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INFLUENCE OF AMF (GLOMUS CORONATUM AND GLOMUS ETUNICATUM) ON PLANT GROWTH AND PHYSIOLOGICAL PARAMETERS OF VINCA MINOR GROWING UNDER HIMALAYAN CONDITION

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ABSTRACT

Physiological performance of plants in general, particularly cultured plant productivity and crops quality depend on the rhizosphere characteristic feature, an area of great interest to plants, producers, consumers and environmental health. Among the rhizosphere components, arbuscular mycorrhizae are one of the most common types of symbiotic associations between some rhizosphere microorganisms and plants roots. AMF leads to the production of reactive oxygen species (ROS) which stimulates formation of highly active signaling compounds capable of striggering production of bioactive compounds (secondary metabolites) that enhances the medicinal value of the plant. *Vinca minor*, a medicinal plant, was selected in the present study. The plant was subjected to AMF namely, Glomus coronatum (10%), Glomus etunicatum (10%) Glomus coronatum + Glomus etunicatum (10%) in order to see their impact on morphological, physiological, biochemical parameters of *Vinca* minor. It is known to be a valuable medicinal plant in folk medicine and it also serves as a natural source for the industrial production of medicaments for stimulating the brain blood flow. V. *minor* contains monomeric eburnamine-type indole alkaloids including vincamine which has modulatory effects on brain circulation and neuronal homeostasis as well as antihypoxic and neuroprotective potencies. The study revealed that inoculation of AMF cause significant increase in length of root and shoot, Leaf area, leaf number Stem diameter, and membrane stability index. Changes in total carbohydrate, total free amino acid, total protein content also occur.

KEYWORDS: AM, Medicinal plants, ROS, Vincamine.

Arbuscular mycorrhizal (AM) fungi have been widely used in agriculture to improve the cultivation of many crops such as medicinal plants. The scarcity and increasing of demand for medicinal plants and their products have promoted the development of artificial cultivation of medicinal plants. The improving the contents of secondary plant metabolites, antioxidant, photosynthesis and mineral nutrition through AM in medicinal plants is quite new. Medicinal plants are the most important source of medicines and play a key role in world health (Kala, 2005). These plants may be considered as famous chemical factory for biosynthesis of a huge array of secondary metabolites (Dhyani and Kala, 2005). The arbuscular mycorrhizal (AM) fungi used to enhance the plant growth and yield of medicinal crops has gained momentum in recent years because of the higher cost and hazardous effects of heavy doses of chemical fertilizers (Srivastava *et al*., 1996).

India with its mega-biodiversity and knowledge of rich ancient traditional systems of medicine (Ayurveda, Siddha Unani, Amchi and local health traditions) provide a strong base for the utilization of a large number of plants in general healthcare and alleviation of common ailments of the people (Pandey *et al*., 2007). AMF have been found to stimulate growth, improve pathogen resistance, contribute to the formation of proper soil structure as well as influence the level of secondary metabolites in plants (Smith and Read, 2008). Nutrient deficiency is one of the most important stresses which can be overcome by mycorrhiza resulting in practical applications concerned with recultivation of marginal and degraded agricultural sites (Bethenfalvay, 1992; Feldmann *et al*., 1995). The extrametrical fungal hyphae can extend several centimetres into the soil and absorb large amounts of nutrients for the host root (Khan *et al.,* 2000). The fungi withdraw glucose from plant roots and act as a significant sink for carbohydrates (Kottke, 2002). Due to their ability to increase nutrient uptake and water transport, AM fungi are being frequently used in sustainable agriculture (Koide and Mosse, 2004). Arbuscular Mycorrhizal Fungi can function in horticulture as a sustainable, biocontrol agent against pathogens, a bioprotectant against toxic stresses, and as a soil-improving anti erosion agent (Vosatka and Albrechtova, 2008). Previous studies revealed that the inoculation of arbuscular mycorrhizal fungi (AM fungi) with agricultural crops has potentially increased the growth of root and shoot biomass. Moreover agricultural importance of AM fungi has been reported by several authors (Anuradha *et al*., 2001; Karagiannidis *et al*., 2012). Several studies have been done by various workers on the role of

AM fungi in medicinal plants (Freitas *et al*., 2004; Pandey *et al*., 2009; Silveira *et al*., 2006). Keeping in view the above information, present investigation was undertaken to investigate the efficacy of two dominant AM fungi i.e. *Glomus coronatum* and *Glomus etunicatum* alone and incombination to find out the best combination having the maximum capability of enhancing AM root colonization, AM spore number, growth and physiological analysis of *V. minor* in pot experiment under greenhouse conditions.

2. MATERIALS AND METHODS

2.1 AM inoculum

The AM fungal species (*Glomus coronatum and glomus etunicatum*) were mass multiplied by using trap plant (*Triticum aestivum*) and were used for inoculation. The starter cultures were procured from Centre for Mycorrhizal Culture Collection, TERI (The energy and resources institute), New Delhi.

2.2 Mass production of AMF

AMF isolated from the soil by Warcup's soil plate method (Warcup, 1950). Air dried soil were placed in sterilized petriplates and 15 ml melted PDA (Potato Dextrose Agar) medium were poured to each petriplate. Then petriplates were incubated at 25±ºC for fungal growth. After inoculation of five to seven days, colonies were identified. These colonies were further purified by sub culturing on PDA medium. After sub culturing, extensive colonies were obtained and further used for mass culturing using wheat bran: saw dust medium. Wheat bran, saw dust and sterilized distilled water were mixed in the ratio of 3:1:4. Mixture will be transferred to conical flasks and sterilized in autoclave at 15 Ib pressure for 30 minutes. After autoclaving the flasks were allowed to cool and fungal colonies grown on petriplates were cut into 8 mm diameter with the help of sterilized cork borer and 5-7 fungal discs were added to each conical flasks containing sterilized medium under aseptic conditions. All the flasks were kept in BOD incubator at 25±2ºC for 7 to 10 days for maximum growth of fungus.

2.3 Preparation of pot mixture

The collected soil were sieved to remove the debris and large organic matter and autoclaved the sieved soil. Three plant cuttings were grown in pots (size 25×25 cm) that depend upon the nature of growth of plants. To each pot 10% inoculum of each AM fungi alone and in combination were added. Plants will be watered regularly as and when required and nourished with 150ml. Hoagland nutrient solution after every 10 days during the course of experimentation. Three replicates wree utilized for each treatment.

2.4 Different treatments

The purpose of using an AM fungus alone and in combination were to assess which one is better for acclimatization and for various growth parameters. Different combinations of AM fungi were utilized for the experimental work and growth parameters for the selected plant will be evaluated, which are mentioned below;

1. Control (Autoclaved soil without any microbial inoculums)
2. Glomus coronatum
3. Glomus etunicatum
4. G. coronatum + G. etunicatum

2.5 Analysis of AM root colonization, AM spore number, various growth, and Physiological parameters

Plants of *Vinca minor* were harvested after 45 and 90 days of AMF inoculation by uprooting them from the soil and AM root colonization was done by Rapid Clearing and Staining Method' (Phillips and Hayman,1970) and AM spores were quantified by grid line intersect method (Adholeya and Gaur, 1994). Various morphological and physiological parameters were measured (Shoot length, Root length, Leaf area, Leaf number and Stem diameter).

2.5.1 PHYSIOLOGICAL ASPECTS

2.5.1.1 Membrane Stability Index (MSI)

Membrane stability index (MSI) was determined by recording the electrical conductivity of leaf leachates in double distilled water at 40º and 100°C (Sairam, 1994). Leaf samples (0.1 g) were cut into discs of uniform size and taken in test tubes containing 10 ml of double distilled water in two sets. One set was kept at 40°C for 30 min and another set at 100°C in boiling water bath for 15 min and their respective electric conductivity"s C1 and C2 were measured by conductivity meter.

$$\text{Membrane stability index} = \left[1 - \left(\frac{C_1}{C_2}\right)\right] \times 100$$

2.5.1.2 Total Amino Acid

Total amino acids were determined according to the method of Moore and Stein (1948). Weighed 500mg of plant sample and grind it in a pestle and mortar with a small quantity of acid-washed sand. To this homogenate, added 5 to 10 ml of 80% ethanol. Centrifuged and saved the supernatant. Repeated the extraction twice with the residue and pooled all the supernatants. Reduced the volume if needed by evaporation and used the extract for the

quantitative estimation of total free amino acids. To 0.1 ml of extract, added 1 ml of ninhydrin solution. Made up the volume to 2 ml with distilled water. Heated the tube in boiling water bath for 20 min. Added 5 ml of the diluents and mixed the contents. After 15min read the intensity of the purple color against a reagent blank in a colorimeter at 570nm. The color is stable for 1h. Prepared the reagent blank as above by taking 0.1 ml of 80% ethanol instead of the extract. For the preparation of standard, dissolved 50mg leucine in 50 ml of distilled water in a volumetric flask. Took 10 ml of this stock standard and diluted to 100 ml in another flask for working standard solution. A series of volume from 0.1 to 1ml this standard solution gave a concentration range 10 to 100mg. Proceeded as that of the sample and read the color. Determined the concentration of the total free amino acids in the sample and expressed as percentage equivalent of leucine.

2.5.1.3 Protein Content

Protein was estimated by method as described by Lowry *et al.* (1951).Weighed 500mg of the sample and grind well with a pestle and mortar in 5-10 ml of the phosphate buffer. Centrifuged and used the supernatant for protein estimation. Pipette out 0.2, 0.4, 0.6, 0.8 and 1 ml of the working standard into a series of test tube. Pipette out 0.1 ml and 0.2 ml of the sample extract in two other test tubes. Made up the volume to 1 ml in all the test tubes. A tube with 1mL of water served as the blank. Added 5 ml of reagent C (alkaline copper solution) to each tube including the blank. Mixed well and allowed to stand for 10 min. Then added 0.5 ml of reagent D (Folin-Ciocalteau Reagent) mixed well and incubated at room temperature in the dark for 30min. Blue color was developed. Took the reading at 660nm.

2.5.1.4 Total Carbohydrates

Total carbohydrates were determined in plant tissue as described by Hedge *et al.* (1962). Weighed 100 mg of the sample into a boiling tube. Hydrolyzed by keeping it in boiling water bath for 3 hours with 5mL of 2.5N HCl and cooled at room temperature. Neutralized it with solid sodium carbonate until the effervescence ceases. Made up the volume to 100 ml and centrifuged at 10,000 rpm for 5 minutes. Collected the supernatant and took 0.5 and 1ml aliquots for analysis. Prepared the standards by taking 0, 0.2, 0.4, 0.6, 0.8 and 1 ml of the working standard. Zero serves as blank. Made up the volume to 1ml in all the tubes including the sample tubes by adding distilled water. Then, added 4 ml of anthrone reagent. Heated for eight minutes in a boiling water bath. Cooled rapidly and read the green to dark green color at 630nm. From the graph calculated the amount of carbohydrate present in the sample tube.

Amount of carbohydrate present in 100mg of the sample $= \frac{\text{mg of glucose}}{\text{Volume of sample}} \times 100$

3. STATISTICAL ANALYSIS

The data was analysed statistically using GraphPad Prism® 5.2. Mean values were calculated from measurements of six replicates and the standard error of means were determined. One-way and two-way analysis of variance (ANOVA) was applied to determine the significance of results between different treatments and Tukey's multiple comparison test and Bonferroni's post tests were performed at the significance level of $P<0.05$.

4. RESULTS AND DISCUSSION

Analysis of the present investigation showed that soil inoculated with AM fungi increased AM root colonization, AM spore number, plant growth, biomass and productivity and physiological parameters of *Vinca minor* a medicinal plant.

4.1 AM spore number and AM root colonization

AM spore number was measured after 45 and 90 days of seed germination. All AMF treatment applied (G. coronatum, G. etunicatum, G. coronatum+ G. etunicatum) adversely affected the seedling growth (Fig. 4.1 (A)). Elevated G. coronatum, G. etunicatum and G. coronatum+ G. etunicatum not increased the AM spore number to greater extend in concentration dependent manner i.e., in 10% G. coronatum by 18%; in 10% G.etunicatum by 19%; in 10% G. coronatum+ G. etunicatum by 25% after 45 days. Similarly, there is increase in AM spore number i.e, 10% G. coronatum by 25%; in 10% G. etunicatum by 35%; in 10% G. coronatum+ G. etunicatum by 55.39% after 90 days and the effect intensified with an increase AM spore number in dual concentration. After 45 days of incubation the degree of increases in AM spore number was minimum in 10% G. coronatum (833) and maximum in 10% G. coronatum+ G. etunicatum (970) as against 246 spore number in control. After 90 days of incubation the degree of increases in spore number was minimum in 10% G. coronatum (1675) and maximum in 10% G. coronatum+ G. etunicatum (2306) as against 423 spore number in control. The two-way ANOVA analysis was performed and means comparison analysis was achieved using Bonferroni post test ($P<0.05$) Bonferroni post test analysis of variance showed significant difference between treated and untreated plants after 45 and 90 days. There was significant difference between G. coronatum, G. etunicatum, G. coronatum+ G. etunicatum treated seedlings.

AM root colonization was measured after 45 and 90 days of seed germination. All AMF treatment applied (G. coronatum, G. etunicatum, G. coronatum+ G. etunicatum) adversely affected the AM root colonization (Fig. 4.1(B)). Elevated G. coronatum, G. etunicatum and G. coronatum+ G. etunicatum increased the concentration in dependent manner i.e., in 10% G. coronatum by 20%; in 10% G. etunicatum by 30%; in 10% G. coronatum+ G. etunicatum by 55% after 45 days. Similarly, in 10% G. coronatum by 15%; in 10% G. etunicatum by 18%; in 10% G. coronatum+ G.etunicatum by 25% after 90 days stem AM root colonization and the effect intensified with an little increase in dual concentration. After 45 days of incubation the degree of increases in spore number was minimum in 10% G. coronatum (69%) and maximum in 10% G. coronatum+ G. etunicatum (83%) as against 15% root colonization control.After 90 days of incubation the degree of increases in root colonization was minimum in 10% G. coronatum (44%) and maximum in 10% G. coronatum+ G. etunicatum (48%) as against 13% root colonization in control.The two-way ANOVA analysis was performed and means comparison analysis was achieved using Bonferroni post test ($P<0.05$) Bonferroni post test analysis of variance showed significant difference between treated and untreated plants after 45 and 90 days. There was significant difference between G.coronatum, G.etunicatum, G. coronatum+ G.etunicatum treated seedlings.

After 45 and 90 days of AMF inoculation of (Glomus coronatum, Glomus etunicatum and *G.coronatum* + *G.etunicatum*) in *Vinca minor* Linn, we observed less increase in spore number and greater increase in AM root colonization (Fig 4.1 (A)(B)).

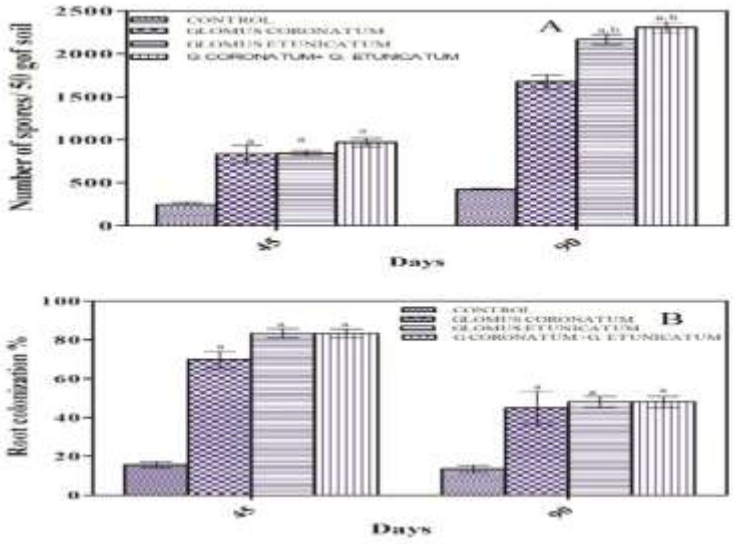

Figure 4.1: Effects of *G.coronatum, G.etunicatum, G. coronatum+ G.etunicatum* AM spore number (A), AM root colonization (%) (B) of *Vinca minor.* Values are mean±SE; N=3 [a]p<0.05 Vs control, [b]p<0.05 Vs 10% *G.coronatum*[c]p<0.05 Vs 10%*G etunicatum* [d]p<0.05 Vs *G coronatum+G etunicatum*; analysed by Two-way ANOVA followed by Bonferroni's multiple comparison test

4.2 Plant Growth Parameter

4.2.1 Plant Shoot and Root length and Leaf Area

Stem length (shoot length) was measured after 45 and 90 days of seed germination. All AMF treatment applied (*G.coronatum, G.etunicatum, G. coronatum+ G.etunicatum*) adversely affected the seedling growth (Fig. 4.2(A)). Elevated *G.coronatum*, *G.etunicatum* and *G. coronatum+ G.etunicatum* increased the length of shoot in concentration dependent manner i.e., in 10% *G.coronatum* by 45.08%; in 10% *G.etunicatum* by 57.25%; in 10% *G. coronatum+ G.etunicatum* by 65.16% after 45 days. Similarly, in 10% *G.coronatum* by 43.82%; in 10% *G.etunicatum* by 58.05%; in 10% *G. coronatum+ G.etunicatum* by 64.39% after 90 days stem length and the effect intensified with an increase in dual concentration. After 45 days of incubation the degree of increases in stem length was minimum in 10% *G.coronatum* (65.2cm) and maximum in 10% *G. coronatum+ G.etunicatum* (93.1cm) as against 42.9cm stem length in control. After 90 days of incubation the degree of increases in stem length was minimum in 10% *G.coronatum* (117.3cm) and maximum in 10% *G. coronatum+ G.etunicatum* (172.6cm) as against 75.6cm stem length in control. The two-way ANOVA analysis was performed and means comparison analysis was achieved using Bonferroni post test (P<0.05) Bonferroni post test analysis of variance showed significant

difference between treated and untreated plants after 45 and 90 days. There was significant difference between *G. coronatum, G. etunicatum, G. coronatum+ G. etunicatum* treated seedlings.

Root length in seedlings of *V. minor* was measured after 45 and 90 days of seed incubation (Fig. 4.3 (A)). The root growth was affected more than the shoot growth. The root length after 45 days of incubation increased with increasing AM dual combination i.e., by 70.60% in10% *G. coronatum*; by 77.88% in 10% *G. etunicatum*; by 91.6% in *G. coronatum+ G. etunicatum*. Similarlily, after 90 days of AM treatment incubation i.e. by 58% in 10% *G. coronatum*; by 68.08% in 10% *G.etunicatum*; by 88.67% *G. coronatum+ G. etunicatum* increases with dual AMF treatments. After 45 days of incubation the degree of increases in root length was minimum in 10% *G. coronatum* (20.0cm) and maximum in 10% *G. coronatum+ G. etunicatum* (22.0cm) as against 15.6cm stem length in control. After 90 days of incubation the degree of increases in root length was minimum in 10% *G.coronatum* (17.6cm) and maximum in 10%*G. coronatum+ G.etunicatum* (27.0cm) as against 15.6cm root length in control. The two-way ANOVA analysis was performed and means comparison analysis was achieved using Bonferroni post test ($P<0.05$) Bonferroni post test analysis of variance showed significant difference between treated and untreated plants. Increase in shoot growth/ shoot length due to AMF inoculation was noticed in the present study. Better results were found in the *Vinca minor* plants inoculated with combination of AMF i.e. *Glomus coronatum + Glomus etunicatum* (Fig. 4.3 (A)).

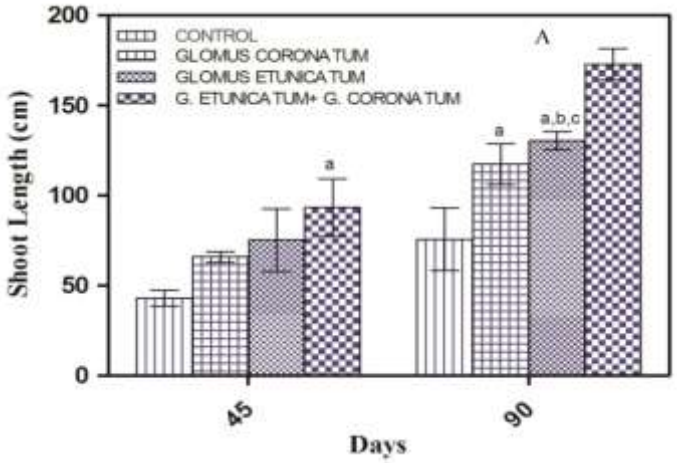

Figure 4.2: Effects of *G.coronatum, G.etunicatum, G. coronatum+ G.etunicatum* stem length (A), of *Vinca minor.*Values are mean±SE;N==3 p[a]p<0.05 Vs control, [b]p<0.05 Vs 10%*G.coronatum*[c]p<0.05 Vs 10%*G.etunicatum*, [d]p<0.05 Vs*G coronatum+G etunicatum*; analysed by Two-way ANOVA followed by Bonferroni's multiple comparison test.

Similiarly increase in the root length of plants inoculated with AMF in combination (*G. coronatum + G. etunicatum*) (Fig 4.3(A)).

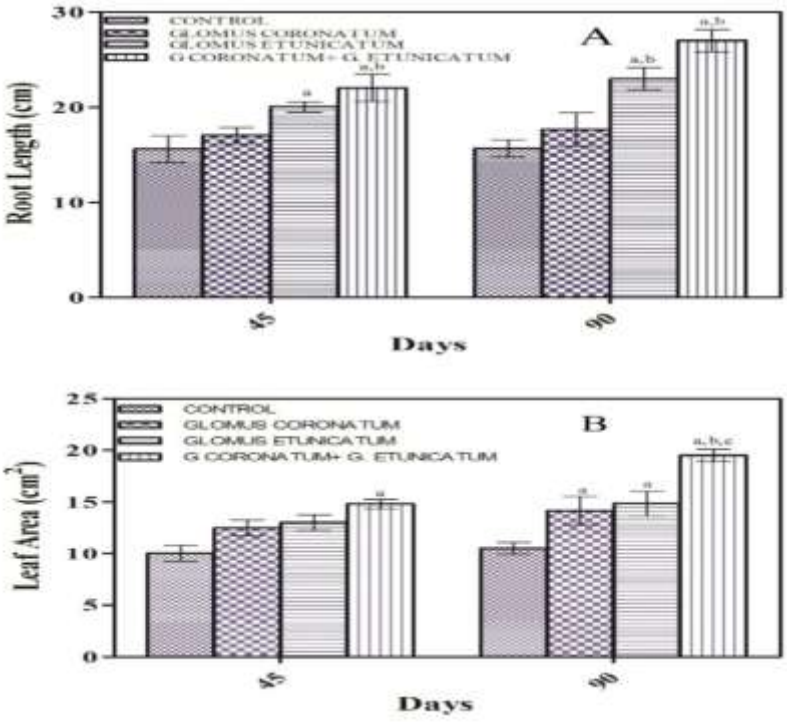

Figure 4.3: Effects of *G.coronatum, G.etunicatum, G. coronatum+ G.etunicatum* root length (A), leaf area (cm^2) (B) of*Vinca minor.* Values are mean±SE; N=3 p[a]p<0.05 Vs control, [b]p<0.05 Vs 10%*G.coronatum*[c]p<0.05 Vs 10%*G.etunicatum*, [d]p<0.05 Vs s*G coronatum+G etunicatum*; analysed by Two-way ANOVA followed by Bonferroni's multiple comparison test.

After 45 and 90 days, there was significant difference between *G.coronatum*, *G.etunicatum*, *G. coronatum+ G. etunicatum* treated seedlings. Leaf area under AM Fungi treatment *G. coronatum*, *G. etunicatum*, *G. coronatum+ G. etunicatum* was observed at an interval of 45 days i.e., 45 and 90 days after sowing of *V. minor* plant cuttings (Fig. 4.3 (B)). As expected, a progressive increase in leaf area was noted during different growth and till flowering stages leaf area after 45 days increased with dual AMF concentration i.e., by 70.94% in 10% *G. coronatum* (minimum increase); by 80% in 10% *G. etunicatum*; by 83% in 10% *G. coronatum+ G. etunicatum* (maximum increase). Leaf area after 90 days increased with dual AMF concentration i.e., by 53.84% in 10% *G.coronatum* (minimum increase); by 70% in 10% *G.etunicatum*; by 74.15% in 10% *G. coronatum+ G. etunicatum* (maximum increase). Two-way analysis of variance (ANOVA) and Bonferroni multiple comparison post test showed significant differences in leaf area after 45 and 90 days between treated and untreated plants There were no significant differences between control vs *G. coronatum* control vs *G. etunicatum* and control vs *G. coronatum+ G. etunicatum*

4.2.2 Stem diameter and Leaf number

Stem diameter under AM Fungi treatments *G. coronatum*, *G. etunicatum*, *G. coronatum+ G. etunicatum* was observed at an interval of 45 days i.e., 45 and 90 days after sowing of *V. minor* plant cuttings (Fig 4.4(A)). As expected,a progressive increase in stem diameter was noted during different growth and till flowering stages stem diameter after 45 days increased with dual AMF concentration i.e., by 31.57% in 10% *G.coronatum* (minimum increase); by 53.12% in 10% *G.etunicatum*; by 61.85% in 10% *G. coronatum+ G.etunicatum* (maximum increase) Leaf area after 90 days increased with dual AMF concentration i.e., by 33.70% in10% *G.coronatum* (minimum increase); by 46.61% in 10% *G.etunicatum*; by 63.47% in 10% *G. coronatum+ G.etunicatum* (maximum increase). Two-way analysis of variance (ANOVA) and Bonferroni multiple comparison post test showed significant differences in leaf area after 45 and 90 days between treated and untreated plants There were significant differences between control vs *G.coronatum* control vs *G.etunicatum* and control vs *G. coronatum+ G.etunicatum.*

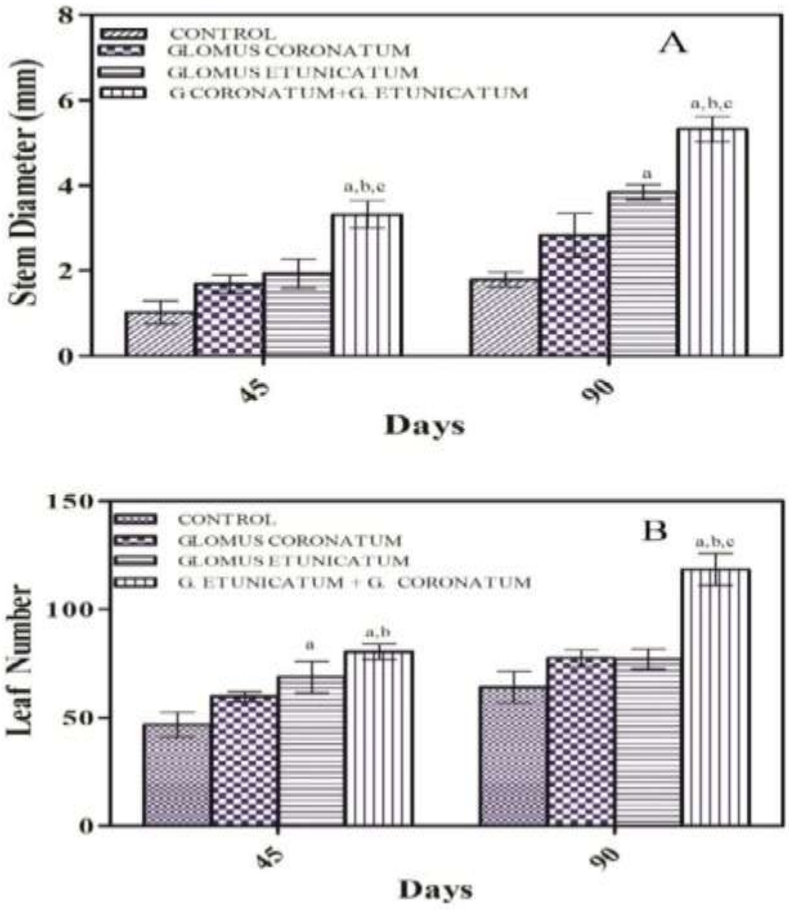

Figure 4.4: Effects of *G.coronatum, G.etunicatum, G. coronatum+ G.etunicatum* stem diameter (mm) (A), leaf number (B) of *Vinca minor*. Values are mean±SE; N=3 [a]p<0.05 Vs control, [b]p<0.05 Vs 10%*G.coronatum*[c]p<0.05 Vs 10%*G etunicatum*, [d]p<0.05 Vs*G coronatum+G etunicatum*; analysed by Two-way ANOVA followed by Bonferroni's multiple comparison test.

Leaf number under AM Fungi was observed at an interval of days i.e., 45 and 90 days after sowing of *V. minor* plant cuttings (Fig 4.4(B)). As expected, a progressive increase in stem diameter was noted during different growth and till flowering stages stem diameter after 45 days increased with dual AMF concentration i.e., by 58.08% in 10% *G. coronatum* (minimum increase); by 67.95% in 10% *G. etunicatum*; by 78.20% in 10% *G. coronatum+ G. etunicatum* (maximum increase) Leaf number after 90 daysincreased with dual AMF concentration i.e., by 54.08% in 10% *G.coronatum*(minimum increase); by 67% in 10% *G.etunicatum*; by 82.76% in 10% *G. coronatum+ G.etunicatum* (maximum increase). Two-way analysis of variance (ANOVA) and Bonferroni multiple comparison post test showed significant differences in leaf area after 45 and 90 days between treated and untreated plants.

There were significant differences between control vs *G. coronatum,* control vs *G.etunicatum* and control vs *G. coronatum+ G.etunicatum.* Growth of all parameters (root length, shoot length, leaf area, leaf number and stem diameter) shows positive effect when inoculated with AMF, but AMF inoculation in combination gave best results.

4.3 PHYSIOLOGICAL ASPECTS

4.3.1 Membrane Stability Index

Membrane stability index (MSI) of *Vinca minor* leaves was measured after 45 and 90 days of plant cutting incubation. All applied AM Fungi (*G.coronatum, G.etunicatum* and *G.coronatum+G.etunicatum*) increased membrane stability index (Fig 4.5(A)). Elevated *G.coronatum, G.etunicatum* and *G.coronatum+G.etunicatum* concentrations increased MSI in dependent manner. After 45 days, it increased by 45.34% in 10% *G.coronatum*; by 53.31% in 10% *G.etunicatum*; by 54.58% in 10% *G.coronatum+G.etunicatum*. After 90 days of AM Fungi treatment again it increased by 53.34% in 10% *G.coronatum*; by 72.46% in 10% *G.etunicatum*; by 76.58% in 10% *G.coronatum+G.etunicatum*. MSI was found to be minimum in plants treated with 10% *G.coronatum* and maximum in plants treated with AMF in combination (*G.coronatum+G.etunicatum*) after 45 and 90 days (fig 4.19(A)). Data analysis was done Two-way analysis of variance (ANOVA) and Bonferroni multiple comparison post test showed significant differences in MSI after 45 and 90 days between treated and untreated. It is concluded that AMF inoculation treatment had significant impact on the stability of membranes in the leaves of plant. After inoculation of AMF *Glomus coronatum, Glomus etunicatum and Glomus coronatum+ Glomus etunicatum* we observed physiological parameters after 45 and 90 days, membrane stability index of *Vinca minor* plant was increased in treatment of AMF in combination (*Glomus coronatum+ Glomus etunicatum*) (fig 4.5(A)).

4.3.2 Total Carbohydrate

Total carbohydrate content under AM Fungi (*G.coronatum, G.etunicatum* and *G.coronatum+G.etunicatum*) was observed after 45 and 90 days of *Vinca minor* (Fig. 4.5(B)). AM Fungi (*G.coronatum, G.etunicatum* and *G.coronatum+G.etunicatum* showed an increase in total carbohydrate content in appropriate manner. After 45 days, it increased by 26.13% in 10%*G.coronatum*; by 38.98% in 10% *G.etunicatum*; by 45.09% in 10% *G.coronatum+G.etunicatum*. After 90 days of AM Fungi treatment again it increased by 36.84% in 10% *G.coronatum*; by 59.44% in 10% *G.etunicatum*; by 62.57% in 10%

G.coronatum+G.etunicatum. Total carbohydrates content was found to be minimum in plants treated with 10% *G.coronatum* and maximum in plants treated with AMF in combination(*G.coronatum+G.etunicatum*) after 45 and 90 days (fig 4.5(B)). After 45 days of incubation the degree of increases in total carbohydrates was minimum in 10% *G.coronatum* (0.59mg/gFW) and maximum in 10% *G. coronatum+ G.etunicatum* (0.88mg/gFW) as against 0.23mg/gFW total carbohydrates in control. After 90 days of incubation the degree of increases in total carbohydrate was minimum in 10% *G.coronatum* (0.47mg/gFW) and maximum in 10% *G. coronatum+ G.etunicatum* (0.76mg/gFW) as against 0.28mg/gFW total carbohydrate in control. Data analysis was done. Two-way analysis of variance (ANOVA) and Bonferroni multiple comparison post test showed significant differences in total carbohydrates after 45 and 90 days between treated and untreated. It is concluded that AMF inoculation treatment had significant impact in the total carbohydrates of plant.

Morphological, nutritional and physiological changes induced in plants colonized with AMF devote to their enhanced resistance to abiotic stresses. In the same manner total carbohydrates content also increased in appropriate manner of *Vinca minor* plants inoculated with AMF other than untreated or control plants (fig 4.5(B).

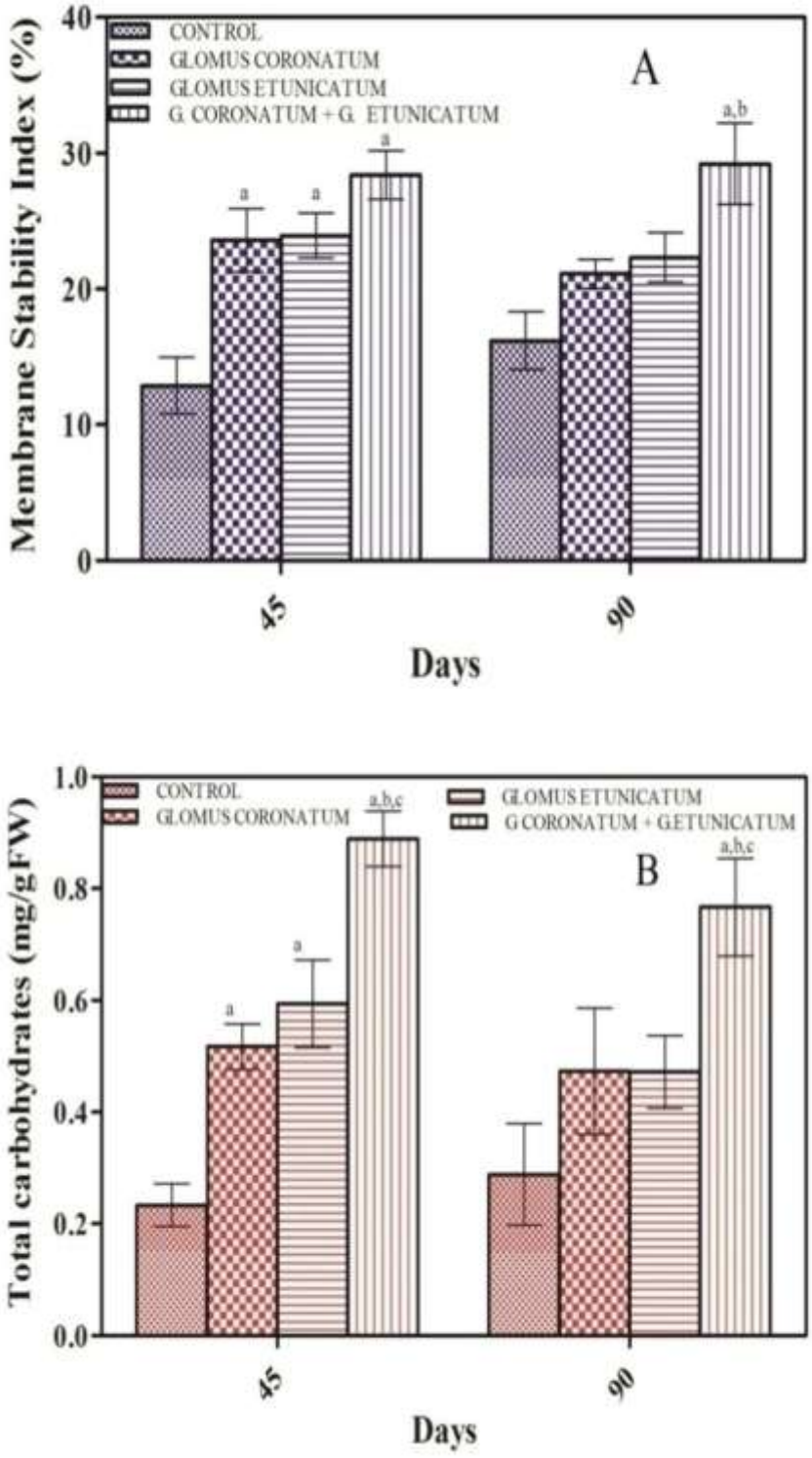

Figure 4.5: Effects of *G.coronatum*, *G.etunicatum*, *G. coronatum*+ *G.etunicatum*. Membrane stability index (A), Total carbohydrate (mg/gFW)(B), of *Vinca minor*. Values are mean±SE; N=3 [a]p<0.05 Vs control, [b]p<0.05 Vs 10% *G.coronatum*[c]p<0.05 Vs 10%*G etunicatum*, [d]p<0.05 Vs*G coronatum*+*G etunicatum*; analysed by Two-way ANOVA followed by Bonferroni's multiple comparison test

4.3.3 Total Amino Acids

Amino acid content of *V. minor* leaves was measured after 45 and 90 days after sowing. All AM Fungi applied (*G.coronatum, G.etunicatum* and *G.coronatum+G.etunicatum*) affected the amino acid content significantly (Fig. 4.6(A)). AM Fungi (*G.coronatum, G.etunicatum* and *G.coronatum+G.etunicatum* showed an increase in total aminoacid content in appropriate manner.After 45 days, it was increased by 37.09% in 10% *G.coronatum*; by 41.81% in 10% *G.etunicatum*; by 56.08% in 10% *G.coronatum+G.etunicatum*. After 90 days of AM Fungi treatment again it increased by 23.80% in 10% *G.coronatum*; by 41.17% in 10% *G.etunicatum*; by 68.29% in 10% *G.coronatum+G.etunicatum*. Total amino acid content was found to be minimum in plants treated with 10% *G.coronatum* and maximum in plants treated with AMF in combination (*G.coronatum+G.etunicatum*) after 45 and 90 days. After 45 days of incubation the degree of increases in total amino acid was minimum in 10% *G.coronatum* (0.041mg/gFW) and maximum in 10% *G. coronatum+ G.etunicatum* (0.062mg/gFW) as against (0.023mg/gFW) total amino acid in control. After 90 days of incubation the degree of increases in total carbohydrate was minimum in 10% *G.coronatum* (0.046mg/gFW) and maximum in 10% *G. coronatum+ G.etunicatum* (0.084mg/gFW) as against 0.028mg/gFW total amino acid in control. Data analysis was done by Two-way analysis of variance (ANOVA) and Bonferroni multiple comparison post test showed significant differences in total aminoacid after 45 and 90 days between treated and untreated. It is concluded that AMF inoculation treatment had significant impact in the total aminoacid of plant.

4.3.4 Protein

Protein content of *V. minor* leaves was measured after 45 and 90 days after sowing. All AM Fungi applied (*G.coronatum, G.etunicatum* and *G.coronatum+G.etunicatum*) affected the protein content significantly (Fig. 4.6(B)). AMFungi (*G.coronatum, G.etunicatum* and *G.coronatum+G.etunicatum* showed an increase in total protein content in appropriate manner. After 45 day It was increased by 46.66% in 10% *G.coronatum*; by 56% in 10% *G.etunicatum*; by 70% in 10% *G.coronatum+G.etunicatum*. After 90 days of AM Fungi treatment again it increased by 45% in 10% *G.coronatum*; by 55% in 10% *G.etunicatum*; by 75% in10% *G.coronatum+G.etunicatum*. Total protein content was found to be minimum in plants treated with 10% *G.coronatum* and maximum in plants treated with AMF in combination (*G.coronatum+G.etunicatum*) after 45 and 90 days. In case of total amino acids and protein content, we again concluded that maximum aminoacids and protein were found in

the treatment in combination and other treatments also gave satisfactorily results as compared to control or untreated plant of *Vinca minor* (fig 4.6(A)(B)).

Fig 4.6 (B). After 45 days of incubation the degree of increases in total protein was minimum in 10% *G.coronatum* (0.45mg/gFW) and maximum in 10% *G.coronatum*+ *G.etunicatum* (0.63mg/gFW) as against 0.35mg/gFW total protein in control. After 90 days of incubation the degree of increases in total protein was minimum in 10% *G.coronatum* (0.47mg/gFW) and maximum in 10% *G. coronatum*+ *G.etunicatum* (0.75mg/gFW) as against 0.33mg/gFW total protein in control.

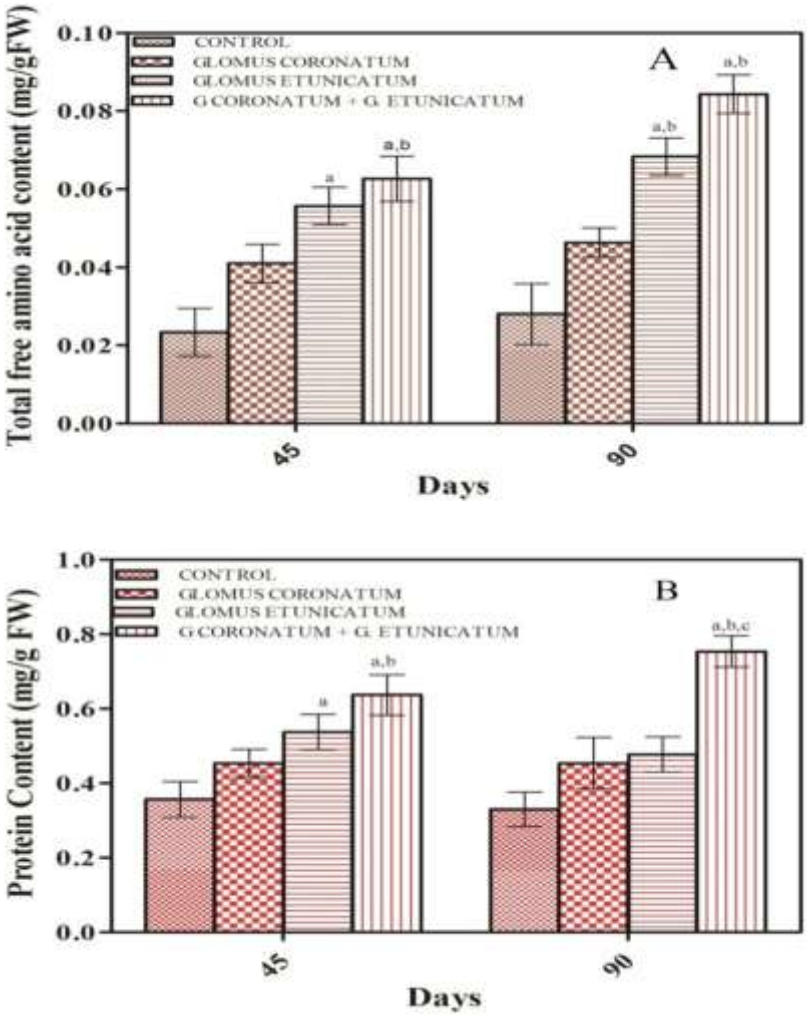

Figure 4.6: Effects of *G.coronatum, G.etunicatum, G.coronatum*+ *G. etunicatum* Total Aminoacids (A), Protein (mg/gFW) (B), of *Vinca minor.* Values are mean±SE; N=3 [a]p<0.05 Vs control, [b]p<0.05 Vs 10% *G.coronatum*[c]p<0.05 Vs 10%*G etunicatum*, [d]p<0.05 Vs *G coronatum*+*G etunicatum*; analysed by Two-way ANOVA followed by Bonferroni's multiple comparison test

Data analysis was done by Two-way analysis of variance (ANOVA) and Bonferroni multiple comparison post test showed significant differences in total protein after 45 and 90 days between treated and untreated. It is concluded that AMF inoculation treatment had significant impact in the total protein of plant.

(a) Mass culture of AMFungi (*Glomus coronatum*)

(b) Mass culture of AMF (*Glomus etunicatum*)

Figure 4.7: Mass production of *G. coronatum* and *G. etunicatum* in plastic pots.

Figure 4.8: Plants after 45 days of AM Fungi treatment ((A) Control, (B) *G.coronatum*, (C) *G. etunicatum*, (D) *G. Coronatum*+*G. etunicatum*).

Figure 4.9: Plants after 90 days of AM Fungi treatment ((A) Control, (B) *G.coronatum*, (C) *G. etunicatum*, (D) *G. Coronatum*+*G. etunicatum*).

DISCUSSION

During cool and dry weather, root colonization was low, but sporulation was relatively high; the opposite occurred at the warm season, with more root colonization and fewer spores. It has been reported that root colonization ordinarily increases until 35˚c (Bowen, 1987). The relative abundance and function of AMF are influenced by the variation of seasons and temperatures (Bell *et al*., 2009) and the effect of elevated temperature on the species richness of AMF in soil can be positive (Liu *et al*., 2012) or have no effect on AMF species composition (Heinemeyer *et al*., 2003). Global climate change induced by human activities influences the development and species composition of AMF. Although root colonization and sporulation seems to be correlated at least in some cases, with plant phenology and physiology (Escudero and Mendonza, 2005). The patterns and timing of AMF development may depend on the edaphic conditions (He *et al*., 2002; Morammad *et al*., 2003) or climatic conditions (Saito and Kato, 1994; Carvalho *et al*., 2001; Muthukumar and Udaiyan, 2002). The development and seasonal fluctuations in AMF has been studied in several plant species

or communities (Merryweather and Fitter, 1998; He *et al*., 2002; Muthukumar and Udaiyan, 2002; Morammad *et al*., 2003).

It was proved that AMF inoculation in combinations significantly improved the shoot length of plants. Arbuscular mycorrhizal fungi symbiotic association have been reported on the growth of plants through enhanced uptake of macro and micronutrient, improved plant resistance against biotic and abiotic stress and valuable alternations of beneficial plant growth regulators (Watts-Williams *et al*., 2014). AMF on vegetative propagation through stem cutting have previously been reported to produce significantly higher rooting and growth parameters than untreated cuttings which acted as a control (Druege *et al*., 2006; Wang *et al*., 2008; Oseni *et al*., 2010). The beneficial effect of inoculation at initial stage of plant development is that it can promote AMF symbiosis resulting to increase plant growth in the nursery and improving performance after planting in the field (Kung'u *et al*., 2008; Wang *et al*., 2008). The improvement of plant growth in AM inoculated plants than in non-inoculated plant. The extraradical phase of the fungi acts as an extension of the root system for the uptake of mineral nutrients, especially immobile nutrients such as P, Cu and Zn. There is considerable evidence that AMF can improve plant growth and nutrition in soils subject to a range of saline soils (Tian *et al*., 2004; Ruiz-Lozano *et al*., 2011; Mbadi *et al*., 2015). Greater root system architechture in AM plants will keep a good contact with soils in favour of better uptake of water and nutrient. Inoculation with AM fungi causes plants to produce coarser root systems, which could be a coordinated response that facilitates a shift from root-driven to mycorrhizae-driven nutrient uptake (Zangaro *et al*., 2007). Arbuscular mycorrhizal fungi (AMF), a kind of soil inhabitant microorganism, can form a mutual symbiont with the roots of 80% of land plants, thus enhancing the uptake of mineral nutrition and water from the soilto the host plant, as well as withstanding various adverseties (Koide and Mosse, 2004). Studies showed that inoculation with AMF could alter the RSM (root system morphology) traits and promote the formation of lateral roots of higher order for the host plant (Yao *et al*., 2009). With increase in shoot length there is a increase in root length by receiving appropriate P and nutrients from the soils through proper root transport system respectively.

Figure 4.3 (B) and 4.4(B) shows increase in leaf area and leaf number due to inoculation by AMF alleviated the detrimental effect of salt stress on photosynthesis on *Vinca minor* plants and it is thought to occur via improved water supply (Sheng *et al*., 2008) and balanced ionic status (Evelin *et al*., 2009). Higher the photosynthesis, particularly under moderate salt

stress.And this may be involved with hormonal and sugar regulation. Higher IAA, ABA and ZR content in mycorrhizal plant leaves might promote photosynthesis due to enhancement in the anabolic processes (especially photosynthesis) as a result of better uptake and mobilization of various essential nutrients and water by AM Fungi, there is increase in leaf number and leaf area by inoculation of AMF. Due to improved osmotic adjustment, the tissue water status was found to be higher in fungal associated plants and therefore, these plants showed greater membrane stability under stress leading to reduced electrolytes leakage. This improvement in membrane stability has also been attributed to P induced changes in membrane phospholipids levels and also in permeability properties (Evelin *et al*., 2011). AMF act as bio-ameliorators of stress and help plants in mollifying stress induced damage (Wu *et al.,* 2014; Hameed *et al.,* 2014). Plants colonized with AMF have been reported to show enhanced growth and vigour (Ahanger *et al.,* 2015; Abd-Allah *et al.,* 2015a).

AMF inoculation also enhanced the total soluble sugar content providing further powerhouse to plant cells in maintaining the turgor dependent processes. AMF triggered accumulation of total soluble sugars in our present study are in confirmation with the results of Mahboobeh and Akbar (2013) for *Nicotiana plumbaginifolia* and Masood *et al.* (2013) for mustard. Plant species that have the ability to accumulate the exopolysaccharides play an important role in plant stress tolerance (Kawakam *et al.,* 2008; Ahanger *et al.,* 2015). Increased accumulation of carbohydrates, prolineand other osmolytes impart tolerance to higher salt concentrations leading to protection of the major metabolic pathways (Hoseini, 2010).

Arbuscular mycorrhizal fungi stimulated the maximum production of protein in experimental plants. Krishna and Bagyaraj, (1983) and Gabriel and Lakshman, (2015) have recorded increased levels of protein in the *Glomus coronatum* and *Glomus etunicatum* inoculated plants of *Niger* could be attributed to either the presence of fungal proteins or post infectional stimulation of protein synthesis in the host plant which supports our results. The biochemical contents such as protein and total carbohydrates content were increased with the advancement age of the plant (Channabasava *et al.*, 2014, Bheemereddy and Lakshman, 2015; Chaitra *et al.,* 2016). Increase in aminoacid contents was observed in roots and leaves of AM fungi inoculated *Vinca minor* plant. Content of arginine and proline was considerably high. These aminoacids are known to play an important role in providing resistance to the mycorrhizated plant against pathogens.

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