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EFFECT OF ROOT INOCULATION WITH PLANT GROWTH PROMOTING RHIZOBACTERIA (PGPR) ON PLANT GROWTH, ALKALOID CONTENT AND NUTRIENT CONTROL OF *CATHARANTHUS ROSEUS* (L.) G. DON.

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Karthikeyan, B., Joe, M. M., Jaleel, C. A. & Deiveekasundaram, M.: Effect of root inoculation with plant growth promoting rhizobacteria (PGPR) on plant growth, alkaloid content and nutrient control of *Catharanthus roseus* (L.) G. Don. Nat. Croat., Vol. 19, No. 1, 205–212, 2010, Zagreb.

The effect of plant growth promoting rhizobacteria such as *Azotobacter, Bacillus* and *Pseudomonas* was tested separately or in combination in *Catharanthus roseus* for two consecutive years (2005 and 2006). The combinations of above mentioned PGPR strains significantly increased plant height, root length, root girth and alkaloid content in *C. roseus* in comparison to the control. In addition, all nutrient contents (N, P, K, Ca and Mg) were also significantly increased as compared to the control. The maximum N, P, K content was obtained from the combination of PGPR treatment. The results of this study suggest that PGPR applied in combination have the potential to increase the plant growth, alkaloid content and nutrient content of *C. roseus.*

Key words: *Catharanthus roseus,* ajmalicine, PGPR, root inoculation

Karthikeyan, B., Joe, M. M., Jaleel, C. A. & Deiveekasundaram, M.: Efekt inokulacije korijena korijenovim bakterijama za poticanje rasta (PGPR) na rast biljke, sadržaj alkaloida i nutrijenata kod biljke *Catharanthus roseus* (L.) G. Don. Nat. Croat., Vol. 19, No. 1, 205–212, 2010, Zagreb.

Testirani su učinci korijenovih bakterija za poticanje rasta, kao što su *Azotobacter, Bacillus* i *Pseudomonas*, zasebno ili u kombinaciji, na biljci *Catharanthus roseus* i to tijekom dviju godina (2005 i 2006).

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Kombinacije gorespomenutih PGPR sojeva značajno su povećale visinu biljaka, duljinu korijena, debljinu korijena i sadržaj alkaloida kod *C. roseus* u usporedbi s kontrolom. Uz to sadržaj svih nutrijenata (N, P, K, Ca i Mg) bio je značajno povišen u usporedbi s kontrolom. Maksimalni sadržaj N, P, K dobiven je kombiniranim tretmanom PGPR. Rezultati ovog rada sugeriraju da PGPR primjenjeni u kombinaciji imaju potencijal povećati rast biljke, sadržaj alkaloida i nutrijenata kod biljke *C. roseus.*

Ključne riječi: *Catharanthus roseus,* ajmalicin, PGPR, inokulacija korijena

INTRODUCTION

Madagascar periwinkle, *Catharanthus roseus* (L) G. Don. is a medicinally important plant that produces anticancer dimeric alkaloids, vinblastine and vincristine in leaves, and accumulates anti-hypersensitive alkaloids (ajmalicine and serpentine) in roots. This plant grows wild in distant tropical and subtropical geographical areas with different agroclimate zones. It contains more than 100 alkaloids, distributed in all parts of the plant in varied proportions. The total alkaloid content in root amounts to 2–3 % or reaches up to 9 % in fibrous roots, whereas leaves contain one per cent of alkaloids, stem contains 0.48 %, fruit 0.40 %, seeds 0.18 % and pericarp contains 1.14 %. This plant has a tremendous export potential and can increase foreign exchange to the amount of several million dollars.

An intensive farming practice that warrants high yield and quality requires the extensive use of chemical fertilizers, which are costly and may create environmental problems. Therefore, more recently there has been a resurgence of interest in environmental friendly, sustainable and organic agricultural practices (Esitken *et al.*, 2005). In this context, the use of biofertilizers containing plant growth-promoting rhizobacteria (PGPR) strains instead of synthetic chemicals may serve as an effective alternative and environmentally friendly practice to improve plant growth through the supply of plant nutrients and soil productivity (O'CONNELL, 1992). Moreover, it has been found that exploiting these PGPR strains for the growth promotion could reduce the need for chemical feritilizers as well as the cost of cultivation.

Among different groups of biofertilizers; nitrogen fixing and phosphorus solubilizing bacteria may be considered to be important since they improve plant nutrition by increasing N and P uptake by plants, and they play a significant role as plant growth promoting rhizobacteria (PGPR) in the biofertilization of crops (KARLIDAG *et al.*, 2007). It has been a well-known fact that these PGPR strains may promote growth either by fixation of atmospheric nitrogen or by solubilization of minerals such as phosphorus (KARTHIKEYAN *et al.*, 2007; 2008) and they can also promote growth through production of plant growth regulators (KLOPPER & SCHROTH, 1978; JALEEL *et al.*, 2007).

This PGPR activity is reported in species belonging to *Azospirillum*, *Azotobacter*, *Pseudomonas*, *Bacillus*, *Acinetobacter*, *Alcaligenes*, *Beijernckia*, *Burkholderia*, *Enterobacter*, *Erwina*, *Flavobacterium*, *Rhizobium* and *Serratia* (RODRIGUEZ & FRAGA, 1999; STURZ & NAWAK, 2000; SUDHAKAR *et al.*, 2000; KARLIDAG *et al.*, 2007).

The occurrence of *Azospirillum*, *Azotobacter* and *Pseudomonas* in the rhizosphere of medicinal plants such as *C. roseus*, *Coleus forskholi*, *Ocmium sanctum* and *Aloe vera* has been documented earlier (KARTHIKEYAN *et al.*, 2008). Furthermore, these strains

are also known to stimulate growth and yield in *Ashwagandha* and other medicinal plants (THOSAR *et al.*, 2005; ATTIA & SAAD, 2001).

However, reports regarding the bioinoculation effect of these PGPR strains in medicinal plants and particularly in *C. roseus* have been scarce. Hence the present study was undertaken to investigate the growth promoting effects of root inoculation of *Azotobacter chrooccocum*, *Pesudomonas fluorescens* and *Bacillus megaterium* on plant height, root length, root girth, ajmalicine content (root alkaloid).

In addition the effect of bacterial treatment of plant leaves in *Catharanthus roseus* variety was also evaluated.

MATERIALS AND METHODS

Bacterial strains, culture conditions, media and treatments

All bacterial strains used in the present study were isolated from *C. roseus* (KARTHIKEYAN *et al.*, 2008) *A. chroococcum* grown in Waksman base medium (WB) for routine use and maintained in Waksman broth with 15% glycerol. *P. fluorescens* and *B. subtilis* were grown on nutrient agar (NA) for routine use and for long-term storage they were maintained in Nutrient broth (NB) with 15% glycerol at –80 °C. The isolates were designated based on the location in Tamilnadu, India, where they were collected as (Virudhanagar – *A. Chroococcum*) – VAzt, (Virudhanagar – *P. fluorescens*) – Vps, (Virudhanagar – *B. subtilis*) – Vbs. For each experiment a single colony was transferred to 500 mL flasks containing WB and NB grown aerobically in flasks on a rotating shaker (150 rpm) for 48 h. The bacterial suspensions were then diluted in sterile water to a final concentration of 10^9 CFU/mL, and the resulting suspensions were treated with *C. roseus* plants.

FIELD EXPERIMENTS

The seedlings of *C. roseus* were raised in the pot culture yard, Department of microbiology, Faculty of Agriculture, Annamalai University in both years 2005 and 2006. Thirty days old seedlings were dipped in the PGPR inoculum and planted in the field. The trial was conducted for two consecutive years with the same treatment. Eight treatment plots (three plants per pot) were prepared and irrigated immediately for a better accomodation. Three replications were maintained for each treatment. Subsequent irrigation was done two times in a week to keep the optimum moisture level of the soil. Application of bacterial treatments with *Azotobacter*, *Pseudomonas*, *Bacillus* and their combinations were performed by using the dipping methods in which the bacterial suspensions were used to inoculate plants (10^9 CFU/mL in sterile water) and control plants were dipped in sterile water. Growth promoting effects of bacterial treatments were evaluated by determining the plant height, root length (for a single primary root), root girth, ajmalicine content (alkaloid content) on 90, 120 and 150 days after planting (DAP), and the plant nutrient element (PNE) of the plant was analyzed on 150 DAP.

Leaf Analysis

Fully developed leaves were sampled on 150 DAP, and to determine the mineral contents of leaves, plants sample were oven-dried at 68°C for 48 h and then ground. The Total Kjeldahl Nitrogen (TKN) method was based on the wet oxidation method as described by Bremmer & Sulvane (1982). Potassium, calcium and magnesium contents were determined after the wet digestion of dried and ground Sub-Samples in H_2SO_4 (1L) – Se (0.2g) – Salicylic acid (25g) mixture (AOAC, 1990). In the diluted digests, P was measured spectrophotometrically by the indophenol blue method after reaction with ascorbic acid (AOAC, 1990). Results are expressed in percentage mineral content of plant samples.

Ajmalicine extraction and quantification

Ajmalicine extraction from the roots was carried out by following the standard extraction method (ZHAO *et al.*, 2000). Identification and quantification of ajmalicine was done by preparation of the Thin layer chromatography using silica gel (Merck) in chlorofrom: methanol (98:2, v/v) (RENOUDIN, 1984) by comparing Rf values with authentic ajmalicine Standard (Himedia, Mumbai). Ajmalicine was spotted with Dragendroffs reagent (STAHL, 1969).

Statistical Analysis

Data were subjected to one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT) as per procedures described by GOMEZ & GOMEZ (1984). Values represent mean ±SD for three samples in each group. P values <0.05 were considered as significant.

RESULTS

Two consecutive years of testing (2005 and 2006) showed that the plant growth and alkaloid content of *C. roseus* was significantly increased by the bacterial treatment in all sampling days (Tab. 1). There was a significant increase in plant height (41.79 %), root length (60.02%), root girth (109.09 %) and alkaloid content (179.41 %) of *C. roseus* obtained with triple combined application of PGPR VAZt + VPs + VPb for 90 days, followed by double combination VAZt + VPs. Testing for two consecutive years (2005 and 2006) showed that bacterial treatments increased growth parameters and alkaloid content compared to the control.

Bacterial root inoculations increased plant nutrient contents of *C. roseus* plants compared to control. In particular biofertilizer combination (VAZt++VPs+VPb) increased all the plant nutrient elements content of *C. roseus* (Tab. 2). The highest N-content (4.20 %) was obtained from a triple combination of VAZt + VPs + VPb, followed by the double combination of the PGPR treatments, when compared with control. Maximum plant phosphorus content was recorded (0.62% P in total plant nutrient content) in a triple combination followed by a double combination, compared to 0.22% in control. This was followed by potassium content, which recorded maximum in biofertilizer combination (0.40%); while the calcium (0.69%) and magnesium (0.22%) content was also found to be at maximum in the same biofertilizer treatment combination on 150 DAP.

Tab. 1. Effect of plant growth promoting rhizobacterial application on growth and alkaloid content of *C. roseus*.

Treatments	Plant height (cm)			Root length (cm)			Root girth (cm)			Alkaloid content (mg/g of root dry wt)		
DAP*	90	120	150	90	120	150	90	120	150	90	120	150
Control	38.23± 0.76 ^{f}	44.12± 0.04 ^{e}	50.12± 0.24 ^{e}	20.14± 0.14 ^{f}	23.24± 0.24 ^{f}	27.11± 0.11 ^{e}	1.54± 0.04 ^{c}	2.14± 0.24 ^{c}	2.62± 0.12 ^{d}	0.34± 0.12 ^{f}	0.50± 0.10 ^{f}	0.70± 0.10 ^{e}
VAZt	43.16± 0.14 ^{d}	48.31± 0.0.09 ^{d}	54.18± 0.14 ^{c,d}	22.12± 0.14 ^{d}	27.26± 0.24 ^{d}	30.25± 0.25 ^{d}	2.42± 0.04 ^{b}	2.82± 0.14 ^{b}	3.14± 0.24 ^{b,c}	0.56± 0.14 ^{e}	0.65± 0.25 ^{d}	0.92± 0.12 ^{c}
VPs	46.12± 0.22 ^{c}	51.46± 0.24 ^{c}	55.12± 0.14 ^{c}	25.15± 0.15 ^{c}	29.12± 0.14 ^{c}	33.23± 0.23 ^{c}	2.53± 0.03 ^{b}	2.92± 0.24 ^{b}	3.24± 0.24 ^{b,c}	0.80± 0.20 ^{b}	0.75± 0.05 ^{c}	0.12± 0.04 ^{f}
VPb	40.40± 0.20 ^{e}	44.12± 0.14 ^{e}	52.14± 0.14 ^{d}	21.13± 0.13 ^{e}	25.15± 0.15 ^{e}	29.12± 0.14 ^{d}	2.24± 0.04 ^{b}	2.64± 0.14 ^{b}	2.94± 0.14 ^{c}	0.40± 0.05 ^{f}	0.60± 0.02 ^{e}	0.80± 0.02 ^{d}
VAZt+VPs	49.33± 0.13 ^{b}	53.04± 0.04 ^{b}	58.24± 0.34 ^{b}	28.32± 0.22 ^{b}	32.23± 0.23 ^{b}	35.26± 0.24 ^{b}	2.64± 0.04 ^{b}	3.25± 0.25 ^{b}	3.62± 0.24 ^{b}	0.60± 0.74 ^{c}	0.80± 0.05 ^{b}	1.00± 0.05 ^{b}
VAZt+VPb	47.03± 0.07 ^{c}	51.26± 0.34 ^{c}	54.12± 0.14 ^{c,d}	26.12± 0.14 ^{b}	30.15± 0.12 ^{c}	34.12± 0.14 ^{b,c}	2.54± 0.14 ^{a,b}	2.93± 0.13 ^{b}	3.22± 0.24 ^{b,c}	0.58± 0.22 ^{c,d}	0.67± 0.03 ^{d}	0.90± 0.05 ^{c}
VPs+VPb	48.62± 0.24 ^{b}	50.12± 0.14 ^{c}	52.46± 0.42 ^{d}	25.14± 0.14 ^{c}	29.12± 0.22 ^{c}	32.14± 0.14 ^{c}	2.34± 0.04 ^{b}	2.74± 0.14 ^{b}	3.42± 0.24 ^{b,c}	0.55± 0.25 ^{d}	0.62± 0.22 ^{d}	0.90± 0.05 ^{c}
VAZt+VPs+VPb	54.21± 0.12 ^{a}	60.12± 0.14 ^{a}	67.13± 0.13 ^{a}	32.23± 0.23 ^{a}	35.14± 0.22 ^{a}	38.42± 0.44 ^{a}	3.22± 0.22 ^{a}	3.62± 0.12 ^{a}	4.24± 0.14 ^{a}	0.95± 0.15 ^{a}	1.35± 0.05 ^{a}	2.00± 0.10 ^{a}

Values are mean of three replications ± SD for five replicates per treatment. Different letters after values in a column indicate that there is a significant difference at P value of 0.05, as determined by DMRT.

*DAP – Days after planting.

Tab. 2. Effect of plant growth promoting rhizobacterial application on the plant nutrient element content of plants in *C. roseus* on 150 DAP.

Treatments	N(%)	P(%)	K(%)	Ca(%)	Mg(%)
Control	2.15±0.15 ^{g}	0.22±0.02 ^{c}	0.26±0.04 ^{c}	0.40±0.05 ^{b}	0.17±0.03 ^{a}
VAZt	3.50±0.10 ^{d}	0.25±0.05 ^{c}	0.32±0.02 ^{b}	0.44±0.02 ^{b}	0.18±0.02 ^{a}
VPs	3.00±0.10 ^{e}	0.38±0.02 ^{b}	0.30±0.05 ^{b}	0.52±0.04 ^{b}	0.17±0.03 ^{a}
VPb	2.62±0.12 ^{f}	0.54±0.04 ^{a}	0.29±0.01 ^{b,c}	0.40±0.01 ^{b}	0.17±0.01 ^{a}
VAZt+VPs	3.80±0.04 ^{b}	0.44±0.06 ^{b}	0.20±0.02 ^{d}	0.48±0.02 ^{b}	019±0.03 ^{a}
VAZt+VPb	3.00±0.12 ^{e}	0.60±0.05 ^{a}	0.32±0.04 ^{b}	0.46±0.04 ^{b}	0.20±0.05 ^{a}
VPs+VPb	3.34±0.08 ^{c}	0.52±0.08 ^{a}	0.33±0.03 ^{b}	0.4912±0.01 ^{b}	0.20±0.05 ^{a}
VAZt+VPs+VPb	4.20±0.10 ^{a}	0.62±0.12 ^{a}	0.40±0.05 ^{a}	0.69±0.01 ^{a}	0.22±0.04 ^{a}

Values are mean of three replications ±SD for five replicates per treatment. Different letters after values within a column indicate that there is a significant difference at P value of 0.05, as determined by DMRT.

* % refers to percentage mineral content of plant samples.

DISCUSSION

Root inoculation with PGPR promoted significant increase in growth and alkaloid content but the growth responses varied between different rhizobacterial strains. However in general the growth response was found to be enhanced when the PGPR strains were applied in combination. This growth response was more effective in terms of an increased plant growth and alkaloid content compared to the control. Earlier reports had shown that combined inoculation of sorghum with *A. brasilense* and phosphate solubilization bacteria; *P. striata* or *B. polymyxa* significantly increased grain yield and dry matter content, N and P uptake as compared with single inoculation of individual organisms (ALGAWADI & GAUR, 1992). The stimulatory effects of this PGPR strains on the yield and growth of these crops were attributed to the N_2 fixation ability, plant growth regulator production and phosphate solubilizing capacity (CAKMAKCI *et al.*, 2007; KEVINVESSEY, 2003; KARLIDAG *et al.*, 2007). For *C. roseus P. fluorescens* is known to enhance biomass yield and ajmalicine alkaloid content under water deficient stress (JALEEL *et al.*, 2007)

The higher N, P and K content in PGPR combination treatment may have resulted from the N_2 fixation and P-solubilizing ability of these strains as reported in previous studies (ATTIA & SAAD, 2001; CAKMAKCI *et al.* 2007; ASLANTAS *et al.*, 2007; KARLIDAG *et al.*, 2007).

In addition bacterial inoculations increased Ca and Mg in plants. We suppose that this increase may be due to organic acid production by bacteria and plants in the rhizosphere, which decrease soil pH and stimulate the availability of Ca and Mg. Similar reports on the role of PGPR strains in increase of the plant nutrient elements has been reported earlier (SUNDRA *et al.*, 2002; SHEN *et al.*, 2004; KARLIDAG *et al.*, 2007).

In conclusion the combinations of PGPR strains were found to have a great potential for use as bioinoculants to increase production in medicinal plants and other crops.

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