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Influence of arbuscular mycorrhiza on antioxidative system of wheat (*Triticum aestivum*) under drought stress

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ABSTRACT

The effect of arbuscular mycorrhizal fungi (AMF) on antioxidative system in drought tolerant (WH 1025) and drought susceptible (WH 1105) wheat varieties was investigated in screen house under control and stress conditions. Mycorrhizal and non-mycorrhizal wheat (*Triticum aestivum* L.) plants were subjected to water stress by withholding irrigation at different stages of plant growth (*i.e.* jointing and heading stages). The antioxidant and antioxidative enzymes were estimated in leaves of water stressed and control plants. It was found that drought tolerant and drought susceptible varieties showed different response under drought conditions. Variety WH 1105 suffered greater damage to cellular membrane due to high level of reactive oxygen species (ROS) as indicated by superoxide radical (O^{-}), hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) content under stress conditions. Antioxidative enzymes *viz*. superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR), catalase (CAT) and peroxidase (POX) were higher in drought tolerant variety. Antioxidative metabolites *viz*. ascorbic acid and glutathione content was increased in both tolerant and susceptible variety under water stress but with higher magnitude in WH 1025 than WH 1105. Results showed that under water stress conditions, mycorrhizal inoculation significantly decreased the O_2^{-}, H_2O_2, and MDA content and enhance the activities of antioxidative enzymes in both the varieties. But it was found that the activity was higher in tolerant variety than susceptible variety under water stress conditions. Hence, overall results suggest that mycorrhizal symbiosis play a vital role in enhancing the activities of antioxidative enzymes and decreasing the ROS content that enables the host plant to sustain the drought conditions.

Key words: Antioxidant, Antioxidative enzymes, Arbuscular mycorrhizal fungi, Drought, Wheat

Drought one of the most frequent and severe abiotic stress factor that limits plant growth and crop productivity in many arid and semi-arid regions (Zhu *et al.* 2012). Dry land ecosystems cover over 35% of the world terrestrial land mass (Housman *et al.* 2006). Furthermore, seasonal droughts often occur even in the non-arid regions. Therefore, it is crucial to understand the mechanisms that plants use to respond to the drought stress (Vankova *et al.* 2012). In many arid and semi-arid regions, water stress affects morphological, physiological, biochemical and molecular processes of plants (Asrar and Elhindi 2011). The effect of drought on plant growth depends on various factors such as plant genetic resistance, stage of growth and duration of plant exposure to drought etc. (Echave *et al.* 2005, Song *et al.* 2011). In plants, metabolism of reactive oxygen species (ROS), such as superoxide radicals (O_2^{-}), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^{-}) is kept in dynamic balance. Under water stress conditions, this balance is disturbed and antioxidant systems are needed to decrease the damage to tissue. Despite their destructive activity, ROS are well-described secondary messengers in a variety of cellular processes including tolerance to environmental stresses (Yan *et al.* 2007). Whether ROS will act as damaging or signalling molecule depends on the delicate equilibrium between ROS production and scavenging. Because of the multifunctional roles of ROS, it is necessary for the cells to control the level of ROS tightly to avoid any oxidative injury and not to eliminate them completely.

Plant cells contain an array of protective and repair systems that minimize the occurrence of oxidative damage. These system can be divided into two categories: systems that react with active forms of oxygen and keep them at a low level (*i.e.* SOD, CAT, POX), and systems that regenerate oxidized antioxidants (glutathione (GSH), glutathione reductase (GR), ascorbate and mono- and dehydroascorbate reductases). The first group of enzymes are involved in the detoxification of O_2^{-} radicals and H_2O_2, thereby preventing the formation of OH radicals. The GR, as well as the GSH, are important components of the ascorbate glutathione

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pathway responsible for the removal of H_2O_2 in different cellular compartments.

To alleviate the problem of drought stress, there are many strategies, of which arbuscular mycorrhiza fungi (AMF) is an efficient and new way to enable plants to grow well under drought-prone environments (Ashraf 2010). The successful association between plants and AMF constitutes a strategy to improve the nutritional status of both associates, which reduces the use of fertilizers (Almagrabi and Abdelmoneim 2012). The AMF absorb carbohydrates compound from their host plant, while the plants benefit from the association by increased nutrients uptake, which improve tolerance to abiotic stresses (Song *et al.* 2011). However, the application of AMF inoculation to mitigate the adverse effect of water stress on antioxidative system is still unexplored. Therefore, the objective of this work was to evaluate the mechanisms by which the mycorrhizal symbiosis protects wheat (*Triticum aestivum* L.) plants against damage induced by drought stress.

MATERIALS AND METHODS

The present investigation was carried out on two varieties of wheat *viz*. WH 1105 (drought sensitive, DS) and WH 1025 (Drought tolerant, DT). Seeds were procured from Wheat and Barley Section, Department of Genetics and Plant Breeding, College of Agriculture, CCS HAU, Hisar. The experiment was laid out in CRD factorial design. Seeds were sterilized with alcohol, washed 3-4 times with distilled water and dried on. Plants of two varieties of wheat were raised in earthen pots (20 × 16 cm) with 5 kg dune sand in natural conditions. In half number of pots seeds were inoculated with AMF and in another half pots, seeds were without AMF. Six seeds were sown in each pot at uniform depth. Thinning was done one week after germination to leave two plants of uniform growth in each pot. The plants were supplied with nutrient solution (Hoagland and Arnon 1950) at regular intervals. Drought stress was created by withholding irrigation before jointing (40-45 days) and heading (80-85 days) stage and leaves samples were collected from control and stressed plants. Soil moisture content was estimated by Gravimetric method. Soil moisture observed was 15.6% in irrigated soil and 6.7 and 5.8% at jointing and heading stage respectively under drought conditions.

The MDA, $O_2^{\cdot -}$ and H_2O_2 content were extracted in chilled 0.8 N $HClO_4$. Malondialdehyde content was estimated according to the method of Heath and Packer (1968). The reaction mixture having 0.5 ml supernatant, 2.3 ml of 20% (w/v) TCA containing 0.5% thiobarbituric acid was heated at 95°C for 30 min. The absorbance was recorded at 532 nm and non-specific absorption at 600 nm was subtracted from it. The concentration of MDA was calculated using extinction coefficient of 155 mM^{-1} cm^{-1}. Superoxide radical was measured following the method of Elstner and Heupel (1976) by monitoring the nitrite formation from hydroxylamine which corresponded to $O_2^{\cdot -}$ production and calculated from standard curve of NO_2^- (0–100 nmol). Hydrogen peroxide was estimated by the method of Sinha (1972). To the mixture having 50 µl sample and 1.95 ml of 0.1 M phosphate buffer (pH 7.0), 2 ml solution of 5% potassium dichromate and glacial acetic acid (1:3v/v) was added. The optical density was read at 570 nm and the quantity of H_2O_2 was determined using standard (10-160 nmol).

Extraction conditions were standardized w.r.t. type, molarity and pH of buffer and concentration(s) of stabilizing agents to achieve the maximum extraction of enzyme. The SOD, CAT, APX and GR were extracted in 0.1 M phosphate buffer (pH 7.5) containing 5% (w/v) polyvinyl polypyrrolidone (PVPP), 1 mM EDTA and 10 mM β-mercaptoethanol. For POX, however, the extraction buffer consisted of 0.1 M phosphate buffer (pH 7.5) containing 3% (w/v) PVPP. All the estimations were carried out in duplicates with three extractions each.

The activity of SOD was determined by quantifying the ability of the enzyme extracts to inhibit light induced conversion of nitroblue tetrazolium (NBT) to formazan (Beauchamp and Fridovich (1971). One enzyme unit was defined as the amount of enzyme which caused 50% inhibition of the photochemical reaction. Catalase and POX activities were assayed at 37°C as described by Sinha (1972) and Shannon *et al.* (1966), respectively. The reaction mixture for catalase contained 0.5 ml of 0.2 M phosphate buffer (pH 7.0), 0.4 ml of 0.2 M H_2O_2 and 0.1 ml enzyme extract. After incubating at 37°C, the reaction was terminated by adding 3 ml mixture of 5% (w/v) $K_2Cr_2O_7$ and glacial acetic acid (1:3v/v) and heated in boiling water bath for 10 min. After cooling the tubes, absorbance was measured at 570 nm and the amount of H_2O_2 oxidized was determined. The POX reaction mixture (2.75 ml) contained 2.5 ml of 50 mM phosphate buffer (pH 6.5), 0.1 ml of 0.5% H_2O_2, and 0.1 ml of 0.2% O-dianisidine and 0.05 ml enzyme extract. The change in absorbance was measured at 430 nm.

One unit of CAT and POX was defined as the amount of enzyme required for oxidation of 100 µmole H_2O_2 per min and one O.D/min, respectively. Method of Nakano and Asada (1981) was employed to assay APX and decrease in absorbance due to oxidation of ascorbic acid at 290 nm was recorded. One enzyme unit was defined as amount of enzyme required to oxidize 1 µmole ascorbic acid per min. Glutathione reductase activity was determined at 30°C by adding 100 µl enzyme extract to 1 ml phosphate buffer (0.2 M, pH 7.0) containing 1 mM EDTA, 0.1 ml 20 mM oxidized glutathione (GSSG) and 0.1 ml of 2 mM NADPH. Oxidation of NADPH was monitored at 340 nm and the rate (nmol/min) was calculated using the extinction coefficient of 6.12 mM^{-1} cm^{-1} (Halliwell and Foyer 1978). One enzyme unit was defined as the amount of enzyme causing 0.01 change in O.D/min.

Ascorbic acid and glutathione were extracted in 6% TCA. Ascorbic acid content was estimated by following the method of Mukherjee and Choudhuri (1983). The concentration of total ascorbic acid was calculated from a standard curve plotted with known concentration of

ascorbic acid. Method of Griffith (1980) was employed for determining the level of total glutathione [total *viz*. GSH + GSSG and oxidized (GSSG)].

The data were statistically analyzed using complete randomized design (CRD) where each observation was replicated thrice and each replicate was estimated in duplicate. The critical difference (CD) among the variance was calculated at P≤0.05 (Panse and Sukhatme 1961).

RESULTS AND DISCUSSION

Effect of drought stress and AMF on oxidative stress

Mycorrhization associated changes in antioxidant enzymes are widely reported. Earlier studies (Ni *et al.* 2013 and Huang *et al.* 2014) using different plant demonstrated that AMF conferred greater tolerance to plants against soil water deficit through an enhancement in their antioxidative enzymes system on consequent decrease in level of oxidative stress. The indices of oxidative stress *viz.* MDA value, H_2O_2 content and $O_2^{\cdot-}$ level increased under drought conditions in both the varieties regardless of AMF inoculation (Fig 1). However, AMF inoculation decreased hydrogen peroxide concentration by 11.2%, 15.2% (Fig 1A), $O_2^{\cdot-}$ concentration by 18.6%, 33.6% (Fig.1B), MDA content also decreased by 24.7, 27.7% (Fig 1C) at jointing and heading stage respectively, as compared to uninoculated plants under stress conditions. The pattern observed in WH 1105 was similar to WH 1025, but $O_2^{\cdot-}$, H_2O_2 and MDA content was higher under stress conditions as compared to WH 1025 irrespective of AMF treatments. The alleviation of $O_2^{\cdot-}$ and H_2O_2 accumulation by AMF inoculation under stress conditions had also been reported by Benhiba *et al.* (2015), Tian *et al.* (2015), Huang *et al.* (2014); Ni *et al.* (2013). In this study although $O_2^{\cdot-}$, H_2O_2 and MDA content increased during drought stress in both WH 1025 and WH 1105. But H_2O_2, $\cdot O_2^-$ and MDA contents were higher in the AMF uninoculated plants than inoculated plants, showing that AMF inoculated plants suffered less oxidative damage than uninoculated plants during water deficit conditions. The less oxidative damage in AMF inoculated plants might be due to better water availability and osmoregulation and higher antioxidative defense systems (Ni *et al.* 2013, Wu *et al.* 2013).

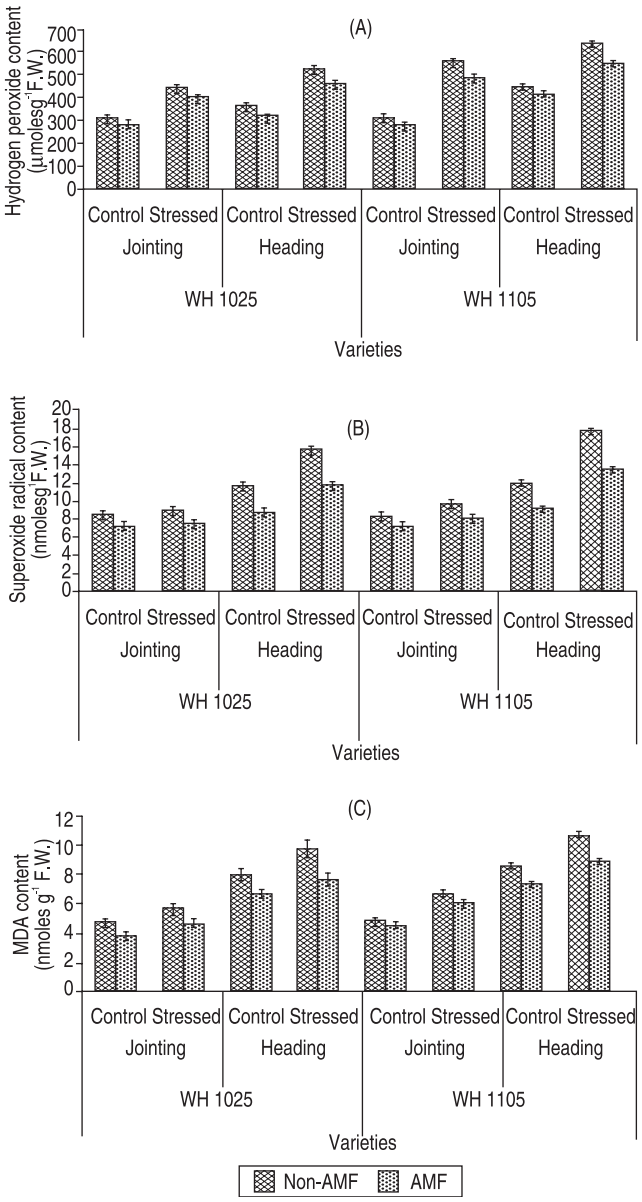

Fig 1 Effect of Arbuscular mycorrhizal fungi on (A) H_2O_2 content, (B) Superoxide radical content ($O_2^{\cdot-}$ content) and (C) MDA content in wheat plants under control and drought stress condition at different growth stages

Effect of drought stress and AMF on antioxidative enzymes

Under drought stress conditions activities of antioxidative enzymes *viz*. SOD (Superoxide dismutase), APX (Ascorbate peroxidase), GR (Glutathione reductase) and POX (Peroxidase) increased but CAT (Catalase) activity decreased in AMF inoculated and uninoculated plants of both the varieties (Fig 2). The SOD activity (108.4 units/g f. wt.) was maximum in mycorrhizal inoculated plants of WH 1025 at heading stage (Fig 2A). Mycorrhizal inoculation increased the activity by 8.5, 21.8% in WH 1025 and 9.7, 16.41% in WH 1105 at jointing and heading stage respectively under stress conditions. Previous reports suggest that mycorrhizal inoculation increased the SOD activity in plants under stress conditions (Huang *et al.* 2014, Ni *et al.* 2013). Mycorrhizal inoculated lettuce plants subjected to drought showed significantly higher SOD activity as compared to uninoculated controls and molecular analyses have confirmed this response at the transcriptional level (Ruiz-Lozano *et al.* 2001).

Catalase activity in uninoculated plants increased by 30.3, 16.6% in WH 1025 but it decreased by 17.4, 19.2% in WH 1105 at jointing and heading stage, respectively under stress conditions (Fig 2B). Arbuscular mycorrhiza fungi inoculation enhanced the enzyme activity in both the varieties irrespective of water stress conditions. Similar results have been reported in date palm (Benhiba *et al*. 2015), trifoliate orange (Huang *et al.* 2014), *Citrus tangerina*

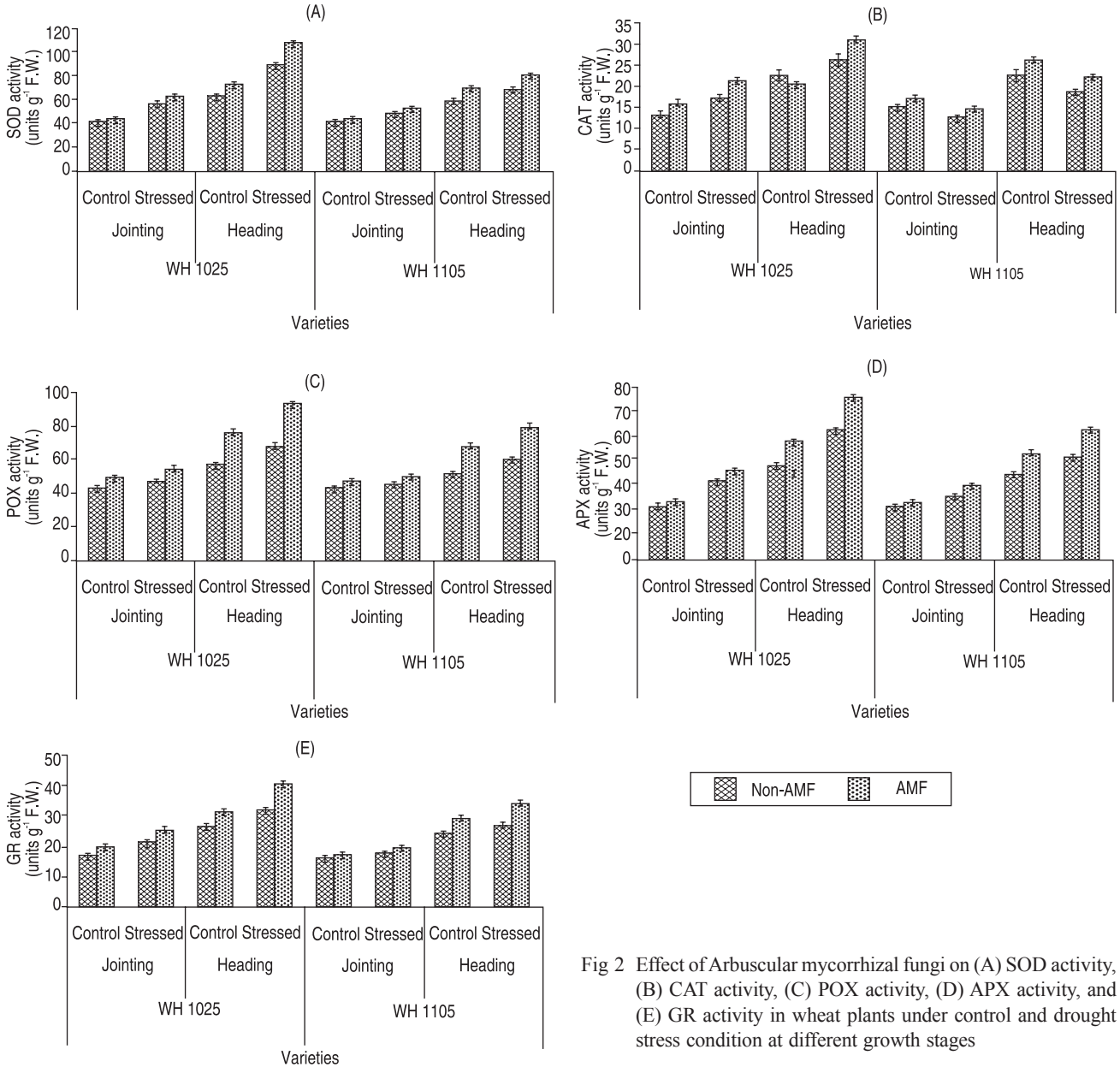

Fig 2 Effect of Arbuscular mycorrhizal fungi on (A) SOD activity, (B) CAT activity, (C) POX activity, (D) APX activity, and (E) GR activity in wheat plants under control and drought stress condition at different growth stages

(Ni *et al.* 2013) and *Allium sativum* (Borde *et al.* 2012). Peroxidase activity significantly enhanced by water stress conditions at both the growth stages of the varieties (Fig 2C). Higher activity was observed at heading stage as compared to jointing stage. The AMF inoculation increased the POX activity of both the varieties irrespective of water conditions. Compared with uninoculted leaves, the POX activity of inoculated leaves in WH 1025 increased 32.1, 34.5% and 30.1, 31.5% in WH 1105 under control and stress conditions, respectively. The beneficial effect of AMF on POX under water stress was also observed by Ni *et al.* (2013) in citrus seedlings and Abbaspour *et al.* (2012) in pistachio plants. The increase in peroxidase activity under stress conditions has been described to either increased expression of POX encoding genes or increased activation of the already existing enzymes. As a result mycorrhizal wheat seedlings that possess significantly higher POX activity might cope with ROS accumulation during drought conditions. The ascorbate peroxidase activity of AMF inoculated and uninoculated leaves increased under stress conditions in DT and DS varieties (Fig 2D). In AMF inoculated DT variety, activity increased by 2.8, 2.1 fold while in DS variety it increased by 1.8, 1.7 fold under control and stress conditions respectively at heading stage as compared to jointing stage. Concomitant with our results, Benhiba *et al.* (2015) also reported that AMF colonization enhanced the APX activity under control and stress conditions in date palm, Porcel *et al.* (2003) in soybean plants. Increase in APX activity has been to be due to transcriptional and translational modifications (Alscher 1989, Sreenivasula *et al.* 2000). The glutathione reductase activity (Fig 2E) increased linearly at both the growth stages under stress conditions. In AMF inoculated WH 1025, activity increased by 32.6% and 28.6% at jointing and heading stage respectively as compared to control plants.

Similar trend was found in WH 1105 but activity was much lower than WH 1025 under stress conditions. This was in agreement with previous reports obtained in date palm AMF inoculation enhanced the GR activity under drought stress (Benhiba *et al.* 2015). Higher GR activity found in AMF inoculated roots than uninoculated roots of citrus (Wu *et al.* 2006) under drought stress. Increase in GR activity may be correlated with increased synthesis of GR protein under stress conditions (Edwards *et al.* 1993).

Effect of drought stress and AMF on antioxidants

Ascorbic acid (Fig 3A) content (ASA) increased under water stress. In AMF inoculated leaves of WH 1025, ascorbic acid content increased by 34.3, 23.0% at jointing stage and heading stage, respectively over their uninoculated plants. Similar trend was observed in WH 1105 under water stress (Fig 3A). The results obtained in present study were in agreement with those obtained in citrus plants in which AMF colonization increased the ascorbic acid content under drought stress (Wu *et al.* 2006). Ascorbic acid may provide protection to membrane by directly scavenging the $O_2^{\cdot-}$, OH^- and by regenerate α-tocopherol from tocopheroxy radicals. Increased ASA content has been shown to confer oxidative stress tolerance in *Arabidopsis* (Wang *et al.* 2010). The reduced glutathione increased in WH 1025 but decreased in WH 1105 under water stress, however, mycorrhizal inoculation increased the GSH content in both WH 1025 and WH 1105 varieties (Fig 3B). The AMF inoculated plants had higher GSH level over uninoculated plants. In AMF inoculated leaves of WH 1025, GSH content increased by 11.8 and 27.3% however, in WH 1105 it decreased by 7.0, 6.0% at jointing and heading stage respectively under water deficit conditions. Arbuscular mycorrhizal fungi inoculation also increased the GSSG level (Fig 3C). Ratio of reduced to oxidized glutathione (Fig 3D) was found to be more in WH 1025, than in WH 1105 under stress conditions in leaves of AMF uninoculated and inoculated plants. In mycorrhizal plants of both the varieties higher level of reduced to oxidized glutathione ratio was reported as compared to uninoculated plants irrespective of water conditions. Glutathione is necessary to maintain the normal reduced state of cells so as to counter act the inhibitory effect of ROS induced oxidative stress. AMF infection increased the GSH and GSSG content was also reported in previous study in plants (Wu *et al.* 2006).

Similar to drought stress, altered ratio of GSH/GSSG has been observed in plants under various stresses (Hefny and Abdel-Kader 2009, Radyuk *et al.* 2009, Maheshwari and Dubey 2009, Tian *et al.* 2015). Eltayeb *et al.* (2011) observed greater protections against oxidative damage imposed by various environment stresses in transgenic potato with higher level of reduced glutathione. Avoidance of oxidative stress through preventing ROS accumulation is the most effective approach used by mycorrhizal plants

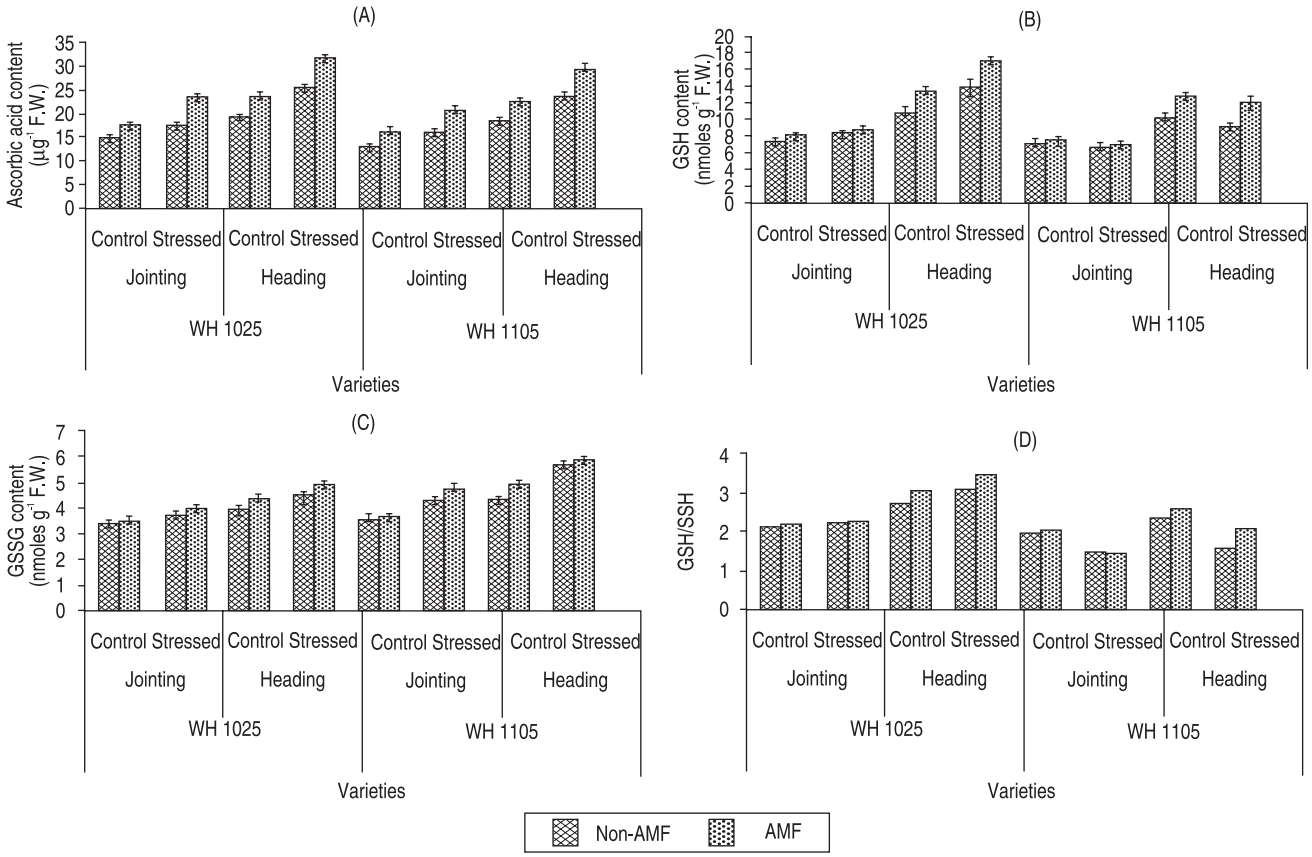

Fig 3 Effect of Arbuscular mycorrhizal fungi on (A) ASA Content, (B) GSH content, (C) GSSG content, and (D) GSH/GSSG in wheat plants under control and drought stress condition at different growth stage

to cope with drought stress. The higher enzymatic and non-enzymatic antioxidant associated with low accumulation of ROS and less peroxidation of lipids would explain the better performance of WH 1025 mycorrhizal plants, giving proof that AMF symbiosis contributes to protect plants against the oxidative damage induced by drought stress.

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