Chapter 15

Microbial Interactions and Activities Affecting Sago Palm Growth

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Abstract Microbes are ubiquitous soil inhabitants. Both aboveground and below-ground parts of plants are associated with diverse and abundant microbes. Such microbes have positive and negative impacts on the plant productivity. Biological nitrogen fixation (BNF) is such a beneficial interaction. To reveal BNF by free-living bacteria in sago palm, different parts (root, rachis, petiole, leaflet, bark, pith, and extracted starch) were collected in the Philippines, and their nitrogen-fixing ability was measured. Almost all the samples showed positive nitrogen fixation. Then, nitrogen-fixing bacteria (NFB) were isolated, belonging to different genera, such as *Klebsiella* sp., *Pantoea* sp., *Burkholderia* sp., *Stenotrophomonas* sp., and *Bacillus* sp. All the isolates preferred simple carbon compounds, like glucose, sucrose, and mannitol, as their substrates for nitrogen fixation, while they showed very low activity in starch, pectin, and hemicellulose media. When NFB were cocultured in such a medium with the polymer-degrading bacteria, nitrogen-fixing ability was markedly increased. Stimulatory effects were observed in Rennie medium by co-inoculation of NFB and indigenous bacterial consortia isolated from sago palm samples. These results indicate that complex microbial interactions may increase in situ nitrogen fixation and contribute to nitrogen nutrition in sago palm. This chapter also introduces characteristics of nitrogen-fixing bacteria and amounts of BNF in palm trees in recent studies.

15.1 Microbial Activities Relating to Plant Growth

Microbes exist everywhere, especially in soil. A classic report in Russia revealed that bacteria are a ubiquitous soil inhabitant, and total bacterial numbers are stable across quite diverse soil environments, from dry and desert steppe through meadow steppe, from forest meadow to taiga, and from 1200 to 3600 m above sea level (Mishustin and Mirsoeva 1968). By contrast, they found that specific groups of bacteria favor certain habitats and show large fluctuations depending on time and

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location. For example, spore-forming bacteria, enumerated as heat-tolerant ones, showed large fluctuations in their numbers, ranging from 0.01 to $1.2 \times 10^6 \text{ g}^{-1}$ soil (more than 100 times difference) depending on the sampling sites, but the differences in total bacterial numbers were only less than five times. Recent studies demonstrate that soil contains as many as 10^{10} – 10^{11} bacteria, 6000–50,000 bacterial species, and up to 200 m of fungal hyphae per 1 g and the soil microbes play key roles in ecosystems (van der Heijden et al. 2008).

In addition to soil, plants harbor a diverse and an abundant microbial community and support their activities as the key primary producers in most terrestrial ecosystems. In particular, the rhizosphere, defined as the soil environment influenced by the presence and activities of plant roots, has a huge impact on soil microbes. Plant roots release organic matter called *rhizodeposition* or root exudate, and the amount of rhizodeposition represents ca. 11% of carbon fixed by the plant and 27% of carbon allocated to roots (Dennis et al. 2010). Rhizodeposition supports the microbial activities in the rhizosphere. A wide range of organisms, i.e., different kinds of bacteria, fungi, protozoa, and nematodes, show higher populations in the rhizosphere than in the non-rhizosphere, which is not affected by plant roots. The rhizosphere consists of endorhizosphere (root tissue area), rhizoplane (root surface), and exorhizosphere (rhizosphere soil: soil directly surrounding the root). According to a report summarizing the results of 22 different plants, the numbers of aerobic bacteria were on average 9.9 times (2.6–24.2) higher in rhizosphere soil than in non-rhizosphere soil (Lochhead and Rouatt 1955), and microbial densities are markedly higher in the rhizoplane than in the endorhizosphere. An example showed that the numbers of total bacteria, Gram-negative bacteria, and fungi were 100–1000 times higher in the rhizoplane than in the endorhizosphere (Fig. 15.1). Rhizosphere soil and the rhizoplane are microbial hot spots, and many microbes colonize even the endorhizosphere.

In addition to belowground, the aboveground parts of plants also support a variety of bacteria, yeasts, and fungi. According to Lindow and Brandl (2003), bacteria are by far the most numerous colonists of leaves, ranging from 10^6 to 10^7 cells cm^{-2} (up to 10^8 cells g^{-1}). The aerial habitat colonized by these microbes is termed the *phyllosphere*, and the inhabitants are called *epiphytes*. The number of epiphytes is affected by surrounding environments. For example, Enya et al. (2007) compared the number of leaf-associated bacteria between field-grown and greenhouse-grown tomato leaves and found that the former harbored 100 times higher densities and the population densities increased with age. Since sago palm grows under field conditions, its leaves and stems are colonized by many microbes.

Microbes affect plant productivity through different mechanisms. First, a typical one is direct effects on plants via plant-associated organisms that form mutualistic or pathogenic relationships with plants. Another is indirect effects via the action of free-living microbes that alter rates of nutrient supply and the partitioning of resources (van der Heijden et al. 2008). Plant productivity is enhanced by different microbial actions, while there are a number of microbial threats to crop production (Table 15.1).

Nitrogen-fixing bacteria contribute to plant productivity to the largest extent, as described below. Arbuscular mycorrhizal (AM) fungi enhance plant productivity, especially in grasslands (van der Heijden et al. 1998). These two symbiotic micro-

Fig. 15.1 The number of microorganisms in the rhizoplane and endorhizosphere of radish grown in soil. *Solid lines*, rhizoplane; *dotted line*, endorhizosphere. All: total culturable bacteria, *G*(-) Gram-negative bacteria, *F* fungi (Toyota et al. unpublished data)

Table 15.1 Influence of soil microbes on various ecosystem processes (From van der Heijden et al. 2008)

Ecosystem process	Microbes involved	Estimated microbial contribution to ecosystem process
Plant productivity	Nitrogen-fixing bacteria, mycorrhizal fungi	0–50%
	Microbial pathogens	–50–0%
Decomposition	Bacteria, fungi	Up to 100%
Plant acquisition N fixation	Rhizobia, actinomycetes, free-living bacteria	0–20% (sometimes higher)
Soil uptake	Mycorrhizal fungi	0–80%
Nitrogen loss denitrification	Denitrifying bacteria and some fungi	Up to 60%
Enhanced leaching because of nitrification	Nitrifying bacteria, archaea	??
Phosphorus cycle	Mycorrhizal fungi, P-solubilizing bacteria	0–90%
Plant phosphorus acquisition		
Phosphorus loss because of leaching		??

organisms have the greatest impact on plant productivity, but such symbiotic interactions have not been reported in sago palm.

Nutrient supply is an important function of soil microbes. Usually, plants do not use organic forms of N, the dominant form in soil, but use inorganic N, which is mineralized by the actions of soil microbes and animals. Even in agricultural crops

Fig. 15.2 Origin of N uptake by crops (Drawn from Fujiwara et al. 1996, paddy rice; Brady and Weil 2008, maize; Kato 2004, tomato)

where fertilization is always done, crops absorb more N from the soil organic matter fraction than from applied fertilizer (Fig. 15.2). Since sago palm is often cultivated under non-fertilized conditions, the most important mechanism for its N acquisition is the mineralization of soil organic matter by soil microbes and animals. Indeed, a pot experiment conducted in a glasshouse showed that sago palm growth evaluated by its plant height did not appear to be affected by urea fertilization (Lina et al. 2010), suggesting the importance of the nutrient-supplying capacity of soil.

Direct yield losses caused by pathogens, insects, and weeds are estimated to range from 20 to 40% of global agricultural productivity (Savary et al. 2012). In the natural environment, there are many airborne and soilborne plant pathogens, including fungi, bacteria, viruses, and nematodes. On the other hand, many studies have reported biological control of plant pathogens by epiphytes and rhizosphere microbes.

In sago palm, infestation with the bagworm larvae (*Pteroma pendula*) was found in the Philippines, but its damage to plant productivity was not estimated (Okazaki et al. 2012). As far as the author knows, there is no study reporting damage to sago palm caused by pathogens.

15.2 Importance of Biological N Fixation

Biologically fixed N is a free and renewable resource that plays a key role in sustainable agriculture. Biological N fixation (BNF) is seen to a varying degree in the soil environment including various parts of plants grown in soil. The potential for N fixation exists in any environment capable of supporting the growth of N-fixing bacteria (NFB). The global terrestrial amount of BNF is estimated between 100 and 290 million mt per year (Cleveland et al. 1999), 40–48 million mt per year of which

is fixed by agricultural crops in fields (Jensen and Nielsen 2003), corresponding to nearly one-half of global fertilizer usage. Symbiotic BNF in leguminous plants is famous, and N-fixing bacterial symbionts of legumes can contribute up to 20% of all plant N that is annually acquired by vegetation (Cleveland et al. 1999), and its amount reaches as high as 300 kg N/ha/crop. In addition to symbiotic relations, BNF through the activities of free-living N fixers is often seen in non-leguminous plants, e.g., sugarcane (Urquiaga et al. 1992), rice (Shrestha and Ladha 1996), and sago palm (Shrestha et al. 2006). Free-living N-fixing bacteria, which are ubiquitous in terrestrial ecosystems, can also contribute significantly to the N budget of some systems. Cleveland et al. (1999) reported free-living N-fixing bacteria fix relatively small amounts of N (<3 kg N/ha/year), but there are studies reporting larger contributions, such as 3.3–7.8 kg N/ha/year in tropical forest ecosystems (Reed et al. 2011) and 150 kg N/ha/crop in sugarcane (Dobereiner 1997). In oil palm plantations, legume covers are sometimes used under tree crops for the purpose of weed suppression, soil erosion control, and biological control of insect pests (Agamuthu and Broughton 1985). In addition to these, symbiotic BNF is expected; a report found 0.3–34.2 kg N/ha fixed by legume covers (Pipai 2014).

15.3 Taxonomy of N-Fixing Bacteria in Palm Trees

Many free-living N-fixing bacteria have been reported in different crops (Table 15.2). In sago palm, Shrestha et al. (2006) reported that different genera of N-fixing bacteria, such as *Klebsiella pneumoniae*, *K. oxytoca*, *Pantoea agglomerans*, *Enterobacter cloacae*, *Burkholderia* sp., *Stenotrophomonas maltophilia*, and *Bacillus megaterium*, were isolated from the root, rachis, petiole, leaflet, bark, and pith collected from sago palms in the Philippines. *Enterobacter* sp., *Klebsiella* sp., and *Pantoea* sp. were isolated from extracted starch (Shrestha et al. 2007). Shipton et al. (2010) also focused on starch extracted from sago palm trees and isolated *Enterobacter oryzae*, *Klebsiella oxytoca*, and *Cronobacter* (former *Enterobacter*) *turicensis* as N fixers. They also isolated *Pectobacterium* (former *Erwinia*) *cypripedii* from the rhizosphere of sago palm. Few studies have been done on N-fixing bacteria colonizing palm trees. Except for the studies by Shrestha et al. (2006) and Shipton et al. (2010), Reis et al. (2000) reported that oil palm was colonized by *Azospirillum brasilense*, *A. amazonense*, and *Herbaspirillum seropedicae*, and Tang et al. (2010) reported that *A. amazonense* was isolated from the root of sago palm and that *Burkholderia vietnamiensis* and *B. kururiensis* from the root of nipa palm. The genus *Azospirillum* is a Gram-negative free-living N-fixing rhizosphere bacterium, and *A. amazonense* was first isolated from roots and rhizosphere soil of Gramineae, in the Amazon region, Brazil (Steenhoudt and Vanderleyden 2000). *Burkholderia vietnamiensis* was the only known N-fixing species of this bacterial genus and was first isolated from the rhizospheres of rice, maize, and coffee plants (Santos et al. 2001). Since then, several species have been isolated from the endophytic environment of a wide range of taxonomically unrelated plants such as maize, sorghum, sugarcane,

Table 15.2 Lists of free-living N-fixing bacteria isolated from different plants

Plants	Isolated bacteria
Sugarcane (<i>Saccharum</i> spp.)	<i>Azospirillum</i> sp., <i>Azospirillum brasilense</i> , <i>Azospirillum lipoferum</i> , <i>Azospirillum amazonense</i> , <i>Azotobacter</i> sp., <i>Beijerinckia indica</i> , <i>Beijerinckia fluminensis</i> , <i>Burkholderia brasiliensis</i> , <i>Burkholderia tropicalis</i> , <i>Derxia</i> sp., <i>Enterobacteriaceae</i> , <i>Gluconacetobacter diazotrophicus</i> , <i>Gluconacetobacter azotocaptans</i> , <i>Herbaspirillum seropedicae</i> , <i>Klebsiella</i> sp., <i>Paenibacillus azotofixans</i> , <i>Pantoea</i> sp., <i>Vibrio</i> sp.
Maize (<i>Zea mays</i>)	<i>A. lipoferum</i> , <i>Azotobacter vinelandii</i> , <i>Bacillus circulans</i> , <i>Burkholderia</i> sp., <i>B. vietnamiensis</i> , <i>Enterobacter</i> sp., <i>Enterobacter cloacae</i> , <i>Klebsiella terrigena</i> , <i>P. azotofixans</i>
Rice (<i>Oryza sativa</i>)	<i>A. lipoferum</i> , <i>Azospirillum irakense</i> , <i>Azotobacter</i> sp., <i>B. brasiliensis</i> , <i>Clostridium</i> sp., <i>E. cloacae</i> , <i>H. seropedicae</i> , <i>K. oxytoca</i> , <i>K. pneumoniae</i> , <i>Pantoea agglomerans</i> , <i>P. azotofixans</i> , <i>Pseudomonas</i> sp.
Wheat (<i>Triticum</i> spp.)	<i>A. lipoferum</i> , <i>Azotobacter</i> sp., <i>Bacillus</i> sp., <i>E. cloacae</i> , <i>Klebsiella oxytoca</i> , <i>Klebsiella pneumoniae</i> , <i>P. agglomerans</i>
Bahia grass (<i>Paspalum notatum</i>)	<i>Acetobacter paspali</i> , <i>Acetobacter halopareferans</i> , <i>P. agglomerans</i>
Banana (<i>Musa</i> spp.)	<i>K. pneumoniae</i> , <i>E. cloacae</i> , <i>H. seropedicae</i> , <i>Herbaspirillum rubrisubulbicans</i>
Cereals	<i>H. seropedicae</i> , <i>A. lipoferum</i> , <i>A. amazonense</i> , <i>A. irakense</i> , <i>A. brasilense</i> , <i>B. brasilense</i>
Coffee	<i>Gluconacetobacter johannae</i> , <i>G. azotocaptans</i>
Dune grass	<i>B. tropicalis</i> , <i>P. agglomerans</i> , <i>Stenotrophomonas maltophilia</i>
Forage grasses	<i>H. seropedicae</i> , <i>A. brasilense</i> , <i>A. lipoferum</i> , <i>B. brasiliensis</i>
Kallar grass (<i>Leptochloa fusca</i>)	<i>A. halopareferans</i> , <i>Azoarcus</i> sp., <i>E. cloacae</i> , <i>P. agglomerans</i>
Mangrove	<i>Azotobacter chroococcum</i> , <i>A. vinelandii</i> , <i>A. beijerinckii</i>
<i>Miscanthus</i> spp.	<i>A. doebereinae</i> , <i>A. lipoferum</i> , <i>H. frisingense</i>
Oak	<i>B. megaterium</i> , <i>Pseudomonas putida</i> , <i>P. fluorescence</i> , <i>Xanthomonas oryzae</i>
Oil palm (<i>Elaeis</i> spp.)	<i>A. brasilense</i> , <i>A. amazonense</i> , <i>H. seropedicae</i>
Pine (<i>Pinus</i> spp.)	<i>Bacillus</i> sp., <i>Burkholderia gladioli</i> , <i>Burkholderia glathei</i>
Pineapple (<i>Ananas comosus</i>)	<i>Acetobacter diazotrophicus</i> , <i>H. rubrisubulbicans</i>
Sago palm (<i>Metroxylon sagu</i>)	<i>B. megaterium</i> , <i>K. pneumoniae</i> , <i>K. oxytoca</i> , <i>E. cloacae</i> , <i>P. agglomerans</i>
<i>Spartina</i>	<i>A. brasilense</i> , <i>A. lipoferum</i> , <i>A. vinelandii</i> , <i>Azotobacter chroococcum</i> , <i>G. diazotrophicus</i> , <i>Herbaspirillum frisingense</i> , <i>H. seropedicae</i> , <i>P. stutzeri</i>
Sweet potato (<i>Ipomoea batatas</i>)	<i>Acetobacter diazotrophicus</i> , <i>P. agglomerans</i>

pineapple, and coffee, and currently nine diazotrophic plant-associated *Burkholderia*, including *B. kururiensis*, have been validly described (Wong-Villarreal and Caballero-Mellado 2010). These studies conclude that palm trees harbor N fixers such as facultative anaerobic enteric bacteria, *Burkholderia* and *Azospirillum*, which have been also isolated from major crops, such as rice and maize.

15.4 Enhancement of N-Fixing Ability by Microbial Interactions

BNF requires many factors; the most important element among them is a carbon substrate. In symbiotic BNF, abundant substrate is provided through photosynthesis by the host plant, while in free-living BNF, a crucial issue is how to obtain enough substrate for the energy source. For that reason, many free-living N-fixing bacteria are associated with plants. In general, NFB utilizes exclusively simple carbon sources like glucose and sucrose (Haahtela et al. 1983). In contrast, the major carbohydrates in plants are complex carbon sources like cellulose, hemicellulose, and pectin (Lack and Evans 2001). Thus the effects of microbial interactions between NFB and indigenous bacteria, both of which were isolated from sago palm, on BNF were investigated. All NFB isolated from sago palm preferred simple sugars, like glucose, sucrose, and lactate, as their substrate for N fixation and showed extremely low levels of N-fixing activity in starch, hemicellulose, and pectin-containing media (Fig. 15.3) (Shrestha et al. 2007). N-fixing activity by NFB was markedly enhanced by the consortium of starch-degrading *Bacillus* sp. strain B1 in a starch medium, although the NFB showed negligible N-fixing activity when inoculated singly (Fig. 15.4). The consortium of hemicellulose-degrading *Agrobacterium* sp. strain

Fig. 15.3 Nitrogen-fixing activity of nitrogen-fixing bacteria in different carbon substrates. *Glu* glucose, *Suc* sucrose, *Lac* lactate, *Pec* pectin (Shrestha et al. 2007)

Fig. 15.4 Effect of co-inoculation of nitrogen-fixing bacteria (S1, S2, R1, and R2) and polymer-degrading bacteria on the nitrogen-fixing activity in polymer media (Shrestha et al. 2007)

Fig. 15.5 Effects of co-inoculation of randomly isolated strains (each group consisted of ten strains) on nitrogen-fixing ability of nitrogen-fixing bacteria (R1 and R2) (Shrestha et al. 2007)

HMC1 or *Flexibacter* sp. strain HMC2 and NFB also showed enhanced N-fixing activity in a hemicellulose medium. Such stimulation was not observed in a pectin medium. These results suggest that N-fixing bacteria may actively fix N in collaboration with degraders of starch and hemicellulose, the major carbohydrates in sago palm plant bodies.

The second important parameter in BNF is an oxygen level which affects nitrogenase activity directly (Robson and Postgate 1980). Coculture of NFB and indigenous bacteria, isolated from sago palm using nutrient agar medium, in a nitrogen-free Rennie medium containing simple organic compounds showed significantly higher N-fixing activity than single inoculations of NFB did in almost all combinations (Fig. 15.5), suggesting that co-inoculated bacteria consumed O₂ and made conditions with deficient O₂. Indeed, we confirmed that a reduced oxygen status itself enhanced the N-fixing activity of NFB (Fig. 15.6) (Shrestha et al. 2007).

Fig. 15.6 Effect of ambient (white bar) and reduced O₂ (gray bar) conditions on the nitrogen-fixing activity of selected nitrogen fixers (R1, R2, R3, and R4) (Shrestha et al. 2007)

These results may indicate that beneficial microbial interactions occur in sago palm to enhance N-fixing activity through collaborative utilizations of starch, hemicellulose, and their degradation products.

15.5 Estimate of Amounts of N Fixation in Palms

Shrestha et al. (2006, 2007) quantified N-fixing activity in different parts of sago palm, such as stem, leaf, root, bark, and starch, and isolated a wide range of N-fixing bacteria. Although the estimation of fixed N based on the acetylene-reducing activity contains some uncertainties, the amount of fixed N in a mature sago palm stand was estimated at 210 kg N/ha/y from calculation based on N-fixing activity in root (221 nM C₂H₄/g/day), pith (213 nM C₂H₄/g/day), and leaf sheath (35.4 nM C₂H₄/g/day) and their mean weights per palm (68, 721, and 97 kg in root, pith, and leaf sheath, respectively) with a sago palm density in a mature forest of 923 sago palms/ha (Toyota 2015).

Yonebayashi et al. (2014) estimated the amount of N fixation in sago palm using a tracer experiment. They measured $\delta^{15}\text{N}$ values of the youngest leaves of sago palms at different stages of growth and found that $\delta^{15}\text{N}$ values were lower with age, indicating higher N fixation with maturing. The contribution of biologically fixed N to total N of sago palm leaves was less than 10% at the beginning of growth stage (3 years), but it increased with age and reached more than 90% at the later stage (13 years).

Amounts of N fixation have been estimated in other palms. De Carvalho et al. (2008) reported that 13–76% of total N in oil palm depended on biological N fixation in a 1-year growth experiment using N poor quartz sand and subsoil. Zakry et al. (2012) found that the contribution of biological N fixation to total N uptake by oil palm was different depending on the plant parts in oil palm (75% in leaflet, 13% in stem, and 13% in root) and was 63% on the whole plant basis. These results reveal that BNF functions as a biofertilizer and is essential in establishing sustainable crop production.

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