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Full Length Research Paper

Effect of salicylic acid (SA) and gibberellic acid (GA_3) pre-soaking on seed germination of stevia (*Stevia rebaudiana*) under salt stress

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The role of salicylic acid (SA) and gibberellic acid (GA_3) pre-soaking were examined on stevia (*Stevia rebaudiana*) seed germination under NaCl or $NaHCO_3$ stress. A set of seeds was pre-soaked in H_2O, another set of seeds in 0, 0.1, 0.5, 1.0, 5.0 and 10.0 mM SA and another set in 200 ppm GA_3 for 24 h. Seeds were then placed in Petri-dishes with 5 ml of 0, 50, 100, 200 and 300 mM NaCl or $NaHCO_3$. Under the highest salinity condition no germination was observed. Seed germination was 65% in H_2O, 0, 5 and 1 mM SA, 33% in 5 mM SA, 12, 5% in 10 mM SA, 90% in 200 ppm GA_3 44% in 50 mM NaCl, 14% in 100 mM NaCl, 40% in 50 mM $NaHCO_3$, 11% in 100 mM $NaHCO_3$. The germination velocity was 10.50 in H_2O, 6.50 in 1 mM SA, 15.00 in 200 ppm GA_3, 7.00 in 50 mM NaCl, 1.50 in 100 mM NaCl, 5.00 in 50 mM $NaHCO_3$, 1.00 in 100 mM $NaHCO_3$. Seed germination and velocity in NaCl was higher than the corresponding ones in $NaHCO_3$. By pre-soaking in 0.5, 1.0 mM SA the seed germination was as much as in H_2O, while in 5.0, 10.0 mM SA they were less than in H_2O. By pre-soaking in all SA concentrations, the 50 or 100 mM NaCl and also in $NaHCO_3$, this resulted in the reduction of germination in all combinations, when compared to their respective one in only 50 or 100 mM NaCl. In all SA concentrations the velocity was lower than the corresponding one in H_2O. Highest velocity was observed in 200 ppm GA_3.

Key words: Stevia *Stevia rebaudiana*, seed germination, NaCl, $NaHCO_3$, salicylic acid (SA), gibberellic acid (GA_3).

INTRODUCTION

Stevia (*Stevia rebaudiana*) is a perennial shrub from the *Asteraceae* family, indigenous to the higher elevations of Northern Paraguay near the Brazilian borders. It is a sweet herb gaining significance in different parts of the world. Due to the non-caloric sweeteners extracted from its leaves, mainly stevioside, this plant has gained importance as a crop for the pharmaceutical and food industries**.** The seeds of stevia show a poor percentage of viable seeds (Carneiro et al, 1997) and a very low germination percentage (Goettemoeller and Ching, 1999). The propagation through seeds is not adequate, leading to a very low seed germination percentage (Taware et al., 2010). Germination rates of stevia seeds vary greatly (Yadav et al., 2011). In the plant life cycle, the seed and seedling stages are key developmental stages conditioning the final yield of crops. Both are very sensitive to environmental stresses (Bewley and Black, 1994; Koornneef et al., 2002). Germination under saline conditions is stimulated by applying dormancy-relieving compounds, which counteract the negative change in growth regulator balance in seeds when they are exposed to salt stress (Khan and Gul, 2006). Plant species differ in their sensitivity or tolerance to salt stress (Cony and Trione, 1998).

In three wild green vegetables (mediterranean hartwort,

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(*Tordylium apulum*), sorrel, (*Rumex acetosa*) and shepherds needle (*Scandix pecten-veneris*)) a reduction of the germination percentage was observed with concentrations of NaCl (Liopa-Tsakalidi, 2010). It has been demonstrated in different plant species that pre-treatment with low concentrations of salicylic acid (SA) enhances tolerance toward most kinds of abiotic stresses due to an enhanced antioxidant capacity (Horváth et al., 2007). (SA; 2-hydroxybenzoic acid) is an endogenous signal molecule, which may play a role in plant responses to various kinds of stresses (Horváth et al., 2007). Borsani et al. (2001) demonstrated that SA plays an important role in determining the sensitivity of plants, notably at the seedling stage. Among abiotic stresses, SA has been reported to counter salinity stress (Khodary, 2004; El Tayeb, 2005). Szepesi et al. (2005) show that SA substantially improved germination vigor under salt stress conditions with tomato plants.

SA is a phenolic compound naturally occurring in plants in very low amounts and a natural signaling molecule, that could be raised to the status of the above phytohormones because it has significant impact on the various aspects of the plant life (Hayat and Ahmad, 2007; Gharib and Hegazi, 2010). SA was also shown to influence a number of physiological processes, including seed germination (Raskin, 1992). Several application methods (soaking seeds in SA prior to sowing, adding SA to the hydroponic solution, irrigating or spraying with SA solution) have been shown to protect various plant species against abiotic stress factors such as salinity (El Tayeb, 2005; Szepesi et al., 2009) by inducing a wide range of processes involved in stress tolerance mechanisms. Rajjou et al. (2006) showed that SA markedly improved germination under salt stress. However, there are some conflicting reports on the effect of SA on seed germination suggesting that this molecule can inhibit germination (for example, as in maize (*Zea mays*) (Guan and Scandalios, 1995)).

Seed pre-soaking in different concentrations of SA induced significant reduction in germination percentage (Aldesuquy, 1999). Macchia et al. (2007) also found that the germination rate of stevia varied over an extensive range (germination percentage from 9 to 83%), mainly due to the divergent specific characteristics of the material examined and the different treatments studied.

GA_3 is found to play an important part in the germination process (Ritchie and Gilroy, 1998) through a multiple regulatory mechanism. The exogenous application of GA_3 on germination and seedling growth under salt stress conditions provides an attractive approach to encounter the effects of salinity. Gibberellins have been shown to promote many facets of plant growth and development including germination, and seed development (Sun and Gubler, 2004). The aim of the present work was to study the effect of SA_3 and GA_3 on the germination of stevia to ameliorate the inhibitory effect of different levels of salinity.

MATERIALS AND METHODS

Experimental details

Seeds of stevia (*S. rebaudiana*) plants were used in this study. Germination experiments were tested using three replications of 25 seeds per each treatment. The dishes were moistened with 5 ml of distilled water (control) or with an equal quantity of the respective test solution. The following treatments were designed: (i) H_2O (control), (ii) 50, 100, 200 and 300 ppm GA_3 solution (GA_3, Sigma), (iii) 0.1, 0.5, 1.0, 5.0, 10.0 mM SA (Serva), (iv) 50, 100, 200 and 300 mM NaCl solution, (v) 50, 100, 200 and 300 mM $NaHCO_3$ solution. Seeds of stevia were pre-soaked in distilled water, in 200 ppm GA_3 or in 0.1, 0.5, 1.0, 5.0 and 10.0 mM SA (Serva), respectively, for 24 h. After soaking, seeds were placed on 9 cm sterilized Petri dishes containing double layered Wathman No.1 filter papers moistened with 5 ml of (vi) 0 (distilled water), 50, 100 200 and 300 mM NaCl and (vii) 0 (distilled water), 50, 100, 200 and 300 mM $NaHCO_3$ and were transferred to a controlled plant growth chamber under a 16 h photoperiod and 23 ± 2°C temperature regime and 75 ± 5% relative humidity (RH). Distilled water, or test solutions were added to each Petri dish during the experiment as required. The number of the germinated seeds was recorded every two days, starting from day 2 after the seeds were initially placed on Petri dishes. The experiment consisted of three replications.

Data analysis

The germination percentage is an estimate of the viability of seeds. The equation to calculate the final germination percentage (GP) is:

$$\mathrm{GP} = \frac{\text{number of germinated seeds}}{\text{number of total seeds}} \times 100$$

The germination rate was estimated by using a modified

$$\text{Timsons index of germination velocity} = \sum G/t,$$

where G, is the percentage of seeds which germinated after 2 day intervals and t is the total germination period (Khan and Ungar, 1984). The analysis of the seed germination data of stevia in different levels of NaCl and $NaHCO_3$ was performed according to the completely randomized design with three replicates. The means of the examined traits were ranked according to Duncan's multiple range tests and the Post Hoc comparison was used alternatively with Dunnett and Turkey methods.

RESULTS

NaCl, $NaHCO_3$, SA and GA_3 effects on germination

The seed germination observation of stevia lasted 14 days at a temperature of 23°C in a controlled plant growth chamber (Figure 1). The radicle emergence from the stevia seeds occurred in day 2 after the seeds were placed in Petri dishes. Data presented in Figures 1a and 2a show that salt stress induced by 50, 100, 200 and 300 mM NaCl led to a progressive gradual decrease of the percentage of germination with increasing the salt concentration as compared to the control. In this work no significant effects of 300 mM NaCl and 200 and 300 mM $NaHCO_3$ treatment were observed on stevia seed

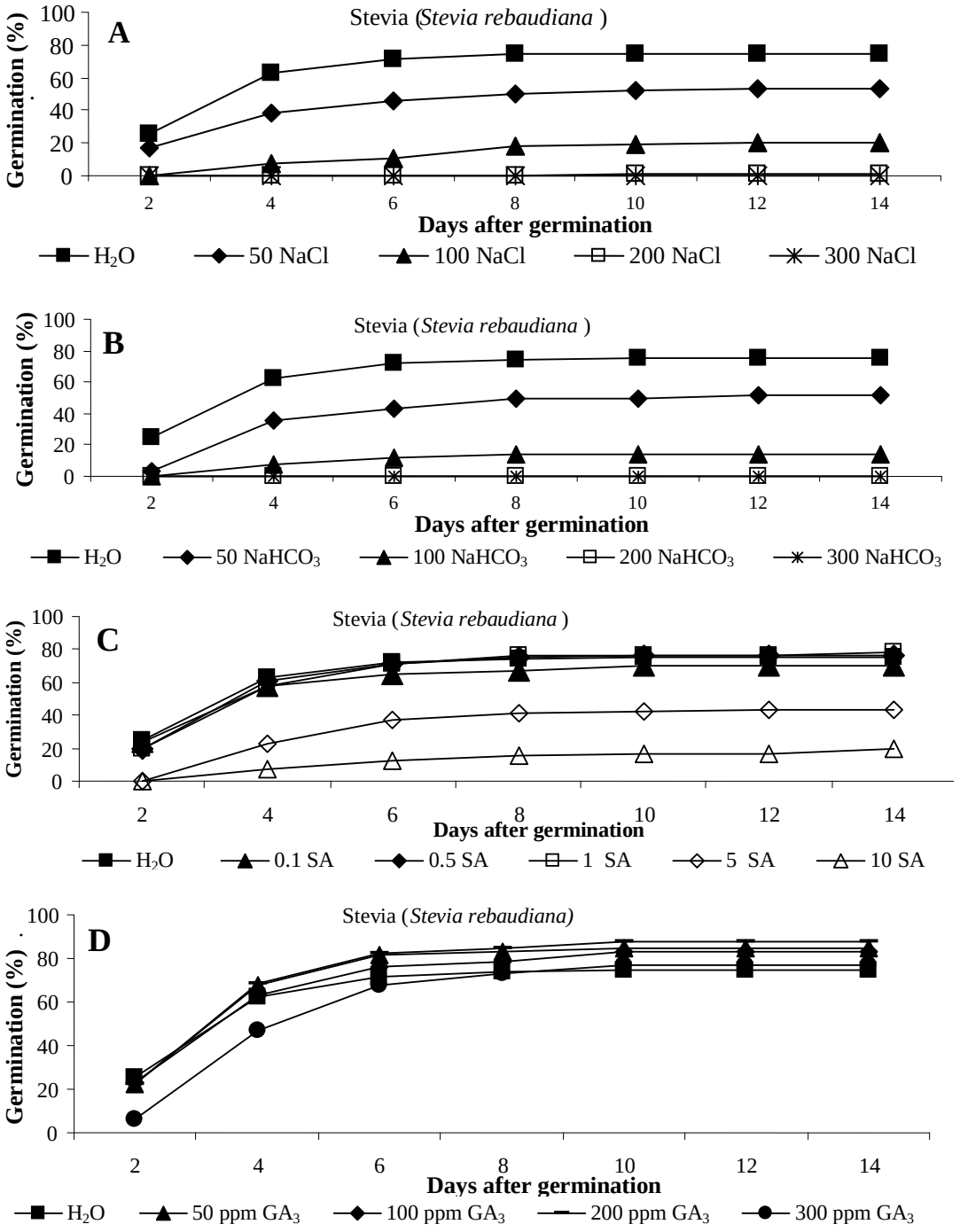

Figure 1. Seed germination time-course of stevia in various NaCl; (A), $NaHCO_3$; (B) SA; (C), and GA_3 (D), concentrations.

germination. Treatment of stevia seeds with 200 mM NaCl concentrations did not produce changes in germination. However, treatment with 50 and 100 mM NaCl reduced the seed germination, as being seen in Figures 1 (A, B) and 2 (A, B). The seed germination of stevia in the control was 65%.

In the 50 mM NaCl concentration there was a significant reduction in the germination (44%) of stevia when compared to the control, in the 100 mM NaCl concentration the germination was 14%, while in the $NaHCO_3$ concentrations the germination reduced (41% at 50 mM $NaHCO_3$, 11% at 100 mM $NaHCO_3$) (Figure 2A and B). The response of stevia seeds to the application of SA at 0.1, 0.5, 1.0, 5.0 and 10.0 mM was determined in

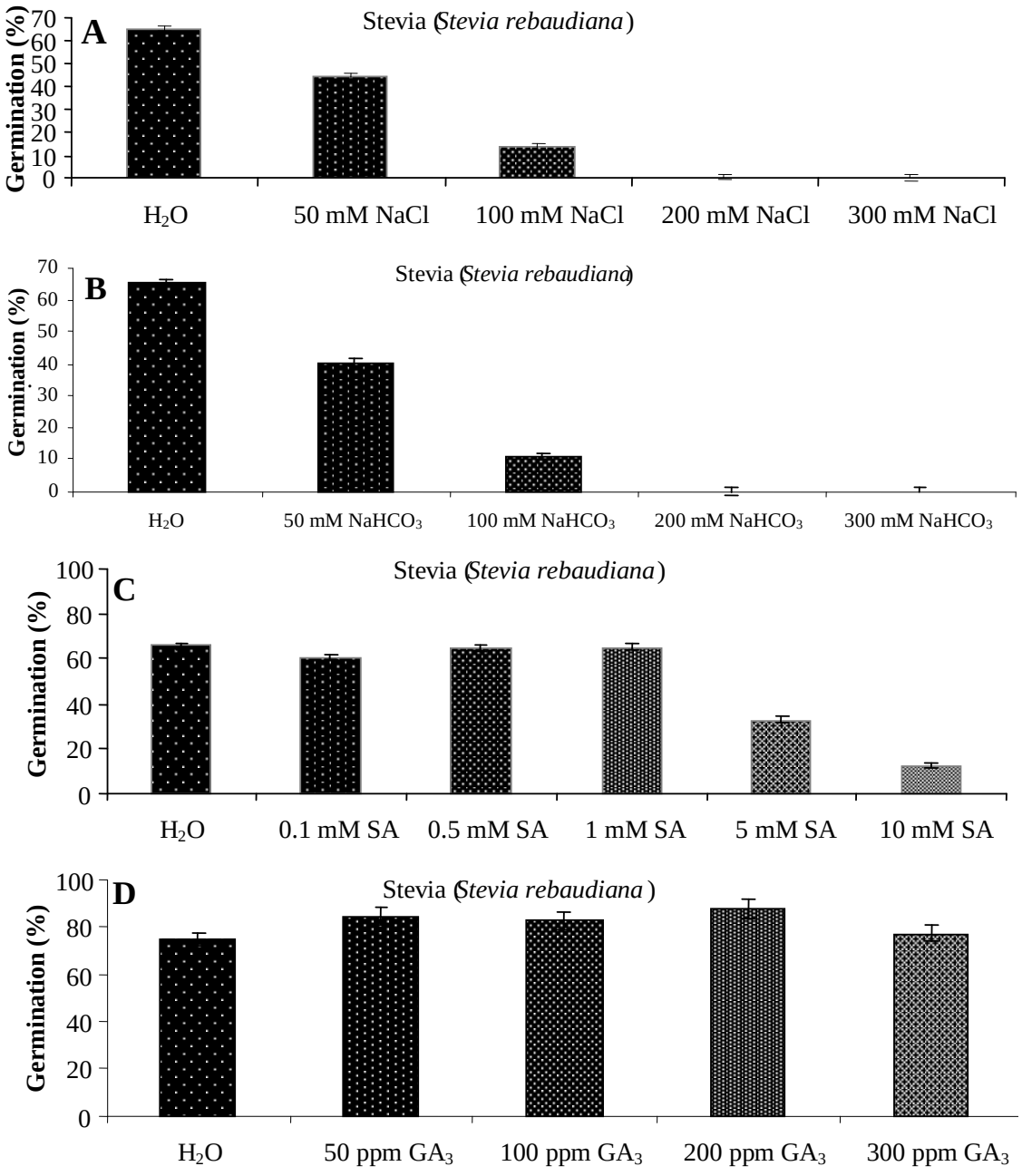

Figure 2. Seed germination of stevia in various NaCl; (A), $NaHCO_3$; (B), SA; C and GA_3 (D) concentrations.

Petri dish experiments. The stevia germination in the substrate at 0.5, 1.0 mM SA was the same as in the control, while in the substrates with higher SA concentrations (5.0 and 10.0 mM) it was significantly lower than the corresponding one in H_2O (Figures 1C and 2C). The seed germination of stevia in all GA_3 concentrations (50 to 300 ppm GA_3) was significantly higher than the corresponding seed germination one in H_2O. The maximum seed germination was observed with the 24 h GA_3 soaked period treatment compared to the other treatments (Figures 1D and 2D). In the low NaCl (50 mM NaCl) concentration, the Timson Index of germination velocity was statistically higher (7.00) and in the high NaCl (100 mM NaCl) concentration and it was lower (1.50) than the corresponding rate in the control (10.5). In all substrates with SA, the germination velocity of stevia was significantly lower than the corresponding velocity in H_2O. The highest Timson Index of the germination velocity was observed in the 200 ppm GA_3 concentrations. The germination velocity in the substrate with NaCl was higher than the corresponding one in $NaHCO_3$ (Table 1).

Table 1. Timson Index of germination velocity mean (± s.e.) of stevia (*Stevia rebaudiana*) with different NaCl, $NaHCO_3$ concentrations pre-soaked in H_2O, SA and GA_3 concentrations.

Treatment		50 mM NaCl	100 mM NaCl	50 mM $NaHCO_3$	100 mM $NaHCO_3$
H_2O	10.50±0.07	7.00±0.07	1.50±0.07	5.00±0.07	1.00±0.07
0.1 mM SA +	4.00±0.07	1.50±0.07	2.00±0.07	3.50±0.07	0.50±0.07
0.5 mM SA +	5.00±0.07	2.50±0.07	1.50±0.07	1.50±0.07	0.00±0.07
1 mM SA +	6.50±0.07	3.00±0.07	0.50±0.07	1.00±0.07	0.50±0.07
5 mM SA +	0.50±0.07	0.50±0.07	1.00±0.07	0.00±0.07	0.50±0.07
10 mM SA +	0.50±0.07	0.00±0.07	0.00±0.07	0.50±0.07	0.00±0.07
200 ppm GA_3 +	15.00±0.07	4.50±0.07	2.50±0.07	11.00±0.07	2.00±0.07

NaCl and $NaHCO_3$ effects on seed germination in SA and GA_3 pre-soaking

Pre-soaking of stevia seeds in SA for 24 h in the 50 and 100 mM NaCl substrates and also in the 50 and 100 mM $NaHCO_3$, respectively, resulted in the reduction of seed germination in all combinations, when compared to their respective one in only 50 and 100 mM NaCl after pre-soaking in water for 24 h (Figure 3). The only exceptions with higher seed germination percentages were in the 0.1 mM SA + 100 mM NaCl, 0.1 mM SA + 100 mM $NaHCO_3$ and 5 mM SA + 100 mM $NaHCO_3$ substrates (Figure 3B and D). The germination velocity in the 50 and 100 mM NaCl substrates with seeds pre-soaked in water was higher than the corresponding ones in the 50 and 100 mM $NaHCO_3$ substrates, respectively (Table 1). The NaCl or $NaHCO_3$ substrates with pre-soaking in SA resulted in the reduction of stevia germination velocity in all combinations when compared to their respective germination velocity in H_2O or only NaCl, or only $NaHCO_3$ (Table 1). The seeds soaked in 200 ppm GA_3 overcame the effect of salt stress and improved the germination percentage only in the low concentration of NaCl (50 mM NaCl + 200 ppm GA_3) (Figure 4A). In the low concentration of 50 mM $NaHCO_3$ the pre-soaked stevia seeds with 200 ppm GA_3 and 200 mM NaCl concentrations did not produce important changes in germination, compared to H_2O (Figure 4B).

DISCUSSION

Stevia has quite a low germination rate and salinity is an important factor of environmental stress which prevents seed germination. There is lack of knowledge about the reaction of stevia to salinity. This paper has shown that the germination percentage of the seeds reduced in the presence of NaCl and $NaHCO_3$ at the temperature of 23°C, as the increase in NaCl or $NaHCO_3$ concentrations gradually decreased the seed germination in stevia. Seed germination decreased significantly with the increasing salinity and stevia can therefore be considered as a salt-sensitive plant. Knowing its reaction to salinity, which is an increasingly severe agricultural problem, will contribute to the agricultural practice. The pre-soaking with SA of stevia seeds with 0.5 and 1 mM SA concentrations did not produce important changes to germination. However, treatment with 5 and 10 mM SA reduced germination. The present study indicated that the SA pre-soaking in the 50 and 100 mM NaCl or $NaHCO_3$ substrates resulted in the reduction of stevia germination. These results are in keeping with those of Guan and Scandalios (1995), showing that SA inhibits germination in maize. But the results of this study are not in agreement with the results of Shakirova et al. (2003), McCue et al. (2000) and Rajjou et al. (2006), who reported that SA markedly improved germination under salt stress.

The relations between the SA addition and stevia seed germination are obviously complex and hard to assess. The aim of this work was to determine whether the pre-soaking with SA concentrations and after imposition of NaCl or $NaHCO_3$ concentrations, improved the response of stevia to this stress. Seed germination is known to be regulated by exogenous hormones. This investigation with growth regulators will help determining which concentration of GA_3 is suitable for the seed germination of stevia. In this study, the maximum germination percentage with pre-soaking was recorded with 200 ppm GA_3, which is in agreement with those obtained by Darra and Saxena (1971), Jagadish et al. (1994), Aoyma et al. (1996), and Asrar (2011), who stated higher germination in pre-soaking with 200 ppm GA_3. Observations in the present study showed that salt stress affected seed germination of stevia. Seed germination was significantly reduced against NaCl and $NaHCO_3$ concentrations. Consequently, these results strengthened the role of NaCl and $NaHCO_3$ as stress factors, and also the susceptibility of stevia to salinity. Seed pre-soaking in SA negatively affected the response of stevia seed germination to NaCl or $NaHCO_3$ concentrations. In all concentration combinations of NaCl or $NaHCO_3$ the pre-soaking in 200 ppm GA_3 resulted in the reduction of germination. Overall, pre-soaking with GA_3 could not be an approach to improve the response of stevia

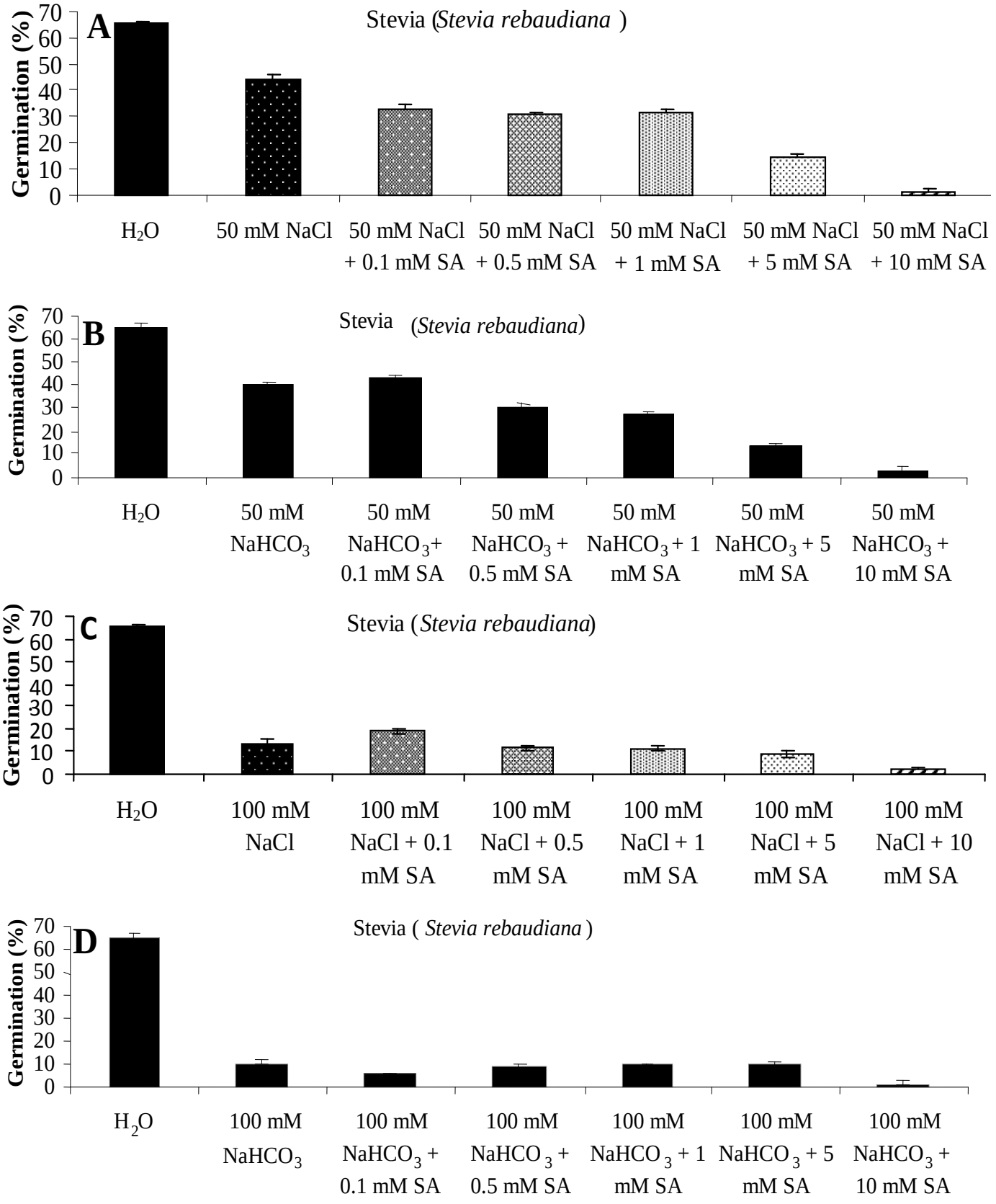

Figure 3. Effect of seed pre-soaking in SA solutions on seed germination of stevia under 50 and 100 mM NaCl; (A); (C), $NaHCO_3$; B); (D) concentrations.

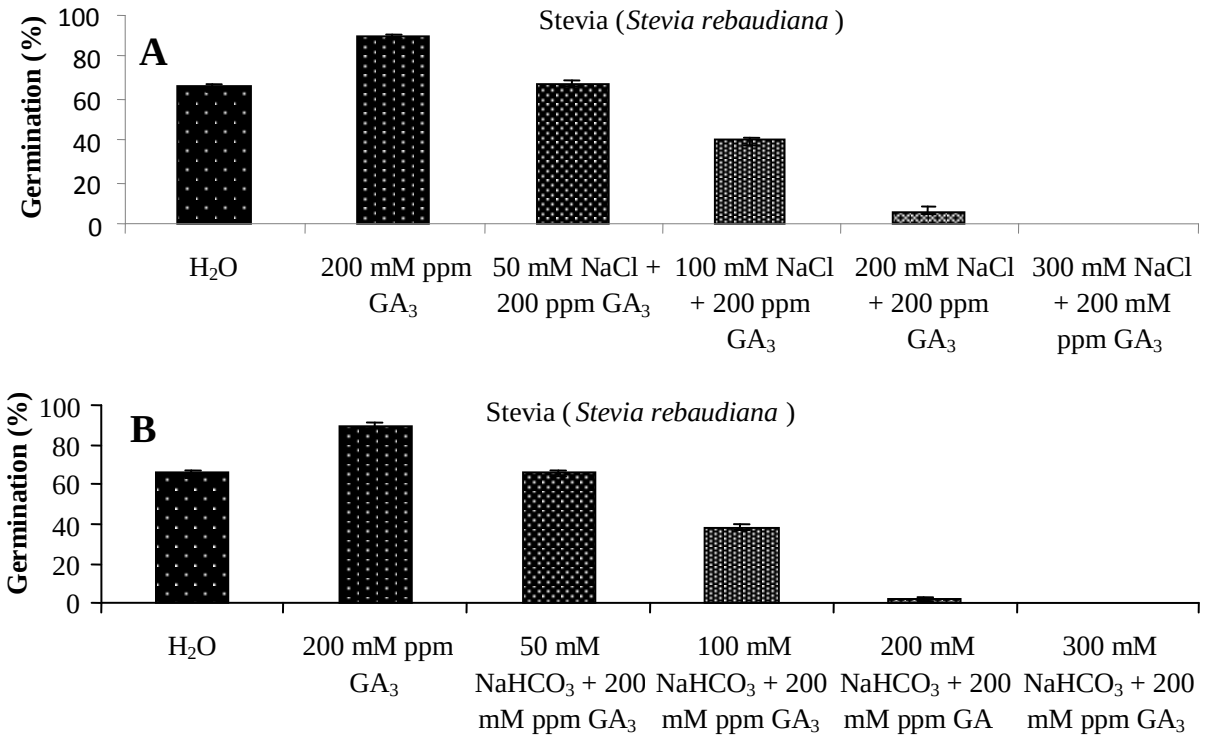

Figure 4. Effect of seed pre-soaking in 200 ppm GA_3 concentrations on seed germination of stevia under various mM NaCl; (A) and $NaHCO_3$; (B) concentrations.

germination to salinity.

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