OPEN ACCESS

Advanced Natural Sciences: Life and Health Sciences,
ISSN:3107-636X, Vol-1, Issue-1, Dec-2024, PP-4-16
https://indjournals.com/index.php

Original Research

Collection and characterization of short-term gamma irradiated *pisum sativum*

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Article Info

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[Received 19-04-2024,
Accepted 22-08-2024,
Published 29-10-2024]

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Abstract:

In the present investigation, *Pisum sativum* (pea) plants subjected to short-term gamma irradiation and various concentrations of ethyl methanesulfonate (EMS) were collected and characterized. The results indicated that increasing EMS concentration generally led to a reduction in plant height, with the exception of the 0.1% EMS treatment. Plants treated with 0.1% EMS exhibited the greatest height (75 cm), which was significantly higher compared to those treated with higher EMS concentrations.

Further, phylogenetic differentiation and geographic distribution of genetic variation, assessed in terms of total seed protein content across three varieties, revealed that lower EMS concentrations (0.1%, 0.2%, and 0.3%) resulted in a notable increase in protein content compared to the untreated control (WB) and the highest EMS treatment (0.4%). At 0.4% EMS concentration, a decline in protein content was observed, which was accompanied by deleterious morphological traits.

Despite existing reports in cereals of a negative correlation between total protein content and seed yield, the present study suggests that the induction of high-protein mutants may result from micromutations with positive effects on protein synthesis, while the observed reduction in seed yield may be attributed to micromutations with detrimental effects. The lack of a statistically significant negative correlation between protein content and yield further indicates that improving protein levels through breeding may not adversely affect the yield potential of the genotypes.

Keywords: gamma irradiation, *pisum sativum, mutation, protein, branches, shoot, root, pea.*

Cite this article: Sarah Hasyim and Anissa Azwar, 2024. Collection and characterization of short-term gamma irradiated pisum sativum, Advanced Natural Sciences: Life and Health Sciences, ISSN:3107-636X, Vol-1, Issue-1, Dec-2024, PP-4-16

I) Introduction:

Pea (Pisum sativum, dicot, 2n=14) is a grain legume crop with global importance. It is also one of the most extensively researched plants in terms of physiological, biochemical, genetic, molecular biology, and breeding studies. Pea has garnered attention as an experimental plant because it possesses big blooms, a self-pollinating sexual system, and a wide array of clearly observable seed, seedling, and adult plant phenotypes. [24]. Several genetic markers have been located on each of its 7 chromosomes [6]. Because of these characteristics, peas are a valuable plant model for molecular genetic research of developmental and biosynthetic processes as well as the structure and control of genes. They may also be used to create new material for breeding improved pea and other crop plant kinds. In this situation, a variety of biochemical gene markers are needed in peas to serve as a foundation for the use of molecular genetics (genetic engineering) techniques. [17]

Rogers Bros. Seed Co., Twin Falls, ID, provided the commercial pea (Pisum sativum) cultivar Sparkle'(S) seeds as a gift. The seed was exposed to 1% ethylmethane sulfonic acid (0.08 M) for one hour. When looking for induced mutants with few or no nodules, the M2 mutant selection E107 was discovered. [20,21]. The plant hormone GA is known to have a considerable effect on primary root development [14]. For instance, when bioactive GA was applied to roots treated with the growth inhibitor ancymidol, the growth of the untreated plants was fully recovered. [34]. The GAs act by destabilizing the growth inhibitory DELLA proteins [27,16,30,3]. In other words, GA acts as an ‘‘inhibitor of an inhibitor’’ [16].

Thylakoid material from chloroplasts has been widely broken down into particles small enough to be resolved by polyacrylamide-gel electrophoresis using sodium dodecyl sulphate and its related detergent sodium dodecylbenzenesulphonate. Therefore, Ogawa et al. [26] and Thornber et al. [37] identified two predominating chlorophyll protein-detergent complexes, which (with the 'free' pigment) accounted for most of the chlorophyll in the original material. A common problem in the investigation of membrane proteins is that they tend to be of a hydrophobic nature and to resist solubilization except in the presence of detergents. The proteins of the chloroplast thylakoids were regarded as typical in this respect [12,38]. Increased utilization of legume protein by the food industry, especially soybean protein [19], has increased research in the utilization of legume or seed proteins in foods [18,15,36,28]. Legume and seed proteins are increasingly utilized as key ingredients in the food industry due to their capacity to enhance nutritional value and impart various functional properties. These properties contribute significantly to the development of favorable structure, texture, flavor, and color in formulated food products. A comprehensive understanding of the protein structure and molecular characteristics across different legume species or seed varieties is essential. Such knowledge facilitates the strategic modification and optimization of protein functionality, thereby supporting the design and improvement of novel food products. [23,18,40,13]. Nutritional and functional qualities of protein are largely determined by its amino acid content and nitrogen solubility [19]. Over the past five decades, the production of major crops has markedly improved due to advancements in plant varieties, enhanced agricultural practices, and optimized agronomic inputs [33]. In addition to conventional breeding methods, mutation breeding has emerged as a viable alternative for enhancing specific traits in crop plants. This approach relies on the induction of genetic variability followed by the selection, assessment, and propagation of desirable genotypes. Among the strategies employed, nuclear techniques for mutation induction have become a well-established component of modern crop breeding programs. Numerous crop varieties with enhanced economic traits have been successfully developed through induced mutagenesis [8,931]. Furthermore, certain mutants have contributed significantly to our understanding of plant genetics and developmental biology [39,7]. In *Pisum sativum* (pea), mutations that alter seed texture—such as wrinkling due to compositional differences—have been linked to variations at the *rugosus* loci [41,42]. Additionally, mutants deficient in phytochrome

chromophore synthesis have played a crucial role in elucidating the function of phytochromes in light-mediated plant development [35]. Singh et al. [32] identified chickpea (*Cicer arietinum*) cultivars using Polyacrylamide Gel Electrophoresis (PAGE) of storage seed proteins. Proteins soluble in sodium chloride were extracted from mature seeds of nine cultivars and analyzed using non-denaturing PAGE. Most cultivars exhibited two to three prominent protein bands along with several minor ones. Further analysis of the G1 protein fraction through denaturing PAGE and SDS-PAGE confirmed the efficacy of combining all three methods for reliable cultivar identification. Genetic relationships within the genus *Cicer* were also examined using SDS-PAGE analysis of seed storage proteins [2]. The total protein profiles of *C. arietinum* and eight other wild annual species were found to be conserved and species-specific. Similarly, phylogenetic variation and geographic distribution of seed protein diversity in 217 accessions of *Setaria italica* from Europe and Asia were assessed via SDS-PAGE [1]. The resulting electrophoretic patterns were grouped into six types, each associated with different geographical regions globally. Structural conservation during evolutionary processes often surpasses sequence conservation. This means that sequences with limited similarity may still assume nearly identical three-dimensional structures. This principle, initially observed by Chothia and Lesk [10] and quantitatively described by Sander and Schneider [29], underscores the significance of protein structure in evolutionary biology.

II) **Materials and Methods:**

1. Plant Material- The wild variety of *pisum* will be collected from Institute for Islamic Studies Ma'arif Nahdlatul Ulama, Indonesia, another will be cultivated one for comparative study.

2. Preparation of Seed Sample: For extraction of protein, individual seeds were ground to fine powder with Mortal and pestle. To extract protein in0.01 g of seed flour, 400microlitre of the protein extraction buffer (0.05M of Tris-HCL, 0.2% SDS, 5 M urea and 1% Beta-mercaptethanol) will be added to the tube and mixed well by vortex. Then centrifuge at 15,000 rpm for 5 min at room temperature. The extracted crude proteins were recovered as clear supernatant and stored at -20 ^{O}C.

3. Preparation of Gel:

Seed proteins were analyzed through slab tyoe SDS-PAGE Followed by Laemmli [22] using 11.25% polyacrylamide gel. Electrophresis will be carried out at 100v for two and half hours. In order to reproducibility of the method to separate gels were run under similar electrophoretic conditions. After elctrophoresis of seed storage proteins [2]. Total seed storage proteins of the cultivated, C. *arietinum* and eight other wild annual species were separated and compared by SDS-PAGE . The seed protein profile was a conservative and species specific trait.

4. Fractionation and isolation of the major protein components:

Peas will be dehulled and the cotyledons milled to pass through a 365 micro metre –mesh-sieve. The meal (50 g) will extract twice with 20mM sodium acetate buffer, pH5.0 at a ratio of 1g of meal to 5 mlof buffer, for 1-2 hour at 4^0C. Extracts will be clarified by centrifugation at 10,000rpm for 30 min and then fractionation by $(NH_4)_2So_4$ precipitation at 0^0C. Precipitation in the ranges 0-50% and 50-90% rel. satn.will be prepared, resolved in 10 ml of 50 mM-Tris/HCL buffer, pH 7.5 and either dialysed against water and freeze dried or will be us eimmdiately for further fractionation.

b. Gamma irradiation treatment to wild varieties of *pisum.*

The seeds collected from the above regions were brought to lab, were properly sun dried to keep away microbial contamination away from them. The set of healthy seeds were selected from each varieties and were treated with different gamma doses (kilorad) viz. 5KR, 10 KR, 15 KR, 20 KR, 25 KR and 30 KR. The treatments were given at cobalt 60 chamber for particular time period.

Effect of gamma radiation on weight of seeds

The weight of total 10 no. of seeds were taken , to find out the changes in the weights. It was observed that the weight of seeds goes on decreasing as the gamma dose (KR) goes on increasing.

c. Petriculture of seeds

The sterile paper towels were prepared into the petridishes. The healthy seeds from each dose were initially soaked in sterile distilled water for 2 hrs. The set of each 10 seeds were kept on wet towel. The each dose set were studies in terms of Percentage of sprouts; Increase of decrease in germination percentage of seeds with different doses i.e LD_{50}; Initiation of root and shoots; Morphology of root and shoots; Heights of plantlet and susceptibility to microbial contamination. The results were recorded after 3 days of interval. The comparatives data were collected and were compared within each dose and with field trail.

d. Field study of gamma dose irradiated wild pisum seeds

The field for the experimentation trail was prepared with incorporating neem cake with small amount of *Trichoderma* powder. The field was fine tilth. The ten seeds from each treatment were taken and dibbled in soil at the distance of 30 cm. The rhizobium powder was applied to the pisum seeds before sowing. The treatments were replicated thrice. The field was irrigated with water at proper intervals. Plant protection measures were applied as per recommendations. The results obtained were statistical analyzed by RBD (Randomized block design).

Method of extraction of total protein

a. The 30 mg of crush sample /powder dissolved in 0.1M Na0H. It was freezed thrice and then centrifuged at 10,000 g for 20 min.

b.Supernatant was treated as protein sample.

3. Estimation of soluble protein by dye-binding method (Bradford's method)

Standard protein solution [BSA 25 ml(1mg/ml)] 0.01% protein reagent (Coomassie Brilliant Blue G-250) Measured absorbance at 595 nm after 5 min.

Results:

Field pea cultivation thrives best under cool climatic conditions, with optimal growth observed when the average temperature ranges between 55°F and 65°F. Under standard agronomic practices, field peas typically require approximately 60 days from sowing to the flowering stage and around 100 days to reach physiological maturity and produce dry seeds [25]. Mutation refers to any inheritable modification in the genetic material and encompasses a wide array of alterations,

Table 1. Effect of gamma radiation on percent germination (M1/G1 generation)

S.No	Gamma dose (KR)	Percent germination
1	Control	92
2	5	95
3	10	100
4	15	95
5	20	90
6	25	80
7	30	75

The table shows that gamma radiation influences seed germination rates. The control group, without radiation, had a germination rate of 92%. Lower doses of gamma radiation (5 and 10 KR) increased germination rates, peaking at 100% germination at 10 KR. However, higher doses beyond 15 KR caused a gradual decline in germination, dropping to 75% at the highest dose of 30 KR. This indicates that moderate gamma radiation can enhance germination, but excessive doses negatively impact seed viability.

Petriculture technique

Radiation has been shown to influence the size and weight of plants. In many radiobiological processes, the effect of a given dose depends on the intensity of radiation and how the total dose is fractionated, known as the time-intensity factor. Gamma rays affect plant growth and development by causing cytological, genetic, biochemical, physiological, and morphogenetic changes within cells and tissues. Growth observations of both control and irradiated seedlings were recorded at 3, 7, and 12 days after germination on petri dishes. Parameters assessed included root length, root type, and the initiation of the first shoot or root. It was noted that on the third day, 60% of control wild pea seeds had germinated, compared to a higher germination rate in treated seeds. By the seventh day, seeds exposed to 5 KR, 10 KR, and 15 KR showed varying germination rates, with germination decreasing at higher doses. While 90% of control seeds had germinated by this time, treated seeds exhibited faster root induction (around 70%) compared to shoot

emergence. Notably, higher doses resulted in 100% root induction but only 10% shoot induction. Furthermore, in the control group, shoot and root sizes were normal, whereas lower gamma doses produced shoots that were longer, greener, and more fleshy, and roots that were longer with some advantageous lateral roots. However, as gamma doses increased, roots became stunted, abnormal, and brown in color. Despite regular watering, plants subjected to higher doses died after 10 days. Cobalt-60 gamma radiation induced several physiological effects, including delayed and reduced germination at higher doses compared to controls. A low dose of 5 KR enhanced germination rates but produced minimal variability, whereas higher doses generated significant morphological variation but resulted in lower seed survival.

Table 2. Effect of gamma doses on seed weight

S.No.	Gamma radiation dose	Weight of 10 seeds (grams)
1	Control	5.1250
2	5KR	5.5565
3	10KR	5.7695
4	15KR	5.4550
5	20KR	5.7435
6	25KR	4.9150
7	30KR	4.8870

It was observed from the Table **2,** that the weight of seeds varied according the treatment given to it. The lower doses i.e 5 KR, 10 KR, 15 KR and 20 KR, showed the corresponding increase in weight of 10 seeds from each dose. The higher doses has the decrease in weight of seeds as compare to control.

Morphological parameters

The mutation affecting the gross morphological changes in branching, growth habit, flower colour, pod size, number of seeds, maturity and plant types etc. were scored as viable mutations. There were differences in the mutation spectrum between mutagenic treatments. Phenotypic changes were recorded after 12 days and analysis was based on randomized complete block design, after 60 days of growth in field. The results of field experiments are given in **Table no 3.**

Gamma Irradiated Pisum Sativum

Land used for sowing the Pisum Sativum (5kR, 10kR, 15kR.20kR, 25kR, 30kR and Wild), prepared seven lines for each type of seed.

Fig1: Land used for sowing the Pisum Sativum (5kR, 10kR, 15kR.20kR, 25kR, 30kR and Wild)

Fig2: The results of field experiments gamma treated at **5kR**

Fig3: The results of field experiments gamma treated at 10**kR**

Fig4: The results of field experiments gamma treated at 15**kR**

Fig5: The results of field experiments gamma treated at 20**kR**

Fig6: The results of field experiments gamma treated at 25**kR**

Fig7: The results of field experiments gamma treated at 30kR

Fig8: Wild/Natural Pisum (Without Gamma Irradiation)

Yield Parameters Influenced by Effect of Gamma Irradiation:

Gamma-irradiation stress induced significant changes in the plant physiological and biochemical processes. The data presented shows that the effect of different doses of gamma rays on the yield components through two generations under the effect of gamma irradiation. The result revealed significant increase in the number of pods per plant in M1 population at lower doses viz 5KR, 10 KR, 15 KR and 20 KR, equivalent to 25 KR and lower at 30 KR doses of gamma rays above unirradiated plants. These variations concerning germination, plant size and seed production, differed by the nature of the irradiated material. However, consequences of irradiation on nutritive quality and possible mutagenic effect on organism consuming then were not well understood. These studies clearly established that an array of qualitatively and functionally different radiomimetic principles might be produced at different doses and after radiation. One of the chief advantages of traditional mutagenesis is that it can give rise to many different mutant alleles with different degree of great modification.

Total protein estimation

The phylogenic differentiation and geographical distribution of genetic variation in term of total seed protein in WB control, germinated shoots and roots. The lower gamma doses were found to increase the considerable protein concentration in 0.1%, 0.2% and 0.3% EMS concentration *pisum* varieties as compare to highest 0.4% EMS dose, were it was associated with decreased or fetal morphological characters.

The plant explants (seed, root, shoot) were ground to fine powder with mortle and pestle. The extract protein in 30mg of seed flour, 1000 l of 0.1M NaoH was added to tubes and mix by vortex. Then centrifuged at 10,000rpm for 20 minutes at room temperature. The extracted crude proteins are recovered as a clear supernatant. The extracted protein sample were estimated for seed protein content using BSA (0.25mg/ml) as a standard. The potential of induced mutations is of importance in genetic improvement of crops. In the present experiments Ethyl methane sulfonate (EMS) applied in variable dose as treatments. To estimation total seed soluble protein in all three wild varieties of *pisum,* the dye-binding method (Bradford's method) is used. The results were presented in Table –4.

Total Protein Content of irradiated pisum seeds

The Microkjeldahl method was carried out to determine the protein content of the mutant wild pisum seeds. The protein samples were

titrated against 0.01N H2s04 along the blank. The calculations were carried out as follows:

1ml of 0.01 N H2S04 = 0.00014 g Nitrogen.

Since average nitrogen content of most protein is 16%

1 g nitrogen = 100/16 gm protein x = titer value of blank

The protein content = x X 0.00014 X100/16 X 100/02

= x X 0.4375 gm

Thus using above formula, the protein content of each set of mutant seeds were calculated and presented in **table -5.**

Separations of proteins

Proteins are separated on the basis of charge using ion exchange chromatography. The first eluted sample was collected and further separated by gel filteration chromatography. The absorbance of each protein sample was taken at 280 nm on UV – Vis spectrophotometer. Which implies that there is presence of increasing amount of fractional proteins in mutants as compared to control set. Legume seed proteins are mainly composed of water soluble albumins and salt soluble globulins.

Estimation of total proteins

The phylogenic differentiation and geographical distribution of genetic variation in term of total seed protein in three varieties shows that the, as compare to WB control, the lower EMS doses were found to increase the considerable protein concentration in 0.1%, 0.2% and 0.3% EMS concentration *pisum* varieties as compare to highest 0.4% EMS dose, were it was associated with decreased or fetal morphological characters. The plant explants (seed, root, shoot) were ground to fine powder with mortle and pestle. The extract protein in 30mg of seed flour, 1000 l of 0.1M NaoH was added to tubes and mix by vortex. Then centrifuged at 10,000rpm for 20 minutes at room temperature. The extracted crude proteins are recovered as a clear supernatant.

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Treatment	Height of plant (cm)	Number of branches	Number of flowers	Number of Pods	Size of pod (cm)	Number of seeds/pod
Control	21	9	8	6	2.45	**3**
5 KR	27	9	10	9	3.0	**2.5**
10 KR	28	8	11	9	3.8	**2.0**
15 KR	35	7	10	10	4.0	**4.0**
20 KR	35	7	9	7	3.0	**2.0**
25 KR	30	5	8	6	2.5	**2.0**
30 KR	**30**	**4**	**4**	**2**	**2.4**	**1.8**

Table 3 : Effect of gamma radiation on morphological parameters (M1 generation)

Gamma dose	WB (Protein conc. g/ml)		
	Seed	**Shoot**	**Root**
Control	390	550	280
5 KR	410	640	250
10 KR	420	800	240
15 KR	480	720	240
20 KR	480	460	250
25 KR	430	720	240
30 KR	500	670	220

Table 4. Effect of variable gamma doses on total protein content in wild *Pisum*

S. No	Gamma dose	Protein content (g)
1	Control	**6.96250**
2	5 KR	**7.09375**
3	10 KR	**7.1875**
4	15 KR	**7.5937**
5	20 KR	**7.8937**
6	25 KR	**6.9813**
7	**30 KR**	**7.9687**

Table -5. The protein content of each set of mutant seeds

EMS concentration	WB (Protein conc. g/ml) Seed	Shoot	Root
Control	26.8	29	17
0.1%	35.4	17	19
0.2%	35.59	17	18
0.3%	35.7	24	42
0.4%	24.8	28	15

Table 6. Effect of variable EMS treatment on total protein content in wild *Pisum*

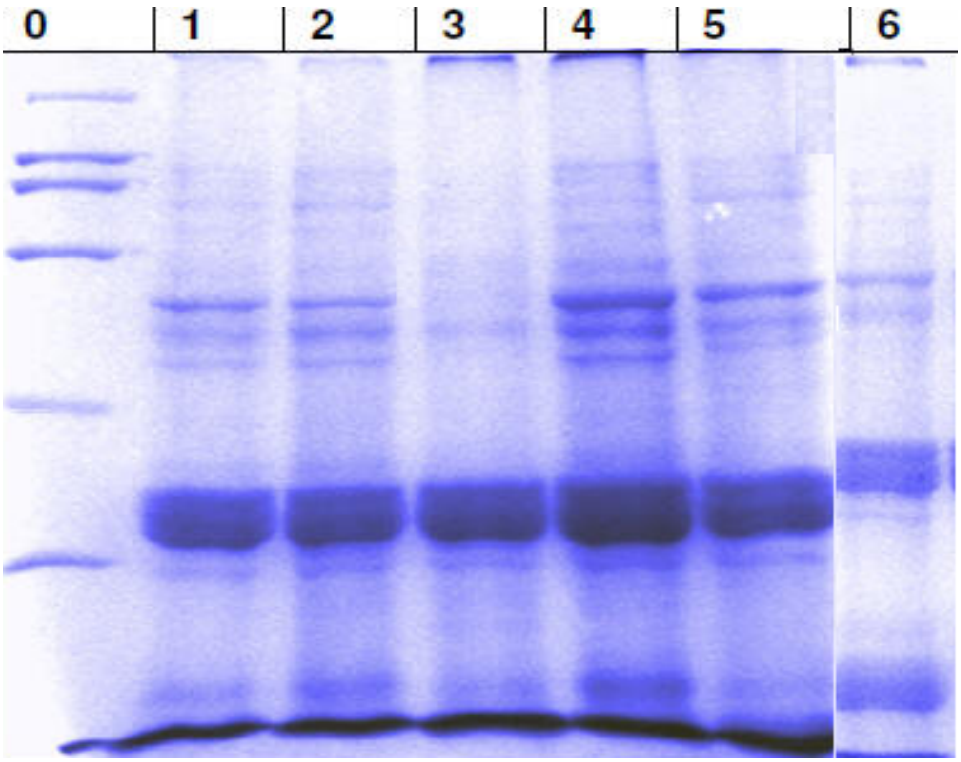

Fig.9. PAGE-SDS (12.5%) electrophoresis of total protein, albumins and globulins of seeds produced by G1 plants. Regardless of the irradiation dose, electrophoretic patterns are qualitatively and quantitatively similar. 0-Control, 1=5KR, 2=10KR, 3=15KR, 4=20KR, 5=25KR, 6=30KR

Morphological traits in cultivated M1 generation

Phenotypic changes were recorded after 10 days and analysis was based on randomized complete block design, after 2 months of growth in field. The results of field experiments are given in Table no.-7. Since various physical and chemical mutagens are known to act in different ways cause DNA

lesion, combined and in various concentrations, the effects can be investigated. It was wildly believed that spontaneous mutations have played a major role in speciation. However, consequences of irradiation on nutritive quality and possible mutagenic effect on organism consuming then were not well understood. These studies clearly established that an array of qualitatively and functionally different radiomimetic principles might be produced at different doses and after radiation. One of the chief advantages of traditional mutagenesis is that it can give rise to many different mutant alleles with different degree of great modification.

Table 7- Induced polygenic variability in M1

S.No	Treatment	No. of pods	No. of seed/pod	Length of pod	Height of plant (cm)	No. of Branches	No. of Flowers
1	Control	7.35	2.02	2.74	70.51	13	13
2	0.1%	11.29	3.62	3.14	75.20	12	18
3	0.2%	9.72	2.81	2.88	66.85	11	16
4	0.3%	4.50	1.53	2.62	58.72	10	11
5	0.4%	5.35	2.11	2.60	46.18	9	12
6	F test	Sig	Sig	NS	Sig	NS	N.S
7	SE	0.48	0.20	0.11	2.20	0.21	0.22
8	CD at 0.05	1.47	0.61	-	4.68	-	-

Sig- significant; **NS**- non significant

Height of plant (in cm) - From the data in Table no. 7 it is seen that height of plant significantly influenced by EMS concentration, As the concentration of EMS increase the height of plant decreased, except 0.1% concentration. Significantly heights eight 75cm was recorded by the plants which were treated with 0.1%, con then rest of the treatments.

Branches and flower per plant – Branches and flower per plants were not significantly influenced as various con. of EMS. Numerically highest flowers were produced by the plants from 0.1% con.(18).

Summary

The set of healthy seeds were selected from each varieties and were treated with different gamma doses (kilorad) of gamma rays for the purpose of isolating protein mutant improved in quantity and quality as compared to initial line. The mutant isolated at M1/G1 0R M2/G2 generation revealed that sufficient improvement in total seed protein content and its quality could be achieved through mutagenesis at lower doses rather than at higher gamma doses.

The mutants show typical type of protein variability. All mutants may significant negative correlation between yield and total protein was exhibited in mutants of higher doses, while the mutant at lower doses shows no significant correlation. Radiation is measured as a useful method to enhance the species genetic variability. Plant breeders assume gamma radiation is the most suitable mutagen.

Discussion

Extensive research has been published on genetic enhancement through applied mutagenesis aimed at developing future varieties with higher protein content and improved quality.

In our study, mutants derived from gamma ray-treated *Pisum sativum* demonstrated a notable increase in total seed protein content. Although previous reports in cereals have shown a negative correlation between total protein content and yield in protein mutants, this phenomenon may be due to micro-mutations exerting either positive effects (leading to high protein) or negative effects (resulting in reduced seed yield).

The presence of a non-significant negative correlation in our findings suggests that breeding for enhanced protein content may not substantially compromise the yield potential of the genotype.

Acknowledgement:
None stated

Conflicts of interest: Authors declared No conflict of interest.

Funding: None

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