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ORIGINAL PAPER

Effect of salinity on germination, phytase activity and phytate content in lettuce seedling

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Abstract Seeds of four lettuce (*Lactuca sativa* L.) varieties (Romaine, Augusta, Vista and Verte) differing in their salt sensitivity were sown at 0 (Control), 50, 100 and 150 mM NaCl. The final germination percentage decreased with the increasing salinity and was annulated at the highest salt concentration in *Vista* and Verte, the most sensitive varieties. However, in the less sensitive ones, *Romaine* and *Augusta*, it was slightly modified at 50 and 100 mM NaCl and then decreased by 50% compared with the control, at 150 mM. The effects of NaCl 100 mM on seedling growth, phytase activities, phytate and inorganic phosphorus contents were studied in *Romaine* and *Vista* showing different behaviours towards salinity. Radicle and hypocotyl length and fresh and dry weights were reduced by salt treatment in both varieties. In addition, radicle phytase activity exhibited an increase in *Romaine* (less sensitive) and a decrease in *Vista* (more sensitive). In hypocotyl, this activity showed no difference with the control in the two varieties. However, in cotyledons, and during early hours after germination, salinity decreased phytase activity in both varieties whereas in the later hours (72–96 h) this activity reached the value of the control in *Romaine*. The enhancement of phytase activity was concomitant with an increase in orthophosphate content and a decrease in phytate reserve. These results suggest that salt presence in the medium delays Pi remobilization from phytate stock, but stimulates assimilation of phosphorus more than its accumulation in the organs of the two lettuce varieties.

Keywords Germination · Lettuce · Phytase · Phytate · Salinity

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Introduction

The germination of seeds is one of the most crucial and decisive phases in the growth cycle of plant species since it determines plant establishment and the final yield of the crops (Beweley 1997). In nature, several environmental constraints, such as salinity (Kashem et al. 2000), water stress (Kebreab and Murdoch 1999) and temperature (Welbaum et al. 1998) can inhibit or reduce seed's ability to germinate. This stage is a major factor limiting the establishment of plants under saline conditions (Al-karaki 2001). However, salt stress affects germination percentage, germination rate and seedling growth in different ways depending on plant species. NaCl decreased germination percentage, speed of germination and seedling dry matter in different types of rice (Khan et al. 1997) and reduced final germination percentage in wheat (Almansouri et al. 2001). However, in pepper, NaCl delayed germination but did not reduce the final germination percentage (Chartzoulakis and Klapaki 2000).

Germination process inhibition may be related to the altered activities of hydrolytic enzymes, including phytases, phytate-specific phosphatases that hydrolyse phytate to inositol and free orthophosphate (Greiner et al. 1998). The phytate is considered as the primary storage form of phosphorus and inositol in almost all seeds (Ravindram et al. 1994). It forms a complex salt called phytin with counter ions including K^+, Mg^{2+}, Ca^{2+} and Zn^{2+}, which represents a significant reserve of minerals in seeds (Mitsuhashi et al. 2005). The use of this phosphorus and mineral stock by germinating seeds is dependent on phytase activity stimulation, generally low during seed maturation and in dry seeds (Laboure et al. 1993). Indeed, a marked increase in phytase activity was reported in germinating seeds of maize, wheat and rice and was associated with a concomitant decrease in phytate content and an increase in that of phosphate (Laboure et al. 1993; Greiner et al. 1998, 2000).

The sensitivity to salinity of a given species or cultivar may change during ontogeny. It may decrease or increase depending on the plant species, cultivar or environmental factors (Foolad and Lin 1997). The results of Zapata et al. (2003) on nine varieties of lettuce indicate a relatively high tolerance during germination, since high levels of NaCl, as 150 mM, delayed the germination, but most seedlings were able to germinate under such conditions. Salt tolerance variability during germination stage is often attributed to differences in the activities of some enzymes like phosphorylitic enzymes as shown in rice (Dubey and Sharma 1990).

The aim of the present work was to study in a first step the effects of a range of NaCl concentrations on germination kinetics of four *Lactuca sativa* varieties then in a second step the variations of phytase activity and phytate content in two selected varieties exhibiting contrasting responses towards salinity.

Materials and methods

Plant material and germination conditions

The seeds of the four lettuce varieties (Romaine, Augusta, Vista and Verte) used in this investigation were provided by the Seed Laboratory of the Tunisian Ministry of Agriculture. For germination, seeds were soaked for 2 h in distilled water or different NaCl concentrations 50, 100 and 150 mM. The seeds were then placed in Petri dishes with double-layer filter paper initially moistened with a solution of the respective salt concentration. The Petri dishes were incubated for 4 days in the dark at room temperature (25 ± 2°C). Each treatment consisted of 25 seeds per Petri dish and was replicated three times. Seeds with emerged radicle were counted daily. Final germination percentage (FG%) was calculated as 100 × number of germinated seeds divided by number of sown seeds. After 4 days, seedlings were divided into radicle, hypocotyl and cotyledons for determination of growth parameters. Fresh weights (FW) of all samples were recorded. Plant material was dried at 60°C for 2 days and dry weights (DW) were measured. Tissue water content was obtained from the (FW − DW/FW) ratio.

Preparation of enzymatic extracts

Germinated seeds were separated into radical, hypocotyl and cotyledons and then ground separately in 0.1 M acetate buffer (pH 5.4). The homogenate was centrifuged at 13,000*g* for 15 min and the supernatant was analyzed for phytase activity, protein determination and phosphate content. All procedures were carried out at 4°C.

Determination of total protein concentration

Total protein concentration of the supernatant was determined according to the method described by Bradford (1976) with bovine serum albumin as a standard.

Determination of phytase activity

Phytase activity was performed by measuring the release of phosphate from sodium phytate (Houde et al. 1990). Phytate and *p*-nitrophenyl phosphate were used as substrates to distinguish between enzymes able to hydrolyse phytate (phytases) and the other acid phosphatases (Greiner et al. 2000). One unit of phytase activity was defined as 1 µmol phosphate per min under the following conditions: pH 5.4, temperature 37°C (Sung et al. 2005). The reaction mixture consists of 500 µl of 1 mM sodium phytate, 400 µl of 0.1 M acetate buffer (pH 5.4) and 100 µl enzyme sample. The enzyme reaction was stopped by adding 1 ml of 10% (w/v) trichloroacetic acid (TCA) after incubation for 1 h 30 min at 37°C. Another aliquot of 100 µl enzyme sample received 400 µl of acetate buffer and 500 µl of the phytic acid substrate, but the reaction was stopped immediately with TCA without incubation. The Pi in the phytic acid substrate was determined as a blank. Pi concentration in the extracts was measured spectrophotometrically at 630 nm using malachite green (Ohno and Zibilske 1991). The phytase activity was calculated as the difference between Pi in the extracts with and without incubation, and expressed in nmol of Pi released per min per organ.

Determination of Pi content

Pi content of the supernatant was determined according to the method described by Ohno and Zibilske (1991) in

different parts of lettuce seedling (radicle, hypocotyl and cotyledons).

Determination of phytate in seeds

Phytate content was determined in the cotyledons, the richest organs of germinated lettuce seeds. The extraction of phytic acid was carried out with 3% sulphuric acid for 60 min. The sample was then centrifuged at 13,000*g* for 20 min and the supernatant was recovered. Extract purification was accomplished by iron (III) phytate precipitation (March et al. 1995). Sample filtrate (1 ml) was mixed with 0.4 ml of 0.1 M $FeCl_3$ $6H_2O$ in 3% H_2SO_4. The precipitate formed was separated by centrifugation (20 min at 13,000*g*) and washed with distilled water. 1 ml of 1.5 M NaOH and 7 ml of distilled water were added to the solid iron (III) salt in order to precipitate hydrated iron (III) oxide and release phytate. After 30 min at 80°C the supernatant, containing the phytate, was separated by centrifugation.

Then the supernatant was mineralized at 150°C. When the precipitate dried, 60% of perchloric acid ($HClO_4$) was added and mineralized at 220°C until a clear supernatant was obtained. Pi concentration in the supernatant was measured spectrophotometrically at 630 nm using malachite green (Ohno and Zibilske 1991).

Determination of different Pi fraction: accumulated and assimilated

Assuming that P phytate which had disappeared from cotyledons had been remobilized and thereafter either accumulated as Pi or assimilated in the different organs, assimilated P was estimated as the difference between initial phytate P and retrieved Pi.

Statistics

Data were presented as the mean of six seedlings for each treatment. Significant differences between treatments were analysed using ANOVA and mean comparison with Duncan test (Statistica®). Values were calculated at the $p = 0.05$ probability level.

Results

Germination kinetics differed according to varieties and salt treatments (Fig. 1). All germination curves reached a plateau ($FG\%_{max}$: maximum FG%), but they differed on the value of $FG\%_{max}$ (Table 1) and on the time necessary to reach this value. For Romaine seeds, $FG\%_{max}$ exceeded 90% at 0, 50 and 100 mM NaCl, but it was reduced to ca. 40% at 150 mM NaCl. Furthermore, the latency for radicle emergence augmented with salt concentration. In this variety, the germination kinetics at 50 mM NaCl was not distinguishable from that at 0 mM NaCl. The Augusta variety displayed germination kinetics similar to those of Romaine, except that $FG\%_{max}$ values were slightly more sensitive to salt at intermediary concentrations (50 and 100 mM) and the plateau was reached more slowly. Vista differed from the two former varieties mainly because $FG\%_{max}$ was strongly lowered by increasing salt concentration, germination being suppressed at 150 mM NaCl. Verte was the most salt-sensitive among the four varieties,

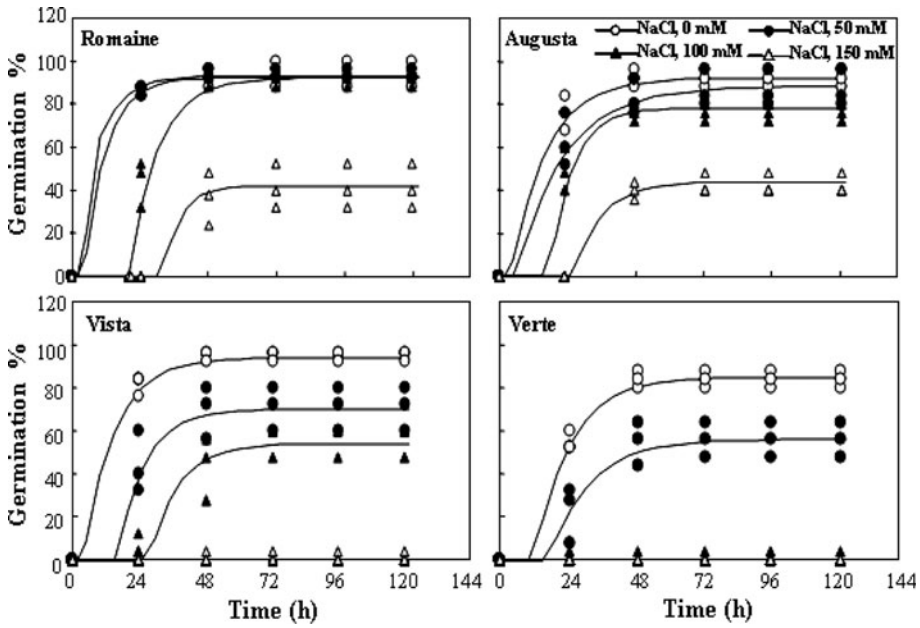

Fig. 1 Evolution of germination percentage of four lettuce varieties under different NaCl treatments. Data are the mean of three samples of 25 seedlings each one

Table 1 Effect of different NaCl treatments on the final germination percentage ($FG\%_{max}$) of four lettuce varieties (Romaine, Augusta, Vista and Verte)

Final germination percentage				
NaCl (mM)	Romaine	Augusta	Vista	Verte
0	93	92	94	85
50	92	88	70	56
100	92	78	54	0
150	42	44	0	0

Values deduced of the results of Fig. 1

with a complete suppression of germination at both 100 and 150 mM NaCl. In this variety, 50 mM NaCl was sufficient to limit $FG\%_{max}$ at two-thirds of its value at 0 mM NaCl.

Romaine (the variety with the most salt tolerant germination) and Vista (moderately salt sensitive germination) were chosen to analyze the effect of salt on growth parameters and enzymatic activities at 100 mM NaCl, 4 days after sowing. At this stage, seedling biomass did not differ significantly between the two varieties in the absence of salt (Table 2). In the presence of 100 mM NaCl, whole seedling biomass was diminished by 32% in Romaine (0.65 ± 0.20 mg DW plant^{-1}) and by 43% in Vista (0.57 ± 0.09 mg DW plant^{-1}), the salt effect being statistically significant. Radicle growth was more affected by salt than was shoot growth. In Romaine, radicle biomass was restricted by −40% as compared with control (no salt), whereas shoot growth was restricted only by −29%. For Vista, these values were −48% for radicle biomass and −40% for shoot biomass. Radicle elongation was still more sensitive to salt inhibition: radicle length was diminished by 74% (Romaine) to 77% (Vista) by salt. However, radicle water content was poorly diminished (Romaine) or even augmented (Vista). In hypocotyl, salt slightly limited water content (−2% of control in Romaine and −17% in Vista) and length (−24% for both varieties).

In cotyledons, phytate P initially amounted to 0.2 µmol seed^{-1} in *Romaine*, and 0.5 µmol seed^{-1} in *Vista* (Fig. 2). In cotyledons of seeds with recently (24 h) emerged radicle phytate amount was diminished by 85–90% in Romaine and by 75–80% in Vista. At later times, cotyledon phytate continued to decrease slowly and eventually disappeared. Parallely, Pi content of the seedlings (including radicle, hypocotyl and cotyledons) increased, reaching high levels at the later time. Assuming that P phytate which had disappeared from cotyledons had been remobilized and thereafter either accumulated as Pi or assimilated in the different organs, assimilated P was estimated as the difference between initial phytate P and retrieved Pi. In the absence of salt, the latter fraction

Table 2 Effect of salinity (NaCl 100 mM) on radicle and hypocotyl length, and the water content and dry weight in radicle, hypocotyl and cotyledons in two lettuce varieties (Romaine and Vista)

	Radicle			Hypocotyl			Cotyledon	
	Length (cm)	Water content (ml g^{-1} MS)	Dry weight (mg)	Length (cm)	Water content (ml g^{-1} MS)	Dry weight (mg)	Water content (ml g^{-1} MS)	Dry weight (mg)
Romaine								
Control	4.03 ± 0.37^a	10.00 ± 2.30^a	0.25 ± 0.06^a	2.77 ± 0.28^a	20.22 ± 3.16^a	0.32 ± 0.12^a	6.75 ± 0.86^a	0.38 ± 0.08^a
NaCl, 100 mM	1.05 ± 0.11^b	9.67 ± 2.47^a	0.15 ± 0.06^b	2.11 ± 0.18^b	19.83 ± 6.45^a	0.25 ± 0.09^b	8.17 ± 2.30^a	0.25 ± 0.09^b
Vista								
Control	5.18 ± 0.49^a	10.81 ± 1.83^a	0.23 ± 0.05^a	2.26 ± 0.17^b	21.97 ± 2.40^a	0.37 ± 0.05^a	5.28 ± 0.86^a	0.40 ± 0.09^a
NaCl, 100 mM	1.18 ± 0.28^b	13.83 ± 3.21^a	0.12 ± 0.04^b	1.71 ± 0.13^a	18.25 ± 2.10^a	0.22 ± 0.04^b	8.31 ± 1.42^a	0.23 ± 0.05^b

Values are means of six replicates ± SD. Means not sharing a common letters (a, b, or c) in column are significantly different ($p \leq 0.05$) as assessed by Duncan's multiple range tests

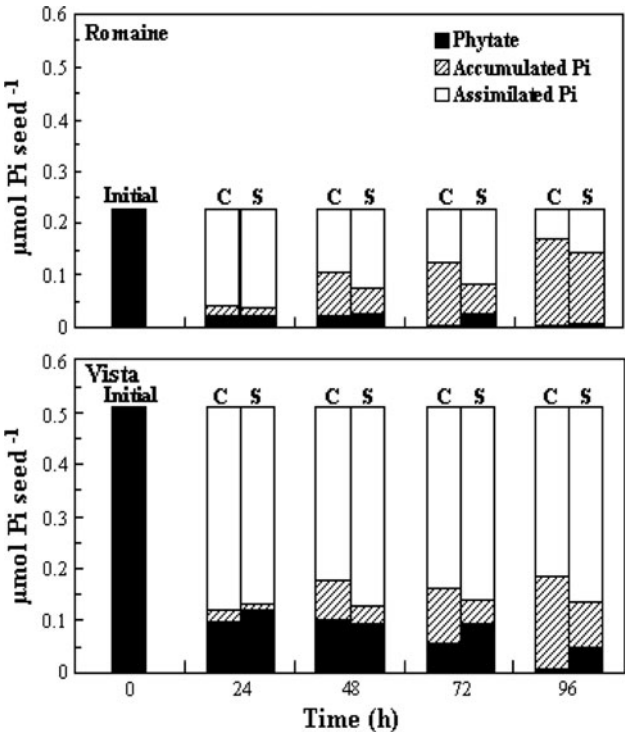

Fig. 2 Phosphorus metabolism in radicle, hypocotyl and cotyledons of two lettuce varieties (Romaine and Vista) during germination. *Phytate* µmol Pi of purified cotyledons phytate during germination. *Accumulated Pi* Pi accumulated in lettuce seedling as a function of time. *Assimilated Pi* calculated as the difference between remobilized and accumulated Pi during germination. (*C* 0 mM NaCl, *S* 100 mM NaCl). Values are means of six replicates

progressively built up, representing after 4 days more than two-thirds of P remobilised from phytate in Romaine, but only 30% in *Vista*. The same trend was observed in the presence of salt, but Pi contents remained lower than in control treatment, and accordingly, represented lower proportion of remobilised P. The two varieties differed on the evolution of assimilated P. This fraction diminished quickly with time in Romaine and more rapidly in the absence of salt. In Vista, the diminution of assimilated P in the absence of salt was slower than in Romaine, and no diminution at all was observed in the presence of salt.

Phytase activity was much higher in cotyledons than in radicle and hypocotyl (Fig. 3). In the former organs, it increased with time and reached a maximum at 2 (Romaine) or 3 (Vista) days. In presence of salt, this enzyme activity was almost completely inhibited in the radicle of the sensitive variety, Vista, and showed an early decline in its cotyledons. These effects seem very slightly marked in the organs of the relatively tolerant variety (Romaine). However, in both varieties, phytase activity was poorly dependent on salt at the last time, identical values being reached in the cotyledons after 3 to 4 days at 0 and 100 mM NaCl.

Discussion

Given the results of this study, it appeared that salt treatment slowered down germination almost in the same way, in all varieties (Fig. 1). On the contrary, it reduced significantly the final germination percentage ($FG\%_{max}$) since the lower NaCl concentration (50 mM) in Vista and Verte varieties, and only at the higher NaCl concentration (150 mM) in Romaine and Augusta ones (Table 1). In accordance with our results Jamil and Rha (2004) reported that germination of sugar beet and Cabbage decreased as the salinity concentration increased and salinity also delayed germination rate. Differences of salt sensitivity, at the germination stage, were also described by Zapata et al. (2003) in nine cultivars of *Lactuca sativa*: under saline conditions, $FG\%_{max}$ was reduced in Iverna and Baby star cultivars, whereas it reached a rate of 100% in the other seven cultivars. On the whole, these results indicated that increased salinity results in decrease in germination percentage and rate of germination (Almansouri et al. 2001). Salt-induced inhibition of seed germination could be attributed to osmotic stress or to specific ion toxicity (Huang and Redman 1995). According to Al-Karaki (2001) the adverse effect of salt stress on seed germination in barley may result from internal osmotic stress or restricted imbibitions rather than from ion toxicity effects.

After germination, salt treatment decreased early growth of young seedlings in the two lettuce varieties (Table 2). Radicle and hypocotyl lengths are the most important characteristics for salt stress because the first organs are in direct contact with soil and absorb water from soil and the second supply the rest of the plant with water. For this reason, radicle and hypocotyl lengths provide an important clue to the response of plants to salt stress (Jamil and Rha 2004). The reason for reduced radicle and hypocotyl development may be due to toxic effects of the Na^+ and/or Cl^- in their tissues as well as unbalanced nutrient uptake induced by salt. High salinity may also inhibit root and shoot elongation due to slowing down the water uptake by the plant (Werner and Finkelstein 1995). Salt stress inhibited radicle growth more than that of hypocotyl in two lettuce varieties. Similar observations have been reported in sugar beet, cabbage (Jamil et al. 2006), barley and *Brassica* species (Huang and Redman 1995). Decreases in seedling vigour under salinity were also mentioned by Zapata et al. (2003) in nine lettuce varieties. Stavir et al. (1998) showed that a salinity of 75 mM NaCl caused a decrease of about 50% in germination and 60–70% in the lengths and weights of roots and shoots of chickpea seeds as compared with control. However, the mechanism of growth inhibition by salt is still unclear.

In the present study, an increase in phosphorus content of lettuce seedling was observed with a significant decrease

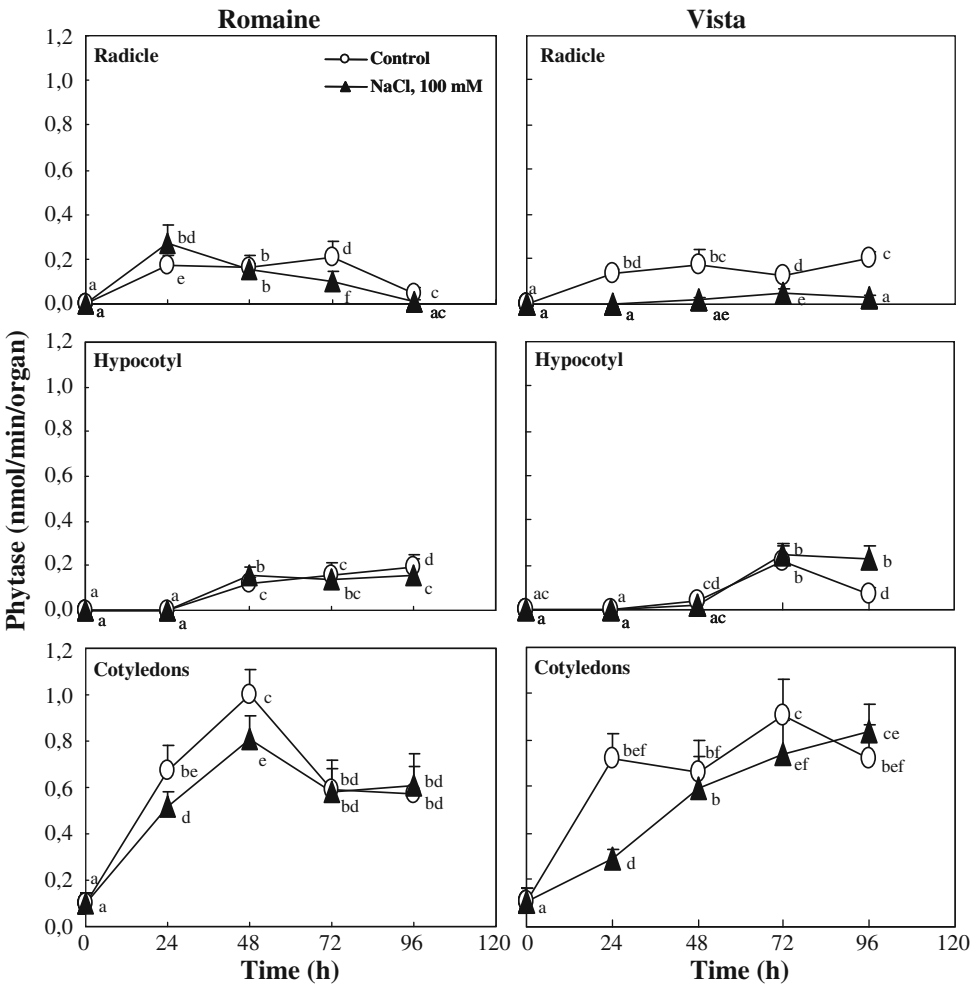

Fig. 3 Phytase activity during germination in radicle, hypocotyl and cotyledons of two lettuce varieties (Romaine and Vista) after imbibitions with distilled water (Control) or salt solution (NaCl, 100 mM). Values are means of six replicates ± SD. Means not sharing a common letters (*a*, *b*, *c*, *d*, *e*, or *f*) are significantly different ($p \leq 0.05$) as assessed by Duncan's multiple range tests

in phytate content in cotyledons (Fig. 2). Phytase activity in the seeds of two lettuce varieties (Romaine and Vista) was high at the beginning of germination process and enzymatic peaks were observed in both varieties. Afterward, a decline in phytase activity was noticed (Fig. 3). Seed phytates constitute a Pi source during the early stages of germination when phosphate supply is a limiting factor or when there is no other phosphate source for this process. Phosphate is released from phytate through the enzymatic action of phytases that are abundant in dry seeds and increases rapidly during germination (Kikunaga et al. 1991). Centeno et al. (2001) observed an increase of phytase activities with a concomitant decrease of phosphorus phytate and an increase in the content of inositol phosphates during the rye and barley germination. In other works, it was reported that processes, such as soaking and germination, activate the endogenous phytases, which are able to hydrolyse Inositol-6-phosphate (Suleiman et al. 2007). Sung et al. (2005) noticed a significant increase in phytase activity up to 7.9-fold, occurred during the first several days of barley germination, followed by a decrease. Phosphate production (viz. phytate degradation) in the barley seedlings occurred rapidly during the early stage of germination.

Abiotic stresses induce various biochemical and physiological responses in plants. Several hundred genes have been identified as the genes that respond to these stresses at the transcriptional level (Zhu 2002; Kreps et al. 2002). Recently, tilling array technology has become a useful tool for analysis of whole genome in response to abiotic stress (Matsui et al. 2008). A number of phytase-encoding genes have been identified in soybean (Hegeman and Grabau 2001), Medicago (Xiao et al. 2005) and tobacco (Lung et al. 2008). By sequence homology, currently known plant phytases are classified into two families, histidine acid phosphatases (HAPs) and purple acid phosphatases (PAPs), which were first discovered in maize (Maugenest et al. 1997) and soybean (Hegeman and Grabau 2001), respectively.

Salt treatment (NaCl, 100 mM) showed different effects on phytase activity in the two lettuce varieties (Fig. 3). In radicle, phytase activity decreased in the sensitive variety (Vista), which was associated with a restriction of the quantity of phosphorus remobilised from phytate reserves.

According to Gopal et al. (1983), the application of salt treatment lowers the phytase activity in groundnut cotyledons during germination, thereby delaying the breakdown of phytate. In Romaine, the variety that exhibited a salt insensitivity at germination stage, salt treatment improved phytase activity, which was due to maintenance of phosphorus remobilisation at the level of the control. These results agree with those found in rice by Dubey and Sharma (1990). The authors suggest that in the tolerant cultivar, salinity may increase phytase activity in endosperm during the early hours of germination. The activated phytase appears to maintain higher cell metabolic status by providing a higher rate of phosphate release and active transport and biosynthetic events in growing embryoaxes. Liao et al. (2003) showed that NaCl stress causes a general induction of a purple acid phosphatase gene expression (GmPAP3) in both roots and leaves of various soybean varieties. Higher activities of phytase enzymes under salt stress conditions in tolerant varieties suggest their direct role in maintaining the much higher energy requirement of the cell to cope with adverse effects of salinity. In the salt-sensitive variety, decreased phytase activity under salt stress may decrease the general metabolic status in germinating seeds. This decrease of endosperm reserves hydrolysis reduced active transport and thereby limited the availability of phosphate in growing embryoaxes, which ultimately led to decreased seed germination and seedling vigor (Dubey and Sharma 1990).

In conclusion, the results of this study demonstrate that salt tolerance during germination exist within lettuce varieties. Indeed, salinity inhibited germination and early seedling growth in sensitive variety. This effect was correlated with reduction in phytase activity and limitation in the energy need of germinating seeds. In tolerant variety, higher germination processes in the presence of salt was correlated with activation of phytase activity and higher Pi remobilized in germinating seeds.

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