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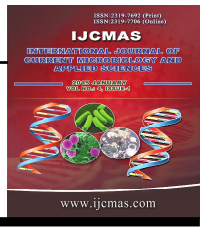

Original Research Article

Influence of Arbuscular mycorrhizal fungi on chlorophyll, proteins, proline and total carbohydrates content of the pea plant under water stress condition

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Keywords

Water stress, AM fungi, chlorophyll, protein, proline, total carbohydrates, pea

A B S T R A C T

This study was conducted to determine the effects of arbuscular mycorrhizal (AM) fungi on chlorophyll, proteins, proline and total carbohydrate contents in the leaves and seeds of pea plant under drought stress. An experiment was carried out where seeds of Pea (*Pisum sativum*) were sown in the pots with and without mycorrhiza. The pots were placed under shade net and watered with normal water for one month at an interval of 4 days. The water stress treatment was started after one month at an interval of 4, 8 and 12 days for next one month. The estimation of chlorophyll, proteins, proline and total carbohydrates from the control and mycorrhizal plants was carried out each time at the interval of 15 days for next one month. The amount of chlorophyll and proteins has been decreased significantly in the leaves with the increase in water stress. The amount of proline and total carbohydrates has been increased significantly in the leaves and seeds with the increase in water stress. However, these contents were more in mycorrhizal plants as compared to control plants. It was evident that mycorrhiza helped the plants during water stress conditions. The mixture of AM fungi used for current experiment included the species of *Acaulospora denticulata, A. gerdemannii, Glomus macrocarpum, G. maculosum, G. fasciculatum* and *Scutellospora minuta*.

Introduction

Pea (*Pisum sativum* L.), is a cool season food legume, has a wide variety of uses and is grown in India and other countries of the Mediterranean region as a cheap source of protein. Pea has high levels of the amino acids, lysine and tryptophan which are low in cereal grains and grain protein in pea can range from 19 to 27 percent but is most commonly 22 to 24 percent. Pea also contains high levels of carbohydrates and is low in fibre and contains 86 to 87 percent total digestible nutrients (Miller *et al.*, 2005).

Drought is undoubtedly one of the most important environmental stresses limiting the productivity of crop plants around the world (Bohnert *et al*., 1995). Drought stress decreases the rate of photosynthesis (Kawamitsu *et al.*, 2000). Plants grown under drought condition have a lower stomatal conductance in order to conserve water. Consequently, CO_2 fixation is reduced and photosynthetic rate decreases, resulting in less assimilate production for growth and yield of plants. Diffusive

resistance of the stomata to CO_2 entry probably is the main factor limiting photosynthesis under drought (Boyer, 1970). The mycorrhizal roots enable plants to obtain more moisture from the surrounding soil than non-mycorrhizal plants. The AM fungi can enhance resistance to drought stress in host plant may include improving the properties of soil in rhizosphere, enlarges root areas of host plants, and improves its efficiency of water absorption, enhances the absorption of phosphorus and other nutritional elements and then improves nutritional status of host plant, activates defence system of host plant quickly, protects against oxidative damage generated by drought. Ommen *et al*., (1999) reported that leaf chlorophyll content decreases as a result of drought stress. Drought stress caused a large decline in the chlorophyll *a* content, the chlorophyll *b* content, and the total chlorophylls content in all sunflower varieties investigated (Manivannan *et al*., 2007). The decrease in chlorophyll under drought stress is mainly the result of damage to chloroplasts caused by active oxygen species (Smirnoff, 1995). Abbaspour *et al*., (2012) found that AM colonization improved the drought tolerance of *Pistacia vera* seedlings by increasing the accumulation of osmotic adjustment compounds, nutritional and antioxidant enzyme activity. It appears that AM formation enhanced the drought tolerance of Pistachio plants, which increased host biomass and plant growth.

Lenin *et al*., (2010) studied the growth and biochemical changes of vegetable seedlings induced by arbuscular mycorrhizal fungus. They have made an attempt to examine the effect of AM fungi on the four vegetable crops such as Tomato (*Lycopersicum esculentum* L.), Brinjal (*Solanum melongena* L.), Chilli (*Capsicum annuum L*.) and Bhendhi (*Abelmoschus esculentus* Moench.). The maximum increase in four plant's morphological parameters like root length, shoot length, fresh weight, dry weight, number of leaves, total leaf area (Patale and Shinde, 2014) and biochemical parameters like chlorophylls, proteins and nutrient content of nitrogen, phosphorus and potassium were observed in AM fungi treated seedlings as compared to non-mycorrhizal seedlings (control). The sugar and starch contents showed decrease in mycorrhizal seedlings than control plants.

Plants can partly protect themselves against mild drought stress by accumulating osmolytes. Proline is one of the most common compatible osmolytes in drought stressed plants. Proline content has increased under drought stress in pea (Sanchez *et al*., 1998; Alexieva *et al*., 2001). Proline accumulation can also be observed with other stresses such as high temperature and under starvation (Sairam *et al*., 2002). Proline metabolism in plants, however, has mainly been studied in response to osmotic stress (Verbruggen and Hermans 2008). Proline does not interfere with normal biochemical reactions but allows the plants to survive under stress (Stewart, 1981). The accumulation of proline in plant tissues is also a clear marker for environmental stress, particularly in plants under drought stress (Routley, 1966). Proline accumulation may also be part of the stress signal influencing adaptive responses (Maggio *et al*., 2002).

Drought increased the peroxidase and superoxide dismutase activities in both shoots of *Juniperus oxycedrus* seedlings inoculated with exotic AM fungi and grown with composted sewage sludge, but the increase was less than in the plants neither inoculated nor treated with sewage sludge. Both the plants inoculated with exotic AM fungi and the plants grown with composted sewage sludge developed additional

mechanisms to avoid oxidative damage produced under water-shortage conditions (Alguacil, 2006). Accumulation of sugars in different parts of plants was enhanced in response to a variety of environmental stresses (Wang *et al*., 1996; Prado *et al*., 2000; Gill *et al*., 2001). In case of water stress (Prado *et al*., 2000; Siddique *et al*., 2000), adaptation to these stresses has been attributed to the stress-induced increase in carbohydrate levels. Sugars also facilitates vitrification (a phenomenon in which intracellular water hardens like glass with no ice crystal formation during freezing or chilling stress) and thus avoids the damage to cells caused by crystallization as water is withdrawn (Williams and Leopold, 1989).

The objective of this work is to investigate the effect of AM fungi on the chlorophyll, protein, proline and total carbohydrates in the leaves and seeds of pea plant at an interval of 4, 8 and 12 days under water stress.

Materials and Methods

Experimental Set Up: A study was conducted to determine the effect of arbuscular mycorrhizal (AM) fungi inoculation on the biochemical contents of pea grown under water stressed pot culture conditions. Water stress treatment was given at the Fergusson College botanical garden. In this experiment, seeds of Pea (*Pisum sativum*) were sown in the pots with and without mycorrhiza. Fifteen replicates of both control and mycorrhizal plants were maintained during present investigation. These plants were watered with normal water for one month at an interval of 4 days. The mixture of AM fungi used for current experiment included the species of *Acaulospora denticulata, A. gerdemannii, Glomus macrocarpum, G. maculosum, G. fasciculatum* and *Scutellospora minuta*. The number of AM propagules per 100 gm soil was 260. Ten gram of mycorrhizal soil was added in the pots at the time of sowing of seeds in mycorrhizal set. The AM fungi have been shown to help in retaining moisture of soil and also help in uptake of important nutrients during stress conditions (Heikham *et al.,* 2009). The water stress treatment was started after one month old pea seedlings at an interval of 4, 8 and 12 days for next one and a half month. Every time biochemical analysis was done at an interval of 15 days. The different parameters studied in mycorrhizal and non-mycorrhizal plants include biochemical analysis of chlorophyll, proteins, proline and total carbohydrates.

Chlorophyll was extracted from the leaves of mycorrhizal and non mycorrhizal plants by Arnon's method (1949). Fresh leaves of mycorrhizal and non mycorrhizal pea plants were plucked and one gram samples were weighed. The leaves were crushed in 20 ml 80% chilled acetone using chilled mortar and pestle. The slurry was centrifuged at 5000rpm for five minutes. The supernatant was collected and the residue was again homogenized with 80% acetone and centrifuged. This was continued till the residue lost all its green pigment and finally turned white. The supernatants were collected and the final volume was made up to 100ml by using 80% acetone. The solvent (80% acetone) was used as a blank and the absorbance of the samples was read at 645 and 663nm using UV- visible spectrophotometer.

The protein content of leaves and seeds was estimated by Lowry *et al*., (1951) method. The mycorrhizal and non mycorrhizal samples were washed and 0.5g of each sample was crushed in 5ml 0.1M phosphate buffer (pH-7). The contents were centrifuged at 5000rpm for 2 minutes. The

supernatants were used as the source of protein. The stock solution was prepared by dissolving 50mg BSA (Bovine Serum Albumin) in 50ml distilled water. Working standard was prepared by diluting 10ml stock to 50ml by distilled water (200µg/ml). This working standard was taken in a series of test tubes and final volume was adjusted to 1ml with distilled water. For protein estimation, 0.1 and 0.2ml of sample was used and the total volume of these test tubes was adjusted to 1ml with distilled water. To all the test tubes, 5ml of reagent C was added including the blank and the test tubes were allowed to stand for 10 minutes after gentle mixing. Then 0.5ml of diluted Folin-Ciocalteau reagent was added to all the test tubes and all the test tubes were incubated for 30 minutes in dark.

Total carbohydrates in the leaves and seeds were determined by phenol sulphuric acid method proposed by Krishnaveni *et al.*, (1984). For the estimation, 100mg of tissue of mycorrhizal and non mycorrhizal plants was weighed. The tissue was hydrolyzed by adding 5 ml 2.5N HCl and boiling in hot water bath for three hours. After cooling, it was neutralized using solid sodium carbonate until effervescence ceases. The final volume was made to 100 ml and centrifuged. From this sample, 0.1 and 0.2 ml was pipette out in two separate test tubes. The volume of the test tube was made to 1 ml by using distilled water. To the test tubes, 1 ml phenol and 5 ml 96% H_2SO_4 was added. The tubes were kept for 10 minutes and shaken well. These test tubes were kept in hot water bath at 20-30^{0}C for 20 minutes. The absorbance was taken at 490nm after cooling by using the mixture of 1 ml water, 1 ml phenol and 96% H_2SO_4.

Assessments of proline content were performed thrice during the experimental period, at 4, 8 and 12 days interval after the completion of one month. Proline was extracted from 0.5g fresh leaf material samples in 3% (w/v) aqueous sulphosalycylic acid and estimated using the ninhydrin reagent according to the method of Bates *et al*., (1973). The absorbance of fraction with toluene aspired from liquid phase was read at a wave length of 520 nm. Proline concentration was determined using a calibration curve and expressed as µ mol proline g-1 FW.

Result and Discussion

The effect of AM fungi on the chlorophyll content in leaves of pea plant was examined under water stress condition. The water stress treatment was given to one month old seedlings at an interval of 4, 8 and 12 days. The chlorophyll a and chlorophyll b contents respond differently to the drought. Drought stress imposed at the plant significantly decreased chlorophyll *a* content, chlorophyll *b* content and total chlorophylls both at the vegetative and flowering stages. At the interval of 4 days, total amount of chlorophyll was more but it started decreasing with increase in water stress interval. The mycorrhizal plants showed higher amount of chlorophylls than control plants in all the three treatments.

Among these three treatments, plants treated with 4 days interval showed maximum amount of chlorophyll a (0.50 mg/g), chlorophyll b (0.28 mg/g) and total chlorophyll (0.7899mg/g) in control and in mycorrhizal plants, chlorophyll a was 0.51 mg/g, chlorophyll b was 0.29 mg/g and total chlorophyll was 0.8133mg/g. The chlorophyll content was recorded minimum at the interval of 12 days, chlorophyll a was 0.46 mg/g, chlorophyll b was 0.25mg/g and total chlorophyll was 0.7166 mg/g in control and in mycorrhizal plants, chlorophyll a was 0.48 mg/g, chlorophyll b was 0.26 mg/g and

total chlorophyll was 0.7436 mg/g (Chart 1). The plants treated with 8 days interval showed intermediate results. The restricted water supply during the entire growth phase of the plant had a mild effect on these contents.

Bhosale and Shinde (2011a) reported similar results in *Zingiber officinale* under water stress condition. Shinde and Khanna (2014) recorded higher amount of chlorophyll pigments in mycorrhizal potato plants as compared to non mycorrhizal potato plants. Our results are in agreement with Nyachiro *et al*., (2001), who described a significant decrease of chlorophyll *a* and *b* caused by water deficit in six *Triticum aestivum* cultivars. The results of present studies also corroborate with findings of Mafakheri *et al.*, (2010). According to them drought stress imposed during vegetative growth or anthesis significantly decreased chlorophyll a, chlorophyll b and total chlorophyll content in three varieties of chickpea. Decreased or unchanged chlorophyll level during drought stress has been reported in other species, depending on the duration and severity of drought (Kpyoarissis *et al*., 1995). A decrease of total chlorophyll with drought stress implies a lowered capacity for light harvesting. Since the production of reactive oxygen species is mainly driven by excess energy absorption in the photosynthetic apparatus, this might be avoided by degrading the absorbing pigments (Herbinger *et al.*, 2002).

The increase in the water stress causes a decrease in the amount of protein in the leaves and seeds of pea. The more amounts of proteins were present in the leaves of pea plants during 4 days water stress interval. At the interval of 4 days, the total amount of protein recorded was high in leaves (2.46 mg/g in control and 2.50 mg/g in mycorrhizal) and seeds (20.6mg/g in control and 34.2 mg/g in mycorrhizal) and with the increase in water stress interval it has been decreased significantly in leaves (2.34mg/g in control and 2.37mg/g in mycorrhizal) and seeds (15.5 mg/g in control and 29.2 mg/g in mycorrhizal) at the interval of 12 days (Chart 2 and 3). It was decreased with the increase in water stress interval. The least amount of protein was present in the plants with the water stress interval of 12 days. The plants treated with 8 days interval showed intermediate results.

Proteins were suggested to have important roles during stress as osmotic adjustment and available sources of carbon and nitrogen. In the present study drought treatments resulted in reduction of the total protein content. Similar findings were observed by Misra and Gupta (2006), Bhosale and Shinde (2011b) and Osman *et al*., (2007). Our results indicated that there was a general decreasing trend in total seed protein of the pea plant due to water deficit which is in agreement with findings of Ashraf and Iram (2005) who reported that in chickpea (*Cicer arietinum*) amino acid content increased under drought conditions apparently due to hydrolysis of proteins. According to Fresneau *et al.*, (2007), drought induces changes in a number of physiological and biochemical processes including inhibition of protein synthesis in the plants.

The amount of proline has been increased with the increase in water stress interval. During 4 days water interval, the amount of proline was less in leaves (0.90 mg/g in control and 0.86 mg/g in mycorrhizal) and seeds (0.75 mg/g in control and 0.76 mg/g in mycorrhizal). At the interval of 12 days, the amount of proline was quite high in leaves (1.96 mg/g in control and 0.92 mg/g in mycorrhizal) and seeds (1.89 mg/g in control and 1.52 mg/g in mycorrhizal). The plants treated with 8 days interval showed intermediate results (Chart4 and 5).

Chart.1 Effect of AM fungi on the chlorophyll content of pea plants at the interval of 4, 8 and 12 days

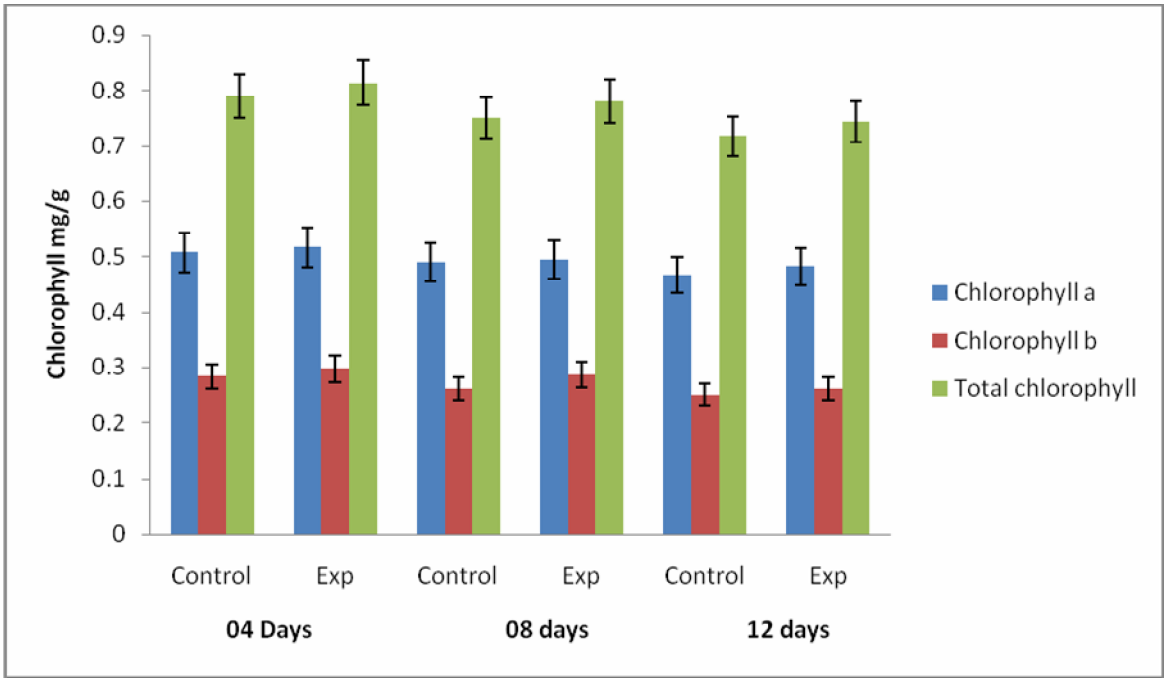


Chart.2 Effect of AM fungi on the protein content of leaves of *Pisum sativum* During water stress

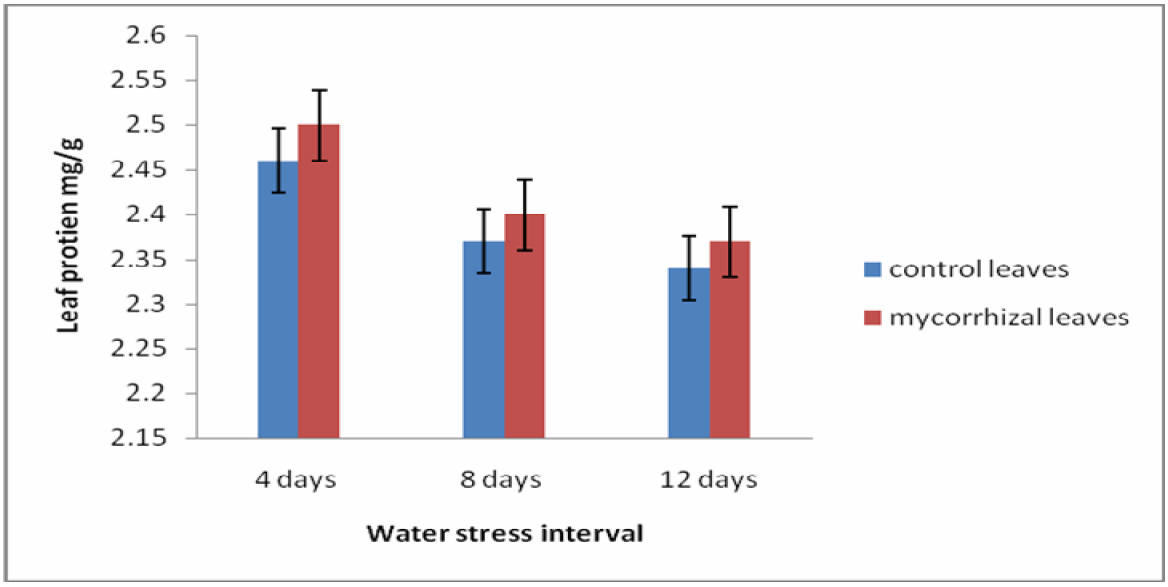


Chart.3 Effect of AM fungi on the protein content in seeds of pea at the interval of 4, 8 and 12 days water stress interval

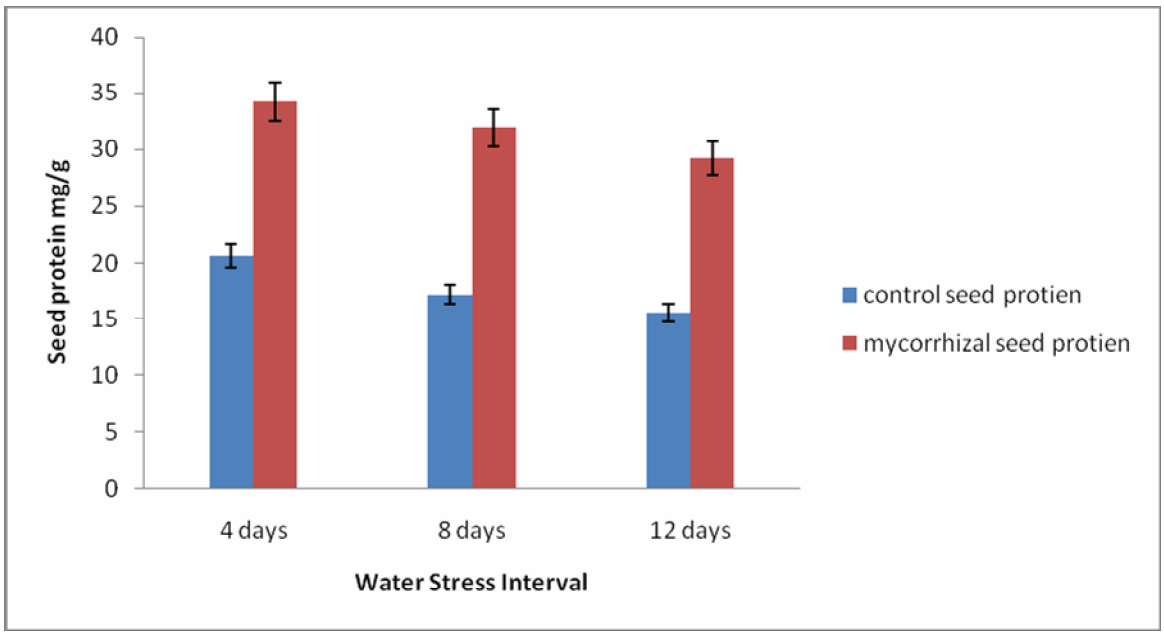


Chart.4 Effect of AM fungi on the proline content in leaves of pea at the interval of 4, 8 and 12 days water stress interval

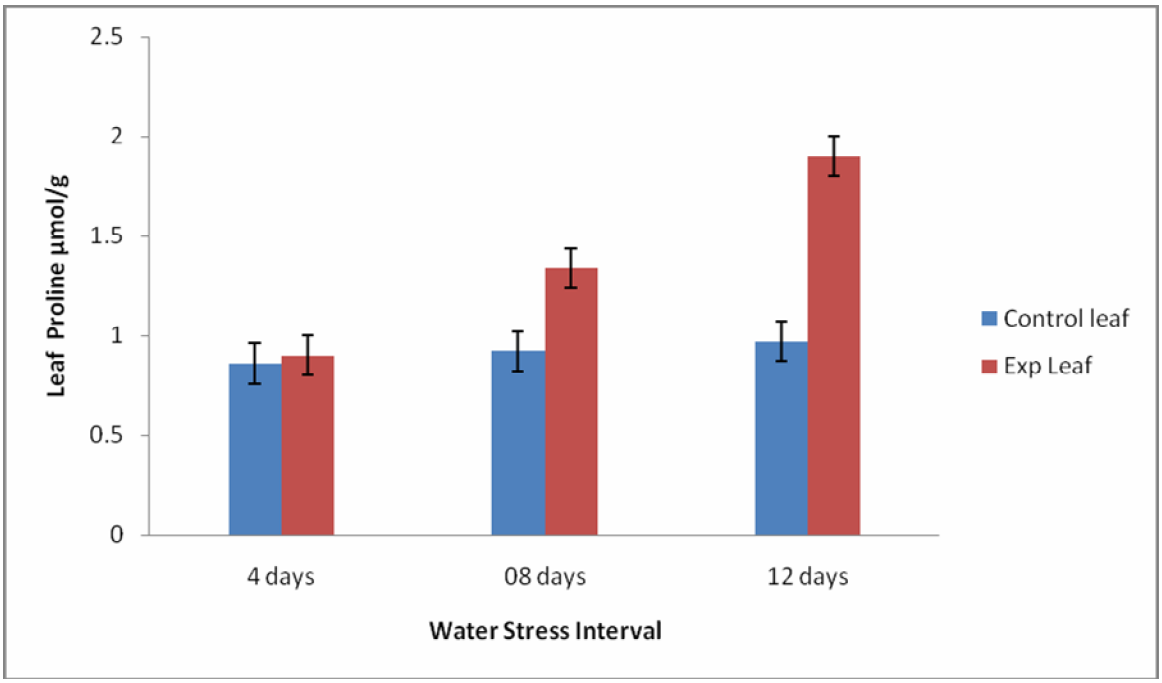


Chart.5 Effect of AM fungi on proline content in seeds of pea at the interval of 4, 8 and 12 days water stress interval

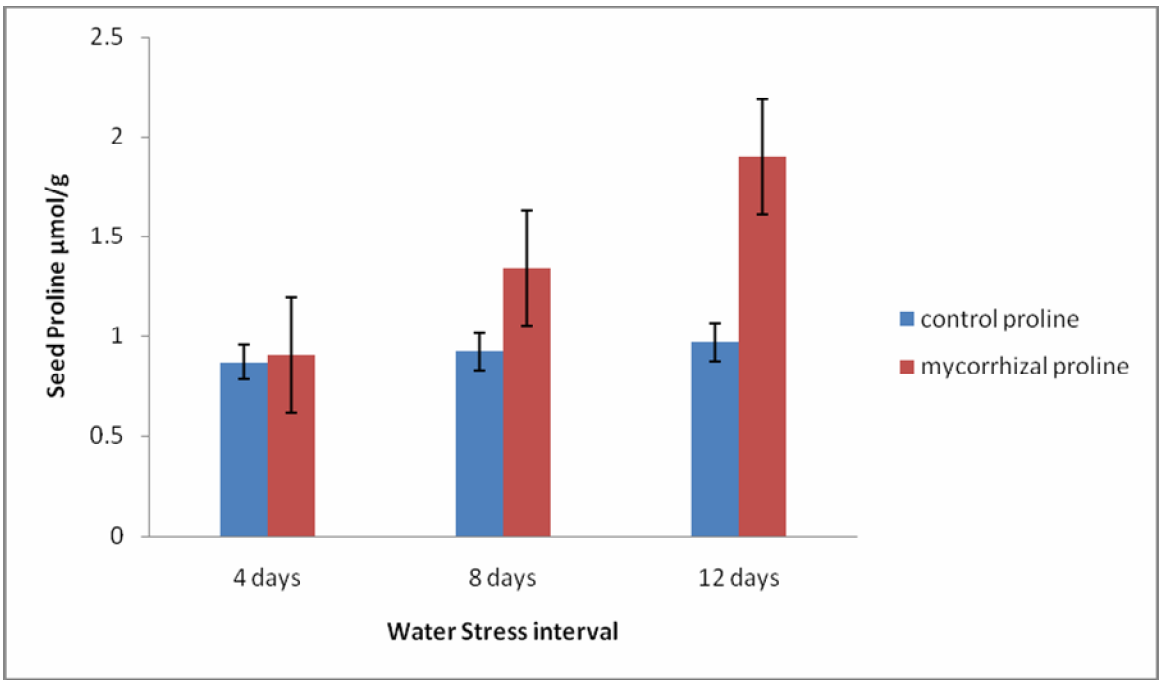


Chart.6 Effect of water stress on the total carbohydrates in leaves of pea at the interval of 4, 8 and 12 days

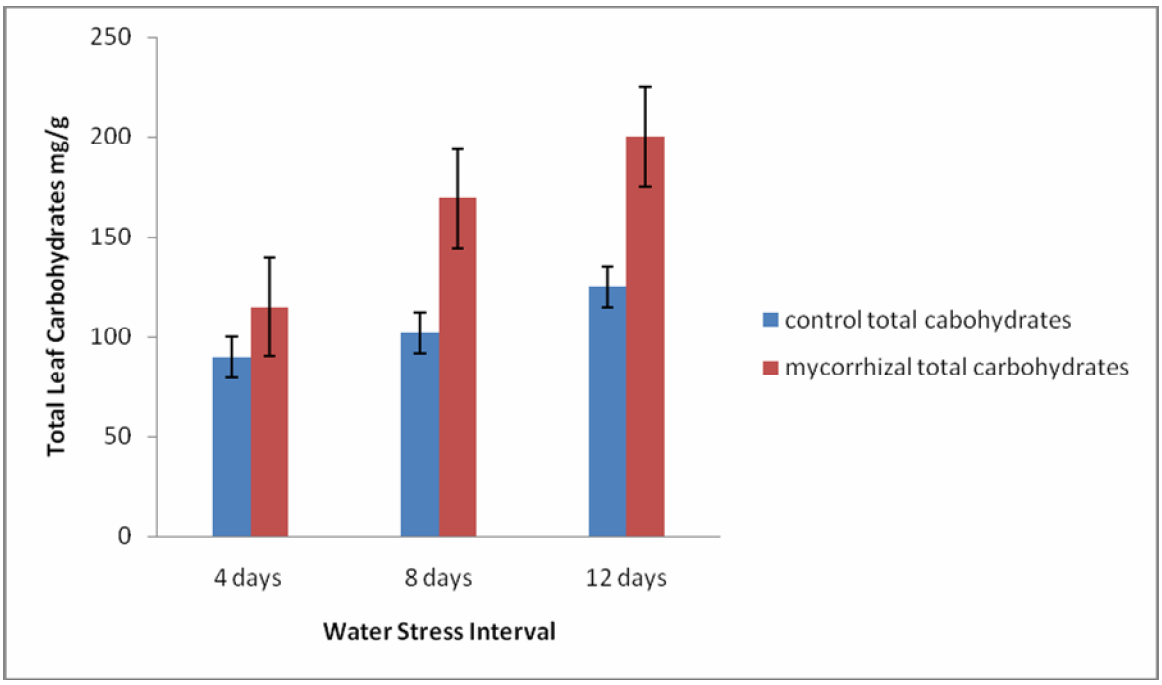
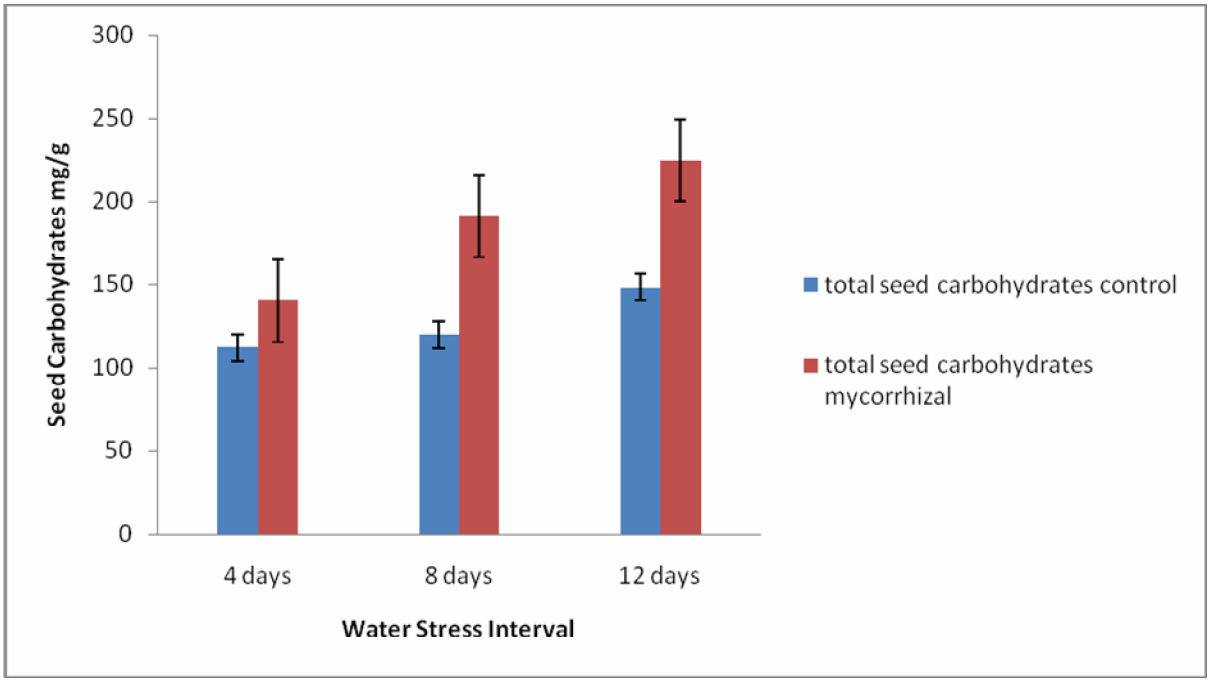


Chart.7 Effect of AM fungi on the total carbohydrates in seed of pea at the interval of 4, 8 and 12 days



These findings are in accordance with the results of Aranjuelo *et al*., (2011) and Bhosale and Shinde (2011a) who found that water stressed plants could invest a large quantity of carbon and nitrogen resources into the synthesis of osmoregulants in the leaves such as proline for maintaining cell turgor. However, the control plants showed comparatively less amount of proline as compared to mycorrhizal plants. Plants can partly protect themselves against mild drought stress by accumulating osmolytes. Proline accumulation can also be observed with other stresses such as high temperature and under starvation (Sairam *et al.*, 2002). Proline metabolism in plants, however, has mainly been studied in response to osmotic stress (Verbruggen and Hermans 2008). Proline is one of the most common compatible osmolytes in drought stressed plants. The proline content increased under drought stress in pea (Sanchez *et al.*, 1998; Alexieva *et al*., 2001). Proline does not interfere with normal biochemical reactions but allows the plants to survive under stress (Stewart, 1981). The accumulation of proline in plant tissues is also a clear marker for environmental stress, particularly in plants under drought stress (Routley, 1966). Proline accumulation may also be part of the stress signal influencing adaptive responses (Maggio *et al*., 2002).

Water stress has increased the total carbohydrates of pea plant. At the interval of 4 days, the carbohydrate content was less in leaves (90 mg/g in control and 115 mg/g in mycorrhizal) and seeds (112 mg/g in control and 140 mg/g in mycorrhizal) and at the interval of 8 days, carbohydrate content was 102 mg/g in control and 169 mg/g in mycorrhizal leaves and in seed, it has been found to be 120 mg/g in control and 191 mg/g in mycorrhizal. At the interval of 12 days, the amount has been increased in both leaves (125mg/g in control and 200 mg/g

mycorrhizal) and seeds (148 mg/g in control and 225mg/g in mycorrhizal). The plants treated with 8 days interval showed intermediate results (Chart 6 and 7).

Hodges and Lorio (1969) found marked increase in sugars and total carbohydrates in the bark of water stressed loblolly pines, but no difference in the degree of starch degradation in stressed versus non-stressed controls. They attributed this to the reduced growth of stressed trees. Soluble sugars also seem to play an important role in osmotic regulation of cells during germination (Bolarin *et al.*, 1995). In addition to this role, sugars also regulate the expression of some genes involved in germination of seeds (Reynolds and Smith, 1995; Yu *et al.*, 1996). Accumulation of sugars, a characteristic of mature seeds appears to be central to the development of desiccation tolerance (Hoekstra *et al.*, 2001).

Iljin (1957) has suggested that reduced growth with water stress may result in sugar accumulation. It has been concluded from the present results that draught stress has increased all the biochemical contents. In VAM induced plants there was significant increase in carbohydrate concentration because mycorrhiza has helped the plant to tolerate the stress without the utility of carbohydrates.

It has been concluded from the above results that draught stress imposed during the growth of pea plant showed increase in proline and total carbohydrates content and there was decrease in chlorophyll and protein content with increase in water stress interval.

Chlorophyll is the green pigment present in all the green plants which help in photosynthesis. The content of chlorophyll in the leaves of both mycorrhizal and non mycorrhizal plants was analyzed at an interval of 15 days when plants became 45 days old. The mycorrhizal plants showed more amount chlorophyll a, chlorophyll b and total chlorophylls than the non-mycorrhizal plants at all the stages of the water stress given at an interval of 4, 8 and 12 days. The amount of chlorophyll was decreased as the water stress interval was increased in *Pisum sativum.*

In the current study, the protein was estimated from the leaves and seeds of non mycorrhizal and mycorrhizal pea plants. Mycorrhizal plants showed higher content of proteins than control plants. The amount of protein was recorded more in seeds as compared to leaves. The amount of proteins in the leaves and seeds were the maximum at the interval of 4 days water stress interval and this level dropped slightly at an interval of 8 days and 12 days. The amount of proteins has also shown decreasing trend with increase in water stress interval.

Plants can partly protect themselves against mild drought stress by accumulating osmolytes. Proline is one of the most common compatible osmolytes in drought stressed plants. The proline content increased under drought stress condition in pea in both control and mycorrhizal plants. The mycorrhizal pea plants showed lower content of proline than control plants. The amount of proline recorded was more in leaves as compared to seeds. The amount of proline has increased with increase in water stress interval. It was recorded maximum in plants watered with 12 days interval.

The amount of total carbohydrate was recorded minimum in leaves and seeds of both mycorrhizal and non mycorrhizal plants which were watered at an interval of 4 days. On other hand the amount of total carbohydrates has been increased

significantly in both leaves and seeds of mycorrhizal and non mycorrhizal plants at an interval of 12 days. The mycorrhizal pea plants showed higher content of total carbohydrates than control plants. The amount of total carbohydrates was recorded more in seeds as compared to leaves. The total carbohydrate content of the plant has increased with the increase in water stress interval in pea plants.

On the basis of above findings, it can be concluded that out of four biochemicals studied proline and total carbohydrates were significantly increased and chlorophyll and protein content were decreased under water stress in *Pisum sativum.* The AM fungi helped pea plants during drought stress which resulted in increase in biochemical contents than control plants.

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