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ORIGINAL PAPER

Responses of *Bruguiera sexangula* propagules to beneficial microbes in the nursery

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Abstract *Bruguiera sexangula* (Lour) Poir., a threatened mangrove tree, was inoculated with beneficial microbes in a nursery to assess any improvements in growth and biomass. From soil samples from the rhizosphere of *B. sexangula* in a mangrove forest in Panangadu of Kerala India, nitrogen-fixing bacteria *Azotobacter chroococcum* and *Azospirillum brasilense* were isolated. The phosphate-solubilising bacterium *Bacillus megaterium* and potassium-mobilizing bacteria *Frateruria aurantia* were also isolated and cultured on suitable media. Later, ripe propagules of *B. sexangula* were collected from matured trees and raised in sterilized soil bags (13 × 25 cm) containing sterilized soil and sand (2:1 ratio). The cultured beneficial microbes were propagated and used to inoculate the ripe propagules of *B. sexangula* and maintained in the nursery for 6 months. After 6 months, growth and biomass of the inoculated propagules were greater than for the uninoculated control propagules. Shoot length, number of leaves, stem girth and root length were also significantly greater than in the controls. This study showed that the mangrove-specific beneficial microbes influenced the growth of *B. sexangula* in the nursery and will help in the establishment of *B. sexangula* in degraded mangrove forests.

Keywords Mangroves · *Bruguiera sexangula* · Beneficial microbes · Nursery · Propagules

Introduction

Bruguiera sexangula (Lour) Poir., is a mangrove tree distributed in the coastal areas of Papua New Guinea, Bangladesh, Myanmar, Vietnam, northern parts of Australia and coastal regions of India. Listed as threatened by the IUCN (Duke et al. 2010), this tree species is mainly threatened by habitat destruction due to shrimp culture, agriculture and by urban development in India. Because *B. sexangula* and other mangrove species are ecologically important for protecting coastal areas and marine ecosystems, their cultivation and the development of growth improvement techniques in the nursery has to be studied. Certain soil bacteria from the rhizosphere of plants can enhance the growth of the plants (Rodriguez and Fraga 1999). These beneficial free-living soil bacteria, known as plant growth-promoting rhizobacteria, include nitrogen-fixing bacteria, phosphate-solubilizing bacteria, potassium-mobilizing bacteria and biocontrol agents (Sudhakar et al. 2000). The beneficial effects are due to the production of plant hormones such as auxins, gibberellins and cytokinins or to biological fixation of nitrogen. They can also improve plant growth indirectly by suppressing bacterial, fungal and nematode pathogens and producing compounds such as siderophores, ammonia, antibiotics, and volatile metabolites (Glick 1995). Beneficial microbes are also used with tree crops in mine spoil recovery (Karthikeyan et al. 2009; Diagne et al. 2013). Because beneficial microbes are

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clearly a prerequisite for successful growth and biomass improvement in tree and agricultural crops, we isolated beneficial microbes to treat propagules of *B. sexanugla* to improve growth and biomass in the nursery. This first assessment of the effect of cultured beneficial microbes on the response of rooted propagules of *B. sexangula* should inform efforts to reforest degraded mangroves.

Materials and methods

Collection of samples

The study site at Panangadu, Kerala, India (10°26′89″N and 76°19′33″E) has a small (ca. 2.5 ha), mixed patch of mangrove forests consisting of *Avicinia officinalis*, *B. sexangula* and *Rhizophora mucronata*. Among these mangrove species, *B. sexangula* is the dominant mangrove species (41% of the density). The average height of *B. sexangula* trees is (16.5 ± 1.4) m. The mean relative humidity was (72.4 ± 1.2)%, and mean temperature was (32.29 ± 1.1) °C.

Rhizosphere soils adhering to the root system from fully grown *B. sexangula* trees were collected carefully and the physicochemical properties assessed according to Jackson (1973); pH was (6.53 ± 0.08), electrical conductivity (1.3 ± 0.001) mS, nitrogen (N) (24.9 ± 1.3) mg kg^{-1}, phosphorus (P) (20.1 ± 1.4) mg kg^{-1} and potassium (K) (19.2 ± 1.2) mg kg^{-1}. Mean relative humidity was (72.4 ± 0.53)%, and mean temperature of the locality was (32.29 ± 45) °C. Propagules of *B. sexangula* were later collected from the mangrove forests at Panangadu during the fruiting season (June–July) and stored in paper bags. These viviparous seedlings had an average hypocotyl length of (14.5 ± 0.55) cm and weighed (12.8 ± 0.58) g.

Isolation of beneficial microbes

Azotobacter chroococcum

Four 0.1-mL samplesof soil, serially diluted in sterile water from 10^{-3} to 10^{-6}, were plated to isolate *Azotobacter* on Jensen's agar (Jensen 1942) containing 20 g of sucrose, 1 g of K_2HPO_4, 0.5 g of $MgSO_47H_2O$, 0.1 g of $FeSO_4$, 0.005 g of Na_2MoO_4, 20 g of agar and 1-L of distilled water (pH 6.9). Thereafter, the plates were then incubated at 32 °C. After 10 days, slimy blackish *Azotobacter* colonies were noticed. The colonies were identified as *Azotobacter chroococcum* based on morphological characters. These *Azotobacter chroococcum* colonies were further multiplied in Jensen's broth and stored at 4 °C.

Azospirillum brasilense

For isolating *Azospirillum*, 1-g of collected soil sample was serially diluted to 10^{-8} dilutions with sterile distilled water and placed on Congo red agar (Rodriguez Caceres 1982) containing 0.05 g of K_2HPO_4, 0.02 g of $MgSO_47H_2O$, 0.01 g of NaCl, 0.005 g of yeast extract, 0.0015 g of $FeCl_36H_2O$, 0.5 g of DL-malic acid, 0.48 g of KOH, 20 g of agar and 1-L of distilled water. Fifteen milliliters of 1:400 Congo red solution was added to the medium as an indicator. The samples were then incubated at 32 °C for 5 days. After 5 days, pinkish scarlet colonies were evident as they absorbed the Congo red, suggesting that the isolated beneficial microbe was *Azospirillum brasilense*. These colonies were multiplied in Congo red broth and stored at 4 °C.

Phosphate-solubilizing bacteria (PSB)

Rhizosphere soils from *B. sexangula*, serially diluted to 10^{-9}, were placed in Petri plates of Pikovskya medium containing 0.5 g of yeast extract, 10 g of dextrose, 5 g of $Ca_3(PO_4)_2$, 0.5 g of $(NH_4)_2SO_4$, 0.2 g of KCl, 0.1 g of$MgSO_47H_2O$, 0.0001 g of $FeSO_4$, 0.0001 g of $MnSO_4$, 20 g of agar and 1-L of distilled water (pH 7.0) (Pikovskaya 1948). The plates were then incubated at 32 °C for 5 days. After 5 days, colonies formed a clear zone on the medium, confirming them as PSB. The white colonies were identified as *Bacillus megaterium* based on their white, slimy colony morphology. They were further multiplied in Pikovskya broth and stored at 4 °C.

Potassium-mobilizing bacteria

Similar soil dilutions were plated on modified Aleksandrov agar (Hu et al. 2006) containing 5 g of glucose, 0.005 g of $MgSO_47H_2O$, 0.1 g of $FeCl_2$, 2 g of $CaCO_3$, 2 g of mica powder, 2 g of $CaPO_4$ and 1-L of distilled water (pH 7.2). The Petri plates containing the soil dilution and Aleksandrov medium were incubated at 28 °C for 3 days. After 3 days of incubation, light-orange colonies surrounded by a clear zone were found on the medium. Based on these morphological characters, the colonies were identified as *Frateuria aurantia*. The colonies were further multiplied in Aleksandrov broth and stored at 4 °C.

Nursery experiments

B. seangula propagules were propagated and inoculated with beneficial microbes at the silviculture nursery of IFGTB, Coimbatore, India. The collected propagules of *B. sexangula* were sterilized with 80% ethanol for 2 min followed by 0.1% $HgCl_2$ for 10 min. Later, the sterilized

propagules were washed 10 times with sterile distilled water. Thus, treated uniformly sized (15 cm) propagules were planted in poly bags (13 × 25 cm) containing sterilized loamy soil + sand (1:1v/v) and maintained in the nursery for 30 days in a shade house and watered twice per day to develop roots. After roots developed, the propagules were treated with the beneficial microbes cultured in broth at a constant concentration as shown below:

T1: *Azospirillum brasilense*@10 mL/propagule
T2: *Azotobacter chroococcum*@10 mL/propagule
T3. *Bacillus megaterium*@10 mL/propagule
T4: *Frateuria aurantia*@10 mL/propagule
T5. *Azospirillum brasilense*@5 mL + *Azotobacter chroococcum*@5 mL/propagule
T6: *Azospirillum brasilense*@5 mL + *Bacillus megaterium* @5 mL/propagule
T7: *Azospirillum brasilense*@5 mL + *Frateuria aurantia* @5 mL/propagule
T8: *Azotobacter chroococcum*@5 mL + *Bacillus megaterium*@ 5 mL/propagule
T9: *Azotobacter chroococcum*@5 mL + *Frateuria aurantia*@5 mL/propagule
T10: *Bacillus megaterium* @5 mL + *Frateuria aurantia* @5 mL/propagule
T11: *Azospirillum brasilense*@2.5 mL + *Azotobacterchroococcum*@2.5 mL + *Bacillus megaterium*@2.5 mL + *Frateuria aurantia*@2.5 mL/propagule
T12: Control (un inoculated propagules)

The 12 treatments including the control were replicated 10 times using 5 bags per treatment. Hence, a total of 600 propagules were used in this study. These treatments were arranged in a randomized block design in the nursery at (32 ± 1.2) °C and (74 ± 2.6)% RH.

Harvest and analyses

Six months after inoculation, the seedlings were harvested with their entire root system intact. For each seedling root length, shoot length, collar diameter were measured, and leaves were counted. Shoots and roots were weighed after they were dried in an oven at 50 °C for 48 h.

Tissue nutrient analysis

Dried (5 g) root and shoot samples were digested with catalyst mixture (potassium sulphate and copper sulphate 5:1) and triple acid (nitric acid + sulphuric acid + perchloric acid; 9:3:1) in Kjeltec digestion system at 420 °C for 1 h. Later, the samples were analyzed for N, P, and K according to the method of Jackson (1973).

Soil nutrient analysis

Air-dried soil samples (1 g) used in the nursery experiments as potting media with the beneficial microbes were also analyzed for N, P, and K (Jackson 1973).

Statistical analysis

All data were statistically analyzed using Duncan' smultiple range test in SPSS ver. 16 (SPPS, Inc., Chicago, USA).

Results

Growth improvement in *B. sexangula*

The microbe-inoculated propagules of *B. sexangula* had significant ($p < 0.05$) increase in growth and biomass compared with the control propagules (Table 1). Treatment T11 (*Azotobacter chroococcum* + *Azospirillum brasilense* + *Bacillus megaterium* + *Frateuria aurantia*) yielded significantly ($p < 0.05$) greater shoot length, root length, number of leaves and collar diameter than any of the other single or multiple species (Table 1). Dual inoculation T9 with *Azotobacter chroococcum* + *Frateuria aurantia* yielded the largest increase (significant at $p < 0.05$) in collar diameter (Table 1). More leaves (significant at $p < 0.05$) were produced on propagules inoculated with multiple microbes (T5, T6, T7, T8, T9, T10) than single beneficial microbes inoculated propagules (Table 1).

Tissue nutrient content

Major tissue nutrients such as N, P, and K increased significantly ($p < 0.05$) for *B. sexangula* propagules inoculated with combination of beneficial microbes (T11). Interestingly, propagules inoculated with *F. aurantia* alone or in combination with other microbes (T4, T7, T9, T10, T11) had significantly ($p < 0.05$) higher K content than propagules inoculated with other beneficial microbes (Fig. 1).

Soil nutrients

The major soil nutrients N, P, and K were significantly higher in soil with beneficial microbes (T1–T11) than in the control (T12) soil (Fig. 2).

Table 1 Growth response of *B. sexangula* seedlings after inoculation with beneficial microbes (mean of 10 replicates)

Treatment	Root length (cm plant^{-1})	Shoot length (cm plant^{-1})	Collar diameter (cm plant^{-1})	Number of leaves (plant^{-1})	Root dry mass (g plant^{-1})	Shoot dry mass (g plant^{-1})	Root/shoot biomass
T1	18.37 ab	32.17 b	2.47 bc	11.33 ab	6.44 b	10.22 b	0.63 a
T2	33.33 cde	35.33 b	2.33 ab	11.00 ab	6.22 b	11.26 b	0.55 b
T3	37.00 def	39.33 bc	2.57 bcd	10.67 ab	6.63 b	9.56 b	0.69 a
T4	50.33 f	39.45 bc	2.43 bc	12.33 b	6.61 b	11.25 b	0.58 a
T5	40.00 def	41.67 cd	2.47 bc	12.01 b	7.76 bc	14.38 c	0.53 b
T6	44.17 ef	42.67 cd	2.77 bcd	12.67 bc	7.79 bc	11.64 b	0.66 a
T7	40.53 def	40.83 cd	2.80 bcd	11.05 ab	8.54 c	14.64 c	0.58 a
T8	38.60 def	42.33 cde	2.80 bcd	16.33 d	9.36 cd	15.28 cd	0.61 a
T9	41.00 def	44.33 cde	3.37 e	11.66 ab	8.58 c	16.11 d	0.53 b
T10	42.33 def	45.67 def	2.87 cd	13.67 bcd	8.67 c	16.27 d	0.53 b
T11	50.67 f	50.10 f	2.90 cde	15.67 d	12.6 e	25.35 e	0.49 c
T12	9.33 a	21.33 a	1.96 a	8.67 a	4.21 a	6.58 a	0.63 a

T1: *Azospirillum brasilense*; T2: *Azotobacter chroococcum*; T3: *Bacillus megaterium*; T_4: *Frateuria aurantia*; T5: *Azospirllum-brasilense* + *Azotobacter chroococcum*; T6: *Azospirillum brasilense* + *Bacillus megaterium*; T7: *Azospirillum brasilense* + *Frateuria aurantia*; T8: *Azotobacter chroococcum* + *Bacillus megaterium*; T9: *Azotobacter chroococcum* + *Frateuria aurantia*; T10: *Bacillus megaterium* + *Frateuria aurantia*; T11: *Azospirillum brasilense* + *Azotobacter chroococcum* + *Bacillus megaterium* + *Frateuria aurantia*; T12: Control. Means followed by the same letter are not significantly different according to Duncan's multiple range test ($p < 0.05$)

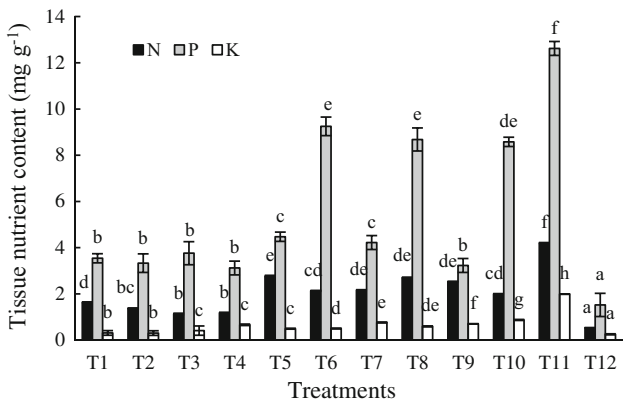

Fig. 1 Tissue nutrient content in *B. seangula* propagules after inoculation with various microbes and their combinations. T1: *Azospirillum brasilense*; T2: *Azotobacter chroococcum*; T3: *Bacillus megaterium*; T4: *Frateuria aurantia*; T5: *Azospirllum brasilense* + *Azotobacter chroococcum*; T6: *Azospirillum brasilense* + *Bacillus megaterium*; T7: *Azospirillum brasilense* + *Frateuria aurantia*; T8: *Azotobacter chroococcum* + *Bacillus megaterium*; T9: *Azotobacter chroococcum* + *Frateuria aurantia*; T10: *Bacillus megaterium* + *Frateuria aurantia*; T11: *Azospirillum brasilense* + *Azotobacter chroococcum* + *Bacillus megaterium* + *Frateuria aurantia*; T12: Control. Means followed by the same letter are not significantly different according to Duncan's multiple range test ($p < 0.05$). Error bar indicates SE (±) of mean

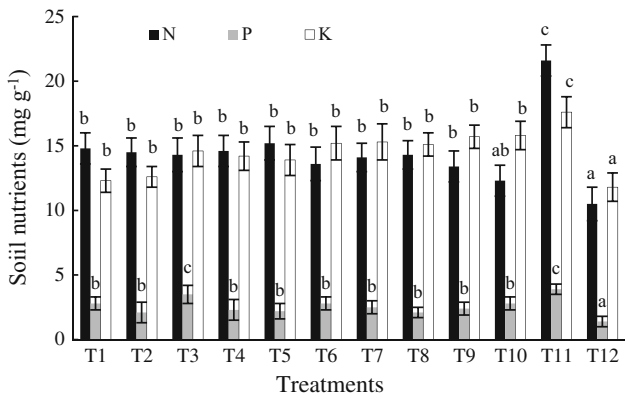

Fig. 2 Mean nutrient content ($N = 10$ replicates)in soil medium used to grow *B. sexangula* propagules after inoculation with various microbes and their combinations. T1: *Azospirillum brasilense*; T2: *Azotobacter chroococcum*; T3: *Bacillus megaterium*; T4: *Frateuria aurantia*; T5: *Azospirllum brasilense* + *Azotobacter chroococcum*; T6: *Azospirillum brasilense* + *Bacillus megaterium*; T7: *Azospirillum brasilense* + *Frateuria aurantia*; T8: *Azotobacter chroococcum* + *Bacillus megaterium*; T9: *Azotobacter chroococcum* + *Frateuria aurantia*; T10: *Bacillus megaterium* + *Frateuria aurantia*; T11: *Azospirillum brasilense* + *Azotobacter chroococcum* + *Bacillus megaterium* + *Frateuria aurantia*; T12: Control. Means followed by the same letter are not significantly different according to Duncan's multiple range test ($p < 0.05$). Error bar indicates SE (±) of mean

Discussion

Mangrove ecosystems are important for marine fauna and protecting coastal regions from natural disorders. The total economic value of mangrove forests is estimated to be US $4,370,000 per hectare (Malik et al. 2015). However, due to anthropogenic pressures and increased land-use for human habitation, the area with mangrove vegetation is being depleted at an alarming rate (Ravishankar and Ramasubramanian 2004), causing a decline in coastal

fisheries in many tropical and subtropical countries (Bashan and Holguin 2002). For restoring the mangroves with suitable mangrove plant species, it is necessary to maintain microbial communities in the mangrove ecosystem that will help to conserve essential nutrients.

Microbes in the mangrove ecosystem are mainly considered to play role intrans forming essential nutrients from soil (Kathiresan and Bingham 2001), and they are also involved in primary production, decomposition and nutrient recycling (Kathiresan 2010). In turn, mangrove roots fuel microbial communities by oxidizing the soil (Sherman et al. 1998). Therefore, we considered that the inoculation of mangrove propagules with beneficial microbes was a prerequisite for the successful establishment of mangroves in degraded areas. Microbial diversity in Indian mangroves, especially for nitrogen fixers (*Azotobacter* and *Azospirillum*) and phosphate solubilizers, were also reported in earlier studies (Kathiresan and Bingham 2001; Kathiresan and Masilamani 2005). However, the inoculation of propagules of endangered and threatened mangrove species such as *B. sexangula* with beneficial microbes in this study revealed a significant increase in subsequent growth and biomass of the plants.

In this present study, N-fixers, P-solubilizer and K-mobilizer were isolated and used toward improving the quality of the planting propagules of *B. sexangula*. Nitrogen-fixing beneficial microbes were isolated by Sengupta and Chauduri (1990, 1991) from the rhizosphere and roots of various mangrove species, and P-solubilising microbes were isolated from the rhizosphere of mangroves (Vazquez et al. 2000). However, the K-mobilising bacterium *F. aurantia* isolated from the roots of *B. sexangula* in our present study is a new finding.

Inoculation of *Rhizophora* seedlings with *Azotobacter chroococcum* isolated from mangrove rhizosphere improved shoot biomass up to 29.49% and increased growth and total biomass (Ravikumar et al. 2004). In the present study, the N-fixers *Azotobacter chroococcum* and *Azospirillum brasilense* significantly increased the collar diameter and shoot biomass. Thus, these microbes are very important for mangroves to convert in soluble forms of N, P, and K into soluble forms (Chen et al. 2008), thereby increasing their uptake by seedlings of *B. sexangula* in the nursery. This result is in accord with Toledo et al. (1995) who found that total N in black mangrove seedlings inoculated with N-fixing microbes was significantly higher than control seedlings, the inoculated seedlings also had significantly more leaves than in the control seedlings. Rojas et al. (2001) found similar results in black mangrove seedlings.

The multiple-species inoculation promoted nutrient uptake and increased biomass and seedling growth of *B. sexangula* better than inoculation with one or two of the microbial species in this study. Inoculation with multiple species similarly increase growth and biomass in multiple ways in *Azadirachta indica* better than any of the species alone, suggesting synergistic actions among the microorganisms were responsible for the enhanced promotion (Muthukumar et al. 2001). Similarly, in soil after combined inoculation with four microbial species increased nutrient uptake by *Casuarina* trees more than any of the individual species or combinations with fewer species (Rajendran and Devaraj 2004). Bashan et al. (2000) found that mangrove rhizosphere bacteria increased P content and various measures of plant growth, but N was significantly lower in *Salicornia bigelovii* at the end of the growing season. Normally, chemical fertilizers such as diammonium phosphate and urea are used to improve the growth of mangroves in nurseries (Ravishankar and Ramasubramanian 2004), but this study provides a method for successful improvement and establishment of mangroves in nurseries without using chemical fertilizers that will help restore degraded mangroves using mangrove-inhabiting beneficial microbes.

Conclusion

Inoculation of mangrove seedlings with beneficial microbes is an ecofriendly approach for restoring degraded mangrove vegetation. The results of the study showed that growth and biomass of *B. sexangula* propagules was the best after inoculation with a combination of beneficial microbes. The beneficial microbes isolated from the mangrove rhizosphere should be further tested for promoting growth and biomass of mangrove propagules in mangrove wetlands to aid restoration programs.

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