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Stable Inheritance of Excellent Agricultural Traits Induced by $^{12}C^{6+}$ Heavy-ions in Lentil (*Lens culinaris* Medik.)

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Abstract

Zhou W., Wu Z., Zhang Y., Wu D., Liu D., Wang Y., Gao Q., Dang B., Li W., Hou S. (2015): Stable inheritance of excellent agricultural traits induced by $^{12}C^{6+}$ heavy-ions in lentil (*Lens culinaris* Medik.). Czech J. Genet. Plant Breed., 51: 29–35.

The lentil (*Lens culinaris* Medik.) belongs to the most important leguminous plants in Asia. It is a very popular and highly nutritious food. However, small pod size and low yield limit its agricultural use. Through irradiation mutagenesis of dry lentil seeds by $^{12}C^{6+}$ ion beam, we found some lines with excellent agricultural traits in M2 progeny at doses of 90 Gy (grey), such as height increase and improvement of the number of branches and three-seed pods as well as seeds with increased grain yield. Six generations of irradiated lentils were screened for these traits. In the M8 generation, the grain yield of three high-yielding lentil lines (lines hyl-1, hyl-2, and hyl-3) reached 212.4%, 195.3% and 190.8%, respectively, of the non-irradiated controls. The results indicated that crop improvement was stable inherited across the generations. Statistical analysis revealed that the increase in grain yield was due to increased total pod number and seeds per pod. Fortunately, adverse effects of irradiation (e.g. reduced germination rates and poor pollen vitality) disappeared over the eight generations. In conclusion, we present a practical method for the improvement of lentils through radiation breeding, leading to high yielding cultivars, which could support the use of this crop in agriculture.

Keywords: $^{12}C^{6+}$ irradiation; crop enhancement; high-yielding lentils; three-seed pods

Heavy-ion beams can be controlled in the form of high-LET (linear energy transfer) radiation to deposit high energy at precise positions of irradiated materials. This technique is very effective at causing mutations in embryos at a particular stage of fertilization, without damaging other plant tissues (Abe *et al.* 2002). Moreover, heavy ion irradiation, which induces a broad spectrum and high frequency as well as high diversity of mutants, has shown a great potential for breeding cultivars (Shikazono *et al.* 2001; Tanaka *et al.* 2010). Existing studies have demonstrated that ion irradiation caused irreparable DNA double-strand breaks (DSB), whereas most damage caused by traditional radiation resources (like X-ray and γ-ray) was repaired (Wada *et al.* 2002). Damage that cannot be easily repaired is vital for mutant induction, and is a major contributor to the high relative biological effectiveness and dominates the biological consequences in plants (Feng *et al.* 2006). Biological effects of high-LET heavy-ion radiation are more pronounced than those of X-rays, γ-rays, and electron beams (Shikazono *et al.* 2001). Mutation

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in plant is an effectual tool to create new breeding varieties and has been widely used in recent years (Feng *et al*. 2006; Roychowdhury & Tah 2013).

Lentil (*Lens culinaris* Medik. $2n = 14$), belonging to the subfamily Papilionaceae, is one of the most important leguminous plants in Asia. Lentil seeds contain large amounts of vitamins and minerals which are important for human health (Mondal *et al*. 2013). Lentil plants have been proved to exhibit high drought and pest resistance, so it may be cultivated in less favourable areas. However, agricultural production of lentils has several issues that require resolving to improve the application and exploitation of this crop, such as undesirable plant types and low seed yields. Some researchers have focused on the contribution of various yield components and hybridization between species to enhance seed yield (Mondal *et al*. 2013; Suvorova 2014). Nevertheless, its small size and low grain yield have not been sufficiently resolved. In this study, we expected to provide new insights into improving the breeding of lentils and other leguminous plants.

MATERIAL AND METHODS

Plant materials. Dry seeds of purebred lentil were collected from the Pingliang region (Gansu, China). Seeds obtained after irradiation were considered as M_1 generation. After they were successively planted with adequate irrigation on the experimental farm of Lanzhou University (GanSu, China), seeds were individually collected from M_1 seedlings (M_2 generation). M_2 seeds were planted, and three lines with three-seed pods from the 90 Gy-irradiated group were selected. Each screened line was reaped and sown individually, while other seeds irradiated at different doses were planted together. Seeds of M_3–M_8 generation were harvested using the same procedure.

Irradiation. Accelerated $^{12}C^{6+}$ ion beams ejected from the terminal of the Heavy-ions Research Facility (HIRFL) in Lanzhou, the detailed irradiation procedures referred to Wu *et al*. (2009) (Figure 1). 1500 dry seeds were irradiated by penetrating $^{12}C^{6+}$ ion beams (80.55 MeV/u) at doses of 0 (control), 30 Gy, 90 Gy, and 180 Gy.

Grain yield and plant height measure. A total of 6000 seeds of control, 30-Gy, 90-Gy, 180-Gy, and three screened lines from the M_3–M_8 generation were planted in respective plots. The experimental plots were laid out in a randomised complete block with four replications. The plot size was 2 × 2 m. Grain

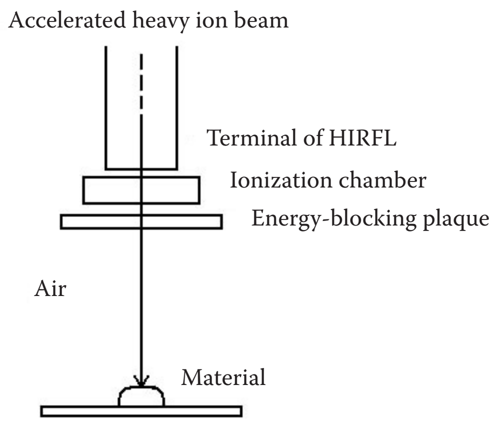

Figure 1. Heavy-ions Research Facility (HIRFL) irradiating instrument

yield per unit area (g/m^2) was calculated after these plants matured. At the seed stage, 50 individual plants of each line were selected randomly, and aerial parts were measured with a ruler to determine plant height, numbers of branches and pods were counted. The number of seeds per pod was counted and weighed together to calculate the single plant yield.

Germination rate. The germination experiment was conducted under natural conditions and 1000 seeds of each line were sown in drills. The number of germinated seeds was counted and recorded after four weeks. Germination rate (%) = number of germinated seeds/total number of seeds × 100.

Pollen vitality. Fully ripe pollen grains from 30 individual plants of the control, 30-Gy, 90-Gy, and 180-Gy groups from M_1–M_8 progeny were stained with triphenyl tetrazolium chloride for 48 h at 36°C. Pollen vitality (%) = number of pollen grains stained red/total number of pollen grains × 100.

Statistical analysis. All samples were randomly collected and data from at least three independent experiments are expressed as means ± SD. All values of the control were general average of eight years. Student's *t*-test was performed to compare with the control.

RESULTS AND DISCUSSION

$^{12}C^{6+}$ heavy-ion irradiation improves lentil yield. Irradiation mutagenesis of dry lentil seeds was conducted with the aim of breeding high-yielding lentils. The seeds were penetrated by $^{12}C^{6+}$ ion beams at doses of 30, 90, and 180 Gy. To investigate the influence of irradiation on yield, we calculated the average yield per plant irradiated at different doses in the M_1 and M_2 generations. The average yield of

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irradiated lines at doses of 90 Gy increased by 21.7% and 33.3% compared to that in the control in the M_1 and M_2 generation, whereas 180 Gy irradiation decreased (Table 1). After steady inheritance for six generations, grain yield at doses of 30, 90, and 180 Gy increased to 135.6%, 170.7%, and 148.8%, respectively, compared to the control in the M_8 generation (Table 2).

In addition to the improvement of grain yield, the irradiated lines showed advantages for other agronomic traits. The average height and number of branches, total pods, and seeds per plant increased in the M_7 and M_8 generations (Table 2) compared to the control. These data showed that 90-Gy irradiation confers the best advantages for agricultural traits and enhances the yield. This finding provides important information about the heavy-ion radiation dosages that are required to obtain desirable variations in agronomic traits of lentils.

Crop yields of three mutants in M_7 and M_8. Unexpectedly, three-seed pods appeared when using 90-Gy irradiation in the M_2 progeny. Three screened mutant lines with three-seed pods were called high-yielding lentil lines 1–3 (hyl-1, hyl-2, and hyl-3). To confirm the improved yield of mutants under natural conditions (Figure 2A), single plant yield and grain yield were calculated. The grain yield of hyl-1, hyl-2, and hyl-3 was improved by 104.9%, 86.7%, and 72.0%, respectively, compared to the control in the M_7 generation (Table 3). In accordance with the grain yield of M_7, those in the M_8 generation increased to 212.2%, 195.3%, and 190.8% (Table 3). Similarly, a considerable grain yield increase was reported in fava bean after irradiation (Podleśny 2002).

Agronomic characteristics of three high-yielding mutants screened from the 90-Gy line. Compared to the control, the mutant lines were much taller and stronger by the M_8 generation (Figure 2B; Table 3). Both at seedling and reproductive stage, the control had fewer branches (Figures 2E, F) than the mutants (Figures 2I, J; Table 3). During the flowering period, the control tended to have one or two flowers on each branchlet which would develop into pods at maturity (Figures 2C, D), whereas the mutant lines usually had two or three flowers (Figures 2G, H). Furthermore, three or four seeds were present in the pods of mutants (Figures 3F–H), whereas the control tended to have only one or two seeds per pod (Figures 3B–D). This phenotype was not previously reported for lentils, whose pods usually contain only one to two seeds (Cui 1998). Though significant enhancement in the total number of seeds was observed (Table 3), there was no obvious difference in the seed size and the 1000-seed weight between the mutants and the control in the M_8 generation (Figure 3A, E; Table 3). In addition, $^{12}C^{6+}$ irradiation increased the number of pods with two seeds in the three mutant lines (Figure 4). More importantly, about 20% of pods had three to four seeds in three mutant lines, which were stably inherited in M_7 and M_8 (Figure 4). Taken together, these results support the conclusion that the combined effects of increased branches, pod numbers, and numbers of seeds contributed to significant enhancement of crop yields of hyl-1, hyl-2, and hyl-3. Although no obviously drought- and disease-sensitive or resistant phenotype to abiotic stress was observed in three mutant lines, irradiation treatment induces a variety of mutations in plants which are very important for plant breeding (Younis *et al*. 2008; Tanaka *et al*. 2010).

Some adverse effects of irradiation disappeared in offspring. The impact of $^{12}C^{6+}$ irradiation on the

Table 1. Effects of different doses of irradiation on yield and yield components of lentils in the M_1 and M_2 generations

	Doses irradiated (Gy)	Average height/plant (cm)	Branches/plant (number/plant)	Total pods/plant (number/plant)	Total seeds/plant (number/plant)	Average yield/plant (g)	1000 seed weight (g)
M_1	30	29.5 ± 1.8	2.6 ± 0.3	41.8 ± 5.2	74.1 ± 6.7	3.49 ± 0.2	29.25 ± 0.8
	90	30.8 ± 1.4	4.0 ± 0.3**	56.8 ± 6.2**	89.5 ± 7.6**	4.27 ± 0.2*	29.73 ± 0.8
	180	27.2 ± 1.3*	3.9 ± 0.2*	37.6 ± 2.1*	58.4 ± 4.5*	2.93 ± 0.1*	28.17 ± 0.7*
M_2	30	31.4 ± 1.1	3.8 ± 0.4*	53.9 ± 4.4*	85.0 ± 10.5	3.22 ± 0.4	29.69 ± 0.9
	90	35.9 ± 1.5*	4.9 ± 0.2**	65.4 ± 3.3**	93.8 ± 10.2**	4.68 ± 0.3*	30.01 ± 0.7
	180	30.5 ± 0.7	4.2 ± 0.2*	50.5 ± 4.3	75.0 ± 11.0	3.34 ± 0.3	29.96 ± 0.8
Control	0	31.4 ± 1.3	2.6 ± 0.3	44.0 ± 3.5	75.4 ± 6.4	3.51 ± 0.3	30.18 ± 0.6

*, ** are significant at the 5% and 1% probability level, respectively; $n = 50$

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Table 2. Effects of different doses of irradiation on yield and yield components of lentils in the M_7 and M_8 generations

Doses irradiated (Gy)	Average height/plant (cm)		Branches/plan (number/plant)		Total pods/plant (number/plant)		Total seeds/plant (number/plant)		1000 seed weight (g)		Grain yield (g/m²)	
	M_7	M_8	M_7	M_8	M_7	M_8	M_7	M_8	M_7	M_8	M_7	M_8
30	36.8 ± 1.4*	35.8 ± 1.6*	4.13 ± 0.3*	3.9 ± 0.4*	75.9 ± 4.3*	91.2 ± 4.2*	127.0 ± 5.8**	146.1 ± 2.7**	30.66 ± 0.6	30.80 ± 0.8	94.8 ± 2.6**	104.3 ± 4.1**
90	37.3 ± 1.7**	38.5 ± 1.4**	5.4 ± 0.5**	5.6 ± 0.5**	95.6 ± 6.3**	107.2 ± 6.7**	181.5 ± 7.8**	190.6 ± 4.9**	29.12 ± 0.8	30.26 ± 0.5	120.7 ± 4.7**	131.3 ± 5.0**
180	35.6 ± 1.5*	37.6 ± 1.9**	4.6 ± 0.4*	4.8 ± 0.5*	86.6 ± 4.5**	96.4 ± 4.6**	156.0 ± 5.2**	163.0 ± 6.7**	29.99 ± 0.4	29.98 ± 0.4	106.4 ± 3.4**	114.4 ± 3.3**
Control	31.4 ± 1.3		2.6 ± 0.3		44.0 ± 3.5		75.4 ± 6.4		30.18 ± 0.6		76.9 ± 3.4	

*, ** are significant at the 5% and 1% probability level, respectively; n = 50

Table 3. Effects of high-yielding lentils lines hyl-1, hyl-2, and hyl-3 on yield and yield components of lentils in the M_7 and M_8 generations

Variety	Average height/ plant (cm)		Branches/plant (number/plant)		Total pods/plant (number/plant)		Total seeds/plant (number/plant)		1000 seed weight (g)		Grain yield (g/m²)	
	M_7	M_8	M_7	M_8	M_7	M_8	M_7	M_8	M_7	M_8	M_7	M_8
hyl-1	39.2 ± 1.9	40.3 ± 1.2	6.2 ± 0.6	6.2 ± 0.4	130.5 ± 5.3	157.7 ± 8.2	252.3 ± 10.9	289.7 ± 7.7	28.98 ± 0.4	30.30 ± 1.2	157.6 ± 5.4	163.3 ± 5.2
hyl-2	38.1 ± 1.1	38.6 ± 1.3	5.7 ± 0.7	6.0 ± 0.5	112.0 ± 6.3	120.4 ± 6.6	218.4 ± 11.0	238.1 ± 3.3	29.64 ± 0.4	29.91 ± 0.8	140.6 ± 4.4	149.4 ± 5.5
hyl-3	37.7 ± 1.1	38.0 ± 1.6	5.6 ± 0.3	6.1 ± 0.7	102.8 ± 3.3	112.7 ± 3.4	193.8 ± 12.0	231.6 ± 3.3	29.74 ± 0.5	29.77 ± 0.9	132.3 ± 5.5	146.7 ± 4.2
t-Test	**	**	**	**	**	**	**	**	NS	NS	**	**
Control	31.4 ± 1.3		2.6 ± 0.3		44.0 ± 3.5		75.4 ± 6.4		30.18 ± 0.6		76.9 ± 3.4	

NS – not significant, **significant at the 1% level of probability; n = 50

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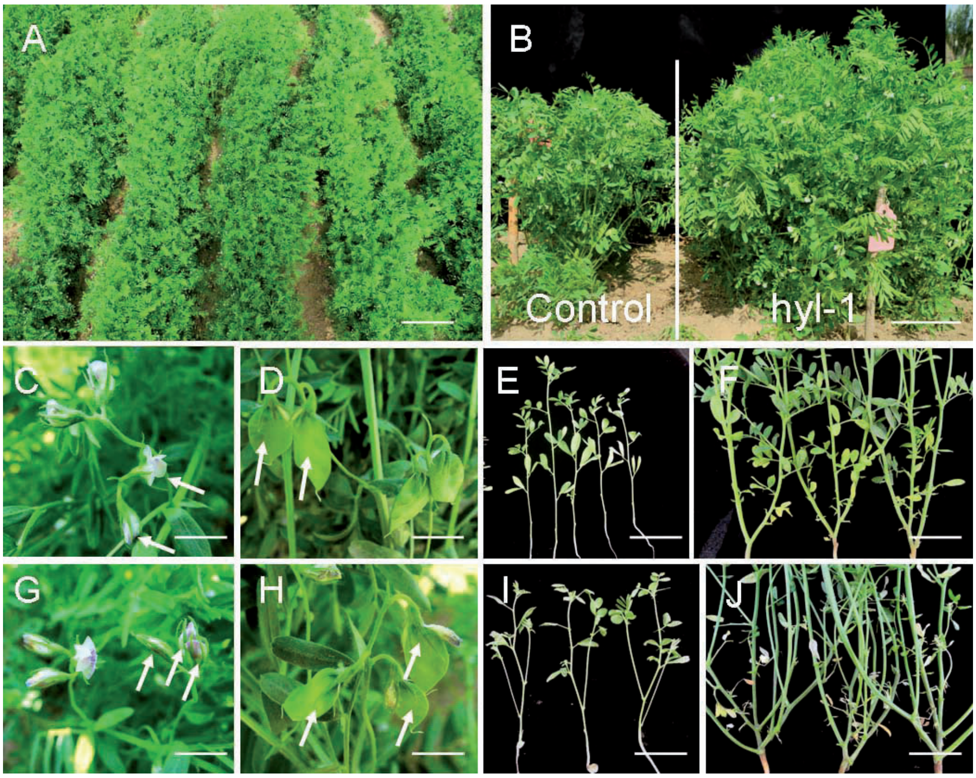

Figure 2. Phenotype of hyl-1 and control plants: (A) plants sown in drills under natural conditions; (B) comparison of plant height of hyl-1 and control, bars = 8 cm; (C–F) control; (G–J) hyl-1; (C, G) lentil flowers, arrows indicate the flowers on one branchlet; (D, H) lentil pods, arrows indicate the pods on one branchlet, bars = 1 cm; (E, I) lentil seedlings; (F, J) lentil branches, bars = 5 cm

germination rate was not detectable in the M_1 progeny (Figure 5A). In contrast, the germination rate of M_2 progeny declined by 11.3%, 14.0%, and 18.4% compared to the control at doses of 30, 90, and 180 Gy, respectively (Figure 5A). This result indicates that irradiation reduced the germination percentage in earlier generations. In contrast to the germination rate, irradiation had a direct negative effect on the pollen vitality of the M_1 generation (Figure 5B); however, this significant decline in pollen vitality was reduced in the M_2 progeny (Figure 5B). Therefore, despite the major influence of irradiation on M_1 and M_2 plants, it had a minimal influence on M_7 and M_8 plants (Figure 5B).

Heavy-ion irradiation may have multiple effects, including DNA damage, chromosome aberration, and gene mutation, leading to different types of phenotypes (Feng *et al*. 2006). These effects might be imperfectly repaired, resulting in heritable excellent agricultural traits, and which are retained by the offspring (Wada *et al*. 2002). The molecular mechanism that causes this phenotype is not clear,

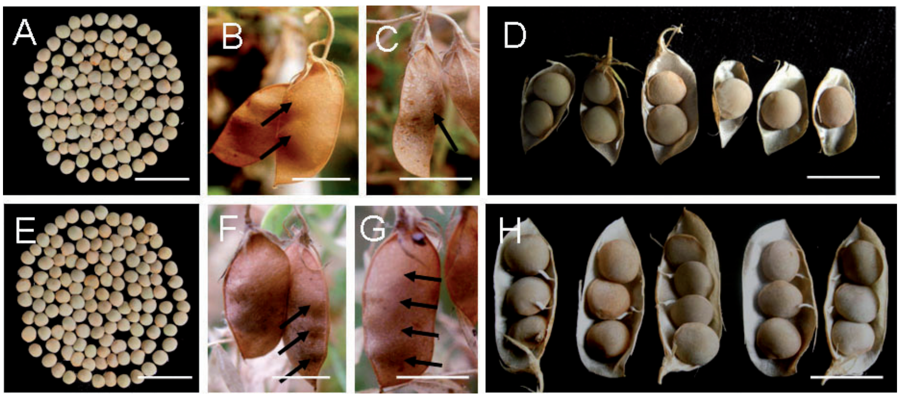

Figure 3. Mature pods and seeds of hyl-1 and the control: (A–D) control; (E–H) hyl-1; (A, E) shape of the seeds, bars = 2 cm; (B, C) one-seed and two-seed (black arrows) pods; (D, H) seeds in pods; (F, G) three-seed and four-seed (black arrows) pods, bars = 1 cm

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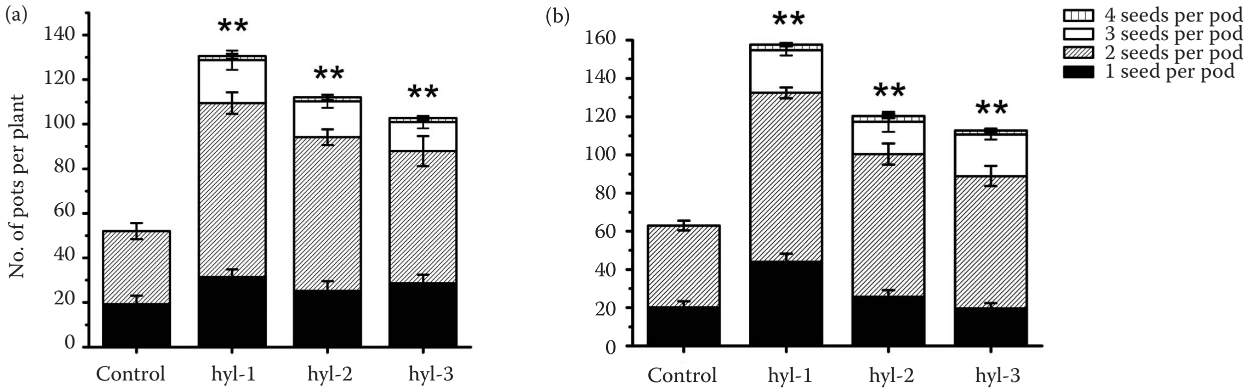

Figure 4. Distribution of different pod types: (A, B) different pod types of hyl-1, hyl-2, and hyl-3 in the M_7 and M_8 generation, respectively; **indicates that the means are significantly different at $P < 0.01$

but Shikazono *et al*. (2001, 2005) reported that carbon ion-irradiated mutants might more likely contain rearrangements and short deletions in the genome of the plant. Tanaka *et al.* (2010) further summarized and clarified that ion beams frequently produce large DNA alterations, including translocations, inversions and deletions. Therefore, we speculate that three *hyl* mutants, which were induced by $^{12}C^{6+}$ heavy-ions, may be caused by the deletion or restructuring of some genes, finally resulting in these genes losing the function. Although it has scarcely been reported about regulating the seed number and development of lentil gene due to a lack of genomic resources, some genes in other cereal grains have been cloned and studied, such as *DEP1* in rice (Huang *et al*. 2009). There may exist some homology according to the similar phenotype speculation. In addition, gene-based molecular markers and SNP markers are emerging as important tools for molecular mapping in lentil (Fedoruk *et al*. 2013). With the deepening study of lentil genome, the mutant gene will be cloned in future. In summary, $^{12}C^{6+}$ irradiation represents a highly effective method to enhance the yield and quality of lentils.

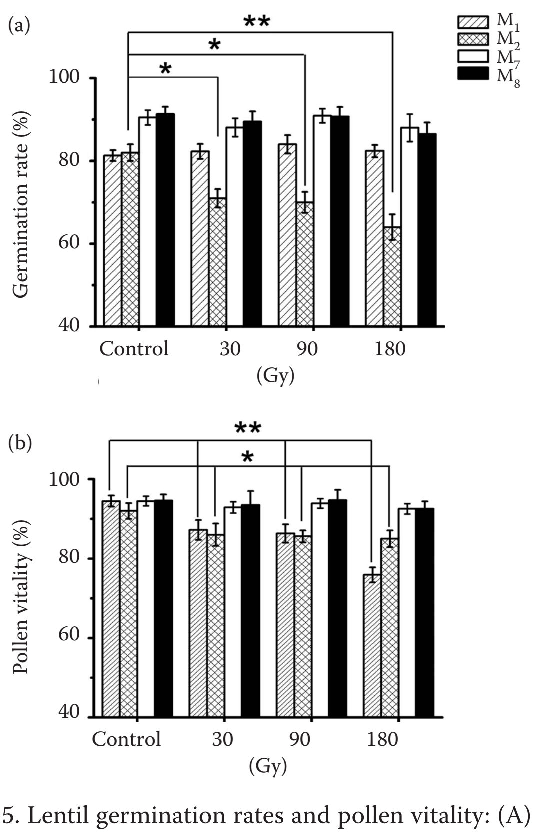

Figure 5. Lentil germination rates and pollen vitality: (A) lentil germination rates; (B) lentil pollen vitality; * and ** are significant at the 5% and 1% probability level, respectively. (n = 30)

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