American Journal of Agricultural and Biological Sciences 8 (1): 1-11, 2013
ISSN: 1557-4989

doi:10.3844/ajabssp.2013.1.11 Published Online 8 (1) 2013 (http://www.thescipub.com/ajabs.toc)

Tolerance of Accessions of Glagah (*Saccharum spontaneum*) to Drought Stress and Their Accumulation of Proline

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Received 2012-06-15, Revised 2013-01-30; Accepted 2013-02-04

ABSTRACT

Glagah (wild sugarcane, *Saccharum spontaneum*) is a perennial grass that grows well in marginal soils. It has a high carbohydrate and fibre content, making it suitable for ethanol production and the study industry. Thus, glagah has potential as a crop species in its own right and may also be used in sugarcane breeding programs. However, glagah germplasm has not been extensively utilised in breeding programs, especially in relation to improving drought tolerance. This study was performed to evaluate the effect of drought stress over an eight week period on plant height, stalk diameter, green leaf number and leaf proline content of eight, two month-old accessions of glagah to identify their drought tolerance and to determine whether proline accumulation can be used as a metabolic marker of drought tolerance. Accessions, BOT-53, BOT-54 and BOT-62, were the most tolerant and productive. Two patterns of proline accumulation were shown in drought-stressed plants. In four accessions, proline increased after both four and eight weeks of drought stress. While, in the others, proline increased after four weeks and then declined. Significant, positive correlations were found between leaf proline contents (after both two and eight weeks of drought) and plant height at all assessment times. A significant, positive correlation was also discovered between proline content after eight weeks and green leaf number after four weeks of drought. Glagah accessions of BOT-53, BOT-54 and BOT-62 show drought-tolerance and have potential for use as a crop for arid regions or in breeding programs to improve production of sugarcane. Drought tolerance in glagah appears to be mediated by proline and accumulation of this amino acid has potential as a metabolic marker of drought tolerance.

Keywords: *Saccharum Spontaneum*, Proline, Metabolic Marker, Glagah, Drought Stress, Drought Tolerance, Proline Contents, Breeding Programs, Proline Accumulation, Ethanol Production

1. INTRODUCTION

Sugarcane (Saccharum officinarum L.) (Poaceae) is an important crop globally primarily for sugar production but is increasingly being used as a source of bioenergy due to its phenomenal capacity for dry matter production; however, a lack of water often limits sugarcane production (Naik, 2001; Silva *et al*., 2008; Tammisola, 2010). Drought stress simultaneously affects a number of morphological and physiological traits in plants thereby causing a loss of productivity (Bray, 1997; Jamaux *et al*., 1997; Yordanov *et al*., 2003). Therefore, traits associated with tolerance of drought stress are suitable criteria for screening germplasm collections for accessions that may contribute to breeding programs to reduce the influence of water deficit on crop yield (Silva *et al*., 2008).

Maintenance of plant water potential during water deficits is essential to continued growth and can be achieved

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by osmotic adjustment resulting from the accumulation of molecules, such as proline, in the cytoplasm (Ingram and Bartels, 1996). Proline in drought-stressed plants is not only used for osmotic adjustment but also for scavenging and detoxifying oxidants (Yamada *et al*., 2005; Vallidoyan and Nguyen, 2006) and for preventing membrane damage and protein denaturation during severe drought stress (Bandurska, 1998; Ain-Lhout *et al*., 2001). Thus, proline content has been proposed as a metabolic marker of drought stress (Dib *et al*., 1994).

Glagah (wild sugarcane, *Saccharum spontaneum* L. (Poaceae) is a tall perennial grass with deep roots and rhizomes that can grow well in marginal soils where no other crop can be cultivated (Amalraj *et al*., 2008) and can established itself in diverse habitats such as rocky regions, deserts and sandy flats (Kandasami *et al*., 1983). Therefore, glagah can be considered as a drought-tolerant, perennial grass and a preliminary, unpublished study by the authors suggested there are considerable differences among accessions of glagah in their resistance to drought stress. Glagah has a high carbohydrate content, making the biomass from this species a suitable substrate for ethanol production (Chandel *et al*., 2009). Glagah also has a high fibre content in its stalk and a dry matter content of 22%. This is higher than in the stalks of cultivated sugarcane, which only have ~14% dry matter, suggesting that this species has potential as a possible renewable fibre resource for the study industry (Amalraj *et al*., 2008). Thus, glagah has not only potential value as a crop species in its own right but also may be used in breeding programs to improve the production of sugarcane.

Crop improvement has played a significant role in yield improvement (Swamy *et al*., 2003). However, to date, the genetic resources in the germplasm of glagah, especially those related to drought stress, have not been extensively utilized in sugarcane breeding programs. To improve drought tolerance, it is necessary to understand the mechanisms by which plants respond to drought and studies on the physiological response of different glagah accessions to drought could be a useful to develop an understanding of these mechanisms. Therefore, in this study, we evaluated the effect of drought stress on plant height, stalk diameter and green leaf number of eight accessions of glagah (BOT-53, BOT-54, BOT-83, BOT-84, IM76-238, BOT-62, BOT-77 and BOT-91) to determine their drought tolerance. As outlined above, changes in proline content have an adaptive role in plants during stress. Therefore, leaf proline contents were also assayed to determine whether proline accumulation is involved with drought tolerance and whether concentrations of this amino acid can be used as a metabolic marker of drought tolerance.

2. MATERIALS AND METHODS

2.1. Plant Material and Experimental Conditions

Eight accessions of glagah, BOT-53, BOT-54, BOT-83, BOT-84, IM76-238, BOT-62, BOT-77 and BOT-91, were used in this study. The accessions were kindly provided by the Indonesian Sugar Research Institute (ISRI/P3GI), Pasuruan, East Java. The accessions were propagated from single node stalk segments (bud sets) in polybags ($400\times200\times400$ mm^3) containing 10 kg of a mixture of soil and manure (2:1) for two months before being subjected to drought stress. The plants were watered every two days until the treatments were imposed and were fertilized three times (1 day, 1 week and 1 month after planting) with 2 g of ammonium sulphate and 1 g of triple superphosphate per polybag. After two months, the plants were transferred from the field to a glasshouse and were then subjected to drought stress by withholding watering for 8 weeks. Untreated, control plants were watered every two days.

2.2. Growth Measurement

Plant height and green leaf number were measured two, four and eight weeks after commencement of the treatment for both control and drought-stressed plants of each accession; stalk diameter was measured after four and eight weeks. Plant height was measured from the soil surface to the first fully-expanded leaf. Green leaf number was calculated from number of green leaves divided by total number of leaves; stalk diameter was measured at 50 mm above the soil surface. A Drought Susceptibility Index (DSI) was calculated as the plant height data according to the formula proposed by Fischer and Maurer (1978): DSI = (1 - Yd/Yw)/(1 - Xd/Xw), where Yd = height of droughted accession, Yw = height of the watered accession, Xd = mean height of all droughted accessions and Xw = mean height of all watered accessions.

2.3. Estimation of Proline Accumulation

Assessment of proline content was performed in Laboratory of Biochemistry, Research Center for Biotechnology, Universitas Gadjah Mada, Yogyakarta. Proline was assayed in the youngest fully-expanded leaves from both stressed and control plants. Proline content was determined using the colorimetric method of Bates *et al.* (1973). DL-proline (Sigma) was used as a standard.

2.4. Experimental Design and Data Analysis

The experiment of was set out using a completely randomized design with two replicates. Data were checked for heteroscedastisity using Bartlett's test before being subjected to ANOVA using Statistica (Version 9.1., StatSoft, Inc. 2010) and means were separated using Fisher's LSD test at P = 0.05. Due to large interactions between the effects of accession and drought treatment, data for plant height, green leaf number and proline content of the control plants were analysed separately from the droughted plants.

3. RESULTS

3.1. Plant Height

Plant height was significantly affected by accession (F = 39.7-64.2, d.f. = 7, p<0.0001) and by treatment (F = 64.2-187.9, d.f. = 1, p<0.0001) and there was a significant accession*treatment interaction (F = 3.8-4.5, d.f. = 7, P = 0.01–0.006) at all assessment times (**Fig. 1**). Accessions, BOT-53, BOT-54, BOT-62 and IM76-238, appeared to show some degree of drought tolerance and, by the end of the eighth week of the drought stress, these accessions were significantly taller than the other accessions. After the eight weeks of drought stress, these accessions had reductions in height of 34.6, 9.6, 31.2 and 21.5%, respectively, compared to their control plants, whereas all of the other accessions had reductions in height of more than 41% compared to their controls.

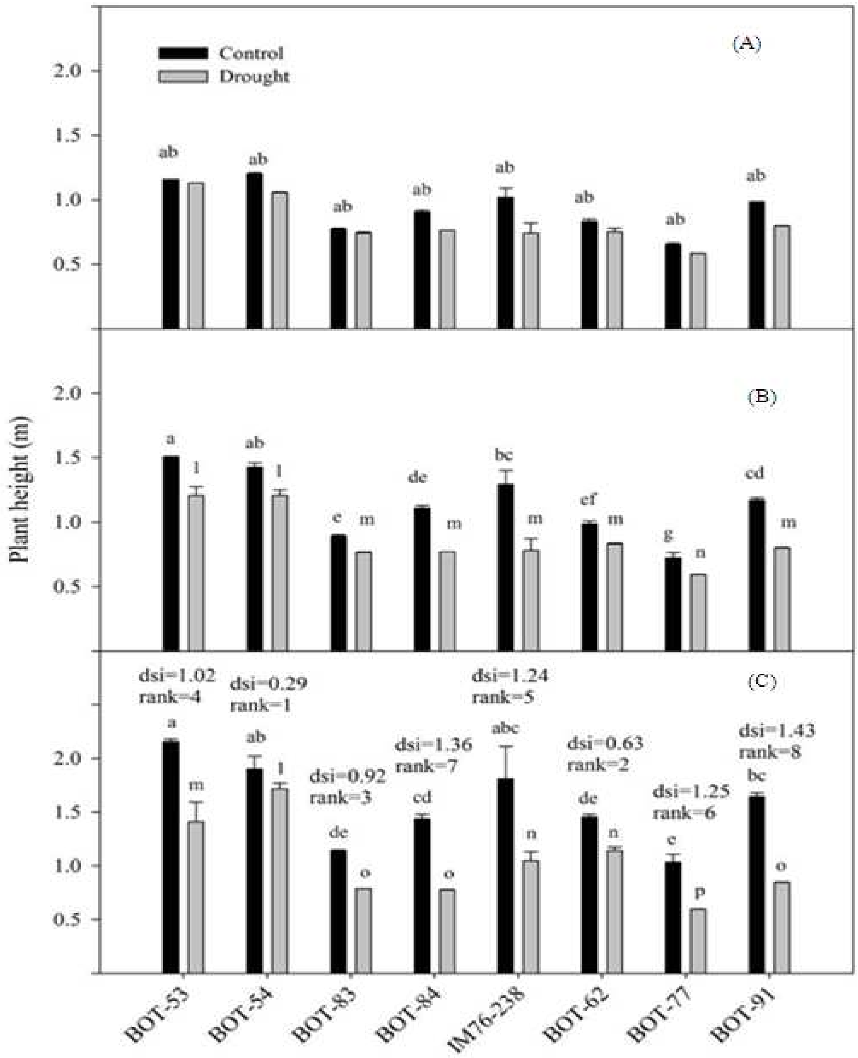

Fig. 1. Plant height of eight glagah accessions subjected to drought stress for two (A), four (B) and eight weeks (C) and the height of well-watered, control plants. Each value is the mean of two independent samples. For each panel, means associated with the same letter are not statistically different from each other according to Fisher's LSD test at P = 0.05. Data for the control and droughted plants at each assessment period were analysed separately. The drought susceptibility index was calculated according to Fischer and Maurer (1978)

The DSI calculated from the plant height data from Week 8 suggests that the accessions can be split into three groups based on this criterion with BOT-54 and BOT-62 in the most drought tolerant group, BOT-83 and BOT-53 in an intermediate group with the remainder being the least tolerant.

3.2. Stalk Diameter

For stalk diameter, there were significant differences due to accession (F = 34.7 and 10.3, d.f. = 7, p = <0.0001) and to treatment (F = 61.4 and 413.7, d.f. = 1, p = <0.0001) at both the fourth and eighth weeks of drought stress (**Fig. 2**). There was no significant accession*treatment interaction. However, despite these statistical differences, the differences in diameter found among the control or drought-treated plants at each assessment time were small in real terms. Nor was there a large amount of variation in the percentage reduction in stalk diameter due to the drought treatment. After eight weeks, all accessions showed a 20-26% reduction in radial growth.

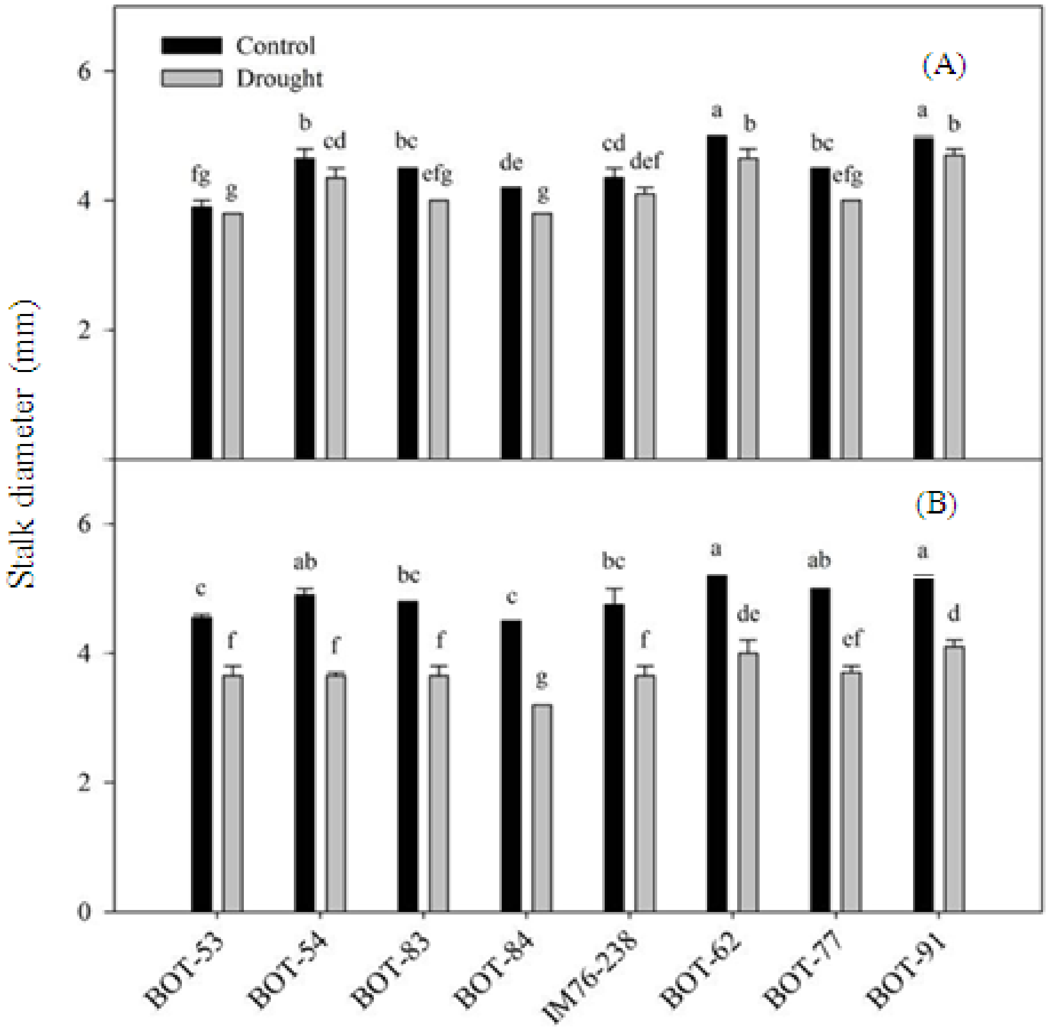

Fig. 2. Stalk diameter of eight glagah accessions subjected to drought stress for four (A) and eight weeks (B) and the diameter of well-watered, control plants. Each value is the mean of two independent samples. For each panel, means associated with the same letter are not statistically different from each other according to Fisher's LSD test at P = 0.05. Data at each assessment period were analysed separately

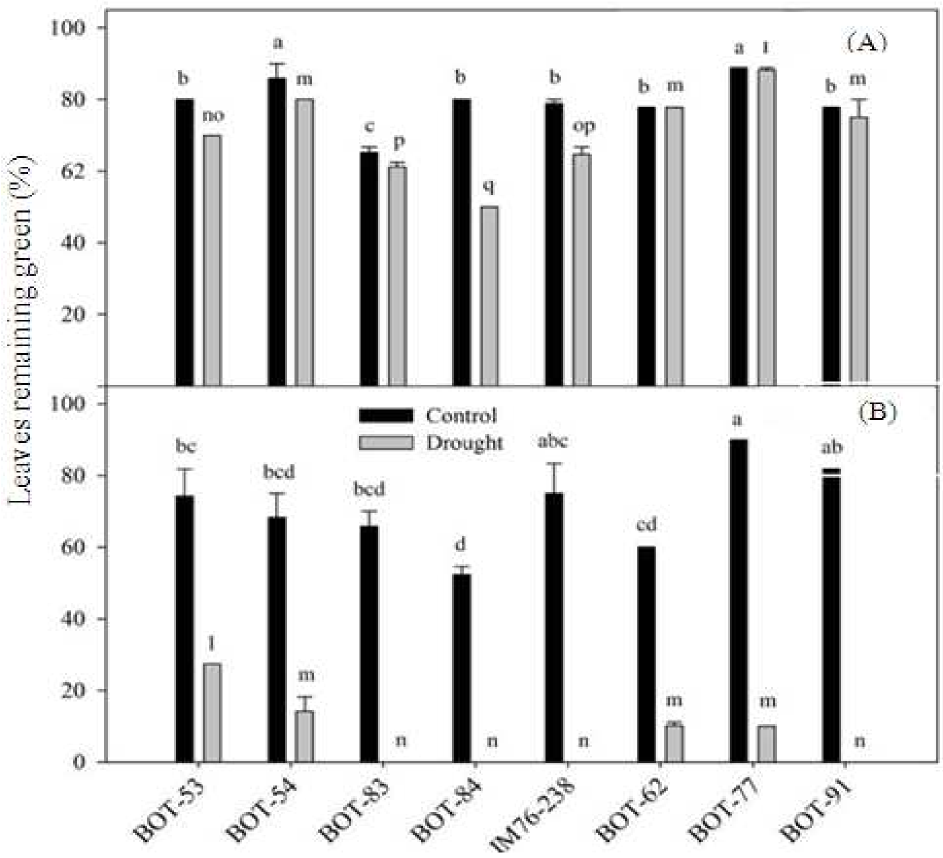

Fig. 3. Percentage of leaves remaining green of eight glagah accessions subjected to drought stress for two (A) and four (B) and the percentage for well-watered, control plants. Each value is the mean of two independent samples. For each panel, means associated with the same letter are not statistically different from each other according to Fisher's LSD test at P = 0.05. Data for the control and droughted plants at each assessment period were analysed separately

3.3. Green Leaf Number

Data concerning the percentage of leaves which remain green are shown in **Fig. 3**. After two weeks of drought, 50-90% of leaves on the droughted plants remained green and there were significant effects due to accession (F = 38.8, d.f. = 7, p<0.0001), to treatment (F = 68.4, d.f. = 1, p<0.0001) and a significant interaction (F = 10.5, d.f. = 7, p<0.0001). After the four weeks of drought, there was a large reduction in the percentage of leaves that remained green and again the effects of accession, treatment and the accession*treatment interaction were all significant (p<0.0001 in all cases). At this assessment point, green leaves only remained on accessions BOT-53, BOT-54, BOT-62 and BOT-77. After eight weeks of drought stress, green leaves only remained on one plant of BOT-53 and BOT-77 (**Fig. 4**).

3.4. Proline Content

The results of the proline analysis are presented in **Fig. 5**. Two weeks after drought treatment, there was little difference among the accessions in terms of proline content. Also, there was little difference between drought-stressed and control plants, with all plants containing between ~7-25 µg g (FW) **Fig. 5A**. The proline content of leaves of the control, watered plants did not increase during the assessment period and did not differ significantly among the accessions. However, differences emerged after four and eight weeks imposition of drought (p<0.0001) (**Fig. 5B and C**) and the leaf proline content significantly increased in plants under drought stress. Two patterns of proline accumulation can be seen in the data from these plants.

(a)

(b)

Fig.4. Influence of drought stress on vegetative growth: (A) watered control plants, (B) potential drought tolerant accessions (BOT-53 and BOT-54) and an accession (BOT-77) with intermediate tolerance showing necrosis eight weeks after the imposition of drought stress

Table 1. Correlation coefficients and their statistical significance between leaf proline contents and plant height of eight droughted glagah accessions after two, four and eight weeks of imposed drought

	Plant height		
Proline content	Week 2	Week 4	Week 8
Week 2	r = 0.5800	r = 0.5900	r = 0.610
	P = 0.0180	P = 0.0170	P = 0.011
Week 4	r = 0.3500	r = 0.2500	r = 0.038
	P = 0.1900	P = 0.3400	P = 0.890
Week 8	r = 0.8400	r = 0.8700	r = 0.910
	p<0.0001	p<0.0001	p<0.0001

The leaf proline content of four accessions, BOT-53, BOT-54, IM76-238 and BOT-62 increased after both four and eight weeks of drought stress (**Fig. 5B and C**). The greatest increases in concentration were 339.25 µg g^{-1} FW (17-fold increase) and 353.25 µg g^{-1} FW (28-fold increase) in accessions, BOT-53 and BOT-54, respectively. In the other accessions (BOT-83, BOT-84, BOT-77 and BOT-91), proline concentrations increased significantly after four weeks of drought stress and then declined. However, at Week 8, the proline concentrations of accessions, BOT-84 and BOT-91, were still higher than in the watered control plants, whereas, the proline concentrations in accessions BOT-77 and BOT-83 were little different from their controls.

3.5. Correlation Between Proline Contents and Morphological Traits

Correlations were found between proline contents and morphological characters assessed in this study. Strong positive correlations were found between proline contents in plants after two weeks of drought and plant height after two, four and eight weeks (**Table 1**). Also, the proline contents after eight weeks showed strong positive correlations with plant heights after two, four and eight weeks. The correlation coefficients were stronger between the proline contents after eight weeks and plant height than between proline contents after two weeks and this character. The only other significant correlation was between proline content at eight weeks and green leaf number at four weeks ($r = 0.59$; $P = 0.016$). No significant correlations were found with stalk diameter.

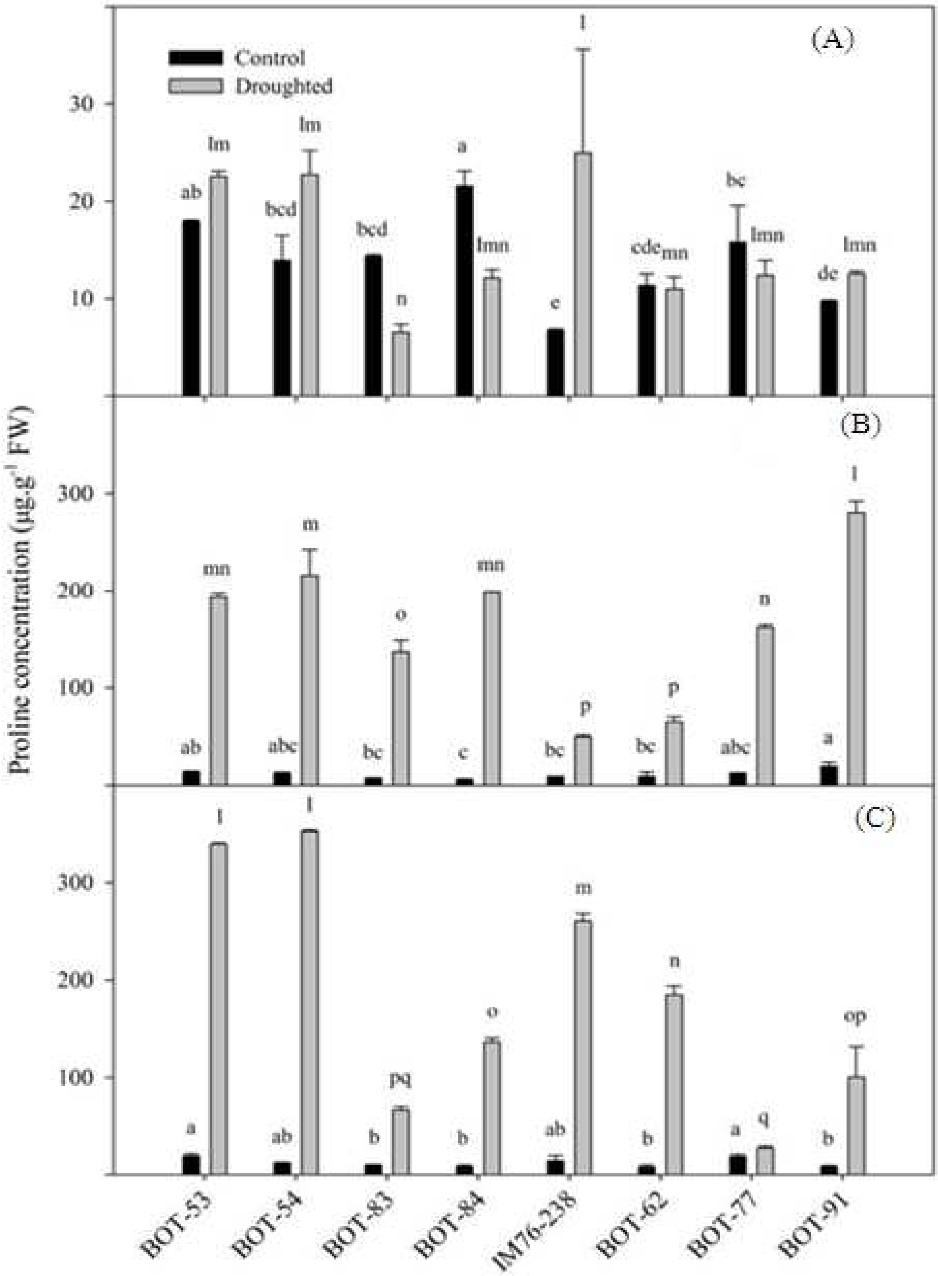

Fig. 5. The proline content of eight glagah accessions subjected to drought stress for two (A), four (B) and eight weeks (C) and the proline content of well-watered, control plants. Each value is the mean of two independent samples. For each panel, means associated with the same letter are not statistically different from each other according to Fisher's LSD test at P = 0.05. Data for the control and droughted plants at each assessment period were analysed separately

4. DISCUSSION

Drought is one of the most important environmental stress factors that can restrict the potential yield of sugarcane and tillering (beginning approximately on the fortieth day after planting and lasting up to 120 days) and the grand growth phase (taking up to 9 months after planting) are known as critical stages of droughtsensitivity due to the high need for water at these times (Zingaretti *et al*., 2012). Plants respond to drought stress at the molecular, cellular and physiological levels and this response depends on the species and genotype, the length and severity of water loss, the age and stage of development, the organ and cell type and the sub-cellular compartment (Barnabas *et al*., 2008). In this study, the first obvious symptoms of stress were observed two weeks after the drought treatment and commenced with changes being leaf discoloration. Chlorosis is a common symptom associated with drought stress and is due to decreases in leaf photosynthetic pigments, as has been shown by Chaves *et al*. (2002) in their studies on Quercus ilex and Q. suber and by Manivannan *et al*. (2008) on sunflower. Studies have shown that the majority of chlorophyll loss in plants in response to water deficit occurs in the mesophyll cells with a lesser amount being lost from the bundle sheath cells (Anjum *et al*., 2011). Low concentrations of photosynthetic pigments can directly limit photosynthetic potential and, hence, primary production. The decrease in chlorophyll content under drought stress has been considered as a typical symptom of oxidative stress and may be the result of pigment photo-oxidation and chlorophyll degradation and may be the cause of the reduction of green leaf number found in this study.

Besides green leaf number, drought stress also decreased plant height and stalk diameter in the eight accessions of glagah studied. However, stalk diameter appears to be a poor character for the assessment of changes in plant growth due to drought stress due to the low variation in this character. The reduction in plant height and stalk diameter may be due to a decline in the cell enlargement and greater leaf senescence under drought stress. Similar results were found in rice (Perez-Molphe-Balch *et al*., 1996), avocado (Chartzoulakis *et al*., 2002), sunflower cultivars (Manivannan *et al*., 2008) and apple (Liu *et al*., 2012).

The data for three morphological characteristics (plant height, stalk diameter and green leaf number) suggested that the accessions can be divided into three groups with respect to drought tolerance. Three accessions, BOT-53, BOT-54 and BOT-62, appear to be the most drought-tolerant and productive. Plants of these three accessions were the tallest at the end of the drought period and still had green leaves. Two accessions, BOT-77 and IM76-238, showed intermediate tolerance. IM76-238 grew to the same height as BOT-62, but all leaves became necrotic between four to eight weeks after the drought treatment commenced. BOT-77 still had green leaves at the end of the experiment; however, it did not grow as tall as the other accessions. The remaining three accessions (BOT83, BOT-84 and BOT-91) showed the least tolerance to drought. They had no green leaves by the end of the experiment and were the shortest of the eight accessions. The ranking of plants according to their drought susceptibility index calculated from the plant height data from Week 8 supported the grouping of the accessions with the exception of BOT-83. This accession did not grow as high as the other accessions even when watered; however, its ranking showed that its DSI was the third smallest. It is necessary to further study these accessions to confirm their tolerance to drought and to establish their recovery after rewatering.

Proline is known to be involved in a plant's response to a various environmental stresses, including drought stress and proline accumulation was first reported in wilting perennial rye grass (Lolium perenne) (Kemble and Macpherson, 1954). An increase in proline content is a common response of plants to drought stress (Mostajeran and Rahimi-Eichi, 2009) and has been found in other species such as wheat (Johari-Pireivatlou, 2010; Keyvan, 2010; Akhkha *et al*., 2011), chickpea (Mafakheri *et al*., 2010), rice (Vajrabhaya *et al*., 2001; Hien *et al*., 2003; Mostajeran and Rahimi-Eichi, 2009), cotton (Parida *et al*., 2008) and potato (Farhad *et al*., 2011) and this study has shown a similar increase in S. spontaneum.

Studies on the use of proline content as a marker of drought tolerance have produced different conclusions. Hanson *et al*. (1979), working with *Hordeum vulgare* L., suggested that proline accumulation was of no practical use in breeding, even though they found a heritable component to this trait. Ceh *et al*. (2009) found no correlation between proline content and drought-resistance in hops (*Humulus lupulus* L.) and Ilahi and Dorffling (1982) found that drought-susceptible cultivars of Zea mays had higher proline contents that drought-resistant ones. In contrast, Singh *et al*. (1972; 1973) working with barley (*Hordeum vulgare* L.), Vajrabhaya *et al*. (2001) working on rice lines, Ma *et al*. (2004) working with *Brassica* spp., Sofo *et al*. (2004) working on olive trees, Bayoumi *et al*. (2008) studying *Triticum aesitivum* L., Naser *et al*. (2010) working on the Persian walnut (*Juglans regia* L.) and Sharada and Naik (2011) working with groundnut (*Arachis hypogaea* L.) all found higher concentrations of proline in drought-tolerant types. Ma *et al*. (2004); Bayoumi *et al*. (2008) and Naser *et al*. (2010) all suggested that proline accumulation could be used as a marker of drought tolerance. Differences in the role of proline with respect to drought tolerance in sugarcane have also been found. Zhao *et al*. (2010) suggested that proline was not a sensitive water stress indicator, whereas Rao and Asokan (1978) found that drought-resistant varieties of sugarcane accumulated more proline than susceptible ones and suggested that proline accumulation could be used as an index of drought tolerance. The significant positive correlations between plant height and proline content found in this study suggest that levels of this amino acid can be used as a marker of drought tolerance in glagah. However, this needs to be further confirmed with a wider range of genotypes. It is not unlikely that proline could act as a marker of drought tolerance in glagah as a mechanism for tolerance which has been suggested by Molinari *et al*. (2007) for sugarcane. These authors reported that transgenic sugarcane containing the P5CS gene from Vigna aconitifolia (Jacq.) Maréchal driven by a stress inducible promoter accumulated significantly higher amounts of proline and biomass under drought conditions. In these plants, there was no clear association between proline accumulation and osmotic adjustment. However, the increased biomass production was associated with lower levels of lipid peroxidation resulting in less damage to the plants' photosynthetic apparatus.

5. CONCLUSION

The results of this study indicated that glagah accessions, BOT-53, BOT-54 and BOT-62, are drought tolerant and have potential for use as a crop for arid regions and for use in breeding programs to improve production of sugarcane. Drought tolerance in glagah seems to be mediated by proline and concentrations of this amino acid have potential use as a marker of drought tolerance.

6. ACKNOWLEDGEMENT

The researchers are grateful to Prof. Dr. Ir. Mirzawan, P.D.N. and Ir. Eka Sugiyarta, MSc. from the Indonesian Sugar Research Institute (ISRI/P3GI), Pasuruan, East Java, Indonesia for kindly providing the glagah accessions. Thanks are also to Prof. Sutiman B. Sumitro, SU, DSc. and Dr. Wahyu Widoretno (Dept. of Biology, Universitas Brawijaya, Malang, Indonesia) for reading this manuscript and giving suggestions to it. Special thanks to Chandra Kirana, MS, MAgrSc. PhD (School of Biological Sciences, Victoria University, Wellington, New Zealand) for her critical review. This research was financially supported by BPPS (DIKTI) and Universitas Brawijaya.

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