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ORIGINAL ARTICLE

Changes in the Activity of Antioxidant and Glyoxylate Cycle Enzymes of Hydro-Primed *Calendula officinalis* (L.) Seeds after Re-Drying Temperature Stress

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In order to study the effect of re-drying temperatures on the enzymatic activation of hydro-primed *Calendula officinalis* (L.) seeds, a completely randomized design with three replications was conducted. Treatments were six different re-drying temperatures including control (without drying), 20, 30, 40, 50, and 60 °C applied after hydro-priming. Results showed that rapid drying at high temperatures (40-60 °C) caused a significant difference comparing control. The best re-drying temperature considering many studied traits was 20-30 °C. At 60 °C, activity of antioxidant enzymes such as superoxide dismutase, catalase and peroxidase decreased 65.5, 75, and 62%, respectively over control. Re-drying had not significant effect on the activity of malate synthase and isocitrate lyase in comparison with control and 20-30 °C that demonstrates no negative and reductive effect of re-drying with these temperatures on seed germination. It seems that seeds are more desiccation tolerant at these temperatures. In conclusion, we can dry hydro-primed seeds slowly at 20 and 30 °C and store them until utilization.

Key words: antioxidant enzymes, C. officinalis, glyoxylate cycle, hydro-priming, re-drying temperature

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Key words: antioxidant enzymes, C. officinalis, glyoxylate cycle, hydro-priming, re-drying temperature

Pot Marigold (*Calendula officinalis* L.) is an annual herb originated from west Asia and Mediterranean. Seeds have 18-22% lipid which 55-60% of it contains calendic acid (Omidbeigi, 2005). Some of scientists believe that re-drying of primed seeds can decrease the efficacy of priming (Armstrong and McDonald, 1992; Butler *et al*., 2009). If this is being true, then finding a technique to overcome the damages is necessary. Re-drying is a critical point to seed quality and damages increase with storage time after priming (Drew *et al*., 1997). Any unfavorable status in the whole plant systems

can lead to the production of reactive oxygen species (ROS) and oxidative stress (Sedghi *et al*., 2012a). Others mentioned that efficacy of priming increases after re-drying and antioxidant systems have sufficient time to affect repair systems and led to physiological stability (Dell Aquila and Tritto, 1991).

Parera and Cantliffe (1992) proposed that rapid drying of primed seeds can decrease the priming efficacy. Re-drying removes the positive effect of priming especially when seeds programmed to be stored (Butler *et al*., 2009). Gurusinghe *et al*. (2002) reported that slow re-drying can induce the desiccation tolerance through synthesis of late embryogenesis abundant (LEA) proteins.

Expression and function of some genes only after slow re-drying are the same with the genes responsible for DNA protection and stress tolerance induction (Soeda *et al*., 2005). It seems that low and moderate re-drying temperatures (slow re-drying) cause to desiccation tolerance and success dryness. Chen (2011) reported that after re-drying, Malondialdehyde (MDA) content increased about 75% in the primed seeds of spinach. Activities of catalase and superoxide dismutase during two days of re-drying increased respectively 100 and 80% over control.

Leprince *et al*. (1994) demonstrated that free radical accumulation and increasing in lipid peroxidation during hydratation and subsequent dehydration can decrease the longevity of primed seeds. Wattanakulpakin *et al*., (2012) reported some different results in maize. They showed that after re-drying MDA content decreases. Davison *et al*., (1991) concluded that priming can repair some damages to rRNA in onion seeds and this repair system maintains active even after seed re-drying.

This experiment conducted to evaluate the effect of slow and rapid re-drying techniques on the maintenance of priming efficacy regarding to antioxidant repair systems and study the glyoxylate cycle enzymes activity after drying. On the best of our knowledge there is no similar study in the literature about the changes in the glyoxylate cycle activity in re-dried primed seeds of *C. officnalis.* This part of study can journalize considering to high lipid content in Pot Marigold's seeds.

MATERIALS AND METHODS

This experiment conducted to evaluate the effect of re-drying temperatures on the changes in the activity of antioxidant and glyoxylate enzymes in hydro-primed *C. officinalis* seeds at the University of Mohaghegh Ardabili, Ardabil, Iran. Statistical design was completely randomized in three replications and treatments were five different temperatures as 20, 30, 40, 50 and 60 °C and a control treatment which hydro-primed seeds were not re-dried after hydro-priming and directly were used for germination test. Freshly harvested seeds were soaked in deionized distilled water without aeration about 24 h at laboratory temperature and hydro-primed. Then, seeds re-dried at different temperatures until their weight was constant at two sequential weighing. Seeds re-dried after 48 h at 20 and 30 °C, but the rest re-dried after 24 h. Standard germination test performed using 50 seeds per each petri dish in the germinator with 25±1 °C and without light. On the 5^{th} day of experiment randomly selected seed residuals (without radicle and plumule) were taken and frozen in liquid nitrogen for subsequent enzyme extraction.

Antioxidant enzymes assay

One g of the frozen seed residual was homogenized in mortar with 5 ml of 50 mM

potassium phosphate buffer (pH 7.5) containing 1 mM EDTA, 1 mM dithiotreitol and 2% polyvinyl pyrrolidone (PVP). The homogenate was centrifuged at 15,000 g for 25 min and the supernatant was used for superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD) assay. The activity of SOD (EC 1.15.1.1) was determined according to Beyer and Fridowich (1987). In small glass tubes, 20 µL of enzyme supernatant were added to 50 mM potassium phosphate buffer (pH 7.8), 9.9 mM L-methionine, 57 µM nitro blue tetrazolium (NBT), and 0.025% triton-X-100. Reaction was started under fluorescent light for 10 minutes by adding 10 µL of riboflavin solution. Absorbance of the solution was measured at 560 nm for both blank and control. SOD activity was expressed as Unit mg^{-1} DW. The activity of CAT (EC 1.11.1.6) was assayed according to Chance and Maehly (1955). A 1.5 mL reaction mixture containing 30 µL water, 50 µL 1M Tris-HCl buffer (pH 8.0), 5 mM EDTA and 900 µL 10 mM H_2O_2 was added to 20 µL of enzyme supernatant. The decrease in the absorbance at 240 nm was recorded for 60 seconds. CAT activity was expressed as absorbance in mg protein per min. The activity of POD (EC 1.11.1.7) was determined spectrophotochemically at 470 nm according to Yamane *et al*., (1999) in a 3 mL reaction mixture containing 1.5 mL 0.1 M potassium phosphate buffer (pH 7.0), 600 µL 10 mM guaiacol, 800 µL 4 mM H_2O_2 and 100 µL crude enzyme. POD activity was expressed as µmols of guaiacol oxidized to tetraguaiacol by a unit of enzyme per min. Lipid peroxidation was measured in terms of malondialdehyde (MDA) content of samples as described by Stewart and Bewley (1980) in a colorimetric method. Samples were homogenized in 2 ml of 0.1% trichloroacetic acid (TCA) and centrifuged. Then, 0.5 ml of supernatant was mixed with 2 ml of 20% TCA containing 0.5% thiobarbituric acid. The mixture was incubated at 95˚C for 30 minutes. The samples were centrifuged at 10,000 g for 10 minutes. The absorbance of the supernatant was read at 532 and 600 nm. The amount of MDA was calculated from the extinction coefficient of 155 mM^{-1} cm^{-1}.

Glyoxylate enzymes assay

The samples were hand homogenized at 4 °C in a pre-chilled mortar and pestle with 0.15 M Tris-HCl (pH 7.5) containing 1 mM EDTA, 2 mM DTT, 10 mM KCl, 10 mM $MgCl_2$, 0.6 M sucrose. The homogenate was centrifuged at 12,000 g for 20 min and the supernatant was used as the enzyme preparation for isocitrate lyase (ICL, EC 4.1.3.1) and malate synthase (MS, EC 4.1.3.2) activity assays.

The ICL activity was determined using the protocol of Dixon and Kornberg (1959) that modified by Ranaldi *et al*., (2000). The assay mixture consisted of 4 mM D, L-isocitric acid, 5 mM $MgCl_2$, 4 mM cysteine, 6 mM phenylhydrazine and 50 mM triethanolamine (pH 7.5). The reaction was started with the substrate, 4 mM D, L-isocitric acid. Activity of ICL was assayed at 25 °C and 324 nm for 2 min using a spectrophotometer.

Malate synthase (EC 4.1.3.2) activity was assayed by a modified method of Cooper and Beevers (1969). The assay mixture in a volume of 1 ml consisted of phosphate buffer (pH 6.5), 100 mM acetyl CoA, 0.5 mM sodium glyoxylate, 3 mM $MgC1_2$, and 100 mM 5,5'-dithiobis-2-nitrobenzoic acid (DTNB). The reaction was started by the addition of the glyoxylate and incubated at 35°C. The absorbance of the mixture was measured at 412 nm after 10 min.

The data were obtained from completely

randomized design (CRD) in three replications. All data subjected to an analysis of variance after normality test and when a significant (P< 0.05) F ratio occurred for treatment effects, comparison of means was carried out using Tukey's honestly test at 5% statistical probability level.

RESULTS

Table 1 indicates the effects of re-drying temperatures on the activity of antioxidant and glyoxylate cycle enzymes in hydro-primed seeds of pot marigold. This table shows a significant effect of treatments on all measured traits. Slow re-drying of hydro-primed seeds at 20 and 30 °C had no significant difference with control on SOD activity (Table 2). This indicates no negative effects of slow re-drying on SOD activity, while rapid re-drying at 60 °C decreased the enzyme activity about 65.5% over control. On the other hand, rapid re-drying caused to decrease the antioxidant activity of SOD for scavenging ROS. CAT activity at 20 °C re-drying temperature was not significant with control, but decreased at 30 °C. Rapid re-drying also decreased 75% of CAT activity in comparison with control (Table 2). POX had greater activity in control treatment and there were 4 and 6% decrease in POX activity at 20 and 30 °C, respectively (Table 2). Rapid re-drying caused 62% reduction in POX activity.

Lipid peroxidation on the basis of MDA production showed that the highest membrane damage was related to rapid re-drying and was 74% higher than control (Table 2). Slow re-drying treatments had no effect on membrane structure and were the same with control.

In this experiment, there were no significant difference on the activity of glyoxylate cycle enzymes between control and slow re-drying at 20 and 30 °C (Table 1), while rapid re-drying decreased 49 and 54% of enzyme activity over control, respectively in MS and ICL (Table 2).

Table 1: Analysis of variance for the effect of re-drying temperatures on the enzymatic activity in hydro-primed *Calendula officinalis* seeds

		Mean of squares					
SOV	**df**	**SOD**	**CAT**	**POX**	**MDA content**	**MS**	**ICL**
Re-drying temperature	5	2054.548**	635.714**	479.791**	0.084**	0.034**	0.023**
Error	12	1.267	0.988	0.197	0.001	0.0001	0.0001
CV (%)		0.015	0.033	0.013	0.044	0.029	0.037

** significant at 1% probability level. SOD, superoxide dismutase, CAT, catalase, POX, peroxidase, MDA, Malondialdehyde content as estimation of lipid peroxidation, MS, malate synthase, ICL, isocitrate lyase.

Table 2: Comparison of means for the effect of re-drying temperatures on the enzymatic activity in hydro-primed *Calendula officinalis* seeds

Re-drying temperature (°C)	**SOD (unit mg^{-1})**	**CAT (unit mg^{-1})**	**POX (unit mg^{-1})**	**MDA (mM g^{-1})**	**MS (unit mg^{-1})**	**ICL (unit mg^{-1})**
Control	96.76 a	43.13 a	45.43 a	0.54 d	0.45 a	0.37 a
20	95.93 a	42.36 ab	43.63 b	0.58 d	0.43 a	0.35 a
30	95.2 a	40.36 b	42.8 b	0.59 d	0.42 a	0.34 a
40	78.36 b	24.13 c	27.7 c	0.78 c	0.28 b	0.21 b
50	55.83 c	15.03 d	20.33 d	0.86 b	0.24 bc	0.18 b
60	33.4 d	10.73 e	17.26 e	0.94 a	0.22 c	0.20 b

In each column means with the same letter are not significantly different. SOD, superoxide dismutase, CAT, catalase, POX, peroxidase, MDA, Malondialdehyde content as estimation of lipid peroxidation, MS, malate synthase, ICL, isocitrate lyase.

DISCUSSION

Bailly *et al*., (2000) reported that during priming, activity of seed antioxidant systems increase and repair processes improve effectively.

Plants produce ROS even at normal conditions and SOD is the first defensive line against ROS. SOD dismutates the superoxide radicle to oxygen and H_2O_2 (Cruz de Carvalho, 2008). Scientists believe that after priming activity of antioxidant enzymes increases and indicates a repair mechanism in seed (Wahid *et al*., 2008; Li *et al*., 2010). Lipid peroxidation increases gradually after re-drying of primed seeds (Schwember and Bradford, 2005; Chen, 2011). Most likely, reduction in the percentage and rate of germination after rapid re-drying of primed seeds (Sedghi *et al*., 2012b) is due to increase in membrane degradation and reducing the activity of antioxidant enzymes. At slow re-drying, there is a recovery in repair and turn over power of defensive systems and antioxidants, and when seed placed in wet medium it can better avoid of oxidative stress. Davison *et al*., (1991) mentioned that priming repairs the rRNA damages and this improvement system maintain in both primed and re-dried seeds.

Re-drying of primed seeds lead to a desiccation stress and if applied slowly, seed can better adapt to stress. In the slow re-drying there is enough time to extension of repair systems and this compensates damages to membrane and the other cell compartments, effectively (Drew *et al*., 1997; Dell Aquila and Tritto, 1991; Gurusinghe *et al*., 2002).

El-Araby and Hegazi (2004) showed that re-drying at room temperature (Slow re-drying) caused to increase in CAT and POX activity. Reduction in membrane damages and lipid peroxidation at slow re-drying probably is related to accumulation of adaptation molecules such as LEA proteins, dehydrins, sucrose and oligosaccharides that conserve the membrane integrity. This hypothesis is supported by Buitink *et al*., (2002). *Bip* protein discovered in tomato seeds likely is responsible for amelioration of stress conditions during re-drying or storage of primed seeds (Gurusinghe *et al*., 2002).

Glyoxylate cycle happens in glyoxysomes during seed germination and early seedling growth before photosynthesis and converts lipids to structural carbohydrates. Glyoxysomes disappear after photosynthesis establishment. Activity of cycle is higher in the seeds containing more lipid storage (Eastmond and Graham, 2001). Seeds of pot marigold have approximately 20% lipid that makes it suitable case for study of glyoxylate cycle activity. It seems that reduction of the activities of MS and ICL with rapid re-drying caused to dramatically decrease in the conversion of lipids to carbohydrates, so storage and membrane lipids switch to a good source of substrate for peroxidation reactions and increase the content of MDA.

Jeng and Sung (1994) reported that priming increases the activity of glyoxylate cycle enzymes. Sedghi *et al*. (2011) also reported such results.

CONCLUSION

C. officinalis only propagated with seeds and they are weak in germination. Sometimes due to environmental and technical difficulties, planting may face with some delays. One method to overcome the problem is priming, but we need to re-dry the seeds before transport to field or use on-farm priming techniques. Results of this experiment showed that we can re-dry primed seeds at 20-30

°C without any significant damages and store them several months before planting.

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