

Organic N-fertilizer, rhizobacterial inoculation and fungal compost improve nutrient uptake, plant growth and the levels of vindoline, ajmalicine, vinblastine, catharanthine and total alkaloids in *Catharanthus roseus* L.

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ABSTRACT

The aim of the study was to replace mineral fertilizers with organic and biological fertilizers to improve nutrient uptake, plant growth and the concentrations of some important secondary metabolites in periwinkle (*Catharanthus roseus* L.). Periwinkle plants were grown under different rates of N supply (0, 20 and 40 mg kg⁻¹ soil) and biological treatments (*Azospirillum*, *Azotobacter*, *Azospirillum* plus *Azotobacter*, *Azospirillum* plus fungal compost, *Azotobacter* plus fungal compost, and fungal compost). The concentrations of pigments and nutrients were measured by spectrophotometry and flame photometry. Secondary metabolites were analyzed by high-performance liquid chromatography (HPLC). Data were recorded for plant growth and development parameters, nutrient uptake and some secondary metabolites of periwinkle plants. The results showed that the N-fertilizer and biological treatments significantly improved most growth attributes and nutrient uptake and increased the concentrations of secondary metabolites as compared to the control. Maximum concentrations of root ajmalicine (0.54 mg g⁻¹ DW), leaf vinblastine (0.96 mg g⁻¹ DW) and root catharanthine (2.38 mg g⁻¹ DW) were obtained from the treatment with *Azospirillum* under N-fertilizer at 20 and 40 mg kg⁻¹ soil. *Azotobacter* along with fungal compost under N-fertilizer at 40 mg kg⁻¹ soil induced the maximum concentration of leaf vindoline (1.94 mg g⁻¹ DW). The highest concentration of root alkaloids (1.11 mg g⁻¹ DW) was obtained from the treatment with compost under 40 mg N kg⁻¹ soil. *Azospirillum*, *Azotobacter* and fungal compost combined with the N-fertilizer improved many morphological and nutrient characteristics. In conclusion, the growth and metabolism of *C. roseus* were significantly positively affected by the organic and biological fertilizers.

Key words: nitrogen fertilizer, periwinkle, plant secondary metabolites, root inoculation, *Vinca rosea*

INTRODUCTION

Periwinkle (*Catharanthus roseus* L., synonym *Vinca rosea*, *Lochnera rosea*), belonging to the family Apocynaceae and native to Madagascar, is

a tropical perennial plant that is cultivated for various purposes such as pharmaceuticals, cosmetics, condiments and spices. It is grown in many tropical and subtropical regions (Łata, 2007). This ornamental and medicinal plant produces

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more than 100 monoterpenoid indole alkaloids, distributed in all parts of the plant, particularly the roots and leaves (Maloney et al., 1968). Three types of action of these compounds are important: anticancer (vincristine and vinblastine), diuretic (vindoline and catharanthine) and hypotensive (ajmalicine and serpentine) (Łata, 2007; Magnotta et al., 2006; Masidur Alam et al., 2012). Vincristine and vinblastine used in the treatment of leukemia and lymphomas are mainly found in the leaves, while ajmalicine, used as antihypertensive substances (to treat high blood sugar and high blood pressure), is present in the roots (Mishra et al., 2001; Łata, 2007). Vindoline, with diuretic properties, is also contained in the leaves (Łata, 2007).

Extensive use of chemical fertilizers is costly and creates environmental problems. Therefore, there has been a need to use environmentally friendly, sustainable, organic and biological fertilizers. The use of biological fertilizers containing plant growth promoting rhizobacteria (PGPR) instead of chemical fertilizers may improve plant growth and development through increased supply of plant nutrients and soil productivity (Karthikeyan et al., 2010). Positive effects of PGPR, such as *Azospirillum* and *Azotobacter*, on improving the yield and quality of some species have been reported by several researchers (Karlidag et al., 2007; Abdul Jaleel et al., 2007; Karthikeyan et al., 2009). Nitrogen fixing and phosphate solubilizing bacteria are among the most important biological fertilizers that improve plant nutrition by increasing N and P uptake and the production of plant growth regulators by plants (Karlidag et al., 2007; Abdul Jaleel et al., 2007). Stimulating effects of PGPR, N supply and fungal compost on plant secondary metabolites in medicinal plants have been demonstrated in some species (Matros et al., 2006; Karthikeyan et al., 2008, 2009; Khattak et al., 2011; Singh and Agrawal, 2015). Certain secondary metabolite pathways are induced by infection with PGPR (Abdul Jaleel et al., 2009; Karthikeyan et al., 2009).

The present study was undertaken to determine the growth promoting effects of root inoculation with *Azospirillum* and *Azotobacter*, and fungal compost as well as N supply on nutrient uptake, plant growth and development, and the concentrations of

vindoline, ajmalicine, vinblastine, catharanthine and total alkaloids in *Catharanthus roseus* L., an important ornamental and medicinal species.

MATERIAL AND METHODS

Source materials, cultivation bed and growth conditions

A pot culture experiment was carried out under natural light in a greenhouse at 28-30°C and 14 h photoperiod. Healthy seeds of periwinkle (*Catharanthus roseus* L.) were obtained from Sabz Fanavar Co. The seeds were sown into plastic pots (15 cm height × 20 cm diameter) filled with 5 kg of a homogenous mixture of sandy loam soil along with an organic N-fertilizer, biological fertilizers and fungal compost as experimental treatments. The concentrations of nutrients in the experimental soil and fungal compost were determined at the Soil and Plant Analysis laboratory prior to the experiment (Tabs 1 and 2). N, P and K were added to the soil prior to the experiment and during the plant growth period based on the fertilization condition of the experimental soil. Growing plantlets were kept free from weeds and irrigated as and when required.

Treatments

The seeds were treated with different concentrations of N-fertilizer, nitrogen-fixing rhizobacteria and fungal compost. The study was conducted with two experimental factors at three and seven levels, respectively, as follows: 1. N-fertilizer (0, 20 and 40 mg kg⁻¹ soil); 2. Nitrogen-fixing rhizobacteria and fungal compost (*Azospirillum*, *Azotobacter*, *Azospirillum* + *Azotobacter*, *Azospirillum* + fungal compost, *Azotobacter* + fungal compost, fungal compost and control). The fungal compost constituted 40% of the pot volume.

Morphological, physiological and biochemical analyses

Determination of morphological characteristics

After a period of 60 days, the following variables were recorded: plantlet height, node number, leaf number, root length, root volume, flower number and flower diameter. Root length, flower diameter and plantlet height were measured with a ruler.

Table 1. Physical and chemical characteristics of the soil used

Sand	Silt (%)	Clay	EC (dS m ⁻¹)	pH	OC (%)	N (%)	P	K	Zn (mg kg ⁻¹)	Fe	Mn
27	43	30	0.69	7.1	0.8	0.08	11.7	176	0.64	4.4	7.12

Table 2. Physico-chemical properties and elemental composition of the fungal compost and their detection method

Parameters	Method	Fungal compost
pH	pH meter	7.43
EC	Conductometer	1.26
Ash content (g kg ⁻¹)	Burning	441
Total OC (g kg ⁻¹)	Walkley black	316
Total OM (%)	Nitration	49
Total N (g kg ⁻¹)	Kjeldahl	28.3
Total P (g kg ⁻¹)	Olsen	13.48
Total K (g kg ⁻¹)	Flame photometry	13.7
C:N ratio	-	11.3
Cu (mg kg ⁻¹)	Atomic	164.8
Fe (mg kg ⁻¹)	Atomic	496.6
Mn (mg kg ⁻¹)	Atomic	248.7
Zn (mg kg ⁻¹)	Atomic	369

The number of organs was counted with a naked eye in intact plants. To measure the volume of the roots, first, the roots were removed from the soil and washed thoroughly under running tap water. Water was poured into a graduated cylinder to a certain level and the roots were immersed up to the crown in the cylinder. The increase in the level of water represented root volume.

Estimation of aerial and root dry matter percentage

Whole shoots of the plants were harvested at maturity and were oven-dried at 70°C for 24 h to determine their dry weight. The roots of the plants were cut away from the crown at maturity and were oven-dried at 70°C for 24 h to determine their dry weight. The aerial and root dry matter percentage was calculated as follows:

$$\text{Aerial and root dry matter percentage} = \frac{\text{Dry weight}}{\text{Fresh weight}} \times 100$$

Estimation of leaf total chlorophyll

To measure the chlorophyll content of the leaves, 0.5 g of leaves were weighed using digital scales, placed in a solution containing 80% acetone and distilled water, and then thoroughly ground in a porcelain mortar. The prepared solution was passed through filter paper and the filtered solution was made up to a volume of 50 ml with 80% acetone and distilled water. Chlorophyll content was calculated according to the following equation (Mazumdar and Majumder, 2003):

$$\text{Total chlorophyll content} = 7.12 (A_{660}) + 16.8 (A_{642.5})$$

where; 'A' is light absorbance at wavelengths of 660 and 642.5 nm.

Estimation of petal anthocyanin

A 0.5 g sample of a fresh petal during the flowering period was ground in liquid nitrogen, using a mortar and pestle. Acidic methanol (pure methanol + HCl) was added to the ground petal to obtain an extract. The anthocyanin concentration was calculated as follows (Singh et al. 2004):

$$\text{Anthocyanin concentration (mg 100 g}^{-1} \text{ FW)} = \frac{e \times b \times c}{d \times a} \times 100$$

where: 'e' is specimen weight, 'b' is specimen volume for measurement, 'c' is whole made solution volume, 'd' is volume of specimen taken, and 'a' is light absorbance at a wavelength of 535 nm.

Estimation of N, P and K content

For macro- and microelement analyses, the youngest mature leaves on the shoots were collected, dried and ground. To measure N, P and K, 0.3 g of leaves dried at a temperature of 75°C for 24 h were placed in 3 ml of mixed acids (100 ml sulfuric acid + 6 g of salicylic acid, and 18 ml distilled water) and digestion was carried out on a heater using H₂O₂. The obtained solution was filtered and the final volume was brought to 50 ml with distilled water. This solution was used to estimate N, P and K. Total nitrogen (N) concentration of leaf blades was determined by the Kjeldahl method (AOAC 1970). The concentration of phosphorus was measured by spectrophotometry. Potassium content was calculated using flame photometry.

Estimation of N, P and K uptake from soil

To estimate N, P and K uptake, we need to know the concentration of these elements in the plant and the

plant's dry weight. N, P and K uptake from soil was calculated according to the following equation:

$$\frac{\text{Element concentration in plant} \times \text{plant dry weight}}{100}$$

Estimation of total alkaloid content in roots

The total alkaloid content in roots was estimated as described by Afaq et al. (1994). The roots were dried using an oven at 80°C for 24 h. The dried roots were powdered and passed through a 72 mesh screen. A 500 mg sample of root powder was transferred into a 100 ml round bottom flask. A known volume of ethyl alcohol was added to this flask and refluxed for 6 h. Then, it was filtered through a Whatman No. 1 filter paper. 50 ml of dilute HCl was added to the filtered solution. Thereafter, it was transferred into a separating funnel, to which 50 ml of diethyl ether was added. This solution was shaken for 20 min. The upper layer was discarded and the lower water layer was decanted into a beaker and made basic by adding an ammonia solution. The decanted content was transferred into a separating funnel with 50 ml diethyl ether layer and was decanted. Anhydrous sodium carbonate was added to the decanted solution. The mixture was again decanted in a pre-weighed dry porcelain dish and was heat-evaporated until dry and weighed again (Masidur Alam et al., 2012). The total alkaloid content (%) was obtained using the following formula:

$$\frac{W_A - W_E}{W_R} \times 100$$

where, W_A is weight of dry porcelain dish and dry alkaloid residue after evaporation (g); W_E is weight of empty, dry porcelain dish (g); and W_R is weight of the tissue powder (g).

Estimation of vindoline, ajmalicine, catharanthine, vinblastine and vincristine

Vindoline, ajmalicine, catharanthine, vinblastine and vincristine were extracted from *C. roseus* according to Luo et al. (2005), with slight changes. The alkaloids were extracted with hot methanol (100%) from dry plant powder. The alkaloid extract was filtered and analyzed by high-performance liquid chromatography (HPLC). The detailed method was as follows: these four kinds of alkaloids were separated on a Diamonsil C18 column (250 mm × 4.6 mm, 5 μm), using 380 mL solution A [water: diethylamide = 986:14 (v/v), pH 7.2] combined with 620 mL solution B [methanol: acetonitrile = 4:1 (v/v)] as the mobile phase. The detection was performed at a flow rate of 1.5 mL min⁻¹ and a wavelength of 220 nm within 40 min.

Experimental design and statistical analysis

The experiment was conducted according to a completely randomized factorial design with three replications. Statistical analyses were performed by using Microsoft Excel 2013 and Statistical Package for Social Sciences (SPSS) v. 16.0. The analysis of variance (ANOVA) procedure for a factorial experiment was used to test for significant effects of treatments, followed by an LSD test and the use of LS means for comparisons of the means of different treatments.

RESULTS AND DISCUSSION

Effect of treatments on morphological characteristics

The effect of bacterial treatments was significant on most morphological characteristics, regardless of N-supply (Tab. 3). However, there was a level of N-supply among the treatments that induced the highest values of these parameters, but the differences were not significant between the 'with' and 'without' applications of N-fertilizer. *Azospirillum* plus *Azotobacter* under 40 mg N kg⁻¹ soil resulted in the greatest plant height (41.43 cm per plant), surpassing the control by 19.80 cm per plant. According to Table 3, very tall plants (33-38 cm per plant) were observed in the treatments containing *Azospirillum*, *Azotobacter* and fungal compost without and/or with 20 mg N kg⁻¹ soil. The application of *Azotobacter* along with fungal compost, and *Azospirillum* along with fungal compost, both under 40 mg N kg⁻¹ soil, resulted in the largest number of nodes (15.00 per plant) and leaves (149.80 per plant) in comparison with the control, which showed the lowest values (9.13 and 28.33, respectively) (Tab. 3). Fungal compost and N-fertilizer at 20 mg kg⁻¹ soil was the best combination for increasing root length (32.30 cm). *Azotobacter* under 20 mg N kg⁻¹ soil was the next best treatment regarding root length. The control plants produced the shortest roots (21.77 cm) (Tab. 3). Plants grown in the medium containing *Azotobacter* plus fungal compost produced the highest number of flowers (27.98 per plant) under N-supply of 20 mg kg⁻¹ soil. The control plants produced the lowest number of flowers (3.13) (Tab. 3). Maximum and minimum flower diameters (35.65 and 27.46 mm per plant) were measured in the plants grown in the media without the N-fertilizer. Fungal compost induced the maximum and *Azospirillum* along with fungal compost induced the minimum flower diameter (Tab. 3). *Azospirillum*

Table 3. Means comparison of the effect of different concentrations of N fertilizer and biological fertilizers on the growth and development characteristics of *C. roseus* L.*

N fertilizer (mg kg ⁻¹ soil)	Biological fertilizers	Parameters									
		Plant height (cm)	Node number	Leaf number	Root length (cm)	Root volume (cm ³)	Flower number	Flower diameter (mm)	Total leaf chlorophyll content	Petal anthocyanin content	
0	AP	28.83 b	9.70 e	40.70 cd	23.83 c	28.33 bc	5.48 c	31.65 bc	12.24 a	92.15 cd	
	AB	20.60 c	9.73 e	59.80 c	25.25 bc	21.66 bed	3.93 c	32.59 bc	16.75 a	106.40 b	
	AP+AB	23.00 c	11.46 cd	46.26 cd	25.66 bc	17.66 cd	7.45 c	32.69 b	13.87 a	94.36 cd	
	AP+FC	33.93 ab	13.33 ab	84.13 b	29.60 a	48.00 a	16.13 b	27.46 e	13.38 a	115.20 a	
	AB+FC	37.66 a	14.73 a	111.75 a	28.15 ab	29.00 bc	26.66 a	30.90 cd	12.18 a	96.22 c	
	FC	34.53 a	13.06 bc	124.66 a	27.60 ab	37.00 ab	23.33 a	35.65 a	16.30 a	87.40 d	
	C	19.80 c	9.13 de	28.33 d	22.53 c	7.00 d	3.13 c	29.52 d	13.42 a	94.19 cd	
LSD (0.05)		5.34	1.7	19.8	3.52	18.87	4.58	1.74	1.51	6.2	
20	AP	28.44 b	10.44 c	44.94 c	30.05 ab	24.00 ab	5.10 c	34.13 a	14.85 ab	89.30 b	
	AB	24.54 bc	10.60 c	49.27 c	31.38 a	22.00 abc	6.10 c	30.01 c	11.22 b	92.80 b	
	AP+AB	22.60 c	10.53 c	63.40 bc	30.88 ab	13.33 bc	8.45 bc	32.90 ab	17.09 a	92.40 b	
	AP+FC	37.62 a	11.77 bc	85.51 b	28.02 b	34.00 a	14.24 b	31.04 bc	13.36 ab	103.30 a	
	AB+FC	37.13 a	14.65 a	125.9 a	29.58 ab	30.66 a	27.98 a	31.53 bc	10.26 b	102.80 a	
	FC	33.86 a	12.95 b	124.0 a	32.30 a	35.66 a	21.50 a	30.17 c	16.68 a	89.78 b	
	C	20.69 c	10.80 c	37.47 c	24.16 c	7.66 c	4.33 c	31.36 bc	16.25 a	90.90 b	
LSD (0.05)		4.17	1.5	26.8	3.23	15.79	6.72	1.23	2.43	9.1	
40	AP	23.88 cd	10.27 b	52.28 c	29.33 a	14.11 a	8.16 bc	33.94 a	16.47 ab	74.16 de	
	AB	24.11 cd	10.83 b	67.73 c	27.10 ab	11.25 ab	5.88 c	33.37 ab	15.03 bc	81.46 cd	
	AP+AB	41.43 a	9.91 b	47.55 c	25.58 b	4.66 c	9.45 bc	32.10 bc	15.23 bc	71.28 e	
	AP+FC	34.73 b	13.55 a	149.8 a	24.71 bc	8.50 bc	21.70 a	32.81 ab	18.83 a	94.19 ab	
	AB+FC	26.60 cd	15.00 a	114.2 b	22.91 cd	6.93 bc	25.26 a	32.27 b	17.20 ab	98.60 a	
	FC	28.70 c	10.53 b	68.95 c	24.91 bc	4.58 c	13.26 b	30.61 c	12.97 c	95.04 ab	
	C	22.97 d	11.58 b	37.94 c	21.77 d	3.61 c	5.00 c	33.13 ab	10.09 c	87.74 bc	
LSD (0.05)		5.11	1.9	33.1	2.54	5.01	6.57	0.86	1.89	7.5	

*Means with different letters in the same column are significantly different ($p < 0.05$) based on LSD test. AP: *Azospirillum*, AB: *Azotobacter*, FC: Fungal compost, C: Control

along with fungal compost without N-fertilizer resulted in the highest root volume (48.00 cm³ per plant) compared to the control, which showed the lowest value (3.61 cm³ per plant) (Tab. 3).

The current study reveals that there was significant improvement in the morphological characteristics as a result of the application of PGPR and fungal compost. Similar results had been reported earlier in *Catharanthus roseus* L. and some other medicinal plants (Siddiqui, 2004; Thosar et al., 2005; Karthikeyan et al., 2010). The stimulating effect of PGPR on plant growth can be due to the increased supply of plant nutrients, particularly by the N₂ fixation ability, and greater soil productivity, especially by the phosphate solubilizing capacity (O'Connell, 1992; Karthikeyan et al., 2010). PGPR can also promote plant growth through the production of plant growth regulators, particularly auxins and gibberellins (Karthikeyan et al., 2009, 2010). It is important to note that the increase in growth characteristics observed following inoculation with PGPR has been found to manifest itself in an increase in root number, root length and root biomass, which in turn can enhance mineral uptake (NPK) in plants (Khalid et al., 2004; Karthikeyan et al., 2009).

Effect of treatments on plant biomass

Statistically significant differences were found between the means for plant biomass and treatments. The maximum value of the biomass of aerial parts (14.84%) was produced by *Azotobacter* without N-supply in comparison with the control, which showed the minimum value (5.39%) (Tab. 3). On the other hand, of the various organic and biological fertilizer treatments, fungal compost under N-fertilizer at 20 mg kg⁻¹ soil induced the maximum value of root biomass (13.73%) in comparison with the control, which showed the minimum value (4.50%) (Tab. 3).

Our findings demonstrated that plant biomass was increased when PGPR or fungal compost were applied separately. Contrary to this finding, earlier reports on *Catharanthus roseus* L. and some other species had shown that combined inoculation with PGPR increased dry matter content (Algawadi and Gaur, 1992; Cakmakci et al., 2007; Karlidag et al., 2007; Karthikeyan et al., 2010). This difference may be due to the type of plant species and applied bacterial strains. Łata (2007) showed that the highest plant biomass in *Catharanthus roseus* L. was obtained at 200 mg N dm⁻³ in the substrate. Plants with a vigorous growth and the highest

biomass contained 2.9-3.0% nitrogen in the dry matter of the leaves (Łata, 2007). The stimulatory effect of PGPR on plant biomass has been attributed to the fixation of atmospheric nitrogen, phosphate solubilizing capacity and the production of plant growth regulators (PGRs) (Vessey, 2003; Cakmakci et al., 2007; Karlidag et al., 2007).

Effect of treatments on leaf and petal pigments

Total chlorophyll and anthocyanin contents were significantly increased under the bacterial and fungal treatments. Analysis of variance revealed that the amounts of chlorophyll and anthocyanins were changed by the organic and biological treatments, separately or in combination. *Azospirillum* along with fungal compost under N-fertilizer at 40 mg kg⁻¹ soil followed by *Azotobacter* along with fungal compost under N-fertilizer at 40 mg kg⁻¹ soil and *Azospirillum* plus *Azotobacter* under N-fertilizer at 20 mg kg⁻¹ soil increased the amount of total chlorophyll to the highest extent. The lowest chlorophyll content was calculated in the control plants (Tab. 3). *Azospirillum* plus *Azotobacter* under 40 mg N kg⁻¹ soil caused a significant reduction in the production of anthocyanins. *Azospirillum* along with fungal compost without N-fertilizer significantly stimulated the production of anthocyanins (Tab. 3).

Different rates of N supply significantly increased the amounts of photosynthetic pigments. Nitrogen is a major component of the chlorophyll structure. Singh and Agrawal (2015) had reported an increase in chlorophyll content of *Catharanthus roseus* L. with N supply, which may be ascribed to enhanced uptake of N. Shangguan et al. (2000) reported a reduction in total chlorophyll content caused by nitrogen deficiency. High soil nitrogen supply enhanced the absorption rate of leaves due to increased chlorophyll content (Singh and Agrawal, 2015). A similar finding had been reported by Zhao et al. (2010). PGPR can have an important role in increasing the amount of nitrogen through fixation of atmospheric nitrogen.

Effect of treatments on uptake and concentration of macro-nutrients

The ANOVA test revealed a significant effect of the treatments and their combinations on the uptake and concentration of N, P and K. Based on Table 4, the maximum concentrations of N, P and K were obtained in the plants treated with rhizobacteria and fungal compost without the application of N-fertilizer. The maximum concentrations of N (3.94%), P (0.16%) and K (15.27%) were induced

Table 4. Means comparison of the effect of different concentrations of N fertilizer and biological fertilizers on ton uptake and concentration of macro-nutrients of *C. roseus* L.*

N fertilizer (mg kg ⁻¹ soil)	Biological fertilizers	Parameters									
		Plant N content (%)	Plant P content (%)	Plant K content (%)	N uptake (g)	P uptake (g)	K uptake (g)	Aerial dry matter percentage	Root dry matter percentage		
0	AP	3.94 a	0.07 b	13.22 c	0.11 b	0.01 d	0.31 c	7.73 bc	10.28 a		
	AB	3.81 a	0.06 b	11.80 d	0.07 bc	0.11 d	0.30 c	14.84 a	8.29 a		
	AP+AB	2.83 bc	0.06 b	14.99 a	0.06 bc	0.14 d	0.37 c	11.50 ab	5.88 a		
	AP+FC	3.44 ab	0.15 a	13.93 b	0.18 a	0.79 b	0.72 b	8.68 bc	9.47 a		
	AB+FC	3.07 ab	0.09 b	14.88 a	0.16 a	0.50 c	0.79 b	6.39 c	6.37 a		
	FC	2.70 bc	0.14 a	15.27 a	0.20 a	1.02 a	1.14 a	6.83 c	4.74 a		
	C	1.96 c	0.01 c	11.66 d	0.05 c	0.16 d	0.22 c	5.43 c	6.90 a		
LSD (0.05)		0.495	0.036	1.3	0.061	0.266	0.198	4.59	7.36		
20	AP	2.58 a	0.14 a	14.33 ab	0.07 b	0.13 c	0.34 e	8.08 a-d	4.54 b		
	AB	2.83 a	0.08 bc	14.10 ab	0.06 b	0.18 c	0.31 e	13.05 a	9.09 ab		
	AP+AB	2.21 a	0.06 d	14.66 a	0.10 b	0.14 c	0.35 e	12.08 ab	6.52 ab		
	AP+FC	3.07 a	0.02 e	13.95 ab	0.17 a	0.45 b	0.78 c	6.78 bcd	7.26 ab		
	AB+FC	2.95 a	0.07 cd	13.60 b	0.20 a	0.50 b	0.91 b	6.30 cd	8.17 ab		
	FC	2.58 a	0.09 b	14.93 a	0.21 a	0.75 a	1.19 a	11.34 abc	13.73 a		
	C	3.07 a	0.03 e	11.91 c	0.05 b	0.08 c	0.46 d	5.39 d	4.50 b		
LSD (0.05)		0.456	0.016	1.21	0.059	0.137	0.116	2.64	6.44		
40	AP	3.07 ab	0.12 ab	14.95 a	0.09 cd	0.36 bc	0.37 c	8.74 a	10.93 a		
	AB	2.95 abc	0.14 ab	13.07 bc	0.07 de	0.36 bc	0.33 c	13.72 a	5.19 b		
	AP+AB	3.57 a	0.06 bc	12.73 c	0.14 bc	0.16 bc	0.74 b	6.22 a	5.68 b		
	AP+FC	1.96 bc	0.10 abc	14.32 a	0.22 a	0.70 ab	1.05 a	7.63 a	10.46 a		
	AB+FC	3.44 a	0.18 a	14.83 a	0.13 bc	1.15 a	0.95 a	5.60 a	6.54 ab		
	FC	2.82 abc	0.11 ab	13.91 ab	0.18 ab	0.58 bc	0.66 b	5.66 a	9.16 ab		
	C	1.59 c	0.02 c	12.06 c	0.04 e	0.11 c	0.31 c	6.75 a	4.60 b		
LSD (0.05)		0.492	0.024	1.37	0.053	0.544	0.143	8.11	4.63		

*Means with different letters in the same column are significantly different ($p < 0.05$) based on LSD test. AP: *Azospirillum*, AB: *Azotobacter*, FC: Fungal compost, C: Control

with *Azospirillum*, *Azospirillum* along with fungal compost, and fungal compost. The minimum concentrations of N (1.59%), P (0.01%) and K (11.71%) were produced in the control plants. Compost notably enhanced the uptake of N, P and K (Tab. 4). The highest uptake of N (0.22 and 0.21 g) was shown by the plants treated with *Azospirillum* along with fungal compost, and fungal compost under N-fertilizer at 40 and 20 mg kg⁻¹ soil, respectively (Tab. 4). This amount is 5 times higher than the uptake of N in the control plants. The highest uptake of P (1.15 g) was obtained from the plants treated with *Azotobacter* along with fungal compost under N-fertilizer at 40 mg kg⁻¹ soil, surpassing the control by 0.08 g (Tab. 4). The maximum uptake of K (1.19 g) was calculated for the plants treated solely with fungal compost. The minimum uptake of K (0.22 g) was shown by the control plants (Tab. 4).

Mineral nutrition is one of the most important elements of field cultivation. Nitrogen is the main yield-determining factor under field conditions (Łata, 2007). *Catharanthus roseus* L., as an alkaloid-rich species, can be classified as a high N demanding plant (Łata, 2007). The current study showed the importance of fungal compost, PGPR and N supply for the uptake of N, P and K from soil. The amounts of these elements in plants were also high with the application of compost. N supply had an important role in determining the amounts of N, P and K in plants but not for the uptake of these elements. Similar to our findings, Łata (2007) showed that an increased N supply stimulated K and P uptake by *Catharanthus roseus* L. Earlier reports had shown that fungal compost was the best for *Catharanthus roseus* L. production (Khataak et al., 2011). A study by Karthikeyan et al. (2010) on *Catharanthus roseus* L. revealed that the maximum N, P and K content was obtained from treatments with PGPR. The higher N, P and K content in PGPR treatments may have resulted from the fixation of atmospheric nitrogen and P-solubilizing and absorption ability of these rhizobacteria and fungal compost (Cakmakci et al., 2007; Karlidag et al., 2007; Karthikeyan et al., 2010). Similar reports on the role of PGPR in the enhancement of plant nutrient elements have been presented (Shen et al., 2004).

Effect of treatments on secondary metabolites

The results showed that all the measured secondary metabolites of *Catharanthus roseus* L. were significantly increased by the bacterial and fungal treatments (Tab. 5). There was a significant increase

in the concentration of root ajmalicine (0.54 mg g⁻¹ DW) and leaf vinblastine (0.96 mg g⁻¹ DW) with the application of *Azospirillum* under N-fertilizer at 20 mg kg⁻¹ soil. The concentration of leaf vinblastine (0.95 mg g⁻¹ DW) was high through the application of fungal compost under N-fertilizer at 40 mg g⁻¹ soil. The concentrations of root ajmalicine (0.24 mg g⁻¹ DW) and leaf vinblastine (0.64 mg g⁻¹ DW) were the lowest in the control plants (Tab. 5). The highest root catharanthine content (2.39 mg g⁻¹ DW) was obtained in the treatment with *Azospirillum* under N-supply at 40 mg kg⁻¹ soil followed by the treatment with *Azotobacter* along with fungal compost without N-supply (2.35 mg g⁻¹ DW). The lowest root catharanthine content (0.71 mg g⁻¹ DW) was obtained from the control plants. *Azotobacter* plus fungal compost under N-supply at 40 mg kg⁻¹ soil produced the maximum leaf vindoline content (1.94 mg g⁻¹ DW). The control plants had the minimum vindoline content (1.05 mg g⁻¹ DW). *Azospirillum* plus *Azotobacter* under 20 mg N kg⁻¹ soil caused the maximum increase in the total root alkaloid content (0.93 mg g⁻¹ D.W.). *Azospirillum* plus fungal compost and *Azotobacter*, both under N-fertilizer at 40 mg kg⁻¹ soil, and also *Azotobacter* plus fungal compost caused the production of total alkaloids of more than 0.90 mg g⁻¹ DW (Tab. 5). Compared with the treatments mentioned above, the plants grown in the cultivation bed without bacterial and fungal treatments produced the lowest amount of total root alkaloids (0.72 mg g⁻¹ DW). The results of the ANOVA test showed that the concentrations of vindoline, ajmalicine, vinblastine, catharanthine and total alkaloids varied due to the organic and biological fertilizers and their interaction.

Nitrogen is a constituent of alkaloids and plays an important role in their synthesis (Łata, 2007). Also, P has the greatest influence on alkaloid synthesis by *Catharanthus roseus* L. (Łata, 2007). The application of *Azospirillum* resulted in the maximum concentrations of ajmalicine, vinblastine, catharanthine and total alkaloids. Similar significant increases in the concentration of alkaloids have been observed in plants exposed to various PGPR, particularly *Azospirillum* (Thosar et al., 2005; Karthikeyan et al., 2009, 2010). It is likely that with the use of PGPR the amount of growth regulators is increased, which causes an increase in the concentration of alkaloids. Our results are in agreement with those of other researchers (Karthikeyan et al., 2010). Corresponding to our results, a positively significant effect of PGPR on the alkaloid content of *Catharanthus roseus* L.

Table 5. Means comparison of the effect of different concentrations of N fertilizer and biological fertilizers on the secondary metabolites of *C. roseus* L.*

N fertilizer (mg kg ⁻¹ soil)	Biological fertilizers	Parameters				
		Leaf vindoline (mg g ⁻¹ DW)	Leaf vinblastine (mg g ⁻¹ DW)	Root ajmalicine (mg g ⁻¹ DW)	Root catharanthine (mg g ⁻¹ DW)	Total root alkaloids (mg g ⁻¹ DW)
0	AP	1.34 bc	0.67	0.28	1.42 b	0.78 a
	AB	1.32 bc	0.66	0.28	1.36 b	0.77 a
	AP + AB	1.64 a	0.7	0.29	1.26 bc	0.81 a
	AP + FC	1.53 ab	0.72	0.3	2.16 a	0.84 a
	AB + FC	1.38 abc	0.67	0.28	2.35 a	0.78 a
	FC	1.25 bc	0.63	0.26	1.39 b	0.74 a
	C	1.20 c	0.73	0.31	0.87 c	0.85 a
LSD (0.05)		0.305	0.049	0.056	0.683	0.259
20	AP	1.55 abc	0.96 a	0.54 a	2.28 a	0.85 abc
	AB	1.38 bc	0.68 cd	0.28 bc	1.23 b	0.79 c
	AP + AB	1.80 a	0.80 b	0.34 b	1.18 b	0.93 a
	AP + FC	1.65 ab	0.76 bc	0.32 bc	2.23 a	0.88 abc
	AB + FC	1.59 abc	0.77 bc	0.32 bc	1.13 b	0.90 ab
	FC	1.32 cd	0.71 bcd	0.30 bc	2.38 a	0.83 bc
	C	1.05 d	0.64 d	0.24 c	1.15 b	0.72 c
LSD (0.05)		0.417	0.121	0.033	0.761	0.078
40	AP	1.41 ab	0.68 cd	0.28 c	2.38 a	0.79 bc
	AB	1.69 ab	0.80 b	0.33 b	2.13 ab	0.93 b
	AP + AB	1.50 ab	0.71 bcd	0.30 bc	2.01 ab	0.83 bc
	AP + FC	1.75 ab	0.79 bc	0.33 b	2.03 ab	0.92 bc
	AB + FC	1.94 a	0.65 d	0.27 c	1.53 b	0.75 c
	FC	1.38 ab	0.95 a	0.40 a	0.85 c	1.11 a
	C	1.17 b	0.67 d	0.27 c	0.71 c	0.80 bc
LSD (0.05)		0.619	0.115	0.038	0.651	0.164

*Means with different letters in the same column are significantly different ($p < 0.05$) based on LSD test. AP: *Azospirillum*, AB: *Azotobacter*, FC: Fungal compost, C: Control

plants was reported by Karthikeyan et al. (2009, 2010), Attia and Saad (2001) and Abdul Jaleel et al. (2007). The alkaloid content of *Catharanthus roseus* L. was significantly increased by the bacterial treatment (Karthikeyan et al., 2010). Similar reports on the role of PGPR in the induction of secondary metabolite synthesis, particularly of alkaloids, had been presented earlier (Khalid et al., 2004; Karthikeyan et al., 2010).

CONCLUSIONS

1. Application of organic fertilizers and plant growth-promoting rhizobacteria (PGPR) and fungal compost as biofertilizers instead of chemical fertilizers is a promising approach to the growth and development of medicinal species.

2. PGPR promote plant growth by triggering the production of PGRs and facilitate the uptake of nutrients from the soil. Fungi enhance nutrient uptake, especially of phosphate.
3. PGPR and fungal compost stimulate the synthesis and accumulation of secondary metabolites in plants.
4. The use of suitable levels of organic N-fertilizers, PGPR and fungal compost can increase the amounts of pigments, improve the morphological and physiological characteristics, and increase the concentration of nutrient elements and some secondary metabolites in *C. roseus* L.

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AUTHOR CONTRIBUTIONS

All the authors contributed equally to all aspects of this study. D.H. – designed the paper; F.S. – performed the experiments and collected the data; B.K. – wrote the paper.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

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