudates on spore germination of *Glomus mosseae*. For each day of observation (days after treatment), the percentages of germination are not significantly different according to the test of proportions. B RE and E RE refer to the root exudates of *Bruguiera gymnorrhiza* and *Excoecaria agallocha*, respectively

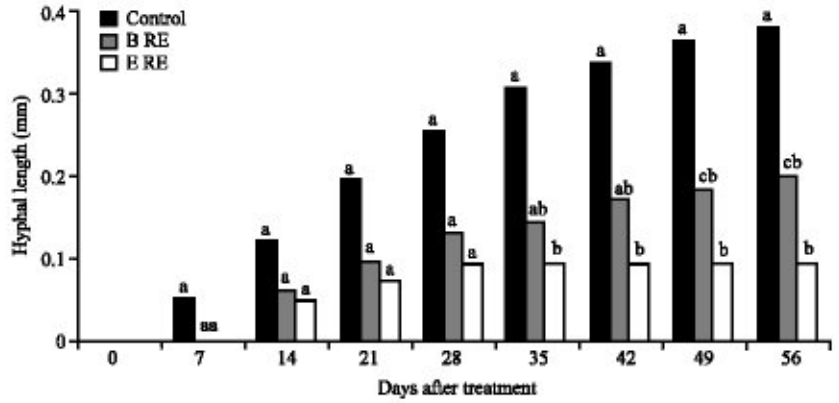

Fig. 2: Effect of root exudates on hyphal growth of *Glomus mosseae*. For each day of observation (days after treatment), the means followed by the same letter are not significantly different (p = 0.05) according to the Tukey test. B RE and E RE refer to the root exudates of *Bruguiera gymnorrhiza* and *Excoecaria agallocha*, respectively

germination rate was of the order Control>B RE>E RE. From 28 to 56 days of incubation the germination rate was in the order B RE>Control>E RE. However, according to the test of proportions the germination percentages were not significantly different. In all the three cases spore germination percentages became constant from 28 to 56 days of incubation.

Hyphal Length

Rate of increase in hyphal length was of the order Control>B RE>E RE (Fig. 2). There were significant differences in hyphal length between the cases at 35, 42, 49 and 56 days, but no significant differences at 7, 14, 21 and 28 days. In case of E RE hyphal length became constant from 28 to 56 days of incubation.

Preliminary Detection of Putative Active Compounds

Fatty and amino acids could not be detected by spot tests in the RE of the two plants. At least two compounds, presumably phenolics could be noticed (Fig. 3) in the RE of *B. gymnorrhiza* and a single one in the RE of *E. agallocha*. Compounds A and C seemed to be the same. The Rf values

Fig. 3: Paper chromatographic run of root exudates of *Bruguiera gymnorrhiza* (B RE) and *Excoecaria agallocha* (E RE). The chromatogram shows two bands (A and B) in case of B RE and a single band (C) in case of E RE. Solvent system: n-butanol: acetic acid: water:4:1:1

of all the three compounds were much lower than those of six standard phenolic compounds namely, 3,4-dihydroxycinnamic acid, vanillic acid, syringic acid, 4-hydroxycinnamic acid, 3-4 dihydroxybenzoic acid and 4-hydroxybenzoic acid, run in the same solvent system and under the same conditions.

DISCUSSION

It is evident from Fig. 1 that the germination percentages of spores in control from 28 to 56 days after incubation was 17.91 and in B RE treated spores the percentage was 22.22, but the difference was insignificant according to the test of proportions. In case of E RE treated spores the percentage was 11.29 from 28 to 56 days after incubation and the difference with control was not significant. Thus the effect of root exudates of both *B. gymnorrhiza* and *E. agallocha* was neither stimulatory nor inhibitory to *G. mosseae* spore germination.

The mean hyphal length in control on 49 and 56 days was 0.37 and 0.38 mm, respectively. In B RE treated spores these were 0.19 and 0.20 mm, respectively, indicating an inhibition. Tukey test suggested that the differences between control and B RE were significant at 49 and 56 days of incubation. Thus B RE had inhibitory effect on hyphal growth. In E RE treated spores the mean hyphal lengths remained the same (0.09 mm) at 35, 42, 49 and 56 days. Tukey test suggested that the differences between control and E RE were significant at 35, 42, 49 and 56 days of incubation. So E RE also had inhibitory effect on hyphal growth. Moreover, the inhibitory effect of E RE on hyphal length of *G. mosseae* was more than that of B RE.

The inhibitory effect of RE might be due to some metabolites. As phenolics have been detected in the RE of many plants (Gianinazzi-Pearson *et al.*, 1989; Seervino *et al.*, 2005; Tsai and Phillips, 1991) and these compounds, are, in fact, the largest group of secondary plant products and growth inhibitory substances (Harborne, 1984) we may first consider these chemicals. As is evident from Fig. 3 there are at least two presumably phenolics in B RE and one in E RE (compound C seemed to

be the same as compound A), the inhibitory effect might be due to this compound. In that case, compound B in B RE perhaps was slightly antagnostic to A(=C). Synergistically, A and B lessened the inhibitory effect of A(=C). However, the effect might also be due to some other unidentified molecule(s) in the root exudates. After identification of the compounds A(=C) and B with the help of GC-MS/MS, standard substances can be tested for inhibition of *G. mosseae* hyphal growth.

The present results suggest that the two mangrove species are unlikely to be the hosts for colonization with *G. mosseae*, despite the fact that the spores of this fungus occur as one of the most predominant species of AMF in the saline soils of the Sundarbans (Sengupta and Chowdhuri, 2002; Kumar and Ghose, 2006). Study on this aspect is being planned on one or two other species of mangroves.

Addendum

Thin layer chromatography was performed with the root exudate of *Bruguiera gymnorrhiza* using the same solvent system [butanol:acetic acid:water (4:1:1)]. A single band was detected after staining with silver nitrate reagent. A similar band (unstained) was eluted in acetone and GC-MS/MS was attempted with it using VARIAN-CP3800 model with injection port temperature of 250°C, DB 5 MS column, MS-70-450 mv, scan time of 0.6 sec and ionization mode-EI+VE. Fifteen peaks were revealed of which two were very small. Most of the compounds did not match with any in the library of 65,000 compounds. However, the only compound indicated with probability of 38% was 9, 12, 15-octadecatrienoic acid, 2, 3-bis [(trimethylsilyl)oxy]propyl ester (Z, Z, Z). The peak at m/z 149 strongly supported the presence of $CH_3CH_2CH = CHCH_2CH = CH\text{-}CH_2^+$ as a fragment of a more extended chain and a small peak at m/z 496 suggested the structure with two carbon-bound silicon atoms.

As the mangroves remain rooted in an anaerobic environment, silicon atoms can likely be incorporated as $SiMe_3$. Si cannot be incorporated in the plant in H-or O-bound form, so Si bound to carbon atoms is probably the only possibility. This might be the first report of a complex Si-containing organic compound in the root exudate of a mangrove species.

The other compounds showing peaks including the presumably phenolic compounds may in future be identified through Alfred Bader laboratory or such other facilities.

ACKNOWLEDGMENT

We thank Mr. K. Sengupta of SGS India Private Limited and Dr. S. Ray of Department of Pure Chemistry, University of Calcutta, India for their helpful discussions on interpretation of GC-MS/MS data. We also thank the DFO, South 24 Parganas, West Bengal for permitting us to collect the samples from the Sundarbans for this study.

REFERENCES

Daniels, B.A. and H.D. Skipper, 1982. Methods for the Recovery and Quantitative Estimation of Propagules from Soil. In: Methods and Principles of Mycorrhizal Research. Schenck, N.C. (Ed.), Am. Phytopathol. Soci. St. Paul., pp: 20-45.

Elias, K.S. and G.R. Safir, 1987. Hyphal elongation of *Glomus fasciculatus* in response to root exudates. Applied Eviron. Microbiol., 53: 1928-1933.

Gerdemann, J.W. and T.H. Nicolson, 1963. Spores of mycorrhizal fungi isolated from soil by wet sieving and decanting. Trans. Br. Mycol. Soc., 46: 235-244.

Gianinazzi-Pearson, V., B. Branzanti and S. Gianinazzi, 1989. *In vitro* enhancement of spore germination and early hyphal growth of a vesicular-arbuscular mycorrhizal fungus by host root exudates and plant flavonoids. Symbiosis, 7: 243-255.

Giovannetti, M. and B. Mosse, 1980. An evaluation of techniques for measuring vesicular-arbuscular mycorrhizal infection in roots. New Phytol., 84: 489-500.

Harborne, J.B., 1984. Phytochemical Methods. 2nd Edn., London New York Press.

Hirosuke, O., K. Tawaraya and T. Wagatsuma, 2002. Inhibition of pre-symbiotic hyphal growth of arbuscular mycorrhizal fungus *Gigaspora margarita* by root exudates of *Lupinus* spp. Soil Sci. Plant Nutr., 48: 117-120.

Kumar, T. and M. Ghose, 2006. Mycorrhizal status (VAM) of some mangroves growing in saline and non-saline soils. J. Mycopathol. Res. (In Press).

Mosse, B., 1962. The establishment of vesicular-arbuscular mycorrhiza under aseptic conditions. J. Gen. Microbiol., 27: 509-520.

Molisch, H., 1937. Der Einfless einor Pflanze auf die and re-Allelopathie. Fischer, Jena.

Mukerji, K.G., C. Manoharachary and B.P. Chamola, 2001. Techniques in Mycorrhizal Studies. Kluwer Academic Publishers, Dordrecht, Boston, London.

Nagahashi, G. and D.D. Jr. David, 2000. Partial separation of root exudates components and their effects upon the growth of germinated spores of AM fungi. Mycol. Res., 104: 1453-1464.

Ratanayake, M., R.T. Leonard and J.A. Menge, 1978. Root exudation in relation to supply of phosphorus and its possible relevance to mycorrhiza formation. New Phytol., 81: 543-552.

Rice, E.L., 1984. Allelopathy. Academic Press Inc., 2nd Edn., Orlando, San Diego, San Francisco.

Scervino, J.M., M.A. Ponce, R. Erra-Bassells, H. Vierheilig, J.A. Ocampo and A. Godeas, 2005. Flavonoids exclusively present in mycorrhizal roots of white clover exhibit a different effect on arbuscular mycorrhizal fungi than flavonoids exclusively present in non-mycorrhizal roots of white clover. J. Plant Interac., 1: 15-22.

Sengupta, A. and S. Chowdhuri, 2002. Arbuscular mycorrhizal relations of mangrove plant community at the Ganges river estuary in India. Mycorrhiza, 12: 169-174.

Stahl, E., 1969. Thin Layer Chromatography. 2nd Edn., Academic Press, London.

Tawaraya, K., S. Watanabe, E. Yoshida and T. Wagatsuma, 1996. Effects of onion (*Allium cepa*) root exudates on the hyphal growth of *Gigaspora margarita*. Mycorrhiza, 6: 57-59.

Tsai, S.M. and D.A. Phillips, 1991. Flavonoids released naturally from alfalfa promote development of symbiotic *Glomus* spores *in vitro*. Applied Environ. Microbiol., 57: 1485-1488.