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Full Length Research Paper

Influence of salicylic acid on *in vitro* propagation and salt tolerance in *Hibiscus acetosella* and *Hibiscus moscheutos* (cv 'Luna Red')

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Salicylic acid (SA) has been reported to improve *in vitro* regeneration as well as induce abiotic stress tolerance in plants. The effects of varying SA concentrations (0, 0.5, and 1 mM) on *in vitro* shoot apices of two *Hibiscus* species, *Hibiscus moscheutos* (cv 'Luna Red') and *Hibiscus acetosella*, grown under various salt (NaCl) concentrations (0, 175, and 200 mM) were assessed for shoot growth and multiplication, root formation, root elongation, plant survival rate, and proline accumulation. Application of exogenous SA, at 0.5 mM had a beneficial effect on all these parameters in both species under saline and non-saline conditions. Results obtained showed that *H. moscheutos* was more salt tolerant than *H. acetosella* and that SA could be used to improve *in vitro* regeneration and salt tolerance in these two species. Furthermore, the *in vitro* regeneration and screening system developed here could be incorporated in a breeding program for a rapid initial screening, further evaluation, and eventual development of salt tolerant *Hibiscus* plants.

Key words: *Hibiscus*, *in vitro,* salt tolerance, salicylic acid.

INTRODUCTION

Salinity is a major abiotic stress increasingly affecting plant health and survival worldwide. More land is becoming salinized through poor local irrigation practices (Winicov, 1998) and natural phenomena such as periodic coastal flooding. Therefore, the identification and selection of salt tolerant plants is of critical importance. However, developing salt tolerant plants using conventional breeding methods is labor intensive because it requires a large amount of resources and space (Vijayan et al., 2003). In addition, salt tolerance in plants could be a quantitative trait (Foolad and Jones, 1993), thus requiring a more tedious process in selecting for this trait. Therefore, other methods of selection, such as *in vitro* culture systems, need to be explored because such systems can be carried out under controlled conditions with limited space and time (Ghosal and Bajaj, 1984).

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Salicylic acid (SA) is a hormone-like substance that plays an important role in the regulation of plant growth and development (Raskin, 1992). For the last two decades, SA has received much attention because of its involvement in plant defense mechanisms under both biotic and abiotic stresses. These defense mechanisms include establishment of systemic acquired resistance (SAR) (Métraux et al., 1990), induction of pathogenesis-related (PR) proteins (Malamy et al., 1990) as well as hypersensitive response (Horváth et al., 2007). The protective effect of SA against abiotic stress factors such as toxic metals (Strobel and Kuc, 1995), heat stress (Dat et al., 1998), low temperature (Janda et al., 1999; Mora-Herrera et al., 2005), and oxidative damage (Strobel and Kuc, 1995; Kusumi et al., 2006) has been demonstrated. Furthermore, the role of SA in inducing salt tolerance has been investigated in detail in many plant species. SA has been reported to induce salinity tolerance in tomato (Stevens et al., 2006), maize (Gunes et al., 2007), carrot (Eraslan et al., 2007), and wheat (Arfan et al., 2007). It has also been used to enhance *in vitro* regeneration in

several plant species (Quiroz-Figueroa et al., 2001; Luo et al., 2001; Hao et al., 2006). However, no such studies have been undertaken in *Hibiscus* species, including *Hibiscus acetosella* and *Hibiscus moscheutos*, native to western tropical Africa (Menzel and Wilson, 1961) and North America, respectively. *H. acetosella*, also known as 'false roselle' or 'red leaf hibiscus', is grown as an annual ornamental for the attractiveness of its deep burgundy red, maple-like leaves. *H. moscheutos* has showy attractive flowers. Both species are grown as ornamenttals in the United States Gulf Coast region where frequent coastal floodings take place because of extreme weather events such as hurricanes, leading to more salinized land. The objective of this study was to determine whether application of various exogenous SA concentrations to *in vitro* grown meristem shoots could improve micropropagation and induce salt tolerance in *H. acetosella* and *H. moscheutos*.

MATERIALS AND METHODS

Plant material and *in vitro* seed germination

Seeds of the two species were scarified for 15 min using 98% sulfuric acid concentration. Following acid treatment, the seeds were rinsed promptly and thoroughly for about 5 min with running tap water to remove acid residues. After, seeds were surface sterilized under a laminar flow hood by dipping them in 100% ethanol for 3 min with gentle shaking. They were transferred into sterilized 250 ml beakers containing 40% (v/v) bleach (NaOCl) solution and one drop of Tween^{TM} 20 (Sigma, MO, USA) and shaken for 20 min at 110 rpm. The seeds were then rinsed with sterile distilled water and stored overnight in distilled sterile water on a shaker at 110 rpm. The next day, the seeds were rinsed again three times with distilled sterile water and transferred into 95 x 15 mm Petri dishes (3 seeds per dish maximum) containing MS (Murashige and Skoog, 1962) basal medium with 20 gl^{-1} sucrose, 0.75 mgl^{-1} $MgCl_2$, and 2 gl^{-1} Gelrite.

SA application

Meristematic shoots were excised from 3 - 5 days old *in vitro* grown seedlings and transferred to 125 ml flasks containing 30 ml of a liquid medium consisting of MS salts and B5 (Gamborg et al., 1968) vitamins, 20 gl^{-1} sucrose, 0.2 gl^{-1} myo-inositol, 1 gl^{-1} casein hydrolysate, 1 mgl^{-1} thiamine, 0.6 µM thidiazuron (TDZ), and 8.9 µM N^6-benzyladenine (BA). All media were autoclaved for 15 min at 121°C. Appropriate volumes from a filter-sterilized stock solution were added to the media to reach the various concentrations (0, 0.5, and 1 mM) needed for treatment. The flasks containing the liquid media were shaken for 24 h at 110 rpm and 22°C.

Salt stress treatments

After 24 h, SA-treated meristems as well as the control (non SA-treated) plants were transferred to 25 x 150 mm test tubes each containing 20 ml of a medium similar to the liquid medium described above, except that 0.75 gl^{-1} magnesium chloride and 2 gl^{-1}Gelrite were added to the latter in addition to various salt treatments, which were 0, 175, and 200 mM NaCl.

Rooting and acclimatization

The plants that did not form roots after 30 days in culture were transferred to a rooting medium made of MS basal medium supplemented with 0.1 gl^{-1} IAA, Gamborg's vitamins, 15 gl^{-1} sucrose, 0.75 gl^{-1} $MgCl_2$ as well as the adequate NaCl concentration for each treatment. Rooted plants were gently washed with tap water to remove residual gelling agent and transferred into moistened Jiffy-7 peat pellets (pellets (Jiffy Products, Ltd, Shippagan, Canada) and covered with clear plastic bags (16.5 x 14.5 cm). After three days, holes were punched in the plastic bags to allow for gradual acclimatization of the plants. Plantlets were watered using salinized (0, 175, 200 mM) water as needed. The plants were kept in a growth room at 22°C with a photoperiod of 16 h light (100 $\mu molm^{-2}s^{-1}$) and 8 h dark.

Proline determination

Proline determination was accomplished essentially as described by Bates et al. (1973). Briefly, 0.5 g of plant tissue was homogenized in 10 ml of 3% sulfosalicylic acid and centrifuged at 11000 rpm for 15 min. Then 2 ml of supernatant was mixed with acid-ninhydrin and 2 ml glacial acetic acid for 1 h at 100°C. The reaction was terminated by placing the mixture on ice. The reaction mixture was then extracted with 4 ml of toluene and mixed vigorously for 15 - 20 s and the chromophore containing toluene was aspirated from the aqueous phase, warmed to room temperature and read at 520 nm (Perkin-Elmer Lambda 3B UV/VIS spectrophotometer, Oak Brook, IL, USA) using toluene as blank. The proline concentration was extrapolated from a standard curve and calculated on fresh weight basis as follows: [(µg proline/ml x ml toluene) /115.5 µg/µmol]/ (g sample) / 5] = µmol proline/g of fresh weight material.

Acid ninhydrin was prepared by warming 1.25 g ninhydrin in 30 ml glacial acetic acid and 20 ml 6 M phosphoric acid and agitating to dissolve.

Experimental design and statistical analysis

The experiment was a completely randomized design with 20 replications (meristem explants) with a factorial arrangement of three SA concentrations (0, 0.5, and 1 mM) and three NaCl concentrations (0, 175, and 200 mM). The parameters used to evaluate *in vitro* salt tolerance of *H. moscheutos* (cv 'Luna Red') and *H. acetosella* included shoot height and number of shoots, root formation expressed as a percentage of the number of explants that developed roots, root length, proline content of regenerated plantlets, and survival rate. Shoot height was measured at 10, 20, and 30 days after transfer of shoot explants in test tubes, and the number of multiple shoots formed was counted at day 30. Root formation and root length were recorded 60 days after culture initiation. Survival rate, both *in vitro* and *ex vitro*, was assessed 90 days following culture initiation. The experiment was repeated three times. Data were subjected to analysis of variance (ANOVA) procedure and treatment means were separated using Tukey's HSD test at P = 5%. All data analyses were performed using the SAS statistical analysis software (SAS Institute, 2003).

RESULTS AND DISCUSSION

Plant growth

Growth responses of the two *Hibiscus* species, as measured by shoot height and shoot numbers, are summarized

Table 1. Effect of various concentrations of salicylic acid (SA) and sodium chloride (NaCl) on plant height (±SE) and the average number (±SE) of shoots on *in vitro* grown meristem explants from *H. moscheutos* ('Luna Red') and *H. acetosella*.

H. moscheutos		**Shoot height (cm)**			
SA (mM)	**NaCl (mM)**	**Day 10**	**Day 20**	**Day 30**	**N° shoots/explant**
0	0	3.7 ± 0.4b	8.1 ± 0.3b	11.5 ± 2.1b	2.7 ± 0.3b
0.5	0	4.7 ± 0.1a	10.1 ± 0.5a	14.1 ± 1.1a	5.7 ± 1.1a
1	0	3.3 ± 1.3b	7.8 ± 1.9b	10.6 ± 2.3b	2.5 ± 1.2b
0	175	2.6 ± 0.5b	5.5 ± 0.1b	5.9 ± 3.2c	2.1 ± 0.1 b
0.5	175	3.3 ± 0.1a	7.3 ± 1.3a	9.5 ± 1.4a	3.0 ± 0.5a
1	175	3.1 ± 0.3a	6.9 ± 2.1a	7.8 ± 2.4b	3.1 ± 1.8a
0	200	1.9 ± 0.2ab	1.9 ± 0.5b	2.3 ± 0.3b	1.0 ± 0.2a
0.5	200	2.5 ± 1.4a	3.5 ± 1.5a	5.4 ± 2.1a	1.8 ± 0.5 a
1	200	2.1 ± 1.1ab	2.9 ± 1.3a	4.2 ± 1.7a	1.0 ± 0.1 a
H. acetosella					
0	0	4.6 ± 0.2a	6.1 ± 0.2b	8.5 ± 1.1b	3.7 ± 0.3b
0.5	0	4.6 ± 0.1a	8.1 ± 0.1a	12.1 ± 1.1a	6.1 ± 0.1a
1	0	4.3 ± 1.3a	5.8 ± 1.9b	7.6 ± 2.4b	3.2 ± 0.6b
0	175	2.1 ± 0.5a	2.5 ± 1.1b	3.0 ± 1.1b	1.3 ± 1.1ab
0.5	175	2.2 ± 1.1a	3.3 ± 1.1a	4.6 ± 1.2a	2.0 ± 0.5a
1	175	1.9 ± 2.3a	3.4 ± 0.1a	4.8 ± 2.4a	1.4 ± 1.8ab
0	200	1.2 ± 0.1b	1.5 ± 1.1b	1.8 ± 0.3b	1.0 ± 0.2a
0.5	200	2.0± 1.1a	3.1 ± 1.3a	3.9 ± 1.9.a	1.1 ± 0.5 a
1	200	1.9 ± 1.1a	2.8 ± 1.3a	3.2 ± 1.8a	1.0 ± 0.1 a

Means with a common letter within the same column and belonging to the same NaCl treatment are not significantly different using the Tukey's test ((P < 0.05). Each value represents mean ± SE. Results are from 20 replicates (shoot meristem explants) and the experiment was repeated three times.

in Table 1. In *H. moscheutos*, the addition of 0.5 mM SA improved shoot growth in the control (0 mM NaCl) and produced more shoots per explant in non-saline environment (Table 1). These results are not surprising since SA is a hormone-like substance that has been reported to enhance *in vitro* regeneration in several plant species, including *Coffea arabica* (Quiroz-Figueroa et al., 2001), *Astragulus adsurgens* (Luo et al., 2001), and *Avena nuda* (Hao et al., 2006). Also, addition of 0.5 or 1 mM SA to media containing 175 mM NaCl significantly improved both shoot growth and multiplication compared to the control treatment (0 mM SA) for *H. moscheutos* (Table 1). On the other hand, inclusion of 0.5 or 1 mM SA to the 200 mM NaCl treatment significantly improved shoot growth but not shoot multiplication (Table 1) in the same species. For *H. acetosella*, inclusion of 0.5 mM SA in non saline media improved both shoot growth and shoot multiplication (Table 1). However, there was no significant difference between 0 mM SA and 1 mM SA treatments. The slightly lower values for both shoot height and number of shoots may be due to the toxic effect of SA which has been reported in some plant species at higher concentrations (Roustan et al., 1989). When *H. acetosella* explants were challenged with 175 mM NaCl, there were significant differences between SA-containing media and the control (0 mM SA) for shoot growth (Table 1). There was a reduction in both shoot height and number of shoots when explants were grown in media containing 200 mM NaCl even though the explants cultured in the SA-containing media performed better than the control (0 mM SA) for shoot height. There were no significant differences between treatments for number of shoots regenerated in this situation. The ameliorative effect of exogenous SA application on *in vitro* shoot growth under saline and non-saline environments was observed for both species. Generally, this effect was more pronounced at 0.5 mM SA. The results obtained in this study are in agreement with other findings in various crops by several authors (Zhou et al., 1999; Tari et al, 2002; Shakirova et al., 2003; Arfan et al., 2007) who reported that exogenous SA application promotes growth and counteracts the growth inhibition induced by abiotic stresses.

Root initiation and growth

In *H. moscheutos*, no differences were found among explants grown in non-saline environments for root formation and growth (Figure 1). Both the percentage of plants that formed roots and the subsequent root growth were adversely affected by the inclusion of 175 mM NaCl in culture media, but this negative effect was significantly attenuated by incorporating SA in the various treatments

(Figure 1B and D). For example, in media containing 0.5 mM SA and 175 mM NaCl, the percentage of shoots that formed roots and the average root length were respectively 3.6 and 5.3 fold higher than those in media with 175 mM NaCl without SA (Figure 1B and D). For the media containing 200 mM NaCl and 0 mM SA, only 4.9% of shoots formed roots, while the average root length was severely reduced. In non-saline environment, 80% of *in vitro* grown explants developed roots in *H. acetosella* with an average root length of about 3 cm (Figure 1A and C). However, root formation (initiation) and root growth or length were more negatively affected by NaCl in *H. acetosella* than in *H. moscheutos*, suggesting that the latter may be more salt tolerant. These adverse effects were more pronounced when salt concentration was increased to 200 mM. However, despite this high level of salinity, inclusion of SA in the media improved root formation, compared to non SA-containing treatments (Figure 1A). The positive effect of SA on root formation and growth is of particular interest since some reports on *in vitro* salt tolerance studies suggest that rooting and root growth are not only highly affected by salt but also positively correlate with salt tolerance at the whole plant level (Martinez et al., 1996; Cano et al., 1998). Furthermore, exogenous SA application through roots induced tolerance to abiotic stresses including tolerance to aluminum in *Cassia tora* (Yang et al., 2003), cadmium in rice (Guo et al., 2007), and salt in wheat (Arfan et al., 2007).

Survival rate

When *H. moscheutos* shoots were cultured in non-saline environment, the survival rate was very high, ranging from 93.5 to 96.3% in media containing 1 and 0 mM SA, respectively (Figure 2B). This survival rate decreased to about 48% in media containing 175 mM NaCl and no SA. However, inclusion of SA in media containing this salt concentration increased plant survival to over 70%. When salt concentration was increased to 200 mM, plant survival dropped to 12.3% in non SA-containing media and to 52.0 and 38.7% in 0.5 mM and 1 mM SA treatment, respectively (Figure 2B).For *H. acetosella* explants grown in non-saline condition, the survival rate (94.1 - 97.0%) was comparable to that obtained for *H. moscheutos* grown under identical conditions (Figure 2A). Under saline (175 mM NaCl) condition, survival rate for *H. acetosella* dropped sharply, in particular when shoot explants were subjected to non SA-containing treatments. However, when 0.5 or 1 mM SA was added to the growth media, a marked improvement was observed as survival rate increased to about 50% (Figure 2A). When salinity was increased to 200 mM, the survival rate decreased dramatically, as shown by the total mortality rate obtained in shoots grown in the non-SA containing treatment. A slight decrease in mortality was achieved when shoot explants were challenged with 200 mM NaCl and 0.5 mM or 1 mM SA. Based on the overall responses of the two *Hibiscus* species, it can be concluded that *H. moscheutos* is a more salt tolerant species than *H. acetosella*. Exogenous application of SA resulted in improved plant growth, root formation and growth, and survival rate for both species under both saline and non-saline environments.

Proline content

When subjected to high salt environment, plants maintain their water content by accumulation of compatible organic solutes, such as proline, in their cytoplasms (Harinasut et al., 2000). These organic solutes act as osmoprotectants in response to abiotic stresses, such as increased salinity. Proline accumulation under salt stress has been reported and suggested to be a biochemical marker for increased salt tolerance in plant species such as potato (Martinez et al., 1996), mulberry (Harinasut et al., 2000), acacia (Yokota, 2003), and sugarcane (Gandonou et al., 2006). In the current study, the free proline content of *H. moscheutos* shoot explants increased as salinity was raised to 175 mM (Figure 2D). This increase was significantly higher when SA (0.5 or 1 mM) was added to the culture media. However, when salt concentration was increased to 200 mM, there was a slight decrease in shoot proline content (Figure 2D). For *H. acetosella*, the proline content of shoots grown in non-saline environments was lower than that of *H. moscheutos* cultured under identical conditions (Figure 2C and D). In addition, as it was the case for *H. moscheutos*, the proline content of *H. acetosella* shoots subjected to saline conditions increased significantly (Figure 2A). This proline accumulation in *H. acetosella* was more pronounced in treatments containing both NaCl and SA even though the overall proline accumulation was higher in *H. moscheutos* than *H. acetosella* (less salt tolerant species). These results are in agreement with those reported by Igarashi et al. (1997), who found that the level of proline accumulation in a salt-tolerant rice species was higher than in a salt-sensitive species under high salinity conditions. However, other researchers have reported a larger accumulation of proline in salt-hypersensitive plants than in salt-tolerant ones (Liu and Zhu, 1997; Yokota, 2003). In the current study, the level of proline accumulation appeared to be related to the degree of salt tolerance as larger proline accumulation occurred in the more salt-tolerant species, *H. moscheutos*, under both non-saline and high saline conditions (Figure 2C and D). In conclusion, the results obtained showed that SA improved shoot regeneration and induced salt tolerance under *in vitro* conditions in the two *Hibiscus* species studied. The improved salt tolerance of SA-treated *H. acetosella* supports the assertion that all plants, including sensitive ones, have in their genomes genes for salt tolerance (Zhu, 2000), so the *in vitro* scheme developed here using SA-treated plants could be a valuable tool in gene expression studies comparing salt tolerant and sen-

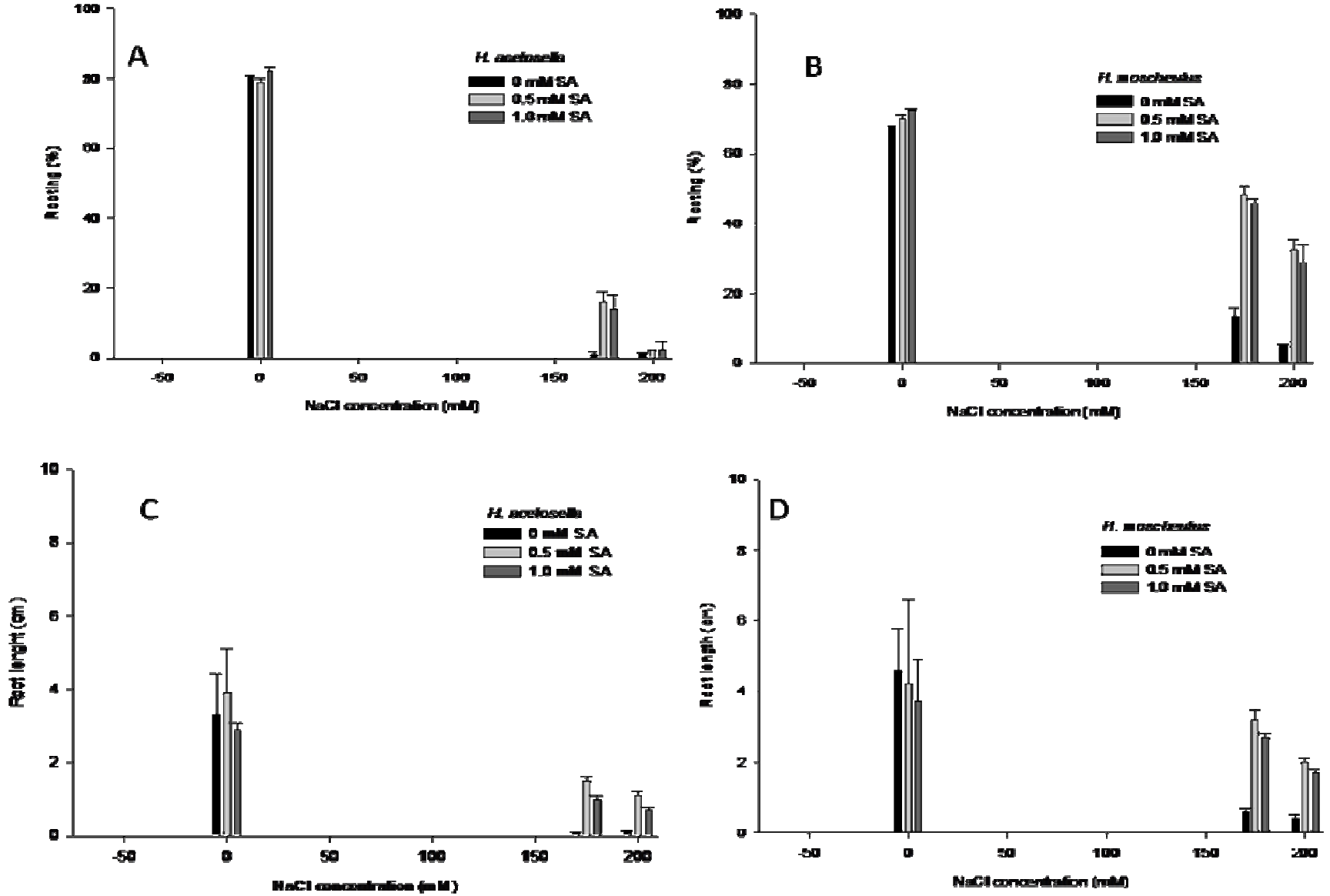

Figure 1. Effect of salicylic acid (SA) and sodium chloride (NaCl) on *in vitro* root formation (A and B) and growth (C and D) of two *Hibiscu*s species, *H. moscheutos* and *H. acetosella* (B). Each value represents mean ± SE. Results are from 20 replicates (shoot meristem explants) and the experiment was repeated three times.

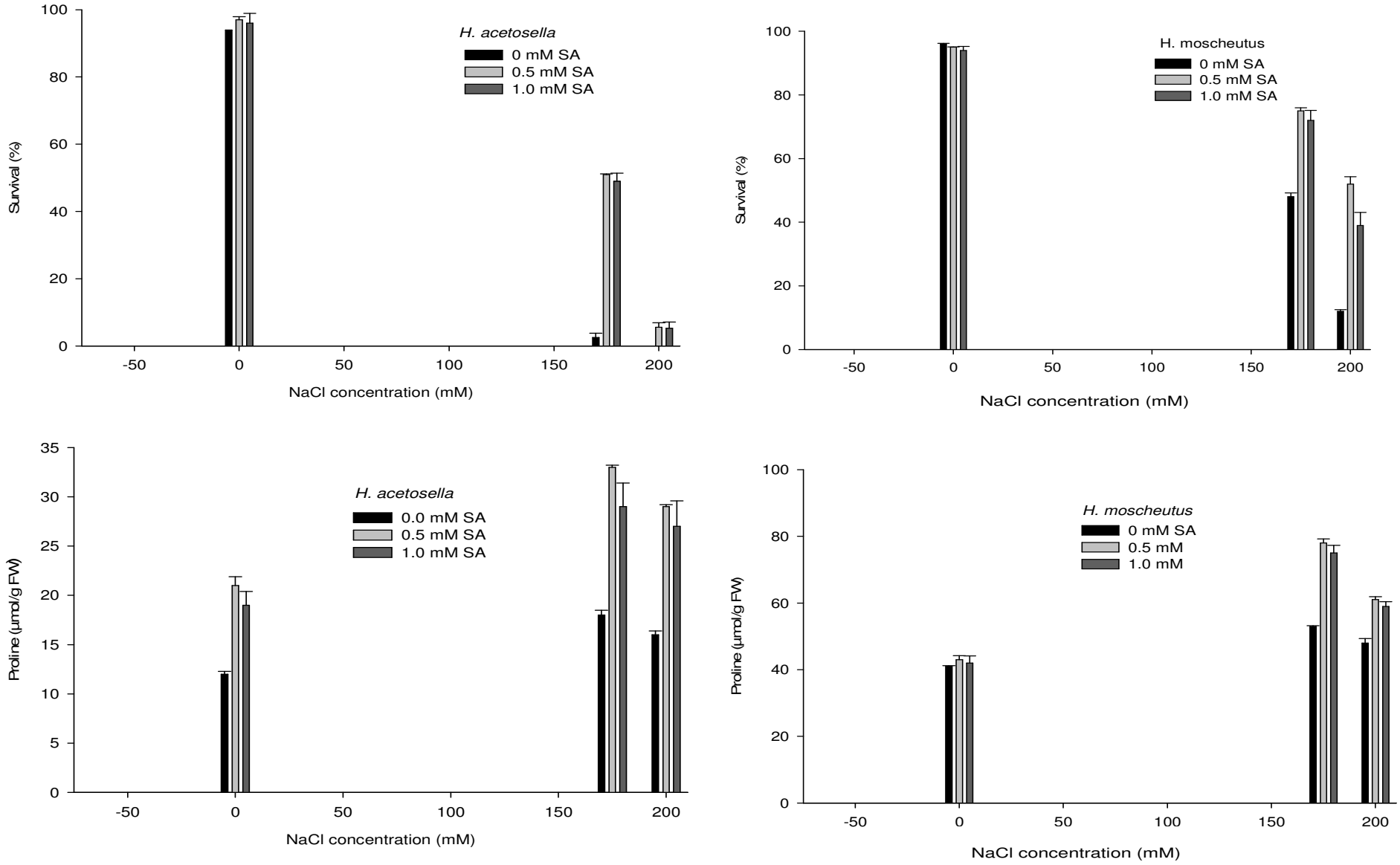

Figure 2. Effect of salicylic acid (SA) and sodium chloride (NaCl) on *in vitro* shoot survival (A and B) and proline content (C and D) of two *Hibiscus* species, *H. moscheutos* and *H. acetosella*. Each value represents mean ± SE. Results are from 20 replicates (shoot meristem explants) and the experiment was repeated three times.

sitive plants. These results also suggest that the approach described in this investigation could be incorporated in a breeding program for a rapid initial screening and development of salt tolerant *Hibiscus* plants. Identified salt tolerant plants could be asexually propagated either using the *in vitro* meristem culture described here or through cuttings for further evaluation in the greenhouse or field. Finally, the improved tissue culture protocol developed using various SA concentrations can constitute an effective mass propagation tool for both *H. acetosella* and *H. moscheutos*.

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