Seed priming alleviated salt stress effects on rice seedlings by improving Na^+/K^+ and maintaining membrane integrity

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Abstract

The potential of seed priming by different chemicals on alleviation of growth inhibition of rice (*Oryza sativa* L.) seedlings under salt stress was investigated. A preliminary experiment using 10 seed-priming chemicals including NaCl, KCl, $CaCl_2$, KNO_3, ascorbic acid (AsA), mannitol, polyethylene glycol (PEG_{6000}), sorbitol, wood vinegar and distilled water revealed that mannitol, KNO_3 and wood vinegar were more effective than the others in alleviating salt-induced growth inhibition of 10-day-old seedlings. Various concentrations of mannitol (1, 2 and 3%), KNO_3 (0.25, 0.5 and 0.75%) and wood vinegar (1:1000, 1:300 and 1:100 dilutions) were subsequently used to prime rice seeds to investigate the effects on mitigation of salt-induced growth inhibition and modulation of physiological responses of 4-week-old rice plants grown in a hydroponic solution. All tested concentrations of mannitol, KNO_3 and wood vinegar resulted in seedlings with significantly higher dry weights than those grown from non-primed and hydro-primed seeds under both controlled and salt-stressed (150 mM NaCl, 7 days) conditions. Under salt stress, enhanced growth of seedlings raised from seeds primed with all three chemicals was attributable to greater membrane stability, higher chlorophyll content and lower Na^+/K^+ ratio.

Introduction

Rice is considered a salt-sensitive cereal crop and most of the attempts to develop salt-tolerant rice cultivars via conventional breeding and biotechnology have not yet been very successful because of the complexity of salt-tolerance traits.[1] This necessitates application of traditional agricultural approaches dealing with the treatments and managements of the soil, plants and plant environments to alleviate the salt stress damage to plants or to induce physiological tolerance.[2] Seed priming stimulates many of the metabolic processes involved in early phases of germination, resulting in an improved seed performance and provides faster and synchronized germination and more vigorous seedlings with higher level of abiotic stress tolerance than seedlings obtained from non-primed seeds.[3,4] Generally, seed priming has been successfully demonstrated to improve the rate and uniformity of seedling emergence in many crops, particularly vegetables, cereals and oilseed crops.[5-8] Hydro-priming in rice improved germination percentage, enhanced subsequent seedling growth resulting in higher grain yield in dry direct-seeded rice.[9] At the subcellular level, seed priming was found to improve germination and seedling vigor by conferring protection to cellular proteins,[10] repairing DNA damage during seed storage,[11] improving the functioning of protein synthesis machinery[12] and increasing energy status by improving mitochondrial integrity.[13] Seed priming of maize and chickpeas by $CaCl_2.2H_2O$, NaCl, KCl and mannitol improved germination percentage, plant survival and growth up to maturity under saline stress.[6,14] Many reports have optimized seed-priming techniques for vigor enhancement and improved salt tolerance in rice.[15-17] In addition, treatments with $CaCl_2$, followed by KCl, were the most effective in enhancing germination capacity and seedling growth of salt-tolerant and salt-sensitive fine aromatic rice cultivars.[18]

The objectives of this study were, firstly, to compare the effectiveness of 10 previously reported seed-priming agents on alleviation of salt-induced inhibition of growth in young seedlings of rice; secondly, to investigate changes in physiological parameters associated with growth alleviation under salt stress in seedlings grown from seeds primed with different concentrations of the three most effective agents selected from the first experiment.

Materials and Methods

Seeds of rice cv. KDML105 were obtained from Khon Kaen Rice Research Center, Khon Kean, Thailand. To primarily investigate the effect of different salts and chemicals as seed-priming agents on seedling growth of rice under salt stress, 10 priming agents (water, salts, osmolytes, antioxidants and wood vinegar) from the previous reports were compared.[3,15-20] Seeds were surface-sterilized by soaking in 10% sodium hypochlorite for 1 min and then rinsed three times with sterile distilled water. Seeds were then soaked in distilled water (hydropriming), 100 mM NaCl, 200 mM KCl and 200 mM $CaCl_2$ for 24 hr; 0.5% of KNO_3, 10 mg L^{-1} ascorbic acid (ASA), 2% mannitol, 250 mM sorbitol and 1:300 dilution of wood vinegar for 48 h; and 20% polyethylene glycol (PEG_{6000}) for 12 h. Wood vinegar made from eucalyptus wood was obtained from the Faculty of Agriculture at Khon Kaen University. After priming treatments, the seeds were washed thrice in distilled water, surface dried on filter paper and then allowed to air-dry for 48 h at room temperature. Primed seeds were germinated in clear plastic boxes (15×21×7 cm in W×L×H; 30 seeds per box) lined with double layers of filter paper wetted with 20 ml of distilled water (control) or 150 mM NaCl. Eight germination boxes (15×21×7 cm in W×L×H; four boxes each for control and NaCl) were set up for each priming treatment. The seeds were germinated in the dark for 3 days and the seedlings were allowed to grow for 7 days under 12/12 h light/dark conditions in the tissue-culture room. Twenty seedlings per treatment (5 seedlings from each replication) were used to determine the shoot length, fresh weight (FW) and dry weight (DW) of shoots and roots.

In the second experiment, various concentrations of mannitol (1, 2 and 3%), KNO_3 (0.25,

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Contributions: PT designed experiment and revised manuscript, NK analyzed data and prepared manuscript, BP performed experiment

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0.5 and 0.75%) and wood vinegar (1:1000, 1:300 and 1:100 dilutions) were used to prime rice seeds. Hydroprimed and non-primed seeds were also included as controls. Healthy rice seeds (cv. KDML105) were primed in mannitol, KNO_3, wood vinegar and distilled water for 48 h; washed; blotted dry on filter paper and air-dried for 48 h at room temperature. Primed and non-primed seeds were germinated for 3 days on filter papers, transferred to holes in styrofoam lined with nylon net floated on nutrient solution[21] in 15-L plastic containers and grown for 21 days, during which time the solution was refreshed every five days. The seedlings obtained were then divided into two groups; the control (fresh normal growth medium) and the salt-stress (growth medium containing 150 mM NaCl). After 7 days of salt treatment, growth (FW and DW) of plants was measured. For physiological responses, electrolyte leakage (EL), proline, chlorophyll and ion content were determined. The experiment was carried out in a completely randomized design (CRD) with three replications and maintained in a greenhouse.

Electrolyte leakage, as an indicator of membrane permeability of the leaves, was measured according to Whitlow *et al.*[22] using freshly collected leaves. Proline content was analyzed according to the procedures of Bates *et al.*[23] The amount of free proline was determined using a standard curve and expressed as µg g^{-1} FW. Chlorophyll content was determined according to the method outlined by Arnon[24] and expressed as mg g^{-1} FW. For ion measurement, shoots of plants were oven-dried for 3 days at 60°C. Approximately 0.5 g powder of each dried sample was subjected to chemical analyses by digesting with tri-acid mixture (HNO_3:$HClO_4$:H_2SO_4=5:2:1) at 200°C. The concentrations of Na^+ and K^+ were determined using atomic absorption spectroscopy (Corning, Model GBC932AAA, UK) and the concentrations were estimated from a standard curve for each ion. Analysis of variance (ANOVA) was performed on all parameters and significant differences between means were analyzed using Duncan's multiple range test (DMRT) at the P≤0.01 level.

Results

Effects of priming of rice seeds with various chemicals on seedling growth

Growth of young seedlings (length, FW and DW of shoots and roots) under salt-stressed conditions (150 mM NaCl), irrespective of priming treatments, was dramatically inhibited when compared with the control group (H_2O) (Table 1). Seedlings grown under the non-saline condition from seeds primed with all chemicals had significantly higher root FW and shoot FW than those from the hydroprimed seeds (except for the root FW of seedlings grown from seeds primed with 20% PEG_{6000}). However, for DW, significantly higher shoot DW were observed only in the case of priming with KNO_3, mannitol and wood vinegar. Furthermore, chemical seed priming showed no significant beneficial effects on root DW. Under saline conditions, priming with wood vinegar resulted in the highest percentage increase in root length (76.05%), shoot FW (27.39%) and root DW (26.31%) compared with seedlings grown from hydroprimed seeds. Priming with KNO_3, mannitol and wood vinegar were equally effective in promoting shoot growth under salt stress (69.83% increase in shoot length and 25.0-31.15% increase in shoot DW). Conversely, priming seeds with NaCl, KCl, $CaCl_2$, AsA, sorbitol and PEG_{6000} either did not promote or worsen seedling growth under saline conditions. This could be attributable to unsuitable concentrations of these chemical for this rice culivar. Therefore, KNO_3, mannitol and wood vinegar were used in the second experiment to evaluate the optimal concentrations to use as seed-priming agents.

Priming-induced physiological changes in seedlings grown from seeds primed with KNO3, mannitol and wood vinegar

In response to salt stress, root FW and shoot FW were reduced in all priming treatments, whereas root DW and shoot DW were reduced in some priming treatment when compared with non-saline conditions (Figure 1). Priming seeds with 0.25% KNO_3, 1:1000 and 1:100 dilu-

Table 1. Growth characteristics of 10-day-old seedlings grown from seeds primed with 10 chemical priming treatments, germinated and grown under control (H_2O) and salt-stressed (150 mM NaCl) conditions.

Priming treatments	Lengths (cm)		Fresh weights (g $plant^{-1}$)		Dry weights (g $plant^{-1}$)	
	Root	Shoot	Root	Shoot	Root	Shoot
Control (H_2O)						
H_2O	5.33^{cd}	6.83^{cd}	0.0075^{f}	0.0163^{e}	0.0032^{ab}	0.0036^{cde}
100 mM NaCl	5.48^{cd}	6.79^{cd}	0.0114^{c}	0.0217^{ab}	0.0032^{ab}	0.0035^{de}
200 mM KCl	5.35^{cd}	6.92^{c}	0.0124^{b}	0.0222^{a}	0.0035^{a}	0.0041^{bcd}
200 mM $CaCl_2$	4.73^{d}	5.84^{f}	0.0089^{e}	0.0177^{d}	0.0029^{b}	0.0032^{e}
0.5% KNO_3	6.9^{a}	8.62^{a}	0.0133^{a}	0.0222^{a}	0.0034^{ab}	0.0048^{a}
10mg/L AsA	3.87^{e}	6.37^{de}	0.0098^{d}	0.0196^{c}	0.0030^{ab}	0.0033^{e}
2% Mannitol	6.72^{a}	8.27^{a}	0.0135^{a}	0.0223^{a}	0.0034^{ab}	0.0046^{ab}
250mM Sorbitol	5.77^{bc}	7.38^{b}	0.011^{c}	0.0224^{a}	0.0032^{ab}	0.0043^{abc}
20% PEG_{6000}	5.31^{cd}	6.17^{ef}	0.0082^{ef}	0.0206^{bc}	0.0030^{ab}	0.0036^{cde}
1:300 Wood vinegar	6.38^{ab}	8.55^{a}	0.0131^{ab}	0.0219^{ab}	0.0034^{ab}	0.0048^{a}
Salt (150 mM NaCl)						
H_2O	3.09^{cd}	2.42^{c}	0.006^{de}	0.0073^{c}	0.0019^{cd}	0.0016^{abc}
100 mM NaCl	3.55^{c}	2.54^{bc}	0.007^{bc}	0.0079^{bc}	0.0015^{ef}	0.0016^{abc}
200 mM KCl	3.21^{c}	2.83^{bc}	0.0076^{ab}	0.0051^{e}	0.002^{c}	0.0012^{cd}
200 mM $CaCl_2$	2.45^{e}	1.64^{d}	0.0052^{ef}	0.0056^{de}	0.0013^{f}	0.0012^{cd}
0.5% KNO_3	4.88^{b}	4.11^{a}	0.0081^{a}	0.0088^{ab}	0.0021^{bc}	0.0020^{a}
10 mg/L AsA	1.57^{f}	3.01^{b}	0.0066^{cd}	0.0076^{c}	0.0019^{cd}	0.0017^{ab}
2% Mannitol	4.76^{b}	4.11^{a}	0.0084^{a}	0.0086^{ab}	0.0023^{ab}	0.0021^{a}
250 mM Sorbitol	2.67^{de}	2.88^{bc}	0.0047^{fg}	0.0064^{d}	0.0015^{ef}	0.0013^{bcd}
20% PEG_{6000}	2.24^{e}	1.78^{d}	0.004^{g}	0.0052^{e}	0.0017^{de}	0.0011^{d}
1:300 Wood vinegar	5.44^{a}	4.11^{a}	0.0082^{a}	0.0093^{a}	0.0024^{a}	0.0021^{a}

Means sharing same alphabets were not significantly different at P≤0.01.

tions of wood vinegar produced significantly greater root FW (Figure 1A) while 1:100 and 1:1000 dilutions of wood vinegar produced seedlings with greater shoot FW (Figure 1B) than the non-priming and other priming treatments. Moreover, based on seedling root DW and shoot DW, 1% mannitol and the wood vinegar solution at the dilution of 1:100 were the most effective priming agents (Figure 1C,D).

Seedlings grown under non-saline conditions showed similar levels of electrolyte leakage percentage (ELP), proline and Na^+/K^+ ratio (Figure 2A,B,D) irrespective of the seed-priming treatments. Under salt stress, the highest ELP was found in seedlings grown from non-primed seeds, which showed an increase of 173% as compared with that under non-saline condition. Meanwhile, all priming treatments alleviated the adverse effects of salt stress on membrane damage leading to lower ELP. The lowest percentage increase in ELP, 17%, was found in seedlings grown from seeds primed with 1:100 dilution of wood vinegar (Figure 2A).

Under salt stress, proline contents in rice seedlings increased dramatically and significantly differed ($P<0.01$) among different priming treatments (Figure 2B). Salt-stressed seedlings grown from non-primed and hydroprimed seeds accumulated approximately 9 folds more proline as compared to the seedlings in the control condition. Priming seeds with 1% mannitol resulted in the seedlings that accumulated the highest amount of proline (14 times more than that in the non-saline seedlings), whereas priming with 1:1000 and 1:300 dilutions of wood vinegar, 0.75% KNO_3 and 3% mannitol showed lower levels of proline accumulation (2.1-4.4 folds increase over the non-saline seedlings) (Figure 2B). Salt stress caused a reduction in chlorophyll contents in seedlings of all priming groups (Figure 2C). Priming seeds with wood vinegar (all 3 dilutions), 0.25 and 0.75% KNO_3 and 3% mannitol produced seedlings (under salt stress) that contained higher chlorophyll content than those from non-primed, hydroprimed and the remaining seed-priming treatments. Seedlings grown from different seed-priming treatments and cultivated under the non-saline conditions showed similar ratios (0.39-0.43) between the toxic Na^+ and the essential K^+ ions (Figure 2D). However, salt stress resulted in a dramatic increase in Na^+/K^+ ratios (0.89-1.17), which significantly differed ($P\leq0.01$) among different seed-priming treatments. Under salt stress, seedlings raised from seeds primed with 1:1000 dilution of wood vinegar, followed by 0.25% KNO_3, had the lowest values of Na^+/K^+ ratio.

Discussion

The present study revealed that rice seed priming promoted growth at the early seedling and vegetative stages, under both non-saline and saline conditions. During priming, meta-

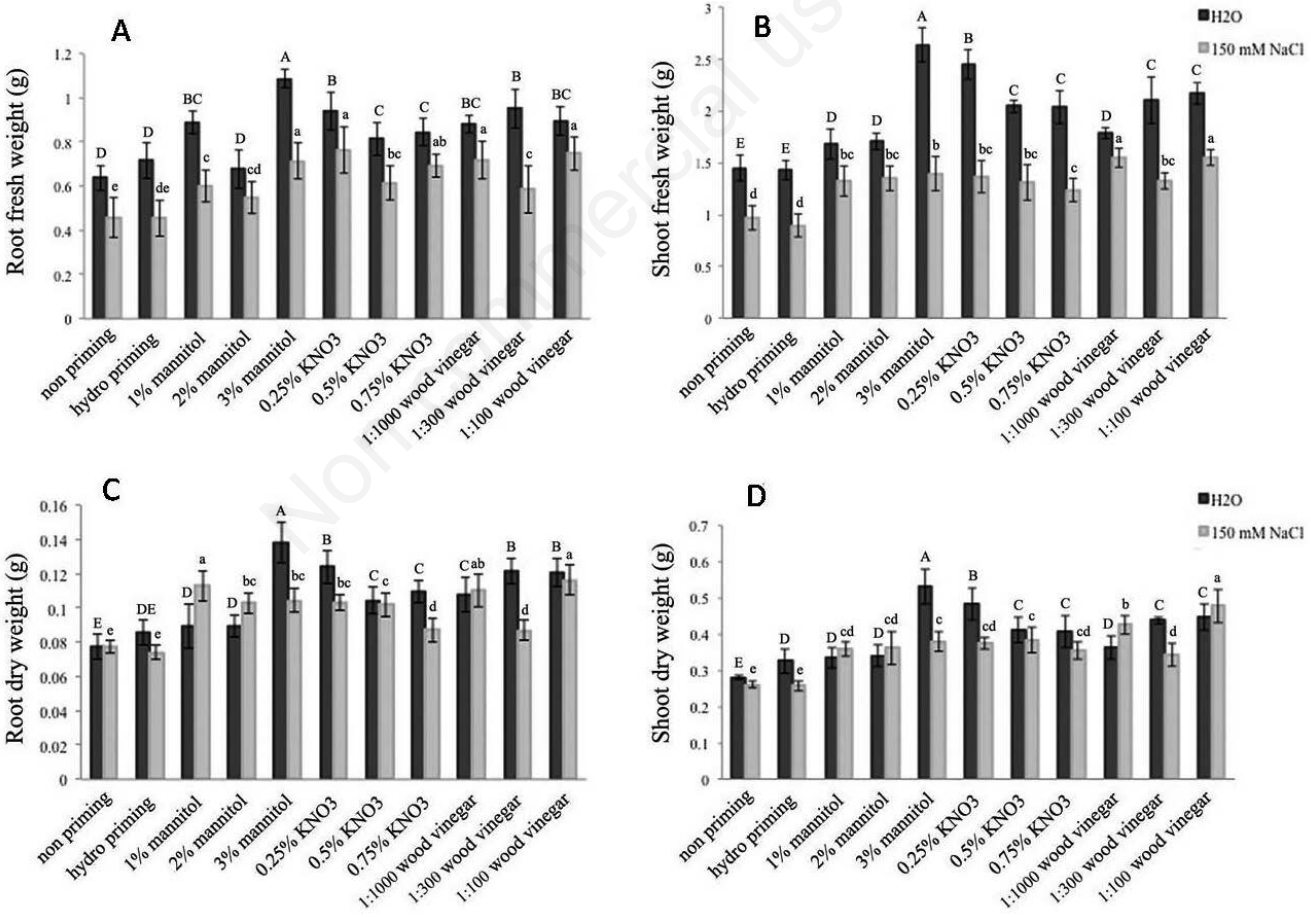

Figure 1. Root fresh weight (A), shoot fresh weight (B), root dry weight (C) and shoot dry weight (D) of 4-week-old rice plants raised from non-primed seeds and seeds primed with water, mannitol (1%, 2% and 3%), KNO3 (0.25%, 0.5% and 0.75%) and wood vinegar (1:1000, 1:300 and 1:100 dilutions) and grown under non-saline and saline condition (150 mM NaCl). Means sharing same alphabets (uppercase, non-saline condition; lowercase, saline condition) were not significantly different P≤0.01.

bolic activities proceed to repair and build up of nucleic acids, increase synthesis of proteins as well as repair membranes, and enhance the activities of anti-oxidative enzymes.[25,26] As a result, the germination capability and tolerance of unfavorable conditions of seeds can be promoted.

Among different priming treatments, KNO_3, mannitol and wood vinegar yielded the most pronounced effects of enhancing seedling growth (Table 1). The enhancement effect of KNO_3 priming was in accord with earlier reports for pepper,[27] tomato[6] under normal growth conditions and sunflower[28] under saline conditions. Beneficial effects of KNO_3 salt as a seed-priming agent include the controlled hydration of seeds, essential roles of K^+ as an osmoregulator and crucial role in metabolic processes.[29] KNO_3 is also an ideal source of two major essential plant nutrients important for metabolic processes during priming. Moreover, under salinity stress, KNO_3 prevents salinity build-up in plant tissues because K^+ counteracts the harmful effects of Na^+ toxicity.[29,30] Priming chickpea seeds with mannitol promoted seedling growth[31] and crop yield[32] under water deficit by enhancing enzymes in sucrose metabolism. Similar promotive effects were also reported in rice.[3]

Reducing the negative effects of salt stress in rice plants by seed priming with wood vinegar was also reported recently.[33] Moreover, wood vinegar has also been reported to improve the yield of KDML105 rice[34] and tomato[20] by foliar spray and soil drench, respectively. The mechanisms that priming by wood vinegar increased seedling vigor and enhanced salt tolerance of rice seedlings could be attributable to its acidity, essential element components, and the presence of butenolide from plant-derived smoke which acted as a germination stimulant.[20,33,35,36]

Priming seeds with various concentrations of mannitol, KNO_3 and wood vinegar (except 0.75% KNO_3) lowered the Na^+/K^+ ratio in rice shoots produced under salt stress, leading to growth promotion (Figures 1 and 2D). Similarly, previous reports[32,37] showed that priming with wood vinegar or smoke water in rice and KNO_3 in wheat, respectively, suppressed Na^+ uptake and stimulated K^+ accumulation in plant shoots. Over-accumulation of Na^+ leads to increased production of oxygen free radicals, lipid peroxidation and finally membrane damage, resulting in reduced photosynthetic and antioxidant activity of plant cells.[38,39] Mannitol, KNO_3 and wood vinegar were all very effective in mitigating the salt-induced increase in membrane damage (Figure 2A). Similar alleviative effects of mannitol were reported in alfalfa that was subjected to 150 mM NaCl stress.[38] The effects of KNO_3 priming on Indian mustard was reported that KNO_3 lowered lipid peroxidation and increased activities of antioxidative enzymes under both the control and saline conditions.[39] Primed seeds with mannitol, KNO_3 and wood vinegar showed varying degrees of stimulating effects on total chlorophyll content (Figure

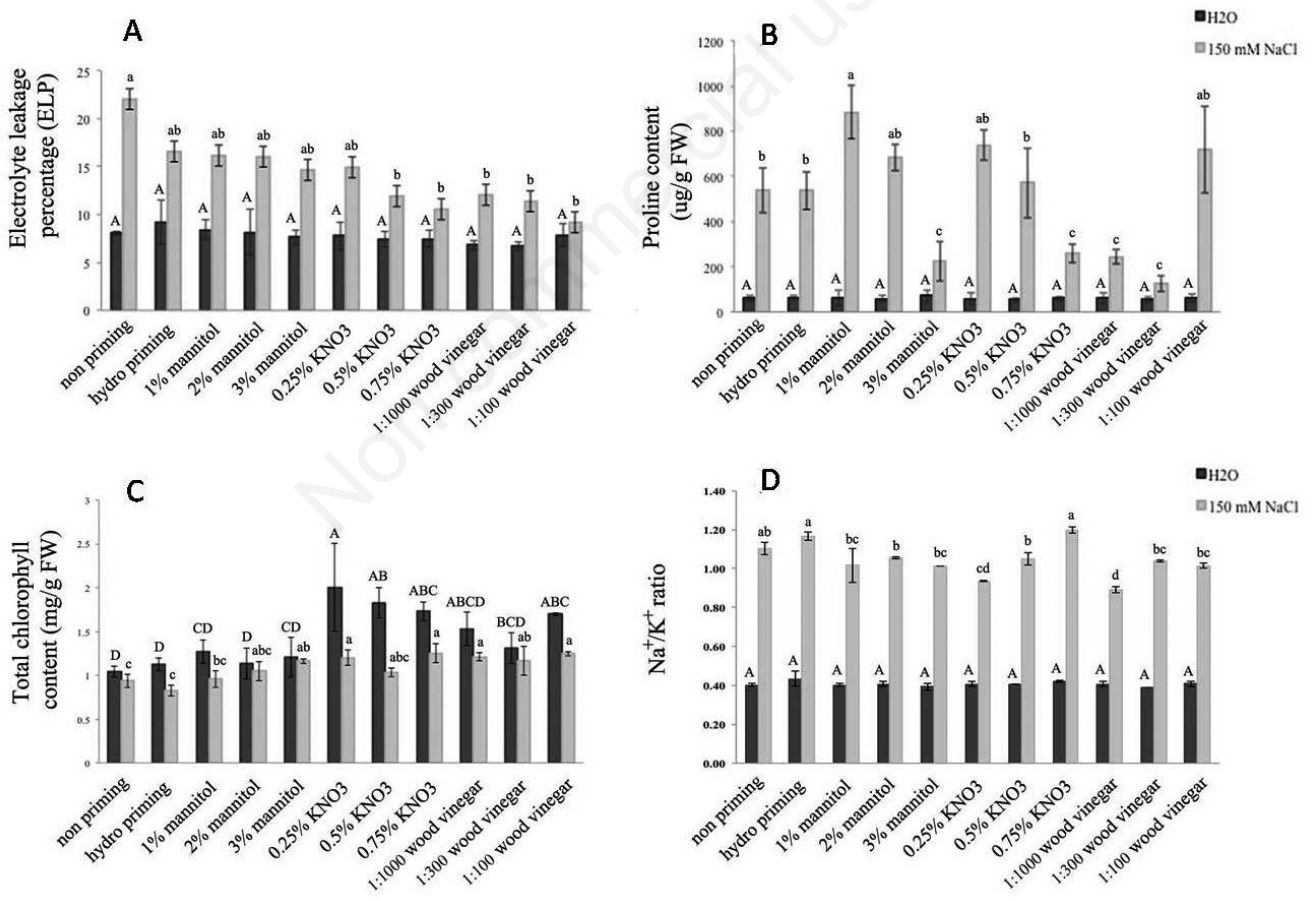

Figure 2. Electrolyte leakage percentage (A), proline content (B), chlorophyll content (C) and Na^+/K^+ ratio (D) of 4-week-old rice plants raised from nonprimed seeds and seeds primed with water, mannitol (1%, 2% and 3%), KNO_3 (0.25%, 0.5% and 0.75%) and wood vinegar (1:1000, 1:300 and 1:100 dilutions) and grown under non-saline and saline condition (150 mM NaCl). Means sharing same alphabets (uppercasee, non-saline condition; lowercase; saline condition) were not significantly different P≤0.01.

2C). Jamil *et al.*[32] have also reported that seed primed with wood vinegar increased the chlorophyll content, fresh and dry weights but reduced Na^+ content in rice seedlings.

Some investigators suggested that proline accumulation was negatively related to the degree of salt tolerance and proline was a symptom of salt stress.[40] The results of the present study showed that under saline conditions, seed priming with the highest concentration of mannitol and KNO_3, and low dilutions of wood vinegar (1:1000 and 1:300) (*i.e.*, under salt stress) reduced proline content (Figure 2B). Our results confirmed the previous findings that rice seedlings raised from seeds primed with various dilutions of smoke water (1:500, 1:1000 and 1:5000) accumulated lower amount of proline under salt-stressed conditions as compared with seedlings grown from non-primed seeds.[41] Anosheh *et al.*[42] also found the negative effects of high proline content on growth of maize grown from KNO_3-primed seeds. Therefore, these seed-priming agents, especially wood vinegar, are promising candidates for practical applications in enhancing salt tolerance of rice seedlings because of their roles in maintaining ion homeostasis, the protection of chlorophyll loss and membrane damage, and a reduction in proline content.

Conclusions

Based on growth parameters, priming rice seeds with all tested concentrations of KNO_3, mannitol and wood vinegar alleviated adverse effects of salt stress on growth compared with the non-priming and hydropriming treatments. These priming agents modulated key physiological processes, leading to an improvement of ion homeostasis, mitigation of membrane damage, protection of chlorophyll degradation, and reduction of proline accumulation.

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