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Potassium Nitrate Priming Affects the Activity of Nitrate Reductase and Antioxidant Enzymes in Tomato Germination

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Abstract

Priming has been used to improve the performance of germination at the field, and potassium nitrate (KNO_3) is a promising compound for this purpose. The nitrate (NO_3) could be absorbed, being used in the metabolism of the embryo, through the enzyme nitrate reductase (NR). Besides, the priming could also activate the response of the antioxidant system, becoming the primed seeds more prepared for possible stresses. Thus, the aim of this study was to evaluate the metabolic effect of nitrate in tomato seed germination by the quantification of NR activity, and also evaluate the activity of some antioxidant enzymes as superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX). Tomato seeds were primed using solutions of polyethylene glycol (PEG 6000) -1,1MPa, 50 mM KNO_3 and PEG+KNO_3. The variables analyzed were germination (germinability, mean germination time, mean germination rate, coefficient of variation of the germination time, uncertainty and synchrony) nitrogen, total proteins and enzymes. The germination data were analyzed using an ANOVA, comparing the averages by Scott-Knott test ($P < 0.05$). To analyze the nitrogen, protein and enzymatic activities, we used a Kruskal-Wallis test ($P < 0.05$). The results show an increase in the NR activity, as well as in the antioxidant enzymes. The germination time (t) and germination rate (v) primed in KNO_3 had a better performance compared to the other treatments. In conclusion, the observed benefits in tomato seeds primed with KNO_3 were related to the activity of the enzyme nitrate reductase in the production of nitrite/nitric oxide, which acted promoting a faster germination.

Keywords: KNO_3, PEG, priming, germination, tomato

1. Introduction

The most sensitive stages, for many crop species submitted to the stress conditions, are seed germination and early seedling growth (Rahimi, 2013). Therefore, the seed germination performance indicators (germinability, germination time, germination rate and synchronization index) are very important for successful crop production.

Seed priming has been used to accelerate the germination, uniform seedling emergence and improve a germination performance under the temperature or drought stresses (Janmohammadi, Dezfuli, & Sharifzadeh, 2008; Jahangir, Amjad, Afzal, Iqbal, & Nawaz, 2009). Priming starts some of the metabolic process to occur in germination without a radicle protrusion. Various seed priming techniques have been developed in different medias: hydropriming (water), osmopriming (low water potential solutions such as polyethylene glycol – PEG), halo-priming (salt solutions) (Hamidi, Pirasteh-Anosheh, & Izadi, 2013; Chen, R. Arora, & U. Arora, 2010). Seed priming with nitrate solutions resulted in better seed quality and stand establishment in maize field grown (Hanegave, Hunje, Nadaf, Biradarpatil, & Uppar, 2011). Nitrate solutions helped in shorting a time required for spread of germination in tomato (Farooq, Basra, Saleem, Nafees, & Chisthi, 2005).

The biological mechanism of priming could be the response of antioxidant systems (Chen & Arora, 2010). The priming with nitrate solutions stimulates the germination, so Hendricks and Taylorson (1974) suggested that its mechanism could be trough a nitric oxide (NO) synthesis. NO breaks a seed dormancy trough the interaction with the phytochrome signaling pathways, the ethylene biosynthesis, and interplays with reactive oxygen species - ROS (Sírová. Sedlárová, Piterková, Luhová, & Petrivalsky, 2011). In germinating seeds, the nitrate reductase (NR) and NO synthase (NOS) are among the strongest candidates for the enzymatic sources of NO. Nitrate broke the seed

dormancy in *Arabidopsis thaliana* seeds by reducing abscisic acid levels (Matakiadis et al., 2009). It is considered NO dependent in mechanism of dormancy breaking in lettuce seeds (Dong, Tong, Xiao, Cheng, & Song, 2012).

Tomato shows the primary seed dormancy, which can reduce germination capacity or/and germination speed. When tomato seeds are imbibed in PEG solution, the seed dormancy is broken (Hilhorst & Downie, 1995).Tomato seeds priming with potassium nitrate (KNO_3), PEG or NaCl have been shown to improve the germination, seedling emergence and the initial growth of various plant species (Govinden-Soulange & Levantard, 2008; Zhang et al., 2012). Salt solution of potassium nitrate (KNO_3) was more effective than PEG in improving the germination speed in tomato (Frett et al., 1991).

The benefit role of nitrate in tomato seed germination had been proved in various studies, but its metabolic involvement is still not clear. The aim of this study was to evaluate the metabolic effect of nitrate (KNO_3) in tomato seed germination by the quantification of the nitrate reductase activity, and the activity of some antioxidant enzymes.

2. Materials and Methods

2.1 Plant material and Priming

The tomato seeds (*Lycopersicon esculentum* Mill.), cv. Santa Clara, from the ISLA Co. Ltd, were priming in polyethylene glycol solution (PEG 6000) -1,1MPa, 50 mM KNO_3 and PEG + KNO_3 (half and half). For the technique of priming, the seeds were treated with continuous aeration at 15 °C on light, for six days. The primed seeds then were washed twice with distilled water and dried to their original moisture content (7.0%) at 25 °C, to perform the germination tests.

2.2 Determination of Nitrogen and Total Proteins

Seeds were dried at 60 °C for 48 hours, weight and subsequently milled. The milled seeds (0.01 g) were placed into 2 mL of concentrated sulfuric acid, and put into the digester block with distillated water, in one micro-distiller. Titration was made with 0.1 N sulfuric acid. The total N was quantified by standard Kjeldahl method (Association of Official Analytical Chemistry [AOAC], 1984). Proteins were quantified by Bradford assay (1976).

2.3 Enzymatic Activities

Nitrate reductase (NR) activity of seeds was assayed *in vivo* by the modified method of Delú Filho, Oliveira, Alves and Purcino (1997). Soaked seeds were weighted (0.6 g), put in vacuum for two minutes, and followed by a bath for 75 minutes at 30 °C. Aliquots of 2 ml were withdrawn and NR activity was measured *in vivo* by formation of NO_2, measured by the absorbance at 540 nm, by using a spectrophotometer BECKMAN, model DU 640. The absorbance values of samples were compared with a standard nitrate curve.

The extracts of the activities of superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) were obtained by a method of Biemelt, Keetman and Albrecht (1998). Ground soaked seeds (0.2 g) were mixed with 100 mM of potassium phosphate buffer (pH = 7.8), EDTA (0.1 mM) and ascorbic acid (1 mM). Extracts were centrifuged at 13,000 g at 4 °C, for 20 min. Supernatant was collected and used for enzyme assay.

SOD activity was measured by method of Giannopolitise and Ries (1977), adapted for a tomato seed analyses. The reaction mixture included: 50 mM of potassium phosphate buffer (pH = 7.8), 14 mM of methionine, 75 µM of nitro blue tetrazolium, 2 µM of riboflavin, 100 nM of EDTA and 20 µl of enzyme extract. The reaction was initiated by adding the riboflavin and placing under a light bank consisted on two15 W fluorescent tubes, 30 cm long. The reaction was allowed to continue for 40 min, and was terminated by switching off the light and covering the tubes with a black cloth. Subsequently, the absorbance of reaction mixture was measured at 560 nm at spectrophotometer BIOTEK, EPOCH model. One unit of SOD activity was defined as the amount of the enzyme required to reach 50% inhibition of the reaction in the "minus enzyme extract" control, which should have the higher absorbance compared to the samples with the enzyme extract.

CAT activity was measured using the protocol of Havir and McHale (1987). The reaction mixture included 200 mM of potassium phosphate buffer (pH 7.0), 20 µl of enzyme extract, and 120 µl of 12.5 mM H_2O_2 solution. One unit of CAT activity was defined as the degradation of 1 µM of H_2O_2 during one minute at 240 nm (using extinction coefficient $\varepsilon = 36\ mM^{-1}\ cm^{-1}$).

APX activity was measured by the adaptation of the Nakano and Asada (1981) method for tomato seed analyses. The reaction mixture was composed of 50 mM of potassium phosphate buffer (pH 7.0), 0.5 mM of ascorbic acid (ASA), 0.1 mM of EDTA, 20 µl of enzyme extract. H_2O_2 (0.1 mM) was added to initiate the reaction. One unit of APX was defined as the conversion of ASA (1 µM) into the monodehydroascorbate at 290 nm, during one minute ($\varepsilon = 2.8\ mM^{-1}\ cm^{-1}$).

2.4 Germination Tests

Four replications with 50 seeds were used as basic sample and placed at each petri dish. The petri dishes were moistened with distilled water and placed in germinator at 20-30 °C and photoperiod of 12 hours. The number of germinated seeds was recorded daily, during the period of seven days. A seed was considered germinated when its radicle emerged. At the end of this period, the germinability (G%), mean germination time (t), mean germination rate (v), coefficient of variation of the germination time (Cv_t), uncertainty (U) and synchrony (Z) were calculated (Ranal, Santana, Ferreira, & Mendes-Rodrigues, 2009).

2.5 Statistical Design

The experiment was arranged as a completely randomized design with four replications and 50 seeds per replicate for each solution. The priming solutions were PEG, KNO_3, PEG + KNO_3 and control (unprimed seeds). Germination percentage and germination rate were subjected to analysis of variance (ANOVA). The differences between the means were compared using Scott-Knott test ($P < 0.05$). Data about the nitrogen and protein concentrations and enzymatic activities were subjected to Kruskal-Wallis test ($P < 0.05$).

3. Results

3.1 Determination of Nitrogen and Total Protein Content

The nitrogen level did not vary with priming (Figure 1A), but concentration of total proteins increased with KNO_3 (Figure 1B and Table 1).

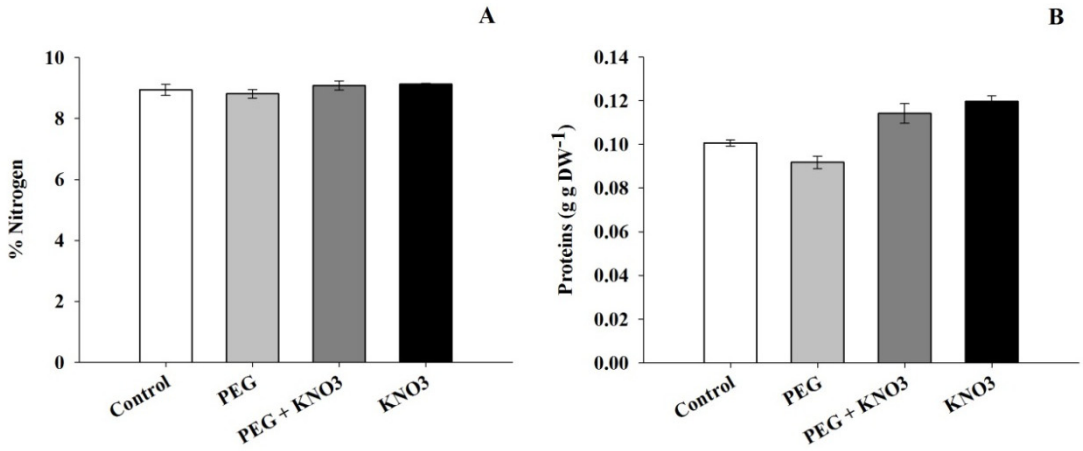

Figure 1. Mean values and standard error for of **A)** nitrogen percentage and **B)** protein concentration in tomato seeds observed for different priming solutions (PEG, PEG + KNO_3 and KNO_3)

Table 1. Mean values and Kruskal-Wallis test for nitrogen percentage and protein concentrations of tomato seeds in different priming solutions (PEG, PEG + KNO_3 and KNO_3)

Variable	Treatment	n	mean	p-value
Nitrogen %	Control	3	8.94	0.1808
	KNO_3	3	8.81	
	PEG	3	9.08	
	PEG+KNO_3	3	9.12	
Proteins	Control	3	0.10	0.02692
	KNO_3	3	0.11	
	PEG	3	0.09	
	PEG+KNO_3	3	0.11	

$p < 0.05$ were considered significant and marked in bold.

3.2 Nitrate Reductase Activity

The supply with KNO_3, increased the NR activity compared to PEG + KNO_3, PEG and control (Figure 2, Table 2), promoting an increase in germination speed (Table 4). When primed with PEG and PEG + KNO_3 the reduction in NR activity was observed in comparison to the control (Figure 2).

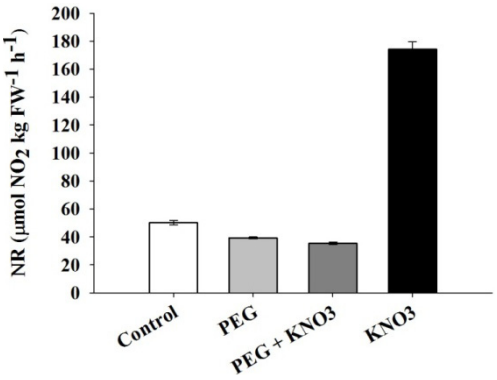

Figure 2. Mean values and standard error for nitrate redutase (NR) activity in tomato seeds priming with different solutions (PEG, PEG + KNO_3 and KNO_3)

Table 2. Mean values and Kruskal-Wallis test of variable nitrate redutase (NR) activity of tomato seeds in different priming solution (PEG, PEG + KNO_3 and KNO_3)

Variable	Treatment	n	mean	p-value
NR	Control	3	50.25	0.01556
	KNO_3	3	174.16	
	PEG	3	39.22	
	PEG+KNO_3	3	35.46	

$p < 0.05$ were considered significant and marked in bold.

3.3 SOD, CAT and APX Activity

In seeds primed with KNO_3, an increase in antioxidant system activity was observed (Figure 3). The seeds priming only with KNO_3 showed the highest SOD activity compared to the treatments with PEG, PEG + KNO_3 and control (Figure 3A, Table 3). Moreover, the CAT activity was highest in KNO_3 priming seeds (Figure 3B, Table 3). The reduction in the activity of APX was observed when a priming treatment with KNO_3 was compared to those with PEG and PEG+KNO_3, while no difference was observed when compared to control (Figure 3C, Table 3).

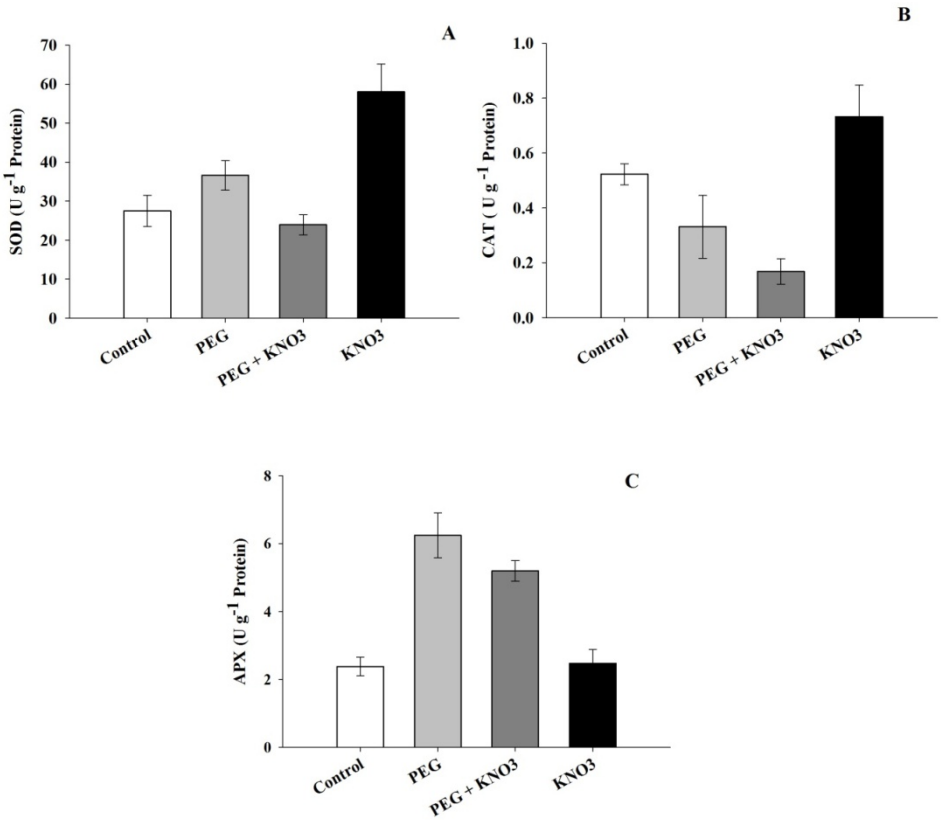

Figure 3. Mean values and standard error for A/ Superoxide dismutase (SOD), B/ catalase (CAT) and C/ ascorbate peroxidase (APX) activities in tomato seeds observed for different priming solutions (PEG, PEG + KNO_3 and KNO_3)

Table 3. Mean values and Kruskal-Wallis test for superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) activities of tomato seeds in different priming solution (PEG, PEG + KNO_3 and KNO_3)

Variable	Treatment	N	Mean	p-value
SOD	Control	3	27.47	0.03069
	KNO_3	3	58.01	
	PEG	3	36.58	
	PEG+KNO_3	3	23.94	
CAT	Control	3	0.52	0.04148
	KNO_3	3	0.73	
	PEG	3	0.33	
	PEG+KNO_3	3	0.17	
APX	Control	3	2.38	0.02998
	KNO_3	3	2.47	
	PEG	3	6.24	
	PEG+KNO_3	3	5.18	

$p < 0.05$ was considered significant and marked in bold.

3.4 Germination Tests

The test of germinability (*G*%) did not show any significant differences among the treatments of priming (Table 4). Seeds priming with KNO_3 had the shortest germination time (*t*) and the highest germination rate (*v*) compared to other treatments. The seeds primed with KNO_3 take less time to germinate and sprout more seeds in a shorter time. The uncertainty (*U*) and synchrony (*Z*) did not show variation among the treatments, indicating that KNO_3 did not interfere in germination synchronization in tomato seeds. Sample homogeneity had not been affected by osmopriming, analyzing the coefficient of variation of the germination time (Cv_t).

Table 4. Germination measurements of tomato seeds in different priming solution (PEG, PEG + KNO_3 and KNO_3)

Treatments	*G (%)*	*T* (day)	CV_t (%)	*v* (day^{-1})	*U* (bit)	*Z*
Control	87a	3.79a	24.00a	0.26c	1.57a	0.36a
PEG	81a	2.93b	30.35a	0.34b	1.57a	0.38a
PEG + KNO_3	84a	2.76b	33.38a	0.36b	1.61a	0.38a
KNO_3	86a	2.37c	38.34a	0.42a	1.67a	0.35a
F(P)	1.25 **(0.35)**	50.73 (0.001)	2.21 **(0.16)**	42.44 **(0.001)**	0.09 **(0.96)**	0.28 **(0.86)**
W(P)	0.96 **(0.85)**	0.94 **(0.48)**	0.95 **(0.64)**	0.97 **(0.92)**	0.89 **(0.12)**	0.87 **(0.07)**

Means followed by the same letters in each column are not significantly different based on the Scott-Knott test at 0.05 probability. (G: germinability; *t*: mean germination time; CV_t: coefficient of variation of the germination time; *v*: mean germination rate; *U*: uncertainty; *Z*: synchrony; F: Snedecor distribution value; boldfaced values indicate significant difference among matrixes (ANOVA; $P < 0.05$); P: probability). W: statistics of the Shapiro-Wilk test; boldfaced values indicate normality for the residuals of ANOVA ($P > 0.01$).

4. Discussion

NR role in seed germination was related to nitrogen assimilation (Hendricks & Taylorson, 1974), but in our study the nitrogen in tomato seeds has not change, while total proteins and NR activity was improved. Nitrate can improve the NR activity by *de novo* synthesis (Somers, Kuo, Kleinhofs, Warner, & Oaks, 1983), thus priming with KNO_3 can increase the synthesis of NR, consequently increase total proteins in tomato seeds. The highest NR activity attended in tomato seeds priming with KNO_3 caused the increased activity of the antioxidant system, and the increment of SOD and CAT activities may have been caused by the formation of NO by NR. NR capacity to form nitric oxide through reduction of nitrate to nitrite creates a pool for NO production in plants (Sírová. Sedlárová, Piterková, Luhová, & Petrivalsky, 2011). In seeds, NO can be formed of a non-enzymatic (Bethke, Bedger, & Jones, 2004), or through enzyme synthesis (NOS) (Simontacchi, Jasid, & Puntarulo, 2007). NO production was observed in sorghum seeds during germination, even in control samples (Simontacchi, Jasid, & Puntarulo, 2004). In primed tomato seeds, the NO is responsible for the positive effects on seed germination at low temperatures (Amooaghaie & Nikzad, 2013).

Although NO is involved in germination, this molecule is also a reactive oxygen species (ROS), which is countered by the antioxidant system, as indirectly observed in our study, by increasing the activity of SOD and CAT in tomato seeds priming with KNO_3. In calluses of two ecotypes of reed (*Phragmites communis* Trin.) under heat stress, the increased activities of superoxide dismutase (SOD) and catalase (CAT) were observed when sodium nitroprusside (SNP), as an NO donor was applied (Song, Ding, Zhao, Sun, & Zhang, 2006). In seeds of alfalfa (*Medicago sativa*) germinated under salt stress, the increased activities of superoxide dismutase (SOD) were recorded when seeds were treated with SNP (Wang, Li, Cui, Xu, Shen, & Wang, 2012).

Although priming with KNO_3 increased the activity of the SOD and CAT in tomato seeds, the reduction in the activity of APX was observed. The reduction of APX activity could be related to small concentrations of H_2O_2 required for its operationality, which is owned to its high affinity for substrate (Mittler & Zilinskas, 1991). The H_2O_2 level in seeds affects the germination due to the increased respiration, and consequently, to the H_2O_2 concentration rise (Bailly, 2004), and KNO_3 primed seeds may have higher H_2O_2 production because of higher germination speed. Tomato seeds reduced germination time and improved germination rate when priming with KNO_3. A reduction in the average time of germination in seeds primed with KNO_3 was observed in other species, as in *Gladiolus alatus* (Musthaq et al., 2012), while the increased germination rate after a KNO_3 priming was recorded in *Brassica napus* L. under salinity conditions (Abdollahi & Jafari, 2012).

The reduction of germination time and the improved germination rate seem to be related to the dormancy breaking through the formation of NO (Dong, Tong, & Xiao, 2012; Jackobsen et al., 2013). Thus, seed dormancy in tomato makes the priming with KNO_3 an useful technique for its germination (Hilhorst & Downie, 1995). Despite the positive results in germination time and germination rate, a KNO_3 priming did not affected the germinability (*G%*), the uniformity (Cv_t) and the synchrony of germination (*U* and *Z*) in this experiment.

The high vigor of tested tomato seeds may be the cause of the lack of priming effect at the germinability. This idea has been evaluated in an experiment with asparagus seeds of low and high vigor. The beneficial effect of priming on germination and vigor was more expressive in the seeds with low physiological quality (Bittencourt, Dias, Dias, & Araujo, 2005).

5. Conclusions

The observed benefits in tomato seeds primed with KNO_3 were related to the activity of enzyme nitrate reductase in the production of nitrite/nitric oxide, which acted removing dormancy and promoting a faster germination.

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