Biochemical changes and seedling growth in primed rice (*Oryza sativa* L.) seed

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ABSTRACT: Seed emergence failure is an important problem leading to low yield in dry direct-seeded rice, especially under rainfed areas of Asia. Seed priming before sowing promotes rice seed metabolism which enhances germination and seedling establishment. This study was conducted under laboratory condition to investigate the biochemical changes and seedling growth of primed seeds. The treatment consisted of two rice cultivars, Khao Dawk Mali 105 (KDML 105) and RD6 and six seed priming treatments were used: 1) hydropriming with distilled water, 2) osmohardening with calcium chloride ($CaCl_2$) 3) osmohardening with potassium chloride (KCl) 4) hormonalpriming with gibberellic acid (GA_3), 5) hormonalpriming with gibberellic acid combined ethylene (GA_3 + ET) and 6) hormonalpriming with wood vinegar compared to non-primed control. Total sugar was highest in hormonalpriming with GA_3 + ET in both rice cultivars. The cultivar (cv.) KDML 105 seeds primed with GA_3, GA_3 + ET, wood vinegar or distilled water and as well as hormonalpriming with GA_3 in cv. RD6 seeds significantly increased α-amylase activity over non-primed control, while priming with $CaCl_2$ or KCl decreased α-amylase activity in both rice cultivars. Seed priming with GA_3 or GA_3 + ET significantly increased shoot biomass and total seedling biomass over non-primed control. These results indicate that seed priming with GA_3, GA_3 + ET or wood vinegar improved biochemical metabolism in the seeds and seedling growth and could potentially be used to improve grain yield in dry direct-seeded rice.
Keywords: rice production, dry direct-seeded rice, seed priming, water stress

Introduction

The conventional production practice of transplanting rice seedlings into a flooded field has declined in rainfed area of Asia. The sustainability of this practice is questionable because water worldwide is becoming scarce, and because this practice is time-consuming, labor intensive and very costly (Pandey and Velsaco, 2002; Tuong et al., 2005). Therefore, most sustainable farmers in Asia have shifted from conventional transplanting to dry direct-seeded rice. Dry direct-seeded rice is broadcasting rice seed onto the moist soil (not saturated soil or water logging soil) during the beginning of rainy season. However, dry direct-seeded rice is frequently affected by drought stress almost stage of rice from seedbed until reproductive stage. Especially, at seedbed and seedling stage, loss of large soil moisture contents in the field likely to be associated with high temperature resulting to rapidly drying soils (Nabi et al., 2001). This stage, seeds rehydrate in preparation for germination, they reach a point at which desiccation is lethal. This point usually coincides with the loss of larger oligosaccharides (Koster et al., 1988), and late embryogenesis (LEA) proteins (Cordova and Burris, 2002) at the time of radicle protrusion can cause poor seed germination, seedling death and poor seedling establishment which can lead to high weed infestation (Du and Tuong, 2002). Weed infestation reduced yield by 50-91% in dry direct-seeded rice under rainfed conditions (Fujisaka et al., 1993). To mitigate the effect of

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drought periods, farmers increase seeding rate but this practice does not reduced weed dry matter at maturity stage in dry direct-seeded rice field (Ghansham and Surjit, 2008). Weed control is achieved through manual weeding and herbicide use but these practices also increase production cost (Pandey and Velasco, 2002). "Seed priming" before sowing has been suggested to improve emergence and seedling establishment. Prior studies found that seed priming reduced emergence time and provided uniform emergence (Ashraf and Foolad, 2005; Farooq et al., 2005); and allowed seeds to better tolerate unsuitable environmental conditions such as drought stress (Harris et al., 1999). Thus, seed priming could be a viable alternative for dry direct-seeded rice cultivation under rainfed conditions.

Seed priming is a seed enhancement technique in which seeds are partially hydrated until the germination process begins, but radicle emergence does not occur (Bradford, 1986). Various seed priming techniques are used to improving seed germination, seedling growth and crop yield such as hydropriming, halopriming, osmopriming, osmohardening and hormonalpriming (Nawaz et al., 2013). After initial seed imbibition, water activates hydrolytic enzymes that degrade stored protein, starch and oil as a source of energy and building block from new biosynthesis. The amylase enzyme is the main enzyme which catalyzes the hydrolysis of starch into sugar. The sugar fuels respiration in the embryo, so seeds can grow (Koning, 1994). Seed priming increased total sugar contents and α-amylase activity content in the seed. Osmoconditioning and osmohardening of normal and aged rice seeds increased total sugar contents and α-amylase activity (Lee and Kim, 2000). Amylase activity improved in the primed rice seeds and the extent of this change was different in the seeds subjected to different priming treatments (Farooq et al., 2006b).

Seed priming also improved rice seedling growth. Osmohardening with KCl or $CaCl_2$ improved seedling growth, stand establishment and grain yield of direct wet-seeded rice (Farooq et al. 2006b; Rehman et al., 2011). Hormonalpriming rice seeds with gibberellic acid (GA_3) or ethylene (ET) increased seedling growth (Watanabe et al., 2007) as well as hormonal rice seeds with wood vinegar improved seedling root growth (Sungwal et al., 2010). To our knowledge, the effect of seed priming on seed physiology and seedling survival under drought stress has not been investigated.

Seed germination and speed of germination are dependent on the biochemical changes occurring in seeds after imbibition. Most studies evaluated seed priming direct-wet seeded rice (saturated soil) but little information is available on dry direct-seeded rice especially under soil moisture stress conditions. Thus, the objectives of these studies were to investigate the effects of different seed priming treatments on total sugar and α-amylase activity content of two rice cultivars (experimental I) and the effects of seed priming on seedling growth of two rice cultivars under with-and without-drought stress conditions (experimental II).

Materials and Methods

These studies consisted of 2 experiments. The first experiment was designed to assess changes in total sugar contents and α-amylase

activity (biochemical changes) of primed rice seed, and the second experiment was designed to determined seedling growth after priming under stimulated drought stress comparison with well watering. These two experiments used the same cultivars and seed priming treatments but were set up slightly different as described in this section. They were conducted during January - November 2014. The rice cultivars used in these experiments were obtained from the Khon Kaen Rice Research Institute, Khon Kaen, Thailand.

Experiment I

Location, experimental design

This laboratory experiment was conducted at National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand. The experiment was designed as a split plot with three replications. Two rice cultivars (KDML 105 and RD6) were assigned as main-plot and seven seed priming treatments were assigned as sub-plot.

Seed treatments

The seeds were surface-sterilized by using 5% (v/v) sodium hypochlorite solution. Seeds were rinsed three times with distilled water and dried with tissue paper (Farooq, 2005) before priming. The ratio of seed weight to sterilizing solution was 1:5 (Rehman et al., 2011). Six seed priming treatments were included: 1) hydropriming with distilled water, 2) osmohardening with $CaCl_2$, 3) osmohardening with KCl, 4) hormonalpriming with GA_3, 5) hormonalpriming with GA_3 + ET and 6) hormonalpriming with wood vinegar compared to non-primed control.

Hydropriming with distilled water was soaked 250 g seeds in 1 L of distilled water for 48 h (Farooq, 2005). The osmohardening treatments consisted in soaking 250 g rice seeds for 24 h in 1 L distilled water mixed with Polyethylene Glycol-6000 (PEG) (Ajax Finechem Pty Ltd., Australia) and a 22% $CaCl_2$ (Inter Education Supply Co., Ltd., Thailand) or a 20% KCl (Inter Education Supply Co., Ltd., Thailand) by equilibrate an osmotic potential of the solution as -1.25 MPa (Farooq et al., 2005). The hormonal-priming with GA_3 consisted of soaking 250 g seeds for 48 h in a solution of 100 ppm of GA_3. (The Institute of Biotechnology and Genetic Engineering, Chulalongkorn University) mixed with 1 L of distilled water (Watanabe et al., 2007). The hormonalpriming with GA_3 + ET was soaked 250 g seeds for 48 h in 1 L distilled water mixed 100 ppm of GA_3 and 50 ppm of ET (2-chloroethylphosphonic acid, Osgar Agro Co., Ltd., Nonthaburi, Thailand) in distilled water (Watanabe et al., 2007). Hormonalpriming with wood vinegar (or pyroligneous acid; TPI Polene Bio-organic Co., Ltd., Bangkok, Thailand) was soaked 250 g seeds in a solution of 1:300 (wood vinegar: distilled water) for 48 h (Sungwal et al., 2010). All priming treatments were soaked at room temperature (25±3 °C) (Ruan et al., 2002). Then, seeds were rinsed 3 times with distilled water and air-dried using an Air-dryer SKK 09 (Ceres International Co., Bangkok, Thailand) for 10 h or until closely with initial seed moisture content under a 30 °C of temperature. Seeds were cooled and sealed in polythene bags and stored in refrigerator at 15 ºC, 50 % relative humidity until the experiments were conducted at day 5 after priming.

Seed moisture contents

Ten grams of seeds were dried at 105 °C in a hot-air oven until constant weight. The seed samples were cooled in desiccators at room temperature and then weighted. The moisture content on a fresh weight basis was calculated by the loss of weight using the following formula:

$$Seed\ moisture\ content\ (\%) = ((M2 - M3)/(M2 - M1))\ x\ 100$$

Where: M_1 = Weight of container + cover, M_2 = Weight of container and seeds before drying and M_3 = Weight of container and seeds after drying

α-amylase activity measurement

The samples were prepared by the methodology of Bailey et al. (1992). Five grams of seed were ground using an electric grinder. A 1 g sample of powder was mixed with 50 ml of distilled water, heated gently at 50 °C while stirring until soluble starch was completely dissolved. Then, 0.2 ml citrate buffer pH 5.5 were added and adjusted to a volume of 100 ml with distilled water, this solution called "substrate solution[1]". Then the substrate solution[1] was diluted, this solution called "enzyme dilution[2]".

Three ml of dinitrosalicylic acid (DNS) was added in 15 ml tube already had a 2.0 ml substrate solution[1], mixed and boiled for 5 minutes in the water bath and cooled in cold water, this solution called "reagent solution[3]". This solution was used to reagent blank at zero spectrophotometer. Then, 3 ml of DNS was added in 15 ml tube already had a 1.8 ml substrate solution[1], then added 0.2 ml of enzyme dilution[2], mixed and boiled for 5 minutes in the water bath and cooled in cold water.

The absorbance of the enzyme solution was determined at 540 nm by Spectrophotometer (SPECORD 250 Plus analytic Jena, Germany) by comparison with the reagent blank. The activity of enzyme in the samples was determined by the method of Miller (1959) by one unit of amylase is the amount of enzyme required to liberate 1 μmole of glucose from starch soluble in one minute, computed by following the formula:

$$\text{Enzyme activity (1 μml/min)} = \frac{Absorbance\ x\ Standard\ factor\ x\ dilution}{Time\ of\ incubation\ x\ Molecular\ weight\ of\ glucose}$$

Total sugar contents measurement

Total sugars were determined by using the phenol sulfuric method (Masuko et al., 2005). One gram of seed from each sample was ground with an electric grinder and mixed with 10 ml distilled water and left for 24 h at room temperature. The mixture was filtered through Whatman filter papers no. 42 and distilled water was added to a final volume of 25 ml. The solution was further diluted with distilled water to a 1:25 ml volume. One ml of this solution was pipette into a test tube mixed with 1 ml of phenol solution and the absorbance was read at 490 nm by Spectrophotometer (SPECORD 250 Plus, analytic Jena, Germany).

Experiment II

Location, experimental design and seed treatments

The laboratory experiment was conducted at Seed Processing Plant, Faculty of Agriculture, Khon Kaen University, Thailand. The experiment was a split-split plot design with 4 replications. Two rice cultivars were assigned as main-plot, two water stress conditions (0 and -0.75 MPa) were assigned as sub-plot and seven seed priming treatments were assigned as sub-sub-plot. The seven seed priming treatments (six treatments and a non-primed control) were the same as those used in experiment I.

Seed Testing and water regimes

Seedling growth was tested using the International Seed Testing Association (ISTA)'s Between Papers Method (ISTA, 2004). The 50 seeds were placed in the papers and treated every day with either 2 ml/day of distilled water (control treatment) for 10 days or -0.75 MPa osmotic-potential solution of PEG (stress treatment). The PEG solution was added fresh daily to maintaining its concentration in the germination test. Tests were placed inside the germination box (SNIJDERS, Economic deluxe, Netherland) at 25 °C of temperature and 12 h/day photoperiod with 40 J m/s of light intensity. The osmotic potential of PEG solution was calculated according to Van't Hoff equation (1884).

$$\text{Water potential} = -iRTCs$$

When; i is number of atom resulting from the disintegration of solution, R is constant of gas (0.0083143 L

MPa mol/K), T is temperature (K) and Cs is concentration of solute (M)

Seedling growth measurements

At 14 days after planting, ten seedlings were recorded for root biomass, shoot biomass, total seedling biomass, root length, shoot length, seedling length and root:shoot ratio. For biomass traits, the seedlings were dried at 80 °C for 48 h or until constant dry weight with a dry-air oven. For root:shoot ratio were determined based on seedling dry weight.

Statistical analysis

The analysis of variance (ANOVA) and correlation coefficient analysis was performed by using Statistix 8 program (Analytical Software). Mean comparisons were performed using Least-Significant Difference (LSD) for all experiments.

Results

Seed moisture contents

The initial seed moisture contents were 10.6 % and 10.9 % for cv. KDML 105 and cv. RD6, respectively. After seed priming, seed moisture content significantly increased. The minimum and maximum mean of seed moisture contents were 24.8 % to 30.4 % for cv. KDML 105 and 24.3 % to 32.2 % for cv. RD6. The seed moisture content of osmohardening were lower (24.1 % to 25.2 %) than those of hormonal- and hydro- priming (29.0 % to 32.2 %). After priming, seeds were dehydrated to closely of initial seed moisture content ranges from 13.0 % to 16.6 %.

Total sugar contents and α-amylase activity content

Rice cultivars were significantly effect on α-amylase activity and total sugar content

(P<0.05) (data not shown). The cv. RD6 observed higher α-amylase activity and total sugar than those of the cv. KDML 105.

The interaction between seed priming and rice cultivars were significantly (P<0.01) different on total sugar content and α-amylase activity content. Therefore, data were reanalyzed by rice cultivar and results are presented in **Figure 1** and **2**.

Seed hormonalpriming with GA_3, GA_3 + ET or wood vinegar significantly increased (P<0.01) total sugar content over non-primed control of both rice cultivars. Seeds osmohardening with $CaCl_2$ significantly decreased total sugar content as compared to non-primed control, while seeds hydropriming with distilled water and osmohardening with KCl did not show any significantly different on total sugar content of both rice cultivars. The total sugar content of both rice cultivars were the highest in hormonalpriming with GA_3 + ET (**Figure 1**).

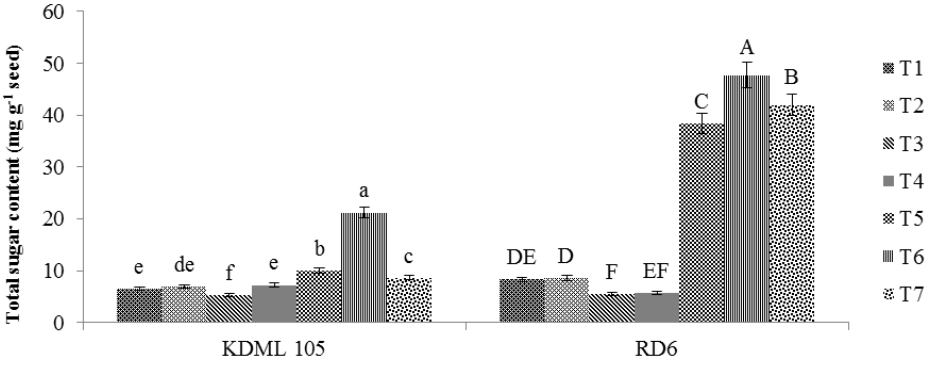

Figure 1 Seed priming and rice cultivar interaction means for total sugar contents (mg/g).
Means labeled with the same letter do not differ significantly at P < 0.05 of each cultivar.
*T1= control, T2 = distilled water, T3 = $CaCl_2$, T4 = KCl, T5 =GA_3, T6 = GA_3 + ET and T7 = wood vinegar

Seeds hormonal priming with GA_3, GA_3 + ET or wood vinegar and hydropriming with distilled water significantly increased (P<0.01) α-amylase activity as compared to non-primed control in cv. KDML 105 (**Figure 2**). Seeds hormonalpriming with GA_3 significantly increased α-amylase activity over non-primed control, but hydropriming with distilled water, hormonalpriming with GA_3 + ET or wood vinegar were not significantly effect on α-amylase activity as compared to non-primed control for cv. RD6. The α-amylase activity of seeds osmohardening with $CaCl_2$ or KCl were significantly lower than those of non-primed control of both rice cultivars (**Figure 2**).

Seedling growth

No significantly interaction was observed between rice cultivar and water regime, rice cultivar and seed priming, water regime and seed priming and as well as cultivar and water regime and seed priming (data not shown) in the present experiment, therefore the results were separately analyzed in each factor.

The rice cultivar was not significantly different (P<0.05) on shoot biomass, root biomass, seedling biomass, root:shoot ratio, shoot length, root length and seedling length (data not shown).

Water regime was not significantly different on shoot biomass, root biomass, total seedling biomass, root:shoot ratio, shoot length, root length and seedling length (data not shown).

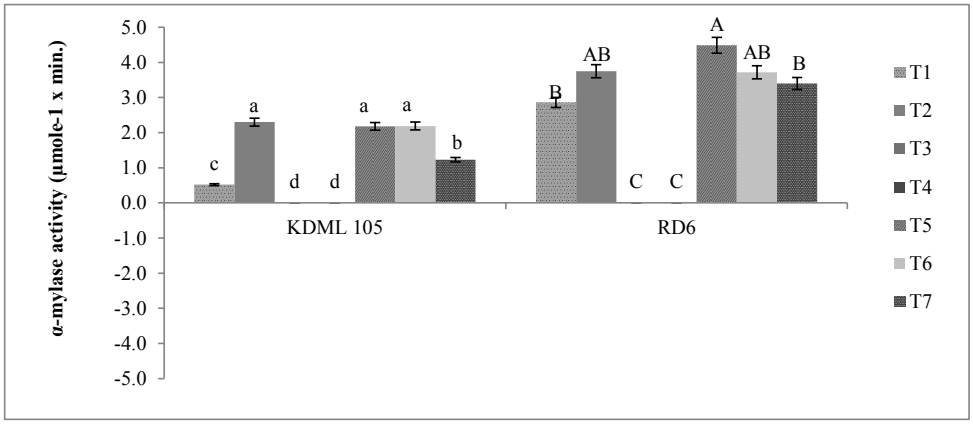

Figure 2 Seed priming and rice cultivars interaction means for α-amylase activity content (µmole^{-1} x min.).
Means of following the same letter do not differ significantly at P<0.05 of each cultivar.
*T1= control, T2 = distilled water T3 = $CaCl_2$, T4 = KCl, T5 = GA_3, T6 = GA_3 + ET and T7 = wood vinegar

Seed priming was significantly different (P<0.05) on shoot biomass, root biomass, root biomass, total seedling biomass and root:shoot ratio. However, there was not significantly different on root length and seedling length. Seeds hormonalpriming with GA_3 or GA_3 + ET were significantly increased (P<0.05) shoot biomass (**Figure 3a**) and total seedling biomass (**Figure 3c**) over non-primed control. However, these treatments showed the lowest on root biomass (**Figure 3b**) and root:shoot ratio (**Figure 3f**). Osmohardening with KCl significantly increased root:shoot ratio when compared to other treatments (**Figure 3f**). While, root length (**Figure 3d**) and seedling length (**Figure 3e**) were not significantly different by seed priming.

Discussions

Difference of rice cultivar on α-amylase activity content and total sugar content

Rice cultivar RD6 and KDML 105 are popular cultivated in rainfed areas of Northeastern, Thailand. In the present experiment, rice cultivar was significantly different on biochemical changes. The cv. RD6 gave higher α-amylase activity content and as well total sugar content than those of cv. KDML 105. In generally, the cv. RD6 is sticky rice or glutinous rice type, which it contain higher sugar content (0.1 g/100 g seeds) (Schenker, 2012), while the cv. KDML 105 is jasmine rice or non-glutinous rice type, which it consist less sugar content (<0.01 g/100 g seeds) (Schenker, 2012) than those of cv. RD6. Previous study reported that total sugar content was positive correlated with α-amylase activity (Lee and Kim, 2000), so that rice cultivar contain high total sugar content would be gave high α-amylase activity as well.

Effect of seed priming on α-amylase activity and total sugar content

Amylase is an important enzyme for seed germination in rice. During seed rehydration, GA_3 is produced in the embryo and released into the endosperm. GA_3 induces aleurone layer cells to synthesize and release α-amylase enzyme into the endosperm. The main storage compound in rice is starch which is located in the endosperm and it is made up of amylose and amylopectin

(Bewley and Black, 1994). The enzyme α-amylase randomly hydrolyzes the α-glyosidic bonds between glucose molecules throughout the amylose and amylopectin chains (Bewley and Black, 1994). The sugar fuels metabolism and respiration in the embryo, so seedlings can grow (Koning, 1994). Ethylene (ET), also released during seed rehydration process, plays a role in promoting seed germination (Matilla, 2000).

As the results, seed hormonalpriming with GA_3, GA_3 + ET or wood vinegar significantly increased total sugar content over non-primed control in both rice cultivars. The total sugar content in both rice cultivars observed the highest in seed hormonalpriming with GA_3 + ET; while hormonalpriming with GA_3 illustrated a significantly increased in α-amylase activity as compared to non-primed control. Previous study reported that ethylene (ET) can stimulate the conversion of starch to sugar (Thammawong, 2010). However, when GA_3 was applied simultaneously with ethylene, no significant effect on amylase synthesis and release was observed (Eastwell and Spencer, 1982). Our results indicated that GA_3 combined with ET increased total sugar contents in rice seeds.

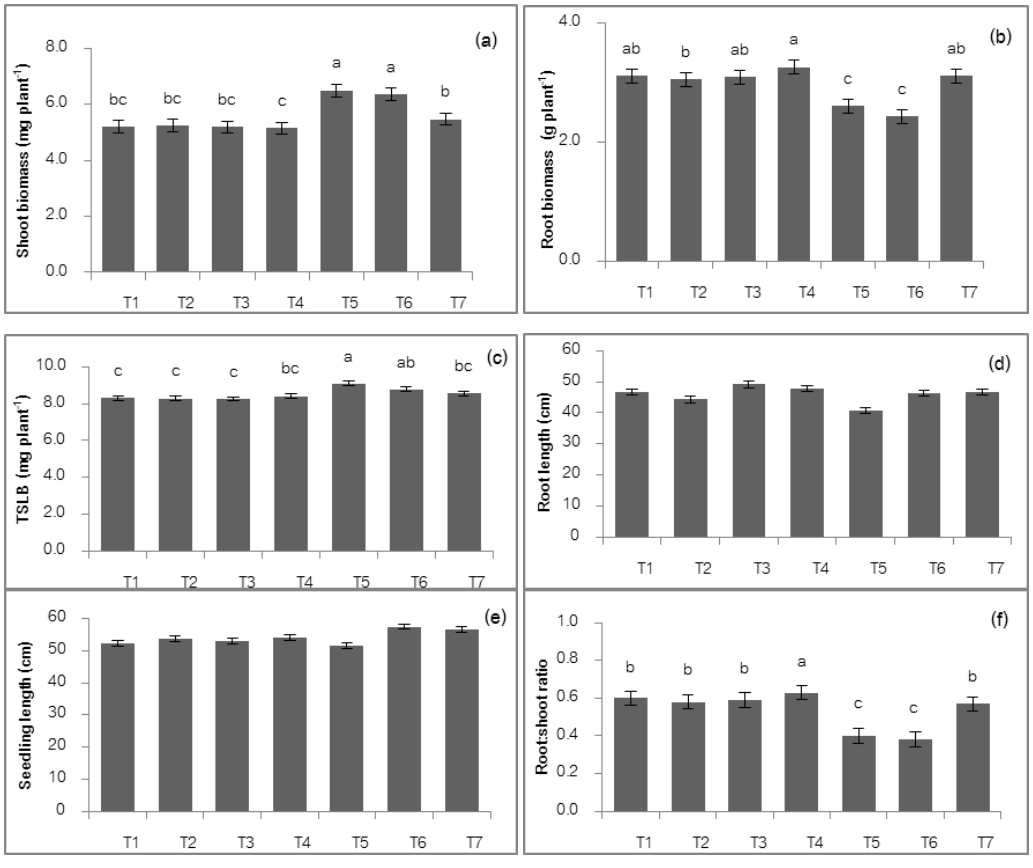

Figure 3 Effects of seed priming on shoot biomass (a), root biomass (b), total seedling biomass (TSLB) (c), root biomass (d), seedling length (e) and root:shoot ratio (f).

Means labeled with the same letter do not differ significantly at $P < 0.05$.

*T1= control, T2 = distilled water, T3 = $CaCl_2$, T4 = KCl, T5 =GA_3, T6 = GA_3 + ET and T7 = wood vinegar

In the present experiment, hydropriming, hormonalpriming and osmohardening can be used to enhance metabolic breakdown of stored starch and to evaluate changes in total sugar and α-amylase activity. The results showed that hydropriming and hormonalpriming increased. While, osmohardening decreased total sugar content and α-amylase activity content in comparison with non-primed control. This was probably due to shorter of soaking period (24 h) and lower water potentials (-1.25 MPa) in osmohardening than those of hydropriming and hormonalpriming (48 h and 0 MPa), leading to inadequate rehydration of the seed for germination. The average seed moisture content of osmohardening was 24.8 %, while it was 30.4 % of hydropriming and hormonalpriming. The lower moisture content in the osmohardened seeds due to the fact that in lower α-amylase activity and total sugar content.

Effect of rice cultivar on seedling growth

The results of this study observed that the cv. KDML 105 was significantly higher root length, shoot length and seedling length than those of the cv. RD6. This was probably due to the difference of the genetic variation characteristic of the two rice cultivars (Kato et al., 2006).

Effect of water regime on seedling growth

Davatgar et al. (2009) found that rice experienced to water stress when soil moisture reached permanent wilting point (PWP) at a water potential of -1.5 MPa. So, in the present experiment was designed for a drought of 50% of PWP or -0.75 MPa compared to non-water stress condition (0 MPa). In the present experiment water stress condition (-0.75 MPa) had no affected on all seedling growth traits as compared to well watering condition (0 MPa). This might be due to rice at seedling growth stage can be adapted to water stress at -0.75 MPa. Further research experiment should be tested at higher degree of water stress conditions in comparison with well watering condition.

Effect of seed priming on seedling growth

As the results of this study, shoot biomass and total seedling biomass were significantly greater only in seed hormonalpriming with GA_3 and GA_3 + ET. The rest treatments were not significantly different from non-primed control. The seedling growth improved due to the effect of low concentrations of GA which promoted seed germination (Takahashi et al., 1991) and inducing synthesis of soluble proteins (Gupta and Mukherjee, 1982). Adomet is the precursor of ET, which regulates seed germination by promoting the weakening and rupture of seed tissues surrounding the radicle (Linkies et al., 2009). Moreover, the roles of GA_3 and ET were described above. Watanabe et al. (2007) observed that hormonalpriming with gibberellic acid (GA_3) or ethylene (ET) increased rice seedling growth.

Seeds osmohardening with KCl significantly increased root:shoot ratio as compared to the other seed priming treatments or non-primed control. Previous study reported that potassium ion (K^+) is an important nutrient for increasing crop yield. K^+ is a cofactor or activator for many enzymes of carbohydrate and protein metabolism (Fageria et al., 2010). K^+ also is needed for ATP production and for sugar transport into the phloem, improves photosynthesis rate and

increases root growth and drought resistance (Potash&Phosphate Institute, 1998). Farooq et al. (2006b) and Rehman et al. (2011) also reported that osmohardening with KCl or $CaCl_2$ improves seedling growth, stand establishment and grain yield of wet direct-seeded rice. However, previous studies observed that seed priming can improves seed germination, growth of seedling and yield under well watering and limited water conditions in lentil (*Lentil culinaris*) (Golezani et al., 2013), sunflower (*Helianthus annus*) (Fatemi, 2014) moreover, seed priming increased drought tolerance due to reduced lipid peroxidation, stabilized the cell membrane, increased osmotic adjustment in sorghum (*sorghum bicolor*) (Zhang et al., 2015).

Conclusions

Seeds hydropriming (distilled water) and hormonalpriming (GA_3, GA_3 + ET or wood vinegar) significantly increased total sugar contents and α-amylase activity in comparison with non-primed control, while seeds osmohardening ($CaCl_2$ or KCl) did not in both rice cultivars (cv. KDML105 and RD6). Based on our results, seed hormonal primed with GA_3, GA_3 + ET or wood vinegar improved biochemical pathways and seedling growth of both rice cultivars and two water stress conditions. These methods could be suggested for improving crop establishment in dry direct-seeded rice cultivation under rainfed conditions.

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