response of reactive oxygen species production and antioxidant metabolism to putrescine under water stress in wheat leaves

BY

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Dedicated
To My Family
and My Nephew
Kushagra...

CERTIFICATE - I

This is to certify that this dissertation entitled, **"Response of reactive oxygen species production and antioxidant metabolism to putrescine under water stress in wheat leaves",** submitted in partial fulfillment for the degree of **Master of Science** in the subject of **Biochemistry**, to the Chaudhary Charan Singh Haryana Agricultural University, Hisar, is a bonafide research work carried out by **Ms. Vinita Bhankar** under my supervision and that no part of this dissertation has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.

Dr. (Mrs.) Veena Sawhney

Major Advisor

CERTIFICATE - II

This is to certify that this dissertation entitled, **"Response of reactive oxygen species production and antioxidant metabolism to putrescine under water stress in wheat leaves",** submitted by **Ms. Vinita Bhankar** to the CCS Haryana Agricultural University, Hisar in partial fulfillment of the requirements for the degree of **Master of Science** in the subject of **Biochemistry**, has been approved by the Student's Advisory committee after an oral examination on the same.

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CHAPTER -1

INTRODUCTION

Drought is among the most limiting abiotic factors affecting wheat production under fragile arid ecosystem**.** It results in a decrease in foliage area, biomass and hence yield (Schwanz and Polle, 2001). In the last decade, our understanding of the processes underlying plant response to drought, at the molecular and whole plant levels, has rapidly progressed. Water stress causes stomatal closure which reduces the CO_2/O_2 ratio in leaves and inhibits CO_2 fixation while it continues to harvest light energy (Schwanz and Polle, 2001). To protect the photosynthetic apparatus from photo-oxidative destruction plants must dissipate excess light energy by down-regulation of the photochemical efficiency via the action of the xanthophyll cycle (Demmig-Adams *et al.*, 1996) or by maintenance of electron flux involving alternative pathways as 'redox valves', for example, photorespiration and the Mehler-peroxidase reaction (Foyer and Harbinson, 1994; Asada, 1999). Under these circumstances cellular redox homeostasis is disrupted which can lead to the generation of reactive oxygen species (ROS), such as superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals (HO) and singlet oxygen ($^1O_2$). ROS are produced naturally during cell metabolism in photosynthesis, photorespiration, fatty acid oxidation,

senescence and other processes (Turpaev, 2002). During these processes ROS concentration are low, but on onset of senescence or intense stress, ROS accumulate and their concentration become above a certain 'threshold' level (Turpaev, 2002). Chloroplasts, mitochondria and peroxisomes are thought to be the most important generators of ROS (Fridovich, 1986; Asada, 1999). During dessication, in chloroplasts electrons leak from PS II and react with O_2 to generate ROS (Vertucci and Farrant, 1995). Isolated mitochondria produce H_2O_2 and O_2^- in the presence of NADH (Loschen *et al.*, 1974). The superoxide radical (O_2^-) and H_2O_2 are themselves deleterious to the cellular constituent and they further potentiate the production of most dangerous hydroxyl radicals (Noctor and Foyer, 1998). The free radicals disrupt normal metabolism through peroxidating lipids, denaturing proteins and nucleic acids (Jiang and Zhang, 2001; Bor *et al.*, 2003). Lipid peroxidation causes degradation and impairment of structural components (Lester and Stein, 1993) leading to change in selective permeability of bio-membranes (Weckx and Clijsters, 1997) and thereby membrane leakage and change in activity of enzymes bound to the membrane (Lester and Stein, 1993). These hasten the loss of membrane integrity and cell metabolites such as sugar, protein, and phenols etc. (Lott *et al.*, 1991). Plants can respond and adapt to water stress by altering their cellular metabolism by invoking various defense mechanisms (Bohnert and Jenson, 1996), such as enzymatic and non-enzymatic

antioxidant system to scavenge ROS (Foyer *et al.*, 1994; Tambussi *et al.*, 2002). Antioxidant system includes low molecular weight compounds like carotenoids, ascorbate, glutathione, α-tocopherols and anthocyanin pigments (Del Rio *et al.*, 2002; Neill *et al.*, 2002) and enzymes such as superoxide dismutase (SOD, E.C.1.15.1.1), catalase (CAT, E.C.1.11.1.6), glutathione peroxidase (GPX, E.C.1.11.1.9), peroxidases (EC 1.11.1.7) and the enzymes of ascorbate-glutathione cycle (Foyer and Halliwell, 1976); ascorbate peroxidase (APX, E.C.1.11.1.1) dehydroascorbate reductase (DHAR, E.C.1.8.5.1), monodehydroascorbate reductase (MDHAR, E.C.1.6.5.4) and glutathione reductase (GR, E.C.1.6.4.2). Though the antioxidant defense system protects the plant from oxidative damage upto some extent, but the defense system can be overwhelmed under higher stress level when it is unable to remove all the toxic reactive molecular species. If at this stage some other antioxidant metabolites are provided to plants exogenously which along with plant's own defense mechanism, ameliorate stress symptoms, Polyamines are one of them. They are implicated in wide range of biological processes (Evans and Malmberg, 1989; Martin-Tanguy, 2001). A number of physiological studies indicated important roles of polyamines in plant defense to a wide array of environmental stresses (Bouchereau *et al.*, 1999; Shen *et al.*, 2000; Bais and Ravishanker, 2002; He *et al.*, 2002). The diamine putrescine, the triamine spermidine and the tetramine spermine are ubiquitous in plant cells, while other polyamines are of limited occurrence (Galston and Sawhney, 1990) and they have

been found to play important role in stress management (Walden *et al.*, 1997). They appear to be localized in the vacuole or cytosol of plant cells (Bagni *et al.*, 1981). They are low molecular weight polycations which are present in cells in very low concentration. The cationic nature of free polyamines (especially putrescine, spermidine and spermine) at physiological pH allows them to interact with negatively charged molecules such as nucleic acids, phospholipids and proteins to protect them from metal induced oxidative damage (Cohen, 1998). They have also been suggested to function as metal chelators (Bors *et al.*, 1989) as well as direct or indirect free radical scavengers (Drolet *et al.*, 1986). Polyamines at physiological pH might serve as counter ions and play important role in maintaining cellular homeostasis (Smith, 1985).

In order to study ROS production and antioxidant metabolism in wheat leaves under water stress and the ameliorating effect of putrescine, the present investigations were carried out with the following objectives:

1. To study the effect of water stress on generation of ROS and their scavenging by antioxidant metabolism.
2. To study the ameliorating effect of exogenous putrescine on production of ROS and antioxidant metabolism.

CHAPTER-2

REVIEW OF LITERATURE

Stress is being defined as any environmental factor capable of inducing a potentially injurious response in plants (Cowan, 1994). With an increase in human population, particularly in developing countries, there is an urgent need for expanding its cultivation from irrigated or relatively high rainfall areas to non-irrigated low rainfall areas where drought stress can be a common phenomenon. Water stress is one of the most important environmental stresses influencing the productivity of agricultural systems around the world. Its impact on losses of production in semi-arid regions of developing countries, can be devastating (Hamada, 2000). It causes reduced plant growth, yield and biomass and there are a complex of responses at molecular, cellular, physiological and developmental levels (Ingram and Bartels, 1996). It has been well reported that much of the injury to plant caused by stress is associated with oxidative damage at cellular level (Allen, 1995). Severe dehydration increased the levels of reactive oxygen species (ROS), such as superoxide anion (O_2^-), H_2O_2, hydroxyl radicals ($HO\cdot$) and singlet oxygen ($^1O_2$), which damage cellular structures and macromolecules (Smirnoff, 1993). At an early stage they also act as signal molecules that activate multiple defense responses (Van Breusegem *et al*., 2001;

Vranova *et al*., 2002). Chloroplast, mitochondria and peroxisomes are the major sites of ROS production in plant cells (Asada, 1999). However plants use enzymatic and non-enzymatic antioxidant defense mechanisms to scavenge ROS (Foyer *et al*., 1994; Tambussi *et al*., 2002). The enzymatic system includes superoxide dismutase, catalase, peroxidase and enzymes of the ascorbate-glutathione cycle, such as ascorbate peroxidase, glutathione reductase and dehydroascorbate reductase. Metabolites such as ascorbate, glutathione, α-tocopherol and carotenoids also contribute to control the levels of ROS in plant tissues (Noctor and Foyer, 1998). Moreover a number of physiological studies have indicated important roles of polyamines in plant defense to a wide array of environmental stresses (Bouchereau *et al*., 1999; Shen *et al*., 2000). The link between polyamines and abiotic stress was first documented through putrescine accumulation in response to salt stress in barley (Watson and Malmberg, 1996). Since then, a connection has been suggested between increased putrescine levels and abiotic stress. Although sufficient information is available for the accumulation of polyamines in stress conditions, very little information is available on their role in the mechanism of stress tolerance.

The available literature on the effect of water stress and putrescine on antioxidant defence system has been reviewed under the following headings:

2.1 Effect of water stress and putrescine on plant water relations.

2.2 Effect of water stress and putrescine on production of various reactive oxygen species.

2.3 Effect of water stress and putrescine on damage to cell membrane.

2.4 Effect of water stress and putrescine on some antioxidant enzymes.

2.5 Effect of water stress and putrescine on antioxidant metabolites.

2.1 Effect of water stress and putrescine on plant water status

2.1.1 Relative water content (RWC)

RWC is considered as an alternative measure of plant water status reflecting the metabolic activity in tissue affected by various types of stresses (Weatherly, 1950). Genotypic variations in RWC of several stressed crop plants were evident from the studies of Nagy *et al.* (1995) and El Hafid *et al.* (1998). Relatively higher RWC have been reported in drought tolerant cultivars of wheat (Martin *et al.*, 1997) and rice (Tyagi *et al.*, 1999). Dalmia and Sawhney (2004) observed that RWC in wheat seedlings decreased progressively with incressing level of water stress. Similar, response was reported under water stress in maize leaves (Jiang and Zhang, 2002), jute plants (Chowdhury and Choudhuri, 1985), Vigna seedlings (Mukherjee and Choudhuri, 1985), French bean cultivars (Upreti and Murti, 2005) and in contrasting genotypes of wheat (Gupta *et al*., 2005). Molnar *et al.* (2004) correlated mild osmotic stress (-0.7 M Pa) to a slight reduction in the RWC of the leaves of *Aegilops biuncialis* and *Triticum aestivum* genotypes. Short term NaCl stress was reported to produce reversible effects on RWC in pea plants

(Hernandez and Almansa, 2002). However significant increase in RWC in the stressed pea plants by 1mM spermine was observed by Upreti and Murti (1999). Meanwhile Vakharia *et al.* (2003) maintained that 0.1 mM putrescine treated groundnut seedlings had greater retension of moisture content as compared to non treated seedlings.

2.1.2 Osmotic potential

Osmotic potential is another physiological parameter used for recording the extent of stress level in plants and it is decreased during water stress (Munns and Cramer, 1996; Maldonado *et al*., 1997; Upreti *et al*., 1998; Singh *et al*., 1972, 1973). Reduction in osmotic potential under drought was observed by Dalmia and Sawhney (2004) in wheat seedlings which was -0.7, -0.96, -1.23 and -1.76 MPa corresponding to 27, 63, 86 and 162% decrease in their osmotic potential after 2, 4, 6 and 8 days of PEG application, respectively. Similarly during drought stress in olive trees, predawn leaf water potential fell from -0.37 to -5.37 MPa (Sofo *et al.*, 2005). It was observed that the tolerant genotype of rice showed lower osmotic potential while susceptible ones had higher (more negative value) osmotic potential under water stress (Tyagi *et al*., 1999). Lower osmotic potential in tolerant genotypes of wheat (Martin *et al*., 1997), chickpea (Deb *et al*., 1996) and rice (Dingkuhn *et al*., 1991) have also been reported.

2.2 Effect of water stress and putrescine on production of reactive oxygen species (ROS)

The production of ROS and their scavenging have been extensively reviewed by Apel and Hirt (2004). Prolonged exposure to the conditions that cause excess excitation energy can result in an increase in the generation of ROS such as $^1O_2$, O_2^-, and H_2O_2 (Karpinski *et al*., 1997, 1999; Yoshimura *et al*., 2000). The formation of O_2^- radicals and H_2O_2 had been reported to increase under various environmental stresses like osmotic stress in spring wheat seedlings (Li *et al*., 2004), drought stress in maize leaves (Jiang and Zhang, 2002), chilling stress in cucumber plants (Lee and Lee, 2000), excess light stress in pea cvs (Hernandez *et al*., 2004), high radiation treatment in intact wheat leaves (Misha *et al.*, 1993) and 50 μM paraquot treatment in maize leaves (Jiang and Zhang, 2002). Damage of leaves due to air pollutants such as SO_2 and O_3 or photodynamic herbicides such as paraquot has also been mediated through the excessive production of ROS (Mehlhorn *et al*., 1990; Foyer and Mullineaux, 1994; Foyer *et al*., 1994; Okpodu *et al*., 1996). Polyamines have ROS scavenging and membrane protecting properties (Roberts et al., 1986; Lovaas, 1991; Groppa *et al*., 2001). For example spermidine accounted for the enhanced tolerance to various environmental stress in transgenic *Arabidopsis* (Yoshihisa *et al*., 2004). Xu *et al.,* (2001) observed that exogenous polyamines application inhibited the accumulation of O_2^- and H_2O_2 in wheat seedlings under osmotic stress.

2.2.1 H_2O_2

H_2O_2 is natural plant metabolites (Halliwell, 1974, 1977), produced as a result of scavenging of O_2^- by superoxide dismutase. It is also formed via photorespiration and during the activity of several oxidase enzymes. The decrease in catalase enzyme which is responsible for its breakdown, allow its accumulation (Kar and Mishra, 1976; Patra *et al*., 1978; Biswas and Choudhuri, 1978). Its higher concentration is injurious as it causes lipid peroxidation and membrane injury (Nayar and Kaushal, 2002). H_2O_2 content was reported to increase in cucumber plants under chilling stress (Lee and Lee, 2000), in pea cultivars under excess light stress (Hernandez *et al*., 2004), in maize leaves (Jiang and Zhang, 2002) and in seedlings of contrasting wheat genotypes under water stress (Gupta *et al*., 2005). The level of H_2O_2 in water stressed tissue has been found to be function of magnitude of water stress (Mukherjee and Choudhuri, 1985). Pretreatment with spermidine and spermine prevented the enhancement of H_2O_2 in acid rain treated bean plants and the protective effect of spermine was comparatively higher (Velikova *et al*., 2000). Similarly, Li *et al*. (2004) observed that exogenous spermine significantly reduced the production rate of H_2O_2 in spring wheat seedlings under osmotic stress. Shen *et al.* (2002) made observation that the pretreatment with spermidine prevented chill induced increase of H_2O_2 content in cucumber leaves. Inclusion of putrescine in nutrient medium reduced H_2O_2 level

considerably (50%) in leaves of control seedlings as well as in stressed mustard seedlings (Verma and Mishra, 2005).

2.2.2 O_2^- radicals

O_2^- radicals production is enhanced by reduction of O_2 at PSI in water stressed plants (Quartacci and Navari-Izzo, 1992). Jiang and Zhang (2002) observed that the production of O_2^- radicals increased in stressed maize leaves by 41% as compared to the controls. However, Drolet *et al.* (1986) reported that O_2^- radicals formed either enzymatically with xanthine oxidase or chemically from riboflavin or pyrogallol were significantly inhibited by exogenous application of polyamines. Similarly its production rate was found to decline by exogenous polyamines in wheat seedlings (Xu *et al*., 2001). Infact its level which was 240 μmol s^{-1} under normal growth condition was increased to 240-720 μmol s^{-1} under various types of stresses (Polle, 2001). Exogenous putrescine also caused 60% reduction in O_2^- radicals production under mild salt stress and 10-17% at higher salinity in Indian mustard seedlings (Verma and Mishra, 2005).

2.3 Effect of water stress and putrescine on cell membranes

2.3.1 Electrolyte leakage

The leakage of electrolyte under stress condition has been widely accepted as selectable marker for indexing stress tolerance (Upreti and Murti, 2005). Increased

electrolyte leakage was reported in Vigna seedlings under water stress (Mukherjee and Choudhuri, 1985) and in rice seedlings under salinity stress (Dionisio-Sese and Tobita, 1998). Choudhuri and Choudhuri (1993) and Liang *et al.* (2003) proposed that accumulation of H_2O_2 led to lipid peroxidation, causing membrane damage and leakage of various micro- and macromolecules and electrolytes out of the cell. Upreti and Murti (2005) demonstrated that the tolerant cultivar of French bean i.e. Contender, maintained stable electrolyte leakage than the susceptible cv. Arka Suvidha. However, Kuk (2003) reported that the electrolyte leakage was consistently lower in cold-acclimated leaves than in non-acclimated leaves during the chilling period. After 3 days in the recovery environment (25ºC), cellular leakage from cold-acclimated leaves returned to the baseline levels observed during acclimation. The effects of polyamines in reducing membrane leakage have been described in various crops under different types of stress (Tiburcio *et al*., 1994; Borrell *et al*., 1997). Spermine has been shown to counter the stress induced electrolyte leakage in mung bean seedlings (Basra *et al*., 1997). Bors *et al.* (1989) also suggested that the root application of polyamines prevented leaf injury of the ozone sensitive tobacco CV Bel W3, caused by ozone treatment. Altman (1982) observed that polyamines significantly decrease the leakage of betacyanin through membrane during ageing of beet root dics in-vitro. Shen *et al.* (2000) also reported that spermidine pretreatment of cucumber seedlings growing under chilling condition checked H_2O_2 production and prevented electrolyte leakage. Verma and

Mishra (2005) showed that in Indian mustard, electrolyte leakage under higher salinity stress, was reversed upto 27% by putrescine application. Velikova *et al.* (2000) proposed that polyamines may prepare the cell to meet and combat stress by stabilizing membranes and inducing higher 'buffering' and antioxidant capacity.

2.3.2 Lipid peroxidation

Sairam *et al.* (2000) reported the rise in lipid peroxidation, which is measured as MDA content, under water stress and similar results were obtained by Singh and Verma (2001). They reported that 5-6 fold increase in MDA content in pB1121, grown on medium containing Na (200 mM) and Cd (20 μM) was an indication of the extent of lipid peroxidation due to free radical generation. The increased lipid peroxidation has also been reported in *Morus alba* (Sudhakar *et al*., 2001), *Lycopersicon esculentum* (Mittova *et al*., 2002), *Beta vulgaris* (Bor *et al*., 2003), *Oryza sativa* (Vaidyanathan *et al*., 2003), *Gossypium hirsutum* (Meloni *et al*., 2003) and *Cicer arietinum* (Kukreja *et al*., 2006) under various types of stresses. Lower membrane stability has also been reported in susceptible genotypes of wheat (Sairam *et al*., 1997, 1998), maize (Pastori and Trippi, 1992) and chickpea (Deb *et al*., 1996). Polyamines inhibit lipid peroxidation, due its ability to form a ternary complex with iron and the phospholipids polar heads (Tadolini, 1988). Exogenous polyamines were able to reduce the level of MDA in dark incubated *Avena sativa* L. cv. Victory leaves, as reported by Borrell *et al.* (1997), thus they had a significant influence on the reduction in lipid peroxidation and they

most likely did so by scavenging free radicals (Drolet *et al*., 1986). Kitada *et al.* (1979) observed that during chilling stress, spermidine pretreated leaves had significantly lower MDA content in cucumber cultivars through prevention of O_2^- generating NADPH oxidase activation. Further, Li *et al.* (2004) studied lipid peroxidation in terms of TBARS content using TBA reaction and membrane stability index (MSI). Treatment with spermine reduced TBARS content and promoted MSI significantly after 4 and 24 hrs of root osmotic stress in spring wheat seedlings. Similarly it was reported that acid rain treatment induced lipid peroxidation in bean leaves and exogenous spermidine and spermine prevented lipid peroxidation and the protective effect of spermine was higher (Velikova *et al*., 2000). Verma and Mishra (2005) also observed that putrescine supplementation to the salt stressed *Brassica juncea* plants reduced the MDA production by 10-40% depending on salinity level.

2.4 Effect of water stress and putrescine on antioxidant enzymes

2.4.1 Catalase

Catalase is an important antioxidant enzyme which is localized in peroxisomes and is involved in detoxification of H_2O_2. Catalase activity has been reported to change under various abiotic stresses like water stress (Jiang and Zhang, 2002; Dalmia and Sawhney, 2004), salinity (Venkateshan and Chellappan,

1999,) chilling (Nayar and Kaushal, 2002), intermittent anoxia (Goggin and Colmer, 2005) and acid rain treatment (Velikova *et al*., 2000). Increased catalase activity had been reported in maize leaves (52%) under 12 hours of water stress (Jiang and Zhang, 2002), in leaves and fine roots of olive trees under severe water deficit stress (Sofo *et al*., 2005) and in *Ipomoea pes-caprae* sweet (a halophyte) at 200mM salinity stress (Venkateshan and Chellappan, 1999) and it was suggested that high catalase activity is essential for the survival of the halophyte (Kalir and Polja Koff-Mayber, 1981). The increase was more conspicuous in the salt tolerant than in the salt sensitive genotypes. Similar results have been observed with *Beta maritime* (halophyte) and the non-halophyte, *Beta vulgaris* (Bor *et al*., 2003), rice (Sudhakar *et al*., 2001) and wheat (Sairam *et al*., 2002), differing in salt tolerance. The tolerant genotypes of chickpea showed significantly higher catalase activity in comparison to susceptible genotypes at both pre and post flowering stages (Singh *et al*., 2005). Kuk (2003) found higher catalase activity in leaves of cold-acclimated plants suggesting a more efficient scavenging of H_2O_2. It was suggested that the higher activities of catalase and ascorbate peroxidase might have removed H_2O_2, produced by water stress (Sairam *et al*., 2000; Gupta and Gupta, 2005). Nayar and Kaushal (2002) also reported that the increased activity of catalase and peroxidase enzymes constitute a potential defense mechanism against chilling induced oxidative damage in germinating wheat grains. In contrast, catalase activity was observed to decline in cucumber plants under chilling stress

(Lee and Lee, 2000), in bean plants after few hours of acid rain treatment (Velikova *et al*., 2000). Venkateshan and Chellappan (1999) reported that NaCl (0.6%) depressed catalase activity in gram but not in wheat. Similarly, 30% decrease in catalase activity after 8 hours of treatment with 0.5 ppm ozone was observed (Tanaka *et al.,* 1985). However exogenous application of spermine inhibited the catalase activity in seeds and roots of chickpea (*Cicer arietinum* L.) CV JG62 (Keshamma *et al*., 2004) though Velikova *et al.* (2000) did not find significant effect of pretreatment with polyamines on catalase activity at pH 5.6, but Li *et al.* (2004) obtained significant increase of catalase activity in pretreated spring wheat seedlings with spermine under 4 and 24 hours of root osmotic stress. Verma and Mishra (2005) also reported increased catalase activity (15-85%) by putrescine depending on salinity level.

2.4.2 Peroxidase (POD)

Peroxidases are non-specific enzymes and are known to utilize different substrates to metabolize H_2O_2 using preferably some phenolic compounds (eg**.** guaiacol) as primary reducing agents (Mehlhorn *et al*., 1996). Peroxidase activity has been shown to be stimulated under various abiotic stresses like drought (Sofo *et al*., 2005), salinity (Venkateshan and Chellappan, 1999), chilling stress (Lee and Lee, 2000), and in iron (Hendry and Brocklebank, 1985) and aluminium toxicity (Cakmak and Horst, 1991). An increased activity of peroxidase in pea and cotton under NaCl stress was also shown by Strogonov (1964). Similar results were

obtained by Sofo *et al.* (2005) in olive trees under drought stress. However guaiacol POD acitivity was not affected in cucumber roots under Fe deficiency stress (Zaharieva *et al*., 1999). Enhancement in POD activity under various stress conditions was linked to protection from oxidative damage, lignification and cross-linking of cell wall to protect from such adverse conditions (Dalal and Khanna-Chopra, 2001). Treatment with spermine promoted the activity of peroxidase significantly after 4 and 24 hrs of root osmotic stress in spring wheat seedlings (Li *et al.,* 2004) and in seeds and roots of chickpea (Keshamma *et al*., 2004). Verma and Mishra (2005) also reported that treatment with putrescine increased peroxidase activity in salt stressed *Brassica juncea* seedlings. However, pretreatment with polyamines had no significant effect on peroxidase activity at pH 5.6 under acid rain (AR) in bean plants (Velikova *et al.,* 2000) while application of spermidine and spermine lead to a reduction of peroxidase activity in AR treated plants amounting to 92% (Spd + AR 3rd hr), 84 and 88% (Spd + AR, 3rd and 5th hr) decrease, respectively.

2.4.3 Ascorbate peroxidase (APX)

Ascorbate peroxidase is one of the most widely distributed antioxidant enzyme in chloroplast and it reduces H_2O_2 to water using ascorbate as the electron donor (Mehlhorn *et al*., 1996). Oxidative stress can induce or enhance APX activity (Mehlhorn *et al*., 1987) as observed by Noctor and Foyer (1998); Knorzer *et al.* (1996); Gullner *et al*. (1991) in various plants. The important role of APX in

relation to increasing oxidative tolerance has been observed in many plants (Feierabend *et al*., 1992; Gupta *et al*., 1993). Dalmia and Sawhney (2004) reported that under drought stress in wheat seedlings, APX activity was 2.3 and 2.7 folds higher at 2 and 8 days after stress treatment respectively. APX activity was found to increase under drought stress in sunflower (Zhang and Kirkham, 1996) and olive trees (Sofo *et al*., 2005). Enhancement in APX activity was observed due to chilling stress in cucumber roots (Zaharieva *et al*., 1999), salt stress in rice (Vaidyanathan *et al*., 2003) and Fe deficiency stress in cucumber roots (Zaharieva *et al*., 1999). Jiang and Zhang (2002) obtained 33% higher APX activity in maize leaves after 12 hours of water stress treatment. Similarly, Kukreja *et al.* (2006) reported that in chickpea roots, ascorbate peroxidase activity increased by 9% at 2.5 dSm^{-1} salinity levels, whereas it increased by 99% at 10 dSm^{-1}. However, in pea plants, short term NaCl stress produced reversible effects on APX activity (Hernandez and Almansa, 2002). Pastori and Trippi (1993) suggested that a drought tolerant wheat (*Triticum aestivum* L.) line with high tolerance to photo-oxidant stress, was correlated with higher level of APX activity. Treatment with spermine promoted the activity of APX significantly after 4 and 24 hrs of root osmotic stress in spring wheat seedlings (Li et al., 2004). Similarly Verma and Mishra (2005) observed that putrescine caused 1.5-2.5 fold increase in APX activity, while the plants exposed to 200 mmol/L NaCl, showed little increase in activity.

2.4.4 Dehydroascorbate Reductase (DHAR)

Dehydroascorbate reductase is the enzyme involved in H_2O_2 detoxification (Dipierro and Borraccino, 1991). Dehydroascorbate is reduced to ascorbate by the action of DHAR, using glutathione as the reductant (Foyer and Halliwell, 1976). Dalmia and Sawhney (2004) showed a linear increase in its activity with various stress levels. Similarly increased DHAR activity was observed in *Sporobolius stapfianus* (Sgherri *et al*., 1994), sorghum and sunflower during water stress (Zhang and Kirkham, 1996). Vaidyanathan *et al.* (2003) also reported an increased DHAR activity in rice under NaCl stress. In contrast to these results Knorzer *et al.* (1996) found a significant decrease in DHAR activity in suspension cultured soybean cells (*Glycine max*) under stress conditions induced by oxyfluorfen concentrations between 50 and 500 mM. DHAR activity was also reported to decline rapidly during germination in wheat seeds (Cakmak *et al*., 1993), in chloroplasts isolated from *Lycopersicon pennellii* plant under salt stress (Mittova *et al*., 2002) and in plant tissues exposed to low temperature stress (Jahnke *et al*., 1991; Kuroda *et al.,* 1991).

2.4.5 Glutathione Reductase (GR)

Glutathione reductase is an essential catalyzer which maintain the redox state of ascorbate and glutathione (Foyer *et al.*, 1994). GR has been implicated as an important protective agent against oxidation damage in many plants (Foyer *et al*., 1991). Schmidt and Kunert (1986) reported that osmotic stress can induce or

enhance GR activity. The activity of GR was found to increase linearly with various water deficit stress levels in wheat (Dalmia and Sawhney, 2004). Similarly the activity of GR increased by 38% in maize leaves after 12 hours of water stress treatment (Jiang and Zhang, 2002), by 50% under both continuous and intermittent anoxia (Goggin and Colmer, 2005), in rice seedlings under salt stress (Vaidyanathan *et al.,* 2003) and in cucumber plants under chilling stress (Lee and Lee, 2000). Verma and Mishra (2005) reported increased (90-110%) GR activity in *Brassica juncea* seedlings with increase in salinity level (100-250 m mol/L). High GR activity had been observed in salt tolerant rice (Dionisio-Sese and Tobita, 1998), cotton (Gossett *et al*., 1994), pea (Hernandez *et al*., 2001) and wheat (Sairam and Srivastava, 2002). Pastori and Trippi (1993) correlated drought tolerant wheat (*Triticum aestivum* L.) lines with higher levels of GR activity. The activity of GR were also induced by eosin (singlet O_2 generating compound) in light dependent reactions (Gullner and Dodge, 2000). In contrast, decreased GR activity was observed in *Scenedesmus bijugatus* under copper stress (Nagalakshmi and Prasad, 2001), whereas GR activity remained unchanged in chloroplasts isolated from *Lycopersicon pennellii* plants subjected to salt stress (Mittova *et al*., 2002). However, Putrescine treatment elevated the GR activity by 37 to 67% under salt stress. It was suggested that the putrescine elevated GR activity could increase the ratio of $NADP^+$/NADPH, thereby ensuring the availability of $NADP^+$ to accept electron resulting into lesser flow of electron to O_2 for ROS generation

(Verma and Mishra, 2005). Increased GR may also maintain a high ratio of GSH/GSSG which is required for the regulation of ascorbate threshold level and activation of several CO_2 fixing enzymes (Foyer and Halliwell, 1976).

2.4.6 Superoxide Dismutase (SOD)

Superoxide dismutase is a group of metalloenzymes that catalyze the dismutation of O_2^- to H_2O_2 and O_2. Thus it plays an important role for protection against superoxide derived oxidative stress in plant cells (Asada and Kiso, 1973; Fridovich, 1986). Various isoforms –Cu/Zn-, Mn- or Fe-SOD regulate the levels of O_2^- produced in chloroplast, mitochondria and cytosol (Bowler *et al*., 1992). Oxidative stress can induce or enhance its activity (Bowler *et al*., 1992; Scandalios, 1990). Increased SOD activity was reported under water stress in leaves and roots of olive trees (Sofo *et al.*, 2005), in maize leaves (Jiang and Zhang, 2002) and in two genotypes of wheat (Gupta *et al.*, 2005). Lee and Lee (2000) also reported that chilling stress induced a significant increase of total SOD activity. Kukreja *et al.* (2006) observed 2-fold increase in SOD activity in chickpea roots under short-term salinization. Induction of SOD have also been obtained in *Distichlis spicata* (Daines and Gould, 1986) under salt stress. However, Cherian and Reddy (2000) observed 85% decrease in SOD activity at low salinity level, but it increased by 304% at higher salinity in the halophyte; *Suaeda nudiflora* MOQ. Bowler *et al.* (1992) suggested that high SOD activity has been associated with

stress tolerance in plants where overproduction of O_2^- is involved. Recent studies have demonstrated that overexperssion of mitochondrial Mn-SOD in transgenic *Arabidopsis thaliana* (Wang *et al*., 2004) and chloroplastic Cu/Zn-SOD in transgenic *Nicotiana tabacum* (Badawi *et al.,* 2004a) can provide enhanced tolerance to salt stress. Similar results have been found in *Morus alba* (Sudhakar *et al*., 2001), *Triticum aestivum* (Sairam *et al*., 2002) and Lycopersicon sp. (Mittova *et al*., 2002). It was proposed that increased SOD activity countered Zn stress in rye grass given 50 mM Zn in the nutrient solution (Bonnet *et al*., 2000). SOD activity was reported to decline in pea plants (Hernandez *et al*., 1995, 1993), in cowpea plants (Hernandez *et al*., 1994), and in rice seedlings (Dionisio-See and Tobita, 1998) under salt stress and in susceptible genotypes under Zn-stress (Cakmak and Marschner, 1988). Its activity was decreased by 20% under continuous anoxia but it ultimately returned to aerated activities under intermittent anoxia (Goggin and Colmer, 2005; Sarkar *et al*., 2001).

Li *et al.* (2004) reported that treatment with spermine promoted the activity of SOD significantly after 4 and 24 hours of root osmotic stress in spring wheat seedlings. Verma and Mishra (2005) observed a steep rise in SOD activity (60-85%) in *Brassica juncea* plants exposed to moderate to high salt stress (100-250 mmol/L NaCl) which was further increased by exogenous putrescine. They proposed that the increased activity of SOD due to putrescine under salinity

might be able to protect the biomolecules from attack of O_2^- radicals. Bouchereau *et al.* (1999) reported that putrescine could bind to SOD and allow them to reach to the sites of oxidative stress within the cells. It was suggested that a covalent putrescine-SOD complex was 20-fold more membrane permeable than SOD alone.

2.5 Effect of water stress and putrescine on antioxidant metabolites

2.5.1 Glutathione

The reduced form of glutathione (GSH) is a tripeptide (glu-cys-gly) that exists interchangeably with its oxidized form, GSSG. GSH is important antioxidant and a redox buffer (Foyer, 1993; Kunert and Foyer, 1993; Law *et al*., 1983; Meister, 1994). Glutathione plays an active role in protecting membrane against free radical damage (Rennenberg and Brunold, 1994; Rennenberg, 1995). It is involved in quenching the free radicals through the ascorbate-glutathione cycle (Smirnoff, 1995). Dalmia and Sawhney (2004) reported that glutathione increased several folds with onset of drought stress in wheat seedlings, but decreased at higher stress level, while ratio of GSH/GSSG decreased from 4.7:1 on 2 days after stress (DAS) to 1:1 on 8 DAS. A similar trend of a rapid increase in glutathione pool sizes as an early response of the plants to the stress has been reported previously (Foyer *et al*., 1994). Implication of glutathione redox pair in the defence against oxidative stress has been given by Potters *et al.* (2002). An elevated level of oxidized glutathione was probably emanated due to scavenging of the reactive oxygen species produced with increasing intensity of stress and

resulting in conversion of GSH to GSSG as suggested by Dhindsa (1987). Vaidyanathan *et al.* (2003) reported that the glutathione content was higher in salt tolerant cultivar of rice than in salt sensitive cultivar under salt stress. Gullner and Dodge (2000) showed that the foliar glutathione levels were considerably increased by singlet oxygen generating substances. Oxidative stress led to an increase in total glutathione content (Mehlhorn *et al*., 1986) and in particularly GSH levels in plants (Komives *et al*., 1998; Noctor *et al*., 1998; May *et al*., 1998). Knorzer *et al*. (1996) obtained nearly 3 fold increase in glutathione content in the presence of 250 nM oxyfluorfen in osmotically stressed soybean cells (*Glycine max*). Under this condition oxidised glutathione (GSSG) was 5 times above the control levels. Karpinski *et al.* (1997) reported that the GSH levels or redox status of glutathione could have a regulatory impact on the signalling pathway from the chloroplast to the nucleus during excess light stress. It was further proposed that glutathione is an indicator of stress resistance and showed almost 2-folds difference between control and transgenic lines of tobacco under oxidative stress (Singh and Verma, 2001). Recently, Yu *et al.* (2002 and 2003) suggested that H_2O_2 induced chilling tolerance in mung bean plants might be mediated by an elevation of GSH contents.

2.5.2 Ascorbic acid

Ascorbate is a major metabolite in plants and is component of the antioxidant system. It protects the plants against oxidative damage resulting from

aerobic metabolism, and a range of biotic and abiotic stresses (Smirnoff, 1996). Ascorbic acid interact directly with activated oxygen (Arrigoni *et al*., 1981). Ascorbate protects the chloroplasts from photoinhibition (Asada, 1999) and suppresses the accumulation of photoproduced H_2O_2 in the stroma. It also acts as the electron donor to ascorbate peroxidase and protect components of the Calvin cycle from inactivation (Asada, 1999; Hernandez *et al.*, 2004). Ascorbic acid content was reported to increase under oxidative stress (Mehlhorn *et al*., 1986). Dalmia and Sawhney (2004) reported several folds increased ascorbate level with onset of drought stress in wheat seedlings, but its level decreased with increasing magnitude of stress. A similar trend of a rapid increase in ascorbic acid pool sizes as an early response of the plants to the stress has been reported previously (Foyer *et al*., 1994). Kukreja *et al.* (2006) reported that ascorbic acid content declined from 16% to 26% with increasing salinity level. The onset of senescence in oat leaf segments was accompanied by a sharp decrease in the ascorbic acid content (Borraccino *et al*., 1994) which could be due to various factors such as its slower synthesis, faster utilization, or a decreased reduction rate of oxidation product (Borraccino *et al*., 1994). The cellular ascorbate concentration was halved, whereas DHA remained roughly constant in suspension cultured soybean cells (*Glycine max*), under stress conditions with more than 250 nM oxyfluorfen (Knorzer *et al*., 1996). However, Gullner and Dodge (2000) observed that though the cellular level of ascorbate was markedly decreased by the herbicides as well as

by the dyes (singlet oxygen generating substances) but no significant accumulation of DHA in any treatment was found. Goggin and Colmer (2005) also did not observe any significant change in ascorbate concentration under both continuous and intermittent anoxia in wheat roots. They reported that the proportion of reduced ascorbate in the total ascorbate pool was 1 in the continuously aerated tissues and around 0.7 in the continuously and intermittently anoxic tissues. Vaidyanathan *et al.* (2003) showed enhanced ascorbate levels in salt tolerant cultivar of rice than the sensitive ones under salt stress. It was observed that ozone tolerant soybean cultivar (cv Essex) exhibited 1.1 to 1.2 times higher levels of ascorbate and it had 1.5 to 2.2 times higher redox ratio of ascorbate / DHA than the leaflets of ozone sensitive cultivar under ozone treatment (Robinson and Steven, 1999).

The redox state of ascorbate was observed to shift towards its oxidized form under excess light stress in two cultivars of pea namely J1281 and J1399 (Hernandez *et al*., 2004). The accumulation of DHA under excess light (EL) stress was recorded while ascorbate levels remained unchanged resulting in a decrease in its redox status (Yoshimura *et al*., 2000). Zaharieva *et al.* (1999) reported that Fe-deficient cucumber roots were enriched both, in ascorbic acid (AA) (by factor of 1.7) and DHA (by factor of 3.2) while the ratio of AA/ (AA+DHA) was decreased by 23% as compared to Fe-sufficient roots. The ratio of ascorbate/

dehydroascorbate was reported to decrease from 0.77:1 after 2 days to 0.29:1 after 8 days of water stress in wheat seedlings (Dalmia and Sawhney, 2004).

2.5.3 Carotenoids

Carotenoids are the lipid soluble antioxidant (Schindler *et al*., 1994; Mishra, 1999), present in chloroplast membrane. Carotenoids quench singlet oxygen which is generated when light energy absorbed by chlorophyll is not dissipated through photosynthesis (Foote, 1968). They are suggested to be one of the required factors for tolerance to salt stress in crop plants (Hernandez *et al*., 1995). Singh *et al.* (1995) reported that carotenoids content at post-flowering stage was higher than pre-flowering stage and efficient genotypes of chickpea had significantly higher carotenoids contents as compared to sensitive ones subjected to Zinc stress. They further proposed that carotenoids are one of the important determinants of chickpea growth under Zn deficient condition. Polyamines strongly prevent loss of chlorophyll in senescing oat leaves (Shih *et al*., 1982). Besford *et al.* (1993) reported that polyamines stabilized the molecular composition of the thylakoid membrane and delayed senescence. It was suggested that polyamines kept a significant part of the thylakoid membrane native by binding with them. Similarly, Borrell *et al.* (1997) observed that treatment with spermidine or spermine prevented the loss of chlorophyll, stabilized the molecular composition of the thylakoid memberane. Verma and Mishra (2005) reported that the salinity caused decrease in carotenoids level in *Brassica juncea*. The reduction in carotenoids

under stress was checked and elevated by putrescine supplementation. It was suggested that putrescine caused reduction in ROS could be through quenching of singlet oxygen ($^1O_2$) and excited chlorophyll by elevated level of carotenoids (Schindler *et al*., 1994; Mishra, 1999) thereby maintaining chloroplastic membrane and $NADP^+$/NADPH ratio. This may reduce chances of electron escaping and generation of free radicals.

Thus exogenously supplied putrescine prevented stress damage and increased stress tolerance and resistance to oxidative stress. Polyamines have been described as modulators of stress tolerance and the foliar application of putrescine improved growth under abiotic stress conditions. They have ROS scavenging and membrane protecting properties and they might be activating antioxygenic enzymes and elevating antioxidants thereby controlling free radicals generation, resulting into improved growth under stress. It has been accepted that polyamines may ‘prepare’ the cell to meet and combat stress by stabilizing membranes and forming a potential of higher ‘buffering’and antioxidant capacity.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

The work presented in this thesis was carried out on two cultivars of *Triticum aestivum* viz. WH147 and UP2338. Seeds of these cultivars were obtained from the Department of Plant Breeding, Chaudhary Charan Singh Haryana Agricultural University, Hisar. All the biochemicals used during the present investigations were purchased either from Sigma Chemical Company (St. Louis, U.S.A.) or from SRL (Sisco Research Laboratories Pvt. Ltd., India), Aldrich Chemical Company (Germany) or BDH and were of highest purity.

3.2 Methods

3.2.1 Growth Conditions

About 50-60 seeds of uniform size of wheat were selected and were surface sterilized with 0.2% (w/v) $HgCl_2$ solution for 5 minutes. They were then washed under running tap water for one hour. The sterilized seeds were kept for germination in plastic bowls (diameter 12cm) containing approximately 300 gm acid washed river sand. These bowls were then kept in growth chambers having light/dark cycle of 15/9 hours with day/night temperature being 22/15^{o}C. The light source was fluorescence tubes having light intensity 2500 Lux.

3.2.1.1 Imposition of water deficit stress

After 5-6 days of germination, control set of seedlings was irrigated with half strength Hoagland nutrient solution while in another set of seedlings, water deficit stress was created by including polyethylene glycol (PEG) 6000, having osmotic potential equivalent to -2 bars in the Hoagland solution. Loss of water due to evaporation and transpiration were compensated daily by saturating the seedlings with respective solutions to a constant weight. As far as possible, identical growth conditions were provided to all the plants. In the initial experiments, the four different concentrations of putrescine i.e. 0.25, 0.5, 0.75 and 1.00 mM were tried on both control and stressed seedlings and the concentrations giving maximum ameliorating effect of water stress were selected and used for further experiments. The criteria of selection was based on their effect on relative water content, osmotic potential and electrolyte leakage in the stressed plants.

The samples were taken after 2 and 6 days of PEG, putrescine and PEG + putrescine treatment. PEG given for 2 and 6 days produced mild and severe stress, respectively.

3.2.1.2 Composition of Nutrient solution.

In the modified Hoagland nutrient solution, $NH_4H_2PO_4$ was replaced by KNO_3 and KH_2PO_4 in such a way that they provided same amount of PO_4^{2-} as in the original media and the concentration of the nitrate in the solution was 20mM. The composition of the nutrient solutions, supplied to the seedlings was as follows:-

(a) Solution A (Macronutrients)

Salt	Molarity of the stock solution	ml of stock solution taken per litre nutrient solution
KNO_3	1.0	10.0
$Ca(NO_3)_2$	1.0	5.0
KH_2PO_4	1.0	4.0
$MgSO_4.7H_2O$	1.0	2.0

(b) Solution B (Micronutrients)

Salt	g per litre of stock solution
H_3BO_3	0.286
$MnCl_2.4H_2O$	0.181
$CuSO_4.5H_2O$	0.08
$ZnSO_4.7H_2O$	0.22
H_2MoO_4	0.09

The above listed salts were dissolved in one litre of distilled water.

(c) Solution C (Iron)

Salt	***g per 100 ml of the stock solution***
$FeSO_4.7H_2O$	0.5
Tartaric acid	0.4

The indicated volumes of various components of solution A were mixed and after adding 1 ml of stock solution B and 0.6 ml of the stock solution C, the final volume was made 1 litre with distilled water.

11.2 gm of polyethylene glycol (6000) was dissolved in 100 ml of half strength Hoagland solution to get solution having osmotic potential equivalent to –2 bars.

3.2.2 Determination of Plant Water Status

3.2.2.1 Relative Water Content (RWC)

RWC was measured by the procedure of Irigoyen *et al.* (1992). 100 mg leaf (tissue) was taken from control, stress and putrescine treated control and stressed plants. They were cut into small discs and were dipped in distilled water in covered petriplates for about 4 hours. The leaf discs were then blotted dry with filter paper and their turgor weight was taken. After drying them in oven at 80^{o}C for 72 hours, their dry weight was taken. The RWC was calculated from the following equation:

$$\% \text{ RWC} = \frac{\text{Fresh Weight} - \text{Dry weight}}{\text{Turgid Weight} - \text{Dry Weight}} \times 100$$

3.2.2.2 Osmotic Potential (ψ_S)

Leaves from the control, stressed and putrescine treated plants were excised from the base and put in dry eppendorf vials and stored at $0^{O}C$ for one week. The frozen leaves in the eppendorf were squeezed with the plunger to extract the cell-sap. 5mM (dia) discs of Whatman No. 1 filter paper were dipped in this sap and placed in the chamber of Vapour Pressure Osmometer (OM-230 B WESCOR, INC. LOGAN, U.S.A.) and readings in osmolality (m mol/kg) were recorded as described by Kumar *et al*. (1984). The osmometer was calibrated with standard solution of NaCl and the osmotic potential was calculated from the following relationship:

40 osmolality = -1 bar

3.2.3. Estimation of cell membrane damage

3.2.3.1 Electrolyte leakage

Leakage of electrolytes from the leaves of control, stressed and putrescine treated plants was determined by the method of Scherbakava and Kacperska (1980).Leaf samples were cut into small discs and placed in test tubes containing 10 ml distilled water. After 4 hours of incubation at room temperature, the conductivity of the water in which leaf discs were kept, was recorded by Direct Reading Conductivity Meter 304 (Systronics). The same samples were then covered with aluminum foil and boiled in water bath for 30 min and refrigerated overnight. Their conductivity was recorded on the following day to estimate the

total electrolytes leached in the water. Electrolyte leakage was expressed in terms of relative injury which was calculated as follows:

$$\text{R.I. (\%)} = \frac{\text{Initial Reading}}{\text{Final Reading}} \times 100$$

A

/ 5 7 0

nm

10 20 30 40 50 60 70 80 90 100

3.2.3.2. Lipid peroxidation

H_2O_2 (μM)

Th

content l

trichloroacetic acid (TCA) containing 0.5% (w/v) thiobarbituric acid (TBA) was added to 0.75 ml of supernatant obtained as described in Section 3.2.6.1. The mixture was heated at 95^0C for 30 min. and then cooled in ice bath. After centrifugation for 10 minutes at 10000 rpm; absorbance of the supernatant at 532 nm was read and the value for the non-specific absorption at 600 nm was subtracted from it. The concentration of MDA was calculated using its extinction coefficient of 155 mM^{-1} cm^{-1}.

Based on effect of putrescine concentrations on RWC, ψs and relative injury, its two concentration giving maximum protection against stress were selected.These were 0.25 mM and 0.5 mM for both the cultivars and were used during all the further experiments.

3.2.4 Measurement of reactive oxygen species (ROS)

3.2.4.1. Hydrogen peroxide (H_2O_2)

H_2O_2 was estimated by the method of Sinha (1972). 200 μl of extract prepared as described in Section 3.2.6.1 was made to 1 ml with 0.1 M phosphate buffer (pH 7.5). 2 ml of 5% Potassium dichromate and glacial acetic acid [1:3 v/v],

was added to it. The mixture was then heated in boiling water bath for 10 minutes and cooled. Its absorbance was read at 570 nm against reagent blank

which was without sample extract. The quantity of H_2O_2 was determined from standard curve prepared similarly with 0-100 n mol H_2O_2 (Fig.1).

3.2.4.2. Superoxide radicals (O_2^-)

O_2^- radicals was measured by monitoring the nitrite formation from hydroxylamine following the method of Elstner and Heupel (1976). 0.5 ml extract prepared as described in Section 3.2.6.1, was incubated with 65mM Sodium phosphate buffer (pH 7.8) and 0.1mM hydroxylamine hydrochloride for 20 minutes at room temperature and then followed by addition of 1% Sulfanilamide and 0.02% N-1 naphthyl ethylenediamine dihydrochloride (NED) in final volume of 2 ml. The reaction mixture was again incubated at room temperature for 20 minutes. The absorbance of coloured complex thus formed was read at 540 nm against blank which was prepared similarly except that leaf extract was replaced by water. Amount of NO_2^- formed which corresponded to O_2^- production was calculated from the standard curve of NO_2^- (0-100 n mol) prepared by the above procedure (Fig. 2).

3.2.5. Estimation of antioxidant enzymes

3.2.5.1 Preparation of cell free extract

One gm of the plant material was homogenized in a pre-chilled pestle and mortar with 4 ml of 0.1M Potassium phosphate buffer (pH 7.0) containing 1%

PVP, 0.1 mM EDTA and 20% glycerol. 0.5mM ascorbate was included in the extraction buffer of ascorbate peroxidase. The homogenate was centrifuged at 12000 rpm for 30 min in refrigerated centrifuge. The supernatant was carefully decanted and used for the assay of different enzymes.

3.2.5.2 Assay procedures :

3.2.5.2.1 Catalase (EC 1.11.1.6)

The activity of catalase was estimated according to the procedure described by Aebi (1984). The reaction mixture in final volume of 3 ml, contained 0.1M phosphate buffer (pH 7.0), 10mM H_2O_2 and 50 µl of cell free extract. Reaction was initiated with the addition of H_2O_2 and enzyme activity was determined by following the consumption of H_2O_2 at 240 nm for 2 minutes. The enzyme activity was calculated using the extinction coefficient value of 39.4 mM^{-1} cm^{-1} for H_2O_2. One unit of enzyme activity corresponded to one µmol H_2O_2 consumed during the reaction.

3.2.5.2.2 Peroxidase (E.C. 1.11.1.7)

The procedure of Siegel and Siegel (1969) was followed for estimating peroxidase activity. 3 ml of reaction mixture contained 0.1 M phosphate buffer (pH 7.0), 0.1 mM guaiacol, 0.1 mM H_2O_2 and 50 µl cell free extract. Reaction was started with the addition of H_2O_2 and increase in absorbance at 470 nm was

recorded for 2 min. The activity was calculated using the extinction coefficient value of 22.6 mM^{-1} cm^{-1} for guaiacol. One unit of enzyme activity was equivalent to μ mol of H_2O_2 oxidized.

3.2.5.2.3 Ascorbate peroxidase (APX) (E.C. 1.11.1.11)

The enzyme activity was determined according to the method described by Nakano and Asada (1981). The composition of assay mixture was 50 mM phosphate buffer (pH 7.0), 0.5 mM Sodium ascorbate, 0.1 M H_2O_2 and 50 μl enzyme extract in 1.5 ml final volume. The reaction was initiated by the addition of H_2O_2. The decrease in absorbance due to oxidation of ascorbate at 290 nm was recorded spectrophotometrically for 2 min. The enzyme activity was determined using extinction coefficient value of 2.8 mM^{-1} cm^{-1} for ascorbate. One unit of enzyme activity is the μ mol of ascorbate oxidized.

3.2.5.2.4 Dehydroascorbate Reductase (DHAR) (E.C.1.8.5.1)

The enzyme activity was assayed according to the procedure of Cakmak *et al*. (1993). 1.5 ml of the reaction mixture contained 0.1M phosphate buffer (pH 7.0), 0.2 mM dehydroascorbate, 0.1mM EDTA, 2.5mM reduced glutathione (GSH) and 100 μl enzyme extract. The reaction was started with the addition of GSH. The DHAR activity was measured by recording increase in absorbance at 265 nm for 2

min. Extinction coefficient of 14 mM^{-1} cm^{-1} was used for calculating enzyme activity. One unit of enzyme activity corresponded to μmol of DHA reduced.

3.2.5.2.5 Glutathione Reductase (GR) (E.C. 1.6.4.2)

GR activity was assayed by the method of Halliwell and Foyer (1978). Incubation mixture for enzyme assay consisted of 0.1M phosphate buffer (pH 7.5), 5mM oxidized glutathione (GSSG), 0.2 mM NADPH and 100 μl enzyme extract in a final volume of 1.5 ml. Addition of GSSG, initiated the enzyme reaction. The decrease in absorbance at 350 nm due to oxidation of NADPH was monitored. Non enzyme oxidation of NADPH was recorded and substracted from it. An extinction coefficient value of 6.2 mM^{-1} cm^{-1} for NADPH was used to calculate the amount of NADPH oxidized which corresponded to GR activity. One unit of enzyme activity was equivalent to μmol of NADPH oxidized.

3.2.5.2.6 Superoxide dismutase (SOD) (E.C. 1.15.1.1)

The activity of SOD was measured by the method of Nishikimi *et al.* (1972) as modified by Kakkar *et al.* (1984). The assay mixture contained 50mM Tris-HCl buffer (pH 8.3), 6.2 μM phenazine methosulfate, 30 μM nitro-blue tetrazolium (NBT), 52 μM NADH and varying amount of cell free extract. The reaction was started by the addition of NADH. After incubation at 30^0C for 90 seconds, the reaction was terminated by the addition of 1ml glacial acetic acid and its absorbance was recorded at 560 nm against blank which was without NADH. The

reaction mixture without extract gave maximum reduction of NBT. One unit of SOD activity was defined as the amount of enzyme required for 50% inhibition of NBT reduction.

3.2.6 Estimation of antioxidant metabolites

3.2.6.1 Preparation of extracts

One gm of the plant tissue was ground in 6 ml of chilled 0.8 N $HClO_4$ and centrifuged at 10000 rpm for 30 minutes. The clear supernatant was decanted and neutralized with 5M K_2CO_3. It was again centrifuged at 10000 rpm for 30 minutes.

The clear supernatant thus obtained was carefully decanted and the corresponding volume of each preparation was recorded. This supernatant was used for estimation of various ROS and antioxidant metabolites.

3.2.6.2 Estimations of metabolites

3.2.6.2.1 Ascorbic acid

The slightly modified procedure of Oser (1979) was followed for estimating total ascorbic acid (reduced and oxidized forms) and dehydroascorbic acid. For estimation of oxidized (DHA) and total ascorbate (ASC + DHA), 1 ml extract for each was treated with equal volume of 10% TCA. It was then allowed to stand in ice for 5 minutes. For estimation of DHA, 5 M NaOH, 150 mM Sodium phosphate buffer (pH 7.4) and distilled water in a final volume of 7 ml were added. During estimation of total ascorbate, 10 mM DTT and 0.5% (w/v) NEM replaced water while rest of the steps were similar for both the estimations. 2% Dinitrophenyl hydrazine in 9N H_2SO_4 and a drop of 10% thiourea in 70% ethanol were added. The tubes were kept in boiling water bath for 15 minutes and then cooled. After cooling 80% H_2SO_4 was added to the tubes at 0^oC and mixed on vortex shaker. Their absorbance at 530 nm was recorded against reagent blank. A reference curve was prepared with (0-100 n moles) dehydroascorbate (Fig. 3). Ascorbic acid content was determined by subtracting the value of DHA from that of the total ascorbate.

A/412

nm

10 20 30 40 50

3.2.6.2.2 Glutathione

Glutathione (n moles)

Tot

Fig. 4 : Standard curve for Oxidized Glutathion

the modifi

in section 3.2.6.1 was neutralized with 360 µl of 1M triethanolamine while 40 µl of 2-vinyl pyridine was added only for measurement of GSSG to derivatize GSH. After vigrous shaking, the mixture was allowed to stand at room temperature for 1 hour. Further procedure was same for both total and oxidized glutathione. 1.5 ml of the reaction mixture consisted of 125 mM Sodium phosphate buffer (pH 7.5) containing 6.3 mM EDTA, 0.5 units of GR enzyme, 0.3 mM NADPH, 6mM DTNB, 100 µl of each of the above treated extracts. Increase in absorbance at 412 nm was recorded for 4 min. Glutathione content was estimated from the standard curve prepared with 1-50 µ mol GSSG (Fig. 4).

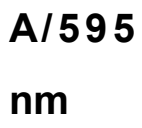
3.2.6.2.3 Carotenoid

Extraction: -

30 mg of the fresh leaves were cut into small discs and dipped in test tubes containing 3 ml dimethyl sulfoxide (DMSO). The tubes were kept at room temperature for overnight.

Estimation: -

The carotenoids extracted in the DMSO was estimated by recording its absorbance at 480, 645 and 663 nm respectively (Wellburn, 1994) and its amount was calculated from the following formula: -



Protein BSA (μg)

Fig. 5: Standard curve for Protein Bovine Serum Albumin

$$\text{Chl } a = \frac{12.19 \times A_{663} - 3.45 \times A_{645} \times \text{Vol.}}{1000 \times \text{Weight}}$$

$$\text{Chl } b = \frac{21.99 \times A_{645} - 5.32 \times A_{663} \times \text{Vol.}}{1000 \times \text{Weight}}$$

$$\text{Carotenoids} = \frac{1000 \times A_{480} - 2.14 \times \text{Chl } a - 70.16 \times \text{Chl } b}{220}$$

3.2.7 Estimation of protein

The protein content of cell free extract was estimated by Bradford method (1976). 50 µl of cell free extract was mixed with 2.5 ml of Bradford reagent and after standing for 5 minutes at room temperature, its absorbance was recorded at 595 nm against reagent blank. Standard curve (0 – 100 µg) of Bovine serum albumin was similarly prepared (Fig. 5).

CHAPTER – 4

RESULTS AND DISCUSSION

4.1 Optimization of Putrescine concentration:

To find out the optimum concentration for ameliorating the deleterious effects of water stress, effects of various levels of putrescine (0.25, 0.5, 0.75 and 1 mM) on relative water content, osmotic potential and relative injury of leaves of stressed plants were studied.

4.1.1 Effect of water stress and putrescine on plant water status

4.1.1.1 Relative water content (RWC)

The water stress decreased the relative water content (RWC) of the seedlings in both the cultivars. In WH147, at 2days after treatment (2DAT), RWC was 93% and it further decreased to 84.1% at 6DAT as compared to their corresponding controls, whereas in UP2338, these values were 97.5% at 2DAT and 94% on 6DAT (Table 1). Thus observed decrease in RWC was relatively more in WH147. Decreased RWC had been reported under water stress in maize leaves (Jiang and Zhang, 2002), jute plants (Chowdhury and Choudhuri,1985**)**, Vigna seedlings (Mukherjee and Choudhuri, 1985), French bean cultivars (Upreti and Murti, 2005), rice (Tyagi *et al.*, 1999) and in contrasting genotypes of wheat (Gupta *et al.*, 2005). A mild osmotic stress resulted in a slight reduction in the RWC of the leaves of *Aegilops biuncialis* and *Triticum aestivum* (Molnar *et al.*, 2004) genotypes. Gupta *et al.* (2005) had also reported significant decrease of RWC in leaf disc of both drought tolerant and sensitive genotypes of wheat under

Table 1: Effect of water stress and putrescine on Relative Water Content (%) in leaves of cultivars WH147 and UP2338 of wheat

Treatment	WH147		UP2338	
	2DAT	6DAT	2DAT	6DAT
Control	88.88 ±1.32 (100.0)	90.62 ±0.89 (100.0)	90.73 ±1.23 (100.0)	92.95 ±0.55 (100.0)
Control+0.25mM Putrescine	89.63 ±1.15 (100.8)	94.12 ±2.96 (103.9)	91.92 ±0.87 (101.3)	94.30 ±0.24 (101.4)
Control+0.5mM Putrescine	89.08 ±0.20 (100.2)	93.88 ±0.15 (103.6)	91.48 ±0.20 (100.8)	94.27 ±0.21 (101.4)
Control+0.75 mM Putrescine	88.95 ±0.32 (100.1)	93.57 ±2.38 (103.2)	91.96 ±0.40 (101.4)	94.52 ±0.29 (101.7)
Control+1.0mM Putrescine	89.14 ±0.05 (100.3)	94.05 ±0.22 (103.8)	93.09 ±0.21 (102.6)	93.80 ±0.27 (100.9)
PEG	82.67 ±1.03 (93.0)	78.19 ±0.45 (84.1)	88.48 ±0.25 (97.5)	87.39 ±0.58 (94.0)
PEG+0.25mM Putrescine	84.97 ±0.13 (95.6)	79.76 ±0.52 (88.0)	89.94 ±0.49 (99.1)	90.45 ±0.85 (97.3)
PEG+0.5mM Putrescine	85.53 ±0.39 (96.2)	80.06 ±0.15 (88.3)	89.89 ±1.74 (99.1)	88.46 ±1.42 (95.2)
PEG+0.75mM Putrescine	83.56 ±0.27 (94.0)	79.66 ±0.48 (87.91)	88.70 ±2.09 (97.8)	87.21 ±0.90 (93.8)
PEG+1.0mM Putrescine	83.04 ±0.31 (93.4)	79.21 ±0.28 (87.4)	89.60 ±0.44 (98.7)	89.15 ±0.25 (95.9)

CD at 5% level	For WH147		For UP2338	
	A	N.S.	A	N.S.
	B	2.0528	B	1.8556
	AB	2.903	AB	N.S.
	A	Days after treatment		
	B	Treatment		

The figures in parenthesis indicates the values as percent of control.

different levels of water stress. However RWC was significantly higher in drought tolerant cultivar of wheat both under nonstress and water stress conditions. It was suggested that the high RWC could help the tolerant genotype to perform physio-biochemical processes more efficiently under water stress conditions than susceptible genotypes. Martin *et al.* (1997) also observed relatively higher RWC in drought tolerant cultivars of wheat.

The exogenous application of varying amount of putrescine (0.25 to 1 mM) increased the RWC of the stressed seedlings of both the cultivars. In WH147, 0.25 mM was able to raise RWC of stressed plants by 2.8 and 4.6% after 2 and 6DAT as compared to stressed plants while the increase with 0.5 mM at similar treatment was 3.5 and 5.1% respectively, whereas 0.75 mM and 1 mM were less effective. Similarly in UP2338, the maximum increase in RWC was observed by 0.25 mM putrescine and which was 1.6 and 3.5% at 2 and 6DAT respectively. Thus the response of putrescine was better in WH147. A significant increase in RWC was also reported in stressed plants by application of spermine in pea (Upreti and Murti, 1999) and by putrescine in groundnut seedlings (Vakharia *et al.*, 2003). It was suggested that increase in RWC following polyamine treatments in the stressed plants were probably due to altered membrane permeability (Upreti and Murti, 1999).

4.1.1.2 Effect of water stress and putrescine on osmotic potential.

In seedlings of WH147, water stress of 2 days decreased its osmotic potential by -2.5 bars which was further accentuated to -5.7 bars as the stress level

Table 2: Effect of water stress and putrescine on Osmotic Potential (-bars) in leaves of cultivars WH147 and UP2338 of wheat

Treatment	WH147		UP2338	
	2DAT	6DAT	2DAT	6DAT
Control	-7.97 ±0.32 (100.0)	-8.36 ±0.20 (100.0)	-6.16 ±0.32 (100.0)	-5.62 ±0.23 (100.0)
Control+0.25mM Putrescine	-8.10 ±0.16 (101.6)	-8.80 ±0.18 (105.3)	-7.08 ±0.14 (114.9)	-6.72 ±0.17 (119.6)
Control+0.5mM Putrescine	-8.58 ±0.26 (107.6)	-9.05 ±0.11 (108.2)	-7.57 ±0.04 (122.9)	-7.11 ±0.08 (126.5)
Control+0.75 mM Putrescine	-9.46 ±0.22 (118.7)	-9.83 ±0.11 (117.6)	-7.91 ±0.29 (128.4)	-7.70 ±0.11 (137.0)
Control+1.0mM Putrescine	-8.08 ±0.30 (101.4)	-8.30 ±0.35 (99.3)	-7.98 ±0.16 (129.5)	-7.35 ±0.28 (130.8)
PEG	-10.48 ±0.14 (131.5)	-14.04 ±0.26 (167.9)	-11.94 ±0.09 (193.8)	-16.18 ±0.13 (287.9)
PEG+0.25mM Putrescine	-8.81 ±0.07 (110.5)	-11.32 ±0.15 (135.4)	-10.05 ±0.05 (163.1)	-13.90 ±0.10 (247.3)
PEG+0.5mM Putrescine	-8.71 ±0.20 (109.3)	-10.37 ±0.24 (124.0)	-10.91 ±0.04 (177.1)	-14.81 ±1.17 (263.5)
PEG+0.75mM Putrescine	-9.02 ±0.17 (113.1)	-12.98 ±0.28 (155.3)	-11.01 ±0.22 (178.7)	-14.40 ±0.08 (256.2)
PEG+1.0mM Putrescine	-10.15 ±0.29 (127.3)	-11.84 ±0.15 (141.6)	-11.78 ±0.17 (191.2)	-15.13 ±0.59 (269.2)

CD at 5% level

For WH147		For UP2338	
A	0.2474	A	0.3042
B	0.5531	B	0.6803
AB	0.782	AB	0.962
A	Days after treatment		
B	Treatment		

The figures in parenthesis indicates the values as percent of control.

progressed. These values corresponded to 31.5% and 67.9% reduction in osmotic potential as compared to their controls, respectively. In UP2338, the decrease in osmotic potential was more severe. Its value lowered by -4.8 and -10.6 bars on 2DAT and 6DAT which were equivalent to 94 and 187% decrease, respectively (Table 2). Osmotic potential had been reported to decline under drought stress in Vigna seedlings(Mukherjee and Choudhuri, 1983), wheat seedlings (Dalmia and Sawhney, 2004), olive trees (Sofo *et al.*, 2005) and rice (Tyagi *et al.*, 1999). Tyagi *et al*. 1999 proposed that decrease in osmotic potential under stress reflected the increased hydrolysis of macromolecules into simpler ones like mono- and disaccharides, amino acids especially proline etc. and consequently higher osmolite concentration. Lowered osmotic potential resulted in decreased availability of water to root cells of chickpea, which ultimately decreased RWC of roots (Kukreja *et al.*, 2006). Similar trend was observed in our results as obvious from Tables 1 and 2.

However exogenous application of various concentrations of putrescine ranging from 0.25 to 1 mM enhanced osmotic potential of the stressed seedlings partly and there was no complete recovery in osmotic potential at any concentration. In WH147, the recovery of 17% as compared to the stressed plants was observed with 0.5 mM putrescine at 2DAT which further increased to 26% on 6DAT. Other concentrations of applied putrescine were less effective in eliminating the stress effect. Similarly in UP2338 also, 0.25 mM putrescine and

0.5 mM were found effective in increasing osmotic potential of stressed seedlings. 0.25 mM putrescine caused 15.8% and 14% recovery at 2DAT and 6DAT respectively, whereas 0.5 mM putrescine was able to relieve by 85% of stress effects at both the stress levels. Higher concentrations of putrescine were less effective in ameliorating the stress level. Putrescine (0.25 – 1mM) itself had slightly negative effect on the osmotic potential of control seedlings of both the cultivars.

4.1.2 Effect of water stress and putrescine on relative injury (RI)

Water stress is injurious to the cell membranes with the result the permeability of the membranes is disturbed causing leakage of electrolytes from the cells. In WH147, there was 35% increase in relative injury even after 2 days of stress which was further intensified to 65% at 6DAT (Table 3). In UP2338, the increase in RI was 23 and 64% at 2DAT and 6DAT respectively. Electrolyte leakage had been reported to increase under water stress in Vigna seedlings (Mukherjee and Choudhuri, 1985), and in rice seedlings under salinity stress (Dionisio-Sese and Tobita, 1998). Salt stress induced electrolyte leakage has also been previously observed in tomatoes (Tal and Shannon, 1983) and melons (Borochov-Neori and Borochov, 1991). Lower membrane stability or higher injury reflects the extent of lipid peroxidation (Dhindsa *et al.*, 1981) which in turn is a consequence of higher oxidative stress due to various environmental stresses

(Leibler *et al.*, 1986). Upreti and Murti (2005) reported stable electrolyte leakage in the tolerant cultivator of French bean than the susceptible cultivar.

Table 3: Effect of water stress and putrescine on Relative Injury (%) in leaves of cultivars WH147 and UP2338 of wheat

Treatment	WH147		UP2338	
	2DAT	6DAT	2DAT	6DAT
Control	17.90 ±0.58	19.24 ±0.15	11.08 ±0.07	11.40 ±0.12
Control+0.25mM Putrescine	17.55 ±1.12 (-2.0)	19.20 ±0.14 (-0.2)	9.86 ± 0.41 (-11.0)	11.01 ±0.05 (-3.4)
Control+0.5mM Putrescine	17.03 ±0.07 (-4.9)	19.12 ±0.06 (-0.6)	10.91 ±0.11 (-1.5)	11.28 ±0.14 (-1.1)
Control+0.75 mM Putrescine	16.67 ±0.35 (-6.9)	18.74 ±0.07 (-2.6)	10.66 ±0.19 (-3.8)	11.35 ±0.16 (-0.4)
Control+1.0mM Putrescine	16.13 ±0.59 (-9.9)	18.49 ±0.18 (-3.9)	10.94 ±0.11 (-1.3)	11.58 ±0.06 (+1.6)
PEG	24.19 ±0.23 (+35.1)	31.88 ±0.80 (+65.7)	13.65 ±0.09 (+23.2)	18.74 ±0.28 (+64.4)
PEG+0.25mM Putrescine	22.08 ±0.14 (+23.3)	27.55 ±0.33 (+43.2)	11.74 ±0.44 (+6.0)	13.53 ±0.44 (+18.7)
PEG+0.5mM Putrescine	21.71 ±0.07 (+21.3)	26.31 ±0.20 (+36.7)	12.48 ±0.21 (+12.6)	13.98 ±0.20 (+22.6)
PEG+0.75mM Putrescine	22.42 ±0.07 (+25.2)	28.64 ±0.06 (+48.8)	12.88 ±0.10 (+16.2)	16.03 ±0.11 (+40.6)
PEG+1.0mM Putrescine	22.16 ±1.06 (+23.8)	27.95 ±0.05 (+45.3)	12.70 ±0.08 (+14.6)	14.19 ±0.18 (+24.5)

CD at 5% level

For WH147		For UP2338	
A	0.4127	A	0.1954
B	0.9229	B	0.4370
AB	1.305	AB	0.618
A	Days after treatment		
B	Treatment		

The figures in parenthesis represents per cent change in values as compared to control.

However exogenous application of different concentrations of putrescine to the rooting medium (0.25 -1 mM), reduced the relative injury in control as well as stressed seedlings of both the cultivars. In WH147, 0.25 mM putrescine decreased RI by 8.7 and 13.6% as compared to their respective stressed plants on 2 and 6DAT respectively, while the corresponding decrease with 0.5 mM putrescine was 10.2 and 17.5% respectively. 0.75 and 1 mM concentrations of putrescine were less protective against water stress. However in UP2338, highest recovery in relative injury of stressed seedlings was observed with 0.25 mM than with 0.5 mM putrescine and there was 14% decrease at 2DAT which further decreased by 28% at higher stress while other concentrations of putrescine were less effective in ameliorating the membrane injury under water stress. These results also indicate that the protective effect of same concentration of putrescine was comparatively higher at 6DAT. Under different types of stress in plants, polyamines have been found to reduce membrane leakage (Tiburcio *et al.*, 1994; Borrell *et al.*, 1997), as polyamines decreased electrolyte leakage in tobacco under ozone stress (Bors *et al.*, 1989), beet root discs (invitro) during ageing (Altman, 1982) and Indian mustard seedlings under salt stress (Verma and Mishra, 2005). Polyamines caused decrease in the conductivity and amount of soluble protein in the seed leachate is suggested to be due to repair of the membrane permeability in germinating groundnut seed (Yuan *et al.*, 1990). It was also suggested that under chilling condition, spermidine pretreatment of cucumber seedlings checked H_2O_2

production and prevented electrolyte leakage. Slocum *et al.* (1984) reported that the highly protonated form of polyamines at physiological pH should favour electrostatic binding of polyamine with the negatively charged phospholipid head groups on membranes, thereby influencing stability and permeability characteristics of these membranes. Also, the inhibition of lipid peroxidation may be one of the mechanisms responsible for the antisenescence effects of polyamines.

Thus from the results of RWC, osmotic potential and relative injury it was concluded that for cv. WH147, 0.5 mM and 0.25 mM putrescine for cv. UP2338 were optimum concentrations in protecting the plants from harmful effects of both mild and severe water stresses. Therefore these two concentrations were used for both cultivars of wheat in all the further studies.

4.2 Effect of water stress and putrescine on production of ROS

4.2.1 O_2^- radicals

Exposure of wheat seedlings to water stress increased the production of superoxide radicals in both the cultivars. In WH147, the O_2^- radical production was enhanced by 3.7 and 11.9% as compared to their corresponding controls at 2DAT and 6DAT respectively (Table 4). Similarly in UP2338, it increased by 27.3 and 47.5% compared to their respective controls. Thus the production of O_2^- under stress was much higher in UP2338 than in WH147. In water stressed plants,

enhanced O_2^- production was reported to be due to reduction of O_2 at PSI (Quartacci and Navari-Izzo, 1992).

Table 4: Effects of water stress and putrescine on O_2^- Radical production (μmoles g^{-1}DWt) in cultivars WH147 and UP2338 of wheat

Treatment	WH147		UP2338	
	2DAT	6DAT	2DAT	6DAT
Control	4.57 ±0.24 (100)	4.61 ±0.11 (100)	3.22 ±0.11 (100)	3.24 ±0.10 (100)
Control+0.25mM Putrescine	4.41 ±0.21 (96.5)	4.56 ±0.20 (98.9)	3.21 ±0.08 (99.7)	3.24 ±0.13 (100)
Control+0.5mM Putrescine	4.64 ±0.31 (101.5)	4.71 ±0.06 (102.2)	3.25 ±0.15 (100.9)	3.38 ±0.11 (104.3)
PEG	4.74 ±0.25 (103.7)	5.16 ±0.06 (111.9)	4.10 ±0.13 (127.3)	4.78 ±0.22 (147.5)
PEG+0.25mM Putrescine	4.01 ±0.28 (87.7)	4.57 ±0.19 (99.1)	3.42 ±0.07 (106.2)	3.84 ±0.19 (118.5)
PEG+0.5mM Putrescine	3.93 ±0.08 (86.0)	4.61 ±0.13 (100)	3.65 ±0.19 (113.3)	4.22 ±0.05 (130.2)

CD at 5% level	For WH147	For UP2338
	A 0.253	A 0.168
	B 0.448	B 0.291
	AB N.S.	AB N.S.
	A Days after treatment	
	B Treatment	

The figures in parenthesis indicates the values as percent of control.

Exogenous application of putrescine reduced the O_2^- production in these two cultivars at both the stress levels. In the stressed seedlings of WH147, 0.25 mM putrescine decreased O_2^- production by 15.4 and 11.4% on 2DAT and 6DAT, whereas it was reduced by 17 and 10.6% with 0.5 mM putrescine, respectively as compared to their stressed plants. Thus the protective effect of putrescine was higher at lower stress level. However in UP2338, 16.6 and 19.7% reduction in O_2^- production as compared to stressed seedlings was observed with exogenous application of 0.25mM putrescine for 2DAT and 6DAT respectively. 0.5mM putrescine decreased O_2^- production correspondingly by 19.7 and 11.7%. In control seedlings, 0.25 mM putrescine caused slight decrease in O_2^- radicals in both the cultivars whereas a small elevation in O_2^- level was observed with 0.5mM putrescine. Putrescine was found to reduce ROS concentration through quenching of singlet oxygen ($^1O_2$) (Verma and Mishra, 2005). Also, the protective effect of polyamines against the damage of superoxides has been considered to depend on their prior conversion to perchloric acid-soluble conjugated polyamines (Bouchereau *et al.*, 1999). The formation of O_2^- radicals might be inhibited in-vitro by polyamine application. The polyamines act as free radical scavengers.

4.2.2 Effect of water stress and putrescine on production of H_2O_2

As seen in Table 5, depriving the seedlings of water caused increased production of H_2O_2 in both the cultivars. In stressed seedlings of WH147, as compared to control, the H_2O_2 formation increased by 17.8% at 2DAT which was further intensified to 20.5% on 6DAT. In UP2338 also, there was 22 and 50%

Table 5: Effects of water stress and putrescine on H_2O_2 content (μmoles g^{-1}DWt) in cultivars WH147 and UP2338 of wheat

Treatment	WH147		UP2338	
	2DAT	6DAT	2DAT	6DAT
Control	1.18 ±0.021 (100)	1.37 ±0.04 (100)	0.59 ±0.021 (100)	0.66 ±0.026 (100)
Control+0.25mM Putrescine	1.36 ±0.017 (115.2)	1.71 ±0.03 (124.8)	0.62 ±0.021 (105.1)	0.74 ±0.021 (112.1)
Control+0.5mM Putrescine	1.22 ±0.020 (103.4)	1.62 ±0.06 (118.2)	0.63 ±0.026 (106.8)	0.82 ±0.030 (124.2)
PEG	1.39 ±0.025 (117.8)	1.80 ±0.06 (131.4)	0.72 ±0.029 (122.0)	0.99 ±0.035 (150.0)
PEG+0.25mM Putrescine	1.07 ±0.035 (90.7)	1.43 ±0.04 (104.4)	0.60 ±0.18 (101.7)	0.82 ±0.029 (124.2)
PEG+0.5mM Putrescine	0.96 ±0.025 (81.3)	1.32 ±0.08 (96.3)	0.60 ±0.010 (101.7)	0.86 ±0.010 (130.3)

CD at 5% level	For WH147	For UP2338
	A 0.051	A 0.068
	B 0.088	B 0.117
	AB N.S.	AB N.S.
	A Days after treatment	
	B Treatment	

The figures in parenthesis indicates the values as percent of control.

increase in H_2O_2 levels at 2DAT and 6DAT, respectively. H_2O_2 production was comparatively more in cv UP2338. Mukherjee and Choudhuri (1985) reported that the increasing level of H_2O_2 in water stressed tissue was a function of increasing magnitude of water stress. Enhanced H_2O_2 content had been observed in pea cultivars under excess light stress (Hernandez *et al.*, 2004), cucumber plants under chilling stress (Lee and Lee, 2000), maize leaves (Jiang and Zhang, 2002) and wheat seedlings under water stress (Gupta *et al.*, 2005). The salinity induced generation of H_2O_2 in Indian mustard seedlings (Verma and Mishra, 2005) is corroborated with other cases (Hernandez *et al.*, 1995, 2001). Increased H_2O_2 led to lipid peroxidation, causing membrane damage and leakage of electrolytes (Verma and Mishra, 2005) which has been reported in many other cases also (Choudhuri and Choudhuri, 1993; Liang *et al.*, 2003). Gupta *et al.* (2005) proposed that in tolerant cultivar of wheat, lower values of MDA and H_2O_2 indicated that this cultivar was better equipped with an efficient free radical quenching system that offered protection against oxidative stress. H_2O_2 is a membrane permeable molecule that has been demonstrated to function as a diffusible intercellular signal (Levine *et al.*, 1994; Foyer *et al.*, 1997; Karpinski *et al.*, 1999; Neill *et al.*, 2002) and an elevation of H_2O_2 by 20-40% seems to be sufficient for inducing cytosolic APX (Karpinski *et al.*, 1999; Morita *et al.*, 1999; Yoshimura *et al.*, 2000).

Both concentrations of putrescine reduced H_2O_2 production in stressed seedlings while it enhanced H_2O_2 in the control plants. In WH147, at 2DAT the

H_2O_2 formation was decreased by 23.0 and 21.0 as compared to stressed plants with 0.25 mM and 0.5 mM putrescine, respectively, and at 6DAT, the ameliorating effect was 20.5 and 26.6% with 0.25 and 0.5 mM putrescine, respectively. However in UP2338, there was 16.6% reduction in its formation at 2DAT with both the concentration of putrescine, whereas at 6DAT the reduction in its content was 17.2% with 0.25 mM and 13.1% with 0.5 mM putrescine respectively. Putrescine has been reported to suppress the level of H_2O_2 in stressed *Brassica juncea* seedlings (Verma and Mishra, 2005). Similarly a decline in H_2O_2 content by exogenous polyamine was reported in bean plants under acid rain treatment (Velikova *et al.*, 2000), spring wheat seedlings under osmotic stress (Li *et al.*, 2004), cucumber under chilling stress (Shen *et al.*, 2002). The H_2O_2 and MDA production had been taken as an indicator of stress sensitivity in case of pea (Hernandez *et al.*, 1993) and rice (Dionisio-Sese and Tobita, 1998). Therefore, putrescine potential to suppress H_2O_2 level and thereby membrane damage is being evaluated in terms of antioxidative system also.

4.2.3 Effect of water stress and putrescine on Malondialdehyde (MDA) production

Increase in Malondialdehyde content is an indicator of peroxidation of the cellular membrane lipids. Higher MDA level might illustrates more leakage of electrolytes from inside the cell. Water stress created by PEG caused an elevation

in MDA content in both cultivars of wheat (Table 6). The MDA content was found to be 35.4% more at 2DAT in WH147 which was further accentuated by 59.9% on

Table 6: Effects of water stress and putrescine on MDA content (nmoles g^{-1}DWt) in cultivars WH147 and UP2338 of wheat.

Treatment	WH147		UP2338	
	2DAT	6DAT	2DAT	6DAT
Control	63.82 ±0.82 (100)	63.07 ±0.15 (100)	39.26 ±0.18 (100)	46.92 ±1.08 (100)
Control+0.25mM Putrescine	51.42 ±0.81 (80.6)	57.47 ±0.32 (91.1)	35.73 ±0.06 (91.0)	42.68 ±0.29 (91.0)
Control+0.5mM Putrescine	53.09 ±0.28 (83.2)	61.01 ±0.16 (96.7)	34.93 ±0.18 (89.0)	43.39 ±0.28 (92.5)
PEG	86.43 ±1.58 (135.4)	100.88 ±0.31 (159.9)	41.89 ±0.13 (106.7)	53.18 ±1.10 (113.3)
PEG+0.25mM Putrescine	71.73 ±0.96 (112.4)	87.98 ± 0.32 (139.5)	38.51 ±1.22 (98.1)	45.27 ±0.41 (96.5)
PEG+0.5mM Putrescine	65.07 ±0.11 (101.9)	91.50 ±0.28 (145.1)	38.10 ±1.42 (97.0)	46.43 ±0.26 (98.9)

CD at 5% level	For WH147	For UP2338
	A 0.8227	A 0.8827
	B 1.4250	B 1.5289
	AB 2.015	AB N.S.
	A Days after treatment	
	B Treatment	

The figures in parenthesis indicates the values as percent of control.

6DAT. In UP2338, MDA production was increased by only 6.7% and 13.3% at 2DAT and 6DAT, respectively. Lipid peroxidation had been reported to increase in pea (Hernandez *et al.*, 2004), *Morus alba* (Sudhakar *et al.*, 2001), *Lycopersicon esculentum* (Mittova *et al.*, 2002), *Beta vulgaris* (Bor *et al.*, 2003), rice (Vaidyanathan *et al*, 2003), cotton (Meloni *et al.*, 2003) of wheat (Sairam *et al.,* 2000) and chick pea (Kukreja *et al.*, 2006) under various types of stresses. A possible cause of increase in lipid peroxidation in chickpea roots with increased level of salinity was due to increased H_2O_2 accumulation (Kukreja *et al.*, 2006). Membranes are main loci affected under water stress conditions. Dhindsa *et al.* (1981) suggested that lower membrane stability or higher injury reflects the magnitude of lipid peroxidation, which in turn is a consequence of higher oxidative damage due to various environmental stresses (Leibler *et al.*, 1986).

Exogenously applied putrescine decrease MDA production in control as well as in stressed seedlings of both the cultivars. MDA content was reduced by 17.0 and 20.4% in 0.25 mM putrescine treated stressed plants of WH147 at 2 and 6DAT, respectively, whereas 0.5mM putrescine caused corresponding decrease of 24.7% and 8.5% as compared to their respective stressed plants. In UP2338, 0.25 mM putrescine decreased MDA content by 8.0% and 14.8% at 2 and 6DAT, respectively and 0.5mM by 9.0% and 12.7% respectively. These results thus indicated that putrescine effect was more pronounced at mild stress (2DAT) in WH147 while in UP2338 which showed lesser degree of lipid peroxidation of

membrane, the protective effect of putrescine was more obvious on 6DAT. Polyamines have been reported to decrease MDA content significantly in callus from leaves of sugarcane (Zhang *et al.,* 1996) and in woody plants (Xie *et al.*, 1999) subjected to osmotic stress and in bean under acid stress (Velikova *et al.*, 2000). The effect of putrescine on stabilization of membrane damage under stress could be due to its polycationic nature as suggested by Smith (1985) and Tiburcio *et al.* (1994). Mishra and Singh (2000) reported that putrescine being rapidly taken up by plants might compete with Pb or Cd uptake through cation channels of the membranes in mustard seedlings. Also, it might stabilize the membranes against stress. Shen *et al.* (2000) proposed that spermidine acted as a cellular membrane protectant against chill-induced lipid peroxidation through prevention of superoxide generating NADPH oxidase activation. Thus, polyamines had a significant influence on the reduction in lipid peroxidation and most likely it did so by scavenging free radicals (Drolet *et al.*, 1986).

4.3 Effect of water stress and putrescine on antioxidant enzymes.

4.3.1 Catalase (EC 1.11.1.6 CAT)

Catalase is H_2O_2 scavenging enzyme present in peroxisomes of the cell. Its activity was increased in both the cultivars on imposition of stress, created by application of PEG to 5 days old seedlings. In WH147, catalase activity was enhanced by 22.1% at 2DAT and only by 5% at 6DAT, whereas in UP2338, increase in activity was higher i.e. 42% at 2DAT and 25% at 6DAT (Table7).

Dalmia and Sawhney (2004) also indicated that higher catalase activity in wheat seedlings under water stress might avert the damage to the cellular machinery by

Table 7: Effect of water stress and putrescine on Catalase activity (units mg $^{-1}$protein min^{-1}) in cultivars WH147 and UP2338 of wheat.

Treatment	WH147		UP2338	
	2DAT	6DAT	2DAT	6DAT
Control	100.73 ±1.75 (100)	112.68 ±1.35 (100)	69.76 ±1.21 (100)	86.93 ±1.40 (100)
Control+0.25mM Putrescine	104.25 ±2.37 (103.5)	108.93 ±2.18 (96.7)	80.07 ±1.07 (114.8)	93.12 ±1.33 (107.1)
Control+0.5mM Putrescine	108.26 ±1.08 (107.5)	123.55 ±0.79 (109.6)	90.41 ±0.81 (129.6)	110.12 ±1.24 (126.7)
PEG	123.04 ±1.34 (122.1)	118.37 ±0.95 (105.0)	99.34 ±1.08 (142.4)	109.14 ±1.88 (125.5)
PEG+0.25mM Putrescine	147.78 ±1.76 (146.7)	123.40 ±1.36 (109.5)	119.81 ±1.12 (171.7)	147.91 ±2.53 (170.1)
PEG+0.5mM Putrescine	152.83 ±1.52 (151.7)	129.69 ±1.01 (115.1)	107.97 ±1.62 (154.8)	121.11 ±0.99 (139.3)

CD at 5% level	For WH147	For UP2338
	A 1.8266	A 1.3442
	B 3.1638	B 2.3282
	AB 4.474	AB 3.293
	A Days after treatment	
	B Treatment	

The figures in parenthesis indicates the values as percent of control.

decomposing H_2O_2, as increase in its activity was being related with higher tolerance to water stress (Lima *et al.*, 2002) and salt stress (Sairam *et al.*, 2002). Increased catalase activity had also been reported in maize leaves (Jiang and Zhang, 2002), wheat (Sairam *et al.*, 2002), chickpea (Singh *et al.*, 2005), *Beta maritime* (halophyte) and *Beta vulgaris* (non-halophyte) (Sudhakar *et al.*, 2001), olive trees (Sofo *et al.*, 2005) under various types of stresses. Sofo *et al.* (2005) reported an increase in catalase activity at mild and moderate water deficit stress in leaves and roots of olive trees but a decline in catalase activity was observed in roots during severe water deficit. It may be due to its inactivation or degradation by severe dehydration (Feierabend *et al.*, 1992). The CAT activity in leaves of stressed plants likely aid in removal of photorespiratory H_2O_2 produced during drought stress (Noctor *et al.*, 2000). Higher CAT activity in leaves of cold-acclimated plants suggested a more efficient scavenging of H_2O_2, which resulted in protection against peroxidation (KuK, 2003). Similarly, CAT activity in cold-acclimated material has been reported to remain similar to (O'Kane *et al.*, 1996) or higher than (Anderson *et al.*, 1995) that of non stressed plants.

Data in the Table 7 illustrates that exogenous application of putrescine further elevated the enzyme activity in both WH147 and UP2338. In WH147, the increase in its activity by 0.25 mM putrescine was 20.1% and 4.25% as compared to PEG treated seedlings at 2 and 6DAT, respectively, but the increase in activity was higher with 0.5mM putrescine i.e. 24.2% and 9.6% respectively as compared

to the corresponding stressed plants. In UP2338, 0.25mM putrescine caused higher stimulation in enzyme activity. It showed 20.6% increase at 2DAT and 35.5% at 6DAT as compared to stressed seedlings. It is obvious from these results that in WH147, 0.5mM putrescine was more effective in increasing enzyme activity at mild stress while in UP2338, severe stress reacted more positively to 0.25mM putrescine. Verma and Mishra (2005) also reported that the tendency of increase in CAT activity under salinity was further increased with putrescine supplementation. In general, 0.25 mM and 0.5 mM putrescine increased the catalase activity in the control plants of both the cultivars, but in WH147, 0.25 mM putrescine slightly decreased the activity at 6DAT.

4.3.2 Peroxidase (EC.1.11.1.7 POD)

Peroxidase is another H_2O_2 scavenging enzyme having non specific donor. Mild water stress, created by application of PEG for 2 days inhibited the enzyme activity in both cultivars while severe stress had marginal effect. In WH147, the enzyme activity was decreased by 8.7% at 2DAT, whereas at 6DAT, water stress had no effect. In UP2338 also, the enzyme activity was found to decrease by 21.7% at 2DAT which was higher as compared to that in WH147. However at 6DAT the enzyme activity increased marginally by 8.7% (Table 8). A significant inhibition of peroxidase at higher NaCl concentration in corn and pea has been reported by Vasile (1963). Similarly peroxidase activity had been reported to decline in alfalfa leaves (Irigoyen *et al.*, 1992) and in sorghum and sunflower

plants (Zhang and Kirkham, 1996) by drought stress. On the contrary an increased POD activity in pea and cotton under NaCl stress was shown by Strogonov (1964).

Table 8: Effect of water stress and putrescine on Peroxidase activity (units mg $^{-1}$protein min^{-1}) in cultivars WH147 and UP2338 of wheat

Treatment	WH147		UP2338	
	2DAT	6DAT	2DAT	6DAT
Control	0.23 ±0.011 (100.0)	0.26 ±0.006 (100.0)	0.184 ±0.011 (100.0)	0.206 ±0.010 (100.0)
Control+0.25mM Putrescine	0.21 ±0.021 (91.3)	0.23 ±0.010 (88.5)	0.141 ±0.006 (76.6)	0.188 ±0.021 (91.3)
Control+0.5mM Putrescine	0.23 ±0.014 (100.0)	0.25 ±0.011 (96.1)	0.142 ±0.011 (77.2)	0.264 ±0.023 (128.1)
PEG	0.21 ±0.011 (91.3)	0.26 ±0.020 (100.0)	0.144 ±0.006 (78.3)	0.224 ±0.010 (108.7)
PEG+0.25mM Putrescine	0.24 ±0.020 (104.3)	0.28 ±0.011 (107.7)	0.165 ±0.015 (89.7)	0.342 ±0.015 (166.0)
PEG+0.5mM Putrescine	0.25 ±0.015 (108.7)	0.30 ±0.010 (115.4)	0.155 ±0.010 (84.2)	0.237 ±0.006 (115.0)

CD at 5% level	For WH147	For UP2338
	A 0.0178	A 0.0161
	B 0.0309	B 0.0280
	AB N.S.	AB 0.040
	A Days after treatment	
	B Treatment	

The figures in parenthesis indicates the values as percent of control.

Increase in POD in salt-tolerant varieties of *Pisum sativum* (Olmos *et al.*, 1994), in the halophytes *Distichlis spicata* (Daines and Gould, 1986) and *Halimione portulacoides* (Kalir and Poljakoff-Mayber, 1981) has also been reported. Increase in POD activity could be due to the release of the enzyme from the membrane structure (Zhang and Kirkham, 1994). H_2O_2, produced at initial stage of stress, act as a signalling compound. Dalal and Khanna-Chopra, (2001) reported that higher peroxidase activity under various stress conditions could protect the plants from oxidative damage by scavenging H_2O_2, as well as cause the lignification and cross linking of cell wall. It was reported that peroxidase along with catalase constituted a potential defense mechanism against chilling induced oxidative damage in germinating wheat grains (Nayar and Kaushal, 2002).

Treatment of stressed seedlings with putrescine increased the enzyme activity in both the cultivars. In WH147, 0.5 mM putrescine enhanced its activity by 19.4% and 15.4% in stressed plants as compared to the increase of 14.2 and 15.4% by 0.25 mM putrescine on 2 and 6DAT respectivity. In UP2338, putrescine increased the enzyme activity to much greater extent at severe stress. 0.25 mM increased peroxidase by 14.6% at 2DAT and 55.7% at 6DAT as compared to corresponding stressed seedlings. Verma and Mishra (2005) observed inconsistent effect of putrescine on POD activity in mustard seedlings under salt stress. Moreover, the activity was elevated considerably at high salinity (150 mmol/L NaCl) but little effect was observed at other stress levels. Therefore upsurge in

POD activity with putrescine under high saline condition with concomittant decline in H_2O_2 level (Verma and Mishra, 2005) suggested putrescine role in H_2O_2 detoxification through peroxidase. 0.25 mM putrescine decreased the POD activity in control plants of both the cultivars whereas in WH147, 0.5 mM putrescine showed no change in POD activity at 2DAT and at 6DAT it decreased slightly. However, in UP2338, the peroxidase activity was found to increase at 6DAT.

4.3.3 Ascorbate peroxidase (EC.1.11.1.11 APX)

Ascorbate peroxidase is another H_2O_2 scavenging enzyme localized in chloroplasts. Its activity is increased under water stress and enhancement was proportional to stress level. In WH147, it increased by only 6.1% at 2DAT and 19.4% at 6DAT whereas corresponding increase in UP2338 was 44.4% and 50%, respectively as compared to their controls (Table 9). Thus, UP2338 was affected to greater extent. APX reduce H_2O_2 to water and have higher affinity for H_2O_2 as compared with catalase (Graham and Patterson, 1982). Higher APX activity had been reported in wheat seedlings under drought stress (Dalmia and Sawhney, 2004), chick pea (Kukreja *et al.*, 2005) and rice (Vaidyanathan *et al.*, 2003) under salt stress and cucumber roots in Fe deficiency stress (Zaharieva *et al.*, 1999). Molina *et al.* (2002) have reported higher APX and glutathione reductase activities in NaCl-adapted tomato cells and suggested their role in higher tolerance to

NaCl-induced oxidative stress. The important role of APX in relation to increasing oxidative tolerance has also been observed in many plants (Feierabend *et al.*, 1992; Gupta *et al.*, 1993). The overexpression of cytosolic APX in tobacco chloroplasts enhanced the tolerance to salt stress (Badawi *et al*, 2004b). Comba *et*

Table 9: Effect of water stress and putrescine on Ascorbate Peroxidase activity (units mg $^{-1}$protein min^{-1}) in cultivars WH147 and UP2338 of wheat

Treatment	WH147		UP2338	
	2DAT	6DAT	2DAT	6DAT
Control	0.98 ±0.026 (100.0)	0.96 ±0.017 (100.0)	0.63 ±0.025 (100.0)	0.78 ±0.021 (100.0)
Control+0.25mM Putrescine	1.06 ±0.021 (108.2)	0.96 ±0.021 (100.0)	0.53 ±0.006 (84.1)	0.72 ±0.011 (92.3)
Control+0.5mM Putrescine	1.10 ±0.038 (112.2)	1.03 ±0.030 (107.3)	0.62 ±0.010 (98.4)	0.76 ±0.010 (97.4)
PEG	1.04 ±0.020 (106.1)	1.17 ±0.015 (121.9)	0.91 ±0.021 (144.4)	1.17 ±0.015 (150.0)
PEG+0.25mM Putrescine	1.14 ±0.021 (116.3)	1.12 ±0.011 (116.7)	1.09 ±0.015 (173.0)	1.44 ±0.017 (184.6)
PEG+0.5mM Putrescine	1.18 ±0.021 (120.4)	1.17 ±0.026 (121.9)	1.00 ±0.030 (158.7)	1.30 ±0.029 (166.7)

CD at 5% level	For WH147	For UP2338
	A N.S.	A 0.0220
	B 0.0464	B 0.0381
	AB 0.066	AB 0.054
	A Days after treatment	
	B Treatment	

Th
e figures in parenthesis indicates the values as percent of control.

al. (1997) reported the increased activity of APX in salt-tolerant while decreased activity in salt sensitive cultivars of soybean exposed to salt stress. It was reported that the leaves of olive trees under drought stress, showed a considerable increase of APX activity, while their roots possessed low levels of activity (Sofo *et al.*, 2005). The huge increase in APX activity observed in leaves can protect chloroplasts, which under stress conditions present sustained electron flow and are the main producers and targets of ROS action (Asada, 1999). However, others have not found the same correlation between increase in APX activity and salt tolerance (Siegel *et al.*, 1982; Dionisio-Sese and Tobita, 1998).

Exogenous application of putrescine further stimulated the enzyme activity in stressed seedlings. In WH147, there was 9.6 and 4.2% enhancement in activity as compared to the stressed plants with 0.25 mM putrescine at 2DAT and 6DAT respectively, whereas 0.5 mM putrescine at 2DAT resulted in 13.5% higher enzyme activity as compared to the stressed plants while at severe stress, it did not show any protective effect. Thus at this stage, either the enzyme was already activated to its full potential or putrescine had no stimulatory effect on this enzyme in this cultivar. However in UP2338, 0.25 mM putrescine created about 20% and 23% enhancement of enzyme activity in the stressed leaves at 2 and 6DAT respectively. While 0.5 mM putrescine was less stimulatory. In the control seedlings, putrescine stimulated the enzyme activity in WH147 but inhibited it in UP 2338.

4.3.4 Dehyroascorbate Reductase (EC.1.8.5.1 DHAR)

The effect of two levels of water stress on activity of DHAR in WH147 and UP2338 is shown in Table 10. It is apparent from the results that enzyme activity in cv. WH147 was not much affected by either levels of stress, while in UP2338, the enzyme activity was stimulated by around 23% at both 2 and 6DAT, respectively (Table 10). The activity of DHAR was reported to increase under drought stress in wheat seedlings (Dalmia and Sawhney, 2004), sorghum and sunflower (Zhang and Kirkham, 1996) and under salt stress in rice (Vaidyanathan *et al.*, 2003). On the other hand, reduced DHAR activity was observed in germinating wheat seeds (Cakmak *et al*, 1993).

Application of putrescine increased the enzyme activity in control as well as in stressed plants of both the cultivars. In WH147, at 2DAT there was about 8.2% increase in enzyme activity as compared to stressed plants with 0.25 mM as well as with 0.5 mM putrescine. At 6DAT there was decrease in enzyme activity with both the concentrations of putrescine. In UP2338, 0.25 mM putrescine treated stressed seedlings showed 14.6% and 15.8% enhancement in enzyme activity at 2 and 6DAT, respectively as compared to the stressed plants. However 0.5 mM putrescine increased the enzyme activity by 4 and 7% on 2 and 6DAT, respectively.

4.3.5 Glutathione Reductase (EC.1.6.4.2 GR)

Water stress stimulated GR activity in both the cultivars which was further accentuated with increase in stress level. In WH147, initially at 2DAT enzyme

Table 10: Effect of water stress and putrescine on Dehydroascorbate Reductase activity (units mg $^{-1}$protein min^{-1}) in cultivars WH147 and UP2338 of wheat.

Treatment	WH147		UP2338	
	2DAT	6DAT	2DAT	6DAT
Control	0.099 ±0.006 (100.0)	0.110 ±0.003 (100.0)	0.039 ±0.002 (100.0)	0.046 ±0.001 (100.0)
Control+0.25mM Putrescine	0.103 ±0.012 (104.0)	0.106 ±0.001 (96.4)	0.040 ±0.001 (102.6)	0.049 ±0.001 (106.5)
Control+0.5mM Putrescine	0.109 ±0.010 (110.1)	0.112 ±0.002 (101.8)	0.041 ±0.002 (105.1)	0.054 ±0.001 (117.4)
PEG	0.098 ±0.021 (99.0)	0.112 ±0.001 (101.8)	0.048 ±0.001 (123.1)	0.057 ±0.001 (123.9)
PEG+0.25mM Putrescine	0.106 ±0.006 (107.1)	0.107 ±0.003 (97.3)	0.055 ±0.001 (141.0)	0.066 ±0.001 (143.5)
PEG+0.5mM Putrescine	0.106 ±0.009 (107.1)	0.109 ±0.003 (99.1)	0.050 ±0.001 (128.2)	0.061 ±0.002 (132.6)

CD at 5% level	For WH147	For UP2338
	A 0.0025	A 0.0020
	B N.S.	B 0.0035
	AB 0.006	AB N.S.
	A Days after treatment	
	B Treatment	

The figures in parenthesis indicates the values as percent of control.

activity was increased only by 15.1% as compared to control plants but as the stress level progressed, GR activity increased by 47.9% at 6DAT. In UP2338, the enzyme activity was higher by 33.3% and 41.2% as compared to their controls on 2 and 6DAT respectively (Table11). GR activity had also been observed to increase under drought stress (Dalmia and Sawhney, 2004; Jiang and Zhang, 2002) and salt stress (Vaidyanathan *et al.*, 2003; Dionisio-Sese and Tobita, 1998; Gossett *et al.*, 1994; Hernandez *et al.*, 2001; Sairam and Srivastava, 2002). Porcel *et al.* (2003) attributed higher GR activity in roots and nodules of mycorrhizal soybean plants under drought stress to decreased oxidative damage to biomolecules, which were involved in pre-mature nodule senescence. In Scenedesmus, its activity was found to decrease under copper stress (Nagalakshmi and Prasad, 2001) and this might be due to inactivation of enzyme by Cu^{2+} ions which decreased the rate at which GSSG is reduced to GSH. Similarly GR activity was decreased in wheat roots under anoxia (Albrecht and Wiedenroth, 1994; Blokhina *et al.*, 2000) and it was suggested that this persuambly reduced the capacity of these tissues to tolerate oxidative stress (Goggin and Colmer, 2005).

Application of putrescine to the stressed seedlings increased GR activity in both the cultivars while it was generally inhibitory in the control seedlings. In WH147, there was 24.6 and 34.4% enhancement in enzyme activity in the stressed plants with 0.25 mM and 0.5 mM putrescine, respectively at 2DAT, whereas at

6DAT when stress level was higher, enzyme activity was not greatly affected by both 0.25 mM and 0.5 mM putrescine treatments. Similarly in UP2338 also, GR

Table 11: Effect of water stress and putrescine on Glutathione Reductase activity (units mg $^{-1}$protein min^{-1}) in cultivars WH147 and UP2338 of wheat

Treatment	WH147		UP2338	
	2DAT	6DAT	2DAT	6DAT
Control	0.053 ±0.012 (100.0)	0.117 ±0.002 (100.0)	0.015 ±0.003 (100.0)	0.017 ±0 (100.0)
Control+0.25mM Putrescine	0.048 ±0.012 (90.6)	0.088 ±0.001 (75.2)	0.012 ±0.001 (80.0)	0.018 ±0.001 (105.9)
Control+0.5mM Putrescine	0.052 ±0.004 (98.1)	0.105 ±0.003 (89.7)	0.016 ±0.001 (106.7)	0.020 ±0.001 (117.6)
PEG	0.061 ±0.015 (115.1)	0.173 ±0.003 (147.9)	0.020 ±0.001 (133.3)	0.024 ±0.002 (141.2)
PEG+0.25mM Putrescine	0.076 ±0.002 (143.4)	0.171 ±0.002 (146.1)	0.023 ±0.002 (153.3)	0.029 ±0.001 (170.6)
PEG+0.5mM Putrescine	0.082 ±0.012 (154.7)	0.181 ±0.001 (154.7)	0.028 ±0.001 (186.7)	0.034 ±0.002 (200.0)

CD at 5% level	For WH147	For UP2338
	A 0.0023	A 0.0021
	B 0.0040	B 0.0036
	AB 0.006	AB N.S.
	A Days after treatment	
	B Treatment	

The figures in parenthesis indicates the values as percent of control.

activity was increased by 15 and 21% on 2 and 6DAT, respectively with 0.25 mM putrescine as compared to their corresponding stressed plants. 0.5 mM putrescine enhanced the enzyme activity by about 40% both on 2 and 6DAT. Verma and Mishra (2005) proposed that increased GR activity by putrescine in leaf of seedlings under salinity might be also potentiating the salt tolerance in *Brassica* seedlings. It could be suggested that the putrescine elevated GR activity could increase the ratio of $NADP^+$/NADPH, thereby ensuring the availability of $NADP^+$ to accept electron resulting into lesser flow of electron to O_2 for generation of ROS (Verma and Mishra, 2005).

4.3.6 Superoxide Dismutase (EC.1.15.1.1 SOD)

Imposition of water stress to wheat seedlings decreased the superoxide dismutase activity in both the cultivars (Table 12). In WH147, it was decreased by 36% and 44.8% as compared to their controls at 2 and 6DAT respectively. In UP2338, the decrease in activity was only 6.9% at 2DAT, but at higher stress level, the activity decreased considerably by 38%. SOD activity was also reported to decline in cowpea plants (Hernandez *et al.*, 1994), pea plants (Hernandez *et al.*, 1995, 1993), and in rice seedlings (Dionisio-Sese and Torbita, 1998), under salt stress and in susceptible genotypes under Zn stress (Cakmak and Marschner, 1988). The basis of susceptibility to Zn toxicity could be due to impaired detoxification of toxic oxygen species, as suggested by Marschner and Cakmak

(1989). Previous studies have also shown that SOD activity in plants was decreased under anoxic conditions in response to lower rates of ROS production

Table 12: Effect of water stress and putrescine on Superoxide Dismutase activity (units mg $^{-1}$protein min^{-1}) in cultivars WH147 and UP2338 of wheat

Treatment	WH147		UP2338	
	2DAT	6DAT	2DAT	6DAT
Control	9.59 ±0.30 (100.0)	10.91 ±1.15 (100.0)	10.27 ±0.27 (100.0)	10.98 ±0.20 (100.0)
Control+0.25mM Putrescine	13.27 ±0.45 (138.4)	14.10 ±0.63 (129.2)	11.41 ±0.39 (111.1)	12.26 ±0.25 (111.7)
Control+0.5mM Putrescine	15.52 ±0.33 (161.8)	17.68 ±0.61 (162.0)	9.79 ±0.20 (95.3)	10.80 ±0.43 (98.4)
PEG	6.14 ±0.47 (64.0)	6.02 ±0.52 (55.2)	9.56 ±0.20 (93.1)	6.81 ±0.16 (62.0)
PEG+0.25mM Putrescine	6.76 ±0.31 (70.5)	7.56 ±0.26 (69.3)	11.10 ±0.71 (108.1)	16.39 ±0.18 (149.3)
PEG+0.5mM Putrescine	8.81 ±0.28 (91.9)	10.59 ±0.35 (97.1)	10.11 ±0.35 (98.4)	11.80 ±0.40 (107.5)

CD at 5% level	For WH147	For UP2338
	A 0.6256	A 0.3669
	B 1.0835	B 0.6355
	AB N.S.	AB 0.899
	A Days after treatment	
	B Treatment	

The figures in parenthesis indicates the values as percent of control.

and it was increased back to aerated levels upon re-aeration (Sarkar *et al.*, 2001). Goggin and Colmer (2005) observed similar results in wheat roots under continuous and intermittent anoxia. On the contrary, oxidative stress had been reported to induce or enhance SOD activity (Bowler *et al.*, 1992; Scandalios, 1990). Higher SOD activity was observed in olive trees (Sofo *et al.*, 2005), rye grass (Bonnet *et al.*, 2000), wheat cultivars (Gupta *et al.*, 2005), *Suaeda nudiflora* MOQ (a halophyte) (Cherian and Reddy, 2000) under various types of stresses. SOD had been proposed to be the first line of defence via detoxification of O_2^- (Siaram *et al.*, 2000). Higher SOD activity has been associated with stress tolerance in plants where overproduction of superoxide is involved (Bowler *et al.*, 1992). Exhibition of higher constitutive and induced levels of SOD in salt tolerant as compared to their salt sensitive counterparts has been demonstrated in cotton (Gosset *et al.*, 1994), barley (Acar *et al.*, 2001), tomato (Shalata and Tal, 1998) and wild beet (Bor *et al*., 2003). This increase might be due to increased enzyme synthesis and thus useful for adaptation under conditions requiring prevention of peroxidation of membrane lipids (Kalir *et al*., 1984).

The exogenous application of putrescine increased the enzyme activity of stressed as well as control plants of both the cultivars. In WH147, 0.25 mM and 0.5 mM putrescine enhanced SOD activity by 10.1 and 43.5% at 2DAT and by 25.6 and 75.9% at 6DAT, respectively. Therefore higher elevation was observed with 0.5 mM putrescine. In UP2338, with 0.25 mM putrescine there was 16.1%

increase in enzyme activity at 2DAT and 140.7% at 6DAT. While 0.5 mM putrescine was not as effective, as there was only 5.7% stimulation at 2DAT and 73.3% at 6DAT. Polyamines increased SOD activity in spring wheat seedlings under osmotic stress (Li *et al.*, 2004) and in Indian mustard seedlings under salt stress (Verma and Mishra, 2005), thus help in protection of the biomolecules from attack of O_2^- radicals. Bouchereau *et al.* (1999) reported that putrescine could bind to SOD and allow them to reach to the sites of oxidative stress within the cells.

4.3.7 Effect of water stress and putrescine on relationship between H_2O_2 producing and H_2O_2 scavenging enzymes

The relationship between the activities of H_2O_2 producing enzymes and H_2O_2–scavenging enzymes (CAT+APX+POD) is presented in Table 13. Water stressed wheat seedlings of both cultivars showed decrease in ratio between H_2O_2 producing enzymes and H_2O_2 scavenging enzymes as compared to their respective control plants. This indicated that water stress decreased the activity of H_2O_2 producing enzyme and/or increased the activities of H_2O_2 scavenging enzymes. In WH147, this ratio decreased by 47.7 and 47.9% on 2 and 6DAT respectively, whereas in UP2338, initially the decrease was less i.e. 34.5% at 2DAT but at higher stress level (6DAT), the decrease in ratio was higher i.e. 50.4%. It thus appears that in WH147 both mild and severe stress had almost similar effect on

both types of enzymes while in UP2338 the effect at 2DAT was mild which then got escalated with progress of stress.

However treatment of stressed seedlings with 0.25 mM putrescine increased the ratio at 6DAT only. 0.5 mM putrescine effectively increased the

Table 13 Relationship of H_2O_2 producing enzymes and H_2O_2 scavenging enzymes.

Treatment	SOD/(CAT+APX+POD)			
	WH147		UP2338	
	2DAT	6DAT	2DAT	6DAT
Control	1:0.094 (100.0)	1:0.096 (100.0)	1:0.145 (100.0)	1:0.125 (100.0)
Control+0.25mM Putrescine	1:0.126 (134.0)	1:0.128 (133.3)	1:0.141 (97.2)	1:0.130 (104.0)
Control+0.5mM Putrescine	1:0.142 (151.1)	1:0.142 (147.9)	1:0.107 (73.8)	1:0.097 (77.6)
PEG	1:0.049 (52.1)	1:0.050 (52.1)	1:0.095 (65.5)	1:0.062 (49.6)
PEG+0.25mM Putrescine	1:0.045 (47.9)	1:0.060 (62.5)	1:0.092 (63.4)	1:0.109 (87.2)
PEG+0.5mM Putrescine	1:0.057 (60.6)	1:0.081 (84.4)	1:0.093 (64.1)	1:0.096 (76.8)

The figures in parenthesis indicates the values as percent of control.

ratio in WH147 by 16 and 55% on 2 and 6DAT respectively. These results demonstrated that putrescine was more effective in inhibiting H_2O_2 production/scavenging system at severe stress. In UP2338, both the concentration of putrescine had no protective effect against mild stress at 2DAT while at higher level of stress at 6DAT, both the concentrations of putrescine ameliorated the stress effect by 75.8% with 0.25 mM and 59.8% with 0.5 mM putrescine. Although SOD is not the only H_2O_2 producing enzyme in plant tissues, the balance between the activity of this enzyme and that of the H_2O_2 scavenging enzymes in cells has considered to be crucial in determining the level of O_2^- and H_2O_2 (Badwai *et al.*, 2004a). It was observed that SOD/(CAT+APX+POD) ratio in the salt sensitive genotype of sorghum increased with salt stress, but it remained same in the salt tolerant genotype of sorghum. The SOD/(CAT+APX+POD) ratio in roots in both salt tolerant and sensitive genotypes increased in response to stress. These results thus showed a strong correlation between salt tolerance and the antioxidant enzymatic system, suggesting that the balance between the activity of H_2O_2 producing enzymes and that of the H_2O_2 scavenging enzymes plays an important role in providing a defense mechanism against salt-induced oxidative damage.

4.4 Effect of water stress and putrescine on antioxidant metabolites

4.4.1 Carotenoids

The carotenoid, an antioxidant metabolite (Schindler *et al.*, 1994; Mishra, 1999), is suggested to be one of the required factors for salt tolerance in crop

Table 14: Effect of water stress and putrescine on Carotenoid content (mg g^{-1}DWt) in cultivars WH147 and UP2338 of wheat

Treatment	WH147		UP2338	
	2DAT	6DAT	2DAT	6DAT
Control	29.08 ±1.02 (100.0)	31.12 ±0.28 (100.0)	16.17 ±0.20 (100.0)	15.91 ±0.08 (100.0)
Control+0.25mM Putrescine	23.39 ±0.13 (80.4)	32.38 ±0.30 (104.0)	11.82 ±0.32 (73.1)	14.10 ±0.15 (88.6)
Control+0.5mM Putrescine	23.97 ±0.27 (82.4)	32.81 ±0.73 (105.4)	14.27 ±0.09 (88.2)	16.94 ±0.04 (106.5)
PEG	26.04 ±0.24 (89.5)	31.27 ±0.33 (100.5)	11.84 ±0.36 (73.2)	11.00 ±0.18 (69.1)
PEG+0.25mM Putrescine	21.07 ±0.26 (72.4)	32.36 ±0.11 (104.0)	12.75 ±0.13 (78.8)	12.01 ±0.29 (75.5)
PEG+0.5mM Putrescine	25.56 ±0.30 (87.9)	34.98 ±0.10 (112.4)	10.41 ±0.21 (64.4)	13.01 ±0.10 (81.8)

CD at 5% level	For WH147	For UP2338
	A 0.5128	A 0.2483
	B 0.8882	B 0.4302
	AB 1.256	AB 0.608
	A Days after treatment	
	B Treatment	

The figures in parenthesis indicates the values as percent of control.

plants (Hernandez *et al.*, 1995). Exposure of wheat seedlings to water stress decreased the carotenoid content in both the cultivars. In WH147, it was found to decrease by 10.5% at 2DAT whereas the higher stress level did not inhibit its content further. In UP2338, the decrease was 26.8% and 30.9% at 2 and 6DAT, respectively, which was higher as compared to that observed in WH147 (Table 14).

The exogenous application of putrescine had differential effect on control as well as in stressed seedlings of both the cultivars. In WH147, both 0.25 mM and 0.5 mM putrescine decreased the carotenoid content of stressed plants at 2DAT by 27.6% and 12.1% respectively, whereas at 6DAT it increased by 4 and 12.4%, respectively. In UP2338, both concentrations of putrescine further decreased the level of carotenoid in the stressed plants. 0.25 mM putrescine was found to decrease its content by 21.2% and 24.5% at 2 and 6DAT, respectively while 0.5 mM putrescine reduced it by 35.6% and 18.2% at these stages, respectively. Verma and Mishra (2005) reported similar results in Indian mustard seedlings under salinity stress. This suggested that putrescine caused reduction in ROS titer could be through quenching of singlet oxygen ($^1O_2$) and of excited chlorophyll by elevated level of carotenoids (Schindler *et al.*, 1994; Mishra, 1999), thereby maintaining chloroplastic membrane integrity and $NADP^+$/NADPH ratio which might reduce the chances of electron escaping and generation of free radicals.

4.4.2 Glutathione

The water deficit stress decreased total glutathione content of the seedlings in both the cultivars. The reduction was proportional to stress level. The reduced from of glutathione was highly affected. In WH147, total glutathione was declined by 35 and 42% at 2 and 6DAT, respectively (Table 15), while there was 28.6% increase in amount of oxidized from of glutathione (GSSG) at 2DAT, which decreased slightly (2.4%) on 6DAT. Reduced glutathione (GSH) was severely affected even at mild stress and its amount was decreased by 76.3% and 83% at 2 and 6DAT as compared to their respective controls. In UP2338, the deleterious effect of stress on total glutathione content was comparatively less. It decreased by 28.3% on 2DAT and 10.6% on 6DAT. However GSH was reduced by 36.1 and 56.4% as compared to controls on 2 and 6DAT respectively (Table 16). GSSG content was more than double in its amount in 6 days stressed seedlings. Glutathione, being a prominent cellular antioxidant plays an active role in protecting membranes against free radical damage (Rennenberg and Brunold, 1994; Rennenberg, 1995). GSH is involved in quenching the free radicals through the ascorbate-glutathione cycle (Smirnoff, 1995). Increased GSH levels were often observed in plants in response to oxidative stimuli (Komives *et al.*, 1998; Noctor *et al.*, 1998; May *et al.*, 1998). GSH accumulation was observed in acifluorfen-treated plant tissues (Gullner *et al.*, 1991; Schmidt and Kunert, 1986).

Dalmia and Sawhney (2004) also reported increased glutathione levels in water stressed wheat seedlings. A possible role of glutathione as an inducer of defense genes has been

Table 15 : Effect of water stress and putrescine on Glutathione content (μmoles g^{-1}DWt) in wheat cultivar WH147

Treatment	WH147					
	2DAT			6DAT		
	Total	GSSG	GSH	Total	GSSG	GSH
Control	43.33 ±1.82 (100.0)	17.13 ±0.99 (100.0)	26.20 (100.0)	76.25 ±1.08 (100.0)	38.87 ±1.45 (100.0)	37.38 (100.0)
Control+0.25mM Putrescine	41.60 ±1.64 (96.0)	15.60 ±0.33 (91.1)	26.00 (99.2)	66.59 ±0.78 (87.3)	33.58 ±1.71 (86.4)	33.01 (88.3)
Control+0.5mM Putrescine	44.91 ±1.47 (103.6)	15.94 ±0.78 (93.0)	28.97 (110.6)	74.43 ±2.59 (97.6)	34.64 ±1.02 (89.1)	39.79 (106.4)
PEG	28.24 ±1.0 (65.2)	22.03 ±2.14 (128.6)	6.21 (23.7)	44.28 ±0.87 (58.1)	37.95 ±2.14 (97.6)	6.33 (16.9)
PEG+0.25mM Putrescine	47.17 ±2.52 (108.9)	16.28 ±0.48 (95.0)	30.89 (117.9)	64.64 ±2.18 (84.8)	31.11 ±1.45 (80.0)	33.53 (89.7)
PEG+0.5mM Putrescine	46.33 ±1.41 (106.9)	12.66 ±0.67 (73.9)	33.67 (128.5)	83.10 ±1.12 (109.0)	31.36 ±1.46 (80.7)	51.74 (138.4)

CD at 5% level	For Total glutathione	For GSSG
	A 1.980	A 1.618
	B 3.430	B 2.803
	AB 4.851	AB N.S.
	A Days after treatment	
	B Treatment	

The figures in parenthesis indicates the values as percent of control.

Table 16 : Effect of water stress and putrescine on Glutathione content (μmoles g^{-1}DWt) in wheat cultivar UP2338.

Treatment	UP2338					
	2DAT			6DAT		
	Total	GSSG	GSH	Total	GSSG	GSH
Control	72.36 ±1.10 (100.0)	14.79 ±0.44 (100.0)	57.57 (100.0)	78.19 ±0.88 (100.0)	18.53 ±0.82 (100.0)	59.66 (100.0)
Control+0.25mM Putrescine	91.07 ±1.11 (125.8)	12.09 ±0.21 (81.7)	78.98 (137.2)	77.07 ±0.87 (98.6)	16.78 ±0.63 (90.5)	60.29 (101.0)
Control+0.5mM Putrescine	84.07 ±0.52 (116.2)	12.33 ±0.50 (83.4)	71.74 (124.6)	82.13 ±0.76 (105.0)	22.05 ±0.28 (119.0)	60.08 (100.7)
PEG	51.9 ± 0.86 (71.7)	15.09 ±1.02 (102.0)	36.82 (63.9)	69.94 ±0.73 (89.4)	43.95 ±0.87 (237.2)	25.99 (43.6)
PEG+0.25mM Putrescine	64.65 ±1.19 (89.3)	15.45 ±0.31 (104.5)	49.20 (85.5)	69.93 ±1.17 (89.4)	37.13 ±1.06 (200.4)	32.80 (55.0)
PEG+0.5mM Putrescine	61.23 ±0.73 (84.6)	15.64 ±0.32 (105.7)	45.59 (79.2)	67.84 ±0.10 (86.8)	39.26 ±1.27 (211.9)	28.58 (47.9)

CD at 5% level

For Total glutathione

A 1.1056

B 1.9150

AB 2.708

A Days after treatment

B Treatment

For GSSG

A 0.8206

B 1.4214

AB 2.010

The figures in parenthesis indicates the values as percent of control.

mooted by Foyer *et al.* (1997). Recently, it has been suggested that H_2O_2 induced chilling tolerance in mung bean plants might be mediated by an elevation of GSH contents (Yu *et al.*, 2002, 2003). On the contrary, decreased glutathione pool have been observed in wheat roots under anoxia (Albrecht and Wiedenroth, 1994; Blokhina *et al.*, 2000) and this presuambly reduced the capacity of these tissues to tolerate oxidative stress during re-aeration (Goggin and Colmer, 2005). Similarly glutathione content decreased to a considerable extent in the presence of copper and this might be one of the mechanisms of toxicity alleviation in *Scenedesmus bijugatus*. Rauser (1999) proposed that enzymes of GSH metabolism participate in the H_2O_2 scavenging pathway. Gullner and Dodge (2000) observed increased content of non-protein thiol and total glutathione in pea leaf discs exposed to subtoxic or slightly toxic concentrations of the xanthene dyes under illumination.

Application of both concentrations of putrescine enhanced total as well as GSH content of stressed seedlings in both the cultivars. In WH147, 0.25 mM putrescine was able to completely restore the level of total glutathione declined under mild stress (2DAT) and of 85% lost at severe stress (6DAT), while 0.5 mM putrescine was fully effective at both 2 and 6DAT. GSH content was increased by both concentrations of putrescine greatly and at 2DAT its content was even more than that observed in irrigated control plants. On the other hand at 6DAT, 0.25 mM putrescine was able to bring GSH level to 90% of the control, thus showing more than 5 fold increase in its concentration as compared to stress plants. While

0.5 mM putrescine was able to completely eliminate the effect of mild and severe stresses on GSH content as it was even higher than that observed in control plants. Putrescine decreased GSSG level which become even lower than that obtained in control plants. In UP2338, putrescine had lesser stimulatory effect. There was some increase in amount of total glutathione and GSH with 0.25 mM putrescine at 2DAT while at 6DAT there was little change in total glutathione content but GSH was increased by 26% with this concentration of putrescine. GSSG remained unchanged with both concentrations of putrescine at 2DAT but at 6DAT its amount was decreased by 15.5% and 10.6% with 0.25 and 0.5 mM putrescine, respectively as compared to stressed plants which was still almost twice than that observed in control plants. Thus recovery in reduced form of glutathione and reduction in GSSG contents by putrescine was less pronounced in UP2338. Putrescine increased the glutathione content in salt stressed *Brassica juncea* seedlings (Verma and Mishra, 2005).

4.4.3 Ascorbic acid

Imposition of water stress, created by PEG, increased total ascorbate content as well as that of reduced ascorbate and dehydroascorbate in both the cultivars. In WH147, total ascorbate content of the stressed plants was more than double as compared to their respective controls. However increase in total content was slightly lesser at higher stress level (Table 16), and both reduced ascorbate and dehydroascorbate level increased correspondingly. In UP2338, total ascorbate

level increased by 86.2% at 2DAT, as stress level increased, its level was raised by 128.3%, while there was 58 and 65% enhancement in level of dehydroascorbate at

Table 17 : Effect of water stress and putrescine on Ascorbic acid content (μmoles g^{-1}DWt) in wheat cultivar WH147

Treatment	WH147					
	2DAT			6DAT		
	Total	DHA	Asc	Total	DHA	Asc
Control	3.31 ±0.18 (100.0)	2.11 ±0.15 (100.0)	1.20 (100.0)	4.29 ±0.23 (100.0)	2.42 ±0.13 (100.0)	1.87 (100.0)
Control+0.25mM Putrescine	3.85 ±0.33 (116.3)	2.50 ±0.22 (118.5)	1.35 (112.5)	4.35 ±0.06 (101.4)	2.67 ±0.04 (110.3)	1.67 (89.3)
Control+0.5mM Putrescine	4.25 ±0.14 (128.4)	2.88 ±0.13 (136.5)	1.36 (113.3)	4.83 ±0.28 (112.6)	3.08 ±0.04 (127.3)	1.75 (93.6)
PEG	7.09 ±0.18 (214.2)	4.27 ±0.05 (202.4)	2.82 (235.0)	8.54 ±0.17 (199.1)	4.30 ±0.09 (177.7)	4.24 (226.7)
PEG+0.25mM Putrescine	5.69 ±0.29 (171.9)	3.60 ±0.13 (170.6)	2.09 (174.2)	6.47 ±0.24 (150.8)	3.60 ±0.22 (148.8)	2.88 (154.0)
PEG+0.5mM Putrescine	6.37 ±0.25 (192.4)	4.56 ±0.17 (216.1)	1.81 (150.8)	7.31 ±0.17 (170.4)	4.47 ±0.13 (184.7)	2.83 (151.3)

CD at 5% level	For Total Ascorbic acid	For DHA
	A 0.2776	A N.S.
	B 0.4808	B 0.3026
	AB N.S.	AB N.S.
	A Days after treatment	
	B Treatment	

The figures in parenthesis indicates the values as percent of control.

Table 18 : Effect of water stress and putrescine on Ascorbic acid content (μmoles g^{-1}DWt) in wheat cultivar UP2338

Treatment	UP2338					
	2DAT			6DAT		
	Total	DHA	Asc	Total	DHA	Asc
Control	6.36 ±0.06 (100.0)	4.63 ±0.20 (100.0)	1.73 (100.0)	6.40 ±0.13 (100.0)	4.27 ±0.13 (100.0)	2.13 (100.0)
Control+0.25mM Putrescine	6.51 ±0.13 (102.3)	5.10 ±0.09 (110.1)	1.41 (81.5)	7.21 ±0.21 (112.6)	5.17 ±0.07 (121.1)	2.04 (95.8)
Control+0.5mM Putrescine	6.33 ±0.25 (99.5)	4.67 ±0.10 (100.9)	1.67 (96.5)	7.05 ±0.15 (110.1)	4.91 ±0.24 (115.0)	2.13 (100.0)
PEG	11.84 ±0.11 (186.2)	7.35 ±0.05 (158.7)	4.49 (259.5)	14.61 ±0.55 (228.3)	7.08 ±0.14 (165.8)	7.52 (353.0)
PEG+0.25mM Putrescine	12.30 ±0.22 (193.4)	8.24 ±0.21 (178.0)	4.06 (234.7)	13.86 ±0.47 (216.6)	7.27 ±0.04 (170.2)	6.58 (308.9)
PEG+0.5mM Putrescine	11.32 ±0.10 (178.0)	7.30 ±0.07 (157.7)	4.02 (232.4)	13.71 ±0.11 (214.2)	6.88 ±0.03 (161.1)	6.82 (320.2)

CD at 5% level	For Total Ascorbic acid	For DHA
	A 0.2967	A 0.1607
	B 0.5139	B 0.2784
	AB 0.727	AB 0.394
	A Days after treatment	
	B Treatment	

The figures in parenthesis indicates the values as percent of control.

2 and 6DAT, respectively. But the corresponding increase in reduced ascorbate content was much higher i.e. 159 and 253%, respectively. Mehlhorn *et al.* (1986) also reported increased ascorbic acid content under oxidative stress. It protected the plants against oxidative damage resulting from aerobic metabolism, and from a range of biotic and abiotic stresses (Smirnoff, 1996). Enhanced ascorbate levels in salt tolerant cultivar of rice than the sensitive ones under salt stress, has been reported by Vaidyanathan *et al.* (2003). With onset of drought stress in wheat seedlings several folds increased ascorbate level was observed but its level decreased with increasing magnitude of stress (Dalmia and Sawhney, 2004). A similar trend of a rapid increase in ascorbic acid pool sizes as an early response of the plants to the stress has been reported previously (Foyer *et al*, 1994). The cellular ascorbate concentration was halved, whereas DHA remained roughly constant in suspension cultured soybean cells (*Glycine max*) under stress, created with 250 nM oxyfluorfen (Knorzer *et al.*, 1996), while Yoshimura *et al.* (2000) reported accumulation of DHA under excess light stress. On the other hand, Borraccino *et al.* (1994) observed decreased ascorbic acid content in senescing oat leaves, which could be due to various factors such as its slower synthesis, faster utilization, or a decreased reduction rate of oxidation product. Similarly declined ascorbic acid was reported in chickpea under salt stress (Kukreja *et al.*, 2006), and in pea leaves by herbicides and dyes (singlet oxygen generating substances) (Gullner and Dodge, 2000). However Goggin and Colmer (2005) did not observed

any significant change in ascorbate concentration under both continuous and intermittent anoxia in wheat roots. Putrescine was partly able to decrease stress-induced elevated level of ascorbate. In WH147, 0.25 mM putrescine decreased total ascorbate of the stressed plants by 19.75 and 24% on 2 and 6DAT, respectively. This decrease was observed in both ascorbate and dehydroascorbate contents. There was reduction of 15.7 and 16.3% in dehydroascorbate content at 2 and 6DAT respectively while ascorbate decline correspondingly by 25.9 and 32%, respectively. 0.5 mM putrescine was less effective but it decreased ascorbate level by 35.8 and 33.3% at 2 and 6DAT respectively. In UP2338, putrescine had very little effect on ascorbate concentration. Putrescine caused induction of total ascorbate level of the control seedlings of both the cultivars (Table 16).

4.5 Effect of water stress and putrescine on Red : Oxi ratio of antioxidant metabolites

4.5.1 Red : Oxi ratio of Glutathione

Water stress in wheat seedlings caused considerable decrease in ratio of GSH : GSSG in both the cultivars though it was much higher in WH147. In this cultivar, the ratio reduced greatly at 2DAT i.e. about 5 folds which was further decreased with increase in stress level. In UP2338, the decrease in ratio was not as high at 2DAT, only about 57% reduction, while at 6DAT the reduction in ratio was as high as observed in WH147. The decrease in ratio of GSH : GSSG in both the cultivars was due to increase in oxidized form of glutathione. Dalmia and Sawhney

(2004) reported that the red : oxi ratio in water deficit wheat seedlings decreased from 4.7 : 1 at 2DAS to 1 : 1 on 8DAS. The decreased ratio suggested

Table 19: Effect of water stress and putrescine on ratio of Reduced : Oxidized Glutathione in cultivars WH147 and UP2338 of wheat

Treatment	WH147		UP2338	
	2DAT	6DAT	2DAT	6DAT
Control	1:0.65 (100.0)	1:1.04 (100.0)	1:0.26 (100.0)	1:0.31 (100.0)
Control+0.25mM Putrescine	1:0.60 (92.3)	1:1.02 (98.1)	1:0.15 (57.7)	1:0.28 (90.3)
Control+0.5mM Putrescine	1:0.55 (84.8)	1:0.87 (83.6)	1:0.17 (65.4)	1:0.37 (119.3)
PEG	1:3.55 (546.1)	1:6.00 (576.9)	1:0.41 (157.7)	1:1.69 (554.2)
PEG+0.25mM Putrescine	1:0.53 (81.5)	1:0.93 (89.4)	1:0.31 (119.2)	1:1.13 (364.5)
PEG+0.5mM Putrescine	1:0.38 (58.5)	1:0.61 (58.6)	1:0.34 (130.8)	1:1.37 (441.9)

The figures in parenthesis indicates the values as percent of control.

greater utilization of the GSH in the stressed seedlings (Dalmia and Sawhney, 2004). Elevated oxidized glutathione was probably emanated due to scavenging of the reactive oxygen species produced as the stress intensity increased and resulting in conversion of GSH to GSSG as suggested by Dhindsa (1987), whereas Gullner and Dodge (2000) observed no substantial increase in the ratio of GSSG to GSH in pea leaves treated with xanthine dyes. Moreover, using the criterion that the GSH : total glutathione ratio is below 0.9 in oxidatively stressed tissues (Noctor *et al.*, 1998), exposure of wheat seminal root segments to H_2O_2 (ratio, 0.2 after 8 h), intermittent anoxia (ratio 0.5 after 72 h) or continuous anoxia (ratio 0.6-0.7 after 24 h) caused oxidative stress. Goggin and Colmer (2005) further proposed that with higher endogenous ascorbate and adequate GR activity, the GSH : total glutathione ratio in the intermittently anoxic tissues was able to remain high. However 0.25 and 0.5 mM putrescine treatment were able to enhance the ratio in the stressed seedlings of both the cultivars. In WH147, 0.25 mM putrescine caused increase in ratio at both 2 and 6DAT, respectively as compared to stressed plants, and effect of 0.5 mM putrescine was still higher. The increase in ratio was probably due to lowering of oxidized glutathione content. Application of both concentration of putrescine were able to reduce the level of GSSG even lower than that observed in control plants. The reduction by 0.25 mM putrescine in stressed seedlings of cultivar UP2338 was 24.4% at 2DAT and 33% at 6DAT. 0.5 mM putrescine was less effective. In this cultivar either concentrations of putrescine

was not as effective in reverting the ratio of GSH : GSSG efficiently at both stress levels.

4.5.2 Red : Oxi ratio of Ascorbic acid

Exposure of wheat seedlings to water deficit stress decreased the ratio of reduced ascorbate : dehydroascorbate in both the cultivars. The reduction in ratio was much less as compared to that observed for glutathione. In WH147, the ratio decreased by only 14.2 and 21.7% at 2 and 6DAT, respectively, whereas in UP2338, the increase was higher as compared to WH147. The reduction in ratio was observed to be 38.8% at 2DAT and 53% at 6DAT. Dalmia and Sawhney (2004) reported a decrease in ascorbate/dehydroascorbate ratio to 0.77 : 1 after 2 and 0.29 : 1 after 8 days of water stress treatment in wheat seedlings. The ozone tolerant soybean cultivar (cv. Essex) exhibited 1.1 to 1.2 times higher levels of ascorbate and it had 1.5 to 2.2 times higher redox ratio of ascorbate to DHA than ozone sensitive cultivar under ozone treatment (Robinson and Steven, 1999). Zaharieva *et al.* (1999) reported that Fe-deficient cucumber roots were enriched both, in ascorbic acid (AA) and DHA while the ratio of AA/(AA+DHA) was decreased by 23% compared to Fe sufficient roots. However exogenously applied putrescine enhanced the ratio in both the cultivars. In WH147, there was 13.9 and 26.8% increase in ratio with 0.25 mM putrescine at 2 and 6DAT respectively, while 0.5 mM putrescine had slightly more stimulatory effect i.e. about 66.8% at 2

and 56.4% at 6DAT, respectively. In UP2338, 0.25 mM putrescine treated stressed seedlings enhanced the ratio by 23.8% at 2DAT and slightly lower at 6DAT. 0.5

Table 20: Effect of water stress and putrescine on ratio of Reduced : Oxidized Ascorbic acid in cultivars WH147 and UP2338 of wheat

Treatment	WH147		UP2338	
	2DAT	6DAT	2DAT	6DAT
Control	1:1.76 (100.0)	1:1.29 (100.0)	1:2.68 (100.0)	1:2.00 (100.0)
Control+0.25mM Putrescine	1:1.85 (105.1)	1:1.60 (124.0)	1:3.62 (135.1)	1:2.53 (126.5)
Control+0.5mM Putrescine	1:2.12 (120.4)	1:1.76 (136.4)	1:2.80 (104.5)	1:2.30 (155.0)
PEG	1:1.51 (85.8)	1:1.01 (78.3)	1:1.64 (61.2)	1:0.94 (47.0)
PEG+0.25mM Putrescine	1:1.72 (97.7)	1:1.25 (96.9)	1:2.03 (75.7)	1:1.10 (55.0)
PEG+0.5mM Putrescine	1:2.52 (143.2)	1:1.58 (122.5)	1:1.81 (67.5)	1:1.01 (50.5)

The figures in parenthesis indicates the values as percent of control.

mM putrescine was even less effective. So putrescine was able to recover the ratio of Asc : DHA only partialy in contrast to complete recovery obtained in case of glutathione.

4.6 Effect of water stress and putrescine on redox state

4.6.1 Glutathione redox state

The redox state of the cell is indicator of cellular metabolism. Imposition of water deficit stress, as created by PEG treatment decreased the glutathione redox state in both the cultivars. However reduction was more obvious in WH147. In this cultivar, the redox state reduced by 81.7% at 2DAT and as the stress level increased on 6DAT the reduction was increased by 82.3% whereas in UP2338, at 2DAT the decrease was only 37.3% and with progressive magnification of stress at 6DAT it decreased considerably by 81.7%. The exogenous application of putrescine to the stressed seedlings increased the redox state in both the cultivars of wheat but the increase was found to be sufficiently higher in WH147 where it was able to increase it to the level even higher than that observed in control seedlings. In UP2338, 0.25 mM putrescine increased the redox state of the stressed seedlings by 30.8 and 49% on 2 and 6DAT, respectively. 0.5 mM putrescine was even still less effective. Glutathione redox was increased in control seedlings of both the cultivars treated with 0.25 mM and 0.5 mM putrescine concentration. However, in UP2338, 0.5 mM putrescine was inhibitory at 6DAT.

Table 21: Effect of water stress and putrescine on Glutathione redox (GSH/GSSG) state of cultivars WH147 and UP2338 of wheat

Treatment	WH147		UP2338	
	2DAT	6DAT	2DAT	6DAT
Control	1.53 (100.0)	0.96 (100.0)	3.89 (100.0)	3.22 (100.0)
Control+0.25mM Putrescine	1.67 (109.1)	0.98 (102.1)	6.53 (167.9)	3.59 (111.5)
Control+0.5mM Putrescine	1.82 (118.9)	1.15 (119.8)	5.82 (149.6)	2.72 (84.5)
PEG	0.28 (18.3)	0.17 (17.7)	2.44 (62.7)	0.59 (18.3)
PEG+0.25mM Putrescine	1.90 (124.2)	1.08 (112.5)	3.18 (81.7)	0.88 (27.3)
PEG+0.5mM Putrescine	2.66 (173.8)	1.65 (171.9)	2.91 (74.8)	0.73 (22.7)

The figures in parenthesis indicates the values as percent of control.

4.6.2 Ascorbic acid redox state

Water stress in wheat seedlings increased the ascorbate redox state in both the cultivars. The increase was higher in UP2338 than in WH147. In WH147, ascorbate redox state enhanced by 15% at 2DAT and 28.6% at 6DAT, whereas in UP2338, the enhancement was 64.9% and 112% at 2 and 6DAT, respectively. However exogenous application of putrescine decreased the redox state in both the cultivars. In WH147, 0.25 mM putrescine lowered the redox state as compared to stressed seedlings by 12.2% and 19.2% at 2 and 6DAT, respectively. A 39.4% and 36.4% reduction was observed by 0.5 mM putrescine treatment at the corresponding stress stages. However in UP2338, reduction in redox state by 0.25 mM putrescine was 19.6% and 15.09% at 2 and 6 DAT, respectively, whereas 0.5 mM putrescine caused only 15.09% and 6.6% decreased at corresponding stages.

The redox state of ascorbate was observed to shift towards its oxidized form under excess light stress in two pea cultivars (Hernandez *et al.*, 2004). Yoshimura *et al.* (2000) also reported that in spinach plants excess light stress, increased DHA level whereas reduced ascorbate level remained unchanged resulting in a decrease in the redox status of the ascorbate (Yoshimura *et al.*, 2000). This indicated that as severity of stress increased, the efficiency of defense mechanism decreased inspite of good functionality of antioxidant enzymes and oxidative stress was intensified as evident from decreased levels of reduced ascorbate in wheat (Dalmia and

Sawhney, 2004). Both the concentrations of putrescine also decreased the ascorbate redox state in control seedlings of both the cultivars of wheat.

Table 22 : Effect of water stress and putrescine on Ascorbate redox (Asc/DHA) state of WH147 and UP2338 of wheat

Treatment	WH147		UP2338	
	2DAT	6DAT	2DAT	6DAT
Control	0.57 (100.0)	0.77 (100.0)	0.37 (100.0)	0.50 (100.0)
Control+0.25mM Putrescine	0.54 (94.7)	0.62 (80.5)	0.28 (90.3)	0.39 (78.0)
Control+0.5mM Putrescine	0.47 (82.4)	0.57 (74.0)	0.36 (97.3)	0.43 (86.0)
PEG	0.66 (115.8)	0.99 (128.6)	0.61 (164.9)	1.06 (212.0)
PEG+0.25mM Putrescine	0.58 (101.7)	0.80 (103.9)	0.49 (132.4)	0.90 (180.0)
PEG+0.5mM Putrescine	0.40 (70.2)	0.63 (81.8)	0.55 (148.6)	0.99 (198.0)

The figures in parenthesis indicates the values as percent of control.

The present investigations as well as those conducted by numerous other workers indicate that putrescine and other polyamines can confer considerable oxidative stress resistance. However, the precise mechanism(s) of this ameliorative effect remains unclear. Various possible mechanisms have been proposed and these may not be mutually exclusive but could be additive. The role of putrescine and other polyamines as scavenger of free radical has been recorded (Drolet *et al.*, 1986; Bors *et al.*, 1989). They could bind to antioxidant enzymes, such as superoxide dismutase, or be conjugated to small antioxidant molecules and allow them to permeable to the sites of oxidant stress within cells. They might also interact with membranes directly either by inhibiting transbilayer movement of phospholipids (Bratton, 1994), or by stabilizing molecular complexes of thylakoid membranes (Popovic *et al.*, 1979; Besford *et al.*, 1993). Exogenous or constitutive putrescine levels might affect the redox state of plant cells. Nagele *et al.* (1994) reported that the superoxide dismutase-mimetic copper complex can dismutate superoxide with high efficiency, and enhance oxidation of glutathione. It can protect against peroxide-induced cell injury. Polyamines may have effects at the gene level, i.e. increased levels of putrescine might either inhibit DNA methylation, permitting the expression of specific genes (Ruiz-Herrera *et al.*, 1995; Martin-Tanguy *et al.*, 1996), or affect gene expression by altering sequence-specific DNA-protein interactions (Burtin *et al.*, 1991; Panagiotidis *et al.*, 1995), or activate or modulate translocation of protein kinases such as CK2 in

signal transduction (Shore *et al.*, 1997).

CHAPTER 5

SUMMARY AND CONCLUSION

The present investigation was carried out primarily to examine the deleterious effects of water-deficit stress due to enhanced production of highly injurious ROS radicals and the subsequent stimulation of antioxidant defence system in leaves of WH147 and UP2238 cultivars of wheat (*Triticum aestivum*). The ameliorating effect of exogenously applied putrescine on formation of ROS radicals and in activation of antioxidant defence mechanism under two levels of water stress was also determined.

In the preliminary experiments, optimal conditions for the extraction and assay of various enzymes of antioxidant defence metabolism were studied. Two concentrations of putrescine (0.25 and 0.5 mM), which exhibited optimum ameliorating effects in both the cultivars, were selected. The salient results obtained during the present investigation have been summarized as below :

1. RWC and osmotic potential of leaves progressively decrease with increasing degree of stress in both the cultivars viz. WH147 and UP2338 of wheat. Exogenous application of putrescine to stressed plants was helpful in maintaining a relatively more favourable RWC and osmotic potential in both the cultivars and 0.25 mM putrescine in cultivar

UP2338 and 0.5 mM putrescine in cultivar WH147 gave maximum recovery.

2. The production of superoxide radical and H_2O_2 was significantly high in the leaves of stressed plants and their contents increased with severity of stress in both the cultivars. However their level was comparatively higher in UP2338. Accumulation of H_2O_2, which is a strong oxidant, led to disruption of cellular membrane integrity. This was obvious from increase in lipid peroxidation (MDA content) and increase in injury to the cellular membrane with increasing levels of stress of both the cultivars. Putrescine reduced the O_2^- radicals and H_2O_2 production under stress in both the cultivars and their levels lower even than that observed in the control plants of WH147. In UP2338, however, putrescine was relatively less effective in inhibiting formation of O_2^- and H_2O_2. Lipid peroxidation was also lowered by applied putrescine and recovery was higher in UP2338 where putrescine was highly effective in preventing lipid peroxidation at both stages of stress.

3. Activities of various H_2O_2 detoxifying enzymes, viz., catalase, ascorbate peroxidase were elevated under water deficit stress in both the cultivars. However, activity of peroxidase declined in both the cultivars at 2DAT only. Putrescine application to the stressed plants further

enhanced the activities of catalase, peroxidase and ascorbate peroxidase. In UP2338, higher stimulation of the activities of these enzymes was observed at 6DAT i.e. at higher stress level.

4. Activity of SOD, the O_2^- radical scavenging enzyme, decreased progressively with intensification of stress level. The reduction in enzyme activity following imposition of stress was lower in UP2338. Exogenous application of putrescine to the stressed seedlings increased its activity in both the cultivars. In WH147, 0.5 mM putrescine caused an almost complete recovery of the enzyme activity at 6DAT. In UP2338 the enhancement in presence of 0.25 mM putrescine was even much higher and, at 6DAT, the enzyme activity was 50% more than that in control plants.

5. The activity of GR was enhanced under stress in both cultivars and increase was greater at higher stress level. Application of putrescine to the stressed plants further increased its activity and the enhancement was higher in UP2338. Dehydroascorbate reductase was not much affected in WH147 either by stress or by exogenous application of putrescine. In UP2338, its activity was enhanced similarly at both levels of stress and further enhancement was observed in response to 0.25 mM putrescine.

6. The concentration of glutathoine, an antioxidant metabolite, decreased progressively with increasing intensity of stress level. The decrease was mainly due to decline in the level of reduced glutathione while the level of oxidized form increased. In case of ascorbate, total ascorbate content as well as that of dehydroascorbate and, reduced ascorbate were enhanced in both cultivars. Exogenous application of 0.5 mM putrescine to stressed plants of cultivar WH147 restored the level of total and reduced glutathione completely but lowered the content of oxidized glutathione. Similar results were obtained in UP2338 but recovery was not as remarkable as in WH147. Putrescine lowered the stress induced elevated level of ascorbate in both cultivars WH147 and UP2338.
7. Water stress resulted in decrease in redox-state of glutathione thus causing oxidative stress which was injurious to plants. However there was increase in ascorbate redox-state in both cultivars and this effect corresponded to stress intensity. Putrescine treatment, however, largely mitigated the unfavourable effect of water stress on redox state of glutathione and ascorbate though the recovery was partial in cv. UP2338.
8. Carotenoids content diminished under water stress in both the cultivars and the reduction was much more pronounced in UP2338. The

putrescine treatment did not show any significant protection against bleaching of carotenoid.

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ABSTRACT

Title of Research Project	:	**Response of reactive oxygen species production and antioxidant metabolism to putrescine under water stress in wheat leaves**
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Title of degree	:	**Master of Science**
Name of Discipline	:	**Biochemistry**
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Degree awarding University	:	**CCS HAU, Hisar – 125 004**
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The reduction in RWC and osmotic potential by water deficit stress in the seedlings of WH147 and UP2338 cultivars of wheat was partially enhanced by putrescine applicaton. The superoxide radicals and H_2O_2 production was high in water deficit plants, also it resulted in the increased lipid peroxidation, but they were decreased on addition of putrescine to the stressed plants, indicating the repair of cellular membranes. Water deficit stress enhanced the activities of H_2O_2 scavenging enzymes viz. catalase, ascorbate peroxidase and enzymes of ascorbate-glutathione cycle but activity of superoxide dismutase, a H_2O_2

producing enzyme, was decreased under stress. Exogenously applied putrescine further elevated the activities of these enzymes along with that of superoxide dismutase in both the cultivars. The level of total and reduced glutatione which was decreased due to water stress was recovered by putrescine application. Redox state of glutathione and ascorbate, disturbed in the stressed plants, was found to recover in putrescine treated stressed plants.

MAJOR ADVISOR **SIGNATURE OF THE STUDENT**

HEAD OF THE DEPARTMENT