J Plant Growth Regul (2012) 31:588–598
DOI 10.1007/s00344-012-9269-5

Evaluation of Multispecies Plant-Growth-Promoting Consortia for the Growth Promotion of *Jatropha curcas* L.

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Received: 19 November 2011 / Accepted: 13 February 2012 / Published online: 21 March 2012

Abstract Direct interactions that occur between members of different microbial types often result in the promotion of key processes that benefit plant growth and health. In the present study, four isolates, *Brevibacillus brevis* (MS1), *Bacillus licheniformis* (MS3), *Micrococcus* sp. (MS4), and *Acinetobacter calcoaceticus* (MS5), were used to develop multispecies consortia. They have the ability to produce IAA, solubilize inorganic P, and produce ACC deaminase and siderophore. The growth profile of MS1 was similar in monospecies and mixed-species cultures, but about a 24 % increase in mean growth rate was recorded for MS5. They enhanced the growth of *Jatropha curcas* in individual trials. Plant growth further improved maximally when the three were applied together. All four strains enhanced *Jatropha* growth in greenhouse and field experiments. Co-inoculation provides the largest and most consistent increases in shoot weight, root weight, total biomass, shoot and root length, total chlorophyll, shoot width, and grain yield.

Keywords *Brevibacillus brevis* · *Bacillus licheniformis* · *Acinetobacter calcoaceticus* · *Micrococcus* sp. · Plant-growth-promoting consortia (PGPC) · Multispecies consortia

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Introduction

Bacteria of different genera, existing in close proximity, are thought to aid each other in growth and survival via gene transfer and metabolic cross-feeding. The latter case has been relatively well studied with bacteria that provide amino acids or vitamins to other strains with biosynthetic deficiencies. More recently, there has been interest in elucidating microbial metabolic cooperation that functions in the catabolism of organic compounds. Direct interactions that occur between members of different microbial types often result in the promotion of key processes that benefit plant growth and health. Syntrophic relationships between different organisms have been demonstrated in several microbial ecosystems. Therefore, mixed inoculants (combination of microorganisms) that interact synergistically to yield better and quick results are currently being devised (Bashan 1998). A microbial consortium for plant growth promotion was suggested (Seneviratne 2003). It has been suggested that development of a plant-growth-promoting consortium (PGPC) could be a feasible strategy for increased activity and better viability of plant-growth-promoting rhizobacteria (PGPR). When these strains are made into an inocula consortium, each of the constituent strains of the consortium not only competes with the others for rhizospheric establishment, but is functionally complementary for plant growth promotion (Shenoy and Kalagudi 2003).

These bacteria were identified by biochemical identification as well as by 16S rDNA sequencing, and after phylogenetic analysis they were identified and the sequences were deposited in the NCBI gene bank. Because we are working on certain aspects of PGPR to find the characterization of the five best cultures as PGPR, we were interested in developing consortia with increased biofertilizer value and the ability to work with leguminous and nonleguminous plants.

Direct interactions that occur between members of different microbial types often result in the promotion of key processes that benefit plant growth and health. It is obvious that all interactions that take place in the rhizosphere are plant-mediated, at least indirectly. However, the plant acts as a "supporting actor" in the rhizosphere. Van Veen and others (1997) critically reviewed the reasons for the poor performance of agricultural bioinocula in natural environments and in the rhizosphere of host plants and suggested that instead of using a single strain for a single trait, multiple microbial consortia could be used for multiple benefits and could thrive together in unique ecological niches in ideal proportions. We used barren land for growing *Jatropha* with our developed consortia. *Jatropha* as a biofuel would replace petrol which is associated with economic and environmental concerns. These consortia helped increase the number of seeds, as discussed in the "Results" and "Discussion" sections below in terms of yield per plant. Thus, productivity of the plant was increased. On the other hand, it has been found that these bacteria would also interact synergistically by providing nutrients, removing some inhibitory products, or stimulating each other through physical or biochemical mechanisms.

Materials and Methods

Bacterial Cultures

Brevibacillus brevis (MS1), *Bacillus licheniformis* (MS3), *Micrococcus* sp. (MS4), and *Acinetobacter calcoaceticus* (MS5) were isolated and characterized according to their plant-growth-promoting attributes and were used in the present study. Only these four cultures were growing together so they were selected for the study; *Enterobacter aerogenes* (MS2) was not able to grow in any combinations.

The combination of isolates that showed growth together are as follows (T = treatment):

- *B. brevis* (MS1) + *B. licheniformis* (MS3): (T1)
- *B. brevis* (MS1) + *A. calcoaceticus* (MS5): (T2)
- *B. licheniformis* (MS3) + *A. calcoaceticus* (MS5): (T3)
- *B. licheniformis* (MS3) + *Micrococcus* sp. (MS4): (T4)
- *B. brevis* (MS1) + *B. licheniformis* (MS3) + *A. calcoaceticus* (MS5): (T5)
- *B. brevis* (MS1) + *B. licheniformis* (MS3) + *Micrococcus* sp. (MS4) + *A. calcoaceticus* (MS5): (T6)

Interaction of Microbes under Culture Conditions (Growth Profile Study)

To observe the interaction of different microbes under liquid and solid culture conditions, 100 ml broth in tryptic soybean broth (TSB) (Hi Media Laboratories, Mumbai, India) was used to inoculate the organisms at 1 % inoculum level. The growth curve of each of the four isolates was determined by the viable cell count method. The growth profiles of the four isolates in different combinations were determined by inoculating the early exponential phase culture in 50 ml of TSB broth. The combinations prepared for this study were T1, T2, T3, T4, T5, and T6 as explained above. In mixed cultures, an equal volume of the early exponential phase of each culture was mixed aseptically. Samples were withdrawn after every 4 h. The mean growth rate constant (K) was calculated using the formula: $K = 3.322\ (\log Z_t - \log Z_0)/Dt$; where Z_0 and Z_t are the initial and final cell populations, and Dt is the difference in culture time (Pandey and Maheshwari 2007). Under solid-culture conditions, lawns of each bacterial culture were prepared using four plates of TSB medium using 0.5 ml of the inoculum of each culture. After 2 h of incubation, wells of 1 mm diameter were made in the center of each plate, the wells were sealed, and culture filtrate/cell supernatant of each organism was added (Suneja and others 2007).

Characterization of Consortia for Plant-Growth-Promoting Potentials

PGPC have the potential to contribute to the development of sustainable agricultural systems. Generally, PGPC function in three different ways: they synthesize particular compounds for the plants, facilitate the uptake of certain nutrients from the soil, and prevent and control plant diseases. The coinoculated strains were also tested in liquid Pikovasky's medium for quantitative phosphate solubilization and change in pH (Gaur 1990). The production of indole-3-acetic acid (IAA) was determined using the method of Bric and others (1991). The IAA concentration in the consortia's broth was determined by using a standard curve of pure IAA (Bano and Musarrat 2003). Siderophore production was determined by performing the chrome azurol S (CAS) assay (Schwyn and Neiland 1987). All the glassware was cleaned with 6 N HCl. The medium was deferrated by extracting with 3 % 8-hydroxyquinoline in chloroform. The medium was then autoclaved to remove any residual chloroform. Consortia were raised in DF minimal medium at 30 °C to a density of 10^8 CFU/ml. Cells in the late log phase were removed by centrifuging at 3,000 rpm, and the filtrate was tested for siderophore in CAS agar medium. The quantitative estimation was performed according to the method of Chambers and others (1996). Specific tests were carried out for identification of hydroxamate and catecholate types of siderophores following standard methods (Arnow 1937).

Seed Bacterization

Jatropha seeds (*Jatropha curcas* SDAU J1 Chhatrapati), collected from the Regional Research Station S.D. Agriculture University, Sardarkrushinagar, Gujarat, were soaked

in 0.02 % sodium hypochlorite for 2 min and washed five times with sterilized distilled water. Seeds were coated with 1 % carboxymethylcellulose as an adhesive. Then seeds were treated with a bacterial strain for 30 min. Each consortium was inoculated in a 150 ml flask containing 60 ml medium and incubated at 28 ± 1 °C for 3 days. An optical density of 0.5 recorded at λ 535 nm was achieved by dilution to maintain uniform cell density (10^8–10^9 CFU/ml) (Gholami and others 2009). A daily record of seeds that had emerged out of the surface of the soil was kept. Germination continued to be recorded for 21–28 days. At the end of 28 days all the seeds that had not germinated were taken out and counted; they were then cut open to find whether they were still viable. The parameters germination percent, germination energy, germination capacity, and seedling vigor were calculated (Abdul-Baki and Anderson 1973).

Seed Surface Survival

Bacterialized seeds were placed in paper towels and air-dried in a laminar flow chamber overnight. Seed samples were placed in 30 × 15 cm^2 brown paper bags and stored at room temperature. After 1 week, a subsample of each seed lot was removed from storage and microbiological analyses of coated seeds and controls were conducted to determine microbial load. For enumeration of attached cells, 1 g of each seed lot was added to potassium phosphate buffer (0.06 M, pH 6.8) and stirred at 200*g* for 2 h at 30 °C. Bacterial viability of PGPR isolates was determined by plating on selective media (Amutha and others 2009).

Biometric Observations of *Jatropha* in Greenhouse Conditions

Ten inoculated seeds of *Jatropha* were sown in each earthen pot filled with sandy loam soil and watered regularly. Three such pots were maintained for each treatment. Uninoculated seeds sown in pots served as controls. For each observation, two plants from each replicate were randomly selected from each treatment and the mean of two plants was used as one replication. The experiment was repeated three times. Observations were recorded on rate of seedling emergence, root length, shoot length, chlorophyll content, leaf area, and dry mass of roots, shoots, and total plants, drawing random samples at 30, 60, 90, and 120 days after showing (DAS) (Tank and Saraf 2008).

Field Experiment

A field study was conducted to assess the coinoculation with different combinations developed for improving the growth and yield of *J. curcas* at the field site provided by Gujarat University, Ahmedabad. The surface soil was collected from the research area, air-dried, mixed thoroughly, passed through a 2-mm sieve, and analyzed for various physicochemical characteristics. Consortia-treated nursery polybags were transplanted in the field in a randomized block design. The row-to-row distance was 3 m and the plant-to-plant distance was 2 m. Plants were uprooted at different time intervals and vegetative parameters and yields were recorded after 2 years in the field.

Data Analysis

All greenhouse and field experiments were arranged in completely randomized block designs with three replications in each treatment and repeated three times. The data were subjected to analysis of variance (ANOVA), and mean values in each treatment were compared using least significant differences at 5 % probability ($P = 0.05$).

Results

Interaction of Microbes under Cultural Conditions (Growth Profile Study)

The five isolates used in the study were grown in parallel in different combinations on the tryptic soya agar plate to study their mutualistic growth. They showed overlapping growth on each other, except MS2, so they were selected for the further consortia study. Coinoculation was done by mixing 1 × $10^{2.3}$ CFU/ml of each isolate to achieve 1 × 10^8 CFU/ml of total population density. The populations of MS1, MS3, MS4, and MS5 were recorded as 3.3 × 10^7, 3.3 × 10^7, 3.2 × 10^7, and 3.6 × 10^7 CFU/ml, respectively. The same method was adopted for coinoculation of seeds, but 1 × 10^4 CFU/ml of each isolate was mixed to achieve 1 × 10^8 CFU/ml of total population density. *B. brevis* (MS1), *B. licheniformis* (MS3), *Micrococcus* sp. (MS4), and *A. calcoaceticus* (MS5) were grown in monoculture and a mixed-species consortium (Fig. 1). All isolates were fast growing. The *K* values of MS1, MS3, MS4, and MS5 were 1.17 ± 0.02, 1.21 ± 0.03, 0.83 ± 0.02, and 1.19 ± 0.04/h, respectively, in single-species cultures. When grown as multispecies mixed cultures, the *K* values of MS1 and MS4 remained unchanged while those of MS5 and MS3 increased to 1.47 ± 0.01 and 1.19 ± 0.05/h, respectively.

Characterization of Consortia for Plant-Growth-Promoting Potentials

Phosphate Solubilization for Coinoculated Strains

Phosphate solubilization for these five isolates was monitored up to 21 days in Pikovaskaya's broth (pH 7).

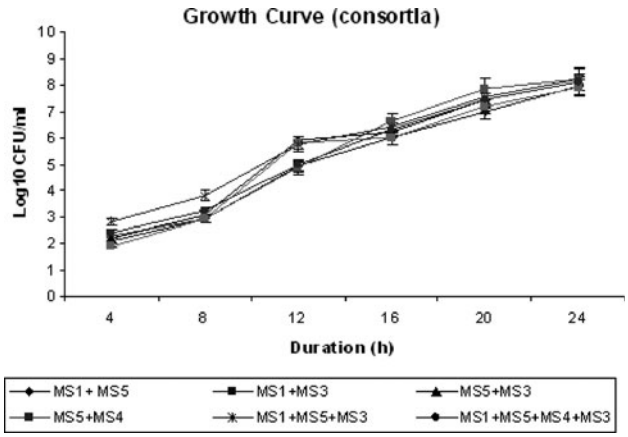

Fig. 1 Growth profile study of the developed consortia

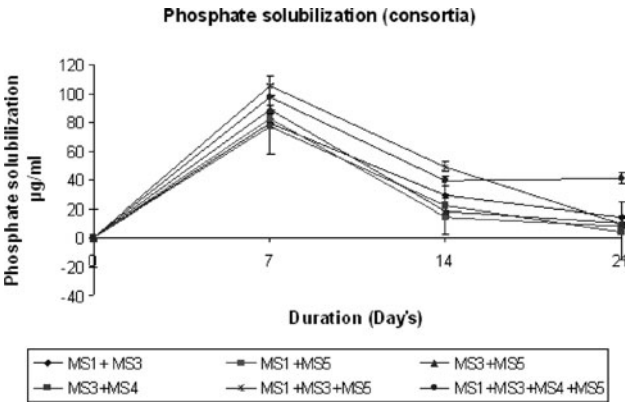

Fig. 2 Phosphate solubilization of the coinoculated strains

Maximum solubilization of P was achieved on the 7th day by MS3. In the mixed cultures, maximum solubilization was achieved by T5 on the 7th day with the level of soluble P gradually increasing up to the 7th day, with a maximum value of 105 µg/ml (Fig. 2). All the mixed cultures were found to lower the pH of the growth medium. A decrease in pH indicates the production of acids, which is considered to be responsible for P solubilization. In the coinoculated consortium, the maximum amount of soluble P by T5 was comparable with that by MS3 alone. In our study we found that in coinoculated culture, the maximum P was solubilized relative to single-species culture.

Indole Acetic Acid Production by Developed Consortia

A significant amount of IAA production was observed in the case of T5: *B. brevis* (MS1) + *B. licheniformis* (MS3) + *A. calcoaceticus* (MS5). The combination produced a maximum of 107 µg/ml (Fig. 3) However, after 72 h of incubation in single inoculation MS1 there was maximum IAA production, that is, 52 µg/ml IAA after 96 h. The IAA production profile of PGPC had a declining

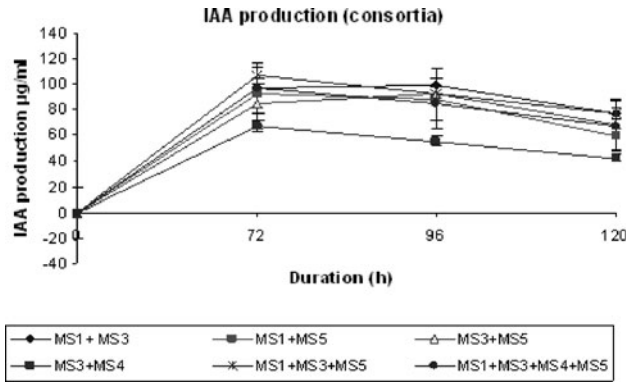

Fig. 3 Indole acetic acid production by the coinoculated strains

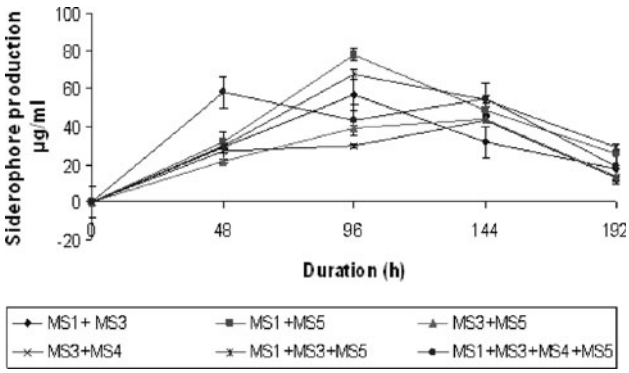

Fig. 4 Siderophore production by the coinoculated strains

trend with a maximum at 72 h, which kept falling subsequently thereafter.

Siderophore Production by Developed Consortia

Maximum siderophore production under the mixed coinoculation of T5, *B. brevis* (MS1) + *B. licheniformis* (MS3) + *A. calcoaceticus* (MS5), was 67.59 µg/ml after 96 h (Fig. 4). MS5 alone produced the maximum amount of siderophore, that is, 32 µg/ml, after 96 h, but in combination it was found to increase to double the quantity. MS3 produced only 22 µg/ml after 96 h but this increased by the mutualistic association with MS5 during coinoculation.

Seed Surface Survival

B. brevis (MS1) + *B. licheniformis* (MS3) + *A. calcoaceticus* (MS5) coinoculated strains exhibited a higher degree of survival in log phase to the seed surface of *J. curcas* compared to the single culture (Fig. 5). These results are in line with the findings of Neyra and others (1995), where *Enterobacter* and *Pseudomonas* sp. showed good survival rates as coflocs of *Azospirillum* when

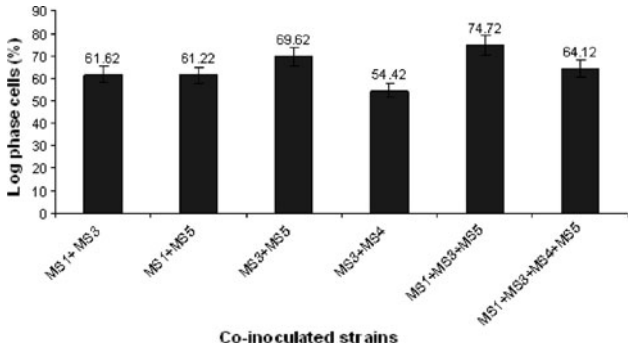

Fig. 5 Percentage survival of growth-promoting consortia on *Jatropha* seed surface

compared to non-flocculated *Pseudomonas* or *Enterobacter*. A viability study was done by direct enumeration count and the plate count method. A known volume of suspension is spread over a known area on the slide. The stained smear is subjected to a direct cell count in several fields to determine the average number of cells per field. The value is multiplied by the total number of fields in the smear area.

Seed Germination Testing during Nursery Conditions and Biometric Observations of *Jatropha* in Greenhouse Conditions

The highest germination percentage (76.66 %) and germination capacity (83.33 %) were observed with T5 followed by T6 (73.33 %) with a germination capacity of 80 %. The lowest germination percentage (63.66 %) was recorded for the control treatment. Consortia helped increase germination from controls between 5.26 and 21.04 %. Similarly, the highest germination energy (32.77) was with T5 followed by 31.52 with T6 and the lowest (28.75) was in controls (Table 1).

The vigor index of the seedlings was calculated according to the method of Abdul-Baki and Anderson (1973) as germination percent × seedling total length, that is, total shoot and root length. In our study the maximum seedling vigor index (1,696.48) was reported with T5 and the minimum (970.84) was in the control test. The effect of consortia showed a 74.74 % increment in the seedling vigor index against the control. The developed consortia with the PGPR characteristics such as production of IAA and ACC deaminase helped in breaking seed dormancy, with mechanical scarification and environmental impacts increasing germination. The plant-growth-promoting activity of the consortia was determined with the seed inoculation experiment in sterile soil with *J. curcas*. All the coinoculated strains proved to be effective when used during pot and field studies. The root length of *J. curcas* seedlings was significantly increased by inoculation with different consortia, with T6 yielding the highest increase at 24.41 % at the 60th DAS (Table 2) compared to uninoculated controls. A similar increase in the fresh root weight of *Jatropha* seedlings compared to that of uninoculated controls was maximum with T2 (181.03 %), T1 (90.80 %), T5 (78.16 %), and T6 (85.06 %) at the 60th DAS, whereas dry weight increased with T2 (155.64 % at the 30th and 200.25 % at the 60th DAS), T1 (119.10 %), T5 (100.99 %), and T6 (101.74 %) at the 60th DAS compared to uninoculated controls.

The shoot length of *Jatropha* seedlings increased with the inoculated consortia, obtaining the maximum (61.43 %) with T3, whereas a steady increase was shown with T6, that is, 28.44, 21.06, 25.57, and 27.93 % in 30th, 60th, 90, and 120 DAS, respectively (Table 3). Similarly, fresh shoot weight increased to a maximum (166.67 %) with T5 at the 30th DAS and a consistent increase with all the consortia was shown at the 60th day: T1 144.08; T3 140.17; T4 83.39; T5 129.42; and T6 127.58 %, respectively. The dry weight also was found to be more consistent on the 60th day, that is, T1 262.83; T3 211.18; T4 244.41; T5 125.66; and T6 180.59 %, respectively. The shoot width of *Jatropha* was calculated and the highest (43.50 %) increase over uninoculated controls was with T5 at 30 DAS. Consortia T5 was also effective in significantly increasing the number of leaves per plant of *Jatropha*, resulting in a maximum 38.57 % increase, and a consistent increase in all other consortia at the 60th day compared with their uninoculated controls also was observed.

Table 1 Germination parameter study shown by the coinoculated strains in comparison with the control

Treatments	No. of seeds shown	No. of seeds germinated	Percentage germination	Germination capacity (%)	Germination energy	Seedling vigor index
Control	30	19	63.33	66.66	28.75	970.84
T1	30	19	63.33	70	28.75	958.18
T2	30	21	70	76.66	23.87	1107.4
T3	30	20	66.66	73.33	29.53	1170.54
T4	30	20	66.66	73.33	22.85	672.83
T5	30	23	76.66	83.33	32.77	1696.48
T6	30	22	73.33	80	31.52	1351.47
Total	210	138	65.71	73.33	29.57	–

These parameters were calculated after the germination count up to the 28th day after the seeds were sown in the pot

Table 2 Vegetative parameters study with the developed consortia in comparison with control 60 DAS

Vegetative parameters	Control	T1	T2	T3	T4	T5	T6
Root							
Length (cm)	17.33 ± 0.1^{ns}	19.7 ± 0.2^{ns}	20.89 ± 1.3^{ns}	22.8 ± 1.7^{ns}	18.74 ± 2.2^{ns}	24.43 ± 0.05^{ns}	21.56 ± 0.2^{ns}
Fresh wt (g)	1.74 ± 0.3^{ns}	3.32 ± 0.1^{ns}	4.89 ± 0.3	3.1 ± 0.04^{ns}	4.28 ± 0.3*	3.1 ± 0.05^{ns}	3.22 ± 0.2^{ns}
Dry wt (g)	0.403 ± 0.02^{ns}	0.883 ± 0.1^{ns}	1.21 ± 0.1**	0.87 ± 0.03*	0.98 ± 0.4*	0.81 ± 0.03^{ns}	0.813 ± 0.04^{ns}
Shoot							
Length (cm)	15 ± 1.5^{ns}	17.16 ± 1.9^{ns}	16.22 ± 0.3**	17.66 ± 1.5^{ns}	15.77 ± 1.3^{ns}	16.83 ± 1.1^{ns}	18.16 ± 2.3^{ns}
Fresh wt (g)	9.21 ± 0.4^{ns}	22.48 ± 0.4*	11.22 ± 0.1^{ns}	22.12 ± 0.5**	18.69 ± 0.3^{ns}	21.13 ± 1.0**	20.96 ± 0.4**
Dry wt (g)	3.04 ± 0.08^{ns}	11.03 ± 0.3**	4.62 ± 0.2^{ns}	9.46 ± 0.2**	10.47 ± 0.7**	6.86 ± 0.2**	8.53 ± 0.04**
Width (mm)	36.66 ± 4.1^{ns}	45.33 ± 2.0^{ns}	36.82 ± 2.1^{ns}	40 ± 0.5^{ns}	38.88 ± 0.2^{ns}	40.33 ± 0.8^{ns}	40 ± 1.1^{ns}
Leaf							
Number of leaves	4.33 ± 0.3^{ns}	5 ± 0.00^{ns}	5.33 ± 0.3^{ns}	6 ± 0.5^{ns}	5.66 ± 0.3^{ns}	5.66 ± 0.3^{ns}	5.66 ± 0.3^{ns}
Length (cm)	6.73 ± 0.9^{ns}	7.3 ± 0.6^{ns}	7.66 ± 0.4^{ns}	9.06 ± 0.6^{ns}	8.42 ± 0.4^{ns}	7.3 ± 0.3^{ns}	7.06 ± 0.4^{ns}
Width (cm)	7.69 ± 0.6^{ns}	7.76 ± 0.8^{ns}	8.01 ± 0.2^{ns}	8.9 ± 0.9^{ns}	8.31 ± 0.2^{ns}	7.93 ± 0.4^{ns}	7.96 ± 0.4^{ns}
Biomass (g)	7.5 ± 0.4^{ns}	13.89 ± 0.5^{ns}	10.28 ± 0.3^{ns}	14.89 ± 0.8*	11.52 ± 0.5^{ns}	16.57 ± 0.8*	14.74 ± 0.4*
Cd at 1 %	3.304	6.14	4.83	4.759	2.605	1.871	6.05
Cd at 5 %	2.6	2.83	2.22	2.349	1.278	0.861	2.78
CV	4.693	4.04	10.46	10.857	6.81	5.583	12.56

Values are means of three replicates ± SE (standard error). Significance test by using ANOVA software at 120 DAS

DAS days after inoculation, *C* control, *Cd* critical difference, *ns* nonsignificant as compared to control (ANOVA)

* Significant at 5 %; ** significant at 1 %

Table 3 Vegetative parameters study with the developed consortia in comparison with control 120 DAS

Vegetative parameter	Control	T1	T2	T3	T4	T5	T6
Root							
Length (cm)	23.53 ± 2.0^{ns}	24.92 ± 0.4^{ns}	23.58 ± 1.74^{ns}	24.19 ± 0.1^{ns}	24.26 ± 0.92^{ns}	27.20 ± 1.5^{ns}	25.72 ± 0.2^{ns}
Fresh wt (g)	5.40 ± 0.2^{ns}	5.95 ± 0.7^{ns}	4.93 ± 2.82^{ns}	5.92 ± 0.3^{ns}	5.21 ± 0.86^{ns}	7.04 ± 0.04^{ns}	6.12 ± 0.5^{ns}
Dry wt (g)	0.88 ± 0.05^{ns}	1.33 ± 0.06*	0.93 ± 0.82^{ns}	1.21 ± 0.03^{ns}	1.11 ± 0.77^{ns}	1.51 ± 0.03*	1.11 ± 0.04*
Shoot							
Length (cm)	18.44 ± 0.4^{ns}	20.54 ± 0.2^{ns}	19.21 ± 1.22^{ns}	20.87 ± 0.9^{ns}	19.54 ± 0.54^{ns}	22.53 ± 0.3*	23.59 ± 1.2*
Fresh wt (g)	10.48 ± 0.3^{ns}	14.51 ± 0.4^{ns}	12.86 ± 0.89^{ns}	13.48 ± 0.5^{ns}	13.52 ± 1.7^{ns}	19.57 ± 0.4**	19.99 ± 0.9**
Dry wt (g)	3.63 ± 0.4^{ns}	3.89 ± 0.3^{ns}	4.27 ± 0.56^{ns}	3.98 ± 0.5^{ns}	3.98 ± 2.1^{ns}	5.40 ± 1.0^{ns}	4.56 ± 0.8^{ns}
Width (mm)	42.55 ± 0.6^{ns}	44.92 ± 0.7^{ns}	44.87 ± 0.2^{ns}	43.97 ± 0.3^{ns}	43.27 ± 0.4^{ns}	48.25 ± 0.5^{ns}	45.87 ± 0.6^{ns}
Leaf							
Number of leaves	5.66 ± 0.3^{ns}	6.33 ± 0.3^{ns}	6.83 ± 0.3^{ns}	6.66 ± 0.5^{ns}	5.83 ± 0.3^{ns}	6.66 ± 0.3^{ns}	6.71 ± 0.8^{ns}
Length (cm)	9.61 ± 0.7^{ns}	8.86 ± 0.3^{ns}	9.62 ± 2.4^{ns}	9.43 ± 0.3^{ns}	8.24 ± 2.6^{ns}	10.69 ± 0.3^{ns}	9.43 ± 0.3^{ns}
Width (cm)	9.87 ± 0.5^{ns}	7.3 ± 0.3^{ns}	9.97 ± 1.8^{ns}	9.89 ± 0.3^{ns}	10.39 ± 1.8^{ns}	10.48 ± 0.5^{ns}	10.8 ± 0.2^{ns}
Biomass (g)	12.35 ± 0.8^{ns}	15.24 ± 0.4^{ns}	12.59 ± 1.43^{ns}	14.21 ± 2.7^{ns}	13.64 ± 2.4^{ns}	10.717 ± 0.9^{ns}	20.5 ± 5.06^{ns}
Cd at 1 %	3.62	7.57	2.654	2.69	3.149	2.687	2.61
Cd at 5 %	2.54	5.33	1.46	1.89	1.105	1.483	2.13
CV	6.64	17.56	9.31	5.64	8.237	3.53	21.94

Values are means of three replicates ± SE (standard error). Significance test by using ANOVA software at 120 DAS

DAS days after inoculation, *C* control, *Cd* critical difference, *ns* nonsignificant as compared to control (ANOVA)

* Significant at 5 %; ** significant at 1 %

Leaf length increased with T3 (32.33 % at the 30th day and 38.57 % at the 60th day) and T5 (30.71 % at the 60th day), and leaf width increased with T3 (36.74 % at the 30th day and 34.62 % at the 60th day) and T5 (26.52 % at the 30th day and 15.73 % at the 60th day) compared with uninoculated controls.

Biomass of *J. curcas* also increased over controls after inoculation with these developed consortia and a maximum (157.46 %) was observed with T5 at the 30th day and 120.93 % at the 60th day, whereas this result was found to be more consistent at the 60th DAS in all other consortia. Chlorophyll content (Chl *a* and Chl *b*) increased maximally from 30 (39.6 %) days to 60 (58.4 %) days with T5, after which the effect seen at 90 and 120 DAS (Fig. 6) was a lower increase over the uninoculated controls. The overall effect of these consortia on *J. curcas* compared to controls is shown in Fig. 7. Pandey and Maheshwari (2007) reported that the length of *Cajanus cajan* increased by 149 % in coinoculated conditions. Similarly, a 47.9 and 61.4 % increase in shoot weight was recorded with *Burkholderia* sp. MSSP and *S. meliloti* PP3, respectively, in individual trials, and a 181.86 % increase in shoot weight was recorded when both were inoculated together.

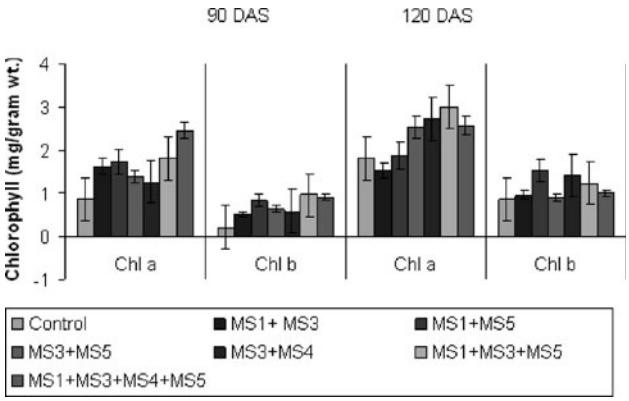

Fig. 6 Chlorophyll (*a* and *b*) content in *Jatropha* leaves after 90 and 120 DAS shows the comparative study with the control

Field Experiments

The effects of consortia treatment on the grain yield of *J. curcas* were found to be significant. Field experiments were conducted to assess coinoculation with different combinations developed for improving the growth and yield of *J. curcas* at the field site provided by Gujarat University, Ahmedabad. Grain yield ranged from 300 g/plant in uninoculated strains to a maximum of 742 g/plant with T5 followed by 697 g/plant with T6 (Table 4), which is the highest rate of grain yield increase (T5 147.33 and T6 132.33 %) compared with controls of *Jatropha* seeds after plantation in the field after one and half years.

The combinations of these treatments with joint traits suggest that MS3 and MS5 have the maximum role in grain yield. Data revealed that not only the yield but also plant height (110.4 and 100.8 %), shoot width (56.81 and

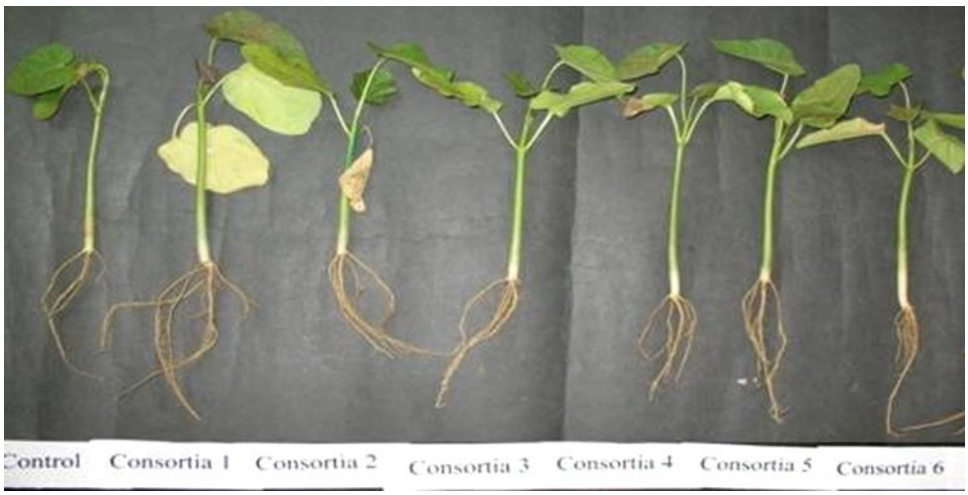

Fig. 7 Vegetative structure study of *J. curcas* plant at 60 DAS

Table 4 Effect of consortia on the growth yield of *Jatropha cucas* under field conditions

Treatments	Plant height	Shoot width (cm)	No. of branches	Yield (g)/plant	Oil content (%)
Control	70 ± 8.6	7.4 ± 5.48	11 ± 2.5	300 ± 7.13	35
TI	85 ± 7.5	9.3 ± 4.18	22 ± 3.8	452 ± 4.15	38
T2	90 ± 4.8	8.43 ± 7.32	19 ± 4.6	499 ± 7.46	38.5
T3	73 ± 3.9	9.57 ± 2.49	20 ± 5.9	598 ± 5.16	38.7
T4	72 ± 5.84	7.8 ± 5.42	18 ± 4.7	459 ± 4.89	39
T5	110 ± 4.67	10.32 ± 3.49	32 ± 3.6	742 ± 6.12	52
T6	100 ± 7.21	9.6 ± 3.28	25 ± 4.1	697 ± 3.46	45

Values are means of three replicates ± SE (standard error)

43.18 %), and number of branches (190.90 and 127.27 %) also increased in the two best consortia: T5 and T6 (Table 4). At final harvest of the seeds, concentrations of total N, P, and Fe in microbe-inoculated soils (T6 and T3) were higher than in uninoculated soils (Tables 5 and 6). However, very little change was evident in the Cl and available Mg content in microbe-inoculated soils. A non-significant response of soil EC was observed with inoculations. The percent increase in the post-harvest soil with coinoculation of 63.68 % for P, 22.28 % for S, and 14.13 % for Fe was observed with T5 and the maximum total N (28.88 %) was observed with T6.

Discussion

When organisms were grown in mixed-species culture with respect to single-species culture, it was shown that MS1 favors the growth of MS3. The growth profile data represent commensalism between the two isolates. The results suggest that the process of *P* solubilization, which is governed by a complex group of mechanisms, is substantially affected by a mixed culture. Although there was no appreciable increment in the maximum soluble P level, the maximum soluble P was released much earlier in the multispecies consortium and the solubilization was two times greater than in the single-species treatment. The pH of the medium also showed a decrease from 6.5 to a maximum of 4.6 after 14 days in the case of T6. However, from the observations it is clear that no correlation could be established between the degree of P solubilization and final pH of the medium. Many PSMs lower the pH of the medium either by H^+ extrusion (Illmer and Schinner 1995) or by secretion of organic acids such as acetic, lactic, malic, succinic, tartaric, gluconic, 2-ketogluconic, oxalic, and citric acids.

In mixed culture, IAA production increased by almost 50 % compared to monospecies cultures. This further extended the findings of growth dependence between the two isolates. Increased phytohormone production has been reported with *Azospirillum* when grown in mixed culture under in vitro conditions (Janzen and others 1992). However, in mixed-species culture, the maximum release of IAA was observed in the initial hours of incubation, which remains unexplained. Production of siderophores results in siderophore-mediated competition among the bacteria, which further results in exclusion of siderophore-non-producer pathogens from the rhizosphere due to lack of iron depletion for sclerotia germination and hyphal growth. This was supported by Singh and Varaprasad (2008) who showed that the rhizosphere isolate *Bacillus subtilis* (BN1) inhibited the growth of *M. phaseolina* by up to 60 %.

Table 5 Soil analysis results from the field study before inoculation of developed consortia on the growth of *J. curcas* (as reported by Gujarat Soil Testing Laboratory, Gandhinagar) before plantation in the field

Tests	pH	Cl^- (mg/kg soil)	Mg^+ (%)	SO_4^- (mg/kg soil)	PO_4 (mg/kg soil)	Na^+ (%)	Salinity (%)	Ec (mmhos/cm)	K^+ (kg/ha)	Total N (%)	TOC	TDS	Fe^{2+} (mg/kg)
Soil	7.6 ± 0.3	310 ± 5.39	0.0059 ± 0.03	83 ± 4.27	11.4 ± 1.22	0.042 ± 0.02	0.32 ± 0.04	0.22 ± 0.07	182 ± 7.28	0.045 ± 0.02	149 ± 3.22	221 ± 4.56	184 ± 8.23

Table 6 Soil analysis results from the field study after the effect of consortia on the growth of *J. curcas* (as reported by Gujarat Soil Testing Laboratory, Gandhinagar) after plantation in the field

Tests	pH	Cl^- (mg/kg soil)	Mg^+ (%)	SO_4^- (mg/kg soil)	PO_4 (mg/kg soil)	Na^+ (%)	Salinity (%)	Ec (mmhos/cm)	K^+ (kg/ha)	Total *N* (%)	TOC	TDS	Fe^{2+} (mg/kg soil)
MS1 + MS3	6.9 ± 0.3	290 ± 14.41	0.0060 ± 0.3	95 ± 2.52	13.5 ± 1.11	0.045 ± 0.2	0.30 ± 0.07	0.28 ± 0.09	174 ± 3.43	0.047 ± 0.02	152 ± 4.76	180 ± 4.38	231 ± 6.95
MS1 + MS5	6.6 ± 0.2	320 ± 17.23	0.0054 ± 0.1	85.3 ± 1.59	14.8 ± 0.98	0.049 ± 0.2	0.31 ± 0.08	0.27 ± 0.07	169 ± 4.32	0.052 ± 0.03	147 ± 3.24	241 ± 5.87	174 ± 6.98
MS3 + MS5	6.8 ± 0.2	288 ± 21.22	0.0048 ± 0.2	94 ± 2.32	16.42 ± 1.28	0.057 ± 0.3	0.27 ± 0.07	0.29 ± 0.03	184 ± 7.35	0.056 ± 0.02	159 ± 4.37	245 ± 4.98	156 ± 4.98
MS3 + MS4	7.1 ± 0.2	327 ± 22.17	0.0059 ± 0.1	87 ± 2.91	13.54 ± 1.17	0.058 ± 0.1	0.24 ± 0.06	0.34 ± 0.03	198 ± 4.37	0.055 ± 0.03	178 ± 4.87	284 ± 2.98	182 ± 5.78
MS1 + MS3 + MS5	6.3 ± 0.3	287 ± 13.32	0.0062 ± 0.01	101.5 ± 4.41	18.66 ± 0.87	0.059 ± 0.07	0.20 ± 0.3	0.38 ± 0.02	188 ± 3.67	0.047 ± 0.04	145 ± 5.37	224 ± 4.98	210 ± 5.28
MS1 + MS3 + MS4 +MS5	6.4 ± 0.2	290 ± 12.44	0.0074 ± 0.03	99.3 ± 3.22	17.45 ± 1.01	0.062 ± 0.04	0.28 ± 0.1	0.37 ± 0.04	222 ± 8.95	0.058 ± 0.03	162 ± 5.48	271 ± 8.47	195 ± 4.28

Siderophore production acts as a biocontrol agent and iron chelator as well. All these isolates individually produce only one type of siderophore (hydroxymate type), so in combination the quantitative efficiency increases. In this study, initial inoculation loads of 1×10^9 CFU/ml and the survival percentage in the seed surface assay after 1 week are shown in the graph for all the coinoculates. Probably the phenomenon of flocculation has provided a safe niche for survival and cell release upon seed sowing in favorable surroundings, which enhances survivability (Kim and others 2005). Flocculation has also provided a microenvironment that is highly protective against physical and chemical stresses.

The germination capacity of one seed, based on a binary answer (germinated or non-germinated), is one qualitative attribute of the germination process that is generally converted to a quantitative attribute, usually percentage. Germinability of one seed sample is the percentage of seeds in which the germination process reaches the end, under experimental conditions, by means of the intraseminal growth that results in the protrusion (or emergence) of one live embryo. In general, germinability is presented as percentage, accompanied by some dispersion measurement, but it is possible to use proportion rather than percentage, and the proportion values for one or several samples can be submitted to statistical tests. Nainar and others (1999) have shown that among the seed pretreatments, including mechanical scarification, hot water treatment (with or without removing the testa), and sulfuric acid treatment (with or without breaking the testa), mechanical scarification gave the highest germination percentage (60 %) in *T. chebula.* However, according to our results, the effect of the multispecies bacterial consortia on the *J. curcas* plant was excellent compared to the untreated control and individual trials of PGPR. Not only was the plant's root and shoot increased but also the biomass, fresh weight, chlorophyll content, and germination frequency per plant were maximized with PGPC treatment. All the developed consortia except T4 enhanced all the vegetative characteristics consistently compared with controls after 60 days of incubation, but they showed a steady growth rate after 90–120 days in the pot because of the limited space in the earthen pot. Development of PGPC, including a core competent strain, has been suggested as a feasible strategy for increasing activity and viability of PGPR (Waish and others 2001). In our experiment, MS3 and MS5 were supposed to be the core competent strain and effective along with other PGPR. Recently, PGPR, in conjugation with effective *Rhizobium* strains, have been reported to affect growth and nitrogen fixation in pigeonpea by inducing the occupancy of introduced *Rhizobium* in the nodules of the legume (Tilak and others 2006). These findings were similar to those of the present work, irrespective of the fact that they were based on *Rhizobium* and a leguminous plant whereas the present work dealt with a nonleguminous biofuel crop *J. curcas* L. These symbiotic relationships are in fact beneficial in the global context because they act to maintain ecological balance (Griffin and others 2004).

We reported on the cooperation between four rhizobacteria that belong to four distant genera of established plant-growth-promoting bacteria due to their PGP ability. However, they showed cooperation while growing together in vitro, which indicates their common ecological niche. Each of the four organisms improved seedling growth, but one curious attempt revealed that seedling growth was further improved when all organisms in different combinations were applied together. This encouraged us to study their behavior in a consortium. This is a well-known strategy that enables organisms to successfully survive and maintain themselves in communities (Andrews 1991). Earlier, microbial studies performed without plants indicated that some combinations allow the bacteria to interact with each other synergistically, provide nutrients, remove inhibitory products, and stimulate each other through physical and biochemical activities that may enhance some beneficial aspects of their physiology (Bashan 1998). Research on the mechanisms by which PGPR enhance nodule formation implicates their production of plant hormones among the coinoculation benefits. For example, Chebotar and others (2001) demonstrated that some *Pseudomonas* strains, but not all, increased nodule number and acetylene reduction in soybean plants inoculated with *B. japonicum*.

Various researchers have reported the synergistic effects produced by plant-growth-promoting rhizobacteria and *Rhizobium* on nodulation and yield of legume crops (Tilak and others 2006). Coinoculation resulted in more N_2 fixation and P solubilization by lowering the soil pH and producing organic acids (Khan and others 2006). Production of organic acids resulted in acidification of the microbial cell and its surroundings (Khan and others 2006). Barea and others (2005) demonstrated that the interactive effect of rhizobia and rhizobacteria mediated the soil processes and thus enhanced availability of nutrients. Previously, many researchers reported an increase in the percent N and available P contents with coinoculation (Suneja and others 2007). Enhanced N–P content of plants due to coinoculation of free-living diazotrophs and rhizobia has been reported by various researchers (Lata and Tilak 2000).

Conclusion

This is the first report regarding the growth promotion of *J. curcas* by applying multispecies consortia. Results with coinoculated seeds were better: shoot weights approximately four times greater than those of controls were

recorded with the coinoculated treatment. Similarly, a 110 % increase in plant length and 147 % increase in grain yield were recorded. Data were higher with respect to controls as well as in individual trials. This supported the in vitro findings of PGP potentials in the multispecies consortium. In the present investigation, the four isolates were studied with the possibility of forming a consortium as an effective bioinoculant formulation. The three isolates *B. brevis* (MS1) + *B. licheniformis* (MS3) + *A. calcoaceticus* (MS5) have the ability to produce IAA, solubilize inorganic P, and produce ACC deaminase and siderophores. They enhanced the growth of *J. curcas* in individual trials. Plant growth was further improved maximally when the three were applied together. Considering the plant-growth-promoting abilities of these four isolates, a nonspecific, multispecies PGPC for bioinoculant preparation is possible.

Acknowledgments We are thankful to British Petroleum International Ltd. for their financial support.

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