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Physiological responses of the M_1 sainfoin (*Onobrychis viciifolia* Scop) plants to gamma radiation

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ABSTRACT

Effects of gamma radiation on physiological responses of the M_1 sainfoin plants were investigated. Seeds of sainfoin ecotype 'Koçaş' were exposed to 0, 400, 500 and 600 Gy from a ^{60}Co source at a dose rate of 0.483 kGy h^{-1}. Irradiated and unirradiated seeds were sown into culture vessels containing MS-basal medium to be cultured for 30 days under in vitro conditions. At the end of this period, seedlings, which germinated from the radiated and unirradiated seeds, were transferred into pots in a growth chamber for 30 days more. Chlorophyll contents, activities of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR), as well as contents of monodehydroascorbate reductase (MDA) and proline were examined in unirradiated and irradiated 60-day-old seedlings. Overall, the activities of the antioxidant enzymes (SOD, CAT and GR) and contents of chlorophyll and proline in the leaves tended to increase after irradiation in a dose-dependent manner. By contrast, the activity of APX decreased. The lipid peroxidation characterized by the MDA content remained unchanged, except after irradiation to 500 Gy. The highest CAT activity and the highest proline content were observed after irradiation to the highest dose of 600 Gy. The highest SOD and GR activities were observed after irradiation to the lowest tested dose of 400 Gy. This is the first study that provided basic information on the impact of gamma radiation on physiological responses of sainfoin and its radiosensitivity. These findings will be useful in development of a mutation breeding program of sainfoin.

1. Introduction

Gamma rays can be used to improve mutant varieties and increase genetic variability in plants (Jan et al., 2013). Gamma rays produce free radicals, which can damage various important compounds in plant cells (Kovacs and Keresztes, 2002; Wi et al., 2005). One of the biological effects of gamma radiation is increased production of reactive oxygen species (ROS). ROS, which include such compounds as superoxide, peroxide, singlet oxygen and the hydroxyl radical, are inevitable by-products of aerobic metabolism. They are produced by electron transfer reactions in mitochondria, chloroplasts, and peroxisomes. ROS are toxic; unless their concentrations are under control, they can damage the protein, membrane and DNA of a cell, which will ultimately kill it (Mittler, 2002). To protect themselves against ROS, plant cells employ antioxidant defense systems. The cellular antioxidant defense mechanism is important in protecting the cell against various stresses, including radiation. The antioxidant defense mechanisms can be either non-enzymatic (such as those involving glutathione, proline, α-tocopherols, carotenoids or flavonoids) or enzymatic, involving various enzymes. The antioxidant enzymes, such as catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR), ascorbate peroxidase (APX), GR or monodehydroascorbate reductase (MDAR), play a major role in scavenging toxic ROS inside plant cells, along with non-enzymatic ROS scavengers, such as ascorbic acid or reduced glutathione. The effectiveness of the protection against ROS depends on the proper balance of all the processes and compounds involved. Pyramiding ROS scavenging enzymes can also be used to produce abiotic-stress-tolerant plants. Therefore, plants with the ability to scavenge cellular ROS or control their levels by other means may prove to be valuable under harsh environmental conditions. Many studies focused on various aspects of the defense mechanisms in plants, such as the activity of antioxidative enzymes, chlorophyll contents, lipid peroxidation, and proline levels under radiation stress (Zaka et al., 2002; Kim et al., 2004, 2012; Alikamanoğlu et al., 2007; Aly and El-Beltagi, 2010; Silva et al., 2011; Jan et al., 2012; Marcu et al., 2013; Vanhoudt et al., 2014; Kim et al., 2015).

ROS are also currently regarded as key regulatory molecules vitally important for cells (Gill and Tuteja, 2010). Rapid cell proliferation and

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active aerobic metabolism are often associated with the production of ROS. Regulation of the reducing and oxidizing (redox) processes is critical for viability, activation, and proliferation of cells, as well as functions of organs (Birben et al., 2012). ROS in plants are important regulators of cell division and can serve as signaling molecules at crossroads of cell proliferation (Feher et al., 2008; Liu et al., 2014). It was suggested that induction or inhibition of cell proliferation depends on the balance between oxidants and antioxidants in a cell (Druckova, 2008). Kim et al. (2004) investigated changes of antioxidant enzymes in growing cells of irradiated plants. Malondialdehyde (MDA) is a natural product of lipid peroxidation. MDA is known to be damaging to plant cells. The toxicity of MDA and its association with lipid oxidation have got significant attention in the literature. However, the fact that MDA is damaging to cells is striking in light of the fact that the aldehyde is widespread in healthy organs of plants. (Schmid-Siegert et al., 2012). Schmid-Siegert et al. (2012) found high levels of MDA in dividing cells in the root tip cell proliferation zones in *Arabidopsis*. Low-molecular-weight antioxidants, such as ascorbate, glutathione, and tocopherol, are information-rich redox buffers, which interact with numerous cellular components. The redox state of the low-molecular-weight antioxidants may modulate the degree of cell proliferation. CAT and APX play major roles in restoring the ROS homeostasis. SOD and GR work at the first and the last step, respectively, of the ascorbate-glutathione cycle scavenging ROS in plant cells. Glutathione disulfide (GSSG) produced in the process is then recycled to glutathione (GSH). Glutathione is essential for the cell life cycle in plants. The balance of ROS is a critical factor in cell proliferations (in the presence of favorable GSH redox state). Kumar et al. (2011) found a decrease in cell divisions in the root tip of *Allium cepa* after a gamma irradiation and noticed that the activities of the antioxidant enzymes remained balanced at the active stage of the developing root, helping to scavenge the excessive free radicals in the dividing cells.

The genus of *Onobrychis* (Fabaceae) Adams consists of approximately 170 species, which are found mostly in southwestern Asia, the Mediterranean region, as well as temperate zones of Eastern Europe and Asia (Cronquist, 1981; Zohary, 1987; Aktoklu, 2001; Sancak et al., 2003; Avcı and Kaya, 2013). Sainfoin *Onobrychis viciifolia* (Scop.) is a perennial forage legume crop well adapted to calcareous, well drained, poor and dry soils (Açikgöz, 2001; Cavallarin et al., 2005). It is highly tolerant to salinity and drought, and improves soil fertility by fixing atmospheric nitrogen (Özaslan-Parlak and Parlak, 2008; Imanparast and Hassanpanah, 2009; Beyaz et al., 2011). Although there is a study of morphological responses of sainfoin to gamma radiation (Bağcı and Mutlu, 2011), there are no reports in the literature on its physiological responses under the same treatment. As physiological characteristics can be changed by gamma radiation (Kiong et al., 2008), we undertook this work to investigate such changes in sainfoin seedlings.

2. Materials and methods

2.1. Plant material

Seeds of sainfoin (*O. viciifolia* Scop.) ecotype 'Koçaş' were used as the plant material.

2.2. Gamma irradiation

In a previous study, Bağcı and Mutlu (2011) observed that high doses (700 Gy and above) of gamma radiation remarkably inhibited germination of seeds and the growth of sainfoin cv. 'Özerbey' seedling at the developmental stage. They found that the lethal dose was 500 Gy and the doses between 400 and 600 Gy could be used in a mutation breeding program without changing the viability of sainfoin. Therefore, we selected the doses of 400, 500 and 600 Gy for this investigation. The seeds of sainfoin (*O. viciifolia* Scop.) ecotype 'Koçaş' were irradiated to 0, 400, 500 and 600 Gy using an experimental ^{60}Co source at the Sarayköy Nuclear Research and Training Center (SANAEM) of the Turkish Atomic Energy Authority (Ankara, Turkey). The dose rate of 0.483 kGy h^{-1} was determined with Fricke and alanine dosimetry. The seeds were irradiated on a plate rotating 360° in a cylindrical radiation field.

2.3. Growth conditions

Irradiated and unirradiated (control) seeds of sainfoin were cultured in the Murashige and Skoog (1962) medium for 30 days in vitro. Seeds were allowed to germinate at 20 ± 1 °C under cool white fluorescent light (27 μmol m^{-2} s^{-1}) with a (16 h light/8 h dark) illumination period. After that, plantlets were transferred to pots for acclimation to external conditions. These plantlets were grown in a growth chamber under controlled conditions for 30 days. Leaf samples from 60-day-old seedlings of sainfoin were collected for a physiological analysis.

2.4. Physiological analysis

Chlorophyll contents, antioxidant enzyme activity, lipid peroxidation characterized by the MDA content, and proline content were measured.

Contents of chlorophyll a, chlorophyll b and total chlorophyll were measured according to the protocol by Curtis and Shetty (1996). To this end, 50-mg portions of fresh leaf material were placed into falcon tubes containing 3 mL of methanol and left there in the dark at 23 °C for 2 h for homogenization. After that, 1.5-mL portions of the solution were transferred into cuvettes to measure absorbances A at 650 and 665 nm with a spectrophotometer Shimadzu UVmini-1240. The contents of chlorophyll a, chlorophyll b and total chlorophyll as micrograms of chlorophyll per gram of fresh tissue were calculated using the following formulas:

$$\text{content of chlorophyll a} = \left(16.5 \times A_{665} - 8.3 \times A_{650}\right) \times 3/0.05;$$

$$\text{content of chlorophyll b} = \left(33.8 \times A_{650} - 12.5 \times A_{665}\right) \times 3/0.05;$$

$$\text{content of chlorophyll} = \left(25.8 \times A_{650} - 4.0 \times A_{665}\right) \times 3/0.05.$$

Lipid peroxidation (MDA content) was determined using the methodology described by Lutts et al. (1996). A 5-mL portion of a 0.1% tricholoroacetic acid (TCA) solution was added to a 200-mg sample of fresh leaves, and the mixture was centrifuged at 12,500 rpm for 20 min. A 3-mL portion of the supernatant was then taken out of the 5-mL extract. After that, a 3-mL portion of a solution containing 20% of tiobarbituric acid and 20% of tricholoroacetic acid was added to 3 mL of the supernatant. The absorbances at 532 and 600 nm were measured with the spectrophotometer.

To measure changes in the antioxidant enzyme activity, 1-g samples of fresh leaves were squeezed with liquid nitrogen in a ceramic mortar and homogenized with 10-mL portions of a 50 mM phosphate buffer solution containing 0.1 mM of Na-EDTA. Homogenized samples were centrifuged at 15,000 rpm for 15 min and then used for enzyme analysis.

The activity of superoxide dismutase (SOD) was measured using the method published by Cakmak and Marschner (1992) and Çakmak et al. (1994). The following solutions were added into glass bottles in the order they are listed: ? ? mL of a 50 mM phosphate buffer (pH 7.6) containing 0.1 mM of Na-EDTA; the enzyme extract (0.025–0.1 mL); 0.5 mL of a 50 mM solution of Na_2CO_3 (pH 10.2); 0.5 mL of a 12 mM solution of L-methionine; 0.5 mL of a 75 μM solution of *p*-nitro blue tetrazolium chloride (NBT); and, finally, 0.01 mL of riboflavin. The absorbances of the solutions at 560 nm were measured 15 min after the mixing.

The activity of catalase (CAT) was determined by the rate of the decrease of absorbance of H_2O_2 at 240 nm. In this procedure, 0.8 mL of a 50-mM phosphate buffer solution (pH 7.6) containing 0.1 mM of Na-EDTA, 0.1 mL of a 100 mM H_2O_2 solution and 0.1 mL of the enzyme extract were added to the reaction medium. The volume of the reaction medium was adjusted to 1 mL (Cakmak and Marschner, 1992; Çakmak et al., 1994). The absorbances of the resulted solutions were measured at 240 nm.

The activity of ascorbate peroxidase (APX) was determined by using a method described by Cakmak and Marschner (1992) and Çakmak et al. (1994). A portion of a 50-mM phosphate buffer solution (0.7 mL, pH 7.6, also containing 0.1 mM of Na-EDTA) was mixed with 0.1 mL of a 12 mM solution of H_2O_2 also containing 10 mM EDTA, 0.1 mL of a 0.25 mM solution of L(-)-ascorbic acid and 0.1 mL of the enzyme extract. The total volume of the mixture was adjusted to 1 mL, and its absorbance at 290 nm was measured.

The activity of glutathione reductase in the plant leaves was determined according to protocols by Cakmak and Marschner (1992) and Çakmak et al. (1994). A 0.7-mL portion of 50 mM phosphate buffer solution (pH 7.6, also containing 0.1 mM of Na-EDTA) was mixed with 0.1 mL of a 0.5 mM oxide glutathione (GSSG) solution, 0.1 mL of a 0.12 mM NADPH solution and 0.1 mL of the enzyme extract. The total volume of the mixture was adjusted to 1 mL, and its absorbance at 340 nm was measured.

The proline assay was based on the method by Bates et al. (1973), which uses 3% sulfosalicylic acid in grinding fresh plant samples. Portions of ninhydrin (1 mL) were placed into tubes containing 0.1-g portions of the grinded samples. The tubes were kept in a 100 °C water bath for 1 h. After cooling, 4-mL portions of toluene were added to the mixtures. The absorbances were measured at 520 nm.

2.5. Statistical analysis

A completely randomized design was used with three replicates of both the treated samples and controls. An analysis of variances of all the examined parameters was performed using the 'IBM SPSS Statistic 21 software. The differences between the mean values were compared using the Duncan's multiple range test ($P < 0.05$). The data presented as percentages were subjected to arcsine transformation before the statistical analysis (Snedecor and Cochran, 1967).

3. Results

Table 1 shows the effect of gamma radiation on the chlorophyll contents in sainfoin. The contents of chlorophyll a, chlorophyll b and total chlorophyll in the leaves of irradiated sainfoin seedlings grew significantly ($P < 0.05$) with the increasing radiation dose. The differences between the contents of chlorophyll a in the seedlings irradiated to 400, 500 and 600 Gy were not statistically significant, but they were statistically significantly different from its content in the leaves of the unirradiated seedlings. The highest chlorophyll a content was observed after irradiation to 600 Gy (28% greater than that of the control). The content of chlorophyll b increased statistically significantly ($P < 0.05$), and a particularly high content was found in the leaves irradiated to 600 Gy (183% of that in the control). The contents of chlorophyll a exceeded those of chlorophyll b. The content of the total chlorophyll increased significantly upon irradiation to 400, 500 and, particularly, 600 Gy (89% in the latter case).

The SOD activity increased after the irradiation, featuring a maximum at 400 Gy (248% of the activity in control plants) (Table 2). However, the SOD activity in the plants exposed to 600 Gy was significantly lower, only about 24% higher than that in the controls.

The change of the GR activity with the dose was also nonmonotonous, with a global maximum after irradiation to 400 Gy and a local minimum after irradiation to 500 Gy (Table 2).

The CAT activity actually decreased after irradiations to 400 and 500 Gy, but it jumped up to a value 95% higher than that in the unirradiated plants after an irradiation to 600 Gy.

Although the final activity of APX after irradiation to 600 Gy was the highest among the activities of all the studied antioxidant enzymes, it still decreased somewhat from the initial value in the controls after irradiations to the lower doses (Table 2).

Radiation stimulated the activities of SOD and GR statistically significantly ($P < 0.05$), and this stimulation reached its maximum at 400 Gy. On the other hand, the activity of CAT was the highest after an irradiation to 600 Gy. It is interesting that the APX activity decreased after irradiations to all the tested doses, whereas the activities of SOD, GR and CAT increased, especially in the plants exposed to 400 and 600 Gy.

The MDA concentrations increased after irradiations to 500 and 600 Gy statistically significantly ($P < 0.05$) (Table 3). The highest MDA concentration was observed after an irradiation to 500 Gy (11.2 μmol/g fw). After an irradiation to 500 Gy, the concentration of MDA grew up to 156% of that of the control.

The proline accumulated statistically significantly ($P < 0.05$) in the plantlets irradiated to all the tested doses (Table 3). An irradiation to 600 Gy increased the content 90%.

4. Discussion

The leaf chlorophyll content, which is a good indicator of the photosynthesis activity, mutations, stress and nutritional state, is of special significance for precision agriculture (Marcu et al., 2013). Chlorophyll a and chlorophyll b in the leaves of higher plants are the main pigments of photosynthesis in the chloroplasts (Kim et al., 2012). Gamma radiation exerts different effects on photosynthetic pigments depending on the plant species and the radiation dose (Kim et al., 2015). In this study, the contents of chlorophyll a, chlorophyll b and the total chlorophyll increased with the radiation dose (Table 1). However, the content of chlorophyll a was higher than the concentration of chlorophyll b at all the doses (Table 1). An increase of the chlorophyll content in a plant material was also observed after an irradiation of pigeon peas to doses from 50 to 250 Gy (Desai and Rao,

Table 1.
Dose-dependent changes of the contents of chlorophyll a, chlorophyll b and total chlorophyll induced by gamma radiation.

Dose (Gy)	Chlorophyll a content (mg/g of fresh issue)	Increase (%)	Chlorophyll b content (mg/g of fresh tissue)	Increase (%)	Total Chlorophyll content (mg/g of fresh tissue)	Increase (%)
0 (control)	1248 ± 114		385 ± 63		848 ± 101	
400	1549 ± 68[a]		708 ± 126[a]		1252 ± 137[a]	
500	1539 ± 25[a]		671 ± 50[a]		1215 ± 53[a]	
600	1603 ± 5[b]	28.5	1087 ± 97[b]	182.5	1602 ± 81[b]	89.0

The values represent the mean ± S.E. calculated on the basis of results of three replicate experiments.
In the same column, values with different subscripts are statistically significantly different from each other at $P < 0.05$ according to the Duncan's multiple range test.
[a] Statistically significant difference at $P < 0.05$ from the value for 0 Gy.
[b] Statistically significant differences at $P < 0.05$ from the values for 0, 400, and 500 Gy.

Table 2.
Effect of the gamma radiation dose on the activities of the antioxidant enzymes SOD, CAT, APX and GR in leaves.

Dose (Gy)	SOD activity (unit/min/mg of fresh tissue)	Increase (%)	CAT activity (unit/min/mg of fresh tissue)	Increase (%)	APX activity (unit/min/mg of fresh tissue)	Increase (%)	GR activity (unit/min/mg of fresh tissue)	Increase (%)
0 (control)	179 ± 31		302 ± 33		5854 ± 202		72 ± 16.7	
400	622 ± 93[a]		200 ± 31		5299 ± 102[a]		210 ± 2.8[a]	
500	209 ± 68		286 ± 59		5712 ± 247		84 ± 6.5	
600	222 ± 8	24.5	589 ± 139[a]	95.4	5175 ± 125	−11.7	109 ± 3.1[b]	52.0

The values represent the mean ± S.E. calculated on the basis of results of three replicate experiments.
In the same column, values with different subscripts are statistically significantly different from each other at $P < 0.05$ according to the Duncan's multiple range test.
[a] Statistically significant difference at $P < 0.05$ from the value for 0 Gy.
[b] Statistically significant differences at $P < 0.05$ from the values for 0, 400, and 500 Gy.

Table 3.
Effect of the gamma radiation dose on the contents of MDA (product of lipid peroxidation) and proline in leaves.

Doses (Gy)	MDA content (μmol/g of fresh tissue)	Increase (%)	Proline content (μmol/g of fresh tissue)	Increase (%)
0 (control)	7.1 ± 0.5		2.7 ± 0.4	
400	7.4 ± 0.8		3.3 ± 0.3	
500	11.2 ± 1.2[a]		2.8 ± 0.3	
600	9.6 ± 0.7[a]	34.0	5.1 ± 0.1[a]	90.0

The values represent the mean ± S.E. calculated on the basis of results of three replicate experiments.
[a] Statistically significant difference at $P < 0.05$ from the value for 0 Gy.

2014). Borzouei et al. (2010) observed significant increases in chlorophyll concentrations in wheat with increasing dose of gamma radiation (they used 100 and 200 Gy). Jan et al. (2013) reported that a gamma dose of 10 kGy significantly increased the concentration of chlorophyll, while doses 15 and 20 kGy decreased it. Melki and Dahmani (2009) stated that a low, 20-Gy, dose of gamma radiation had a positive effect on the chlorophyll concentration in wheat. An increase in the chlorophyll concentration in seedlings of red pepper cultivars irradiated to doses from 2 to 16 Gy was reported by Kim et al. (2004). Also, El-Beltagi et al. (2013) found that irradiating seeds with gamma rays to 50 Gy increased the concentration of the photosynthetic pigment in cowpea (*Vigna Sinensis*) plants under salt stress (25 and 50 mM NaCl). However, ionizing radiation can sometimes decrease the chlorophyll concentrations in some plants. Kebeish et al. (2015) reported that gamma rays ranging from 1 to 15 kGy greatly reduced the chlorophyll concentrations in *Allium sativum*. Similarly, Kim et al. (2012) noted that 200 Gy was enough to decrease the chlorophyll content in *Oryza sativa* L. Sengupta et al. (2013) showed that concentrations of the photosynthetic pigments as markers of stress in *Vigna radiata* Wilczek decreased with increasing dose. Similar results were obtained with plantlets of *Citrus sinensis* irradiated to 40 and 50 Gy (Ling et al., 2008). According to Kiong et al. (2008), the chlorophyll content dropped significantly when seedlings were exposed to 70 Gy of gamma radiation. Çelik et al. (2014) used a ^{137}Cs source to irradiate soybean seeds to 300 Gy and observed a decrease in the chlorophyll content as a result. The chlorophyll concentrations in fenugreek plants decreased remarkably after irradiation to 150 Gy (Moussa and Jaleel, 2011). Fan et al. (2014) reported that the concentrations of chlorophyll a, chlorophyll b and total chlorophyll in *Zizania latifolia* plants decreased significantly after gamma irradiations to 50 and 100 Gy. These contradictions (increases vs. decreases of the chlorophyll concentrations) were attributed to differences in the developmental and/or reproductive stages of the plants (Kim et al., 2004, 2011). Here in sainfoin, the seedlings were at the early developmental stage, only 60-day-old. On the other hand, Kim et al. (2004) reported that gamma irradiation altered the photosynthetic pigments, but this action was not directly related to acceleration of the early growth in the irradiated plants. The higher chlorophyll concentrations might have been due to the efficacious activity of the chlorophyll a/b binding (Cab) protein gene, which codes for chlorophyll protein (Arulbalachandran et al., 2007). An influence of physical and chemical mutagens could lead to differences in the chlorophyll concentrations (Arulbalachandran et al., 2007). The magnitude of the increase of the chlorophyll concentration may have enhanced photosynthesis and improved crop yields in *Vigna mungo* (Arulbalachandran et al., 2007). From this perspective, gamma irradiation (especially to 600 Gy) could have enhanced photosynthesis in sainfoin, which may improve yields of sainfoin.

Exposure of cells to ionizing radiation results in formation of reactive oxygen species (ROS), which are related to radiation-induced cytotoxicity (Sun et al., 1998). Antioxidant enzymes and antioxidants are capable of scavenging cytotoxic ROS generated at high levels, particularly under environmental constraints (Fan et al., 2014). Antioxidant enzymes protect plants against the ionizing radiation stress (Wada et al., 1998). Antioxidative defense systems in sainfoin were altered by ionizing radiation. However, the responses of the antioxidant enzyme activities in sainfoin to gamma radiation were different. CAT is an important antioxidant enzyme in oxidative defense systems against various environmental stresses. Although our results indicate that the activity of CAT was suppressed by gamma radiation, especially at the doses of 400 and 500 Gy, there were no statistically significant differences between the activities in the plants irradiated to 0, 400 and 500 Gy (Table 2). Previous studies showed different CAT activity change patterns under radiation stress in different dose ranges. Al-Rumaih and Al-Rumaih (2008) reported that the activity of CAT in leaves of *Trigonella* decreased 48% after irradiation to 1 kGy. A significant, 62.4% decrease of the CAT activity was observed after an irradiation of soybeans to 120 Gy (Moussa et al., 2009). A significant decline in the CAT activity was observed after developing seedling of *Psoralea corylifolia* L. were irradiated to 20 kGy (Jan et al., 2012). Similarly, a significant decrease in the activity of this enzyme was observed in *Arabidopsis thaliana* irradiated to 58.8 Gy (Vanhoudt et al., 2014). Fan et al. (2014) reported a significant CAT activity decrease in *Zizania latifolia* plants after their irradiation to 100 Gy. Wada et al. (1998) observed that gamma irradiation up to 500 Gy decreased CAT activity in two species of *Nicotania*.

On the other hand, some papers reported that the activity of CAT increased after gamma irradiations depending on the dose. Thus, Aly and El-Beltagi (2010) stated that the activity of CAT in *Vicia faba* L. was significantly stimulated by irradiation to 5 kGy. Increased activity of CAT was also observed in two rice cultivar seeds irradiated to 200 Gy (Silva et al., 2011). Qi et al. (2014) and Kim et al. (2015) reported that the activities of CAT increased significantly after Arabidopsis seedlings were gamma irradiated to 50 Gy and *Brachypodium distachyon* plants were irradiated to 150 Gy.

In our study, the APX activity decreased with the increasing dose of gamma radiation (Table 2). This is not in line with the previous studies,

which found increased activity of APX in irradiated plants. So, Jan et al. (2012) reported that the APX activity in irradiated seedlings of *Psoralea corylifolia* L.increased after their irradiation to 20 kGy. Fan et al. (2014) stated that gamma radiation induced substantial increase in the ascorbate peroxidase (APX) activity depending on the chronic gamma radiation dose (100 Gy). Vanhoudt et al. (2014) reported that an irradiation to 58.8 Gy increased the APX activity in *Arabidopsis thaliana*. Kim et al. (2015) reported that the activity of APX in *Brachypodium distachyon* increased with the dose of gamma radiation increasing from 0 to 150 Gy. Similar results were obtained by Silva et al. (2011). They observed an increase in the APX activity in seedling of rice as a result of a high radiation dose (200 Gy). Kim et al. (2005) found that APX plays an important role in improving stress resistance under gamma irradiation. An observed ineffectiveness of APX in improving photosynthesis in response to gamma radiation was presumably due to elevated ROS production, which led to an inactivation of PSII (Fan et al., 2014).

Both APX and CAT are enzymes that catalyze conversion of H_2O_2 into water (Gratao, 2005; Silva et al., 2011). However, APX has greater affinity for H_2O_2 than CAT (Silva et al., 2011; Graham and Patterson, 1982). Peroxidase is believed to be the key enzyme to decompose H_2O_2, especially when CAT is inactivated (Jan et al., 2012; Abedi and Pakinayat, 2010). Therefore, the decreased CAT activity and increased peroxidase activity induced by oxidative stress seem to constitute a general mechanism to overcome the stress and protect the cells from damage. We found a negative correlation between the activities of CAT and APX, which depends on the dose statistically significantly (Table 2). Regarding the activity, the key role seems to change between these two enzymes during scavenging ROS in the seedlings grown from irradiated seeds. A similar concept of substitution of one ROS scavenger enzyme with another was proposed by Kim et al. (2012). According to them, higher plants have developed a diverse antioxidant response system (ARS) against the ROS induced by a broad spectrum of environmental stress factors. They also believe that the ARS in plants exposed to different ionization treatments have different mechanisms for scavenging the ROS generated by water radiolysis. The toxic effects of ROS are usually kept at balanced levels by a synchronized action of antioxidant enzymes (Silva et al., 2011). However, it is also known that gamma radiation changes the enzyme capacity, but the result depends on the radiation dose and the plant species used (Kim et al., 2005; Wada et al., 1998; Zaka et al., 2002; Vanhoudt et al., 2014).

SOD is the first enzyme in the detoxification process converting superoxide radicals into hydrogen peroxides (Kim et al., 2012). SOD may play a central role among the antioxidant enzymes in protecting cells against the ROS injury during ionizing radiation exposure (Sun et al., 1998). Upon irradiation of the sainfoin seedlings, activities of SOD and GR increased more significantly than the activities of the other antioxidant enzymes, and their highest activities were observed after the irradiation to 400 Gy. SOD and GR were stimulated statistically significantly ($P < 0.05$) by 400 Gy. That was in line with observations by Similarly, Aly and El-Beltagi (2010) that some antioxidant enzymes (POD, APOX, CAT and SOD) were positively stimulated by 5 Gy of gamma radiation. It could be speculated that the increase of the antioxidant enzyme activities may be one of the mechanisms of the hormetic effects of gamma radiation. Furthermore, our results demonstrated that SOD and GR are the major antioxidant enzymes in the chloroplast. These two enzymes are the first and the last components, respectively, in the ascorbate-glutathione cycle that occurs mainly in chloroplasts (Mullineaux and Creissen, 1997; Asada, 1999; Kim et al., 2004). In this study, we observed an increase in the SOD and GR activities observed in leaves of sainfoin plantlets with decreasing radiation doses. In contrast to this result, we also observed an increase in the chlorophyll contents in the leaves of sainfoin plantlets depending on the radiation dose. Therefore, it can be speculated that the increased chlorophyll content in the leaves of sainfoin plantlets was due to the high activities of SOD and GR.

Moussa (2008) reported that gamma radiation doses up to 100 Gy increased the activities of SOD and GR in *Vicia faba*. According to Al-Rumaih and Al-Rumaih (2008), the SOD and GR activities in *Trigonella* grew up after irradiations up to 1 kGy. Stajner et al. (2009) stated that the SOD and GR activities in soybeans increased after irradiations to 150–200 Gy. On the other hand, Fan et al. (2014) reported that the SOD activity in *Zizania latifolia* plants declined after irradiations to 50 and 100 Gy. We emphasize that the significant SOD activation following low doses of gamma rays depends on the dose and the plant developmental stage or just the plant age in the original environment of plants. According to Jan et al. (2012), significant activation of SOD is a function of the severity of the irradiation and depends on the plant growth stage or age of plants in their original environment. Similar acquisition of the SOD gene regulation was observed for GR and APX under the same conditions.

We also emphasize that changes in the activities of the antioxidative enzymes and the chlorophyll content depend not only on the dose of gamma radiation, but also on the age or the development stage and the cultivar of the plants (Kim et al., 2004; Silva et al., 2011; Jan et al., 2012). Therefore, further studies of antioxidant enzymes are needed to understand how a specific enzyme changes after an exposure to gamma radiation. The plantlets of sainfoin investigated in this study were 60 days old and in the development stage. Moreover, transcript levels of genes of the antioxidant enzymes can change under radiation stress (Al-Rumaih and Al-Rumaih, 2008).

MDA is the end product of lipid peroxidation in biomembranes. The MDA content usually reflects the level of lipid peroxidation and also indirectly reflects the extent of the membrane injury (Wang et al., 2010). Intensification of the lipid peroxidation, which is a major indicator of oxidative stress, results from accumulation of the $HO^{\cdot}$ radicals during irradiation (Stajner et al., 2009). Our results showed that the MDA content increased with the radiation dose (Table 2). That suggests that the membrane injury in sainfoin visible from the increase in the MDA production needs to be monitored closely if ionizing radiation is to be used.

Stajner et al. (2009) reported that 200 Gy of gamma radiation enhances the MDA content in soybean. Aly and El-Beltagi (2010) found that irradiation up to 20 kGy increased levels of lipid peroxidation in *Vicia faba* L. Fan et al. (2014) noted that the content of lipid peroxidation products increased in *Z. latifolia* seedling exposed to 50 and 100 Gy. However, the maximal decrease of the leaf MDA content was observed in chickpea genotypes irradiated to 700–1000 Gy by Hameed et al. (2008). According to Kim et al. (2012), 200 Gy of gamma radiation was sufficient to decrease the contents of the lipid peroxidation products in irradiated rice. Similarly, El-Beltagi et al. (2013) reported that the low-dose (50 Gy) gamma irradiation of cowpeas decreased the contents of the liquid peroxidation products.

Our data showed that ionizing radiation increased the proline content, suggesting that proline plays an important role in the defense systems against gamma rays. Jan et al. (2012) reported that concentrations of proline were significantly elevated in *P. corylifolia* plants gamma irradiated to 15 and 20 kGy. Fujımaki et al. (1968) noted that the proline content increased in potatoes gamma-irradiated to 150 and 300 Gy. An increase in the proline content was also observed in pigeon peas gamma-irradiated to 250 Gy (Desai and Rao, 2014). These results agree with those reported by Kebeish et al. (2015), who observed that gamma irradiation to 15 kGy increased the proline content in *Allium sativum* L. In a similar study, a low dose (100 Gy) of gamma radiation was found to stimulate the proline content in wheat (Borzouei et al., 2010). In addition, Al-Enezi and Al-Khayri (2012) reported that X-rays increased the proline content in *Phoenix dactylifera* L. seedlings. There was a correlation between the gamma radiation dose, the oxidative stress and antioxidant enzymes activity in developing seedlings of *Psoralea corylifolia* L. (Jan et al., 2012). El-Beltagi et al. (2013) reported that low doses of gamma radiation (50 Gy) increased tolerance of *Vigna sinensis* to the salt stress by means of accumulation of

proline in tissues. A low-dose (50 Gy) gamma irradiation produces proline in *Arabidopsis* seedlings (Qi et al., 2014). Radiation induces formation of ROS, which is highly toxic to plant cells. Proline is a scavenger of ROS. It can stabilize the structure and function of macromolecules such as DNA, protein and membranes (Akshatha et al., 2013). The molecular mechanism of proline accumulation is still unclear and needs to be investigated under radiation stress (Qi et al., 2014). The hermetic effect may be a result of increased proline accumulation in the plant cell.

Overall, the full set of data on antioxidant enzyme activities, chlorophyll contents, lipid peroxidation and proline contents seems to suggest that 600 Gy could be a suitable dose for a mutation breeding program for sainfoin. Wehr (1987) suggests that, in plant mutation breeding studies using seeds as materials, 50% of irradiated seeds would be germinated and, also, mature plants germinated from these seeds must be non-sterile. For sainfoin, Bağcı and Mutlu (2011) reported that gamma radiation doses between 400 and 600 Gy could be used in a mutation breeding program. However, they reported that the germination rates of seeds increased under 50% at 600 Gy. Therefore, to determine the most suitable gamma radiation dose for such a program for sainfoin (Ecotype 'Koçaş'), additional studies are needed that would focus on such characteristics as biomass parameters, seed germination, seedling survival, as well as pollen and ovule sterility.

5. Conclusion

Our study has shown that radiation stress in sainfoin results in changes in a number of physiological parameters. Radiation stress causes an increase in the chlorophyll content, MDA, proline content, activities of antioxidative enzyme, such as SOD, GR and CAT, and a decrease in the APX activity. Even though the highest SOD and GR activities were observed at 400 Gy, the highest chlorophyll and proline contents and the highest CAT activity occurred after irradiation to 600 Gy. In addition, this study enabled us to propose a basic mechanism of response of the plant to radiation and oxidative stress produced by ROS. It has provided basic information for future studies of the gamma-irradiated mutagenesis in sainfoin. However, further studies are needed to understand whether or not gamma irradiation of sainfoin can result in better crop yields.

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