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# Role of Trehalose during Recovery from Drought Stress in Micropropagated Banana (*Musa* spp.) Transplants.

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**ABSTRACT**

Micropropagated banana (*Musa* spp.) plants are widely accepted by growers as valuable, pathogen-free stock materials. Water stress is believed to adversely affect growth and yield of banana plants, as banana plants requires water in sufficient quantity for their normal metabolic activities. Trehalose is one of the organic substances currently known to be involved in osmotic adjustment, a significant strategy for plant drought tolerance. The current study was aimed to assess the role of trehalose as a presoaking treatment at 0, 20, 60 or 100 mM in improving tolerance of banana plantlets under *in vitro* drought stress conditions and following transitional period required in preparation for planting in the field. The pretreatment of 20 mM trehalose enhanced growth of banana plantlets under *in vitro* drought stress conditions in terms of root system and fresh weight. Non of *in vitro* stressed un-pretreated plantlets succeeded to continue grow during recovery stage in the green house. Results showed markedly higher indicators of growth recovery in banana transplants previously supplied with 20 mM trehalose. This growth improvement was accompanied with enhancement of chemical composition in terms of increasing pigments and reducing total free amino acids contents to reach almost normal levels in addition to higher total phenols content and overall antioxidant activity even more than control plants. Sodium Dodicyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) analysis showed over expressed protein band of 32 kDa molecular weight in recovered transplants pretreated with the highest trehalose concentration (100 mM). Recovered transplants pretreated with trehalose at the lowest concentration (20 mM) showed balanced up regulation of both trehalose-6-phosphate synthase (TPS) and trehalase genes over control, while recovered transplants pretreated with trehalose at the highest concentration (100 mM) showed also over expression of TPS gene but down regulation of trehalase gene under control.

**Keywords:** Banana, Trehalose, Drought stress, Recovery, Antioxidant activity, SDS-PAGE, TPS, Trehalase.

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## INTRODUCTION

Micropropagated banana (*Musa* spp.) plants are widely accepted by growers as valuable, pathogen-free stock materials. Several plant tissue culture labs are engaged in clonal propagation of banana in Egypt, producing at least 1.5 million vitroplants per year [1]. After a period of *ex vitro* acclimatization following *in vitro* culture, the weaned plants should be raised in the green house to obtain plants large enough to be grown in the field.

Drought is one of the most complex natural global phenomena, that is hard to quantify and manage, and has multiple and severe social and economic impacts in both developed and developing countries, the latter still suffer from droughts the most. Ever-increasing exploitation of water resources and associated water scarcity coupled with the growing concern that future climate change will exacerbate the frequency, severity, and duration of drought events and associated impacts explains the increasing attention that individual countries are paying to drought-related issues [2].

According to [3], the gap between water requirements for different sectors and the actual water resources currently available for use in Egypt is about 20 BCM/yr. This gap is overcome by recycling. The overall efficiency of the Nile system in Egypt is about 75%. By the year 2020, water requirements will most likely increase by 20% (15 BCM/yr).

Plants accumulate a variety of organic and inorganic substances (such as sugars, polyols, amino acids, alkaloids, and inorganic ions) to increase their concentration in the cytochylema, reduce the osmotic potential, and improve cell water retention in response to water stress. This phenomenon is defined as osmotic adjustment (OA), a significant strategy for plant drought tolerance. OA has been documented to sustain cell structure and photosynthesis at low water potentials, and to delay leaf senescence and death and improve root growth as water deficits become severe. Trehalose is one of substances currently known to be involved in OA [4]. Tolerance to abiotic stresses can be acquired by pre-treatment with such a protective organic compound [5].

Main pathway of trehalose metabolism in plants and yeast could be summarized as follow: Synthesis of trehalose-6-phosphate from UDP-glucose and glucose-6-phosphate is catalyzed by trehalose-6-phosphate synthase (TPS) activity. Trehalose-6-phosphate is then converted to trehalose by trehalose-6-phosphate phosphatase (TPP) activity. Finally, trehalose can be hydrolyzed by trehalase generating two glucose molecules [6].

Water stress is believed to adversely affect growth and yield of banana plants, as banana plants requires water in sufficient quantity for their normal metabolic activities. There is a dire need to improve water stress tolerance of such valuable crops. Thus, the current study was aimed to assess the role of trehalose as a presoaking treatment in improving tolerance of banana plantlets under *in vitro* drought stress conditions and following transitional period required in preparation for planting in the field.

## MATERIALS AND METHODS

This study was conducted during 2015-2016 at Biotechnology Research Laboratory and the green house of Horticulture Research Institute, Agricultural Research Center, Giza, Egypt in collaboration with Faculty of Biotechnology, October University for Modern Sciences and Arts, Egypt.

### *In vitro* trehalose and drought stress applications

*In vitro* raised shoots resulting from proliferation of Grand Nain banana shoot-tips were used as starting experimental materials. Before a subculture, explants were transferred to half strength MS medium supplemented with trehalose at different concentrations (0, 20, 60 and 100 mM). After two days of growing in trehalose medium, the stress treatment was applied.

Medium specifically formulated to induce drought stress supplemented with modified MS salts and vitamins [7], 3% sucrose, BA at 22 µM and 3.0% PEG [8], as compared to PEG free medium. The pH of the prepared medium was adjusted at 5.7 prior to addition of agar at 5 g/L. The medium was distributed into the

culture jars (325 ml.), where each jar contained 30 ml. of the medium. The culture jars were autoclaved at 121°C at 15 lb/inch$^2$ for 20 min. Cultures were allowed to grow for 8 weeks at 27°C of day and night temperature. Light was provided by white fluorescent tubes giving intensity of about 1500 Lux at the explants' level.

After the stress treatment, proliferated shoots were re-cultured, each on free or PEG supplemented medium as previous, on a free growth regulators modified MS medium and allowed to grow for another 8 weeks under growth room conditions. Plantlet height (cm), leaves No., roots No, average root length (cm) and fresh weight (gm) were measured by the end of incubation period.

**Acclimatization (recovery)**

Produced plantlets were thoroughly separated from culture medium, washed with a distilled tap water, then dipped in "Rhizolix" solution (1.0 g/L) as a fungicide for 2 min just before transplanting in plastic pots (300 ml) containing autoclaved transplanting medium (sand: peat moss at 1:1, by volume). Pots were covered with transparent polyethylene bags to maintain high relative humidity around the plants in the green house. After two weeks, the polyethylene bags were partially removed to allow air circulation, and later removed after another two weeks. Plantlets were irrigated with half strength MS maintenance medium (free hormone medium) during the period of acclimatization. The irrigation was carried out according to the requirement of plantlets. Pests and disease control program was followed as recommended [9]. After one month, fully acclimatized plantlets were transplanted to culture bags (20 cm) filled with the same medium and maintained in the green house for another five months. Uniform application of water and nutrients given by a commercial 19-19-19 N-P-K fertilizer plus micronutrients (1.0 g/L solution) applied daily as 100 ml/culture bag [1]. Data on plant height (cm), green leaves No., total leaves No., corn diameter (cm), leaf area (cm$^2$), roots No. and average root length (cm) were recorded after five months of transplanting. Samples of leaves were subjected to further analysis.

Obtained data were subjected to analysis of variance according to [10] and significant difference was determined using L.S.D. values at P = 0.05.

**Chemical analysis**

**Pigments; Chlorophyll A (mg/g), Chlorophyll B (mg/g) and Carotenoids (mg/g)**

The content of total chlorophylls (chlorophyll a and chlorophyll b) and total carotenoids were determined by [11] and [12].

**Total soluble sugars (%)**

Extraction of soluble sugars were carried out according to [13], then total soluble sugars was measured colorimetrically.

**Reducing sugars (%)**

Reducing sugars was determined according to [14].

**Total free amino acids (mg/100g)**

Total free amino acids was determined by the ninhydrin method [15].

**Total phenols (%)**

Total phenolics was analyzed spectrophotometrically using the method described by [16].

**Antioxidant activity %**

The antioxidant activity of plant methanol extracts was determined based on the radical scavenging ability in reacting with a stable DPPH (2, 2-diphenyl-1- picrylhydrazyl) free radical according to [17].

**SDS-PAGE protein electrophoresis**

Separation of proteins was performed by using Sodium Dodicyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE), according to the method of [18].

**Expression of trehalose-regulated genes**

Leaf samples of control (normal banana) and recovered drought stressed transplants as affected by trehalose pretreatments (20, 60 and 100 mM) were subjected to detection and quantification of trehalose-regulated genes.

Total RNA extraction was carried out according to [19]. Quality of isolated RNA was displayed on 1.4% agarose formaldehyde gel electrophoresis. cDNAs were synthesized using QIAGEN One-Step RT-PCR Kit. The primers used for the targeted two trehalose-regulated genes were; TPS (Gene ID: 103969073) with the forward as 5`-cttgctcccgattacttggc-3`and the reverse as 5`-aacatcatccactcccagca-3`and Trehalase (Gene ID: 103982784) with the forward as 5`-caactgctgaatcgggatgg-3`and the reverse as 5`-ttctgaggtcacactgtccc-3`. For quantification of the targeted genes, Applied Biosystems 7500 Real-Time PCR System was used and SYBR Green Real-Time PCR Master Mix was incorporated in the reaction. The levels of expression were calculated using the high-efficiency assays enable relative quantization to be performed using the comparative threshold cycle ($C_T$) method ($\Delta\Delta C_T$), [20].

## RESULTS AND DISCUSSION

***In vitro* growth of banana plantlets**

*In vitro* growth of trehalose pretreated plantlets at different concentrations (0, 20, 60 and 100 mM) as affected by PEG (3%) inducing drought stress compared with those grew under normal conditions is presented in Table (1) and Figure (1).

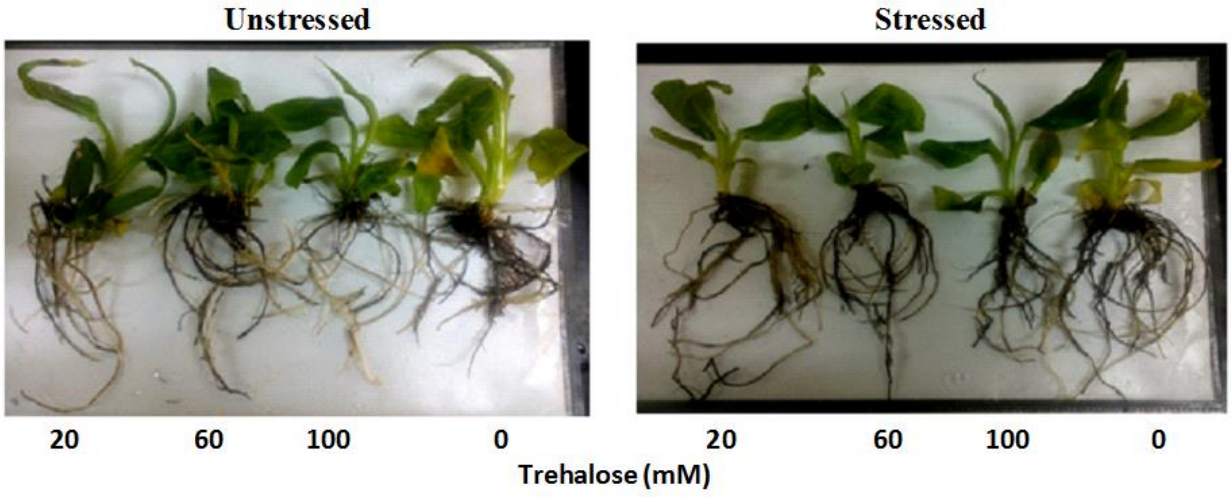


**Figure 1. *In vitro* growth of trehalose pretreated plantlets at different concentrations (mM) as affected by drought stress compared with normal conditions.**

Concerning vegetative growth, no significant differences were recorded between any of trehalose pretreatments neither in normal conditions nor under drought stress. On the other hand, root system was negatively affected in response to drought conditions specially root length which gave 7.57 cm in average

when PEG was incorporated in the culture medium compared to 9.14 cm in average under normal conditions. Trehalose pretreatments had no significant effects on number and average length of roots. Generally, banana plantlets which did not receive any trehalose pretreatment produced more roots (10) while those pretreated with 60 mM trehalose gave the tallest ones (10.18 cm), all under normal conditions. On contrary, plantlets pretreated with the highest trehalose dose (100 mM) and grew under drought stress was the least (7) in number and shortest (6.82 cm) in length of roots. In respect to plantlet fresh weight, drought stress significantly reduced plantlets fresh weight to reach 2.37 gm as a mean value compared to 3.01 gm under normal conditions. On the other hand, plantlets pretreated with the lowest trehalose concentration (20 mM) recorded the highest weight (2.98 gm) while un-pretreated ones gave the least (2.42 gm) in average. As for the interaction, highest concentration of trehalose (100 mM) under normal conditions resulted in heaviest plantlets (3.19 gm) while lacking trehalose in combining with drought stress produced lightest ones (2.01 gm).

**Table 1. *In vitro* growth of trehalose pretreated plantlets at different concentrations (mM) as affected by drought stress compared with normal conditions.**

| Growth parameters | Plant height (cm) | | | No. leaves | | | No. roots | | | Average root length (cm) | | | Fresh weight (gm) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| PEG (%) Trehalose (mM) | 0 | 3 | Mean | 0 | 3 | Mean | 0 | 3 | Mean | 0 | 3 | Mean | 0 | 3 | Mean |
| **0** | 9.33 | 8.50 | **8.92** | 6.00 | 5.00 | **5.50** | 10.00 | 8.00 | **9.00** | 8.50 | 7.36 | **7.93** | 2.82 | 2.01 | **2.42** |
| **20** | 10.17 | 10.50 | **10.33** | 6.33 | 6.33 | **6.33** | 9.33 | 9.67 | **9.50** | 9.75 | 8.60 | **9.18** | 2.96 | 3.00 | **2.98** |
| **60** | 10.50 | 9.83 | **10.17** | 6.33 | 6.33 | **6.33** | 8.67 | 7.67 | **8.17** | 10.18 | 7.49 | **8.83** | 3.08 | 2.34 | **2.71** |
| **100** | 10.67 | 8.83 | **9.75** | 6.67 | 4.67 | **5.67** | 8.33 | 7.00 | **7.67** | 8.12 | 6.82 | **7.47** | 3.19 | 2.14 | **2.66** |
| **Mean** | **10.17** | **9.42** | | **6.33** | **5.58** | | **9.08** | **8.08** | | **9.14** | **7.57** | | **3.01** | **2.37** | |
| **L.S.D.5%** | | | | | | | | | | | | | | | |
| **Trehalose** | **ns** | | | **ns** | | | **ns** | | | **ns** | | | **0.512** | | |
| **PEG** | **ns** | | | **ns** | | | **ns** | | | **1.396** | | | **0.374** | | |
| **Interaction** | **ns** | | | **ns** | | | **2.644** | | | **2.792** | | | **0.724** | | |

**Acclimatization (recovery)**

**Growth responses**

Non of plantlets that didn't receive any trehalose pretreatment and were grown *in vitro* under drought stress succeeded to continue grow during recovery stage in the green house where they were lost consecutively during the period of acclimatization (Figure 2A).

It could be clearly notice that, the pretreatment of lowest trehalose concentration (20 mM) enhanced growth of recovered transplants produced under drought stress in terms of plant height, number of total and green leaves, leaf area, corn diameter, number and average length of roots (Figure 3) recording 52 cm, 12, 9, 415.5 $cm^2$, 4.8 cm, 22 and 20 cm, respectively, with no significant differences as compared to normal banana transplants which didn't receive any trehalose pretreatments and micropropagated in absence of PEG where they gave 47.5 cm, 11, 9, 450.6 $cm^2$, 4.9 cm, 20 and 16 cm, respectively (Figure 2B, C, D, E, F, G and H, respectively). On contrary, recovered transplants pretreated with the highest trehalose dose (100 mM) and grew *in vitro* under drought stress showed severe decline with regard to all growth measurements (11.5 cm, 7, 4, 48 $cm^2$, 2.5 cm, 11 and 8.5 cm, respectively).

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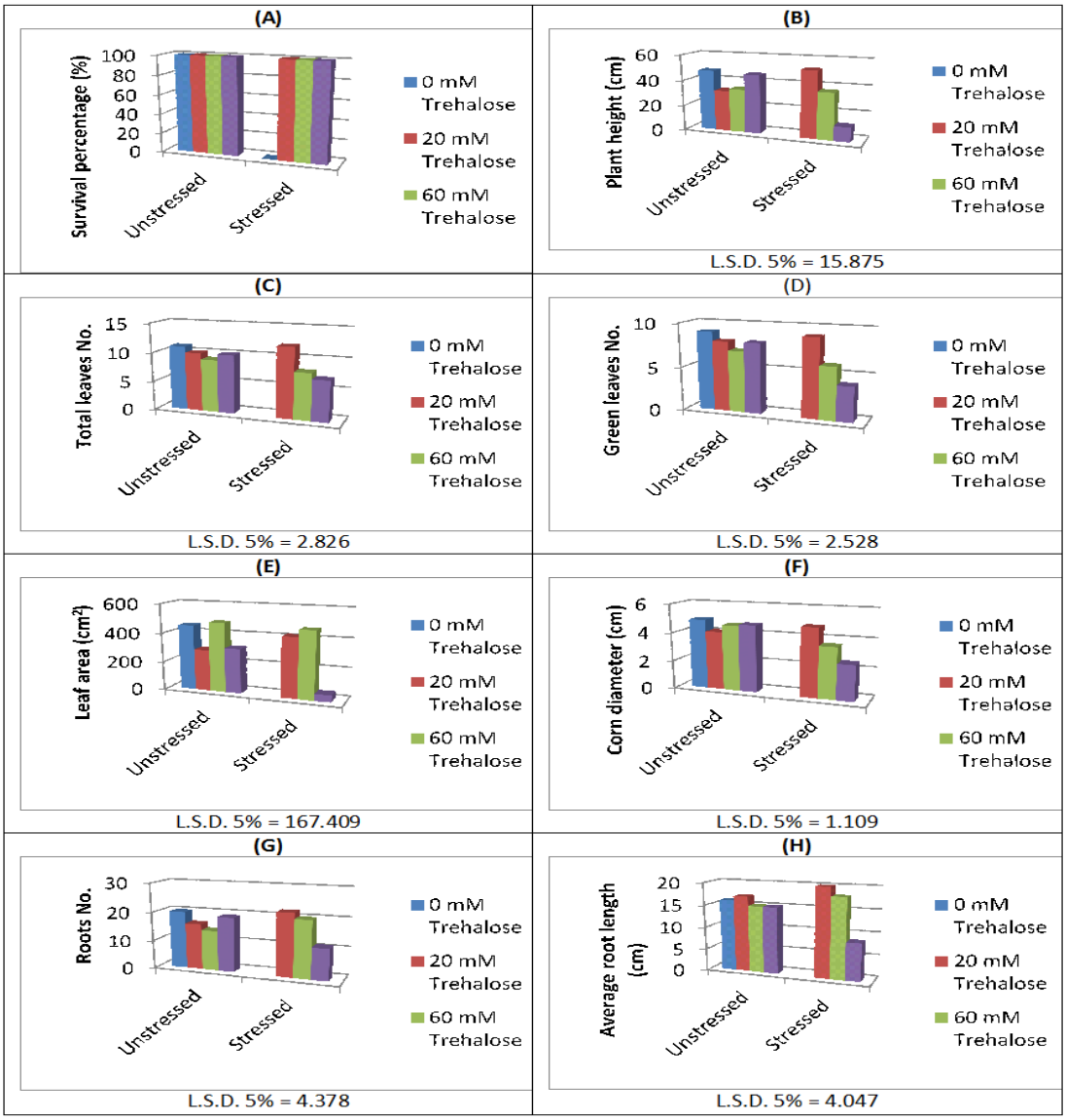


**Figure 2. Growth responses of recovered trehalose pretreated Grand Nain banana transplants micropropagated under drought stress as compared to normal conditions.**

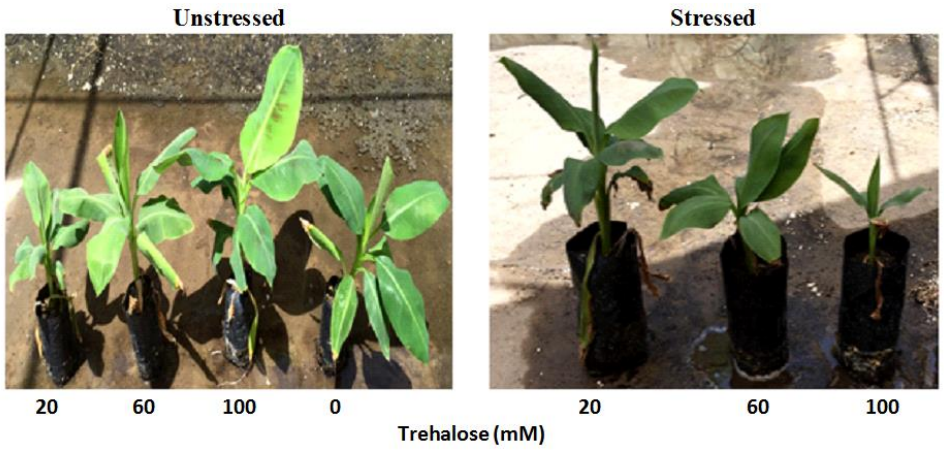


**Figure 3. Growth of recovered trehalose pretreated Grand Nain banana transplants micropropagated under drought stress as compared to normal conditions.**

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**Chemical composition**

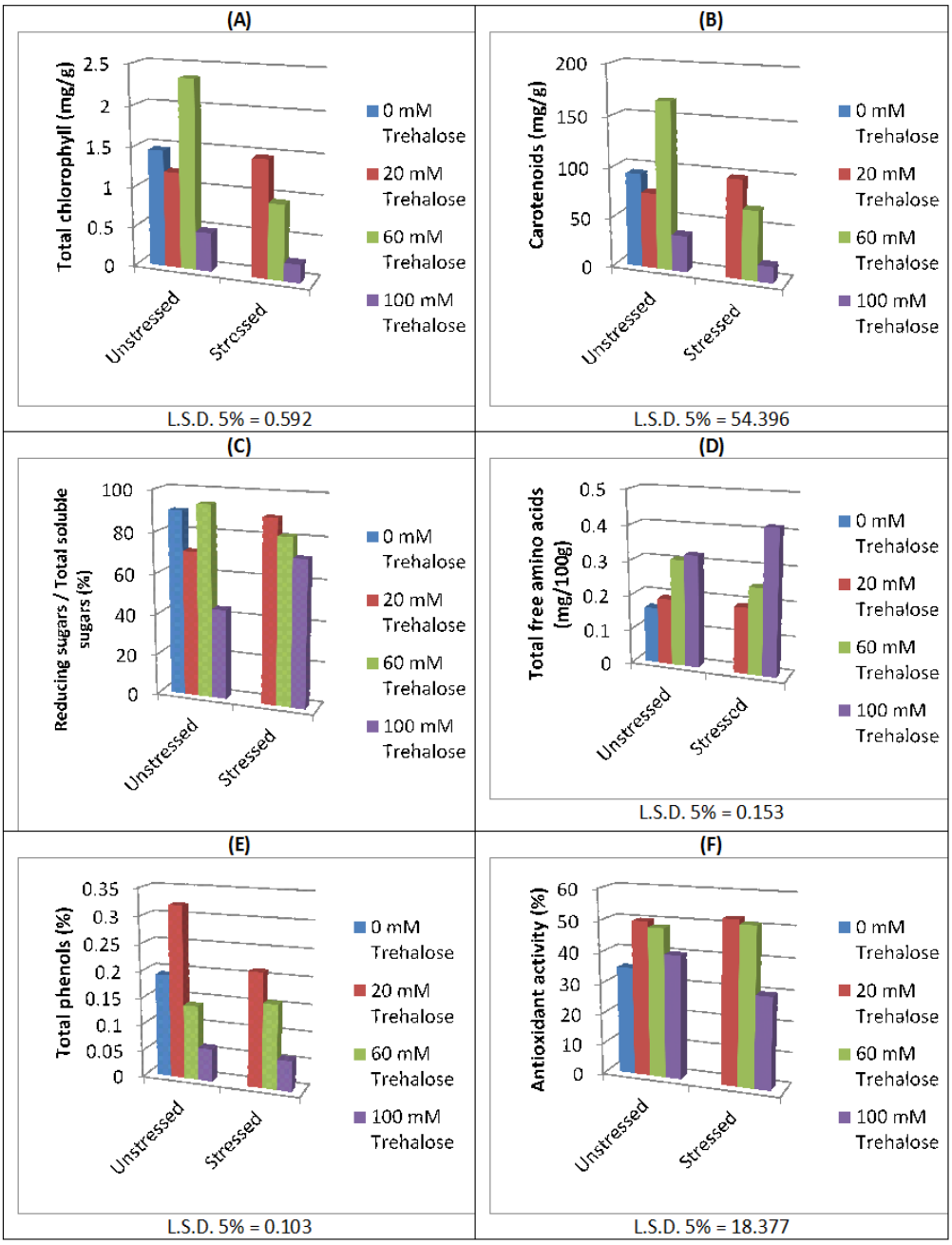


**Figure 4. Chemical composition of recovered trehalose pretreated Grand Nain banana transplants micropropagated under drought stress as compared to normal conditions.**

As shown in Figure (4A and B), transplants pretreated with trehalose at the concentration of 60 mM and micropropagated in absence of PEG had the highest pigments content (2.345 and 166.34 mg/g for total chlorophyll and carotenoids, respectively).The pretreatment of lowest trehalose concentration (20 mM) enhanced pigments content of recovered transplants produced under drought stress in terms of both total chlorophyll and carotenoids representing 1.462 and 97.88 mg/g, respectively, with no significant differences as compared to normal banana transplants which didn't receive any trehalose pretreatments and micropropagated in absence of PEG where they recorded 1.456 and 93.78 mg/g, respectively. On the other hand, transplants pretreated with trehalose at the highest concentration (100 mM) contained low pigments when they grew *in vitro* either in absence or presence of PEG (0.488 and 0.23 for total chlorophyll, 36.13 and 16.39 mg/g for carotenoids, respectively).

Concerning reducing sugars/total soluble sugars ratio (Figure 4C), recovered transplants exposed to severe drought stress *in vitro* recorded in descending order 89.38, 81.15 and 71.6% when they were pretreated with 20, 60 and 100 mM trehalose, respectively. While, recovered transplants pretreated with the highest trehalose concentration (100 mM) prior exposure to long term severe drought stress *in vitro* contained more total free amino acids (0.415 mg/100g) as compared to those received lower trehalose concentrations (20 and 60 mM) which recorded 0.19 and 0.249 mg/100g, respectively (Figure 4D).

Transplants pretreated with trehalose at the lowest concentration (20 mM) and micropropagated in absence of PEG contained the highest total phenols% (0.32). Least total phenols contents (0.061 and 0.057%, respectively) were observed as a result to using the highest trehalose concentration (100 mM) for soaking banana explants prior to culture either in absence or presence of PEG in the *in vitro* culture medium (Figure 4E). Under drought stress, trehalose pretreated transplants at the lowest concentration (20 mM) contained higher total phenols% (0.212) when recovered. As for the overall antioxidant activity (Figure 4F), recovered transplants pretreated with trehalose at lower concentrations (20 and 60 mM) and micropropagated in presence of PEG had higher antioxidant activities (52.42 and 50.96%, respectively) as compared to those received the highest trehalose dose of 100 mM which recorded the least value of 29.87%.

Results showed markedly higher indicators of growth recovery in banana transplants previously supplied with the lowest trehalose concentration (20 mM) and micropropagated under severe drought stress conditions. This growth improvement was accompanied with enhancement of chemical composition in terms of increasing pigments and reducing total free amino acids contents to reach almost normal levels as in control plants which didn't receive any trehalose pretreatments and grew *in vitro* in absence of PEG, in addition to higher total phenols% and overall antioxidant activity even more than control plants.

[21] showed that, exogenous application of trehalose promoted a stronger ability of rice plants to recover from salt-stress. The beneficial effect of trehalose on growth recovery was clearly demonstrated in salt-sensitive cultivar with enhancement of ascorbate peroxidase (APX) antioxidant enzyme activity. Substantial enhancement of APX during recovery presumably led to lower $H_2O_2$ level [22].

Moreover, in a previous study it was observed that, exogenous trehalose promoted drought stress tolerance of Grand Nain banana plantlets *in vitro*. A positive correlation between enhancement growth and DPPH' scavenging effects (%) was observed in trehalose pretreated shoots at the lowest concentration of 20 mM [8]. This observation could be explain by the findings of [5] who suggested that, the protective action of trehalose against drought stress might be through its preservative action on membranes by scavenging of Reactive Oxygen Species (ROS).

**SDS-PAGE protein electrophoresis**

SDS-PAGE analysis (Figure 5) revealed a total number of 16 bands with molecular weights (MW) ranging from about 8.89 to 91.8 kDa. Analysis of data showed over expressed protein band of 32 kDa molecular weight was observed in recovered transplants pretreated with the highest trehalose concentration (100 mM) and micropropagated under severe drought stress conditions (3% PEG).

[23] identified a novel type of plant thioredoxin named CDSP 32 (Chloroplastic Drought-induced Stress Protein of 32 kDa). They observed the accumulation of a 32 kDa polypeptide in *Solanum tuberosum* plants subjected to water deficit. Further, substantial accumulations of CDSP 32 mRNA and protein were revealed upon oxidative stress in *Solanum tuberosum* plants subjected to drought or oxidative treatments [24]. They concluded that, CDSP 32 may preserve chloroplastic structures against oxidative injury upon drought. These findings can explain observed over expression of the 32 kDa protein which was accompanied with reduction of antioxidant activity in recovered banana plants previously supplied with the highest trehalose concentration (100 mM) and grew *in vitro* under severe drought stress conditions, where accumulation of Reactive Oxygen Species (ROS) caused oxidative stress inducing over expression of that specific protein as an additional defense mechanism.

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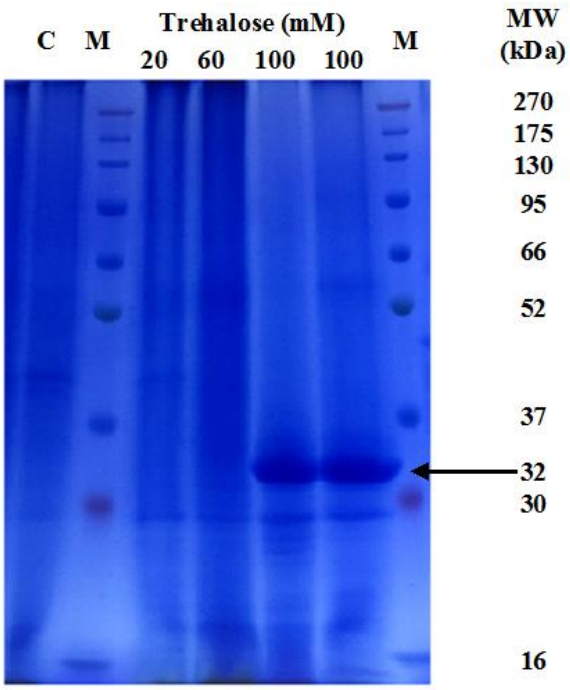


**Figure 5: Electrophoretic banding patterns of Grand Nain banana recovered transplants pretreated with different trehalose concentrations (mM) as compared to control (C: normal banana). M: marker protein.**

**Expression of trehalose-regulated genes**

Total RNA with high quality of normal banana, didn't receive any trehalose pretreatments and grew *in vitro* in absence of PEG, and recovered trehalose pretreated transplants were isolated and visualized (Figure 6A). RT-PCR products were displayed and are shown in Figure (6B,C). The amplification of the two genes fragments were accurate, distinguishable and at the expected size of the designed primers (~215 and 173bp for TPS and Trehalase genes, respectively).

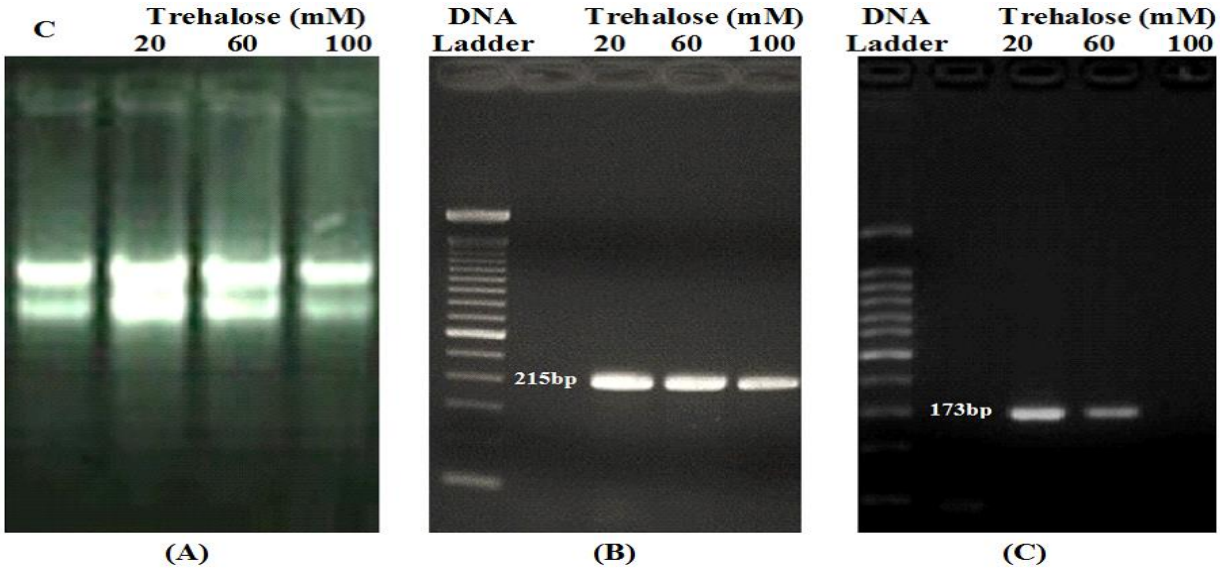


**Figure 6. (A) The 1.4% formaldehyde agarose gel electrophoresis analysis of total RNA of Grand Nain banana recovered transplants pretreated with different trehalose concentrations (mM) and normal banana (C: control). (B) 1.4% agarose gel electrophoresis analysis for TPS gene of recovered banana transplants pretreated with different trehalose concentrations (mM). (B) 1.4% agarose gel electrophoresis analysis for trehalase gene of recovered banana transplants pretreated with different trehalose concentrations (mM).**

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Figure (7) illustrates the relative expression folds of both targeted genes; TPS and Trehalase, in recovered trehalose pretreated transplants as compared to normal banana which didn't receive any trehalose pretreatments and grew *in vitro* in absence of PEG. Recovered transplants pretreated with trehalose at the lowest concentration (20 mM) showed balanced up regulation of both targeted genes over control, while recovered transplants pretreated with 60 mM trehalose had higher over expressed TPS and lower over expressed Trehalase. On the other hand, recovered transplants pretreated with trehalose at the highest concentration (100 mM) showed also over expression of TPS gene but down regulation of Trehalase gene under control.

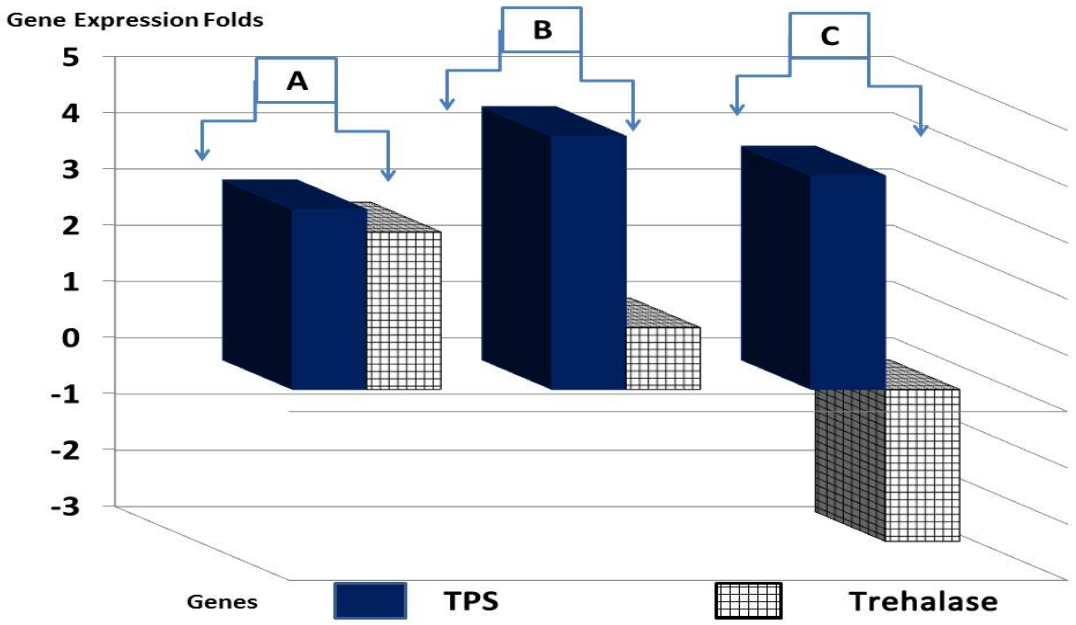


**Figure 7. Change in regulation of TPS and trehalase genes in recovered Grand Nain banana transplants pretreated with different concentrations of trehalose and exposed to drought stress relatively to control (normal banana)**
**A: 20 mM trehalose, B: 60 mM trehalose, C: 100 mM trehalose.**

The non-reducing disaccharide trehalose commonly found in a wide variety of fungi, bacteria, yeasts, and algae, as well as in some invertebrates and vascular plants [25]. Biosynthesis of trehalose is a two-step process consisting of the conversion of UDP-Glc and Glc-6-P into trehalose-6-phosphate by TPS and subsequent dephosphorylation by TPP resulting in trehalose [26]. Trehalase enzyme breaks down the trehalose into two molecules of glucose [6].

Accumulation of high level of trehalose should be expected in recovered transplants pretreated with the highest trehalose dose of 100 mM due to over expressed TPS and down regulated Trehalase gene. These results confirms sugar analysis where recovered transplants pretreated with trehalose at the highest dose (100 mM) contained the lowest reducing sugars/total soluble sugars ratio due to higher rate of trehalose (non-reducing disaccharide) biosynthesis and lower degradation into glucose molecules were observed.

These findings can explain the delay in growth of recovered transplants pretreated with the highest trehalose concentration (100 mM) where constitutive overexpression of TPS and/or TPP genes was found to has negative effect on development of transgenic tobacco plants in terms of stunted growth and altered metabolism under normal growth conditions [25, 27, 28].

On the other hand, banana transplants previously supplied with the lowest trehalose concentration (20 mM) and grew *in vitro* under severe drought stress conditions kept up regulating TPS gene over control during recovery. This treatment was found to be the most effective in regard to combating drought stress where tolerance was accompanied with higher trehalose content under drought stress conditions [8]. In the

case of recovery, higher TPS gene expression was accompanied with up regulation of trehalase gene for undertaking normal trehalose level and avoid the negative effect of trehalose accumulation under normal growth conditions. Balanced up regulation of both targeted genes can explain the obtained higher reducing sugars/total soluble sugars content of recovered transplants pretreated with the lowest trehalose concentration (20 mM).

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