BIOLOGICA NYSSANA
11 (1) September 2020: 45-54
DOI: 10.5281/zenodo.4060294

A real-time PCR assay for evaluation of drought tolerance in a new tea clone

Original Article

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Abstract:
Drought stress induces oxidative stress with subsequent increases in reactive oxygen species ROS in plants. To evaluate oxidative stress intensity with re-irrigation, changes in superoxide dismutase (SOD) expression were investigated by Real-Time PCR in two tea clones, DN and 100. Results showed that SOD expression and other measured factors, except for carotenoids, increased under drought stress in clone DN, but the changes were not significant in clone 100, compared to control. All of the meaningful increases in evaluated enzymatic and non-enzymatic antioxidant factors were eliminated with re-irrigation. The observed changes indicate that clone DN activated its antioxidant defense system in response to drought. In contrast, the lack of response in clone 100 indicates higher levels of tolerance of this new clone against drought.

Key words:
drought, oxidative stress, proline, superoxide dismutase, tea

Received: September 14, 2019
Revised: February 24, 2020
Accepted: March 23, 2020

Apstract:
Ukupan sadržaj fenola i antioksidativni potencijal različitih varijeteta *Brassica oleracea*
Stres izazvan sušom indukuje oksidativni stres sa naknadnim povećanjem ROS-a u biljkama. Da bi se procenio intenzitet oksidativnog stresa ponovnim navodnjavanjem, ispitivane su promene ekspresije superoksid dismutaze (SOD) pomoću PCR-a u realnom vremenu u 2 čajna klona DN i 100. Rezultati su pokazali da ekspresija SOD-a i drugih merenih faktora, osim karotenoida, povećava stres izazvan sušom u klonu DN, dok promene nisu bile značajne u klonu 100, u poređenju sa kontrolom. Sva značajna povećanja procenjenih enzimskih i neenzimskih antioksidativnih faktora eliminisana su ponovnim navodnjavanjem. Uočene promene pokazuju da je klon DN aktivirao svoj antioksidativni odbrambeni sistem kao odgovor na sušu. Suprotno tome, nedostatak odgovora u klonu 100 ukazuje na viši nivo tolerancije ovog novog klona na sušu.

Ključne reči:
suša, oksidativni stres, prolin, superoksid dizmutaza, čaj

Introduction

Plants are often exposed to many stresses, such as drought, high temperatures, and high salt levels, heavy metals and finally oxidative stress. Drought is one of the environmental stresses which has harmful effects on all stages of growth and triggers the production of ROS in plants (Farooq et al., 2009). The amount of damage caused by water shortage depends on the plant species, genotype, duration and intensity of water deficit, soil characteristics, age and developmental stage of the plant (Sánchez et al., 2001). In addition to the physiological changes that occur in plants due to water shortages, stress also inhibits photochemical activity and reduces Calvin cycle activity (Monakhova & Chernyad'Ev, 2004) Accumulation of ROS is one of the many biochemical changes which occurs in plants under stress, as an inevitable and normal product of cell metabolism and oxidative damage caused by ROS is the major limiting factor for plant growth (Allen & Ort, 2001). While there are several sources of ROS production, and whereas some of the most common sources of ROS are the electron transport chains in mitochondria and chloroplasts, chloroplasts are the main source of ROS production in plants (Noctor et al.,

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2014). Plants respond to increased ROS amounts with activation of their enzymatic and non-enzymatic defense systems (Zhu, 2001; Mukhopadhyay ,2014).

Among the most important crops subjected to stress in Iran is tea. The total area under tea cultivation in the north of Iran is over 40 thousand hectares. 4-4,5 percent of the total tea in the world is produced in Iran. It is essential to elevate the amount of product produced per unit to match the increasing consumption of tea. One important strategy for achieving this goal is reducing the environmental damage caused by stresses such as dehydration. Although most tea gardens in Iran are located in areas with high rainfall, reduced crop yields are nevertheless encountered due to the unequal distribution of rainfall in different seasons, especially in the growing season. Research has shown that in areas where the annual rainfall is less than 1150 mm, tea plant growth is reduced due to stress. To reduce losses due to water deficits, varieties should be carefully selected to resist this stress, because damage to the plant is dependent greatly on the plants' stress tolerance.

The first intracellular line of defense is SOD. Phospholipid membranes are non-permeable to O_2- (Asada, 2006). It is therefore essential that SOD be present to remove them in places where these compounds are formed. Based on required metal co-factors, SOD enzymes are classified into 4 groups: Fe-SOD, Mn-SOD, Cu/Zn-SOD, and Ni-SOD. These isoforms are located in different cellular compartments: Fe-SOD is in chloroplasts as is Cu/Zn-SOD, the latter of which is also present in cytosol and extracellular spaces. Mn-SOD is found in mitochondria and peroxisomes (Jamal et al., 2006), and the fourth isoform of SOD, Ni-SOD, which contains nickel in its active site has been found in several types of soil bacteria (Bannister et al., 1987; Bowler et al., 1992).

Many attempts have been made to produce transgenic plants, variously using all 3 isoforms of SOD enzymes. Most of these reports have noted that SOD over-expression led to increased tolerance towards oxidative stress (Faize et al., 2011). However, only in transgenic plants containing Mn-SOD protection was induced against, for example, reduced biomass and leaf damage (Van Breusegem et al., 1999; Samis et al., 2002). Many studies have been consistent with this data and Mn-SOD has been purified from pea (Sevilla et al., 1980; Palma et al., 1998), corn (Baum & Scandalios, 1981), pine (Streller et al., 1994) and tea under cold stress (Vyas & Kumar, 2005). However, research has not been carried out on the role of this isoform in the improvement of drought tolerance in tea. In light of the results in other plants, Mn-SOD is expected to play a crucial role in the improvement of drought tolerance in tea. Hence, in this study, the role of this isoform in resistance to drought was examined. Complex responses of plants to abiotic stresses are manifested in the expression of multiple genes and various biochemical and molecular pathways. Understanding these differences will lead to the identification of genes and molecular processes that play roles in a variety of conditions within biological systems (Moody, 2001). In this study, the expression levels of SOD transcripts were measured by real-time PCR. To evaluate the adaptation capacity of the two clones investigated - 100 and DN - against oxidative stress caused by drought, some indicators of oxidative stress, such as malondialdehyde (MDA), carotenoid and proline content, chlorophyll *a* and total chlorophyll were also investigated.

Material and methods

Sampling

Two selected genotypes belonging to the Tea Research Center, namely DN and 100, were used in this study. Farm sampling was done from 8th July till 25th July for 17 days at the Fashalam Research Station, Iran. The experiment was arranged with a randomized complete block design with 3 replicates. 10-year-old vegetatively propagated tea plants (70-80 cm, height) were used under farm conditions (at ~24-33°C day/night and relative humidity of 75-80%) at longitude and latitude 49°55′10″ and 37°8′20″, respectively. Soil was sandy-loam with pH 5.8 and electric conductivity = 270. Based on data obtained previously, a 10-day drought stress was imposed on the plants. This was followed by a one week irrigation period (twice a week, according to the usual practice of the Research Station) to exit from the stress condition. Soil moisture content (SMC) was measured on days 0, 5, 10 and 15th after withholding water began (Black, 1965). Sampling from the third terminal leaf was carried out in 2 steps, at day 10 and day 17. These samples provided data showing the effects of drought stress and exit from drought conditions, respectively. Leaves were moved in liquid nitrogen and kept at -70 °C until needed.

Total RNA extraction

Fresh leaf tissue, 0.1 g, was used for RNA extraction according to kit instructions (CinnaGen Co.), with slight modification. Because high levels of secondary metabolites in tea prevent high-quality RNA extraction, Isoamyl - chloroform (1:24) was used to minimize the deposition of these compounds in RNA isolates, and sodium acetate (2 M) was applied to optimize RNA precipitation. After extraction, the resulting pellet was dissolved in 50 µl DEPC-treated water and maintained at -70 °C. To determine the

Table 1. Sequence of designed primers for the RT-PCR analysis

Accession number	Putative function	Tm (°C)	Primer sequence (5´-3´)	Size of amplicon
AY641734.2	Manganese Superoxide dismutase	60.5	F: CGGAGGGCATATCAACCACT R: CACCCATCCAGAGCCTCGT	121 bp
AY563528.1	rRNA 18	57.7	F: CAACACGGGGAAACTTACCAG R: TAACCAGACAAATCGCTCCAC	178 bp

quality of the extracted RNA, 1% agarose gel electrophoresis was used. To determine the quantity and the degree of contamination of the RNA, absorbance was read at A260/280 and A260/230 nm. RNAs concentration in µg/µl was calculated as:

$$RNA\ conc. = \frac{A(260\ nm) \times dilution\ ratio\ \times 40}{1000}$$

cDNA synthesis

By the protocol included with the Accupower RT premix kit (BIONEER), one microgram of RNA was used for cDNA synthesis. First, 1 µg of RNA was mixed with 0.5 µg of Oligo dT primer and the mixture was incubated at 70 °C for 5 min to complete annealing. The product was then transferred to AccuPower RT PreMix microtube and the volume was brought to 20 µl with DEPC-treated water. This was vortexed for a few seconds and placed for 60 min at 42 °C. Finally, the mixture was placed at 94 °C for 5 min and the synthesized cDNA was maintained at -20 °C.

Primer Design

Primers used for Mn-SOD gene and the 18S rRNA reference gene were designed using Oligo7 software (Molecular Biology Insights, Inc) (**Tab. 1**). Amplifications were performed in a total reaction volume of 25 µL containing 0.5 mM of dNTP mix, 1 µL of each primer, 2.5 µL of 10X PCR buffer, 0.75 mM MgCl2 25mM, 0.3 unit Taq DNA polymerase and 4µL of template cDNA and 14.95 µL Sterile distilled water with an initial denaturing step of 95 °C for 5 min, followed by 40 amplification cycles of 95 °C for 45 s, 60.5±5 °C for 45 s, and 72 °C for 90 s and a final extension step of 72 °C for 5 min. Finally 60.5 °C was selected for annealing tempreture. RT-PCR was used to determine the appropriate annealing temperature of the primers. PCR products were electrophoresed on a 2% agarose gel.

Real-Time PCR

Various approaches can be used for understanding gene expression in different circumstances. Using of Real-Time PCR allows precise quantization of gene expression with a minimum amount of nucleic acid sample. Measurement of Mn-SOD gene expression was performed on treated and control, untreated samples. The reaction mixture contained 12.5 µl Real-Time Master Mix, 2 µl each of forward and reverse primers, 6 µl template cDNA and 2.5 µl sterile distilled water, with a final volume of 25 µl by 35 amplification cycles. 3 replicates were prepared from each sample and, for standardization, the housekeeping gene (18S rRNA) was used. Finally, after standardizing the data using the expression level of genes in the samples, the actual amount of target gene expression (Mn-SOD) was determined using model 2- CTΔΔ (Livak & Schmittgen, 2001) at the level of mRNA synthesis.

Statistical analysis using One Way ANOVA and Duncan test were performed in SPSS 18 and charts exhibiting the changes in gene expression were plotted in Excel.

Measurement of leaf relative water content

Relative water content (RWC) was measured on days 0 (control), 5, 10 and 17 based on Barrs and Weatherley (1962). After calculating the wet (or fresh) weight (FW), dry weight (DW) and saturated weight (SW), the percentage of leaf RWC in the two clones was obtained in triplicate and finally, RWC percentage was calculated by following formula:

$$RWC = (FW - DW/SW - DW) \times 100$$

Measurement of proline

Proline measurement was carried out with ninhydrin reagent according to Bates et al. (1973). For this purpose, 0.5 g of fresh leaf tissue, glacial acetic acid and toluene were used. After preparation of the extracts, absorbance of the upper layer (containing toluene and proline) was read at 520 nm. By using the standard curve in terms of micrograms per gram fresh weight of leaves, proline content was determined.

Measurement of lipid peroxidation

To determine the extent of lipid peroxidation, MDA concentration was measured using the method of Heath & Packer (1968). For this purpose, 0.5 g of

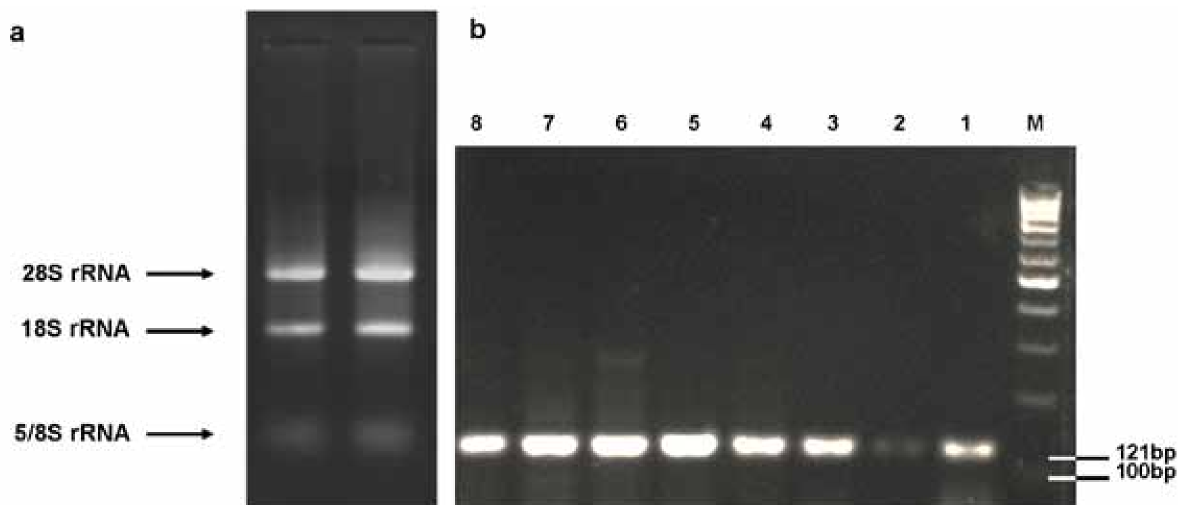

Fig. 1. a) Total RNA extracted from tea leaves and electrophoresed on 1% agarose gel with bands of the 5/8S, 18S, and 28S rRNA, indicating the absence of RNA degradation and lack of protein and DNA contamination in the samples; **b)** 2% agarose gel of Mn-SOD gene RT-PCR product in clones 100 and DN. Bands 1-4 and 5-8 are related to the 121 bp fragment of Mn-SOD gene in clones DN and 100, respectively. The annealing temperature

fresh leaf tissue was ground with 5 ml of 5% trichloroacetic acid. The extract was centrifuged at 14000 rpm for 15 minutes at room temperature and the supernatant was separated using a micropipette. An equal volume of 20% TCA containing 0.5% TBA was then added to the supernatant. The mixture was placed in a boiling water bath at 100°C for 30 min. and immediately cooled on ice. Then, 2 ml of the supernatant was transferred to a microtube and centrifuged for 5 min at 7500 rpm. MDA + TBA complex absorbance was read at 532 nm and the non-specific absorption of the pigment was determined at 600 nm and subtracted from absorption at 532 nm. The extinction coefficient of 155 mM^{-1} cm^{-1} was used to calculate the concentration of MDA. Finally, the reaction product of lipid peroxidation (malondialdehyde) was calculated as nM per gram of fresh weight.

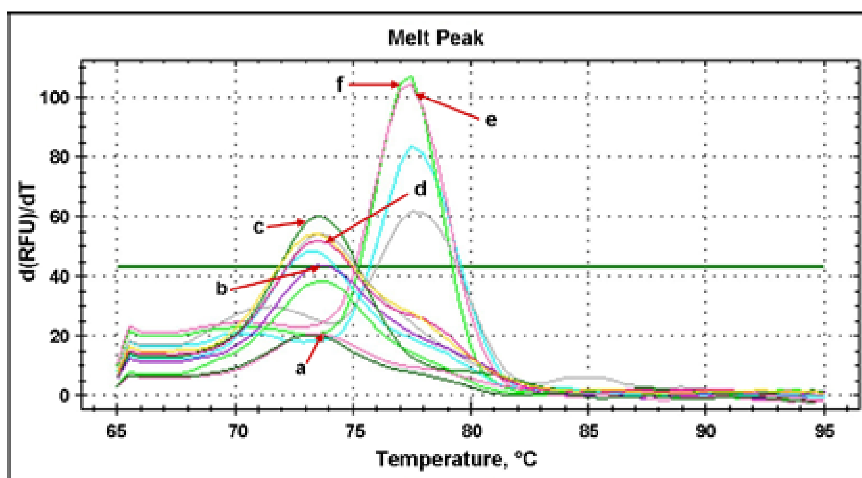

Fig. 2. Melting curves of RT-PCR Product (Mn-SOD gene) in clones 100 and DN. DNA was detected with Cyber Green. a: Melting curve of Mn-SOD gene related to clone DN in control treatments. b: Melting curve of Mn-SOD gene related to clone DN in 10 days drought treatment. c: Melting curve of Mn-SOD gene related to clone 100 in control treatments. d: Melting curve of Mn-SOD gene related to clone 100 in 10 days drought treatment. e: Melting curve of Mn-SOD gene related to clone DN in 17-day treatment (retrieved by re-watering). f: Melting curve of Mn-SOD gene related to clone 100 in 17-day treatment (retrieved by re-watering).

Measurement of chlorophyll and carotenoids

Pigment measurement was performed based on Lichtenthaler (1987). Chlorophyll and carotenoid concentrations were obtained in terms of µg/cm^2 of leaf area.

Results

Extraction of RNA from tea leaves

The extracted RNA bands can be seen in **Fig. 1a**. 3 bands of rRNA, corresponding to 5.8S 18S and 28S, are present in the RNA extract, with the width and intensity of the 28S band higher than the other bands. The extracted RNA sample was used for cDNA synthesis in RT-PCR (**Fig. 1a**).

Electrophoresis and melting curve of RT-PCR products of Mn-SOD gene

As is shown in **Fig. 1b** and **2**, the melting temperature for the 121 bp fragment of Mn-SOD gene RT-PCR product and of the rRNA 18S gene are 73.5 °C and 77.5 °C, respectively (**Fig. 1b, Fig. 2**).

As shown in **Fig. 3** measurement of Mn-SOD gene expression was carried out in leaf tissue from 2 clones 100 and DN at 0, 10 and 17 days after treatment. Standardization of gene expression data was carried out using 18S rRNA. Examining gene expression during drought stress and re-irrigation yielded different results. Results indicate that the greatest amount of Mn-SOD expression was observed in clones of DN after undergoing drought conditions for 10 days. Significant increases in mRNA synthesis of Mn-SOD gene occurred during this period and expression level decreased significantly by day 17 day, i.e., after the 7 day alleviation of the drought conditions, compared to expression levels after the 10 day exposure to drought conditions in the same clone. In contrast, Mn-SOD gene expression did not significantly change in clone 100 in any of the 2 treatments (**Fig. 3**).

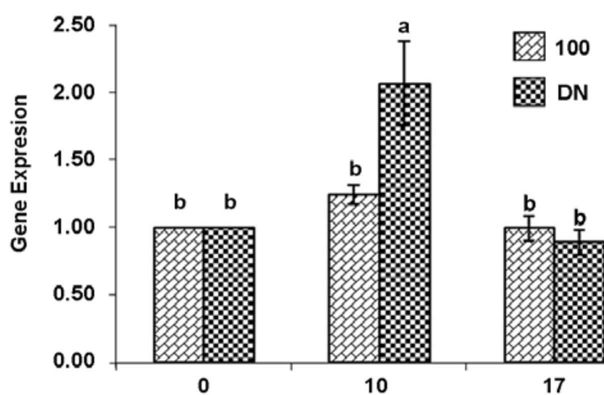

Fig. 3. Changes in mRNA level of the Mn-SOD gene in clones 100 and DN in treatments of control (0), 10 days without watering (10) and treatments recovered with re-irrigation for 7 days (17). Data is average of three replicates ± standard error (SE) respectively. Different letters indicate significant differences between treatments according to Duncan's test with P <0.05.

Measurement of relative water content and soil moisture content

Measurement of SMC showed that soil moisture was decreased meaningfully in all treatments which were inducted by imposition of drought conditions (**Fig. 4**). The effect of drought on RWC changes in the 100 and DN clones showed that responses were similar in both clones and RWC was reduced meaningfully after 10 days of drought stress. In the recovery treatment, RWC levels increased in both clones. This increase was similar in the 2 clones (**Fig. 4**).

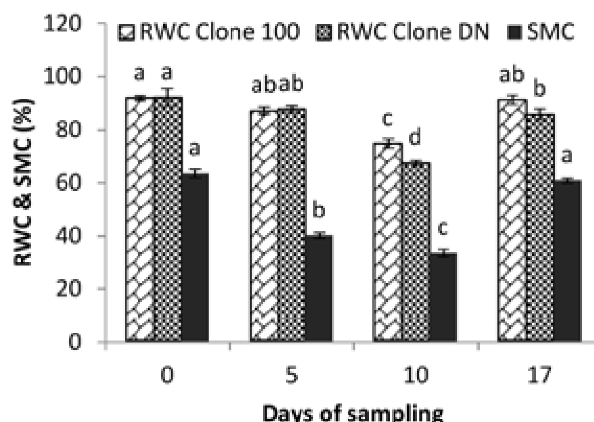

Fig. 4. Changes in RWC of clones 100 and DN in treatments of control (0), 10 days without watering (10) and treatments recovered with re-irrigation for 7 days (17). Data is average of three replicates ± standard error (SE) respectively. Different letters indicate significant differences between treatments according to Duncan's test with P <0.05.

Proline measurement

A comparison of proline content in clones DN and 100 indicates that the responses of the two clones differed in samples under drought and recovery conditions. Proline levels increased under drought stress in clone DN, whereas significant increases were not observed in clone 100 compared to control. Proline content of clone DN was also reduced significantly after the recovery treatment; however, changes of proline content were again not statistically significant in clone 100 at this point (**Fig. 5**).

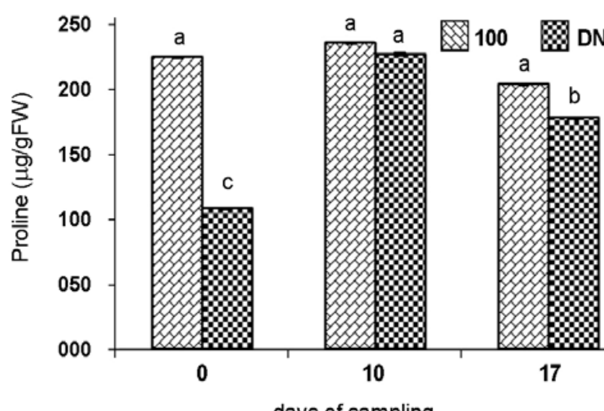

Fig. 5. Changes of proline content of clones 100 and DN in treatments of control (0), 10 days without watering (10) and treatments recovered with re-irrigation for 7 days (17). Data is average of three replicates ± standard error (SE) respectively. Different letters indicate significant differences between treatments according to Duncan's test with P <0.05.

Measurement of lipid peroxidation

As seen in **Fig. 6** changes in malondialdehyde levels in clone 100 are different from those in clone DN under drought stress. MDA content does not increase in clone 100 under drought stress, MDA levels are very substantially increased in clone DN after 10 days of drought treatment. MDA levels in clone DN dropped dramatically in the 7 day recovery period, as assayed on day 17 day of the overall experiment (**Fig. 6**).

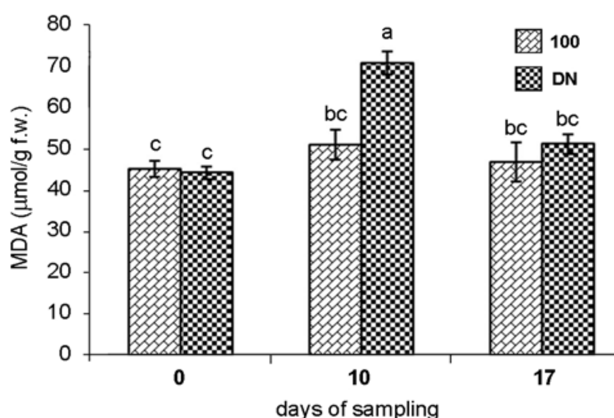

Fig. 6. Changes of MDA content of clones 100 and DN in treatments of control (0), 10 days without watering (10) and treatments recovered with re-irrigation for 7 days (17). Data is average of three replicates ± standard error (SE) respectively. Different letters indicate significant differences between treatments according to Duncan's test and with P <0.05.

Measurement of photosynthetic pigments

Measurement of carotenoids

Data shows that the highest concentrations of carotenoids occurred in clone 100 in the 10 day treatment.

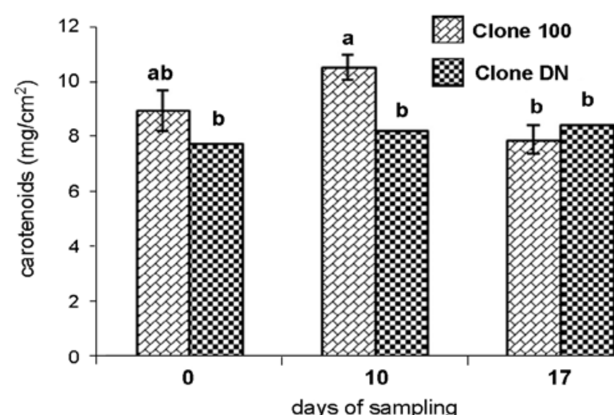

Fig. 7. Changes in carotenoid content in clones 100 and DN in treatments of control (0), 10 days without watering (10) and treatments recovered with re-irrigation for 7 days (17). Data is average of three replicates ± standard error (SE) respectively. Different letters indicate significant differences between treatments according to Duncan's test with P <0.05.

Changes in carotenoid level in clone DN are substantially lower than in clone 100 (**Fig. 7**).

Measurement of chlorophyll *a*

Measurement of chlorophyll *a* in the 2 clones showed that in clone DN levels of chlorophyll *a* were increased after 10 days of drought stress, whereas significant changes were not observed in clone 100. The amount of chlorophyll *a* in clone DN was significantly greater than in clone 100 after the 10 day drought treatment. By day 17, i.e., after 7 days recovery, the amount of chlorophyll *a* in clone DN had dropped dramatically and the levels in it and DN were not significantly different (**Fig. 8**).

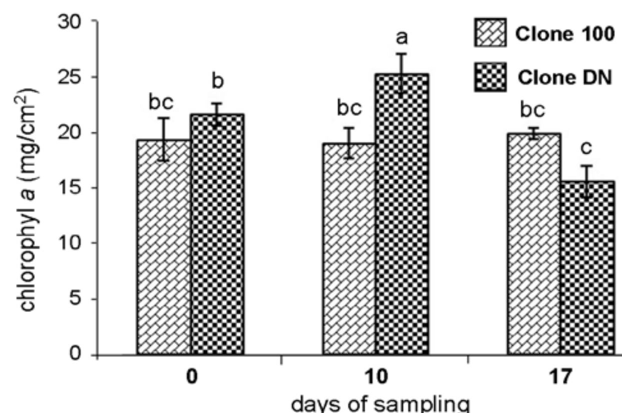

Fig. 8. Changes of chlorophyll *a* content in clones 100 and DN in treatments of control (0), 10 days without watering (10) and treatments recovered with re-irrigation for 7 days (17). Data is average of three replicates ± standard error (SE) respectively. Different letters indicate significant differences between treatments according to Duncan's test with P <0.05.

Measurement of total chlorophyll

Measurement of total chlorophyll content from the 2 clones indicated that chlorophyll content in clone 100 did not vary throughout the experiment. Although total chlorophyll content appeared higher at Day 10 compared to Day 0, these differences were not statistically significant. However, there were significant differences in clone DN after the recovery period, i.e. at day 17 compared to day 10 days and the control (**Fig. 9**).

Discussion

Many studies have shown that in photosynthetic plants there is a strong relationship between tolerance to oxidative stress induced by environmental stresses and increasing concentrations of antioxidant enzymes (Sairam & Saxena, 2000; Sairam & Srivastava, 2001). Researchers have shown that the concentrations of antioxidant enzymes double under stress, resulting, in particular, in increased resistance

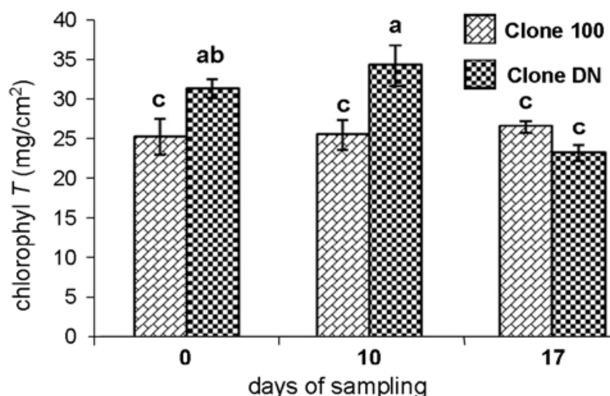

Fig. 9. Changes of total chlorophyll content in clones 100 and DN in treatments of control (0), 10 days without watering (10) and treatments recovered with re-irrigation for 7 days (17). Data is average of three replicates ± standard error (SE) respectively. Different letters indicate significant differences between treatments according to Duncan's test with P <0.05.

to oxidative stress. Drought stress increases the activity of glutathione reductase and SOD especially (Gamble & Burke, 1984; Lascano et al., 2001).

This is not to say that all cultivars of a particular species are stressed to the same degree by drought conditions. In the present study, a significant increase was observed in Mn-SOD gene expression in clone DN during 10 days of drought treatment, while no significant increase in the expression of this gene was found in clone 100. This correlated well with the other physiological parameters assayed and reflected Clone 100's greater resistance to drought in the first place. Clone DN activates resistant mechanisms against oxidative stress caused by drought by increasing the expression of Mn-SOD because it was more greatly stressed by the drought conditions. Malondialdehyde content rose, also, in only clone DN and not in clone 100. Increases in this product represent an increase in lipid peroxidation due to ROS production and there is a positive correlation (r^2=0.66) between the amount of malondialdehyde and SOD gene expression. It can be said, therefore, that SOD gene expression in clone DN was increased to mitigate the effects of generated ROS. Again, neither MDA content nor SOD gene expression were significantly increased in clone 100, indicating that a lower level of cellular stress was induced by drought conditions in this cultivar. On the other hand, appropriate conditions are provided for the growth of clones with re-watering, which is accompanied by reduced expression of the SOD gene in clone DN, indicating a reduction in oxidative stress in this clone. Ten days of drought applied to clone 100 did not result in considerable stress responses involving a significant stimulation of the antioxidant defense system.

Many reports are indicating Mn-SOD expression and activity increases during stress (Shah & Nahakpam, 2012). For example, increased resistance to cold stress was correlated with a simultaneous increase in the expression of Mn-SOD in *Chlorella ellipsoidea* (Clare et al., 1984). Similarly, transformation of wheat plants with the Mn-SOD gene from *Brassica* species and its overexpression increased resistance to oxidative stress and aluminum toxicity (Gachon et al., 2004) in the transgenic wheat. This method to increase SOD activity has also been reported in a study by Bowler et al. (1992). They found that transformation of maize chloroplasts with the tobacco Mn-SOD gene enhanced cold tolerance. Also, increased expression of Mn-SOD in tobacco considerably enhances resistance against oxidative stress. In studies conducted by Upadhyaya et al. (2008), performed on 4 tea clones, a decrease in SOD activity occurred during 10 days drought stress in clone TV 1 that is resistant to water stress, while an increase in activity was seen in the sensitive clone TV 20.

RWC is a very important index of plant water status and dehydration tolerance, and consequently reflects the activity of metabolites in the tissue. RWC is at its highest level in the early stages of leaf development and decreases with increasing leaf dry matter and maturation. This physiological parameter has a direct relationship with water uptake of root and loss of water through respiration (Anjum et al., 2011). Different cultivars show different levels of RWC, depending on duration of stress and ability of the genotypes to maintain osmotic potential in drought conditions at different stages of development (Siddique et al., 2000; Allen & Ort, 2001). Interestingly, a comparison of RWC in clones DN and 100 revealed that RWC was the same in both and was similarly reduced after 10 days of drought stress; whilst re-watering caused an essentially identical increase in RWC in the leaves of both clones. That the decrease in leaf water content happens equally in both clones but causes a different response in these clones strongly indicates that lowered RWC does not necessarily result in oxidative stress. Although not receiving adequate moisture is observed in both examined clones, similar water deficits will not necessarily stimulate defense responses in the two clones equally. Each clone can activate its defense system tailored to its needs and ability. It will be very interesting to learn what the bases of this difference are. Perhaps cytoplasmic proline levels are important here. Clone 100 showed much higher basal proline than did clone DN.

Relationships between the production of free radicals and proline accumulation in plants indicate

a role for proline in reducing toxicity, a non-enzymatic defense mechanism for removing free radicals (Matysik et al., 2002). On the other hand, increases in proline, which reduces the acidity of the cytosol, can be due to various stresses (Kurkdjian & Guern, 1989). Removing excess H^+ as a positive effect of proline synthesis results in a reduction of stress. The current study suggests that a change in proline content in tea plant is regulating the level of osmotic potential that ultimately controls the water absorption. The same phenomenon has been previously observed in cotton (Parida et al., 2008), rice (Pirdashti et al., 2009), maize (Valentovic et al., 2006) and in tea (Upadhyaya & Panda, 2013). Our investigation shows that higher drought resistance of clone 100 compared to clone DN is due to its higher proline level. This capability of clone 100 might be more effective in weak or moderate drought and probably less effective in extreme drought conditions.

Clones DN and 100 differed also in their chlorophyll *a* profiles throughout the experiment. Chlorophyll *a* levels rose under drought conditions in clone DN but were unchanged in clone 100. Similarly, upon relief of drought, chlorophyll *a* levels (and, in this case, total chlorophyll as well) dropped in clone DN and, again, did not significantly change in clone 100. Changes in chlorophyll levels during stress are related to the duration and intensity of the stress (Zhang & Kirkham, 1996). Chapman & Barreto (1997) believe that the growth stage, cultivar and environmental conditions may be important factors influencing the amount of chlorophyll pigments under stress. Sairam et al. (2000 and 2001) have also stipulated that higher levels of carotenoids and chlorophylls in plants under drought stress conditions can be associated with different resistance genotypes. Machado & Paulsen (2001) found that rapid physiological changes such as tubular leaves, reduced leaf area, and increased stomatal resistance may be factors in the apparent increase in chlorophylls content, as part of an avoidance mechanism to drought stress. During stressful conditions, plants reduce transpiration levels to prevent water loss through lower leaf surfaces. Consequently, the chlorophyll content increases per unit leaf area, despite the decrease in total chlorophyll content.

Clone DN is a drought resistant cultivar and is considered as a reference strain for evaluating new Iranian clones used at Tea Research Institute of Iran. Perhaps at least a part of the changes in chlorophylls in this cultivar was related to the higher oxidative stress intensity experienced by this clone during the drought conditions. After the 7 day re-watering period the morphological changes mentioned above were reversed but chlorophyll levels nevertheless fell in clone DN to values lower than in control. It is possible that enhanced chlorophyll synthesis could not be upgraded along with these morphological changes and/or not enough time was available for chlorophyll synthesis to reach levels present in control samples. Clone 100 displayed better tolerance to drought and changes in chlorophyll content in these clones were minimal. Again, it will be very interesting to learn the basis for the lack of apparent oxidative stress in clone 100 as contrasted to the clear oxidative stress experienced by clone DN under conditions of drought.

Conclusion

10-day drought stress was imposed on two clones of tea plants. This was followed by one week irrigation period (twice a week, according to the usual practice of the Research Station) to exit from the stress condition. The results showed that 10-day stress caused significant changes in metabolism and activity of antioxidant system in DN clone, while these changes were not observed in clones 100. Measurement of membrane lipid peroxidation, as an important physiological indicator to detection oxidative stress intensity and check out the Mn-SOD gene expression as the first defense enzyme against oxidative stress, showed that during the 10 days of water stress, the clone 100 is not affected, whereas DN clone showed increase in proline, MDA and also increased expression of superoxide dismutase. With the re-irrigation of the samples, the expression of superoxide dismutase and the levels of malondialdehyde are also decreased in DN clone similar to clone 100, indicating that this clone has been out of stress. Therefore, in terms of some responses to drought stress, clone 100 can be introduced as a more compatible clone for planting in the north region of Iran.

Acknowledgements. The authors acknowledge for the plant material provided by Tea Research Institute of Lahijan, Iran.

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