Biochemical and Molecular Analysis of *in vitro* conserved Microplants of *Solanum tuberosum* L.

Nasiruddin M.*, Karim R., Kabir A.H., Hossain M.M. and Islam A.K.M.R.
University of Rajshahi, Rajshahi 6205, Faculty of Biological Sciences, Department of Botany, BANGLADESH
*mnuddin@ru.ac.bd

Abstract

The present investigation deals with the biochemical and molecular analyses of potato microplants in response to osmotic stress. The findings of this study showed that the chlorophyll content of osmotically stressed cells of potato microplants was significantly decreased compared to the microplants cultivated under non-stressed conditions. In contrast, the total soluble sugar, amino acids and proline concentrations were significantly increased in stressed microplants relative to non-stressed plants.

The results also suggested a substantial induction of antioxidants such as non-enzymatic scavenging, superoxidase dismutase (SOD) activity, in osmotic stressed microplants compared to untreated controls. However, the expression of MnSOD showed no induction which suggests that another form of SOD might be attributed to this enzymatic activity in potato microplants. Furthermore, the osmotic stress induced free proline concentrations consistent with upregulation of the proline-associated gene P5CS. This study implies that regulation of reactive oxygen species through the modulation of proline might be associated with osmotic adjustment in potato microplants.

Keywords: *In vitro* conservation, *MnSOD*, Osmotic stressed microplants, Proline concentration, *P5CS*, *SOD*.

Introduction

In the backdrop of climate change, the conservation of plant genetic resources is one of the major issues of discussion. Plant genetic resources have been continuously dwindling at a rapid pace through deforestation, human activities and monoculture-based modern agriculture practices along with climate change[20,42,58]. Conservation of genetic diversity lies at the heart of improvements in breeding and resilience in potato cultivation[4].

Potato is one the most important tuber crops globally, exceeded only in its contribution to human subsistence by the cereals wheat, rice and maize[9,19]. Potato is highly heterozygous and segregates on sexual reproduction. Thus, its genotypes cannot be used for long time conservation[28]. The preservation of live microplants *in vitro* for mid-term conservation has been used as a source of disease-free potato germplasm and for the international exchange of planting materials[48,62]. In short or mid-term conservation, various techniques have been used to increase interval-time of sub-culture and genetic stability. The substitution of the carbon source *in vitro* by osmotically active solutes instead of sucrose has been shown to provide a rich carbon source and act as an osmotic regulator[41].

In addition, *in vitro* storage of the microplant is also affected by chemical factors such as O_2, CO_2 and ethylene concentration present in the culture environment[7,26].

Potato micropropagation under standard condition (MS0 medium with 3% sucrose, 16 h photoperiod and 24±1 °C) requires sub-culture at 4-8 week intervals[23]. Different approaches have been used for slowing plantlet growth for increasing interval time of sub-culture by employing osmotic stress, growth retardant, low temperature and low light intensity for germplasm conservation.[13,15-18,24,33,34,38,40,47,52,60,61]

In CIP, potato germplasms have been conserved through micropropagation onto MS0 medium supplemented with 4% sorbitol and incubating the culture at 6-8 °C under low light intensity (1000 lux)[47]. However, in the tropics and subtropics, the ambient summer temperature may go up to 45-50 °C. Therefore, the maintenance of growth temperature at 6-8 °C in tropical and subtropical conditions is energy demanding and costly.

In addition, this low temperature driven osmotic stress method of potato conservation resulted in a high-frequency of phenotypic abnormalities[33]. Protocols for medium-term conservation of potato germplasm at 24±1 °C have also been reported by inducing water deficit stress through fortifying culture medium with non-metabolizable sugar alcohol for retarding plantlet growth[55,56].

However, the physiological and molecular basis of the defense response against osmotic stress as well as metabolic and physiological functions of conserved potato microplants, remains poorly understood[22].

Generally, plants under abiotic and biotic stresses manifest augmented proline accumulation through overexpression of the gene *P5CS*[32]. Wang et al[59] reported increased SOD activity in plant cells under stress conditions which are positively associated with gene *MnSOD*.

In the present work, we studied the effect of different formulations of stress-inducing culture on *in vitro* microplants. Along with the conservation efficiency, a few biochemical and molecular parameters were also evaluated.

Table 1
Different concentrations and combinations of sucrose/mannitol/sorbitol used in the culture media

Treatments	Codes	Sucrose (g/l)	Mannitol (g/l)	Sorbitol (g/l)
T1 (Control)	T1Sc30	30	0	0
T2	T2M	0	30	0
T3	T3Sb30	0	0	30
T4	T4Sc15+M15	15	15	0
T5	T5Sc10+M20	10	20	0
T6	T6Sc5+M25	5	25	15
T7	T7Sc15+Sb15	15	0	15
T8	T8Sc+Sb20	10	0	20
T9	T9Sc2+Sb25	5	0	25

Material and Methods

Potato tubers (*Solanum tuberosum* var. Diamond) were collected from Akafuji Agrotechnologies, Rajshahi, Bangladesh. *In vitro* culture for the production of disease-free microplants was established through meristem culture and maintained through node cuttings[23].

The node cuttings of *in vitro* grown plantlets were used as plant materials. The node cuttings from 3-week-old plantlets were cultured (10 nodes/culture bottle containing 20 ml of medium) in MS0 (growth regulator free)[39] medium supplemented with different concentrations and combinations of sucrose, mannitol and sorbitol (Table 1).

The cultures were incubated at 24±1 °C under 16 h photoperiod (cool white fluorescent lamps, approx. 20 µmol m^{-2} s^{-1} light intensity). The experiment had a completely randomized design with nine treatments, comprising all combinations of three sugars at three different concentrations (Table 1).

Data recording on morphological characters: Data on survival percentage, microplant condition (on a visual 0-5 preference scale: 0=dead, 1=very poor, 2=poor, 3=moderate, 4=good and 5=very good) for suitability of sub-culturing, root growth (on a visual 0-5 preference scale: 0=nil, 1=very poor, 2=poor, 3=moderate, 4=good and 5=very good), shoot length (cm) and the number of nodes per microplants were recorded at 12 months of incubation for the stressed microplants and 3 months of incubation for the non-stressed microplants. At the same time, the presence or absence of aerial roots, microtubers or any phenotypic abnormality was recorded.

Biochemical and molecular analysis: After recording the morphological data, the *in vitro* conserved microplants from different osmotic stress treatments were used for biochemical and molecular analysis. In case of control (T1), 3 months old *in vitro* microplants were used and for rest of the treatments (stressed microplants), one year old *in vitro* microplants were used. In addition, the non-stress culture (T1) was used as a control and grown concurrently and harvested at the time of biochemical and molecular study.

Determination of chlorophyll concentration: Total chlorophyll contents from fresh microplants (leaves, stems and shoots) were determined using dimethyl sulphoxide (DMSO) developed by Hiscox and Israelstam[21]. Briefly, 50 mg tissues of fresh microplants were taken and cut into small pieces and suspended in test tubes containing 4 ml of dimethyl sulphoxide (DMSO). Test tubes were incubated at 60 ˚C for 20 min in a water bath. The supernatant was decanted and another 3 ml of DMSO was added to the residue and incubated at 60 ˚C for 20 min. The supernatants were pooled and the volume was made up to 10 ml with DMSO. Then 3 ml of the chlorophyll extract was transferred to a cuvette and the absorbance read in a spectrophotometer (Spectronic 20, Thermo Scientific, USA) at 645 nm and 663 nm against DMSO blank. Total chlorophyll content was calculated following Arnon[3].

Determination of soluble sugar: The total soluble sugar of the microplants was estimated using the anthrone method described by Plummer[44]. Fresh microplants (100 mg) grown in different culture conditions were dried in an incubator for 24 h at 60 ˚C and homogenized in 10 ml of hot ethanol with the help of a mortar and pestle. The homogenized tissues were extracted twice with a small volume of ethanol, filtered through Whatmann filter paper (Type 1) and the final volume of the filtrate was adjusted to 10 ml. A standard glucose solution (200 µg/ml) was serially diluted into nine test tubes and the volumes were adjusted to 1 ml with ethanol. In separate tubes, 1 ml extract of microplants was added to each tube and 5 ml of anthrone reagent was added and mixed well by vortexing and then cooled and subsequently incubated for 10 min at 100 ˚C in a water bath.

The mixture was taken out and cooled in running water. The absorbance was checked at 620 nm in a spectrophotometer against a blank. The amount of soluble sugar in the microplant sample was determined by plotting a standard curve of A_{620} on the Y-axis and µg of glucose on the X-axis.

Amino acids estimation: Amino acids were estimated by the modified ninhydrin method developed by Rosen[50]. The dry microplant powders from different samples (50 mg) were homogenized in 5 ml of 80% methanol and

centrifuged at 5000 rpm for 10 min. The supernatants were transferred into separate test tubes containing the same volume of petroleum ether. The residues were extracted twice and pooled. The methanolic sample extracts (0.1 ml) were transferred into a separate tube and then 1 ml ninhydrin solution was added into each tube and final volumes were adjusted to 2 ml with distilled water. The tubes were incubated in a boiling water bath for 20 min. A standard glycine solution (1 mg/ml) was serially diluted with distilled water into different concentrations. Standard curves of absorbance (at 570 nm) vs. standard glycine solutions were plotted and the amount of total free amino acid was calculated as percentage equivalent of glycine.

Proline estimation: Proline was estimated by the standard method as described by Bates et al[5]. The microplant samples (200 mg) were homogenized in 10 ml of 3% aqueous sulphosalicylic acid and filtered through Whatmann no. 1 filter paper. Two ml of filtrates of each sample were transferred into separate test tubes containing an equal volume of glacial acetic acid and acid ninhydrin. The mixture was placed in a boiling water bath for 1 h and then transferred into an ice bath. Four ml toluene was added to each reaction mixture and stirred well for 20-30 s. The toluene layer was separated and the absorbance measured at 520 nm against the blank. The results were plotted with standard proline solutions and the amount of proline was calculated.

Enzymatic SOD extraction and activity estimation: For SOD extraction, samples of the microplants (0.2-0.5 g) were crushed and homogenized in ice-cold 0.1 M phosphate buffer (pH=7.5) containing 0.5 mM EDTA with pestle and mortar (pre-chilled). Each homogenate was centrifuged at 4 ˚C at 15000 rpm in a Beckman refrigerated centrifuge for 15 min. The supernatant was used to assay the enzyme activity using the method developed by Esfandiari et al[14].

DPPH free radical scavenging assay for antioxidant activity: The DPPH free radical scavenging activity of the extract of microplants was estimated as previously described by Kaneria et al[30] with some modifications. The DPPH solution (0.004% w/v) was prepared in 95% methanol. The methanol extract of microplants was mixed with 95% methanol to prepare the stock solution (5 mg/ml). Freshly prepared DPPH solution (0.004% w/v) was put into test tubes and microplants extracts were added followed by serial dilutions to every test tube so that the final volume was 3 ml. The mixture was left to stand for 30 min before reading the absorbance at 517 nm using a spectrophotometer. Mixer of methanol and DPPH solution was used as control. A standard curve was made using an ascorbic acid solution of different concentrations. The % inhibition of the extract was determined as follows:

$$\text{inhibition \%} = [(Ac-As)/Ac]\times 100$$

where Ac is the absorbance of the control and As is the absorbance of the sample.

Semi-quantitative detection of P5CS and MnSOD expression: The expression level of genes *Actin*, *P5CS* and *MnSOD*) was studied with semi-quantitative RT-PCR (reverse transcription PCR) of the microplants of control culture (T1) after 3 months and microplants grown in different osmotic stress media (T4, T7, T8 and T9, respectively) were studied after 12 months of culture. The total RNAs were extracted from 200 mg microplants using the Total RNA Isolation System (Cat. no. Z3100, Promega Corporation, United States) according to the manufacturer's manual. The quality and quantity of RNA were tested with Micro Nanodrop ND 1000 spectrophotometer (Wilmington, USA). The integrity of RNA was checked by denaturing 1% agarose gel electrophoresis. The total RNA was used for first-strand cDNA synthesis using GoScript™ Reverse Transcription System (Cat no. A5001, Promega Corporation, USA), according to the manufacturer's manual.

The *P5CS* and *MnSOD* genes were amplified from the first-strand cDNA using the following gene-specific primers (*Actin*: F-GAATCCATGAGACCACCTAC, R-AATCCAG ACACTGTACTTCC; *MnSOD*: F-GAGGGTGCTGC TTTACAAGG, R- TGCAGGTAGTACGCATGCTC; *P5CS*: F- CATAAGCTGAGCTGAGGTTACATCC, R-CAAGGCTTGTGCGGCTGTAG) designed by NCBI Primer designing tool with MultiGene™ OptiMax Thermal Cycler (Labnet International Inc).

The PCR products of *P5CS* and *MnSOD* genes were visualized through 1% agarose gel electrophoresis followed by ethidium bromide staining. The image was taken with the Gel Documentation Systems (Thermo Fisher, United States). The expression levels of *P5CS* and *MnSOD* genes were determined using visual intensity detecting Image J software. The PCR program used was as follows: 4 min at 95 ˚C, 35 cycles of 30 s at 95 ˚C, 45 s at 58 ˚C, 1 min at 72 ˚C and 10 min at 72 ˚C.

Statistical analysis: Treatment means were assessed using Duncan's multiple range test (DMRT) as outlined by IBM SPSS software version-20 (SPSS Inc. USA) at 5% probability level.

Results

Morphological variation in response to osmotic stress: The microplants grown *in vitro* under different culture regimes showed various types of phenotypic abnormalities e.g. flaccidity, vitrification, abnormal swelling of the stem, excessive leaf senescence, aerial rooting and overall growth reduction. In the case of microplant survival, a significant difference was observed between the control and osmotically stressed microplants (Table 2a). The highest survival was observed in the control culture (T1) (after 3 months of culture) and it was followed by stress treatments (after 12 months of culture) T8 and T9. The lowest survival was found in T2 and T3. However, among the treatments, T4, T6, T8 and T9 showed no visible differences (Table 2a).

Almost similar results were observed in all microplants regardless of the culture conditions. Here, T6, T9 and T4, T8 showed no visible differences (Table 2a).

The highest root growth was observed in T8 followed by the control (T1), T6 and T9, with the lowest observed in T2 and T3 (Table 2b). The longest shoot length was observed in control (T1) and T8 followed by T7 and T9 and the shortest was found in T2 and T3. The highest number of nodes/microplants was observed in T7 followed by the control (T1), T4 and T8 with the lowest number in T2 and T3 (Table 2b).

Biochemical characters: The results showed that the highest chlorophyll content was observed in the microplants of the control (T1) followed by T6, T8 and T9. The lowest amount of chlorophyll was found in T7 (Table 3a). Amino acid tests showed that the highest was observed in the microplants of the control (T1) followed by T9, T8, T6 and T2. The lowest amounts of amino acids were recorded in T7 (Table 3a). It was shown that the maximum proline concentration was observed in T9 and T7 followed by T8 and T4 and T6 and T5, with the lowest concentration in the control (T1) (Table 3a). The highest concentration of soluble sugar was recorded in T8 with lower levels in the other treatments and the lowest concentration in the control (T1) microplants (Table 3b).

The highest level of antioxidant percentage was in T5 and T3 followed by the other treatments with a much lower level in the control (Table 3b). The results for superoxide dismutase (SOD) (enzymatic antioxidant) activity showed that T9 had the highest SOD activity followed by T4, T8 and T7, with the lowest activity in the control (T1) (Table 3b).

Expression of genes associated with osmotic stress tolerance: Among the 9 treatments, 5 treatments were used for molecular study because of the higher level of proline and SOD involvement in these five treatments correlated with *P5CS* and *MnSOD* genes. Semi-quantitative reverse transcription PCR was used for sampling *P5CS* and *MnSOD* transcripts from the osmotic stress treatments. For gene expression study, microplants of stressed media T4, T7, T8 and T9 were used after one year of culture and control (T1) treatment was used after three months of culture.

Table 2a
Microplant survival % and microplant condition under different culture treatments. Data were recorded after 12 months of culture for (T2-T9) and 3 months after culture for T1 (control)

Treatments	Microplant survival (%)	Microplant condition
T1 (Control)	93.53±2.30^{a}	4.50±0.08^{a}
T2	20.59±0.61^{f}	1.02±0.02^{f}
T3	12.41±0.31^{g}	0.38±0.00^{g}
T4	77.63±0.60^{c}	2.59±0.73^{c}
T5	69.03±1.43^{d}	2.76±0.72^{c}
T6	75.07±0.70^{c}	2.33±0.14^{d}
T7	57.80±0.58^{e}	1.50±0.14^{e}
T8	83.15±0.41^{b}	3.54±0.98^{b}
T9	82.04±0.86^{b}	2.33±0.04^{d}

The different letters indicate significant differences between means of treatments according to DMRT at 5% level of significance

Table 2b
Shoot length, no. of culturable nodes/microplant and root growth of the microplants under different osmotic treatments. Data were taken after 12 months of conservation except in control (T1) (control was taken after 3 months of culture)

Treatments	Shoot length (cm)	No of culturable nodes/microplants	Root growth
Control (T1)	4.61±0.10^{a}	4.93±0.23^{d}	2.55±0.24^{b}
T2	0.66±0.01^{d}	0.8±0.06^{g}	0.39±0.02^{d}
T3	0.65±0.03^{d}	1.4±0.15^{f}	0.18±0.01^{d}
T4	2.26±0.08^{c}	6.86±0.12^{b}	1.65±0.05^{c}
T5	2.41±0.01^{c}	4.8±0.07^{d}	1.50±0.09^{c}
T6	2.23±0.09^{c}	5.68±0.17^{c}	2.61±0.04^{b}
T7	3.25±0.02^{b}	7.8±0.05^{a}	1.77±0.10^{c}
T8	4.23±0.10^{a}	5.15±0.10^{c}	3.30±0.08^{a}
T9	3.36±0.08^{b}	4.08±0.02^{e}	2.42±0.13^{b}

The different letters indicate significant differences between means of treatments according to DMRT at 5% level of significance.

Table 3a
Total Chlorophyll, amino acids and proline concentration of the microplants grown under different culture conditions. Data were taken after 12 months of conservation except in control (T1) (control was taken after 3 months)

Treatments	Total Chlorophyl (mg/g)	Total amino acids (µmol/g)	Proline (µg/g)
Control (T1)	12.48±0.32^{a}	12.31±0.16^{a}	0.75±0.05^{d}
T2	3.61±0.38^{f}	2.65±0.05^{f}	0.76±0.08^{d}
T3	6.44±0.27^{e}	4.03±0.19^{e}	0.81±0.07^{d}
T4	6.11±0.24^{e}	3.84±0.31^{e}	1.26±0.06^{b}
T5	3.54±0.34^{f}	2.49±0.05^{f}	0.97±0.03^{c}
T6	8.92±0.18^{c}	7.15±0.05^{d}	1.01±0.08^{c}
T7	3.24±0.19^{f}	2.40±0.05^{f}	1.40±0.05^{a}
T8	7.41±0.28^{d}	8.41±0.08^{c}	1.30±0.05^{b}
T9	9.65±0.48^{b}	9.20±0.05^{b}	1.45±0.05^{a}

The different letters indicate significant differences between means of treatments according to DMRT at 5% level of significance

Table 3b
Soluble sugar, antioxidant and superoxide dismutase concentrations of the microplants under different osmotic treatments. Data were taken after 12 months of conservation except in control (T1) (control was taken after 3 months)

Treatments	Soluble sugar (%)	Antioxidant (%)	SOD (Umg/protein)
Control (T1)	1.92±0.56^{f}	21.35±1.01^{f}	3.34±0.19^{f}
T2	3.75±0.34^{cd}	76.71±1.89^{c}	4.42±0.63^{e}
T3	2.92±0.39^{e}	87.43±1.42^{a}	5.60±0.73^{cd}
T4	2.65±0.60^{ef}	67.00±1.57^{e}	7.09±0.51^{b}
T5	4.28±0.51^{c}	89.36±1.44^{a}	5.14±0.55^{de}
T6	3.04±0.28^{de}	72.19±2.17^{d}	4.22±0.69^{ef}
T7	2.34±0.36^{ef}	75.96±1.25^{c}	6.51±0.54^{bc}
T8	10.61±0.47^{a}	82.12±1.21^{b}	6.71±0.49^{b}
T9	5.95±0.28^{b}	86.81±0.96^{a}	8.71±0.60^{a}

The different letters indicate significant differences between means of treatments according to DMRT at 5% level of significance

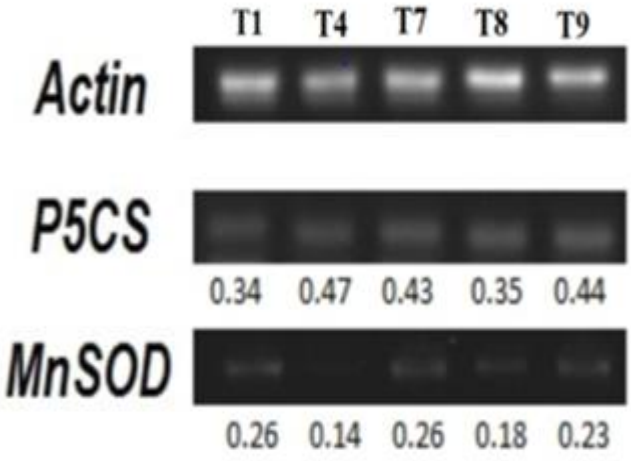

Figure 1: Semi-quantitative RT-PCR detection of relative *P5CS* and *MnSOD* genes. Visual intensity of the two genes was compared with Actin gene

In contrast with control microplants, *P5CS* was more expressive in stressed *in vitro* microplants. Treatment T4 was shown to have the highest *P5CS* expression level followed by T9, T7 and T8. Control (T1) (Figure 1) showed the lowest expression. Furthermore, *MnSOD* showed higher expression in control (T1) microplants than in stressed microplants except in those of T4 where its expression was similar to that of the control microplants. The lowest expression was found in stressed treatment T4 (Figure 1). The expression patterns of the *MnSOD* gene in stressed and non-stressed (control) microplants were not correlated with SOD contents.

Discussion

The main objective of this study was to monitor the biochemical and molecular condition of the conserved microplants by reducing their growth using different types of osmoticum and conserving them up to 12 months. It is known that under control (T1) culture, microplants need to be sub-cultured every 4-8 weeks[23]. However, it is possible to keep the microplant in the culture vessel for more than 3 months without any physical abnormalities under stress culture. Thus, it was of interest to investigate *in vitro* conservation of potato microplants and the underlying physiological and molecular mechanism under different types of osmotic stress and combination of sugar alcohol.

An osmotic agent reduces osmotic potential and restricts water to the *in vitro* cultured explants. Plant physiological and metabolic functions immediately decrease in a growing plant when slightly short of water[35]. Sucrose, sorbitol and mannitol are osmotic regulators that cause osmotic pressure to act as a plant growth retardant[13]. The plant applies osmotic adjustment mechanisms to keep up its water relations under osmotic stress conditions. Some osmotically active molecules/ions have been shown to be stored in these plants including soluble sugars, sugar alcohols, prolines, glycine betaine, organic acids, calcium, potassium and chloride ions[1].

When plants are under abiotic and biotic stresses, they augment proline accumulation and strongly express the gene *P5CS*[32]. SOD activity also increases and has been positively associated with the gene *MnSOD*[59]. The appropriate physiological and molecular mechanisms for defense reactions against osmotic stress as well as metabolic and physiological functions of conserved potato microplants, remain poorly understood[22]. In contrast, *in vitro* technique can be used to achieve medium-term conservation so that genetic material can be maintained for several months to 2-3 years without sub-culture[11].

In this investigation, it was observed that the control (T1) treatment and stressed treatments T2 and T3 were not feasible for mid-term conservation whereas, other stressed treatments were considered to be effective as the microplants displayed longest survivability, healthy and well-rooted plantlet and higher shoot length and number of nodes/microplant. Among these stressed treatments, T8 was found to be the best as the microplants exhibited the highest survival level with better microplants condition, decreased root growth and sufficient number of nodes for sub-culturing following a period of 12 months of conservation (Table 2a and 2b).

The decrease of chlorophyll concentration triggered by abiotic stresses is also in accordance with a previous report[2]. In the present investigation, the reduction in chlorophyll concentration by stress condition is supported by the previous report of Jung[29]. It was found that in the case of osmotic stress conditions, chlorophyll concentration of cells of potato microplants reduced in treated microplants in comparison to control culture which is agreement with the report of Muller et al[37] and Din et al[12].

The functions of amino acids in plants include osmolyte, ion transport control, modulated stomatal opening and heavy metal detoxification[46]. Several studies have reported the increase of amino acids in stressed microplants which is in agreement with our findings[27].

The content of soluble sugars in the microplants has increased significantly under stress. Sugar deposition is also well-documented in plants under stress conditions[25]. We found an increase in soluble sugars of the microplants under stress (e.g. T8 and T9 treatments). The increases in soluble sugars have been reported to enhance plant tolerance to abiotic stresses such as drought[46], but in some cases, the increased levels of compatible solutes may have negative effects on plant growth/development. According to Rosa et al[49], sugar changes do not follow a static model and vary with the genotype and the stress factor. Moreover, different soluble sugars play different roles in plant metabolism under stress e.g. sucrose and glucose are substrates for cellular respiration and fructose is a substrate for secondary metabolism. Soluble sugars can be a typical osmoprotectant that stabilizes the cell membranes and maintains the turgor pressure.

In addition to the proline and soluble sugar accumulations, gene ontology features in chilly genes were noticeably improved implying that the metabolic pathway was important in drought stress response. Indeed, several of those techniques to drought tolerance have been empirically proven by transgenic experiments[57].

The results showed that after one year of conservation in combined stressed treatments, the best antioxidant activity percent occurred in treatment T5. Microplants of control (T1) were found to have the lowest level. A fundamental determinant of their resistance to these stresses seems to be the ability of plant life to scavenge the poisonous effects of live oxygen. The first line of protection from radical damage is antioxidants. In the scavenging of active oxygen in plants, many antioxidants, peptides and metabolites are involved and it is expected that once oxidative stress is detected, their activation will increase[54].

The higher SOD activity was recorded in stressed microplants and the lowest SOD activity in the control (T1) treatment. SOD activity increased with an increasing proportion of osmotic pressure. ROS, especially superoxide radicals and scavenging was declined when SOD activity was intense, resulting in decreased oxidative stress damage to membranes and improved oxidative stress sensitivity.

The level of superoxides in cells was increased as a result of osmotic stress. The biomolecules are vital[36] in disorder if this radical is not frequently scavenged by SOD.

Proline concentration is used as a salt stress tolerance selection parameter. In plants it is used under salinity conditions to be one of the most widely recognized osmoprotectants[53]. It can also act as a hydroxylic scavenger, a carbohydrate and a nitrogen source and as cell membrane stabilizer. It also has two synthetic routes in plant life. Proline is a crucial amino acid that functions against osmotic and oxidation stresses along with metabolizing carbon and nitrogen[31]. One formation mechanism used glutamine as the number one precursor through delta-1-pyrroline-5-carboxylate synthase (*P5CS*) movement while the other pathway utilizes ornithine through ornithine-aminotransferase (OAT) action. These pathways are involved in the stress-induced process of proline accumulation.

However, glutamate proline biosynthesis is considered the primary path, particularly under stress conditions[31]. The aim of this study was to compare the transcript stages in osmotic stress-tolerant microplants of proline genes associated with metabolism. The correlations between proline accumulation and proline metabolism-associated genes (*P5CS*) were statistically examined for osmotically stress tolerant of potato microplants.

Physical factors including temperature, light and culture vessel are the most critical ones to strongly delay microplant growth for *in vitro* storage[6,10]. Also, *in vitro* storage of the microplant is affected by chemical factors such as O_2, CO_2 and ethylene concentration present in the culture environment[7,26]. However, the present investigation emphasized only the effects of osmotic regulator on the conserved microplants. Other physical or environmental factors may be involved here but we are not taking into consideration.

Many species have documented increased activity of antioxidant enzymes during stress. This enzymatic reaction in plants will be an adaptive advantage to defend against oxidative stress. SOD activity analyses of special SOD isoforms show that *MnSOD* accounted for approximately 60 percent of overall SOD value with a marginal decrease in drought progression[43]. Higher expression of *MnSOD* was found in non-stressed (control) microplants than stressed microplants. This may be attributed to other genotypical characteristics.

In vitro conserved (after 12 months) microplants were taken out (T8) and node cuttings were sub-cultured onto MS0 with 30 g/l sucrose. Eighty percent of node cuttings were found to grow into normal microplants. Microplants were acclimatized and planted into field. Noticeable abnormality was not to be found among microplants derived plants grown under field conditions.

Conclusion

Conservation of the plant genetic resources *in vitro* is an important issue in the present time. There are many approaches applying globally for the *in vitro* conservation of crop plants. CIP conserved potato microplants using MS0 with 4% sorbitol and cultured them in low temperature (6-8 °C), but in the tropic and subtropics, it is energy demanding and costly.

Thus, in the present investigation, it was tried to slow the growth of the microplants and to extend their sub-culture time employing osmotic stress using growth retardant. Physiological and molecular basis on defense response against osmotic stress of the conserved microplants are poorly understood.

The results revealed that chlorophyll concentrations of the conserved microplants are decreased and amino acids, total soluble sugar, proline concentrations are significantly increased compared to non-stressed microplants. Enzymatic and non-enzymatic antioxidant scavenging were also induced compared to non-stressed or control microplants. Molecular study also revealed the up-regulation of proline related gene *P5CS* but surprisingly, the expression level of *SOD* related gene *MnSOD* was not changed, or slightly down-regulated. More research is needed to know in details of the concluding points of the physiological and molecular basis of the conserved microplants.

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