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Occurrence of *Azospirillum* spp. in coconut-based farming systems*

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Abstract

Occurrence of Azospirillum was investigated in coconut-based farming systems, such as high-density multispecies cropping (15 crops), multi-storeyed cropping (3 crops), mixed cropping with tea and coffee (2 crops), intercropping with tropical tubers (5 crops), mixed farming with grasses (3 crops) and in 3 crops, arecanut, *Mimosa invisa* and sugarcane from other plots. A total of 26 plantation crops and intercrops were included in the study. Incidence of Azospirillum was determined by 2,3,5-triphenyl-tetrazolium chloride reduction and by culturing root fragments in N-free semisolid malate medium. Root samples from guava, mango and mimosa did not show any tetrazolium reduction or sub-surface pellicular growth. The extent of occurrence of Azospirillum seemed to depend upon the crop combinations. In a mixed farming system where guinea grass was one of the component crops, more root fragments of coconut and pepper demonstrated tetrazolium reduction activity than when guinea grass was absent. *Azospirillum lipoferum* and *A. brasilense* constituted 42% and 45% of the isolates, respectively, in the coconut-based cropping systems. Isolates from guinea grass, sugarcane and jackfruit exhibited higher nitrogenase (C_2H_2 reduction) than those isolated from plantation crops, tuber crops and spices. The large variation in the extent of association and nitrogenase activity of isolates from different crops indicated the need for inoculation with efficient cultures in a number of crops in coconut-based cropping systems.

Introduction

In the last 10 years there have been a considerable interest in the study of the associative dinitrogen fixer, *Azospirillum*. The ecology and distribution of the organism (Döbereiner *et al.*, 1976; Rao and Venkateshwarlu, 1985) and fixation of nitrogen in association with a number of annual crops has been well established (Albert *et al.*, 1978; Barber *et al.*, 1979; Rennie and Larson, 1979). There is only scanty information available on the occurrence of Azospirillum in perennials especially plantation crops (Subba Rao, 1983).

Coconut is one of the important plantation crops grown in India, and intercropping has been recommended for effective utilization of soil and sunlight in coconut gardens (Nair, 1979). Nair and Subba Rao (1977) reported that mixed cropping of coconut and cacao stimulated the population of non-symbiotic N_2 fixing bacteria in the rhizosphere of coconut. The association of Azospirillum with coconut and other crops grown together in different cropping systems has not been investigated so far. The purpose of this study was to record the occurrence and the degree of association of *Azospirillum* in many plantation crops and intercrops growing in 7 different coconut-based farming systems and to study the species composition and activity of isolates from different crops.

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Table 1. Farming systems and crops included in the study

Farming system	Component crops
1. High-density multispecies cropping (HDMSC) system	Coconut, nutmeg, pepper, sapota, pineapple, clove, lime, banana, jackfruit, guava, breadfruit, elephant foot yam, mango, leucaena, coffee.
2. Multistoreyed cropping	Coconut, cacao, pepper
3. Mixed farming	Coconut, guinea grass, pepper
4. Mixed cropping with tropical tree spices	Cinnamon, nutmeg
5. Mixed cropping with tea/coffee	Tea, coffee
6. Intercropping with tropical tubers	Elephant foot yam, sweet potato, turmeric, cassava, cocoyam.
7. Miscellaneous crops	Arecanut, mimosa, sugarcane

Materials and methods

Root samples

The roots of 26 different plantation crops and intercrops were collected from 7 different coconut-based farming systems (Table 1), permanently laid out at research farms of Central Plantation Crops Research Institute, Kasaragod. Kasaragod, situated in the west coast of India (12°31′N, 75°E), receives an annual rainfall of 3600 mm and the relative humidity in this area ranges from 40 to 90%. Maximum temperature in this area ranges from 29 to 34°C and experiences a minimum temperature of 20°C. The soil of the farms of the institute is an acidic (pH 5.1–5.5) sandy loam (*Aeric Peleustalf*). The experimental plots were fertilized with NPK fertilizers (CPCRI, 1983; Nair, 1979). Coconut palms were cultivated as the main crop at a distance of 7.5 m × 7.5 m and interspaces were planted with other crops.

The root samples were collected from randomly selected plants in the months of July–August, 1985 from the surface layer (0–15 cm) at a distance of one meter for coconut and 0.5 m for other crops from the base of the plants. Six replicate plants were sampled for each plant species from each cropping system, and in cases where six plants were not available the number of plants sampled are

Table 2. Tetrazolium reduction and subsurface pellicular growth in semisolid N free medium from root fragments of different crops in coconut-based high density multispecies cropping system (HDMSCS)

Plantation crop/intercrop		2,3,5-Triphenyl-tetrazolium chloride reduction		Subsurface pellicular growth in N-free semisolid malate medium	
Common name	Botanical name	No. of root bits stained (No. of plants)	% positive	No. of root bits inoculated (No. of plants)	% positive
Coconut	*Cocos nucifera* L.	76(6)	71	36(6)	25
Nutmeg	*Myristica fragrane* Houtt.	36(3)	11	18(3)	17
Pepper	*Piper nigrum* L.	72(6)	86	36(6)	33
Sapota	*Manilkara achras* (Mill.) Forsberg	48(4)	29	24(4)	17
Pineapple	*Ananas comosus* (L.) Merr.	72(6)	54	36(6)	19
Clove	*Eugenia caryophyllus* (Sprengel) Bullock & Barrison	72(6)	19	36(6)	17
Lime	*Citrus aurantifolia* (Christm.) Swing.	72(6)	13	36(6)	8
Banana	*Musa sapientum* L.	72(6)	67	36(6)	39
Jackfruit	*Atrocarpus heterophyllus* Lam.	24(2)	88	12(2)	42
Guava	*Psidium guajava* L.	48(4)	0	24(4)	0
Breadfruit	*Atrocarpus altilis* (Park) Forsberg	24(2)	63	12(2)	25
Elephant foot yam	*Amorphophallus campanulatus* (Rexb.) Blume	24(2)	50	12(2)	33
Mango	*Mangifera indica* L.	24(2)	0	12(2)	0
Leucaena	*Leucaena leucocephala* (Lam.) de Wit	72(6)	22	36(6)	0
Coffee	*Coffea arabica* L.	72(6)	78	36(6)	88
	Mean		42		18

mentioned in Table 2. A total of 12 root fragments were taken from each replicate plant. The roots were cleaned with water and surface sterilised with 0.1% $HgCl_2$. After washing thoroughly with sterile water, the roots were cut into 1 cm long fragments.

Incubation with tetrazolium, sectioning and observation

Each root fragment was incubated (at 30°C) with malate-containing tetrazolium phosphate buffer solution (Patriquin and Döbereiner, 1978). This solution consisted of 0.05 *M* phosphate buffer (pH 7.0) containing 0.625 $g l^{-1}$ sodium malate (autoclaved) and 1.5 $g l^{-1}$ triphenyltetrazolium chloride (PBMT). For observation under the microscope, roots were sectioned, placed in water, covered with coverslip, and examined.

Isolation and purification of cultures

The procedure of Döbereiner and Day (1976) was followed to isolate bacteria from surface-sterilised root fragments. To assay the acetylene

Table 3. Tetrazolium reduction and subsurface pellicular growth in semisolid N free medium from root fragments of different crops in coconut-based multistoreyed cropping, mixed farming and mixed cropping systems

System/plot	Plantation crop/intercrop		2-3,5-Triphenyl-tetrazolium chloride reduction		Subsurface pellicular growth in N-free semisolid malate medium	
	Common name	Botanical name	No. of bits stained (No. of plants)	% positive	No. of root bits inoculated (No. of plants)	% positive
Multistoreyed cropping	Cacao	*Theobroma cacao* L.	72(6)	88	36(6)	25
	Pepper	*P. nigrum* L.	72(6)	29	36(6)	19
	Coconut	*C. nucifera* L.	72(6)	46	36(6)	17
			Mean	54		20
Mixed farming	Guinea grass	*Panicum maximum* Jacq.	72(6)	100	36(6)	75
	Pepper	*P. nigrum* L.	72(6)	96	36(6)	17
	Coconut	*C. nucifera* L.	72(6)	85	36(6)	17
			Mean	94		36
Mixed cropping with tropical tree spices	Cinnamon	*Cinnamomum zeylanicum* Breyn.	48(4)	71	24(4)	50
	Nutmeg	*M. fragrane* Houtt.	24(2)	13	12(2)	0
			Mean	42		25
Mixed cropping with tea/coffee	Tea	*Camellia sinensis* (L.) O. Kuntze	72(6)	31	36(6)	17
	Coffee	*Coffea canaphora* Pierre ex Frochner	72(6)	19	36(6)	11
			Mean	25		14
Intercropping with tropical tubers	Elephant foot yam	*A. campanulatus* (Rexb.) Blume	36(3)	25	18(3)	22
	Sweet potato	*Impomoea batatas* (L.) Lam.	36(3)	11	18(3)	22
	Turmeric	*Curcuma domestica* valet	36(3)	17	18(3)	11
	Cassava	*Manihot esculenta* Crantz.	36(3)	8	18(3)	6
	Cocoyam	*Xanthosoma sagittifolium* (L.) Schott.	36(3)	11	18(3)	17
			Mean	14		16

Table 4. Tetrazolium reduction and subsurface pellicular growth in semisolid, N-free medium from root fragments of three miscellaneous crops grown in coconut garden

Plantation crop intercrop		2,3,5-Triphenyl-tetrazolium chloride reduction		Subsurface pellicular growth in N-free semisolid malate medium	
Common name	Botanical name	No. of root bits stained (No. of plants)	% positive	No. of root bits inoculated (No. of plants)	% positive
Arecanut	*Areca catechu* L.	24(2)	29	12(2)	25
Mimosa	*Mimosa invisa* Mert.	36(3)	0	18(3)	0
Sugarcane	*Sacharum officinarum* L.	36(3)	100	26(6)	75

reduction activity (ARA), 3 ml of semisolid N-free malate media (Cote, 1984) in a 15 ml test tube were inoculated with the cultures and incubated for 60 h. Cotton plugs were replaced with subaseals following incubation, and acetylene was injected (10% of the atmosphere). After incubation for 4 h (at 30°C) ethylene production was assayed using a gas chromatograph (Shimadzu). The analysis was done with Porapack T column (2 m × 2.4 mm) using N_2 as carrier gas at a flow rate of 35 ml min^{-1}. Column and detector temperatures were adjusted to 100°C and 150°C, respectively. The procedure adopted was that of Masterson and Murphey (1979). The bacterial isolates were identified to the species level based on the morphological and physiological tests proposed by Tarrand *et al.* (1978).

Results and discussion

Tetrazolium reduction reaction

A 2,3,5-triphenyl-tetrazolium chloride solution is colourless and turns red upon reduction producing triphenyl formazone. Tetrazolium reduction activity (TRA) has been used to identify the presence of nitrogen-fixing bacteria in the cortex of surface sterilised roots (Döbereiner and Day, 1976; Subba Rao, 1983). We have used this property to study the occurrence of similar bacteria in the roots of plantation crops and intercrops.

Surface-sterilised roots upon immersion in PBMT showed red spots within 1–2 h. Sections of these red spots revealed bacteria. Upon overnight incubation, the cortex area of plants, when infected with bacteria, was an intense red. Certain plant cell constituents like starch granules also showed red colouration upon incubation with PBMT and have a size similar to that of bacteria, but these spots do not darken or enlarge upon prolonged incubation. The bacterial spots could be distinguished by a characteristic morphology, motility and colony type.

Twenty-six plantation crops and intercrops from 7 different coconut-based farming systems were studied. In the case of coconut-based high density multispecies cropping (HDMSC) system, root fragments from 15 plantation crops and intercrops were examined (Table 2). Seventy one percent of root fragments of coconut showed positive TRA. There were large variations in the TRA of root fragments from different intercrops. Crops like jackfruit and pepper had a high proportion of roots (88% and 86%, respectively) showing TRA. Root fragments from only mango and guava did not show TRA. Comparatively less number of root fragments from nutmeg (11%) and lime (13%) showed TRA.

In the 5 other cropping models and the miscellaneous crops there was a high proportion of root bits showing TRA in some intercrops whereas some others had only a small percentage roots positive for this test (Tables 3, 4). From coconut-based multistoreyed cropping plot, 88% of roots from cacao showed TRA whereas for pepper this value was low (29%). In the mixed farming plots (milk cows are one of the components of this system), all the root fragments from guinea grass showed TRA. The other two component crops pepper and coconut also showed a high proportion of root bits with TRA. Guinea grass is a C_4 plant which has been reported to be very good at forming associative symbiosis with *Azospirillum* sp. (Garcia *et al.*, 1978; Döbereiner, 1983). Perhaps this could be the reason for higher number of root bits with TRA in pepper and coconut that are growing in combination with guinea grass.

In the mixed cropping plots with tropical tree spices, a fairly high number (71%) of root fragments from cinnamon showed TRA, whereas nutmeg showed less TRA activity. In the plot where mixed cropping was followed with tea and coffee, higher (81%) proportion of tea root fragments showed TRA than coffee roots (19%). In the intercropping plot where tropical tubers were grown, the roots showing TRA were relatively less; 8% for cassava to 25% for elephant foot yam root fragments.

Roots from 3 miscellaneous crops, arecanut, *Mimosa invisa* and sugarcane were also examined. All the root sections from sugarcane exhibited TRA, whereas 29% of those from arecanut were positive (Table 4). On the other hand, none of the root bits from *M. invisa* exhibited TRA. In order to

Table 5. Nitrogenase activity and speciation of *Azospirillum* cultures

Cropping system/crop from which isolated		Mean nitrogenase activity $nMC_2H_4\,ml^{-1}$ media h^{-1}	Speciation+ (No. of isolates)
I. HDMSC System			
Jackfruit		10.45	Al(4)
Breadfruit		1.89	Al(2)
Clove		0.30	NI(1)
Lime		1.21	Ab(2)
Pineapple		7.11	Al(3), Ab(1)
Pepper		7.81	Al(1), NI(1)
Banana		6.20	NI(2)
Coffee		0	NI(1)
Coconut		4.04	Ab(5)
	Mean	4.33	
II. Multistoreyed cropping			
Cacao		6.75	Al(1)
Coconut		9.37	Ab(1)
Pepper		6.67	Al(1)
	Mean	7.60	
III. Mixed farming			
Panicum		15.59	Al(4), Ab(4)
IV. Mixed cropping with tree spices			
Cinnamon		1.35	Ab(4)
Nutmeg		0	Al(1), Ab(1)
	Mean	0.68	
V. Mixed cropping with tea/coffee			
Coffee		1.31	Ab(2)
Tea		1.51	Al(1)
	Mean	1.41	
VI. Intercropping with tropical tubers			
Sweet potato		5.54	Al(2)
Cocoyam		3.03	Ab(2)
Turmeric		0.38	Al(4), NI(1)
Elephant foot yam		3.78	Al(1), Ab(1)
	Mean	3.18	
VII. Miscellaneous crops			
Sugarcane		10.05	Ab(3)
Arecanut		1.31	NI(2)
Mean of coconut isolates		4.93	
Mean of pepper isolates		7.43	
Mean of coffee isolates		0.87	

Al, *Azospirillum lipoferum*; Ab, *Azospirillum brasilense*; NI, Not identified.

see whether symbiotic N_2 fixation is active, *M. invisa* plants were uprooted carefully (at 3 months growth) and roots examined for nodulation. The legume was found to be well nodulated (42 nodules on an average) in a plant which weighed 422.3 mg dry weight (after drying at 70°C till constant weight).

Large variability in TRA of different plant species growing together in different cropping systems was observed. Döbereiner and Day (1976) observed that strongly reducing sites in the root cortex were packed with *A. lipoferum* like tetrazolium reducing bacteria. In our study, the occurrence of associative diazotrophs was confirmed by incubation in semisolid N-free malate medium. This was followed by isolation, identification and testing of nitrogenase activity of cultures from the root fragments that showed positive TRA.

Subsurface pellicular growth in N-free malate medium

A maximum of 75% of guinea grass and sugarcane root fragments showed subsurface pellicular growth when cultured in N-free malate medium (Tables 3, 4). Maximum TRA was also shown by roots of these crops. *Azospirillum* was reported as the major N_2 fixing bacteria associated with these two crops (Döbereiner *et al.*, 1976; Patriquin *et al.*, 1980), and the association was found to be fixing significant amounts of N_2 in a number of studies (Berg *et al.*, 1980; Bouton and Zuberer, 1979). Subsurface pellicular growth was not shown by root fragments of guava, mango and *M. invisa* in N-free media (Tables 2, 4). The roots of these 3 crops also did not show TRA activity.

The percentage of root fragments giving pellicular growth was in general lower than the percentage for TRA, but the trend for the majority of crops matched with TRA. A significant correlation ($r = 0.71$) was obtained between the values recorded for TRA and subsurface pellicular growth. However, the results obtained in the two tests were not similar in two crops; leucaena (in HDMSC) and nutmeg (in mixed cropping with tree spices). In Leucaena and nutmeg, 22% and 13% root fragments reduced tetrazolium but none of them showed subsurface pellicular growth. This indicated that though TRA is a quick test showing significant correlation with subsurface pellicular growth for majority of cases, it may also give positive results in some cases in the absence of associative diazotrophic bacteria. The situation may be similar in case of diseased plants since some of root borne pathogens may also show TRA.

Isolation, identification and nitrogenase activity of cultures

Sixty cultures were isolated during this study, out of which 25 were identified as *Azospirillum lipoferum* and 27 cultures as *Azospirillum brasilense* on the basis of morphological and physiological tests suggested by Tarrand *et al.* (1978). Eight cultures did not fall in either of the above two species (Table 5). These did not belong to *Azospirillum amazonense* as these could not use sucrose as C source and did not show any deep, diffused pellicular growth in LGI medium (Cote, 1984) as suggested by Magalhaes *et al.* (1983). There were some plants that yielded only one species of Azospirillum, while some others had both the species in the root system. All 5 cultures from jackfruit, 2 from breadfruit, 2 from sweet potato and 4 from turmeric were *A. lipoferum*, whereas all the 6 cultures from coconut, 2 from lime, 2 from cocoyam, 2 from elephant foot yam and 2 from sugarcane were *A. brasilense*. In the case of cultures from other plants, both *A. lipoferum* and *A. brasilense* were present.

The cultures were tested for nitrogenase activity by acetylene reduction method (Table 5). Only 8 cultures did not show acetylene reduction activity (ARA). The ARA varied from culture to culture, even those from the same species. Isolates from guinea grass, sugarcane and jackfruit exhibited higher nitrogenase activity when compared to the activity shown by isolates from the other crops. Azospirillum cultures from C_4 plants (guinea grass and sugarcane) were reported to be very efficient in N_2 fixation in earlier studies also (Boddey and Döbereiner, 1984; Döbereiner, 1983). Cultures from breadfruit, clove, lime, tea, coffee, cinnamon and arecanut showed very low nitrogenase activity. Crops having high percentage of roots with Azospirillum can contribute substantial amounts of N_2 to the cropping system by way of biological fix-

ation. It may be worthwhile to test in coconut gardens a combination of crops which have high level of association with Azospirillum to improve associate N_2 fixation in the coconut-based cropping system.

Conclusion

The present study revealed the occurrence of Azospirillum in different levels in plantation crops and intercrops in different coconut-based farming systems in an acidic, tropical soil. Though Azospirillum isolates from a few intercrops showed high nitrogenase activity, the majority of the crops did not yield efficient cultures. It will be worthwhile to conduct inoculation trials on crops which have low incidence of Azospirillum and those having cultures with poor activity, to get maximum benefit from associative diazotrophs in the different coconut-based cropping systems.

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