TÜBİTAK

Turkish Journal of Botany

http://journals.tubitak.gov.tr/botany/

Research Article

Turk J Bot
(2020) 44: 214-221

doi:10.3906/bot-2002-28

The role of *BADH* gene in oxidative, salt, and drought stress tolerances of white clover

Gürkan DEMİRKOL*
Department of Field Crops, Faculty of Agriculture, Ordu University, Ordu, Turkey

Received: 16.02.2020 • **Accepted/Published Online:** 12.04.2020 • **Final Version:** 06.05.2020

Abstract: The aim of this study was to confirm the presence of *BADH* gene and control whether it plays an important role to protect white clover (*Trifolium repens* L.) against oxidative, salt, and drought stresses. Three genotypes possessing *BADH* and 1 genotype lacking *BADH* were selected, considering the expression levels of *BADH* in response to methyl viologen mediated oxidative stress. The genotypes possessing *BADH* displayed significantly less ion leakage under oxidative stress treatment, compared to that lacking *BADH*. In order to test salinity tolerance, the plants were treated with 300 mM NaCl for 2 weeks, and to mimic drought conditions, plants were grown without irrigating for 5 days. Under the salt stress, the plants possessing *BADH* contained lower malondialdehyde levels and higher chlorophyll contents than that of plants lacking *BADH*. Similarly, under the drought conditions, the plants possessing *BADH* were found to have increased levels of glycinebetaine, proline, and high relative water contents than the plant lacking *BADH*. These findings indicate that the plants having *BADH* exhibited enhanced tolerance to salt and drought stresses. These enhanced tolerances of plants possessing *BADH* gene is attributed to its ability to induce glycinebetaine synthesis. Moreover, the plants possessing *BADH* were found to exhibit higher relative feed value under both stress and normal conditions. These plants could be used as forage crops on marginal areas having salinity or drought problems.

Key words: *BADH* gene, betaine aldehyde dehydrogenase, drought stress, glycine betaine, salinity stress, white clover

1. Introduction

Salinity and drought stresses, also members of abiotic stress factors, are the most important problems threating agriculture in many areas of the world, as semiarid and arid areas, which account for 30% of the world, and almost 50% of the irrigated lands of the world are under the threat of salt stress (Mali et al., 2012).

Plants have evolved various strategies to deal with stress. "Compatible solutes" with low molecular weight and high-water solubility are one of the stress responsive molecules in plants. These compounds generally stabilize highly ordered biomolecules under stress conditions (Hare et al., 1998). Glycine betaine (GB) is an effective protectant for various types of abiotic stresses (Chen and Murata, 2011). It effectively stabilizes the structures of proteins and enzymes, thereby protecting the integrity of the membrane under drought and salinity conditions. GB is naturally found in plants, also in animals and microorganisms (Chen and Murata, 2008). The GB biosynthesis is stress-inducible and its concentration in vivo varies among plant species. Moreover, the GB level has been shown to correlate with stress tolerance (Saneoka et al., 1995). Many species, such as wheat, arabidopsis, and barley are not GB accumulators because they do not naturally produce GB with significant amounts under stress or nonstress conditions (Chen and Murata, 2008; Ke et al., 2016).

Increasing evidences from the studies suggest that GB performs crucial functions in plants which expose to abiotic stresses (Ahmad et al., 2008; Li et al., 2014). Thus, generating transgenic plants that can synthesize GB at higher amounts have been the main target for many studies (Hayashi et al., 1997; Sakamoto and Murata, 2000; Chen and Murata, 2011; Li et al., 2014). *BADH* (betaine aldehyde dehydrogenase) is a gene involved in the glycine betaine production and plays an important role in abiotic stress tolerance (Bao et al., 2011).

White clover (*Trifolium repens* L.) is a forage legume with great importance for agronomy in the world due to its high nutritional value. On the other hand, from the agricultural perspective, one of the drawbacks of this plant is its susceptibility to salinity and drought stress conditions. Previous studies have demonstrated that yield and quality of white clover grown under these stress conditions significantly decreased (Wang et al., 2010; Khalid et al., 2017). Therefore, there is a need for developing tolerant white clover lines that can tolerate such abiotic stresses without having any quality and yield loss. Developing white clover germplasm with enhanced drought and salt

* Correspondence: gurkandemirkol@odu.edu.tr

tolerance would bring a long-term solution to forage cultivation on marginal lands.

The aim of this study was to understand the effect of *BADH* gene on physiological and biochemical properties of white clover grown under oxidative, salt, and drought stresses. In addition, the relative feed value (RFV) was evaluated. This is the first report about the *BADH* gene in white clover and its relation with RFV.

2. Materials and methods

2.1. Plant materials

Three genotypes (possessing *BADH*) and 1 genotype as a control (lacking *BADH*) were selected out of 32 white clover genotypes that were collected from the natural flora of Turkey considering the expression levels of *BADH* in response to oxidative stress for further evaluations.

The seeds were plated on ½MS medium (Murashige and Skoog, 1962) containing 1% sucrose and 1% agar (pH 5.8). The cultures were then placed in a temperature-controlled chamber at 25 ± 1 °C where the samples were exposed to photoperiod for 16 h daily. Rooted plants were placed in pots for 1 week before being transferred to soil in the growth chamber.

2.2. Oxidative stress experiment

Oxidative stress analyses were conducted as previously described by Kwon et al. (2002).

Six leaf discs collected from white clover genotypes at the same position were floated on a solution containing 0.4% (w/v) sorbitol and 5 µM methyl viologen, followed by incubating in the dark for 12 h to allow diffusion of the methyl viologen into the leaves. The samples were then subjected to continuous light treatment (150 µmol $m^{-2}s^{-1}$) at 25 °C. The loss of cytoplasmic solutes that could possibly formed after the methyl viologen treatment was measured with an ion conductivity meter over a time period ranging from 0 to 24 h. These conductivity measurements were then compared with the total conductivity of the solution obtained after tissue destruction. The extent of cellular damage was quantified by ion leakage (Li et al., 2014).

2.3. Salt and drought stress experiment

The plantlets were transferred to pods grown in a growth chamber having the following conditions: 16 h photoperiod with an intensity of 100 µmol m^{-2} s^{-1} light and 60% relative humidity at 25 °C. Twenty-eight days old plants that have visually similar were selected and they were irrigated with 300 mM NaCl once in every 3 days for 15 days, followed by irrigation with tap water for 7 days. The plants were exposed to drought stress by withholding the water supply under the conditions of 25 °C and 100 mmol photons m^{-2} s^{-1} light intensity. Before withholding, the plants were irrigated with an equal amount of water for 7 days. After 5 days of withholding, the plants were allowed to recover drought conditions. Salt treatment (300 mM NaCl) and drought time (5 days) were planned based on the preliminary study where the most appropriate upper limits of salt and drought were determined. Moreover, similar treatments have also been used in previous studies focusing on salt and drought stress in various plants (Li et al., 2014; Wei et al., 2017).

2.4. Polymerase chain reaction (PCR) analysis

CTAB method was used to isolate the DNA from white clover plants (Murray and Thompson, 1980). The extracted genomic DNA were mixed with convergent primers complementary to the *BADH* gene (F: 5′-TCCAAATCCACTACGC-3′, R: 5′-TTCTGCTGCCCAACTA-3′) for PCR reaction. The PCR protocol was; 1 cycle (94 °C for 5 min), 30 cycles (94 °C for 1 min, 60 °C for 30 s), 72 °C for 1 min, and a final extension cycle of 10 min at 72 °C. Agarose gel (1%) was used to separate PCR products. Four weeks old leaves grown in soil were treated with 300 mM NaCl, 5 mM methyl viologen, or 5 days water withholding to activate expression of *BADH*. Total RNA was extracted and was treated with DNase I (RNase-free) to remove any contaminating genomic DNA. The actin gene was used for semiquantitative and quantitative *BADH* expression analysis. Moreover, the actin gene was also used to generate the first-strand cDNA using a reverse transcription PCR (RT-PCR) kit (Sangon Biotech) following the manufacturer's instructions. For semiquantitative RT-PCR analysis, the PCR conditions were the same for both actin and *BADH* as expressed in the genomic PCR analysis, except for the extension time. Moreover, the PCR products were separated and visualized as described above. Quantitative RT-PCR (q-RT-PCR) was performed by following the manufacturer's instructions in a fluorometric thermal cycler (Biorad Versafluor, Canada) using the Ever-Green fluorescent dye. Transcript levels were measured relative to the controls.

2.5. Chlorophyll content

A portable chlorophyll meter was used to measure the chlorophyll contents from leaves. After salt stress, the relative chlorophyll content was determined under growth chamber conditions (photoperiod: 16 h, light intensity: 100 mmol $m^{-2}s^{-1}$, relative humidity: 60%, temperature: 25 °C).

2.6. GB content

Four weeks old plants were irrigated with 300 mM NaCl or exposed to drought conditions for 5 days in order to induce the expression of *BADH* gene. After 3 and 5 days of salt and drought treatments, respectively, the samples were collected for GB content measurements. GB extraction was performed with the method described by Grieve and Grattan (1983). Glycine betaine was extracted

from the dry leaf material with 70 °C distilled water. The extract of 0.25 mL was mixed with 0.25 mL of 2 N HCl and 0.2 mL of potassium triiodide solution. The contents were then shaken and cooled in an ice bath for 90 min, followed by addition 2.0 mL of distilled water and 20 mL of 1.2-dichloromethane. The 2 layers were formed in the mixture. The upper aqueous layer phase was then discarded and optical density of the organic layer was measured at 365 nm (Malekzadeh, 2015).

2.7. Malondialdehyde (MDA) and free proline analysis

The lipid peroxidation was estimated by determining the MDA contents with the thiobarbituric acid method described by Horie et al. (2005) The absorbances were measured with a UV-Vis Spectrophotometer (Shimadzu UV-160A, Kyoto, Japan) at 450, 532, and 600 nm.

Proline content in leaves was determined using the method described by Bates et al. (1973) at 520 nm using toluene as the blank and calculated as μg/g FW via using the ninhydrin acid reagent method.

2.8. RFV equation

The RFV, which is included in different quality indices for the determination of hay quality, was calculated by determining the acid detergent fibre (ADF) and neutral detergent fibre (NDF) contents and by estimating the potential energy value for ruminants. NIRSystem (6500, Minnesota, USA) spectroscopy was used for the analysis of ADF and NDF fractions (%). RFV was calculated using the following equations (Mertens, 1987; Linn and Martin, 1989);

Digestible dry matter (DDM) = 88.9– (0.779 x ADF)
Dry matter intake (DMI) = 120/NDF
RFV = (DDMxDMI)/1.29

2.9. Statistical analysis

All analyses were performed in triplicate. Statistical analysis was carried out using SPSS version 22 (IBM Corp, Armonk, NY, USA). In order to determine the differences between samples, analysis of variance (ANOVA) was done at $\alpha = 0.05$. Tukey's test was performed at the $\alpha = 0.05$ level to see whether mean differences were statistically significant.

3. Results

3.1. Determination of the genotype whether it contains *BADH*

The initial screening of plants was achieved using *BADH* specific primers. PCR analysis revealed that 7 genotypes contained the *BADH* gene, whereas it was not found in the control genotype (Figure 1). Moreover, the plants were tested with quantitative RT-PCR analysis with primer (*BADH* gene-specific) from methyl viologen-treated leaf discs. After the methyl viologen treatment, the strongest induction of *BADH* expression was determined in T1, T2, and T3 genotypes. Therefore, these genotypes were selected for further analyses.

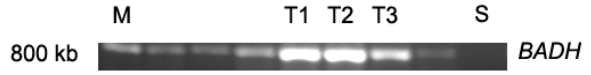

Figure 1. Genomic DNA PCR analysis of the *BADH* gene from white clover genotypes after methyl viologen treatment. *S: the genotype lacking *BADH* gene as a sensitive, T1, T2, T3: the genotypes possessing *BADH* gene as a tolerant.

3.2. Tolerance to methyl viologen based oxidative stress

To evaluate the oxidative stress tolerance of the genotypes, leaf discs obtained from 1- month-old genotypes were treated with 5 μM methyl viologen solution for 24 h. T1, T2, and T3 genotypes (possessing *BADH*) exhibited significantly lower ion leakage against methyl viologen stress (25%, 18%, and 28% in T1, T2, and T3, respectively), compared to S genotype (lacking *BADH*) (54%) (Figure 2a). After oxidative stress, the expression patterns of *BADH* were evaluated using q-RT-PCR. In T1, T2, and T3 genotypes, the *BADH* transcript levels were significantly higher than S genotype (Figure 2b), providing further proof that the *BADH* expression enhances the tolerance to methyl viologen based oxidative stress.

3.3. Tolerance to high salinity stress

In order to determine the salt stress tolerance, 28-days-old plants were subjected to 300 mM NaCl for 15 days, followed by visually evaluating the plant growth. Although salt stress negatively altered the growth of all plants, T1, T2, and T3 genotypes were found to show enhanced tolerance to 300 mM NaCl stress, compared to S genotype.

After the salt treatment, the *BADH* transcript levels significantly increased in T1, T2, and T3 genotypes (1.62, 1.88, and 1.96 before the treatment; while 6.14, 4.23, and 8.76 after the salt stress, respectively), as evaluated by q-RT-PCR ($P < 0.05$) (Figure 3a). The GB contents of the plants were also quantified to evaluate whether increased *BADH* expression after the salt treatment led to any changes in GB content. Before the salt treatment, the GB content in the leaves of S genotype was 6.12 μmol g^{-1} (Figure 3b) (indicating that white clover species is a natural GB producer), and it was found that salt stress did not change the GB content of S genotype, T1, T2, and T3 genotypes revealed significantly increased GB contents after salt stress treatment (the GB contents of T1, T2, and T3 genotypes were 6.20, 8.57, 8.75 μmol g^{-1} before the treatment; while 15.12, 14.99, and 23.44 μmol g^{-1} after the salt stress, respectively) (Figure 3b). These results show that the *BADH* gene had a dramatic impact on GB contents.

In addition, chlorophyll and MDA contents were measured to evaluate the effects of salt stress. Before salt stress, there was no significant difference between

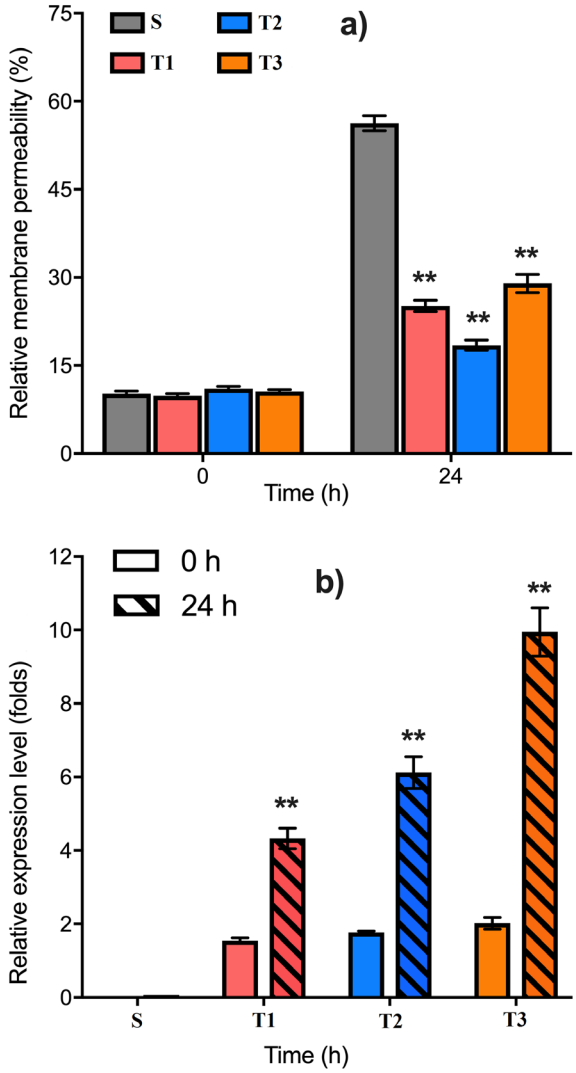

Figure 2. Effects of oxidative stress for white clover genotypes. a) Analysis of ion leakage in the genotypes under 5 µM methyl viologen treatment for 0 and 24 h. b) Transcript levels of *BADH* gene expression (The *BADH* expression level was normalized to that of the actin gene as the internal control). Data represent mean ± SD of triplicates. Asterisks indicate a significant difference between S and T genotypes (Figure a) or control and stressed genotypes (Figure b) at *P < 0.05 or **P < 0.01. *S: the genotype lacking *BADH* gene as a sensitive, T1, T2, T3: the genotypes possessing *BADH* gene as a tolerant.

chlorophyll contents of T1, T2, and T3 genotypes (97.11, 97.96, and 98.19%, respectively) and S genotype (98.54%) (Figure 3c). Salt stress conditions caused a drastic decrease (3 times) in chlorophyll contents of S genotype (33.11%). Collectively, these results indicate that the expression of the *BADH* gene protected the photosynthetic mechanism of T1, T2, and T3 genotypes under the salt stress conditions.

The MDA contents were similar to those of S genotype in T1, T2, and T3 genotypes before salt treatment. However, S genotype were significantly higher than those of T1, T2, and T3 genotypes after salt treatment in terms of MDA contents (Figure 3d).

3.4. Tolerance to drought stress

The performance of T1, T2, and T3 genotypes against drought stress were evaluated to determine whether the *BADH* expression enhances drought tolerance of white clover. After drought stress, the *BADH* transcript levels in T1, T2, and T3 genotypes significantly increased (Figure 4a). Moreover, after the drought stress, GB contents of the leaves of T1, T2, and T3 genotypes significantly increased, compared to nonstress conditions; whereas there was no change observed in S genotype (Figure 4b).

Since the drought conditions cause plants to lose water, the relative water contents (RWC) of the plants were analysed after drought stress. T1, T2, and T3 genotypes were found to show higher RWC than S genotype (Figure 4c), showing that T1, T2, and T3 genotypes were able to preserve more water during the drought conditions which is required to maintain the growth.

Proline content of the plants was also measured in drought treated plants. In normal conditions, there was no significant difference in all genotypes in terms of the free proline contents. However, after water withholding for 5 days, T1, T2, and T3 genotypes were found to contain higher free proline amounts than S genotype (Figure 4d).

3.5. RFV in genotypes after stress conditions

Here, the effects of salt and drought stress on RFV, which is an important parameter to determine the forage quality in plants, were determined. In normal conditions, T1, T2, and T3 genotypes showed significantly higher RFV values, compared to S genotype (Figure 3e, 4e), further demonstrating that the presence of *BADH* in white clover may increase the digestibility. After the salt and drought stresses, T1, T2, and T3 genotypes were able to protect their RFV. However, a dramatic decrease was observed in the RFV of S genotype (Figure 3e, 4e), indicating that the *BADH* gene plays a significant role in determining the forage quality of plants.

4. Discussion

The increasing popularity of white clover as an important forage is attributed to its high nutritional value and its ability to fix nitrogen from the atmosphere. On the other hand, its susceptibility to salinity and drought stress conditions restrict its cultivation in marginal lands. Therefore, there is a need for breeding of white clover plants tolerating abiotic stress conditions. In this study, white clover genotypes possessing *BADH* gene were confirmed for the first time to show enhanced oxidative, salt, and drought stress tolerances.

The current results revealed that T1, T2, and T3

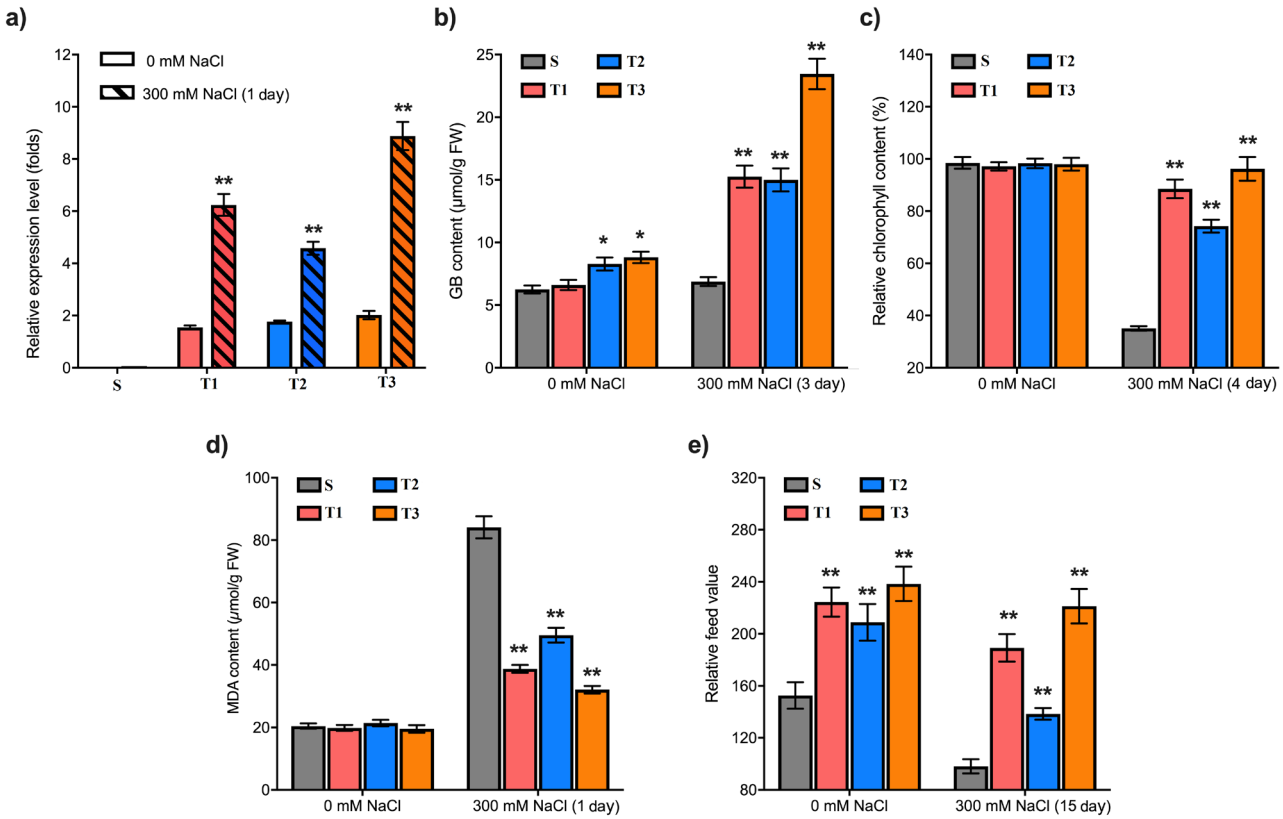

Figure 3. Salinity stress analyses of white clover genotypes. a) Relative expression levels for *BADH* gene (The *BADH* expression level was normalized to that of the actin gene as the internal control) b) GB contents of the leaves of the genotypes c) Relative chlorophyll contents of the leaves of the genotypes d) MDA contents of the leaves of the genotypes e) RFV of the shoots of the genotypes. Data represent mean ± SD of triplicates. Asterisks indicate a significant difference between control and stressed genotypes (Figure a) or S and T genotypes (Figure b, c, d, e) at *P < 0.05 or **P < 0.01. *S: the genotype lacking *BADH* gene as a sensitive, T1, T2, T3: the genotypes possessing *BADH* gene as a tolerant.

genotypes had lower extent of visible damage in leaf discs and lower levels of ion leakage under methyl viologen mediated oxidative stress, further indicating that these genotypes had enhanced tolerance to oxidative stress. These are consistent with previous findings (Li et al., 2014).

The results showed that T1, T2, and T3 genotypes exhibited an enhanced tolerance to oxidative, salinity, and drought stress. This was mainly attributed to the fact that *BADH* gene stimulates the GB synthesis. Similar results were also observed for transgenic plants overexpressing the *BADH* gene (Zhou et al., 2008; Fu et al., 2011; Fan et al., 2012; Di et al., 2015). Moreover, *BADH* gene was found to promote more GB synthesis under the stress conditions in T1, T2, and T3 genotypes.

Cell membrane homeostasis of the plants was also compared by measuring ion leakage and MDA contents of white clover genotypes. Although there was no difference observed under normal conditions, T1, T2, and T3 genotypes had lower ion leakage and MDA contents, compared to S genotype under stress conditions; further indicating that increased GB synthesis protects the cell membrane from stress-based damages (Meloni et al., 2003).

In this study, T1, T2, and T3 genotypes showed higher chlorophyll contents than S genotype under the salinity stress conditions. Having higher photosynthetic activity was the result of GB synthesis in T1, T2, and T3 genotypes compared to S genotype. This indicates that GB protects the photosynthetic system in chloroplasts. GB's mechanistic effects on stress signalling remain unclear due to the lack of direct evidences supporting the impacts of GB on the antioxidative defence system (Li et al., 2014).

There are evidences that high levels of salinity cause ion toxicity and osmotic stress in plants (Aung et al., 2015; Bao et al., 2016; Asci and Acar, 2018; Kaymak and Acar, 2020). The high salt concentrations cause problems for roots to take water. In addition, they can be toxic for protein synthesis and enzyme activity. A variety of plants accumulate osmolytes such as proline when they were subjected abiotic stress. These osmolytes allow the plant to tolerate stress factors (Rajasheker et al., 2019). In this study, the proline contents were measured, and the stress conditions were found to cause increases in proline

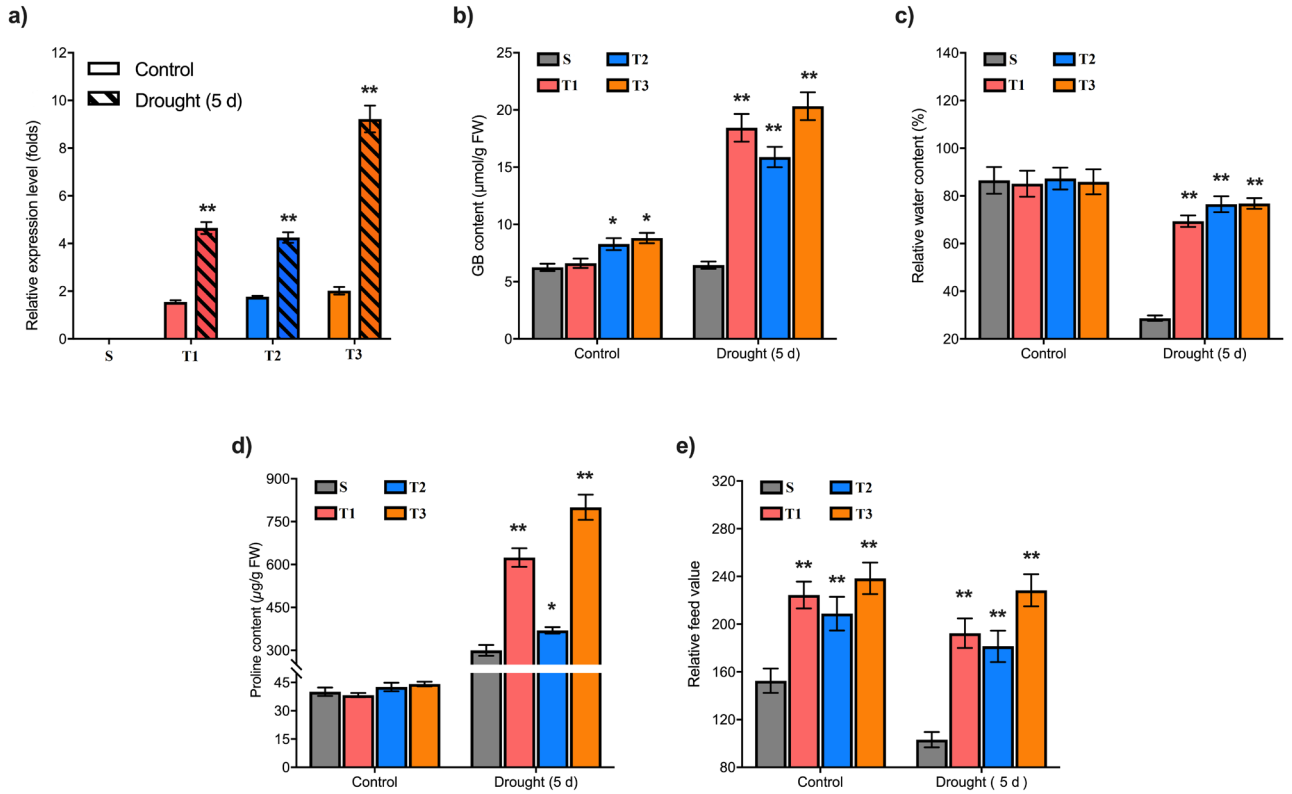

Figure 4. Drought stress analyses of the white clover genotypes. a) Relative expression levels for *BADH* gene (The *BADH* expression level was normalized to that of the actin gene as the internal control) b) GB contents of the leaves of the genotypes c) Relative water contents of the genotypes d) Free proline contents of the leaves of the genotypes e) RFV of the shoots of the genotypes. Data represent mean ± SD of triplicates. Asterisks indicate a significant difference between control and stressed genotypes (Figure a) or S and T genotypes (Figure b, c, d, e) at *P < 0.05 or **P < 0.01. *S: the genotype lacking *BADH* gene as a sensitive, T1, T2, T3: the genotypes possessing *BADH* gene as a tolerant.

contents. Furthermore, the highest increase in free proline contents was observed in T1, T2, and T3 genotypes, which was statistically higher than those detected in S genotype. This suggests that increased free proline contents possibly contributes to enhanced drought tolerance in white clover plants. The high levels of free proline could be the result of enzyme activities in the presence of GB under abiotic stress conditions from the protective effects. A variety of studies indicated that GB stabilizes membranes, and enzymatic activity in stress conditions (Sakamoto and Murata, 2000; Quan et al., 2004; Goel et al., 2011; Li et al., 2014; Ke et al., 2016).

In the present study, T1, T2, and T3 genotypes had higher RWC, which could be another contributing factor to its higher tolerance against drought stress conditions, suggesting that T1, T2, and T3 genotypes exhibited less water loss than S genotype during the dehydration process. Similar results were indicated in GB-synthesizing plants (Li et al., 2014; Ke et al., 2016).

Another important finding of the current study is that T1, T2, and T3 genotypes possessing *BADH* gene were shown for the first time to have higher RFV in stressed and nonstressed conditions, compared to S genotype. This is mainly due to the lower fibre contents of T1, T2, and T3 genotypes. This finding suggests that increased *BADH* expression affected the fibre synthesis. On the other hand, an increase in the fibre content was observed in S genotype. The increased fibre content might be due to the existence of lignin as a component of plant defence system, being distinct from developmental fibre, which is induced by stress factors such as salinity and drought (Stange Jr et al., 2001). This is in an agreement with a previous study where alfalfa plants were shown to possess increased fibre content as a result of defensive response against abiotic stress (Zhang et al., 2016).

5. Conclusions

The results reveal that T1, T2, and T3 genotypes used in this study have great potentials for white clover production in marginal areas. The T1, T2, and T3 genotypes possessing *BADH* gene had significantly enhanced RFV and abiotic stress tolerance. Moreover, it was shown for the first time that *BADH* expression increases RFV in plants. Such plants might have greater importance for

digestibility. Moreover, GB was demonstrated to have essential roles in photosynthetic mechanism under stress conditions. Such information could be used to develop new white clover plants possessing *BADH* that tolerate oxidative, salt and drought conditions; thus, it can be used as a forage or cover crop for marginal lands. The yield parameters of the T1, T2, and T3 genotypes should be further evaluated in field conditions under salt and drought stresses.

References

Ahmad R, Kim MD, Back KH, Kim HS, Lee HS et al. (2008). Stress-induced expression of choline oxidase in potato plant chloroplasts confers enhanced tolerance to oxidative, salt, and drought stresses. Plant Cell Reports 27 (4): 687-698. doi: 10.1007/s00299-007-0479-4

Asci OO, Acar Z (2018). Kaba yemlerde kalite. Ankara, Turkey: TMMOB Ziraat Mühendisleri Odası Yayını (in Turkish).

Aung B, Gruber MY, Amyot L, Omari K, Bertrand A et al. (2015). Ectopic expression of LjmiR156 delays flowering, enhances shoot branching, and improves forage quality in alfalfa. Plant Biotechnology Reports 9 (6): 379-393. doi: 10.1007/s11816-015-0375-2

Bao AK, Du BQ, Touil L, Kang P, Wang QL et al. (2016). Co-expression of tonoplast Cation/H^+ antiporter and H^+-pyrophosphatase from xerophyte *Zygophyllum xanthoxylum* improves alfalfa plant growth under salinity, drought and field conditions. Plant Biotechnology Journal 14 (3): 964-975. doi: 10.1111/pbi.12451

Bao Y, Zhao R, Li F, Tang W, Han L (2011). Simultaneous expression of *Spinacia oleracea* chloroplast choline monooxygenase (*CMO*) and betaine aldehyde dehydrogenase (*BADH*) genes contribute to dwarfism in transgenic *Lolium perenne*. Plant Molecular Biology Reporter 29 (2): 379-388. doi: 10.1007/s11105-010-0243-8

Bates LS, Waldren RP, Teare I (1973). Rapid determination of free proline for water-stress studies. Plant and Soil 39 (1): 205-207. doi: 10.1007/BF00018060

Chen TH, Murata N (2008). Glycinebetaine: an effective protectant against abiotic stress in plants. Trends in Plant Science 13 (9): 499-505. doi: 10.1016/j.tplants.2008.06.007

Chen TH, Murata N (2011). Glycinebetaine protects plants against abiotic stress: mechanisms and biotechnological applications. Plant Cell and Environment 34 (1): 1-20. doi: 10.1111/j.1365-3040.2010.02232.x

Di H, Tian Y, Zu H, Meng X, Zeng X et al. (2015). Enhanced salinity tolerance in transgenic maize plants expressing a *BADH* gene from *Atriplex micrantha*. Euphytica 206 (3): 775-783. doi: 10.1007/s10681-015-1515-z

Fan W, Zhang M, Zhang H, Zhang P (2012). Improved tolerance to various abiotic stresses in transgenic sweet potato (*Ipomoea batatas*) expressing spinach betaine aldehyde dehydrogenase. PLoS One 7 (5): 1-14. doi: 10.1371/journal.pone.0037344

Fu XZ, Khan EU, Hu SS, Fan QJ, Liu JH (2011). Overexpression of the betaine aldehyde dehydrogenase gene from *Atriplex hortensis* enhances salt tolerance in the transgenic trifoliate orange (*Poncirus trifoliata* L. Raf.). Environmental and Experimental Botany 74, 106-113. doi: 10.1016/j.envexpbot.2011.05.006

Goel D, Singh AK, Yadav V, Babbar SB, Murata N et al. (2011). Transformation of tomato with a bacterial *codA* gene enhances tolerance to salt and water stresses. Journal of Plant Physiology 168 (11): 1286-1294. doi: 10.1016/j.jplph.2011.01.010

Grieve CM, Grattan SR (1983). Rapid assay for determination of water soluble quaternary ammonium compounds. Plant Soil 70 (2): 303-307. doi: 10.1007/BF02374789

Hare PD, Cress WA, Van Staden J (1998). Dissecting the roles of osmolyte accumulation during stress. Plant Cell and Environment 21 (6): 535-553. doi: 10.1046/j.1365-3040.1998.00309.x

Hayashi H, Mustardy L, Deshnium P, Ida M, Murata N (1997). Transformation of *Arabidopsis thaliana* with the *codA* gene for choline oxidase; accumulation of glycinebetaine and enhanced tolerance to salt and cold stress. The Plant Journal 12 (1): 133-142. doi: 10.1046/j.1365-313X.1997.12010133.x

Horie T, Motoda J, Kubo M, Yang H, Yoda K et al. (2005). Enhanced salt tolerance mediated by *AtHKT1* transporter-induced Na^+ unloading from xylem vessels to xylem parenchyma cells. The Plant Journal 44 (6): 928-938. doi: 10.1111/j.1365-313X.2005.02595.x

Kaymak G, Acar Z (2020). Determination of salinity tolerance levels of tedera (*Bituminaria bituminosa* L.) genotypes. Anadolu Journal of Agricultural Sciences 35 (1): 51-58. doi: 10.7161/omuanajas.608600

Ke Q, Wang Z, Ji CY, Jeong JC, Lee HS et al. (2016). Transgenic poplar expressing *codA* exhibits enhanced growth and abiotic stress tolerance. Plant Physiology and Biochemistry 100: 75-84. doi: 10.1016/j.plaphy.2016.01.004

Khalid M, Bilal M, Hassani D, Iqbal HM, Wang H et al. (2017). Mitigation of salt stress in white clover (*Trifolium repens*) by *Azospirillum brasilense* and its inoculation effect. Botanical Studies 58 (5): 1-7. doi: 10.1186/s40529-016-0160-8

Kwon SY, Jeong Y, Lee H, Kim J, Cho K et al. (2002). Enhanced tolerances of transgenic tobacco plants expressing both superoxide dismutase and ascorbate peroxidase in chloroplasts against methyl viologen-mediated oxidative stress. Plant Cell and Environment 25 (7): 873-882. doi: 10.1046/j.1365-3040.2002.00870.x

Li H, Wang Z, Ke Q, Ji CY, Jeong JC et al. (2014). Overexpression of *codA* gene confers enhanced tolerance to abiotic stresses in alfalfa. Plant Physiology and Biochemistry 85: 31-40. doi: 10.1016/j.plaphy.2014.10.010

Linn JG, Martin NP (1989). Forage Quality Tests and Interpretation Minnesota Ext. Service AG-FO-2637. Saint Paul, MN, USA: University of Minnesota.

Malekzadeh P (2015). Influence of exogenous application of glycinebetaine on antioxidative system and growth of salt-stressed soybean seedlings (*Glycine max* L.). Physiology and Molecular Biology of Plants 21 (2): 225-232. doi: 10.1007/s12298-015-0292-4

Mali B, Thengal S, Pate P (2012). Physico-chemical characteristics of salt affected soil from Barhanpur, MS, India. Annals of Biological Research 3 (8): 4091-4092.

Meloni DA, Oliva MA, Martinez CA, Cambraia J (2003). Photosynthesis and activity of superoxide dismutase, peroxidase and glutathione reductase in cotton under salt stress. Environmental and Experimental Botany 49 (1): 69-76. doi: 10.1016/S0098-8472(02)00058-8

Mertens DR (1987). Predicting intake and digestibility using mathematical models of ruminal function. Journal of Animal Science 64 (5): 1548-1558. doi: 10.2527/jas1987.6451548x

Murashige T, Skoog F (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiologia Plantarum 15: 473-497. doi: 10.1111/j.1399-3054.1962.tb08052.x

Murray M, Thompson WF (1980). Rapid isolation of high molecular weight plant DNA. Nucleic Acids Research 8 (19): 4321-4326. doi: 10.1093/nar/8.19.4321Z

Quan R, Shang M, Zhang H, Zhao Y, Zhang J (2004). Engineering of enhanced glycine betaine synthesis improves drought tolerance in maize. Plant Biotechnology Journal 2 (6): 477-486. doi: 10.1111/j.1467-7652.2004.00093.x

Rajasheker G, Jawahar G, Jalaja N, Kumar SA, Kumari PH et al. (2019). Role and regulation of osmolytes and ABA interaction in salt and drought stress tolerance. Plant Signaling Molecules. Woodhead Publishing. doi: 10.1016/B978-0-12-816451-8.00026-5

Sakamoto A, Murata N (2000). Genetic engineering of glycinebetaine synthesis in plants: current status and implications for enhancement of stress tolerance. Journal of Experimental Botany 51 (342): 81-88. doi: 10.1093/jexbot/51.342.81

Saneoka H, Nagasaka C, Hahn DT, Yang WJ, Premachandra GS et al. (1995). Salt tolerance of glycinebetaine-deficient and-containing maize lines. Plant Physiology 107 (2): 631-638. doi: 10.1104/pp.107.2.631

Stange RR, Ralph J, Peng J, Sims JJ, Midland SL et al. (2001). Acidolysis and hot water extraction provide new insights into the composition of the induced 'lignin-like' material from squash fruit. Phytochemistry 57 (6): 1005-1011. doi: 10.1016/S0031-9422(01)00096-6

Wang J, Drayton MC, George J, Cogan NO, Baillie RC et al. (2010). Identification of genetic factors influencing salt stress tolerance in white clover (*Trifolium repens* L.) by QTL analysis. Theoretical and Applied Genetics 120 (3): 607-619. doi: 10.1007/s00122-009-1179-y

Wei Y, Xu Y, Lu P, Wang X, Li Z et al. (2017). Salt stress responsiveness of a wild cotton species (*Gossypium klotzschianum*) based on transcriptomic analysis. PLoS One 12 (5): 1-25. doi: 10.1371/journal.pone.0178313

Zhang Z, Shao L, Chang L, Cao Y, Zhang T et al. (2016). Effect of rhizobia symbiosis on lignin levels and forage quality in alfalfa (*Medicago sativa* L.). Agriculture Ecosystems and Environment 233: 55-59. doi: 10.1016/j.agee.2016.08.035

Zhou S, Chen X, Zhang X, Li Y (2008). Improved salt tolerance in tobacco plants by co-transformation of a betaine synthesis gene *BADH* and a vacuolar Na^+/H^+ antiporter gene *SeNHX1*. Biotechnology Letters 30 (2): 369-376. doi: 10.1007/s10529-007-9548-6