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Antioxidant Enzyme Activities and Abiotic Stress Tolerance Relationship in Vegetable Crops

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Abstract

Climatic changes can cause serious reductions in yield and crop quality. Under the threat of climatic changes, one of the precautions to cope is selection and development of resistant vegetable genotypes to abiotic stresses. Several physiological and biochemical reactions and different tolerance levels can occur according to plant species. When plants are subjected to environmental stresses such as salinity, drought, temperature extremes, herbicide treatment and mineral deficiency, the balance between the production of reactive oxygen species (ROS) and the quenching activity of antioxidants is upset, often resulting in oxidative damage. Since activated oxygen species can disrupt normal metabolism through oxidative damage to lipids, protein and nucleic acids, plants possess a number of antioxidant enzymes that protect them from these cytotoxic effects. To control the level of ROS and to protect cells under stress conditions, plant tissues contain several enzymes for scavenging ROS. The high levels of antioxidative enzyme activities were determined in the tolerant genotypes of tomatoes, eggplant, peppers, cucumbers, melons, squash, beans, okra, etc. to several abiotic stress factors. Both the whole plant and in vitro callus culture experiments gave similar results. Antioxidant enzymes can be useful for screening to determine the tolerant and sensitive plant genotypes against abiotic stresses.

Keywords: Drought, salinity, oxidative stress, ROS, chilling

1. Introduction

Stress has a strictly defined physical science definition describing the force per unit area acting upon a material, inducing strain and leading to dimensional change. Biologically, stress has also been defined as the overpowering pressure that affects the normal functions of individual life or the conditions in which plants are prevented from fully expressing their genetic potential for growth, development and reproduction. In the agricultural regard, stress has been

described as a phenomenon that limits crop productivity or destroys biomass. It has become traditional to divide stresses experienced by plants into two major categories: biotic and abiotic stresses. Biotic stresses originate through interactions between organisms, while abiotic stresses are those that depend on the interaction between organisms and the physical environment. Abiotic stresses include potentially adverse effects of salinity, drought, flooding, chilling, metal toxicity, nutrient deficiency, UV exposure, air pollution, etc. [1]. The abiotic stresses represent the factors that most limit the agricultural productivity worldwide. These stresses not only have an impact on current crop species, but they are also significant barriers to the introduction of crop plants into areas that are not currently being used for agriculture [2].

When plants are subjected to environmental stresses such as salinity, drought, temperature extremes, herbicide treatment and mineral deficiency, the balance between the production of reactive oxygen species (ROS) and the quenching activity of antioxidants is upset, often resulting in oxidative damage.

In plants, there are a number of possible ROS sources. These include reactions such as photosynthesis and respiration found in the normal metabolism of plants. This is parallel with the well-known idea that ROS are certain to be one of the products output by aerobic respiration. Pathways that are embellished during abiotic stress also result in ROS production, such as during the photorespiration reaction, where glycolate oxidases in peroxisomes result in superoxidase production. Nonetheless, new sources of ROS have been found recently in plants, such as NADPH oxidases, cell wall-bound peroxidases and amine oxidases. They are involved in ROS production in such processes as cell death and are highly regulated [3]. The ROS are associated with several forms of cellular damage. Since activated oxygen species such as superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($\cdot OH$) can seriously disrupt normal metabolism through oxidative damage to lipids, protein and nucleic acids, plants possess a number of antioxidant enzymes that protect them from these potential cytotoxic effects [4–7].

Plant species and cultivars within a crop species differ greatly in their response to environmental stress. Plants with high levels of antioxidants, either constitutive or induced, have been reported to have greater resistance to oxidative damage [6–9]. Reports suggest that the extent of oxidative cellular damage in plants exposed to abiotic stress is controlled by the capacity of their antioxidant systems [10].

In general, two classes of nonenzymatic antioxidants are found. They are lipid-soluble membrane-associated antioxidants (e.g. α -tocopherol and β -carotene) and water-soluble reductants (e.g. glutathione, phenolics and ascorbate). Ascorbate peroxidase (APX), superoxide dismutase (SOD) and glutathione reductase (GR) compose enzymatic antioxidants and they are thought to search for H_2O_2 in chloroplast and mitochondria. Catalase (CAT) and peroxidase (POD) are the other enzymatic antioxidants and are able to remove H_2O_2 and can neutralise or scavenge oxyintermediates and free radicals [11]. Key enzymes involved in the detoxification of ROS are, namely, SOD, CAT, POD, APX and other enzymes implicated in the Halliwell and Asada cycle (ascorbate–glutathione pathway). Under stress conditions, these antioxidants enhance the activity of almost all of these enzymes [11]. Superoxide radicals that emerge as a result of stress in the plant tissues are transformed into hydrogen peroxide (H_2O_2)

by the SOD enzyme [12, 13]. The accumulation of H_2O_2 , which results from the canalisation reaction of the SOD enzyme and is a powerful oxidant, is prevented by the ascorbate–glutathione cycle. The hydroxyl radical (OH), which is very reactive and the most toxic oxide, can react with all macromolecules without discrimination. SOD and CAT, by combining their actions, can prevent or decrease the formation of this oxide. Even though the particular scavengers are not fully known of the single radical oxygen or the hydroxyl radical, it is thought that SOD functions in removal via chemical reaction [11]. In the defence against intracellular antioxidants, SOD and GSH work together and SOD prevents the radical-mediated chain oxidation of GSH, thus helping GSH in its role as a free radical scavenger physiologically, without the accompaniment of oxidative stress [11, 14]. It was observed that with continued stress conditions, SOD enzyme activity, which acts by decreasing the oxidative oxygen species derived from stress, continued to increase. Even though the linearity of increased stress duration and the increase of SOD activity is concurrent, it was shown that genotypes with more tolerance are superior in this area. The CAT enzyme changes oxidative stress-induced reactive oxygen derivatives, like H_2O_2 , into water and molecular oxygen [15]. CAT, found mostly in glyoxysomes of lipid-storing tissues in plants, contains a tetrameric haeme that catalyses the conversion of hydrogen peroxide, produced from the β -oxidation of fatty acids, into water and oxygen [11, 16]. The GR and APX enzymes, which are a part of the defence mechanism of tolerant genotypes against salt, drought and chilling stress, are generally effective in the reduction of hydrogen peroxide to water in chloroplasts and mitochondria, thereby detoxifying them [17, 18]. APX is one of the most important antioxidant enzymes of plants that detoxify H_2O_2 by using ascorbate for reduction. Different isoforms of APX are active in chloroplasts, cytosol and microsomes [11]. In the ascorbate–glutathione cycle, APX reduces H_2O_2 into water by oxidising ascorbate into monodehydroascorbate (MDHA), which is then converted into ascorbate via the MDHA reductase enzyme; thus, two MDHA molecules are changed into MDHA and dehydroascorbate (DHA) as a non-enzymatic side product in unequal amounts. Subsequently, the reduction of DHA occurs and ascorbate is produced by the action of dehydroascorbate reductase (DHAR) and GR. DHAR can then convert GSH into glutathione disulphide (GSSG) which then is reduced back into GSH by GR [18, 19]. Due to APX activity resulting in the need for regenerating ascorbic acid, it is thought that concurrently an increase in various other components of the antioxidative defence system is needed so that the protective mechanisms of plants can increase as necessary [11]. POD, CAT and APX appear to play an essential protective role in the scavenging process when coordinated with SOD activity. They scavenge H_2O_2 generated primarily through SOD action [11, 20].

The research indicates that APX, CAT, GR and SOD enzyme activities in large variation among cotton varieties [21], tomato [22–24] and melon genotypes [25] in their response to salinity have been observed.

The tolerance of plants to stress has been widely shown to vary with physiological growth stage, developmental phase and size of plants. There is also growing evidence of multiple tolerances to stress in plants with plants showing tolerance to more than one stress. Genetic variability within a species is a valuable tool for screening and breeding for higher stress tolerance.

2. Salinity stress

Salinity is one of the most important abiotic stresses that cause reduction in plant growth, development and productivity worldwide in arid and semi-arid regions, where soil salt content is naturally high and precipitation can be insufficient for leaching. The FAO estimates that 34 million hectares of irrigated land are salt-affected worldwide, and an additional 60–80 million hectares are affected by waterlogging and related salinity [26].

Salt stress changes the morphological, physiological and biochemical responses of plants. There is evidence that high salt concentrations cause an imbalance in cellular ions, resulting in ion toxicity and osmotic stress, leading to the generation of ROS, which cause damage to DNA, lipids and proteins. At the same time, ROS cause chlorophyll degradation and membrane lipid peroxidation, decreasing the membrane fluidity and selectivity. To prevent the negative effects of ROS, plants have developed various antioxidant enzyme systems including non-enzymatic antioxidants (e.g. ascorbic acid, glutathione and carotenoids) and antioxidative enzymes (e.g. GR, SOD and APX). While CAT and peroxidases detoxify the toxic hydrogen peroxide, superoxide is broken down into water and oxygen by catalyses from the SOD enzyme. APX reduces H_2O_2 using ascorbate as an electron donor in the ascorbate–glutathione cycle. Oxidised ascorbate is then reduced by GSH generated from GSSG catalysed by GR at the expense of NADPH. Previous studies showed that the level of antioxidative enzymes increases when plants are exposed to oxidative stress including salinity [27–29].

Plants with high levels of antioxidants, either constitutive or induced, have been reported to have greater resistance to oxidative damage. Genetic variability within a species is a valuable tool for screening and breeding for higher salt tolerance. Some authors have reported large variation among cotton varieties and tomato genotypes in their response to salinity. The response of plants to salt stress is variable and dependent on various factors, particularly plant genotype, extent of stress, age of plant and stage of plant growth when stress is experienced. Plants manifest themselves with various adaptive mechanisms (morphological, biochemical, enzymatic and physiological) to survive under stressed conditions. The extent of these mechanisms' adaptability is unique with every plant genotype [30].

In one recent study, the relationship between antioxidant enzymes and salt tolerance in the leaves of eggplant seedlings of two salt-tolerant varieties (Burdur Bucak and Mardin Kızıltepe) and two salt-sensitive genotypes (Giresun and Artvin Hopa) was examined. In salt-tolerant eggplants, APX, CAT, GR and SOD activities increased significantly when the seedlings were grown in the hydroponic system containing 150 mM NaCl [31]. It was observed, in the results of pumpkin salt tolerance studies, that in the salt-sensitive genotypes (CU-7 and A-24) less of the aforementioned enzymes showed activity as opposed to the increased activity seen in higher salinity levels and in the salt-tolerant genotypes (Iskenderun-4 and AB-44). It was also shown that these enzymes played a role in salinity tolerance of melons [32, 33], green bean [6] and soybean [8].

The salinity experiment in okra genotypes shows that NaCl-induced stress caused decreases in plant biomass, green pigments, photosynthetic activity, stomatal conduction, transpiration rate, number of stomata and stomatal size and resulted in alterations in enzymatic activities

(SOD, POD and CAT) and osmolyte accumulation (proline, glycine betaine, total free amino acids and total soluble sugars). The increase in Na and Cl and lipid peroxidation under saline conditions is the indication of ion toxicity and oxidative damage. However, the oxidative damage is controlled by a defensive system comprising various antioxidants, such as SOD, POD and CAT. The results depicted that salt-tolerant and salt-sensitive genotypes exposed to NaCl stress showed the highest activities of SOD, POD and CAT, both in root and leaf tissues of okra genotypes [30].

According to Yadav et al. [34], plants with high levels of antioxidants have been reported to have greater resistance to this oxidative damage and an increase in the activity of antioxidative enzymes in plants under salt stress. It was observed that increased levels of antioxidants in plants resulted in them having a stronger resistance against the oxidative damage, while plants under salt stress were shown to have the activity of antioxidative enzymes to become greater and that a correlation was seen between tolerance against salt levels and the amount of enzymes. It was also observed that in pea cultures, antioxidant enzyme activity increased when in saline environments, but SOD was unaffected in cucumber. The variations in these observations maybe due to the fact that the effects of salinity depend on a number of factors, for example, salt type, their concentration, plant genotype, growth stage and/or environmental conditions. The mechanism by which salinity affects the antioxidant responses is not yet clear. However, proposed that it might be via the change in membrane integrity caused by high Na⁺ to Ca²⁺ ratio [34].

Salinity inhibition of plant growth is the result of low osmotic potential of soil (water stress), nutritional imbalance, specific ion effect (salt stress), or a combination of these factors. Grafted plants also exhibit phenotypic variations from scion and rootstock plants in terms of salinity tolerance, and grafting onto salt-tolerant rootstocks capable of inducing salt tolerance in the grafted shoots has been an effective method for improvement of salt tolerance in agricultural practices [35]. In parallel, *Solanum lycopersicum* L. ('Elaziğ') grafted on *Nicotiana tabacum* L. ('Samsun') and *Nicotiana rustica* L. ('Hasankeyf'), namely "Tomacco" plant (patent no. TR-2008-05391-B), to 10-d high NaCl irrigation. Physical development, chlorophylls a and b, total chlorophyll, total carotenoid and anthocyanin levels were evaluated [36]. During an increase in osmotic stress levels, plants utilise various antioxidant enzymes and increase their activity; thus these enzymes play vital roles in ROS removal. SOD, which is an important scavenger, is used for producing water and oxygen by catalysing O₂⁻; afterwards the ROS hydrogen peroxide is catalysed by POD and CAT to produce water and oxygen as well [37]. It was reported by Azevedo Neto et al. [38] that antioxidative enzymes (e.g. POD, SOD, GR, CAT and APX) in addition to low molecular mass antioxidants make up the complicated antioxidative defence system seen in plants. It was observed that grafted seedlings when under stress by over the normal amount of Ca(NO₃)₂, contents of hydrogen peroxide and malondialdehyde (MDA), had a much lower percentage of electrolyte leakage and O₂ production rate when compared to those of non-grafted seedlings from which can be deduced that there was less damage to the membrane of grafted seedlings than that of non-grafted seedlings when under excess Ca(NO₃)₂ stress. It was also observed that in grafted seedlings, free radical scavenging systems had a significant job when battling salinity stress, thus resulting in salinity tolerance [38]. In parallel with these results, Wei et al. [39] determined that due to the efficient

scavenging system of free radicals in addition to the mechanisms that utilise antioxidative enzymes and polyamines for protection, eggplant seedlings that were grafted had a greater resistance against stress of over the normal amount of $\text{Ca}(\text{NO}_3)_2$ than when compared to non-grafted eggplant seedlings. For rootstock, they used the eggplant cultivar, Swartz cv. Torvum Vigor, which were tolerant to salinity [39]. Plant tissue culturing has been important in many fields including both agriculturally and commercially and has a significant role in the production of ornamental plants. It has also been invariably useful in manipulating plants for enhanced agronomic performances. In vitro culturing of plant cells provides a means for conducting many studies and improvement in scientific research, including helping to study plant physiology and genomics and their processes and enhancing genetic variability by providing the possibility to help in the breeding of improved cultivars. Thus, it has attracted a large amount of interest in later years. Regenerated plants are expected to have the same genotype as the donor plant; however, in some cases, somaclonal variants have been found among regenerated plants [40]. According to Kusvuran et al. [18], the responses to salt and drought stress of four pumpkin varieties (A-24, CU-7, Iskenderun-4 and AB-44) were investigated under in vitro culture conditions. In this study, it was observed that the tolerant genotypes had less damage on their cell walls (by lipid peroxidation) than that of the sensitive genotypes. While, it was observed that salinity stress had greater effect on the pumpkin genotype than stress by drought. The results of this study suggest that antioxidative defence mechanisms were effective in the pumpkin callus tissues during salt and drought stress. Thus, the increased activity of antioxidative enzyme activity as well as the lower increasing MDA content in the salt- and drought-adapted cells compared with the unadapted cells may contribute to salt and drought tolerance. The results obtained with the callus tissues are in agreement with those observed in studies using seedlings. The results demonstrate the selection of tolerant genotypes for oxidative stress such as drought; salinity could be used for in vitro methods [18]. Similarly, other research on squash, eggplant and melon indicated that SOD, CAT and APX enzyme activities in salt-tolerant genotypes are higher compared to salt-susceptible genotypes in both seedling and callus tissues [41–43].

Antioxidant enzymes such as SOD, POX and CAT are known to substantially reduce the levels of superoxide and hydrogen peroxide in plants. It is one of the most important enzymes used against oxidative stress in the plant defence system, and it occurs ubiquitously in every cell of all types of plants. The most common isoforms of SOD known in the literature are copper–zinc containing SOD (Cu/Zn-SOD), manganese containing SOD (Mn-SOD), iron containing SOD (Fe-SOD) and nickel containing SOD (Ni-SOD) [44]. Moharramnejad and Valizadeh [45] observed the three isoforms for SOD and POX and one isoform for CAT. Authors showed that in salt stress, the main activities of SOD, POX and CAT isozymes are significantly higher than normal conditions in red bean (*Phaseolus vulgaris* L.).

3. Drought stress

Drought stress is one of the most serious abiotic stresses that cause a reduction in plant growth, development and yield in many parts of the world [46–48]. However, plants have developed

different morphological, physiological and biochemical mechanisms to withstand drought stress [49–51]. It is of considerable value to understand the reactions and responses of plants in drought environments as it is also a major part in making crops more tolerant towards stress. It is important to note that responses of plants towards water stress are seen to be considerably different at organisational levels according to the intensity and time they spend under the stressful environment as well as the period of growth they are in and their species [52]. When water becomes limited, the plant generally experiences stomatal closure in an effort to prevent further water loss, limiting the carbon dioxide available for fixation by photosynthesis and reducing NADP⁺ regeneration by the Calvin cycle [53]. These converse conditions increase ROS, such as hydrogen peroxide, superoxide, singlet oxygen and hydroxyl radicals [6, 54]. These ROS attack lipids, proteins and nucleic acids, causing lipid peroxidation, protein denaturing and DNA mutation. Plants possess several antioxidant enzyme systems that protect their cells from the negative effects of ROS. The role of antioxidant enzymes (APX, CAT, SOD and GR) as the components of the main tolerance mechanism is developed in response to different stress conditions. Many reports suggest that the extent of oxidative cellular damage in plants exposed to abiotic stress is controlled by the capacity of their antioxidant systems and the relationship between enhanced or constitutive antioxidant enzyme activities and an increased resistance to drought stress [55–57].

In one study [58], the effects of drought stress on plant growth, relative water content (RWC), ion concentration and activities of the antioxidant enzymes, APX and GR, in eight okra genotypes were investigated. Drought-resistant genotypes exhibit a better protection mechanism against oxidative damage by maintaining a higher inherited and induced activity of antioxidant enzymes than the sensitive genotypes. Previously, 31 different melon genotypes grown under salt and drought conditions were classified according to some growth parameters (i.e. shoot length, plant leaf area, leaf number, fresh and dry weight, leaf water content, ion accumulation and membrane injury index), as well as some antioxidant enzymes activities *in vivo*. At the end of the study, two salt- and drought-tolerant genotypes and two sensitive genotypes were selected according to the growth parameters measured. The aim of this study was to determine the activities of antioxidative stress enzymes in some salt- and drought-sensitive or salt- and drought-tolerant melon varieties grown in *in vitro* culture under salt and drought stress conditions. Another aim of this study was to determine whether *in vitro* callus culture can be used as a screening method for salt and drought stresses in a melon screening study. In contrast with the control, in all the different 8-day salinity and drought stress, it was discovered that growth prevention occurred in callus tissues within media containing 100 mM NaCl or 15% (w/v) PEG-6000 (polyethylene glycol). It was observed in the study from the MDA measurements, from which the amount of lipid peroxidation of the cell wall can be deduced, that the amount of damage to the cell wall (by lipid peroxidation) was more in the sensitive genotypes than that of the tolerant genotypes. The antioxidant enzyme (APX, CAT, SOD and GR) activities were investigated in the callus tissues of four melon genotypes under salt and drought stress. This study suggested that antioxidative defence mechanisms were effective in the melon callus tissues during salt and drought stress. The results demonstrate the selection of tolerant genotypes for oxidative stress such as drought; salinity could be used for *in vitro* methods [59]. Drought tolerance of tomato genotypes [60] was investigated and found

biochemical changes (drought stress index, MDA content and antioxidant enzyme activities) that occur as a result of stress in plants were investigated. In salt-tolerant varieties, T-1 and T-2, the decrease of ions occurred at lower levels under drought conditions. APX, CAT, SOD and GR enzyme activities have increased in drought stress conditions. The four varieties showed an increase in MDA content under drought conditions, especially in the sensitive genotypes. The results indicate that the tomato seedlings respond with enzymatic defence systems against drought-induced oxidative stress.

Water stress tolerance is seen in all plant species but its extent varies from species to species. Effects of different PEG concentrations with drought stress on the activity of antioxidant enzymes, CAT and APX, were investigated in two melon genotypes. Drought tolerant (CU-196) and drought sensitive (CU-3) were grown in hydroponic conditions. Recently, PEG has been used as osmotic pressure inducer in drought physiology studies. In the study, 15, 30 and 45 mM PEG-6000 doses (-0.15 MPa, -0.52 MPa and -1.50 MPa, respectively) were compared to CAT and APX antioxidant enzymes in tolerant and sensitive melon genotypes. At the end of the study, CAT and APX enzyme activities significantly increased in CU-196 than CU-3 [61]. In the other study was conducted for determination of tolerance levels to drought of melon genotypes (Midyat, Şemame, Yuva and Ananas) that have determined the levels of tolerance to salt stress. In this study, three different irrigation methods have been applied to plants. (S0: control-plant-available water, 40% is consumed for irrigation, S1: plant-available water, 90% is consumed for irrigation, S2: during the period of 3–4 leaves of plants completely cut off from the irrigation). Morphological and biochemical changes that occur as a result of stress in plants were investigated. Drought stress applied to the visual scale evaluation of melon genotypes in terms of Midyat and Şemame melons had values close to controls. However, Yuva and Ananas genotypes were found to be more pronounced losses caused by drought. Under drought stress, for Midyat and Şemame genotypes that are tolerant to salt stress, plant fresh and dry weight, the values shown in chlorophyll were closer to control values. At the same time, SOD, CAT, GR and APX enzyme activities have increased in drought stress conditions. However, the susceptible varieties (Yuva and Ananas) compared to the control plants in terms of the parameters studied enzyme activities decreased to varying degrees. In general, it also drought-tolerant melon genotypes found to be tolerant groups, respectively. In particular, these melon varieties have enhanced levels of antioxidant enzyme activities by activating the tolerant concluded [62]. Kiran et al. [60] investigated that determination of tolerance levels to drought of tomato genotypes (TR-68516, Rio Grande, TR-63233, TR-63233 and H-2274). Authors indicated that SOD, CAT, GR and APX enzyme activities have increased in drought stress conditions. On the other hand, in the susceptible varieties (TR-63233 and H-2274) compared to the control plants in terms of the parameters studied enzyme activities decreased to varying degrees [60].

Smirnoff [63] indicated that oxidative damage is also manifest in effects on to proteins and nucleic acids, although these are rarely measured and can be affected by other factors. Oxidation of amino acid residues can be followed by the loss of catalytic activity and denaturation. The damaged proteins may be more susceptible to proteolytic degradation. DNA repairing enzymes may also be induced as a result of oxidative damage to DNA. The various

repair and protection systems found in plants decrease the amount of open oxidative damage and consist of two groups. One of the groups includes CAT, ascorbate, PODs and SOD where in these systems react with oxygen forms that are active and keep them at a minimum level. The second group consists of GR, mono and DHARs, glutathione (GSH) and ascorbate, which are involved in the regeneration of antioxidants that are oxidised. For the purpose of maintaining the superoxide concentration at a minimum, and thus decreasing the production of hydroxyl radicals by the Haber–Weiss reaction of which the catalysis are done by metals, superoxide is converted into hydrogen peroxide by the SOD enzyme catalysis from the first group. Three types of SOD occur in plants: Cu/Zn-SOD, Mn-SOD and Fe-SOD. The latter two have similar amino acid sequences. SOD isoforms occur in most of the subcellular compartments (hydrogen peroxide is broken down to water by CAT which is located in peroxisomes and glyoxysomes). In the chloroplast, this function is fulfilled by ascorbate that also has a cytosolic isoform. In plants, a large amount of the PODs can have major roles other than antioxidants, while ascorbate, with both superoxide and singlet oxygen, has the ability to react non-enzymatically too. In the second group, GSH and ascorbate are the key players in the reactions involved in antioxidants becoming regenerated. In the reaction involving APX, the MDHA radical is the major product and goes on to react with NAD(P)H-dependant monodehydroascorbate reductase (MDHAR) to get reduced to ascorbate. Or, ascorbate and dehydroascorbate (DHA) can be produced by the non-enzymatic reaction between two molecules of MDHA, after which ascorbate is produced by the reduction of DHA by GSH and where the enzyme DHAR catalyses the reaction, with the second product as oxidised GSH (GSSG). An NADP-dependant GR can then reduce the oxidised GSH into GSH. In the chloroplast, these reactions, sometimes known as the Halliwell–Asada cycle, result in the catalysis of the light-dependant reduction reaction from hydrogen peroxide into water by the action of the reductant (NADPH) that is produced by photosynthesis. The key players of this cycle ascorbate and GSH, along with their isoforms (GR, DHAR and MDHAR), are found in large amounts in the chloroplast in addition to other subcellular compartments. The oxidation of GSH pools, which are also essential in keeping sulphhydryl groups of enzymes in reduced forms, could result in enzymes that rely upon these reduced SH groups being inactivated [63].

Yasar et al. [64] investigated 38 genotypes of different pumpkin species for the relationship between the drought tolerance capacity and antioxidant enzyme activity. As a result, it was observed that the enzyme activities are extremely vital in the drought tolerance of the pumpkin genotypes, such as under dry conditions, the drought-tolerant pumpkin genotypes use antioxidative enzymes more actively compared to the drought susceptible genotypes. The genotypes exposed to drought stress had relatively inferior SOD enzyme activity compared to their controls. However, the CAT enzyme activities of these genotypes were found to be increased. Alternatively, the opposite situation was also observed; if the CAT enzyme activities were decreased compared to the controls, the SOD enzyme activities were observed to be increased compared to the genotypes in control group. However, such a relationship was not established for the APX enzyme activities [64].

Drought tolerance in black pepper is attained through osmotic adjustment and better ROS scavenging machinery, functioning through different antioxidant enzymes. The activities of antioxidant enzymes such as SOD and POD become higher during stress in tolerant variety [65].

4. Chilling stress

Low-temperature, or chilling, stress (damage caused by low, but above-freezing temperatures) has been recognised as a unique environmental impact on crop plant physiology [66]. The damage resulting from the symptoms of the chilling stress includes a decrease in growth and yield of the plant. These symptoms consist of the prevention of metabolic processes, rise in the permeability and seepage through the cell membrane due to alterations in the order of the molecules or in the physical form, wilting and chlorosis [67]. Prasad et al. [68] has suggested that mitochondria are critical organelles in the metabolic production of energy in the cell. The competence and the stability of mitochondria are very important for the seedlings to survive low-temperature stress, especially during early seedling growth. Low temperature induces oxidative stress in the cell [69]. Under aerobic conditions, superoxide radicals and H_2O_2 are found to be normal metabolites of plant cells [70, 71] as well as animal cells [72, 73] and are kept at low, steady-state levels by the action of antioxidant enzymes such as SOD, CAT, GSH POD and APX located in the organelles and cytosol [74–76].

Active oxygen species (AOS) has been proposed to be responsible for cold-induced injury because they are produced at higher concentration during cold stress and may initiate degradative reactions, causing lipid peroxidation, membrane deterioration, protein degradation and chlorophyll quenching. An efficient antioxidant activity is essential in order to maintain the concentration of AOS at relatively low levels [67]. On the other hand, the damage that occurs during chilling stress accompanying illumination was thought to be mediated by an oxygen radical. The defence mechanisms of the cell against oxidative stress involve antioxidants that can be found in many plant organs in large amounts to perform vital biological functions. These include the enzyme systems CAT, SOD and numerous PODs, e.g. APX and guaiacol peroxidases (POX) [69]. During photosynthesis, superoxide and hydrogen peroxide are produced as side products and need to be removed. This is achieved by the SOD enzyme and the enzymes GSH and ascorbate from the ascorbate–glutathione cycle. It was shown by Aroca et al. [77] that due to ROS being produced under chilled environments during light-induced photo-oxidation, the major damage from chilling stress occurs during this time. The reason for ROS production under these circumstances is because of the slowing down of the enzymes involved in the Calvin–Benson cycle, thus resulting in the limitation of the NADP⁺ supplements receiving the electrons from the electron transport chain and inducing oxygen to absorb more energy than needed. To decrease photo-oxidation under chilling, there are three important mechanisms. The first one involves avoiding production of ROS by diminishing electron transport chain; the second one involves scattering surplus energy in the form of heat via violaxanthin de-epoxidation, and the third one involves scavenging ROS produced by antioxidant compounds and enzymes. Additionally, the water-water cycle in the chloroplast, where electrons flow in photosystem II from water to photosystem I to reduce oxygen without

O₂ levels having a net change, is said to be an active mechanism that can disperse energy from over excitation when there is an environmental stress. SOD, GR and APX are also some of the antioxidant enzymes involved in the function of this cycle [77]. The enzyme SOD is located in the cytoplasm, chloroplast, mitochondrion and peroxisome and acts as the first line of defence mechanism against ROS by dismutating O₂ into H₂O₂ [78]. Furthermore, the dismutation of superoxide radicals into H₂O₂ and oxygen is an important step in protecting the cell, and in that conversion, SOD is considered a key enzyme [69]. CAT also played a significant role in chilling tolerance and is especially important for removal of H₂O₂ in C3 plants. Exposure to low temperature may increase the amount of AOS not only in cold-sensitive but also in cold-tolerant plants. There was a correlation between the reduction in CAT activity and H₂O₂ accumulation [79].

Oxidative free radicals can be highly reactive towards cell components, and therefore, the ability of the cell to remove these undesirable species might be viewed as an important feature in improved resistance to chilling stress. The increases in the activities of CAT3 provide evidence for the increased production of superoxide and H₂O₂ in mitochondria of maize seedlings. Increases in superoxide and H₂O₂ can be expected in cases in which there is either high O₂ uptake or decreased ability of the electron transport pathway, which increases potential for higher electron leakage to O₂ for subsequent production of superoxide and H₂O₂ [68].

One major antioxidant that plays a role in the detoxification of ROS and plant protection against oxidative damage is glutathione. There are two versions in which glutathione can exist in which are the oxidised disulphide version (GSSG) and the reduced version (GSH). The function of glutathione as an antioxidant is mainly assigned to its reduced (GSH) version as this form is oxidised to form the oxidised (GSSG) version during its function as an antioxidant. Therefore, keeping the concentration of reduced glutathione, from the ratio GSH/GSSG, high is important for plants. The production of GSH can occur both in cytosol and the chloroplast in the leaves of the plant. Furthermore, in the ascorbate–glutathione cycle, GR catalyses GSSG reduction into GSH via donation of electrons from NADPH molecules. ROS detoxification in the chloroplast is known to be mostly carried out by the ascorbate–glutathione cycle, which is thus accepted as the main pathway in this process. In this cycle, ascorbate is also considered to be a major antioxidant in addition to GSH [80]. According to Prasad [81], rapidity with which GR enzyme was induced during the early stages of acclimation and remained induced during chilling and recovery clearly suggests that acclimation uniquely induces the antioxidant defence mechanism that is necessary for protecting the seedling from oxidative stress injury.

Chilling-sensitive pepper cultivars were investigated for SOD, CAT and POD enzyme activity under chilling stress condition. The results showed that the activity of CAT decreased, and both SOD and POD activities raised in two cultivars, However, permeability of plasma membrane was positively related to MDA content, SOD and POD activity and also negatively related to CAT activity variation. As low temperature treating was extended, permeability of plasma membrane, MDA content and POD activity increased and SOD and CAT activity decreased in two cultivars: Xiza No. 7 (less chilling-sensitive cv.) could maintain a higher protective enzyme activity, and permeability of plasma membrane and MDA content were low; Hajiao No. 1 (chilling-sensitive cv.) was quite the contrary [82].

Anderson et al. [83] indicated that the response of ascorbate and glutathione in mesocotyls to acclimation and chilling was generally the same as that in the coleoptile + leaf, although the increase in the total glutathione pools in response to acclimation was not as extensive. Induction of other antioxidants in the mesocotyl may reduce the need for GSH synthesis. In the roots, there was no effect of acclimation or chilling on any of the antioxidants tested. However, the fact that H_2O_2 levels in the roots were not greatly increased by chilling suggests that a modification of antioxidants was unnecessary to prevent oxidative stress [83]. Likewise, Lee and Lee [84] established that APX is also an important antioxidant enzyme in scavenging or utilising H_2O_2 . Total APX activity increased when chilling stress occurred in the leaves of the cucumbers and seemed to be because of favoured induction of the isozymes APX-5 and APX-4. However, 24 h after the stress, the increase seen in APX activity was because of the favoured expression of the isoform APX-3 [84]. Chilling stress causes many physiological and biochemical changes. Kang and Saltveit [85] investigated that chilling tolerance in cucumber seedling radicals. Chilling seedlings with radicles 20-mm long for 48 h at 2.5°C inhibited subsequent growth by 36%, while it reduced the growth of 70-mm-long radicles by 63%. APX activity was higher in 20-mm-long radicals before chilling than in 70-mm-long radicles. It appears that higher APX, CAT and DPPH (the stable free radical 1,1-diphenyl-2-picrylhydrazyl)-radical scavenging activities, and sustained APX activity during chilled and during subsequent growth at 25°C following chilling in 20-mm-long radicals corresponds with higher chilling tolerance. The activities of APX, CAT and DPPH appear to be positively correlated with chilling tolerance [85].

A study on the effects of chilling stress on two salt- and drought-tolerant and two sensitive pumpkin genotypes in callus culture [18] found that the tolerant genotypes showed lower increase in lipid peroxidation and a greater increase in APX, CAT and GR than the tolerant genotypes under stress conditions. It was observed that even though the increase of glucose, proline and fructose concentration went up with prolonging of the chilling effect in all the pumpkin genotypes, a much more significant increase was observed in the tolerant genotype than that of the sensitive genotype. Thus it can be concluded from these results that in pumpkin genotypes, chilling stress results in an increase in the peroxidation of lipids and in oxidative stress, due to reactive oxygen radical production. Song et al. [86] in their study observed that chilling stress, SOD and CAT activities decreased in some extent in both cultivars, in comparison to control in tomato cultivars. Compared to control, chilling stress resulted in significantly higher POD activity in cv. Mawa on day 6, whereas no significant changes of POD activity caused by chilling stress were observed in cv. Moneymaker at all time points tested. On the other hands, APX activities were increased in the two cultivars under chilling stress. GR activities increased in cv. Mawa after chilling stress, but almost no change was observed in cv. Moneymaker [86].

5. Nutrient deficiency and toxicity of heavy metal

The micronutrients essential for the normal growth and development of plants, as it is known to be required in several metabolic processes [87]. Deficiency of nutrients such as Zn, Mn, Cu,

Fe, Mg, B and K can modify the activities of several antioxidative enzymes [88]. Kosesakal and Unal [89] indicated that Zinc (Zn) is one of the essential micronutrients playing a significant role in many vital metabolic processes. Zinc deficiency is a major global problem hindering plant cultivation, and this problem is especially exacerbated in acidic calcareous soils, which is the most common soil type in arid and semi-arid regions of the world. It is known that magnesium deficiency results in the decrease of chlorophyll amounts in beans. It was observed by Welkie et al. [90] that in peppers, the amounts of chlorophyll and iron in leaves were directly proportional, while zinc was also shown to be beneficial in the synthesis of carotenoids and chlorophyll, thus being proved to be essential in the photosynthetic process in plants. Iron (Fe) is a cofactor of many antioxidant enzymes and could act as a pro-oxidant factor because free or loosely bound it catalyses free radical generation in the presence of reductants and peroxides through the Fenton reaction. The growth of sunflower plants under iron deficiency conditions affects POD isoforms differently, inducing a preferential reduction in activity of those isoforms involved in the detoxification processes [91].

Metal toxicities have received widespread attention as large amounts are released into the environment and affect living organisms. Heavy metal intoxication, especially by lead, cadmium, arsenic and mercury, constitutes serious threat to human health [92, 93]. Although information focussed on the relationship between heavy metals and oxidative stress in plants has been available in recent years, it is still difficult to draw a general conclusion about critical toxic metal concentrations in soils [94]. Heavy metals cause oxidative damage to plants, either directly or indirectly through AOS formations which are extremely toxic to living cells. Redox metals such as Cu or Fe appear to act directly on the production of AOS. Copper is among the major heavy metal contaminants in the environment with various anthropogenic and natural sources. Human health risk from heavy metal bioaccumulation in vegetables has been a subject of growing concern in recent years. Excess Cu inhibits plant growth and seed germination, induces chlorophyll degradation and interferes with photosystem activity. At the molecular level, Cu ions generate ROS. These reactive radicals cause oxidative damage of lipids, proteins and nucleic acids. Cu ions also are responsible for alterations of membrane integrity in plant cells. Cu-mediated membrane lipid peroxidation causes membrane damage, thus changing membrane permeability and leading to electrolyte leakage. Plants have evolved several antioxidant defence mechanisms to protect themselves from oxidative damage [95].

Zn is the second most abundant transition metal after iron (Fe) and is involved in various biological processes in organisms. Due to this, the results of the presence of Zinc were investigated, including zinc deficiency, hyperaccumulation and its protective role in plants. However, it is not clear what the implications of zinc stress are on antioxidant responses and the uptake of nutrition, though it is known that excess of zinc is not beneficial and can result in negative symptoms in plants. The symptoms that can be observed at the organism level include prevention of seed germination, of root development and of the growth of the plant, and chlorosis can be seen in the leaves. At the cellular level, excess Zn can significantly alter mitotic activity, affect membrane integrity and permeability and even kill cells. Investigates showed that Zn stress on the activity of many antioxidative enzymes (APX, SOD, POD and CAT) and antioxidant contents (ascorbate and GSH) in plants [96–105].

The availability of manganese (Mn) to plants is governed by redox processes, which depend on soil's Mn reserve, pH and the availability of electrons. However, excess Mn disturbs the metabolism of plants and inhibits the plant growth. Mn causes deficiency of Fe, Mg and Ca and induces inhibition of chlorophyll biosynthesis and a decline in the photosynthetic rate. The toxic effects of heavy metals, both essential and nonessential elements, have been linked to the production of ROS. To quickly get rid of ROS, which result in the disruption of cellular metabolism due to damage by oxidative stress to important molecules, numerous enzymatic and non-enzymatic ways have evolved in living organisms. As Mn^{2+} plays a role in numerous processes, it is thought that an excess of it results in oxidative stress [106]. Cadmium (Cd) is a non-redox metal unable to participate in Fenton-type reactions. Naturally occurring amounts of Cd are normally low, however, the concentration can be significantly increased by anthropogenic activities. The impact of the uptake of Cd by living cells has been shown to be drastic, inducing oxidative stress and normally leading to cell death depending on the metal dose and time length of exposure [107]. In general, Cd in plants reduces growth, both in roots and stems, due to suppression of the elongation growth rate of the cells [94]. According to Dinakar et al. [93], cadmium is easily translocated from plant roots to above-ground tissues and potentially threatens human health. Cadmium in plants interferes with physiological processes, resulting in declined productivity. Cadmium can harness photosynthetic activity, chlorophyll content, plant growth and induce oxidative stress. ROS are efficiently eliminated by non-enzymatic (glutathione, ascorbate, α -tocopherol and carotenoids) and enzymatic defence systems such as SOD, APX, POD and GR, which protect plants against oxidative damage. The detoxification of O_2 occurs due to the SOD enzyme, while H_2O_2 is detoxified by the enzymes CAT and PODs and thus OH radicals are not formed. In the detoxification of hydrogen peroxide from different compartments in the cell, glutathione reductase (GR) and APX are key players in the ascorbate–glutathione cycle. Glutathione is also the substrate for the biosynthesis. A constitutively high antioxidant capacity or increase in antioxidant level could prevent oxidative damage and improve tolerance to the oxidative stress established [108, 109]. Sandalio et al. [108] investigated effects of cadmium on antioxidative enzyme activity in pea. They said that the level of oxygen radicals in cells could be enhanced by a decrease of the enzymatic antioxidants involved in their detoxification, such as SOD.

It was recorded by Schützendübel and Polle [110] that antioxidative enzymes were prevented from functioning and that GSH was depleted for a short period by cadmium and other metals. It was also put forth that hydrogen peroxide accumulation resulted from the depletion in these antioxidants. These results were obtained by accessing models of antioxidative capacity. As more Cd tolerance was observed in plants when more GSH was synthesised, it can be deduced that the decrease in GSH levels is an important step for cadmium sensitivity [110]. Dong et al. [111] investigated that effect of Cd concentration in tomato seedling for antioxidative enzymes. From the results, it can be observed that POD and SOD activities significantly increased in plants that were given Cd with a concentration of 1–10 μM and that MDA levels also showed a significant increase, indicating that oxidative stress response was the result of Cd stress in tomato plants. Tanyolac et al. [112] reported that tolerance and protective mechanisms have evolved to scavenge free radicals such as superoxide, hydroxyl radicals and peroxides generated during various metabolic reactions. Antioxidative enzymes such as APX play a key

role in controlling the cellular level of these radicals and peroxides. They found that APX activity was increased with Cu treatment [112]. Zhao et al. [113] investigated the different tolerance mechanisms to Cd stress between YSL189 and HZ903 at the seedling stage. When Cd concentration was $>20 \mu\text{M}$ in the growing medium, the uptake rate of Cd was significantly higher in roots of YSL189 than in the roots of HZ903. When plants were supplied with 50- and 100- μM Cd in the growing medium, there were higher Cd concentration, higher biomass and plant height, shorter roots and higher expression levels of transporter genes natural resistance associated macrophage proteins (Nramp)2, Nramp3 and zinc and iron regulated transporter (ZIP) in roots of YSL 189 compared to HZ903. The high Cd accumulation in YSL189 was partly due to the higher Cd uptake rate and higher expression levels of Nramp2, Nramp3 and ZIP in its roots. At the same time, the degree of cell injury indicated by thiobarbituric acid reactive substance showed no significant differences in roots and stems between the two genotypes. The higher activities of SOD, POD and CAT in roots and stems of YSL189 were compared to HZ903 [113].

Xiong and Wang [95] indicated that Cu phytotoxicity in *Brassica pekinensis*. Cu treatments increased electrolyte leakage and POD activity, showing a significant correlation between Cu concentration in shoots with electrolyte leakage and POD activity. Oriental melon IVF09 was used as a scion, while the pumpkin Jinxinzhen No. 3 was used as stock to research the physiological characteristics of grafted melon (*Cucumis melo*) seedlings when copper stress was induced. It was observed from the results that copper stress resulted in the inhibition of the physical characteristics of the melon seeds. In the grafted seeds, as opposed to self-rooted seedlings, an increase was seen in the levels of glucose, photosynthetic pigments, fructose, the photosynthetic parameters, biomass, the phosphate and sucrose synthase activities, acid invertase and neutral invertase. When levels of Cu decreased and the levels of P, NA and K increased nutrients were taken up more easily. The concentration of CU in the leaves decreased by 31.3%, while a 15.2% decrease was seen in roots of the grafted seedlings when the levels of copper ion (Cu^{2+}) stress became 800 μM and it was shown that grafting resulted in better endogenous hormone balance in the seedlings. When compared to the control, it was observed that grafted seedlings had a higher concentration of IAA and that POD activity was increased, while concentrations of ABA and maleic dialdehyde and the CAT and SOD activities became less. Thus, it could be deduced that grafting of melon seedlings was beneficial to them when under copper stress and relieved the resulting physiological characteristics from the stress, showing that the resistance of the grafted seedlings to copper stress increased due to grafting [114].

Shi and Zhu [106] indicated that the accumulation of ROS significantly increased in cucumber leaves exposed to excess Mn. It was observed that cucumber leaves in the presence of excess Mn resulted in higher activity of SOD, DHAR, POD and GR while adding SA (salicylic acid) resulted in the inhibition of the activities of APX and CAT, thus showing that different antioxidant enzymes had different changes. When the cucumber leaves were treated with SA, in the presence of excess Mn, the concentrations of the essential glutathione and ascorbate antioxidants increased [106]. Human health risk from heavy metal bioaccumulation in vegetables has been a subject of growing concern in recent years. It was observed by Kiran et

al. that when under abiotic stress, mainly heavy metal applications, the Burdur Merkez and Burdur Bucak genotypes that were salt-tolerant had a higher resistance as opposed to the sensitive genotypes. The results also showed that drought, heavy metal and salinity stress resistance was observed to have evolved in similar ways in plants [115]. Another heavy metal lead (Pb) exerts adverse effects on morphology, growth and photosynthetic processes of plants; causes inhibition of enzyme activities, water imbalance and alterations in membrane permeability; and disturbs mineral nutrition [116]. Wastewater, which is used in agriculture in order to provide growing water demand, might be included heavy metal and trace elements. Lead is one of the most hazardous heavy metals, and it causes an extensive pollution in the environment, and also it has adverse effect on the growing of plants. In the other study was conducted to evaluate the effects of Pb stress in on lettuce (*Lactuca sativa*). It was found that SOD and GR were increased with oxidative stress [115].

6. Conclusions

In conclusion, both the callus tissue and whole plant studies show a positive correlation between increased antioxidant activity and different abiotic tolerance. Antioxidative enzyme activities play an important role against stress. The tolerance level against salt, drought and chilling stress in callus culture can be utilised as an effective criterion in the plants with other physiological criteria. Therefore, it can be said that antioxidative defence mechanisms and effective working systems in the aspect of tolerance against stress conditions in the plants. The literature suggests that tolerant and sensitive genotypes show different responses under abiotic stress conditions, that antioxidative enzyme activities play a protective role against abiotic stress and that antioxidative defence mechanisms are effective in providing resistance to stress in plants. The results of the studies showed that the young plants of the tolerant genotypes may have better protection against stress by increasing the activity of antioxidant enzymes under different abiotic stresses.

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