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Seed Priming With Leaf Aqueous Extract of Lesser Bulrush Improves Resistance against Salt Stress in Pea

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Abstract

This study was conducted to evaluate the effect of seed priming with aqueous extract of lesser bulrush on the resistance against salt stress in pea. For priming, seeds of pea variety Douce Provence were soaked in aqueous extract of lesser bulrush leaves. Salt stress was imposed as 0, 240 and 320 mM NaCl for germination and 0 and 120 mM for growth. Primed (P) and non primed (NP) seeds were sown in Petri plates. Salt stress suppressed the germination, growth, and reduced the membrane integrity, respiration, mineral composition (potassium and phosphorus) of pea. However, salt stress caused significant increase in the accumulation of sodium, proline, total soluble sugars and secondary metabolites (phenols, flavonoids and alkaloids). Seed priming, with leaf aqueous extract of lesser bulrush, helped in mitigating the adverse effects of salt stress on pea. Plants developed from primed seeds showed better response to salt stress by the protection of membrane integrity and the maintenance of the osmotica (proline, total soluble sugars, potassium and phosphorus. In conclusion, the seed priming with extracts of lesser bulrush could be considered as an effective alternative method to improve the resistance salt stress in pea.

***Key words**: Pea, salinity, seed priming, aqueous extracts, lesser bulrush.*

1. Introduction

Among abiotic stresses, salinity stress is an important stress as it causes significant reduction in crop production (Saha et al. 2010). Salinity affects some physiological and biochemical processes of the plants and reduces significantly the yield (Munns and James, 2003).

Salt stress affects all major processes and plant metabolism such as growth, flowering, photosynthesis, respiration, water potential, enzymatic activity, absorption of minerals and nutrient balance (Cassaniti et al. 2013; Parihar et al. 2015). In most plant species salinity affects germination and development of the seedling, which is considered the developmental stage that is most sensitive and vulnerable to abiotic stresses (Sosa et al. 2005; Belaqziz et al. 2009). Delay of germination (Foolad, 2004) and growth inhibition due to salinity are caused by low external water potential, ion imbalance and specific ion toxicity (Munns, 2002; Miranda et al. 2010).Under these conditions there is a decrease in water absorption and an excessive absorption of ions (Akram et al. 2010).

Pea belongs to the Leguminosae family which represents the second most important family of crop plants after Poaceae, accounting for approximately 27% of the world's crop production (Graham and Vance, 2003). Field pea, like other pulses, is comparatively sensitive to a number of abiotic stress factors, particularly involving soil nutrition such as salinity (Dita et al. 2006).Its productivity was markedly reduced at elevated levels of salt stress (Najafi et al. 2007).

Seed priming is one of the physiological methods that improves seed performance and provides faster and synchronized germination. It is an easy, low cost and low risk technique and recently being used to overcome the salinity problem in agricultural lands (Neto and Tabosa, 2000). It stimulates many of the metabolic processes involved with the early phases of germination and it has been noted that seedlings from primed seeds emerge faster, grow more vigorously and perform better in adverse conditions (Cramer, 2002).

Seed priming is a technique used for plants to give them good start and to enhance the productivity with increase in germination rate, germination percentage, early emergence and vigor (Basra et al. 2005a; Farooq et al. 2005). Different techniques have been carried out, like hydropriming, osmopriming, matripriming, hardening, osmohardening, and hormonal priming (Hardegree and Emmerich 1992a, 1992b; Basra et al 2004, 2005b), to improve the germination rate, field emergence, seedling vigor, stand establishment, and economic yields in many crops (Du and Tuong, 2002; Harris et al. 2002; Farooq et al. 2008), and to induce tolerance against biotic and abiotic stresses (Senaratna et al. 2000; Shakirova et al. 2003). Each treatment has advantages and disadvantages and may have varying effects depending upon plant species, stage of plant development and concentration/dose of priming agent and incubation period (Ashraf and Foolad, 2005). Bio-priming

(hydration using biological compounds) is a new technique designed to introduce organic and inexpensive seed priming tools like plant extract and jute mat as natural and easily adaptable priming agent sources to improve germination and seedling growth (Nouman et al. 2012).

Lesser bulrush (*Typha angustifolia* L.) is commonly known as Elephant grass or cattail from family Typhaceae. It is a perennial herbaceous plant of genus *Typha* commonly found in the Northern hemisphere in brackish locations.

Pea variety Lincoln and Douce Provence are widely cultivated in Tunisia. In a recent study, we found seed priming with that aqueous extract of lesser bulrush improved the resistance against salt stress in pea variety Lincoln by modulating membrane stability, photosynthetic pigments, sugar metabolism and ionic homeostasis (Ghezal et al. 2016). This study was conducted to evaluate the influence of seed priming with aqueous extract of lesser bulrush in improving the resistance against salt stress in pea variety Douce Provence and its mode of action.

2. Material and methods

2.1. Plant material

Mature leaves of lesser bulrush were collected during spring, and were washed several times with tap water and dried in hot-air oven at 60°C for 72 h. Then, they were powdered in blender and sieved through 40 mesh (420 µm) sieve. 60 grams of powder were soaked in 1 L of sterile distilled water in the dark at room temperature for 24 h. The extract was filtered and sterilized using 0.25 µm filters. This extract (60 g/L) was used for bio-priming essay.

2.2. Seed priming

Pea (*Pisum sativum* L.) variety Douce Provence seeds, selected from a crop cultivated in the center of Tunisia, were used in this experiment. The seeds were disinfected with 10% (w/v) sodium hypochlorite for 5 min and then washed 3 times with distilled water. For priming, seeds were soaked in leaves aqueous extracts (60 g/L) for 48 h at room temperature in dark. Then washed with distilled water and left to dry in air between two filter papers. The ratio of seed weight to solution volume was 1:5 (Farooq et al. 2006). After priming, seeds were given 3 washings with distilled water and re-dried to near their original weight (Basra et al. 2002). The primed seeds (P) were stored at 4 °C until use. Non-primed seeds (NP) are used as control.

2.3. Pea growth and treatment conditions

Three concentrations of NaCl were used to evaluate the effect of priming on its tolerance by pea germination and seedling growth. For germination, seeds were exposed to 240 and 320 mM NaCl (provoking respectively, 50% and 0% of germination rate) and for growth, pea seedlings were exposed to 120 mM NaCl (inducing 50% inhibition of root and shoot length).

Primed (P) and non-primed seeds (NP) (control) were placed in Petri dishes and irrigated with 5 mL of each solutions of mM NaCl. Germinated seeds, with 1mm root length, were, for a first batch, weighed before and after drying at 60 °C for 2 days, and placed in a freezer (−80 °C) for a second batch, for biochemical analysis.

For seedling growth, primed (P) and non-primed (NP) seeds were germinated in Petri dishes at room temperature in the dark irrigated with distilled water. Seven-day old seedlings uniform were subsequently cultured individually in a complete Hoagland's medium (Hoagland and Arnon, 1950) diluted by half in a greenhouse (16 h light/8 h dark at 20/17 °C). After 10 days of acclimation, plants were cultivated in the presence of 120 mM NaCl which was the concentration inducing a reduction of 50% root and shoot length. Salt treatment was initiated by adding increments of 40 mM NaCl daily until the desired concentration of 120 mM NaCl to avoid osmotic shock. Plants were grown in the final salinity solutions for 12 days. The culture media were aerated continuously and the renewal was done every 2 days. At the end of the treatment period, the plants were harvested and separated into roots and shoots, fresh or dry materials was used for the determination of different parameters.

2.4. Electrolyte leakage

The electrolyte leakage (EL) was determined as described by Lutts et al. (1996a). Germinated seeds, roots or aerial parts of fresh pea seedlings were cut and placed in test tubes containing 15 mL of distilled water. The tubes were incubated at room temperature for 24 h and 48 h and the initial electrical conductivity of the medium (EC1) was measured using a digital conductivity meter (type BCT- 4308). The samples were autoclaved at 121 °C for 20 min to release all electrolytes, cooled down to 25 °C and the final electrical conductivity (EC2) measured. The electrolyte leakage (EL) was calculated according to the following formula (Lutts et al., 1996a):

$$EL = (EC1/EC2) \times 100.$$

2.5. Lipid peroxydation

Frozen samples (200 mg) (germinated seeds, roots and aerial parts) were homogenized with a mortar kept on ice and thoroughly mixed with 2.5 mL of 67 mM phosphate buffer (pH=7) and 0.05 g PVP. After centrifugation (2000 g for 15 min at 4 °C), the supernatant was used to determine lipid peroxidation (Doblinski et al. 2003). A 750 µL of enzyme extract was added to 3 mL of 0.5% TBA (prepared in 20% TCA). The homogenate was incubated at 90 °C for 10 min. The reaction was stopped quickly by cooling the mixture in ice. Then, the mixture was centrifuged and the supernatant absorbance was measured at 532 and 600 nm, and the MDA concentration was calculated using the extinction coefficient of 155 $mM^{-1}cm^{-1}$ (Doblinski et al. 2003).

2.6. Cell metabolic activity

The fresh plant material (100 mg) was washed and dried quickly between blotting paper, then incubated in 5 mL of TTC (0.2%, pH= 7) at 37 °C for 4 h in the dark. The reaction was stopped by adding 0.5 mL of sulfuric acid (1 M). Thereafter, the plant material was removed, washed with distilled water, dried quickly between filter paper and ground in a mortar placed in ice containing 3.5 mL of ethyl acetate. The homogenate was filtered and the volume was adjusted to 7 mL with ethyl acetate. The absorbance was measured at 485 nm and the amount of formazan was calculated as follows (Sampietro et al. 2006):

Formazan content (%) = DO_{485} treatment/DO_{485} control.

2.7. Total soluble sugars content

The total soluble sugars rate was determined according Dubois (1956). The fresh plant material (100 mg) was incubated in 2 mL of 80% ethanol for 48 h in the dark. Thereafter, all the alcohol was evaporated by putting the test tubes in a water bath at 70 °C. Test tube was cooled and 20 ml of distilled water were added. 1 mL of the solution was taken and reactive with 1 mL of 5% phenol, taking care to shake well. Then 2 mL of concentrated sulfuric acid were added and the test tubes were placed in a bath ice for 25 min. The absorbance was measured at 490 nm. L-glucose was used as standard.

2.8. Proline content

Proline in pea germinated seeds, roots and aerial parts was extracted and analyzed according to Bates et al. (1973). Ten milligram (10 mg) of dry plant material was mixed with 1.5 mL aqueous sulfosalicylic acid (3%, w/v). The homogenate was centrifuged at 13 000 tr/min for 10 min, and the supernatant was transferred to a fresh 1.5 mL tube. The extracted solution was reacted with an equal volume of glacial acetic acid and ninhydrin reagent (1.25 g ninhydrin in 30 mL glacial acetic and 20 mL 6M H_3PO_4) and incubated at 100 °C for 1 h. The reaction was terminated by placing the tube in an ice bath. The reaction mixture was vigorously mixed with 2 mL of toluene. After warming at 25 °C, the chromophore was measured at 520 nm. L-Proline was used as a standard.

2.9. Determination of sodium, potassium and phosphorus contents

After drying and grinding, 1 g of plant material was weighed in capsules and was placed in a muffle furnace type (Flli Manfredi) at 220 °C for 2 h and then at 550 °C for 6 h, to be well calcined. Then, 2 mL of concentrated chlorhydric acid were added in each capsule and heated until the acid total evaporation. 5 mL of hydrochloric acid were added to sample for 10 minutes and the obtained solutions were filtered and brought back to 100 mL with distilled water. The absorbance was read by flame spectrometry. The % of Na and K was determined by calibration solution (Martin-Prével et al. 1984).

For the determination of the phosphorus (P) content, 5 mL of each solution was react with 5 mL of nitrovanadomolybdic reactive in a flash of 25 mL and then distilled water was added to the gauge mark. The absorbance was read at 430 nm and the P content was determined by a calibration curve (Martin-Prével et al. 1984).

2.10. Secondary metabolites production in pea

Pea seedlings grown in the presence of 120 mM NaCl harvested and frozen (separating roots and aerial parts) were homogenized in 80% methanol for 24 h. The homogenate was centrifuged at 10 000 g for 20 min at 4 °C and the supernatant was used to determine the levels of secondary metabolites (García-Sánchez et al. 2012).

2.10.1. Total phenolic (TP) content

The TP content was measured using the modified Folin–Ciocalteau method (Velioglu et al. 1998). Sample extract (100 µL) was mixed with 500 µL of 1/10 diluted (in Milli-Q water) Folin–Ciocalteau phenol reagent and allowed to react for 5 min in the dark at room temperature. Then 400 µL of sodium bicarbonate (7.5%) were added to the mixture. After 90 min of incubation in the dark at 30 °C, the absorbance was read at 765 nm. TP content were expressed as mg gallic acid equivalent/g dry matter (mg GAE/g DW) using gallic acid calibration curve (R^2= 0.998).

2.10.2. Total flavonoid (TFd) content

The TFd content was determined spectrophotometrically according to standard method (Quettier et al. 2000). Briefly, 0.5 mL of 2% solution of $AlCl_3$ in methanol was mixed with the same volume of extract. Absorption readings at 430 nm were taken after 30 min against a blank. TFd content was expressed as mg quercetine equivalent/g dry weight (mg QE/g DW) using quercetine calibration curve (R^2 = 0.988).

2.10.3. Total precipitable alkaloid (TA) content

The TA content was determined by spectrophotometric method with Dragendorff reagent (Stumpf, 1984). Principally, 300 µL of plant extract was mixed with 100 µL of Dragendorff reagent. After centrifugation at 7000 g for 1 min, the supernatant was removed and dissolved in 1 mL of 2.45M NaI. An aliquot of 10 µL of each tube was added to 1 mL of 0.49 M NaI, after which the absorbance was read at 467 nm. TA content was expressed as mg papaverine hydrochloride equivalent/g dry weight (mg PAHE/g DW) using papaverine hydrochloride calibration curve (R^2 = 0.951).

2.11. Statistical analysis

The laboratory bioassays and pots culture were conducted in a completely randomized design with four replications. Duncan, Student t and ANOVA tests were performed using PASW statistics 20.00, for Windows program, to analyze treatment differences. The means were separated based on least significant differences at the 0.05 probability level.

3. Results

3.1. Influence of seed priming on the germination and seedling growth of pea

NaCl affected pea germination which was reduced by half with 240 mM and completely inhibited at 320 mM. The priming with aqueous extracts of lesser bulrush leaves ameliorated significantly the germination and the rate was increased by 13.75% and 11.25% respectively at 240 and 320 mM. The germination index (IG), reflecting the germination speed, was also accelerated. IG was increased by 28%, 43.8% and 45% for primed seeds in absence and in presence of NaCl at 240 and 320 mM, respectively (Table 1).

Table 1. Influence of seed priming, with leaf aqueous extract of lesser bulrush, on germination percentage and germination index of pea under salt stress.

Salt stress (mM NaCl)	Seeds	Final germination (%)	Germination index
0	NP	100a	6.47b
	P	100a	8.29a
240	NP	57.5c	2.35d
	P	71.25b	3.38c
320	NP	-	-
	P	11.25d	0.45e

Means with the same letters in a column are not significantly different at $p \leq 0.05$. NP = Non-priming; P = Seed priming.

At 120 mM NaCl, a concentration reducing 50% of root and shoot length for NP, the priming has ameliorated the root and shoot length respectively by 20% and 26.5%. In the absence of salt stress this stimulation percentages were 91% and 74.4% respectively for the two organs (Fig 1).

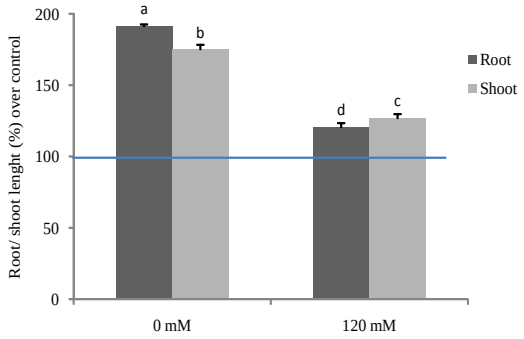

Figure 1. Influence of seed priming, with leaf aqueous extract of lesser bulrush, on root and shoot length of pea seedling under salt stress. Each bar indicates mean ± S.D., n = 4. Bars with different letters differ significantly at $p \leq 0.05$. NP = Non-priming; P = Seed priming.

3.2. Influence of seed priming on electrolyte leakage of pea germinated seeds and pea seedlings

The electrolyte leakage (EL) from pea germinated seeds and seedlings (roots and shoots) were determined by measuring the conductivity of the medium where they were immersed (Fig 2). Pea germination was carried out in the presence of 0, 240 and 320 mM NaCl and growth with 0 and 120 mM NaCl.

Results showed that the priming was beneficial; hence EL was reduced in all cases (in the absence and in the presence of NaCl). Indeed, after 48h of incubation, the priming induced a 70% reduction of EL (against 33.8% registered after 24h) for germinated seeds in the absence of NaCl. With NaCl, the reduction percentage was 44% and 49.8% after 24h and 70.1% and 113.7% after 48h respectively at 240 and 320 mM (Fig 2A).

For seedlings, Fig 2B showed the variation of EL for pea roots and shoots cultivated in the presence of 0 and 120 mM NaCl. The priming reduced the EL in all cases. Thus, for roots, after 24 h, the priming decreased significantly the EL by 20.4% and 271.2% respectively with 0 and 120 mM NaCl, these values were 14.5% and 252.5% for shoots. After 48 h, priming influenced slightly the leakage of ions from roots, which was recorded with decrease values by 98.8% and 934.7%, respectively at 0 and 120 mM. For shoots, these reductions were 25.2% and 244.7% (Fig 2B).

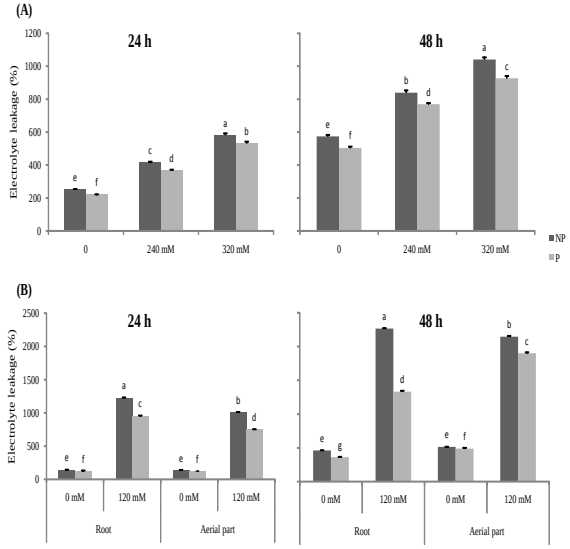

Figure 2. Influence of seed priming, with leaf aqueous extract of lesser bulrush, on electrolyte leakage (%) of (A) germinated seeds and (B) root and shoot of pea under salt stress after an immersion of 24 and 48 h in distilled water. Each bar indicates mean ± SD; n = 4; Bars with different letters differ significantly at $p \leq 0.05$. NP = Non-priming; P = Seed priming.

3.3 Influence of seed priming on lipid peroxidation in pea germinated seeds and seedlings

Polyunsaturated fatty acids are the major lipid component of the membrane susceptible to peroxidation and degradation. Indeed, the increase in membrane permeability under salt stress corresponds

to an increase in lipid peroxidation estimated by malondialdehyde (MDA) accumulation. This assay was performed on primed (P) and non-primed (NP) seeds.

For germinated seeds, the results showed that MDA content was lower in P compared to NP seeds in the absence and under salt conditions. This reduction was, respectively, 5.3, 11 and 20 $U.g^{-1}$ FW at 0, 240 and 320 mM NaCl. In addition, MAD content increased in NP when NaCl concentration enhanced, however in primed seeds there is no significant difference between the three treatments (Fig 3A).

For seedlings, Fig 3B shows the MDA contents in roots and shoots of seedlings pea developed from P and NP seeds, in the absence and in the presence of 120 mM NaCl. An average increase of 6.5 $U.g^{-1}$ FW was recorded in NP seedling at 120 mM compared to NP seedling at 0 mM NaCl. While, priming decreases this content of MDA by 2.8 and 2.1 $U.g^{-1}$ FW in roots and by 5.9 and 5 $U.g^{-1}$ FW in aerial parts respectively at 0 and 120 mM NaCl (fig 3B).

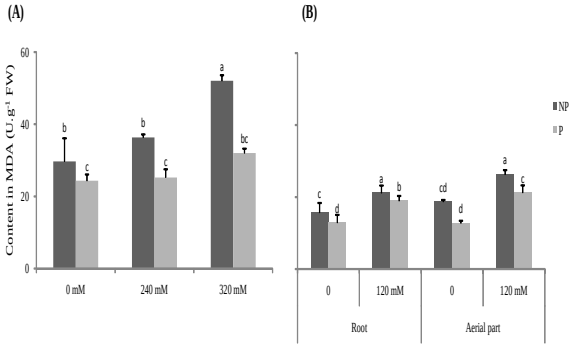

Figure 3. Influence of seed priming with leaf aqueous extract of lesser bulrush, on malondialdehyde (MDA) content (U/g FW) in (A) germinated seeds and (B) in root and shoot of pea under salt stress. Each bar indicates mean ± SD; n = 4; Bars with different letters differ significantly at $p \leq 0.05$. NP = Non-priming; P = Seed priming.

3.4. Influence of seed priming on cell metabolic activity in pea germinated seeds and seedling

Figure 4 reports the formazan content, expressed in percent of control in pea P seeds and seedlings, in the absence and the presence of NaCl. These contents reflect the metabolic activity of cells, mainly the activities of dehydrogenase enzymes and thus mitochondrial respiration. The results showed a more or less significant decrease of formazan production under the effect of salt stress. However, the priming ameliorated the cell metabolic activity in all cases.

The formazan content in primed seeds showed a stimulation of 46.8%, 23.2% and 42.8% at 0, 240 and 320 mM NaCl respectively (Fig 4A).

The formazan content, expressed as a percent of control (NP seedling), in P pea roots and aerial parts grown in the presence of 0 and 120 mM NaCl is shown in (Fig 4B). The results showed amelioration in cell metabolic activity with primed seeds in the absence and also in the presence of NaCl. For the roots, the formazan level was greatly increased in all cases. In the absence of salt, the content was recorded (37.5%) followed by the presence of NaCl (17.7%). For aerial parts, these levels were 52.7% and 16.3%, respectively (Fig 4B). The formazan level was higher in pea seedling indicating greater metabolic activity caused by the effect of the priming.

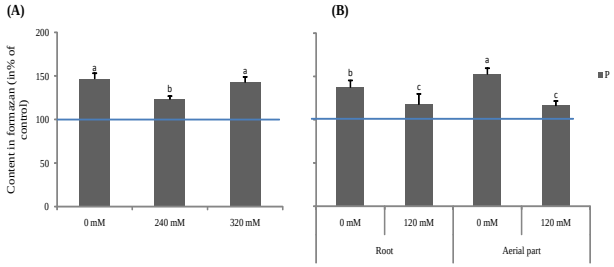

Figure 4. Influence of seed priming, with leaf aqueous extract of lesser bulrush, on formazan content (in % of control) in (A) germinated seeds and (B) in root and shoot of pea under salt stress. Each bar indicates mean ± SD; n = 4; Bars with different letters differ significantly at $p \leq 0.05$.NP = Non-priming; P = Seed priming.

3.5. Influence of seed priming on proline accumulation in pea germinated seeds and seedlings

Figure 5 shows that salt application enhanced significantly ($p<0.05$) proline accumulation in pea germinated seeds and in seedlings. As response to priming, proline content rose more even by application of salinity in almost cases.

Indeed, proline amounts were 8.10^{-3}, 16.10^{-3} and 13.10^{-3}µmol/g DM respectively in NP germinated seeds at 0, 240 and 320 mM (Fig 5A). These contents increased 1.4 times in absence of NaCl and 1.1 times at 240 mM, but decrease by 0.8 times at 320 mM for primed seeds (Fig 5A).

In seedlings developed from not primed seeds, the proline amount passed from 43.10^{-3}µmol/g DM to 58.10^{-3}µmol/g DM for roots and from 46.10^{-3}µmol/g DM to 62.10^{-3}µmol/g DM for shoots, in the presence of 120 mM of NaCl. This content was stimulated by an average of 1.2 times in seedlings developed from primed seeds (Fig 5B).

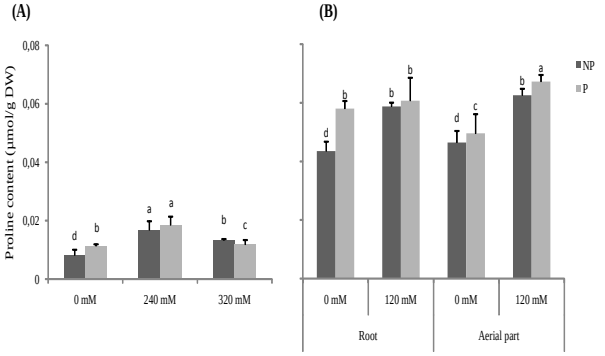

Figure 5. Influence of seed priming, with leaf aqueous extract of lesser bulrush, on proline content (µmol/g DW) in (A) germinated seeds and (B) in root and shoot of pea under salt stress. Each bar indicates mean ± SD; n = 4; Bars with different letters differ significantly at $p \leq 0.05$.NP = Non-priming; P = Seed priming.

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3.6. Influence of seed priming on accumulation of total soluble sugars in pea germinated seeds and seedlings

Data of figure 6 showed significant ($p \leq 0.05$) effect of salinity and seed priming on the accumulation of soluble sugars which increased with enhanced NaCl concentration (Fig 6).

The priming increased this accumulation in the absence of NaCl, where primed seeds had 31.8 mg/g FW which presented 2.6 times the level of not primed seeds (Fig 6A). In the presence of NaCl, sugars content did not vary significantly at 240 mM, and slightly decreased at 320 mM in primed seeds compared to non-primed ones (Fig 6A).

In seedlings, salinity has enhanced sugars content in the two organs and the enhancement was more important in seedlings developed from primed seeds (Fig 6B). Over all, an average of 2.5 mg/g FW was noted in roots and shoots of pea developed from primed seeds (Fig 6B).

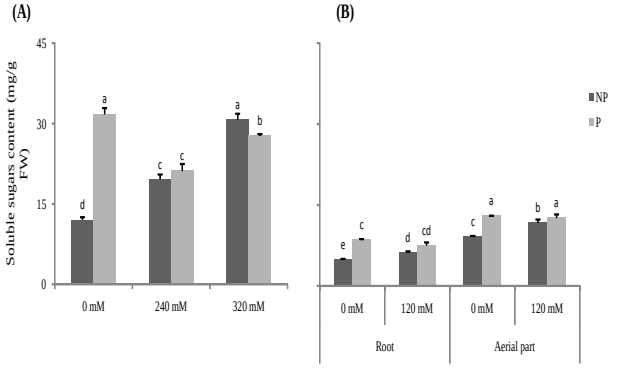

Figure 6. Influence of seed priming, with leaf aqueous extract of lesser bulrush, on total soluble sugars content (mg/g FW) in (A) germinated seeds and (B) in root and shoot of pea under salt stress. Each bar indicates mean ± SD; n = 4; Bars with different letters differ significantly at $p \leq 0.05$.NP = Non-priming; P = Seed priming.

3.7. Influence of seed priming on Sodium, Potassium and Phosphorus content of pea germinated seeds and seedling

Mineral composition in pea was significantly ($p \leq 0.05$) affected by salt stress and priming. Overall, increasing salinity produced increasing in Na accumulation but decreasing in K and P content in pea germinated seeds and shoots, and the priming effect was different (Fig 7).

For pea germinated seeds, the figure 7A showed that Na content was increases by 4.5 and 5 times respectively at 240 and 320 mM in not primed seeds. While, priming decreased this amount by an average of 0.9 times, in all cases. Salinity decreases the K content in NP seeds by 0.9 times, when priming slightly ameliorated this content without and under salt conditions. Also, priming increased the P content at 0 and 240 and 320 mM by 1.3 times compared to non-primed seeds (Fig 7A a, b, c).

In shoots neither developed from non-primed seeds, Na content was increased, significantly, by 5.9 times at 120 mM NaCl. An average decrease of 0.9 times was noted for Na content at 0 and 120 mM NaCl in shoots developed from primed seeds (Fig 7B a). For K content, it decreased in presence of salt (0.9 times), however, an amelioration of its level was obtained in shoots of primed seeds (1.1 times) in salt conditions (Fig 7B b). Similarly, for P content, its amount was slightly ameliorated by priming in the absence and in the presence of NaCl (Fig 7B c).

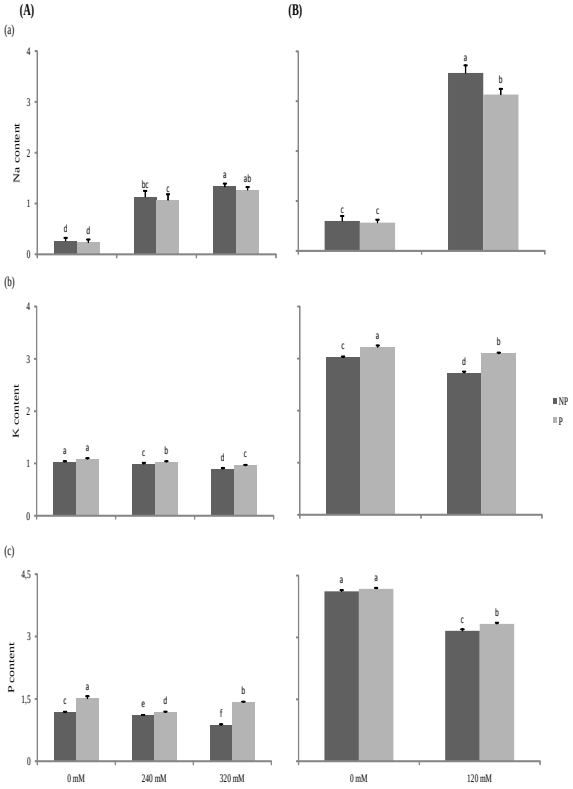

Figure 7. Influence of seed priming, with leaf aqueous extract of lesser bulrush, on sodium (a), potassium (b) and phosphorus (c) content (in %) in (A) germinated seeds and (B) in shoot of pea under salt stress. Each bar indicates mean ± SD; n = 4; Bars with different letters differ significantly at $p \leq 0.05$. NP = Non-priming; P = Seed priming.

3.8. Influence of seed priming on accumulation of secondary metabolites in pea seedlings

The amounts of secondary metabolites were determined in roots and shoots of seedlings developed from primed and non-primed pea seeds in the absence and in the presence of salt (120 mM NaCl) (Fig 8, 9 and 10).

3.8.1. Total phenolic (TP) content

Total phenols were slightly accumulated in pea roots and shoots at 120 mM NaCl where the amounts were increased by an average of 1.1 times, in the absence of priming. The same trend was registered for material from primed seeds (Fig 8).

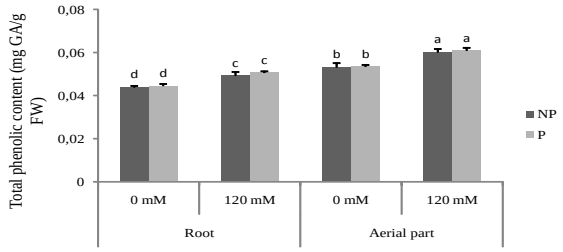

Figure 8. Influence of seed priming, with leaf aqueous extract of lesser bulrush, on total phenolic content (mg GA/g FW) in root and shoot of pea under salt stress. Each bar indicates mean ± SD; n = 4; Bars with different letters differ significantly at $p \leq 0.05$. NP = Non-priming; P = Seed priming.

3.8.2. Total flavonoid (TFd) content

The accumulation of total flavonoids in roots and shoots was accumulated in all cases by the priming. The presence of NaCl in the medium increased this accumulation in seedling of non-primed seeds by an average of 1.5 times. For seedling developed from primed seeds, this accumulation was more important in shoot than in root (Fig 9).

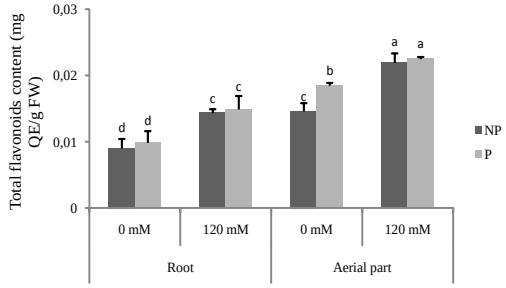

Figure 9. Influence of seed priming, with leaf aqueous extract of lesser bulrush, on total flavonoid content (mg QE/g FW) in root and shoot of pea under salt stress. Each bar indicates mean ± SD; n = 4; Bars with different letters differ significantly at $p \leq 0.05$. NP = Non-priming; P = Seed priming.

3.8.3. Total precipitable alkaloid (TA) content

The figure 10 shows that TA levels increase in pea seedling from not primed seeds under salt conditions (1.2 times). The same trend, the priming increases this accumulation at 0 mM (1.2 times). However, it decreased this content by 0.9 times in root and shoot at 120 mM (Fig 10).

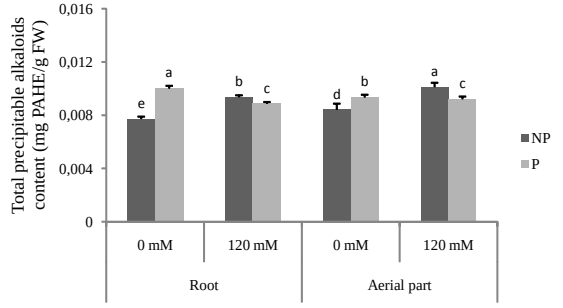

Figure 10. Influence of seed priming, with leaf aqueous extract of lesser bulrush, on total precipitable alkaloids content (mg PAHE/g FW) in root and shoot of pea under salt stress. Each bar indicates mean ± SD; n = 4; Bars with different letters differ significantly at $p \leq 0.05$. NP = Non-priming; P = Seed priming.

4. Discussion

The present study investigated the response of pea (*Pisum sativum* L.) variety Douce Provence to salinity after pretreatment of its seeds by leaf aqueous extract of lesser bulrush. Data showed that salinity had significantly ($p \leq 0.05$) affected germination and seedling growth. Indeed, at 240 mM NaCl, only 50% of pea seeds could germinate, while, at 320 mM the germination was completely stopped. For growth, 120 mM NaCl was responsible of 50% of inhibition of pea root and shoot length. This shows that pea was considered as moderately tolerant to NaCl (Subbarao and Johansen, 1994). Salinity could affect germination in two ways, by decreasing the osmotic potential which retard or prevent the water uptake or by a toxic accumulation of ions which damages the embryo (Soughir et al. 2013). In this respect, Ansari and Sharif-Zadeh (2012) stated that increasing salt concentration decreases the germination percentage and increases germination time. In the present study, salt stress caused a significant reduction in seedling length (Fig 1) and 120 mM NaCl reduced by half the length of pea root and shoot. Bandeoglu et al. (2004) indicated also that this retarded growth is due to inhibition of cell elongation due to higher concentration of Na^+ which causes membrane disorganization, inhibition of cell division and expansion (Deivanai et al. 2011).

Studying the effect of seed priming on pea germination and growth, results demonstrated that this treatment limited the negative impact of salinity because plants raised from primed seeds recorded better germination and growth than plants derived from unprimed seeds where they showed significant amelioration in the absence and in the presence of NaCl (Table 1 and Fig 1). The positive effects of seed priming under salinity conditions have been reported in many crops, such as lettuce (*Lactuca sativa* L.) (Nasri et al. 2011), tomato (*Solanum lycopersicum* L.) (Pradhan et al. 2014), maize (*Zea mays* L.) (Tabatabaei, 2014), pepper (*Capsicum annuum* L.) (Aloui et al. 2014), and milk thistle (*Silybum marianum* L.) (Zavariyan et al. 2015).Seed priming improves seed performance and provides faster and synchronized germination with certain physiological, biochemical, cellular and molecular changes (Di Girolamo and Barbanti, 2012; Siri et al. 2013). These changes include cell division and elongation, plasma membrane fluidity, the induction of stress-responsive proteins, changesin transcriptome and proteome, H^+-ATPase activity and changes in the antioxidant system activity (Varier et al. 2010). Priming is also thought to increase the activity of many enzymes involved in metabolism of carbohydrates (α and β amylases), proteins (proteases) and lipids mobilization (isocitrate lyase) that are implicated in the stored reserves mobilization (Varier et al. 2010; Di Girolamo and Barbanti, 2012). These enzymes are essential in the breakdown of macromolecules for the development and growth of the embryo that ultimately result in early

and higher seedling emergence (Varier et al. 2010). There are reports that priming facilitates the repair of chromosomal damage (Varier et al. 2010), permits early DNA replication and repair, increases RNA and de novo proteinsynthesis and reduces the leakage of metabolites (Paparella et al. 2015).

Salinity made also oxidative stress manifested by electrolyte leakage (EL) and the lipid peroxydation (MDA). In fact, damage of cell membrane caused by salt stress is expressed by its ion permeability, which can be easily measured by the leakage of electrolytes (Lutts et al. 1996b).Data of the present study reveal that membrane permeability was increased in pea seeds germinated and seedling with increasing NaCl stress and with time of incubation (Fig 2). Similar results were recorded on sensitive rice varieties (Lutts et al. 1996b).The priming was beneficial in all cases where the electrolyte leakage was reduced compared to unprimed seeds. This is in agreement with the results of Stevens et al. (2006) on tomatoes and Yildirim et al. (2008) on cucumber.This reduction in EL seems due to better membrane repair during the re-drying process following priming (Farooq et al. 2011).

Membrane lipid peroxidation results in malondialdehyde (MDA) accumulation. The content of MDA is a sign of membrane damage at the cellular level under salt stress (Weisany et al. 2012). Thus, the accumulation of MDA can serve as an important oxidative stress indicator (Gong et al. 2008). Data demonstrated that MDA content of pea germinated seeds and seedling increased significantly in the presence of salt stress (Fig 3) which is consistent with the results of Keutgen and Pawelzik, (2008) and Falleh et al. (2012). Priming used in this study reduced significantly this accumulation (Fig 3) reflecting a reduction in lipid peroxidation and consequent of preservation of cell membranes. The priming strategies reduce malondialdehyde accumulation under salt stress condition in seedlings (Dong et al. 2014; Paparella et al. 2015; Younesi and Moradi, 2015). These results were observed in wheat (Zheng et al. 2008), bean (Azooz, 2009) and alfalfa (Younesi and Moradi, 2014).

Salinity also decreased the activity of dehydrogenases which could be a reflection of cell damage due to exposure to NaCl. The priming was beneficial in all cases, as for, data showed that the formazan level was higher in primed pea germinated seeds and seedling indicating greater metabolic activity (Fig 4). Similar results were reported by Nasri et al. (2011) on lettuce, Mahajan et al. (2011) on rice and Kubala et al. (2015a) on oilseed rape (colza).

It is reported that seed priming can activate processes related to germination by activation of ATPase as well as other enzymes promoting the availability of ATP (Nasri et al. 2011). The activities of several enzymes associated with the germination process have been improved following priming including dehydrogenases (Badek et al. 2014).

As a response to salt stress, pea germinated seeds and seedling accumulated more proline, an organic solutes synthesis for an osmotic adjustment in response to the decreased external water potential (Zavariyan et al. 2015). Proline, also protects the structure of proteins against denaturation, stabilizes cell membranes, and functions as a hydroxyl radical scavenger (Aspinal and Paleg, 1981).Thus, proline content increased significantly in pea seeds and seedlings under salt conditions (Fig 5). This accumulation promotes the reduction of cell osmotic potential and allows an osmotic adjustment to the stress condition (Matias et al. 2015; Kubala et al. 2015b).

In this study (Fig 5), the synthesis of proline was more significant in the material developed from primed seeds; in almost cases. Priming pepper (*Capsicum annuum* L.) seeds caused a marked increase in proline accumulation (Aloui et al. 2014). Moreover, priming enhanced proline content in sunflower (*Helianthus annuus* L.) seedlings under stress conditions (Moghanibashi et al. 2013) In fact, priming may increase the proline content, which can increase crop resistance to salinity (Kazemi and Eskandari, 2012; Kubala et al. 2015 a; b).

Similarly, total soluble sugars content in pea seeds and seedling was also touched by salinity stress and priming treatment (Fig 6). Plants overcome salinity-induced osmotic effects through the accumulation of organic solutes such as sugars; compatible with plant metabolism is an important mechanism of salt tolerance in plants. This mechanism promotes the reduction of cell osmotic potential and allows an osmotic adjustment to the stress condition (Matias et al. 2015; Kubala et al. 2015b).

After priming, the total soluble sugars amounts in pea seeds and seedling increased more proving the benefit impact of this method like it was observed on wheat (Farooq et al. 2011b), barley (Anwar et al. 2011) and coriander (Ben Fredj et al. 2014). This increase was attributed to an increase in α-amylase activity (Lee and Kim, 2000). Thus, it is believed that the priming of the seeds would have improved the vigor of the seedlings, as a result of an increase in the hydrolysis of the starch. Also, the induction of de *novo* synthesis or an increase in hydrolytic enzyme activity, thus producing germination metabolites in required amounts, has been reported (Lee and Kim, 2000).

Salinity and priming had moreover significant impact on mineral balance in pea. In this study, increase in Na while decrease in K and P were observed, but with significant ($P \leq 0.05$) differences in the presence of NaCl (Fig 7). Seed priming alleviates the adverse effects of salinity stress on germination and seedling growth of pea by promoting K and P accumulation and decreasing Na accumulation in the seedlings (Fig 7). The present result is in agreement with the work of Meneguzzo et al. (2000) and Zeid and

Al- Semary (2001). This decreases the osmotic potential of the plant and increases the uptake of water (Ashraf, 2004). Potassium plays an important role in balancing membrane potential and turgor, activating enzymes, and regulating osmotic pressure in cells (Cherel, 2004). The accumulation of these ions and the restriction of the entry of toxic ions into the cytoplasm (Na^+ and Cl^-) are among the strategies for adapting plants to salinity and improving their tolerance to this stress (Lauchli and Epstein, 1990).

Measurement of total phenols, flavonoids and alkaloids content in roots and aerial parts of pea demonstrated that it is also affected by salinity and priming treatment (Fig 8, 9 and 10).

Many authors report that polyphenols are involved in plant responses to salt stress (Wahid and Ghazanfar, 2006). Indeed, it is reported that polyphenols play an important role in protecting the photosynthetic apparatus against photo-oxidative damage (Tattini et al. 2006) and photo-inhibition (Steyn et al. 2002) in salt conditions. Flavonoids which are glycosides localized in the vacuole also display antioxidant activity (Nijveldt et al. 2001). Also, flavonoids, particularly anthocyanins, have a very powerful antioxidant capacity and are involved in the trapping of reactive oxygen species (ROS) accumulated as a result of salt stress (Tsuda et al. 1996). They reduce lipid peroxidation and preserve the integrity of membrane structures (Tsuda et al. 1996).

In addition to these non-enzymatic antioxidants, there are other metabolites with antioxidant properties, such as alkaloids, phenolic acid, and diterpenes, but their exact function in the cell detoxification mechanism is still poorly defined (Ashraf, 2008).

These findings of this study are in agreement with those of Ghezal et al. (2016) where seed priming with leaf aqueous extract of lesser bulrush helped in alleviating the inhibitory effect of salt stress on germination and growth of pea variety Lincoln. Indeed, primed seeds were better able to germinate and develop into a seedling under salt stress by modulating membrane stability, photosynthetic pigments, sugar metabolism, and ionic homeostasis (Ghezal et al. 2016).

5. Conclusion

Seed priming alleviated the inhibitory effect of salt stress of pea seeds and seedling and all of them positively responded to seed priming in the absence and also in the presence of NaCl. Primed seeds become more tolerant to salt stress, probably thanks to the membrane integrity protection and the maintaining of the highest values of osmotica (proline and total soluble sugars, K and P). These findings suggest that priming pea seeds with leaf aqueous extract of lesser bulrush may be considered as an effective alternative method to improve the salt tolerance of pea (*Pisum sativum* L.) variety Douce Provence. In perspective, it is important to continue the study by testing the reproducibility of these results in field crops and in other stages of plant development, as well as on their yield.

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