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Drought- induced oxidative stress and activities of boiling soluble antioxidants in seedlings of drought tolerant and susceptible cultivars of *Triticum aestivum*.

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Abstract

Drought is one of most important abiotic stress that affects plant growth and productivity. Various antioxidants are known to maintain oxidative stress induced-ROS at sub-lethal levels in plants under abiotic stress conditions, but, studies documenting how drought regulates boiling soluble antioxidants at vegetative phase is still a matter of conjuncture. In this study, changes in total Chlorophyll content and its different fractions(Chl a and Chl b), total protein content (TPC), relative water content (RWC), H_2O_2,$O_2^{\cdot-}$, malondialdehyde (MDA), Membrane injury index(MII), membrane stability index(MSI) and ROS scavenging boiling soluble antioxidants were studied in the shoots of two cultivars (PBW 175 and PBW 621) of wheat at different stages of development. Simultaneous analysis of ROS induced oxidative damage and activities of ROS-scavenging boiling soluble antioxidants gave an integrative view of physiological state and detoxifying potential under conditions of sensitivity and tolerance. Drought stress increased TPC in a developmental stage dependent manner and decreased RWC in both the cultivars of wheat. H_2O_2 and $O_2^{\cdot-}$ content increased considerably under drought stress in both the cultivars. As a result, MDA content, a product of lipid peroxidation along with MII also increased in both the cultivars. There was a differential response of boiling soluble antioxidants in both the cultivars under stress. The BsSOD activity declined considerably in the cv. PBW 621, whereas in the cv. PBW 175, BsSOD activity remained unchanged upon water imposition. The BsAPX activity declined considerably in both the cultivars under drought stress, with a sharper decrease in the cv. PBW 621. The BsGPX activity increased in response to elevated ROS levels in both the cultivars in a developmental stage dependent manner. The BsCAT activity showed negligible values upon water imposition in the cvs. PBW 175 and PBW 621. Based upon our results, it can be inferred that tolerant cv. PBW 175 have more of a biological antioxidative capacity to combat drought- induced oxidative stress.

Key words: Boiling soluble proteins, drought stress, H_2O_2 metabolizing enzymes, wheat.

Introduction

Water deficit conditions due to drought, is one of the main limiting factor for plant growth and productivity, resulting in large agricultural and economic losses in different parts of the world [5]. Drought leads to oxidative stress through an increase in reactive oxygen species (ROS), such as superoxide (O_2 $^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH•).These ROS are highly reactive and can trigger phytotoxic reactions like lipid peroxidation, protein degradation and DNA mutations [32]. Plants respond to oxidative stress through a host of biochemical, physiological and metabolic changes. These changes mainly adaptive, include a host of biochemical pathways associated with ROS detoxification, signal perception, transduction and gene expression regulation in a temporal and spatial pattern [15]. Out of this, timely turnover of ROS (ROS detoxification) is achieved by a complex defense antioxidative system, including low-molecular mass antioxidants as well as antioxidative enzymes, such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), guaiacol peroxidase (GPX) and glutathione reductase (GR) [14]. These antioxidant enzymes work in concert and act synergistically to scavenge ROS, as not one of them can single-handedly detoxify the various forms of all the ROS. The measure of the specific antioxidant enzyme activities upon water imposition is considered to be an approach to assess the involvement of the scavenging system during water stress [8]. The differences in the subcellular localization, biochemical properties of antioxidant enzymes and the distinct responses in gene expression, result in a versatile and flexible antioxidant system able to control the optimum ROS levels]. SOD is the major O_2 $^{\bullet-}$ scavenger and its enzymatic action results in H_2O_2 and O_2 formation. The H_2O_2 produced is then scavenged by several H_2O_2 detoxifying enzymes like CAT, APX and GPX. Out of this, CAT, is found in peroxisomes, cytosol and mitochondria and facilitates the dismutation of H_2O_2 into H_2O and O_2 . Peroxidases (APX and GPX) are distributed throughout the cell and catalyze the reduction of H_2O_2 to H_2O. APX uses ascorbate as electron donor in the first step of the ascorbate–glutathione cycle and is considered the most important plant peroxidase in H_2O_2 detoxification. GPX, which is less specific to electron donor substrate, decomposes H_2O_2 by oxidation of co-substrates such as phenolic compounds and/or ascorbate [14].

Besides, these drought responsive antioxidants, when plants are exposed to osmotic or treated with ABA, a novel set of proteins accumulates, which represent just 0.2% of the total genome in plants. These are characterized by high degree of hydrophilicity, enabling them to remain in solution even after boiling the extracts in vitro. Previous studies in E. coli and yeast have demonstrated that hydrophilins may be necessary in these organisms to adapt themselves against adverse abiotic stress like conditions. In silico analysis of BSPs from several kingdoms like : plant, bacteria and fungi have revealed the conservation of lysine rich regions in these proteins, thus , suggesting an evolutionary role for these cellular boiling soluble proteins during water – deficits [13]. So, it can be concluded that hydrophilins have evolved independently in different protein families and in different organisms, but with the similar goal of protecting the plants under

water stress. There have been several reports that authenticate the role of antioxidants under water stress conditions [10], but studies are lacking wherein the role of these hydrophilic antioxidants have been provided in a genotype and developmental stage dependent manner. Because plant responses to different abiotic stress conditions are complex and multigenic, the function of many of the stress-induced genes including boiling soluble antioxidants is still a matter of conjuncture. Therefore, the aim of this study was to evaluate the effects of drought- induced possible induction of oxidative stress and likely alterations in the behaviour of multiple boiling soluble antioxidants in leaves of two wheat genotypes differing in drought tolerance. In order to better understand the physiological and biochemical mechanisms involving boiling soluble antioxidants under drought stress, a factorial experiment was conducted with two genotypes and two watering regimes at vegetative phase. This study will provide documentation for the selection of higher drought resistant wheat genotypes to generate genetically transformed tolerant wheat plants in arid regions based upon the boiling soluble antioxidants stress responses. We focused our studies on the vegetative phase since seedling emergence is one of the critical stages for plant establishment in crops grown in drought affected areas and hence, crop density and final yield is determined at this stage. We selected wheat for our study because of the natural genetic variation in its traits related to water deficit tolerance. In order to facilitate the detection of BSPs, boiling soluble or soluble (Bs) fractions that resists coagulation upon heating at 100°C were focused.

Materials and Methods

Growth and Stress conditions

The seeds of *Triticum aestivum* L. cvs. 'PBW 175' (drought tolerant) and 'PBW 621 ' (drought sensitive) differing in degree of drought tolerance [45] were procured from PAU Ludhiana, Punjab, India. The seeds were surface sterilized with 1% (w/v) mercuric chloride and 70% ethanol. Plants of the two cultivars were raised in 5L pots and kept in a net house under natural conditions. Shoots at vegetative phase {53 and 76 Days Post Sowing (DPS)} were harvested in triplicates, pooled and used for further analysis. Water stress was imposed by withholding water for consecutive 5-6 days while the control plants were watered daily. Relative Water Content (RWC) was calculated as per equation= (FW-DW/ TW-DW) X 100.

Extraction of boiling soluble proteins

The boiling soluble proteins were extracted as described previously [39].

Estimation of total Chlorophyll and Chl a/b contents

The total Chlorophyll and its different fractions (Chl a,b) were estimated according to the method described in [38].

Estimation of hydrogen peroxide, superoxide anion,MDA content,MII and MSI

These parameters were measured by the method described in [39].

Estimation of BsAPX, BsSOD, BsGPX and BsCAT activity

These were estimated as described previously in [39].

Results and Discussion

In the present study, drought- induced changes in the activities of various boiling soluble antioxidative enzymes (BsSOD,

BsCAT, BsAPX, BsGPX), indices of oxidative stress ($O_2^{\cdot-}$, H_2O_2, MII,MSI, MDA) and related physiological parameters were investigated in seedlings of drought tolerant (PBW 175) and susceptible (PBW 621) cultivars of wheat at two different developmental stages (53 DPS and 76 DPS).

Changes in physiological parameters

RWC is an important physiological parameter, indicating the health and sturdiness of the plants and is decreased in the state of stress. RWC as observed in our study, declined significantly upon imposition of water stress in both the cultivars at 53 and 76 DPS (Fig.1 A). Similar findings have been reported in leaf discs of maize genotypes, roots of Zea mays, in shoots and roots of wheat and in leaves of mulberry under different stress conditions [19], [12], [34]. Also, in a study by [42] on four different caprifig genotypes (Dane Sephid, Pouz Doubali, Shah Anjiri and Khormaaei), they observed a reduction in RWC in all the cultivars under water stress period.

The increase in Total Protein Content (TPC) was observed in the cvs. PBW 175 and 621 under drought conditions in a developmental stage dependent manner (Fig. 1B). At 53 DPS, TPC increased significantly only in the tolerant cv. PBW 175 while at 76 DPS, TPC increased drastically in the sensitive cv. PBW 621. Consistent with this finding, [47] reported an increase in the protein content of *Orthosiphon staminneus* under PEG- induced water stress and NaCl stress. The increase in the TPC can be regarded as an adaptive mechanism of survival of the plants under stress since proteins supports the leaf structure during the wilting process. Increased TPC can be attributed to the activation of some stress-responsive genes encoding boiling soluble proteins under drought stress conditions and also decreased protein degradation.

The Chlorophyll content of leaf is an indicator of photosynthetic capacity of plant tissues and the decline in its activity is the first indicator of leaf senescence in plants subjected to drought [16].The results in the present study, also revealed that there was a considerable decline in the total Chlorophyll content in both the cvs. PBW 175 and PBW 621 at 53 and 76 DPS due to significant reductions in both Chl a and Chl b fractions (Fig. 1C,D,E). However, as compared to the tolerant cv. PBW 175, there was a greater decrease in the total Chl and its different fractions in the sensitive cv. PBW 621 at 53 and 76 DPS. Decreased chlorophyll content, as observed in this study, has also been reported earlier in other plant species under drought stress depending upon the duration and the severity of the stress [44]. A decrease in chlorophyll content at different developmental stages may be attributed to the strong reductions of membrane-bound chloroplast antioxidant defence, which indicates oxidative stress in chloroplasts and production of Reactive Oxygen Species (ROS) like $O_2^{\cdot-}$ and H_2O_2 causing lipid peroxidation and consequently chlorophyll destruction [35].Also, photo-inhibition and photo-destruction of pigments may contribute to such changes. Besides this, photosynthetic apparatus may show acclimation responses like changes in the relative proportion of stacked and unstacked membrane domains [2]. From the findings, it can be speculated that a lesser decrease in the Total Chl and its fractions under drought conditions in the tolerant cv. PBW 175 might enable it to avoid drought stress by

maintaining a high photosynthetic activity and can be considered as an essential factor of the adaptation of this cultivar under drought conditions.

Fig.1: Effect of drought stress on relative water content (A) , total protein content (B), Total Chl and its different fractions (C, D, E) in shoots of drought-tolerant (PBW 175) and drought-sensitive (PBW 621) cultivars of *Triticum aestivum* at 53 and 76 Days Post Sowing (DPS). Data shown are Mean ± SE of three replicates. Each replicate contain a set of 3 three shoots. ^{d} indicates significant difference vs. control at $P \leq 0.05$.

Changes in ROS.

During normal cellular metabolism, reactive oxygen species (ROS) like singlet oxygen (O_2^1), superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot}$) are generated as natural by-products of many metabolic reactions like photosynthesis, photorespiration [14]. The exposure of the plants to the different environmental stresses leads to enhanced generation of ROS, which in turn orchestrates signal transduction pathways that activate gene expression. Among the different ROS, H_2O_2 is relatively stable and able to penetrate the plasma membrane as an uncharged molecule. Upto certain concentration, H_2O_2 seems to be a master hormone, controlling a variety of stress responses and ROS/ hormonal homeostasis in the cell [46]. On the other hand, $O_2^{\cdot-}$ is more reactive, short lived, unstable and impermeable to biological membranes. In our study, ROS ($O_2^{\cdot-}$ and H_2O_2) levels increased tremendously under drought conditions in cvs. PBW 175 and PBW 621 at 53 and 76 DPS (Fig. 2A, B). Similar to this study, ROS including $O_2^{\cdot-}$ and H_2O_2 have been reported to increase in wheat [28] and maize [24] under osmotic stress conditions. [41] reported that heavy metal Cd stress resulted in the over- production of ROS (H_2O_2 and $O_2^{\cdot-}$) in *Pisum sativum*. Enhanced production of ROS under stress can pose a threat to cells, but it is also thought that ROS serve as signal molecules to activate the stress responses and defense pathways [27]. Thus, drought- induced increase in ROS, as evident in our study, can be viewed as cellular indicators of stress and as secondary messengers involved in the stress – response signal transduction pathways.

Lipid peroxidation, measured in terms of malondialdehyde (MDA) content happens to be a crucial effect of ROS and is a biochemical marker for the free radical-mediated injury. Free radical- induced peroxidation of membranes is both a reflection and an indicator of stress- induced damage at the cellular level [48]. MDA content was observed to increase significantly in cvs. PBW 175 and PBW 621 under drought at 76 DPS only, whereas non-significant difference under drought was observed at 53 DPS in both the cultivars (Fig.2 C). The rise in MDA content at 76 DPS under stress conditions in both the cultivars suggests that water stress could induce membrane lipid peroxidation by means of ROS [43]. The increase in the rate of ROS production under drought stress is mainly responsible for membrane lipid peroxidation, rendering the cell incapable of carrying out its metabolic activities [22]. MDA is mainly formed by the ROS- induced degradation of the polyunsaturated lipids and hence a clear link is provided between observed increase in ROS in drought stressed wheat seedlings and the analogous increased cellular damage. Based upon our

findings, it is speculated here that increased MDA accumulation is correlated with the reduction of the physiological parameters (RWC, Total Chl along with its fractions) under drought stress as has been postulated by [23]. The results of the present study corroborate the previous reports [31], [49] showing higher levels of MDA content and hence lipid peroxidation in different plant species under stress conditions.

In addition, increase in Membrane Injury Index (MII) or decrease in Membrane stability index (MSI) reflects the extent of lipid peroxidation, which in turn is a consequence of higher oxidative stress due to water stress conditions. Cell membrane stability plays a critical role in maintaining cell turgor and physiological functions during drought and electrolyte leakage (EL) has been used to estimate MII and MSI [37]. In our study, MDA content at 76 DPS, in both the cultivars was observed to be positively and negatively correlated with MII and MSI respectively. Similar to a significant increase in MDA content at 76 DPS in both the cultivars under stress, MII and /or MSI registered a substantial increase and decrease respectively at 76 DPS in the cvs PBW 175 and 621 upon water imposition (Fig. 2 D/E). High concentrations of MDA, increases lipid peroxidation and oxidation of cell membrane fatty acids which finally increases membrane injury and decreases cell membrane stability [9]. Concomitant to this study, [18] also reported that MDA content in drought tolerant (IR 50 and Xiangzhongxian) and susceptible cutivars (Xiangnuo no.1 and Zimanuo) of rice increased upon drought imposition and was positively correlated with the increased electrolyte leakage (MII) in both the cultivars. All these observations point toward the damaging effects of ROS in causing membrane injuries in the cvs. PBW 175 and 621 at 76 DPS leading to non-selective ion leakage under drought stress. The cause of increased MII under drought stress can be attributed to the decreased cellular volume which causes aggregation and increases the viscosity of cellular components. This, in turn, enhances the likelihood of the molecular interactions among different components leading to the membrane fusion and protein denaturation [40].

Fig.2: Changes in H_2O_2 content (A), $O_2^{\cdot-}$ content (B), MDA (C), MII (D), and MSI (E) content in shoots of drought-tolerant (PBW 175) and drought-sensitive (PBW 621) cultivars of *Triticum aestivum* at 53 and 76 Days Post Sowing (DPS). Data shown are Mean ± SE of three replicates. Each replicate contain a set of 3 three shoots. ^{d} indicates significant difference vs. control at $P \leq 0.05$.

Changes in BsAntioxidants

H_2O_2 scavenging enzyme (CAT, APX and GPX) activities, in conjunction with SOD, seems crucial for determining the steady state balance of $O_2^{\cdot-}$ and H_2O_2 and hence, is important to prevent the formation of highly toxic hydroxyl radicals [32]. The active involvement of these enzymes is related, atleast in part, to the drought- induced oxidative stress tolerance in wheat plants [4].

SOD is one of the ubiquitous key enzymes in the aerobic organisms and plays a key role in the active oxygen scavenging mechanism [3]. SOD activity modulates the relative amounts of $O_2^{\cdot-}$ and H_2O_2 and decreases the risk of $OH^{\cdot}$ radical formation which may cause severe damage to membranes, protein and DNA. The ability of plants to overcome

oxidative stress partly relies on the induction of SOD activity and subsequently on the up-regulation of other downstream antioxidant enzymes [1]. BsSOD activity was observed to decrease significantly under the effect of drought stress only in the susceptible cv. PBW 621 at 53 DPS while in the tolerant cv. PBW 175, the BsSOD activity remained unchanged upon water imposition (Fig. 3A). SOD activity was also reported to decrease in the creeping bent grass after exposure to high temperature stress [29]. At 76 DPS, BsSOD activity remained unchanged in both the cvs. PBW 175 and 621 under the effect of drought stress. Since, SOD activity was not found to increase significantly in either of the cultivars under the effect of drought stress, the $O_2^{\cdot-}$ dismutation into H_2O_2 by the action of BsSOD was inhibited, therefore, the levels of $O_2^{\cdot-}$ under drought stress were found to be significantly higher in both the cultivars at 53 and 76 DPS. The H_2O_2 concentration showed negative correlation with the BsSOD activity, since H_2O_2 was found to increase in the seedlings of both the cvs. PBW 621 and 175, indicating some other source of H_2O_2 production besides the action of SOD.

H_2O_2, a toxic species, must be eliminated by conversion to H_2O, involving H_2O_2 metabolizing enzymes (APX, POD, and CAT), functioning in different sub-cellular compartments [33]. CAT is an oxidoreductase present only in the peroxisomes but it is indispensable for ROS detoxification during stress, when elevated levels of ROS are produced [50]. This enzyme does not require a reducing power and has a high reaction rate but a low affinity for H_2O_2, thereby removing the high concentrations of H_2O_2 [14]. The BsCAT activity was found to decline considerably, reaching nil values under drought effect in the cv. PBW 621 at 53 and 76 DPS. However, in the cv. PBW 175, drought- induced decrease to nil values in the BsCAT activity was only detected at 76 DPS (Fig. 3B). The decline in the BsCAT, suggest delay in the removal of ROS (H_2O_2 and peroxides) and in turn, an enhancement in the free- radical mediated lipid peroxidation and membrane injury at 76 DPS in both the cvs. PBW 175 and 621 (Fig. 2). The reduction in BsCAT activity under drought conditions can either be due to the inactivation of enzyme protein due to ROS ,reduced rate of protein turnover, decrease in the enzyme synthesis or change in the assembly of the enzyme subunits. Study by [51] also reported a decrease in the CAT activity in the shoots of rice cvs. Ratna and Jaya in response to 1000 μM Pb treatment. In another study,[21]reported that CAT activity decreased in the seedlings of the salt tolerant rice cv. Pokkali as well as in the salt sensitive cv. BRRI dhan 29 under the effect of salt stress. [26] also reported that foliar levels of CAT activity was decreased by chilling stress in the cucumber.

GPXs are involved not only in scavenging H_2O_2 but also in plant growth, development, leaf expansion, fruit growth, modification of the cell wall (lignification, suberization, and cross-linking of cell wall compounds)[7]. Roles played by GPX in cell wall toughening and in the production of secondary metabolites, along with its simultaneous oxidant and antioxidant capacities, make it an important factor in the integrated defense response of plants to drought stress. The BsGPX activity was found to increase tremendously upon water stress imposition in the cv. PBW 175 at 53 DPS. Conversely, a marked decline in the BsGPX activity was

observed in the sensitive cv. PBW 621 at 53 DPS (Fig. 3C). Drought stress- induced BsGPX activity in the tolerant cv. PBW 175, was associated with an increase in the ROS generation, suggesting an induction of antioxidant defenses regulated by ROS mediated signalling pathways. Our findings indicate an enhancement in BsGPX activity, thereby suggesting, that this enzyme serves as an intrinsic defense tool to resist drought-induced oxidative damage and to develop adaptive metabolic means of tolerance to drought stress. The observations, strongly imply a possibility that BsGPX activity is being utilized in tolerant cv. PBW 175 to alleviate oxidative stress caused by drought, thus protecting the cells from oxidative damage. Decline in the BsCAT activity and an increase in the BsGPX activity indicated that BsGPX might be more active in the protection of the leaf tissues of the tolerant cv. PBW 175 against membrane injury. Contrary to this, at 76 DPS, the BsGPX activity remained unchanged under drought stress in the tolerant cv. PBW 175 while in case of susceptible cv. PBW 621, the BsGPX activity was found to increase significantly under drought stress. [21] reported that GPX activity increased in the seedlings of the salt tolerant rice cv. Pokkali as well as in the salt sensitive cv. BRRI dhan 29 under the effect of salt stress. Some previous studies, as parallel with our results, reported increased GPX activity under drought stress in various plants, like Arabidopsis [25], wheat [20], sunflower [17]. Although BsGPX activity increased in the susceptible cv. PBW, it was incapable to destroy the free radicals, as seen from increased MDA levels at 76 DPS (Fig. 2). In other words, we can say that increased BsGPX activity in the sensitive cv. PBW 621, alone could not compensate for suppressed BsCAT and BsAPX activity.

A major hydrogen- peroxide detoxifying system in plant cells is the ascorbate-glutathione cycle, in which ascorbate peroxidase (APX) enzymes are an indispensable components, that play a key role catalyzing the conversion of H_2O_2 into H_2O [30]. APX has high affinity to its substrate H_2O_2 and even small increase in its activity may play a crucial role in allowing ROS scavenging capacity [14]. Different APX isoforms are present in distinct subcellular compartments, such as chloroplasts, mitochondria, peroxisome, and cytosol. APX are involved not only in scavenging H_2O_2 but also in plant growth, development, regulation of redox signalling pathways, lignifications, suberization and cross linking of cell wall compounds [36]. The BsAPX activity decreased considerably under drought stress in both the cultivars PBW 175 and 621 at 53 DPS, with a more drastic decrease in case of the susceptible cv. PBW 621. Contrary to this, at 76 DPS, BsAPX activity remained unchanged upon imposition of water stress in case of the tolerant cv. PBW 175. Conversely, in sensitive cv. PBW 621, nil BsAPX activity was observed under control as well as drought conditions (Fig. 3D). Decrease in the BsAPX in both the cultivars, may be responsible for oxidative stress as it is the potential scavenger of oxy radicals [11]. [6], stated that ROS- detoxifying enzyme APX has been shown to be inefficient in plants subjected to drought- induced oxidative stress. Taken together, we can postulate that in the tolerant cv. PBW 175, lesser decrease in BsAPX as well as unchanged values in response to drought at 53 and 76 DPS might be responsible for the drought tolerant nature.

Fig.3: Changes in the activities of boiling soluble (Bs) SOD(A), CAT(B),GPX(C), APX(D), in shoots of drought-tolerant (PBW 175) and drought-sensitive (PBW 621) cultivars of *Triticum aestivum* at 53 and 76 Days Post Sowing (DPS). Data shown are Mean ± SE of three replicates. Each replicate contain a set of 3 three shoots. ^{d} indicates significant difference vs. control at $P \leq 0.05$.

Conclusions

A perusal of the data indicates that water stress was responsible for the induction of oxidative stress and thus, related damage, as shown by RWC decrease, increased MDA content, H_2O_2 accumulation and degradation of Chlorophyll in both the cultivars. Also, boiling soluble antioxidants were almost completely suppressed in case of the sensitive cv. PBW 621 while in case of tolerant cv. PBW 175, boiling soluble antioxidant levels were maintained better in response to oxidative stress. Drought tolerant cv. PBW 175, maintained unchanged BsSOD activity, increased BsGPX along with a lesser decrease in the BsAPX activity in response to drought at 53 and 76 DPS and hence, may have a better protection mechanism against drought-induced oxidative damage as compared to the sensitive cv. PBW 621.

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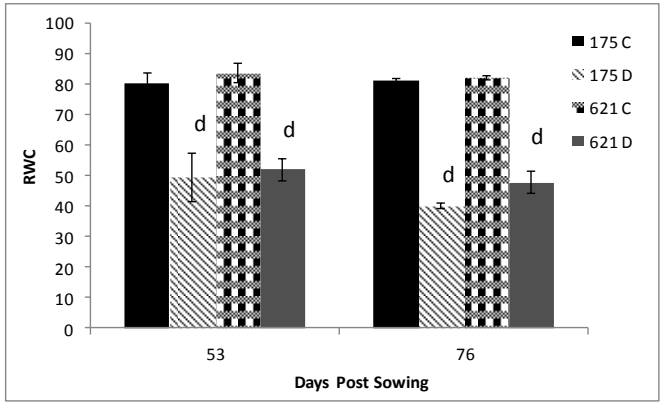
Fig-1

A



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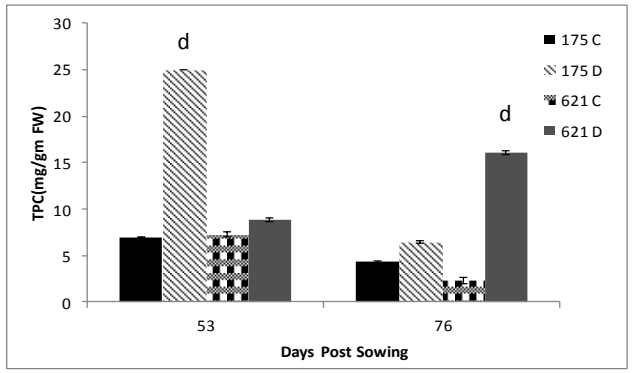

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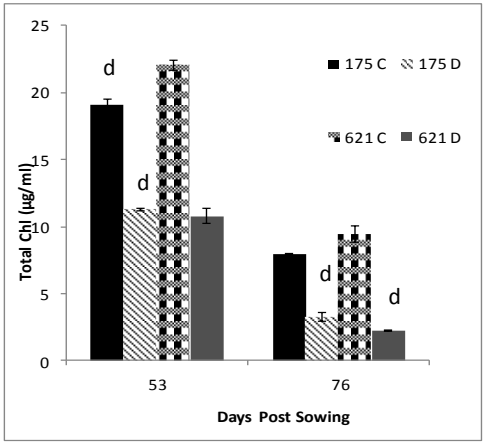

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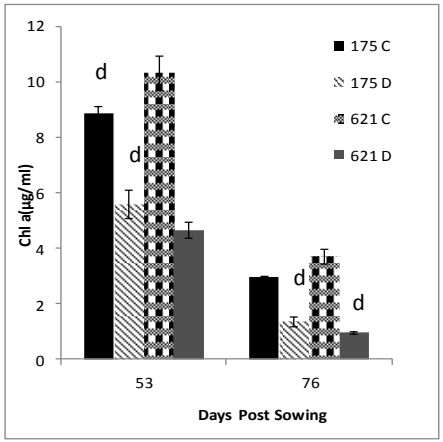

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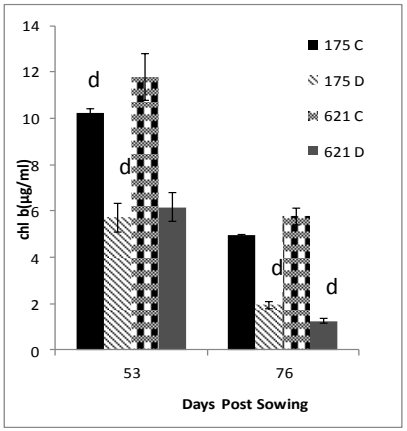
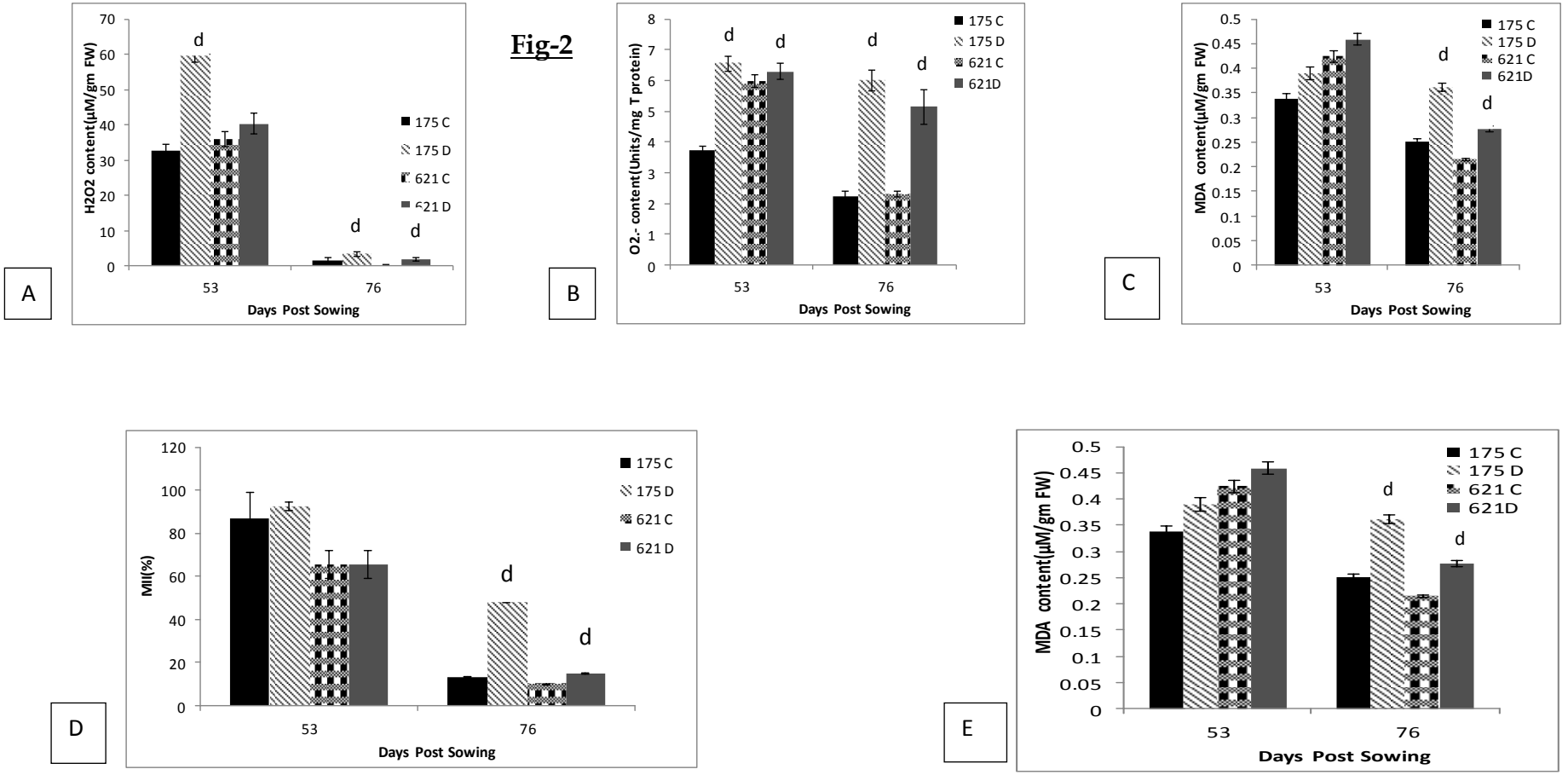


Fig-2



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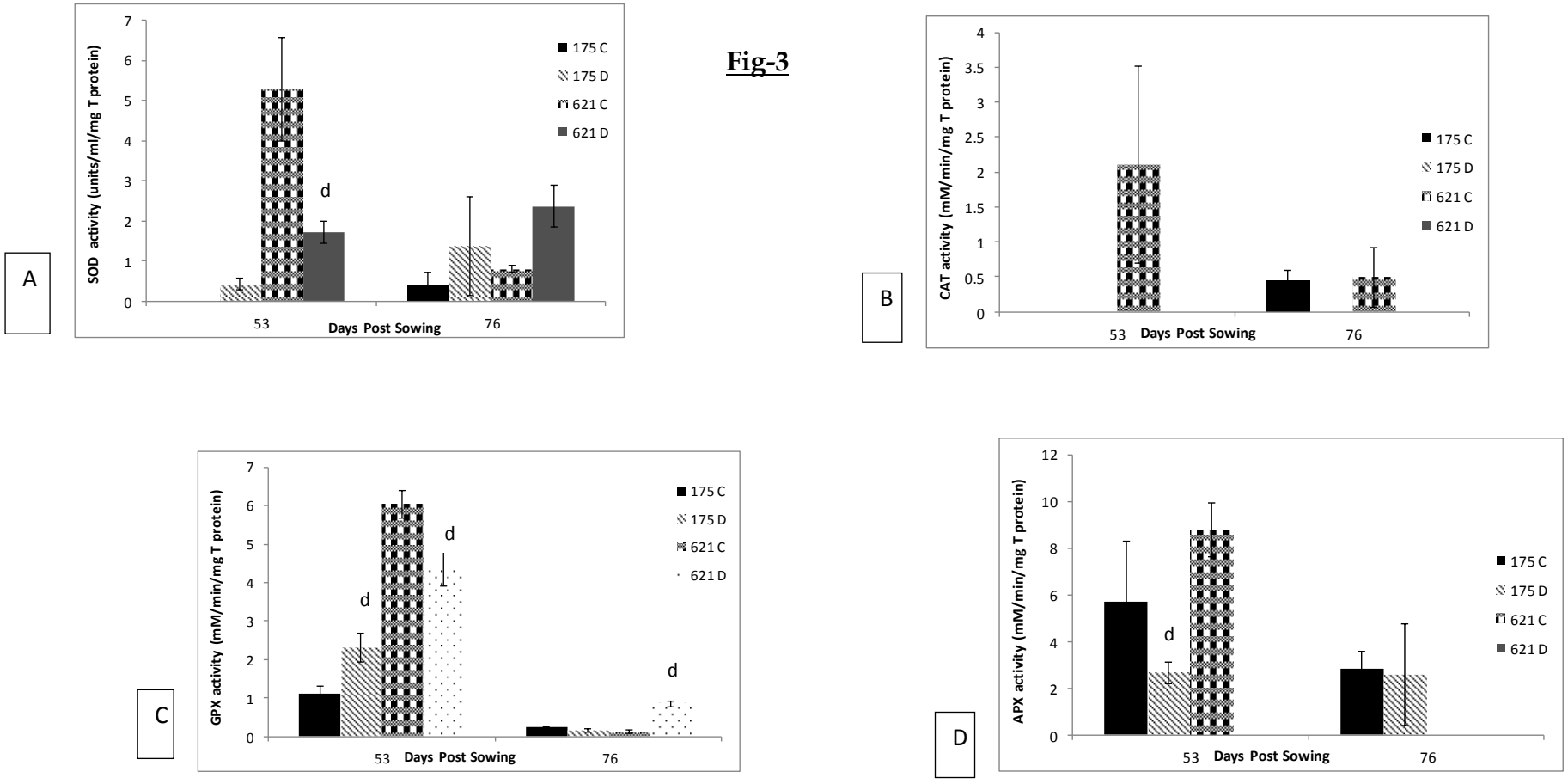

Fig-3

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