Effect of *Glomus* species on physiology and biochemistry of *Catharanthus roseus*

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Received: 9 April 2008 / Accepted: 18 January 2009
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Abstract The present study on efficacy of different *Glomus* species, an arbuscular mycorrhizal (AM) fungus (*G. aggregatum*, *G. fasciculatum*, *G. mosseae*, *G. intraradices*) on various growth parameters such as biomass, macro and micronutrients, chlorophyll, protein, cytokinin and alkaloid content and phosphatase activity of pink flowered *Catharanthus roseus* plants showed that all *Glomus* species except *G. intraradices* enhanced the chlorophyll, protein, crude alkaloid, phosphorus, sulphur, manganese and copper contents of *C. roseus* plants along with phosphatase activity significantly over uninoculated plants. However only *G. mosseae* and *G. fasciculatum* exhibited superior symbiotic relationship with the plant. *G. mosseae* was found to be the best for increasing the crude alkaloid content (8.19%) in leaf and also in increasing the quantity of important alkaloids vincristine and vinblastine.

Keywords *Catharanthus roseus* · *Glomus* species · Cytokinin · Alkaloids · Phosphatase activity

Introduction

Natural plant products have served as the basis of man's medicinal arsenal since time immemorial. Scientists all over the world are concentrating on the use of herbal medicine to immune defenses of the body against cancer. Seventy-five to 80% of the world's population depends on traditional herbal medicine. Herbal medicines are proving their value in cancer treatment. Synthetic vincristine used to treat leukemia is only 20% as effective as the natural product derived from *Catharanthus roseus*. Two alkaloids vincristine and vinblastine from *C. roseus* are effective against childhood lymphocytic leukemia and Hodgkin's disease. Folkloric usage cited the plant as possessing properties that lowered blood sugar thereby making it a possible source of an oral insulin substitute.

Catharanthus roseus (L.) G. Don is cultivated on a commercial scale in many parts of the world. It contains over 200 naturally occurring alkaloids, of which catharanthine and vindoline are monomeric precursors of the two important anticancer dimeric alkaloids vinblastine and vincristine. The plant is cultivated as an ornamental but mainly for its alkaloids. The physiologically important alkaloids are the chemotherapeutic antineoplastic dimeric oncolytic alkaloids vinblastine and vincristine used in cancer chemotherapy. Alkaloids present in this plant are distributed in different parts of the plant viz. roots, leaves, stems and flowers and are used for the treatment of various cancers [1]. Vincristine and vinblastine are mainly present in the aerial parts and the alkaloids of the *Rauvolfia* group viz. ajmalicine (raubasine), serpentine and reserpine are found in the roots of *C. roseus* plant. Vinblastine sulfate sold as velban is used particularly to treat Hodgkin's disease besides lymphosarcoma, neuroblastoma and carcinoma of the breast, lungs and other

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organs in acute and chronic leukemia. Vincristine sulfate sold as oncovin arrests mitosis in metaphase and is very effective for the treatment of leukemia in children and is also used against Hodgkin's disease, Wilm's tumor, neuroblastoma and reticulum cell sarcoma. Whereas vindoline and other alkaloidal fractions from the leaves are active against several pathogenic bacteria.

Earlier studies reported absence of arbuscular mycorrhizal fungi in medicinal plants possessing alkaloids [2], because the biosynthesis of alkaloids takes place in the roots. But later reports indicated presence of AM fungi in medicinal plants [3, 4]. Association of AM fungi have been reported to improve the nutritional uptake and general health of the plants [5]. So the present study was conducted to know the effect of AM fungi in the improvement of physiological and biochemical parameters of the plant and alkaloid production.

Materials and methods

A soil culture of *G. aggregatum* Schenck and Smith emend Koske, *G. fasciculatum* Gerdemann and Trappe (Thaxter), *G. mosseae* (Nicolson and Gerdemann) Gerdemann and Trappe and *G. intraradices* Schenck and Smith maintained, on Rhodes grass in sterile soil for 90 days was used as inoculum. *G. aggregatum*, *G. fasciculatum* and *G. mosseae* were identified by Gupta and Janardhanan [6] and *G. intraradices* was borrowed from Dr D. J. Bagyaraj, Head, Department of Agricultural Microbiology, University of Agricultural Sciences, GKVK, Bangalore. The infected root fragments and rhizospheric soil of the Rhodes grass containing 125 spores/g of soil was used as inoculum and spread 2 cm below the surface of soil of Rhodes grass. Effect of *G. aggregatum* Schenck and Smith, *G. fasciculatum* (Thaxter) Gerdemann and Trappe emend Walker and Koske, *G. mosseae* (Nicolson and Gerdemann) Gerdemann and Trappe and *G. intraradices* Schenck and Smith was studied on the plant. Plants were harvested after 90 days. Dry weight of shoot, root and leaf was recorded after drying in an oven at 40°C for 7 days. Fresh weight of root, shoot, leaves and height of the plants was recorded at the time of harvesting.

Plant growth conditions and methods of inoculation

Surface sterilized seeds of *C. roseus* (L.) G. Don were sown in earthen tray containing sterilized sand-vermiculite (1:1) mixture. Each tray was inoculated with 10 g of soil-based AM inoculant (four species of *Glomus*) containing approximately 125 chlamydospores of *Glomus aggregatum*, *G. fasciculatum*, *G. mosseae* and *G. intraradices* each at the

time of sowing. Seeds sown in sterilized sand-vermiculite mixture without AM fungi served as controls. Each treatment had five replications. Twenty seeds were sown in each tray and were grown for 30 days in glasshouse with day/night regimes of 12 h at 30°C ± 2°C and 45–60% relative humidity. Germinated seedlings were transplanted in earthen pots one plant in a pot (6" size) filled with autoclaved soil (pH 8.0, EC 0.832 dsm⁻¹, available P 16.70 µg/g soil). The soil was collected from the experimental fields of Department of Botany, University of Lucknow where the experiment was conducted. Prior to sterilization the soil was air dried, sieved through 20 mm sieve and autoclaved at 121°C for 1 h twice over a 3-day period. There were five replicates for each treatment. Plants were watered when necessary.

Roots of the seedlings were periodically checked for AM colonization by standard method of Phillips and Hayman [7]. Thirty-day-old mycorrhizal and non-mycorrhizal seedlings were transplanted in earthen pots filled with steam sterilized soil.

Analysis of phosphatase activity, macro and micronutrients, protein and chlorophyll content

Macro and micronutrients were analyzed from stem, root and leaf parts of the plant by the standard method of Jackson [8]. Assay of acid and alkaline phosphatase activity was done by the modified method of Bergmeyer [9]. Phosphatase activity was expressed as µm/mg fresh wt/min. Protein content was estimated by the standard method of Lowery [10]. Chlorophyll content was estimated by the age old method of Arnon [11].

Extraction of crude cytokinin content

Crude cytokinin was estimated by the method of Vreman and Corse [12]. Cytokinin was extracted from thoroughly washed thin feeder roots (5 g) of both mycorrhizal and non-mycorrhizal plants. Arbuscular mycorrhizal fungi were reported to increase cytokinin content [13].

Extraction and assay of alkaloid

Alkaloid extraction and analysis was done by the method of Uniyal et al. [14]. Crude alkaloid contents were analyzed by high performance liquid chromatography (HPLC) using analytical reverse phase Nova Pak C₁₈ column (250 × 4.6 µm). The data were analyzed using analysis of variance (ANOVA) least significant difference (LSD) as described by Cochran and Cox [15]. Statistical significant differences among the treatments were tested by critical difference (CD) test at 5% probability level.

Percentage root colonization and number of spores/100 g soil

The root samples were cleared using 10% KOH for 1 h at 90°C and stained with trypan blue in lactoglycerol by standard method [7]. The percentage of infected root length was evaluated by the gridline intersect method of Giovannetti and Mosseae [16]. Spores were isolated from the soil samples by wet sieving and decanting method [17] and quantified by eelworm counting slide.

Results and discussion

The three species of *Glomus* viz. *G. aggregatum*, *G. fasciculatum*, *G. mosseae* were found to have significant effect on various physiological parameters of *C. roseus* plants (Tables 1–3). Out of the four AM fungi *G. mosseae* and *G. fasciculatum* had relatively superior symbiotic relationship with *C. roseus* than other species. Plant growth, chlorophyll content, macro and micronutrient content, phosphatase activity and protein content were significantly higher in the plants inoculated with these two species.

Plant height was 34.8% higher than control in *G. mosseae* treatment while it was 17% and 8% higher in *G. fasciculatum* and *G. aggregatum* treatment, respectively. *G. intraradices* has no significant effect on height. The root, shoot and leaf dry weight was enhanced in all mycorrhizal treated plants as compared to control. Shoot, leaf, root dry weights were 117.65, 132.47 and 67.90% higher than control in *G. mosseae* inoculated plants. Total dry weight was 108.07% higher than control in the same treatment. Total dry weight was 27.09, 42.36 and 16.14% higher in *G. aggregatum*, *G. fasciculatum* and *G. intraradices* treatment as compared to control, respectively.

Phosphorus (P) content in stem, leaf, root was 31.78, 21.17 and 34.86% higher in *G. mosseae* treatment as compared to control. Sulphur, manganese (Mn) and copper (Cu) content followed the same trend. Sulphur content was

0.27, 1.12 and 1.0% in *G. mosseae* inoculated shoot, leaf and root of *C. roseus* plant, respectively as compared to 0.13, 0.15 and 0.50 of control treatment. Manganese content was 192, 225 and 195 µg/g dry weight in the same treatment as compared to 151.4, 219.6 and 175 in control treatment. Cu content was 93.94, 102.82 and 88.51% higher in *G. mosseae* treatment as compared to control. The contents of P, S, Mn and Cu were found increased in AM inoculated treatments over controls, and they are statistically significant at 5%. Mycorrhizal fungi improved P availability by solubilizing inorganic phosphorus. Solubilization of phosphorus is mediated through release of organic acids and phosphatases [18]. Mycorrhizal fungi was reported to increase both phosphatase activity and cytokinin content in cowpea roots and these biochemical changes might be responsible for improved growth of host plants [19].

G. mosseae increased cytokinin content in roots by 6.29% as compared to non-inoculated control plants (Fig. 1). Enhancement of P uptake and other nutrients by fungal hyphae is the primary mechanism responsible for plant growth stimulation by AM fungi [5]. However, mycorrhizal benefits may not be limited to improved nutrient uptake, the production of plant hormones by these mutualistic fungi can also be involved with their effects on plant metabolic process [20]. Cytokinins have been reported to stimulate protein and chlorophyll synthesis as well as cell division and expansion in plants [21].

Total alkaloid content was 3.47, 8.19 and 4.24% in *G. mosseae* treatment as compared to 1.76, 7.27 and 3.76 in control, while it was 3.45, 8.0 and 3.89% in *G. fasciculatum* treatment, respectively. Crude alkaloid in different parts of *C. roseus* plant inoculated with *G. mosseae* (Fig. 2) were found to be more in leaf tissue (8.19%), followed by flower (8.02%), root (4.24%) and stem (3.47%), respectively. Effect of *G. mosseae* treatment on the alkaloid productivity of *C. roseus* showed significant increase in vincristine, vinblastine, catharanthine and vindoline contents in leaf tissue over control plants. Although the alkaloid profile in leaf tissue of both inoculated and uninoculated plant

Table 1 Effect of four *Glomus* species on height and dry weight of *C. roseus* plant

Treatment	Plant height (cm)	DW (g)			Total DW/plant (g)	Root colonization (%)	Chalmydosporae/100 g soil
		S	L	R			
Control	57.4	3.74	1.54	1.62	6.94	–	–
<i>G. aggregatum</i>	62.0	4.5	2.36	1.96	8.82	77	510
<i>G. fasciculatum</i>	67.2	5.04	2.64	2.2	9.88	78	664
<i>G. mosseae</i>	77.4	8.14	3.58	2.72	14.44	84	620
<i>G. intraradices</i>	57.8	4.18	2.0	1.88	8.06	56	408
LSD (p = 0.05)	2.83	0.89	0.51	0.31	–	–	–

LSD = Least significant difference; DW = Dry weight; S = Stem; L = Leaf; R = Root.

Table 2 Effect of four *Glomus* species on P, S, Mn, Cu and total alkaloid content of *C. roseus* plant

Treatment	Phosphorus (mg/g DW)			Sulphur (%)			Manganese (μ g/g DW)			Copper (μ g/g DW)			Total alkaloid (%)		
	S	L	R	S	L	R	S	L	R	S	L	R	S	L	R
Control	1.29	1.37	1.09	0.13	0.15	0.50	151.4	219.6	175	13.2	14.2	17.4	1.76	7.27	3.76
<i>G. aggregatum</i>	1.39	1.87	1.29	0.21	0.25	1.0	176.2	231.8	167.4	18.8	30.4	35.8	2.50	7.83	3.95
<i>G. fasciculatum</i>	1.46	1.84	1.15	0.24	0.31	0.99	180.6	230	177.2	16.4	24	54.8	3.45	8.0	3.89
<i>G. mosseae</i>	1.7	1.66	1.47	0.27	1.12	1.0	192	225	195	25.6	28.8	32.8	3.47	8.19	4.24
<i>G. intraradices</i>	1.32	1.60	1.25	0.21	0.30	0.80	177.4	224.2	169.2	11.6	16.4	18.6	2.69	7.32	3.82
LSD (p = 0.05)	4.51–02	4.12–02	4.58–02	4.40–02	0.16	0.14	5.04	5.80	6.27	3.32	3.34	3.62	4.27–02	0.23	5.11–02

LSD = Least significant difference; DW = Dry weight; S = Stem; L = Leaf; R = Root.

Table 3 Effect of four *Glomus* species on chlorophyll content, phosphatase activity and protein content in leaf of *C. roseus* plant

Treatment	Chlorophyll content mg/g fresh wt			Phosphatase activity μ m p-nitrophenol/mg fresh wt/min		Protein content mg/g fresh wt
	a	b	Total	Alkaline		
				Acid	Alkaline	
Control	0.39	0.18	0.56	0.59	1.65	6.04
<i>G. aggregatum</i>	0.40	0.26	1.01	0.90	1.70	7.31
<i>G. fasciculatum</i>	0.45	0.32	1.09	0.91	1.72	7.49
<i>G. mosseae</i>	0.68	0.31	1.21	0.88	1.84	8.27
<i>G. intraradices</i>	0.29	0.17	0.57	0.61	1.70	7.08
LSD (p = 0.05)	3.84–02	4.49–02	0.06	0.15	0.05	5.09–02

LSD = Least significant difference.

Fig. 1 Effect of *G. mosseae* inoculation on crude cytokinin content in *C. roseus* plant.

Fig. 2 Effect of *G. mosseae* inoculation on alkaloid percent in different parts of *C. roseus* plant.

was qualitatively similar, still quantitative difference was recorded among them (Fig. 3). Vindoline the precursor of bisindole alkaloid was in the traces in the leaf tissue of *C. roseus* plant. Miura et al. [22] found vindoline as major alkaloid, but in our study we found vinblastine and catharanthine major alkaloids in leaves of *C. roseus* plant. The production of secondary metabolites was considered to be part of the defence mechanism of the plant cells in response to an offending foreign substance [23]. Thus an increase in the accumulation of vincristine, vinblastine, catharanthine and vindoline in the leaves was recorded due to the presence of AM fungi in the plants.

Acid and alkaline phosphatase activity was 49.15%, 11.52% higher in *G. mosseae* treatment as compared to control. Acid phosphatase activity was highest (54.24%

Fig. 3 Effect of *G. mosseae* inoculation on different alkaloids from leaf of *C. roseus* plant. VCR (vincristine), VLB (vinblastine), CATH (catharanthine), VINDO (vindoline).

higher than control) in *G. fasciculatum* treatment followed by *G. aggregatum* (52.54% higher) and *G. mosseae* (Table 3). Mycorrhizal fungi has been reported to increase phosphatase activity in other plants also [24]. Phosphatase activity can be used as a potential indicator for the phosphorus status of the plants [25]. Increase in acid phosphatase in the mycorrhizal plants is strongly influenced by the fungus [26]. Increased phosphatase activity was correlated with increased phosphate uptake [27].

Leaf protein level expressed as mg protein/g root fresh weight increased in the mycorrhizal plants as compared to non-mycorrhizal plants. *G. mosseae* inoculated plants of *C. roseus* exhibited higher level of protein (8.27 mg/g) than non-mycorrhizal (6.04) and other mycorrhizal inoculated plants. *G. mosseae* induced more leaf protein than *G. aggregatum* and *G. fasciculatum*. In general leaves of mycorrhizal plants contained significantly high protein level. Accumulation of protein in the mycorrhizal roots is reported in many plants and are referred to as mycorrhizins [28].

The root colonization and spore number at the end of experiment were 84%, 620 in *G. mosseae* where it was highest while it was 78,664, 77,510 and 56,408 in *G. fasciculatum*, *G. aggregatum* and *G. intraradices*, respectively (Table 1). In conclusion, the present study indicate the potential of *Glomus* species as a useful symbiont to enhance growth and other useful traits such as alkaloid and cytokinin content of *C. roseus* plants, thus can be exploited for use after some more studies on confirmation under field conditions.

Acknowledgements The author Neelima Ratti is thankful to DST, New Delhi for awarding Young Scientist fellowship. CIMAP, Lucknow is also acknowledged for help in HPLC analysis of crude alkaloid.

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