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Individual and Combined Effects of *Azospirillum brasilense* and *Pseudomonas fluorescens* on Biomass Yield and Ajmalicine Production in *Catharanthus roseus*

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Abstract: In the present study, the effect of different plant growth promoting rhizobacteria (PGPR) like *Azospirillum brasilense* and *Pseudomonas fluorescens* on growth parameters and the production of terpenoid indole alkaloids are investigated in two varieties 'rosea' and 'alba' of *Catharanthus roseus*. The production of IAA by the PGPR has taken as main criteria in the selection of PGPRs for the seed and seedling treatments. The maximum ajmalicine content recorded in the combined inoculation of *Azospirillum brasilense* + *Pseudomonas fluorescens* in 'rosea' variety on 90 DAP (0.700 mg g^{-1} dry weight). The control plants performed far lower than the individual and combined inoculation of PGPR treatments in both seed and seedling treatment in 'rosea' and 'alba' varieties. From the results of this investigation, it can be concluded that, the seed priming and seedling treatments of native PGPRs can be used as a good tool in the enhancement of biomass yield and alkaloid contents in medicinal plant cultivation, as it provides an eco-friendly approach.

Key words: *Catharanthus roseus*; IAA, Ajmalicine, Seed priming, Plant growth promoting rhizobacteria (PGPR), *Azospirillum brasilense*, *Pseudomonas fluorescens*

INTRODUCTION

Numerous microorganisms live in the portion of soil modified or influenced by plant roots so called 'rhizosphere' [1]. Among these microorganisms, some have positive effects on plant growth promotion constituting the plant growth promoting rhizobacteria (PGPR) such as *Azospirillum*, *Azotobacter*, *Pseudomonas*, several gram positive *Bacillus* sp [1,2]. The diazotrophic rhizobiocoenosis is an important biological process that plays a major role in satisfying the nutritional requirements of the commercial medicinal plants. Studies on the diazotrophic population in the rhizosphere region and testing the suitability of the isolated diazotroph as seed inoculant will be highly useful in improving the productivity of these commercially important medicinal plants. Diazotrophs secrete plant growth hormones such as auxins, gibberellins and cytokinins [3]. The strong and rapidly stimulating effect of fungal elicitor on plant secondary metabolism in medicinal plants attracts considerable attentions and research efforts [4]. The reasons responsible for the diverse stimulating effects of fungal elicitors are complicated and could be related to the interactions between fungal elicitors and plant cells, elicitor signal transduction and plant defense responses [5]. In plants certain secondary metabolite pathways are induced by infection with microorganisms [6].

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Mediculture, the scientific cropping of industrially important medicinal plants, has become the need of the day to improve the productivity of the critical alkaloid components. *Catharanthus roseus* (L.) G. Don. (Madagascar periwinkle) is a perennial tropical plant belonging to the family Apocynaceae that produces more than 100 monoterpenoid indole alkaloids (MIAs) including two commercially important cytotoxic dimeric alkaloids used in cancer chemotherapy [7]. Periwinkle, native to Madagascar is now found in many tropical and sub-tropical regions of the world. The derivatives of its economic importance from its highly situated anti-cancer leaf alkaloids, vincristine (VCR) and vinblastine (VLB) and antihypertension root alkaloid, ajmalicine. In addition to anticancer activity, the total alkaloid and chloroform fraction of crude drug, showed hypotensive and selective transquilizing properties [8]. In medicinal plants, the content of the economically important metabolite is more than the yield of the plant part containing the metabolite, as it determines the cost of its extraction [9]. The cell, tissue cultures and biotechnological aspects of this plant are being extensively investigated to increase the yield of the alkaloids [10].

In this context, the cultivation of periwinkle is becoming popular among the farmers. The major problem in the cultivation of periwinkle is poor yield and establishment at field level. Many investigations have been already carried out in this plant on its medicinal importance [11-16], but the role of PGPR in this medicinal plant had attracted little attention. The plant growth promoting rhizobacteria such as *Azospirillum*, *Azotobacter* and *Pseudomonas* were isolated from *C. roseus*. In this experiment, an attempt is made for the improvement of alkaloids through the application of PGPRs. We systematically investigated the effect of PGPR (*Azospirillum* and *Pseudomonas*) on growth parameters and indole alkaloid (ajmalicine) production in *C. roseus* plant. Two distinct varieties of this plant, the pink flowered 'rosea' and white flowered 'alba' were taken for the present study.

MATERIALS AND METHODS

IAA Production: The IAA production by the microbial inoculants was used as main criteria for the selection of inoculants for further seed and seedling treatments in 'rosea' and 'alba' varieties. *Pseudomonas fluorescens*, *Azospirillum brasilense* individually and both combined in separate culture filtrates were estimated.

Preparation of PGPR Broth for Seed Priming: The seeds of both the varieties of *C. roseus* were collected from J.P. Laboratories, Rajapalayam, Tamil Nadu, India. The PGPR like *Azospirillum brasilense* and *Pseudomonas fluorescens* were taken for the study. The isolates of *Azospirillum brasilense* and *Pseudomonas fluorescens* were isolated from the rhizosphere were used. The both of them were separately grown in Nfb and King's 'B' broth. Seeds were treated with 10 ml of broth culture with an initial population of 10^7 cells ml^{-1} of *Azospirillum brasilense* and *Pseudomonas fluorescens* as individual and combined treatments. The seeds were treated for 30 minutes and then shade dried. Prior to the treatment, the seeds were surface sterilized in aqueous solution of 0.1% $HgCl_2$ for 60 seconds to prevent microbial contamination and washed with sterile water for several times. The seedlings were raised in the potculture yard of Microbiology Department, Faculty of Agriculture, Annamalai University during February-May 2006. Before transplantation into main field, the seedlings were dipped in PGPR inoculum and planted in the field. The nursery beds were watered twice a day and weeded regularly in order to ensure healthy growth of the seedlings. The land was prepared repeatedly ploughed and brought to fine tilth and divided into four plots for transplantation.

Four plots for each variety were prepared, 20 plants plot^{-1}, with a spacing of 30x45 cm and irrigated immediately for better establishment. Subsequent irrigation was done two times in a week to keep the optimum moisture level in the soil.

***Azospirillum* and *Pseudomonas* Treatment:** Each variety had been subjected to seed treatment of *Azospirillum brasilense*, *Pseudomonas fluorescens* and combination of *Azospirillum* + *Pseudomonas* and untreated control. The plants were uprooted randomly on 30th, 60th and 90th days after planting (DAP) for analysing morphological parameters and estimation of ajmalicine content.

Ajmalicine Extraction and Quantification: Ajmalicine extraction from the roots was carried out by following the standard extraction method explained in [7].

Statistics: Each treatment was analysed with at least seven replicates and a standard deviation (SD) was calculated and data are expressed in mean ± SD of seven replicates.

RESULTS

Effect of PGPR on IAA Content: IAA studies on the selected rhizosphere microorganisms (Table 1) revealed that *Azospirillum brasilense* of 1.985 mg IAA/100 ml culture filtrate, *Pseudomonas fluorescens* of 2.017 mg of IAA/100 ml of culture filtrate. When the combined inoculation of both isolates had resulted in 2.264 mg of IAA/100 ml of culture filtrate. The studies are further can be correlated to the alkaloid production abilities when reported in the rhizosphere of rosea and alba varieties of periwinkle.

Table 1: IAA production capability of the selected rhizosphere microorganisms

Isolate strains	Quantity of IAA* 10th day
Azospirillum brasilense	1.985
Pseudomonas fluorescens	2.017
Azospirillum + Pseudomonas	2.264

* Expressed as mg/100 ml culture filtrate

Effect of PGPR on Growth Parameters of *C. roseus*: The variation in plant height and root length growth parameters of *C. roseus* 'rosea' variety seedlings treated with different PGPR treatment when compared with *C. roseus* 'alba' variety (Table 1). In combined inoculation, *Azospirillum + Pseudomonas* was recorded the highest plant height (34±1.68; 41±1.42 and 50±1.24), root length (20±0.42, 31±0.66 and 39±0.28) at all sampling periods in 'rosea' variety (30, 60 and 90 DAP). In individual inoculation of *Pseudomonas fluorescens* resulted an increase in plant height and root length of rosea variety at all sampling periods (30, 60, 90 DAP) followed by *Azospirillum brasilense*. The control treatment performed for below the rest of the treatments.

Effect of PGPR on Number of Leaves: The number of leaves was recorded more in the treated combined inoculation of *Azospirillum* and *Pseudomonas* on 90 DAP in the *C. roseus* 'rosea' variety (34±0.42) followed by individual inoculation of *Pseudomonas fluorescens* of

Table 2: Growth parameters of *C. roseus* variety as influenced by different PGPR

		Rosea variety						Alba variety					
		Plant height (cm plant^{-1})			Root length (cm plant^{-1})			Plant height (cm plant^{-1})			Root length (cm plant^{-1})		
Sl. No.	Treatments	30 DAP	60 DAP	90 DAP	30 DAP	60 DAP	90 DAP	30 DAP	60 DAP	90 DAP	30 DAP	60 DAP	90 DAP
1.	*Azospirillum*	28±1.44	34±1.32	42±1.44	16±0.64	22±0.42	31±0.44	24±1.23	32±1.56	39±1.60	14±0.22	20±0.32	29±0.24
2.	*Pseudomonas*	30±1.26	38±1.66	48±1.88	18±0.86	27±0.14	32±0.66	28±1.44	36±1.60	44±1.20	16±0.66	22±0.84	30±0.24
3.	*Azospirillum + Pseudomonas*	34±1.68	41±1.42	50±1.24	20±0.42	31±0.66	39±0.28	32±1.24	39±1.66	47±1.54	19±0.26	25±0.23	32±0.33
4.	Control	25±1.64	30±1.66	36±1.36	14±0.24	18±0.64	24±0.14	20±0.44	25±0.66	34±0.60	12±0.44	17±0.21	22±0.14

Values are mean ± S.E. of three samples

Table 3: Growth parameters and Ajmalicine content (mg g^{-1}DW)of *C. roseus* variety as influenced by different PGPR

		Rosea variety						Alba variety					
		No. of leaves plant^{-1}			Ajmalicine content			No. of leaves plant^{-1}			Ajmalicine content		
Sl. No.	Treatments	30 DAP	60 DAP	90 DAP	30 DAP	60 DAP	90 DAP	30 DAP	60 DAP	90 DAP	30 DAP	60 DAP	90 DAP
1	*Azospirillum*	14±0.16	18±0.43	26±0.12	0.35±0.18	0.40±0.44	0.60±0.18	12±0.35	16±0.12	23±0.84	0.30±40	0.35±0.06	0.40±0.14
2	*Pseudomonas*	16±0.50	20±0.88	29±0.78	0.375±0.44	0.425±0.22	0.65±0.24	14±0.46	19±0.43	26±0.56	0.35±0.12	0.39±0.12	0.45±0.26
3	*Azospirillum + Pseudomonas*	19±0.66	25±0.62	34±0. 42	0.45±0.23	52.5±0.14	0.70±1.20	18±0.79	22±0.66	29±0.44	0.40±0.44	0.475±0.40	0.51±0.06
4	Control	12±0.42	17±0.20	25±0.27	0.30±1.24	0.35±3.4	0.42±0.18	10±1.10	14±0.55	20±0.62	2.50±0.20	0.31±0.10	0.37±0.40

Values are mean ± S.E. of three samples

C. roseus 'rosea' variety (29±0.42) when compared with *C. roseus* 'alba' variety. The low number of leaves recorded in the control of *C. roseus* 'alba' variety on DAP (20±0.62) (Table 2).

Effect of PGPR on Ajmalicine Content: During the early stages of plant growth the alkaloid ajmalicine content was less in both varieties. The lowest content was recorded in roots of control of 'alba' variety on 30 DAP. But the content increased with age of plant in both control and treated plants. The highest content (0.700 mg g^{-1} dry weight) was recorded in the roots of combined inoculation of PGPR (*Azospirillum* + *Pseudomonas*) treated 'rosea' variety on 90 DAP (Table 2).

DISCUSSION

The seed treatment with PGPR (*Azospirillum brasilense* and *Pseudomonas fluorescens*) increased the plant height, root length, number of leaves and Ajmalicine content of *C. roseus* 'rosea' variety and *C. roseus* 'alba' varieties when compared to control. The occurrence of *Azospirillum*, *Pseudomonas* in and around the root system of cereals, vegetables and the beneficial effect upon inoculation has been well established. In the present study, the increased growth parameters in *C. roseus* 'rosea' and 'alba' variety due to seed treatment of PGPR might be due to the production of growth hormones (IAA, gibberellins, auxins), by the bacteria. It is worth noting that the increase in growth parameters observed on inoculation of PGPR strains usually have been found to increase the root length and root biomass [17-19] and this better developed root system may increase the mineral uptake in plants.

In the present study, the PGPR were tested for their effect on growth parameters of *C. roseus* 'rosea' and 'alba' variety and the results clearly indicated the PGPR isolates significantly improved plant growth, root length, number of leaves etc. This was in confirmation with early report that *Azospirillum* and *Azotobacter* isolates to the medicinal plants. Siddique [20] reported that tomato crop inoculated with *Pseudomonas fluorescens*, *Azotobacter chroococcum* and *Azospirillum brasilense* either alone or in combination recorded higher plant growth or controlled the nematode, *Meloidogyne incognita*. Khalid *et al*. [17] reported that due to PGPR inoculation besides increasing yield also enhanced the alkaloid contents of roots especially Withaferin-A in ashwagandha due to the production of IAA. In the present study, increase in Ajmalicine content in the root may be due to the inoculation of PGPR. Since the PGPR which normally induces the production of growth promoting substances like IAA, gibberellins, auxins and thereby by the rhizobacteria enhanced proliferation of root system which in turn enhanced mineral uptake (NPK) and consequently increased dry matter production [21].

In conclusion, PGPR's seed treatments had improved number of leaves, plant height and Ajmalicine of rosea and alba varieties. A similar trend has been reported by Thosar *et al*. [22] in Ashwagandha. Continuous availability of growth regulators induced different alkaloids with variable effects among the regulators (El-Sayed and Verpoorte, 2004). Further studies are required to confirm whether IAA or Gibberellin or both are involved in total alkaloid production in the *Catharanthus roseus* varieties. Since the alkaloids production determines the rhizosphere microbial population.

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