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Effect of the Ascorbic Acid, Pyridoxine and Hydrogen Peroxide Treatments on Germination, Catalase Activity, Protein and Malondialdehyde Content of Three Oil Seeds

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Abstract

Oil seed production has an important role in human nutrition and industry. Success in oil plant cultivation is related to seed production with high viability and rapid germination, because these seeds rapidly loose their viability by fats oxidation. Thus, in this work we studied the effects of ascorbic acid, pyridoxine and hydrogen peroxide solutions on germination quantitative traits, catalase activity, protein and malondialdehyde content of three old oil seeds (sunflower, rape seed and safflower). The results showed that ascorbic acid and pyridoxine stimulated significantly the sunflower and rape seed germination. These vitamins, however, didn't have any effect on safflower germination. Hydrogen peroxide strongly increased safflower germination. Ascorbic acid and pyridoxine decreased catalase activity in sunflower and rape seed, whereas hydrogen peroxide increased it. Ascorbic acid and pyridoxine prevented protein degradation and lipid peroxidation in germinated seeds. Consequently, we understand that ascorbic acid and pyridoxine can increase sunflower and rape seed germination and stimulate rate of growth. Also safflower germination increased due to germination inhibitor oxidation by hydrogen peroxide. In conclusion, this report shows that oil seeds treated with ascorbic acid, pyridoxine and hydrogen peroxide remarkably increase the capacity of germination. We suggest that treatments with such substances can improve the old oil seed germination during storage.

Keywords: ascorbic acid, pyridoxine, hydrogen peroxide, germination, oil seed, catalase, malondialdehyde

Introduction

Oil seeds have a special place in food security and industry throughout the world. One of the most important factors for oil seed cultivation is seed product with high vigor and rapid seed germination, because these factors are critical to crop production under different conditions. In many crop species, seed germination and early seedling growth are the sensitive stages; particularly oil seeds are very sensitive to storage and store conditions such as temperature, moisture and gaseous exchanges. Fast oxidation of fatty acids and proteins; reduce of viability, vigor and germination percentage in these seeds. Oil seeds are more sensitive to lipid peroxidation of plasma membrane and protein degradation. Oxidation of lipids and another biomolecules generate free radicals that cause disorder in the cells (Dell and Tritto, 1989). Use of old oil seeds and those stimulate to germination and increase their germination percentage can be one way to those renewed apply. Priming is a common method for increase germination rate and resistance to stresses (Taylor and Harman, 1990). Under different conditions particularly environmental stress, reactive oxygen species, such as superoxide anion radicals, hydrogen peroxide, and hydroxyl radicals, are generated (Zhu, 2000). Reactive oxygen species can damage essential membrane lipids as well as proteins and nucleic acids (Noctor and Foyer, 1998). However, some authors have shown that the production of reactive oxygen species during seed germination may be a beneficial biological reaction, linked with high germination capacity, seedling development and protective function against parasitic organisms during germination (Schopfer et al., 2001). Ascorbic acid is (vitamin C) an important metabolite involved in many cellular processes, including cell division (De Gara et al., 2003). Several reports indicate that a large amount of ascorbic acid is utilized during the initial stages of germination by both zygotic (Arrigoni et al., 1997) and somatic embryos (Stasolla and Yeung, 2001). Furthermore, exogenous applications of ascorbic acid have been found to induce mitotic activity in several systems, including *Zea mays* and *Allium cepa* roots (Kerk and Feldman, 1995) and *Lupinus albus* seedlings (Arrigoni et al. 1997), possibly by inducing the progression of cells from G_1 to S (Liso et al., 1988). Ascorbate has also been utilized in *in vitro* systems. It is a common nutritional additive to tissue culture media, and it can also serve as an antioxidant to reduce oxidative stress and alleviate tissue browning (Dodds and Roberts, 1995). Morphogenetically, ascorbic acid has been shown to promote growth of tobacco cells (De Pinto et al., 1999) and enhance shoot organogenesis in tobacco (Joy et al. 1988) and somatic embryo germination in white spruce (Stasolla and Yeung, 1999). Ascorbic acid is an antioxidant molecule that acts as a primary substrate in the cyclical pathway for detoxification and neutralization of superoxide radicals

and singlet oxygen (Noctor and Foyer, 1998). Ascorbate has been shown to play multiple roles in plant growth, such as in cell division, cell wall expansion, and other developmental processes (Pignocchi and Foyer, 2003). Pyridoxine (Vitamin B_6), in its active form pyridoxal 5'-phosphate, it is an essential metabolite in all organisms. It can act as a coenzyme for numerous metabolic enzymes and has recently been shown to be a potent antioxidant. Plants and microorganisms have a *de novo* biosynthetic pathway for vitamin B_6. It requirements for growth and differentiation of some plant species have been reported (Proebsting et al., 1990). Although roots of some plant species are capable of vitamin B synthesis, roots of other plants can not synthesize this vitamin (Mozafar and Oertli, 1992) and are dependent on transport from the shoot. Absorption by plant roots has been reported (Mateikene et al., 1988). It is an essential cofactor for numerous metabolic enzymes including amino acid metabolism and antibiotic biosynthesis. Most interestingly, it has recently been found that the vitamin is a potent antioxidant with a particular ability to quench reactive oxygen species such as superoxide and singlet oxygen. The hydrogen peroxide has generally been viewed as a toxic cellular metabolite. However, it is now clear that it may also function as a signal molecule in both plant and animal cells (Neill et al., 2002). The generation of hydrogen peroxide is increased in response to a wide variety of abiotic and biotic stresses, and some authors have suggested that hydrogen peroxide plays a dual role in plants: at low concentrations, it acts as a messenger molecule involved in acclamatory signaling, triggering tolerance against various abiotic stresses, and at high concentrations it orchestrates programmed cell death (Vandenabeele et al., 2003). Several studies have produced evidence for a signaling role for hydrogen peroxide (Foyer et al., 1997). The addition of hydrogen peroxide to plant tissues or its experimental generation has been demonstrated to act as a signal in the induction of gene expression of the enzymes catalase (Polidoros and Scandalios, 1999), Changes in hydrogen peroxide homeostasis also induces synthesis of heat shock proteins and activates the mitogen-activated protein kinase cascade (Van Breusegem et al., 2001). Endogenous hydrogen peroxide production has been shown to increase in response to chilling stress in maize seedlings, and exogenously applied hydrogen peroxide increased chilling stress tolerance. This increase in tolerance was partly due to an enhanced antioxidative system that prevents the accumulation of reactive oxygen species during chilling stress (Prasad et al., 1994). Considering that changes in hydrogen peroxide homeostasis may be a pivotal signaling event of the acclimation phenomenon and a key component in the stress survival process, the aim of this study was to evaluate the effects of hydrogen peroxide pre-treatment on seed germination of three old oil seed. In this work we studied the change in the germination, lipid peroxidation, protein content and activities of catalase during seed germination, and the effect of ascorbic acid, pyridoxine and hydrogen peroxide on these parameters. Our aim was to follow the expression of particular parts of antioxidative systems in the seeds during the early stages of germination, to see whether vitamins and hydrogen peroxide has any effect on these processes and can they to increase germination?

Materials and methods

Plant material and growth conditions

Old seeds of sunflower (*Helianthus annus* L.), rape seed (*Brassica napus* L.) and safflower (*Carthamus tinctorius* L.) were surface-sterilized for 5 minute in sodium hypochlorite solution and then in 96% ethanol for 30 second and then rinsed by distilled water. Sterilized seeds were transferred in to sterile plates contain papers germinating and added 10 ml ascorbic acid (0, 100, 200 and 400-ppm), pyridoxine (0, 100, 200 and 400-ppm) and hydrogen peroxide (0, 1%, 3% and 5%) solution to each plates. For prevent of infection and evaporation of solution, all of plates were closed with parafilm. All operations were done under laminar flow. Plates were put in the germinator at 25°C for 8 days.

Seed germination and sampling

After ten days; number of total germinated seed, length of radical, length of seedling and their dry matter weight were recorded. Germination Percentage was calculated. The remains of seedlings were frozen in liquid N_2 and stored under -80°C until biochemical analysis.

Extract preparation

Seedlings (0.2g) were homogenized in a mortar and pestle with 3 ml of ice-cold extraction buffer (25mM sodium phosphate buffer, pH 7.8). The homogenate was centrifuged at 18,000×g for 30 minute at 4°C and then supernatant filtered through watman paper. The supernatant fraction was used as crude.

Catalase activity

Catalase activity was estimated by the method of Cakmak and Horst (1991). The reaction mixture contained 100µl of crude enzyme extract, 500 µl of 10 mM H_2O_2 and 1400 µl of 25 mM sodium phosphate buffer and the decrease in the absorbance was recorded at 240 nm for 1 minute. Catalase activity of the extract was expressed as catalase units per mg protein.

Protein assay

Total protein content was determined using bovine serum albumin (BSA) as a standard, according to the method of Bradford (1976), using 1 ml Bradford solution and 100 µl crude extract. The protein concentration was calculated from a BSA standard curve.

Lipid peroxidation assay

The level of membrane damage was determined by measuring MDA as the end product of peroxidation of membrane lipids De Vos et al (1991). In brief, samples were homogenized in an aqueous solution of trichloroacetic acid (10% w/v), and aliquots of the filtrates were heated in 0.25% thiobarbituric acid. The amount of MDA was determined from the absorbance at 532 nm, followed by correction for the non-specific absorbance at 600 nm. The concentration of MDA was determined using the extinction coefficient of malondialdehyde (ε=155 μM^{-1} cm).

Statistical analyze

All data were analyzed using SAS software SAS Institute Inc (1997). Each treatment was analyzed in three replications. When analysis of variance (ANOVA) showed significant treatment effects, Duncan's multiple range test was applied to compare the means at $P<0.05$.

Results and discussion

There were differences ($p<0.01$) among solution and concentration levels for germination (Table 1). The results showed that ascorbic acid treatment increased germination percentage in sunflower and rape seed in compared to control (Table 2, 3) but, didn't have any positive effect on safflower germination. Exogenous application of ascorbic acid may influence on range of diverse processes in plants, including seed germination, ion uptake and transport, membrane permeability. In fact exogenous ascorbic acid increased level of ascorbic acid uptake by different tissues as reported by Arrigoni and Detullio (2000). Length of shoot and root, their dry weight and seedling total dry weight in sunflower and rape seed increased significantly by ascorbic acid than control treatment (Table 2, 3). One of the remarkable roles of ascorbic acid in seed germination and cell growth is its anti-oxidant activity, rather than its possible utility as an organic substrate for respiratory energy metabolism. We have found that ascorbic acid increased length of root and shoot in treated seeds. In fact increase of root and shoot length by ascorbic acid might be due to the cell division and differentiation of meristem cells (Liso et al., 1988). In control treatment of rape seed, germination percentage was very few, consequentially dry weights, catalase activity, protein and malondialdehyde content weren't calculable. In contrast ascorbic acid treatment increased germination and stimulated root and shoot growth in rape seed. The effect of additional ascorbic acid on plant survival is associated with the partial inhibition of a few interactions in reactive oxygen species production (Shalata and Neumann, 2001). Neither of ascorbic acid and pyridoxine treatments didn't have any positive effect on safflower germination (Table 4). Catalase activity was decreased under ascorbic acid treatment in sunflower and rape seed, but at 400-ppm ascorbic acid treatment, catalase activity was increased in sunflower seedlings. In old seeds hydrogen peroxide generate by lipid peroxidation processes it is converted to oxygen and water by catalase. Catalase use ascorbate as the hydrogen donor (Hegedus et al. 2001). 100 and 200-ppm ascorbic acid prevented of protein degradation in sunflower but 400-ppm ascorbic acid decreased it. In rape seed seedlings, protein content increased by raised ascorbic acid concentration. Ascorbic acid decreased lipid peroxidation in sunflower and rape seed (Table 2, 3). One of the best known toxic effects of reactive oxygen species is damage to cellular membranes, lipids and proteins. The inhibitory effect of ascorbic acid on lipid peroxidation is related to plant survival but its actual mechanism(s) are not yet clear. Exogenous ascorbic acid inhibited lipid peroxidation because ascorbic acid is a scavenger of reactive oxygen species (Noctor and Foyer 1998). Similar to ascorbic acid, pyridoxine increased germination percentage in sunflower and rape seed. Increase of germination in rape seed by pyridoxine was obviously significant. Antioxidant effects of pyridoxine reported too but biosynthesis pathway and the function of vitamin B6 in plants are not well elucidated yet. Pyridoxine increased significantly length of shoot and root in sunflower and rape seed seedlings in compared to control treatment. It seems that pyridoxine play a key role in cell division. We showed that pyridoxine stimulated hairy root growth in sunflower. Dry weights increased in both of them; whereas pyridoxine didn't have any effect on shoot dry weight of rape seed. Enhancement of dry weight is due to increase of length. In control treatment of rape seed, germination was very few, on the other hand different concentration of pyridoxine increased total dry weight (Table 3). Catalase activity was decreased by pyridoxine treatments in sunflower and rape seed seedlings whereas protein content was increased. Pyridoxine like to ascorbic acid prevented of lipid peroxidation and decreased malondialdehyde content. Hydrogen peroxide (1% and 3%) increased seed germination in sunflower than control treatment, but hydrogen peroxide 5% decreased it (Table 2). In rape seed hydrogen peroxide 1% was effective and increased seed germination but 3% and 5% inhibited germinations (Table 3). Hydrogen peroxide stimulated root and shoot growth in sunflower and increased length of root and shoot. Sunflower root dry weight increased by applying of hydrogen peroxide in compared to control. Hydrogen peroxide 5% decreased shoot dry weight and total dry weight in sunflower. Hydrogen peroxide stimulated lipid peroxidation and catalase activity in sunflower and didn't effect on protein content. In control and hydrogen peroxide 3% treatments of rape seed dry weight, catalase activity, protein and malondialdehyde content weren't calculable. Highest concentration of hydrogen peroxide destroyed seeds completely (Table 3). Safflower germination increased by hydrogen peroxide only (Table 4). Most of root and shoot length was showed in hydrogen peroxide 3% treatment. Safflower dry weight was increased by hydrogen peroxide treatments. Hydrogen peroxide increased catalase activity and lipid peroxidation

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Table 1 Analyze of variance on germination, length of root and shoot, dry weight, catalase activity, protein and malondialdehyde content of three oil seeds

	S.O.V	df	Germination percentage	Radical length (cm)	Shoot length (cm)	Radical dry weight (gr)	Shoot dry weight (gr)
Sunflower	Sol.	2	22.54	0.16*	1.52**	0.00000169	0.01548719**
	Conc.	3	209.39**	3.06**	3.63**	0.00010071**	0.00322951*
	Sol * Conc.	6	32.82	0.05	0.42**	0.00001871*	0.00368490**
	Error	24	26.22	0.05	0.03	0.0001	0.001
Rape seed	Sol.	2	3298.76**	115.80**	99.71**	0.00001245**	0.00025013**
	Conc.	3	2921.99**	47.94**	40.69**	0.00000765**	0.00011160**
	Sol*Conc.	6	967.53**	16.17**	11.40**	0.00000197**	0.00002906**
	Error	24	37.65	0.415	0.309	0.0001	0.0001
Safflower	Sol.	2	13741.30**	8.545**	3.336**	0.00011592**	0.02702188**
	Conc.	3	833.57**	0.722**	0.652**	0.00000431**	0.00005262**
	Sol*Conc.	6	1609.38**	1.070**	0.396**	0.00001314**	0.00304412**
	Error	24	18.212	0.003	0.004	0.0001	0.0001

Table 1 Continue

Seed	S.O.V	df	Seedling dry weight (gr)	Catalase activity (ΔA/ mg protein/min)	Protein (mg/g/FW)	Malondialdehyde (μM^{-1} cm)
Sunflower	Sol.	2	0.01549582**	8027.88**	0.063**	0.008**
	Conc.	3	0.00419734**	920.27**	0.051**	0.021**
	Sol * Conc.	6	0.00399815**	1575.31**	0.104**	0.004**
	Error	24	0.001	13.13	0.008	0.0001
Rape seed	Sol.	2	0.00037275**	2.00	1.35464189**	0.032**
	Conc.	3	0.00017130**	165.62**	0.42261827**	0.069**
	Sol * Conc.	6	0.00004369**	27.07**	0.44437949**	0.012**
	Error	24	0.0001	0.725	0.0001	0.0001
Safflower	Sol.	2	0.03067752**	3752.37**	3.71345747**	0.136**
	Conc.	3	0.00007650**	509.91**	0.43160564**	0.18**
	Sol * Conc.	6	0.00345685**	509.91**	0.43160564**	0.018**
	Error	24	0.0001	1.826	0.0001	0.0001

*, ** - significant at the 1% and 5% probability level, respectively

Table 2 Means value of sunflower germination traits, catalase activity, protein and malondialdehyde content

Seed	Sol.	Conc.	Germination	Root length	Shoot length	Root weight	Shoot weight	Total weight	Catalase activity	Protein	Malondialdehyde
Sunflower	AsA	0	86.66b	1.40d	1.48e	0.006c	0.188bcd	0.195c	36.49d	0.94b	0.3221b
		100	98.89a	2.55ab	2.60bcd	0.006c	0.171d	0.177cd	24.86ef	1.35a	0.1875c
		200	95.55ab	2.54ab	2.85b	0.011bc	0.236ab	0.247ab	18.38g	1.23a	0.1858c
		400	94.44ab	2.87a	3.74a	0.017a	0.230ab	0.247ab	36.22d	0.91b	0.1868c
	B_6	0	86.66b	1.40d	1.48e	0.006c	0.188bcd	0.195c	36.49d	0.94b	0.3221b
		100	96.66a	2.20bc	2.69bc	0.010bc	0.252a	0.262a	28.57e	0.97b	0.1779c
		200	94.44ab	2.56ab	2.79b	0.014ab	0.269a	0.283a	26.41ef	0.94b	0.1789c
		400	98.89a	2.71a	2.73bc	0.012ab	0.266a	0.279a	20.91fg	1.34a	0.1872c
	H_2O_2	0 %	86.66b	1.40d	1.48e	0.006c	0.188bcd	0.195c	36.49d	0.94b	0.3221b
		1%	98.89a	2.12c	1.64e	0.012b	0.181cd	0.193c	54.12c	1.04b	0.3287b
		3%	94.44ab	2.17c	2.43cd	0.014ab	0.195bcd	0.209bc	87.04b	0.92b	0.3705a
		5%	86.66b	2.72a	2.31d	0.011b	0.123e	0.134d	115.68a	0.95b	0.3886a

Table 3 Means value of rape seed germination traits, catalase activity, protein and malondialdehyde content

Seed	Sol.	Conc.	Germination	Root length	Shoot length	Root weight	Shoot weight	Total weight	Catalase activity	Protein	Malondialdehyde
Rape seed	AsA	0	2.22d	0.36d	0.33c	0	0	0	0	0	0
		100	40c	3.51c	5.96b	0.0017c	0.0086b	0.0104c	7.84b	1.09g	0.2331a
		200	40c	6.80b	6.40ab	0.0023b	0.0101a	0.0124bc	6.09c	1.09g	0.2362a
		400	47.77bc	7.76b	7.13a	0.0033a	0.0113a	0.014a	2.13d	1.22b	0.196b
	B_6	0	2.22d	0.36d	0.33c	0	0	0	0	0	0
		100	44.44c	7.70b	7.13a	0.0015c	0.0109a	0.0124bc	6.21c	1.08e	0.2458a
		200	56.67b	7.58b	6.73ab	0.0018c	0.0112a	0.013ab	5.72c	1.16c	0.2063b
		400	68.88a	9.03a	7.33a	0.0034a	0.0108a	0.014ab	1.05de	1.25a	0.2048b
	H_2O_2	0 %	2.22d	0.36d	0.33c	0	0	0	0	0	0
		1%	38.88c	0.30d	0.36c	0	0	0	13.56a	1.06f	0.2214ab
		3%	1.11d	0.06d	0.06c	0	0	0	0	0	0
		5%	0	0	0	0	0	0	0	0	0

Table 4 Means value of safflower germination traits, catalase activity, protein and malondialdehyde content

Seed	Sol.	Conc.	Germination	Root length	Shoot length	Root weight	Shoot weight	Total weight	Catalase activity	Protein	Malondialdehyde
Safflower	AsA	0	7.78c	1.16c	0.93d	0.0037c	0.038b	0.042c	0.2635b	1.39a	0.1623b
		100	0	0	0	0	0	0	0	0	0
		200	0	0	0	0	0	0	0	0	0
		400	0	0	0	0	0	0	0	0	0
	B_6	0	7.78c	1.16c	0.93d	0.0037c	0.038b	0.042c	0.2635b	1.39a	0.1623b
		100	0	0	0	0	0	0	0	0	0
		200	0	0	0	0	0	0	0	0	0
		400	0	0	0	0	0	0	0	0	0
	H_2O_2	0 %	7.78c	1.16c	0.93d	0.0037c	0.038b	0.042c	0.2635b	1.39a	0.1623b
		1%	65.55b	1.68b	1.06c	0.0065b	0.10b	0.10b	27.24b	1.38b	0.2585a
		3%	86.66a	2.44a	1.44a	0.0077a	0.11a	0.12a	46.66a	1.37c	0.2706a
		5%	82.22a	1.72b	1.15b	0.0073a	0.11a	0.12a	48.60a	1.08d	0.2523a

in safflower instead decreased protein content. Hydrogen peroxide is a toxic molecule in biological systems in addition, it act as a signal molecule in the cells (Neill et al., 2002). At low concentrations, it acts as a messenger for catalase gene expression (Polidoros and Scandalios, 1999). Hydrogen peroxide increased germination via influence on seed coat. Safflower seeds are containing inhibitor substance. These substances prevent of germination. Hydrogen peroxide oxidized inhibitors and stimulates germination.

Means with similar letter(s) in each trait are not significantly different at 5% probability level according to Ducan's Multiple Range Test.

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