

## Accumulation of Glycinebetaine and Betaine Aldehyde Dehydrogenase Activity in *Eucalyptus camaldulensis* Clone T5 Under *in vitro* Salt Stress

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### ABSTRACT

A widely distributed adaptation to counteract abiotic stress in plant is an accumulation of compatible organic solutes. There are many different types of salts and almost an equally diverse set of salt-tolerant defense mechanisms. Glycinebetaine (GB) is a major organic osmolyte that accumulates in a variety of plant species in response to salt stress. This research focused on the elucidation of the GB accumulation in the *E. camaldulensis* clone T5, under NaCl salinity. The results showed that there was a high correlation between GB accumulation and NaCl concentration, for a range of 0 to 500 mM NaCl. The biosynthesis of GB in the plant involved betaine aldehyde dehydrogenase (BADH) as a key enzyme, which also increased as the concentration of NaCl increased. The BADH protein contents in the extract were analyzed by SDS-PAGE gel. The result showed BADH protein expression with molecular mass 60 kDa. BADH activity was correlated with an accumulation of GB in response to salt stress. Total chlorophyll content in multiple shoots was unchanged in the first 13 days with 200 mM NaCl.

**Key words:** glycinebetaine, betaine aldehyde dehydrogenase, eucalypt

### INTRODUCTION

Salinity is one of the major abiotic stresses affecting plant growth and productivity globally. Many eucalypts are well known as multipurpose tree species that tolerate ambient soil salinity during reclamation of salt-affected soils. One of them, *Eucalyptus camaldulensis*, has a high adaptation ability, survival percentage and growth rate under saline soil conditions. One of the responses of plants that helps them to become acclimated to unfavorable environmental conditions, such as salinity, is the accumulation

of organic compounds with low molecular weight that are known collectively as compatible solutes. In this research, *E. camaldulensis* clone T5 was used as a model plant to study the regulation of glycinebetaine (GB) accumulation against salt stress. It is thought that accumulated GB under environmental stress does not inhibit biochemical reactions and plays a role as an osmoprotectant during osmotic stress. The accumulation of GB has been found in many organisms, including higher plants, playing a part in the tolerance mechanism (Shen *et al.*, 2002).

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Plants synthesize GB *via* a two-step oxidation of chlorine: chlorine→betaine aldehyde→GB. The first step is catalyzed by choline monooxygenase (CMO, EC 1.14.15.7), while the second step is mediated by betaine aldehyde dehydrogenase (BADH, EC 1.2.1.81). The protein and mRNA levels of BADH increase in parallel with an accumulation of glycinebetaine. The aim of this study was to examine the salinity response of *E. camaldulensis* in an attempt to gain increased understanding of its salinity tolerance mechanism.

## MATERIALS AND METHODS

### Plant materials

Single node-cuttings of *E. camaldulensis* clone T5 were propagated on MS media (Duriyapong, 2004) with a 45 day-interval subculture. Single shoot-cuttings were transferred to the media containing 0, 50, 100, 150, 200, 300, 400 and 500 mM NaCl for 45 days.

### Glycinebetaine determination

GB was determined by the method described previously by Grieve and Grattan (1983) with some modifications. Dried, finely powdered, plant material (0.5 g) was shaken with 20 ml of deionized water for 24 h at 25°C. The extracts were diluted 1:1 with 2 N H<sub>2</sub>SO<sub>4</sub>. Aliquots of 0.5 ml were put in test tubes, cooled in ice water for 1 h, before a cold KI-I<sub>2</sub> reagent (200 µl) was added. The tubes were stored at 0-4°C for 16 h and then centrifuged at 10,000 rpm for 15 min at 4°C. The supernatant was aspirated. The periodite crystals were dissolved in 5 ml of 1,2-dichloroethane. After 2-2.5 h, the absorbance was measured at 365 nm.

### Determination of BADH activity

BADH activity was assayed as described by Daniell *et al.* (2001) with some modifications. To obtain crude protein extracts, plant materials were homogenized in 250 µl homogenization

buffer containing 50 mM HEPES-KOH, pH 8.0, 1 mM EDTA, 20 mM sodium metabisulfite, 10 mM sodium borate, 5 mM ascorbic acid, 5 mM dithiothreitol, and 2% (w/v) PVPP. Homogenates were then centrifuged at 10,000 rpm for 15 min at 4°C and supernatants were used for determination of BADH activity. The BADH activity was assayed by monitoring the absorbance at 340 nm with 0.05 mM betaine aldehyde chloride as substrate. The activity was calculated using the extinction coefficient of 6220 M<sup>-1</sup>cm<sup>-1</sup> for NADH.

### Determination of chlorophyll

The procedure for chlorophyll a (Chl<sub>a</sub>), chlorophyll b (Chl<sub>b</sub>) and total chlorophyll determination was based on the work of MacKinney (1941). The Chl<sub>a</sub>, Chl<sub>b</sub> and total chlorophyll concentrations in the leaf tissues were calculated according to Equations 1-3:

$$[\text{Chl}_a] = 12.7D_{663} - 2.69D_{645} \quad (1)$$

$$[\text{Chl}_b] = 22.9 D_{645} - 4.68D_{663} \quad (2)$$

$$\text{Total chlorophyll} = 8.02 D_{663} + 20.2 D_{645} \quad (3)$$

where D<sub>i</sub> is the absorbance at wavelength i.

### Gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel separation was performed according to the method of Laemmli (1970) using 9% (w/v) acrylamide gels. Gels were stained with Coomassie brilliant blue (R-250).

## RESULTS

### Glycinebetaine accumulation in *Eucalyptus camaldulensis* clone T5 under NaCl salinity

After both five and seven days, multiple shoots exposed to 50, 100, 150, 200 and 300 mM NaCl produced more GB than the control (0 mM NaCl). At 200 mM NaCl, in stressed eucalypt plants, the GB level increased 1.86 times from the 1-day-treated to 7-day-treated level. After seven days of NaCl treatment, the GB content peaked

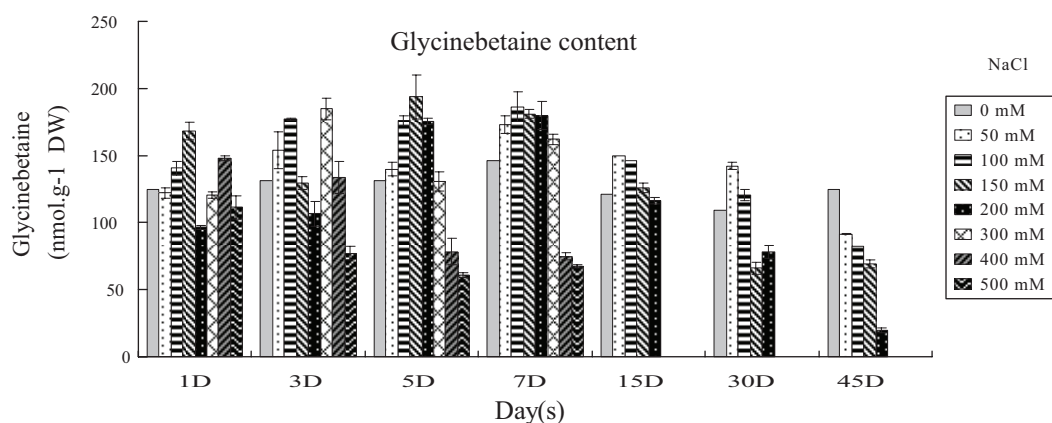
(179.87 nmol g<sup>-1</sup> DW) and then decreased to a minimum concentration of 19.58 nmol g<sup>-1</sup> DW at 45 days (Figure 1). Note that after 15 days, the GB content at NaCl concentration levels higher than 200 mM could not be detected because the multiple shoots could not withstand such conditions. The salinity condition of 200 mM NaCl was used throughout the experiment. In addition, the GB concentration at 0 mM NaCl (control) did not increase significantly within 45 days (Figure 1).

The plant material of *E. camaldulensis* is a GB accumulator, which reached 146 nmol g<sup>-1</sup>

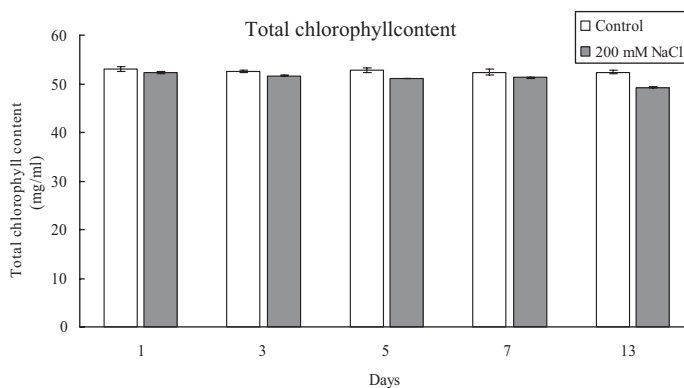
DW under control conditions. In addition, the salt exposure time was positively related to the GB content in *E. camaldulensis* plantlets.

### Effect of NaCl on total chlorophyll content

To determine the potential of the GB in a protective role on chloroplasts, the total chlorophyll content in plantlets was examined under 0 mM (control) and 200 mM NaCl (salt stress) treatments after 1, 3, 5, 7 and 13 days (Figure 2). There was no significant difference in total chlorophyll content between the control and salt-stressed plantlets (Figure 2).



**Figure 1** Effects of various concentrations of NaCl (0, 50, 100, 150, 200, 300, 400 and 500 mM) on the concentration of GB in *E. camaldulensis*, in culture media for 45 days. The data represent means  $\pm$  standard error (SE) of six replications.



**Figure 2** Effect of NaCl on total chlorophyll content of *E. camaldulensis* clone T5. The data are means  $\pm$  SE of three replications.

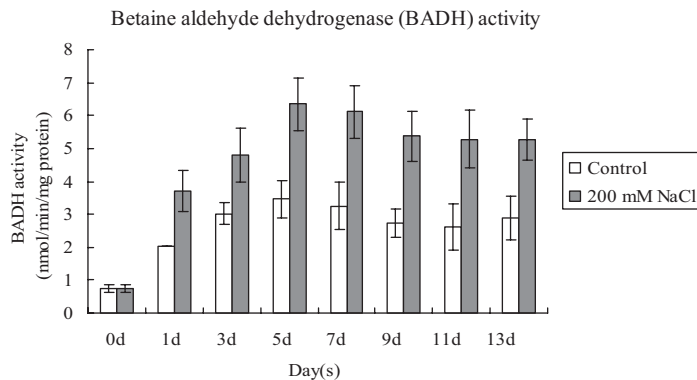
### Effect of NaCl on BADH enzyme activity in T5 eucalypt multiple shoots

The relationship between the level of BADH enzyme activity and the exposure time to 200 mM NaCl is shown in Figure 3. The maximum response was observed at the seventh day after treatment. The level of BADH enzyme activity under saline conditions was higher than in the control throughout the 13 days of treatment (Figure 3).

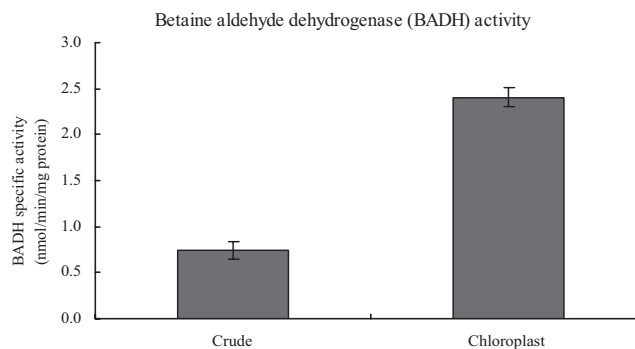
The molecular activity for oxidation of betaine aldehyde by eucalypt clone T5 BADH under saline conditions (200 mM NaCl) was higher than in the control (0 mM NaCl) (Figure 3). BADH

enzyme activity was enhanced to a maximum 2.03 times under 200 mM NaCl when compared to 0 mM NaCl (Figure 3). This result, suggested that BADH enzyme activity was induced by salinity stress in *E. camaldulensis* clone T5.

To determine the localization of BADH in the eucalypt T5 chloroplast, the BADH enzyme activity of crude extract and chloroplasts of *E. camaldulensis* clone T5 were determined as in Figure 4. Extracts from chloroplast showed an elevated level of BADH activity compared to that of crude extracts from multiple shoots. The specific activity of BADH in chloroplast was 32% higher than in the crude extract (Figure 4).



**Figure 3** The relationship between BADH enzyme activity and days of treatment. Soluble protein of multiple shoots from *E. camaldulensis* clone T5 was extracted from the control (0 mM NaCl) and salt treatment (200 mM NaCl) at the indicated times. The data represent means  $\pm$ SE of six replications.



**Figure 4** BADH activity from crude extract and chloroplasts of *E. camaldulensis* clone T5. The data represent means  $\pm$ SE of six replications.

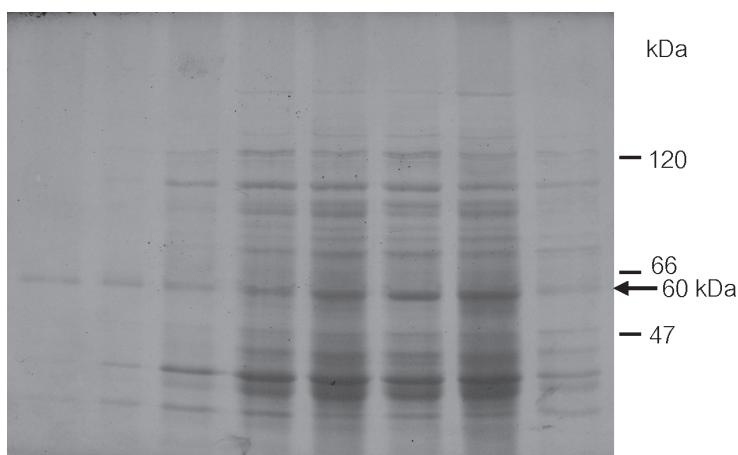
To further confirm the results of BADH activity in saline-treated multiple shoots of eucalypt clone T5, SDS-PAGE (9% gel), analysis was performed using crude extracts of multiple shoots grown under 200 mM NaCl for 0, 1, 3, 5, 7, 9, 11 and 13 days (Figure 5). After five days, stained bands of the BADH subunit increased up until 11 days after treatments and decreased at 13 days after treatments. It is suggested that the BADH activity was increased after five days of NaCl treatment corresponding to the result in Figure 3. The BADH enzyme has a subunit molecular mass of approximately 60 kDa as shown in SDS-PAGE (Figure 5).

## DISCUSSION

The salinity levels created were representative of single salt (NaCl) saline environments, though there is always a mixture of different ions under natural saline conditions (Niazi *et al.*, 2004). Eucalypts have also been examined for several aspects of salt tolerance *in*

*vitro*. Shoot cultures of salt-tolerant *E. microcorys* were able to grow in higher levels of salinity (150 mM NaCl) when compared to salt sensitive shoots (Chen *et al.*, 1998). In the present study, the results indicated that *E. camaldulensis* was a salt tolerant species during *in vitro* growth.

GB is considered a good indicator of salt tolerance. The presence of higher amounts of GB in the plant organs indicated a higher degree of salt tolerance. The GB content in the salt-stressed plantlets was normally enhanced depending on the salt concentration and exposure times (Figure 1). Even though the accumulation of GB was quite high, the osmoprotective mechanism in combination with other mechanisms, such as antiport, may produce plants with even higher levels of salt tolerance (Daniell *et al.*, 2001). The accumulation of GB may function as an alternative defense mechanism to saline environments. NaCl concentrations of 100 and 150 mM in the medium produced GB accumulation in barley leaves (Jageendorf and Takabe, 2001).



**Figure 5** SDS-PAGE analysis of the effect of salinity on the expression of BADH protein of *E. camaldulensis* clone T5. Protein extracts (30 µg) were electrophoresed in a 9% SDS-PAGE gel. The subunit of BADH was detected in 0, 1, 3, 5, 7, 9, 11 and 13 days after NaCl treatment. The arrow indicates the position of the BADH subunit protein (60 kDa). Proteins were stained with Coomassie brilliant blue (R-250). Ovalbumin (47 kDa), bovine serum albumin (66 kDa),  $\alpha$ -galactosidase (120 kDa) were used as standard proteins.

In the salt treatment of spinach, there was no difference in photosynthesis in the last fully expanded leaves compared to the control during the first 10 days of treatment (Martino *et al.*, 2003). At the same time, the total chlorophyll content was unchanged. This outcome corresponded with the results from the current study (Figure 2), where the total chlorophyll content in the salt-stressed plantlets after 13 days was unchanged compared to the control. Isolated chloroplasts of various species have also been shown to contain high GB concentrations (McNeil *et al.*, 1999). From this result, it is suggested that GB plays an important role in the protection of chloroplasts under salt stress.

In plants, the BADH enzyme is a dimeric protein with 60 kDa monomers (Weigel *et al.*, 1986). In eucalypt clone T5, the BADH protein was detected at the position relative to the 60 kDa protein mass (Figure 5) and BADH enzyme activity (Figure 4) was evidently expressed in the chloroplast. The observed increase in BADH activity (Figure 3) was accompanied by increases in the levels of BADH protein (Figure 5). At lower NaCl concentrations (75 and 150 mM), other proteins can be induced by salinity, such as P5CS activity (Claudia *et al.*, 2007), superoxide dismutase (SOD) (Yu and Rengel, 1999) and hormones (Gupta *et al.*, 1998), in agreement with Figure 5 that shows an increase in almost every band of protein under saline conditions.

## CONCLUSION

The GB concentration of *E. camaldulensis* was directly correlated with the mild (50, 100, 150 and 200 mM) NaCl concentrations. An increasing GB content was positively related to the NaCl salt exposure time. Total chlorophyll content was not affected by salt stress (200 mM NaCl) until 13 days, when compared to the control (0 mM NaCl). The GB accumulation in salt-stressed plantlets was

positively correlated with the total chlorophyll content after exposure to 200 mM NaCl for 13 days. BADH protein levels were expressed in the *E. camaldulensis* clone T5 under NaCl salinity, in parallel with an accumulation of GB in response to salt stress. The level of BADH enzyme activity also peaked after exposure to 200 mM NaCl for seven days. The soluble protein with a 60 kDa subunit may be identified as one of BADH subunits. The BADH soluble protein in salt-stressed plantlets possibly corresponded to a molecular mass of approximately 60 kDa, and was increased after exposure to 200 mM NaCl for 5-11 days.

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