Bioengineering of *Dioscorea nipponica* with rhizospheric *Proteus* spp. for enhanced tuber size and diosgenin content

John C. Jimtha¹ · Jyothis Mathew¹ · E. K. Radhakrishnan¹

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Abstract Rhizobacterial production of plant hormones, ACC deaminase, fixation of nitrogen, solubilization of phosphate and antimicrobial metabolites play very important role in the health and growth of plants. Hence exploration of plant probiotic perspectives of promising rhizobacterial isolates from biodiversity rich areas can have enormous applications to engineer both the biomass and active ingredients of plants. In the present study, rhizospheric *Proteus* spp. R6 and R60 isolated from *Pseudarthria viscida* and *Glycosmis arborea* were analyzed for tuber and diosgenin enhancement effects in *Dioscorea nipponica* under field conditions for 1 year. Among the two *Proteus* spp. selected, both were positive for ACC deaminase, siderophore, nitrogen fixation, IAA and ammonia production. However, the isolate R6 was found to have additional phosphate solubilizing activity. Quantitative analysis of IAA by HPTLC showed its maximum production by *Proteus* sp. R60 ($714.47 \pm 8.7 \mu\text{g/mL}$) followed by *Proteus* sp. R6 ($588.06 \pm 7.0 \mu\text{g/mL}$). The tubers formed from the *Proteus* sp. R6 treated samples were identified to have significant enhancement in size, root number and diosgenin content when compared to control. Interestingly, HPLC analysis has confirmed twofold higher diosgenin content in *Proteus* sp. R6 treated samples than control during 1 year period of its field growth. The obtained results are of great importance as it involved the utilization of rhizospheric bacteria to improve tuber size which suggests its potential use in developing cost-effective, eco-friendly and multifunctional biofertilizer.

Keywords Plant growth promoting rhizobacteria (PGPR) · *Dioscorea* · Indole 3 acetic acid (IAA)

Introduction

Plant root exudate mediated enthrallment of plant growth promoting rhizobacteria (PGPR) towards the rhizosphere and the PGPR support towards plant growth involve a good deal of chemico-biological interactions (Ahmad et al. 2008a, b). Hence the rhizosphere microbiome of wild plant species appears to be a marina of potential PGPR. This is because, these PGPR are expected to have heavy deposition of plant growth promoting features accumulated over generations. So an in-depth understanding on chemical biology of rhizosphere microbiome is expected to provide immense opportunities to engineer both growth and secondary metabolite content of plants. Plant growth promotion by PGPR (Compant et al. 2005) occurs mainly by 1-aminocyclopropane-1-carboxylate (ACC) deaminase production (Glick 2005), nitrogen fixation, production of plant growth promoting hormones like indole acetic acid (IAA) (Ali 2015), solubilization of phosphorus and other trace elements. Furthermore, they have been reported to have an array of additional phytoprotective mechanisms, including production of antibiotics, siderophores and cyanide which protect the plants from infections (Bouizgarne 2013). Therefore, application of PGPR as commercial plant probiotic is getting much attention. However, their yield enhancement effect on economically important parts of the plant and its modulatory effect on natural product biosynthesis is least explored.

Yam (*Dioscorea*) is cultivated for its tuber which is rich in carbohydrate, minerals of calcium, phosphorus, iron and also vitamins such as riboflavin and thiamin. Some species

✉ E. K. Radhakrishnan
radhakrishnanek@mgu.ac.in; radhakrishnanek@gmail.com

¹ School of Biosciences, Mahatma Gandhi University, Priyadarshini Hills PO, Kottayam District, Kottayam 686560, Kerala, India

of yam have been used medically to treat diseases like diabetes mellitus, to increase coronary flow and to prevent hypercholesterolemia (Undie and Akubue 1986). Natural saponins like diosgenin have mainly been isolated from Mexican yam (*Dioscorea* species) tubers. The saponins of *Dioscorea* are important precursors of drugs such as cortisone, and contraceptive estrogens. Also they possess cytotoxicity, anti-tumor, anti-mutagenic, anti-inflammatory, anti-viral and anti-helminthic activities (Kareru et al. 2007). However, the major problem with *Dioscorea* cultivation and purification of diosgenin is the time required for the tubers to grow to a size to possess sufficient concentration of diosgenin to be used for pharmaceutical purposes (Acharya et al. 2008). This on an average is 1 year and hence *Dioscorea* was selected in the current study to analyze the effect of PGPR on its tuber size and diosgenin content.

Various bacterial genera have been reported to have rhizospheric association with plants. These include *Pseudomonas*, *Bacillus*, *Azospirillum*, *Aerobacter*, *Aeromonas*, *Agrobacterium*, *Chryseomonas*, *Curtobacterium*, *Enterobacter*, *Erwinia*, *Flavimonas* and *Sphingomonas* (Sturz et al. 1997). However, identification of other bacterial genera with multipotent plant probiotic effect is highly demanding as enormous diversity can be expected among the rhizobacteria from biodiversity rich areas. In the case of organisms like those of *Proteus* spp., only limited reports are there on its plant beneficial features. Tsigie et al. (2011) have previously reported enhanced growth rate of rapeseed by the inoculation with *Proteus vulgaris*. *Proteus* spp. present on rhizosphere of wild plant species can offer highly promising agronomical application as plant probiotics and hence studies on its plant growth promoting properties and long term interaction with plant are of great significance to be exploited. In the current study, two *Proteus* spp. strains of rhizosphere origin were selected as candidate organisms to study their plant beneficial applications. Results of the study have demonstrated beneficial impact of selected *Proteus* spp. on tuber size and diosgenin content of *Dioscorea* after one time inoculation followed by 1 year field growth. Their remarkable influence on enhanced diosgenin content provide new avenues to explore rhizobacteria to engineer the biosynthesis of medicinally significant plant secondary metabolites.

Materials and methods

Source of bacterial strains and its molecular identification

Bacterial strains R6 and R60, which were isolated from the rhizosphere of *Pseudarthria viscida* and *Glycosmis*

arborea of Western Ghats, were selected for the study. Identification of the selected bacterial isolates was done by 16S rDNA based sequencing method. Genomic DNA was isolated from the bacterial isolates and was used as template for PCR. Primers used for the amplification of part of 16S rDNA were 16SF (5'-AgA gTTTgA TCM Tgg CTC-3') and 16SR (5'-AAG gAg gTg WTCCAR CC-3'). PCR was done in a 50 µL reaction volume containing 50 ng of genomic DNA, 20 pmol of each primer, 1.25 units of *Taq* DNA polymerase (Bangalore Genei), 200 µM of each dNTPs and 1X PCR buffer. PCR was carried out for 35 cycles in a Mycycler™ (Bio-Rad, USA) with the initial denaturation at 94 °C for 3 min, cyclic denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s and extension at 72 °C for 2 min with a final extension of 7 min at 72 °C. The PCR product was checked by agarose gel electrophoresis, purified and was further subjected to sequencing. The sequence data was checked by BLAST analysis (Zhang et al. 2000).

Screening of *Proteus* spp. R6 and R60 for plant growth promoting properties

The selected *Proteus* spp. R6 and R60 were screened for 1-Aminocyclopropane-1-carboxylate (ACC) deaminase activity by culturing on DF minimal media. For indole-3-acetic acid (IAA) production analysis, Salkowski method was done using culture supernatant of R6 and R60. Phosphate (P) solubilization property was checked by culturing R6 and R60 on Pikovskaya medium. Amount of phosphate solubilized was calculated from standard curve of phosphate solubilization prepared using known concentration of phosphate (Baykov et al. 1988). For this, one volume of the malachite green based color reagent was mixed with four volumes of the solution to be analyzed. The mixture was allowed to stand for 10 min, and the absorbance at 630 nm was measured using spectrophotometer. Siderophore production feature of R6 and R60 was analyzed using modified blue agar CAS medium containing the indicators chrome azurol S (CAS) and hexa decyl trimethyl ammonium bromide (HDTMA). Ammonia and HCN production by R6 and R60 were analysed as per previous methods (Sharifi Noori and Mohd Saud 2012). For nitrogen fixation, nitrogen free Jensen's media was used (Franché et al. 2008).

Characterization of indole-3-acetic acid (IAA)

The bacterial isolates R6 and R60 were cultured in 200 mL nutrient broth supplemented with 0.2% tryptophan. After a period of 10 days incubation, culture supernatant from R6 to R60 were separated out and extracted with ethyl acetate. Presence of IAA in the prepared extracts of R6 and R60 was checked by reverse phase HPLC analysis on a Supelcosil LC-18 column as described previously

Fig. 1 HPLC analysis for detection of IAA production. **a** Positive control (indole 3 acetic acid only), **b** Crude extract of *Proteus* sp. R60 and **c** Crude extract of *Proteus* sp. R6

(Aswathy et al. 2012). The extracts were further used along with known concentration of standard IAA (Sigma) for HPTLC analysis on silica gel 60 F254 pre-coated plates as per previous methodology (Jasim et al. 2014).

The crude extracts from R6 and R60 were also checked for IAA by Fourier transform infrared (FT-IR) analysis (IRP Shimadzu prestige 21 FTIR Shimadzu) (Huddedar et al. 2002). Further the extracts were subjected to liquid chromatography–mass spectroscopic (LC–MS) analysis (Kowalczyk and Sandberg 2001).

Treatment of *Dioscorea nipponica* with *Proteus* spp. R6 and R60

For this, *Proteus* spp. R6 and R60 were cultured for 10 days in 50 mL nutrient broth containing 0.2% additional tryptophan (Rahman et al. 2010). Tubers of *D. nipponica* were collected during November 2014 from Kerala Agricultural University, Mannuthy. Tuber pieces of 100 gm weight were made and surface sterilized using 2% sodium hypochlorite and 70% ethanol. This was followed by washing with sterile distilled water. After this, tuber pieces were treated with

10 mL cultures of *Proteus* spp. R6 and R60. The tubers were then sown in soil and growth was observed for 12 months. Tubers treated with uninoculated media were used as control. The experiment was conducted in triplicate in which each set contained 6 tubers. After 1 year growth, plants were collected and the tuber size, and root numbers were measured. The tubers were then dried at 65 °C, powdered and equal quantity from each treatment was used for extraction.

Measurement of diosgenin in treated plants

Dried and powdered dioscorea tuber (100 mg) was extracted with ethanol. This was followed by hydrolysis with 1 mol/L sulfuric acid (20 mL). This was further extracted three times with petroleum ether. The solution from this extraction was washed twice with 1 mol/L of NaOH solution followed by distilled water. The petroleum ether solution was then concentrated, dissolved in acetonitrile, and filtered through a 0.22 µm filter.

Diosgenin quantification was carried out by HPLC system (Shimadzu, Japan). The peak area of diosgenin was calibrated to diosgenin content of standard diosgenin

Fig. 2 HPTLC analysis of IAA production. *L1* Crude extract of *Proteus* sp. R60, *L2* Crude extract of *Proteus* sp. R6; *L3* STD IAA (500 µg/mL), *L4* Control

(Sigma) and the diosgenin concentrations in the different samples were calculated (Bharti et al. 2012). All treatments were performed in triplicate, and experimental data were subjected to statistical (SPSS Inc., Chicago, IL, USA) analysis. Data were used for analysis of variance (ANOVA) and means separated by Duncan's multiple range test (* $P < 0.05$).

Results

PCR amplification of 16S rDNA of R6 and R60 gave product of 1500 bp size. BLAST analysis of the sequence showed its maximum identity with *Proteus mirabilis* (93%) and *Proteus vulgaris* (99%), respectively.

Plant growth promoting properties of *Proteus* spp. R6 and R60

Among the two *Proteus* spp. selected, both were positive for ACC deaminase, siderophore, nitrogen fixation, IAA and ammonia production. However, the isolate R6 was found to have additional phosphate solubilizing activity. Growth of isolate R6 in NBRIP medium showed pH drop to 3.5 and hence it was used for quantitative determination

of phosphate solubilization by malachite green method. A maximum of 132 mg/L phosphate was found to be solubilized in tricalcium phosphate by *Proteus* sp. R6.

IAA production by R6 and R60 were confirmed by HPLC due to formation of peaks at 14.3 min retention time comparable to that of pure IAA (Fig. 1). Quantitative analysis of IAA by HPTLC showed its R_f value near to 0.76 with maximum IAA production by *Proteus* sp. R60 (714.47 ± 8.7 µg/mL) followed by *Proteus* sp. R6 (588.06 ± 7.0 µg/mL) (Fig. 2). The IR spectrum of standard IAA was found to have OH frequency at 3385 cm^{-1} and a C=O frequency at 1689 cm^{-1} which was also detected in the IR spectrum of samples. LC-MS analysis in negative mode revealed a peak of m/z 174 (M-H) which was same as that of authentic standard of IAA (Figs. 3, 4).

D. nipponica inoculated with *Proteus* sp. R6 and R60 performed better in terms of tuber size under the experimental conditions used. The plants inoculated with *Proteus* sp. R6 recorded an increase of 3.75-fold (156.67 gm) in the tuber size when compared to the uninoculated control plants (41.67 gm). The same for *Proteus* sp. R60 treated tubers showed an increase of 2.16-fold in tuber size (90 gm). The plants inoculated with *Proteus* sp. R6 were found to have 2.43-fold increase (65) in root number when compared to control plants (26.67). The same for *Proteus* sp. R60 treatment was 1.68-fold (45) higher than control (Figs. 5, 6). *Proteus* sp. R6 and R60 were found to have significant modulatory effect on biomass enhancement in *D. nipponica* as revealed by the comparative analysis of various parameters. Even though, the isolate *Proteus* sp. R6 exhibited highest tuber biomass enhancement activity, *Proteus* sp. R60 treatment also enhanced tuber size when compared to control (Fig. 5). All the data were subjected to statistical analysis for testing the significance of variance with control using SPSS Software and found to be significant at 0.05 level.

HPLC based quantification of diosgenin

The extracts were subjected to quantitative comparison of diosgenin content by HPLC. HPLC chromatogram of pure diosgenin showed a peak at RT 2.9 and the R6 and R60 treated samples also showed peak at the same retention time (Fig. 7). In the HPLC result, R6 treated samples showed twofold higher (10.08%) diosgenin content when compared with the uninoculated control (4.73%).

Discussion

Over the period of last 40 years, PGPR are shown to have the promises to support sustainable agriculture (Goswami et al. 2016). They have been reported to have wide array of

Fig. 3 FT-IR analysis of: **a** authentic IAA, **b** Crude extract of *Proteus* sp. R60, **c** Crude extract of *Proteus* sp. R6

chemically adapted mechanisms which are plant beneficial (Wong et al. 2015). The ability of plant to attract promising rhizobacteria is considered as an important factor which determines both its growth and disease control properties (Goswami et al. 2015). Hence the potential of isolated PGPR to interact with taxonomically diverse and metabolically distinct plants open up immense opportunities to explore their agricultural applications. Even though many PGPR formulations have been studied for plant growth promoting properties, its modulatory effect on natural product biosynthesis is least investigated. As the chemical biology of rhizosphere is just begin to explore, such approaches will have hopeful applications in rhizobacteria based metabolite engineering. But the major challenge involved is the selection of suitable rhizosphere source for the isolation of potential organisms. Various natural and man-made processes can have significant

impact on the presence of key rhizobacterial species. So unexplored and biodiversity rich areas like Western Ghats can expect to harbor PGPR with multipotent plant probiotic properties. Their identification is of significant importance to utilize their applications in the growth and yield of plants where the economically important part of the plant can be the underground tubers or rhizomes. So the plants like *D. nipponica* provide interesting model systems where the role of PGPR is least explored. Here it takes longtime for the formation of underground tuber and also for the synthesis and accumulation of medicinal natural product like diosgenin. Hence the use of potential PGPR may expect to provide enhancement effect to tuber size as a result of its plant growth promoting features and may also increase diosgenin content due to its modulatory effect on natural product biosynthesis. Considering this novelty and applicability, the study selected rhizospheric *Proteus* spp.

Fig. 4 LC/MS analysis of IAA. **a** LC/MS analysis of authentic IAA. **b** LC/MS analysis of crude extract of *Proteus* sp. R60 and **c** LC/MS analysis of crude extract of *Proteus* sp. R6

Fig. 5 Tuber enhancement of *D. nipponica* upon treatment with selected *Proteus* spp. **a** Control; **b** *Proteus* sp. R60 treated and **c** *Proteus* sp. R6 treated

Fig. 6 Effect of *Proteus* sp. R6 and *Proteus* sp. R60 on the tuber size and root numbers of *D. nipponica*. Data represented as mean $\pm$ SE; where $n = 6$. (Mean values followed by different alphabets are significantly different by Duncan's multiple range test at $P < 0.05$)

from Western Ghats as candidate organisms to study its bioengineering effect on *D. nipponica*.

One of the major properties studied in detail for the selected *Proteus* spp. was its ability to synthesize IAA. IAA dependent plant growth promotion mechanisms have previously been described (Maheshwari et al. 2015; Rybakova et al. 2015). In the current study, the selected *Proteus* sp. R6 and *Proteus* sp. R60 were found to perform tryptophan dependent IAA synthesis and the quantitative analysis showed R6 and R60 to have the maximum IAA yield of 588.06 ± 7.0 and 714.47 ± 8.7 $\mu\text{g/mL}$, respectively. However, R6 was observed to have highly influential effect on the tuber growth of *Dioscorea* and may likely be due to synergistic effect of various plant growth promoting

properties. The bacterial IAA increases root surface area which provides increased access to soil nutrients. Hence rhizospheric microorganisms and its IAA production can have regulatory effect on plant growth (Glick 2012). This rhizobacterial property can also influence other plant-bacterial interactions, which synergistically can determine the outcome to be either plant growth promotion or root nodulation (Kudoyarova et al. 2015). Hence, in addition to the rhizobacterial production of IAA, its interaction with other factors may also determine its impact on specific plant. This may likely to be dependent on nature of both the rhizobacteria used and the involved plant. In the current study, the *Proteus* sp. R6 which was found to have higher tuber inducing property and diosgenin enhancement effect was remarkable for its phosphate solubilisation property. The major mechanism associated with the solubilization of insoluble phosphate is the production of organic acids and the present results are also in agreement with previous reports (Sharma et al. 2013). The increased mobilization of nutrients due to this property might have favored formation of enhanced tuber biomass. Thus, the increased tuber growth and diosgenin content could be considered as a result of cell elongation and multiplication and nutrient mobilization, particularly phosphorus mediated by the applied *Proteus* sp. R6.

For analyzing the effect of *Proteus* sp. R6 and *Proteus* sp. R60 on the diosgenin level of the plant, HPLC based method was used. Here both the isolates were found to induce significant increase in the biosynthesis of diosgenin. The plants treated with R6 was found to have highest amount of diosgenin which was 10.08% while untreated plants showed only 4.73% diosgenin. Synthesis of saponins like secondary metabolites has been suggested as an adaptive strategy in plants to survive in adverse environmental conditions. The studies of Vacheron et al. (Vacheron et al. 2013) clearly demonstrated the role of PGPR on regulation of plant secondary metabolism. As role of rhizobacteria on secondary metabolism enhancement is least investigated, the current study is highly impressive with extensive agriculture applications for the cultivation of tuber crops to increase both the yield and metabolite content.

Conclusion

In the present study, two rhizospheric *Proteus* spp. were used for studying their enhancement effect on plant growth and tuber biomass of *D. nipponica*. The isolate *Proteus* sp. R6 was found to have a high impact on tuber size and diosgenin content of *D. nipponica*. Enhancement of diosgenin content is with much application as it opens ways to enhance medicinal natural product biosynthesis.

Fig. 7 HPLC analysis showing the difference in the diosgenin content of *D. nipponica* when treated with *Proteus* sp. R6 and *Proteus* sp. R60. **a** Diosgenin standard; **b** control (untreated plantlet); **c** *Proteus* sp. R60 treated plant; **d** *Proteus* sp. R6 treated plant

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest in the publication.

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