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PERICARP LOOSENING AND REDUCED LIPID PEROXIDATION ENHANCES SPINACH SEED PERFORMANCE AND BIOMASS PRODUCTION UNDER NORMAL AND SALINE CONDITIONS

Khurram Ziaf [1,*], Muhammad Faheem Ahmad[1], Muhammad Amjad[1], Muhammad Muzammil Jahangir[1] and Qumer Iqbal[2]

[1]Institute of Horticultural Sciences, University of Agriculture, Faisalabad, Pakistan; [2] Nuclear Institute of Agriculture and Biology (NIAB), Faisalabad, Pakistan.
[*]Corresponding author's e-mail: khurramziaf@uaf.edu.pk

Spinach seeds show low germination percentage and speed due to pericarp imposed physical and chemical hindrance; the problem is further aggravated under saline conditions. To assess seed vigor and repress salinity induced lipid peroxidation, seeds of spinach cultivar 'Desi Palak' were subjected to various treatments *viz*., soaking in distilled water (for 4 h), washing of seeds with bleach i.e. sodium hypochlorite (for 10 min, 50% of commercial grade), seed priming (30 h) using distilled water i.e. hydropriming (with or without soaking), kinetin (25 ppm, after bleach and soaking; hereafter mentioned as kinetin only for simplicity) or Indole–3–Acetic Acid (IAA 100 ppm, after bleach and soaking; hereafter mentioned as IAA only for simplicity). The effect of seed treatments on seed vigor and salinity tolerance was evaluated through germination test (at 0, 50, 100, 150, and 200 mM NaCl induced salinity) and emergence test (at 0 and 200 mM NaCl induced salinity). Seed priming using IAA showed better final germination and emergence percentage, germination index, germination and emergence energy, seedling biomass, chlorophyll contents and vigor index than control and other seed treatments. All parameters were negatively affected by salinity but were altered to significantly lesser extent in seeds primed with distilled water or IAA, especially IAA at higher (200 mM) salinity levels. Bleach and soaking treatments seemed to soften the pericarp as evident by enhanced germination/emergence, germination energy and index, while reducing mean germination/emergence time. Further seed enhancement and increased biomass production in IAA or hydro- primed seeds (after bleach and soaking) can be attributed to reduced lipid peroxidation as evidenced by low MDA contents in seeds primed with IAA and distilled water, particularly under saline conditions.
Keywords: *Spinacia oleracea*, seed bleaching, priming, seed vigor, indole acetic acid, kinetin.

INTRODUCTION

Demand for the food and feed is increasing with the tremendous increase in population. The possible ways to satisfy future nutritional requirements are to increase the area under cultivation and yield per unit area. But, both options become less feasible due to various devastating problems, salinity is one of those. According to an estimate, global salt affected land is about 190 million hectares; 48 million hectares is in South and Southeast Asia (FAO, 2010). Salinity causes degradation of agricultural land and reduces productivity of crop plants (Viswanathan *et al.,* 2005) by decreasing water uptake due to lower osmotic potential and change in metabolic activity (Yupsanis *et al.,* 1994). Salinity reduces and delays seed germination, especially in direct seeded crops (Shannon, 1998).

Spinach (*Spinacia oleracea* var. *Inermis*), a direst seeded vegetable crop from family *Chenopodiaceae*, is a rich source of antioxidants, vitamins (A, B_2, B_6, C, E and K), and some minerals. It has been cultivated in Pakistan on 8635 hectares with production of 102.5 thousand tonnes, during 2013-14 (MNFSR, 2015). It is mostly used in fried snacks, locally known as pakora. Spinach has been classified as moderately salt tolerant vegetable crop (Langdale *et al.,* 1971; Hussain *et al*., 2016). But, germination percentage and germination rate of spinach seed was decreased while mean germination time was increased up to 100 mM NaCl concentration (Keshavarzi *et al.,* 2011). Salinity decreased uptake and endogenous level of K^+, Ca^{2+} and NO^{3-} (Turhan *et al.,* 2013), and oxygen uptake (75% reduction) thereby inhibiting the synthesis of enzymes like alkaline phosphatase in spinach plant (Ahmad and Huq, 1974) and thus affected various growth stages. Mwazi (2012) reported that saline irrigation water reduced germination, vegetative growth and yield of Spinach but increased leaf sugar contents. Moreover, seedling growth is also affected to variable extent at different levels of salinity. Further, germination of spinach seed is low even under favorable conditions. It has been attributed to chemical inhibitors such as abscisic acid (ABA) and non–hormonal inhibitors (coumarin, phenolic compounds) present in its pericarp (Leskovar and Esensee, 1999; Jaskani *et al*., 2006). Furthermore, pericarp also imposes physical hindrance by restricting emergence of radicle and/or reducing the availability of oxygen to germinating embryo (Katzman *et al*., 2001). Seed germination under optimal conditions may be improved by removing the pericarp (Leskovar and Esensee, 1999) but results in atypical germination at 30°C (Katzman *et al.*, 2001). Soaking of seeds in bleach alone and in combination with hydrogen peroxide has been reported to improve germination of spinach (Ku *et al.*, 1996; Katzman *et al.,* 2001). But, these authors' have not mentioned seedling size and vigor in response to bleach and hydrogen peroxide treatments, which is equally important to evaluate the overall effect of any treatment. Seed priming has the potential to improve the vigor under stress conditions by strengthening the antioxidant system (Chen and Arora, 2011) and therefore can be used as shotgun approach to combat abiotic stresses.

Wang *et al*. (2001) reported decline in indole-3-acetic acid (IAA) under saline conditions. So, its exogenous application can be a good strategy and has been employed by several researchers to alleviate the injurious effects of salts (Gulnaz *et al.,* 1999; Khan *et al.,* 2004). In addition, exogenous IAA showed high stimulatory effect on the root and shoot growth of wheat seedling in saline condition (Egamberdieva, 2009). Other plant growth regulators, such as GA_3 (Iqbal and Ashraf, 2010) and cytokinins (Iqbal *et al.,* 2006; Zahir *et al.,* 2001) have also been reported to improve germination and plant growth under saline stress.

At present, the information regarding the role of priming with auxins or cytokinins, and their effect on salt stress tolerance in spinach are scarce. Moreover, the impact of bleach and soaking treatments (to remove physical and chemical hindrance by pericarp) on seed invigoration and biomass production has not been exploited in previous studies (Katzman *et al.*, 2001). The objective of this study was to estimate the impact of bleach and/or soaking treatments in combination with seed priming on spinach seed invigoration and biomass production under normal and saline conditions.

MATERIALS AND METHODS

The seeds of spinach cultivar Desi Palak (having beet type seed–ball) were kept in water for a few minutes to remove all the impurities and floating non–viable seeds. Healthy seeds (those sank in the bottom of container) were selected for seed treatments as mentioned in Table 1. These treatments were selected on the basis of results of screening experiments. IAA and kinetin were purchased from Acros-Organics and Sigma-Aldrich, respectively. Seed priming was done in temperature controlled incubator (Isuzu, Japan) in darkness at 25±1°C. Treated and untreated seeds were used in germination and emergence tests.

Germination test: Seeds were germinated under saline conditions (at 25±1°C) by placing 20 seeds on two folds of filter paper in Petri dishes moistened with different concentrations (0, 50, 100, 150 and 200 mM) of NaCl.

Emergence test:Treated and untreated seeds were sown in trays filled with loam soil and saturated with water or 200 mM NaCl solution. Trays were kept at room temperature (20±2°C) and irrigated as per requirement.

Both experiments were laid according to completely randomized design under factorial arrangements with four replications of each treatment. The data were recorded for final germination and emergence percentage, time to 50% germination (Farooq *et al*., 2004), mean germination emergence time (Ellis and Roberts, 1981), germination index (summation of number of germinated seeds on each day divided by the corresponding day number, germination and emergence energy (germination and emergence percentage on 4^{th} and 10^{th} day of test, respectively; Ruan *et al.,* 2002), vigor index (product of seedling length and germination percentage; Abdul-Baki and Anderson, 1973), seedling length and seedling fresh and dry weights, number of leaves, chlorophyll contents (Wellburn, 1994) and MDA contents

Table 1. Chlorophyll and MDA contents in response to seed treatments at two salinity levels (Mean ± SD).

Salinity Level	Seed Treatments	Chlorophyll A (mg/g FW)	Chlorophyll B (mg/g FW)	Total Chlorophyll (mg/g FW)	MDA (µmol/g FW)
0 mM	T_1 = Untreated Seed (Control)	3.56±0.46 c	3.00±0.13 c	6.57±0.32 d	1.66±0.21 bc
	T_2 = Washing of seeds with bleach (B)	5.18±0.48 c	6.06±1.94 c	11.25±2.43 cd	1.96±0.19 bc
	T_3 = Seed soaking in distilled water for 4 hour (dH_2O 4h)	5.73±0.71 c	4.14±0.77 c	9.88±1.48 cd	1.63±0.12 bc
	T_4 = B+ 30 hours hydropriming	9.24±1.06 b	6.97±1.20 bc	16.22±1.88 bc	1.761±0.10 bc
	T_5 = B + dH_2O 4h + 30 hours hydropriming	9.00±0.70 b	10.22±0.66 ab	19.23±1.30 ab	1.57±0.62 bc
	T_6 = B + dH_2O 4h + 30 hours priming with 100 ppm IAA	12.99±0.27 a	11.62±0.43 a	24.62±0.70 a	1.30±0.12 c
	T_7 = B + dH_2O 4h + 30 hours priming with 25 ppm kinetin	11.15±2.51 ab	10.73±3.88 ab	21.89±6.33 ab	1.47±0.78 bc
200 mM	T_1 = Untreated Seed (Control)	4.80±0.67 c	3.93±0.96 c	8.74±0.96 d	2.10±0.13 bc
	T_2 = Washing of seeds with bleach (B)	4.36±0.29 c	2.39±0.26 c	6.75±0.53 d	2.98±0.69 a
	T_3 = Seed soaking in distilled water for 4 hour (dH_2O 4h)	4.20±0.70 c	3.37±0.31 c	7.57±1.01 d	2.29±0.47 ab
	T_4 = B+ 30 hours hydropriming	4.90±0.54 c	3.41±0.24 c	8.32±0.65 d	2.11±0.62 bc
	T_5 = B + dH_2O 4h + 30 hours hydropriming	6.10±0.60 c	3.67±0.51 c	9.77±1.10 cd	2.02±0.13 bc
	T_6 = B + dH_2O 4h + 30 hours priming with 100 ppm IAA	5.99±0.94 c	4.15±1.49 c	10.15±2.25 cd	1.83±0.13 bc
	T_7 = B + dH_2O 4h + 30 hours priming with 25 ppm kinetin	5.37±0.89 c	4.51±0.48 c	9.89±0.65 cd	1.71±0.83 bc

(Hodges *et al.,* 1999). Experiment was performed twice to confirm the results. Data collected were analyzed statistically using Fisher's analysis of variance (ANOVA) techniques and treatments means were compared by using LSD test at 5% probability level by using Statistica (version 5.5).

RESULTS

***Germination and vigor indices in response to seed treatments and salt stress*:** Various seed treatments improved seed germination and vigor indices to varying extent at different salinity levels. Although, difference among various seed treatments for values of the parameters studied was evident even at 0 mM yet, most striking difference was recorded at 200 mM NaCl salinity level (Fig. 1). Seeds primed in IAA (T_6) resulted in maximum germination (100% vs. 22.50% in control [T_1]) (Fig. 2a)., GE (86.25% vs. 78.78% in control [T_1]) (Fig. 2b), GI (35.16 vs. 3.55 in control [T_1]) (Fig. 2c), and VI (700.50 vs. 87.33 in control [T_1]) (Fig. 2d), at highest salinity (200 mM NaCl) level as well as all other salinity levels (0, 50, 100 and 150 mM NaCl). Seeds hydro–primed after bleach treatment (T_4) also performed better than all other seed treatments and had higher value of vigor index at 0, 50 and 150 mM salinity levels followed by the seeds washed with 50% solution of

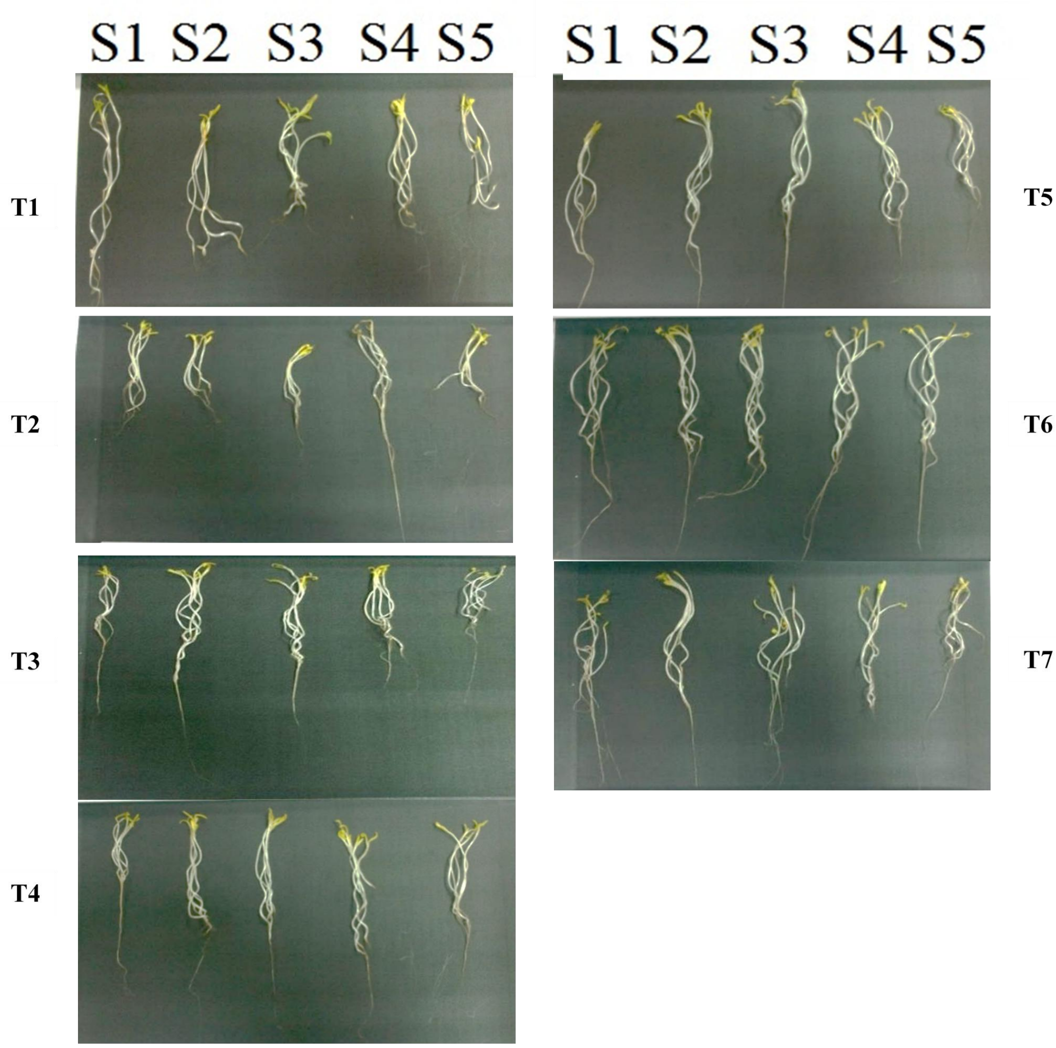
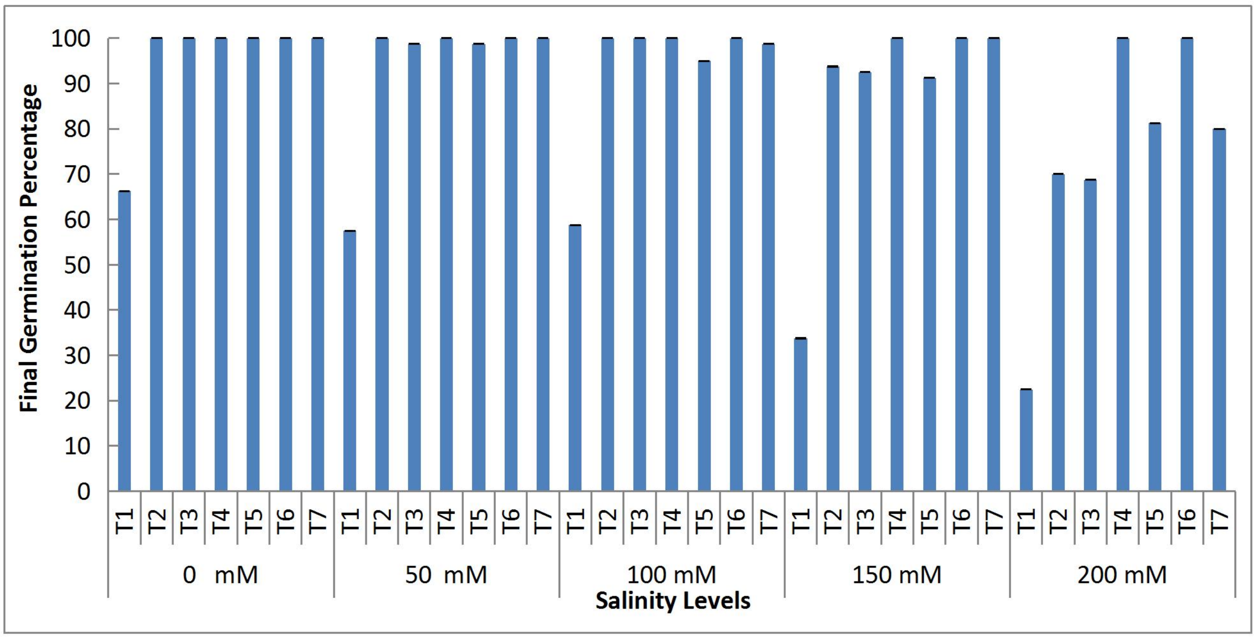


Figure 1. Germination of Spinach seed under saline conditions; S1, S2, S3, S4 and S5 stands for 0, 50, 100, 150 and 200 mM, respectively. (T_1= Untreated seeds (control); T_2= Washing of seeds with bleach (B); T_3= Soaking of seeds in distilled water for 4 hour (dH_2O 4h); T_4= Washing of seeds with bleach + 30 hours hydropriming; T_5= B + dH_2O 4h + 30 hours priming; T_6= B + dH_2O 4h + 30 hours priming with 100 ppm IAA; T_7= B + dH_2O 4h + 30 hours priming with 25 ppm kinetin).

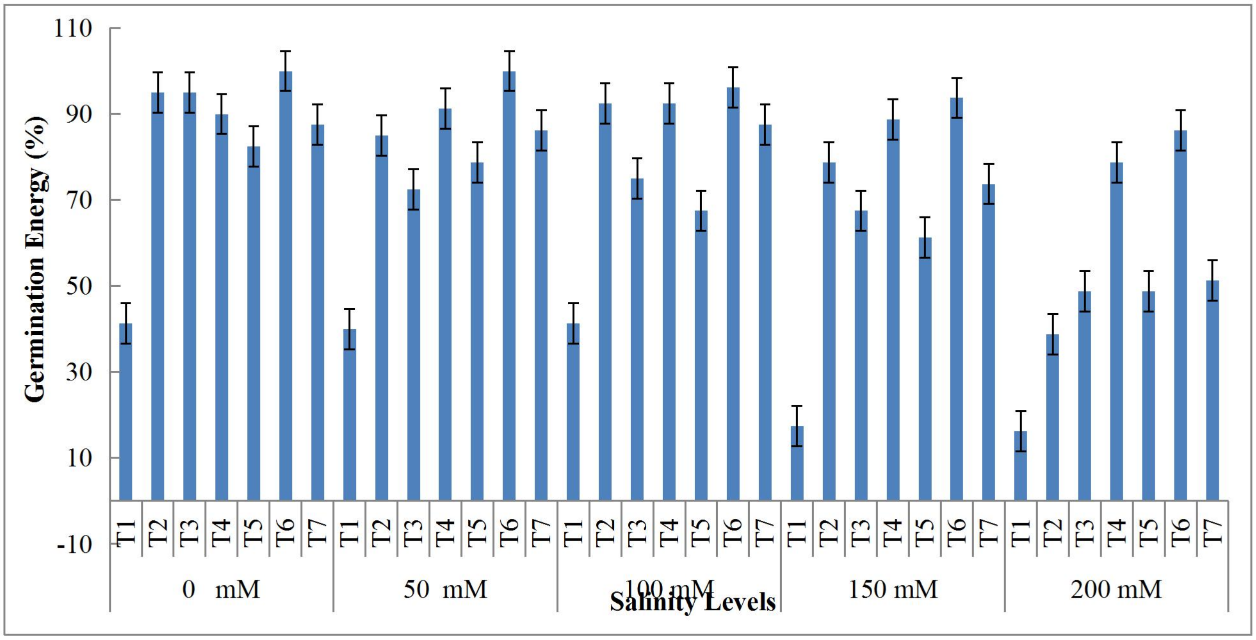
commercial bleach (T_2) at the same salinity levels. At low to moderate salinity levels (0, 50 and 100 mM) seed priming in kinetin (T_7) and seed washing with bleach (T_2) also gave good results. Minimum vigor index for all salinity levels was noticed in untreated seeds (T_1) (Fig. 2d).

When impact of seed treatments on alleviation of salt stress in terms of T_{50} was analyzed, performance of seeds primed in IAA (T_6) was better than all other seed treatments at all salinity levels followed by hydro–primed seeds after bleach treatment (T_2). At 200 mM salinity level, seeds primed in IAA (T_6) took less time to 50% germination (1.54 days) followed by those primed in kinetin (T_7; 1.62 days) and untreated seeds (T_1; 1.65 days). At the same (200 mM) salinity level, seeds soaked in water (T_3; 1.82 days) and treated with bleach (T_2; 1.81 days) attained maximum value of T_{50} (Fig. 2e). Non–primed seeds showed minimum MGT value (6.45 days) followed by seeds hydro–primed after bleach treatment (T_4; 7.14 days) at 150 and 200 mM. While, seeds primed in kinetin (T_7) or treated with bleach (T_2) showed maximum value of MGT at 150 and 200 mM salinity level, indicating delay in germination (Fig. 2f).

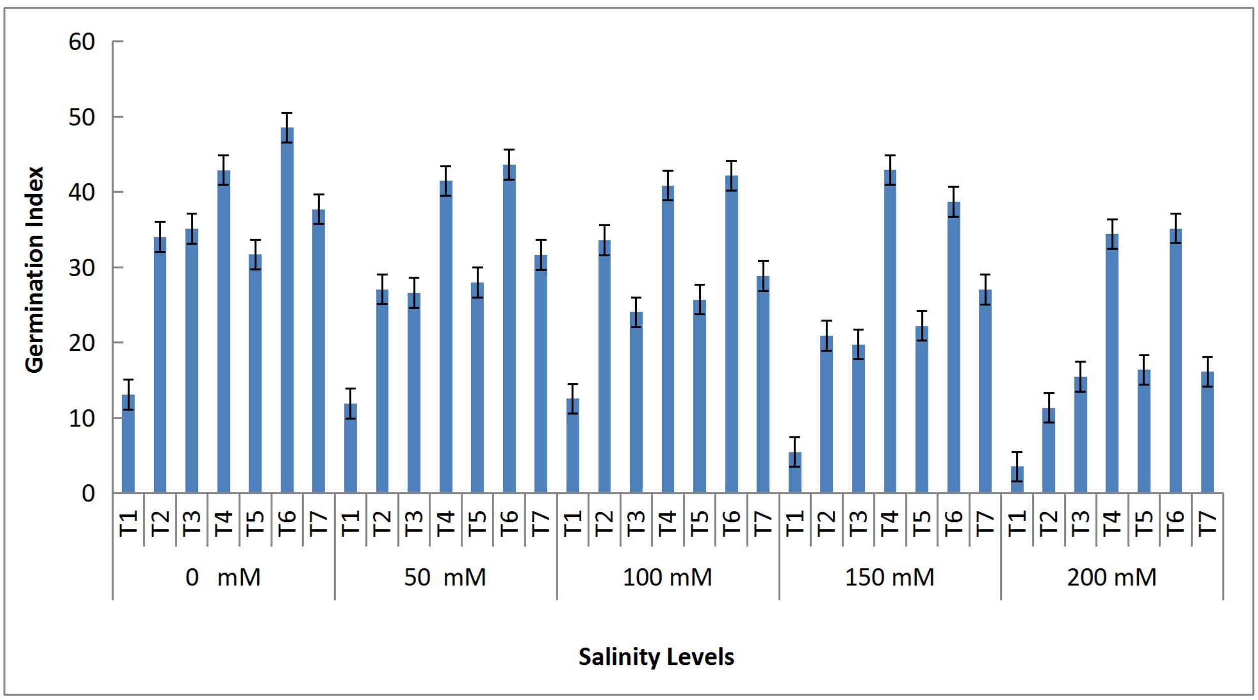
(a)



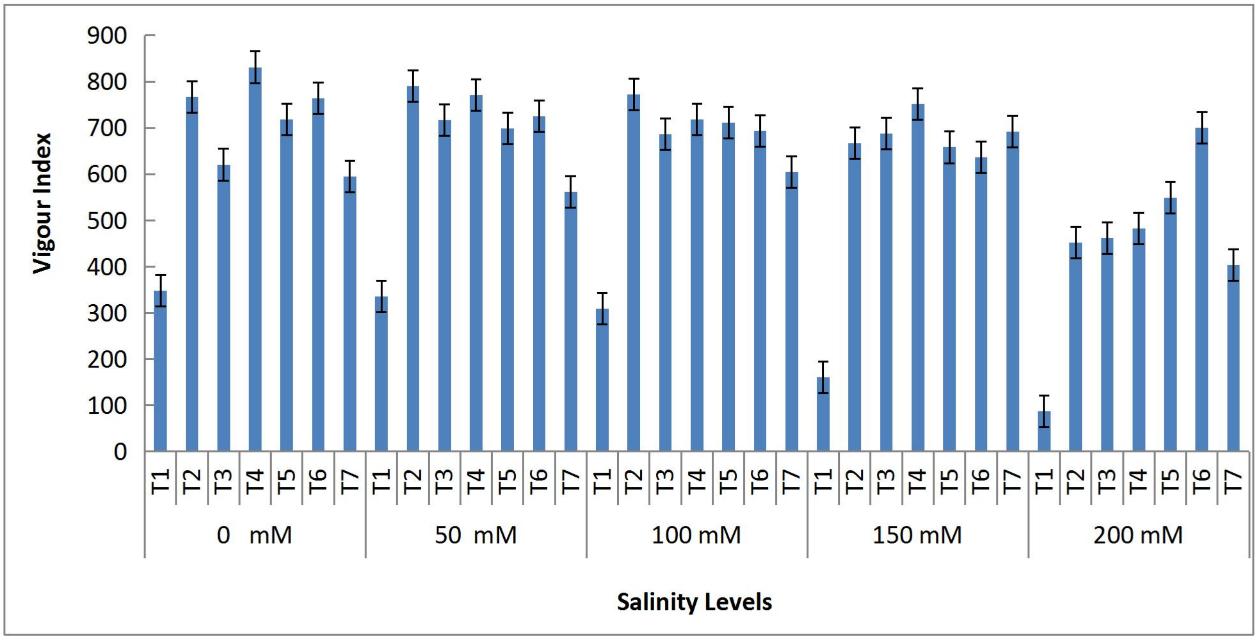
(b)


Germination Energy (%)
110
90
70
50
30
10
-10
T1 T2 T3 T4 T5 T6 T7
0 mM
50 mM
100 mM
150 mM
200 mM
Salinity Levels

(c)

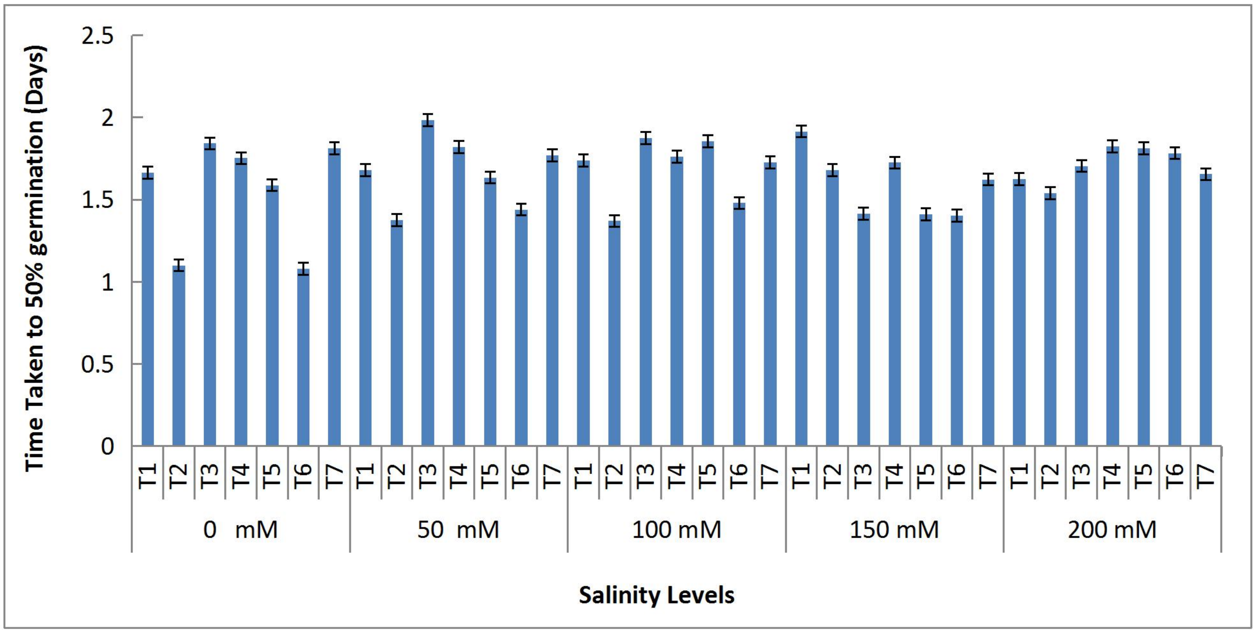

Germination Index
60
50
40
30
20
10
0
T1 T2 T3 T4 T5 T6 T7
0 mM
50 mM
100 mM
150 mM
200 mM
Salinity Levels

(d)



Vigour Index
900
800
700
600
500
400
300
200
100
0
T1
T2
T3
T4
T5
T6
T7
0 mM
50 mM
100 mM
150 mM
200 mM
Salinity Levels

(e)



(f)

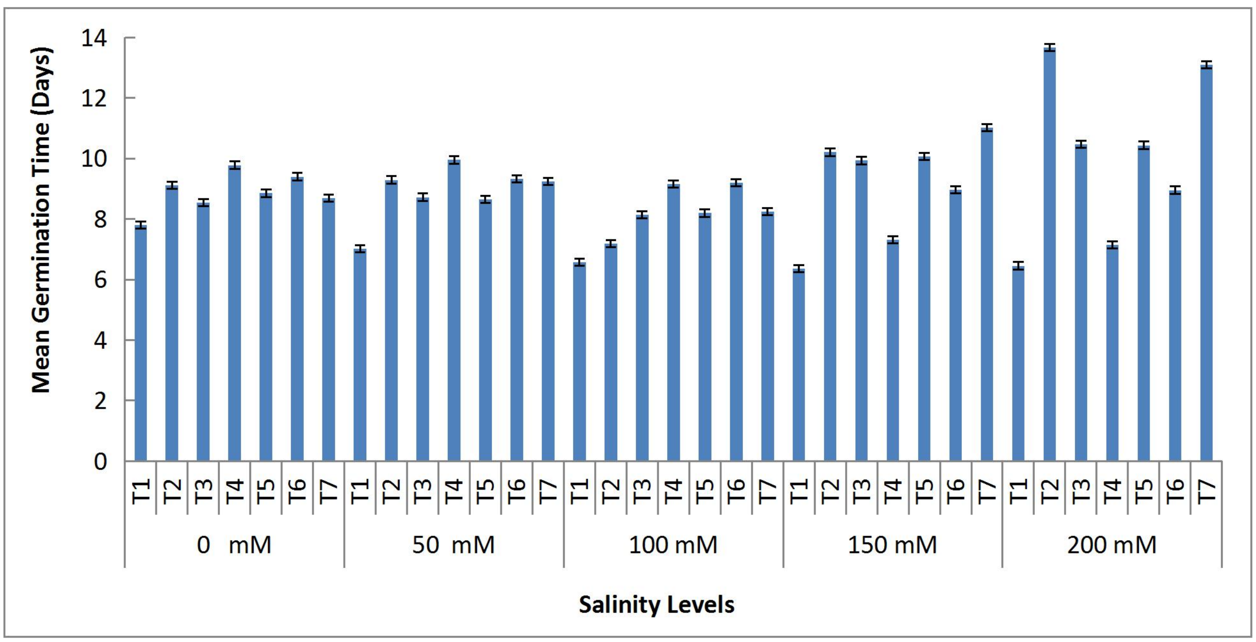


Figure 2. Effect of various seed treatments on final germination percentage (a), germination energy (b), germination index (c), vigor index (d), time taken to 50% germination (e), and mean germination time (f) of spinach. (T_1= Untreated seeds (control); T_2= Washing of seeds with bleach (B); T_3= Soaking of seeds in distilled water for 4 hour (dH_2O 4h); T_4= Washing of seeds with bleach + 30 hours hydropriming; T_5= B + dH_2O 4h + 30 hours priming; T_6= B + dH_2O 4h + 30 hours priming with 100 ppm IAA; T_7= B + dH_2O 4h + 30 hours priming with 25 ppm kinetin).

(a)

(b)

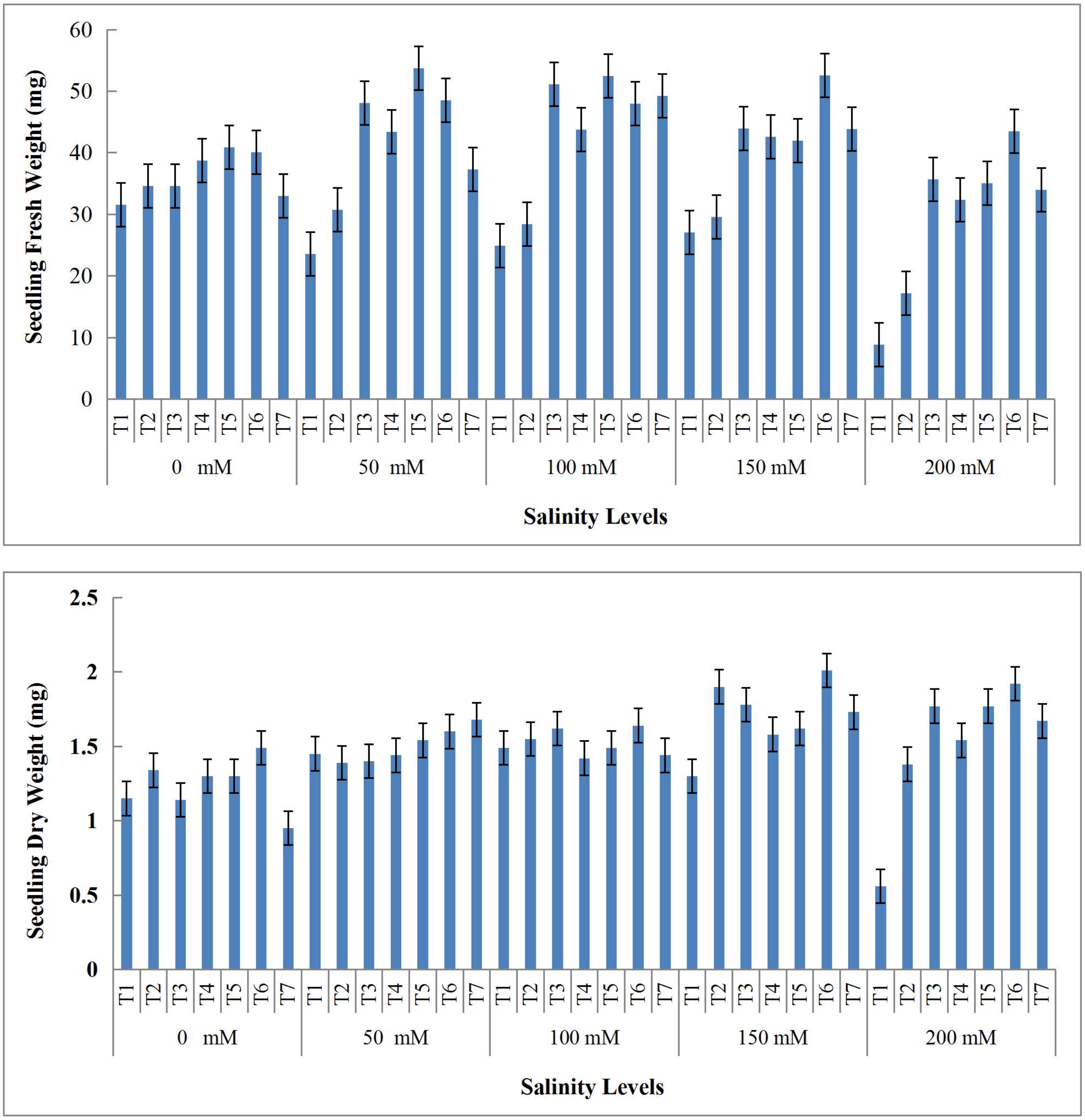


Figure 3. Fresh weight (a) and dry weight (b) of ten spinach seedlings in response to different seed treatments at various salinity levels. (T_1= Untreated seeds (control); T_2= Washing of seeds with bleach (B); T_3= Soaking of seeds in distilled water for 4 hour (dH_2O 4h); T_4= Washing of seeds with bleach + 30 hours hydropriming; T_5= B + dH_2O 4h + 30 hours priming; T_6= B + dH_2O 4h + 30 hours priming with 100 ppm IAA; T_7= B + dH_2O 4h + 30 hours priming with 25 ppm kinetin).

All seed treatments significantly improved seedling fresh and dry weight at all salinity levels. Maximum SFW was observed for seed priming with IAA (T_6) at 150 mM and 200 mM but, at lower salt concentrations hydropriming after bleach and soaking treatments (T_5) resulted in more SFW. At 200 mM, priming in IAA resulted in 72% more SFW as compared to control (Fig. 3a). All seed treatments also improved seedling dry weight at all salinity levels except priming in kinetin (T_7) at 0 mM salt concentration. Priming in IAA (T_6) excelled over all other seed treatments at all salinity levels. It resulted in higher SDW value at 200 mM than control (Fig. 3b).

***Emergence, growth, and chlorophyll and MDA contents in response to seed treatments and salt stress*:** The treatments used in experiment No. 1 were used in this experiment to confirm the efficacy of seed treatments in improving seed vigor and alleviation of salt stress. There was significant difference among different treatments at two salinity levels (0 and 200 mM NaCl) for various parameters except mean emergence time (Fig. 4a). It is evident from results that salinity reduced emergence percentage, emergence energy, number of leaves, fresh and dry weight of leaves, and chlorophyll contents. Salinity delayed emergence and enhanced lipid peroxidation. But, some seed treatments significantly improved emergence, growth and chlorophyll contents by reducing lipid peroxidation. It was observed that spinach seeds primed in IAA (100 ppm) after bleach and soaking treatments (T_6) showed maximum emergence percentage followed by seeds primed in kinetin (25 ppm; T_7), both under normal (81.33%) and saline (74%) saline conditions (Fig. 4b). Emergence energy (Fig. 4c), determined on 10th day, was maximum, while mean emergence time (Fig. 4a) was minimum for seeds primed in IAA (100 ppm) after bleach and soaking treatments (T_6)

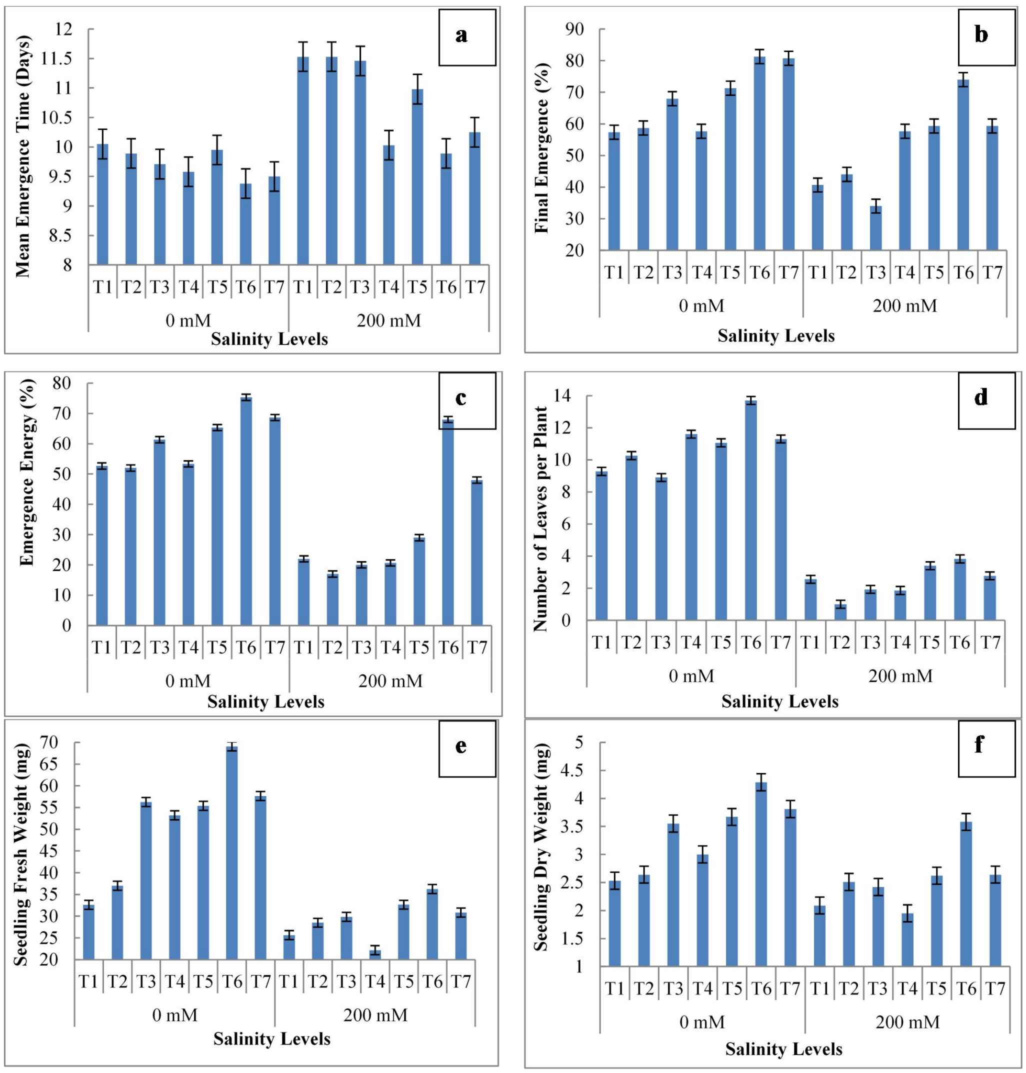


Figure 4. Effect of various seed treatments on mean emergence time (a), final emergence percentage (b), emergence energy (c), number of leaves (d), seedling fresh weight* (e) and seedling dry weight* (f) of spinach. (T_1= Untreated seeds (control); T_2= Washing of seeds with bleach (B); T_3= Soaking of seeds in distilled water for 4 hour (dH_2O 4h); T_4= Washing of seeds with bleach + 30 hours hydropriming; T_5= B + dH_2O 4h + 30 hours priming; T_6= B + dH_2O 4h + 30 hours priming with 100 ppm IAA; T_7= B + dH_2O 4h + 30 hours priming with 25 ppm kinetin). * Data of 10 seedlings.

both under normal and saline conditions. Seeds primed in distilled water after bleach and soaking treatment (T_5) also performed better than the control and other seed treatments. Maximum number of leaves was also recorded in IAA (T_6) primed seeds under both normal (13.7) and saline (3.83) conditions (Fig. 4d). Fresh and dry weight of seedlings was maximum in response to IAA treatment at 0 mM (69.06 mg and 4.29 mg) and 200 mM (36.27 mg and 3.58 mg) salinity levels, respectively (Fig. 4e, 4f).

To estimate the effect of seed treatments on pigments and lipid peroxidation both at (0 and 200 mM) salinity levels, chlorophyll '*a*', '*b*' and total chlorophyll contents as well as MDA contents were assayed. Chlorophyll '*a*', '*b*' and total chlorophyll contents were at peak in response to seed priming with IAA 100 ppm (T_6) under normal and saline conditions, respectively (Table 1). Seeds primed in distilled water (T_5) and kinetin 25 ppm (T_7) after bleach and soaking treatments increased chlorophyll contents over the untreated seeds under non–saline and saline conditions. Moreover, salinity reduced chlorophyll '*b*' contents to somewhat greater extent than the chlorophyll '*a*' contents. This increase in chlorophyll contents seemed to be correlated with lipid peroxidation of membranes as evidenced by MDA. Minimum MDA contents were recorded in response to seed priming with IAA (T_6), followed by seeds primed in kinetin (T_7) and distilled water (T_5) under non–saline and saline conditions. Maximum MDA contents were found in response to seed treatment with bleach (T_2) both at 0 mM (1.96 µmol g^{-1} FW) and 200 mM (2.98 µmol g^{-1} FW), even higher than that of untreated seeds (T_1) (Table 1).

DISCUSSION

Poor and non–uniform seed germination under saline conditions is a big problem in direct seeded vegetable crops with intact pericarp, for example spinach (Leskovar *et al.,* 1999). Besides soil management, there are various seed enhancement techniques, which can alleviate the deleterious effects of salinity in several vegetable crops (Amjad *et al.,* 2007; Chen and Arora, 2011). Spinach is a directly seeded vegetable crop which shows low germination percentage and speed under normal conditions (Katzman *et al*., 2001; Leskovar *et al.*, 1999). Germination and emergence percentage was less than 60% and 50% in untreated seeds under normal (0 mM NaCl) and saline (150 and 200 mM NaCl) conditions. Results also showed that speed of germination (T_{50}, MGT, GE and GI values) and emergence (MET and EE), vigor, seedling size, and fresh and dry weights decreased under saline (150 and 200 mM NaCl) conditions. Keshavarzi *et al.* (2011) reported reduction in germination (% and speed), seedling length as well as fresh and dry weight of spinach at 150 mM salt concentration in the growing medium as compared to control, as observed in our studies. Reduction in GE and GI even at 50 mM in our experiment corresponds with the results of Turhan *et al*. (2011). Moreover, decline in fresh and dry weight of seedlings in salt stress was in accordance to the report of Xu and Mou (2016), and Mwazi (2012) who also noticed reduction in growth of spinach under saline conditions.

Spinach seeds are enclosed in pericarp in the form of a seed ball, which delays germination due to physical and chemical inhibition (Katzman *et al.,* 2001). Removal of pericarp can improve germination (%) (Leskovar *et al.,* 1999), but it increases the number of atypical seedlings (Katzman *et al*., 2001), and also this option seems to be impractical for farmers. Katzman *et al*. (2001) also reported that soaking/washing in NaCl/water remove inhibitors from the pericarp and also enhance seed germination and vigor to perform well under high temperature and normal conditions. Use of growth regulators has ameliorated the drastic effect of salinity in several crops (Egamberdieva, 2009; Iqbal *et al.,* 2006; Iqbal and Ashraf, 2010). Therefore, we tried several seed treatments to enhance germination and vigor of spinach seeds. It is obvious from results that all seed treatments improved germination and emergence as compared to control except washing of seeds in water for 4 hours under saline conditions. Results of emergence test also depicts that bleach treatment is more effective when combined with other seeds treatments, particularly bleach+soaking+priming in IAA (100 ppm), bleach+soaking+priming in kinetin (25 ppm) and bleach+soaking+priming in distilled water. Bleach might have softened the pericarp and thus improved germination. Soaking and/or various priming treatments might have helped in leaching of abscisic acid (ABA) and other inhibitors from seed ball and thus improved seed germination, emergence and vigor and was in correspondence to Atherton and Farooque (1983 a, b). Significant improvement in germination of spinach due to seed treatment with bleach and hydrogen peroxide was also recorded by Katzman *et al.* (2001), which further support our results. Germination enhancement in response to priming using distilled water (Amjad *et al.* 2007), IAA (Afzal *et al.,* 2005) and cytokinins (Iqbal *et al.*, 2006) has been reported in various crops. Priming with IAA might have increased the endogenous level of GA (Wolbang *et al.*, 2004), that is supposed to reduce the endogenous ABA (Gonai *et al.*, 2004), and thus promoted seed germination, emergence and vigor of spinach seeds. It is a well-established fact now that salinity reduces the endogenous level of IAA (Iqbal *et al.,* 2006; Prakash and Prathapasenan, 1990; Wang *et al.,* 2001). IAA priming might have replenished the endogenous IAA concentration and thus enhanced germination, emergence and seedling vigor as compared to control (untreated) and other seed treatments. Akbari *et al*. (2007) reported improvement in seedling length as well as fresh and dry weights of wheat seedlings in Petri dishes containing salt solution and IAA as compared to seedlings without IAA and thus corroborates our findings.

Inclusion of IAA in priming solution enhanced seed germination and vigor and reduced germination time under saline conditions, particularly at 200 mM salt concentration, was in accordance to the previous reports on wheat (Afzal *et al.*, 2005; Gherroucha *et al.*, 2011; Gulnaz *et al.,* 1999), maize (Kaya *et al.*, 2010), *Brassica* species (Ozturk *et al.*, 2006), sugar beet (Eisa *et al.*, 2012). Chen and Arora (2011) concluded that priming strengthens the antioxidant system of the seeds and suppresses the lipid peroxidation of membranes. Higher concentration of photosynthetic pigments (total chlorophyll and chlorophyll '*a*' and '*b*' contents) and less MDA contents reflects improvement in antioxidant system of primed spinach seeds, particularly those primed in IAA. Thus, protection of membranes and photosynthetic pigments increased fresh and dry mass of spinach. So, priming in IAA and distilled water not only enhances seed germination and vigor but also the biomass under normal and saline conditions.

In present results, we observed soaking and bleach treatments enhanced seed germination, vigor and biomass of spinach seedlings by relieving physical and chemical hindrance due to pericarp. Priming in IAA (T_6) and distilled water (T_5) (after bleach and soaking) further reinforced antioxidant system to protect membranes of seeds and seedlings and therefore, seems to be a better option than washing only with bleach solution. This study provides an easy and farmers friendly approach to improve crop stand and the ultimate yield of spinach in normal as well as in saline conditions.

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