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Contents

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Research Article (9 pages), Article ID 5686484, Volume 2023 (2023)

Improvement of Polyethylene Glycol, Sorbitol, Mannitol, and Sucrose-Induced Osmotic Stress Tolerance through Modulation of the Polyamines, Proteins, and Superoxide Dismutase Activity in Potato
Zahoor Ahmad Sajid and Faheem Aftab
Research Article (14 pages), Article ID 5158768, Volume 2022 (2022)

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Research Article

Molecular Cloning, Subcellular Localization, and Abiotic Stress Induction Analysis of a Polyamine Oxidase Gene from *Oryza sativa*

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Abiotic stress is a major bottleneck for crop productivity. To counter abiotic stresses, plants have developed several strategies, and the accumulation of polyamine (PA) serving as an osmolyte is one of them. The cellular pool of PAs is primarily regulated by polyamine oxidases (PAOs) either by terminal catabolism or by back conversion (BC) of polyamines. The role of PAO in abiotic stress tolerance has also been reported. Polyamine oxidases are primarily localized in the cytosol, cell wall, and apoplastic regions; however, lately, their peroxisomal localization has also been demonstrated. In this study, we reported the detection of polyamine oxidase isoform 3 from *Oryza sativa* (OsPAO3) in peroxisome as early as 12 h post-transformation under *in vitro* conditions using fluorescent microscopy. The gene was also found to be significantly upregulated by salinity, dehydration, cold, and heat stress. We have also demonstrated that the expression of *OsPAO3* gene was mediated by a second messenger, calcium. The upregulation of *OsPAO3* by salinity, dehydration, cold, and heat stresses suggests that it could be a suitable candidate for providing tolerance to plants against abiotic stress combination or stress matrix, which is a common feature in agricultural field conditions. Furthermore, the data provided here would be valuable in understanding the abiotic stress-mediated signal transduction network of PAOs.

1. Introduction

Being sessile in nature, plants are not able to escape from the adversaries of the various abiotic stresses. To withstand these harsh conditions, plants have developed sophisticated mechanisms of perception, signaling, and counter-response. One such response is the accumulation of polyamines (PA), which are small aliphatic amines present in almost all organisms starting from prokaryotes to eukaryotes [1]. They can be diamine (putrescine), triamine (spermidine), or tetramine (spermine). Usually, these three types of PAs are predicted to have similar biological functions; however, their signatures may differ [1–3]. In plants, PAs have been suggested to be involved in a wide range of functions, from embryogenesis to senescence, including stress tolerance [1, 4].

The cellular PA levels primarily depend upon two types of amine oxidases; copper-containing amine oxidases (CuAOs) and FAD-dependent polyamine oxidases (PAO). The role of PAOs has been implicated in both catabolism and biosynthesis of PAs, referred to as terminal catabolism (TC) and back conversion (BC) type PAO, respectively [1]. The TC-type PAO catalyzes the oxidative deamination of PAs. The end product of this conversion is 4-aminobutanal and N-(3-aminopropyl)-4-aminobutanal, respectively, for spermidine (Spd) and spermine (Spm), along with concomitant production of 1,3-diaminopropane and hydrogen peroxide (H_2O_2) [5–7]. The BC-type PAO leads to the conversion of tetramine (Spm and thermospermine) to triamine (Spd) and triamine to diamine (putrescine) [5–8].

Multiple isoforms of PAOs have been reported in various plant species. The model plant *Arabidopsis thaliana* and *Oryza sativa* have been reported to contain five and seven PAO isoforms, respectively [3, 4]. They have been found to be localized in the cytosol, cell wall, apoplastic region, and peroxisomes [3, 4]. The role of PAOs has also been

implicated in abiotic stress tolerance [9–12]. A common after-effect of all the abiotic stresses is an increase in the cellular concentration of reactive oxygen species (ROS), and in plant cells, peroxisome in addition to chloroplast and mitochondria acts as one of the nodal centers for ROS homeostasis [13]. Owing to the significance of peroxisomes and PAOs in abiotic stress tolerance, the peroxisomal isoforms of PAOs are of much significance. The model plant *A. thaliana* and *O. sativa* contain three such isoforms each, namely, AtPAO2, AtPAO3, and AtPAO4 [3, 6, 14] and OsPAO3, OsPAO4, and OsPAO5 [4].

Furthermore, in abiotic stress signaling, after the perception of the signal, the second messenger plays a critical role in further transducing the message to the downstream components. Normally, calcium has been proposed as a potential second messenger in the majority of abiotic stress signal transduction pathways [15, 16]. The indirect involvement of calcium in PAO-mediated signaling has also been observed [17, 18].

The peroxisomal localization of certain PAOs and their involvement in abiotic stress tolerance have already been documented. Peroxisomes being a nodal center for ROS homeostasis play a significant role in abiotic stress tolerance. A BC-type PAO leads to back conversion of PAs, thereby increasing the cellular concentration of PAs and helping in abiotic stress acclimation. However, it would be interesting to understand at what stage (either early or late) of stress acclimation PAOs play a role, and how their signaling is mediated. In order to understand these, we have used OsPAO3, a BC-type peroxisomal PAO from *O. sativa*. In this study, we studied the time-dependent subcellular localization combined with detailed expression analysis of *OsPAO3* to investigate the involvement of PAO in the early or late stage of stress acclimation. Furthermore, an attempt was made to investigate the underlying second messenger in order to speculate the probable mode of abiotic stress-mediated PAO signaling.

2. Materials and Methods

2.1. Plant Material and Stress Treatment. For all experiments, 11-day-old *O. sativa* IR 64 (Indica rice variety) seedlings were used. The seeds of *O. sativa* were obtained from the National Rice Research Institute, Cuttack, India. This is an abiotic stress-sensitive variety. The seedlings were grown in a plant growth chamber maintained at $28 \pm 2^{\circ}$C, 60% humidity, and a light/dark cycle of 16/8 h. The abiotic stresses were induced by treatment with 200 mM sodium chloride (saline stress), 15% PEG 6000 (dehydration stress), $4 \pm 2^{\circ}$C (cold stress), and $45 \pm 2^{\circ}$C (heat stress). Treatments were given for 0.5, 1, 2, 4, 8, 16, and 24 h. The untreated seedlings served as a control and were used for the calculation of the relative transcript level of *OsPAO3*. All the experiments have been performed a minimum of three times.

2.2. RNA Extraction and Cloning of OsPAO3. Extraction of RNA was conducted using RNeasy plant mini kit from Qiagen as per the manufacturer's protocol, with the modification of inclusion of in-column DNase digestion step (RNase free DNase set, Qiagen, 79254). The extracted RNA was checked for its quantity and quality using a microvolume spectrophotometer (Thermo Fisher Scientific) and agarose gel electrophoresis (Bio-Rad), respectively.

The extracted RNA was converted to cDNA using Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) as per the manufacturer's manual. The obtained cDNA was used for polymerase chain reaction amplification using the primers: forward AATTGCGGCCGCGATGGC GAACAACAGTTCATATGGTG and reverse TATGCC GCGGTCATCACAGCCGGGAGATGAGCAGTG, using HiFidelity polymerase (Qiagen), with PCR cycle of denaturation at 94°C for 20 s, annealing at 60°C for 30 s, and extension at 72°C for 90 s. The obtained PCR product was checked for amplification using agarose gel electrophoresis followed by digestion using NotI and SacII restriction enzymes. The digested product was resolved on an agarose gel followed by gel purification using the GenJET gel extraction kit (Thermo Fisher Scientific). The digested product was cloned in a pCAT plant expression vector under the control of a double 35 S cauliflower mosaic virus (CaMV) promoter [19]. The insert was verified using an automated DNA sequencing. No mutations were detected.

2.3. Subcellular Localization of OsPAO3. The gene of interest (*OsPAO3*) was cloned as a fusion product of enhanced yellow fluorescent protein (EYFP). DsRed-SKL was used as a peroxisomal marker to decipher the identity of the organelle [20]. In the case of single transformation experiments, pCAT plasmid containing EYFP-*OsPAO3* was coated with gold particles, while in the case of double transformation experiments, both the pCAT plasmid containing EYFP-*OsPAO3* and peroxisomal marker (Ds-Red-SKL) were coated with gold particles and bombarded to onion epidermal cells [21]. The onion slices were placed on a wet blotting paper in Petri dishes and stored at room temperature in the dark for approximately 12 h/18 h/24 h, followed by analysis using fluorescent microscopy. Image capture and analysis were conducted as explained [22].

2.4. Protoplast Isolation. Green tissues from the stem to sheath of about 40–60 *O. sativa* seedlings were cut together into approximately 1 mm strips using a sharp razor blade. The strips were incubated in 0.5 M mannitol for 10 min in the dark, followed by O/N incubation in an enzyme solution containing 1.5% cellulase and 0.75% macerozyme. After the enzymatic digestion, an equal volume of W5 solution (154 mM NaCl, 125 mM $CaCl_2.2H_2O$, 5 mM KCl, and 5 mM Glucose) was added, followed by vigorous shaking for 10 s to detach the protoplasts. Protoplasts were filtered through nylon meshes into 15 mL tubes with 3–5 washes of the strips using the W5 solution. The pellet was collected by centrifugation at 1,500 rpm for 5 min at 4°C in a swinging bucket rotor. The pellet was resuspended in 5 mL of W5 medium and centrifuged again at 1,500 rpm for 5 min at 4°C. The pellet obtained was resuspended in 5 mL of W5 medium and incubated in ice for 1 h. The protoplast concentration was

determined by using a hemocytometer. After incubation in ice for 1 h, the suspension was centrifuged (1,500 rpm, 5 min, 4°C), and the pellet obtained was resuspended in ice-cold MaMg solution (0.5 M Mannitol, 15 mM $MgCl_2.6H_2O$, 0.1% MES). The protoplast obtained was used for various treatments; 200 mM sodium chloride [23, 24], 50 μM calcium ionophore A23187 [25, 26], 2 mM calcium channel blocker verapamil [27], and 2 mM calcium chelator BAPTA (1,2-bis(o-amino phenoxy) ethane-N,N,N′,N′-tetra acetic acid) [26, 28]. The treatments were given for 2 h. After treatment, RNA extraction was performed as described in Section 2.2.

2.5. Real-Time PCR. The total RNA was extracted from stress-treated and control (untreated) seedlings as explained above (Section 2.2), its concentration was determined and equalized, and 1 μg of total RNA was converted to cDNA using a high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific) as per the manufacturer's manual and was further used for quantitative real-time PCR analysis using TaqMan™ Open Array™ Real-Time PCR Master Mix (Thermo Fisher Scientific). The reactions were carried out by multiplexing. The *OsPAO3* was labeled with FAM, and endogenous control eukaryotic elongation factor 1 (*OseEF1*) α was labeled with VIC reporter dye, respectively. The TaqMan probes were custom synthesized by Thermo Fisher Scientific. Thermal cycling conditions were as follows: hold stage of 2 min at 50°C, followed by 10 min at 95°C, 40 cycles of denaturation at 95°C for 15 s, and annealing and extension at 60°C for 1 min. The data were captured during the annealing and extension stage. The relative transcript level or "fold change" (degree of change in expression between the treated sample and untreated sample) was calculated by the ΔΔCT method using the formula, relative transcript level/fold change = $2^{-\Delta\Delta CT}$, where ΔΔCT = ΔCT test sample − ΔCT calibrator sample (ΔCT test sample = CT test − CT reference, ΔCT calibrator = CT calibrator − CT reference; test sample = treated sample, calibrator = untreated sample, and reference = endogenous control) [29]. The data provided represent an average of three biological replicates of independent samples, and the values depicted represent mean ± standard deviation.

3. Results

3.1. Molecular Cloning and Subcellular Localization of OsPAO3. The cDNA of the *O. sativa* was used as a template for PCR-based cloning of *OsPAO3*. The primer sequences and PCR cycle are explained in the materials and method section (Section 2.2). The PCR amplified product was found to be of 1500 bp and was cloned in the pCAT vector under the control of a double 35 S CaMV promoter. The gene was cloned as a C-terminal fusion of EYFP. The sequence of the cloned gene was verified using an automated DNA sequencing. No mutations were detected.

For subcellular localization studies, the biolistic bombardment method was used, in which onion epidermal cells were transformed with plasmid constructs coated on gold particles, which were further examined under a fluorescent microscope after an appropriate incubation time. Under standard conditions, the transformed onion epidermal cells are examined after 18–24 h post-transformation (p.t.). Onion epidermal cells transformed only with EYFP, which served as a negative control, showed uniform cytosolic fluorescence (Figure 1(a)). The OsPAO3 which was expressed as a fusion product of EYFP was observed in small punctuate structures after 12 h and 18–24 h p.t. (Figures 1(b) and 1(c)). The identity of fluorescent punctuate structures was confirmed by double transformation experiments coexpressing peroxisomal marker DsRed-SKL. The double transformation experiments demonstrated the overlapping of both green and red fluorescence from EYFP and peroxisomal marker, respectively (Figures 1(d) and 1(e)), confirming the identity of the punctuate fluorescing organelle to be peroxisome. The finding suggests that the PAO3 from *O. sativa* was detectable in peroxisome as early as 12 h p.t.

3.2. Expression Analysis of OsPAO3. For *OsPAO3* gene expression analysis, the abiotic stress-treated seedlings were used for RNA extraction, followed by cDNA synthesis and real-time PCR. The untreated seedlings were used as a control. The expression data were represented as relative transcript level or "fold change" which essentially means the change in the degree of expression between the treated sample and untreated samples. The details are explained in the materials and methods (Section 2.5).

3.2.1. Salt Stress Analysis. The expression pattern of *OsPAO3* was checked at 0.5, 1, 2, 4, 8, 16, and 24 h of 200 mM sodium chloride treatment. The *OsPAO3* mRNA expression rose to 3.7-fold (meaning 3.7 times higher than that of the untreated condition) at 0.5 h, followed by a drop in the transcript level to 1.7-fold at 1 h. The expression of *OsPAO3* again increased to 12-fold at 2 h followed by a steady decline with time and finally reaching 3.2-fold at 24 h (Figure 2(a)). The peak of transcript accumulation was observed at 2 h of sodium chloride treatment. The transcript level of *OsPAO3* increased at 0.5 h followed by a drop at 1 h, and a second surge (increase) in transcript accumulation suggests that *OsPAO3* expression follows a pulsating pattern.

3.2.2. Cold Stress Analysis. Cold stress was given at 4 ± 2°C for 0.5, 1, 2, 4, 8, 16, and 24 h. At 0.5 h, the expression of *OsPAO3* increased to 2.5-folds followed by a drop in the transcript level of *OsPAO3* at 1 h. The transcript level at 1 h was found to be lesser than at the control level. However, after 1 h, the *OsPAO3* transcript accumulation increased to 1.4-fold at 2 h and then peaked to 3-fold at 4 h. Beyond 4 h of cold treatment, the expression reduced, and a basal level of expression of 1.8–2.0-fold was maintained (Figure 2(a)). The cold stress treatment also showed a pulsating pattern of expression, i.e., expression increased at 0.5 h, followed by a drop, and again increased in transcript accumulation at 4 h.

3.2.3. Drought Stress Analysis. Dehydration stress was given by treating the seedlings with PEG6000, which led to the withdrawal of water. The *OsPAO3* expression increased to a 4.2-fold at 0.5 h, followed by a reduction in the transcript

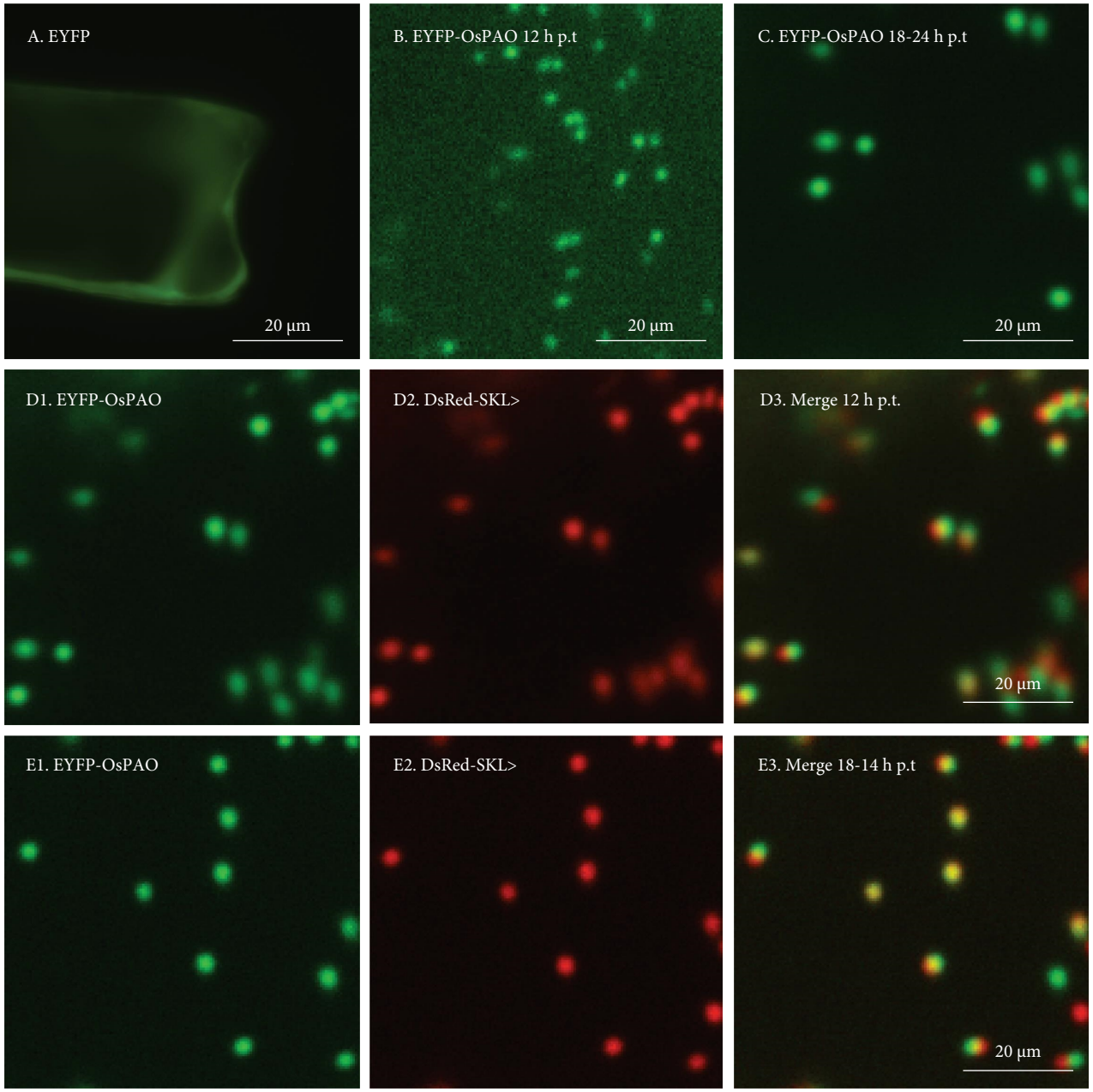

Figure 1: Subcellular localization of OsPAO3. The *OsPAO3* was cloned in the plant transient expression vector pCAT as an EYFP fusion construct. DsRed SKL was used as a peroxisome marker. The plasmids of interest were coated with gold particles followed by biolistic bombardment of onion epidermal cells. The onion epidermal cells were incubated at room temperature followed by analysis using fluorescence microscopy. (a) The cytosolic EYFP fluorescence. (b, c) The EYFP fluorescence localized in punctate structure after 12 and 18 h p.t., which were confirmed to be peroxisomes upon colocalization with peroxisomal marker DsRed-SKL. (d, e) Imaging 12 h and 18–24 h p.t., respectively.

level to 1.5-fold at 1 h. The expression further again increased to 3.6-fold at 2 h, followed by a decrease in transcript accumulation to approximately 2-fold till further time points (Figure 2(a)). The peak of transcript accumulation was observed at 2 h.

In the case of salinity, cold, and dehydration stress, a similarity in the expression pattern was observed. The expression of *OsPAO3* was found to be pulsating in nature, i.e., the expression increased at initial time points (0.5 h), followed by a reduction in the expression, and again a second surge (increase) in expression (2/4 h) was observed.

3.2.4. Heat Stress Analysis. Heat stress treatment was given at 45 ± 2°C. In the initial time points, a basal level of expression of 2.1 to 5.4-fold was maintained, and the peak of

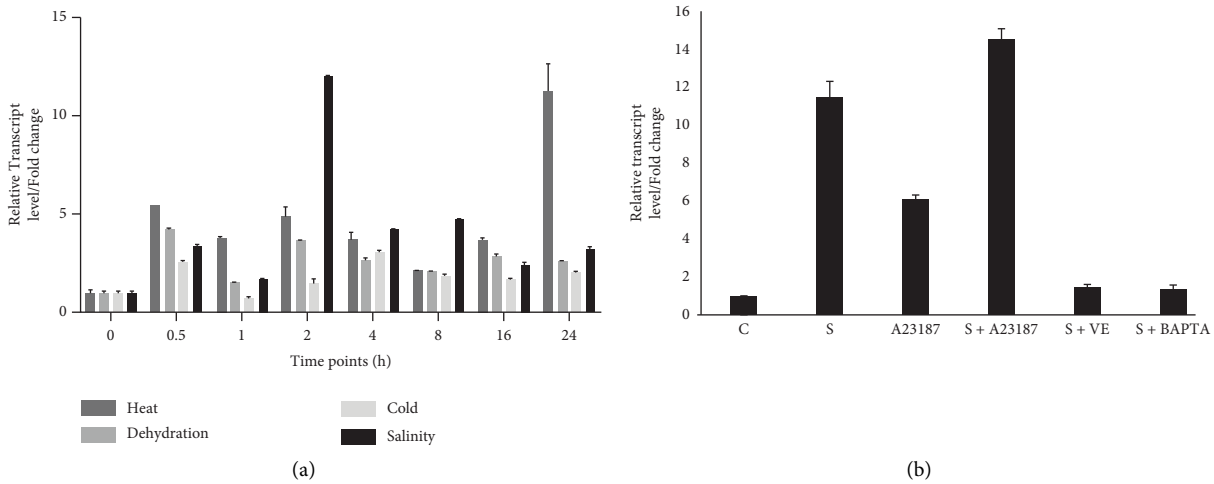

Figure 2: Expression analysis of *OsPAO3*. The expression analysis was performed from the *O. sativa* seedling/protoplast after the treatments. Extraction of RNA was conducted by RNeasy plant mini kit (Qiagen), followed by cDNA synthesis (high-capacity cDNA reverse transcription kit, Thermo Fisher Scientific). The cDNA obtained was used for expression analysis by real-time PCR using TaqMan probes. *OsPAO3* and *OseEF1* were labeled with the FAM MGB probe and VIC MGB probe (custom TaqMan™ gene expression assay), respectively. The assay was conducted by multiplexing. The assays were repeated with a minimum of three replicates, and ΔΔCT values were calculated; relative transcript level (fold change) was determined, and the graph was plotted. The *y*-axis shows the fold change while the *x*-axis in (a) shows the time points of abiotic stress treatment, and (b) shows various chemical treatments: C, control; S, sodium chloride (200 mM); A23187 (calcium ionophore, 50 μM); VE, verapamil (calcium channel blocker, 2 mM); and BAPTA, calcium chelator (2 mM). The data represent three biological replicates, and error bars have been shown.

expression of 11.2-fold was reached at 24 h of heat stress treatment (Figure 2(a)). In case of the heat stress, the peak of transcript accumulation was observed at late hours of treatment.

3.3. Calcium-Dependent Expression of OsPAO3. Furthermore, the probable involvement of calcium as a second messenger in the abiotic stress-induced PAO signal transduction was investigated. The studies were performed on protoplasts isolated from the 11-day-old *O. sativa* leaves. For calcium-related studies, the protoplast system was used, instead of seedlings due to the issue of permeability of specific chemicals used in the studies [25, 30, 31]. The protoplast obtained was treated with calcium channel ionophore A23187 (50 μM) [25, 26], calcium channel blocker verapamil [27], and calcium chelator BAPTA (1,2-bis(o-amino phenoxy) ethane-N,N,N′,N′-tetra acetic acid, 2 mM, [26, 28] in combination with abiotic stress. After the treatment, RNA extraction, cDNA synthesis, and real-time PCR analysis were performed as described earlier. Since the upregulation of *OsPAO3* transcript was the highest with respect to saline stress, hence for calcium-related studies, only saline stress was applied. The peak of *OsPAO3* transcript accumulation due to saline stress was observed at 2 h; hence, all the treatments were given for 2 h only. Calcium channel ionophore A23187 leads to the opening of calcium channels and the release of calcium from cellular storehouses [25, 26, 29], and verapamil is a calcium channel blocker that closes the calcium channel and does not allow the calcium to come out of the cellular storehouse [27], and BATPA is a calcium chelator which sequesters all the free calcium already present in the system [26, 28].

Upon treatment with calcium ionophore A23187, even in the absence of any kind of stress, the expression of *OsPAO3* was found to be increased to 6.1-fold (Figure 2(b)). Since A23187 leads to the opening of cellular calcium channels, suggesting that calcium might be involved in the expression of *OsPAO3*, when protoplasts were co-treated with ionophore A23187 and sodium chloride, the expression of *OsPAO3* further increased to 14.2-fold. The additive effect in expression suggested that ionophore A23187 and sodium chloride stress conditions both led to the release of calcium from the cellular storehouse, followed by an increase in the expression of *OsPAO3*. To further verify the involvement of calcium, the plant cells were treated with calcium channel blocker verapamil and calcium chelator BAPTA. The former obstructs the calcium channels, and hence, calcium was not able to release from the cellular storehouse despite the presence of stimuli, while the latter chelates all the free calcium in the cell. When the plant cells were treated either with verapamil or BAPTA in combination with sodium chloride, the expression level decreased drastically and remained comparable to untreated conditions (Figure 2(b)). This could be due to the fact that when sodium chloride treatment was provided either with a calcium channel blocker (verapamil) or calcium chelator (BAPTA), the stress treatment might have provided the signal for the release of calcium, but in the former scenario, the channels were blocked by the calcium channel blocker; hence, calcium was unable to come out of the cellular storehouse, while in the latter scenario, calcium was able to come out of cellular storehouse, followed by getting chelated due to the presence of calcium chelator; hence, the further downstream signaling could not take place, and *OsPAO3* expression did not occur. As per the data obtained, we suggest that upon the

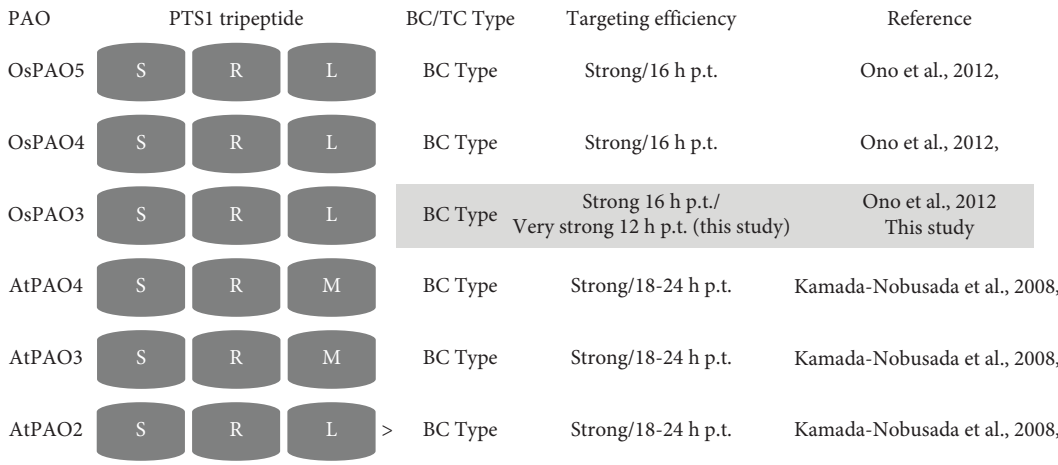

Figure 3: The figure shows the comparative information of *A. thaliana* and *O. sativa* peroxisomal PAOs. The grey boxes represent the PTS1 tripeptide. The greater sign (>) indicates the C-terminal end of the peptide. BC, back conversion; TC, terminal catabolism; p.t., post-transformation. The last column shows the literature reference. Except for OsPAO3, others have not yet been verified at 12 h p.t.

perception of stress conditions by plant cells, the stimuli led to an increase in the cellular concentration of the second messenger (calcium), which in turn led to the upregulation of *OsPAO3*.

4. Discussion

Polyamines have been known to play a major role in protecting the plant against environmental stresses [32]. The cellular homeostasis of PAs is largely controlled by PAOs [33], which are found in multiple subcellular locations: cytosol, cell wall, apoplastic regions, and peroxisomes [11, 34, 35]. Peroxisomes have been known to play a pivotal role in cellular ROS homeostasis [36]. In plant cells, the increase in the concentration of ROS is a common after-effect of abiotic stress conditions [32, 37]. If the accumulation of ROS is left unchecked, it may become detrimental to cells. Due to the considerable implication of PAOs and peroxisomes in abiotic stress-induced ROS homeostasis, the peroxisome-localized PAOs interest us particularly. In our studies, we have reported a PAO isoform 3 from *O. sativa* to be localized in the peroxisome within 12 h p.t. under *in vitro* conditions. The peroxisome localization of PAO has earlier been demonstrated; however, none of the previously reported peroxisomal PAOs were found to be targeted to the peroxisome as early as 12 h p.t. [4, 14], making OsPAO3 the only reported PAO targeted to the peroxisome within a very short span of time under *in vitro* studies, making it "early" peroxisome targeted PAO.

All the peroxisomal PAOs are targeted via peroxisome targeting signal (PTS) type 1, which is primarily represented by the last three amino acids, present at C-terminus [38]. The PTS1 signal in the case of OsPAO3, OsPAO4, and OsPAO5 was found to be SRL> (">" denotes the end of the polypeptide chain) in all three proteins, while the same in the case of AtPAO2, AtPAO3, and AtPAO4 is represented by SRL>, SRM>, and SRM>, respectively [4, 14] (Figure 3). The presence of two different types of PTS1s in *A. thaliana* suggests the occurrence of a greater diversity of PTS1 in *A. thaliana* as compared to *O. sativa*. Traditionally, depending upon the efficiency of PTS1, they have been categorized as canonical (strong) and noncanonical (weak). The classifications have been made on the basis of the time required for the reporter protein (fused with PTS1-containing protein) to be detected in peroxisomes under in vitro conditions [22, 39, 40]. The canonical PTS1 leads to "strong" targeting of reporter proteins and usually has a consensus sequence of (SA), (KR), and (LMI)> at −3, −2, and −1 positions, respectively. These largely do not depend upon the upstream residues. These have been found to occur frequently in the PTS1 proteins of higher plants and have been detected in peroxisomes within 18 h–24 h p.t. [22, 39, 40]. The noncanonical PTS1s require a comparatively longer time to be detected in the peroxisome. Usually, they are detectable in peroxisome 5–7 d p.t. The noncanonical ones are considered "weak" peroxisome-targeting signals [40]. However, recently, Skoulding et al. [41] further elaborated on the classification regarding the efficiency of PTS1s: very strong, strong, moderate, and weak. PTS1-carrying proteins fused to the reporter protein, leading to the detection of fluorescence in peroxisome within 12 and 18–24 h p.t., were categorized as very strong and strong, respectively, while proteins containing PTS1 requiring an extended time of 48 h and 5–7 d p.t. for the fluorescence to be detected in peroxisome have been referred as moderate and weak, respectively.

As per Skoulding et al. [41], SRM> has been demonstrated to be a very strong PTS1. In our studies, we reported OsPAO3 to be a very strong peroxisome targeting protein that contains SRL> as its PTS1, making SRL>, in addition to SRM>, another very strong PTS type 1. However, earlier targeting efficiency studies conducted by Skoulding et al. [41] have been performed on the last ten amino acids fused to a reporter protein, EYFP. This is a standard technique employed for the detection of peroxisomal protein [21, 39, 42–44]. Here, we have performed the localization studies using the full-length OsPAO3 protein fused to the reporter protein EYFP. We could detect OsPAO3 to be in

peroxisome 12 h p.t., suggesting it to be very strongly targeted. This is the first report of a full-length protein fused to EYFP to be detected in peroxisome 12 h p.t. and hence categorized as a very strong peroxisome-targeting signal containing protein.

All the peroxisomal PAOs have been reported to be BC-type. The BC-type PAOs lead to the back conversion of tetramine to triamines and triamines to diamines, thereby increasing the subcellular concentration of PAs, which help the plant in stress acclimation [45]. Furthermore, the expression pattern of *OsPAO3* was analysed in detail with respect to various abiotic stresses, namely, sodium chloride (salinity), cold, dehydration, and heat stress. It was found to be upregulated in the early time point (0.5–2 h) of stress treatment. Peroxisomal PAOs from *Citrus sinensis* and *Solanum lycopersicum* were also found to be upregulated by multiple abiotic stresses [10, 46]. Accumulation of higher levels of *OsPAO3* mRNA in earlier time points of abiotic stress treatment suggests that this particular peroxisomal PAO may have a defensive role in the early stages of stress acclimation and tolerance. This is further supported by the fact that OsPAO3 was found to be an early peroxisome targeting (12 h post-transformation) protein, as shown in Section 3.1. Furthermore, *OsPAO3* was found to be upregulated in case of sodium chloride, cold, dehydration, and heat stress, suggesting it to be a universal abiotic stress acclimation candidate. In the agricultural field, stresses do not act in isolation rather they work synchronously and in combinations. The extent of damage caused to standing crops by two or more abiotic stresses necessitates to the development of plants that are tolerant to multiple abiotic stresses. Agriculturally, important stress combinations, which usually impact the crops together and work synchronously, have been termed as "stress combination" [47] or "stress matrix" [48]. Salinity, dehydration, and cold stresses have been found to be acting synergistically against crop plants [48]. In the present study, *OsPAO3* mRNA was found to be accumulated to a higher extent in response to salinity, dehydration, and cold in earlier time points of treatment, suggesting that it could be a potential candidate for imparting tolerance against stress matrix/stress combination and for engineering plants against multiple abiotic stresses.

In plant cells, calcium has been known to act as a second messenger in a wide range of signal transduction pathways [16]. A transient increase in calcium has also been reported in response to abiotic stresses. The increase in cytosolic calcium concentration occurs due to calcium influx through the plasma membrane from extracellular sources and calcium release from intracellular stores [16]. In our studies, we could demonstrate that the expression of *OsPAO3* is mediated via second messenger calcium (Section 3.3). Previously, Monroy et al. [30] reported that the expression of cold acclimation-specific (cas) genes mediated by calcium using similar means. This would be the first report of its kind, where the involvement of calcium, as a second messenger, has been demonstrated in the PAO-mediated abiotic stress signal transduction network. However, previously, Ca^{2+} has been demonstrated to be linked with PA. In *A. thaliana*, Spd-dependent increase in cytosolic calcium has been demonstrated, [18] and an imbalance in calcium homeostasis in the absence of Spm resulting in hypersensitivity to saline stress has also been observed [49]. The export of PAs to apoplast leads to an influx of calcium, leading to an increase in cytosolic calcium concentration [50].

5. Conclusions

Polyamine oxidases have been reported to play a significant role in abiotic stress tolerance in plants [51]; however, the mechanism by which they impart tolerance remains elusive. It is believed that the BC-type PAOs lead to an increase in the cellular concentration of PA, thereby helping in abiotic stress acclimation. Incidentally, most of the BC-type PAO has been found to be peroxisomal in localization. Peroxisomes play a crucial role in cellular ROS homeostasis, which tends to get disrupted due to stress conditions. In this study, we have investigated a BC-type peroxisomal PAO from *O. sativa*, namely, OsPAO3. The OsPAO3 protein was detected in peroxisomes at 12 h p.t. under in vitro conditions, as demonstrated by fluorescent microscopy results. Hence, it was categorized as a very strong PTS1-containing protein meaning that it is an early peroxisome-targeting protein. The expression analysis of *OsPAO3* revealed that it was upregulated in the early time points of stress (salinity, dehydration, and cold) treatment. The early peroxisome targeting combined with the early upregulation of *OsPAO3* suggests that it could be instrumental in imparting abiotic stress acclimation to *O. sativa* plants at the initial stages of stress acclimation. In order to understand the signaling mechanism, we further studied the probable involvement of second messengers, and it was observed that calcium could be a probable second messenger in the PAO-mediated abiotic stress signal transduction. However, the performance of OsPAO3 further needs to be investigated in the *O. sativa* plants grown under field conditions where multiple abiotic stresses may be applied simultaneously to better understand the involvement of OsPAO3 in imparting tolerance against stress matrix or stress combinations. The stable transgenic *O. sativa lines*, overexpressing *OsPAO3* may also be generated.

Data Availability

The data (microscopic images and real-time PCR data) used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

GC conceptualized the idea, arranged for funding, and wrote the manuscript. IS and SC primarily performed the experiments. PCR helped with the microscopic image analysis.

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Research Article

Improvement of Polyethylene Glycol, Sorbitol, Mannitol, and Sucrose-Induced Osmotic Stress Tolerance through Modulation of the Polyamines, Proteins, and Superoxide Dismutase Activity in Potato

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The present study was planned to investigate the changes in morphological and biochemical parameters of *in vitro*-grown potato (cultivar Cardinal and Desiree) plants under osmotic stress conditions induced by various concentrations of sorbitol, mannitol (0, 0.025, 0.05, 0.10, or 0.15 M), sucrose (0, 2, 3, 4, 6, and 8%), and polyethylene glycol (PEG: MW-4000: 0, 5, 10, 15, and 20%). Nodal segments (ca. 1.0 cm) from healthy *in vitro*-grown potato plantlets were inoculated on Murashige and Skoog's medium consisting of various levels of above mentioned drought stress-inducing agents. Data was recorded on 60^{th} day of incubation exhibited a severe reduction in most of the growth parameters at 0.10 and 0.15 M of sorbitol and mannitol, respectively, and at 5–10% PEG. Similar results were observed when the sucrose level varied from 3% except for the number of roots and plant dry weight, which exhibited an increase in increasing the sucrose level. Data collected for total soluble protein content and activity of an antioxidant enzyme (superoxide dismutase) unveiled an overall increasing trend in osmotically stressed potato plants suggesting their major action in detoxification of active oxygen species produced under osmotic stress. Polyamines (putrescine, spermidine, and spermine) increased significantly in both the cultivars of potato by using osmotic stress-inducing agent in the present investigation indicating their positive role in stress alleviation. Overall results indicated that potato cultivar Desiree was more stress-tolerant than the cultivar Cardinal.

1. Introduction

Yield reduction of major staple food crops under abiotic stresses such as drought stress is becoming a serious problem worldwide affecting food security. It has been a catalyst for great famines of the past and is reported to affect important crops such as soybean and maize in dry Savanna; chickpea and groundnut in Mexico and Central America; and potatoes in various countries of Asia [1]. Drought stress impairs plant growth and ultimately the crop yield by affecting cellular processes such as mitosis, cell expansion, and enlargement [2]. It also creates oxidative stress in plants by generating reactive oxygen species (ROS) including hydroxyl radicals (OH^-), superoxide anions (O_2^-), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) [3]. These ROS cause lipid peroxidation, denaturation of cell membrane, damaging protein structure, and destruction of DNA [4, 5]. Plants have a very strong antioxidant defense mechanism (enzymatic as well as nonenzymatic) to lessen the harmful effects of these ROS. Enzymatic antioxidants include catalase (CAT), peroxidase (POX), ascorbate peroxidase (APX), glutathione reductase (GR), and superoxide dismutase (SOD) [6]. Amongst these, SOD has a central role in the antioxidant defense network as it catalyzes the conversion of superoxide radicals (O_2^-) to hydrogen peroxide and molecular oxygen thus acting as the first line of detoxification against ROS [7]. It has also been observed that an increase in its activity is correlated with increased protection against the

damaging effect of environmental stress [8]. Polyamines (PA's) are important low molecular weight compounds involved in various metabolic processes in plants under both normal and extreme environmental stress conditions [9]. PA's play's regulatory role in plants under osmotic stress conditions by increasing the Ca, K, Fe, Mn, Zn, and NO_3^- ions in Lettuce leaves and reduced stomatal aperture [10]. Involvement of polyamine was also reported by Elsayed et al. [11] under salt stress by enhancing antioxidant enzymes in wheat.

Potato (*Solanum tuberosum* L.) of family *Solanaceae* ranks first amongst nongrain staple food crops with a worldwide production of 388 million tons [12]. It is a good source of dietary starch which is having an adhesive property, binder, texture agent, and filler. Starch content of potato peel is used to produce fuel-grade ethanol. Although, there is a considerable increase in cultivation and production of this crop worldwide, the average yield is still considered as far below the existing potential owing to drought stress as a major limiting factor [13]. Potato has been considered as a drought-sensitive plant because plants have shallower root systems [14]. Its growth and productivity decline sharply with an increase in water stress in countries like Pakistan, where a total cropped area is some 23.63 million hectares. Out of this, 170.3 thousand hectares are being used for potato cultivation with an annual production of 23.4 tons per hectare [15]. In Pakistan, most of the land area is classified as arid and semiarid and water scarcity is a limiting constraint for agricultural production. Improvement in agricultural productivity is, therefore, imperative to ensure higher crop yield under such unfavorable environmental stresses and limited resources.

Plant tissue culture is quite an amenable technique to understand the various aspects of drought tolerance in potatoes [16, 17]. Plants when grown under abiotic stress conditions accumulate various compatible solutes or osmolytes in their cells which include sorbitol and mannitol, polyethylene glycol, etc. [18]. These are highly soluble and low molecular weight organic molecules and mostly accumulate in the cytosol without disturbing the metabolism of cells even at high concentrations. In addition to this, these osmolytes also perform the function of stabilizing protein and structure of cell membranes under drought stress. Unlike drought stress that causes osmotic stress in plants, accumulation of these compounds decreases osmotic potential and hence maintains the cellular turgidity and increases uptake of water. Apart from this they also play an important role in protecting cells against oxidative stress by scavenging reactive oxygen species in plants [19]. Similarly, sucrose has also been found to accumulate in response to water stress conditions in plant tissues [20]. In addition, polyethylene glycol (PEG) has also been reported as a suitable osmoticum to modify the osmotic potential of plants. PEG is not being used up in the cellular metabolism of plants, but it increases water stress by decreasing the water potential of nutrient solutions and has thus been found to be effective in reducing the *in vitro* growth of plants [21].

Several researchers have used different concentrations of abovementioned osmotica to understand the mechanism of drought stress in different plants [22–24]. However, the information about the precise level suitable for effective *in vitro* screening of potatoes is scanty. Considering this, the present study was undertaken with an objective to partially characterize biochemically the effect of some selected osmotica (sorbitol, mannitol, sucrose, and PEG) on *in vitro* cultures of two potato cultivars (Cardinal and Desiree). In doing so, the relative efficacy of abovementioned osmotica was also determined to induce *in vitro* drought like conditions. Considering its significance, work was also carried out on changes in polyamines activity (putrescine, spermidine, and spermine) on exposure to drought stress in potato cultivars used in the present investigation.

2. Materials and Methods

2.1. Plant Material and Experimental Layout. MS basal medium [25] was used with various concentrations of sorbitol, mannitol, sucrose, and polyethylene glycol (PEG; Sigma-Aldrich, St Louis, and MO) to mediate drought stress to potato plants. Potato germplasm of both cultivars (Cardinal and Desiree) was procured from seed center, University of the Punjab, Lahore, Pakistan. For osmotic stress treatments on potato plants, five different concentrations of sorbitol and mannitol (0, 0.025, 0.05, 0.10, or 0.15 M); and six concentrations of sucrose (0, 2, 3, 4, 6, or 8%) were used. In case of PEG: MW-4000, five different levels (0, 5, 10, 15, and 20%) were added to MS liquid medium directly. Single nodal portions of approximately 1.0 cm from earlier raised 30 days-old potato plantlets under *in-vitro* conditions were inoculated on MS medium containing various concentrations of abovementioned osmotica. Ten culture vessels with single nodal segments were inoculated for each treatment. The cultures were incubated for 16 hours under cool white florescent light (40 μmole $m^{-2}{\cdot}s^{-1}$) at $25 \pm 2^{\circ}$C. All the experiments were set up in a complete block design and repeated thrice with 10 replicates to study the effect of sorbitol, mannitol, sucrose, and PEG on potato plants.

2.2. Morphological and Biochemical Analyses. After 60 days of explants inoculation, data were recorded for various growth attributes, i.e., root/shoot length, root, shoot, number of nodes, and fresh/dry weight of plantlets. Estimation of protein content and SOD activity was also carried out at the conclusion of the experiment. Growth parameters were recorded by taking out plants from the culture tubes with intact roots and washing with tap water to remove all the traces of agar. The numbers of shoots/roots, nodes, and tubers were counted carefully. Shoot and root lengths were measured by using a suitable scale (excluding 1 cm; size of explants). Fresh/dry weight of plantlets was recorded by using an electric balance (Scientech 5220) and then by drying in an oven at 60°C for 72 hours.

For the estimation of protein content and superoxide dismutase activities, 1 gm of plant material was ground in liquid nitrogen with 0.01 mL of Tritone X-100 (Sigma-Aldrich, St Louis, MO) and 0.1 g *P*oly*V*inyl *P*olypyrrolidone

(PVP; Sigma-Aldrich). The resulting fine powder was dissolved in 2 mL of Phosphate buffer (0.1 M, pH 7.2) to make a slurry and centrifuged (Sorval RB-5) at $15400 \times g$ for 30 minutes at 4°C. The supernatant was used for the estimation of protein contents and SOD activities.

Total protein contents were estimated by following Biuret method of Racusen and Johnstone [26] with few modifications. Control and experimental samples were prepared. The experimental or reaction mixture in a tube consisted of 2 mL of Biuret reagent and 0.2 mL of supernatant or plant extract. Control comprises of 0.2 mL water and 2 mL of Biuret reagent. Both tubes were kept at room temperature for 20 minutes for the completion of the reaction. Finally, the absorbance was observed at 550 nm on a spectrophotometer (Hitachi U1100). Bovine serum albumin was used for the preparation of the standard curve. The amount of total protein contents was calculated by following formula (Eq. 1):

$$\text{protein contents (mg/g)} = \frac{\text{CV} \times \text{TE}}{\text{EU} \times \text{Wt} \times 1000}, \quad (1)$$

where CV is the curve value, TE is the total extract, EU is the extract used, and Wt is the fresh weight of tissue.

Superoxide dismutase (1.15.1.1) activity was measured spectrophotometrically as purposed by Maral et al. [27] with few modifications. Assay of SOD was based on recording its capability to inhibit the reduction of nitro blue tetrazolium (NBT; Sigma-Aldrich, St Louis, MO) by O_2^- produced photochemically. Experimental and control samples were prepared in separate test tubes. The experimental tube consisted of 2.0 mL of the reaction mixture (50 mM phosphate buffer (pH 7.8), 13 mM methionine, 75 μM nitro blue tetrazolium, 0.1 mM ethylenediaminetetraacetate (EDTA), and 2 μM riboflavin) with 20 μL enzyme extract. The control sample consisted of only a reaction mixture without enzyme extract. Both tubes (experimental and control) were placed under the white fluorescent light of two 30-W tube lights (Philips Pakistan) for 15 minutes to irradiate simultaneously. The absorbance was recorded at 560 nm on a spectrophotometer (Hitachi U1100). SOD activity was calculated by using the following formula (Eq. 2):

$$\%\ \text{inhibition} = \frac{\text{absorbance of control sample} - \text{absorbance of experimental sample}}{\text{absorbance of experimental sample}} \times 100. \quad (2)$$

2.3. Polyamine Extraction and Estimation. Fresh leaves samples (0.5 g) of potato were used for the estimation of polyamine contents. Polyamines were extracted in 1.5 mL ice chilled perchloric acid (5%) as described by Redmond et al. [28]. After centrifugation at 14,000 rpm, 500 μL of supernatant was mixed in Benzoyl chloride (10 μL) followed by adding 1 mL of NaOH (2 molL^{-1}) and vertexing for 20 seconds. After 20 minutes of reaction at 37°C, 2 mL NaCl was added. This mixture was extracted again with 2 mL ether and then centrifuged at 15,000 rpm for 5 minutes at 4°C. The organic phase 1 mL was dried under nitrogen stream and the dried extract was redissolved in 1 mL methanol and filtered (0.22 μm) and stored at −20°C until analysis by high-performance liquid chromatography using an Agilent 1200 Infinity LC on a C18 column (250 × 4.6 mm) (Agilent Technologies, USA). The mobile phase was methanol: water (60 : 40) at a flow rate of 0.7 mL·min^{-1}, column temperature 30°C, and detection wavelength 230 nm. The standard curves were created for the derivatives of putrescine, spermidine, and spermine (put, spd, and spm) standards (Sigma USA) between 1 and 100 μgmL^{-1}.

2.4. Statistical Analysis. Univariate analysis was employed by using SPSS Version 22.0.0 to analyze the data. The standard error of means was calculated for each treatment. To compare the means values, Duncan's multiple range test was used.

3. Results

3.1. Effect of Various Concentrations of Sorbitol on Potato Plants. A significant decrease was observed in shoot length, number, and root length in both potato cultivars (Cardinal and Desiree) by increasing the concentration of sorbitol (Table 1). In case of Cardinal plants, a decrease in shoot length from 14.833 to 6.094 cm was recorded by a gradual increase of sorbitol from 0 to 0.15 M in MS medium. Similarly, root length was reduced from 10.294 to 4.766 cm and this reduction in root length was significant at 0.10 and 0.15 M concentration of sorbitol. As in the case of cv. Cardinal, both shoot and root length in Desiree decreased from 13.527 to 4.244 cm and 10.444 to 5.794 cm, respectively, with an increasing sorbitol concentration in MS medium. Number of roots and nodes also showed a decreasing trend in both the cultivars. Number of shoots, however, exhibited an increase in Cardinal, and a decrease in Desiree. Sorbitol treatment also significantly decreased the total fresh/dry masses of Desiree plants. Fresh weight was decreased from 0.910 to 0.293 g and dry weight from 0.072 to 0.039 g at 0.15 M concentration. However, the effect of different concentrations of sorbitol was nonsignificant in the case of dry weights of Desiree plants. Overall, plants that were grown on MS medium without any sorbitol treatment were healthier compared with the plants grown over various sorbitol treatments. Furthermore, a change in leaf morphology was also observed at higher sorbitol levels (0.15 M), where leaves appeared relatively smaller in size and yellowish green in color (Figure 1).

There was a significant difference in sorbitol treatment on total protein contents in both the cultivars. Proteins increased gradually by a value of 0.23 mgg^{-1} to 0.85 mgg^{-1} at 0 to 0.10 M sorbitol concentration. However, at a higher concentration of sorbitol (0.15 M), a decrease (0.79 mgg^{-1}) in its content was recorded as compared to 0.10 M. SOD

Table 1: Growth parameters of potato cultivars in response to osmotic stress induced by various levels of sorbitol.

Medium	Sorbitol (M)	Shoot length (cm)		No. of shoots		Root length (cm)		No. of roots		No. of nodes		Fresh wt. (g)		Dry wt. (g)	
		Car	Des	Car	Des	Car	Des	Car	Des	Car	Des	Car	Des	Car	Des
*S1	0	14.83 ± 0.41^{a}	13.52 ± 0.71^{a}	1.83 ± 0.18^{c}	3.11 ± 0.26^{a}	10.29 ± 0.51^{a}	10.44 ± 0.69^{a}	7.44 ± 0.47^{a}	8.72 ± 1.02^{a}	20.33 ± 0.41^{a}	16.27 ± 0.49^{a}	0.42 ± 0.03^{ab}	0.91 ± 0.09^{a}	0.03 ± 0.00^{a}	0.07 ± 0.01^{a}
*S*2	0.025	14.68 ± 0.61^{a}	10.69 ± 0.81^{b}	3.44 ± 0.23^{a}	2.50 ± 0.28^{ab}	9.63 ± 0.49^{a}	9.92 ± 0.41^{ab}	6.33 ± 0.59	6.72 ± 0.66^{a}	20.27 ± 0.73	13.33 ± 1.08^{b}	0.54 ± 0.05^{a}	0.83 ± 0.08^{ab}	0.03 ± 0.00^{a}	0.06 ± 0.00^{a}
*S*3	0.05	12.250 ± 0.77^{b}	8.58 ± 1.10^{b}	2.50 ± 0.18^{b}	2.33 ± 0.32^{bc}	8.92 ± 0.80^{a}	9.21 ± 0.69^{ab}	6.55 ± 0.67^{a}	6.61 ± 0.87^{a}	19.72 ± 0.83^{a}	9.77 ± 0.96^{c}	0.29 ± 0.02^{b}	0.68 ± 0.10^{ab}	0.05 ± 0.03^{a}	0.05 ± 0.01^{a}
*S*4	0.10	8.58 ± 0.87^{c}	9.07 ± 0.73^{b}	3.33 ± 0.25^{a}	1.72 ± 0.22^{cd}	7.14 ± 0.68^{b}	8.53 ± 0.32^{b}	5.61 ± 0.81^{ab}	6.38 ± 0.55^{a}	15.94 ± 0.90^{b}	13.27 ± 0.90^{b}	0.32 ± 0.05^{b}	0.59 ± 0.08^{b}	0.03 ± 0.00^{a}	0.05 ± 0.01^{a}
*S*5	0.15	6.09 ± 0.69^{d}	4.24 ± 0.60^{c}	2.33 ± 0.22^{bc}	1.05 ± 0.05^{d}	4.76 ± 0.54^{c}	5.79 ± 0.84^{c}	3.94 ± 0.59^{b}	3.88 ± 0.61^{b}	13.11 ± 0.90^{c}	7.83 ± 0.88^{c}	0.29 ± 0.05^{b}	0.29 ± 0.04^{c}	0.02 ± 0.00^{a}	0.03 ± 0.00^{a}
Significance ($P \leq 0.05$)		*	*	*	*	*	*	*	*	*	*	*	*	NS	NS

Results are mean from at least ten to replicate cultures ± S.E. Similar letters within the column do not differ significantly ($P \leq 0.05$) according to DMRT. Significant (*) or nonsignificant (NS) at $P \leq 0.05$. *MS [25] contains different levels of sorbitol (designated as *S*1 to *S*5; sorbitol level as shown in the next column).

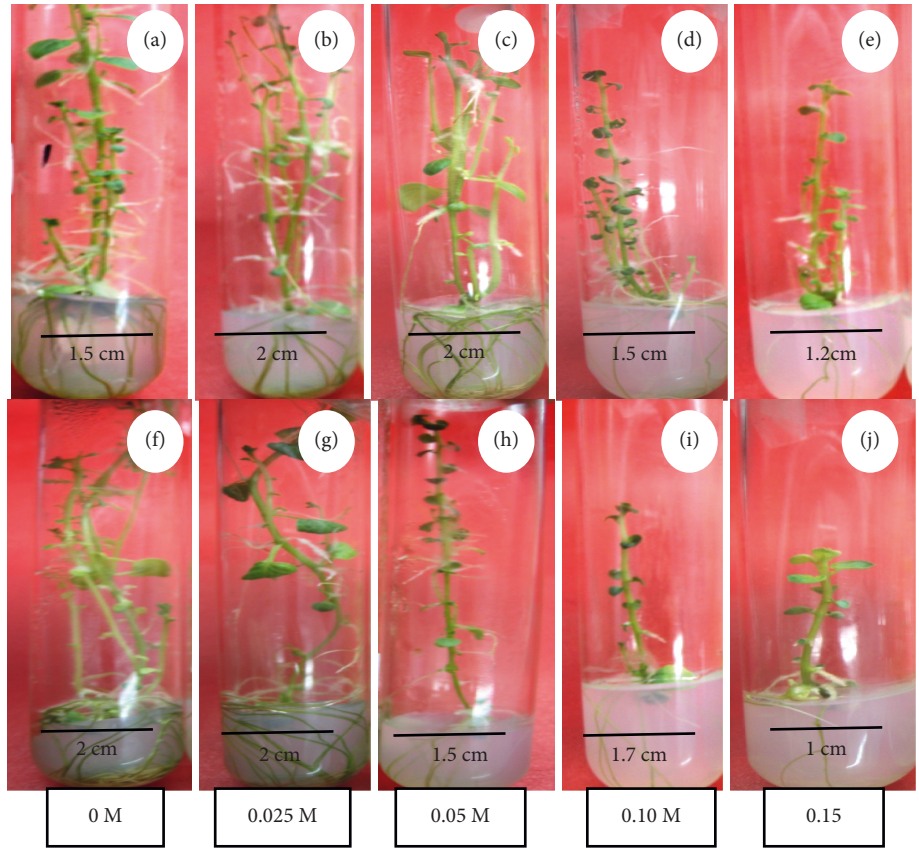

FIGURE 1: Growth of CVS. Cardinal (a)–(e) and Desiree (f)–(j) at different sorbitol concentrations. Scale bar. (a) = 1.5 cm, (b)–(c) = 2.0 cm, (d) = 1.5 cm, (e) = 1.2 cm, (f)–(g) = 2.0 cm, (h) = 1.5 cm, (i) = 1.7 cm, and (j) = 1.0 cm.

activity was also found to increase significantly in sorbitol-treated Cardinal plants as compared to control. However, the increase was not gradual as the maximum value for SOD activity (32.25 Umg^{-1} of protein) was observed at 0.025 M sorbitol concentration, which was followed by a progressive reduction in SOD activity with increasing sorbitol concentration in the medium. As indicated in Figures 2(a) and 2(b), SOD activity increased gradually by increasing sorbitol concentration in case of Desiree plants as compared to control ones. However, its value was decreased to 14.29 Umg^{-1} of protein at 0.15 M sorbitol level as compared to plants without any sorbitol treatment, i.e., 19.51 Umg^{-1} of protein.

The contents of endogenous polyamines in both cultivars of potato increase significantly by adding various concentrations of sorbitol in MS medium and the highest contents of all the investigated polyamines were observed in plants growing on 0.15 M of sorbitol. putrescine contents increased from 125.23 (control) to 150.89 $nmol{\cdot}g^{-1}$ FW (10 M) in cv. Cardinal, however, in Desiree this increase in putrescine contents was not as higher as in case of cardinal. Spermidine also showed an increasing trend by increasing concentrations of sorbitol from 2.5 to 10 M. Similarly, spermine also showed an increasing trend but its amount was less as compared to putrescine and spermidine. Potato cv. Cardinal showed less increase in polyamine contents as compared to Desiree (Table2).

3.2. Effect of Various Concentrations of Mannitol on Potato Plants. Maximum shoot/root length (12.565 and 9.145 cm, respectively) was observed at 0 M and minimum (2.360 and 2.896 cm, respectively) at 0.15 M mannitol concentration. Similar results were observed for a number of roots and nodes. However, the results were different in case of number of shoots, where an increase in their number was observed with increasing stress levels in MS medium (Figure 3). It is evident from the data given in Table 3 that mannitol treatment to Desiree plants had a significant effect on all the studied growth parameters. Reduction in the growth of several parameters including shoot/root length, shoot/root, and number of nodes was recorded as the concentration of mannitol was higher in the MS medium. Table 3 also indicates that mannitol treatment had a significant effect on the fresh weight of both the cultivars. Dry weight of plants was also affected by mannitol applications; however, it was not as significant as was in case of fresh weights in cardinal but significant in case of Desiree.

An overall increase in total proteins was recorded at all mannitol levels in both the cultivars of potato. In case of control Cardinal plants, protein content (0.26 mgg^{-1}) increased gradually to 0.85 mgg^{-1} at 0.15 M treatment. Similar increase in its value was also observed for Desiree where the change in its values was from 0.16 (in control plants) to 1.15 mgg^{-1} at the highest (0.15 M) mannitol level. SOD activity was greatly influenced by the addition

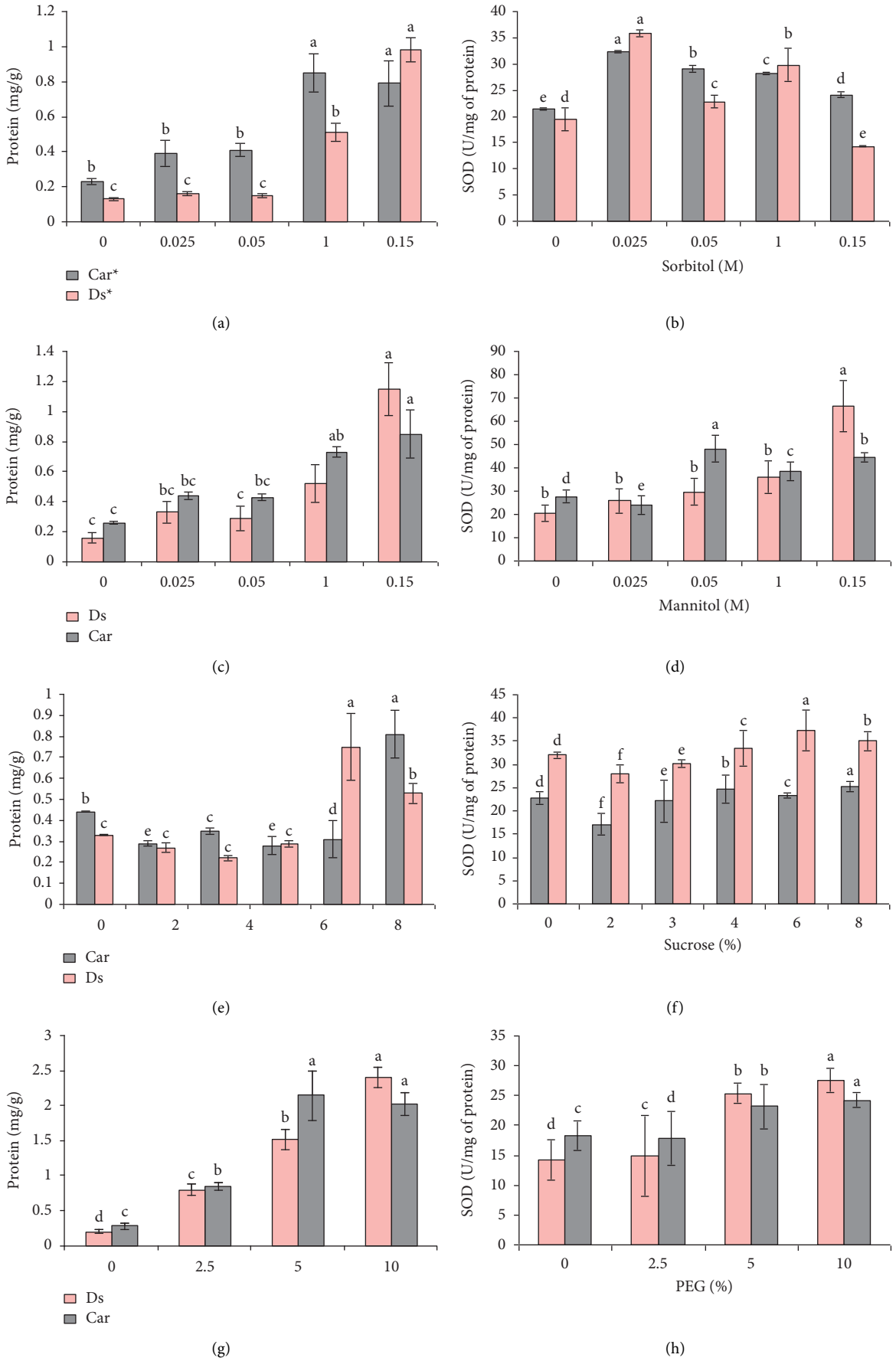

FIGURE 2: Protein contents and superoxide dismutase activity in potato cultivars cardinal (Car) and Desiree (Ds) in response to sorbitol (a) and (b) mannitol (c) and (d), sucrose (e) and (f) and PEG (g) and (h) induced osmotic stress. Results are the means ± S.E. of ten replicates cultures. Bars with similar letters are not significantly different at $P \leq 0.05$ by Duncan's multiple range test.

Table 2: Effect of various levels of sorbitol, mannitol, sucrose, and PEG on polyamines contents (putrescine, spermidine, and spermine) of potato plants.

Medium	Putrescine (nmol·g^{-1} FW)		Spermidine (nmol·g^{-1} FW)		Spermine (nmol·g^{-1} FW)	
Cultivar	Car	Des	Car	Des	Car	Des
(Control) without sorbitol	145.13 ± 3.42^{b}	143.56 ± 3.67^{a}	132.62 ± 3.23^{c}	122.45 ± 3.24^{c}	118.75 ± 2.78^{e}	119.28 ± 2.33^{d}
MS + 0.025 M sorbitol	135.35 ± 2.07^{c}	148.34 ± 3.25^{a}	141.30 ± 2.27^{b}	131.73 ± 3.27^{b}	124.45 ± 1.15^{d}	124.46 ± 3.79^{c}
MS + 0.05 M sorbitol	147.22 ± 1.18^{b}	142.28 ± 2.42^{a}	142.27 ± 2.14^{b}	133.65 ± 2.12^{b}	130.25 ± 3.13^{c}	129.33 ± 2.53^{b}
MS + 0.10 M sorbitol	155.80 ± 2.27^{a}	145.39 ± 2.23^{a}	151.13 ± 2.28^{a}	142.35 ± 2.38^{a}	139.29 ± 3.20^{b}	130.13 ± 1.21^{b}
MS + 0.15 M sorbitol	158.23 ± 1.37^{a}	147.32 ± 2.53^{a}	155.15 ± 2.78^{a}	143.05 ± 2.38^{a}	149.29 ± 3.20^{a}	140.03 ± 1.31^{a}
(Control) without mannitol	115.23 ± 3.42^{e}	113.26 ± 3.67^{c}	122.20 ± 3.23^{c}	122.15 ± 3.34^{b}	128.95 ± 2.28^{d}	119.18 ± 2.23^{c}
MS + 0.025 M mannitol	119.15 ± 1.27^{d}	118.34 ± 1.35^{b}	131.30 ± 2.27^{b}	124.72 ± 3.27^{b}	124.95 ± 1.13^{d}	114.27 ± 3.19^{c}
MS + 0.05 M mannitol	127.29 ± 1.28^{c}	121.28 ± 2.12^{b}	129.25 ± 2.14^{b}	131.35 ± 1.22^{a}	130.15 ± 1.12^{c}	124.73 ± 2.53^{b}
MS + 0.10 M mannitol	133.89 ± 2.37^{b}	121.29 ± 2.23^{b}	132.35 ± 2.28^{b}	131.15 ± 2.18^{a}	139.20 ± 3.20^{b}	129.35 ± 1.34^{a}
MS + 0.15 M mannitol	140.89 ± 1.47^{a}	132.99 ± 1.33^{a}	134.25 ± 2.28^{a}	132.05 ± 2.28^{a}	149.20 ± 1.31^{a}	130.15 ± 1.22^{a}
(Control) without sucrose	145.29 ± 2.42^{c}	143.26 ± 1.67^{d}	132.10 ± 3.43^{e}	132.25 ± 3.24^{c}	128.15 ± 2.78^{c}	129.08 ± 2.63^{c}
MS + 2% sucrose	151.25 ± 1.07^{b}	144.34 ± 1.25^{bc}	134.20 ± 2.37^{d}	131.72 ± 2.97^{c}	129.25 ± 1.95^{c}	124.31 ± 2.79^{d}
MS + 3% sucrose	157.21 ± 2.18^{b}	147.38 ± 2.42^{b}	139.05 ± 2.24^{c}	134.35 ± 2.12^{c}	130.35 ± 3.33^{c}	129.93 ± 2.53^{c}
MS + 4% sucrose	159.80 ± 2.27^{a}	140.29 ± 1.23^{bc}	141.15 ± 2.08^{b}	142.15 ± 2.08^{b}	139.22 ± 3.30^{b}	140.22 ± 1.34^{b}
MS + 6% sucrose	160.82 ± 2.27^{a}	150.29 ± 2.23^{a}	139.35 ± 2.28^{d}	142.15 ± 1.98^{b}	139.91 ± 3.32^{b}	140.23 ± 1.24^{b}
MS + 8% sucrose	162.83 ± 2.27^{a}	152.19 ± 2.23^{a}	151.15 ± 1.28^{a}	152.25 ± 2.08^{a}	149.23 ± 3.31^{a}	150.21 ± 1.14^{a}
(Control) without PEG	125.23 ± 3.42^{c}	123.76 ± 3.67^{c}	112.60 ± 3.23^{c}	102.35 ± 3.24^{b}	110.95 ± 2.78^{bc}	109.28 ± 2.63^{b}
MS + 2.5% PEG	135.15 ± 2.07^{b}	128.64 ± 3.25^{bc}	121.90 ± 2.27^{b}	111.70 ± 3.27^{ab}	115.95 ± 1.15^{b}	114.37 ± 3.79^{b}
MS + 5% PEG	137.29 ± 1.18^{b}	132.78 ± 2.42^{b}	122.25 ± 2.14^{b}	121.65 ± 2.12^{a}	122.15 ± 3.13^{b}	121.93 ± 2.53^{a}
MS + 10% PEG	150.89 ± 2.27^{a}	140.99 ± 2.23^{a}	131.15 ± 2.28^{a}	122.05 ± 2.38^{a}	129.20 ± 3.30^{a}	120.25 ± 1.24^{a}

Results are mean from at least ten to replicate cultures ± S.E. Similar letters within the column do not differ significantly ($P \leq 0.05$) according to DMRT. Significant ($*$) or non-significant (NS) at $P \leq 0.05$. *MS [25] contains different levels of sorbitol (designated as *S*1 to *S*5; sorbitol level as shown in the next column).

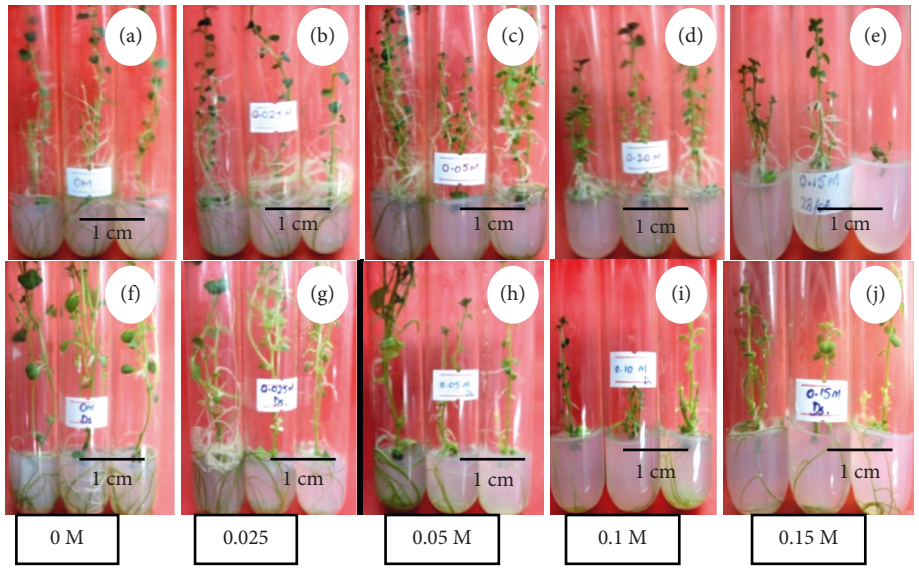

Figure 3: Growth of Cardinal (a)–(e) and Desiree (f)–(j) at different mannitol concentrations. Scale bar (a)–(j) = 1 cm.

of mannitol in the MS medium. In case of Desiree, mannitol treatment resulted in an increase of SOD activity, with its highest value (66.6 Umg^{-1} of protein) observed at 0.15 M. With some minor variations, osmotic stress (induced by mannitol) also resulted in increased activity of SOD in Cardinal plants (Figures 2(c) and 2(d)). In this case, SOD activity was decreased (24.14 Umg^{-1} of protein) at 0.025 M mannitol as compared to control ones (27.64 Umg^{-1} of protein).

All the tested polyamines were increased by increasing concentrations of mannitol in MS medium. Both cultivars of potato plants showed a significant ($P \leq 0.05$) increase in polyamines however, in Desiree plants, this increase was higher than in the cardinal plants. The

Table 3: Growth parameters of potato cultivars in response to osmotic stress induced by various levels of mannitol.

Medium	Mannitol (M)	Shoot length (cm)		No. of shoots		Root length (cm)		No. of roots		No. of nodes		Fresh wt. (g)		Dry wt. (g)	
		Car	Des	Car	Des	Car	Des	Car	Des	Car	Des	Car	Des	Car	Des
*M1	0	12.56 ± 1.02^{a}	14.21 ± 0.51^{a}	1.65 ± 0.23^{a}	3.22 ± 0.19^{a}	9.14 ± 0.86^{a}	11.63 ± 0.70^{a}	6.35 ± 0.70^{a}	10.27 ± 0.73^{a}	16.90 ± 1.40^{a}	16.72 ± 0.35^{a}	0.36 ± 0.03^{a}	0.99 ± 0.06^{a}	0.03 ± 0.00^{ab}	0.06 ± 0.01^{abc}
*M*2	0.025	10.36 ± 0.96^{a}	12.59 ± 0.72^{a}	2.05 ± 0.23^{a}	2.61 ± 0.14^{ab}	7.23 ± 0.78^{ab}	9.86 ± 0.51^{ab}	5.05 ± 0.60^{ab}	8.38 ± 0.61^{b}	17.20 ± 1.49^{a}	15.94 ± 0.51^{a}	0.35 ± 0.04^{a}	0.91 ± 0.08^{a}	0.03 ± 0.00^{a}	0.09 ± 0.01^{a}
*M*3	0.05	6.80 ± 0.99^{b}	10.08 ± 0.65^{b}	2.10 ± 0.28^{a}	2.88 ± 0.26^{ab}	6.82 ± 0.87^{b}	10.67 ± 0.61^{ab}	4.95 ± 0.88^{ab}	7.44 ± 0.53^{bc}	13.65 ± 0.69^{ab}	11.16 ± 0.60^{b}	0.28 ± 0.05^{a}	0.81 ± 0.07^{a}	0.03 ± 0.00^{ab}	0.08 ± 0.01^{ab}
*M*4	0.10	4.49 ± 0.66^{bc}	5.35 ± 0.51^{c}	2.25 ± 0.27^{a}	2.27 ± 0.33^{b}	4.26 ± 0.54^{c}	9.56 ± 0.64^{b}	5.10 ± 0.03^{ab}	5.94 ± 0.80^{c}	10.30 ± 0.23^{bc}	9.33 ± 0.70^{c}	0.24 ± 0.04^{ab}	0.40 ± 0.05^{b}	0.03 ± 0.00^{ab}	0.05 ± 0.01^{bc}
*M*5	0.15	2.36 ± 0.38^{c}	5.13 ± 0.42^{c}	1.90 ± 0.27^{a}	2.16 ± 0.25^{b}	2.89 ± 0.54^{c}	7.25 ± 0.58^{c}	3.35 ± 0.75^{b}	3.77 ± 0.29^{d}	7.25 ± 0.97^{c}	10.27 ± 0.51^{c}	0.13 ± 0.02^{b}	0.31 ± 0.02^{b}	0.02 ± 0.00^{b}	0.04 ± 0.01^{c}
Significance ($P \leq 0.05$)		*	*	NS	*	*	*	NS	*	*	*	*	*	NS	NS

Results are mean from at least ten to replicate cultures ± S.E. Similar letters within 7the column do not differ significantly ($P \leq 0.05$) according to DMRT. Values are significant (*) or nonsignificant (NS) at $P \leq 0.05$.
*MS [25] was supplemented with different levels of mannitol (designated as *M*1 to *M*5).

highest putrescine contents were observed in plants treated with 0.15 M of mannitol. Spermidine contents was 122.20, 131.30, 129.25, 132.35, and 134.25 nmol·g^{-1} FW in cardinal plants at 0, 0.025, 0.5, 0.1, and 0.15 M of mannitol, respectively. In Desiree plants, this increase in spermidine contents was 122.15, 124.72, 131.35, 131.15, and 132.05 nmol·g^{-1} FW at 0, 0.025, 0.5, 0.1, and 0.15 M of mannitol, respectively. Spermine contents also showed a similar increasing trend by increasing mannitol concentration in MS medium (Table 2).

3.3. Effect of Sucrose-Induced Osmotic Stress on Potato Plants. The effect of sucrose was statistically significant on shoot growth, its number, and on number of roots in both the cultivars of potato with a maximum value at 3% sucrose level; and a gradual decrease as the concentration varied. Highest value of root length was recorded at 4% sucrose in Cardinal followed by 2% sucrose concentration (Table 4). All the abovementioned parameters showed a decreasing trend by increase or decrease in sucrose concentration from 3% except for the root number, where an increase was recorded at 8% sucrose. For Cardinal, number of nodes decreased significantly with a gradual increase in sucrose concentration from 3%. Fresh weight exhibited in general a decrease, while dry weight exhibited an increasing trend in both the cultivars as the concentration of sucrose varied from 3 to 8%.

In Cardinal plants, total protein contents decreased gradually at 2, 4, and 6% sucrose concentration and exhibited an increase at 0 and 8% sucrose levels while in Desiree, protein content increased at all the stress levels induced by sucrose. SOD activity was also significantly increased in both potato cultivars at different sucrose concentrations except at 2%, where a decrease in its level was observed. Highest SOD activity was observed at 8% sucrose concentration in Cardinal and at 6% in Desiree (Figures 2(e) and 2(f)).

Polyamines (putrescine, spermidine, and spermine) contents increased significantly ($P \leq 0.05$) in potato plants by increasing sucrose concentration in MS medium however, this increase was less sharp in case of Desiree as compared to cultivar cardinal. The highest tested polyamines contents were observed when 8% sucrose was added in MS medium and the lowest contents of polyamines were observed at control in both cultivars of potato (Table 2).

3.4. Effect of Polyethylene Glycol (PEG) on Potato Plants. All the tested PEG concentrations greatly influenced most of the morphological growth parameters in both the cultivars. In Cardinal, a significant reduction was recorded in shoot/root length as the concentration of PEG was increased gradually in the MS medium from 0–10%. Shoot/root and node numbers also reduced gradually with increasing PEG concentrations (Table 5). Growth responses were similar in Desiree plants where a continuous reduction in shoot/root length was recorded by increasing PEG concentrations in the medium. Fresh/dry weights of potato plants also exhibited a decreasing trend with increasing PEG concentration. There was a statistically significant difference in total protein contents of both the cultivars at various PEG treatments. Maximum values of protein content (2.157 and 2.419 mgg^{-1}) were observed at 5% PEG concentration in Cardinal and at 10% concentration in Desiree, respectively. SOD activity also showed an overall increase with some exceptions in Cardinal, where 2.5% PEG treatment reduced the activity of SOD (Figures 2(g) and 2(h)).

All the tested polyamines were increased by increasing concentrations of Polyethylene glycol (PEG; MW-4000) in MS medium. The highest putrescine contents (150.89 nmol·g^{-1} FW) were observed in plants treated with 10% of PEG. Spermidine contents were 112.60, 121.90, 122.25, and 131.15 nmol·g^{-1} FW in cardinal plants at 0, 2.5, 5, and 10 M of PEG, respectively. In Desiree plants, this increase in spermidine contents was 102.35, 111.70, 121.36, 131.15, and 122.05 nmol·g^{-1} FW at 0, 2.5, 5, and 10 M of PEG, respectively. Spermine contents also showed a similar increasing trend by increasing PEG concentrations in MS medium. Spermine contents were 110.95, 115.95, 122.15, and 129.20 nmol·g^{-1} FW in cardinal plants, and in Desiree plants, this increase in spermidine contents was 109.28, 114.37, 121.93, and 120.25 nmol·g^{-1} FW at 0, 2.5, 5, and 10 M of PEG, respectively (Table 2).

4. Discussion

Drought stress is usually found to reduce morphological characteristics of the plants including leaf area, stem height, root number, and tuber yield under field and in vitro conditions [24, 29]. This is because cell expansion and growth are suppressed due to loss of turgor pressure or osmotic imbalance, which in turn reduces the plant growth and activity of all the metabolic processes [30]. It is evident from the literature that plant maintains the osmotic equilibrium by producing and enhancing the level of many osmotica for instance pinitol, mannitol, sucrose, sorbitol, trehalose, etc., [31, 32]. According to them, accumulation of these omotica resulted in an increase in solute concentration significantly. Furthermore, they also play a vital role in the protection of the cells from damage caused by dehydration [33]. However, it was also observed that when these osmotica accumulate in higher concentrations, they impair the growth in plants [34] and work as stressing agents [35]. Results of the present study indicate that all the studied osmotica (sorbitol, sucrose, mannitol, and PEG) induce osmotic stress in potato plants under in vitro conditions in both cultivars. These osmotic agents are known to produce drought like conditions as prevailing under field conditions [36]. It is well evident from the literature that sorbitol, sugar alcohol, was used in various *in vitro* experiments to induce osmotic stress in the medium [37–39]. In the present study, treatment of potato plants with various levels of sorbitol resulted in a decrease in most of the growth parameters in both the tested potato cultivars. This decline in growth due to sorbitol-induced stress was also reported by Gopal and Iwama [24], who studied the same pattern in several growth parameters. The growth reduction of *in vitro*-grown plants at higher concentrations of sorbitol could also be due to the accumulation of phenolic compounds in the medium

Table 4: Growth parameters of potato cultivars in response to osmotic stress induced by various levels of sucrose.

Medium	Sucrose (%)	Shoot length (cm)		No. of shoots		Root length(cm)		No. of roots		No. of nodes		Fresh wt.(g)		Dry wt.(g)	
		Car	Des	Car	Des	Car	Des	Car	Des	Car	Des	Car	Des	Car	Des
*S1	0	3.16 ± 0.47^{d}	7.06 ± 0.93^{c}	1.11 ± 0.07^{b}	0.55 ± 0.12^{c}	0.18 ± 0.18^{b}	4.48 ± 0.92^{b}	0.05 ± 0.05^{b}	1.44 ± 0.42^{b}	11.61 ± 0.93^{c}	11.22 ± 0.85^{a}	0.05 ± 0.00^{c}	0.09 ± 0.01^{c}	0.00 ± 0.00^{c}	0.00 ± 0.0^{b}
*S*2	2	8.98 ± 0.51^{bc}	11.64 ± 0.90^{ab}	5.30 ± 1.18^{a}	6.51 ± 1.91^{ab}	5.03 ± 0.91^{a}	8.35 ± 1.66^{a}	3.77 ± 0.28^{a}	3.94 ± 0.59^{b}	13.38 ± 0.78^{bc}	11.61 ± 0.74^{a}	0.178 ± 0.01^{b}	0.32 ± 0.03^{b}	0.01 ± 0.0^{bc}	0.01 ± 0.0^{b}
*S*3	3	13.31 ± 0.68^{a}	13.47 ± 0.57^{a}	6.04 ± 1.30^{a}	10.20 ± 2.48^{a}	4.13 ± 0.63^{a}	5.46 ± 1.01^{ab}	5.11 ± 0.46^{a}	7.83 ± 0.74^{a}	17.44 ± 0.77^{a}	12.50 ± 0.60^{a}	0.35 ± 0.02^{a}	0.81 ± 0.09^{a}	0.02 ± 0.0^{ab}	0.05 ± 0.0^{a}
*S*4	4	11.37 ± 0.15^{ab}	11.80 ± 0.84^{ab}	4.23 ± 0.90^{a}	5.00 ± 0.88^{b}	5.49 ± 0.90^{a}	6.04 ± 1.06^{ab}	4.88 ± 0.52^{a}	8.33 ± 1.49^{a}	15.16 ± 1.05^{ab}	10.33 ± 0.63^{a}	0.34 ± 0.05^{a}	0.84 ± 0.11^{a}	0.03 ± 0.0^{a}	0.08 ± 0.1^{a}
*S*5	6	8.42 ± 1.36^{c}	11.15 ± 0.85^{ab}	4.43 ± 0.78^{a}	4.28 ± 0.82^{bc}	4.08 ± 0.63^{a}	4.82 ± 0.75^{b}	3.83 ± 0.64^{a}	7.61 ± 1.08^{a}	12.33 ± 1.18^{bc}	11.11 ± 0.79^{a}	0.28 ± 0.04^{ab}	0.75 ± 0.09^{a}	0.03 ± 0.0^{a}	0.07 ± 0.1^{a}
*S*6	8	6.96 ± 0.90^{c}	9.83 ± 0.83^{b}	3.82 ± 0.73^{a}	5.17 ± 1.07^{b}	3.95 ± 0.85^{a}	4.88 ± 0.77^{b}	6.44 ± 1.13^{a}	9.83 ± 1.30^{a}	11.88 ± 1.24^{c}	10.55 ± 0.77^{a}	0.35 ± 0.06^{a}	0.69 ± 0.05^{a}	0.04 ± 0.01^{a}	0.08 ± 0.0^{a}
Significance ($P \leq 0.05$)		*	*	NS	*	*	NS	NS	*	*	NS	*	*	NS	NS

Results are mean from at least ten to replicate cultures ± S.E. Similar letters within the column do not differ significantly ($P \leq 0.05$) according to DMRT. Significant (*) or nonsignificant (NS) at $P \leq 0.05$. *MS [25] medium supplemented with different levels of sucrose (designated as *S*1 to *S*6).

Table 5: Growth parameters of potato cultivars in response to osmotic stress induced by various levels of polyethylene glycol.

Medium	PEG (%)	Shoot length (cm)		No. of shoots		Root length(cm)		No. of roots		No. of nodes		Fresh wt. (g)		Dry wt. (g)	
		Car	Des	Car	Des	Car	Des	Car	Des	Car	Des	Car	Des	Car	Des
*$P1$	0	10.20 ± 0.42^{a}	13.76 ± 0.65^{a}	2.60 ± 0.22^{a}	2.35 ± 0.20^{a}	10.65 ± 0.46^{a}	8.38 ± 0.90^{a}	8.85 ± 0.78^{a}	9.23 ± 0.53^{a}	16.25 ± 0.91^{a}	12.95 ± 0.74^{a}	0.67 ± 0.06^{a}	1.02 ± 0.06^{a}	0.06 ± 0.00^{a}	0.07 ± 0.0^{a}
$P2$	2.5	5.15 ± 1.07^{b}	8.64 ± 1.24^{b}	1.90 ± 0.17^{b}	1.70 ± 0.17^{b}	3.20 ± 0.73^{b}	3.83 ± 0.51^{b}	4.35 ± 1.15^{b}	4.36 ± 0.79^{b}	10.80 ± 1.26^{b}	9.45 ± 0.75^{b}	0.24 ± 0.07^{b}	0.24 ± 0.04^{b}	0.01 ± 0.00^{b}	0.02 ± 0.0^{b}
$P3$	5	1.19 ± 0.18^{c}	2.78 ± 0.41^{c}	1.25 ± 0.14^{c}	1.65 ± 0.22^{b}	0.36 ± 0.33^{c}	2.40 ± 0.58^{bc}	0.15 ± 0.10^{c}	1.90 ± 0.53^{c}	4.15 ± 0.58^{c}	6.50 ± 0.75^{c}	0.05 ± 0.01^{c}	0.09 ± 0.02^{c}	0.00 ± 0.00^{b}	0.01 ± 0.0^{b}
$P4$	10	0.89 ± 0.07^{c}	0.99 ± 0.13^{c}	1.15 ± 0.08^{c}	1.05 ± 0.08^{c}	0.17 ± 0.17^{c}	0.84 ± 0.29^{c}	0.20 ± 0.20^{c}	0.65 ± 0.24^{c}	3.75 ± 0.31^{c}	3.60 ± 0.71^{d}	0.04 ± 0.00^{c}	0.05 ± 0.00^{c}	0.00 ± 0.00^{b}	0.01 ± 0.0^{b}
Significance ($P \leq 0.05$)		*	*	NS	*	*	*	NS	*	*	*	*	*	NS	*

Results are mean from at least ten to replicate cultures ± S.E. Similar letters within the column do not differ significantly ($P \leq 0.05$) according to DMRT. Significant (*) or nonsignificant (NS) at $P \leq 0.05$. * MS [25] basal medium supplemented with different levels of polyethylene glycol (designated as $P1$ to $P5$).

[40, 41]. This may be due to the fact that drought can lead to disruption of metabolism and cell structure and ultimately to the termination of enzymatic reactions of the plants [42]. Sucrose is an important carbohydrate source in tissue culture medium besides being an osmoticum [43, 44]. Significant differences in growth parameters of potato plants grown on sucrose stressed media exhibited a similar picture as previously reported by Custodio et al. [43] those high concentrations of sucrose as compared to other carbon sources were responsible for an increased rooting index of Ceratonia siliqua. An increase in fresh/dry weights of plants in response to sucrose-induced osmotic stress might be due to the accumulation of sucrose in the plant tissues with its increase in the medium. Sucrose acts as a fuel source for sustaining photo mixotrophic metabolism [45]. This increase in root number at high sucrose level might be due to the fact that high sucrose level favors the formation of storage roots at high frequencies [46].

In the present study, the addition of PEG to the culture medium affected most of the growth parameters negatively in both cultivars. A similar effect of PEG on growth has also been reported in other plant species, e.g., mulberry [47], *Lycopersicon esculentum* Mill [48], and cherry plants [49]. This decrease in growth parameters of plants in the present study might be due to the low water content at high PEG treatments, which results in less availability of water for cell expansion. During the present study, tuber formation was observed in osmotic-stressed potato plants, especially when higher levels of sorbitol, mannitol, and sucrose were added to MS medium. Maximum number of tubers was recorded at 0.10 and 0.15 M sorbitol and mannitol and at 6 and 8% sucrose concentrations in Desiree and Cardinal, respectively. Tuber formation at higher concentrations of these osmotica is a general phenomenon because of their absorbing nature in plant tissue.

Quantitative analysis of the protein content revealed an increasing trend generally in both the potato cultivars (Cardinal and Desiree) when subjected to osmotic stress induced by various osmotic. The reason for an increased level of protein content under stressed conditions induced by different osmotica in the present study might be due to the synthesis of some stress-associated proteins in response to drought stress. These stress-associated proteins have been reported to be soluble in water, which contributes to stress tolerance by cellular dehydration [50]. Zang and Komatsu [51] reported the expression of some new proteins in rice in response to mannitol-induced drought stress. Under various stress conditions, it was observed that several reactive oxygen species (ROS) are also formed in plant cells, for instance, superoxide radicals (O_2^-), singlet oxygen, hydroxyl radicals (OH), and hydrogen peroxide. SOD plays a vital role in the scavenging of superoxide radicals [48]. Previously, several workers have reported that the activity of SOD increases significantly in plants as a result of various stresses, especially water stress [52–55]. In our work, an overall increase in SOD activity was recorded under sorbitol, mannitol, sucrose, or PEG-induced water deficit. This increase in SOD activity was also reported in mannitol or sorbitol-treated apple plants by Molassiotis et al. [19]. Wang and Li [56] also reported an increase in total leaf and chloroplastic SOD activity in white clover, which were grown on a medium containing different concentrations of PEG. Similar results were also observed by Wang et al. [57] in some plant species of *Trifolium*, where the application of PEG to the medium resulted in an enhancement of SOD activities and its various isozymes. On the basis of our results, it might be suggested that SOD is one of the major scavenger enzymes within the antioxidant defense system of potatoes under drought stress induced by sorbitol, mannitol, sucrose, or PEG. During the present investigation polyamines increase significantly in both cultivars of potato by using various osmotica. Polyamines have a protective role on plants under various abiotic stress by regulating and increasing the uptake of various inorganic ions directly, which in turn enhances stress tolerance in plants [10, 58]. Furthermore, an increase in the activity of SOD under various osmotic stress-inducing agents during the present study may justify the role of polyamines in modulating the homeostasis of reactive oxygen species.

5. Conclusions

In conclusion, all measured growth parameters were sensitive to osmotic stress induced by sorbitol, mannitol, sucrose, or PEG with severe reduction observed at higher levels of these osmotica. This reduction was more in the case of PEG-treated potato plants as compared to others. The increase in the level of protein content and superoxide dismutase activity suggested their major role in the detoxification of reactive oxygen species and compensation of drought stress conditions. Desiree plants performed comparatively better under osmotic stress by showing less reduction in growth. The use of compounds that do not interact with plants in any other way than lowering the water potential of the medium like PEG is more valuable to study the drought stress mechanism as compared to the compound having absorbing nature like sorbitol, mannitol, or sucrose. Viscous solutions of PEG limit the movement of oxygen ultimately resulting in oxygen deficiency and other toxic effects. Keeping all these points in view, one may conclude that the use of sorbitol or mannitol might be a better choice for further studies on drought stress mechanisms in potatoes. However, there is a need for further studies on these and related parameters before conclusive evidence could be presented.

Data Availability

The data used to support the findings of this study are included in this manuscript.

Conflicts of Interest

The authors declare that they have no conflicts of interest regarding the publication of this paper.

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