



Metabolic profiling during germination of hydro primed cotton seeds

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ABSTRACT

This work tries to study the effect of the cotton seed hydropriming on its metabolic status during germination. Two groups of cotton seed (primed with water and non-primed) were germinated for 7 days. Through this period; the antioxidant enzymes, H₂O₂, Malonyl dialdehyde, lipid hydrolysis, shikimic acid, aldolase, and the isocitrate lyase were determined at 1st, 4th and 7th day of sowing. The study ensured that the hydropriming improved the germination of the cotton seed. Improved germination of primed seed combined with the enhancement of the stored lipid metabolism through the activation of lipase, the lipoxygenase and isocitrate lyase activity increased. The significant decrease in the shikimic acid content ensured the active metabolic status of the primed seed. Also priming increased considerably the antioxidant enzymes activity. This helped in maintaining the balance in the reactive oxygen species inside the primed germinated seed cells. This is illustrated in reducing H₂O₂ content in the primed germinated seed than that in the non-primed germinated seed. The control of reactive oxygen species in content helped in minimizing the lipid peroxidation in the primed germinated seed than that in the non-primed seed.

1. Introduction

Cotton (*Gossypium hirsutum* L.) is one of the most important crops for human all over the world. It is called the “white gold” and “king of fibers”. The cultivation of cotton has many economic benefits. Cotton is the most important source of natural fiber used in textile industry. Rapid and health field germination is necessary to give a high yield of good quality and quantity in annual crops (Yari et al., 2010). Cotton seed highly deteriorates as their oil content readily oxidizes, which decrease the seed health in storage (Kausar et al., 2009).

Priming is one of the easy methods to improve seed performance and provide faster and healthier germination (Dolatabadian and Modarres, 2008). It is a widely used technique for improving seed germination and vigor (Varier et al., 2010).

During priming the seed is taken through the first biochemical processes within the initial stages of germination bringing the seed close to the point of germination (Mustafa et al., 2017).

Seed priming causes an advancement of metabolic status during the prolonged phase of imbibition that prepares the radicle protrusion. Mainly during seed priming portion of the seed endosperm is hydrolyzed that permits faster embryo growth (Afzal et al., 2012).

Hydro-priming is a simple, environmentally friendly and cheap technique used for improving pre-germination processes, seedling growth and establishment (Ghassemi-Golezani et al., 2008). Although the enhanced effect of priming of seed to seedling emergence and

growth is well reported by various workers, little is known about the metabolic changes in seed priming. Therefore this study will investigate the effect of hydropriming on the metabolic status during germination of cotton seeds.

2. Material and methods

2.1. Priming and germination

The present experiment was conducted in the laboratory of plant physiology and tissue culture in the Botany Department, Faculty of Science, Zagazig University during the year 2017. The experiment was conducted in a complete randomized design with five replications.

Seed was conditioned in between two towel papers wet with distilled water for 12 h for hydropriming and after that seeds were air dried to avoid excess moisture and proceed for the germination test after a month. Dry seeds were invoked as the control. 20 seed from each treatment was germinated on moist filter paper in the incubator at 28 °C for 7 days. After 7 days, all parameters were observed, measured and recorded. Germination %, vigor index length and seed vigor mass were calculated with the following formula:

$$\text{Germination\%} = \frac{\text{Total number of germinated seeds}}{\text{Total number of planted seeds}} \times 100$$

$$\text{Vigor Index I} = \text{Germination \%} \times \text{seedling length (cm)}$$

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Seed Vigor II = $\text{Germination \%} \times \text{seedling dry wt (mg)}$

Samples for biochemical studies were collected every 3 days beginning from the first day after sowing (i.e. 3 stages at the 1st, 4th and the 7th day after sowing).

2.2. Lipids hydrolysis

2.2.1. Total lipids content

The lipids were extracted according to Folch et al. (1957). The tissue is homogenized with chloroform/methanol (2/1) (1 g in 20 mL of solvent mixture). The homogenate is filtrated. The filtrate is washed with water (4 mL for 20 mL). After vortexing some seconds, the mixture is centrifuged at low speed (2000 rpm) to separate the two phases. Remove the upper phase by siphoning. The lower chloroform phase containing lipids is evaporated under vacuum in a rotary evaporator. Total lipids content was identified according to Marsh and Weinst (1966). 2 mL of concentrated sulfuric acid was added to 3 mL lipid extract. The tubes were enclosed in an aluminum heating block at 200 °C for 15 min. After incubation the tubes were placed in water at room temperature for 15 s and then were transferred to an ice bath for 5 min; 3 mL of water was added to each tube carefully. When cool, the tubes were removed from the ice and left standing until all bubbles had disappeared. The optical density was recorded at 375 nm. The total lipids content was expressed as mg/g fresh weight.

2.2.2. Lipase assay

The titrimetric method of Maliks et al. (2000) was used for the determination of lipase activity. Olive oil emulsion was prepared in 180 mL distilled water containing 20 mL olive oil, 0.4 g of sodium benzoate and 1 g gum-arabic. Assay mixture contained 5 mL olive oil emulsion, 5 mL 0.1 M Tris buffer (pH 8), and 1 mL crude enzyme and incubated at 35 °C for 10 min. The reaction was stopped by 10 mL of acetone and methanol mixture (1:1). Each sample was titrated against 0.025 N NaOH using 1% phenolphthalein as indicator. The volume of NaOH employed in the titration was used for enzyme activity calculations. Lipase activity was reported as percent (%).

2.2.3. Lipoxygenase activity

The lipoxygenase activity was measured spectrophotometrically using linoleic acid as substrate (Axelord et al., 1981). The assay mixture consists of 10 µL of linoleic acid and 50 µL of Tween 20 in 10 mL of 0.1 M phosphate buffer, pH 9.0. The reaction was initiated by the addition of 0.1 mL of enzyme extract to 1 mL of standard assay mixture. LOX activity was measured by monitoring the change in absorbance at 234 nm over a period of 5 min. LOX activity was expressed as µM min⁻¹ mg⁻¹ protein.

2.3. Glyoxylate cycle marker (Isocitrate lyase)

Isocitrate lyase assay was modified from the procedure described by Dixon and Kornberg (1959). The reaction buffer was 50 mM potassium phosphate, 4 mM MgCl₂, 4 mM Phenylhydrazine, 12 mM Cysteine, pH 7.0, and it was kept on ice until use. The mixture of 2 mL reaction buffer and 4 mL enzyme solution was incubated at 37 °C for 10 min, and the change of absorbance at 324 nm was measured after the addition of 0.1 mL isocitrate. Specific activity was defined as the change in the optical density min⁻¹ mg⁻¹ protein.

2.4. Metabolic activity marker (shikimic acid) content

Shikimic acid concentration was determined according to Zelya et al. (2011). The samples were homogenized in 0.25 M HCl (1 mL/100 mg biomass). The homogenate was shaken (2 min) and then centrifuged at 3000 rpm for 30 min. The supernatant (50 µL) reacted with 0.5 mL of a 1% periodic acid. After 3 h at room temperature, 0.5 mL of

1 M sodium hydroxide and 0.3 mL of 0.1 M glycine were added per sample. Samples were centrifuged again and absorbance valued at 380 nm. The shikimic acid content was expressed as µg g⁻¹ fresh weight.

2.5. Determination of reactive oxygen species and antioxidant machinery

2.5.1. Determination of hydrogen peroxide (H₂O₂) content

H₂O₂ content was determined according to the method of Alexieva et al. (2001). 1 g fresh sample was homogenized in 0.1% trichloro acetic acid (TCA). The homogenate was filtered through Whatman No.1 filter paper. 0.5 mL 100 mM K-phosphate buffer (pH 6.8) and 2 mL reagent (1 M KI in fresh double-distilled water H₂O) were added to 0.5 mL of the extract filtrate. The reaction was established for 1 h in darkness and absorbance measured at 390 nm. H₂O₂ content was expressed as µg g⁻¹ fresh weight.

2.5.2. Determination of malonyl dialdehyde (MDA) content (a lipid peroxidation product)

MDA determination was performed according to the method described by Li (2000). 5 g fresh seedlings were homogenized in 5 mL 5% TCA. The homogenates were centrifuged at 3000 rpm for 20 min. A reaction mixture of the supernatant (0.5 mL) and 1 mL 20% TCA and 1 mL 0.5% TBA (Thiobarbituric acid) was incubated at 95 °C in a water bath for 25 min. Cooled immediately before centrifugation. Absorbance of the supernatants was determined at 450, 532 and 600 nm, respectively.

Calculation of MDA was based on the following formula:

$$\text{MDA}(\mu\text{m}/\text{mL}) = 6.45(A_{532} - A_{600}) - 0.56 A_{450}$$

2.5.3. Estimation the activities of antioxidant enzymes (Superoxide dismutase (SOD), catalase (CAT), polyphenol oxidase (PPO) and peroxidase (POX))

5 g fresh seedlings were homogenized in 0.05 M cold phosphate buffer (pH 6.5) containing 1 mM EDTA, Na₂ and centrifuged at 3000 rpm for 10 min. The supernatant was completed to a total known volume (5 mL) and used as the enzymes and the soluble protein source (Ma et al., 2012). SOD activity was measured by the reduction of nitro blue tetrazolium (NBT) method (Beyer and Fridovich, 1987) Test tubes containing reaction solution with 3 mL of assay buffer, 60 µL of crude enzyme and 30 µL of riboflavin were illuminated for 7 min in an aluminum foil lined box containing two Fluorescent lamps at 25 °C. After reaction, the absorbance of the blank solution and reaction solution was measured at 560 nm. SOD activities were calculated according to the following equation:

$$\text{SOD activity}(\%) = (1 - A/B) \times 100$$

(A: absorbance of sample; B: absorbance of blank)

Assay of polyphenol oxidase activity (PPO) was performed according to Kar and Mishra (1976). 5 mL of assay mixture containing 125 µM of phosphate buffer (pH 6.8), 100 µM of pyrogallol and 1 mL of crude extract were prepared. After incubation at 25 °C for 5 min, the reaction was stopped by the addition of 1 mL 10% H₂SO₄. The absorbance of the produced color was measured at 430 nm and the enzyme activity was expressed as the change in OD min⁻¹ mg protein⁻¹.

Assay of peroxidase activity (POX) was performed according to Saroop et al. (2002). 5 mL of assay mixture containing 300 µM of phosphate buffer (pH 6.8), 50 µM catechol, 50 µM H₂O₂ and 1 mL of crude enzyme extract were prepared. After incubation at 25 °C for 5 min, the reaction was stopped by the addition of 1 mL 10% H₂SO₄. The absorbance of the produced color was measured at 430 nm and the enzyme activity was expressed as the change in OD min⁻¹ mg protein⁻¹.

Table 1
Mean Comparison of germination behavior and vigor traits in cotton seeds either non-primed or primed.

Germination parameter	Germination %	Seedling length (cm)	Seedling dry wt. (mg)	Vigor index I	Vigor index II
Non-primed	80.00 ± 0.00	23.9 ± 0.173	99.56 ± 0.179	1912.00 ± 13.8	7964.80 ± 14.62
Primed	100.00 ± 0.00*	29.70 ± 0.175*	120.98 ± 0.642*	2970.00 ± 17.53*	12098.00 ± 63.79*

Values are given as means of 5 replicates. Within a column, Means followed by asterisks are significantly different according to the paired-samples *t*-test.

3. Results

3.1. Germination parameters

Hydro priming significantly increased the germination ratio by 25% more than that of non-primed seeds. Also, the priming increased considerably the seedling length and of 24.27% more than that of non-primed seeds. Also, the dry weight of the primed seedling was 21.51% more than that of the control. The priming seedling has vigor index I and II higher than that of non-primed seeds of 55.33%, and 5189% respectively as showed in Table (1).

3.2. Lipid hydrolysis

Hydropriming enhanced the lipid hydrolysis as described in the Table (2). Total lipids content in the primed germinated seed was significantly lower than that in the non-primed germinated seed. Total lipids at the 1st day decreased in the primed group of 45.84% less than that in the non-primed one. This is associated with the significant enhancement of the lipase and the lipoxigenase in the primed group more than that in the non-primed group. The two enzymes increased on the 1st day in the primed group of 104.74% and 68.87% more than that in the non-primed one respectively.

3.3. Shikimic acid content

Priming significantly decreased the shikimic acid content less than that in the non-primed germinated seeds. On the 1st day, its content decreased by the highest decreasing ratio about 66.87% less than that in the non-primed germinated seeds as shown in Table (2).

3.4. Isocitrate lyase activity

Results in the Table (2) show that priming significantly increased the isocitrate lyase activity. Its activity in primed germinated seed increased by 69.02%, 38.18% and 52.42% more than that in the non-primed germinated seed in the 1st, 4th and 7th day respectively.

3.5. Change in reactive oxygen species and antioxidant machinery

In non-primed germinated seed H₂O₂ content increased at the 4th day more than that in the 1st day. In the 7th day, H₂O₂ content decreased less than that in the 4th day. In the primed, it decreased continuously with time. Also H₂O₂ content in primed germinated seed was

Table 2

Lipid hydrolysis (Lipase (%), lipoxigenase (μM min⁻¹ mg⁻¹ protein) and total lipids (mg g⁻¹ fresh weight)), Shikimic acid content (μg g⁻¹ fresh weight) and isocitrate lyase (change in OD min⁻¹ mg⁻¹ protein) in either non-primed or primed germinated cotton seeds after 1, 4 and 7 days from sowing.

Parameter	Non-primed			Primed		
	1	4	7	1	4	7
Lipase	26.55 ± 0.121	53.82 ± 0.119	89.46 ± 0.344	54.36 ± 0.217*	81.42 ± 0.097*	154.98 ± 0.480*
lipoxigenase	10.28 ± 0.034	18.39 ± 0.073	20.97 ± 0.144	17.36 ± 0.044*	22.29 ± 0.019*	25.89 ± 0.026*
Total Lipids	89.37 ± 0.020	63.54 ± 0.139	48.59 ± 0.031	48.40 ± 0.056*	35.78 ± 0.040*	30.49 ± 0.058*
Shikimic acid	3.00 ± 0.260	0.993 ± 0.005	0.893 ± 0.005	0.994 ± 0.004*	0.654 ± 0.005*	0.598 ± 0.003*
Isocitrate lyase	0.041 ± 0.004	0.0647 ± 0.0002	0.0847 ± 0.0003	0.0693 ± 0.0005*	0.0894 ± 0.0004*	0.1291 ± 0.0003*

Values are given as means of 5 replicates. Within a column, Means followed by asterisks are significantly different according to the paired-samples *t*-test.

much lower than that of the non-primed germinated seed.

Priming significantly decreased the malonyl dialdehyde (MDA) in the primed germinated seed. In primed germinated seed, MDA decreased with time. In the non-primed seed, MDA content increased at the 4th day more than that in the 1st day. In the 7th day, MDA content reduced less than that in the 4th day.

The priming caused a significant increase in the activities all studied antioxidant enzymes (SOD, POX or PPO) more than that of the non-primed germinated seed. SOD showed the lowest increase ratio of 29.15% on the 1st day. POX showed the highest increase ratio of 164.43% on the 4th day (Table 3).

4. Discussion

The process of seed germination is a tri-phase phenomenon which includes an initial phase of rapid early water uptake, followed by a plateau phase with little change in water content; and subsequently, the last phase which coincides with radicle emergence and resumption of growth. Primed seed completed first two phases during the priming process so immediately germinate after sowing (Ahmadvand et al., 2012).

The results of this study ensured that hydropriming significantly increased the germination percentage and improved the seed vigor index. This agreed with Farooq et al. (2011) and Shabbir et al. (2014) who reported that Priming improves seed vigor and vigorous seed, uniform emergence and good stand establishment. The beneficial effects of priming have also been demonstrated for many field crops such as wheat, sugar beet, maize, soybean barley chickpea lentil milk thistle and sunflower (Sadeghian and Yavari, 2004; Rashid et al., 2006; Ghassemi-Golezani et al., 2008; Hosseinzadeh-Mahootchi, Ghassemi-Golezani, 2013).

The positive effects of hydropriming are probably due to its stimulating property in the early stages of the germination process by the mediation of cell division in germinating seeds (Mustafa et al., 2017). According to McDonald (2000), experiment the major pre-germination steps such as DNA and RNA synthesis are accomplished in the seed during the priming states consequently the seeds are physiologically close to germination and have fewer steps to complete than non-primed seeds.

The germination of lipid-rich seed involves, among other processes, the rapid transport of stored triacylglycerols (TAGs) in the cotyledons. Such hydrolysis of TAGs is catalyzed by highly active lipases. This is the first step in TAG conversion to the sugar required for growth of the germinating embryo (Sebei et al., 2007).

Table 3

H₂O₂ content ($\mu\text{g g}^{-1}$ fresh weight), Malonyl Dialdehyde (MDA $\mu\text{g g}^{-1}$ fresh weight) and Antioxidant enzymes activity (Superoxide dismutase (SOD %), Polyphenoloxidase (PPO) and Peroxidase (POX) (change in OD min^{-1} mg protein⁻¹) in cotton germinated seeds at 1st, 4th and 7th day after sowing.

Parameter	Non-Primed			Primed		
	1	4	7	1	4	7
H ₂ O ₂	18.29 ± 0.024	19.203 ± 0.009	14.952 ± 0.005	9.283 ± 0.005*	8.352 ± 0.007*	6.059 ± 0.0003*
MDA	1.98 ± 0.066	2.56 ± 0.136	1.09 ± 0.087	0.937 ± 0.006*	0.902 ± 0.0196*	0.721 ± 0.015*
SOD	16.98 ± 0.222	17.12 ± 0.147	22.87 ± 0.263	21.93 ± 0.452*	26.04 ± 0.430*	32.02 ± 0.211*
PPO	0.009 ± 0.0005	0.01 ± 0.002	0.0194 ± 0.0007	0.0198 ± 0.0012*	0.0203 ± 0.001*	0.0498 ± 0.0008*
POX	0.0109 ± 0.0005	0.0149 ± 0.0005	0.0255 ± 0.0007	0.0198 ± 0.0009*	0.0394 ± 0.0006*	0.0438 ± 0.0007*

Values are given as means of 5 replicates. Within a column, Means followed by asterisks are significantly different according to the paired-samples *t*-test.

The increase in germination in the primed seed is attributed to the enhancement of the stored lipid metabolism. The results indicated higher activity of the lipase and the lipoxygenase enzyme in the primed seed than that in the non-primed seed. Thus, total lipids content in the primed seed was less than that in the non-primed seed.

This agreed with Farooq et al. (2011) who reported that hydro-priming breaks down seed dormancy by the activation of hydrolytic enzymes.

Seed germination in fat-storing species requires a functional glyoxylate cycle to affect net gluconeogenesis from the acetyl-CoA obtained from the oxidation of stored triglycerides. The number of fully functional glyoxysomes and the activity of the glyoxysomal enzymes, catalase, malate synthase, and isocitrate lyase are very important for energy production required for metabolic pathways during the germination process (Ooi et al., 2015). These findings have manifestly appeared in the current study in the increase of the isocitrate lyase activity in the primed cotton seed than that in the non-primed seed in the current study.

Basra et al. (2005) reported that priming increased the germination rate due to metabolic repair during imbibition and a buildup of germination enhancing metabolites. Mustafa et al. (2017) also showed that Presowing hydration treatments improved seed germination by delivering various metabolites by enhancing the enzyme action. The active metabolic status of the priming seed is ensured by the lower content of the shikimic acid in the primed seed than that in the non-primed one in this study.

Active metabolic status is combined with the increase in reactive oxygen species (Gill and Tuteja, 2010). This explains why the H₂O₂ content increased in the non-primed seeds in 4th day after sowing. The continuous decrease in H₂O₂ content in the primed seed combined with the higher increase in the studied antioxidant enzymes than that in the non-primed seed. This is agreement with the fact that the balance between production and elimination of reactive oxygen species at the intracellular level must be tightly regulated and/or efficiently metabolized. This is necessary to avoid potential damage caused by reactive oxygen species in cellular components as well as to maintain growth, metabolism, development, and overall productivity of plants. This equilibrium between the production and detoxification of reactive oxygen species is supported by enzymatic and non-enzymatic antioxidants (Mittler et al., 2004).

Improved germination of primed seed was attributed to enhanced counteraction of free radicals and re-synthesis of membrane-bound enzymes compared to unprimed seed (Mustafa et al., 2017). Similar findings were also pointed out by Draganic and Lekic (2012) in Sunflower seed, Lara et al. (2014) in tomato. Comparable results were published by Hosseinzadeh-Mahootchi and Ghassemi-Golezani (2013) for chickpea.

Accelerated aging also resulted in increased lipid peroxidation, decreased levels of anti-oxidants and reduced activity of several enzymes involved in scavenging of free radicals and peroxides (Bhattacharjee, 2005). Thus, reduced antioxidative activities, along with other mechanisms, may contribute to the increased susceptibility to

deterioration of primed seed.

The peroxidation of lipids is considered as the most damaging process known to occur in the action of the accumulation of reactive in every living organism. The concentration of MDA was a sign of lipid peroxidation in plant cells (Cai et al., 2011). The decline of the MDA concentrations in priming samples is attributed to the increase in the antioxidant enzymes activity in the primed grains more than that in the non-primed seeds.

This study has found that the priming of cotton seed increases the germination ratio and the vigor indexes. The increase is linked to the enhancement of the lipid metabolism. This enhancement ensured in increasing the activity of the lipase, lipoxygenase and isocitrate lyase enzymes activities. The high metabolic status in the primed seed clearly appeared in the low shikimic acid content. The reactive oxygen species produced through the high metabolic status are captured by the active antioxidant enzymes. This control in reactive oxygen species production helps in maximizing the lipid peroxidation through germination.

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