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### ASSOCIATION OF DIAZOTROPHIC AND PLANT GROWTH PROMOTING RHIZOBACTERIA WITH COCONUT PALM (*COCOS NUCIFERA* L.)

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#### ABSTRACT

Coconut palm (*Cocos nucifera* L.) has been found to favour the build up of a diverse population of asymbiotic and associative symbiotic beneficial bacteria capable of nitrogen fixation, phosphate solubilisation and plant disease suppression in the root zone as in the case of cereals and grasses. Major nitrogen fixers isolated include *Beijerinckia indica*, different species of *Azospirillum*, *Azoarcus* spp., *Bacillus* spp., *Burkholderia* spp., *Herbaspirillum frisingense*, *Arthrobacter* spp. etc. Root and leaf tissues have also been shown to harbour low numbers of endophytic *Bacillus* spp. capable of plant growth promotion and disease suppression. Preliminary studies have confirmed that inoculation with efficient strains of various diazotrophs and P-solubilizers can enhance plant growth and nut yield. In various coconut-based cropping systems and farming systems, higher incidence of these beneficial bacteria were observed.

#### INTRODUCTION

India is one of the major coconut producing countries ranking first in production in the world coconut scenario. Despite this premier position, the national average is only about 40 nuts per palm per year, the half of what realized in experimental fields under rainfed conditions. This is because of the fact that only limited number of farmers use chemical fertilizers and even among them, a very low percentage apply the recommended dose. Recent increase in prices, uncertain supplies and concerns on the environmental and health effects of chemical fertilizers have paved way for interest in biological alternatives to fertilizers in coconut cultivation. Fortunately, the task is more simple in the case of tropical soils which are able to ensure good plant nutrition despite their low organic matter and nutrient contents owing to their better capacity for biological management for higher yields. Already interest has been generated in sustainable coconut production by adopting biological methods (Thomas *et al.*, 2001, Prabhu *et al.*, 2000). All these technologies greatly rely on a wide variety of functional processes carried out by soil microbial communities (Pankhurst *et al.*, 1995). In the past few years, it became clear that a number of beneficial microorganisms in soils can enhance nutrient availability, promote growth and manage diseases (Glick, 1995). Such beneficial microbes are also of great interest in coconut cultivation. Coconut palms can be seen growing and yielding along the coastal tracts with sandy or sandy loam soils in Kerala. Substantial amounts of

nutrients are removed by these palms each year, but the mechanisms of soil fertility maintenance over the years are largely unknown. But it is strongly believed to be due to the activity of beneficial microorganisms. Unfortunately, over the years very little attention has been paid to N-fixing and other beneficial bacteria in the rhizosphere and roots of perennial plantation and orchard plants (Subba Rao, 1983). This paucity of emphasis on tree-associated beneficial microbes reflects lack of information rather than lack of importance. A biological mechanism to supply nutrients to the palm directly through beneficial microorganisms associated with the root system as in grasses and cereals (Baldani *et al.*, 1997) would be of much interest and relevance to the resource-poor coconut growers. As palms classified under the family Palmae represent yet another group of perennial monocots, it is reasonable to hypothesize that these monocots also benefit naturally from such diazotrophic associations. Ouvrier and Ochs (1978) after a detailed study on mineral export by hybrid coconut palms growing in coastal sandy soils in Cote d'Ivoire noted that even though nitrogen applications were well below requirements, the palms were without slightest deficiency symptoms. The authors concluded that the soil is capable of furnishing a large part of the need, in spite of its poverty in total nitrogen. They suspected that biological nitrogen fixation was responsible for this phenomenon. The celebrated researcher, Dr. Johanna Dobereiner first reported the occurrence of associative symbiotic diazotroph, *Azospirillum* in the rhizosphere soil of coconut palm (Dobereiner, 1978). Subsequently it was isolated from rhizosphere, rhizoplane and endorhizosphere of the palm (Sylvester-Bradley *et al.*, 1980, Govindan and Purushothaman, 1985, Ghai and Thomas, 1989).

A number of recent studies conducted by the authors at the Central Plantation Crops Research Institute, Kasaragod revealed that coconut soils harbour abundant and diverse population of beneficial microorganisms active in nutrient transformation and plant growth promotion. These include asymbiotic, associative symbiotic and endophytic nitrogen fixers, plant growth promoters, phosphate solubilisers and disease suppressive bacteria (Prabhu *et al.*, 2000, Moosa *et al.*, 1998, Thomas *et al.*, 1991, George, 1990). The studies also revealed the interesting fact of evolution of adaptive strategies to adverse soil conditions specific to coconut growing soils such as acidity, low nutrient and moisture conditions. Most of the beneficial bacteria isolated were highly acid loving and had the capability to produce resistant bodies such as cysts, arthrospores and endospores to survive the adverse soil situations. Another important observation was that the activity of these beneficial microbes enhanced many times in coconut plantations which adopted a variety of cropping and farming systems. These observations should permit scientists to make recommendations to farmers to greatly benefit from the services of a variety of rhizobacteria without much expenditure by altering agronomic practices.

### **Coconut soils and root environments as ideal habitats for beneficial microbes**

Coconut root regions have great potential to harbour associative symbiotic bacteria, as it has a well developed fibrous root system and a fully grown palm produces 1500-8000 roots, which produce lateral branches capable of branching and rebranching. The root system is known to produce exudates with a number of sugars, organic acids and amino acids (Bopaiah *et al.*, 1987), favouring build up of population of rhizosphere bacteria. From the surface of roots, small outgrowths

known as pneumatophores develop and act as organs for gas exchange (Davis, 1968). Most of the recently described microaerophilic associative symbionts are known to prefer high temperatures of tropical regions (Dobereiner *et al.*, 1976) and have preferential advantages over other bacteria under conditions of low nutrient supply (Hegazi *et al.*, 1979). All these factors should make the nutrient -poor coconut soils in Kerala, where mean day temperature is above 30 °C a best habitat for diverse groups of aerobic and microaerophilic beneficial bacteria. Pronounced rhizosphere effect has been consistently observed for most of the beneficial bacteria and in coastal sandy soils, coconut roots have clear rhizosheaths, which are thought to be produced by the activity of microorganisms.

### **Beneficial Bacteria in Coconut Soils and Roots**

Different levels of association between coconut plant and various groups of beneficial microbes have been observed. The organisms isolated included non-symbiotic, associative symbiotic and endophytic bacterial groups. Maximum microbial population was observed on rhizoplane and endophytic population was very low.

Using aerobic solid media, a number of plant-beneficial bacteria have been isolated from the rhizosphere soils of coconut. A number of phosphate solubilizing bacteria belonging to the genera *Bacillus*, *Pseudomonas*, and *Micrococcus* were isolated. Inoculation of these bacteria to coconut soils increased the available phosphorus content, indicating that bioinoculants based on these bacteria can help to reduce phosphatic fertilizer use in coconut cultivation. Acidophilic *Beijerinckia* spp. was found to be the most conspicuous aerobic nitrogen fixer in coconut soils. Two subspecies were consistently isolated, *B. indica* subspecies *indica* and *B. indica* subspecies *lacticogenes*. Even though this diazotroph is a typically non-symbiotic, it has been consistently observed in higher numbers in coconut rhizosphere soil and rhizoplane, indicating a closer association with the palm. Bioassays and coconut seedling inoculation studies indicated a positive response on plant growth. Wide range of acetylene reduction ability varying from 4.47 to 590.38 nmoles C<sub>2</sub>H<sub>4</sub>/3ml medium/hr in isolates isolated from the root regions of different coconut cultivars indicated that there is scope for isolating and using much efficient strains for inoculation purposes. As most of these isolates produced copious amounts of gummy polysaccharides, these diazotrophs must be multifunctional in coconut soils and might be playing a role in improving soil aggregate stability and soil structure in addition to improving nitrogen status.

### **Occurrence of New Diazotrophs in Coconut Roots**

Recent studies using different semi-solid media revealed the occurrence of diazotrophs such as *Azospirillum lipoferum*, *Azospirillum brasilense*, *Azospirillum amazonense*, *Herbaspirillum frisingense*, *Arthrobacter* spp., *Burkholderia* spp., *Bacillus* spp., *Azoarcus* spp., *Xanthobacter* spp., *Pseudomonas* spp., a new species of *Azospirillum* and many new types which are yet to be classified. The new species of *Azospirillum* utilized glucose without acidification and growth in malate medium didn't result in large, immotile pleomorphic forms. This may be an intermediary form with characteristics of both *A. lipoferum* and *A. brasilense*. Diazotrophs isolated in our laboratory greatly differed in their carbon source preference and pH requirements for growth. N-free semisolid malate (NFb) medium enriched *Azospirillum lipoferum*,

*A.brasilense*, *Arthrobacter* spp., *Herbaspirillum frisingense*, *Azoarcus* spp., *Xanthobacter* spp. and *Pseudomonas* spp. More efficient isolation of *A.lipoferum*, *Arthrobacter* and *Herbaspirillum frisingense* was observed when N-free JNFb with pH 5.8 was used. Isolation within 3 days of pellicle formation also enhanced the chance of their isolation. This was related to their high sensitivity to alkalinity. Use of semisolid N-free sucrose medium (LGI) resulted in isolation of *Azospirillum amazonense*, *Bacillus* spp. and *Burkholderia* spp., which were rarely isolated with malic acid as carbon source. Use of mannose or inositol as the sole carbon source in N-free semisolid media resulted in highly selective isolation of *Azospirillum amazonense*. Use of L-tartarate as the sole carbon source in N-free semisolid media resulted in preferential isolation of *Herbaspirillum frisingense*. This study proves that studies to isolate diazotroph diversity in coconut growing soils will be complete only with the use of large number of isolation media containing as many number of carbon sources as possible.

A survey conducted throughout Kerala in farmers gardens revealed ubiquitous distribution of these bacteria in coconut soils. A simple medium, TYG medium containing 5 grams each of tryptone, yeast extract and glucose was found to be highly useful for maintaining and mass multiplication of most of the diazotrophs. Mass multiplication techniques for these beneficial bacteria were developed and biofertilizers developed using low cost carrier materials such as composted and vermicomposted coir pith. Bioassays, pot trials and preliminary field trials revealed beneficial effects on growth and yield of palms. Inoculation with efficient isolates of diazotrophs significantly enhanced number of main roots, and favoured the production of root branches in coconut seedlings. Preliminary results from field trials with nitrogen fixers and P-solubilizers also gave some indications that these bacteria can enhance nut yields.

### **Endophytes in Coconut Palm**

Plant beneficial endophytes are capable of invading and living inside plant tissues and can positively affect plant growth and development. The possibility of association of endophytic bacteria with coconut palm tissues was examined in this study. A method for surface sterilizing root tissues was standardised, which includes two minutes each treatment with 0.1 % HgCl<sub>2</sub> and 95% ethanol followed by washing with sterile distilled water five times. This treatment was highly successful in eliminating all bacterial growth from rhizoplane and phylloplane and all the bacteria isolated from ground tissues after the treatment were real endophytes. The root tissues harboured 8000 to 32000 bacteria per gram dry weight, while the counts in leaf tissues were less than 1000. Isolations were made even from 25 year old tall palms. Gram positive bacteria belonging to the genus *Bacillus* spp. were found to be the main component of the bacterial population in the root as well as leaf tissues (Prabhu *et al.*, 2000). Most of the isolates were immotile and sporulated profusely. The cultures grew poorly in liquid media, but the growth on agar media was extremely fast. All the isolates were capable of excreting GA-like substances and bioassays confirmed their plant growth promoting ability. Another species of *Bacillus* isolated from surface sterilized leaves was capable of almost completely inhibiting the growth of *Phytophthora palmivora*, the causative organism of the lethal bud rot disease of the palm (Moosa *et al.*, 1998).

### **Effect of Management Practices on Beneficial Microflora**

The studies so far conducted at CPCRI have confirmed that coconut plantation soils are rich in beneficial microbial species diversity. The limiting factor in these impoverished soils is the organic resources available for maintaining and sustaining the microbial activity and the sleeping beauty paradox (Lavelle and Spain, 2001), which explains the striking contrast between the potential for an extremely fast turnover and field reality of microbial biomass must be true for coconut soil microbial communities as well. Our studies confirmed that by the addition of organic manures such as coir pith, cow dung and green manures, the survival and nitrogenase activity of diazotrophs such as *Beijerinckia* can be enhanced to a very great extent (Thomas and Prabhu, 1998). Coconut is a small holders crop and is usually grown as a mixed crop. Most of the farmers also integrate other enterprises such as dairy and poultry with coconut cultivation. A number of studies at CPCRI indicated that the above-ground diversity of plants usually resulted in increased microbial biomass and higher incidence of beneficial microbes such as diazotrophs and P-solubilizers in coconut plantations. These observations are based on the fact that increased plant diversity in plantations results in increased organic matter addition through crop residues and root exudates (Bopaiah and Shetty, 1991). Inclusion of a grass component in coconut-based farming system always enhanced beneficial microbial activity in coconut soils, which has also been subsequently found to favour better colonization of coconut roots by the root-associated diazotrophs such as *Azospirillum* spp. (Ghai and Thomas, 1989). Because of increased microbial activity in these systems, the palms yielded better at lower doses of chemical fertilizers than with full dose, which reduced microbial activity. Further investigations should permit us to define the best crop combinations and chemical fertilizer doses to derive maximum benefits from the beneficial microbes and finally to completely shift to organic cultivation.

### **Conclusion and future line of work**

Now there is enough evidence to confirm that coconut soils and roots favour the development of a highly diverse beneficial microflora. Based on the experience of successful isolation of effective strains of diazotrophs closely associated with coconut palm, it could be predicted that commercial viability of biofertilizers for coconut will be a reality in the near future. The data on higher incidence of beneficial microbes at lower doses of chemical fertilizers and in mixed cropping and mixed farming systems should enable researchers to make recommendations to farmers to derive maximum benefits from biosources of nutrients by making alterations in agronomic practices. Based on the preliminary observations on the incidence of high diversity of new diazotrophs in root regions of coconut palm, it would be worthwhile to undertake microbial community study at molecular level. This should reveal the incidence of much higher biodiversity. There is some evidence that different coconut hybrids harbour different beneficial microbial populations. It would be interesting to know specific microbial association between different hybrid combinations, which would permit selection of best combinations that favour higher nitrogen fixation.

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