Effect of drought and combined drought and heat stress on polyamine metabolism in proline-over-producing tobacco plants

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1. Introduction

Drought stress affects water retention in plants at the cellular, tissue and organ levels, causing both specific and unspecific reactions as well as potentially damaging tissues and inducing adaptive responses [1]. Plants cope with droughts by activating defense mechanisms against water deficits [2]. These include stomatal closure, which reduces the rate of transpiration and photosynthesis [3]; the synthesis of new proteins; and the accumulation of osmolytes [4]. Plants’ interactions with the environment are mediated, at least in part, by phytohormones. The most important phytohormone in dehydration responses is abscisic acid (ABA) [5]. ABA mediates both rapid responses based on modulating the activity of ion channels to induce stomatal closure and slower metabolic changes that coincide with the activation of defense pathways. Recently, an increasing amount of evidence has emerged to indicate that cytokinins and auxins, plant hormones that are
primarily associated with the stimulation of cell division and the
control of plant growth and development, play important roles in
stress responses [6].

The exposure of plants to environmental stresses such as heat
stress and drought causes the generation of reactive oxygen species
(ROS [7]). High levels of ROS seem to function as a signal that
triggers protective responses; however, they are also potentially
harmful to all cellular components and negatively influence cellular
metabolic processes [8]. In order to maintain redox homeostasis,
plant cells respond to elevated ROS levels by increasing the
expression and activity of ROS-scavenging enzymes [9] and by
increasing their production of lipophilic and hydrophilic scav-
enging molecules [10].

Osmotic adjustment is a mechanism for the maintenance of
water potential in plant cells during periods of water deficiency. It
involves the accumulation of a range of osmotically active mole-
cules/ions such as soluble sugars, sugar alcohols, proline, organic
acids, calcium, potassium, and chloride ions within the cell. Proline
accumulation has been demonstrated to correlate with tolerance of
drought and salt stress in plants [11], and occurs due primarily via
de novo synthesis [12] together with the suppression of proline
oxidation [13]. A common method for obtaining plants with
elevated proline levels involves overexpression of the Δ1-pyrroline-
5-carboxylate synthetase (P5CS) gene. However, the native P5CS
protein is subject to feedback inhibition by its product (proline).
This feedback loop can be circumvented by site-directed muta-
genesis [14]. In P5CS from Vigna aconitifolia, this was achieved by
substituting the phenylalanine residue at position 129 with alanine.
Using this construct, tobacco plants with significantly elevated
proline levels were prepared [15].

Another group of osmotically active substances that is impor-
tant in drought tolerance is the polyamines (PAs). Their roles in
osmotic adjustment, the maintenance of membrane stability and
free radical scavenging have been emphasized [16]. The expression
of many genes that encode enzymes involved in polyamine meta-
bolism has been analyzed under various stress conditions [17].
Biosynthesis of the three most common PAs – putrescine (Put),
spermidine (Spd) and spermine (Spm) – is initiated either by the
direct decarboxylation of ornithine by the enzyme ornithine
decarboxylase (ODC) or by the decarboxylation of arginine by
arginine decarboxylase (ADC) via the intermediacy of agmatine and
hydroxycinnamic acid amides. Stress-tolerant plants generally have
a greater capacity to synthesize polyamines in response to stress
than do susceptible plants, and can increase their endogenous
polyamine levels by a factor of two or three in response to stress
[19]. It has been demonstrated that treatment with exogenous Spm
confers enhanced tolerance of drought and salt stress in Arabidopsis
[20]. Moreover, it has been suggested that transgenic plants with
elevated levels of proline and PAs exhibit enhanced stress tolerance
[21]. However, the levels of PAs and proline may be interrelated
because their biosynthetic and catabolic pathways have some in-
termediates in common [22].

The study presented herein was undertaken to investigate the
roles of proline and PAs in the drought responses of tobacco plants
and to determine how (if at all) elevated proline levels affect
drought tolerance and PA metabolism in tobacco plants. The stress
responses of plants from a tobacco line that over-expresses a
modified P5CS gene (P5CSF129A) and therefore has elevated proline
levels were compared to those for the corresponding wild type
(WT), enabling us to estimate the impact of increased proline levels
on the dynamics of PA levels under stress conditions.

2. Results

2.1. Relative water content

The relative water content values (RWC) did not differ signi-
ficantly between the two genotypes under control conditions
(Table 1). A minor decrease (3–8%) in RWC was observed in
transformant plants subjected to a 5-d drought period; under the
same conditions, the RWC of the WT plants decreased by 14–18%.
After a prolonged drought stress of 10-d, much more significant
reductions in RWC were observed in both genotypes. In WT plants,
the RWC fell by 33% and 50% in the upper and lower leaves,
respectively; the corresponding percentages for the transformants
were 23 and 42%. The water deficit was thus much more pro-
nounced in lower leaves. The application of a combined drought and
heat stress (10-d drought + 2 h at 40 °C) caused a further decrease in
RWC, with only minor differences between the tested
genotypes. During the 24-h recovery period, the RWC values for
both genotypes rose more rapidly after a 10-d drought than they
did after the combined drought and heat stress. Notably, the RWC of
the transformants returned to approximately the level observed in
control leaves within 24 h of the termination of the drought stress
(Table 1).

2.2. Plant growth under stress conditions

Changes in the fresh weights of the shoots of WT and trans-
formant plants were monitored during the 10-d drought stress
period, at the end of the combined stress period, and after rewa-
tering (Table 2). The reductions in shoot fresh weight observed after
drought periods of 5 and 10 days and after the combined stress
treatment were in good agreement with the corresponding RWC
values. The inhibitory effects of both stress treatments on shoot
fresh weight were more pronounced in WT plants.

<table>
<thead>
<tr>
<th>RWC (%)</th>
<th>Control</th>
<th>5-d D</th>
<th>10-d D</th>
<th>10-d D + HS</th>
<th>Rew D</th>
<th>Rew D + HS</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>U</td>
<td>84.35</td>
<td>73.06</td>
<td>56.29</td>
<td>48.68</td>
<td>79.61</td>
<td>74.18</td>
</tr>
<tr>
<td>L</td>
<td>85.13</td>
<td>70.09</td>
<td>42.68</td>
<td>40.34</td>
<td>76.52</td>
<td>73.11</td>
</tr>
<tr>
<td>M51-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>U</td>
<td>83.96</td>
<td>77.91</td>
<td>48.92</td>
<td>40.85</td>
<td>82.35</td>
<td>78.24</td>
</tr>
</tbody>
</table>

Table 1

Relative water content (RWC) in tobacco wild type (WT) and M51-1 transformants
determined in upper (U) and lower (L) leaves at control conditions, after 5-d, 10-d
drought stress (D), combined drought and heat stress (D + HS) and after rewater-
ting (Rew).

<table>
<thead>
<tr>
<th>Relative shoot fresh weight (%)</th>
<th>Control</th>
<th>5-d D</th>
<th>10-d D</th>
<th>10-d D + HS</th>
<th>Rew D</th>
<th>Rew D + HS</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>100</td>
<td>78.9</td>
<td>50.9</td>
<td>46.2</td>
<td>85.3</td>
<td>74.2</td>
</tr>
<tr>
<td>M51-1</td>
<td>100</td>
<td>93.0</td>
<td>61.4</td>
<td>54.3</td>
<td>87.6</td>
<td>75.7</td>
</tr>
</tbody>
</table>
2.3. Proline content

Under control conditions, the transformants accumulated 40 and 25 times more proline in their upper and lower leaves, respectively, than did the WTs, and 10 times more in their roots (Fig. 1). In both genotypes, water deficits caused considerable increases in proline content (P < 0.05 relative to the values observed in the controls). After 5 days of water stress, the amount of proline in the upper and lower leaves and roots of the WTs had increased substantially relative to those in the controls (by factors of 25, 6 and 10, respectively). The proline levels in the leaves and roots of the transformants also increased relative to the corresponding controls, albeit less dramatically: they rose by factors of 3, 9 and 7 in the upper and lower leaves and roots, respectively. Extending the duration of the water deficit to ten days caused further increases in the proline contents of the leaves and roots in both the wild type plants and the transformants. The combined stress treatment, in which plants were subjected to a ten day drought and then to a 2 h period of heat stress, did not significantly affect the proline levels in the upper leaves and roots but did cause further increases in the lower leaves in both genotypes. During the 24 h post-stress recovery period (rehydration at 25 °C), the amount of proline increased significantly in the leaves of the WT plants. However, the proline levels in the lower leaves of the transformants were higher than those immediately after the end of the combined stress treatment. The proline levels in the leaves and roots of both cultivars remained higher than those in the control plants throughout the post-stress recovery period. Proline levels fell more slowly in the transformants and in plants that had been subjected to the combined stress.

2.4. Activities of ODC, ADC and SAMDC

The activities of ODC and ADC in the upper leaves of the WT control plants were almost identical to those in the upper leaves of the transformant controls. However, the enzyme activities for the two genotypes differed slightly in the lower leaves and significantly in the roots (Fig. 2). The variation in ODC activity over the course of the stress period in the WT plants was very similar to that observed in the transformants: ODC activity in the leaves decreased slightly during the first five days of drought stress but then increased slightly over the full ten day period. Subsequent exposure to high temperatures during the combined stress treatment then reduced ODC activity. In contrast, drought and the combined stress treatment caused a pronounced increase in ODC activity in the roots of both the WT plants and the transformants. During the post-stress recovery period, the ODC activities in the leaves were lower than those in the untreated controls for both genotypes. There were no significant changes in the ADC activity in the upper leaves of either the WT plants or the transformants during the stress period. However, in the lower leaves of both genotypes, ADC activity increased over the ten day drought period and then decreased at the end of the combined stress period. In the roots of both genotypes, ADC activity rose significantly during the first five days of the drought period and peaked on the tenth day (Fig. 2). The increase was more pronounced in the WTs than in the transformants. In contrast to the ODC activity, the ADC activity in the roots of both cultivars remained high until the end of the post-stress recovery period. Furthermore, the ADC activity in the lower leaves of the transformants increased rapidly and substantially after rewatering; by the end of the recovery period, it was almost 25% higher than that in the corresponding untreated controls. Drought and the combined stress treatment also caused a significant increase in SAMDC activity in the roots of both cultivars. This increase in activity persisted through the post-stress recovery period. In lower leaves of both genotypes, SAMDC activity decreased slightly during the stress period but then rose during the recovery period.

2.5. Activities of DAO and PAO

The highest DAO and PAO activities in the WT and transformant control plants were observed in the roots (Fig. 3). In both genotypes, these enzymes were more active in the lower leaves than the upper ones. Five days of drought stress caused decreases in DAO activity in the roots of WT plants and the lower leaves of both genotypes, but the DAO activity in the roots of both genotypes remained significantly higher than in the leaves (Fig. 3). Longer periods of drought caused significant (P < 0.05) and pronounced reductions in DAO activity in the leaves of both genotypes relative to the levels observed in control plants. In both genotypes, the changes in PAO activity over time were similar to those observed for DAO during the stress period, i.e. its activity declined markedly in the roots and lower leaves and slightly in the upper leaves. During the post-stress recovery period, DAO activity increased noticeably (relative to their levels at the end of the drought period) in the lower leaves of both genotypes; this increase was more pronounced in WT plants than in transformants. However, this was accompanied by a reduction in DAO activity in the roots. Rewatering also caused a significant increase in PAO activity (relative to that observed in stressed plants) in the lower leaves of the transformants. The activities of both amine oxidases in the leaves and roots of both genotypes at the end of the 24 h rehydration period

![Fig. 1. Proline levels in the upper (U) and lower leaves (L) and roots (R) of wild type (WT) tobacco plants and M51-1 transformants under control conditions, during 5- and 10-day periods of drought stress (D-5d, D-10d), after a combined stress treatment (D-10d followed by a heat shock at 40 °C for 2 h; D + HS), after rewatering (RewD), and during the recovery phase after combined stress (RewD + HS). Data indicate the means ± SE (n = 3). There were significant differences (P < 0.05) between the proline levels observed in stressed plants and those at the end of the recovery phase, and between treated and control plants throughout the experiment.](image-url)
were low. The DAO activity in the upper and lower leaves and the roots of the WT plants decreased by about 70%, 30% and 65%, respectively, relative to those observed in control plants; the corresponding reductions in the transformants were 60%, 50%, 25%, respectively. The PAO activity in the leaves and roots of the WT plants decreased relative to those in the controls, by 85%, 75% and 70% in the upper, lower leaves and roots, respectively, while those in the transformants fell by 60%, 75%, 65% relative to the controls (Fig. 3).

2.6. Polyamine content

Under non-stressed conditions, the transformants had higher PA contents than the WT plants. After 5 d of drought stress, the levels of Put in the WT plants did not change significantly but those of Spd decreased \( (P < 0.05) \) relative to those observed in the controls. The levels of Spm in the lower leaves and roots increased significantly \( (P < 0.05) \) relative to the controls; those in the upper leaves also increased but not significantly. There were further statistically significant \( (P < 0.05) \) changes in the polyamine content of the WTs (relative to those for the control plants) at the end of the ten day drought and combined stress periods: Spd and Put became less abundant while Spm and 1,3-diaminopropane (Dap) became more abundant in the leaves and roots (Fig. 4). After rewatering, the Put and Spd contents of both genotypes increased substantially relative to the levels seen in the stressed plants. The Put and Spd contents of the upper leaves of both genotypes increased to approximately the levels seen in the controls. However, the lower leaves of both genotypes and the roots of the WT plants had significantly \( (P < 0.05) \) higher Put concentrations than were found in non-stressed plants. The levels of Spm and Dap in the leaves and roots of both genotypes remained higher than in the controls. In both genotypes, the increase in PA levels during the post-stress recovery period was faster in plants that had only experienced drought stress than in those that had been subjected to the combined stress.
2.7. Malondialdehyde content

Under control conditions, the reactive oxygen species contents (calculated based on their MDA contents) of the upper leaves and roots of the WT plants were almost identical to those for the transformants, and the two genotypes differed only slightly with respect to the ROS contents of their lower leaves (Fig. 5). Five days of drought stress caused slight reductions in the MDA contents of the upper leaves of the transformants and WT plants relative to controls; the change was significant ($P < 0.05$) for the WT plants. This was accompanied by a slight increase in the MDA contents of the lower leaves in both genotypes. The MDA contents of the lower leaves of the WT plants increased substantially after ten days of drought stress and after the combined stress period; similar but less pronounced increases occurred in the transformants. After the post-stress recovery period, the MDA levels in the leaves of both genotypes remained higher than those observed in the corresponding controls.

3. Discussion

Proline plays an important role in plants’ defenses against abiotic stresses, and plants with elevated proline levels are known to be particularly tolerant to such stresses [31,32]. Similarly to previous reports (e.g. Ref. [33]), the tobacco plants examined in this work responded to water stress by accumulating large quantities of proline, particularly in their upper leaves. The Pro level was notably higher in the stressed 35S:PSCSF129A transformants than in the WT plants. However, due to the higher basal proline content of the transformants, the relative increase in Pro levels in response to drought stress was more pronounced in the WT plants (Fig. 1). The application of a combined drought and heat stress, in which plants were first subjected to ten days of drought and then exposed to high temperatures for 2 h caused further increases in the proline contents of the lower leaves. In both genotypes, drought stress caused a reduction in RWC, although this reduction was less pronounced in the upper leaves than in the other parts of the plants. Notably, in the transformant line, the RWC fell at a later stage during the drought period than it did in the WT plants, and returned to its starting levels more rapidly during the recovery period. By the end of the recovery period, the RWC values for the transformants’ leaves were approximately the same as those for control plants (Table 1). This strongly suggests that the transformant is more stress-tolerant than the WT. Moreover, the fresh weight of the transformants’ shoots did not decrease as much or as rapidly as was observed for the WT plants during the drought period; this also indicates their greater stress tolerance (Table 2). Recently, several studies have demonstrated that PA metabolism is important in plants’ responses to abiotic stresses, especially salt and drought stress [16,34]. As was the case in our studies on tobacco BY-2 cells [35], the results obtained in this work strongly suggest that ODC is the most important enzyme in Put biosynthesis in tobacco (Fig. 3). Prolonged drought stress caused increases in the activity of both ODC and ADC in the lower leaves and roots of WT plants; however, in the transformants, the activities of these enzymes only increased significantly in the roots (Fig. 2). A moderate water deficit caused by five days of drought stress reduced the Spd content of the WT plants (relative to controls) while increasing their Spm contents. No significant changes in the PA contents of the transformants occurred under these conditions, with the exception of a slight reduction in the Put contents of their roots (Fig. 4). After ten days of drought, the levels of Spd and Put in both the WT plants and the transformants decreased significantly relative to those seen in control plants. This was accompanied by corresponding increases in the levels of Spm and Dap, which are formed by the oxidative deamination of Spd and Spm. The combined stress...
treatment caused a further reduction in the levels of Put and Spd in the lower leaves and roots of the transformants; again, this was accompanied by increases in the abundance of Spm and Dap, which is indicative of enhanced oxidative stress. The decline in Spd levels may have been due to its use in Spm biosynthesis or it may have been oxidized by PAO to form Dap, the levels of which increased during the drought stress period. These results suggest that the oxidation of Spm by PAO was partially responsible for the fall in its levels after rewatering (Figs. 3 and 4). This is consistent with the results of a previous study on bean seedlings under salt stress [36]. Although the

![Graph](image1)

**Fig. 4.** Levels of free polyamines in the upper (U) and lower leaves (L) and roots (R) of wild type (WT) tobacco plants and M51-1 transformants under control conditions, during 5- and 10-day periods of drought stress (D-5d, D-10d), after a combined stress treatment (D-10d followed by a heat shock at 40 °C for 2 h; D + HS), after rewatering (RewD), and during the recovery phase after combined stress (RewD + HS). Data indicate the means ± SE (n = 3). Asterisks above the bars indicate significant differences (P < 0.05) between the contents observed in stressed plants relative to those for the corresponding controls.

![Graph](image2)

**Fig. 5.** Content of malondialdehyde in the upper (U) and lower leaves (L) and roots (R) of wild type (WT) tobacco plants and M51-1 transformants under control conditions, during 5- and 10-day periods of drought stress (D-5d, D-10d), after a combined stress treatment (D-10d followed by a heat shock at 40 °C for 2 h; D + HS), after rewatering (RewD), and during the recovery phase after combined stress (RewD + HS). Data indicate the means ± SE (n = 3). Asterisks above the bars indicate significant differences (P < 0.05) between values observed in stressed plants relative to those for the corresponding controls.
precise role of Put in plants under adverse stress conditions is still controversial, many reports have shown that Spm helps to protect plants against drought stress [37,20]. It has been suggested that the accumulation of free Spm is characteristic of plants’ responses to long-term salt stress [38]. The high levels of Spm observed in drought-stressed leaves persisted even after the 24 h recovery phase, during which the RWC of the plants was returning to the levels seen in the controls (Fig. 3); this may reflect the role of Spm in modulating the activity of ion channels [39]. In this context, it is noteworthy that PAs such as Spm inhibit stomatal opening and induce their closure by regulating K⁺ channel activity, as was observed in *Vicia faba* guard cells [40]. Marked increases in the levels of Put and Spd coincided with increases in the activity of PA biosynthetic enzymes in the leaves and roots after the rewatering of the plants. These increases were more pronounced in the transformants than in the WT plants (Fig. 2) and were probably due to general increases in metabolic activity. The early stages of recovery are associated with elevated metabolic activity due to a sharp increase in the demand for resources throughout the plant. Proline degradation may serve as an important source of reducing agents in this process, which would support oxidative phosphorylation in the mitochondria and thus the formation of the ATP that is required by the processes involved in active stress recovery [41]. Some of the accumulated proline might be degraded by proline dehydrogenase, which catalyzes the conversion of proline to pyrroline-5-carboxylate and whose expression is stimulated during stress recovery [42]. In light of the well-known correlation between the proline and PA biosynthetic pathways, it is possible that glutamate might serve as a substrate in PA biosynthesis and could also be involved in the pronounced increase in Put and Spd levels during the recovery period (Figs. 1 and 4, [43]).

Drought stress is one of the most important types of abiotic stress and induces the production of various ROS. The nature of the relationship between PAs and ROS is rather controversial. On one hand, it has been suggested that PAs may protect cells against ROS; on the other, that their catabolism generates ROS [44]. It has also been proposed that the protective effect of exogenous PAs against oxidative damage caused by superoxides is dependent on their prior conversion to conjugates [45]. We believe that the decreases in Put and Spd levels that occurred during the drought stress periods in our experiments were primarily due to their consumption in PA conjugation reactions. This hypothesis is supported by the substantial decreases in DAO activity that occurred in the upper and lower leaves of both genotypes during prolonged droughts and the combined stress period, i.e. under conditions of high oxidative stress (Fig. 3). The results presented by [46] suggest that Put oxidation by DAO occurs only during the early stages of drought treatment in *Arabidopsis*. The importance of PA conjugation in the control of free PA levels in tobacco BY-2 cells under oxidative stress has been further discussed by [47]. In addition, we have previously confirmed the ability of PA conjugates to modulate free PA levels in alfalfa cell suspension cultures and in oak embryogenic cultures [48,49]. However, further studies will be required to properly investigate the potential roles of hydroxycinnamic acid amides in the control of PA homeostasis in response to stress.

In general, ROS are produced on a continuous basis in plants (even under physiological steady state conditions) in cell organelles involved in active electron transport. These ROS must be efficiently scavenged and maintained at non-damaging levels in order to permit cell survival. However, they are also useful signaling molecules that are important in stress signal transduction and the activation of acclimation and defense mechanisms [50]. Measured rates of lipid peroxidation (which are determined by monitoring changes in the levels of MDA) can be related to the antioxidant activity balance within a given cell or tissue. The MDA levels in the leaves of the transformed and the WT plants did not vary greatly under control conditions. However, the MDA contents of the lower leaves of the transformants increased substantially over the course of the ten day drought period, and a similar but much less pronounced increase was observed in the transformants. The imposition of a 2 h period of heat stress at the end of the ten day drought period caused a significant increase in oxidative stress, as demonstrated by pronounced increases in the MDA contents of the upper and lower leaves of the WT plants and the lower leaves of the transformants. After the post-stress recovery period, the MDA levels in the leaves of both genotypes decreased but nevertheless remained higher than those in the corresponding controls (Fig. 5). The higher levels of MDA observed after rewatering may indicate persistent stress-induced damage. No significant increases in MDA levels were observed in the upper leaves of the transformants during the stress period, which may indicate that the plants’ defense mechanisms are particularly active in these tissues.

In summary, the results of this study indicate that the elevated proline and PA levels in the transformed tobacco plants had mild but distinct positive effects on their abiotic stress tolerance. The upper leaves seem to be preferentially protected, as demonstrated by their near-constant levels of MDA during the drought periods. This also demonstrates the efficiency of the plants’ native defense mechanisms against oxidative damage and is consistent with the suggestion that the young and reproductive tissues are particularly well protected.

4. Materials and methods

4.1. Plant material and stress application

Wild-type (*Nicotiana tabacum* L. cv. MS1) and transgenic 35S::P5CSF129A tobacco plants (for detailed information see Ref. [15]) were grown in soil in a growth chamber (SANYO MLR 350H, Osaka, Japan) for 6 weeks with a 16-h photoperiod at 130 μmol m⁻² s⁻¹, day/night temperatures of 25/23 °C, and a relative humidity (RH) of ca. 80%. Drought stress was imposed by the cessation of watering for 10 days at reduced RH (35%). A combined stress was imposed by transferring the plants to high temperature conditions (40 °C, 2 h) at the very end of drought period. The recovery of the stressed plants was determined 24 h after re-watering at 25 °C. Samples of the upper leaves (specifically, the two youngest unfolded leaves) and lower leaves were collected, cut into pieces, and immediately frozen in liquid nitrogen after removal of the main vein. Root samples were shaken to remove residual soil, washed briefly with cold tap water (cca. 1 min), dried with filter paper and frozen in liquid nitrogen. For each treatment, a total of four (control) or five (stress) plants were harvested. Three independent experiments were performed.

4.2. Determination of relative water content

The relative water content (RWC) of the sampled leaves was measured as follows: individual cut leaves were weighed to determine their fresh mass (FM), saturated with water in beakers for 12 h, and re-weighed to determine their water-saturated mass (SM), dried at 80 °C, and finally weighed again to give the dried mass (DM). The RWC was then calculated as: RWC (%) = ([FM – DM]/[SM – DM]) × 100.

4.3. Determination of free proline levels

The levels of free proline in the samples were determined according to the method of [23].

4.4. Ornithine decarboxylase, arginine decarboxylase and S-adenosylmethionine decarboxylase assays

The activities of ornithine decarboxylase (ODC; EC 4.1.1.17), arginine decarboxylase (ADC; EC 4.1.1.19) and S-adenosylmethionine
decarboxylase (SAMDC; EC 4.1.1.50) activities were determined by the radiochemical method developed by [24], modified according to [25]. The protein contents of the samples were measured with Bradford’s method using bovine serum albumin as a standard [26].

4.5. Diamine oxidase assay

Diamine oxidase (DAO, EC 1.4.3.6) activity was assayed by a spectrophotometric method based its ability to catalyze the conversion of cis-1,4-diamino-2-butene into pyrrole via the corresponding amino aldehyde [25]. Samples were homogenized in 0.1 M Tris–HCl buffer, pH 8.5, containing 2 mM mercaptoethanol and 1 mM EDTA, and centrifuged at 20,000 × g for 15 min at 4 °C. The reaction mixture contained 0.1 M Tris–HCl buffer, pH 8.5, catalase (25 μg) and 0.01 M cis-1,4-diamino-2-butene. The reaction was started by adding 0.2 ml of supernatant, incubated for 1 h at 37 °C and stopped by adding 1 ml of Ehrlich’s reagent. The reaction mixture was then incubated at 50 °C for 5 min after which it was chilled on an ice bath before reading the absorbance of the newly-formed pyrrole at 563 nm. Enzymatic activity is expressed in pkat mg⁻¹ prot.

4.6. Polyamine oxidase assays

The activity of polyamine oxidase (PAO; EC 1.5.3.11) was estimated using a modification of the radiometric method of [27], with [1,4-¹⁴C] spermidine (4.37 GBq mmol⁻¹, Amersham Pharmacia Biotech, UK) as the substrate. Samples for the measurement of PAO activity were extracted in 3 volumes of ice-cold 0.1 M Tris–HCl buffer (pH 8.5) containing 2 mM β-mercaptoethanol and centrifuged at 20,000 × g for 30 min at 4 °C. Both the supernatant (soluble fraction) and the resuspended pellet (particulate fraction) were used to determine the sample’s PAO activity. The assay mixtures contained 0.3 ml of extract, 1 mM unlabeled Spd, and 7.4 kBq of [1,4-¹⁴C]Spd. After incubation at 37 °C for 30 min, the reaction was terminated by adding 60 μl of saturated sodium carbonate. The Δ¹⁴C pyrrole in the samples was immediately extracted using 4 mltoluene. The radioactivity of the extracted toluene solution was counted using a Tri-Carb 2900 TR liquid scintillation analyzer (Packard). Enzymatic activity is expressed in pkat mg⁻¹ prot.

4.7. Polyamine analysis

Extraction and HPLC analysis of benzoylated polyamines was performed according to [28].

4.8. Malondialdehyde assay

The malondialdehyde (MDA) content of the samples was determined using the NWLSS-Malondialdehyde Assay Kit (cat. no. NWK-MDA01, Northwest Life Science Specialties, LLC, Vancouver, Canada) as described in detail by [29]. The assay is based on the reaction of MDA with thiobarbituric acid (TBA), which forms an MDA–TBA2 adduct that absorbs strongly at 532 nm. The absorbance of the supernatant was measured at 532 nm and corrected for non-specific turbidity by subtracting the sample’s absorbance at 600 nm [30]. The quantity of MDA in the sample was determined by reference to a 600 nm standard. The quantity of MDA in the sample was determined by reference to a 600 nm standard.

4.9. Statistical analyses

Three independent experiments were carried out, in which similar results were obtained. The mean values (± the standard error, SE) obtained in one experiment with three replicates are shown in the figures. Data were analyzed using Student’s t-test.

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