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LOW DOSE OF GAMMA IRRADIATION ENHANCED DROUGHT TOLERANCE IN SOYBEAN

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Abstract

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Drought stress is the main limiting factor of soybean production. However, no work has been done on how application of low-dose of gamma rays could help to overcome water deficits during critical stages of soybean development. Gamma rays at doses 0.0 and 20 Gray (Gy), from a cobalt source (^{60}Co) with strength of 500 Ci and the dose rate of 0.54 Gy/min^{-1}, were applied to dry seeds of soybean before planting. Two levels of soil moisture (80% field capacity for well-watered control and 35% for drought-stressed treatment) were applied at pod initiation. Thereafter, the interaction effects of low dose of gamma irradiation and water stress on some growth, biochemical, anatomical and antioxidative parameters of soybean plants were investigated. Low dose of gamma irradiation increased biomass accumulation and seed yield for both treatments. Drought stress depressed chlorophyll content and photosynthetic activity ($^{14}CO_2$-fixation), while chlorophyll content, leaf water potential and photosynthetic activity of plants irradiated with gamma rays at a dose 20 Gy were greater than that of drought-stressed plants. Water deficit decreased the enzyme activities of phosphoenol pyruvate carboxylase and ribulose-1,5-bisphosphate carboxylase/oxygenase. However, application of low dose of gamma irradiation (20 Gy) increased the activities of these enzymes, except for phosphoenol pyruvate carboxylase under drought stress. Gamma irradiation dose at 20 Gy increased the concentration of soluble sugars, protein and proline content and the activities of peroxidase and superoxide dismutase of soybean leaves when drought-stressed. However, it decreased the malondialdehyde concentration and electrical conductivity of leaves under drought stress. The following physicochemical characteristics of chloroplasts were chosen as indicators of drought-stressed effects: average size, and ultrastructure. The results suggest that gamma irradiation at dose 20 Gy can partly counterbalance the destructive effects of water deficits. This protective action led to an increase of chloroplast size reduced by drought treatment and rebuilt, to some extent, the chloroplast ultrastructure. Overall, the results indicated that pre-treatment with low dose of gamma rays (20 Gy) to dry seeds of soybean before planting can be used to enhance drought tolerance and minimize the yield loss caused by water deficits. Thus, it may be a useful management tool in afforestation projects in arid and semiarid areas as a promising technique for agricultural improvement.

Key words: drought stress, gamma irradiation, antioxidative enzymes, soybean, proline

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Abbreviations: MDA– malondialdehyde; POD– peroxidase; SOD– superoxide dismutases; ROS– reactive oxygen species; H_2O_2– hydrogen peroxide; Ψ_{leaf}– leaf water potential; RuBPcase– ribulose-1,5-bisphosphate carboxylase/oxygenase; PEPcase– phosphoenol pyruvate carboxylase; Gy– Gray

Introduction

Soybean is one of the most economical and nutritious foods, which may be of help to counter malnutrition and under nutrition in developing countries. Drought limits plant growth on a large proportion of the world's agricultural land. Soybean is considered sensitive to drought stress, especially during critical periods of plant development (Liu et al., 2004). Water stress results in yield reduction by decreasing seed number and seed weight. Intermittent drought is most certain to occur during soybean ontogeny (Dornbos et al., 1989). Drought stress is the primary constraint for increasing soybean yield, particularly when it triggers an early switch from vegetative to reproductive development (Desclaux and Roumet, 1996). Drought is an important environmental factor, which induces significant alterations in plant physiology and biochemistry. The most common symptom of water stress injury is the inhibition of growth, which is reflected in a reduction in the dry matter yield (Le Thiec and Manninen, 2003). Water deficit inhibits photosynthesis as it causes chlorophyll content alterations, harms the photosynthetic apparatus (Costa et al., 1997). In addition, it modifies the activity of some enzymes and the accumulation of sugars and proteins in the plant (Gong et al., 2005), resulting in lower plant growth and yield (Costa et al., 1997). Drought stress was found to decrease the relative water content of plant leaves (Sánchez-Blanco et al., 2002) and total chlorophyll (Shaddad and El-Tayeb, 1990), increase the accumulation of H_2O_2, lipid peroxidation, soluble proteins and free amino acids, including proline, in various plants (Gunes et al., 2008). Drought induces the generation of reactive oxygen species (ROS), causing lipid peroxidation, and consequently membrane injury, protein degradation, enzyme inactivation and the disruption of DNA strands (Becana et al., 1998). The MDA content is often used as an indicator of the extent of lipid peroxidation resulting from oxidative stress (Smirnoff, 1993). Drought stress may lead to stomatal closure, which reduces CO_2 availability in the leaves and inhibits carbon fixation, exposing chloroplasts to excessive excitation energy, which in turn could increase the generation of reactive oxygen species that are responsible for various damages to macromolecules and induce oxidative stress (Reddy et al., 2004). The reduced activity of RuBPC induced by biotic and abiotic stresses is well documented in plants (Allen and Ort, 2001).

Gamma rays have been proved economical and effective as compared to other ionizing radiations because of its easy availability and the power of penetration. This penetration power of gamma rays helps in its wider application for the improvement of various plant species (Moussa, 2006). Sjodin (1962) reported that the material and energy necessary for initial growth are already available in the seed, and so the young embryo has no need to form new substances, but only to activate those already stored in the cotyledons. Low doses of γ-radiation may increase the enzymatic activation and awakening of the young embryo, which results in stimulating the rate of cell division and affects not only germination, but also vegetative growth and flowering. Exposing the dry seeds to low γ-irradiation doses resulted in the increasing yield of some plants such as sunflower (Abo-Hegazi et al., 1988) and *Ammi visnage* (El-Shafie, 1993). Also, Patskevich (1961) came to the conclusion that irradiation of seeds prior to sowing held a great promise from the viewpoint of its practical application in agriculture. It was generally agreed that low doses of gamma rays stimulate cell division, growth, and development of various organisms, including animals and plants. This phenomenon, named hormesis, was analyzed and discussed by various authors for various species (Korystov and Narimanov, 1997). Very low doses of gamma irradiation have been shown to stimulate plant growth (Watanabe et al., 2000). Previous studies have shown that relatively low-doses ionizing irradiation on plants and photosynthetic microorganisms are manifested as accelerated cell proliferation, germination rate, cell growth, enzyme activity, stress resistance and crop yields (Chakravarty and Sen, 2001). The objective of this work is to investigate whether pre-treatment with low dose of gamma rays (20 Gy) to dry seeds of soybean plants before planting may be a protectant agent to nullify the influence of drought stress.

Material and Methods

Plant material, growth conditions, and stress treatments

A homogenous lot of soybean seeds (*Glycine max* L.), cv. Giza 83; was obtained from the Crop Institute, Agricultural Research Center, Giza, Egypt. The caryopsis was kept at 4°C. They were surface sterilized in 0.1 % (w/v) sodium dodecyl sulphate solution and then thoroughly rinsed with sterile deionized water. Dry seeds were exposed to doses of gamma irradiation, 0.0 and 20 Gy, using a gamma source (^{60}Co), Vinderen-Oslo 3-Norway, at the Middle Eastern Regional Radioisotope Center for the Arab Countries (Dokki, Cairo, Egypt) with strength of 500 Ci and the dose rate of 0.54 Gy/min. Seeds were allowed to germinate in pots 35 cm by 30 cm diameter. Each pot was filled with 15 kg sandy loam soil with 2.5% organic matter and available N, P and K concentration of 170, 80 and 200 mg kg^{-1}, respectively. Pots were arranged in a completely randomized design with two factors, two gamma irradiation doses (0.0 and 20 Gy) and two soil water levels (well-watered and drought-stressed) with 20 pots per treatment that were replicated four times. The 320 pots for the experiment were placed in a field sheltered from rain by a removable polyethylene shelter, at a day/night temperature of 24/18°C, with 70% relative humidity, 14-h light and a photon flux density of 400 µmol m^{-2}s^{-1}. Cultural practices, such as weed control and irrigation, were performed as needed. Ten seeds were sown per pot. After the seedlings reached the first true leaf stage, they were thinned to four plants per pot. Two levels of soil moisture were applied by controlled watering beginning at pod initiation until harvest at full maturity. The well-watered and drought-stressed treatments were maintained at 80% and 35% soil field capacity respectively, following the methods of Desclaux and Roumet (1996). The water deficit was initiated by withholding water. The pots were weighed daily to maintain the desired soil water levels by adding appropriate volumes of water. All biochemical estimations were carried out using three leaflets per newly expanded trifoliolate leaves. Samples were collected 10 days after the water treatment was applied, between 9:30 and 10:30 a.m., and kept in liquid nitrogen until analyzed. Effects of treatments on growth and yield were determined by measurement of accumulated biomass of the various organs. At harvest, the plants were removed carefully from the pots. The biomass and seed weights were determined with harvested organs being dried for 48 h at 70°C.

Enzymes assay. Ribulose-1,5-bisphosphate-carboxylase/oxygenase (RuBPcase, EC 4.1.1.39) was determined by Warren et al. (2000). Peroxidase (POD, EC 1.11.1.7), was assayed as given by Macheix and Quessada (1984). Superoxide dismutases (SOD, EC 1.15.1.1), was determined as described by Dhindsa et al. (1981). The activity of phosphoenol pyruvate carboxylase (PEPcase, EC 4.1.1.31) was determined as described by Gonzalez et al. (1998).

Chemical analysis. Total soluble protein contents were measured using Bradford's method (Bradford, 1976). Free proline was determined according to the method described by Bates et al. (1973). Lipid peroxidation was measured in terms of malondialdehyde content using the thiobarbituric acid reaction as described by Madhava Rao and Sresty (2000). Soluble sugars were evaluated using the anthrone method described by Fales (1951). Electrical conductivity was measured with a with a digital conductivity meter (JENWAY, Model 4070, Essex, England). Leaf water potential (Ψ_{leaf}) was measured with a pressure chamber (Model 3000, Soil Moisture Equipment Corp, Santa Barbara, CA, USA).

Total chlorophyll. The total chlorophyll content of fresh leaves was estimated following the method suggested by Barnes et al. (1992).

Photosynthetic activity ($^{14}CO_2$-fixation). Photosynthetic activity was measured in the atomic energy authority, radioisotope department, Cairo, Egypt, with the method of Moussa (2008). The seedlings from each treatment were placed under a Bell jar, which was used as a photosynthetic chamber. Radioactive $^{14}CO_2$ was generated inside the chamber by a reaction between 10% HCl and 50 µCi (1.87×10^6 Bq) $NaH^{14}CO_3$ + 100 mg Na_2CO_3 as a carrier. Then the samples were illuminated with a tungsten lamp. After 30 min exposure time, the leaves were quickly detached from the stem, weighed and frozen for 5 min to stop the biochemical reactions, then subjected to extraction by 80% hot ethanol. The ^{14}C was assayed from the ethanolic extracts in soluble compounds using a Bray Cocktail (Bray, 1960) and a Liquid Scintillation Counter (LSC2-Scaler Ratemeter SR7, Nuclear Enterprises, Edinburgh, UK).

Isolation of chloroplasts. Chloroplasts were isolated from fresh leaves in chloroplast isolation buffer containing 50 mM Tris–HCl, 5 mM EDTA, 0.33 M sorbitol, pH 7.5 using the method of Block et al. (1983). Crude chloroplasts were purified by centrifugation using 40%/80% Percoll gradient (Schwertner and Biale, 1973). Intact chloroplasts were collected from the gradients, diluted three to four times, and centrifuged at 2070 g for 2 min. Next, chloroplasts were resuspended in the isolation buffer and kept in darkness until future use. All procedures were carried out at 0–4 °C.

Electron transmission microscopy. For microscope observations, the lower epidermis was stripped off from the leaves. Samples were prepared as described by Coulomb et al. (1996). Briefly, after fixation in glutaraldehyde and post-fixation in osmium tetroxide, they were dehydrated in acetone and embedded in araldite. The sections, stained in uranyl acetate and lead citrate, were examined by transmission electron microscopy (TEM, Jeol Jem 1200 EX II, Tokyo, Japan).

Chloroplasts size determination. Chloroplast size distribution was determined by dynamic light scattering (DLS) technique (Beckmann, Coulter N4 Plus apparatus).The scattering angle was equal to 90°. A unimodal distribution was assumed for the mean particle size calculation.

Statistical analysis. All data were subject to ANOVA and means were compared using Duncan's multiple range tests ($P<0.05$).

Results

Average sizes of chloroplasts isolated from control soybean seedlings were about 1,200nm and were not noticeably different from the size of the chloroplasts obtained from plants pretreated by 20 Gy dose of gamma irradiation (Figure 1). Chloroplast sizes obtained from drought stressed plants were almost twice as small. This drastic decrease of the average chloroplast size was partly reduced in plants irradiated with gamma rays (20 Gy).

Transmission electron microscopy of the chloroplasts of untreated soybean seedlings revealed the typical ultrastructure with well-organized envelope and internal membrane structure with normally developed grana and stroma thylakoids (Figure 2A). The same chloroplast organization was observed in plants pretreated with gamma irradiation at dose 20 Gy (Figure 2B). Chloroplasts of drought stressed plants showed an altered shape, with wavy grana and stroma thylakoids and enlarged intrathylakoidal spaces. In addition, envelope membranes were not visible at microscopic pictures of most chloroplasts (Figure 2C). The

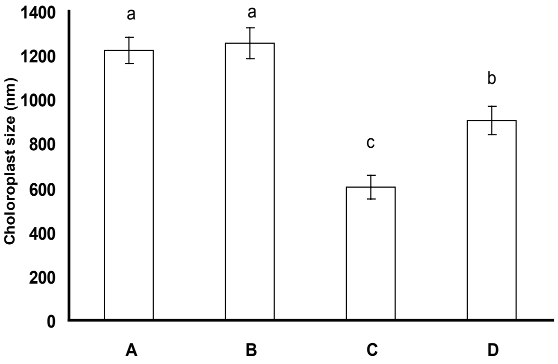

Fig. 1. DLS size of chloroplasts isolated from soybean leaves: (A) control; (B) plants irradiated with gamma rays (20 Gy); (C) drought stressed plants and (D) drought stressed plants pre-exposure to gamma irradiation (20 Gy). Values represent the means of four replicates±SE. Different letters indicate significant differences (P<0.05) between treatments

changes in chloroplasts originated from drought stressed plants pre-exposure to low dose of gamma rays (20 Gy) were not as drastic as those observed for drought stressed plants only. However, some reorganization of the thylakoids and stroma was observed (Figure 2D). Water deficits decreased the chlorophyll content by 12% and photosynthetic activity by 42%. However, the chlorophyll content and photosynthetic activity of plants irradiated with gamma rays (20 Gy) were higher than those of plants under drought-stressed conditions. Under well-watered conditions, the photosynthetic efficiency of the plants irradiated with gamma rays (20 Gy) was higher than the control plants (Table 2).

Water deficits decreased RuBPcase activity by 37% and PEPcase activity by 38%. However, plants irradiated with gamma rays (20 Gy) increased the activity of RuBPcase and PEPcase, except for PEPcase under drought stress (Table 1). Water deficit decreased the total soluble protein concentration by 9% (Table 2).

However, the total soluble protein content of plants pre-exposure to low dose of gamma rays (20 Gy) were higher than those of plants under drought-stressed conditions by 11%. Water deficits decreased Ψ_{leaf} (Table 2). The Ψ_{leaf} for well-watered plants was 0.45 to -0.50 MPa, while that for drought-stressed plants reached -1.8 to -2.3 MPa. Application of low dose of gamma rays (20 Gy) increased Ψ_{leaf} under drought-stressed conditions, but there is no difference in the Ψ_{leaf} between irradiated plants and control under the well-watered conditions. Water deficit treatment increased the concentrations of soluble sugar, proline, protein, the enzyme activities of POD and SOD and electrical conductivity of leaves (Tables 1 and 2).

Under drought-stressed conditions, pre-exposure to gamma rays increased the concentrations of soluble sugars, protein, proline and the enzyme activities of POD and SOD, but not the electrical conductivity of leaves or the concentration

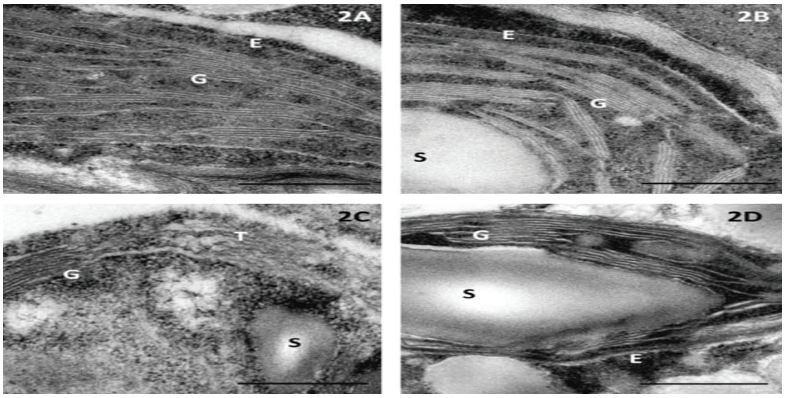

Fig. 2. Chloroplast structure of soybean leaves: (A) control ; (B) plants irradiated with gamma rays (20 Gy); (C) drought stressed plants and (D) drought stressed plants pre-exposure to low dose of gamma rays (20 Gy). E– envelope, G– grana, S– starch and T– thylacoid. Bars correspond to 200 nm

of MDA. For example, application of low dose of gamma rays (20 Gy) increased soluble sugar by 17% and proline by 12%, and increased SOD activity by 28% and POD activity by 30%, but MDA concentration decreased by 13% along with the electrical conductivity by 9% compared with the drought control (Tables 1 and 2).

Water deficits decreased the dry weight of stems and leaves, total biomass and seed yield, but did not affect the dry weight of roots (Table 3). Application of low dose of gamma rays (20 Gy) increased the dry mass of roots, stems and leaves, and seed yield under both water levels, with the exception of the dry weight of stems and leaves under drought stresses conditions. Under well-watered conditions, Gamma rays treatment also increased

Table 1

Effect of gamma irradiation (20 Gy) on enzyme activities of RuBPcase (µmol CO_2 mg^{-1} protein min^{-1}), PEPcase (µmol CO_2 mg^{-1} protein min^{-1}), POD (units mg^{-1} protein) and SOD (units mg^{-1} protein), MDA (nmol g DW^{-1}), proline (µmol g DW^{-1}), and total soluble protein (mg g FW^{-1}) of soybean under well-watered and drought-stressed treatments[A]

Treatments	RuBPcase	PEPcase	POD	SOD	MDA	Proline	Protein
Well-watered	28.6^{b}	2.9^{b}	7.7^{c}	2.8^{c}	98^{c}	33^{c}	65^{b}
Well-watered + γ-irradiation	29.7^{a}	3.2^{a}	7.8^{c}	3.9^{b}	102^{c}	34^{c}	71^{a}
Drought-stressed	20.9^{d}	2.1^{c}	11.0^{b}	4.2^{b}	130^{a}	45^{b}	56^{d}
Drought-stressed + γ-irradiation	23.1^{c}	2.2^{c}	14.3^{a}	5.4^{a}	115^{b}	51^{a}	62^{c}

[A] Well-watered treatment was 80% of soil field capacity, and drought-stressed treatment was 35% of soil field capacity. Values followed by the same letter within columns are not significantly different according to Duncan's multiple range tests ($P<0.05$). Data are the means of four replicates.

Table 2

Effect of gamma irradiation (20 Gy) on photosynthetic activity (*KBq mg FW^{-1}), chlorophyll content (mg g FW^{-1}), the concentration of soluble sugar (mg g FW^{-1}), electrical conductivity (%) and Ψ_{leaf} (MPa) of soybean under well-watered and drought-stressed treatments[A]

Treatments	Photosynthetic activity	Chlorophyll content	Soluble sugar	Electrical conductivity	Ψ_{leaf}
Well-watered	16.8^{d}	52.7^{a}	117^{d}	9.6^{c}	-0.50^{a}
Well-watered + γ-irradiation	19.7^{c}	53.0^{a}	149^{c}	7.2^{d}	-0.45^{a}
Drought-stressed	11.8^{b}	47.2^{c}	182^{b}	14.4^{a}	-2.3^{c}
Drought-stressed + γ-irradiation	14.9^{a}	49.6^{b}	213^{a}	13.2^{b}	-1.8^{b}

[A] Well-watered treatment was 80% of soil field capacity, and drought-stressed treatment was 35% of soil field capacity. Values followed by the same letter within columns are not significantly different according to Duncan's multiple range tests ($P<0.05$). *kilo Becquerel (10^3 Bq). Data are the means of four replicates.

Table 3

Effect of gamma irradiation (20 Gy) on the dry weight of roots, stems plus leaves, seed yield and total biomass of soybean (g/plant) under well-watered and drought-stressed conditions^{A}

Treatments	Roots	Stems plus leaves	Seed yield	Total biomass
Well-watered	1.8^{c}	11.6^{b}	11.3^{b}	24.7^{b}
Well-watered + γ-irradiation	2.8^{a}	13.3^{a}	13.8^{a}	29.9^{a}
Drought-stressed	1.8^{c}	8.1^{c}	7.9^{d}	17.8^{d}
Drought-stressed + γ-irradiation	2.2^{b}	9.4^{c}	9.6^{c}	21.2^{c}

^{A} Well-watered treatment was 80% of soil field capacity, and drought-stressed treatment was 35% of soil field capacity. Values followed by the same letter within columns are not significantly different according to Duncan's multiple range tests ($P<0.05$). Data are the means of four replicates.

the dry weight of roots by 55%, stem plus leaves by 15%, total biomass by 21% and seed yield by 22% compared to unstressed control plants. Under drought-stressed conditions, gamma rays treatment also increased the dry weight of roots by 22%, stem plus leaves by 16%, total biomass by 19% and seed yield by 21% compared to the stressed control plants (Table 3).

Discussion

The effect of drought stress on the photosynthesis process is a subject of intensive investigation. The typical consequence of water deficits action on soybean seedlings consists of a decrease in chloroplast size and changes in the chloroplasts' inner structure, registered by microscopic observations. Drought stress causes a degradation of internal chloroplast membranes, leaving the integrity of chloroplast envelopes. Similar findings have bean reported by Dimitrina et al. (2002). The changes in chloroplasts originated from drought stressed plants pretreated with gamma irradiation (20 Gy) were not as drastic as those observed for drought stressed plants only. However, some reorganization of the thylakoids and stroma was observed.

These results support the findings of Wi et al. (2007). Although no conclusive explanations for the stimulatory effects of low-dose gamma radiation are available until now, papers support a hypothesis that the low dose irradiation will induce the growth stimulation by changing the hormonal signaling network in plant cells or by increasing the antioxidative capacity of the cells to easily overcome daily stress factors such as fluctuations of light intensity and temperature in the growth condition (Kim et al., 2004). In this study, the drought-stressed soybean plants irradiated with gamma rays (20 Gy) had higher biomass and seed yield than the stressed control plants. These beneficial effects resulted in higher leaf area, biomass production, grain yield and yield-related parameters in the treated plants (Moussa, 2006).

Plants irradiated with gamma rays (20 Gy) before the onset of water stress in the present study improved leaf photosynthesis and chlorophyll content of soybean during the period of water stress. Abu et al. (2005) stated that an increase in chlorophyll a, b and total chlorophyll levels was observed in *Paulownia tomentosa* plants that were exposed to gamma irradiation. The plants irradiated with gamma rays (20 Gy) induced increase in photosynthesis due to improvements in leaf water balance as indicated by increased Ψ_{leaf} under water deficits suggesting that leaves lose less water.

The results support the findings of previous workers, Khodary and Moussa (2003), they reported that treatment with low dose of gamma rays

(20 Gy) to dry seeds of lupine increased the total chlorophyll content, soluble sugars and photosynthetic activity. Low doses of gamma rays highly significantly increased the level of carbohydrate constituents (Nouri and Toofanian, 2001). SOD and POD are important antioxidant enzymes that detoxify active oxygen species. Treatment of soybean with gamma rays (20 Gy) was effective in increasing SOD and POD activity under drought stress. Similar findings have bean reported in *Vicia faba* by Moussa (2008), who reported that by exposing three-week-old seedlings to γ-irradiation at the dose of 20 Gy increased the antioxidant enzyme activities of SOD and POD.

In the study by Wi et al. (2006), the induction of POD by the irradiation would be one of the defense systems activated through the ROS-mediated cellular signaling. Enhancement in peroxidase activity by radiation has also been reported by Omar (1988) in sunflower, Sah et al. (1996) in barley and Stoeva (2002) in *Phaseolus vulgaris*. Meanwhile, the activities of peroxidase in radish (*Raphanus sativus*) leaves were enhanced by gamma irradiation at 10 Gy (Lee et al., 2003). Our results also indicated that the plants irradiated with gamma rays (20 Gy) promoted the accumulation of osmoprotectants, such as soluble sugars, protein and proline, and decreased accumulation of MDA and electrical conductivity under drought-stress condition. Osmotic electric conductivity, soluble sugars, proline and antioxidative components are used as physiological indices of membrane stability (Reddy et al., 2004). The accumulation of soluble sugars and free amino acids, including proline, protects the cell under stress by balancing the osmotic strength of the cytosol with that of the vacuole and the external environment (Kerepesi and Galiba, 2000).

The results support the findings of previous workers, presowing γ-irradiation at the dose of 20 Gy can be used for increasing total protein content, total soluble sugars concentration, growth hormone (kinetin and GA_3), total yield and yield quality improvement of *Eruca vesicaria* (Moussa, 2006). Proline as a cytosolic osmoticum and a scavenger of $OH^{\cdot}$ radical can interact with cellular macromolecules such as DNA, protein and membranes and stabilize the structure and function of such macromolecules (Kavir Kishor et al., 2005). Owing to gene expression altered under gamma stress, qualitative and quantitative changes in total soluble protein content was obvious (Corthals et al., 2000). These proteins might play a role in signal transduction, anti-oxidative defense, anti-freezing, heat shock, metal binding, anti-pathogenesis or osmolyte synthesis which were essential to a plant's function and growth (Gygi et al., 1999). Anna et al. (2008) reported that low dose of gamma irradiation (30 Gy) enhanced protein synthesis in *Citrus sinensis.*

Conclusion

In conclusion, applying gamma irradiation at a dose 20 Gy to soybean seeds prior to water deficit stress could partially alleviate the detrimental effect of water stress on growth through increasing photosynthesis, improving antioxidant system and promoting dry weight accumulation.

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