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ORIGINAL ARTICLE

Pseudomonas fluorescens R68 assisted enhancement in growth and fertilizer utilization of *Amaranthus tricolor* (L.)

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Abstract Plant probiotic potential of rhizosphere microbiome and its role in phytofertilizer mobilization are largely unexplored. In the current study, the rhizobacterium *Pseudomonas fluorescens* R68 (PFR68) isolated from Western Ghat was analyzed for its growth enhancement effect on the leafy vegetable *Amaranthus tricolor* (L.). One month of field growth of PFR68 inoculated *A. tricolor* has found to have enhanced growth parameters such as leaf number (1.57 fold), root number (1.76 fold), shoot length (1.28 fold) and fresh weight (2.31 fold). The treatment also improved soil fertility in terms of Nitrogen, Phosphorus and Potassium content. Most remarkably, application of PFR68 alone and 50% of recommended NPK dose along with PFR68 has resulted in enhanced growth of *A. tricolor* comparable to plants treated with full dose of NPK. In addition to this, application of PFR68 along with 50% NPK augmented the available Nitrogen and Phosphorus content in soil. This indicates the potential of selected organism in enrichment of soil health and enhancement of crop productivity. In conclusion, field performance of PFR68 on growth of *A. tricolor* confirms its promises to develop into plant probiotic formulation.

Keywords Biofertilizer · NPK fertilizer · Nutrient accumulation · Soil nutrient status · *Amaranthus tricolor*

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Introduction

Amaranthus tricolor is one of the most commonly used leafy vegetables in Southern part of India. Cooked leaves of the plant are used along with the main dish of cereals or tubers. The leaves are also dried and powdered to be used in traditional sauces during the dry season. The plant is a good source of iron (38.5 mg/100 g), calcium (350–400 mg/100 g), essential micronutrients, vitamins and various minerals (Beswa et al. 2016). Because of this, development of methods to improve the biomass and yield of the plant in the limited area of cultivation is highly demanding.

Recent trends in plant microbiome have demonstrated the potential impact of plant growth promoting rhizobacteria (PGPR) on growth improvement of plants and fertility of soil (Ahemad and Kibret 2014). Plant growth promotion by PGPR may involve phytostimulation, biofertilization or biocontrol mechanisms (Zahid et al. 2015). The chemical basis of these processes can have enhancing effect on soil fertility also (Figueiredo et al. 2016). The well known plant beneficial features of PGPR involve the production of phytohormones, nitrogen fixation, phytopathogen antagonism, cyanogenesis, phosphate solubilization and ACC deaminase activity (Beneduzi et al. 2012). As microbiological methods for plant growth improvement have tremendous potential to explore, in the current study we have selected leafy vegetable *Amaranthus tricolor* as the plant system.

The excessive and uncontrolled use of chemical fertilizers have resulted in various adverse effects to living systems (Adesemoye and Egamberdieva 2013). Hence, PGPR have significant role to generate environmentally sustainable bioformulations either alone or as supplement with low concentration of fertilizers. Among the various plant growth

promoting microorganisms, *Pseudomonas* spp. have diverse chemical means to support plant growth. *Pseudomonas* spp. have been reported to have the potential to improve plant biomass, relative water content, leaf water potential and root adhering soil/root tissue ratio (Sandhya et al. 2010). Positive responses on wheat yield with reduction in the requirement for inorganic fertilizers have previously been described for *Pseudomonas fluorescens* (Ahemad and Kibret 2014). PGPR like *Pseudomonas fluorescens* and *Pseudomonas putida* have also been reported to improve wheat yield to 96% with reduced dependence on inorganic nitrogen (Selvakumar et al. 2012). Inoculation of *Pelargonium graveolens* with *P. fluorescens* have also been shown to have enhancing effect on growth and biomass (Zulueta-Rodriguez et al. 2014). However, identification of microorganisms with potential agricultural applications is highly challenging. Unexplored biodiversity rich areas such as Western Ghats can be treasure trove of organisms with promising plant growth enhancement effect.

Remarkably, the isolate PFR68 used in this study was previously found to have significant effect on hardening period reduction in *Musa acuminata* cv. Grand Naine plants (Suada et al. 2015). The same isolate was also characterized for broad-spectrum plant probiotic effect on *Vigna radiata*, *Phaseolus vulgaris* and the medicinal plant *Bacopa monnieri* (John Jimtha and Radhakrishnan 2016). Because of the impressive plant growth promoting potential of the selected microorganism to interact with taxonomically distinct plants, it was selected in the current study to analyze the biomass enhancement effect on *Amaranthus tricolor.* As comprehensive investigation on effect of rhizospheric microorganisms on vegetative growth of *Amaranthus tricolor* and its effect on available soil nutrients post treatment has not previously been reported, the present study is significant.

Materials and methods

Bacterial strain

Previously isolated rhizospheric *P. fluorescens* R68 from Western Ghat regions of Kerala was used in the study. This strain was previously reported for the ability for IAA and ACC deaminase production, phosphate solubilization, Nitrogen fixation, ammonia and HCN production (John Jimtha and Radhakrishnan 2016).

Soil testing

The soil used for the pot experiment was collected from Adichira, Kottayam. The soil was air dried, grounded and passed through a 4 mm sieve and mixed thoroughly to check the pH. For determining the organic carbon level, the soil was made to fine earth and passed through a 0.2 mm sieve (80 mesh) and 0.5 g from this was made to 500 mL in a wide mouthed Erlenmeyer flask, and analysis was carried out as described previously (Sato et al. 2014). For determining the available nitrogen, microdiffusion method was used with 2 g soil in a glass bottle (Risgaard-Petersen et al. 1995). Available phosphorus was determined by Bray and Kurtz's method using 5 g soil in 100 mL shaking bottle (Sarker et al. 2014). Available potassium was analyzed by adding 5 g soil to a shaking bottle followed by the addition of 50 mL neutral 1 N ammonium acetate solution. This was followed by further incubation for 5 min in the shaking incubator with 180 rpm. The analysis was then carried out (Risgaard-Petersen et al. 1995).

Green house study with selected PGPR

Three methods were applied for the treatment of *Amaranthus tricolor* with PFR68 in which triplicates of ten seeds per treatment was used.

1. Seed inoculation with *P. fluorescens* R68
   For seed inoculation, pure culture of *P. fluorescens* R68 was grown in Luria–Bertani (LB) broth at room temperature (Stefan et al. 2013). Seeds were surface sterilized with 70% ethanol for 2 min and with 2% sodium hypochlorite solution for 10 min and then washed 10 times with sterile distilled water. Surface sterilized seeds were soaked in the bacterial suspension for 30 min under sterile conditions.
2. Supernatant application
   The rhizospheric *P. fluorescens* R68 was inoculated into LB broth supplemented with 0.2% (v/v) of L-tryptophan and incubated for five days at 28 °C. After incubation, the culture was centrifuged at 3000 rpm for 20 min and the supernatant was collected. Then 200 µL of the supernatant was added into each of the germinating seedlings grown in soil for 3 consecutive days (Parmar and Dadarwal 1999).
3. Immobilization using sodium alginate beads
   Here, bacterial culture was prepared by adding a loopful of *P. fluorescens* R68 into 200 mL of LB broth and was incubated for 24 h at 28 °C. Then 4% sodium alginate was prepared by dissolving 4 g of sodium alginate in 100 mL of distilled water with continuous stirring at 60 °C in water bath for 1 h. Bacterial culture was mixed with sodium alginate in the ratio 1:2. Then it was dropped into 0.1 M calcium chloride solution to form calcium alginate beads with bacterial cell entrapped. The beads were kept for 30 min to solidify and 3 beads per plantlet was used for treatment (Schoebitz et al. 2014).

Plant study

The experiment consisted of four treatments each with three replications, plots were distributed in a completely randomized design in which Treatment T1a contained surface sterilized seeds treated with *P. fluorescens* R68 in the form of LB cultures, T1b contained seeds treated with *P. fluorescens* R68 in the form of bacterial supernatant, T1c contained seeds treated with *P. fluorescens* R68 in immobilized form and all the T1 treatments were applied with 50% NPK fertilizer. In T2 treatments seeds were sown in soil with full dose of NPK fertilizer (100%). T3a contained seeds treated with *P. fluorescens* R68 applied in the form of culture suspension in LB culture medium, T3b contained seeds having *P. fluorescens* R68 applied in the form of bacterial supernatant and T3c contained seeds having *P. fluorescens* R68 applied in immobilized form. T4 contained seeds treated with sterile LB medium which served as control (Adesemoye and Egamberdieva 2013; Bhardwaj et al. 2014). After 25 days of sowing, plants were uprooted from the plots carefully and biometric parameters such as root length, shoot length, leaf length, fresh weight, leaf height, leaf numbers and dry weight were recorded. The soil parameters were also checked after collection of plant material.

Statistical analysis

Analysis of variance (ANOVA-One way) was performed on all experimental data, and means of growth parameters of control plants were compared with means of treated plants using the Duncan's multiple range tests with SPSS software at 5% level of significance.

Results and discussion

To achieve successful and reproducible results following the introduction of beneficial rhizobacteria into soil, its survival in the heterogeneous soil environment is very important. Owing to the constrains associated with the inoculum formulation, various introduction methods were attempted in the current study. The processing of microorganisms in various ways can have remarkable impact on microbial viability during its storage, transportation and field application (Suada et al. 2015). The success of the study may likely be due to the easy mixing of *P. fluorescens* R68 with soil without interfering with the environmental constrains.

Role of selected strain on the shoot length, leaf number, leaf length, root number, root length, fresh weight and dry mass of *Amaranthus tricolor* was recorded after 25 days of its growth. The growth parameters of PFR68 treated plants were analyzed with untreated plants and plants of other treatments. Remarkably, the rhizobacteria combined with 50% NPK fertilizer and the rhizobacteria alone led to a significant increase in most of the analyzed plant growth parameters when compared to the sole application of full dose of NPK (Fig. 1). PFR68 treatment (T3) was found to enhance leaf number (1.57 fold), root number (1.76 fold), shoot length (1.28 fold) and fresh weight (2.31 fold) of *A. tricolor* when compared with the plants treated with LB broth control (T4). Similarly, PFR68 treatment with 50% NPK (T1) showed enhancement in root number (1.65 fold), root length (1.8 fold), shoot length (1.59 fold) and fresh weight (1.52 fold) compared to control. The increase in yield and yield attributes due to the application of

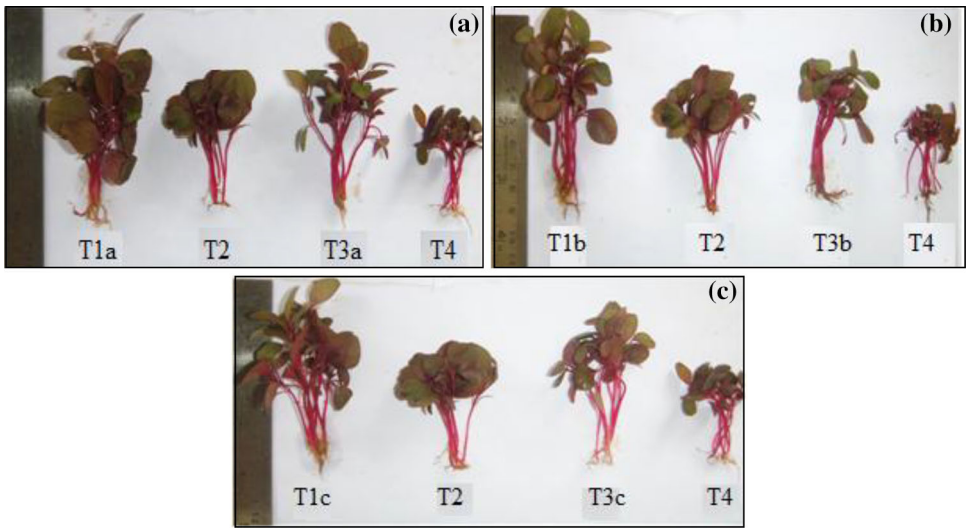

Fig. 1 In vivo study of *Amaranthus tricolor* seedlings under the influence of plant growth promoting rhizobacterium *P. fluorescens* R68. *T1a* soil + 50% NPK + *P. fluorescens* (seed inoculation), *T1b* soil + 50% NPK + *P. fluorescens* (bacterial supernatant), *T1c* soil + 50% NPK + *P. fluorescens* (immobilization), *T2* soil + 100% NPK, *T3a* soil + *P. fluorescens* (seed inoculation), *T3b* soil + *P. fluorescens* (bacterial supernatant), *T3c* soil + *P. fluorescens* (immobilization), *T4* seeds treated with sterile LB broth (Control)

biofertilizer supplemented with chemical nitrogen fertilizer can be due to mechanisms such as Nitrogen fixation, ammonia excretion, phosphate solubilization (Babalola 2010) and production of growth hormones. The result obtained was in accordance with previous studies where growth of cotton, maize and black gram was reported to be higher in treatments of biofertilizer combined with organic and chemical fertilizers (Abbasi and Yousra 2012). Zahir et al. (2000) have conducted a series of laboratory experiments with bacterial strains on wheat and reported an increase in shoot length (38%), shoot dry weight (36%), root length (20%) and root dry weight (13%) due to bacterial effect. A similar increase in wheat growth due to rhizobacterial strains has also been reported by Cakmakci et al. (2007). Effects of root colonization by PGPR on vegetative growth of *Origanum majorana* L., have showed significant increase in shoot length, shoot weight, number of leaves, number of nodes and root dry weight in comparison to controls (Banchio et al. 2008).

Augmentation of fresh weight of *A. tricolor* after treatment with PFR68 showed the plant growth promoting potential of the isolate. Most of the plant growth enhancement parameters of PFR68 treatment and PFR68 with 50% NPK treatment were in a comparable range. Hence the organism treatment alone is having the promises to minimize NPK fertilizer use without affecting the yield. Most remarkably, the application of PFR68 alone was found to have superior performance when compared to application of 100% NPK. Application of *P. fluorescens* R68 alone (T3a, T3b, T3c) has resulted in significant enhancement of leaf number (1.1 fold), root number (1.19 fold), shoot length (1.19 fold) and fresh weight (2.77 fold) when compared with the plants supplemented with a recommended full dose of NPK (T2) (Table 1). The plants treated with *P. fluorescens* R68 in combination with 50% of NPK (T1a, T1b, T1c) (Bhardwaj et al. 2014) also exhibited increase in root number (1.12 fold), root length (1.32 fold), shoot length (1.49 fold), dry weight (1.92 fold) and fresh weight (1.82 fold) compared to those with full dose NPK (T2). Results of current study was in accordance with previous study on cotton, where the use of half dose of mineral NPK with effective microorganisms (EM) and organic matter (OM) saved mineral N fertilizer by almost 50% compared to a system with only mineral NPK application (Abbasi and Yousra 2012). The observed result may likely to be due to the plant growth promoting features of *P. fluorescens*, which might have positively regulated nutrient uptake or resulted in the enhanced production of endogenous phytohormone when compared to treatments where only fertilizer was present (Table 1). Application of PFR68 alone in nutrient rich soil can expect to enhance the growth and yield of leafy vegetables by rapid mobilization of nutrients to plants. In less fertile soil, PFR68 with low fertilizer combination may expect to provide plant growth enhancement by limiting application of large quantity of fertilizer. Result of the current study revealed the potential of PFR68 to reduce the use of NPK fertilizer by half of recommended dose. This also offers flexibility in the development of formulation containing either PFR68 alone or its combination with 50% NPK due to its comparable plant probiotic effect. This is of significant application in its performance on soil with diverse nutrient profile.

The rhizobacteria with ACC-deaminase production are well known for their effect to improve root growth of plants due to decreased ethylene synthesis (Nadeem et al. 2010). Inoculation of PGPR with ACC-deaminase activity have also been reported to increase the growth of wheat and maize under field conditions (Nadeem et al. 2010). The PFR68 selected in the study has already been proved to have ACC-deaminase activity along with IAA

Table 1 Effect of *Pseudomonas fluorescens* R68 and NPK on growth of *Amaranthus tricolor*

Treatments	LN	LL	RN	RL	SL	DW	FW
T1a	4.71 ± 0.61^{de}	1.75 ± 0.39^{cde}	2.33 ± 0.7^{bc}	2 ± 0.59^{bcd}	3.39 ± 0.51^{bc}	0.017 ± 0.003^{a}	0.139 ± 0.036^{bc}
T1b	4.66 ± 0.36^{de}	2.16 ± 0.32^{f}	2.16 ± 0.58^{bc}	1.84 ± 0.78^{abcd}	2.6 ± 0.4^{a}	0.016 ± 0.013^{ab}	0.137 ± 0.01^{bc}
T1c	3.66 ± 0.69^{ab}	1.86 ± 0.15^{def}	2.16 ± 0.62^{bc}	1.58 ± 0.37^{abc}	3.74 ± 1.33^{c}	0.025 ± 0.03^{b}	0.116 ± 0.02^{abc}
T2	4.49 ± 0.69^{cde}	2.04 ± 0.44^{ef}	2.08 ± 0.59^{abc}	1.51 ± 0.37^{abc}	2.51 ± 0.33^{a}	0.013 ± 0.013^{ab}	0.076 ± 0.039^{ab}
T3a	4.88 ± 0.77^{e}	1.92 ± 0.18^{def}	2.49 ± 0.27^{c}	2.16 ± 0.58^{cd}	3 ± 0.77^{ab}	0.011 ± 0.002^{bc}	0.134 ± 0.027^{bc}
T3b	4.6 ± 0.13^{de}	1.48 ± 0.28^{bc}	2.38 ± 0.32^{bc}	1.15 ± 0.2^{a}	2.92 ± 0.28^{ab}	0.008 ± 0.001^{a}	0.139 ± 0.015^{bc}
T3c	4.98 ± 0.4^{cde}	1.87 ± 0.34^{def}	2.21 ± 0.4^{bc}	2.17 ± 0.17^{cd}	2.85 ± 0.45^{ab}	0.007 ± 0.0007^{a}	0.211 ± 0.024^{c}
T4	3.16 ± 0.18^{a}	0.95 ± 0.16^{a}	1.41 ± 0.34^{a}	1.11 ± 0.36^{a}	2.34 ± 0.2^{a}	0.003 ± 0.0004^{a}	0.091 ± 0.0601^{ab}

Data represented as mean ± SE where $n = 6$

T1a soil + 50 % NPK + *P. fluorescens* (seed inoculation), *T1b* soil + 50 % NPK + *P. fluorescens* (bacterial supernatant), *T1c* soil + 50 % NPK + *P. fluorescens* (immobilization), *T2* soil + 100 % NPK, *T3a* soil + *P. fluorescens* (seed inoculation), *T3b* soil + *P. fluorescens* (bacterial supernatant), *T3c* soil + *P. fluorescens* (immobilization), *T4* seeds treated with sterile LB broth (Control) (*LN* leaf numbers, *LL* leaf length, *RN* root number, *RL* root length, *SL* shoot length, *DW* dry weight, *FW* fresh weight)

Mean values followed by the same alphabet are not significantly different by Duncan's multiple range test at $^{*}P \leq 0.05$

Table 2 Influence of chemical fertilizer and biofertilizer on available nitrogen (kg/ha), available Phosphorus, available potassium (kg/ha) and total organic carbon (%) in fertile soil

Treatment	pH	Available nitrogen (kg/ha)	Available phosphorus (kg/ha)	Available potassium (kg/ha)	Total organic carbon (%)
Control soil (B)	5.2 ± 0.09	491 ± 23	35 ± 1.05	224 ± 3.4	3.0 ± 0.04
Control soil (A)	6.4 ± 0.13	486 ± 15	37 ± 0.17	123 ± 3.7	2.09 ± 0.04
Soil + 100% NPK(B)	5.4 ± 0.04	590 ± 23	44 ± 0.96	234 ± 1.06	2.9 ± 0.08
Soil + 100% NPK(A)	5.9 ± 0.09	554 ± 9	38 ± 1.08	201 ± 1.17	2.1 ± 0.03
Soil + 50% NPK + BF(A)	5.19 ± 0.07	637 ± 18	42 ± 0.62	175 ± 1.6	2.9 ± 0.02

B before planting, *A* after harvesting the plant, *BF* biofertilizer

production and phosphate solubilization (John Jimtha and Radhakrishnan 2016). So it is not surprising to confirm the observed result as due to the multiple plant growth promoting properties of selected organism. But the ability of the organism to impart plant growth promotion in taxonomically diverse plants is highly significant. Because the same isolate has been described to have growth enhancement effect in *Vigna radiata*, *Phaseolus vulgaris*, *Bacopa monnieri* and *Musa acuminata* cv. Grand Naine plants (Suada et al. 2015; John Jimtha and Radhakrishnan 2016).

In soil analysis, the highest organic carbon content was found in samples collected from the post treatment of rhizobacteria along with 50% dose of NPK which was 1.45 fold higher when compared to the control. For the same sample, available N content was 1.3 fold higher than the control. In a previous study, *Azotobacter* present in biofertilizer had been reported to produce a variety of growth-promoting substances (Hayat et al. 2010). This was also explained to stimulate the production of root exudates to transfer nearly 5–21% of all photosynthetically fixed carbon (Bhardwaj et al. 2014). Ammonia production by selected PFR68 and applied NPK might have favored higher available nitrogen content of soil collected from post treatment of *P. fluorescens* and 50% NPK combined application. The soil used in this experiment was fairly poor in available P (35 kg/ha). However, available P (Olsen-P) in soil significantly increased when rhizobacteria along with half the recommended dose of NPK (Table 2) was used. Therefore, use of *P. fluorescens* with fertilizer in the study might have played an important role in improving P bioavailability. The increase in soil P content might be due to the P-solubilizing potential of *P. fluorescens* used. Several authors attribute the production of organic acids, chelating oxoacids from sugars, and exchange reactions in the growth environment to the solubilization of inorganic insoluble phosphates by microorganisms (Sharma et al. 2013). Soil K content showed a similar response to different amendments as that recorded for N and P. Hence, biofertilizer in combination with 50% NPK significantly increased the soil nutrient content. The relative increase in soil nutrients due to the application of *P. fluorescens* is considered as a result of its decomposition of organic wastes and residues present in the soil or through applied materials. The novelty of current study is the observation on both biomass enhancement of *Amaranthus* and improved soil fertility by application of combination of *P. fluorescens* with 50% of NPK recommended for *Amaranthus* cultivation when compared to soil without addition of NPK and *P. fluorescens*. The economic analysis (data not shown) has indicated this as cost-effective due to application of only limited dose of fertilizer as soil amendments. Moreover, these microbial formulations could reduce dependence on chemical fertilizers with improved soil health. The findings of current study clearly showed the ability of selected rhizobacterial inoculant to bring about the enhancement of available nitrogen and phosphorus and potassium in *Amaranthus* rhizosphere.

Conclusion

A field study was conducted to analyze the plant probiotic potential of *P. fluorescens* R68 alone or its combination with half dose of chemical fertilizer over the full dose of recommended NPK fertilizer. *Amaranthus* plant when treated with rhizobacteria alone or with 50% dose of NPK was found to perform better as with full dose of NPK fertilizers. This study indicates distinct benefit on the application of rhizobacteria combined with chemical fertilizer compared to a full supply of NPK alone. The increased concentration of nutrients such as nitrogen and potassium in post treatment soil demonstrates the ability of rhizobacterial combination with NPK to have the ability to enrich soil with nutrients. Thus, supplementing biofertilizer with low rates of chemical N fertilizer may compensate for nutrient deficiency also. The use of biofertilizer, therefore, has the potential to decrease the input costs of agricultural production, and may be applied to increase yield potential of low commercial value sites by improvement of nutrient status and physical conditions of poor soils. The results of the study showed the ability of bacteria to support growth

promotion of *Amaranthus tricolor* even with different mode of processing.

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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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