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Priming with Ascorbic Acid, Salicylic Acid and Hydrogen Peroxide improves Seedling Growth of Spring Maize at Suboptimal Temperature

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Abstract: Early sowing of maize crop can contribute to increase maize yield but poor stand establishment at low temperature is main hindrance in its productivity. Seed priming with ascorbic acid, salicylic acid and hydrogen peroxide improves seedling establishment at suboptimal temperature. Maize seeds were soaked in 20 and 40 mg L^{-1} aerated solution of ascorbic acid, salicylic acid and hydrogen peroxide for 24 hour and were dried back to its original weight. Primed and non-primed seeds were sown in pots containing sand under net house conditions. At low temperature, seed priming with hydrogen peroxide, ascorbic acid and salicylic acid improved seedlings' growth probably through inducing superoxide dismutase (SOD) activities, better chlorophyll contents and enhanced nutrient contents. Seed priming with ascorbic acid, salicylic acid and hydrogen peroxide improved seedling establishment by inducing the antioxidants defense system and nutrient homeostasis. Seed priming with either 20 mg L^{-1} or 40 mg L^{-1} solution of H_2O_2, AsA and SA showed maximum seed invigoration and better performance in maize through inducing SOD activity and improving nutrient contents in root and shoot. The 20 mg L^{-1} of H_2O_2, salicylic acid and ascorbic acid seem to be suitable concentration for seed priming.

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1. Introduction

Maize is chilling sensitive crop during early growth stage. Chilling stress is a key environmental factor which limits growth of crop plants in response to the over production of reactive oxygen species (ROS). ROS are known to exacerbate imbalance between light absorption and its utilization by inhibiting Calvin-Benson cycle activity (Logan et al., 2006). ROS also reduce content and activity of ribulose bisphosphate carboxylase oxygenase (RUBISCO) enzyme which leads to higher electron flux to O_2 coupled with reduced CO_2 accumulation (Zhou et al., 2006). Chilling-induced oxidative stress in maize can lead to increased accumulation of ROS which ultimately damage the macromolecules like lipid and protein (Sokolnik et al., 2009; Prasad, 1997). In addition, responses to chilling-induced oxidative stress are alteration in activities of enzymes of antioxidant defense system. However, if the duration of chilling stress is too long, the defense system may not remove overproduced ROS effectively, which may result in severe oxidative damage or even death of cells (Sokolnik et al., 2009). Several endogenous defense mechanisms both enzymatic and non-enzymatic are involved in scavenging ROS (Rayduk et al., 2009). Enzymatic system consists of a number of enzymes such as superoxide dismutase, ascorbate peroxidase and catalase and in nonenzymic mechanism; metabolites like ascorbic acid, salicylic acid and low concentration of H_2O_2 are of prime importance that scavenge these radicals and protect membranes from injurious effects of ROS (Sharma et al., 2012; Ahmad et al., 2012). Many comparative studies using chilling tolerant and sensitive genotypes have shown that greater antioxidant capacity was exhibited in chilling-tolerant species compared to sensitive ones (Jahnke et al., 2009).

Table 1: Weekly average atmospheric temperature recorded during course of studies.

Development stage	Days after sowing	Atmospheric temperature (°C)		
		Maximum	Minimum	Mean
Sowing (1 Feb 2008)	0	18.5	0.0	9.25
Germination stage	7	15.2	5.6	10.40
	14	18.6	2.5	10.55
Seedling stage	21	22.1	6.8	14.45
	28	22.6	6.9	14.25
	35	26.0	12.9	18.45
Final harvest	36	27.0	13.2	20.10

Seed priming is an important tool to improve (Batool et al., 2015) crop vigor. Seed Priming has been used to improve the crop establishment in many crops at suboptimal temperature (Yoon et al., 1997; Dashtamian et al., 2014). Seed priming with salicylic acid improved chilling tolerance by increased germination (Sedghi et al., 2010), activation of antioxidants, maintenance of tissue water contents and reduced membrane permeability (Farooq et al., 2008). Seed priming with ascorbic acid, salicylic acid and H_2O_2 induced salinity tolerance (Athar et al., 2008; Gautam and Singh, 2009; Wahid et al., 2007; Kumar et al., 2010; Ahmad et al., 2012). Thus, it seems that ascorbic acid, salicylic acid and hydrogen peroxide are promising materials for seed treatments.

In the view of previous work done on horticultural crops like pansy and pepper, it can be assumed that seed priming can provide early establishment of vigorous and healthy maize seedlings at suboptimal temperature. To the best of our knowledge, limited work has been reported regarding biochemical and physiological changes in maize induced by seed priming. Therefore, the present study was carried out to investigate the effect of seed priming with different concentration of ascorbic acid, salicylic acid and hydrogen peroxide on enhancing germination and seedling vigor of maize hybrid and to explore the biochemical basis of this enhancement.

2. Material and Methods

The experiment was carried out in the wire house of Department of Crop Physiology, University of Agriculture, Faisalabad, Pakistan by employing completely randomized design (CRD) with three replications. Healthy and uniform seeds of maize hybrid (HI GEN Sawn 9697) were selected for experimental study.

2.1 Seed Priming

The solution of 20 and 40 mg L^{-1} of ascorbic acid (AsA), salicylic acid (SA) and hydrogen peroxide (H_2O_2) were prepared in distilled water. For priming, maize seeds were soaked in aerated solution of respective osmoticum for 24 h at room temperature. Seed weight to solution volume ratio was 1:5 (w/v). During priming fresh air was supplied with aeration pump continuously. After each treatment, seeds were rinsed thoroughly with distilled water, spread on thin layer of filter paper and dried back closer to original weight. These seeds were packed in polythene bags and stored in a refrigerator at 5°C until use.

2.2 Seed Sowing

Primed seeds as per treatment were sown in pots containing sand while untreated seeds were taken as control. Hoagland solution was used to nourish the plants uniformly throughout course of study. The atmospheric temperature was recorded from sowing to harvesting and weekly average were computed and presented (Table 1). At 4th leaf stage plants were harvested and tested for seedling vigor, antioxidants and nutrient analysis.

2.3 Seedling growth and vigor

Five seedlings were carefully harvested from each pot, shoot and root length of every plant was measured with measuring tape and their averages were recorded for statistical analysis. Root length obtained in above parameter was divided by the shoot length to get the root: shoot ratio.

2.4 Determination of Superoxide Dismutase

To extract antioxidant enzymes, 0.5 g fresh leaves randomly sampled from plants in each pot were harvested and immediately ground using a tissue grinder in 8 mL of cooled phosphate buffer [pH 7.0, containing 1% (w/v) polyvinylpyrrolidone] and 0.2 g quartz sand in test tubes that were placed in an ice bath. The homogenate was centrifuged at 15000-x g for 20 minutes at 4°C. The supernatant was used for assays of enzyme activity. The activity of SOD was determined by measuring its ability to inhibit the photoreduction of nitro blue tetrazolium (NBT) following the method of

Giannopolitis and Ries (1977). The reaction solution (3 mL) contained 50 uM NBT, 1.3 uM riboflavin, 13 mM methionine, 75 nM EDTA, 50 mM phosphate buffer (pH 7.8) and 20 to 50 uL enzyme extract. Test tubes containing the reaction solution and leave were irradiated under light bank (15 flouresent lamps) at 78 u mol m^{-2} s^{-1} for 15 minutes. The absorbance of irradiated and non-irradiated solution at 560 nm was determined with spectrophotometer (T_{60} spectrophotometer). One unit of SOD activity was defined as the amount of enzyme that would inhibit 50 % of NBT photo reduction.

2.5 Determination of Chlorophyll Contents

The fresh leaves were cut into 0.5 cm segments and extracted overnight with 80 % acetone at -10°C. The extract was centrifuged at 14000 g for 5 min and the absorbance of supernatant was recorded at 645 and 663 nm using a spectrophotometer (T60 U spectrophotometer PG Instruments, Limited). The chlorophyll *a* (***Chl a***) and *b* (***Chl b***) contents were calculated by using formulae given by Nagata and Yamashita (1992).

2.6 Determination of Nitrogen Contents

Total nitrogen was estimated by Kjeldhal apparatus. 10 mL of digested sample was taken in Kjeldhal flask and placed it on the Micro-Kjeldhal ammonium distillation unit and then 10 mL of 40% sodium hydroxide solution were added and immediately flask was connected to distillation apparatus. 10 mL 4% boric acid was taken along with mixed indicator in 100 mL conical flask. When distillate was approximately 40-50 mL, conical flask was removed and distillation was turned off. The distillate was cooled for a few minutes and titrated against 0.01N H_2SO_4 up to end point. Nitrogen was calculated by the formula given by Singh et al. (2005).

2.7 Determination of Phosphorus Contents

Phosphorus was determined by using a spectrophotometer. The digestion material (2 ml) was dissolved in 2 ml of Barton reagent and maintains volume up to 50 ml. The samples were then subjected to spectrophotometer (Spectrophotometer AnA-720 W Japan) and recorded the absorbance at 470 nm for P ions. Run a blank (without P) simultaneously. Prepared standard solution of KH_2PO_4 (Potassium Di-Hydrophosphate) and formed the standard curve by plotting P-concentration on X-axis and percent transmission/ spectrometer readings on Y-axis. P was calculated by following formula produced by Singh et al. (2005).

2.8 Determination of Potassium Contents

Potassium concentration was determined by using Flame photometer-410 (Corning Model). A graded series of standards (10 to 40 mg L^{-1} of K) were prepared and standard curve for standard was plotted by K-concentration on X-axis and flame photometer on Y-axis. The O.D values of K^+ from flame photometer were compared with standard curve and concentration of K^+ element was computed (Singh et al., 2005).

2.9 Statistical Analysis

The data collected was analyzed statistically using Fisher's analysis of variances technique and treatment means showing F-values significant compared using least significance difference at 0.05 probability level (Steel et al., 1997).

Table 2 Seedling dry weight, seedling length, root length, shoot length and root shoot ratio *Chl a*, *Chl b* and SOD activity of maize as influenced by different seed priming treatments.

Seed priming strategies	Seedling dry weight (g)	Seedling length (cm)	Shoot length (cm)	Root length (cm)	Root shoot ratio	*Chl a* (mg 100 mL^{-1})	*Chl b* (mg 100 mL^{-1})	SOD (unit mg^{-1} Protein)
Control	8.92	63.8	45.72 d	18.08 b	0.395 a	2.35 b	0.93 b	12.89 b
AsA (20 mg L^{-1})	10.76	74.29	54.82 bc	19.47 a	0.355 b	2.67 a	1.03 a	15.24 a
AsA (40 mg L^{-1})	10.94	77.92	58.34 a	19.58 a	0.336 b	2.65 a	1.04 a	15.37 a
SA (20 mg L^{-1})	10.48	70.66	52.94 c	17.72 b	0.335 b	2.68 a	1.05 a	15.25 a
SA (40 mg L^{-1})	10.68	74.05	54.54 bc	19.51 a	0.358 b	2.67 a	1.05 a	15.26 a
H_2O_2 (20 mL L^{-1})	10.53	74.43	54.36 b	20.07 a	0.369 b	2.64 a	1.06 a	15.29 a
H_2O_2 (40 mL L^{-1})	10.59	78.36	58.53 a	19.83 a	0.339 b	2.66 a	1.05 a	15.27 a
LSD	NS	NS	5.2767	0.5577	0.022	0.1039	0.0734	1.1712

Figures sharing same letter, in each column, did not differ significantly at 0.05 level of probability; *Chl a* = Chlorophyll *a* content; *Chl b* = Chlorophyll *b* content; SOD = Superoxide dismutase; AsA = Ascorbic acid; SA = Salicylic acid and H_2O_2 = Hydrogen peroxide. AsA = Ascorbic acid; SA = Salicylic acid and H_2O_2 = Hydrogen peroxide.

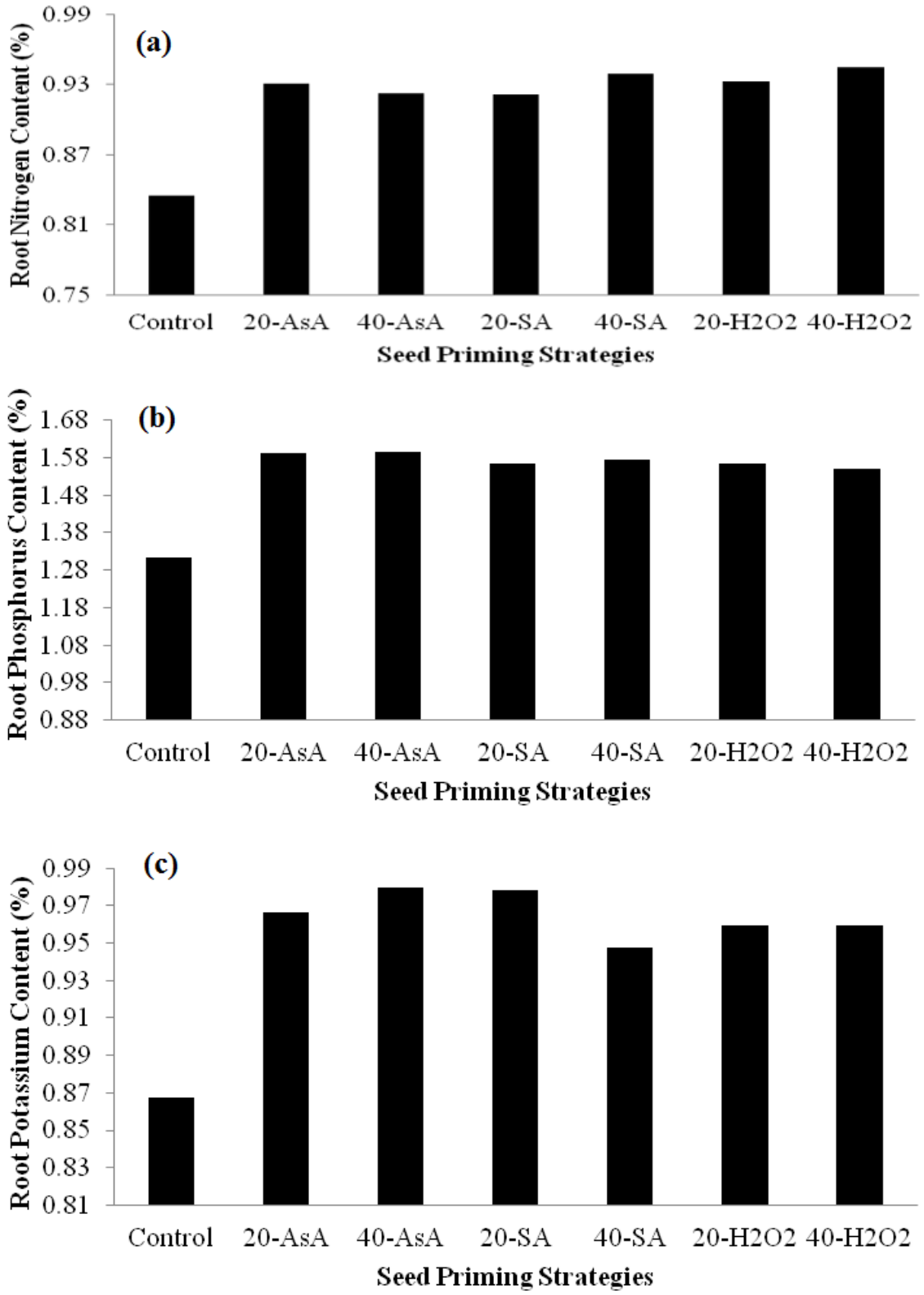

Figure 1. Root nitrogen (a), phosphorous (b) and potassium (c) contents in response to different priming strategies

3. Results

3.1 Seedling vigor and biochemical analysis

Seed priming with different levels (20 and 40 mg L^{-1}) of ascorbic acid, salicylic acid and hydrogen peroxide, significantly affected root and shoot length. Seedlings dry weight and length were improved by different priming strategies but seed primed with AsA-40 H_2O_2-40 were leading in improving dry weight and seedling length, respectively. It is evident from comparison means that all priming strategies improved root and shoot length (Table 2). This increase in root length and shoot length were in order of H_2O_2-20> H_2O_2-40> AsA-40> SA-40> AsA-20 and H_2O_2-40> AsA-40> AsA-20> H_2O_2-20> SA-40> SA-20, respectively. The comparison of different priming techniques indicates that all priming strategies improved root length in maize except SA-20 with similar root length (Table 2).

Root shoot ratio was reduced significantly by difference priming treatments. The lowest root shoot ratio in seedling was produced when seeds were treated with ascorbic acid and salicylic acid (Table 2).

3.2 Chlorophyll contents (mg 100mL^{-1})

Plant photosynthetic efficiency depends on pigments like *Chl a* and *Chl b* involved in photosynthesis. All priming strategies improved leaf *Chl a and b* contents (Table 2). Maximum *Chl a* content (2.68 mg 100mL^{-1}) were observed from seeds primed with SA-20 which was statistically at par with pre-soaked seeds with AsA-20, SA-40, H_2O_2-40, AsA-40 and H_2O_2-20. However, minimum *Chl a* contents (2.35 mg 100mL^{-1}) were noted for control. Data depicts that all the priming treatments improved *Chl b* contents (Table 2) except non-primed control with minimum *Chl b* contents of 0.93. Nonetheless, there was no difference among priming treatments for this trait.

3.3 Superoxide dismutase (unit mg^{-1} Protein)

Superoxide dismutase (SOD) is the most effective enzyme in preventing cellular damage by converting superoxide radical to H_2O_2.In present study, chilling stress caused reduction in SOD activity of maize but the seed priming with ascorbic acid, salicylic acid and hydrogen peroxide enhanced SOD activity. Higher SOD activity in leaves under chilling stress suggests a more efficient scavenging system which may result in better protection against ROS during stress (Table 2).

3.4 Nutrient analysis

Root and shoot nitrogen (N), phosphorus (P) and potassium (K) contents were significantly reduces by chilling stress. However, seed primed with ascorbic acid, salicylic acid and hydrogen peroxide significantly improved the absorption of N, P and K in root (Fig. 1a, b &c) and uptake in shoot (Fig. 2a, b& c). Different priming strategies significantly increased N, P and K contents in root and shoot, but different priming techniques showed similar results in improving nutrient contents.

4. Discussion

4.1 Seedling vigor and biochemical analysis

Chilling stress is a key environmental factor which limits growth of crop plants in response to the over production of ROS by inhibiting activity of Calvin-Benson cycle (Logan et al., 2006), enhancing over reduction of respiratory electron transport chain (Hu et al., 2008) and by reducing activity of RUBISCO (Zhou et al., 2006). Seedling growth in term of both weight and length was increased in seed primed treatment with ascorbic acid, salicylic acid and hydrogen peroxide possibly by ameliorating injurious effects of ROS on light harvesting apparatus in maize plants subjected to chilling stress (Table 2). This view is supported by a positive relationship between seedling dry weight and *Chl a* and SOD activity (Fig. 4 and 5). H_2O_2 at low concentration in biological systems acts as a signal molecule in cells (Neill et al., 2002; Kumar et al., 2010), ascorbic acid (AsA) is the most important antioxidants (Shalata and Neumann, 2001). Salicylic acid (SA) regulates cell growth by expansion and protecting the cell structure (Kang et al., 2007).

Low temperature lowers cell elongation (Ben-Haj-Salah and Tardieu, 1995) but in the present study shoot and root length increased significantly by different priming strategies and increased in shoot and root growth might be due to higher cell expansion in primed seeds as compared to unprimed seeds (Table 2). Similar findings were described by Kaydan et al, (2007) who reported that primed seeds with SA enhance the root and shoot growth under salinity stress.

Priming strategies improved leaf *Chl a* and *b* contents (Table 2). Low temperature (<10^{o}C) induce chilling injury through production of reactive oxygen species (ROS) which causes oxidative damage to chlorophyll molecule (Guan et al., 2009). This was confirmed by increased SOD activities in seeds that were primed with SA, AsA and H_2O_2 (Table 2). These results suggested that seed priming with AsA, SA and H_2O_2 could be used to minimize the harmful effect of low temperature on leaf chlorophyll contents which consequently may enable the plant to withstand at suboptimal conditions.

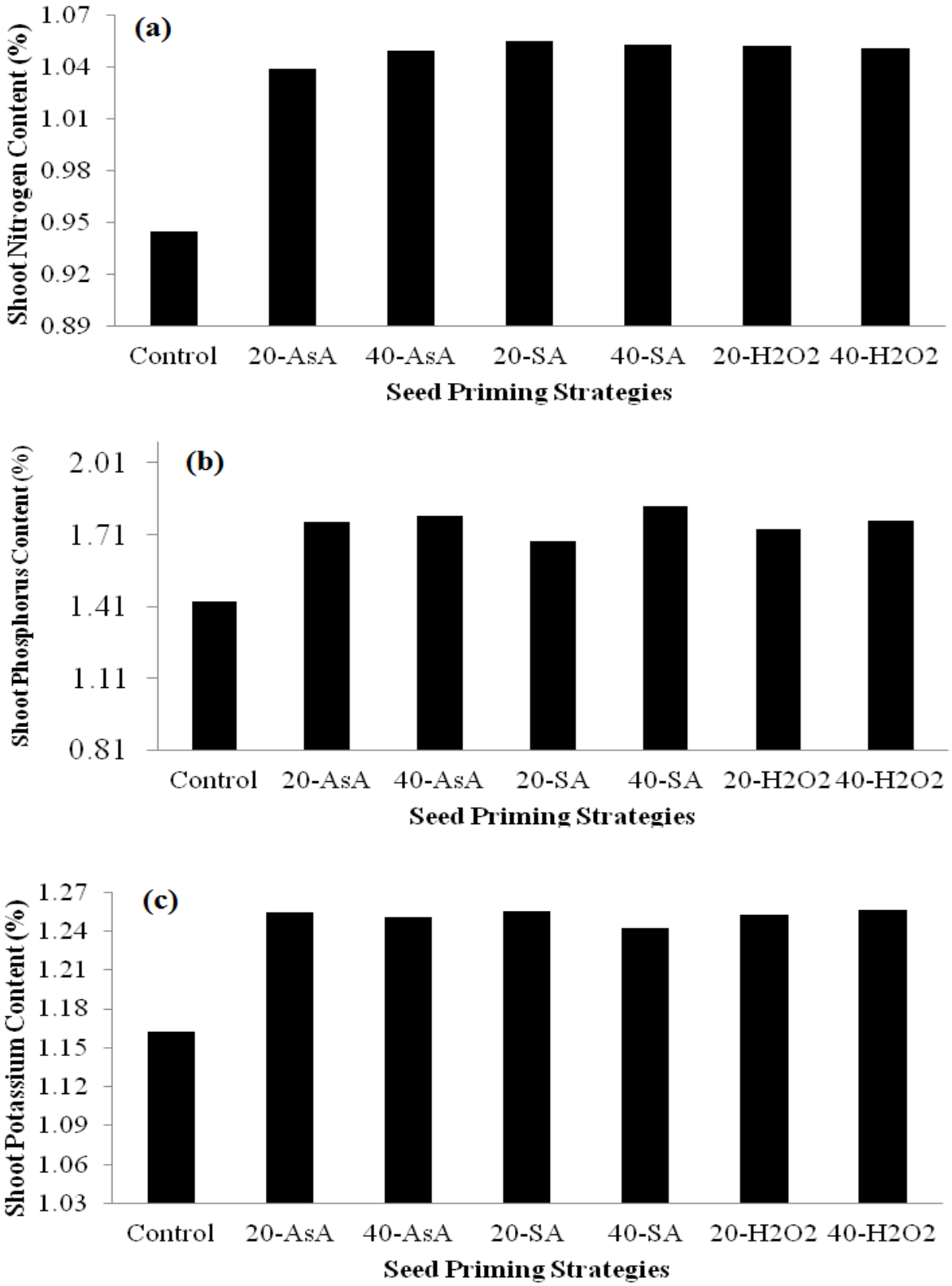

Figure 2. Shoot nitrogen (a), phosphorous (b) and potassium (c) contents in response to different priming strategies

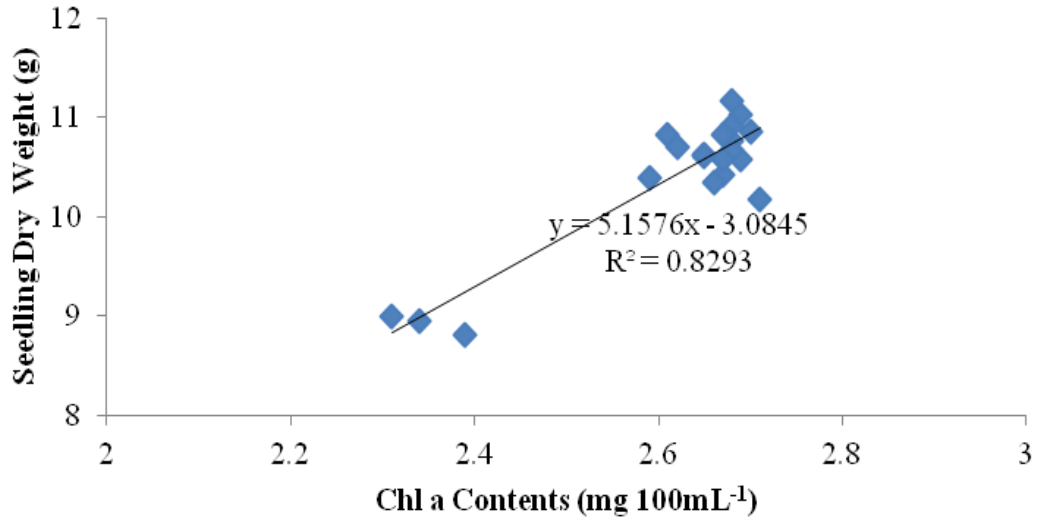

Figure 3. Relationship between Chlorophyll a and seedling dry weight

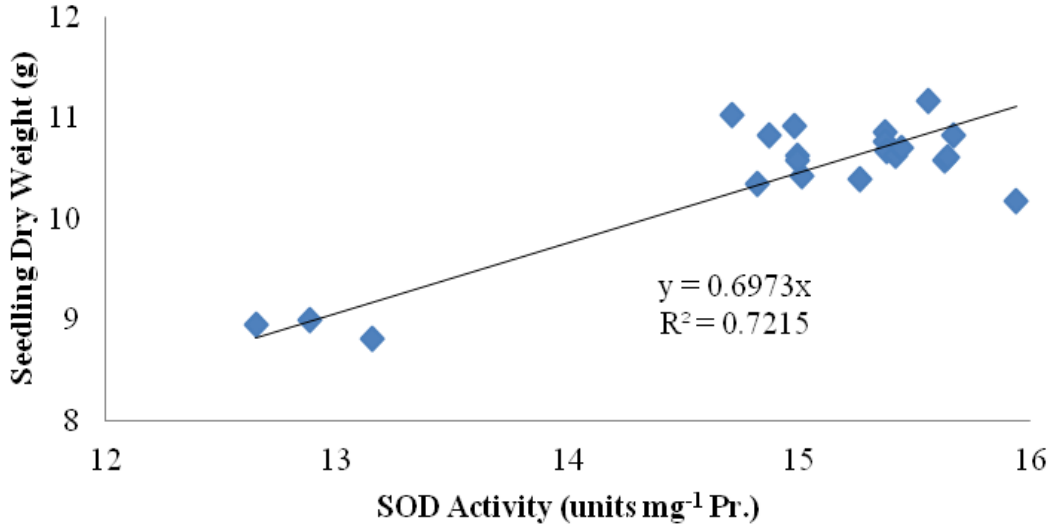

Figure 4. Relationship between superoxide dismutase and seedling dry weight

Responses to chilling-induced oxidative stress include alteration in activities of enzymes of antioxidant defense system. However, chilling stress caused reduction in SOD activity of maize but the seed priming with ascorbic acid, salicylic acid and hydrogen peroxide enhanced SOD activity (Table 2). Higher SOD activity in leaves under chilling stress suggests a more efficient scavenging system which may result in better protection against ROS during cold stress. Many studies indicated that chilling-tolerant genotypes have greater antioxidant capacity than sensitive ones (Jahnke et al., 2009; Hodges et al., 1996; Huang; Chen and Arora, 2012; Guo, 2005). Seed priming with AsA (Athar et al., 2008), SA (Gautam and Singh, 2009) and H_2O_2 (Kumar et al., 2010; Wahid et al., 2007a) also proved that these priming techniques induce SOD activity which quench oxygen radicals under stress (Foyer and Noctor, 2003: Orabi et al., 2015).

Chilling-induced oxidative stress evident by increased accumulation of ROS, including H_2O_2 and $O_2^{\bullet-}$, lipid peroxidation, and protein oxidation is a significant factor in relation to chilling injury in plants (Fryer et al., 1996; Prasad, 1997; Sokolnik et al., 2009). Protein carbonyl content, an indication of oxidative damage, was increased 2-fold in maize seedlings when exposed to chilling temperatures (Prasad, 1997). Lipoxygenase activity as well as lipid peroxidation was increased in maize leaves during low temperatures, suggesting that lipoxygenase-mediated peroxidation of membrane lipids contributes

to the oxidative damage occurring in chill stressed maize leaves (Fryer et al., 1996).

Low temperature limits root growth, which reduce water and nutrient absorption (Stamp et al. 1997). Seed priming treatments with AsA, SA and H_2O_2 significantly improved the root N, P and K contents significantly (Fig.1a, b and c) by reducing the deteriorative oxidative injury caused by ROS in root system. Results of present experiment clearly indicated that primed seeds effectively improved nutrient absorption and root growth, possibly by enhancing SOD activity. These results are consistent with those Gunes et al. (2007) and Wahid et al. (2007) who reported that seed primed with SA increased N and K contents in maize while seed primed with H_2O_2increased P contents in wheat under saline conditions, respectively.

Low temperature reduced root length (Kaspar and Bland, 1992) and shorter roots limited the phosphorous uptake efficiency (Enns et al. 2006) but pre-soaking seed treatments with ascorbic acid, salicylic acid and hydrogen peroxide increased shoot N, P and K content at suboptimal growth temperature (Fig. 2a, b and c). Improved shoot N, P and K contents may be explained by improved and well developed root system, which resulted in improved nutrients uptake and its translocation towards shoot parts. These results also support the findings of Wahid et al. (2007) and Sakr and Arafa (2009) who reported that seed primed with H_2O_2 and SA reduced NO_3^-,P and K^+ in wheat and canola under stress conditions (Gunes et al., 2007; Wahid et al., 2007; Sakr and Arafa , 2009). These results also support the findings of Wahid et al. (2007), and Sakr and Arafa (2009) who reported seed primed with H_2O_2 and SA reduced membrane permeability and leakage of ions like NO_3^- in wheat and canola under saline conditions. In the present study the increased accumulation of potassium (K^+) in maize seedlings seems to be responsible for their survival under chilling stress playing important role in osmotic adjustment.

5. Conclusion

On the basis of results obtained from this investigation it may be concluded maize responded similarly to different seed priming treatments. Seed priming with either 20 mg L^{-1} or 40 mg L^{-1} solution of H_2O_2, AsA and SA showed maximum seed invigoration and better performance in maize through inducing SOD activity and improving nutrient contents in root and shoot. The 20 mg L^{-1} of H_2O_2, salicylic acid and ascorbic acid seem to be suitable concentration for seed priming.

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Competing Interests

Authors declare that they have no competing interests.

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