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Research Article

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Isolation and identification of spore associated bacteria (SAB) from *Glomus* and *Gigaspora* spp. in coconut and arecanut based cropping systems

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Abstract

Arbuscular mycorrhizal symbiosis is exhibited by 80% of the terrestrial plants. The spores of arbuscular mycorrhizal fungi (AMF) form a unique microhabitat for the colonization by many species of bacteria. The present study was undertaken to isolate and identify spore associated bacteria (SAB) and evaluate their functional role in AMF-host interactions with respect to germination of spores. Coconut and arecanut based cropping systems under organic management practices in farmer's field in Kasaragod district, Kerala and high density multi species cropping system (HDMSCS), CPCRI farm were selected for the study. The results revealed that AMF spore load of a particular cropping system increase with the number of intercrops. Spore associated bacteria (SAB) were isolated from the cytoplasm of surface sterilized spores of *Glomus* and *Gigaspora* spp. Identification based on BIOLOG and 16S rRNA sequencing revealed the presence of bacteria - *Citrobacter amalonaticus, Staphylococcus arlettae*, *Bacillus subtilis, Bacillus amyloliquefaciens,* in association with spores of *Glomus* spp. *Corynebacterium coyleae, Bacillus cereus* and *Bacillus subtilis* were found to be associated with *Gigaspora* spp. *In vitro* studies to determine the germination potential in spores showed the maximum results with *Bacillus cereus* GiPHD1 and *Citrobacter amalonaticus* GLNCB1 with 40% increase over control.

Keywords: Arbuscular mycorrhizae, arecanut, coconut, cropping systems, *Gigaspora* spp., *Glomus* spp., SAB

Introduction

Arbuscular mycorrhizal fungi (AMF) are soil microorganisms that establish mutual symbiosis with the majority of higher plants by usually colonising the host roots by forming inter and intracellular hyphae and intracellular arbuscules. Under phosphate-limited conditions, AMF can influence plant community development, nutrient uptake and aboveground productivity. Arbuscular mycorrhizal association was observed in plantation crops mainly coconut (Thomas *et al*., 1993) and arecanut (Bopaiah, 1991).

The ecological importance of bacteria associated with AMF with regard to their interaction with AMF hosts is still far from known. There are some reports on the bacteria associated with AMF spores indicating that they have the ability to influence spore germination and hyphal growth (Azcon, 1987). Bacteria associated with AMF spores are found mainly in the outer wall layer (Bonfante-Fasolo and Schubert, 1987). They could stimulate spore germination by eroding spore walls or by influencing AMF phosphorus acquisition (Maia and Kimbrough, 1998). Interest in research on spore-associated bacteria has increased because these have shown the potential to support AMF to complete spore production *in vitro* in the absence of a host (Azcon, 1987). The overall aim of this study was to determine the role played by bacteria associated with AMF in the interaction of AMF with its plant hosts. This study focused on exploring the composition of cultivable bacteria associated with AM fungal spores (SAB), characterise them and to investigate the interactions between AMF and spore associated bacteria *in vitro*.

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Materials and Methods

Study site: Samples were collected from coconut and arecanut based cropping systems in farmers' fields in Kasaragod and also from coconut based cropping system at high density multiple species cropping system (HDMSCS) of CPCRI, Kasaragod (12°31'32''N, 74°58'4''E) maintained under organic management practices. Coconut cropping system at HDMSCS had coconut, banana, pepper and pineapple as intercrops. Cropping system at Patena (12°15'0''N, 75°6'0''E) had only banana as the intercrop. Arecanut based cropping system includes Neerchal plot (12°35'0''N, 75°2'0''E) with banana, cardamom and rasna (*Pluchea lanceolata*) as intercrops and Mogral Puthur plot (12°33'18.70''N, 74°57'43.08''E) which had only banana as intercrop.

Rhizospheric soil samples as well as root bits were collected at a depth of 0-25 cm from the rhizosphere of each crop to study the mycorrhizal association. Three replications per crop from each cropping system were analysed.

Rhizospheric edaphic features

Soil samples were analysed for different physico chemical parameters namely; soil pH, organic carbon (Walke and Black, 1934), soil available phosphorous (Bray and Kurtz, 1945), available potassium (Hanway and Heidel, 1952) and soil nitrogen (Kjeldhal, 1883).

Mycorrhizal studies

Isolation of arbuscular mycorhizal spores was done by wet sieving decanting technique (Gerdemann and Nicholson, 1963). The identification was based on the keys provided by International Collection of Vesicular and Arbuscular Mycorrhizal Fungi (http://invam.caf.wvu.edu) and the original species descriptions. Roots were stained for mycorrhizal infection (Phillips and Hayman, 1970). Percentage of root colonization was calculated using the formula.

Frequency of root colonization = (Number of root bits having colonization ÷ Number of root bits observed) x 100

Statistical Analysis

Correlation coefficient was calculated for AMF parameters and edaphic factors by computational method using SPSS.

Isolation of spore associated bacteria (SAB)

Dominant *Glomus* and *Gigaspora* spp. from coconut and arecanut cropping system were used for the study. Spores were surface sterilized with PBS saline and washed in several changes of distilled water, followed by thirty minutes of spore decontamination with 5% Chloramine-T and 300 µg/ml of Streptomycin (Lisette *et al*., 2002). The surface sterilized spores were crushed, serially diluted and plated on nutrient agar plates and growth was checked after 24 h of incubation.

Identification of SAB

SAB was identified by differential utilization of carbon source using Gen III BIOLOG. The organisms were first streaked on the BUG Medium and incubated at 33°C for 22 h. Using the BIOLOG inoculators (a sterile incubator disposable swab) the grown cultures were transferred to the inoculation fluid (IF) and the inoculum density was adjusted to 98% T using Biolog Turbidity meter. It was then inoculated into BIOLOG GEN III Microplates using multi channel pipette and incubated at 33ºC. The optical density at 590 nm in each well was read after 24 h using a Biolog Microplate reader (version 5.1.1) in conjunction with the Microlog software. Identification was attained by comparison of the carbon source signature of each strain against the carbon source signatures of known strains in the database. A species ID was called, if the SIM and DIST values were >0.500 and < 5.00, respectively.

DNA isolation and 16S rRNA sequencing

Genomic DNA of the twenty three spore associated bacteria was isolated by GenElute Bacterial Genomic DNA Kit (Sigma, USA). Bacterial 16S rRNA genes were amplified from all DNA samples by the universal 16S rRNA gene primers F27 and R1492. The amplifications were performed using Eppendorf Master Cycler gradient. These 16S rRNA gene sequences were used to carry out BLAST with the nrdatabase of NCBI genbank database.

Response of *Glomus* spores to SAB *in vitro*

Surface sterilized *Glomus* spores from coconut and arecanut rhizosphere of HDMSCS and Neerchal plots respectively were used for *in vitro*

studies of spore germination by AMF. The response of *Glomus* spores were analysed by two methods.

Crude extract assay (Mayo *et al*., 1986): 24 h bacterial cultures in nutrient broth were centrifuged and the supernatant was pippetted onto a sterile filter paper placed on Petriplate. *Glomus* spores were inoculated (n = 5), and kept for 10 days incubation. Filter paper moistened with nutrient broth without bacterial cultures were kept as control. All treatments were replicated 3 times.

Diffusable assay (Lisette *et al*., 2002): Using a sterile cotton swab, SAB cells from a stock culture were streaked on triplicate 1.5% Tryptic Soy Agar plates which were incubated at 27°C for 24 h to obtain a uniform lawn of bacterial growth. Clean decontaminated *Glomus* spores were introduced on a sterile filter paper arranged over a 1.5% agarose block, which was placed on a sterile 90 mm diameter filter paper laid on the bacterial lawn. Plates were incubated at 27°C for 10 days and examined for spore germination and hyphal growth.

Results and discussion

Cropping systems studied had laterite soil, except in HDMSCS plot with red sandy loam. Soil pH was slightly acidic except in Mogral Puthur which had a pH level close to neutral (6.95). The cropping systems harboured a very low phosphorous concentration of 19.8 ppm (Patena) to 42.16 ppm (HDMSCS) and a low level of organic carbon content between 0.115 and 0.845%. Soil analysis revealed the available potassium content ranging from 20.47 ppm (HDMSCS) to 28.32 ppm (Mogral Puthur) and nitrogen % between 0.0952 to 0.143. The data presented in Table 1 revealed the highest spore load in coconut based mixed cropping system, HDMSCS (56.83 ±10.81spores/ 10g soil). Spore load was comparatively less in cropping system which had only one intercrop. Occurrence of *Glomus* spp. as the predominant AMF was observed in both the cropping systems. However, more species diversity was observed in mixed cropping system with *Glomus*, *Gigaspora* and *Acaulospora* spp. in HDMSCS (Fig. 1) and *Glomus* and *Acaulospora* spp. at Neerchal. In coconut cropping system, root colonization frequency was found to be the highest in Patena cropping system, 69.66±14.46. The arecanut cropping system at Neerchal recorded 15±0.34 frequency of mycorrhizal root colonization.

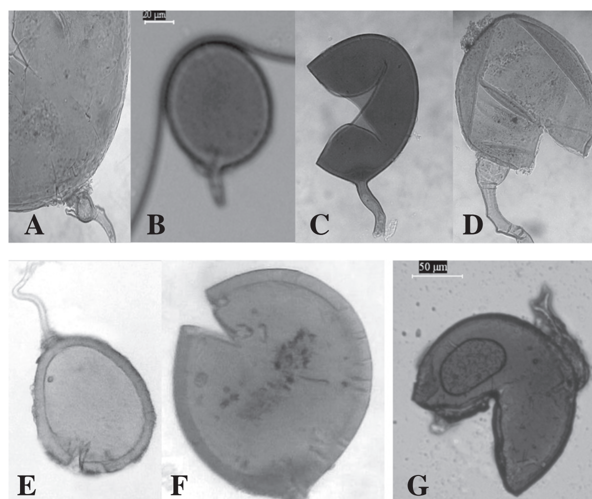

Fig. 1. **A - *Gigaspora* spp., coconut rhizosphere, HDMSCS, B - *Glomus* spp., coconut rhizosphere Patena C - *Glomus* spp., pepper rhizosphere, HDMSCS, D - *Gigaspora* spp., from banana rhizosphere,HDMSCS plot, E - *Glomus* spp., arecanut rhizosphere, Mogral Puthur plot, F - *Glomus* spp., arecanut rhizosphere, Neerchal plot, G - *Glomus* spp., banana rhizosphere, Mogral Puthur plot**

Pearsons correlation analysis of rhizospheric edaphic factors with mycorrhizal parameters is shown in Table 2. A positive correlation was observed with soil pH and AMF root colonization

Table 1. Soil edaphic factors and mycorrhizal parameters

Location	Cropping system	Soil pH	Organic carbon (%)	Nitrogen (%)	Available phosphorous (ppm)	Available potassium (ppm)	Mycorrhizal spore load $10g^{-1}$	Frequency of root colonization
*HDMSCS	Coconut	5.43±0.28	0.84±0.37	0.137±0.69	41.36±6.73	20.47±9.05	56.83±10.83	59.33±5.16
Patena	Coconut	5.58±0.12	0.52±0.15	0.112±0.06	20.47±0.67	21.6±3.67	40.14±1.15	69.46±14.46
Mogral Puthur	Arecanut	6.95±0.49	0.12±0.39	0.143±0.01	22.56±4.76	28.32±8.09	21.33±0.35	---
Neerchal	Arecanut	5.29±0.17	0.76±0.03	0.104±0.06	36.34±8.76	36.83±1.15	36.83±1.15	15.00±0.34

*HDMSCS - High density Multiple Species Cropping System; under organic management practices with husk burial and vermicompost as the main fertilizer input

frequency with a significant positive correlation at Neerchal (P <0.05). Nitrogen content was found to be positively correlated with spore load and frequency of root colonization with a highly significant positive correlation at Neerchal (P<0.01). No correlation could be drawn between AMF colonization and soil K (Table 2) which was in contradiction to the reported works showing a stimulatory effect of soil K on AMF variables (Furlan *et al*., 1989). Soil available phosphorous was negatively correlated with AMF, with a significant correlation at Mogral Puthur plot (P <0.05).

Table 2. Pearson's correlation between rhizospheric edaphic features with AMF parameters

Parameters	pH	OC (%)	N (%)	P	K
High Density Multiple Species Cropping System					
F%	0.127	0.456	0.378	-0.330	0.277
Sporeload	0.188	-0.399	0.463	-0.230	-0.419
Patena					
F%	0.569	0.610	0.731	0.402	-0.299
Sporeload	-0.570	-0.270	-0.662	0.690	-0.696
Mogral Puthur					
F%	-	-	-	-	-
Spore load	- 0.009	0.664	0.746	* -0.219	-0.235
Neerchal					
F%	*0.604	-0.249	0.470	-0.306	0.413
Spore load	*0.694	0.042	**0.833	-0.049	0.359

F% - Frequency of root colonization
Significant at *P<0.05
Significant at ** P<0.01

Twenty three cultivable SAB were obtained from *Glomus* and *Gigaspora* spp. of coconut and arecanut based cropping systems (Table 3 and Fig. 2). Of the twenty three cultivable SAB run through the BIOLOG procedure, thirteen were identified upto the species level (Table 4). The majority of SAB isolates were identified as *Bacillus subtilis* (8), followed by *Bacillus cereus* (1) *Bacillus amyloliquefaciens* (1), *Citrobacter amalonaticus* (1) *Staphylococcus arlettae* (1) and *Cornyebacterium coyleae* (1). Among the twenty three sequenced isolates, eight showed similar identity to that revealed by BIOLOG analysis. Of the 10 isolates, which were considered unidentified by BIOLOG analysis at Similarity Index ≤0.5, were identified as *Bacillus* sp. (1) *Bacillus subtilis* (5), *Bacillus cereus* (1), *Bacillus oleronius* (2) and *Lysnibacillus fusiformis* (1) with 99% identity (Table 5). The type of bacteria differed with respect to the AMF as well as the host plant. *Gigaspora* spp. associated with coconut and pepper rhizosphere showed association with *Bacillus cereus* and *Cornyebacterium coyleae*. *Glomus* spp. isolated from the same cropping system showed association with *Bacillus subtilis*, *Bacillus amyloliquefaciens* and *Staphylococcus arlettae*. In arecanut cropping system *Bacillus subtilis* was observed in *Glomus* spores isolated from both arecanut and banana rhizosphere. While in Neerchal mixed cropping system, *Bacillus subtilis* and *Citrobacter amalonaticus* were found to be associated with the same *Glomus* sp. Specificity of bacteria to a particular type of AMF might be due to the secretion of specific exudates by specific AMF species (Azcon, 1987).

Table 3. Details of spore associated bacteria isolated from AMF spp.

Host plant identity	AMF species	No of SAB isolates
Coconut, HDMSCs	*Gigaspora* sp.	3
Pepper, HDMSCs	*Gigaspora* sp.	4
Pepper, HDMSCs	*Glomus* sp.	2
Banana, HDMSCs	*Glomus* sp.	1
Pineapple, HDMSCs	*Glomus* sp.	3
Coconut, Patena	*Glomus* sp.	1
Arecanut, Mogral Puthur	*Glomus* sp.	4
Banana, Mogral Puthur	*Glomus* sp.	3
Arecanut, Neerchal	*Glomus* sp.	1
Banana, Neerchal	*Glomus* sp.	1

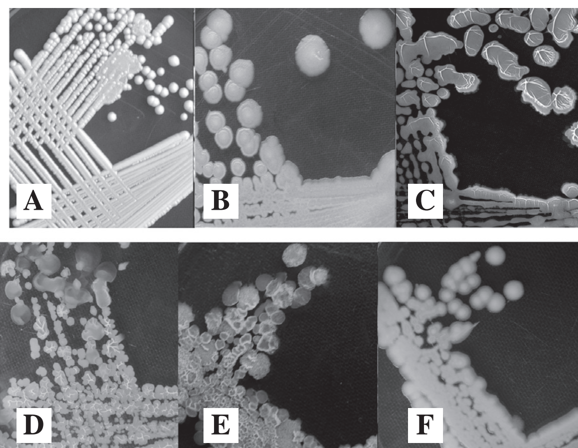

Fig. 2. A, B, C - Spore associated bacteria isolated from *Glomus* spp. and *Gigaspora* spp. of coconut based cropping systems; D, E, F - Spore associated bacteria isolated from *Glomus* spp of arecanut based cropping systems

Table 4. Identification details of spore associated bacteria (SAB) by the differential utilization of carbon source on Gen III BIOLOG plates

Host plant ID	AMF spp. ID	Sample ID	BIOLOG ID	SIM	DIST
Coconut, HDMSCS	*Gigaspora* sp.	GiK2HD	*B. subtilis*	0.781	3.055
Coconut, HDMSCS	*Gigaspora* sp.	GiCHD1	*Corynebacterium coyleae*	0.423	2.372
Pepper, HDMSCS	*Gigaspora* sp.	GiPHD1	*B. cereus*	0.621	4.705
Banana, HDMSCS	*Glomus* sp.	GLBHD1	*B. amyloliquefaciens*	0.333	5.354
Pineapple, HDMSCS	*Glomus* sp	GLPAHD1	*Staphylococcus arlettae*	0.687	5.659
Pinapple, HDMSCS	*Glomus* sp	GLPAHD2	*B. subtilis*	0.702	3.467
Arecanut, Mogral	*Glomus* sp	GLMP2	*B. subtilis*	0.750	3.208
Arecanut, Mogral	*Glomus* sp	GLMP4	*B. subtilis*	0.831	2.704
Arecanut, Mogral	*Glomus* sp	GLMP6	*B. subtilis*	0.688	3.926
Banana, Mogral	*Glomus* sp	GLBMP8	*B. subtilis*	0.812	2.756
Banana, Mogral	*Glomus* sp	GLBMP9	*B.subtilis*	0.641	4.207
Banana, Neerchal	*Glomus* sp	GLNCB1	*Citrobacter amalonaticus*	0.821	2.601
Arecanut, Neerchal	*Glomus* sp	GLNC3	*Bacillus subtilis*	0.777	3.640

Table 5. Identification details of spore associated bacteria (SAB) by 16 S rRNA sequence analysis

Host plant ID	AMF spp. ID	Sample ID	Molecular ID	% Identity
Coconut, HDMSCS	*Gigaspora* sp.	GiCHD2	*B.subtilis*	99
Pepper, HDMSCS	*Gigaspora* sp.	GiPHD2	*B. cereus*	99
Pepper, HDMSCS	*Gigaspora* sp.	GiPHD3	*Bacillus sp/B.oleronius*	99
Pepper, HDMSCS	*Gigaspora* sp.	GiPHD4	*B.subtilis*	99
Pepper, HDMSCS	*Glomus* sp	GLPHD1	*Bacillus sp/B.oleronius*	99
Pepper, HDMSCS	*Glomus* sp	GLPHD2	*B.subtilis*	99
Pineapple, HDMSCS	*Glomus* sp	GLPAHD3	*B.subtilis*	99
Coconut, Patena 1	*Glomus* sp	GLCP1	*Bacillus sp/B.oleronius*	99
Arecanut, Mogral	*Glomus* sp.	GLMP1	*Bacillus sp*	99
Banana, Mogral	*Glomus* sp	GLMP5	*Lysinibacillus fusiformis*	99

Table 6. Effect of spore associated bacteria (SAB) on mycorrhizal spore germination by diffusion (D) and crude extract (CE) methods

SAB	Max. no. of spores germinated/15 spores		Max. no. of spores germinated /15 spores in uninoculated control		Time taken for germination (Days)		% increase in spore germination over control	
Method	D	CE	D	CE	D	CE	D	CE
B.amyloliquefaciens GLBHD 1	3	9	1	4	3	7	13.3	33.3
B. cereus GiPHD1	1	6	0	0	7	4	6.7	40.0
B. subtilis GiK2HD	2	3	0	0	4	5	13.3	20.0
B. subtilis GiCHD 2	0	0	0	0	10	10	No effect	No effect
B. cereus GiPHD 2	2	1	0	0	7	7	13.3	6.7
Bacillus sp/ B.oleronius GiPHD 3	0	2	0	0	10	8	0.0	13.3
B.subtilis GiPHD4	0	2	0	0	10	4	No effect	13.3
Staphylococcus arlettae GLPAHD1	3	3	2	1	10	8	6.7	13.3
B. subtilis GLPAHD3	4	6	2	1	10	9	13.3	33.3
B. subtilis GLPAHD2	5	5	2	1	10	6	20.0	26.6
Bacillus sp./ *B.oleronius* GLCP1	4	0	2	1	10	2	13.3	No effect
Bacillus sp/*B.oleronius* GLPHD1	1	3	2	1	5	8	No effect	13.3
B. subtilis GLPHD2	6	4	2	1	10	8	26.7	20.0
Corynebacterium coyleae GiCHD1	7	7	2	1	10	10	26.7	40.0
Lysinibacillus fusiformis ATMP5	7	9	3	6	4	6	26.7	20.0
B. subtilis GLMP2	1	2	0	0	4	9	6.7	13.3
Bacillus sp. GLMP1	2	3	1	1	5	5	6.6	13.3
B. subtilis GLMP 4	1	2	1	1	5	6	No effect	6.6
B. subtilis GLMP6	2	1	1	1	8	9	6.6	No effect
B. subtilis GLMP 9	3	5	1	1	4	5	13.3	26.7
B. subtilis GLNC 3	2	2	0	0	10	6	13.3	13.3
B. subtilis GLMP 8	2	3	2	2	10	8	No effect	13.3
Citrobacter amalonaticus GLNCB1	3	7	2	2	8	8	6.7	40.0

*Germination of spores was observed over a total period of 10 days

The role of SAB on germination of spores was studied by crude extract and diffusion methods (Table 6). Twenty two SAB out of the twenty three studied was found to show an increase in spore germination over control. In this study, 77% of the spore associated bacteria gave better results with crude extract method. *Bacillus cereus* GiPHD1 gave the highest germination rate of 40% increase over control by crude extract method, whereas only 6.7% increase in spore germination was observed in diffusion method. In arecanut cropping system, *Citrobacter amalonaticus* GLNB1 associated with *Glomus* sp. isolated from arecanut rhizosphere, Neerchal, showed a 40% increase in spore germination with respect to control by crude extract method when compared with the diffusion method which showed an increase of 6.67% only with respect to control. The increased germination rate of AMF spores by crude extract method may be due to the direct contact of bacterial exudates than in diffusible assay. AMF spore germination was stimulated in the presence of bacterial cell-free supernatants, which suggests a stimulatory effect by the chemicals produced by the bacteria (Azcon, 1987).

This study explores the beneficial effects of SAB on mycorrhizae. The information available to support the effect of SAB on mycorrhizal plants is relatively less for AMF compared to ectomycorrhizal fungi. Future prospectives includes assessing the co-inoculation response of host plants to AMF and these growth promoting spore bacteria as an additional strategy for enhanced plant productivity.

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