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Symbiotic and Synergistic Efficiency of Bioinoculants on *Catharanthus roseus* (L.) G. Don

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Abstract: ***Catharanthus roseus*** **(L.) G. Don widely known medicinal herb, belongs to Apocynaceae family has been cultivated for its wide range of therapeutic values. Arbuscular mycorrhizae (AM) are helpful symbionts for growth of the plant and its development, offering a possible substitute for high input agricultural technology employed for production of environmentally hazardous fertilizers. Therefore, the present investigation was focused on to analyze the effect of AM fungi (*** ***Acaulospora laevis*** **and** ***Glomus mosseae*** **) along with** ***Pseudomonas fluorescens*** **, alone and in combinations, on different growth parameters of** ***Catharanthus roseus*** **in a pot experiment with autoclaved sterilized soil under polyhouse condition. AM inoculum and** ***P. fluorescens*** **showed significant increase in different growth parameters after 120 days of inoculation. Among all treatments, consortium of** ***G. mosseae, A. laevis*** **plus** ***P. fluorescens*** **was most effective for increased shoot height, root length, leaf area, shoot and root biomass, percent root colonization and AM spore number. Moreover, chlorophyll content, phosphorous and phosphatase activities were found to be maximum when AM fungi and** ***P. fluorescens*** **were applied in consortium.**

Keywords: Glomus mosseae, Pseudomonas fluorescens, Catharanthus roseus, **Leaf area, Phosphatase activities.**

I. INTRODUCTION

Catharanthus roseus (L.) G. Don (family: Apocynaceae), commonly known as "Madagascar periwinkle", is an important ornamental as well as medicinal herb growing in many tropical, subtropical and temperate regions of the world (Lata, 2007). *C. roseus* is being extensively studied and commercially exploited plant species due to its antitumor indole alkaloids vinblastine and vincristine, used in treatment of leukemia and Hodgkin's disease. Vincamine and vinpocetine have vaso-dialating and memory enhancing properties and have been shown to alleviate Alzheimer's diseases and vascular dementia. Moreover, ajmalicine is being widely used to treat blood circulation disease (Noble, 1990; Hindmarch et al., 1991; Fischhof et al., 1996; Leveque et al., 1996; Sottomayor and Ros- Barcelo, 2006; Rabbinani-Chadegani et al.,2009; Risinger et al., 2009; Jaleel et al., 2007). The plant parts have varying concentrations of alkaloid like leaves accompanied with 1% and roots have 9%; therefore, to achieve desired compound, a large amount of plant sample is required to obtain commercial quantities (Tyler, 1988). Many attempts have been made to enhance the synthesis of secondary metabolites by using cell suspension cultures, but all are unsuccessful (Bonzom et al., 1997) therefore, researchers are trying to find out efficient methods to enhance the biomass production, in order to compensate for a very low content of active ingredients.

Strategies to enhance biomass production included use of chemical fertilizers, growth regulators and growth retardants (Bhattacharjee and Gupta, 1984; Choudhury and Gupta, 1996). The inadequate and non-mobilized nutrients in the soil also affect the productivity of plants. Moreover, direct application of nutrients by using synthetic fertilizer to the soil, increases crop production and its cost. In spite positive effects, excessive use of synthetic fertilizer starts displaying their adverse effects like soil leaching, water pollution, destroying microbial diversity and friendly insects, increases crop susceptibility towards disease and reducing soil fertility. On other hand, use of bio-fertilizer is cost effective as well as eco-friendly approach to enhance biomass production without affecting natural ecosystem. Bio-fertilizers are microbial inoculants consisting of living cells of micro-organism like fungi, algae and bacteria which may help in increasing production by enhancing biological activities in the rhizosphere of plants (Tilak and Reddy, 2006).

Arbuscular Mycorrhizal fungi and plant growth promoting rhizobacteria are soil microbes that trigger the production of growth regulating substances, facilitate absorption of immobilized nutrients from the soil, thereby conferring additive affects on morphology, biochemistry and yield of some plants (Kasliwal et al., 2016; Vittal Navi et al., 2006). Arbuscular mycorrhizal fungi forms the major group of microorganisms that mainly known to improve phosphorus uptake in their host (Toussaint, 2008, Smith and Read, 2008) by improving exploitation of the soil (Cavagnaro et al., 2005), hydrolization of organic P (Richardson et al., 2009) and solubilization of inorganic P forms (Tawaraya et al., 2006).The plant growth-promoting rhizobacteria (PGPRs) have positive influence over mycorrhizal establishment like AM spore germination, root infestation with AM fungi and their hyphal growth as well as functioning (Ranveer Kamal et al., 2014).

II. MATERIAL AND METHOD

A. Study site:

The study was carried out in Poly-house at Botany Department, Kurukshetra University, Kurukshetra, Haryana during December 2016 to March 2017. The poly-house received natural sunlight with controlled temp (20°±5°C) and humidity (50-70%). The soil characteristics are as follows: sand-64.2%, silt-21.81%, clay-3.90%, pH-8.08 ±0, EC-0.25 dS/m, organic carbon-0.40%, total N-0.042%, P-0.017 %, K-0.022 Kg/ m^2.

B. Isolation, identification and mass multiplication of AM spores:

AM fungal (*Glomus mosseae* and *Acaulospora laevis*) spores were isolated from the rhizospheric soil of field grown *C. roseus* plants by using wet sieving and decanting technique (Gerdmann and Nicolson, 1963). The isolated spores were passed through microscopic examination to record their morphological features like size, shape, colour, wall structure and surface ornamentation, size of subtending hyphae, bulbous suspensor, number, and arrangement of spores in sporocarp. These spores were identified by using keys (Schenck and Perez, 1990), *Glomus mosseae* (Walker and Schubler) and *Acaulospora laevis* (Gerd. and Trappe) were found to be the most dominant AM fungal strains.

C. Bioinoculant preparation:

The dominant AM fungal spores were multiplied with wheat as host by Funnel technique (Menge and Timmer, 1982). Initially inoculum was grown in earthen funnels followed by big earthen pots up to 3 months. The inoculum of *Pseudomonas fluorescens* (MTCC NO. 103) was obtained from the Institute of Microbial Technology, Chandigarh, India, and multiplied by using nutrient broth medium (beef extract: 3g, peptone: 5g, NaCl: 5g, 1000 mL distilled water), incubate for 48 hours at 32°C to obtain concentration of 1×10^9 colony forming units (cfu) ml^{-1}.

D. Plant material:

One month old plantlets were procured from Chaudhary Devi Lal Herbal Nature Park Yamuna Nagar, Haryana India. The roots of all plants were washed with distilled water before treatment. In case of control and treatment without *P. fluorescens*, plants were planted after washing. In treatment with *P. fluorescens*, roots were placed in cell suspension of *P. fluorescens* for 2 to 3 minutes before transplanting them in to earthen pots.

E. Pot preparation:

Soil from investigational site was collected and mixed with sand in a proportion of 1:3 (sand:soil). Prior to sterilization the soil mixture was air dried, sieved through 20 mm sieve and autoclaved at 121°C for 1 h twice over a 3-day period. The two plantlets were transplanted in each of the earthen pots (size 25×25cm) having sterilized soil and 10% w:w selected inoculums of AM fungi. Plants were grown under natural illumination and watered regularly in a poly house.

F. Experimental design:

The experiment was performed with below listed treatments in three designs i.e. single inoculation, double inoculation, triple inoculation and one control.

1. Control (autoclaved soil mixture without any bioinoculant)

2. *Acaulospora laevis* (A)

3. *Glomus mosseae* (G)

4. *Pseudomonas fluorescens* (P)

5. *A. laevis* + *G. mosseae* (A +G)

6. *A. laevis* + *P. fluorescens* (A + P)

7. *G. mosseae* + *P. fluorescens* (G + P)

8. *G. mosseae* + *A. laevis* + *P. fluorescens* (G+ A + P)

Each treatment had three replicates and 2 plants were raised in each pot of replicate. The effects on various treatments were recorded after an interval of 120 days after transplant (DAT) for different parameters. The root and shoot were harvested separately and weight for their fresh and dry weight.

G. Experimental analysis:

The morphological parameters including Plant height (cm), Root length (cm), Plant biomass (g) and Leaf Area (cm^2) were recorded after 120 days of treatment. The AM spores isolation and quantification were carried out by using wet sieving and decanting technique (Gerdmann and Nicolson, 1963); Grid line intersect method (Gaur and Adholeya, 1994); while Rapid Clearing and Staining Method (Phillips and Hayman, 1970) and root slide technique (Giovannetti and Mosse,1980) were utilized for assessment of root colonization.

The quantification of root colonization was done by the following formula:

Percentage root colonization = (number of root segments colonized / Number of root segments studied) ×100

The biochemical parameters like Chlorophyll Estimation (Arnon method, 1949). Phosphorus content of shoot and root were determined by vanado-molybdate-phosphoric yellow color method (Jackson, 1973), while Phosphatase activity (Tabatabai and Bremner, 1969).

H. Statistical analysis:

The data was statistically analyzed by using analysis of variance (ANOVA) followed by post hoc test performed by SPSS software package SPSS 16.0 (SPSS Inc. Chicago, IL). Duncan's multiple-range test was performed at $P \leq 0.05$ on each of the significant variables measured.

III. RESULT AND DISCUSSION

A. *Effect on Plant Height:*

Inoculation of *C. roseus* plant with AM fungi (*Glomus* and *Acaulospora*) and *Pseudomonas fluorescens* increased the height of plants as compared to control plant (Table 1). After 120 days of transplantation, increase in plant height was observed maximum in GAP (58.39±2.342cm) followed by double combination GP (58.31±2.407cm) and than in *Acaulospora* (58.29±2.419 cm). Significant enhancement of shoot height may be due to AM fungal colonization as it is known to increase plant growth by providing increased surface area for nutrient absorbtion from soil and by secreting plant growth hormones. *P. fluorescens* was found to be efficient microbe, which improved shoot length in dual combination (with *Glomus* 58.31±2.407 cm) and triple combination (58.39±2.342cm) as compared to alone. *P. fluorescens* bacterium is also concerned with production of growth regulators that increases plant growth by cell elongation, cell division and differentiation of plant cells. The plant disease resistance, suppression of plant pathogenic microbes and phosphate solubilization might be the causative factors behind increment of shoot height. Kavatagi and Lakshman (2014) observed increment in shoot length, when *Solanum lycopersicu* grown with AM, *Azotobacter chrococcum and P. fluorescens* in pot condition.

B. *Effect on Shoot and Root Fresh and Dry Weight:*

Table 1 showed that inoculated *C. roseus* with various bioinoculants tends to increased the root and shoot biomass in the form of fresh and dry weight of root/shoot. GAP treatment was reported to be effective for maximizing fresh (35.54 ±3.969) and dry weight (2.536±0.274) of shoot. It is evident that increased phosphorous uptake leads to improve shoot biomass (Othira et al., 2012) and root development (Al-Qarawi and Alshahrani, 2010). GAP treatment was also observed to be efficient in increment of fresh (4.912±0.437) and dry weight (1.56±0.334) of root. Highly branched roots were observed in combination of GAP. Mycorrhizal fungi lead to an increase in number of fibrous roots by decreasing the meristematic activity of root apices (Berta et al., 1990) and *P. fluorescens* leads to produce changes in the root morphology by synthesized phytohormones, that increase absorptive surface area of roots (Bashan et al., 2004) for nutrients as well as water. Increase in plant biomass has been reported by different workers because AM inoculations

assisted with PGPR in various medicinal plants like *C. roseus* (Lenin and Jayanthi, 2012), *Plectranthus amboinicus* (Kasliwal et al., 2016), *Salvia officinalis* (Kumar et al., 2009).

C. *Root length:*

In the present investigation, it is evident from Table 1 that after 120 days of transplantation, root length of treated plants of *C. roseus* increased significantly. Highest increment was found in GAP (14.360±1.134 cm) treatment over control plant (4.024±0.559cm). The mycelial network of AM fungi might be responsible for increase in root length because of deeper extension of extra radical mycelia to invade nutrients depletion zone (Torrisi et al., 2002; Turk et al., 2006). The present findings are in conformity with results of (Neetu et al., 2015), who observed increment in root length of *Gossypium arboreum* when inoculated with AM fungi and *P. fluorescens.*

D. *Leaf area:*

As depicted in Table 2 the maximum leaf area was observed in triple combination of GAP (13.84±0.887 cm^2) followed by double combination GP. (Yadav et al., 2015) also observed positive impact of AM fungi and *P. fluorescens* on leaf area of sunflower plants. Higher leaf area in AM treated plant might be due to enhanced nitrogen assimilating enzymes (Cliquet and stewart,1993), nitrogen acquisition by external hyphal tr**a**nsport of NO_3 (Tobar et al.,1994a, b) and improved water uptake (Yadav et al., 2012; 2013a, b).

E. *Percent AM root colonization and AM spore number:*

The status of AM root colonization and spores was studied under different treatments as well as in control plant and data depicted in Table 2 showed that the least number of AM spores and degree of colonization was observed in control plant. Except control plant, GAP treatment was found with maximum spore number (85.48±6.309) and highest degree of percentage root colonization (81.3±10.132). The second highest spore number were recorded in *Acaulospora* (83.54±7.512) whereas root colonization in *Glomus* (81.24±4.306) treatment. There is positive relationship between AM spores number and root colonization percentage. *P. fluorescens* might have influenced the root exudation of host plant that resulted in the stimulation of AM spores in the rhizosphere and thus behaved as mycorrhiza helper bacteria because they promoted higher AM spore number and root colonization rate which contribute to the P cycling through solubilization of mineral phosphate and, thus promoting nutrient supply to the crop plants in a sustainable way. (Boer et al., 2005) also observed that root colonization was increased in combined inoculation of *P. fluorescens* and AM fungi.

F. *Chlorophyll content:*

As depicted in Table 3 that after 120 days of transplant, chlorophyll content improved significantly in co-inoculated plants in comparison to control. The co-inoculation of plant with GAP treatment induced maximum increment in Chlorophyll a, b and total chlorophyll content (1.932±0.187, 1.66±0.358, 3.592±0.335 mg g^{-1} FW). This increasing trend was further followed by double inoculation of GP and AP as compared to un-inoculated plantlets of *C. roseus*. The increment observed in chlorophyll content is associated with the increased uptake of phosphorus in the mycorrhizal roots, which indicates essential role of phosphorus in increasing photosynthesis and increasing chlorophyll content. So, high content of chlorophyll in inoculated plants can be associated with improving the host plant nutrition, especially phosphorus. Babaei et al. (2012) demonstrated a positive relationship between AM fungi and *P. fluorescens* and also found highest nutrient uptake in inoculated plants of *Helianthus annuus.* According to Baslam et al. (2012) that the plant has better ability to synthesize chlorophyll and accessory pigments under the influence of better nutritional and environmental conditions for the growth and thus produced more energy.

G. *Phosphorous content:*

According to result shown in Table 4, it is evident that inoculation of *C. roseus* with AM fungi and *P. fluorescens* markedly improved phosphorous content in comparison to the control. Maximum phosphorous content in both shoots and roots was reported in plant treated with GAP (0.612±0.120%) for root and (0.474±0.031%) for shoot followed by dual inoculation of GP (0.605±0.082%) for roots and (0.462±0.023 %) for shoots. According to (Kremer, 2007; Kavatagi and Lakshman, 2014) AMF and PGPR co-inoculations supported each other in terms of the improvement of plant growth and nutrient uptake, particularly phosphorus uptake. AM fungi plays a dominant role in increasing solubilization of phosphorus and its uptake along with other immobile nutrients like Cu and Zn (Hodge et al., 2010) by plants. The fungal hyphae transport phosphate over large distance into the root cortical cells assisted by their ability to grow through soil

pore and phosphate absorption beyond the depleted zone. The AM fungi absorb phosphorus from soil solution in phosphate form which is accumulated as polyphosphate (Poly-P) granules in the vacuoles of the fungus. These (Poly-P) granules are broken down by enzymatic activities into fine branches of arbuscules and releasing inorganic phosphorus in the cytoplasm (Parkash and Aggarwal, 2011).

H. *Phosphatase activity:*

As illustrated in Table-4, the root phosphatase activity was significantly higher in inoculated *C.roseus* plants after 120 days over control. Highest value of acidic (0.190±0.017 $IUg^{-1}FW$) and alkaline (0.206±0.005 $IUg^{-1}FW$) phosphatase activities were observed in the plants inoculated with GAP, which was in accordance with the findings of (Tanwar et al., 2012; Neetu et al. 2012) who observed enhancement in phosphatase activity in GAP treated *Capsicum annuum*, *Linum usitatissimum* plant with low level of Phosphorus fertilizer. According to the results alkaline phosphatase activity was found higher as compared to acidic phosphatase activity. Among different treatments single inoculation of *A. laevis* was least effective for increasing acidic (0.034±0.006 $IUg^{-1}FW$) as well as alkaline (0.031±0.007 $IUg^{-1}FW$) phosphatase activity. It was found in the present investigation that plants having higher mycorrhizal root colonization had maximum phosphatase activity. The increased acidic and alkaline phosphatase activities in mycorrhizal plants linked with increased phosphate uptake by means of the internal hyphae of mycorrhizal fungi has been suggested by (Saito, 1995; Capaico and Callow, 1982).

Table 1: Interactive effect of AM fungi and *P. fluorescens* on growth response of *C. roseus* after 120 days

Treatments	Plant height(cm)	Shoot weight (g)		Root length (cm)	Root weight(g)	
		Fresh	Dry		Fresh	Dry
Control	32.62±2.492^{d}	12.24±2.719^{f}	1.336±0.216^{c}	4.024±0.559^{e}	1.560±0.160^{e}	0.482±0.239^{d}
A	58.29±2.419^{a}	20.92±2.525^{cd}	2.044±0.182^{b}	7.420±0.725^{d}	3.682±0.420^{c}	1.104±0.253^{bc}
G	52.04±4.493^{b}	17.90±1.856^{de}	1.648±0.703^{bc}	7.988±1.297^{d}	4.472±0.217^{ab}	1.448±0.517^{ab}
P	32.84±2.398^{d}	15.50±2.501^{ef}	1.902±0.746^{b}	6.900±0.484^{d}	2.848±0.188^{d}	1.010±0.031^{c}
AG	48.84±2.398^{b}	19.30±1.839^{de}	1.768±0.152^{b}	10.74±0.629^{c}	4.838±0.376^{a}	1.524±0.182^{ab}
AP	43.00±3.685^{c}	24.24±3.745^{bc}	1.776±0.171^{b}	12.62±1.010^{b}	3.986±0.780^{bc}	1.470±0.285^{ab}
GP	58.31±2.407^{a}	25.58±3.067^{b}	2.074±0.254^{b}	14.28±0.860^{a}	4.652±0.251^{a}	1.488±0.335^{ab}
GAP	58.39±2.342^{a}	35.54±3.969^{a}	2.536±0.274^{a}	14.36±1.134^{a}	4.912±0.437^{a}	1.560±0.334^{a}
L.S.D ($P\leq0.05$)	3.094	3.701	0.250	1.134	0.517	0.386
ANNOVA F(7,32)	35.314	31.23	6.416	92.50	41.54	7.81

G†: *G. mosseae*, A: *A. laevis*, P: *P. fluorescens*, ‡: Each value is a mean of five replicates, ±: Standard deviation, AM: Arbuscular mycorrhizae, Values in columns followed by same letter are not significantly different, $P\leq0.05$, least significant difference test.

Table 2: Interactive effect of AM fungi and *P. fluorescens* on Leaf area and mycorrhization in *C. roseus* after 120 days

Treatments	Leaf Area (cm^2)	AM spore number/10 g of soil	AM root colonization (%)
Control	4.840±0.955^{e}	18.96±1.588^{d}	18.44±1.192^{d}
A	6.600±0.463^{d}	83.54±7.512^{a}	79.36±5.05^{a}
G	8.500±0.953^{c}	82.46±5.178^{a}	81.24±4.306^{a}
P	8.740±0.698^{c}	66.16±5.602^{b}	63.02±5.712^{b}
AG	9.860±0.673^{b}	47.44±4.443^{c}	50.28±6.281^{c}
AP	10.24±0.952^{b}	52.64±5.192^{c}	56.64±2.472^{bc}
GP	12.50±0.612 ^{a}	52.62±4.686^{c}	56.82±5.683^{bc}
GAP	13.84±0.887^{a}	85.48±6.309^{a}	81.30±10.13^{a}
L.S.D ($P\leq0.05$)	1.023	6.84	7.32
ANNOVA F(7,32)	67.25	93.33	69.05

G†: *G. mosseae*, A: *A. laevis*, P: *P. fluorescens*, ‡: Each value is a mean of five replicates, ±: Standard deviation, AM: Arbuscular mycorrhizae, Values in columns followed by same letter are not significantly different, $P\leq0.05$, least significant difference test.

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Table 3: Interactive effect of AM fungi and *P. fluorescens* on chlorophyll content of *C.roseus* after 120 days

Treatments	Chlorophyll content (mg. / gm. fresh wt.)		
	Chlorophyll a	Chlorophyll b	Total Chlorophyll
Control	0.683±0.299^{c}	0.120±0.014^{c}	0.805±0.288^{d}
A	1.160±0.513^{bc}	1.022±0.494^{b}	2.186±0.990^{c}
G	1.244±0.470^{bc}	1.082±0.542^{b}	2.326±0.946^{bc}
P	1.068±0.190^{bc}	1.050±0.410^{b}	2.118±0.592^{c}
AG	1.100±0.440^{bc}	1.026±0.445^{b}	2.126±0.840^{c}
AP	1.262±0.281^{b}	1.306±0.393^{ab}	2.568±0.553^{bc}
GP	1.630±0.572^{ab}	1.658±0.423^{a}	3.288±0.834^{ab}
GAP	1.932±0.187^{a}	1.660±0.358^{a}	3.592±0.335^{a}
L.S.D ($P \leq 0.05$)	0.508	0.533	0.926
ANNOVA F(7,32)	4.549	6.845	6.889

G†: *Glomus mosseae*, A: *Acaulospora laevis*, P: *Pseudomonas fluorescens*, ‡: Each value is a mean of five replicates, ±: Standard deviation, AM: Arbuscular mycorrhizae, Values in columns followed by same letter are not significantly different, $P \leq 0.05$, least significant difference test

Table 4: Interactive effect of AM fungi and *P. fluorescens* on phosphorus uptake and phosphatase activity of *C. roseus* after 120 days

Treatments	Phosphorus content (%)		Phosphatase activity ($IUg^{1}FW$)	
	Root	Shoot	Acidic	Alkaline
Control	0.163±0.022^{c}	0.131±0.023^{f}	0.028±0.005^{e}	0.030±0.003^{d}
A	0.379±0.109^{b}	0.182±0.016^{e}	0.034±0.006^{de}	0.031±0.004^{d}
G	0.600±0.091^{a}	0.245±0.0089^{d}	0.042±0.0041^{d}	0.102±0.013^{c}
P	0.362±0.128^{b}	0.221±0.0082^{d}	0.040±0.0043^{d}	0.041±0.007^{d}
AG	0.398±0.111^{b}	0.309±0.0115^{c}	0.112±0.0081^{c}	0.119±0.019^{b}
AP	0.566±0.084^{a}	0.426±0.0116^{b}	0.126±0.0089^{b}	0.130±0.004^{b}
GP	0.605±0.082^{a}	0.462±0.023^{a}	0.121±0.006^{b}	0.127±0.012^{b}
GAP	0.612±0.120^{a}	0.474±0.031^{a}	0.190±0.017^{a}	0.206±0.005^{a}
L.S.D ($P \leq 0.05$)	0.024	0.127	0.008	0.015
ANNOVA F(7,32)	253.347	13.493	492.082	115.880

G†: *G. mosseae*, A: *A. laevis*, P: *P. fluorescens*, ‡: Each value is a mean of five replicates, ±: Standard deviation, AM: Arbuscular mycorrhizae, Values in columns followed by same letter are not significantly different, $P \leq 0.05$, least significant difference test.

IV. CONCLUSION

Inoculation of *P. fluorescens* and AM fungi improved morphological and biochemical parameters of *C.roseus* under pot condition. So AM fungi and phosphate solubilisation bacteria are beneficial microbes to succeed the excessive use of chemical fertilizer, and thus considered as important component for biomass production in a sustainable way. However, further field trials are required to assess efficacy of microbes and to develop strategies for microbes-assisted biomass production.

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