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Alterations in seedling vigour and antioxidant enzyme activities in *Catharanthus roseus* under seed priming with native diazotrophs

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Abstract: An experiment was conducted on *Catharanthus roseus* to study the effect of seed treatments with native diazotrophs on its seedling growth and antioxidant enzyme activities. The treatments had significant influence on various seedling parameters. There is no significant influence on dry matter production with the diazotrophs, *Azospirillum* and *Azotobacter*. However, the vital seedling parameters such as germination percentage and vigour index were improved. *Azotobacter* treatment influenced maximum of 50% germination, whereas *Azospirillum* and *Azotobacter* were on par with *C. roseus* with respect to their vigour index. There was significant difference in the population of total diazotrophs. *Azospirillum* and *Azotobacter* between rhizosphere and non-rhizosphere soils of *C. roseus* had the same trend and were observed at various locations of the study. The activities of antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POX) and catalase (CAT) were increased to a significant extent due to the treatment with diazotrophs.

Key words: Rhizosphere, Non-rhizosphere, *Azospirillum*, *Azotobacter*, Antioxidant enzyme, *Catharanthus roseus*
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INTRODUCTION

The strong and rapidly stimulating effect of fungal elicitor on plant secondary metabolism in medicinal plants attracts considerable attention (Zhao *et al*., 2005). 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 (Nurnberger *et al*., 1994; Somssich and Hahlbrock, 1998). Certain secondary metabolite pathways in plants are induced by infection with microorganisms (Verpoorte *et al*., 2002). Diazotrophic rhizobiocoenosis is an important biological process that plays a major role in satisfying the nutritional requirements of 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 commercially important medicinal plants. Diazotrophs secrete plant growth hormones such as auxins, gibberellins and cytokinins (Deka *et al*., 1992).

Plants are equipped with oxygen radical detoxifying enzymes such as superoxide dismutase (SOD), peroxidase (POX), catalase (CAT) and antioxidant molecules like ascorbic acid and reduced glutathione in order to survive under stress conditions (Prochazkova *et al*., 2001). Generation of reactive oxygen species (ROS) such as superoxide, H_2O_2 and hydroxyl molecules causes rapid cell damage by triggering off a chain reaction (Imlay, 2003). Plants under stress produce some defense mechanisms to protect themselves from the harmful effect of oxidative stress. ROS scavenging is one among the common defense responses against abiotic stresses (Vranova *et al*., 2002). ROS scavenging depends on the detoxification mechanism provided by an integrated system of non-enzymatically reduced molecules like ascorbate

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and glutathione and enzymatic antioxidants (Prochazkova *et al*., 2001).

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 (Magnotta *et al*., 2006). Roots of this plant are the main source of an anti-hypertension alkaloid ajmalicine (Jaleel *et al*., 2006). It is also a popular ornamental plant. There are commonly two varieties in this plant based on the flower colour viz., pink flowered rosea and white flowered alba (Jaleel *et al*., 2007). All parts of the plant are rich in alkaloids, with maximum concentrations found in the root bark, particularly during flowering. An infusion of the leaves is used to treat menorrhagia. The juice of the leaves is applied externally to relieve wasp stings. All parts of the plant have hypoglycaemic properties and are used to treat diabetes (Kar *et al*., 2003). The major practical problem in the cultivation of *C. roseus* is the poor germination percentage at field level. To the best of our knowledge, no information on the germination, seedling vigour and antioxidant potentials of *C. roseus* under diazotrophs treatment is available. The purpose of this study was to provide additional information on the germination and seedling vigour and enzymatic (SOD, POX and CAT) antioxidant constituents of *C. roseus* under seed priming with native diazotrophs such as *Azospirillum* and *Azotobacter*.

MATERIALS AND METHODS

Seed collection and diazotrophs isolation

The seeds of *Catharanthus roseus* (L.) G. Don. were collected from Jaya Priya Laboratories, Rajapalayam, Tamilnadu, India. The rhizosphere and non-rhizosphere soil samples of *C. roseus* were collected from three locations viz., horticultural farm of Annamalai University, microbiological potculture yard of Annamalai University and Horticultural Research Station, Pondicherry, Tamil Nadu, India with the samples being denoted as AHF, AMG and HRS. The population of the total diazotrophs was estimated as suggested by Watanabe and Barraquio (1979). Diazotrophic *Azospirillum* are isolated from the surface sterilized roots of *C. roseus* and the free-living diazotrophic *Azotobacter* was isolated from the rhizosphere soil samples of *C. roseus*.

Germination and seedling vigour

To find out the effect of these isolated diazotrophic *Azospirillum* and *Azotobacter* on the germination and vigour index of *C. roseus*, 100 seeds were taken in a sterile petriplate and treated with 10 ml broth culture of (with an initial population 10^7 cells/ml) *Azospirillum* and *Azotobacter* as separate treatments. The seeds were treated for 30 min and then shade dried. Then, these inoculated seeds were tested for the germination rate using paper towel method (ISTA, 1976). The germination percentage was calculated from 8 days after sowing (DAS) to 12 DAS. The morphological parameters like shoot length and root length were measured on 20 DAS. The vigour index (*VI*) of the seedlings was estimated as suggested by Abdul-Baki and Anderson (1973):

$$VI=RL+SL\times GP,$$

where *RL* is root length (cm), *SL* is shoot length (cm) and *GP* is germination percentage.

Antioxidant enzyme extractions and assays

1. Superoxide dismutase (SOD, EC 1.15.1.1)

The activity of SOD was assayed as described by Beauchamp and Fridovich (1971). The reaction mixture contained 1.17×10^{-6} mol/L riboflavin, 0.1 mol/L methionine, 2×10^{-5} mol/L KCN and 5.6×10^{-5} mol/L nitroblue tetrazolium (NBT) salt dissolved in 3 ml of 0.05 mol/L sodium phosphate buffer (pH 7.8). Three millilitres of the reaction medium was added to 1 ml of enzyme extract. The mixtures were illuminated in glass test tubes by two sets of Philips 40 W fluorescent tubes in a single row. Illumination was started to initiate the reaction at 30 °C for 1 h. Identical solutions that were kept under dark served as blanks. The absorbance was read at 560 nm in the spectrophotometer against the blank. SOD activity is expressed in U/(mg protein). One U is defined as the change in 0.1 absorbance per hour per mg protein.

2. Peroxidase (POX, EC 1.11.1.7)

POX was assayed by the method of Kumar and Khan (1982). Assay mixture of POX contained 2 ml of 0.1 mol/L phosphate buffer (pH 6.8), 1 ml of 0.01

mol/L pyrogallol, 1 ml of 0.005 mol/L H_2O_2 and 0.5 ml of enzyme extract. The solution was incubated for 5 min at 25 °C after which the reaction was terminated by adding 1 ml of 2.5 mol/L H_2SO_4. The amount of purpurogallin formed was determined by measuring the absorbance at 420 nm against a blank prepared by adding the extract after the addition of 2.5 mol/L H_2SO_4 at zero time. The activity was expressed in U/(mg protein). One U is defined as the change in the absorbance per 0.1 min per mg protein.

3. Catalase (CAT, EC 1.11.1.6)

The activity of CAT was measured according to the method of Chandlee and Scandalios (1984) with small modification. The assay mixture contained 2.6 ml of 50 mmol/L potassium phosphate buffer (pH 7.0), 0.4 ml of 15 mmol/L H_2O_2 and 0.04 ml of enzyme extract. The decomposition of H_2O_2 was followed by the decline in absorbance at 240 nm. The enzyme activity was expressed in U/(mg protein). One U is defined as 1 mmol/L of H_2O_2 reduction per min per mg protein. The enzyme protein was estimated by the method of Bradford (1976) for all the enzymes.

Statistical analysis

Statistical analysis was performed using one way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT). The values are mean±*SD* for six samples in each group. *P* values ≤0.05 were considered as significant.

RESULTS AND DISCUSSION

Significant differences have been observed in germination rate, root length, shoot length, dry matter production and vigour index between untreated seeds and treated seeds of *C. roseus*. The maximum germination percentage (70%) was recorded in *Azotobacter* treatment followed by *Azospirillum* (66%). The native isolates of *Azotobacter* and *Azospirillum* significantly increased the germination rate in *C. roseus* which was 70% as against 35% recorded by untreated control (Table 1). Treatments with these diazotrophs resulted in significantly higher dry matter production than control. Similar trend has been observed for the increase in vigour index of *C. roseus* for the *Azospirillum* and *Azotobacter* seed treatment. There is no significant difference in the dry matter production between *Azospirillum* and *Azotobacter* treatments in *C. roseus*.

Significant difference in the population of total diazotroph *Azospirillum* and *Azotobacter* between rhizosphere and non-rhizosphere soils of *C. roseus* was found. On different location of sampling of soils exhibited difference significantly. The rhizosphere soil sample of *C. roseus* recorded the maximum mean population of total diazotroph (72.33×10^3 CFU/g). The population of *Azotobacter* was also found to be high. It was maximum in the rhizosphere soil samples of *C. roseus* in all the locations such as AHF, AMG, HRS viz., 60×10^3, 50×10^3 and 44×10^3 CFU/g, respectively (Table 2).

Table 1 Effect of *Azospirillum* and *Azotobacter* seed treatment on seedling parameters of *Catharanthus roseus*

Treatment	Germination (%)	Root length (cm)	Shoot length (cm)	Dry matter production (mg/seedling)	Vigour index
Untreated control	35.0	4.5	2.0	28.4	227.5
Azospirillum	66.0	10.6	6.2	30.6	1108.8
Azotobacter	70.0	12.6	8.4	32.4	1484.0
CD (*P*=0.05)	3.6179	1.8090	1.5077	351.7499	4.6230
SD	1.7999	0.9000	0.7501	175.0000	2.3000

CD: Critical difference; *SD*: Standard deviation

Table 2 Population of total diazotrophs *Azotobacter* and *Azospirillum* sp. on the rhizosphere and non-rhizosphere samples of *Catharanthus roseus* at different locations

Soil sample	Total diazotroph ($\times10^3$ CFU/g)				*Azospirillum* ($\times10^3$ CFU/g)				*Azotobacter* ($\times10^3$ CFU/g)			
	AHF	AMG	HRS	Mean	AHF	AMG	HRS	Mean	AHF	AMG	HRS	Mean
Rhizosphere	85	70	62	72.31	44	38	34	38.66	60	50	44	51.33
Non-rhizosphere	28	20	18	22.00	12	10	10	10.66	18	16	12	15.53

The seed treatment with native isolates of *Azospirillum* and *Azotobacter* increased the germination percentage, root length, shoot length and vigour index of the *C. roseus*. The occurrence of *Azospirillum* and *Azotobacter* in and around the root system of cereals and the beneficial effect upon inoculation have been well established. In the present study, the increased seedling parameters in *C. roseus* may be due to the production of growth hormones (auxins, gibberellins and cytokinins) by the heterotrophic nitrogen-fixing bacteria. It is worth noting that earlier research determined that the increase in plant growth observed on inoculation with *Azotobacter* was not caused by nitrogen fixation, but by bacterial production of plant hormones (Brown and Burlingham, 1968).

In the present study, isolates of *Azospirillum* and *Azotobacter* were tested for their effect on seedling parameters of *C. roseus* and the results clearly indicated that the native isolates significantly improved the seed germination and related seedling parameters. This confirmated earlier report by Govindarajan and Kavitha (2001) that the *Azospirillum* was isolated to the paddy seedlings under axenic conditions. There was another report by Lakshmanan *et al*.(2005) which confirmed that the medicinal plant such as Senna and Ashwagandha significantly increased germination percentage, root length, shoot length and dry weight of seedlings and more importantly the homologous isolates of *Azotobacter* and *Azospirillum* had a significant effect with host.

There was a significant increase in SOD, POX and CAT activities under *Azotobacter* and *Azospirillum* treatments (Fig.1). SOD activity directly modulates the amount of ROS. It catalyses the dismutation of superoxide anion radical ($O_2^- \cdot$) with great efficiency, resulting in the production of H_2O_2 and O_2 (Lin and Kao, 2000). In our study, an increased level in SOD activity was found. According to Prochazkova *et al*.(2001), many stress situations caused an increase in the total antioxidant activity. The SOD activity showed an increase and in some, a reduction under abiotic stresses (Muthukumarasamy *et al*., 2000; Sairam *et al*., 2002). The changes in SOD activity under *Azotobacter* and *Azospirillum* treatments can be also a consequence of an altered synthesis and accumulation of less active enzymes and/or of a higher turnover of SODs (Chaparzadeh *et al*., 2004).

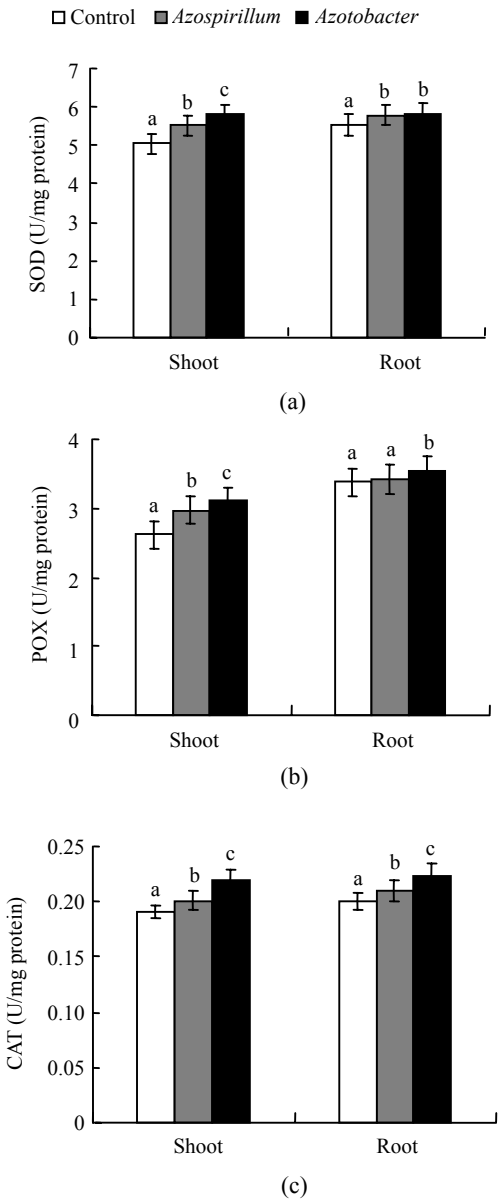

Fig.1 Effect of native diazotrophs on SOD (a), POX (b) and CAT (c) activities of *Catharanthus roseus*
Values are given as mean±*SD* of six experiments in each group. Bar values which are not sharing a common superscript (a, b, c) differ significantly at $P \leq 0.05$ (DMRT)

Variations are recorded in antioxidant enzyme activities under various stresses and treatments of pea (Hernandez and Almansa, 2002) and wheat (Sairam *et al*., 2002). Muthukumarasamy *et al*.(2000) showed that a reduction in POX activity in radish. We observed an increase in CAT activity in *C. roseus* seedlings subjected to *Azotobacter* and *Azospirillum* treatments. This result coincides with the observation in rice leaves under NaCl treatments (Lin and Kao, 2000). The changes in CAT may vary according to the

intensity of stress, time of assay after the stress and induction of new isozyme(s) (Chaparzadeh *et al*., 2004). The level of antioxidative response depends on the species, the development and the metabolic state of the plant, as well as the duration and intensity of the stress (Reddy *et al*., 2004). It is well known that treatment of plants with elicitors, or attack by incompatible pathogens, causes an array of defense reactions, including the accumulation of a range of plant defensive secondary metabolites (Zhao *et al*., 2005). From the results it can be concluded that the application of native diazotrophs could be well used to promote growth and plants' innate antioxidant defense potentials.

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