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The Effect of Gamma Radiation on Freezing Tolerance of Chickpea (*Cicer aretinum* L.) at *In Vitro* Culture

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ABSTRACT

Freezing stress as one of the abiotic stresses limits the plant growth in some areas. Therefore, increasing the tolerance to freezing stress is necessary for surviving in winter. Plant diversity can be produced by mutation in plant breeding programs. This research was carried out with the purpose of applying gamma-ray as a mutagen for screening freezing tolerant chickpea genotypes. At first, 50 percent of lethal temperature (LT_{50}) of branches after cold acclimation and freezing stress (0 to -20°C) were determined by calculating the survival percentage. Then seeds of three chickpea genotypes were treated by different doses of gamma radiation (60, 100, 140 and 180 Gy) and the shoots were placed in their LT_{50} temperatures. The LT_{50} of three genotypes of MCC477, MCC495 and MCC741 were -11.5, -12.3 and -12.5° C, respectively. Significant differences were observed ($P \leq 0.01$) among doses and genotypes according to their survival percentage. The highest survival percentage, 80.1 and 64.6, were observed in the two genotypes of MCC741 in 180 Gy dose and MCC495 in 140 Gy dose, respectively. The increased survival of these two genotypes up to more than 50 percent, could show the increased tolerance to freezing. The radiation may effect the expression of genes which are responsible for freezing tolerance. The results of this experiment showed that gamma radiation can be a suitable strategy for obtaining the freezing tolerant lines in chickpea.

Key Words: Chickpea, Cold acclimation, Freezing tolerance, Gamma radiation, LT_{50}, Survival percentage.

INTRODUCTION

Chickpea (*Cicer arietinum* L.) is the second grain and edible legumes in the world that in addition to protein, has been considered for having essential elements such as calcium, phosphorus, iron and vitamins such as niacin and riboflavin (Morno and Cubero, 1998; Gaur et al, 2008). Biotic and abiotic stresses can be one of the most important barriers for the production of this crop. Drought, high temperature and cold stresses, reduce yields and its stability. Exposing plants to lower temperatures than the optimum temperature can be a cause of tension in plants (Stoddard et al, 2006). Freezing stress is a kind of compound stress which effects plants by exposing them to low temperatures and mechanical stress due to ice formation in plant tissues (Bourion et al, 2003; Martin & Jones, 2004). Growing cold legumes especially chickpea, are done in high areas depending on the temperature in late winter or early spring because of the hard winter cold. In this type of cultivation, the legumes face to high temperatures in late growing season and soil moisture deficit, so the yield is decreased (Adamsen & Coffelt, 2005; Mousavi et al, 2007). If the legumes were planted in autumn water consumption efficiency and plant growth will increase during the season (Cook et al, 1998; Xiao & Han, 2004). But this cultivation system is impossible in high lands because the cold tolerant varieties are not available. Therefore, increasing freezing tolerance in high lands is one of the most important prerequisite for planting autumn-winter chickpea. Even in spring cultivation, cold tolerance is necessary at the early stages of germination (Kanouni et al, 2009). Besides conventional breeding techniques, tissue culture technology can be used for breeding purposes especially for the selection of stress tolerance (Lang et al, 2002; Dita et al, 2006). Tissue culture can provide the possibility of selection in controlled environment and it is not limited by seasonal and environmental constraints, so it will reduce the duration of selection (Jain, 2001). In this regard, one way for increasing genetic diversity under *in vitro* conditions is the application of chemical and physical mutagens such as Ethyl Methane Sulfonate (EMS), Ethyl Ethane Sulfonate (EES), ionized radiation such as gamma radiation and rays of neutron (Kim et al, 2004). The application of mutagens at *in vitro* culture increases the genetic diversity for selecting favorable genotypes and thereby gives speed up breeding programs (Ahloowalia & Maluszynski, 2001; Ahloowalia et al, 2004). Application of these mutagens is reported for cold and salt tolerance of rice (Sallem et al, 2005; Rakotoarisoa et al, 2008), cold tolerance of canola (Madinchey & Kottliama, 2007), drought-resistance in wheat (Khan et al, 2001), tolerance to heat, salinity and disease in potato (Gosal et al, 1997; Sharabash, 1998; Das et al, 2000), and tolerance to cold and salinity in cauliflower (Fuller et al, 2006). Results show that the production of mutants in legumes with tolerance to biotic and abiotic stresses was not very successful, because of recalcitrant nature of

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legumes for regeneration (Roy et al, 2001; Dita et al, 2006). Recently, improvement of legume can help in the application of *in vitro* mutagens in legume breeding systems (Bhatia et al, 2001; Zare, 2007; Moshtaghi, 2008; Yousefiara et al, 2008). Considering how little information is available on the application of mutagens at *in vitro* culture and the importance of selection for freezing tolerant lines in legumes especially chickpea, this study is quite necessary. Therefore, this research was done to aim the application of gamma radiation as mutagen in chickpea for selecting freezing tolerance at *in vitro* culture.

MATERIALS AND METHODS

In this study, the effect of gamma radiation was investigated as a physical mutagen to increase freezing tolerance of chickpea genotypes. For this purpose, experiments were performed in two separate phases. The first stage was the determination of the lethal temperature for the 50 percent of shoots (LT_{50}) in chickpea genotypes. In the second experiment, the effect of gamma radiation doses were investigated on the chickpea genotypes for achieving shoots with freezing tolerance.

First Experiment

Seeds of three chickpea genotypes with partial resistance to cold, including of MCC477 (Flip93-244c), MCC495 (Flip93-262c) and MCC741 (Sel93TH24467) were selected and surface sterilized. Then the seeds were cultured in medium ½ MB (MS salts and B_5 vitamins), 15 $g.l^{-1}$ sucrose with a pH=5.8 and were maintained at dark conditions in growth chamber with temperature of 24 ± 1° C for two days. Then the decapitated embryonic axis with single cotyledon were separated and were cultured in MB medium containing 0.5 $mg.l^{-1}$ BAP, 0.5 $mg.l^{-1}$ kinetin and 0.05 $mg.l^{-1}$ NAA with 30 $g.l^{-1}$ sucrose and 8 $g.l^{-1}$ agar and pH=5.8 for induction of shoots in growth chamber with photoperiod 16/8 (light/dark) at 24 ± 1° C with a light intensity of 30 $\mu mol.m^{-2}.s^{-1}$. After two weeks the cotyledons were removed and the explants were transferred to second medium with no auxin and containing 0.5 $mg.l^{-1}$ BAP and kinetin for multiple shooting. Shoots were subcultured in the third medium include 0.1 $mg.l^{-1}$ BAP and kinetin (Moshtaghi, 2008; Yousefiara et al, 2008). In order for browning inhibition of shoot tips, the shoots were subcultured in ½MB medium containing %2.5 sucrose. All shoots were transferred to growth chamber with temperature of 4 ± 1°C due to cold acclimation for 12 days. Then all of them were transferred to thermogradient freezer in order to apply freezing temperatures from zero to -20°C (with a decline of two degrees per hour). Before freezing treatments, shoots were placed in -2° C for 24 hours for induction of freezing and creating ice nucleation on plants. After that the shoots were kept at 4 ± 1°C for 24 hours, and then were transferred to growth chamber with temperature of 24 ± 1°C. To evaluate the survival rate of shoots and determination of their LT_{50}, the survival percentage of shoots were calculated by the following formula after three weeks:

Percent survival= [(number of plants before freezing / number of survived plants three weeks after freezing) ×100]

Lethal temperature for the 50 percent of shoots (LT_{50}) was determined based on probit analysis of survival percentage and linear equations obtained for all treatments.

Second Experiment

The maximum number of the available seeds of chickpea genotypes (Approximately 2000 seeds from each genotype) were treated with different doses of gamma radiation including 60, 100, 140 and 180 Gray (Gy) (Yasar Ciftcl et al, 2006) by source of Cobalt 60 in Atomic Energy Organization of Iran in Tehran. Preparation phases of shoots took place according to the first experiment. The initial step of culture has been named M_1V_0 and also later steps named by the same method (Suprasanna et al, 2008). Thus the first cycle has been named as M_1V_0, the second, and the third shooting medium and ½ MB (no hormone) were M_1V_1, M_1V_2 and M_1V_3 cycles, respectively. After the last subculture (M_1V_3), all samples were used for applying cold acclimation and freezing treatment as first experiment. But in these experiments the shoots were harvested in determined LT_{50} temperature for each genotype and were maintained in growth chamber for 24 hours at 4 ± 1ºC and then were transferred to growth chamber (24 ± 1°C). Survival percentages of shoots were investigated three weeks after applying stress.

Survived shoots were transferred to the root induction medium (MB containing 0.1 $mg.l^{-1}$ IAA plus 30 $g.l^{-1}$ sucrose with pH=5.8) and then were transferred to hydroponics culture for adaptation for 4 weeks. The seedlings were transferred to the greenhouse for flowering and seed production. Some survived shoots were transferred to the average minimum temperature of 20 past years (1989-2009) which has been determined -14°C in Mashhad, Iran. Like the previous stage, their survival percentage was evaluated three weeks after the stress. Survived shoots were transferred to the root induction medium and adaptation. Statistical analysis of data was performed by using SAS (9.1) software and the comparison of means was achieved using the Duncan test.

RESULTS AND DISCUSION

First Experiment

The results showed that there is significant difference ($P \leq 0.01$) between temperature treatments for survival percentage. Survival percent of shoots decreased with the decrease in temperature. Maximum survival of plants was observed at higher temperatures, but a significant difference in survival index was observed at -10° C and survival of shoots was decreased strongly. In all three genotypes, the survival difference between -4° C to -14° C was more than %70. Very low temperatures (-18 and -20° C) caused no survived plants (Table 1).

Table 1. Mean survival percentage of shoots in three chickpea genotypes after three weeks of freezing stress (T: Temperature, G:Genotype)

T / G	0	-4	-6	-8	-10	-12	-14	-16	-18	-20
MCC477	100.0 a	100.0 a	96.3 a	88.9 ab	77.8 bc	59.2 de	22.2 g	3.7 i	0.0 i	0.0 i
MCC495	100.0 a	100.0 a	100.0 a	96.3 ab	81.5 bc	51.8 e	18.5 gh	3.7 i	0.0 i	0.0 i
MCC741	100.0 a	100.0 a	100.0 a	100.0 a	88.9 ab	70.3 cd	37.0 f	29.6 fg	7.4 hi	0.0 i

*The same letters show no significant difference

Cold and freezing tolerance in plants is one of the most important factors for their winter survival. Therefore, the survival percentage of the plants after being exposed to freezing temperatures has been introduced as one of the indicators of cold tolerance (Hofgaard et al, 2003). Reduced survival of the shoots can be attributed to the effect of low temperatures. Also, by locating shoots in lower temperatures, the plant tissues paled to light green and yellow and were brown at -18 and -20° C temperatures that indicating the loss of photosynthetic activity in freezing stress. Also, low temperatures produce with formation of ice crystals which prevent food exchange between different tissues and mechanical stress, so death will occur (Mittler, 2006).

Mcwilliam (1994) reported that low temperature has direct effects on the photosynthetic system and causes the loss of normal color in plants. In these temperatures, the accumulation of carbohydrates occurred in chloroplast and the photosynthesis completely stopped. Also, the plasma membrane is the first place that is exposed to freezing stress and will be damaged (Uemura et al, 2006). Therefore, continuing integration of plasma membrane is one important factor for survival of plants under freezing stress and any disturbance in membrane structure causes damage and death (Hana & Bischofa, 2004). Considering the relative tolerance of all three chickpea genotypes to cold stress, it was observed that there is not significant difference between genotypes in the survival percentage and also there is not significant interaction among genotypes and temperatures. Rugienius and Stanys (2001) evaluated cold injury and survival rate of regenerated strawberry seedlings. Their results showed that 90% of plants survived in -8° C. While 90% of the samples were dead in -12° C. Maximum difference in tolerance of genotypes was in the range of -9 to -12° C temperatures which depends on the genotype and plants survived between 20 to 100%. The most reliable and simple method for evaluating cold tolerance is determining LT_{50} point or critical temperature for survival of plant (Hofgaard et al, 2003). Fowler et al (1981) showed that LT_{50} has the highest accuracy and flexibility in the inheritance among all tests to determine the rate

of cold tolerance**.** LT_{50} values determined in each chickpea genotypes MCC477, MCC495 and MCC741 were -11.5, -12.3 and -12.5^{o} C respectively (Fig.1).

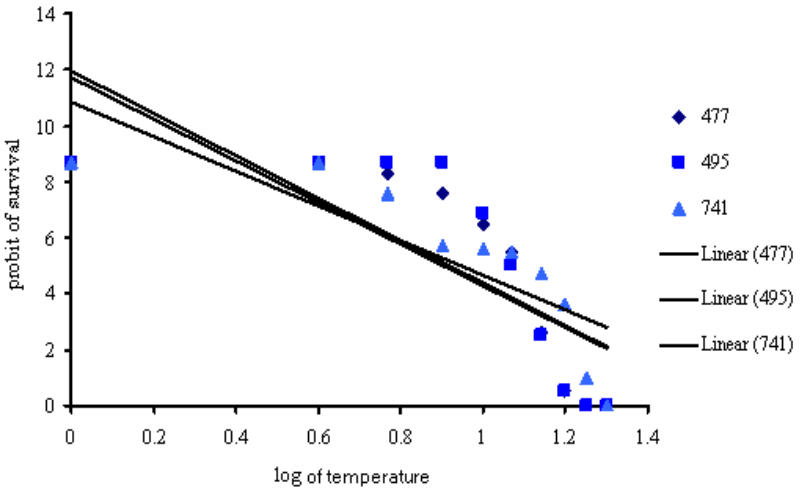

Figure 1. Evaluation of LT_{50} based on the survival probit data in different freezing temperatures in three weeks after stress.

Second Experiment

Morphological changes of shoots

After two weeks of culturing the explants in medium containing auxin, the induced shoots were investigated for morphological changes.

Among the induced shoots, a few albino shoots (no pigment and white color) were observed in MCC495 genotype which was treated by 180 Gy dose (Fig.2A).

This albino plant is showing the effect of the radiation on the plant genetic structure, that possibly has a disturbtion in photosynthetic system. These visible morphological changes due to radiation were reported in some plants. For example, in the experiment of Girija and Dhanavel (2009) bean seeds were treated by 15, 20, 25, 30 and 35 K Rad doses of gamma rays. In their experiment a range of morphological mutants such as albino plants (white) were observed almost in all mutation treatments. Similar to these results has been reported on *Curcuma alismatifolia* (Tohirah Lee, 2009).

One week after the subculturing of samples in third subculture medium (containing low concentrations of BAP and Kin) new morphological change was observed in three genotypes. The change was root growth on aerial part of plants. This phenomenon was observed on three shoots of MCC741 genotype in 180 Gy dose, a plant of MCC495 in 60 Gy dose, and one plant of MCC477 in dose of 60 Gy (Fig.2B). Root production in the second node of shoots lead to abnormal shape of the plant and prevented from its further growth. When shoots were transferred into the fourth medium, the shoots became gradually brown and died. The radiation is able to effect different parts of the genome and cause structural, biochemical and functional changes in plant and produce new forms of plants with modified traits (Selvi et al, 2007).

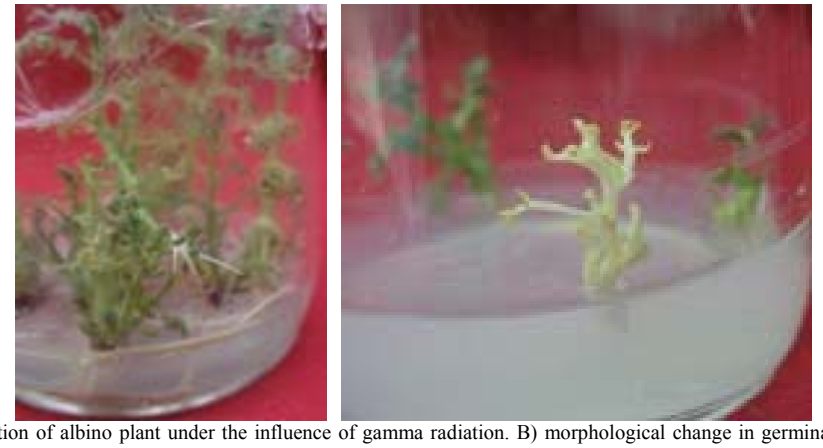

Figure 2. A) production of albino plant under the influence of gamma radiation. B) morphological change in germination and root growth from the second node on the main stem by induction of gamma radiation in three genotypes of chickpea.

Barakat et al (2010) were treated the middle floret of *Chrysanthemum morifolium* by two doses (0.5 and 1 Gy) of gamma radiation. The percentage of callus induction and shoot formation was different between doses of gamma rays. 0.5 Gy radiation dose was more effective than the other for inducing mutation in flower shape and number of middle floret.

The effect of gamma radiation on freezing tolerance of chickpea genotypes

There was significant difference between radiation doses for shoots survival ($P \leq 0.01$). The highest percentage of survival was observed in 180 Gy radiation dose (Fig.3). High dose radiation by affecting on responsible genes for cold, not only causes an increase in their activity but also changes in the gene expression lead to the activation of new genes. In other words, increasing radiation dose over 100 Gy has a positive effect on the survival percentage of the samples in freezing stress.

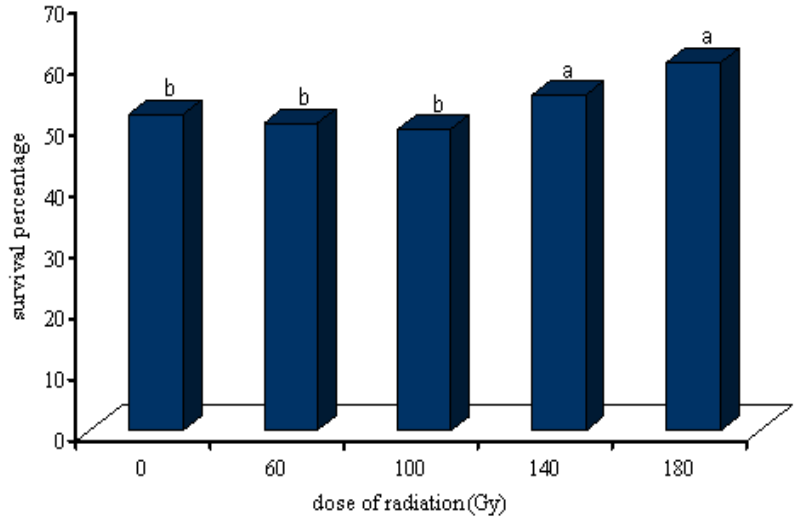

Figure 3. Effect of gamma radiation on the survival rate of chickpea genotypes.

Plant breeding through radiation is a rapid method for improving breeding programs (Kharkwal et al, 2004). The survival of shoots was different in various genotypes (Fig.4). Maximum survival was observed in MCC741 genotype and MCC477 genotype in which up to 55 percent of samples were able to tolerate freezing

temperatures. In other words, by placing shoots in low temperature (in extent determined LT_{50}), mechanisms of cold tolerance was more active in these genotypes, that its outcome has been the increase of samples, survival in these genotypes. At the end of the recovery period, it was observed that some shoots with high damage began growing again by creating new shoots from torque, that this phenomenon show the tolerance of them to the low temperature in these samples.

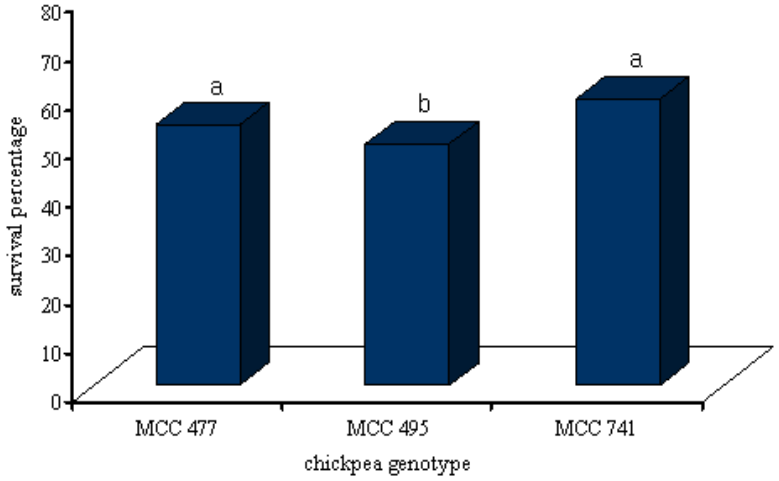

Figure 4. The survival percentage of chickpea genotypes by exposuring them to LT_{50} temperature.

By investigating the effect of gamma radiation on the genotype survival, it was observed that different doses of radiation had different effects on the shoots survival of each genotype. So that between 4 dose, the maximum survival was observed in MCC741 genotype in 180 Gy dose, MCC477 genotype in 140 Gy dose and MCC495 genotype in 180 Gy dose (Table 2). Increased survival of these genotypes to more than 50 percent shows the tolerance increase in them towards freezing stress.

Table 2. Mean survival percentage of chickpea genotypes, in different doses of gamma radiation (D: Dose of radiation, G: Genotypes of chickpea).

D / G	60	100	140	180
MCC477	51.10 c	49.41 c	64.61 b	49.69 c
MCC495	49.27 c	49.44 c	48.87 c	50.28 c
MCC741	50.08 c	49.81 c	51.37 c	80.01 a

*Similar letters show non-significant difference at α=%5

Remaining shoots were placed in the minimum average of absolute temperature in the region since 20 past years which was estimated -14°C. Three weeks after stress, many shoots of different doses of gamma rays were destroyed. Only 23 shoots remained from 180 Gy dose in MCC741 genotype. Remaining shoots were transferred to the rooting medium similar to the previous phase. Only, 16 shoots were rooted and seedlings were transferred to hydroponic condition (Fig.5A and B).

Figure 5. A) Rooting of tolerant branches of chickpea genotypes with the transition to rooting medium. B) The growth of produced seedling from in vitro culture by transferring to hydroponics.

The most important effect of radiation in response to stress (biotic or abiotic) can be the improvement of defense mechanisms in plants. This will lead to the regulation of specific defense and metabolic pathways in plant (Zolla et al, 2003).

CONCLUSIONS

Natural or induced genetic variation is an essential mean for plant breeding programs. Breeders have successfully used germplasm resources for improvement of new varieties or desirable traits like high yield, resistance or tolerance to biotic and abiotic stress. However, farm lands have been limited by increasing human populations but new ways have been provided for desirable agriculture through using of natural or induced genetic variation. Overall results indicated the possibility of obtaining some chickpea lines which have more freezing tolerance than their parents. But the produced mutants from first generation are not adequate for studying the genetic stability, so this trait should be investigated for the desired traits in subsequent generations and in field conditions. Therefore it is necessary to investigate the conditions for adaptation of produced seedlings from tissue culture with the natural environment to gain freezing tolerant genotypes.

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