A 'six-step-strategy' to evaluate competence of plant growth promoting microbial consortia

N. Mishra and S. Krishna Sundari*

Plant and Microbial Biotechnology Group, Biotechnology Department, Jaypee Institute of Information Technology, Sector-62, Noida 201 307, India

In this study a stepwise, statistically verifiable scientific protocol – 'six-step-strategy' – to develop a consortium is presented. Additionally, it introduces a novel *in vivo* plant bioassay – 'tube-in-tube' method – that gives faster (< three weeks) and reproducible results for selecting the most desirable consortia combinations. The study employs eight plant growth promoting microbes (PGPMs) with pre-established growth supporting abilities and no mutual antagonism. Following a two-factorial design, 15 consortia combinations (CCs) were developed from these PGPMs. Applying the principles of the 'six-step-strategy', combinations CC11, CC13 and CC4 showing significant increments (>100%) in root length and dry weight were recognized as the best performing consortia. The method thus shortlists the best and manageable number of consortia for further field trials.

Keywords: Bioassay, consortia combinations, plant growth promoting microbes.

RECENT evidence on the advantages of sustainable agricultural practices promotes chemical-free agricultural production[1]. There is also a growing awareness about the detrimental effects of chemicals used in agriculture, both among the farming community and consumers. Technical market research reports reflect these sentiments, predicting exponential growth to global bioinoculant market, quoting >12% compound annual growth rate (CAGR), i.e. from approximately US $7.1 billion in 2012 to 14 billion by 2019 (refs 2, 3). Rhizosphere bacteria and fungi with beneficial effects upon plant growth have long since been accepted as successful bioinoculants (biofertilizers and biopesticides). These microbes promise growth opportunity for biotechnology-based solutions to increase crop productivity while providing an alternative to agricultural chemicals.

Widely popularized and scientifically explored bioinoculants include symbiotic/free-living/associative nitrogen-fixing bacteria, phosphate-solubilizing bacteria, arbuscular mycorrhizal fungi, *Trichoderma* species and *Metarrhizum* species based formulations[4,5]. Many growth promoting rhizobacteria have also been developed as eco-friendly bioinoculants for crop improvement[6]. In the last decade, despite impressive increase in production (increase 44,833 tonnes), only one-third of the estimated demand potential is as yet met for biofertilizers in the country. This huge gap between potential market demand and production provides an opportunity for the biofertilizer market[7,8]. Presently, the available biofertilizers in the market are single-species inoculants that are not perceived by farmers as a lucrative alternative to their chemical counterparts. This could be due to a variety of reasons, including narrow-range species-specific inoculants, inconsistent performance, lack of quality products, long-term impact on soil fertility, etc.[9].

The expanding biofertilizer market is looking for alternatives that can offer more inclusive benefits over single-species, narrow-host-range biofertilizers. The concept of having a combination of microbes providing broader, non-specific growth promotion across different types of hosts and overall soil health improvement is gaining importance. In a consortium, some microbes may provide nutrient mobilization, release growth hormones in the rhizosphere, and increase available N and P, while others may provide protection against root pathogens, thus contributing to holistic plant growth. However, these consortia combinations would be able to produce synergistic effects only if the candidate microbes survive together as a consortium when inoculated on host plants[10]. Existing literature on consortia studies gives an impression of mixed impact regarding the growth promotional effects of consortia. While some studies report significantly positive impact, certain others report no significant impact. There are yet others who even report inconsistent and contradictory impact of consortia over single-species inoculations[4,10].

Though the concept of consortium is theoretically feasible, developing a consortium is a challenge for the researchers due to aspects like mutual compatibility of microbes, their dependency on each other and the task of maintaining their inoculum potential while not causing any undue depletion of plant resources during mutualism/symbiosis. There is no standard or experimentally validated protocol for screening and selecting promising consortia from a large number of proposed theoretically possible consortia. The customary hit and trial approach results in a large number of combinations and

*For correspondence. (e-mail: krishna.sundari@jiit.ac.in)

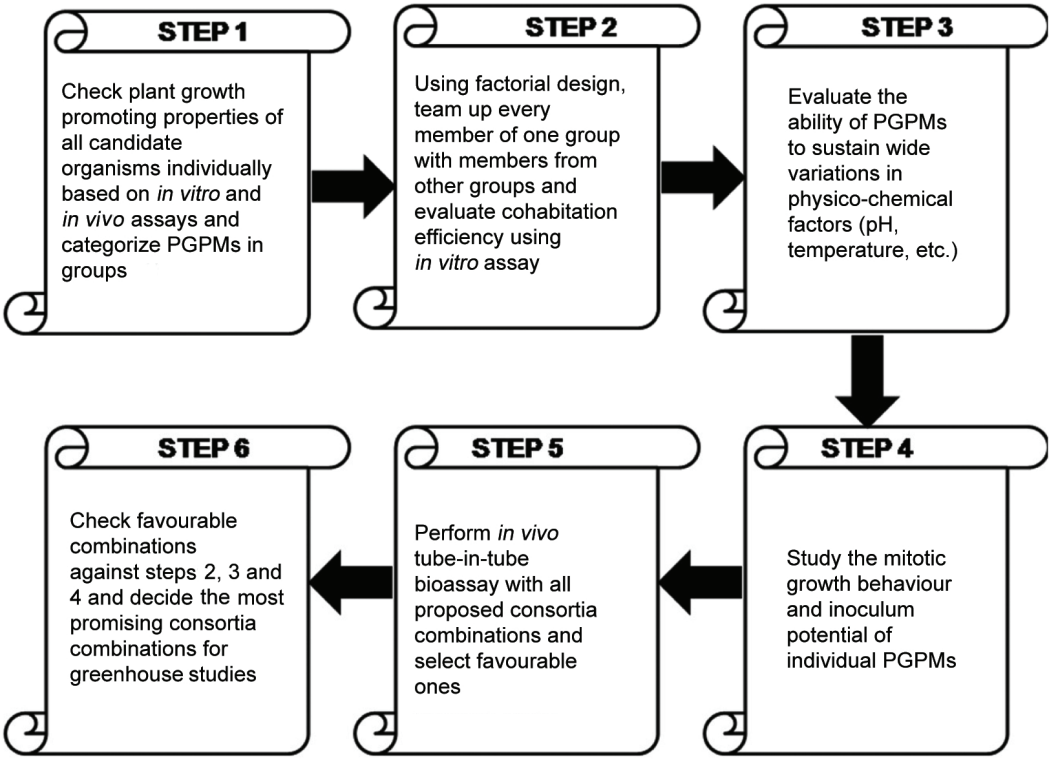

Figure 1. A 'six-step-strategy' to select the most promising consortia of plant growth promoting microbes.

Table 1. Coding of various consortia combinations tested

	PGPM1	PGPM2	PGPM3
PGPM 6	CC 1	CC 2	CC 3
PGPM 7	CC 4	CC 5	CC 6
PGPM 8	CC 7	CC 8	CC 9
PGPM 9	CC 10	CC 11	CC 12
PGPM 10	CC 13	CC 14	CC 15

time-consuming methods (four months at the least), applied randomly, leaving room for scientific improvization[11].

To address this specific problem, we present an experimentally verifiable strategy called 'six-step-strategy', where different PGPMs having host growth-supporting abilities were tested in specific combinations to find whether they can act in cohesion and provide cumulative effect towards better plant growth promotion. We have also devised a robust methodology, 'tube-in-tube', to evaluate the benefits of consortia and choose the most preferable combinations that can be further studied for field applications.

Materials and methods

The six-step-strategy was developed to ascertain cohabitation amongst PGPMs selected for the present study and to derive the most promising consortia combinations for further applications (Figure 1).

Culture and maintenance of selected PGPM isolates

Eight bacterial cultures from laboratory collection were pooled in two groups namely group-I (representing fluorescent pseudomonads: PGPM 1–PGPM 3) and Group-II (comprising PGPMs of diazotrophic nature: PGPM 6–PGPM 10). PGPM1 (MTCC-2421) and PGPM 6 (MTCC-2306) were the reference isolates from IMTECH, Chandigarh. Plant growth promoting potential of these isolates has already been demonstrated using *in vitro* biochemical tests as well as *in vivo* plant bioassays[12].

Cohabitation efficiency studies

To assess the presence of any antagonism amongst individual members of the two PGPM groups, a pairwise growth performance study was conducted *in vitro*. The cohabitation assay was designed such that every PGPM member of group-I was challenged with every member of group-II (Table 1). Overnight-grown broth culture of the respective PGPMs (one each from groups I and II) were streaked opposing each other in two halves of nutrient agar plates. Control plates contained either group-I or group-II streaked on one half of the petri plate, while

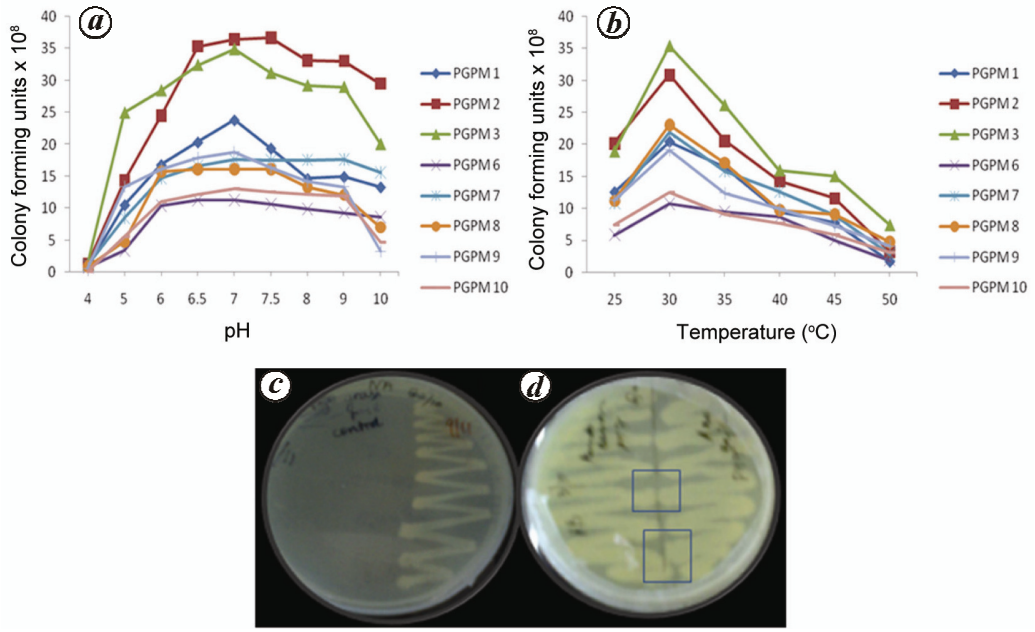

Figure 2. Physiology and compatibility of PGPM isolates. ***a***, Effect of pH; ***b***, Effect of temperature; ***c***, Control plate showing growth of individual group representative; ***d***, Cohabitation plate depicting growth of representatives from both groups. Note. Area inside square shows no zone of inhibition and isolates from both groups merging at the meeting point.

leaving the adjacent segment blank (Figure 2 *c*). All co-culturing experiments were carried out in triplicate. After incubation (30°C for 48 h), the plates were checked for the presence of any zones of inhibition at the colony margins where the two cultures meet.

Response to external stimulus

Temperature response was recorded in the range 25–45°C (at 5°C interval), while pH was studied at 30°C, in the range 5–11 (with unit interval). 100 µl of overnight-grown PGPM cultures (@10^8 cfu/ml) was used as inoculum in both experiments conducted in triplicate. Following overnight incubation of the respective PGPMs in 10 ml nutrient broth (NB), absorbance was measured at 610 nm and results represented graphically (Figure 2 *a* and *b*).

Growth and mitotic activity of PGPM isolates

Growth kinetics of each PGPM isolate was studied at the most optimal temperature and pH conditions (30 ± 2°C; pH 7) as evidenced from Figure 2 *a* and *b*. Overnight-grown PGPM cultures were seeded individually in 25 ml NB in triplicate and incubated in BOD incubator maintaining 120 rpm. Then, 1 ml of culture aliquot was collected at periodic intervals and growth curve plotted with time (in hours) on the *x*-axis and absorbance (OD measured at 610 nm) on the *y*-axis. Doubling time and growth rate were calculated using standard formulae[13] and the results tabulated (Table 2).

Design of consortia

Using factorial design approach, a total of 15 consortia combinations (Table 1) were studied for their plant growth promoting efficiency ensuring that each consortium has one member from the pseudomonad group and one member from the diazotrophic group. Equal volumes of overnight-grown cultures of the respective PGPMs (~10^8 cfu/ml) were mixed together under aseptic conditions to prepare consortia combinations 1–15 (CC1–CC15) and immediately used for host treatment.

A novel and rapid plant bioassay

Sorghum bicolor cultivar CSH-16 procured from the Directorate of Sorghum Research (DSR), Hyderabad, was the host for all *in vivo* plant growth studies. Seeds of *S. bicolor* were surface-sterilized and coated with the respective consortia combinations (CC1–CC15) and placed for germination following an established protocol[10]. Germinated seeds with healthy radical, and plumule were selected and transferred to the novel experimental set-up called 'tube-in-tube' method developed in our laboratory. The cap of the sterilized eppendorf tube (1.5 ml) was removed and bottom was cut to make an aperture. Germinated seeds were transferred aseptically into this eppendorf and the assembly was placed in an autoclaved glass test tube (50 ml capacity), thus drawing its name 'tube-in-tube' method. The test tube was filled with 10 ml of half-strength modified Melin–Norkrans medium, sans glucose and malt. The complete 'tube-in-tube' system was plugged with sterile cotton to maintain aseptic condition and incubated for

Table 2. Comparative mitotic activities of plant growth promoting microbes (PGPMs) tested

	Group I (pseudomonads)			Group II (diazotrophs)				
Parameters studied	PGPR1	PGPR2	PGPR3	PGPR6	PGPR7	PGPR8	PGPR9	PGPR10
Growth rate constant	0.49	0.42	0.34	0.27	0.31	0.30	0.40	0.32
Generation time (g) in hrs	0.23	0.20	0.16	0.13	0.15	0.14	0.19	0.15
Growth rate	4.26	5.01	6.12	7.66	6.80	7.07	5.27	6.51

Table 3. Growth properties of various consortia combinations tested

Consortia combinations	Average root length (cm)	Average shoot length (cm)	Root dry weight (mg)	Shoot dry weight (mg)
Control	08.28 ± 0.82^{a}	12.16 ± 0.98^{a}	33	84
CC 1	22.00 ± 3.02^{b}	14.29 ± 1.69^{b}	49	127
CC 2	15.01 ± 2.89^{c}	13.27 ± 1.01^{a}	52	129
CC 3	13.25 ± 3.03^{c}	06.55 ± 1.0^{d}	16	36
CC 4	19.57 ± 1.69^{b}	13.07 ± 0.72^{a}	87	200
CC 5	13.18 ± 2.00^{c}	07.96 ± 0.72^{c}	12	26
CC 6	13.34 ± 1.24^{c}	11.78 ± 0.66^{a}	24	62
CC 7	19.26 ± 2.23^{b}	13.04 ± 0.68^{a}	52	132
CC 8	19.38 ± 1.44^{b}	12.53 ± 1.26^{a}	49	122
CC 9	14.83 ± 2.31^{c}	09.31 ± 1.33^{c}	24	62
CC 10	20.37 ± 2.63^{b}	13.33 ± 1.00^{a}	28	76
CC 11	18.71 ± 1.29^{b}	11.74 ± 1.15^{a}	71	141
CC 12	13.92 ± 1.44^{c}	06.87 ± 2.96^{c}	16	29
CC 13	19.97 ± 1.00^{b}	13.02 ± 1.47^{a}	77	177
CC 14	18.58 ± 2.00^{b}	12.47 ± 1.03^{a}	50	131
CC 15	15.13 ± 1.10^{c}	05.70 ± 1.20^{d}	20	47

All root/shoot length values indicate mean (± SD) of 12 replicates. Different letters besides the values indicate significant difference among treatments (at $P \leq 0.01$). All root/shoot dry weights presented are from pooled values of 12 replicates.

10 days at 30 ± 2°C, with no daylight regulation. The bottom portion of the entire test tube rack carrying the set-up was wrapped in a black sheet of paper to keep the root system in the dark (Figure 3). All the 15 treatments (CC1–CC15) along with uninoculated host (control) were placed in 12 replicates. Upon harvest, scanning electron microscopy (SEM) was carried out for visual confirmation of bacterial association with plant roots (Figure 4). The impact of different PGPM consortia on plant growth was evaluated based on four parameters: root length (RL), shoot length (SL), root dry weight (RDW) and shoot dry weight (SDW). For RL and SL, geometrical mean for the respective combinations was calculated and variations among replicates expressed as SD (Table 3). For RDW and SDW, all replicates from one treatment were pooled together, dried in a hot-air oven (60°C) till the weight stabilized and readings were expressed per plant (Table 3). Data were subjected to statistical analyses: ANOVA and Student's *t*-test (at $P \leq 0.01$) using XLstat Microsoft software.

Results and discussion

We present a stepwise strategy to assess whether the PGPMs included in the present study can yield promising consortia for further applications, and if so, how the best combinations can be identified.

To exert synergistic effects on plant growth, it is necessary for individual PGPMs in the consortia to grow in harmony. To satisfy this objective, cohabitation studies were conducted following a pairwise co-culturing pattern. No zones of inhibition were observed at the points of contact between the bacterial species in the culture plates (Figure 2 *d*). Compared to control, presence of the second isolate in the adjacent half of the petri plate (pseudomonads/diazotrophs) did not exhibit any visual growth reduction in the counterpart. This indicates absence of any diffusible toxins or volatile compounds that may lead to antagonism towards each other[14]. The experiment thus provided evidence that the tested PGPMs are growing in a mutually non-inhibiting manner, allowing further studies on the consortia.

PGPM isolates favoured pH ranging between 6 and 8.5 for growth and registered maximum growth at pH 7 (Figure 2 *a*). Majority of the isolates showed tolerance to a broader pH range, except for PGPM 1 which exhibited high pH sensitivity. Isolates PGPM 2 and PGPM 9 were particularly resistant to varying pH, showing no significant growth reduction between pH 6 and 10. Though all isolates were able to grow in the range 25–45°C, there was a temperature optima (30°C). A sharp reduction was observed in growth of isolates beyond 40°C (Figure 2 *b*). From the results, it is evident that all the isolates have a

common temperature (30°C) and pH (7) optima. This could prove to be advantageous for consortia-building and facilitating co-survivability of these microbes when in consortia.

In a consortium, comparable generation times of co-habitating microbes would ensure a balance in relative inoculum density of individual isolates. If one consortium member grows faster, it may deplete nutrients available in the medium and create unsuitable growth conditions for others[15]. All bacterial isolates showed generation time ranging between 0.13 and 0.23 h (Table 2). Maximum difference between the generation times was 0.10 h. PGPM 6 emerged as the fastest ($g = 0.13$ h) and PGPM1 the slowest ($g = 0.23$ h) growing isolates, with the rest showing generation times between the two values (Table 2). Group-II organisms were comparatively fast-growing in NB medium. Comparable mitotic growth behaviour of PGPMs, supports co-survival ability and their suitability as prospective candidates for consortia development.

To establish our hypothesis that cumulative synergistic action of carefully selected PGPMs may improve the plant growth promoting benefits of microbes, we designed the 'tube-in-tube' method. This method proved to be effective for screening a large set of consortia combinations in a short span of time. The proposed method draws its idea from the traditional hydroponic system. However, the traditional system requires elaborate space, specific equipment and lacks provision to maintain aseptic conditions[16]. The present method offers economy of space and resources, employs regular lab ware (test tubes, eppendorfs, etc.) and more importantly, facilitates aseptic handling. Media requirement in the present system is also minimal and saves on cost. Compared to conventional pot culture-based screening methods that incur loss in root biomass during removal and recovery, the 'tube-in-tube' system ensures complete biomass harvest with no loss of fragile root material. The cut eppendorf placed inside the larger test tube offers support to growing seedlings, allows for extended vertical growth of roots and assists easy harvest at the end of the experiment. The system allows for continuous monitoring of root and shoot development (Figure 3). Further, the clean and aseptically grown root system can help in studying the host–microbe interactions using advanced techniques employing vital/fluorescent stains, immuno-labelling of microbiont, etc. with zero interference. Minimum pre-processing requirement due to absence of any other inert/live matter helps in SEM/TEM studies using these root samples.

Earlier lab analyses (*in vivo* host inoculation experiments) have confirmed plant growth promotion effects of PGPM isolates included in the study. Individual isolates showed host-specific growth impact[12], providing room for exploring the concept of consortia. The data are a part of the thesis of one of the present authors. Only crucial and representative results of individual PGPM inoculations are presented here, where the data were used to understand the contribution of partnering consortia members (Figure 5).

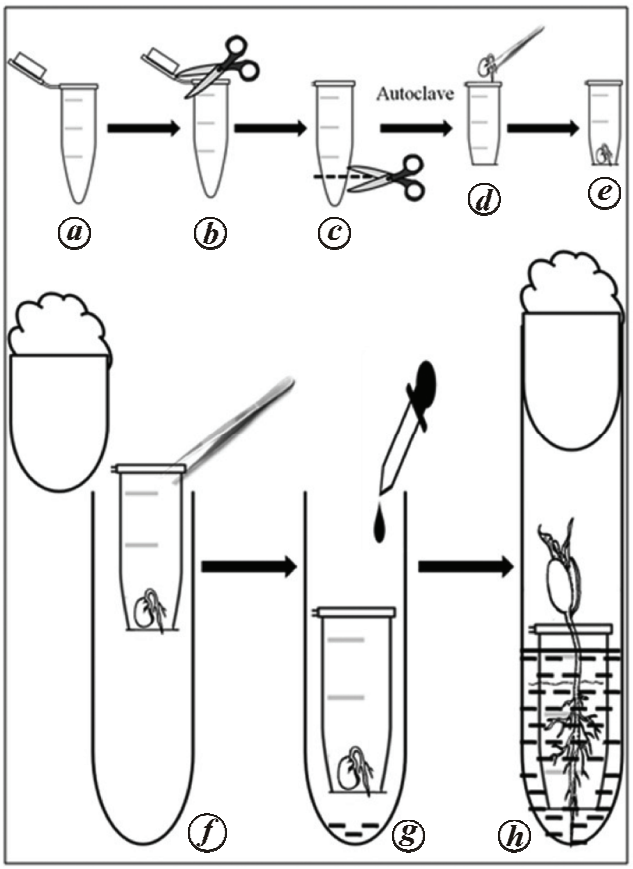

Figure 3. The 'tube-in-tube' methodology. ***a***, Autoclaved eppendorf (1.5 ml); ***b***, ***c***, Cap and bottom portions of the eppendorf excised. ***d***, ***e***, Germinated seedling in eppendorf. ***f***, Eppendorf with seedling placed in autoclaved test tube; ***g***, Glass test tube filled with media; ***h***, 'tube-in-tube' system plugged to maintain aseptic conditions for plant growth.

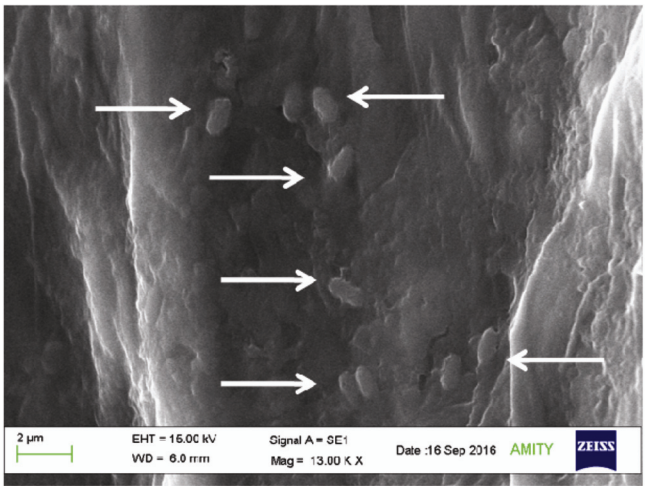

Figure 4. SEM image showing bacterial association with plant roots.

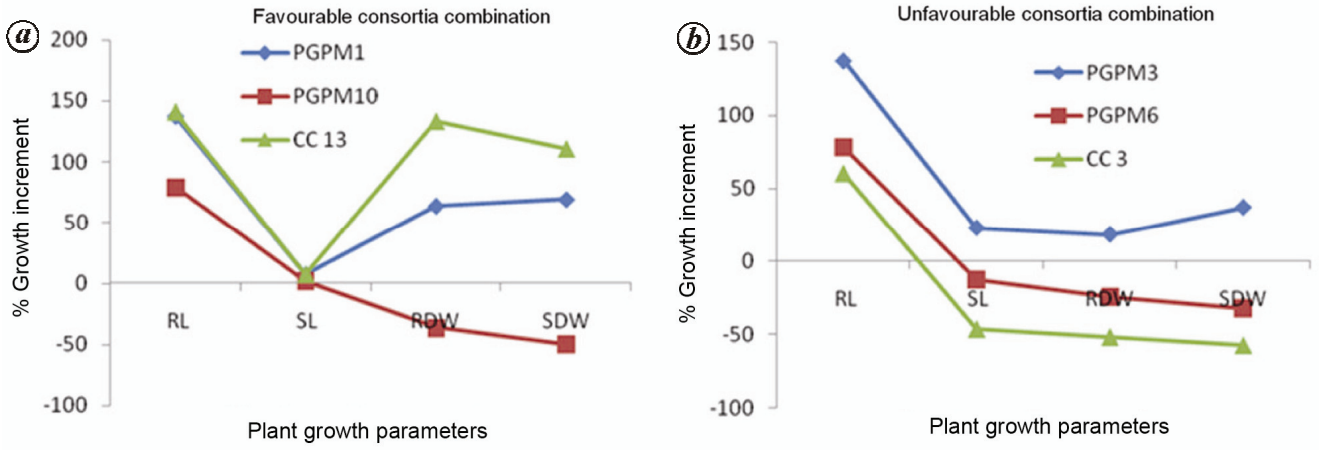

Figure 5. Contribution to plant growth: inoculation by single isolate versus consortium. ***a***, ***b***, Representative favourable (***a***) and unfavourable (***b***) consortia combinations. Note: All values are per cent increase or decrease compared to control.

Most of the PGPM consortia studied have registered statistically significant growth increments over uninoculated control plants (Table 3). Consortia treatments showed more pronounced effect on RL and RDW than on SL and SDW. This is perfectly agreeable in case of mutualistic/symbiotic associations, as early signs of growth improvement would be prominent primarily, in belowground (i.e. root) portions[17]. Growth increments in aboveground architecture take more time to manifest. However, in case of the most favourable associations, improvements in SL and SDW can also be observed (Table 3). Significant increase in RL and SL could be due to PGPM-triggered production of various metabolites, hormones like indole-3-acetic acid (IAA) and other enzymes that are directly or indirectly involved in mobilization, availability and uptake of nutrients in host plants[1]. Combinations CC1, CC10, CC13 and CC4 resulted in maximum increase in RL (≥ 20 cm) and also showed best impact on SL (≥ 13 cm). The root and shoot dry weight measurements reflected more clearly the preference of the host to certain PGPM combinations over others. CC4 gave highest biomass increase (164% RDW and 138% SDW increment) followed by CC13 (133% RDW and 110% SDW increment) and CC11 (115% RDW and 68% SDW increment) compared to uninoculated control. Combinations CC3, CC5, CC6, CC9, CC10, CC12 and CC15 recorded reduction in RDW and SDW compared to uninoculated control. This could be because of non-compatibility between the hosts and/or between members of the consortia that were probably draining out the host nutrient resources for their own establishment in an effort to outcompete the consortium counterpart[4]. In a simple two-factorial design, the geometrical mean of the columns reflects the impact on a particular parameter (RL, SL, RDW and SDW) irrespective of the treatment (CC1-15). Geometrical mean of the rows spells out the overall influence on host for a given treatment (CC1-15), independent of any one specific parameter[18]. Based on the overall growth impact (geometrical row mean), consortia combinations reporting higher cumulative row mean (Table 3) over control (CC1, CC2, CC4, CC7, CC8, CC10, CC11, CC13 and CC14) were considered favourable combinations. Also, combinations showing lower value for row mean than control (Table 3) were categorized as poor performers (CC3, CC6, CC5, CC9, CC12 and CC15).

Six-step-strategy to identify the best consortia combinations

In order to identify the best consortia from the nine favourable combinations shortlisted above, principles of the 'six-step-strategy' were applied (Figure 1). Considering the response of individual PGPM isolates (step 1 of the strategy) vis-à-vis the consortium (Table 3) and partnering members in the respective consortia (Table 2), it is evident that all combinations where PGPM3 was one of the partnering members, showed invariably poor response (CC3, CC6, CC9, CC12 and CC15). Combinations with PGPM1 were better performers, except in CC10, which showed average performance (10% increase in RL, SL; 20% decrease in RDW and SDW). Combinations with PGPM2 also showed good performance, except for CC5 that performed poorly (20% decrease in RL, SL; 70% decrease in RDW and SDW). This implies that one may opt for combinations with PGPM1 and PGPM2 and discard combinations with PGPM3. The next step of the analysis was to observe any antagonism between members of group-I and group-II. Experimental results showed no competitive inhibition amongst consortia members (Figure 2 *d*) leading to step 3, i.e. to evaluate temperature and pH responses. From Figure 2 *a* and *b*, one can decipher that at any given pH, PGPM2 had the highest colony

forming units (cfu) than PGPM1. Moreover, PGPM2 exhibited better performance at all pH levels, while PGPM1 showed a narrow pH tolerance. Though 30°C was optimal for both PGPM1 and PGPM2 (Figure 2 *b*), the latter was a better choice as it had higher inoculum potential (higher cfu) than the former at any given temperature. Therefore, with better pH, temperature tolerance and high inoculum potential, PGPM2 appears to have an advantage over PGPM1. Now considering mitotic growth behaviour (step 4), doubling time of isolates PGPM1, PGPM2 and PGPM9 were in sync showing comparable multiplication rates (g = 0.19–0.23 h), whereas other PGPMs multiplied faster (g = 0.13–0.16 h). Furthermore, PGPM1, the slowest growing of all isolates (completes approximately 4 generations/h) when paired with group-II members (that produce 7 generations/h) could face a more pronounced masking effect from partnering consortium members. Though PGPM2 is also a slow-growing isolate (completes 5 generations/h), it was still a better option over PGPM1 and exhibited better growth partnership with group-II isolates in maintaining its inoculum potential (Table 2). Thus, considering physiological responses of individual isolates, PGPM2 exhibits a competitive edge over PGPM1 as consortium partner.

The next step was to understand whether individual PGPMs are acting in a mutually complementary manner to offer host benefits. In a two-factorial design, dependency curves give an idea of the interdependency of treatments (inoculation with PGPMs) for any given response (RL, SL, RDW and SDW). An intersection among parameters used for evaluation represents a mutually complementary activity and absence of any intersection reflects non-complementation of treatments[18]. To discern the mutual complementarities between partnering consortia members, dependency curves were drawn taking into account host growth response to inoculation with members of the consortium individually and then together as consortium. A clear intersection in growth parameters was observed in consortia combinations considered favourable, whereas non-favourable consortia combinations showed no intersection in any of the growth parameters. Figure 5 depicts representative examples for favourable and unfavourable combinations. As mentioned earlier, cumulative row means in a two-factorial design help in the interpretation of overall efficiency of each of the treatments (CC1–CC15). For the purposes of calculating geometrical row mean, the per cent increment values (over control) of the respective quantitative parameters were chosen (Table 3). Combinations CC4 (PGPM1 : PGPM7), CC11 (PGPM2 : PGPM9) and CC13 (PGPM1 : PGPM10) with highest geometrical row means (Table 3) can thus be regarded as the best combinations. Long-term greenhouse studies can be planned with these combinations to develop consortium-based bioinoculants. We presume that CC11 gives sustained performance as the partnering members PGPM2 and PGPM9 have comparable mitotic activity (growth rate being 5.01 and 5.27 respectively), better tolerance to pH and temperature variations and high inoculum potential. On the other hand CC4 and CC13 have one weak consortium partner each (PGPM7 and PGPM10 respectively). Not only do PGPM7 and PGPM10 have less physiological potential (pH, temperature tolerance, total cfu), there is also a large difference in the mitotic activity between isolates PGPM7 (growth rate 6.8), PGPM10 (growth rate 6.51) and PGPM1 (growth rate 4.26). This physiological imbalance may have a compounding effect in the long-term experimentation. However, this can be proved only after greenhouse studies.

Summary

This study presents a consortium-screening protocol as a six-point schematic to construct, evaluate and shortlist the most promising consortia. *In vivo* plant bioassays are mandatory to evaluate the performances of microbial consortia even when the isolates exhibit similar preference to physiological growth conditions, synergy in co-culture and high mitotic activity. The present 'tube-in-tube' method provides a simpler, faster and economic *in vivo* bioassay compared to conventional pot-culture experiments. The study proposes a factorial design involving two representative groups and eight PGPMs, which can be expanded adding 3–4 different groups and as many isolates to facilitate selection of the most promising combinations for larger greenhouse trials before developing bioinoculants.

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ACKNOWLEDGEMENTS. This work was supported by the Department of Biotechnology, Government of India through a research grant (BT/PR11470/AGR/21/276/2008). We thank students of JIIT, Noida,, Anumeha Shah, Jyoti Chauhan and Kanika Sharma for their contribution in cohabitation experiments. We also thank Jaypee Institute of Information Technology, Noida, for providing the necessary facilities to conduct this study.

Received 19 September 2015; revised accepted 27 February 2017

doi: 10.18520/cs/v113/i01/63-70