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Exogenous Application of Glycine Betaine Alleviates Salt Induced Damages More Efficiently Than Ascorbic Acid In *In Vitro* Rice Shoots

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ABSTRACT

Considerable amount of research have indicated that exogenous application various compounds such as compatible solutes, plant hormones and antioxidants could enhanced salt stress tolerance in plants. However, the salt stress alleviating effect of these compounds on Malaysian rice varieties has not been investigated. Therefore, this study was undertaken with the objective to examine the salt stress mitigating effect of glycine betaine and ascorbic acid on rice shoot apices of two Malaysian rice varieties under NaCl stress. Results clearly indicated that supplementation of glycine betaine at 5 and 10 mM effectively ameliorates salt stress induced damages resulted in improved plant height, root length, biomass and total chlorophyll in both varieties tested, MR220 and MR253 as compared to salt stress condition. However, further increased of glycine betaine to 15 and 20 mM did not showed further enhancement on the overall plant growth. Supplementation of 5 mM glycine betaine increased the plant height of MR220 and MR253 to 17.0 cm and 13.3 cm as compared to 9.8 cm and 10.3 cm in salt stress media. It was noted that supplementation of of glycine betaine successfully increase the total chlorophyll content from 7.0 mg/mL FW to 13.4 and 9.1 mg/mL FW for MR220 and 8.7 and 13.8 mg/mL FW for MR253 at 5 and 10 mM respectively. Conversely, supplementation of ascorbic acid at all concentrations tested did not showed salt stress mitigating effect on the growth of rice shoot. Furthermore, ascorbic acid supplemented at 10, 15 and 20 mM was found exerted negative impact on plant growth with reduction in all growth parameters measured. In short,results from the study clearly indicated the potential of glycine betaine in mitigating salt-stress induced damages. However, more studies need to be carry out in the near future based on glasshouse and field scale trials to study the changes in the rice agronomic traits, especially the yield component should be evaluated because tolerance in stress could not represent the final yield.

INTRODUCTION

Rice (*Oryza sativa* L.) is the staple food which provides contributes around 60 to 70 per cent of daily calorie intake for more than two billion people in Asia(Alagappan and Venkistawamy, 2016).Unfortunately, enormous quantity of rice is being destroyed annually due to various environmental stresses (Varshikar *et al*., 2016).In Malaysia, most of the major rice cultivation areas are located along the coastal areas and high level of salinity in irrigation due to continuous intrusion of sea water can be considered as one of the biggest challenge for crop production in coastal areas. (Hakim *et al*., 2014;Idress *et al*., 2016).In most cases, salinity stress will lead to both ionic and osmotic stresses that affect the growth and crop yield (Horie *et al*., 2012). One of the initial effect of these stresses was the disruption of cellular homeostasis which subsequently led to a series of

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negative effects such as membrane damage, metabolic dysfunction and nutrient imbalance that interfered with the normal functioning of plants (Hasanuzzaman *et al*., 2012; Keshtiban *et al*., 2015).Therefore, maintaining the cellular homeostasis through efficient osmotic adjustment is one of the strategies adopted by plant to combat with stress condition (Giri, 2011). This can be achieved predominantly by the synthesis and accumulation of low molecular weight water-soluble compounds known as osmoprotectants which was favored under water-deficit or salinity stress to provide stress tolerance to cell without interfering cellular machinery (Bouchabke *et al*., 2008; Suleiman, 2016).

Unfortunately, the biosynthesis of osmoprotectant is at a high carbon cost process. As a consequence, this often leads to severe yield penalties even plants were able to survive under salinity stress (Shabala and Shabala, 2011). In addition, some of the crops were unable to synthesize certain osmolytes that are naturally accumulated by stress-tolerant organisms. For example, rice, mustard and tomatowere unable to produce glycine betaine naturally (Ashraf and Foolad, 2007; Wani *et al*., 2013). Plant tissue culture technique provides an easy access on the responses in plant towards salinity stress (Ghomari *et al*., 2015). In the recent past, studies have demonstrated that increased abiotic stress tolerance can be achieved in a number plants through exogenous application of various compound such as glycinebetaine, ascorbic acid, abscisic acid, salicyclic acid and mineral such as Zinc (Nounjan *et al*., 2012;Gurmani *et al*., 2011; Tabatabai *et al*., 2014;Boukraâ *et al*., 2015).

For example, it was reported that the application of glycine betaine through foliar spray method able to improve proline content, water use efficiency and photosynthetic efficiency in rice under salt stress (Cha-um and Kirdmanee, 2010). It was found glycine betaine application increased the antioxidant activities in salt-sensitive rice cultivar under salt stress (Demiral and Türkan, 2004). As for ascorbic acid, exogenous application of ascorbic acid was found to enhance antioxidant activities, proline content and growth of sugarcane under salt stress (Ejaz *et al*., 2012). Therefore, this research was undertaken to elucidate the effectiveness of exogenous glycine betaine and ascorbic acid in mitigating salt stress-induced damages in two Malaysian *indica* rice varieties, MR220 and MR253.

MATERIALS AND METHODS

Plant material and culture condition:

The preparation of explant, basal medium and culture condition were carried out as previously described in Teh *et al*. (2015). To investigate the salt stress mitigating effect of exogenous stress-protectants on the growth of rice shoot apices under salt-stress, glycine betaine, and ascorbic acid were selected. These chemicals were prepared as 1M stock solution and sterilized through filtration using 0.22 µM membrane filter (Millipore Co.). Analytical grade NaCl was used as artificial tension simulating agent (Keshtiban *et al*., 2014). Calculated amount of each solution was added into warm basal medium supplemented with 150 mM NaCl inside a laminar air-flow to generate medium contained 5, 10, 15 and 20 mM of respective stress-protectants. The basal medium contained solely 150 mM NaCl served as the positive control.

Plant Growth Assessment:

In order to determine the plant growth, several parameters including the plant height, the longest root length, fresh weight and dry weight were recorded to compare the effectiveness of exogenous glycinebetaine and ascorbic acid in recovering rice shoot apices cultured under NaCl media. The plants from each treatment were removed from culture bottles, gently washed under running tap water to remove gel residue and subsequently dried on tissue paper. Plant height, fresh weight and longest root length were determined. The samples were oven-dried at 70 °C for one week until a constant weight obtained in order to determine the dry weight. As for the biochemical changes, the total chlorophyll content, proline content and MDA content were carried out as previously described in Teh *et al*. (2015).

Statistical analysis:

The experiments were arranged in a completely randomized design with 10 replications and each replication included 3 explants per treatment. Data were analyzed with SPSS software version 21.0. Differences were determined by analysis of variance, and significant (P<0.05) differences among mean values were estimated using Duncan's new multiple range test.

RESULTS AND DISCUSSION

Effect of exogenous glycine betaine (GB) on the growth of rice shoot apex under salt stress:

Results from the study showed that GB supplemented at 5 and 10 mM resulted in salt-stress mitigating effect in both varieties with improved in plant height, fresh weight and dry weight measured (Figure 1 and 2). However, further increased in GB concentrations (15 and 20 mM) did not showed enhancement on the stress-alleviating effect. The effect of supplementation of GB on the root growth was not significant. (Figure 1B).Also, results revealed that supplementation of GB at 5 and 10 mM significantly improved the shoot fresh weight and dry weight than those in medium supplemented with 15 and 20 mM GB. Quantification of the total chlorophyll content revealed that the beneficial effect of GB was not proportional with the concentrations applied. MR220 showed a decreasing trend in total chlorophyll content with increased GB concentration (Figure 1E). Meanwhile, MR253 showed enhancement in total chlorophyll content with the supplementation of 5 and 10 mM GB with 8.74 mg/g FW and 13.81 mg/g FW respectively as compared to 7.02 mg/g FW in salt-stressed medium. As for the proline content, results indicated that the proline content was generally lower in MR220 than MR253 in all treatments (Figure 1F). Supplementation of GB from 5-20 mM had little effect on the proline content quantified in MR220. In contrast, supplementation of 5 mM GB significantly increased the proline content in MR253, but further increased in GB concentration did not give rise to further increment. Results from MDA content showed that the supplementation of GB resulted in higher MDA content in MR253. In contrast, the MDA content in salt-stressed medium for MR220 was 1.89 nmole/g FW, and supplementation of GB resulted in slight reduction of the MDA content with 1.47, 1.30, 1.59 and 1.56 nmole/g FW for 5, 10, 15 and 20 mM GB respectively.

Glycine betaine, is a quaternary ammonium compound which is widely distributed and its natural accumulation is often related with abiotic stress tolerance in various organisms including microorganisms, animals and higher plants (Giri, 2011). Salinity stress was known to reduce plant growth through disruption of many cellular processes in plants (Dehkordi *et al*., 2015).From the results, it was clear that supplementation of GB resulted in improved shoot growth under salt-stress condition, and the salt-stress alleviating effect was more effective in 5 mM GB than higher concentrations of it. Previously, Cha-um and Kirdmanee (2010) revealed that exogenous application of 50 mM GB through foliar-sprayed significantly improved the plant height, proline content, photosynthetic performance and water use efficiency of Pathumthani 1 (PT1), a salt-sensitive *indica* rice seedlings when exposed to 150 mM NaCl. However, they also found that GB applied at high dose (200 mM) caused reduction in the overall growth performance of PT1 rice. In another research, Demiral and Türkan (2006) revealed that exogenous application of 15 mM GB increased the shoot fresh weight and photosynthetic pigments in Pokkali (salt-tolerant) variety but could not prevent shoot desiccation and photosynthetic pigments reduction in IR-28 (salt-sensitive) variety when exposed to 120 mM NaCl. Furthermore, they found that GB treatment caused an increase and decrease of proline content in Pokkali and IR-28 respectively when exposed to salt stress as compared to non-GB treated group. Our results showed that MR253 variety followed a similar trend with salt-tolerant variety in which shoot biomass, chlorophyll content and proline content were enriched when supplemented with 5 and 10 mM GB. In contrast, the responses in MR220 were comparable to IR-28, the salt-sensitive variety. It was demonstrated that GB was readily taken up by leaves and roots tissues when applied exogenously in many plant species including pea, tomato, spring wheat, soybean and rice (Park *et al*., 2006; Chen and Murata, 2008).Thus, it could be suggested that the higher induced level of proline accumulation together with GB uptake has participated in cellular osmotic adjustment thus reduced the extent of salt stress-induced damages.MDA is generally considered as an indicator of the of plant cell membrane damage status (Fan and Sokorai 2005). Nonetheless, results showed that the MDA content in MR220 was unaffected with different concentrations of GB applied whereas the MDA content was increased in MR253 as compared to salt-stress medium. There are many reports on the reduction of MDA content with GB application under various abiotic stress conditions. Demiral and Türkan (2004) revealed that MDA levels of GB-applied groups in two rice varieties were lower than those in the non-GB treated groups. In maize, Chen *et al*. (2000) reported reduction of MDA content in maize cells supplied with GB when exposed to chilling stress. Instead, there are also reports that did not showed a positive correction between GB and MDA content.

From other point of view, Rasheed *et al*. (2011) revealed that heat stress exaggerated the normal cells differentiation resulted in reduced size of mesophyll cells and deformed vascular bundle in sprouting sugarcane buds. However, presoaking in GB and proline solutions significantly reversed the heat stress effects on these tissues. Similar to sugarcane bud, rice shoot apex harboring the apical meristem comprising a ground mass of actively dividing cells that can be regenerate into a whole plants. The normal development of various tissues is very important to ensure the growth of either sugarcane bud or shoot apex. Hu *et al*. (2012) reported the application of 20 mM GB successfully suppressed the Na^+ accumulation at the same time increased the K^+ in perennial ryegrass under salt stress conditions. Besides that, mineral analysis showed that GB application increased the N, K, Ca, Mg, P, S, Fe, Cu, Zn content in lettuce when exposed to 100 mM NaCl while decreasing the Na content in the leaves tissue (Yildirim *et al*., 2015). Although not quantified, it could be postulated that the GB application has certainly altered the minerals content in the rice shoot that lead to ion homeostasis and proper Na^+/K^+ has led to salt tolerance in the treated plants.

Effect of exogenous ascorbic acid on the growth of rice shoot apices under salt stress:

Exogenous of ascorbic acid resulted in reduction in all plant growth parameters measured including plant height, root length, fresh weight and dry weight (Figure 3A-D). Besides that, application of ascorbic acid also did not improved the total chlorophyll content. As we can see from the morphological appearance, ascorbic acid applied at 20 mM resulted in died shoot in both varieties (Figure 3E). The chlorophyll content quantified was low in medium supplemented with ascorbic acid as compared to those in salt-stressed medium. As for the proline content, results from the study showed that the proline content in MR253 was generally higher than MR220 in ascorbic acid supplemented medium (Figure 3F). However, the proline content quantified was significantly higher in medium supplemented with 5 and 10 mM ascorbic acid than those in 15 and 20 mM ascorbic acid. Quantification of MDA content showed that ascorbic acid applied at 5 mM caused a reduction in MDA content in MR220 as compared to salt-stressed medium. However, further increased in ascorbic acid strength resulted in higher MDA content quantified. On the other hand, supplementation of ascorbic acid resulted in higher MDA content for MR253 as compared to salt stressed medium.

Salinity increase the sodium and chloride ions concentration in plant cells and caused ioninc stress (Al Gehani and Ismail, 2016).Undue amount of ROS is counteract by both enzymatic and non-enzymatic antioxidant systems (Rahman *et al*., 2014). Among them, ascorbic acid can be detected in majority of plant tissues, with higher concentration in photosynthetic cells, meristems and some fruits (Smirnoff, 2000). Recently, Alhaswani *et al*., (2015) reported that the supplemented of ascorbic acid from 0.5-1.5 mM successfully alleviate the deleterious effect of salt-stress in two rice varieties (MRQ 74 and MR 269) when exposed to 200 mM NaCl in an *in vitro* culture system. Similarly, Wang *et al*. (2014) revealed that exogenous ascorbic acid able to alleviate the oxidative stress induced by salt-stress in two rice cultivar, and effects were more prominent in salt-tolerant (Pokkali) than salt-sensitive (Peta) cultivar. However, results from our study was not in line with the reports mentioned. Under normal circumstances, the ascorbate pool is kept reduced by the action of ascorbate-glutathione cycle, monodehydroascorbate reductases (MDHARs) and dehydroascorbate reductases (DHARs) activities (Lisko *et al*.,2014). Through whole plant autoradiography, Franceschi and Taryln (2002) demonstrated that the ^{14}C-ascorbate applied on leaves was detected in other plant tissues suggesting that plant can uptake ascorbate from the surrounding and translocate to other tissues. However, high concentration of ascorbate that exceed the normal amount may not beneficial and could perturb the redox state of plant cells and substantially leading to negative impacts on plant growth. For example, it was reported that the cell division in the quiescent center (a group of slowly dividing cells in root meristem of all angiosperm) of maize was arrested by high level of oxidised ascorbate which subsequently disrupt root hair growth (Jiang *et al*., 2003). In addition, the present of extreme amount of ascorbate also will activate the ascorbate-glutathione cycle resulting in increased of GSSH (oxidised glutathione) and altered the redox state of glutathione (Noctor *et al*., 2000; Smirnoff, 2005).

Also, it has been reported that ascorbate can possibly act as a pro-oxidant by reducing transition metals like iron, copper and manganese and subsequently lead to generation of hydroxyl radicals in the presence of hydrogen peroxide that can degrade wall polysaccharides and impaired DNA (Poulsen *et al*., 2004). In addition, it is also reported that the stability of ascorbic acid can be affected by light and pH whereby it is most stable at pH 4.5 and in the dark condition (Nisyawati and Kariyana, 2012). Since the MS medium used this experiment contained transition metals mentioned above and the pH was 5.75. Thus, it could be postulated that the combination of these factors have led to the negative impact of ascorbic acid on the growth of rice shoot in this experiment.The detrimental of exogenous ascorbic acid on plant growth also being reported in other plant species. For example, supplementation of 4 mM ascorbic acid to chickpea (*Cicer arietinum* L.) exposed to salt stress (20 and 40 mM NaCl) resulted in reduction in stem length, root length, number of leaves, stem and root fresh weight as compared to the non-supplemented groups (Beltagi, 2008). Also, Nisyawati and Kariyana, (2012) reported that the supplementation of ascorbic acid at 100-300mg/L in the regeneration medium did not improved the shoot regeneration efficiency of *in vitro* banana (*Musa acuminate*. L) as compared to the ascorbic acid free medium. Furthermore, the number of shoots produced was lower than the control medium. Based on the contradictory reports on the effect of exogenous ascorbic acid and explanations provided above, we could suggest that the effectiveness of ascorbic acid strongly dependent on a number of factors including the plant species, concentration, pH, lightning as well as the technique of apply. It can be considered as a double-edges sword that could bring either beneficial or deleterious effect to the plant when supply exogenously.

Conclusions:

The salt-stress alleviation effect of glycinebetaine was significant at 5 mM and 10 mM. However, higher concentration of glycine betaine did not further enhance the growth of rice shoot apices. Lastly, supplementation of ascorbic acid did not exert beneficial on the growth of rice shoot apices under salt-stress. Moreover, increased concentration of ascorbic acid resulted in deleterious effect on the growth of rice shoot apices.The results obtained from the study provided clear information on the potential of glycine betaine in mitigating salt-stress induced damages in rice. This could contribute to future study in the glasshouse or field conditions whereby the changes in the rice agronomic traits, especially the yield component should be evaluated with application of glycine betaine because tolerance in stress could not represent the final yield.

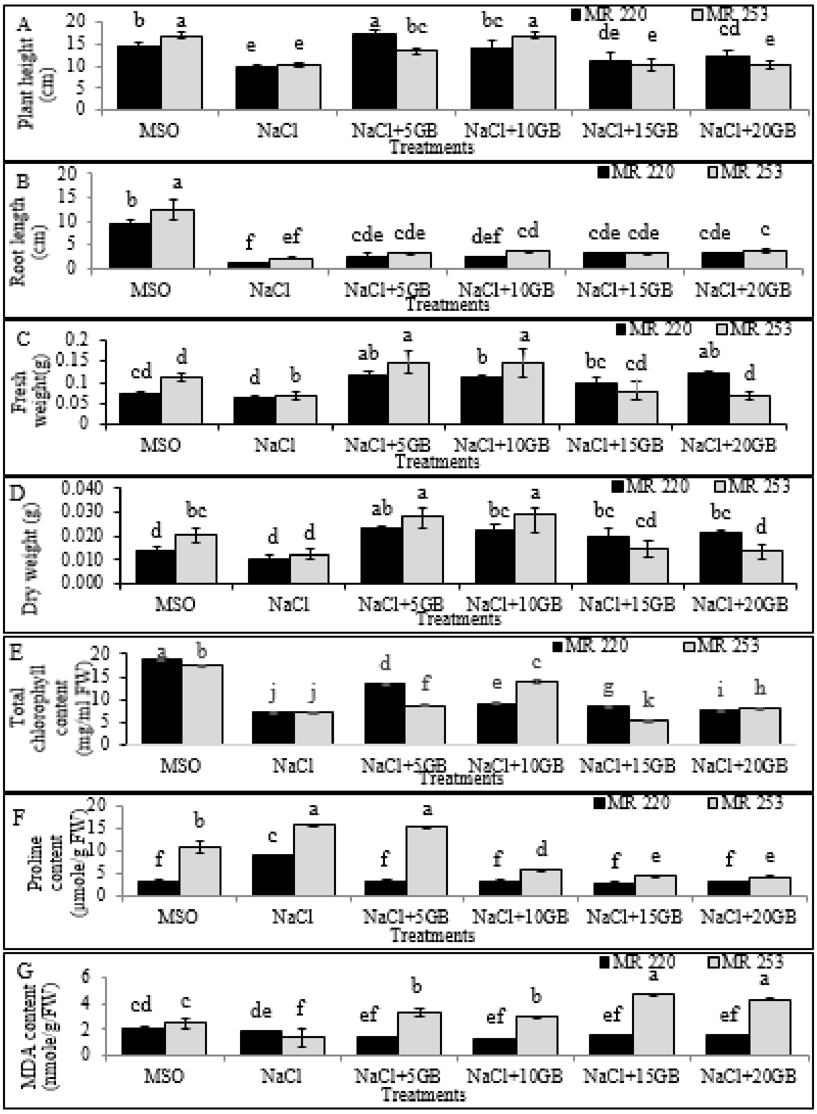

Fig. 1: Effects of different concentrations (5-20 mM) of GB in 150 mM NaCl media on (A) plant height (B) root length (C) fresh weight and (D) dry weight (E) chlorophyll content (F) proline content and (G) MDA content after 30 days of culture. Bars represent mean-SE of three replications and different letters indicate their relative significant at p<0.05 probability level

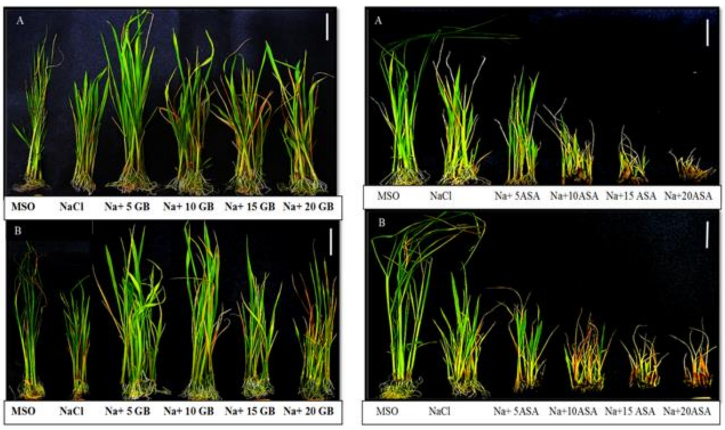

Fig. 2: Comparative effects on the (A) MR220 and (B) MR253 rice shoots performance after 30 days of culture in media supplemented with different concentration of glycine betaine (GB, mM) and ascorbic acid (ASA, mM) . Bar represent 2cm, each clump consists of 5 shoots.

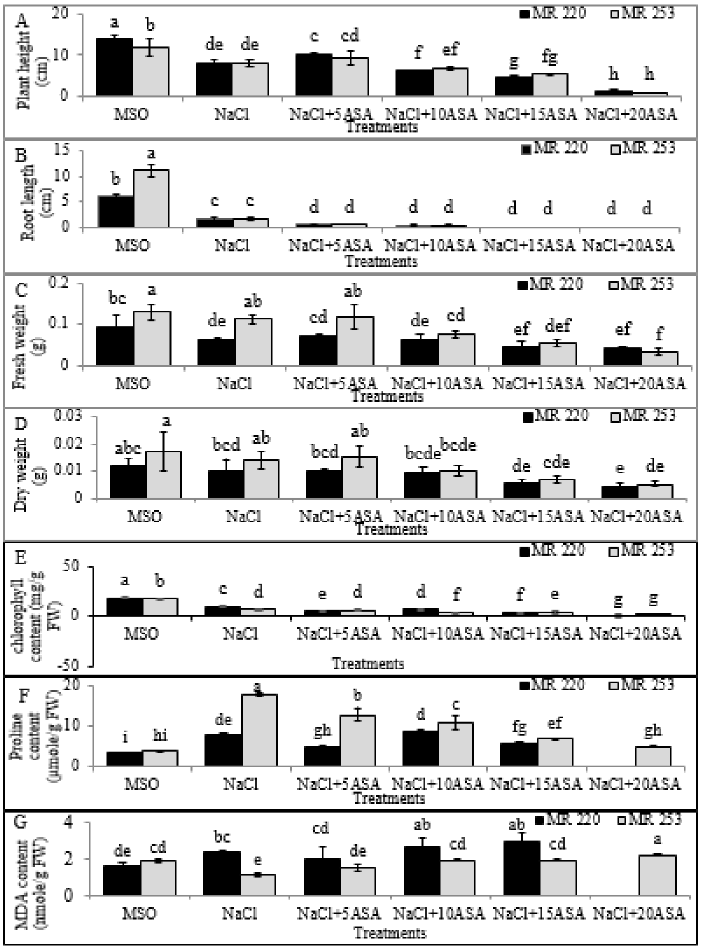

Fig. 3: Effects of different concentrations (5-20 mM) of ascorbic acid (ASA) in 150 mM NaCl media on (A) plant height (B) root length (C) fresh weight and (D) dry weight (E) chlorophyll content (F) proline content and (G) MDA content after 30 days of culture. Bars represent mean-SE of three replications and different letters indicate their relative significant at $p<0.05$ probability level.

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