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Priming-induced metabolic changes in sunflower (*Helianthus annuus*) achenes improve germination and seedling growth

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ABSTRACT. Seed priming improves vigor, but priming agents may differ greatly in their effectiveness. The present study was performed to unravel the physiological basis of vigor improvement by priming sunflower achenes with pre-optimized levels of hydrogen peroxide (H_2O_2), salicylic acid (SA), thiourea (TU), gibberellic acid (GA_3), ascorbic acid (AA), sodium chloride (NaCl), freezing and heating. Most of the treatments induced *de novo* synthesis of peptides with low (37 kDa for H_2O_2, SA and NaCl treatments, and 57 kDa for SA and TU treatments) and high (157 kDa for H_2O_2, SA, TU, GA_3 and AA treatments and 167 kDa for SA treatment) molecular mass, reduced solute leakage, and an enhanced soluble sugar pool in the achenes. Priming reduced days to 50% germination (T_{50}) and mean germination time (MGT) and improved germination energy (GE) and final germination percentage (FGP). Shoot length was improved by priming with H_2O_2, GA_3, and NaCl; root length with NaCl and H_2O_2; shoot and root dry weight with H_2O_2, SA and AA. Positive correlations between GE and FGP and expressed peptides, soluble sugars, shoot and root length, and dry weight and negative ones with EC of leachate suggested that pre-germination changes in primed achenes, in addition to improve germination, show lasting effects in promoting seedling growth. Of the treatments, H_2O_2, SA, TU and GA_3 were the most effective. Overall, the effects of priming treatments are related to *de novo* protein synthesis, an improved repair mechanism, and germination substrates for vigorous and earlier production of seedlings.

Keywords: Leachate; Protein synthesis; Seedling vigor; Signaling; Sunflower.

INTRODUCTION

Greater and better synchronized germination is crucial for achieving an optimal crop stand and better productivity, but several environmental constraints are great impediments. One pragmatic approach to increase crop production is seed invigoration (Lee and Kim, 2000, Basra et al., 2004; Farooq et al., 2006). Seed invigoration strategies include hydropriming, osmoconditioning, osmohardening, hardening, hormonal-priming, matripriming, and others (Chiu et al., 2002; Kao et al., 2005; Windauer et al., 2007). The invigoration persists under adverse field conditions like salinity (Wahid, 2004; Ahmad et al., 2005; Abdul Jaleel et al., 2007; Wahid et al., 2007), temperature extremes (Pill and Finch-Savage, 1988; Bradford et al., 1990, Wahid and Shabbir, 2005), hypoxia (Ruan et al., 2002), and drought (Du and Tuong, 2002).

Seed priming, accomplished through different means and methods, enhances pre- and post-germination activities. Hydroprimed maize seeds showed rapid seedling emergence and improved field stand (Nagar et al., 1998), and osmoprimed seeds with PEG, K_2HPO_4 or KNO_3 showed accelerated germination (Basra et al., 1989). Nerson and Govers (1986) found that 2-3% solutions of KH_2PO_4 + KNO_3 (1:1) synchronized and increased germination rate in muskmelon seeds. Sunflower seeds treated with PEG-8000 solution at 15°C had an increased germination rate (Bailly et al., 1998, 2000). The use of plant growth regulators during pre-soaking, priming, and other seed pretreatments improved crop performance (Miyoshi and Sato, 1997; Basra et al., 2006). GA_3 and ethylene stimulated the elongation of embryonic tissues and internodes of rice seedlings while ABA promoted mesocotyl elongation (Lee et al., 1999). Dry heat treatment broke seed dormancy to ensure better seed germination (Dadlani and Seshu, 1990). Incubation of cotton seed at 60°C markedly improved seedling emergence and vigor (Basra et al., 2004).

The synchronization and promotion of germination with seed priming may take place for several reasons, but changes in metabolite levels are important events during

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seed priming. As revealed from microarray studies, seed protein synthesis is a global phenomenon that initiates the up- or down-regulation of a number of germination related genes (Gallardo et al., 2001; Soeda et al., 2005). Natural or artificial seed priming induces the mobilization and solublization of globulins and the synthesis of late embryogenesis abundant proteins (Capron et al., 2000; Gamboa-deBuen et al., 2006). Antioxidant enzymes—including superoxide dismutase, catalase, and glutathione reductase—were also expressed during seed priming (Bailly et al., 2000). Among other pre-germination metabolic changes, seed priming decreased the level of malondialdehyde (Bailly et al., 1998, 2000), changed saturated and unsaturated fatty acids (Walters et al., 2005), and induced α-amylase to increase the soluble sugar pool, thereby enhancing seedling emergence and other related attributes (Mwale et al., 2003; Farooq et al., 2006).

The achene of sunflower (*Helianthus annuus* L.) is an important source of edible oil. However, sunflower is an oilseed crop and its germination is very susceptible to changing field conditions. Its productivity has not improved appreciably over the past eight years in Pakistan, despite the introduction of improved germplasm, and this is largely due to hampered seed/seedling vigor under field conditions (Anonymous, 2005). In this respect, the use of seeds with enhanced vigor can be a practicable strategy to obtain healthy seedlings and a better crop stand under a range of environmental conditions. Reports showing priming-induced vigor enhancement are numerous, but those highlighting the physiological and biochemical basis of such changes are scarce. We hypothesize that metabolic changes and effects produced by seed priming treatments are specific to each treatment, and may be related to a signaling action. In view of this hypothesis, we evaluated the physiological basis of pre- and post-germination changes produced by the most effective priming agent levels (in terms of improved vigor in sunflower achenes) and their relationships to germination and seedling growth.

MATERIALS AND METHODS

Plant material and treatment selection

Achenes of sunflower (*Helianthus annuus* L. cv. Hyson-33) were obtained from Oilseed Research Institute, Faisalabad. For the selection of effective priming treatments, the achenes were pretreated with varying levels of hydrogen peroxide (H_2O_2), salicylic acid (SA), thiourea (TU), gibberellic acid (GA_3), ascorbic acid (AA), sodium chloride (NaCl), freezing and heating, followed by drying at 27°C to the original (8-10%) moisture. The most effective levels of these treatments, based on greatest final germination percentage (FGP), selected for this study were: H_2O_2 (100 µM for 8 h), SA (50 mg L^{-1} for 8 h), TU (10 mg L^{-1} for 8 h), GA_3 (150 mg L^{-1} for 8 h) AA (500 mg L^{-1} for 8 h), NaCl (1000 mg L^{-1} for 8 h), heating (40°C for 48 h), and freezing (-19°C for 24 h).

Priming-induced metabolic changes in achenes

For protein determinations, frozen seed material (0.5 g) was ground in a pre-chilled pestle-mortar in phosphate buffer saline (PBS) containing NaCl (137 mM), KCl (2.7 mM), Na_2HPO_4 (2 mM), and cocktail protease inhibitors (1 mM). The pH was adjusted to 7.2 (Sambrook and Russell, 2001). The amount of total proteins was determined using a Bradford assay (Bradford, 1976). For fractionation of proteins, 20 µg of each sample was treated with lysis-buffer (sodium dodesyl sulphate 10%, Tris 0.5 M with pH 6.8, glycerol 80% and bromophenol blue), loaded on a 12.5% polyacrylamide gel, and electrophoresed at 100 V (Laemli, 1970). The gel was stained in a 0.1% solution of 20% methanolic Coomassie brilliant blue G-250 overnight. After destaining for 20 min in 20% methanol, the gel was wrapped in a permeable membrane, dried in the dark for three days, and scanned, and molecular mass of the expressed peptides was ascertained by comparing them with protein markers, which were run alongside the unknown samples.

For the assessment of solute leakage, the achenes were put on a double layer of filter paper (Whatman No. 1) in pairs of petri dishes (9 cm diameter) containing 10 mL of deionized water and kept overnight at 25°C. The leachate was collected after washing the filter papers. The final volume was made up to 10 mL, and the sample's electrical conductivity (EC) was determined. To estimate soluble sugar contents, 1 g of the sample was mixed with 10 mL distilled water and left for 24 h at 25°C (Lee and Kim, 2000). The mixture was filtered through Whatman No. 42 filter paper, and the final volume was made up to 10 mL with distilled water. Total soluble sugar contents were estimated by anthrone regent (Yoshida et al., 1976).

Achene germination

Achenes were sown in petri dishes on a double layer of moist filter paper and kept at 26±1°C in a plant growth chamber (Eyelatron, FLI-301N, Tokyo Rikakikai Co., Ltd., Tokyo, Japan). An achene was considered germinated when radicle was 5 mm long. Counts of germinating seeds were taken daily up to six days after the start of germination. Time to 50% germination (T_{50}) and mean-germination time (MGT) were calculated according to Coolbear et al. (1984) and Ellis and Roberts (1981), respectively. Energy of germination (GE), determined on the experiment's 4th day, represented the ratio of germinated seeds to total seeds. Final germination percentage (FGP) was determined at the end of experiment (AOSA, 1983).

Seedling growth

The primed achenes were sown in 2 kg capacity pots containing sand, which was washed thrice with distilled water. The seedling growth was supported by supplementing half strength Hoagland nutrient solution (Epstein and Bloom, 2005) twice during whole of the growth period. Fifteen days after sowing, the seedlings

were harvested. Length and fresh weight (FW) of shoot and root were determined immediately after harvesting while dry weight (DW) was determined after drying these tissues at 80°C in an oven for a week.

Statistical analysis

All the experiments were conducted in a completely randomized design with three replications. The data recorded for various attributes was subjected to statistical analysis using COSTAT computer software (COHORT software, 2003, Monterey, California). For significant ANOVA tests ($P < 0.01$), Duncan's new Multiple Range Test was applied for the comparison of means, which were indicated by alphabets on data sets. Correlations of germination attributes were established with achene metabolic changes (number of peptides expressed, EC of leachate and soluble sugars) and seedling growth (shoot and root length and dry weight) attributes.

RESULTS

Priming-induced metabolic changes in achenes

Fractionation of proteins indicated the appearance of low and high molecular mass peptides by priming treatments, except for control, freezing and heat. Among peptides of low molecular mass, a 37 kDa peptide was induced by H_2O_2, SA and NaCl, and another 57 kDa peptide was evident in SA and TU treatments. A high molecular mass peptide, 157 kDa, was induced by H_2O_2, SA, TU, GA and AA priming while a 165 kDa peptide was induced by SA (Figure 1). The data indicated significant differences ($P \leq 0.01$) among the priming treatments for the EC of leachate and soluble sugar concentrations. EC of achene leachate was highest in control and freezing, but the lowest in SA and H_2O_2. On the other hand, soluble sugars were greatest in the SA and H_2O_2 treatments (Figure 1).

Data on germination attributes of achenes revealed significant ($P \leq 0.01$) differences among priming treatments (Table 1). Except for freezing and heating, although all treatments were effective in curtailing the T_{50}, H_2O_2 and TU were the most effective. MGT was highest in control and freezing treatments, but it was lowest in SA and TU (Table 1). Achene priming greatly improved GE (potential of achene to germinate vigorously) as compared to control, being greatest in SA, followed by H_2O_2, TU and GA. Likewise, FGP was improved by all the priming treatments but most of all by SA, H_2O_2 and TU (Table 1).

Seedling growth attributes

Shoot and root length, which showed significant ($P \leq 0.01$) difference among treatments, was promoted by all the priming treatments. Increase in shoot length was greatest in GA_3, H_2O_2, and NaCl primed achenes. Root length was greatly improved with NaCl priming, followed by H_2O_2 and SA, while freezing was at the bottom (Figure 2). All the priming treatments, with significant ($P \leq 0.01$) differences, improved shoot and root dry weight, but H_2O_2 and SA were the most effective in this regard (Figure 2).

Correlations

Parallels were drawn between germination attributes and priming-induced changes in the achenes and seedling growth attributes (Table 2). T_{50} and MGT showed no association while GE and FGP were positively related to

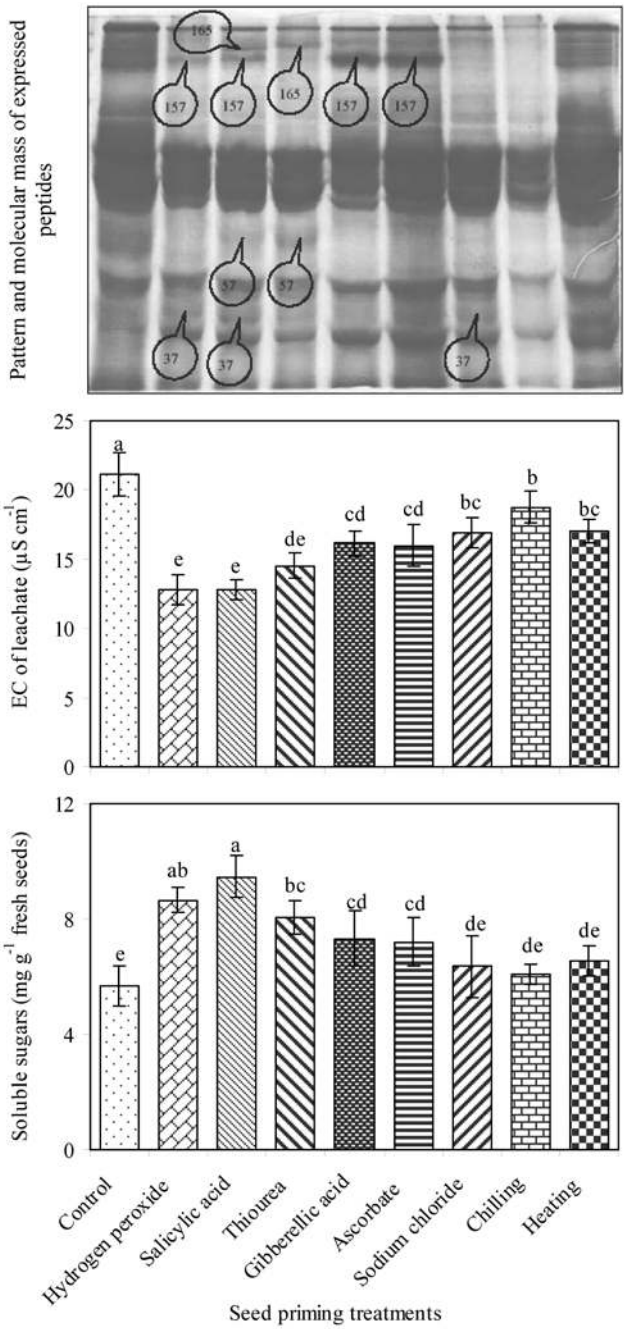

Figure 1. Pattern and molecular weight of the expressed peptides, electrical conductivity of leachates and soluble sugars concentrations of sunflower achenes primed with most effective levels of priming treatments. Bars with same alphabets were not significantly different ($P \geq 0.05$).

Table 1. Some germination attributes of sunflower achenes at the most effective levels of priming treatments

Treatment	Days to 50% germination	Mean germination time (days)	Energy of germination (%)	Final germination percentage
Control	4.80±0.44a	8.43±0.15a	45.67±4.04d	55.67±5.03d
Hydrogen peroxide	2.27±0.17d	6.33±0.12cd	85.33±2.31ab	90.67±2.31ab
Salicylic acid	2.64±0.27d	6.13±0.23d	89.33±6.11a	95.67±2.31a
Thiourea	2.39±0.20d	6.37±0.12d	82.67±8.08ab	89.33±7.37ab
Gibberellic acid	2.41±0.10d	6.40±0.10cd	82.00±8.66ab	86.00±6.93b
Ascorbic acid	2.42±0.21d	6.47±0.21cd	80.00±6.93ab	85.33±2.31b
Sodium chloride	2.48±0.15d	6.50±0.44cd	78.33±4.61b	83.33±5.03b
Freezing	3.93±0.31b	7.20±0.10ab	58.00±4.00c	66.00±4.00c
Heating	3.17±0.25c	6.83±0.38bc	62.33±4.16c	68.67±2.31c

Means sharing same alphabet were not significantly different (P≥0.05).

peptide expression. EC of leachate was positively related to T_{50} and MGT but negatively to GE and FGP. However, soluble sugars were negatively correlated to T_{50} and MGT but positively to GE and FGP. Shoot and root length and dry weights were negatively correlated with T_{50} and MGT but positively with GE and FGP (Table 1).

DISCUSSION

This study was performed to determine the varied responses of germination and seedling growth attributes to priming-induced metabolic changes in sunflower achenes in order to obtain a better crop stand in the field. Priming of seed is an effective tool in enhancing the emergence and vigor of seedlings under both optimal (Demir and van de Venter, 1999; Farooq et al., 2006) and suboptimal conditions (Wahid and Shabbir, 2005; Wahid et al., 2007). Nevertheless, such effects are linked to priming-induced metabolic changes. This study, involving eight different treatments, revealed that sunflower achene priming led to differential expression of certain low (37 and 57 kDa) and high (157 and 165 kDa) molecular weight peptides together with reduced ion-leakage and an enhanced soluble sugars pool (Figure 1). The H_2O_2, SA and TU treatments produced these changes most effectively.

Although all priming strategies curtailed T_{50} and MGT and enhanced GE and FGP; SA, H_2O_2 and TU were the most effective (Table 1), leading to an earlier and energetic seedling start. Primed achenes evaluated for seedling production revealed substantial improvement in elongation and dry mass of both shoot and root (Figure 2), as reported in earlier studies (McDonald, 2000; Mwale et al., 2003;

Table 2. Relationships of germination attributes of sunflower achenes with priming-induced changes in the achenes and seedling growth attributes

Characteristics	Days to 50% germination	Mean germination time	Energy of germination	Final germination percentage
Pre-germination changes				
No. of peptides	-0.573ns	-0.658ns	0.795*	0.834**
EC of leachate	0.889**	0.923**	-0.930**	-0.936**
Soluble sugars	-0.701*	-0.742*	0.837**	0.866**
Seedling characteristics				
Shoot length	-0.840**	-0.782*	0.806*	0.768*
Root length	-0.833**	-0.765*	0.777*	0.747*
Shoot dry weight	-0.670*	-0.727*	0.787*	0.805**
Root dry weight	-0.696*	-0.741*	0.759*	0.767*

Significant at ** P≤0.01; * P≤0.05; ns non-significant.

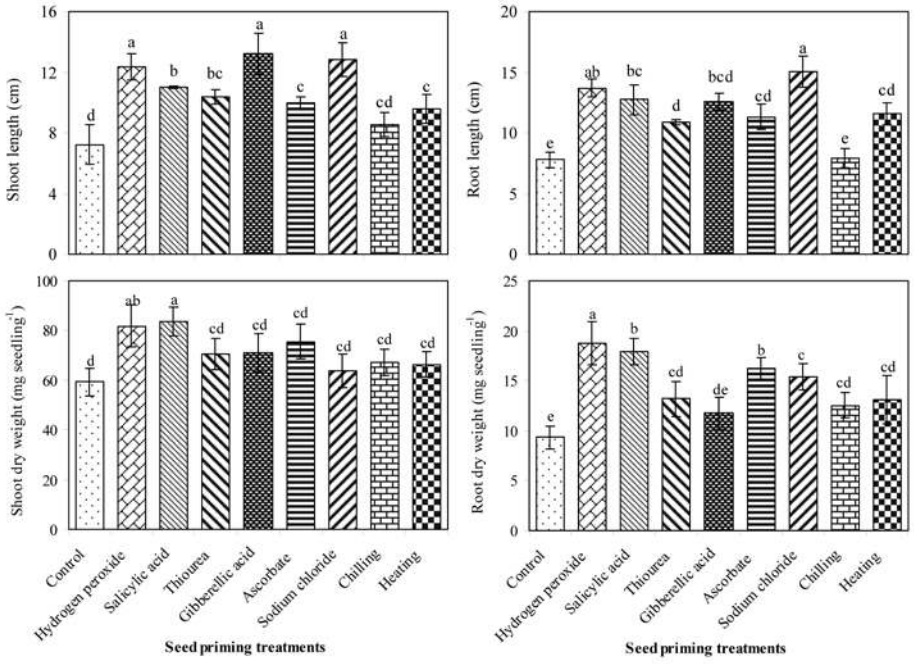

Figure 2. Seedling growth attributes of sunflower seedlings grown from achenes primed with most effective levels of various treatments. Bars with same alphabets were not significantly different (P≥0.05).

Farooq et al., 2006). For seedling elongation, H_2O_2, NaCl and GA_3 were the best while for shoot and root dry weight SA and H_2O_2 were promising. A greater improvement in elongation and dry weight of shoot than root (Figure 2) revealed that changes in primed achenes diverted a greater part of the cotyledonary resources towards the shoot, which was crucial to its earlier establishment and photosynthesis for vigorous growth. The causes of above effects produced by achene priming treatments may be different. Since H_2O_2, SA, TU and GA_3 are signaling molecules (Taiz and Zeiger, 2006), it is plausible that they reprogrammed the gene expression (Cruz-García et al., 2003; Soeda et al., 2005; Gamboa-deBuen et al., 2006), leading to *de novo* protein synthesis, a membrane repair mechanism, and more storage proteins and other substrates available for improved and synchronized germination (Table 1).

As evident from the above, some priming treatments were more effective in improving achene germination attributes than others, a fact which is possibly associated with pre-germination metabolic changes in the achenes and post-germination seedling performance. Data showed positive correlations between the expressed peptides and soluble sugars with GE and FGP, and negative ones between soluble sugars and T_{50} and MGT. EC of leachate was positively correlated with T_{50} and MGT and negatively with GE and FGP (Table 2). This revealed that improvement in germination was closely associated with *de novo* protein expression, repair mechanisms, and a greater availability of germination substrates (Table 1), which resulted in a rapid and energetic start (Mwale et al., 2003; Wahid et al., 2007). Likewise, a stronger negative correlations between T_{50} and MGT and a positive one between GE and FGP separately for shoot and root length and dry weight further substantiated that the priming-induced metabolic changes in the achenes had lasting influence on the seedling growth (Farooq et al., 2006; Wahid et al., 2007). Thus, curtailed time to emergence and vigorous seedling start are beneficial effects of seed priming.

It is concluded that priming-induced improvements in achene germination and seedling growth were associated with proteins synthesis, membrane repair mechanisms, and greater substrate availability for germination. From the metabolic changes in achenes during priming, it is plausible that the priming treatments reprogrammed the gene expression for antioxidant synthesis and mobilized germination substrates in greater amounts. Among the treatments, SA and H_2O_2, probably using a common signaling mechanism, proved to be the most effective. Our results suggest that these strategies can be of great help in the production of sunflower seed capable of growing vigorously under contrasting field conditions.

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在向日葵瘦果以先期誘導技術所引起之代謝改變可以改善種子萌發及幼苗生長

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種子之先期誘導可增進生長勢，唯其效果因所用藥劑而異。本研究分別以低於極大化程度之 H_2O_2，水楊酸，thiourea，GA_3，維他命 C，氯化鈉，冷卻，及熱處理之先期誘導嘗試揭開生長勢（vigor）提高之生理基礎。大多數之處理誘發瘦果的新合成之胜肽，具低分子量（37 kDa: H_2O_2，水楊酸及氯化鈉處理；57 kDa：水楊酸及 thiourea 處理）及高分子量（157 kDa: H_2O_2，水楊酸，thiourea，GA_3，維他命C處理；167 kDa：水楊酸處理）；減少溶質漏失；及擴大水溶糖含量。先期誘導縮短了 50% 發芽之日期，縮短了平均發芽時間（MGT），改善發芽所需能量（GE），及最終發芽百分比（FGP）。莖長度可被 H_2O_2，GA_3，及氯化鈉先處理所改善；而根長度以氯化鈉及 H_2O_2 先處理改善；莖及根之乾重可被 H_2O_2，水楊酸及維他命 C 先處理改善。GE 及 FGP 都分別與表現之胜肽，水溶糖，莖長，根長，莖重及根重具正相關；而與漏出液之濃度呈負相關。以上之結果綜合顯示：在先處理過之瘦果由於發芽前之代謝潛在改變不但改善發芽過程，而且是長效地改善後來之幼苗生長。在上述許多先處理當中，H_2O_2，水楊酸，thiourea，及 GA_3 較為有效。綜合言之，先期誘導（priming）之效果在於新胜肽之合成，改善之修補機制，及足夠之發芽基質可供活躍的及更早的幼苗生長。

關鍵詞：向日葵；滲出液；幼苗強勢；訊號傳遞；胜肽（或蛋白質）合成。