Proceedings

International Conference on Nuclear Science and Technology

Nuclear Society Of Iran
Atomic Energy Organization of Iran
Nuclear Sciences and Technologies Research Institute

Papers on:

Nuclear Agriculture

In this booklet, you will find the selected papers presented at the **First International Conference on Nuclear Science and Technology**, held from May 6-8, 2024, in Isfahan, Iran.
We hope you find it informative and enjoyable!

ICNST 2024

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بسم الله الرحمن الرحیم

The Conference President's Message
ICNST 2024

Attendees, guests, and colleagues

I would like to warmly welcome you to the first International Conference on Nuclear Science and Technology (ICNST 2024). It has been a real honor and privilege to serve as the president of this conference. The conference this year has brought together an incredible diversity of authors and speakers from universities, government, and industry to share ideas and new perspectives on a wide range of radiation applications, nuclear reactors, particle accelerators, radiation measurements, fusion and plasma, stable and radioactive isotopes, radiation safety and security, nuclear agriculture, fuel cycle, lasers, education and training and nuclear governance.

Climate change, a new topic which has been added to this year's agenda as an important worldwide issue. a matter that has been brought up as a critical concern at the majority of IAEA conferences and nuclear scientific assemblies in recent years.

Panel discussions and exhibitions are being introduced as side activities in an attempt to keep this scientific meeting from becoming one-dimensional and increase its effectiveness.

More than 520 complete papers have been approved for this conference; when combined with the additional panels, get-togethers, and side activities, it is anticipated that over 1000 people will attend in person in the historical and touristic city of Isfahan. We look forward to welcoming participants to share their practical ideas and to enjoy an academical and cultural three days in Isfahan.

I'll close by wishing you everyone an incredible, instructive, and transformative experience during ICNST2024 and I hope that this conference can pave the route for academic materials to be used in industry and everyday life.

Prof. Javad Karimi-Sabet
President of ICNST2024
Javad Karimi-Sabet

welcome statment of scientific secretary **ICNST 2024**

"In the name of God, the Merciful,

Prior to giving the stage to address this distinguished forum, let me take this opportunity to express our deep gratitude, on behalf of all attendees, for His Excellency Mr. Islami's scientific, educational, and motivational remarks, as well as for his excellent organization of this conference.

I would also like to express our appreciation to His Excellency Dr. Mortazavi, Governor-General of Isfahan Province, for his constructive and useful support in enabling this meeting to take place.

This is a great pleasure and honor to extend a warm greeting to each and every one of you for the International Conference on Nuclear Science and Technology, scheduled from May 6th to May 8th, 2024, in the historic city of Isfahan, Iran.

With the aim of advancing our knowledge of nuclear science and technology, this conference is a major global convergence of experts, researchers, and practitioners. It is a platform for the sharing of creative concepts, the presentation of state-of-the-art research, and the formation of cooperative alliances.

As the scientific secretary of this prestigious event, I am particularly excited about the diverse array of participants expected to grace us with their presence. From the esteemed scientists and engineers of Russian universities and research centers to representatives from Islamic countries, friendly nations, and beyond, this conference promises to be a melting pot of perspectives, experiences, and expertise.

The extensive coverage of this conference is another aspect of its uniqueness. We have nearly 900 participants representing 22 countries around the world. Of the 900 participants, 620 are authors covering 13 major topics. There are 421 papers for oral and poster presentations, with additional documents for publication in ISC journals. There will be 3 plenary sessions, 16 panel discussions, 20 parallel oral presentation sessions, and 3 poster sessions.

Prof. Hosein Afarideh
Scientific Secretariat of ICNST2024

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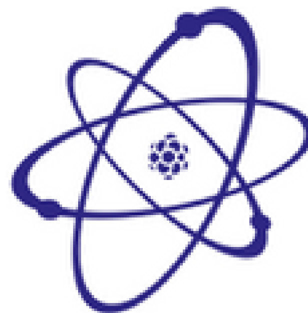

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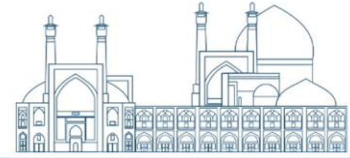

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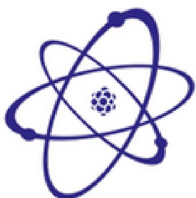

Journal of Nuclear Science and Technology (JonSat)

Radiation Physics and Engineering journal

Nuclear Watch

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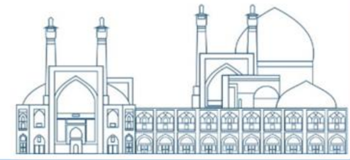

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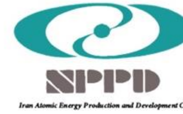

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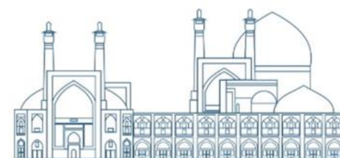

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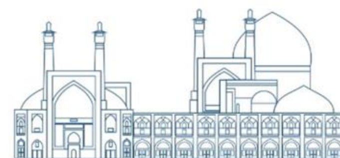

Nuclear Agriculture

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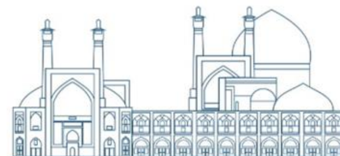
Nuclear Agriculture



Investigating Gamma Rays Effects on Rapeseed and Alfalfa Seed Germination (Paper ID: 1011)

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Abstract

Nowadays, irradiation, especially using gamma ray, is widely used in food and agricultural industries. One of the most important uses of radiation in agriculture is to create useful mutations in plants to increase productivity and enhance crop resistance to water stress. This study aimed to determine the effect of different doses of gamma radiation on rapeseed (Architect cultivar) and alfalfa (Bami and Ranjbar cultivars) germination.

Seeds were exposed to gamma rays from a cesium-137 source. The seeds received doses of 0–450 Gy in steps of 25 Gy each. Afterward, radiated seeds were placed in Petri dishes containing filter paper and filled with distilled water, and the number of germinated seeds was counted every 24 h and for 7 days.

The results showed that the highest total germination was related to the categories of 50 Gy for rapeseed, 400 Gy for the Bami cultivar of alfalfa, and 100, 125, and 300 Gy for the Ranjbar cultivar of alfalfa seeds. In addition, the highest speed of germination compared to the control group, which had not been irradiated, was recorded in rapeseed and alfalfa of Bami cultivars with 50 Gy, and 200 Gy, respectively, and in alfalfa of Ranjbar cultivar treated with 300 Gy.

Although the total germination of rapeseed and alfalfa (Bami cultivar) in one irradiated group is significant compared to the control group and other irradiated groups, in the case of alfalfa (Ranjbar cultivar), the behavior of the irradiated groups fluctuated.

Keywords: Gamma, Agriculture, Germination, Rapeseed, Alfalfa, Mutation

Introduction

Induced mutations have been accepted as a useful tool in plant breeding programs [1]. Nuclear techniques, gamma irradiation, are widely used in agriculture to improve genetic diversity. In contrast to conventional breeding methods that involve the production of new genetic combinations from existing parental genes, nuclear technology creates an exclusively new genetic combination with a high mutation frequency [2]. Mutagenicity has already been used to improve many beneficial traits affecting plant size, flowering time, fruit ripening, increasing resistance to salinity and water stress, resistance to pathogens, etc. [3-5].

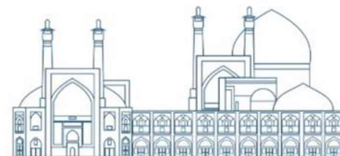
The Rapeseed plant with the scientific name Brassica napus is one of the important oilseed plants that is widely cultivated in Iran. This family is cosmopolitan, but the major centers of diversity are Southwest and Central Asia, and the Mediterranean region [6]. Rapeseed oil seed has been one of the most important sources of edible oil for hundreds of years [7, 8]. Rapeseed is now the second-largest oilseed crop in the world after soybean. Rapeseed is not only the main source of edible oil, margarine, vegetable oil, etc.; it is also an important source of biodiesel [9].

The effects of different gamma doses on the genetic variation, plant height, harvest index, seed weights, number of branches, number of leaves, protein digestibility, and physicochemical of this seed have been investigated by different investigators [6, 10-12].

The alfalfa plant, with the scientific name Medicago sativa, is one of the two-purpose plants that is used as a medicinal plant, food source for humans, and as fodder for livestock due to its high protein content [13]. According to studies, it has been shown that alfalfa contains non-cellulosic polysaccharides that have immune system-modulating, anti-inflammatory, anti-cancer, and growth-promoting properties [14-20].

Some investigators studied the alfalfa seed growth, morphology, and their tolerance to radiation exposure, when exposed to different radiation doses. [21-24].

The purpose of this research was to investigate the effect of cesium-137 gamma radiation on rapeseed and alfalfa seed germination.



Research Theories

In this study, to investigate the effect of gamma radiation on seed germination, the following parameters were compared for irradiated, and non-irradiated seeds.

The germination percentage is obtained as:

$$G_T = [\frac{N_T}{N} * 100] \quad \text{Eq. 1}$$

here N_T is the number of germinated seeds until the last measurement and N is the number of seeds used in the biological assay [30-32].

In order to determine the seed germination rate, equation 2 is used, where $N_1,...,N_n$ are the number of germinated seeds until days 1,...,n [32,33].

$$S = (N_1 * 1) + \frac{1}{2}(N_2 - N_1) + \frac{1}{3}(N_3 - N_2) + \cdots + \frac{1}{n}(N_n - N_{n-1}) \quad \text{Eq. 2}$$

Equation 3 is used to determine the accumulative germination rate of seeds

$$AS = [\frac{N_1}{1} + \frac{N_2}{2} + \frac{N_3}{3} + \cdots + \frac{N_n}{n}] \quad \text{Eq. 3}$$

In this equation, $N_1,...,N_n$ is the accumulative number of germinated seeds at the time of 1,...,n days after the start of the experiment [32,34].

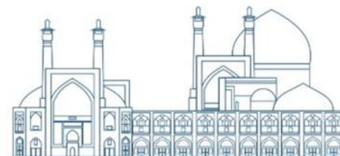
The germination rate coefficient is calculated from equation 4, where N_n is the number of germinated seeds in time T_n [32,35]. Its value increases when the number of germinated seeds increases and the time required for germination decreases [36, 37].

$$CRG = \frac{[N_1 + N_2 + \cdots + N_n]}{(N_1 * T_1) + (N_2 * T_2) + \cdots + (N_n * T_n)} * 100 \quad \text{Eq. 4}$$

Experimental

The experimental work was performed to investigate the effects of the radiation on the Rapeseed seeds of the Architect cultivar and alfalfa cultivars of Ranjbar and Bami. For this purpose, the seeds were packed in small packages, and irradiated by the 662keV gamma rays of Cs-137 source, in the Radiation Research Center of Shiraz University.

The seed packages were irradiated to the doses from 0 to 450 Gy, with 25 Gy increments. For each seed, a package was used as the control group (that was not exposed to irradiation). Each batch of



seeds was placed in a petri dish containing filter paper and thereafter, filled with distilled water. For each batch of irradiated seeds with a specific dose, 3 Petri dishes were used to increase the accuracy of the test from a statistical point of view. 24 hours after placing the seeds in the Petri dish environment, their germination was checked and the number of germinated seeds was counted. This process was repeated every 24 hours for several days. After the measurements, equations 1-4 were calculated and analyzed for each cultivar.

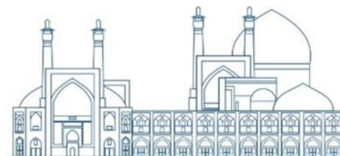
Results and Discussion

The results obtained for rapeseed of the Architect cultivar are shown in table 1.

Table 1. The irradiation effects on germination for the rapeseed, Architect cultivar.

Dose (Gy)	Total germination (%)	Speed of germination	Speed of accumulated germination	CRG (%)
0 (control)	82.00±5.47	10.08±2.18	23.20±3.05	66.07±3.30
25	75.33±5.02	9.06±2.11	20.46±2.95	57.38±2.87
50	88.67±5.91	11.40±2.34	26.01±3.28	67.80±3.39
75	70.67±4.71	8.55±2.01	19.57±2.80	61.54±3.08
100	70.67±4.71	8.07±1.94	18.39±2.70	55.17±2.76
125	73.33±4.89	8.86±2.04	20.48±2.84	66.00±3.30
150	80.00±5.33	8.67±1.97	20.22±2.71	58.06±2.90
175	77.33±5.16	9.53±2.11	22.09±2.95	68.63±3.43
200	70.67±4.71	9.00±2.10	20.52±2.94	64.15±3.21
225	80.00±5.33	9.77±2.13	22.70±2.97	69.23±3.46
250	70.67±4.71	8.45±1.97	19.90±2.72	71.11±3.56
275	64.00±4.27	8.30±1.99	19.32±2.77	78.38±3.92
300	77.33±5.16	9.93±2.18	22.99±3.05	74.47±3.72
325	68.67±4.58	8.52±2.01	19.67±2.8	68.89±3.44
350	70.67±4.71	7.80±1.86	18.40±2.56	61.54±3.08
375	70.67±4.71	9.38±2.13	21.65±2.98	78.05±3.90
400	75.33±5.02	8.88±2.02	20.85±2.79	68.00±3.40
425	75.33±5.02	9.48±2.14	21.52±3.00	62.96±3.15
450	75.33±5.02	9.66±2.15	22.14±3.02	69.39±3.47

In the analysis of the results obtained for rapeseeds of the Architect cultivar, it can be seen that the sensitivity of rapeseed to gamma rays is not very high and the difference between the irradiated



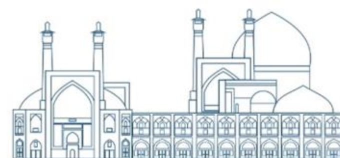
groups and the control group is negligible. However, the seeds received 50 Gy dose had a better performance than the control group, both in terms of total germination and speed of germination.

The calculated quantities for alfalfa seeds of Bami, and Ranjbar cultivar can be seen in Table 2. In the analysis of the obtained results for the Bami cultivar of alfalfa seeds, it can be seen that the samples with the doses of 250 and 400 Gy have had a higher total germination rate than the control group. According to the speed of germination, it can be concluded that irradiation of the Bami cultivar of alfalfa seeds generally increases the speed of germination, which is the highest value at 200 Gy. Based on the analysis of the obtained results for alfalfa seeds (cv. Ranjbar), it can be concluded that the behavior of this seed against gamma rays is completely fluctuating and unpredictable in other words, fitting a function to its behavior is impossible. However, in general, the group with the received dose of 300 Gy has the highest total germination and the highest germination speed among the other irradiated groups and the control group. By comparing the results obtained for these two genotypes of alfalfa, it can be said that in samples irradiated doses between 75 -175 Gy physiological changes in the Ranjbar cultivar are better than Bami cultivar.

in the dose range between 175-275 Gy Bami cultivar has a better function and in doses more than 275 to 450 Gy it seems the Ranjbar cultivar is better physiologically-wise.

Table 2. The irradiation effects on germination for the Bami and Ranjbar cultivars of alfalfa.

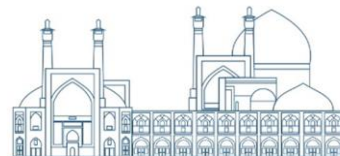
Dose (Gy)	**Total germination (%)**		**Speed of germination**		**Speed of accumulated germination**		**CRG (%)**	
	Bami cultivar of alfalf	Ranjbar cultivar of alfalf	Bami cultivar of alfalf	Ranjbar cultivar of alfalf	Bami cultivar of alfalf	Ranjbar cultivar of alfalf	Bami cultivar of alfalf	Ranjbar cultivar of alfalf
0 (control)	88.87±2.43	82.20±2.34	7.20±1.67	9.64±2.11	21.37±2.19	26.03±2.94	45.98±2.30	61.67±3.08
25	91.13±2.46	80.00±2.31	9.61±2.05	9.26±2.07	26.88±2.80	24.98±2.88	57.75±2.89	52.05±2.60
50	86.67±2.40	77.80±2.2	9.23±2.03	8.34±1.92	25.42±2.79	23.06±2.65	54.17±2.71	56.45±2.82



75	82.20±2.34	82.20±2.34	9.32±2.06	9.14±2.04	25.18±2.86	24.69±2.84	55.22±2.76	53.62±2.68
100	82.20±2.34	93.33±2.49	9.06±2.00	10.44±2.18	25.31±2.74	28.58±3.01	63.79±3.19	57.33±2.87
125	84.47±2.37	93.33±2.49	8.83±1.97	9.49±2.04	24.43±2.70	25.98±2.81	56.06±2.80	47.19±2.36
150	84.47±2.37	86.67±2.40	7.70±1.77	10.17±2.18	22.08±2.38	27.28±3.04	48.10±2.41	57.25±2.86
175	68.87±2.14	88.87±2.43	7.62±1.85	9.82±2.12	20.93±2.56	26.54±2.94	58.49±2.92	52.94±2.65
200	86.67±2.40	73.33±2.21	10.55±2.21	8.17±1.93	28.56±3.07	22.17±2.67	67.24±3.36	55.00±2.75
225	75.53±2.24	84.47±2.37	9.03±2.04	8.72±1.96	24.61±2.82	24.18±2.70	68.00±3.40	53.52±2.68
250	91.13±2.46	84.47±2.37	9.53±2.04	8.95±2.00	26.83±2.78	24.49±2.76	57.53±2.88	52.05±2.60
275	84.47±2.37	80.00±2.31	9.53±2.07	8.99±2.05	26.23±2.86	23.93±2.86	62.30±3.11	50.00±2.50
300	77.80±2.28	93.33±2.49	8.74±1.99	12.00±2.39	23.84±2.75	32.04±3.34	58.33±2.92	62.24±3.11
325	84.47±2.37	80.00±2.31	9.50±2.08	10.28±2.21	25.95±2.87	27.45±3.09	59.38±2.97	73.47±3.67
350	68.87±2.14	86.67±2.40	8.78±2.03	10.33±2.19	23.62±2.83	28.04±3.04	75.61±3.78	66.10±3.31
375	86.67±2.40	80.00±2.31	9.80±2.11	9.50±2.12	26.93±2.91	25.34±2.95	61.90±3.10	50.98±2.55
400	93.33±2.49	88.87±2.43	9.20±1.98	9.17±1.99	26.03±2.69	25.73±2.72	53.16±2.66	56.34±2.82
425	75.53±2.24	82.20±2.34	8.77±2.01	10.22±2.19	23.73±2.79	27.48±3.06	59.65±2.98	69.81±3.49
450	82.20±2.34	91.13±2.46	8.47±1.93	10.58±2.20	23.26±2.66	28.77±3.06	49.33±2.47	62.12±3.11

Conclusions

The gamma radiation effects observed at the individual plant level include: partial or complete inhibition of growth, physiological disorders, various pathological phenomena, sterility, and even lethality [24], [25]. Radiation treatment is fast, convenient, and extensive compared to other physical correction methods, as ionizing radiation penetrate quickly through polysaccharide granules [24, 26, 27]. The advantages of irradiation compared to other methods are less damage to food, especially proteins, no formation of indigestible products, removal of microbial and fungal



contamination from feed, and no residual effects after irradiation [28]. Gamma radiation causes physiological changes in crops, but its potential use in agriculture is limited mainly due to the lack of information about the optimal dose of radiation that varies from one crop to another. Morphological changes caused by radiation in a plant are controlled by the intensity and duration of gamma radiation. When ionizing radiations are absorbed in biological materials, they directly change vital cells or indirectly change important cell components, like germination percentage, GT, germination speed S, Speed of Accumulated germination, AS, and Coefficient of rate of germination CRG [29].

Irradiation of seeds is one of the methods of creating mutations to produce plants with desirable characteristics. In addition to causing useful mutations, irradiation of seeds can also cause destructive effects. since the sensitivity of seeds and cultivars to radiation is different, it is important to determine the optimal amount of irradiation. In this research, an attempt was made to investigate the effect of gamma rays on the rapeseeds of the Architect cultivar and alfalfa of the Bami and Ranjbar cultivars.

In this paper, the seeds of rapeseeds and alfalfa were exposed to a caesium-137 radiation source to determine their germination response to different doses of radiation. Among the rapeseeds, the group with the dose of 50 Gy had the highest total germination and germination speed compared to other doses The alfalfa seeds of the Bami cultivar exposed to 400 Gy had the highest total germination and the group with the dose of 200 Gy had the highest germination speed. However, the total germination of the Ranjbar cultivar of alfalfa seeds showed fluctuating and unpredictable behavior against gamma rays, and the group with the 300 Gy dose had the highest speed of germination. The results showed that plant’s germination responded differently to radiation. Therefore, further experiments in the greenhouse/field are recommended to evaluate the response of plants (radiated seeds) to different stresses during its growing cycle and also the amount of their production.

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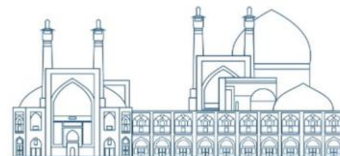

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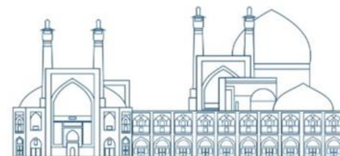

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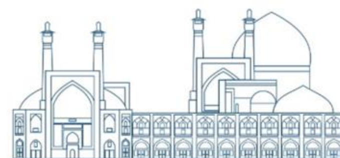

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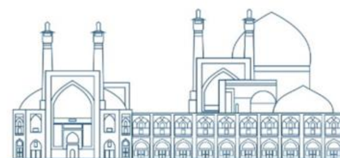

Investigating the effect of decontamination with the optimal dose of gamma rays on the chemical and physical properties of strawberry fruit (Paper ID: 1054)

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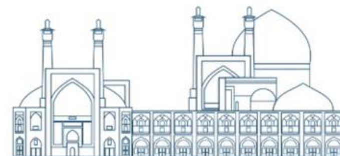
Abstract

Strawberries are among the foods that are susceptible to microbial infection after harvesting because of their high moisture and soluble sugar content. The occurrence of these contaminants causes fruit waste after harvesting, and its quality declines during the storage period. Given that strawberries are consumed fresh, it is crucial to limit contamination elements by radiation processing, which is one of the most effective ways to preserve food items' quality and extend their storage capacity without leaving harmful residues. This study investigated how gamma radiation can extend strawberry storage times without compromising the fruit's quality (chemical and physical). Strawberry fruit was exposed to an optimal dose of 1250 Gy of radiation. Four periods of 0, 3, 6, and 9 days after treatment were used to compare the physical and chemical indices of the fruit: total phenol, anthocyanin, antioxidant enzymes, solids solution, sugar, and color index L, a, and b. The outcomes demonstrated that strawberry fruit's shelf life was extended by gamma radiation irradiation at an optimal dose in comparison to the control group. This was achieved by reducing the growth of bacterial and fungal spoilage microorganisms and enhancing the fruit's skin's physical markers. Additionally, it was noted that the fruit treated with the optimal dose of gamma radiation exhibited higher levels of anthocyanin, total soluble sugar, soluble solids, and acidity in comparison to the control.

Keywords: strawberry, physical effects of irradiation, chemical effects of irradiation, gamma irradiation.

Introduction



Fragaria ananassa, the scientific name for strawberries, are tiny, delectable fruits that are high in phenolic compounds, such as anthocyanins, which give them their vibrant red color. The parenchymal cells of strawberries have thin, occasionally thick walls, and they have a luscious texture with a thin skin. Its fruit is less transportable and storable than other fruits because of its thin peel and high moisture content in the fruit tissue. [1] Fungi that quickly damage a significant portion of strawberries, such as gray mold or Botrytis cinera, are another issue while storing strawberries. [2] Strawberries may be kept for up to two weeks at a temperature of around 2 degrees Celsius and 90% relative humidity. [1] Food may be decontaminated and stored for longer periods using the safe, efficient, and very energy-efficient method of radiation treatment.[3] Cobalt 60 is one of the sources of gamma rays that is used to reduce the microbial load of food. It has a very high penetrating power, which can be used to reduce the microbial load after food packaging.

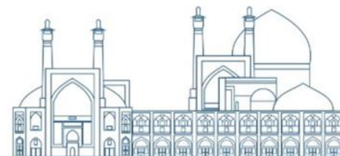
Experimental

Sample Preparation

Strawberry fruits (Fragaria × ananassa Duch. cv. Camarosa) were harvested from a commercial greenhouse in Qazvin province, Iran. The strawberries were sorted and packed in 250 g amounts in plastic containers with lids, then stored appropriately in dry and dark conditions at 4 ± 1 °C until analysis. All chemicals and solutions were purchased mainly from Merck (Darmstadt, Germany) and Sigma-Aldrich (St. Louis, MO, US).
and Sigma-Aldrich (St. Louis, MO, US).

Gamma irradiation

Strawberry fruits packs were exposed to different doses (750, 1000, 1250, 1500, and 2000 Gy) of gamma ray in a Co^{60} gamma resource in triplicate using a Gamma cell 220 irradiator (MDS Nordion, Ottawa, Canada) located at the Radiation Applications Research School, Nuclear Science and Technology Research Institute, AEOI, Tehran, Iran, at dose rate of gamma irradiation was 5.4 kGy/h. Dosimetry was performed with Fickerefrence standard dosimetry system. The temperature and relative humidity during the irradiation process were 30 ± 1 °C and 45% to 55%, respectively. A Red-Perspex dosimeter (Hrwell Dosimeters, UK) was used to assess the absorbed dose.



Evaluation of total fungi count

The viable fungi population count was done by preparing a suspension of 50 gr of each irradiated strawberry sample in 9 ml sterile saline solution (8.5 g NaCl in 1000 ml distilled water) and serially diluted. A certain amount (100 µl) of each dilution was cultured in three plates containing PDA medium (Merck, Germany) (including 50 ppm chloramphenicol) by surface plating method in triplicates. Fungal colonies were counted after incubation at 28 °C for 3–5 days. The results were expressed as log colony-forming units per gram (log CFU/g) [1].

Physicochemical changes of irradiated strawberry fruits

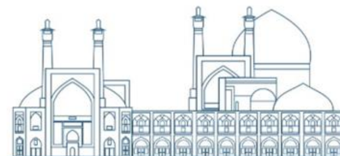
The pH was measured in a digital bench pH meter (SANA SL-901), according to the methodology indicated by AOAC (2005). The determination of the total titratable acidity was done by titrimetry, according to the modified AOAC Methodology (1995), using a solution of 0.1 N NaOH . The samples were to a beaker of 10 ml distilled water. The mixture was filtered and transferred to an Erlenmeyer for titration. The results were expressed in milligrams of citric acid 100 mg^{-1} of strawberry [4].

Firmness of the fruit (Brix)

The firmness of the strawberry fruit was measured with a Force Gauge Model FG-5020 Lutron equipped with a load cell of 20 kg by performing a penetration test to a depth of 6 mm with a cylindrical probe of 4 mm and a probe speed of 2.0 mm/s on the skin of the fruit. [5].

Instrumental color

The color parameters of strawberry samples were directly determined utilizing a Minolta Colorimeter CR-400 (Konica Minolta, Inc., Osaka, Japan) with a D65 illuminant and an observation angle of 10 at 25 °C. The color values of L*, a*, and b* were measured based on the International Commission on Illumination (CIE). L* value represents lightness-darkness, and a* and b* describe redness-greenness and yellowness-blueness of color, respectively. The L*, a*, and b* color value measurements were carried out in five replicates. Chroma was also analyzed. [6]



Total soluble protein

Total soluble protein determination was carried out using the Bradford method. Strawberry extract was prepared by mixing about 50 grams of fruit tissue, then the mixture was centrifuged (5000×g for 5 min), and the supernatant was collected and stored at -20 °C for further use. The collected supernatant was diluted and used for a reaction with Bradford reagent. The absorbance was measured at 595 nm, and bovine serum albumin (BSA) was used as the standard. The results were reported as mg of protein per g of fruit (mg/g) [7].

Total reduced sugar

Total reduced sugar was determined using the DNS method in strawberry extract. The absorbance was measured at 595 nm, and bovine serum albumin (BSA) was used as the standard. The results were reported as mg of protein per g of fruit (mg/g) [8].

Total phenol content (TPC) determination

Phenolic compounds in strawberry fruit samples were extracted by the method described by Jiang and others [9].A sample of 0.5 g of freeze-dried strawberry powder was mixed with 15 mL of an 80% methyl alcohol solution, and then the mixture was sonicated at 45°C for 30 min in an ultrasonic bath. After sonication, a-methanolic extract supernatant was obtained by centrifugation at 7500 g for 5 min. This extract was used to quantify TPC and evaluate its antioxidant capacity. The TPC was [10] according to the Folin-Ciocalteu colorimetric method. Folin-Ciocalteu (100 µL)reagent was mixed with 1160 µL 144 distilled water and 20 µL extract solution, followed by the addition of 300 µL of 20% (w/v) aqueous sodium carbonate solution after 10 min. The mixture was incubated at 40 °C for 1 h in darkness, and then the absorbance was measured at 765 nm. Gallic acid was used as a standard compound. TPC was calculated on a dry weight basis for strawberries as mg gallic acid equivalent (GAE) per gram of strawberry sample.

Statistical analysis

The statistical analysis was performed using SPSS 13.0 software (SPSS Company, Chicago, IL, United States) based on a one-way analysis of variance (ANOVA). Differences were significant at $p < 0.05$ using Duncan’s test. All experiments were conducted in a completely randomized

design, and the data were expressed as the means ± standard deviations of three replicate experiments (SD).

Results and discussion

Fungi decontamination by gamma radiation

The initial fungi contamination levels of the strawberry samples were determined, and the sterilization effect of gamma radiation at 0–2 kGy was evaluated. Gamma irradiation was able to reduce the number of viable colonies of yeasts and fungi in strawberries compared to untreated ones. The dose of 1250 Gy of gamma radiation reduced the amount of strawberry mushrooms to zero, so this dose was considered the optimal dose of gamma radiation. The results are in coherence with Jesus Filho et al. (2018) [2], who reported a significant interaction of gamma irradiation doses on strawberries (1–4 kGy).

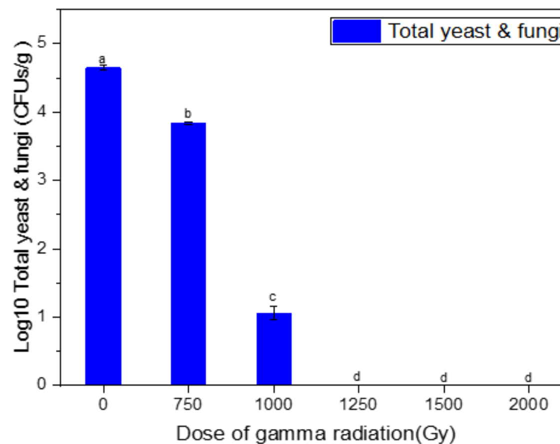
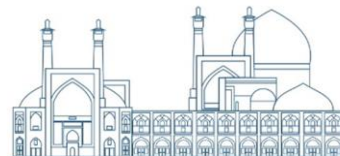


Fig. 1. Decontamination of total yeast and fungi by different doses of gamma radiation on strawberries.

Effect of gamma radiation on instrumental color

Color is the first criterion used in the acceptance or rejection of the product by the consumer. [12] The L*, a*, and b* color parameters of irradiated and non-irradiated strawberry and chroma values are presented in Figure 2 (a). The L color index of strawberries exposed to radiation at a suitable dose is compared to the control sample as a function of storage duration. Based on the results



obtained in Figure 2(b), the L index of the control and irradiated samples changed significantly over time ($p<0.05$), and the irradiated treatment increased. The results shown for index a in Figure 2 (b) show that the changes are significant ($p<0.05$). With the increase in the holding time, the redness of the irradiated treatment increases.The color (b index) of irradiated strawberries varies with storage time compared to the control sample.The results showed that the b index in Figure 2 (a) was significant for the control and irradiated treatments ($p < 0.05$). Increasing the storage period increased the yellowness indices of the control and irradiated samples. The results demonstrate in Figure 2 (b) that, whereas the chroma index of the control samples does not vary significantly over time, the chroma index of the irradiation samples does change significantly. Furthermore, no significant difference ($p < 0.05$) was observed between the control and irradiated treatments during storage. Increasing the storage period increased the color chroma index of the control and radiation treatments.Throughout the process of fruit ripening, strawberries continue to change color. This process continues during the storage period, and the color of strawberry fruit darkens over time [11]. The obtained results show the changes in the color of strawberries, which include an increase in redness and a decrease in brightness after irradiation compared to the control treatment. Meanwhile, Ki-Nam Yoon (2023) [12] stated that irradiation caused a reduction of redness in tomatoes. Yeong-Seok Yoon (2020) [13] said that electron beam radiation prevents strawberry color changes.

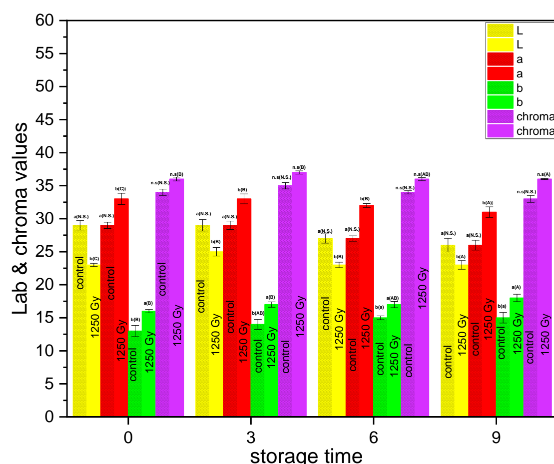
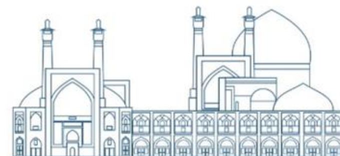


Fig. 2. The effect of the optimal dose of gamma radiation on instrumental L color parameters , a color parameters , b color parameters & choroma color parameters of strawberries.



Effect of gamma radiation on anthocyanin content and reduced sugar concentration

The effect of gamma radiation on the anthocyanin content of freeze-dried strawberry powder is shown in Figure 3 at different storage times. The anthocyanin content of the irradiation sample and the control strawberry differed significantly ($p < 0.05$) for all storage periods. In the control sample, there was also a slight increase in anthocyanin content with time. Samples of strawberries exposed to gamma radiation showed significant changes in anthocyanin concentration over time, along with a slight rise with extended storage periods. Anthocyanin is responsible for the red color of strawberries, and the loss of anthocyanin pigments causes color changes in strawberries [14]. Yeong-Seok Yoon (2020) [13] stated that during the storage period, after electron beam irradiation, the anthocyanins of strawberries increased. Increasing the anthocyanin content of strawberries also makes the product look redder [15].

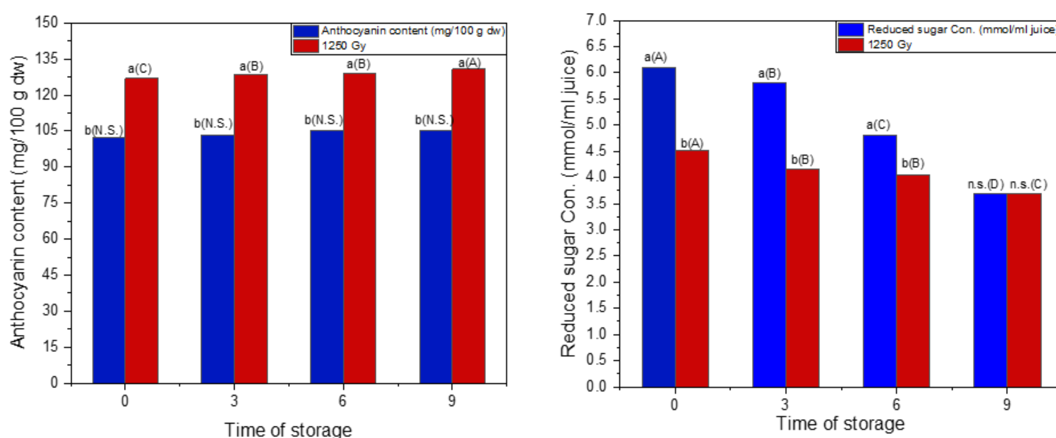
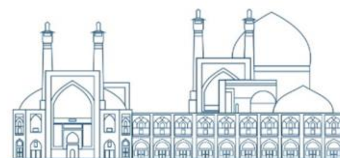


Fig. 3. The effect of optimal doses of gamma radiation on the amount of anthocyanin and the sugar concentration in strawberries.

The reduced sugar concentration of strawberries treated with gamma radiation is shown in Figure 3. As the storage time increased, the amount of reducing sugar in the irradiated sample and the control sample decreased significantly. The results obtained are in line with the theory that fruit sugar is reduced by gamma irradiation [12]. The primary cause is the rise in product respiration following radiation exposure. [13]. However, the data of the storage period demonstrated that there was no difference between the Gamma irradiated and the sugar content of the control group.



Effect of gamma radiation on soluble protein concentration and total phenol content (TPC)

Figure 4 shows the protein changes during storage for the gamma-irradiation treatment and the control. It compares the difference between the optimal dose of radiation and the control in terms of the soluble protein concentration of strawberry fruit as a function of storage time. In all the storage times, the soluble protein concentration was significant ($p < 0.05$) between the gamma irradiation treatment and the control. Longer storage time led to a significant increase in the soluble protein content of strawberry fruit. Fruits that were stored longer had the highest levels of soluble protein.

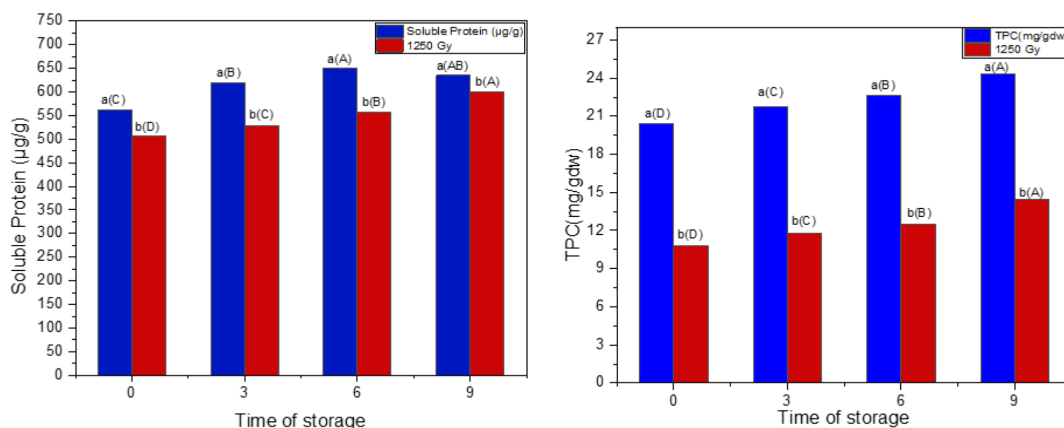
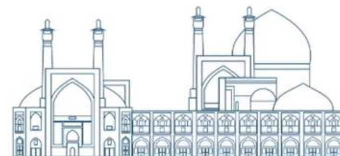


Fig. 4. The effect of the optimal dose of gamma radiation on soluble protein concentration and the total phenolic content of freeze-dried strawberries.

Changes in total phenol for gamma irradiation treatment and control during storage times are shown in Figure 4. Longer storage periods led to a significant increase in total phenol content; the changes between the irradiated treatment and the control were significant ($p < 0.05$). Throughout the storage period, strawberry fruit's phenolic component content can be altered or maintained [18]. The variations in these chemicals during the course of storage are determined by the breathing rate [19]. The concentration of total phenol rises as strawberries are stored [20]. Total phenol is reduced when there is a low oxygen content [21].



Effect of gamma radiation on firmness of strawberry texture and Brix

The changes in dissolved solids (Brix) during the storage period for the irradiated treatment and the control are shown in Figure 5. The amount of change in dissolved solids for the control and irradiated treatments was significant, and we did not see any significant changes. The results obtained are consistent with Wani's (2018) research [16]. Following gamma irradiation, the amount of soluble solids in cherries dropped. This is due to the increase in respiration [12]. However, the data on the storage time demonstrated that there was no difference between the Gamma radiation dose and the soluble solids of the control treatment. The hydrolysis of proteins, the breakdown of glycosaccharides into smaller constituent units during respiration, and the breakdown of carbohydrates and pectin substances are likely the causes of the observed changes in pH and total organic acid, as well as the decrease in the content of soluble solids in strawberry fruits [17].

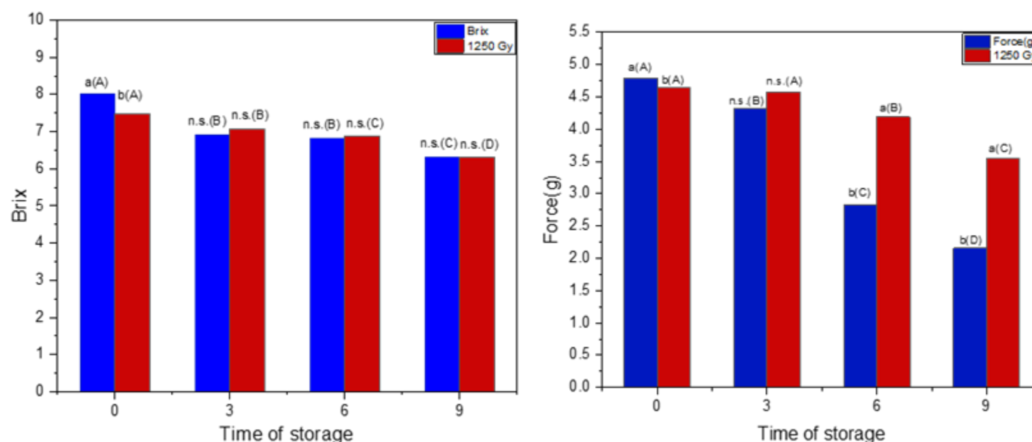
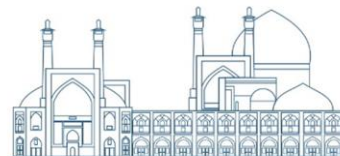


Fig. 5. The effect of the optimal dose of gamma radiation on the soluble solids (Brix) and strawberry tissue strength on strawberry tissue strength strawberries.

Changes in strawberry texture during storage for the irradiated and control treatments are shown in Figure 5. The firmness of strawberry tissue was significantly reduced in the irradiated and control treatments. During the storage period, the irradiated treatment maintains its tissue stiffness



more than the control.The dissolution and breakdown of pectin and other cell wall components by the action of enzymes like polygalactronase and pectin methylesterase is the primary cause of the loss in firmness of strawberry fruit tissue [22]. The findings are comparable to those of a study conducted in 2023 by KN Yoon, which discovered that gourds' resilience was reduced when gamma radiation exposure was increased [12]. Fruit tissue becomes softer due to structural alterations brought about by the action of enzymes that hydrolyze the cell wall, such as the decrease of hemicellulose and galactose as well as the dissolution and depolymerization of pectin [23].

Conclusions

Gamma rays reduce strawberry rot by destroying the microbial load, fungi and mold of strawberries thus increased the storage time. The dose that reduced the strawberry fungi to zero was the dose of 1250 Gy of gamma irradiation, which was evaluated as the optimal dose during storage.anthocyanin, soluble solids, and sugar in the control treatment and the optimal dose of gamma radiation exhibit the same values at the conclusion of the storage time.The amount of protein decreased during the storage period in the control treatment, but increased in the gamma radiation treatment. The amount of total phenol for the gamma irradiation treatment and the control showed an increasing trend during the storage time. The increase in the storage time caused an increase in the tissue resistance of the gamma radiation treatment compared to the control treatment.

Acknowledgments

The authors are grateful to express their gratitude to the Radiation Application Development Co.; Atomic Energy Organization of Iran (AEOI) (Project No. IRAD-PRJ-TR-GI-0104-00) for supporting this research

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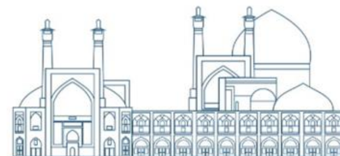

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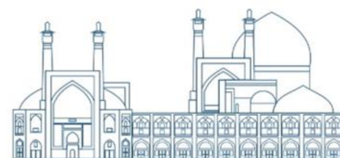

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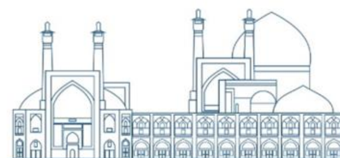

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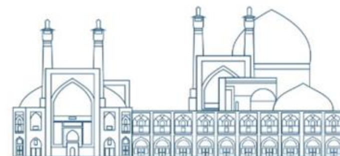

Sterility of the beet armyworm, Spodoptera exigua Hübner (Lepidoptera: Noctuidae), using Co60 gamma radiation (Paper ID: 1202)

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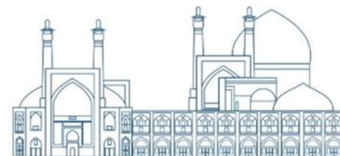
Abstract

The beet armyworm *Spodoptera exigua* Hübner, is a most important pest of sugar beet which damages a variety of fields and vegetable crops all over the world. In the current study, the effect of Co^{60} gamma ray at the sterility of *S. exigua* were investigated in the laboratory condition. For this purpose, 6-day-old male and female pupae were irradiated with doses of 60, 120, 180, 250, and 400 Gy. The emerged adults were mated in three combinations; normal females mated with treated males, treated females mated with normal males and treated females mated with treated males. Their fecundity, fertility and sterility, also, percent of emergence and longevity were analyzed. Results showed irradiation of pupae with different doses of gamma rays significantly reduced the number of eggs per female and their hatchability and increased their sterility rates. This effects on reproduction parameters were positively correlated with the dose level and observed in all three combinations. The fully sterile female and male were observed at 250 and 400 Gy treatments, respectively. Radiosensitivity of females was evident as compared with males. No significant differences were found between 250 and 400 Gy treatments in all combinations and reproductive parameters. The male's adult emergence and longevity were not affected by radiation exposure. However, the female's adult emergence after irradiation at 400 Gy was 67.27% which was apparently different from the control rate of 85%, indicating a significant decrease. Also, females' longevity was prolonged significantly up to 20% of control. According to the results, the employing sterility of moths by using of 250 Gy Co^{60} gamma ray to pupae at laboratory condition were recommended for management of *S. exigua* by sterile insect technique.

Key words: Gamma irradiation; reproductive biology; *Spodoptera exigua;* Sterile insect technique.



Introduction

The beet armyworm, *Spodoptera exigua* Hübner (Lepidoptera: Noctuidae), is a serious pest of sugerbeet which feeds on a variety of vegetables and field crops. Resistance of this insect to many conventional insecticides has been recorded [1]. Considering the economic impact of this pest across a range of crops, and the emergence of resistance to most of the pesticides and the environmental consequences of them, there is a pressing need to implement a safe approach for controling this pest to promote sustainable management. Sterile insect technique (SIT) is a biological pest control method in which sterile insects are injected in large quantities over a wide area to reduce the fertility of field population of the same species. SIT involves rearing a large number of target pest species, exposing them to gamma or X-ray radiation to induce sexual sterility, and then releasing them on a large scale into the target pest population. The released sterile males mate with wild females to prevent them from reproducing [2].

SIT has been studied and performed on a various species of lepidoptera, currently. There are many studies on the *Spodoptera* spp. in the past to recent literature: The effects of sterilizing and substerilizing doses of gamma rays were investigated on the Egyptian cotton leaf worm, *S. littoralis* Boisd. [3-11]; the cotton leafworm *S. litura* Fabr. [12-20]; the fall armyworm *S. frugiperda* J. E. Smith [21-23]. Nevertheless, there were only some ancient published papers on effects of radiation on four different developmental stages of *S. exigua* [24-26]. The objective of this research was finding appropriate male and female sterilizing doses for *S. exigua.*

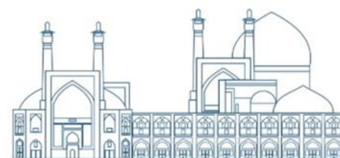
Research Theories

Considering that according to the Bourtzis and Vreysen [27], availability of basic data related to irradiated insects, ideally in a two-year period before intervention, is one of the prerequisites of SIT programs, in this research radiobiological investigations on the developmental and reproductive biology of *S. exigua* were conducted to control it based on SIT in the future.

Experimental

Insect rearing

Spodoptera exigua larvae were collected from a sugar beet field in Karaj, Iran, transported to the laboratory along with host plant leaves, and kept individually in airtight 50 mL plastic cups on



beet leaves until pupation. Adult moths were released into egg-laying boxes. These oviposition boxes were transparent plastic containers (8.5 cm diameter, 6.5 cm height) with air-permeable caps and wrapped on the inside with a white polypropylene non-woven filter cloth to facilitate egg laying and contained cotton balls soaked with 10% sucrose solution in the lid of a bottle for the adult feeding. Larvae were reared on an artificial diet as described previously by Ashouri et al. [28] for three generations at 27 ± 1 ºC, 30 ± 5 % relative humidity and photoperiod 16:8 (L:D) h.

Irradiation

Six-day-old pupae were harvested from the rearing containers. Male and female pupae were separated according to Bandoly and Steppuhn [29]. Thirty female and 35 male pupae were transferred to different Petri dishes (100 mm × 15 mm) and treated with gamma radiation of 60, 120, 180, 250 and 400 Gy. These doses were selected according to the results of preliminary studies, included a wide dose range (30, 60, 90, 120, 150, 180, 200, 250, 300, 350, and 400 Gy). The irradiation was performed at the Nuclear Agriculture School, Nuclear Science and Technology Research Institute, Karaj, Iran. Gamma rays derived from a Co^{60} 265 curie (Gamma cell Issledovatle PX30) was used to irradiate the pupae at ambient air temperature with the Fricke dosimeter. This process was conducted in four replications. Irradiated male and female pupae were transferred individually into separate transparent plastic containers with moistened cotton balls with 10% sucrose solution until adult emergence.

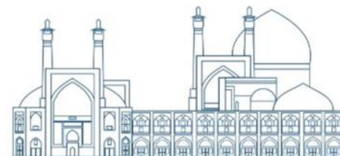
Gamma irradiation effect on the adult's emergence of parental generation

The percentage of emerged male and female moths was calculated after two days of radiation untile control adults completly emerged. Unenclosed pupae and emerged adults with unflyable wings were considered as pupal mortality.

Gamma irradiation effect on reproductive biology of parental generation

The effects of different doses of gamma rays were evaluated on the fecundity and fertility of emerged adults. For these trials, the enclosed adults were paired (5 males: 5 females and four replications for every dose) in oviposition boxes in the following four combinations:

Unirradiated Female (UF) × Unirradiated Male (UM)



Unirradiated Female (UF) × Irradiated Male (IM)

Irradiated Female (IF) × Unirradiated Male (UM)

Irradiated Female (IF) × Irradiated Male (IM)

The non-woven filter cloths were collected daily and the egg batches were brushed into 50 mL airtight plastic cups labeled with the replication number and date. The number of eggs per female and the number of hatched eggs were recorded daily for all replications. This process was continued until the last female died. Additionally, the percentage of sterility index was calculated according to Toppazada et al. [30]:

$$\% \text{ Sterility } = 100 - \left(\frac{a \times b}{A \times B} \times 100\right)$$

Where:
a: number of eggs per female in treatment,
b: % hatching eggs in treatment,
A: number of eggs per female in control,
and B: % hatching eggs in control.

Gamma irradiation effect on adults' longevity of parental generation

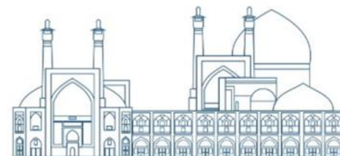
The longevity of male and female adults was recorded daily until the last moth died. These two sexes were distinguished by the shape of the last segment of abdomen, male's narrow abdomen versus female's bloated abdomen.

Data analysis

The effects of radiation on biological and reproductive parameters were assessed by a one-way ANOVA. A completely randomized design with four replications was used. The mean values were compared by Duncan's multiple range test. The data were analyzed via SPSS version 22.0. (IBM ©) software. Microsoft Excel was used for creating graphs.

Results and discussion

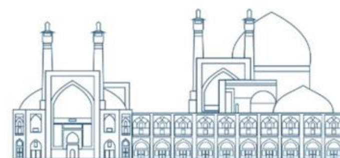
Irradiation of 6-day-old male and female pupae with different doses of gamma rays markedly decreased adults' fecundity and fertility, and increased sterility rates (Table 1). This significant reduction in the number of eggs per female was observed in all three combinations; IM×UF



($F_{(5,23)}$=14.189, *P<0.01*), UM×IF ($F_{(5,23)}$=152.181, *P<0.01*), and IM×IF ($F_{(5,23)}$=141.226, *P<0.01*). Additionally, the percentage of hatching eggs was diminished in all three combinations; IM×UF ($F_{(5,23)}$=89.289, *P<0.01*), UM×IF ($F_{(5,23)}$=95.602, *P<0.01*) , and IM×IF ($F_{(5,23)}$=161.362, *P<0.01*). The decrease in the egg number and hatchability induced by gamma radiation were seen to be positively correlated with the dose level. These two parameters were used to calculate sterility which was increased significantly by increasing the gamma dose in all three combinations; IM×UF ($F_{(4,19)}$=67.447, *P<0.01*), UM×IF ($F_{(4,19)}$=44.52, *P<0.01*), and IM×IF ($F_{(4,19)}$=17.239, *P<0.01*). Differences in radiosensitivity between males and females was evident. The fully sterile female and male were observed at 250 and 400 Gy treatments, respectively. However, no significant differences were found between 250 and 400 Gy treatments in all combinations and reproductive parameters.

Hosny et al. [4] claimed that the fecundity of *S. littoralis* adult was decreased with increasing gamma radiation doses applied to pupae of different ages. Hassan et al. [11] found that 300 Gy could be the sterile dose for *S. littoralis*. Mochida and Miyahara [12] said complete sterility of *S. litura* was induced in mating of unirradiated females with irradiated males at 80 or more gamma ray doses. Ramesh et al. [14] indicated when male pupa of *S. litura* treated by 70 Gy gamma ray and paired with unirradiated female resulted 10.1% reduction in oviposition and 37.24% decline in fertility. Sengupta et al. [18] noted reduction in fecundity and fertility of *S.litura* with increasing radiation dose (100–200 Gy) compared to control and identified 130 Gy for female and 200 Gy for males as a suitable sterilization dose. Wendell Snow et al. [22] claimed that the radiation dose required to sterilize adult male *S. frugiperda* was established at 350 Gy, while females could be sterilized with a dose of 150 Gy.

The emergence of adults (Fig. 1) and their longevity (Fig. 2) resulting from irradiated fully grown pupae were recorded. The pupal irradiation did not affect the male adult emergence, significantly ($F_{(5,23)}$=2.558, *P=0.064*). However, the female adult emergence after irradiation at 400 Gy was 67.27% which was apparently different from the control rate of 85%, indicating a significant decrease ($F_{(5,23)}$=3.348, *P=0.026*). The males longevity was unaffected by radiation exposure ($F_{(5,23)}$=0.118, *P=0.987*), whereas females longevity was prolonged by increasing radiation dose, significantly ($F_{(5,23)}$=6.213, *P<0.01*) (Fig. 2).



Ramesh et al. [14]found that after irradiating pupae with 70 Gy, *S. litura* matured by 75.5% compared to 83.3% in control. Hosny et al. [4] and Hassan et al. [11] stated that the sub-sterilizing doses had no effect on the adult emergence of *S. littoralis*, but the reduction in the appearance of adults was significant at the sterilizing dose. However, in current research, only female emergence at 400 Gy treatment was significantly reduced compared to the control.

Mochida and Miyahara [12] argued that the longevity of *S. litura* adult males irradiated in the pupal stage gradually decreased with increasing radiation dose. However, Hosny et al. [4] stated that *S. littoralis* adults' longevity was not affected by the sterilizing doses. In the current research, only longevity of female adults was extended when radiation doses increased. There may be a relation between reduced fertility and longer life expectancy in females exposed to gamma irradiation. In other words, irradiated females lay fewer fertile eggs and therefore live longer than control.

Tables

Table 1. Fecundity, fertility and sterility (Mean ± SE) of parental generation of *Spodoptera exigua* adults that emerged from pupae irradiated at different gamma ray doses (Gy).

Combination	Dose (Gy)	Eggs/Female ± SE	Egg hatch (%) ± SE	Sterility (%) ±SE
IM×UF	0	517.05±18.54 a	75.44±5.00 a	
	60	505.30±18.75 a	45.08±4.09 b	41.70±5.46 a
	120	355.65±25.53 b	28.25±3.13 c	74.39±2.80 b
	180	344.80±27.15 b	13.24±1.56 d	88.36±1.66 c
	250	344.10±23.79 b	3.12± 1.37 e	97.26±1.20 d
	400	349.55±18.57 b	0.00±0.00 e	100±0.00 d
UM×IF	0	517.05±18.54 a	75.44±5.00 a	
	60	312.35±9.59 b	39.13±4.97 b	68.52±4.44 a
	120	239.30±13.77 c	19.05±1.78 c	88.49±0.39 b
	180	133.70±12.77 d	3.70±1.74 d	98.59±0.77 c
	250	100.75±16.85 de	0.00±0.00 d	100±0.00 c
	400	57.75±8.33 e	0.00±0.00 d	100±0.00 c
IM×IF	0	517.05±18.54 a	75.44±5.00 a	
	60	286.80±10.59 b	12.86±2.62 b	90.51±2.06 a
	120	223.85±15.11 c	4.09±1.06 c	97.56±0.77 b
	180	162.25±10.35 d	0.29±0.16 c	99.88±0.07 b
	250	110.10±15.58 e	0.00±0.00 c	100±0.00 b
	400	95.35±2.86 e	0.00±0.00 c	100±0.00 b

U: unirradiated, I: irradiated, M: male, and F: female.
Means followed by the different letters in each separate column are significantly different at $P<0.01$.

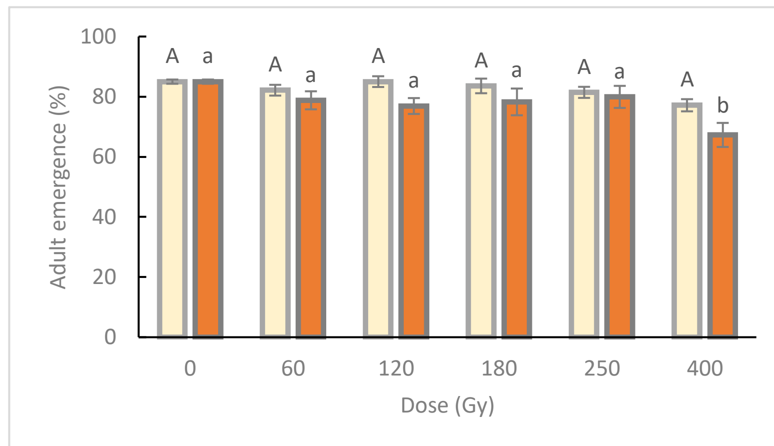


Fig. 1. Male and female adult emergence of *Spodoptera exigua* resulting from irradiated pupae with different gamma radiation doses (Gy). Means followed by the same uppercase or lowercase letters in each separate column are not significantly different at $P<0.05$ and means followed by the different lowercase letters are significantly different at $P<0.01$.

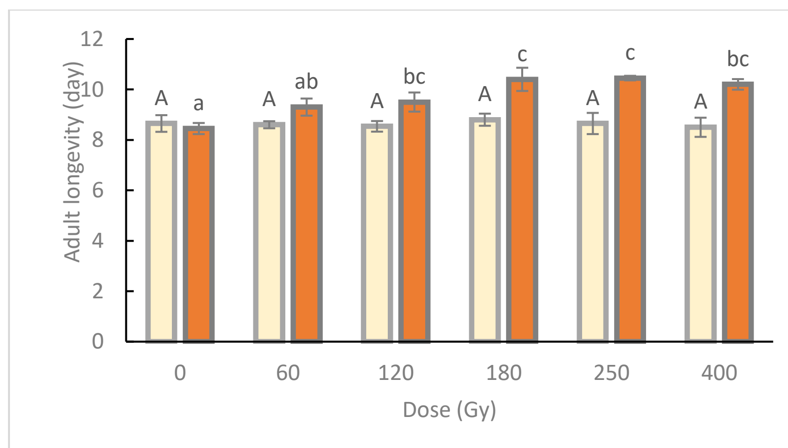
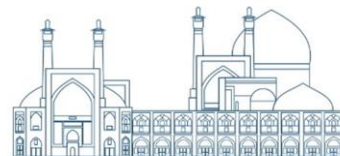


Fig. 2. Male and female adult longevity (day) of *Spodoptera exigua* resulting from irradiated pupae with different gamma radiation doses (Gy). Means followed by the same uppercase or lowercase letters in each separate column are not significantly different at $p < 0.05$ and means followed by the different lowercase letters are significantly different at $P<0.01$.

Conclusions

Gamma radiation affected the reproductive performance of *S. exigua*. It was concluded that a dose of 250 Gy can induce the full sterility of adults. Furthermore, radiation of pupae at 250 Gy had



favorable impacts on adult emergence and longevity. According to these results, the employing sterility of moths by the administration of 250 Gy Co^{60} gamma ray to parent pupae were recommended for management of *S. exigua* by sterile insect technique.

Acknowledgements

The author gratefully acknowledges a grant from the Atomic Energy Organization of Iran (AEOI).

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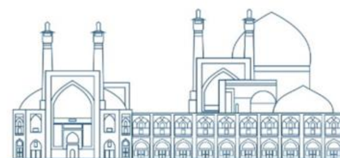

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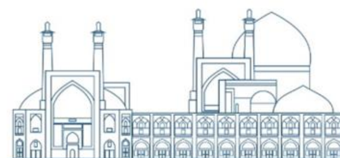

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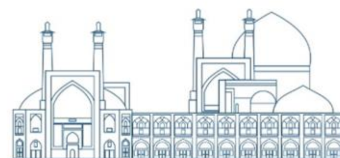

Priming with a DNA Vaccine and Boosting with an Electron Irradiated Vaccine to Induce Neutralizing Antibody Response against FMD Virus Type O (Paper ID: 1279)

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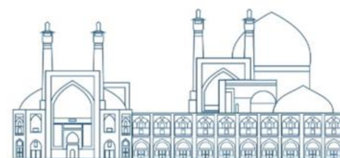
Abstract

More than 100 countries suffer from the economic damage of *Foot and Mouth Disease Virus* (FMDV); therefore, it has a huge economic impact worldwide. *FMDV* belongs to the *Picornaviridae* family and the *aphthovirus* genus and is transmitted to susceptible animals by direct contact or infectious droplets. In this research a DNA vaccine based on VP1 gene was prepared by amplifying the pcDNA3.1 plasmid included VP1 gene. The pcDNA3.1+VP1 cassette was prepared and confirmed as a DNA vaccine. The virus was inactivated by electron beams at 55 kGy as EIV. In addition, the plasmid encoding the granulocyte-macrophage colony-stimulating factor gene (GM-CSF) was used as a molecular adjuvant. Seven groups of five mice each were selected, and the vaccines were administered as homologous and heterologous strategy prime-boost (PB) in three doses two weeks apart. Ten days after the last injection vaccine, blood samples were collected and sera samples were separated from blood samples. Antibodies to *FMDV type O* were then tested using a serum neutralization test (SNT). SNT assays for FMDV antibodies were measured as a humoral immunity assay according to the instructions in the OIE Terrestrial Manual. SNT is important in the development of protection against FMD, and several studies have been performed to demonstrate the association between vaccine-induced antibody titers and levels of protection against live virus challenges. The SNT is serotype-specific and is considered highly sensitive to antibodies against FMDV. Additionally, the SNT is currently the standard gold test for FMDV vaccine matching. B lymphocytes produce neutralizing antibodies in response to infections with the FMDV or vaccine injection. The highest SNT titer was observed in inactivated vaccine groups (Electron Irradiated Vaccine and Conventional Vaccine), thus this increase was significant ($P \leq 0.05$) compared to other vaccinated groups. Acceptable antibody titers were also observed in



prime-boost (PB) groups, but in the PB2 group, it was higher and more significant compared to PB1 group. In addition, the equality antibody titer was reported in the PB1 PB2, DNA vaccine plus GMCSF groups. In general, our results demonstrated that in all vaccinated groups, except for the DNA vaccine group, the antibody titer was higher than 1.2, indicating an acceptable protective response. Nevertheless, a better titer was reported in EIV, and CV groups, confirming an acceptable humoral response. Generally, the SNT titer among the vaccinated groups was as EIV, CV ≥ PB1 ≥ PB2 ≥ DNA vaccine plus GMCSF.

Keywords: Electron Irradiation, DNA, Vaccine, Foot and Mouth Disease Virus

Introduction:

The FMD virus (FMDV) is classified into the Picornaviridae family and genus Aphthovirus and is transmitted to susceptible animals through direct contact or infective droplet. FMDV has a non-enveloped, positive-sense, and single-stranded RNA. It is further surrounded by a protein integument that includes 60 copies of four various structural proteins (VP1-VP4). VP1-VP3 are surface exposed on the virus capsid, and the VP4 is in the inside capsid [1, 2]. The Capsid protein and the carboxyl-terminal of the VP1 region have a G-H loop, which is highly immunogenic [3, 4, 5]. The most common technique for inactivating viruses is chemical methods: Inactivation by formaldehyde and inactivation by aziridines such as Binery Ethylenimine (BEI), N-acetylethylenimine (AEI). The most common technique for inactivating viruses is chemical methods e.g. inactivation by formaldehyde and inactivation by aziridines such as Binery Ethylenimine (BEI), N-acetylethylenimine (AEI) [6, 7]. Ionizing irradiation is another method for inactivating a virus that has been considered for several years [8, 9]. Nearly 50 years ago, the collaborative IAEA/FAO plan first supported radiation methods for inactivating pathogens in preparation killed vaccines for using in livestock. The viral inactivated vaccines by ionizing radiation are secure and non-toxic, and the virus particles cannot escape from inactivation. The antigenic properties of the virus are preserved due to minor damage to its protein structure. Hence, the potency of the vaccine is higher. Using this irradiation method, we can potentially produce

vaccines that tackle constantly evolving, new strains of viruses faster and more effectively compared with the conventional methods [9, 10, and 11].

Materials and Methods:

A DNA vaccine based on VP1 gene was prepared by amplifying the pcDNA3.1 plasmid included VP1 gene. The pcDNA3.1+VP1 cassette was prepared and confirmed as a DNA vaccine. FMD virus type O/2007/IRN was propagated on BHK21 cell line. The suspensions of infected cells were centrifuged at 3000 rpm for 10 min, until the cell debris was deposited at the bottom of the centrifuge tube and separated from the viral suspension. Finally, the viral suspension was aliquoted into 5 mL vials and stored in a freezer at -70 °C for inactivation with electron beam, and BEI. Electron beams are generated from electron accelerators. The emitted electrons by the filaments are accelerated in the vacuum in a strong electric field, creating electron beams. The main difference between gamma-ray and electron is the speed of action and penetration power. Thus, the gamma-ray has a low velocity with high power penetration, and electrons have a high velocity and low penetration power in matter. However, its effect on the virus is similar to that of gamma-rays. An electron accelerator (IBA Company, model Rodotron TT200) with the energy of 10-Mev and a current of 2 mA was used in the present study [9, 12]. Eventually, the optimal dose of the electron beam for FMDV inactivation was calculated based on dose response curve and $TCID_{50}$ titration of un-irradiated and irradiated viral samples. The electron irradiated FMDV was used as an electron irradiated vaccine (EIV). In addition, the plasmid encoding the granulocyte-macrophage colony-stimulating factor gene (GM-CSF) was used as a molecular adjuvant. Seven groups of five mice each were selected, and the vaccines were administered as homologous and heterologous strategy prime-boost (PB) in three doses (100 µl/mouse, each dose of vaccine) two weeks apart. The mice groups are shown in Table1.

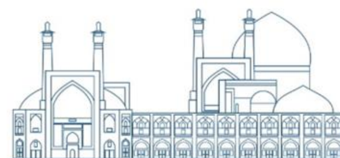


Table 1: The Vaccinated Mice Groups

No	Vaccine Group	Prime Vaccine 1 day	Boost1 Vaccine After 2 weeks	Boost2 Vaccine After four weeks
1	Conventional vaccine (CV)	CV	CV	CV
2	Electron Irradiated Vaccine (EIV)	EIV	EIV	EIV
3	Prime Boost1 (PB1)	DNA Vaccine + GMCSF	DNA Vaccine + GMCSF	CV
4	Prime Boost2 (PB2)	DNA Vaccine + GMCSF	DNA Vaccine + GMCSF	EIV
5	DNA Vaccine	DNA Vaccine	DNA Vaccine	DNA Vaccine
6	DNA Vaccine + GMCSF	DNA Vaccine + GMCSF	DNA Vaccine + GMCSF	DNA Vaccine + GMCSF
7	Negative Control (PBS)	Negative Control (PBS)	Negative Control (PBS)	Negative Control (PBS)

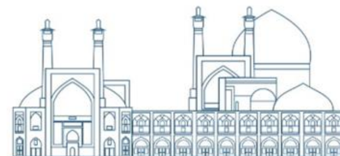
Ten days after the last injection vaccine, blood samples were collected and sera samples were separated from blood samples. Antibodies to *FMDV type O* were then tested using a serum neutralization test (SNT). SNT assays for FMDV antibodies were measured as a humoral immunity assay according to the instructions in the OIE Terrestrial Manual. SNT is important in the development of protection against FMD, and several studies have been performed to demonstrate the association between vaccine-induced antibody titers and levels of protection against live virus challenges.

Statistical Analysis

The results were analyzed by the statistical analysis of one-way ANOVA and Duncan's multiple range test, and the significant difference was set to be $P \leq 0.05$.

Results and Discussion:

The piece of pcDNA3.1+*VP1* was prepared and confirmed by sequencing and digestion with the restriction enzymes Kpn I and BamH I. There were 672 nucleotides and 224 amino acid residuals in the *VP1* coding region. The data were recorded in GenBank with the accession number JF28876. The specific band expression of VP1 protein was detected in Western blotting analysis. Using 50%



endpoint titers of irradiated and un-irradiated samples (Table 2), a dose-response curve (Figure 1) was generated using OriginPro 6.1 software and used to calculate the D10 value, i.e., the electron beam dose required to reduce viral infectivity by one log10 the virus infectivity. Based on the dose-survival curve (Figure1) and the log-linear regression equation (Y= 5.82-0.12X), the D_{10} value and the optimal dose of the electron beam for FMDV inactivation were set at 8.33 and 55 kGy, respectively.

Table 2: $TCID_{50}$ of irradiated and un-irradiated FMD virus samples

Dose of Electron Irradiation (kGy)	0	10	20	25	30	35	40	45
$TCID_{50}$	**$10^{6.318}$**	**$10^{4.318}$**	**$10^{3.314}$**	**$10^{2.318}$**	**10^{2}**	**$10^{1.66}$**	**$10^{1.33}$**	**$10^{0.5}$**

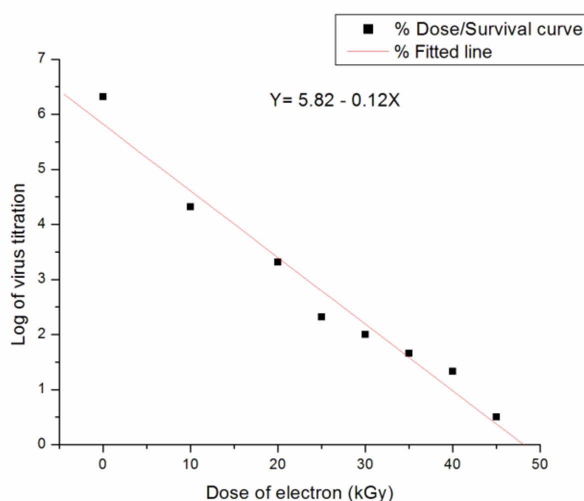
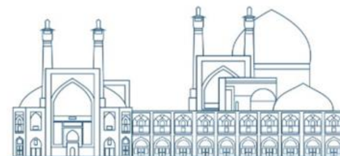


Figure 1: The dose-survival curve of irradiated and un-irradiated FMD virus samples

SNT is important in the development of protection against FMD, and several studies have been performed to demonstrate the association between vaccine-induced antibody titers and levels of protection against live virus challenges [13]. The SNT is serotype-specific and is considered highly sensitive to antibodies against FMDV. Table 3 displays SNT results in vaccinated mice 10 days

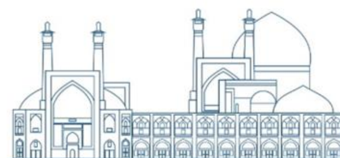


after the last vaccination. The protective titration was considered as PT=1.2 [14, 15]. The highest SNT titer was observed in inactivated vaccine groups, thus this increase was significant (P≤0.05) compared to other vaccinated groups. The highest SNT titer was observed in inactivated vaccine groups (Electron Irradiated Vaccine and Conventional Vaccine), thus this increase was significant (P≤0.05) compared to other vaccinated groups. Acceptable antibody titers were also observed in prime-boost (PB) groups, but in the PB2 group, it was higher and more significant compared to PB1 group. In addition, the equality antibody titer was reported in the PB1 PB2, DNA vaccine plus GMCSF groups**.** The neutralizing antibody of the inactivated vaccine groups (EIV, CV) was significantly higher than in the control group. This indicates that the antigenic structure virus is preserved, and the 146s particle of the virus does not change after the inactivation process. Moreover, it confirms that electron beam, like the BEI, does not cause damage to VP1-VP3 peptides, which was also confirmed in the CF test. These vaccines (EIV, CV), after injection, are detected subcutaneously in mice by dendritic cells. Its immunogenic peptides are delivered to Th2 cells through MHC II, leading to induce the production of neutralizing antibodies by activated B lymphocytes.

Table 3: The mean of SNT titer in vaccinated mice groups

No	Vaccine Group	SNT
1	Conventional vaccine (CV)	2.1
2	Electron Irradiated Vaccine (EIV)	2.1
3	Prime Boost1 (PB1)	1.7
4	Prime Boost2 (PB2)	1.8
5	DNA Vaccine	0.9
6	DNA Vaccine + GMCSF	1.4
7	Negative Control (PBS)	0.9

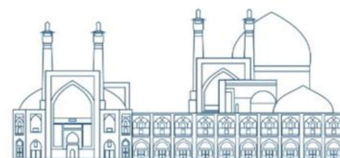
The most common technique to prepare inactivated viral vaccine is chemical methods such as use Binary Ethylenimine (BEI), N-acetylethylenimine (AEI), propylamine and inactivation by formaldehyde. Ionizing irradiation is another way to inactivate a virus; inactivation of the virus by radiation has been considered for several years. The viral inactivated vaccines by ionizing radiation



are secure and non-toxic; the virus particles impossible to escape from inactivation. Due to minor damage to the protein structure of the virus, its antigenic properties are preserved. Hence, the potency of the vaccine is higher. With this irradiation method, we can potentially produce vaccines that tackle constantly evolving, new strains of viruses faster and more effectively than with methods used in the conventional methods. One of the challenges facing governments is the spread of emerging viruses, which require prompt action to produce a suitable vaccine. Irradiated vaccines could help produce vaccines to fight new virus variants relatively quickly and cost-effectively. FMDV Inactivated vaccines elicit a high protective immune response by producing neutralizing antibodies. However, the cellular immune response is weak, so the duration of the immune period is short. The results obtained by previous research suggest that a humoral immune response is necessary to protect animals against FMDV. There is a potent correlation between the circulating humoral antibody titer against FMDV and defense against the virus [16, 17].

Conclusion

The results of this research demonstrated that in all vaccinated groups, except for the DNA vaccine group, the antibody titer was higher than 1.2, indicating an acceptable protective response. Nevertheless, a better titer was reported in EIV, and CV groups, confirming an acceptable humoral response. Generally, the SNT titer among the vaccinated groups was as EIV, CV $\geq$ PB2 $\geq$ PB1 $\geq$ DNA vaccine plus GMCSF.



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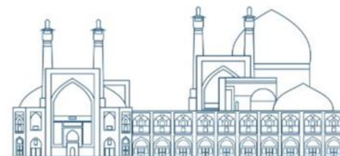

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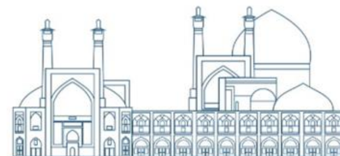

Analysis of flower phenology, flowering and stigma performance of saffron as a result of indirect treatment with surface DBD plasma under salinity stress (Paper ID: 1340)

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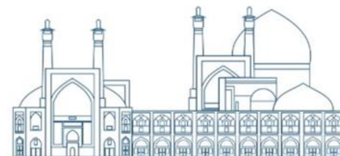
Abstract

Saffron (*Crocus sativus* L.) is cultivated in environments with very different climatic conditions and with very different corm rates from place to place, and it is appreciated for its red dried stigmas used as cooking spice and flavoring agent. To enhance the flower morphological and colorimetric traits under environmental stresses, the applications of non-thermal plasma based techniques are increasingly being investigated in the field of agricultural science as an alternative to conventional pre-germination treatments. Therefore, a field study was conducted to compare four levels P_1 (no treatment), P_2 (5 minute treatment), P_3 (10 minute treatment), P_4 (20 minute) and the salinity stress factors in three levels were 2, 4 and 6 dSm^{-1} according a factorial design with 3 replications. Flowering beginning in saffron seems to be influenced by the combination of plasma and salinity stress. High concentration of salinity (6 dSm^{-1}) resulted in a lower flower and stigma production. The highest number of leaf per plant was found when corms treated to P_4 and 2 dSm^{-1} 6 salinity stress condition. Exposure of saffron corn to plasma treatments of 20 minutes showed the most stimulating effect regarding the flower phenology and stigmas yield in saffron under salinity stress.

Keywords: Cold plasma, Flowering, Saffron corn, Salinity, Stigma yield

Introduction

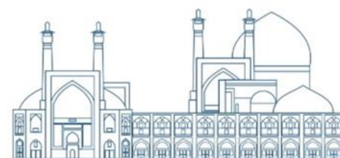
Climate change has emerged as a significant threat, impacting the performance of plants through the occurrence of abiotic stresses (Kumar and Saddhe, 2018). One fast environmental process in



drylands is the soil salinity dynamics, i.e. soil salinization due to surficial accumulation of water-soluble salts. This is caused by an annual rainfall deficit that hinders salt leaching into the subsoil, and strongly affects the physical and chemical soil properties leading to severely changing ecological site conditions (Arabasadi et al., 2024). The extensive Iranian drylands, partly intensively populated today, are characterized by an ongoing aridification trend. Globally, these drylands are between those being most affected by soil salinization (Arabasadi et al., 2024). Soil salinization is one of the important limiting factors for the growth of plants. A saline soil is defined as one, which has an electrical conductivity of 2 dS m^{-1} and above. A significant decline in the yield of corn was observed when electrical conductivity was increased to 1 dS m^{-1} in sandy loam and to 2 dS m^{-1} in clay loam soil (Hopmans et al., 2021).

Saffron (*Crocus sativus* L., Iridaceae) is cultivated in Iran, India, Greece, Italy, Spain and France. It has been reported that saffron production in Iran has decreased significantly, possibly due to effect of weather (Majid et al., 2023). There is a growing need to enhance the activity of plant defense mechanisms and promote plant growth and development, in order to adapt to these changing environmental conditions (Kumar and Saddhe, 2018). Saffron, the most expensive cultivated herb, also known as Red Gold, has extensively been used as an antidepressant, antitumor, anticonvulsant, and antiinflammatory agent (Kothari et al., 2021). Despite the fact that this crop has been grown in the Iran region for an extensive span of time, there has not been much development in the technology used for its production or processing. At present, there exist no strategies to mitigate the adverse impacts of climate variability (Majid et al., 2023).

The application of CP has a profound impact on diverse developmental and processes in plants (Amini et al., 2017). Plasma is an ionized gas, which contains active species, electrons, ions, radicals, excited atoms and molecules. Plasma can be used for temperature sensitive materials, such as biological cells, for the purpose of sterilization, improvement of seed performance and crop yield (Liao et al.2017). The cold plasma treatment process is inexpensive because the plasma is generated in atmospheric pressure and does not need a vacuum system. The input gas (Ar) is less expensive and any chemical or material is not required (Amini et al., 2017). Various types of cold plasma such as dielectric barrier discharge (DBD), plasma jet and cold low



pressure plasma such as radiofrequency (RF) and microwave (MW) discharge, have been used in food science and industry (Kim et al., 2017).

According to the high economic value of saffron, in this study, phenology, growth, and yield of saffron were examined in different plasma treatments to investigate the feasibility of cultivating saffron in saline soils.

Material and methods

Site description and climatic characteristics

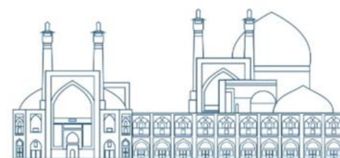
A field study (2024) was conducted at Agricultural Research Farm of Urmia University (latitude 37˚33′09″ N,45˚05′53″ E and 1362 m above sea level), Iran. The elevation of the area ranges from 1300 to 1800 mm and the level of the groundwater table is greater than the adjacent areas. This region enjoys a semi-arid climate.

Experimental setups and plasma treatment process

The experiment was laid out in a factorial experiment based on completely randomized design with three replications for each treatment. The three levels of salinity (2, 4 and 6 dSm^{-1}) were applied for saffron plants as the first factor. Four levels of plasma treatments (P_1, P_2, P_3, P_4) were considered as the second factor.

The plasma source is the surface non-thermal dielectric barrier discharge (DBD) shown in Figure 1. The plasma device is made of Pyrex glass dielectric tubes (diameter 14 mm, thickness 1.5 mm and length 320 mm). Steel rod electrodes with a diameter of 10 mm and a length of 250 mm are located in the center of the glass tubes. The distance between the rod electrodes is 16.7 mm. The AC power supply was an alternating current with a discharge voltage 10 kV and a frequency of 14 kHz and air was inserted into the reactor vessel as the carrier gas with a gas-flow rate of 1.5 L/min^{-1}.

As it is clear in the Fig. 1, the treatment is of an indirect type, which was chosen according to the size of the saffron corns and the more effective plasma treatment. The saffron corns are placed on a shaker plate which is 50 mm away from the glass dielectric tubes. Plasma discharge was performed at different treatment times 5, 10, and 20 minute.



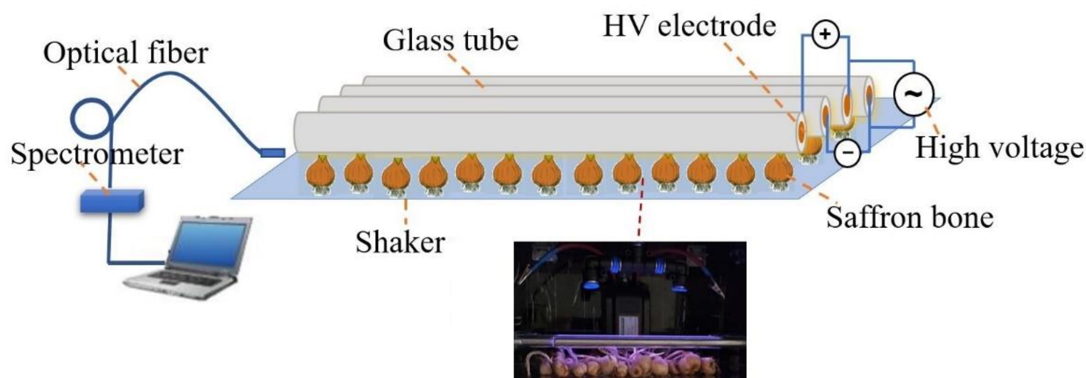
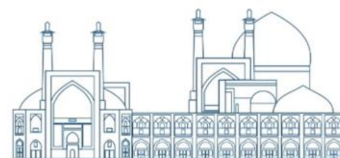


Fig. 1: Schematic diagram of the DBD plasma system for seed treatment

Optical emission spectroscopy (OES) analysis

A computer-controlled Stellar V900 spectrometer (manufactured in Iran) was used to determine the different active species in the air discharge inside the DBD plasma reactor. OES spectra were recorded in the ultraviolet-visible spectroscopy (UV–VIS) wavelength range of 200 to 900 nm, with a spectral resolution of 2 nm and an integration time of 5 s. Fig. 2 shows the plasma OES spectrum with emission lines of reactive oxygen and nitrogen species (RONS). It has been found in various studies before that ROS have an impact on signaling pathways. As a result of the interaction of reactive species with various signaling molecules (due to oxidative stress or reduction), a number of processes, such as differentiation, iron hemostasis, and DNA and nucleic acid cycles, are affected.



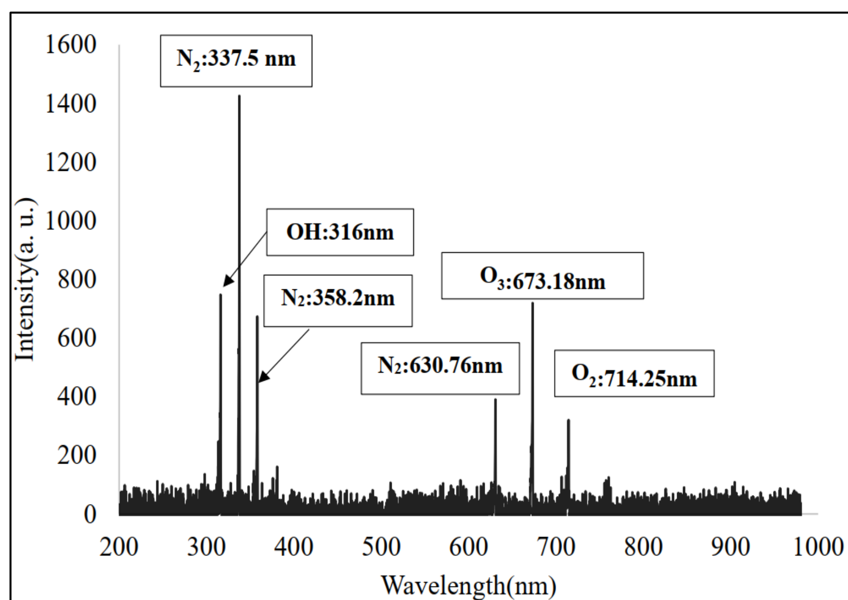


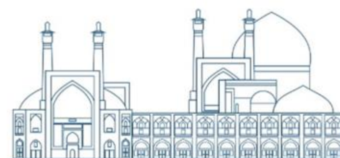
Fig. 2: The emission spectrum of air plasma

Statistical analysis

Statistical analysis and means comparison of data was performed with SAS Statistical Package Program (SAS Institute, Cary, NC). The Least Significant Difference (LSD) test was used to analyze the differences between means comparison of the main effects and interactions.

Results and discussion

It is noteworthy that S_1P_4 obtained higher leaf dry weight (0.035 g/plant) and the lowest of it observed in S_3P_1. Leaf number of saffron grown in low salinity stress was also significantly higher than in high salinity (6dSm^{-1}) when plant exposed to plasma treatment for 20 minutes. In effect, leaf number per plant was higher by 38% in S_3P_4 compared to S_3P_1 (Table 1). Moreover, there was a great variability of in dry weight of flower and stigma obtained in P4 plasma treatment. Dry weight of flower and stigma were higher in S_1P_1 (0.033 and 0.01 g respectively) (Table 1). In saffron, plasma treatments play a role in flowering induction and flower appearance. In addition, the plant exposed to long-term plasma duration for flower initiation lies in the range of 122-125 days, but short exposure to these plasma treatment results in delayed flowering which was the 130



days (Table 1). The utilization of cold plasma as functional stimuli to enhance plant tolerance to salinity stress represents a burgeoning field of research that has already yielded promising outcomes.

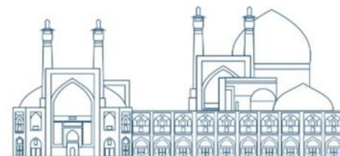
Table 1. The effect of different time of plasma on some characteristics of saffron under salinity stresses.

Treatments		Leaf dry weight (g/plant)	Leaf number (per plant)	Dry weight of stigma (per plant)	Dry weight of flower (per plant)	Start of flowering (number of days after planting)
S_1	P_1	0.025^{d}	8^{g}	0.0085^{c}	0.028^{c}	125^{c}
	P_2	0.017^{f}	9^{f}	0.0090^{bc}	0.026^{d}	130^{b}
	P_3	0.032^{b}	13^{b}	0.0097^{ab}	.031^{b}	122^{d}
	P_4	0.035^{a}	14^{a}	0.010^{a}	0.033^{a}	122^{d}
S_2	P_1	0.017^{f}	8^{g}	0.0044^{e}	0.024^{e}	130^{b}
	P_2	0.02^{e}	10^{e}	0.0088^{bc}	0.024^{e}	130^{b}
	P_3	0.030^{bc}	12^{c}	0.0094^{abc}	0.025^{de}	122^{d}
	P_4	0.031^{b}	14^{a}	0.0097^{ab}	0.030^{d}	122^{d}
S_3	P_1	0.012^{g}	8^{g}	0.0022^{f}	0.018^{g}	130^{b}
	P_2	0.014^{g}	8^{g}	0.0022^{f}	0.021^{f}	132^{a}
	P_3	0.025^{d}	9^{f}	0.0044^{e}	0.020^{f}	125^{c}
	P_4	0.028^{c}	11^{d}	0.0064^{d}	0.025^{de}	125^{c}
	Salinity (S)	**	**	**	**	**
	Plasma (P)	**	**	**	**	**
	S × P	**	**	**	**	**

$^{ns, *, **}$ show non-significant and significant differences at 0.05, 0.01 probability level, respectively.
S_1: 2 dSm^{-1}; S_2: 4 dSm^{-1}; S_3: 6 dSm^{-1}. P_1: control; P_2: 5 min; P_3: 10 min; P_4: 20 min.

Acknowledgments

Authors would like to thank the Plasma Technology Development Company for financial support.



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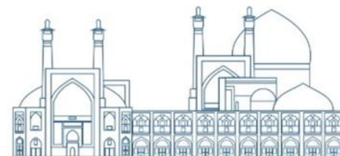

Effects of Gamma Radiation on Germination and Tube Elongation of Cucumber Pollen (Paper ID: 1382)

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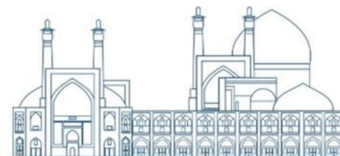
Abstract

Irradiated pollen of cucumber never performed normal fertilization, but pollen germination is crucial for the produce doubled haploids process in Cucumis species, which strongly depends on the gamma- radiation dose. We investigated the effects of different gamma radiation doses (300,400,500Gy) on the pollen germination of Cucumis sativus var. Beith Alpha, Super Dominus and Dastjerdi local accession in vitro condition. Cucumber plants were grown in the greenhouse of Nuclear Agriculture Research School Alborz_ Iran. Cucumber pollens incubated on the medium containing (agar, H3BO3, Ca (NO3)2, and sucrose) to determine pollen germination rate. . Pollen germination and pollen tube length of the cultivars ranged from 12 to 85.71 % and 11 to 120 mm, respectively. Based on Duncan's multiple range test (DMRT), the highest pollen germination rates (62.53% and 45.58%) were found in 400 and 300 Gy, Dastjerdi. There was no significant difference (P = 0.05) between 300 and 400 Gy doses in germination and pollen tube growth. The lowest rate (16%) was observed in 500 Gy, var.Super Dominus. The study suggests that 300 and 400Gy, for Dastjerdi, and 400Gy dose, for Cucumis sativus var. Beith Alpha, supports in achieving the highest pollen germination percentage when radiation. In Dastjerdi local accession, all the three doses of radiation supported the pollen germination but higher radiation dose (500Gy) considerably inhibited the pollen germination. Therefore, doses of 500Gy and more appeared to eliminate the ability to compete for germination, this makes 300 and 400 G-irradiated maternal pollen suitable to serve as inducer pollen in parthenogenesis haploid embryo for doubled haploid plant production and recommended.



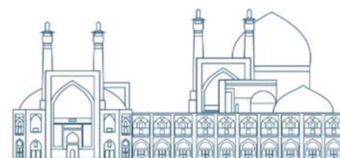
Keywords: Pollen germination, Cucumis sativus, mentor pollen, gamma-radiation, pollen germination,

Introduction

Homozygous lines are very valuable in plant breeding process and genetic researches in agricultural crops. Production of inbreed line in a conventional breeding program self-fertilization takes a long time and requires high costs, but still may not be 100% homozygous. Alternative biotechnological approaches are more efficient and sustainable than traditional methods. Double haploid (DH) technology is a tool used to increase the rate of developing pure lines and genetic gain by shortening breeding cycles. The most common and best-known method of obtaining double haploid cucurbit plants is pollination with irradiated pollen, which induces parthenogenetic development of haploid embryos in plants [1].

Gamma-induced parthenogenesis has shown some success in obtaining DH plants; application of gamma radiation as important factors is very practical for successful haploidization techniques in Cucurbitaceae through pollen grain deactivation and then immature embryo rescue [2]. Gamma radiation is easy to apply and, at the same time, it has good tissue penetration, low fatality [3]. Non-lethal gamma-irradiated pollen has been used to induce parthenogenesis in vivo from many species such as apple, cacao, cucumber, watermelon, muskmelon, and so forth [4-8]. It is known, however, that a large amount of irradiated pollen applied to stigmas can stimulate a parthenogenetic response by increasing the number of pollen tubes reaching the egg cell. This phenomenon was observed in melon [9]. Researchers in all over the world were successful in inducing haploids in many cucurbit plants through pollination with irradiated pollen [1].

The irradiated pollen can germinate on the female stigma and grow pollen tubes to reach the embryo sac. However, this pollen is genetically inactive and unable to fertilize the egg-cell and the polar nuclei. Therefore, irradiated pollen stimulates egg-cell division and parthenogenetic embryo induction [10]. Overall, the dose of ionizing radiation can range from 25 to 500 Gy, depending on the species and can yield less parthenogenetic haploid embryos at higher or more diploid embryos at lower dosages. A low dose of gamma radiation, typically about 300 Gy, was shown to be effective in experiments carried out on melon [9], and was also optimal in cucumber haploid

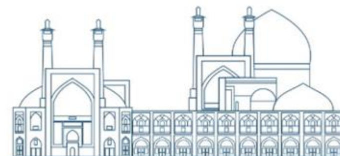


production [11]. Faris et al. [12] examined the effect of the radiation dose on haploid embryo development of cucumber. they applied doses ranging from 50 to 300 Gy and found that 100 Gy was the optimal dose to induce haploid embryo development. Claveria et al. [13] obtained higher induction levels with a higher dose of 500 Gy. Cuny et al. [14] used doses of gamma radiation ranging from 150 to 2,500 Gy, and did not observe a significant effect of the dose on the induction of melon haploids. Although increased levels of gamma ray doses appeared to be more efficient for DH offspring production, the number of DH plants obtained was low, not to mention that many higher gamma ray doses resulted in zero DH plant production. Therefore, based on these results, the efficiency of this process is highly affected by genotype, growth condition, irradiation dose and developmental stage of embryos at the time of excision, exhibiting different regeneration capacity.

Since the pollen sensitivity of each species to radiation resistance is different, determine the appropriate radiation so as to inactivate the pollen grain and induce the embryo to obtain embryos and haploid plants, it is necessary. According to previous researches, the optimal dose of radiation in different varieties of cucurbitaceae from 25Gy to 500 Gy, but the best results were obtained in doses of 250 to 500 for cucumber. Therefore, specific doses of gamma radiation should be optimized for different genotypes. This study was conducted with the aim of determining the appropriate dose of pollen irradiation in cucumber genotypes. Since the Dastjardi local cucumber has not been studied for dosage, this accession with two Cucumis sativuse L. varities were evaluated to determine the appropriate dose for pollen grain irradiation and further research to reach homozygous plants.

Experimental

Cucumber (Cucumis sativus L.) plants were grown in the greenhouse of Nuclear Agriculture Research School Alborz_ Iran. Male flower buds were picked early morning before anthesis from Cucumis sativus var. Beith Alpha, Super Dominus and Dastjerdi local accession. They were irradiated with a range of gamma ray doses, including 300,400,500 Gy. In previous studies on other cucumber varities, shown that irradiation in these doses has obtained more embryos. After irradiation, male flowers were kept at room temperature during the night.

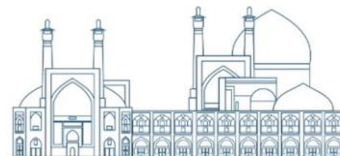


Pollen was collected from three flowers and germinated in cucumber pollen medium agar plates (5 ml) containing 3 mM H3BO3, 1.7 mM Ca (NO3)2, and 14 % sucrose(the medium used in germination experiments was ‘basic germination medium’ consisting of 15% sucrose (w/v), 0.25 mg/l H3BO3 and 1 mM Ca(NO3)2 at pH 7.0. Variations of the basic germination medium involved either altered carbohydrate content or altered pH: sucrose replaced by 15% maltose or 15% glucose, and pH lowered to 5.0 or raised to 8.5). The plates were placed in the growth chamber at 24°C and after 6 hours pollen germination and pollen tube length were determined by microscopic observation. Germination percentages and tube lengths were quantified. To analyze data, use ImageJ software. Means were compared with Duncan's multiple range test (DMRT) in SAS (9.4).

Results and discussion

The results showed that there is no significant difference between 400 and 300 doses that study on the germination percentage and the pollen tube length. However, a significant difference was observed in terms of the traits measured in the dose of 500 with other radiations. Moreover, there is a significant difference between different genotypes in terms of pollen germination under irradiation.

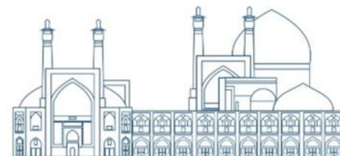
According to the result, dose of 300 Gy gave 45.5% germination in Dastjerdi and 16% in Super Dominus, no germination was obtained in Beith Alpha genotypes on this gamma radiation. 400 Gy dose was found to be the most successful irradiation gamma, with 62.53% germination in Dastjerdi and 83.33% in Beith Alpha that no significant difference between those. No germination was obtained in genotypes Super Dominus irradiated with the dose of 400 Gy. It seems that this dosage is limiting for Super Dominus varity. Godbole and Murthy [16] tested five irradiation doses (150, 200, 250, 300, and 350 Gy) to induce parthenogenesis in snapmelon (Cucumis melo var.momordica). The best results were obtained for the 250 Gy irradiation dose. In a study conducted by Yanmaz and *et al* [17] the effects of gamma irradiation (250, 300, 350 Gy) on in vitro pollen viability in inbred snake cucumber were investigated, pollen viability was higher in pollens irradiated with 250 and 300 Gy doses (28.8 % and 28.0 % respectively) than with 350 Gy (19.4 %). Pollen viability decreased by the increase of the pollen age besides the doses and embryo formation ratios, 300 and 350 Gy doses seemed better than 250 Gy dose. Base on the result



germination percentage of pollen grains in Dastjerdi accession irradiated with a dose of 500 Gy was 26.25%, however, germination could not be provided, for two genotype, Super Dominus, Beith Alpha. Our results showed that no pollen germination occurred at 500 Gy. We concluded that this dose was not suitable for haploid parthenogenesis in these cucumber genotypes. Also, according to Shariatpanahi *et al*.'s results [18], it was no embryo and plant was obtained in genotypes Beith Alpha irradiated with the dose of 500 Gy, while the number of parthenogenic embryo were observed in Karim irradiated with dose of 500 Gy (1.75). This experiment confirms our result and that the interaction between dose and genotype is effective in the germination of pollen grains. Genotype and especially interaction effect of dose and genotype were significant on germination pollen irradiated. Therefore, should be used appropriate dose for each genotype.

Considered together with irradiation dose and genotypes, maximum percentage of germination ranged from 83.33% (Beith Alpha) to 45.5 % (Dastjerdi), lowest observed in genotype Super Dominus irradiated with dose of 300 Gy (16%). Among tested irradiation doses on pollination and tube length, 300 and 400 Gy were better than 500 Gy, with high-rate germination. Induction of maternal haploids embryos by pollination with irradiated pollen (300-400 Gy) has been an effective method for doubled haploid plant production in cucumber. Our results are in accordance with Ebrahimzadeh et al., [19] who reported that different doses of Gamma ray (25, 50, 75, 100, and 200 Gy) were used to irradiate pollen grains for induction of parthenogenetic haploid embryos in oilseed pumpkin. Base on result of this research similar to previous studies, it was confirmed that, different genotypes exhibited different responses to various doses applied and parthenogenic embryogenesis can be induced when optimal dose of irradiation was applied in each genotype.

Figure 1 shows the variation for pollen germination and tube length of three pollen cucumber varieties in response to gamma radiation. The pollination of a female flower stigma with irradiated pollen stimulates an in situ parthenogenetic response when pollen tube reaches the egg-cell [10]. Pollen tube length ranged from 3µm to 120 µm. Pollen tubes remained stable without rupturing for 24h after germination on the in vitro medium. The maximum average pollen tube length was obtained from 300 Gy irradiation gamma dose in Super Dominus pollens (40µm) and fewest average tube length was obtained with 500 Gy, with 10.78 µm. The mean Dastjerdi tube length in



300 and 400 doses was 37.5 µm, 34 µm respectively. Result indicated that the mean tube length in Beith Alpha was 32.5 µm on gamma ray 400 dose. However, no significant difference observed among doses and gynotype on tube length except 500 Gy, which restricted pollen tube grows. The effect of gamma rays on germination and pollen tube length were reported in different species of Cucurbita, irradiating with the doses of 25 and 50 Gy [20], 50 Gy for C. moschata [21], 50 and 100 Gy for C. maxima [3].

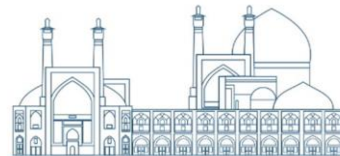
Results obtained from this study reveal that genotype and irradiation dose were effective on germination in pollen cucumber. These results suggest that the preferred radiation dose is specific to each particular experiment.

Considering the interaction between irradiation dose and genotype, gamma irradiation of pollen in Dastjerdi Cv. at 300 or 400 Gy was found to be the most effective. This study has provided important clues for the development of haploidization protocols in cucumber.

Table1. The percentage of pollen germination and pollen tube length of the cucumber genotypes

			varieties	
Gy	Means (%, µm)	dastjerdi	SD	B. A
300	Germination	45.58[a]	16[b]	-
	tube length	37.5[a]	40[a]	-
400	Germination	62.53[a]	-	83.33[a]
	tube length	34[a]	-	32.5[a]
500	Germination	26.25[b]	-	-
	tube length	10.78[b]	-	-

For each means followed by the same letter are not significantly different at level 5%. Based on Duncan's multiple range test. SD: Super Dominus, BA: Beith Alpha



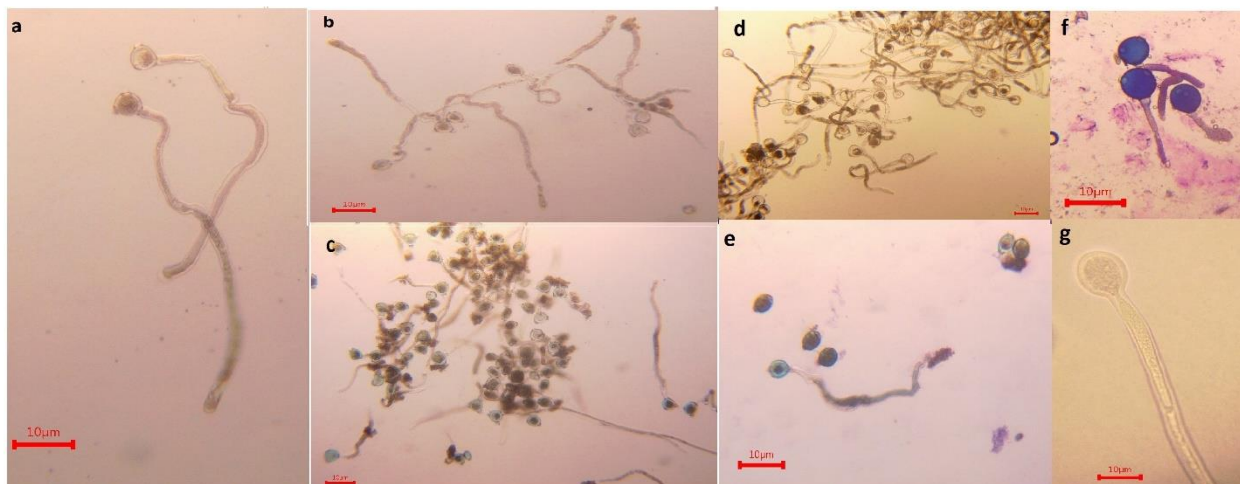


Fig. 1.a) Dastjerdi pollen -300Gy. b) Dastjerdi pollen-400Gy. C)Beith Alpha pollen -400Gy. d) controll test pollen. e) Super Dominus pollen -300Gy. f) Dastjerdi pollen - 500Gy. g) Dastjerdi pollen -4

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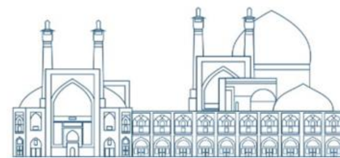

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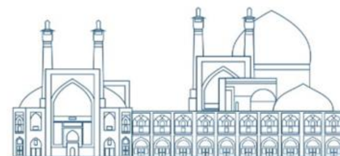

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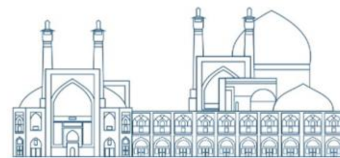

Enhancing Seed Quality in Drought-Tolerant Mutant Rice Genotypes: A Comparative Analysis of Seed Dimensions (Paper ID: 1465)

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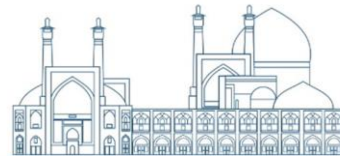
Abstract

This research investigated the impact of drought stress on the seed characteristics of various rice genotypes, with a particular focus on seed length (SL), seed width (SW), and the seed length-to-width ratio (SL/SW Ratio). Our investigation unearthed distinct genotype-specific responses to drought stress, particularly among the mutants, where significant shifts in seed morphology were observed. Notably, the FM-200-E5 and KM-200-17-1 mutants stood out for their remarkable increase in the SL/SW ratio under drought stress condition, indicative of a strategic morphological adaptation to drought stress. The HM-250-E-18-1 mutant exhibited the highest SL/SW ratio among the studied mutants under drought stress, signaling its inherent capability to enhance seed quality in response to reduced water availability. The data reveal substantial genotypic variability in the drought stress response, with certain mutants, such as TM-230-1-2 and HM-250-7-5-2, showcasing exceptional resilience by nearly maintaining their seed dimensions and ratios akin to their performance under normal condition. This resilience hints at a genetic foundation for drought tolerance, which could be leveraged in future rice breeding initiatives. The generalized trend towards longer and narrower seeds under drought condition, particularly pronounced in the superior mutant genotypes FM-200-E5, KM-200-17-1, and HM-250-E-18-1, underscores the potential for these genotypes to contribute to breeding strategies aimed at enhancing drought tolerance and seed quality. By identifying and characterizing these promising mutant genotypes, our study lays the groundwork for developing rice cultivars better equipped to withstand the challenges posed by drought, thereby supporting sustainable agricultural practices in the face of global climate change.

Keywords: Drought stress response, Rice genotypes, Seed morphology, Mutant rice breeding, Climate resilience

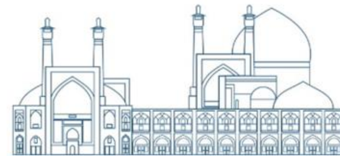


Introduction

In the quest to fortify agriculture against the escalating threat of climate change, enhancing the resilience of staple crops to environmental stresses has emerged as a critical focal point of research [1]. Among these crops, rice (*Oryza sativa*) occupies a pivotal role in global food security, serving as a primary food source for over half of the world's population [2]. However, the increasing prevalence of drought condition worldwide poses a significant challenge to rice production, necessitating the development of drought-tolerant varieties to sustain and potentially increase yields under adverse conditions [3]. This manuscript presented a comprehensive study on the impact of drought stress on the seed characteristics of various rice genotypes, with a particular emphasis on evaluating seed quality through dimensions such as seed length, width, and the length-to-width ratio. By comparing the responses of several mutant rice genotypes to drought stress, our research shed light on the intricate dynamics of seed morphology adaptation and offers a promising pathway for enhancing drought resilience in rice. Through a detailed analysis of genotype-specific responses, this study identified mutant genotypes with remarkable morphological adaptations conducive to improved seed quality under drought condition. These findings not only contribute to our understanding of the genetic mechanisms of drought tolerance but also lay a solid foundation for future breeding programs aimed at securing rice production against the backdrop of climate change.

Plant Materials and Experimental Design

This study utilized an extensive collection of rice genotypes to evaluate drought tolerance, encompassing two renowned Iranian commercial rice landraces (Tarom Mahalli and Hashemi), five Iranian rice cultivars (Khazar, Fajr, Ali Kazemi, Sepidrood, Sangjoo), and an international high-yield standard cultivar named HP. Additionally, 56 mutant rice lines were included, derived from gamma irradiation in 2009 and selected from the M6 generation for their promising characteristics. These comprised 13 lines originating from Tarom Mahalli landrace, 31 from the Hashemi landrace, 10 from Khazar cultivar, and two from the Fajr cultivar. The evaluation of these genotypes' drought tolerance was conducted at the Rice Research Institute of Iran, Rasht, over the



years 2010-2013, with drought stress levels varying from two to three weeks at the onset of flowering.

Drought Tolerance Evaluation

To identify the most drought-tolerant lines among the mutants, an experiment was set up to impose a drought stress of 35 days duration. This period extended from approximately two weeks before to three weeks after the flowering stage. The experimental setup involved 64 rice genotypes, arranged in a three-replicate Square Lattice 8×8 Design to maximize the efficiency of the evaluation process. Soil moisture was maintained at field capacity before the initiation of the water stress regime.

Seed Dimension Measurements

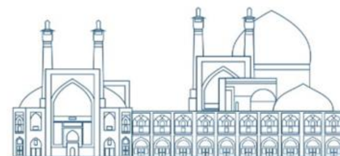
At maturity, seeds from each genotype were harvested, cleaned, and air-dried to a constant weight. A random sample of 100 seeds from each genotype, per replicate, was used for the measurement of seed dimensions. Seed length (SL) and seed width (SW) were measured using a digital caliper with a precision of 0.01 mm. The seed length-to-width ratio (SL/SW Ratio) was calculated for each seed to assess the aspect ratio, an indicator of seed shape and potentially a factor in seed quality.

Statistical Analysis

The collected data on seed length, seed width, and the SL/SW ratio were subjected to statistical analysis using the software package SPSS. Analysis of variance (ANOVA) was performed to determine the significance of differences among genotypes and between treatment groups. Post hoc tests (Tukey's HSD) were conducted to identify specific genotype comparisons where significant differences occurred.

Results and Discussion

Our analysis focused on evaluating the impact of drought stress on seed dimensions, specifically examining seed length (SL), seed width (SW), and the seed length-to-width ratio (SL/SW Ratio) across various rice genotypes, including mutant lines and commercial cultivars.



Seed length (SL)

The response to drought stress varied significantly among genotypes in terms of SL. Notably, HM-250-E-18-1 mutant exhibited a remarkable increase in SL under drought condition, from 10.3mm to 11.2mm, highlighting an exceptional adaptation strategy. In contrast, the FM-200-E6 mutant showed a reduction in SL from 11.2mm under normal condition to 10.6mm under drought stress. On average, mutant genotypes demonstrated a resilience in maintaining SL, with several showing slight increases despite adverse conditions.

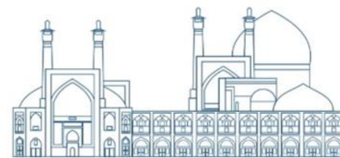
Seed width (SW)

SW adjustments under drought stress were less pronounced than those observed for SL. Most genotypes exhibited minor reductions in SW, indicative of a general trend towards narrower seeds under drought condition. For instance, Fajr cultivar saw its SW decrease marginally from 2.18mm in normal condition to 1.65mm under drought. Among mutants, HM-250-E-18-1's SW decreased from 2.35mm to 2.12mm, aligning with the trend of SL increase, thus affecting the SL/SW ratio.

Seed length-to-width ratio (SL/SW)

The SL/SW ratio under drought stress condition revealed significant insights into genotype adaptation. Several mutants, including FM-200-E5 and HM-250-E-18-1, showed increases in their SL/SW ratios, indicating a strategic morphological adaptation to reduced water availability. FM-200-E5's SL/SW ratio increased from 5.14 to 5.42, and HM-250-E-18-1 from 4.38 to 5.28 under drought condition. This suggests that these genotypes may allocate resources towards elongating the seed while minimally increasing its width, potentially as an adaptation mechanism to drought stress.

In comparison, Sepidrood cultivar demonstrated the most significant increase in the SL/SW ratio among commercial cultivars, from 5.61 under normal condition to 7.03 under drought, suggesting it possesses inherent drought tolerance characteristics potentially valuable for breeding programs. Comparing across all studied genotypes, the data illustrates a wide range of responses to drought stress, highlighting the complex genetic basis of drought tolerance. Mutants exhibited a diverse array of changes in seed dimensions, with some showing notable improvements in SL/SW ratios,

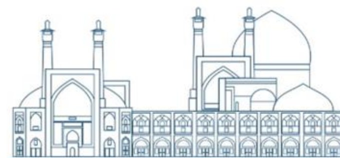


suggesting these traits can be leveraged in breeding programs to develop rice varieties with enhanced drought tolerance. The variability observed in commercial cultivars, alongside the significant adaptations seen in mutants, underscores the potential for genetic improvements to rice seed morphology as a strategy to combat the effects of drought stress.

The findings of our study on the seed dimensions (SL, SW, and SL/SW Ratio) of various rice genotypes under normal and drought stress condition offer insightful revelations into the physiological and molecular mechanisms underlying drought tolerance in rice. The observed variations in seed length, width, and their ratios across different genotypes under stress condition point to complex genetic and environmental interactions influencing seed morphology and, by extension, potential yield and quality.

From a physiological standpoint, the adaptive responses in seed morphology, such as increased seed length-to-width ratio under drought condition, can be interpreted as a survival strategy [4]. This morphological adaptation could be aimed at minimizing transpiration/surface area to conserve water, a critical aspect under water-limited conditions [5]. Such a strategy might not only help the plant to survive under drought stress but could also ensure the reproductive success of rice plants by optimizing seed size and shape for better dispersal and germination under adverse conditions [6]. The genotype-specific responses observed, particularly among the mutant lines, suggest that these adaptations are mediated by distinct physiological pathways [7]. For instance, the increased SL/SW ratio in genotypes like FM-200-E5 and HM-250-E-18-1 under drought stress could be attributed to differential allocation of resources towards seed elongation rather than widening, possibly as a response to the optimization of seed filling and energy storage mechanisms in a water-scarce environment [8].

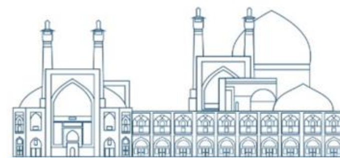
At the molecular level, the variability in drought stress responses among the genotypes suggests the involvement of a complex network of genes regulating seed development and drought tolerance [9]. Previous studies have identified several key genes and transcription factors involved in the ABA (abscisic acid) signaling pathway, which plays a pivotal role in plant response to drought stress, including the regulation of seed size and shape [10,11]. The differential expression of these genes could account for the observed variations in seed morphology under drought stress.



Moreover, genes involved in the synthesis of plant hormones like gibberellins and cytokinins, which are known to influence seed development, could also be differentially regulated under drought condition, leading to the observed changes in seed dimensions [12]. The enhanced SL/SW ratio under drought stress in certain genotypes might thus reflect a molecular adaptation involving the upregulation of genes that promote cell elongation over cell expansion, or perhaps a downregulation of genes that normally inhibit elongation in favor of width [13].

Conclusion

Our comprehensive study investigating the impact of drought stress on seed dimensions across a diverse set of rice genotypes, including mutant lines derived from traditional landraces and commercial cultivars, has illuminated the significant variability in how these plants respond to water scarcity. Through meticulous measurement and analysis of seed length (SL), seed width (SW), and the seed length-to-width ratio (SL/SW Ratio), we have identified distinct genotypic responses that showcase the complex interplay between physiological adaptation and molecular regulation under drought condition. The findings reveal that certain mutant genotypes possess remarkable adaptive traits, such as increased SL/SW ratios under drought stress, pointing towards their potential utility in breeding programs aimed at enhancing drought tolerance in rice. These morphological adaptations, likely underpinned by intricate genetic mechanisms, not only contribute to the plants' survival and reproductive success under adverse conditions but also open new avenues for improving rice resilience to environmental stresses through genetic enhancement. As we move forward, the insights gained from this research will be instrumental in guiding future efforts to develop rice varieties that can withstand the challenges posed by global climate change. By harnessing the genetic diversity and adaptive traits uncovered in our study, it is conceivable to enhance the sustainability of rice production systems, ensuring food security for populations reliant on rice as a staple food. The journey towards creating more drought-resilient rice varieties is complex and demands continued exploration and innovation, yet the potential benefits for agriculture and food security worldwide are profound and compelling.



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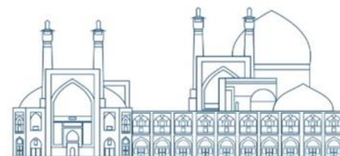

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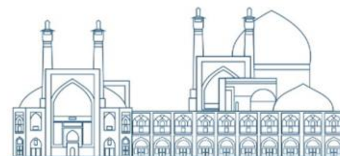
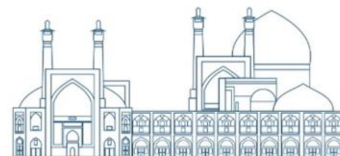
Table 1. Characteristics of seed shape of studied rice genotypes under normal and drought stress conditions

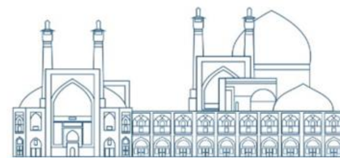
Symbol	Genotype	Seed length-Drought	Seed length-Normal	Seed width-Drought	Seed width-Normal	Seed length/Seed width Ratio-Drought	Seed length/Seed width Ratio-Normal
M1	**TM-220-10-4-1**	9.8	9.78	2.4	2.5	4.08	3.91
M2	**TM-230-VE-7-5-1**	10.2	9.89	2.9	2.57	3.52	3.85
M3	**TM-230-VE-8-4-1**	9.63	9.64	2.68	2.95	3.59	3.27
M4	**TM-250-10-7-1**	9.57	9.48	2.6	2.8	3.68	3.39
M5	**TM-250-15-5-1**	10.4	9.73	2.65	2.52	3.88	3.86
M6	**TM-B-2-1-E**	10.4	9.69	2.92	2.88	3.56	3.36
M7	**TM-B-7-1**	9.58	9.86	2.72	2.92	3.52	3.38
M8	**TM-B-19-1-E**	9.98	9.58	2.62	2.78	3.81	3.45
M9	**TM-B-19-2**	9.88	9.53	2.65	2.82	3.73	3.38
M10	**TM-230-1-1**	10.2	9.79	2.52	2.45	4.05	4
M11	**TM-230-1-2**	10.1	9.03	2.17	2.45	4.65	3.69
M12	**TM-300-5-1**	10.3	10.1	2.23	2.63	4.62	3.84
M13	**TM-250-11-5**	9.53	9.65	2.5	2.7	3.81	3.57
M14	**FM-200-E6**	10.6	11.2	1.98	2.23	5.35	5.02
M15	**FM-200-E5**	10.3	11.3	1.9	2.2	5.42	5.14
M16	**HM-250-7-4-2**	10.7	10.2	2.43	2.47	4.4	4.13
M17	**HM-250-7-4-3**	10.8	10.3	2.5	2.45	4.32	4.2
M18	**HM-250-7-5-2**	10.1	10.5	2.27	2.43	4.45	4.32



M19	**HM-250-7-7-2**	10.3	10.3	2.18	2.5	4.72	4.12
M20	**HM-250-25-1-3**	10.4	9.76	2.37	2.45	4.39	3.98
M21	**HM-250-26-1-E-1**	9.97	9.82	2.22	2.3	4.49	4.27
M22	**HM-250-42-1-E-1**	10.2	10.4	2.68	2.78	3.81	3.74
M23	**HM-250-E-1-1**	9.8	10.4	2.5	2.7	3.92	3.85
M24	**HM-250-E-3-2**	9.92	9.71	1.98	2.32	5.01	4.19
M25	**HM-250-E-5-2**	10.1	10.3	1.97	2.37	5.13	4.35
M26	**HM-250-E-18-1**	11.2	10.3	2.12	2.35	5.28	4.38
M27	**HM-250-2-4**	10.3	10.7	2.13	2.37	4.84	4.51
M28	**HM-250-3-1**	10.7	10.3	2.02	2.38	5.3	4.33
M29	**HM-250-5-1**	10.7	9.96	2.2	2.45	4.86	4.07
M30	**HM-250-6-6**	10.7	10.2	2.12	2.45	5.05	4.16
M31	**HM-250-7-1**	9.8	10.8	2.17	2.55	4.52	4.24
M32	**HM-250-7-6**	10.2	10.6	1.98	2.57	5.15	4.12
M33	**HM-250-12-1**	10.2	10.2	2.02	2.38	5.05	4.29
M34	**HM-250-23-2**	9.12	10.3	1.85	2.52	4.93	4.09
M35	**HM-250-26-1**	10.2	10.8	1.88	2.48	5.43	4.35
M36	**HM-250-34-1**	9.68	10.3	1.88	2.4	5.15	4.29
M37	**HM-250-41-1-E**	9.73	10.3	1.73	2.42	5.62	4.26
M38	**HM-250-41-2-E**	9.45	10.1	2.12	2.8	4.46	3.61
M39	**HM-250-42-1-E**	10	10.9	2.07	2.82	4.83	3.87
M40	**HM-300-E-1**	10.1	10.2	1.88	1.97	5.37	5.18
M41	**HM-300-3-1**	9.5	10	2.05	2.78	4.63	3.6
M42	**HM-300-3-2**	9.65	9.97	2.05	2.83	4.71	3.52
M43	**HM-300-5-1**	9.78	10.3	2.13	2.63	4.59	3.92
M44	**HM-300-5-3**	9.6	10.6	2.15	2.8	4.47	3.79
M45	**HM-300-6-E**	9.6	10.9	2.05	2.38	4.68	4.58
M46	**HM-300-16-1**	9.32	10.4	2.02	2.53	4.61	4.11
M47	**KM-200-4-2-E**	10.4	10.9	1.88	2.57	5.53	4.24
M48	**KM-200-17-1**	10.9	11.7	1.8	2.48	6.06	4.72
M49	**KM-200-19-1**	10.4	10.7	1.88	2.65	5.53	4.04
M50	**KM-200-19-2**	10.4	10.5	1.82	2.42	5.71	4.34
M51	**KM-200-21-1**	10.4	11.2	1.83	2.55	5.68	4.39
M52	**KM-200-24-1**	9.9	10.2	1.82	2.48	5.44	4.11
M53	**KM-200-43-1-E**	10.5	11.1	1.87	2.63	5.61	4.22
M54	**KM-250-2-2-E**	9.7	10.3	1.88	2.55	5.16	4.04
M55	**KM-250-3-1-E**	9.92	10.6	2.08	2.85	4.77	3.72
M56	**KM-250-5-1**	10.2	10.7	1.78	2.42	5.73	4.42



H	**Hashemi**	9.85	10.3	1.78	2.43	5.53	4.24
Kh	**Khazar**	9.82	10.5	1.8	2.47	5.46	4.25
F	**Fajr**	10.7	11.4	1.65	2.18	6.48	5.23
TM	**Tarom Mahalli**	9.88	10.5	1.85	2.53	5.34	4.15
HP	**H.P.**	10.1	10.7	1.78	2.48	5.67	4.31
Se	**Sepidrood**	11.6	12.5	1.65	2.23	7.03	5.61
AK	**Ali Kazemi**	10	10.6	1.82	2.42	5.49	4.38
Sa	**Sangjo**	9.93	10.6	1.87	2.48	5.31	4.27



Effects of Direct Current Diode plasma and plasma-activated water on barley seed germination and seedling growth (Paper ID: 1564)

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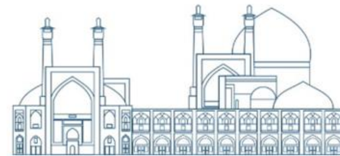
Abstract

Application of cold plasma due to the active species of oxygen and nitrogen in seed priming to improve the germination characteristics of strategic and economic plants especially cereals has recently received much attention. Barley (Hordeum Valgare) is an important crop for human and animals' food source that is widely cultivated in most parts of the world. However, Barley germination is difficult due to the non-nutritive outer coverings. in the present article, the effect of direct treatment of dry seeds by direct current diode plasma at both positive and negative poles as well as indirect treatment of seeds using plasma-activated water on the barley germination characteristics including seed germination rate and seedling growth have been investigated for the first time. The experimental results show a significant effect of increasing the germination percentage under negative pole plasma treatment by 93.3% compared to the positive pole (60%) and the untreated barley seeds (66.6%). The presence of electrons makes seed dormancy break faster. Also, it can be seen that deionized water activated by plasma with 80% seed germination has a greater effect on increasing the germination rate of barley than plasma-activated tap water with 73.3%. The results indicate there is a significant difference in the percentage of germination between two types of plasma-activated water in comparison with the control sample, which is due to the active nitrogen species present in the water treated by plasma. In the competition between the effect of direct treatment by plasma and indirect treatment of seeds using plasma-activated water, the negative pole of direct current diode plasma can have an optimal effect. Furthermore, plasma treatment has been found to enhance seedling growth of barley seeds. It is concluded that cold plasma can be a promising way to improve the germination and seedling growth of barley seeds.

Keywords: Direct Current Diode plasma, Barley, Plasma-Activated Water



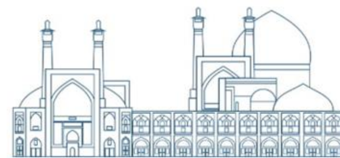
Introduction

One of the important crops supported by agricultural institutions is barley [1]. As one of the most important small grains, according to the latest United Nations agricultural statistics [2], the production of barley until 1400 has been 159 million tons in the world, 3.6 million tons in Iran, and 123,698 tons in Golestan province (6% of the total). Barley plays an important role in human nutrition in the form of bread, biscuits and cakes, soup, baby food, and other foods. Barley farming is important because there are large areas in our country that are only suitable for barley production due to soil salinity, shallow soil depth and salinity of agricultural water. Therefore, finding methods to solve the challenges of barley cultivation, especially its late germination, is considered as one of the important issues in agriculture and industry.

Germination is one of the stages of plant growth that can be affected by many factors. Among cereals, barley is usually difficult to germinate due to its non-nutritive outer sheaths, which constitute approximately 10% of the total seed weight [3]. For a long time until now, various traditional methods such as mechanical scraping, chemical treatment, cold layer, and seed soaking have been used to break the stagnation and increase the germination of seed plants, but each of these methods is associated with problems. One of the main problems in the aforementioned traditional methods is biological contamination and prolongation of seed germination time. For this reason, in recent years, many new methods have been proposed to improve seed germination and increase plant growth, and one of these methods is the use of plasma technology [4-6].

After confirming plasma as an environmentally friendly and safe technology, promising reports of increased germination efficiency and plant growth by cold plasma have been reported. Therefore, the use of cold plasma technology in increasing the germination of plants can have a great effect on increasing production efficiency [7-9]. With direct plasma radiation to the seed, its surface layers are changed and due to the presence of ions and free radicals, functional groups containing oxygen and nitrogen, carboxylic acids, hydroxyls and amines are formed on the surface of the seed [5, 6, 10].

One of the most attractive applications of cold plasma is the production of a new type of water called plasma-activated water, which has unique capabilities in disinfection and improving germination. Creating organic and nitrogen-rich fertilizers for agricultural purposes is one of its



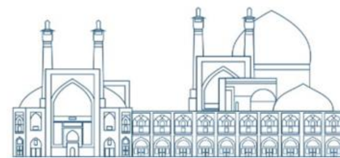
potentials. Experiments conducted on plant samples grown with plasma water show that the nitrogen and oxygen produced in the water increase the growth rate, improve the quality and yield of the plants, and reduce water consumption. Active species of reactive oxygen and nitrogen, especially stable species such as nitrate ion, nitrite and hydrogen peroxide present in plasma water is a clear reason for its antimicrobial ability [11]. The positive effects of potassium nitrate on the stress of seeds have been proven before, so the role of NO_3 ions in plasma water can be considered important [12]. However, its complex mechanism needs more research.

In recent years, the use of plasma technology has been significant in improving the germination and growth of various grains, especially barley. Since the effect of plasma on germination and plant growth depends on factors such as the type of gas, the amount of voltage used and the duration of plasma processing, and on the other hand, depending on the type and condition of the seed, each seed may show different characteristics and grow differently. Therefore, in this article, the effect of plasma produced by a Direct-Current Diode Plasma (DC-DP) generator and plasma-activated water on the germination and growth characteristics of barley seeds has been investigated experimentally. In addition, by measuring the percentage and speed of germination and growth traits such as the length of roots and stems of barley under the influence of different plasma treatments, the optimal condition has been determined.

Materials and Experimental Methods

In this paper, the germination of barley seeds treated with cold plasma produced by the DC-DP method has been investigated. The plasma system used for this experiment is direct current diode plasma (DC-DP) with nitrogen gas, available in the Plasma and Nuclear Fusion Research Institute, Nuclear Science and Technology Research Institute, (Fig. 1). The device has two flat parallel electrodes made of stainless steel with a diameter of 10 cm, which are placed at a distance of 6 cm from each other [13]. One of the electrodes is the anode and the other is the cathode.

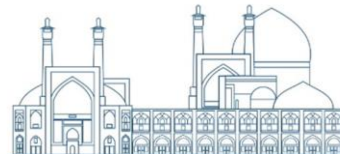
In this experiment, in order to investigate the direct effect of plasma irradiation, we consider the treatments separately for ions and electrons. In order to investigate the effect of ions and electrons, 15 seeds for each treatment were placed on the anode and cathode respectively and were subjected to plasma irradiation for 60 seconds. For this purpose, vacuuming was done in the chamber to a

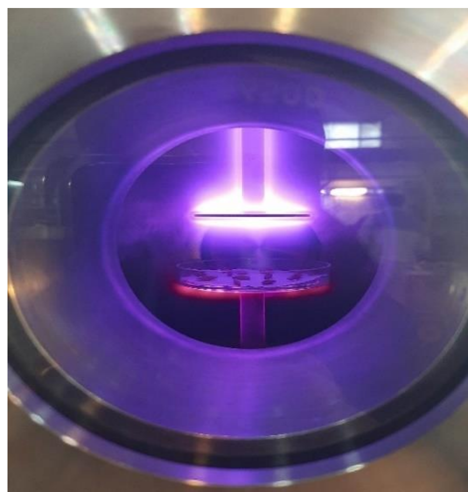


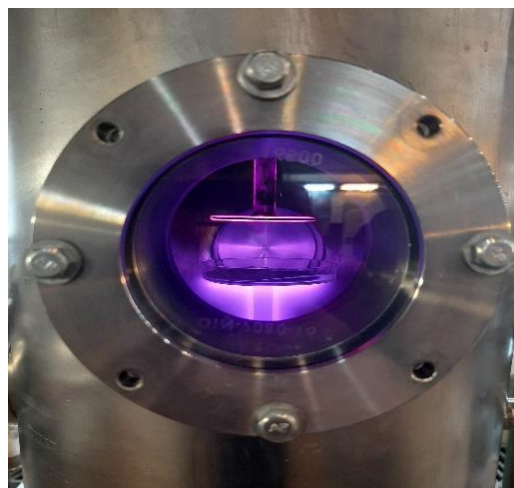
pressure of 1×10^{-2} torr, and after nitrogen gas was injected up to 0.6 torr and a potential difference of 200 V was established between the electrodes, plasma was formed and electrons moved towards the anode and nitrogen ions moved towards the cathode while a plasma halo forms around the cathode.

Fig. 1. Experimental setup of Direct-Current Diode Plasma (DC-DP)

For the negative pole, where the upper electrode is the cathode and the lower electrode is the anode, the seeds are located on the anode and are affected by the electrons (Fig. 2a). To check the effect of positive ions, the place of the electrodes is changed and the seeds are placed on the cathode. In this way, the samples are affected by positive nitrogen ions as shown in Fig. 2b.



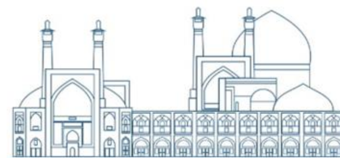




(a) (b)

Fig. 2. (a) Samples irradiated by DC-DP electrons, (b) Samples irradiated by DC-DP nitrogen ions.

For the preparation of activated water, nitrogen plasma at atmospheric pressure has been used by the dielectric barrier discharge method. In this method, nitrogen gas passes between two cylindrical electrodes that are separated from each other by a quartz dielectric, and due to the creation of an electric field caused by a high-frequency power source with a frequency of 15 kHz and a voltage of 15 kV, Nitrogen plasma is formed and enters the water. In order to investigate the effects of plasma water, a certain amount of normal water and ionized water were placed in a sterilized bottle, and negative and positive poles were placed inside the bottle as anode and cathode. Then, two samples of normal water and deionized water were separately affected by this nitrogen plasma for 10 minutes (Fig. 3).



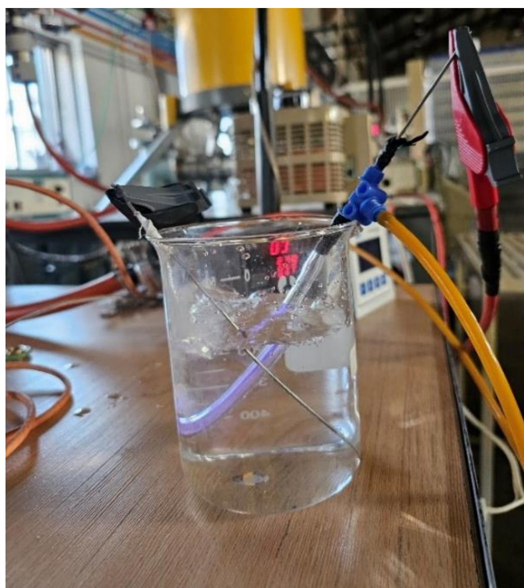
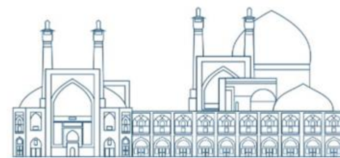


Fig. 3. Preparation of activated water with plasma.

After processing, the seeds of each treatment were transferred to Petri dishes with a diameter of 9 cm containing a filter paper at the bottom of the Petri dish. Seeds without plasma treatment were considered as control samples. One hour after the application of plasma, the control sample and plasma treatment with positive pole and negative pole were moistened with 3 ml of normal water. Also, to check the effect of plasma-treated water, two treatments were humidified with ionized water and ordinary water activated with plasma. To measure the germination of barley seeds, after 24 hours, the samples were transferred to the growth chamber with a constant temperature of 20°C with a relative humidity of 50% for 14 days. The treatments were examined daily at a certain time and the seeds and their germination rate were recorded according to the standards of the International Seed Testing Association (ISTA).

Generally, the germination index is one of the important parameters in determining seed germination, which has a direct relationship with the quality and vitality of seeds. In other words, the better the quality of the seeds, the higher the percentage of germination and the number of germinated seeds, and as a result, the germination index will be higher. Germination in crops is defined as the emergence of radicle from the seed, and the criterion for germination in crops such as barley is that the radicle comes out of the seed approximately 2 mm long or more. According to



the static standard criterion, the percentage of germination on the 7th day for each treatment is calculated using the following equation:

$$\text{Final germination rates} = \frac{\text{number of germinated seeds}}{\text{total number of seeds}} \times 100$$

The length of the shoot and the root are measured on the 9th and 14th days with a ruler with an accuracy of 1 mm (Fig. 4). Then the effects of seed germination and seedling growth on characteristics such as final germination, root length, shoot length and vigor index are evaluated. The vigor index represents the percentage and germination potential, the lower the seed quality, the lower the germination percentage and the vigor index decreases. The vigor index is obtained from the product of the percentage of final germination in the length of the seedling.

$$\text{vigor index} = \text{Seedling length (cm)} \times \text{final germination percentage}$$

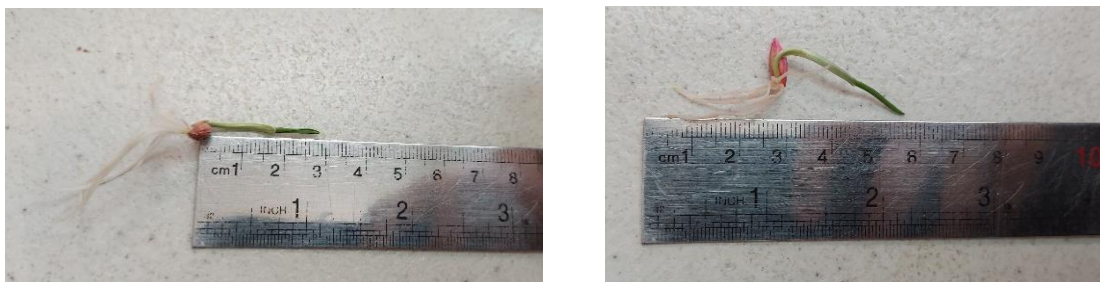
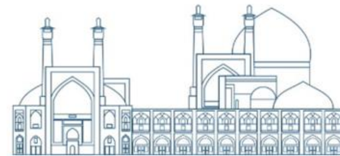


Fig. 4. Measuring the length of shoot and root of barley on the 9th and 14th day.

Results and Discussions

Table 1 presents the results of final germination rates for the treated and untreated seeds after 7 days. The results show a significant effect of increasing the germination percentage under negative pole plasma treatment by 93.3% compared to the positive pole (60%) and the untreated barley seeds (66.6%). The presence of electrons makes seed dormancy break faster. Also, it can be seen that deionized water activated by plasma with 80% seed germination has a greater effect on increasing the germination rate of barley than plasma-activated tap water with 73.3%. The results



indicate there is a significant difference in the percentage of germination between the two types of plasma-activated water in comparison with the control, which is due to the active nitrogen species present in the water. In the competition between the effect of direct treatment by plasma and indirect treatment of seeds using plasma-activated water, the negative pole of direct current diode plasma can have an optimal effect (Fig.5).

Table 1. The percentage increase of seed germination compared to untreated seeds for different plasma treatments

Treatment	**negative pole of DC-DP (P1)**	**positive pole of DC-DP (P2)**	**plasma-activated deionized water (PW1)**	**plasma-activated Tab water (PW2)**	**untreated (W)**
Final germination rates	93.3	60	80	73.3	66.6

It can be stated that due to the interaction of seed cells with plasma, the activity of enzymes is accelerated and the internal nutrients of the seeds are decomposed. Therefore, in addition to the growth of the seedling, a reserve for the seeds is provided. Plasma promotes amylase and protease activity in soluble sugar and protein metabolism, which is essential for the germination process.

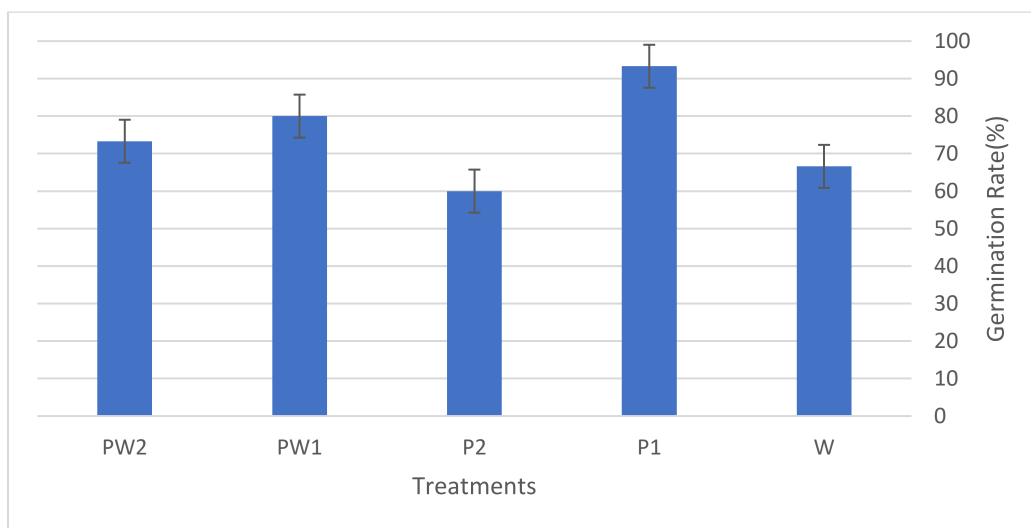


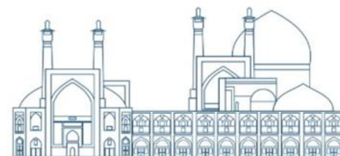
Fig.5. Final germination percentage of barley seeds for different treatments

Table 2 presents the comparisons between the seed germination characteristics of treated and untreated samples after 9 and 14 days. The results show that plasma has a significant effect on the seedling growth of barley, so the growth of the seeds treated with a negative pole of DC-DP is more than the control, and plasma-activated water had an optimal effect on both shoot and root growth.

Table 2. Shoot and root length of barley seeds in different treatments (mm)

Treatments	**W**	**P1**	**P2**	**PW1**	**PW2**
shoot length on day 9	31	41	30	29	45
shoot length on day 14	84	98	81	83	92
root length on day 9	28	33	25	22	34
root length on day 14	39	46	36	30	45

According to the obtained results (Fig. 6), treatment of barley seeds with negative pole cold plasma and plasma-activated water has increased the length of the plant by 21% and 32% compared to the control (Fig. 6a), respectively. Also, the negative pole and plasma-activated water treatment increased the root length by 18% and 21%, (Fig. 6b), respectively. The longer root will help absorb more water and nutrients. The reason for the increase in the length of the root is that the plasma raises the hydrophilicity of the seed by scratching the surface of the seed. These scratches make the shell more fragile for the bud so that the bud with less energy can push the shell aside and grow.



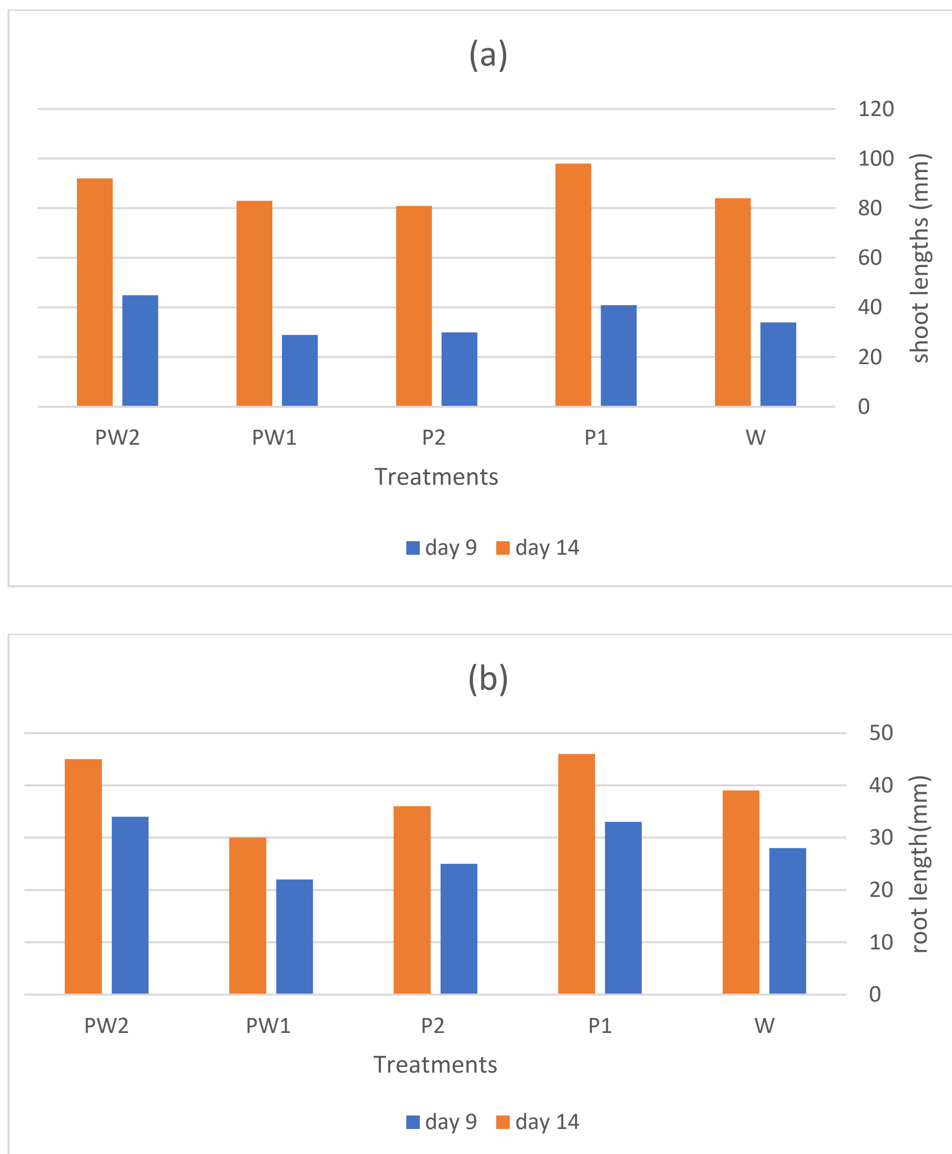
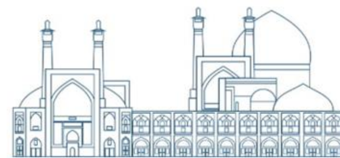


Fig. 6. (a) shoot length and (b) root length of different barley seed treatments after 9 and 14 days

Fig. 7 shows the values of the vigor index for different treatments. It can be seen that the negative pole treatment causes a 71% increase in the vigor index compared to the control, and the plasma-treated tap water causes a 47% increase in the vigor index compared to the control sample.



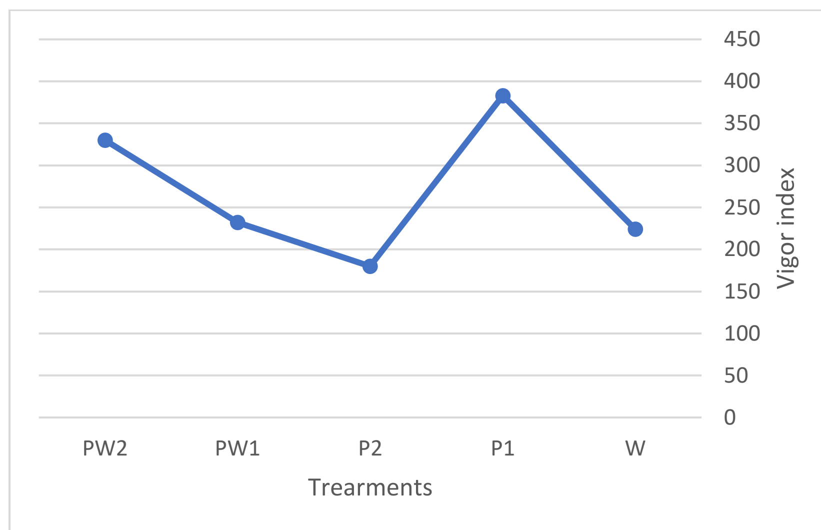
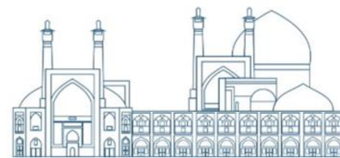


Fig.7. Vigor index for different barley seed treatments

Conclusions

Recent research shows that cold plasma technology has many applications in seed germination and seedling growth, so the improvement of growth and germination through the treatment of seeds with plasma has been studied in many researches. this technology is discussed as a new method for seed germination and increasing the growth rate. Among the types of cereals, barley is usually difficult to germinate due to its non-nutritive outer and inner sheaths. Since the active species of oxygen and nitrogen in cold plasma have a significant effect on the seeds germination stage, therefore, in this research, the germination of barley seeds treated with cold plasma produced by direct current diode method in both positive and negative poles, as well as deionized and tap water activated with nitrogen plasma is investigated and the effects of direct current diode plasma and plasma-activated water on germination and growth rate of barley seeds has been investigated. The results show the significant effect of increasing the percentage of germination under different treatments with plasma. Among the different treatments, negative pole plasma treatment has increased the germination percentage compared to the control, because of the presence of electrons, seed dormancy has been broken faster. Also, the results show that the negative pole of DC-DP and activated tap water treatment increase the growth parameters. Therefore, the simultaneous use of DC-DP treatment and plasma-activated water can be a promising method to increase barley seed germination. In conclusion, Plasma technology can reduce costs by increasing



biomass at a high level of cultivation. This means that cold plasma, as a new and environmentally friendly technology, can be a suitable alternative method in traditional agriculture to increase the growth of plants such as barley.

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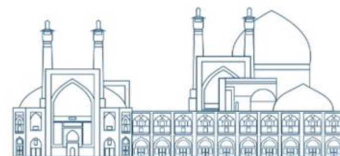

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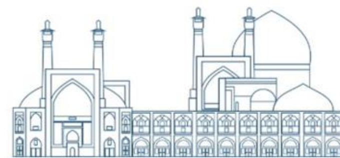

Characterization of Aspartate Kinase in mutant Corynebacterium Glutamicum created by gamma irradiation (Paper ID: 1607)

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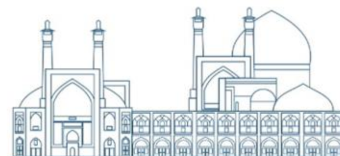
Abstract

One of the microorganisms used in the production of lysine is bacterium Corynebacterium Glutamicum. Using this bacterium to produce amino acids was done for the first time by Kinoshita and colleagues in 1957. In this study, wild bacteria ATCC13032 and its mutant were cultured in broth and their proteins were extracted. To determine the protein subunits of the wild and mutant bacteria's enzymes, protein electrophoresis was performed using electrophoresis of polyacrylamide gel technique Laemmli method. Fermentase protein marker was used to determine the molecular weight of subunits under study. Protein electrophoresis results showed changes in concentration of key enzymes in lysine biosynthesis pathway (aspartate kinase). Pattern of wild type and mutant protein subunits of Corynebacterium Glutamicum shows changes in small subunit of aspartate kinase in the mutant strain. These changes can explain allosteric feedback regulation changes. Changes in the small subunit of aspartate kinase could be due to an increase of the production rate of lysine in these strains.

Keywords: Corynebacterium Glutamicum, aspartate kinase, lysine

1. Introduction

Amino acid metabolism is very complex. Lysine in bacteria is synthesized during the course of d-amino Pimelic. This complexity does not allow the use of genetic engineering methods because there are many biochemical steps that must be modified separately, each by genetic engineering methods such as Site-directed mutagenesis to create an appropriate economic level of lysine value (23) Lysine in bacteria is synthesized using the course of d-amino Pimelic through 7-10 levels of oxaloacetate precursor. Lysine in Corynebacterium Glutamicum is made from pyruvate, oxaloacetate and two molecules of ammonia (to provide four NADH as reducing agents) (1).



Allosteric enzyme is a regulator in aspartate kinase path. In allosteric enzymes, besides a special place for substrate binding, there are places to connect molecules that increase or decrease the enzyme activity. This enzyme catalyzes the first step passes in all aspartate amino acids (16).

2. Materials and Methods

1.2 Bacterial preparations order to radiation

C. glutamicum wild-type strain ATCC 13032 was grown aerobically on a rotary shaker 30˚C in TSB medium. Samples in 20 replicate gamma irradiated with doses of 0, 100, 200 and 300 Gy using a cobalt-60 gamma cell (AEOI, Tehran Irradiation Application Center, Iran) at a dose rate of 300 Gy per min.

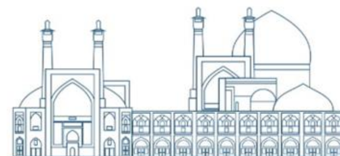
2.2 Bacteria selected suitable

After 24 hours of fermentation in bacterial isolates from the irradiated samples, bacterial population was obtained using standard log CFU/ml against optical density. Using an index calculated by dividing the amount of lysine production (mg/mL) by log CFU/ml (lysine production divided by population), samples with index values above control levels (at 200 Gy) were selected.

3.2 sample preparation

In order to extract the bacterial protein; *C. glutamicum* ATCC 13032 was grown on a rotary shaker at 30˚C the cells were harvested by centrifugation, in a centrifuge during each supernatant was discarded and the pellet was collected. The resulting pellet was washed in phosphate buffered saline. The cell lysate using a variety of physical and chemical methods, including the use of lysis buffer, sonicator, glass beads and freeze- defreeze was used. Then the proteins was extracted from the mutant and wild strains of bacteria were lyophilized.

Optimal protein concentration for polyacrylamide gel electrophoresis was determined according to the Bradford method [1] and patterns of bacterial protein was analyzed using the technique polyacrylamide gel electrophoresis by Lammeli method [8].



3. Results

1.3 Create high-yielding strains

Due to the low amount of lysine production in *Corynebacterium glutamicum* wild type, mutant bacterial strains were created using various doses of gamma ray and density of bacteria and the production of lysine in bacteria and compared to other bacteria in the mutant. Wild type had the lowest population density and the lowest amount of lysine. Irradiation results showed changes in bacterial density and lysine production in the mutant strain. Bacterial population increased in irradiated samples. Also, lysine production was higher in mutant bacteria than in the wild strain and the trend was linear up to dosage 200 Gy and decreased at dose level 300 Gy.

The mutant strains had the highest population densities of strain were irradiated with 100 Gy and the maximum amount of lysine was observed in strain were irradiated with 200 Gy.The findings showed that the highest amount of lysine production was 16/38 mg/mL, which was observed in bacteria irradiated with gamma radiation at dose level 200 Gy (table1).

2.3 Polyacrylamide Gel Electrophoresis

The pattern of protein subunits in the wild strain of *Corynebacterium glutamicum* was studied (figure2). Protein electrophoresis showed changes in key enzyme of lysine biosynthesis

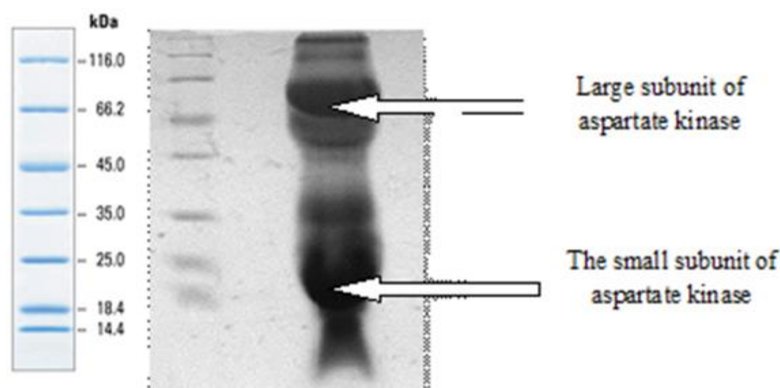
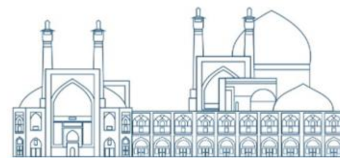


figure2 - pattern protein subunits of wild type *Corynebacterium glutamicum*



(aspartate kinase). The calculated molecular weight of the subunits in this study were close to with The results reported by , Kochhar et al (13). Protein subunit structure of wild and

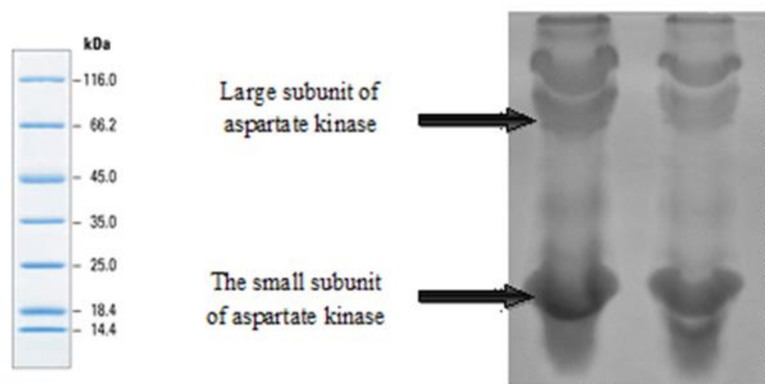
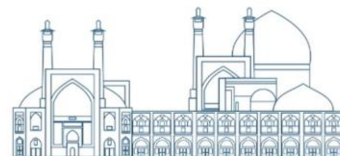


figure3 - pattern protein subunits of wild type (left) and mutant (right) *Corynebacterium glutamicum*

mutant strains of *C. glutamicum* indicate that the small subunit of aspartate kinase undergoes some changes in the mutant strain (figure3). Increase in lysine producing mutant bacteria can be related to changes in the protein pattern of aspartate kinase.

In this study, three major protein types were observed in C. glutamicum, pertaining to enzymes aspartate kinase (58 and 18 kDa), homoserine dehydrogenase (46 kDa), and homoserine kinase (28 kDa). According to Kochhar et al. (13), aspartate kinase protein has four small subunits (17 kDa) and four large subunits (53 kDa). Furthermore, Kochhar et al. (13) reported that aspartate kinase subunits are connected to each other by disulfide bridges, producing a protein with molecular weight of 280 kDa. The β-mercaptoethanol present in the extraction buffer results in the reduction of disulfide bonds and separation of aspartate kinase subunits.

Densitometry findings suggest that three main proteins of C. glutamicum, i.e., aspartate kinase (28%), homoserine dehydrogenase (16.8%) and homoserine kinase (18%), constitute about 62.8% of the total proteins of C. glutamicum. The comparison of key Enzyme in wild and mutant strains indicated a 38 percent decrease of Density of Aspartate kinase large subunit in mutant strain compared to the wild strain, and 60 percent increase of Density of the same enzyme's small subunit in mutant strains.



Protein electrophoresis results to changes in key enzymes of lysine biosynthesis (aspartate kinase) showed. Change in the small subunit of aspartate kinase could be due to an increase of the rate of production of lysine in these strains. So that in this mutant strain is capable of producing 38 g L-lysine for 3 days, respectively, whereas the wild type produced in the same period, 13 g L-lysine.

3.Discussion

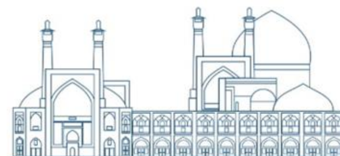
In different strains of Corynebacterium Glutamicum, there is only one type of aspartate kinase enzyme which is controlled through inhibition of concerted feed back of threonine. In mutant strains of bacteria, lysine (final product) does not have inhibitory effect on dehydroepiandrosterone and dehydroepiandrosterone picolinate reductase In addition, no feedback inhibition is done for aspartate kinase or toxic aspartate aldehyde dehydrogenase enzymes aldehyde dehydrogenase, in lysine-producing bacterial strains. Encoding gene of Lysine decarboxylase is altered, whose result is the removal of the mentioned gene in the bacteria that produces large amounts of lysine (20).

Aspartate kinase which is coded by LysC, is the key enzyme in the biosynthesis of lysine and is feedback inhibited by lysine and threonine (10,24). The result of overexpression of LysC, and specifically encoding alleles in the type of aspartate kinase which are not feedback inhibited by lysine, is improved lysine production (21).

table1.bacterial density and the amount of produced lysine before and after irradiation

bacteria population logarithm (cfu/ml)	lysine amount (mg/ml)	irradiation dose (Gy)
8.92	13.70^{cd}	0 (Control sample)
9.07	15.84^{ab}	100
8.99	16.38^{a}	200
8.96	12.26^{d}	300
0.08	1.81	The standard error

Different characters in every column indicated the significance of difference between averages (P<0/05).



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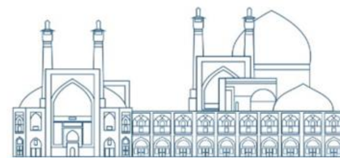

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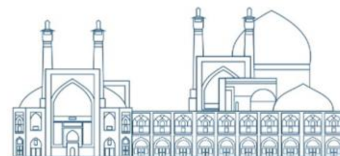

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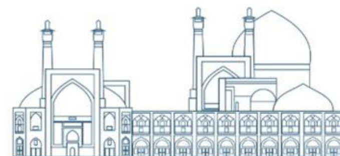

Gynogenesis by gamma irradiation on growth and hematologic indices of the ship sturgeon, Acipenser nudiventris (Paper ID: 1618)

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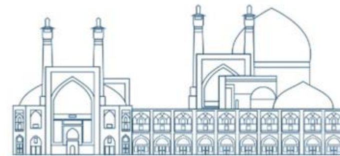
Abstract

The physiological investigation of chromosome-manipulated fish is important. The aim of the study was therefore to investigate the innate immune system in cloned gynogenetic sturgeons. Ten specimens of the gynogenic cloned sturgeon (*Acipenser nudiventris* Lovetsky, 1828) GCS, the hybrid sturgeon [♀ ship sturgeon (*A. nudiventris*) ×♂Persian sturgeon (*Acipenser persicus* Borodin, 1897)], the HS group and the normal ship sturgeon CS (5.60±1.24 g) were transferred into 2000-liter glass fiber tanks with continuous water flow. After a one-year breeding period, the growth and hematological values of the different groups were examined. The result showed no significant difference in the hematological indices between the different groups. Growth traits improved in the GCS group compared to the control (CS) and HS groups. Feed conversion ratio was significantly higher in the control (CS) and HS groups than in the GCS group. The specific growth index was significantly lower in the control (CS) and HS groups than in the GCS group. According to these results, the application of the cloning technique not only had a positive effect on growth improvement, but also had no negative effect on hematological indices; therefore, the use of cloned sturgeons for rearing in sturgeon aquaculture was suggested.

Keywords: gynogenesis, growth indices, haematological indices, ship sturgeon

Introduction

Sturgeons are among the most economically valuable aquatic species native to the Earth's northern hemisphere, particularly the Caspian Sea [1]. This family belongs to the subclass Chondrostei, which separated from the bony fishes about 200 million years ago [2]. Caviar is a valuable commercial commodity, but sturgeon stocks are currently threatened with extinction for a number



of reasons, including loss of spawning grounds, river sand fishing, and illegal and substandard fishing. Therefore, the group of sturgeons is classified as endangered by the International Union for Conservation of Nature (IUCN) [3].

Therefore, great efforts are being made to increase sturgeon aquaculture. Therefore, it is necessary to apply new techniques to increase the efficiency of farming in aquaculture facilities. The use of different sturgeon populations produced by different breeding techniques such as cloning (gynogenesis and androgenesis) and hybridisation (fertilization of the egg of a particular species by the sperm of a different but close species) could improve efficiency in rearing systems [4]. Nuclear technology is one of the technologies that could be used to increase the quantity and quality of aquatic products by producing only female populations. [5-8].

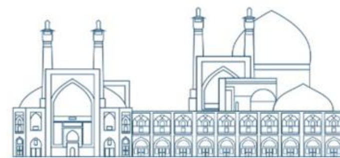
In this technique, the all-female population is generated by irradiating the sperm and destroying their genetic content. Nevertheless, this method is directly effective in homozygous female species (the sex determination system in sturgeon is male zz and female zw.); but the importance of this method in sturgeon species is that this process produces valuable superfemales (WW) in the population, because by fertilizing superfemales with a normal male, they could produce a valuable all-female population [9, 10].

This technique is a type of cloning and causes defects due to inbreeding [11]. This process could have further effects on the immune system, growth and malformations. Since many microorganisms, including opportunistic and pathogenic ones, live in the aquatic environment, studying the effects of inbreeding on the immune system in the F1 generation is very important. Therefore, the aim of this study was to investigate the effects of cloning using gynogenesis on the haematological and growth indices of the ship sturgeon.

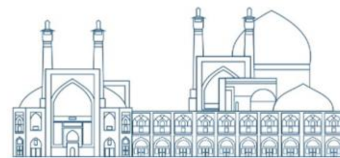
Material and methods

Production of cloned gynogenesis and hybrid sturgeons

Two female ship sturgeons (Acipenser nudiventris) and two male Persian sturgeons (Acipenser persicus) were prepared with hormone therapy for fertilization and the corresponding experiments. GnRH for male broodstock was 10 mg / kg in one step and for female broodstock in two steps 10% in the first injection and 90% after 8 hours [12] at International Caspian Sturgeon Research



Institute. Then the spleen and eggs were extracted. First, the spleen was centrifuged at a speed of 5000 rpm and the sperm fluid was separated. Then the eggs were diluted with sperm fluid (1 sperm per 9 sperm fluids) [9] and were divided into 6, 15 mL conical centrifuge tubes and transferred to the Radiation Application Research Institute in the presence of ice out of reach of water (controlled temperature 4-5 ° C) and the sperm 15 ml Falcon and each tube containing the sperm sample separately with doses of 0,45, 0.6, 0.75, 0.9 and 1.05 kGy of gamma radiation from Cobalt 60 using gamma cells (gamma cell PX-30-ISSIE, Russia). The samples were then sent to the International Caspian Sea Sturgeon Research Institute for fertilisation and incubation. Then 10000 eggs were collected from marine sturgeon breeders through micro-cezarian procedures [13]. To measure the duration and percentage of sperm motility, 300 microlitres of water was added to a slide using a pipette. Then, 50 micrograms of each irradiated sperm and the percentage of motility, motility duration and type of sperm motility at different doses were examined using the CASA system [14, 15]. To assess the maturity of fish oocytes before fertilization, samples were collected and the rate of nuclear migration to the animal pole was measured. GVBD analysis, eggs were collected and fixed in 10% formalin. Then the eggs were removed from the formalin and boiled on an alcohol burner for 2 minutes and poured over with cold water, and then the animal and plant poles were determined. The cut was made with a scalpel from the animal-plant axis. The core disappears during final processing [16]. The incubators were divided for transfer and random distribution of eggs from the different treatments. To induce ploidy in the eggs (cloning), a water bath with dry ice was set at 4°C and the fertilized eggs were subjected to cold shock at this temperature for 10 minutes 40 minutes after fertilization. [17]. The fertilised eggs were then transferred to an incubator with a flow rate of 2 to 3 litres per second, aerated and set at a temperature of 24 °C until they hatched. To determine the optimal conditions, a Control (CS)group with normal fertilization of eggs and sperm without heat shock was considered. Favorable conditions for heat shock of a haploid Control (CS)group resulting from fertilization of irradiated sperm and a normal egg but without heat shock (haploid), and to evaluate the heat shock performance of a triploid group as a heat shock Control (CS)group by applying heat shock with normal sperm. (Invisible beam) was considered. In the hybrid group, normal sperm from Persian sturgeon were fertilised with normal eggs from oyster fish. 1000 eggs were used for each treatment



[16]. After hatching of the larvae, the larvae of the hybrid, Control (CS)and clone groups were treated in tanks with 3 replicates. After demonstrating the success of cloning using the microsatellite markers Afu9 and Afu68, the cloned fish were selected for further information on growth, immunology and haematology at a dose of 900 Gy due to their high efficiency and suitable heritability [18].

Growth indicators

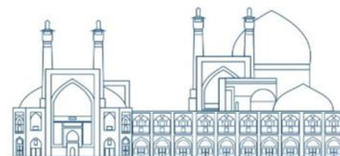
Ten pieces of gynogen of the selected cloned ship sturgeon (*Acipenser nudiventris*) GCS, hybridisation sturgeon [♀ Ship sturgeon (*Acipenser nudiventris*) ×♂Persian sturgeon (*Acipenser persicus*)], HS and uncloned (control) ship sturgeon, groups (5.60±1.24 g), were transferred to 2000-litre glass fibre tanks with continuous water flow and fed a commercial sturgeon diet at 3% of their body weight twice daily. Water factors such as water temperature, dissolved oxygen, pH and temperature were measured weekly by Eutech 300 (Singapore), as were concentrations of ammonia, nitrite and nitrate by Palin test every month. At the end of the breeding period, the growth indices of the fish, including body weight gain, feed conversion and specific growth rate, were determined and compared between the different treatments. (SGR, %/day) =100× (LnW2-LnW1)/T

Feed conversion ratio (FCR) = Feed Intake (G) / Weight Gain

(G(W1, W2 and T Initial Weight (G), Final Weight (G), Number of Days in the Feeding Period.

Blood collection

At the end of the rearing period, after 24 hours of starvation, 3 fish from each experimental unit were randomly caught for haematological parameters (red blood cells, white blood cells, haemoglobin, haematocrit, differential white blood cell count, MCH and MCV). For this purpose, the fish were first anaesthetised and completely dried with clove powder (200 ppm). Blood samples were taken from the tail vein using a heparin syringe. Haematological indices (including MCV, MCH and MCHC, haemoglobin, haematocrit, red blood cell count and white blood cell count) were measured and calculated. For differential white blood cell count, a smear was prepared from each blood sample on a slide and after fixation with methanol, stained with Giemsa solution for



20 minutes and washed. After washing and drying the slides, the type and number of white blood cells were counted under a light microscope. The blood samples were then centrifuged at 5000 rpm for 10 minutes to isolate the serum and stored at -70 ºC.

Statistical analysis

The experimental design was used as a completely randomized design. Normality of the data was checked using the Smirnov kolmograph test. To compare the mean of the data, one-way analysis of variance (ONE-WAY-ANOVA) was performed, and the significance level between treatments was determined by Duncan's test at the 5% probability level. Statistical analysis was performed using SPSS 17 and Excel 2010 in the Windows environment to plot the graphs.

Ethics

Ethical permission (Consent Ref Number 7507001/6/36) for the research obtained from the Ethics Committee of the University of Tehran.

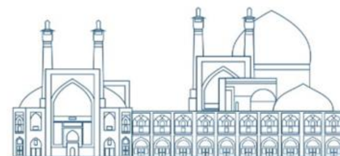
Results

Growth indices

The result showed that growth indices were improved in the groups of GCS compared to the other experimental groups. Final weight, FCR and SGR showed statistical differences between Control (CS)and HS groups. FCR and SGR showed no statistical differences between HS and the Control (CS)groups. There were no statistical differences in survival between the experimental groups (Table 1).

Table 1. Comparison of growth performance (mean ± SD) and survival rate in the cloned ship sturgeon (GCS), hybridation sturgeon (HS) and uncloned (control) ship sturgeon, groups (n =3).

	GCS	HS	Control
Initial weight (g)	5.45±1.02	5.71±1.26	5.65±1.45
final weight (g)	460.54±11.23^{c}	430.51±10.42^{b}	390.42±8.42^{a}
FCR	1.03±0.23^{a}	1.21±0.42^{b}	1.24±0.53^{b}
SGR (% day - 1)	1.92±0.42^{b}	1.87±0.22^{a}	1.83±0.64^{a}
Survival%	99.42±0.98	99.82±1.23	99.79±0.85



The table shows values of mean ± SD of three experimental repetitions. Values within the same row with different superscript differ significantly ($p<0.05$)

Hematological indices

The results showed that the white blood cell count had no significant difference between the Control (CS)and GCS groups, the index was increased in the HS group compared to the other group ($p > 0.05$). There was no statistically significant difference between the erythrocytesRBC and haemoglobin in the Control and GCS groups and the haematocrit in the different experimental groups ($p< > 0.05$). The erythrocytes and haemoglobin in the HS group showed the lowest value compared to the other groups. The haematocrit showed no statistical difference between the GCS and HS groups. There were no significant differences in the MCV, MCH and MCHC indices between the experimental groups GCS and Control (CS)group ($P > 0.05$). (Table 2).

Table 2. Performance of haematological indices (mean ± SD) in the cloned sturgeon (GCS), hybrid sturgeon (HS) and non-cloned sturgeon (control) groups (n=3).

	GCS	HS	Control
WBC	14353±98.22^{b}	14100±110.51^{a}	14291±112.54^{b}
RBC	964000±2230.21^{b}	800000±2365.04^{a}	979500±3102.12^{b}
Haemoglobin	7.74±0.42^{b}	6.42±0.55^{a}	7.3±0.29^{b}
Haematocrit	16.9±1.53^{a}	17.1±1.02^{a}	17.4±1.29^{b}
MCV	165.86±15.21^{a}	211.45±21.22^{b}	162.49±18.54^{a}
MCH	87.91±7.41^{b}	55.025±3.54^{a}	78.63±8.01^{b}

The table shows values of mean ± SD of three experimental repetitions. Values within the same row with different superscript differ significantly ($p<0.05$).

Complete blood count

Lymphocyte levels in the HS group were higher than in the other groups ($p > 0.05$). The percentage of eosinophils in the different groups showed no statistical difference between the groups. The highest percentage of neutrophils was observed in the CS group and there was a statistical difference between the other groups. The lower percentage of monocytes was observed in the HS group. (Table 3).

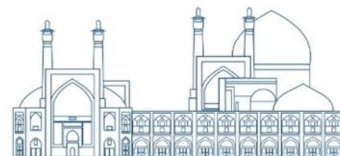


Table 3. Differential Leucocyte Count (DLC) (mean ± SD) in the cloned ship sturgeon (GCS), hybridation sturgeon (HS) and uncloned (control) ship sturgeon groups (n =3).

	GCS	HS	Control (CS)
Neutrophil	48.5±2.12[b]	45.4±1.89[a]	49.3±3.01[a]
Lymphocyte	37.95±2.99[a]	46.5±3.12[b]	35.5±2.89[a]
Monocyte	7.23±1.12[b]	2.53±0.89[a]	8.42±1.22[b]
Eosinophil	5.06±0.21[a]	5±0.29[a]	5.5±0.34[a]

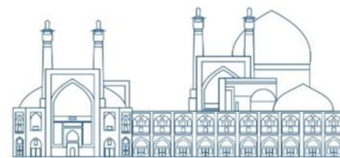
The table shows values of mean ± SD of three experimental repetitions. Values within the same row with different superscript differ significantly ($p < 0.05$)

Discussion

Gynogenesis is a type of reproduction in which the presence of sperm is only necessary to initiate the growth of a new organism, but the DNA of the father is not transferred to the zygote. As a result, the offspring of genetically modified fish have only maternal genetic material [19]. This phenomenon occurs naturally in some fish species such as common carp (*Cyprinus carpio*) or pond fish (*Carassius auratus gibelio*) [20]. In Iran and other countries, studies have been conducted with sturgeon material, most of them using UV light. In a study on miotic fertilisation in ship sturgeon using UV-irradiated sperm from Siberian fish, the results of this study showed that successful geogenesis using Siberian sperm was 60% possible in ship sturgeon [12].

In another study, the eggs of female albino sturgeon were irradiated with sperm that had been irradiated with normal male sterilised UV light. In that study, the percentage of hatching was less than 5% [19]. Other species, including Siberian sturgeon *Acipenser baerii*, white sturgeon *Acipenser transmontanus*, shortnose sturgeon *Acipenser brevirostrum* and sturgeon *Acipenser ruthenus*, may have a ZW sex determination system in which females may be heterogamous. Induced [21, 22]. Some offspring are WW 'superfemales' that, when mated with normal males (ZZ), produce fully female (WZ) offspring. The sex chromosomes of sturgeons have not been identified. Therefore, "supermodels" can only be detected by gonadal analysis. In this study, we used fish that were 100% inherited from their mother [18].

In the study, the different parameters between the different groups evaluated changes in haematological factors mentioned in cloned treatments could be due to genetic degradation and



lack of adequate synthesis of blood cells, which during life stages can increase the sensitivity to weaken the immune system.

In this study, the MCV and MCH indices showed a significant difference between the different groups. However, these indicators did not show a significant difference in other treatments compared to the control group. Low MCV and MCH values in triploid fish as well as in hybrid fish indicate a type of anaemia in these fish, possibly due to genetic defects in the fish groups.

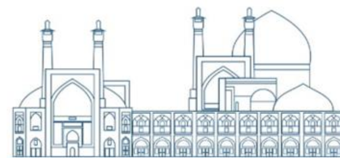
Differentiation of white blood cells, including neutrophils, monocytes and lymphocytes, shows a significant difference between all groups, which may be due to genetic differences between the different hybrids, clones and control groups. Considering that any chromosome manipulation may change the physiological parameters but not the biochemical indicators. Moreover, the primary purpose of chromosome manipulation in fish is to increase yield. However, this increase may be accompanied by changes in immunology, haematology, physiology, etc.[23].

This is because fish have a more basic immune system than other vertebrates, and on the other hand, the aquatic environment as an aquatic habitat is a suitable environment for the transmission of pathogens. Safety is doubly important in the chromosome manipulations of this group of vertebrates [24, 25].

The results of the present study showed that cloning with gamma rays on haematology (haematocrit, haemoglobin, red cell volume, MCH and MCHC) Different experimental treatments are effective, but since this is the first study done on this fish, it cannot be said with certainty that these changes are due to irradiation and cloning, or that they are due to specific environmental and breeding conditions. Note that cloning and hybridisation are among the methods used to increase yields in a breeding period [10].

However, in this group of fish, the effects of this chromosomal alteration on haematological indices should be investigated. In many cases, the sensitivity of cloned fish is increased compared to other fish, and to achieve maximum efficiency, normal and special conditions should be provided for this group of fish [23].

Since the supermaterial of this group of fish is used as breeding line in the next generation and on the other hand cloned fish resemble identical twins, it is very important to study blood indicators and diagnostic immunology in them.



In this study, the sturgeon hybrid group showed changes in haematological parameters including haematocrit, haemoglobin and red blood cells in this group compared to other groups (Table 1) that this may reduce the resistance to changing environmental conditions [10, 26].

The results of this study show that this method of chromosome manipulation affects haematological and immunological indices, but the reason for these changes is not due to materiality or other biological conditions and is not well understood. In some cases, these changes were positive, and in some cases in high dose groups such as 1050 Gy these changes were negative, so this method of chromosome manipulation can be used without changes in immunological and haematological parameters. However, sufficient accuracy, experience and knowledge in selecting the desired dose are the main problem in introducing cloning with this method [27].

Conclusion

This method can be used to develop this part of aquaculture, but only if the effects of inbreeding on various systems of the body, including haematology, are assessed before use. Research can be conducted on other sturgeon species as a stimulus for further work. New generation sequencing for these or other species can be used to find markers. In addition, it is possible to purify the remaining fish for the next generation and use the technique of mating and lineage assignment in the field of breeding this valuable species to create a lineage.

Acknowledgement

The authors would like to thank all colleagues at the Nuclear Science and Technology Research Institute, especially the Nuclear Agriculture Research school, who helped us carry out this research project.

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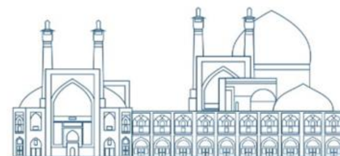

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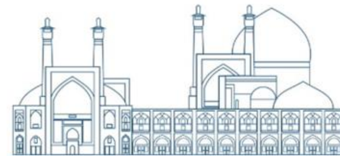

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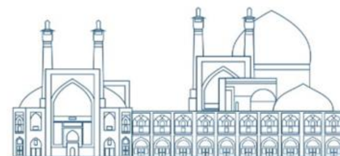

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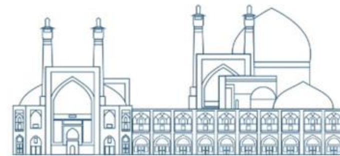

Gamma-irradiated gynogenesis in rainbow trout and sex determination by multiplex PCR (Paper ID: 1619)

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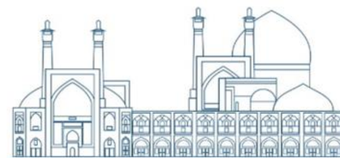
Abstract

The sperm were collected and mixed with a dilution solution of glassine and irradiated with cobalt 60 gamma rays at a dose of 450 Gy. Fertilization was performed with 24000 oocytes in dry form by mixing egg and sperm. After washing, the fertilized eggs were placed in the incubator for 20 minutes and then brought to a temperature of 26-28 degrees for 10 minutes to induce a temperature shock. After hatching, the eggs were fed every 2 hours and the number of losses was counted daily. Genomic DNA extraction of samples from specific female and male and gynogenic larvae was performed in the vicinity of the enzyme proteinase K. Subsequently, sex was determined by the multiplex PCR method using the primers SDy as the male-specific gene and 18S as the reference gene. The results showed that two bands were clearly formed in the males, with the upper one related to 18s and the lower one related to SDY. This band, which is related to SDy, is also not observed in the females' groups. The results also show that the band associated with the male-specific segment SDY was not observed in any of the larvae belonging to the gynogenic group.

Keywords: Gynogenesis, Sex determination, Sdy gene, Rainbow trout

Introduction

Fish meat is the best and most harmless protein of animal origin (far more useful and less dangerous than red meat and chicken meat), which can be increased in quantity and quality through proper propagation. One of the problems in the aquaculture industry is early sexual maturity [1-3].



In rainbow trout, the phenomenon of sexual maturity occurs much earlier in males than in females (between 6 months and a year in males, but at least 18 months in females), and the possibility of males maturing during the breeding process is very high. It is high, whereas the female rarely matures during this period [4-6]. Therefore, the use of whole populations of female produced in different ways increases production efficiency. Breeding only one species in fish farms allows this species (preferably the female species) to be bred in a calm and stress-free environment (due to the presence of opposite-sex pheromones in the aquatic fish's living environment). should be paid for and the result will naturally be good efficiency and weight gain. Another advantage of producing all-female populations is their use as a productive herd, because in breeding and mass reproduction, the number of female breeders is greater than that of males [7].

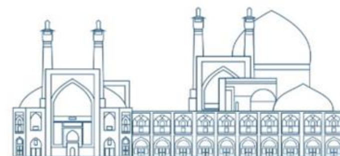
The sex-determining genes are the most important genetic switches that control the cascade of sex differentiation in the gonads and lead to the development of ovaries or testes. In mammals, which have an XX/XY system, the Sry gene (sex-determining region of the Y chromosome) was identified in the early 1990s (Sinclair et al. 1990) as the first sex-determining gene in vertebrates [8, 9].

The recent discovery of sdY as the main sex-determining gene in rainbow trout [10] now makes it possible to discuss this question anew. This is particularly important because the sdY gene is included in the genomic sequence of the rainbow trout Y chromosome (OmyY1), which shows strong similarities to that of the Chinook salmon, Oncorhynchus tshawytscha (OtY3) [11] In this study, our aim was to sex determination of all female rainbow trout gynogenized by gamma irradiation with the male-specific Y chromosome gene sdY.

Material and methods

Trout Breeders

Female breeders of rainbow trout were evaluated in different provinces. Then, the female offspring from a farm in Shahrekord, which had approved offspring and were healthy in appearance, were selected for propagation. At first, sperm was collected from male progenitors in the research institute. At this stage, sperm from 5 pieces of male breeding fish (healthy in terms of disease and



appearance and average weight 951±210 grams) and after evaluating the sperm quality with light microscope, the irradiated sperm on ice were transferred to the female breeding workshop in Shahrekord.

Collection of eggs and sperm from farmed fish, irradiation of sperm, quality testing of sperm and eggs from brood trout.

The spermatozoa were collected and diluted with a glassine solution (CaCl2.2H2O 0.735 g/l and NaCl 7.705 g/l with a pH of 7.7), poured into a 50 ml plastic falcon and irradiated with gamma rays emitted by Cobalt at 60 with a dose of 450 Gy. The irradiation was carried out at the Nuclear Agriculture Research Institute at a dose rate of 0.00105 Gy per second. After checking the percentage of sperm motility and the type of motility, suitable sperm were selected and mixed together and then transferred to nuclear agriculture research school.

Fig 1. Transfer of sperm on ice for reproduction

Eggs were obtained from 13 mature breeders (healthy in terms of disease and appearance and average weight of 2,894 ± 210 grams) and fertilization with 24000 oocytes for the entire female group in dry form by mixing egg and sperm (with a ratio of 0.5-1 3 sperm per 1000 oocytes) was performed. For sperm activation, hatchery water (9°C temperature) was used for 2-3 minutes [4, 10, 12, 13]. After washing, the fertilized eggs were placed in the incubator for 20 minutes and then brought to a temperature of 26-28 degrees for 10 minutes to create a temperature shock (the duration of fertilization is about 35 minutes). 300 eggs for the triploid treatment (without

irradiation) and 300 eggs for the haploid treatment (without heat shock) were considered to control the temperature and radiation conditions.

Transfer of the eggs to the incubator

The fertilized eggs were incubated at 9 degrees Celsius and after they hatched under optimal conditions, they were transferred to the Agricultural Research Institute. After isothermalization, the hatched eggs were transferred to the vertical incubator (Figure 2).

Fig 2. Egg transfer to the incubator

During incubation and hatching of the larvae, the quality characteristics of the incubation water were examined and the unfertilized eggs were isolated. In addition, unfertilized eggs were removed from the incubator daily.

Rearing and feeding larvae

After the larvae had absorbed two thirds of the yolk sac (Figure 3) and started to swim actively, the vertical incubators were transferred to the horizontal boxes and feeding with starter food was started.

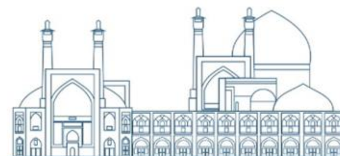
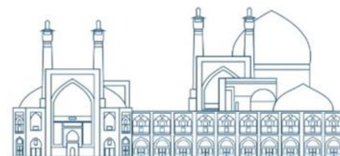


Fig3. Larva with yolk sac

Feeding took place every 2 hours. The number of victims was counted daily. The central aerator of the blower is used for the aeration of the fish larvae rearing system, as is the inlet water overflow of each fish larvae storage tank. In this rearing system, the amount of oxygen was monitored daily. Feeding took place daily and 3-8 times per day depending on the appetite of the fish. The initial feeding was done with size food up to a weight of 0.3 grams. The juvenile fish from 0.3 to 1.5 grams were fed with size 2 and 3 food. After feeding, the fish were released from the vertical incubators into the troughs and kept there until they were transferred to the Alborz Caspian fish farm.

Sampling at the breeding site during the course and conducting molecular tests

To extract the genomic DNA of the samples, chemical digestion was first performed at 56 degrees for 12 hours in the presence of the enzyme proteinase K according to the instructions of the purchased Pars Tous kit (Figure 4). After shaking the samples and adding the homogenization buffer, sedimentation and washing operations were performed using a centrifuge at 12000 rpm, and finally the quality and quantity of DNA samples were measured using an agarose gel (Figure 5) and the Nanodrop device, and samples of suitable quality (average concentration 50-100 ng/µl) were selected. Subsequently, sex was determined by preparing the molecular marker SDY (Table 1).



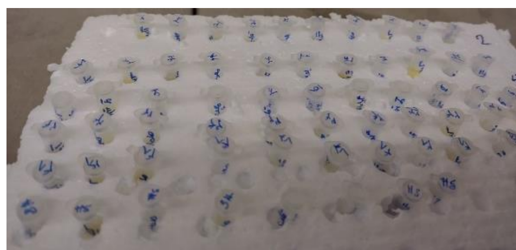


Fig 4. Samples of rainbow salmon fins after digestion at 56°C.

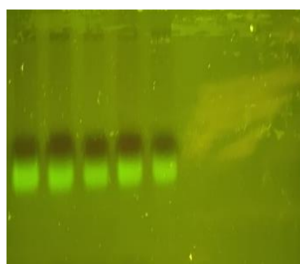


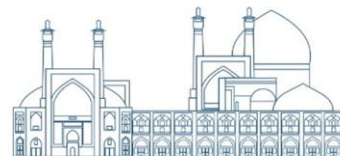
Fig 5. Measuring the quality of extracted DNA on agarose gel

In this study, the multiplex PCR method was used with sdY primers as a specific gene for male fish and 18S as a general gene [10]. Each reaction in each microtube contains 1 sdY primer (sdY E2S1 and sdY E2AS2; 1 µM of each sdY roundtrip primer and 18S and 10 µM Taq master mix, 3 µL sample DNA (male and female samples), 7 microliter distilled water were already in the PCR tube.

Table 2. Primers used for Multiplex PCR test to identify the success rate of gynogenesis

Related Gene	Primer	Sequences
SDY	sdY E2S1 F	5’CCCAGCACTGTTTTCTTGTCTCA3’
	sdY E2AS2 R	5’CTGTTGAAGAGCATCACAGGGTC3’
18S	18S S F	5’GTYCGAAGACGATCAGATACCGT3’
	18S AS R	5’CCGCATAACTAGTTAGCATGCCG3’

The PCR conditions were as follows: Denaturation for 3 min at 95 °C, followed by 35 cycles of denaturation at 95 °C for 30 s, 60 °C annealing for 30 s, and extension at 72 °C for 30 s, with a



final extension of 3 min. The temperature was 72 degrees Celsius. The PCR products were electrophoresed on a 2% agarose gel.

Statistical analysis of the information

The normality of the data is checked using the colmograph Smirnov test. The analysis of variance (ONE-WAY-ANOVA) is used to compare the mean data and the significance level between treatments is determined by the Duncan test at a probability level of 5%. Statistical analysis is performed using SPSS 19 software in the Windows 10 environment.

Results

Percentage of fertilization and survival

The results showed that the percentage of fertilization was statistically significant between the groups ($p < 0.05$). This index was 0% in the haploid group and was significant compared to the other groups ($p < 0.05$). This indicates the appropriate destruction of the 450-degree dose. In the control group, the fertilization rate was 83%, indicating good quality of oocytes and sperm in the above reproduction. The results showed that in the dose of 450 Gy and triploid, the percentage of fertilization decreased and it was significant compared to the control group ($p < 0.05$) and the percentage of survival in the dose of 450 Gy and triploid was not significant ($p < 0.05$), but there was a significant decrease. compared to the control group ($p < 0.05$) (Charts 1 and 2).

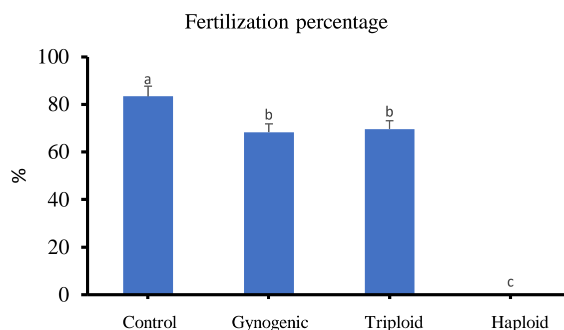
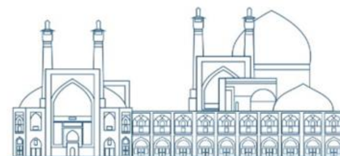


Fig 6. Fertilization percentage of different experimental groups



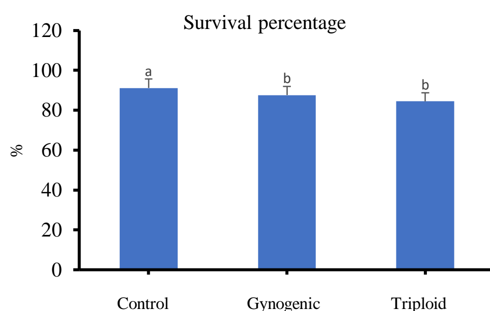


Fig 7. Survival percentage of fry of different groups

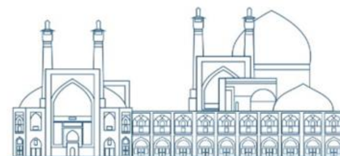
The results show that the number of eggs in the female spawned group at the eyed stage has decreased compared to the control group ($p<0.05$) (Table 2).

Table 2. Number of eggs in the initial stage, hatching, hatching and survival

	Egg	Eyed Egg	Hatched	Larvae Survival	Fry Survival
Gynogenic	٢٤٠٠٠	١٦٥٢٠	١٦١٢٠	١٦٤٢	١٦٠٠٠
Haploide	٣٠٠	٠	٠	٠	٠
Triploide	٣٠٠	٢٠٨	٢٠١	١٨٤	١٨٠
Control	٣٠٠	٢٥١	٢٤٢	٢٢٠	٢١٤

Growth

The results show that there is no significant difference in growth up to the fourth biometric trait ($p < 0.05$), but a slight increase in growth from the third biometric trait onwards was observed in the female and triploid group compared to the control group, which was not statistically significant ($p < 0.05$). (Chart 3).



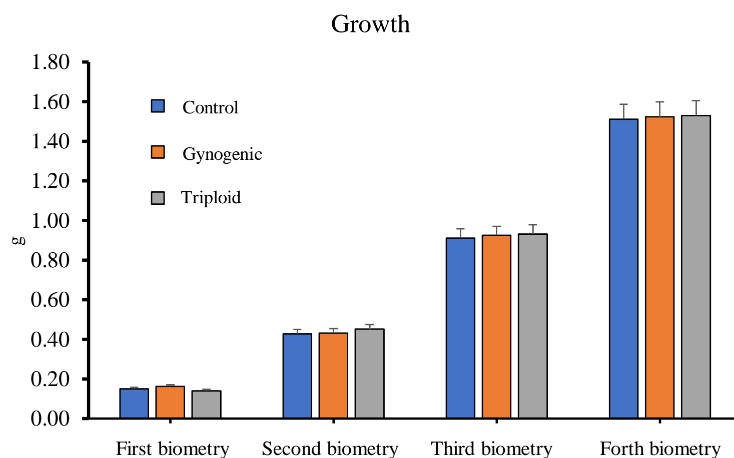
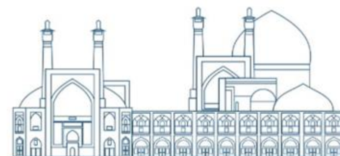


Fig 8. The average growth of fry of different groups in 4 periods of weighing, the first period of biometrics 1 week after yolk sac ingestion, the second period 2 weeks after yolk sac ingestion, the third period 3 weeks after hatching, the fourth period 4 weeks

Molecular analysis

The results show that 2 bands have clearly formed in the second and third rows of the males in the 13th image on the left, of which the upper one is related to 18s and the lower one to SDY. This band, which is related to SDy, is also not observed in the material groups in the fourth and fifth rows. The results also show that in none of the larvae belonging to the feminized group, the band associated with the specific part of the male sex, SDY, was observed (Figure 9, right).



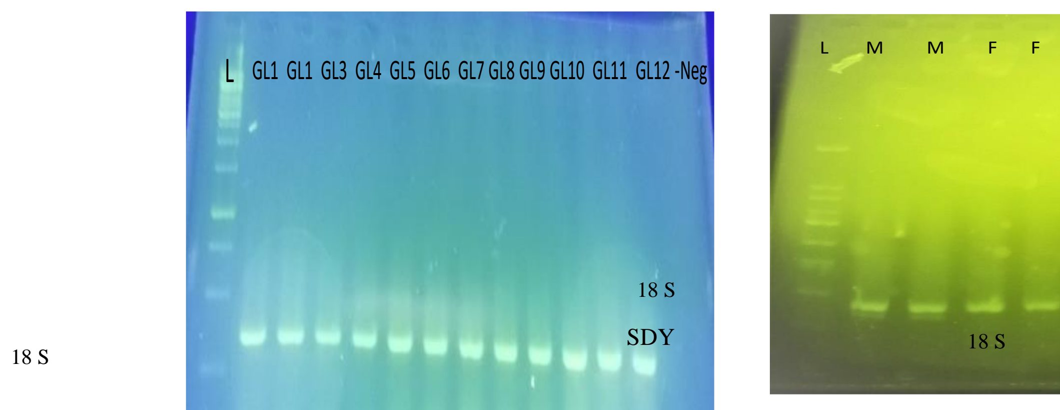
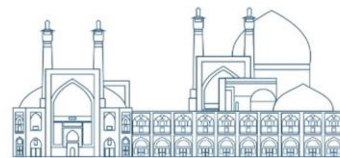


Fig 9. The image related to the PCR product of the feminized group (right), pre-specified female and male samples (left) (ladder=L, female=F, male=M, female generated larva=GL)

Discussion

Meiosis Gynogenesis of trout or successful crossbreeding with second polar globules and optimization of triploid function in this species depends on the time of onset of heat shock, which is basically about 10-15 minutes after fertilization in which triploid conditions can be created. By creating optimized conditions, meiotic gynogenesis can be produced [14]. Due to lower efficiency of mitotic gynogenesis [15, 16]. In this study, the meiotic form was used to produce a population of all females. In previous studies, high yields of meiosis and heat shock were observed as a factor in ploidy manipulation in Atlantic salmon [16].

In this study, the heat shock-induced triploid group was not significantly different from the materialized groups in terms of growth indicators, and since the percentage of hatching and survival was significantly reduced in this group, the use of this group is limited. However, it is possible that the fish that survive and provide the normal living conditions for this category of fish, which requires much higher quality conditions than normal fish, have good growth and survival rate, which is consistent with other information from other studies [۱۸,۱۷]. It can be concluded that it is necessary to use appropriate methods for self-sufficiency and production of hatching eggs and unisexual female indigenous fish in the country and also to consider the importance of production of free-running fish in the Caspian Sea. The application of this method in trout



spawning is feasible and practical, and the dose of Gy 450 in this type of fish is suggested as an appropriate dose for the application of this method.

Conclusions

The results show that the application of sex determination using the multiplex method and the SDY gene is proposed as a method to determine the sex of gynogenic salmon.

Acknowledgement

The authors would like to thank all colleagues at the Nuclear Science and Technology Research Institute, especially the Nuclear Agriculture Research school, who helped us carry out this research project.

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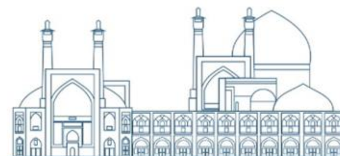

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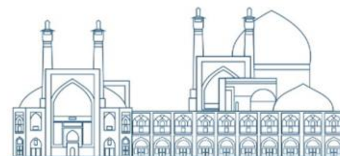

Evaluation of the destruction of irradiated Bacillus subtilis DNA in the freeze-dried state using the comet assay (Paper ID: 1622)

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Abstract

The aim of this study was to evaluate the effect of gamma radiation on the integrity of the DNA structure of *Bacillus subtilis* by the comet assay. *B. subtilis* ATCC 23857 single colonies were transferred to a test tube containing 5 ml broth and incubated at 37°C for one night. The cells were collected in 50 ml tubes and these mixtures were transferred to freeze-drying overnight. The freeze-dried form of the bacteria was irradiated with doses of 1-20 kGy. Based on the irradiation dose, the D10 value was calculated using the above formula and the inactivation dose. DNA changes in the irradiated bacterium *B. subtilis* evaluated by the comet assay at different gamma radiation doses. In this study, the D10 value was 16.32 and the inactivation dose was 18 kGy. In the study, *B. subtilis* were examined with different doses, including the final radiation dose. The results show that the bacterial cells have up to 80% of DNA tails at the inactivation dose of 18 KGy, indicating an appropriate radiation dose for this strain. The results of this study indicate that the use of gamma rays as ionizing radiation effectively destroys the activity of Bacillus subtilis endospore-containing bacteria in freeze-dried form at a dose of 18 KGy. This dose is recommended for the production of the freeze-dried form of this strain.

Keywords: gamma irradiation, comet assay, bacillus subtilis

Introduction

The inactivation of bacterial cells is carried out for many purposes, including disinfection, the production of vaccines against pathogenic bacteria and the synthesis of para-probiotics for beneficial bacteria [1].

Despite numerous studies on the positive effects of probiotic microbes, there are concerns about their use. Some of these concerns include survival in the product/food, different colonization patterns and resistance in the gut, and even the possibility of acquiring virulence genes from pathogenic bacteria through horizontal gene transfer.

When probiotics and dominant organisms are in sufficient contact, gene transfer may even occur in the gastrointestinal tract. Although the composition of the gut microbiota may be different for each individual, microorganisms act as a barrier between the host and the environment and play an essential role in protecting the host from pathogens and harmful nutrients [2].

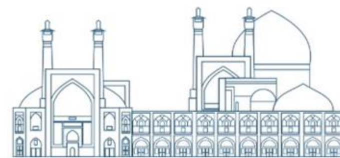
However, genes can be transferred between gut microbiota, gut microbiota and different pathogens or between pathogens to adapt to a constantly changing environment. The transfer of genes between bacteria is well documented and research has focused on horizontal gene transfer, e.g. the spread of drug resistance [3].

However, it is possible that the same properties that make a good probiotic are not only inherited by other organisms but also transferred to other organisms, including pathogens. There is evidence of horizontal gene transfer between probiotics and gut bacteria as well as between probiotics [4].

These issues, as well as new information on the beneficial effects of non-living probiotic microbes that resemble living species, have drawn attention to the use of non-living probiotics, known as paraprobiotics [5].

Various methods such as heating and the use of chemicals can be used to inactivate bacteria. More recently, the irradiation method has been used to inactivate cells and produce paraprobiotics. However, the production of paraprobiotics by ionizing radiation has its limitations and there are very few studies in this field. However, it has been shown that paraprobiotics produced with gamma radiation have at least the same effect as paraprobiotics produced with thermal methods. [6,7].

Studies have shown that the first target of ionising radiation in cells is nucleic acids [8]. Ionizing Irradiation affects DNA in both direct and indirect ways. In the direct method, electrons are removed from the DNA, which leads to fundamental changes by breaking covalent bonds at critical points or creating new bonds between nucleotides. In the indirect method, the DNA is damaged by free oxygen radicals (ROS) [8]. Free oxygen radicals cause the oxidation of purine and pyrimidine



bases, cause breaks in one or both strands, create sites without bases, and form cross bridges between DNA and protein [8-12]

Although inactivation methods using ionizing radiation such as gamma radiation are not available for all of the laboratories and industries, but are more efficient compared to other methods as the shape of the bacterial cells and their immune response are preserved [13-15].

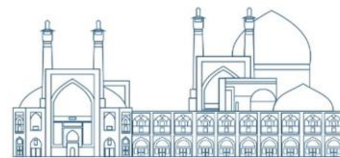
Proper inactivation of bacterial cells requires inactivation of the genome and prevention of growth on the culture medium. Therefore, the most important step in inactivation is to determine the appropriate ra [13,16,17]. This is because low doses are not able to completely inactivate the genome. High doses, on the other hand, destroy the morphology of the bacterial broth and lead to a reduction in the ability to develop beneficial para-probiotic properties [18,19].

Freeze-drying is a common method of incorporating probiotics into food. However, the viability of freeze-dried probiotic bacteria is impaired during processing and storage. Freeze-dried probiotic organisms are protected by the addition of cryoprotectants, and the identification of protective agents that improve cellular survival during storage and food application is the main challenge [20,21].

To use the inactivated bacterial cells with radiation, the method of irradiation of living cells or irradiation in freeze-dried form can be used. Due to the low availability of radiation sources and the higher concentration of radiation in the freeze-dried solid state, this form is preferred for the mass production of inactivated bacterial cells. Of great importance in the inactivation of these cells with gamma radiation is that they cannot regrow, as evidenced by a corresponding disruption of the genetic content [22,23].

Bacillus subtilis, Escherichia coli, and Saccharomyces cerevisiae are microorganisms that have been thoroughly investigated for various biotechnological tasks. These organisms and their cellular associations serve as models for interpreting and understanding the cellular physiology of other microbes [24-26].

Bacillus species can develop into an endospore, a dormant life stage that is highly resistant to heat, chemicals, and desiccation and can survive for an extraordinarily long time [27-29]. Therefore, the use of this species for deactivation in the freeze-dried state with gamma rays is very important.



In the past, it has been difficult to assess DNA damage due toof the high compaction of nuclear chromatin [9,30,31], and one of the main methods for determining DNA damage is single cell gel electrophoresis assay SCGE, (comet assay) which is based on the measurement of breaks. is a DNA strand [32,33], and its use in studies related to bacteria has been limited to recent years.

In recent decades, single cell gel electrophoresis (SCGE) has become one of the standard methods for DNA damage assessment used in genotoxicity testing, human biomonitoring, molecular epidemiology and ecotoxicology, and basic research on damage and DNA repair [34,35].

The percentage of DNA breaks is the best way to describe the frequency of DNA breaks, which is clearly reflected in the extent of damage revealed by the comet tail [36-38].

After irradiation, the microbial cells retain functional gene expression and metabolic activities, but do not initially proliferate due to nucleic acid damage [39]. Previous work has shown that irradiated bacteria mobilize a protective immune response, a T-cell response, more effectively than bacteria killed by heat or formalin [15,40-42].

Due to the formation of free radicals and peroxides as a result of bacterial irradiation, it is not possible to use bacteria in the liquid phase for irradiation [43]; moreover, for the mass production of paraprobiotics, bacteria must be irradiated in a freeze-dried state.

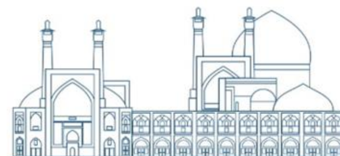
Microbes preservation methods like freez drying are mainly designed for culture collections where only a small proportion of cells need to survive, to improve the survival of the preserved cells [44].

The aim of this study was to evaluate the effect of different doses of gamma radiation on the inactivation of the freeze-dried form of *Bacillus subtilis* as a model microorganism for the production of paraprobiotics and the effect of radiation on the integrity of the DNA structure of this bacterium by the comet assay.

Results

Determination of the inactivation dose

In this study, the freeze-dried form of *Bacillus subtilis* bacteria was used and then irradiated. D10 value was 16.32 in this research .The results showed that a dose of about 18 kGy was eligible for inactivation by gamma radiation (Figure 1).



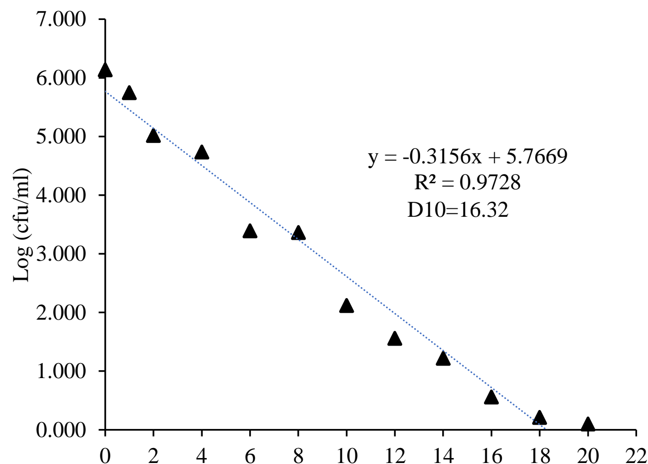
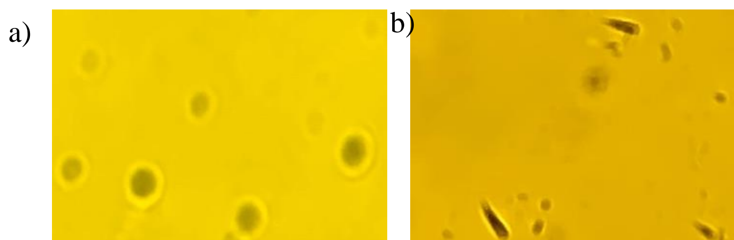


Figure 1. resistance curve of *Bacillus subtilis* against gamma radiation

Comet assay

The results show that cells without radiation do not have comet tail DNA (Figure 2a). In addition, the results show that at a dose of 18 KGy, most of the comet tail is associated with it (Figure 2b). The results show that as the radiation dose increases, the amount of comet tail increases (Figure 2c).

a) b)



c)

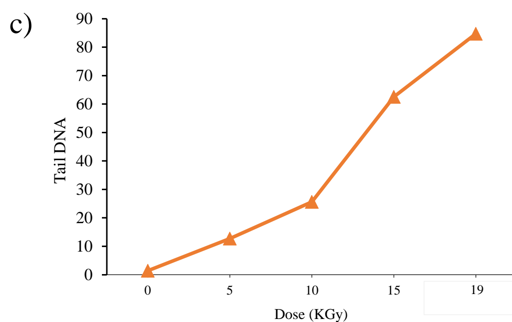


Figure 2. a) The shape of tail DNA in the absence of radiation b) The shape of DNA in a dose of 18 KGy Irradiation. c) It shows the relationship of comet tail with irradiation dose

Discussion

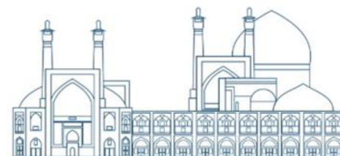
The most important goal of ionizing radiation is to destroy the DNA molecule of microorganisms. Any alteration or destruction of the DNA molecule can cause the cell to lose its ability to survive or reproduce. Biological changes can be attributed to direct and indirect effects of radiation. The direct effect is caused by the removal of electrons from the DNA, which damages it (double-strand or single-strand breaks, chemical modification of bases and cross-linking with matrix proteins or associated nucleotides). The indirect effect is the result of the attacks of radiolytic radicals (hydrogen and hydroxyl), which are already formed during the ionization of water molecules in the cell. The extent to which radiolytic radicals are responsible for damage depends on the number of DNA reactions. Any factor that can prevent reactions, such as the immobilization of radicals by freezing food, reduces the indirect effect of radiation [45,46].

In recent decades, single cell gel electrophoresis (SCGE) has become one of the standard methods for the assessment of DNA damage. It is used in genotoxicity testing, human biomonitoring, molecular epidemiology, ecotoxicology and basic research on damage and DNA repair [47].

It is proposed to express the frequency of DNA breaks as a percentage of the DNA sequence, since the damage caused by the DNA sequence is clearly visible [48]. In the above study, Comet was evaluated at different doses including the final radiation dose of *B.subtilis.*

Irradiation is considered an effective method for inactivating bacteria [49,50]. The results show that bacterial cells inactivated by gamma radiation have up to 80% of the DNA sequence, indicating the appropriate radiation dose for this strain.

Irradiated microbiological data at doses as high and as low as 10 kGy and found no toxicological evidence even at these high doses and concluded that such a limit is not necessary [51]. The effect of radiation on microorganisms varies depending on the strain, environment, temperature and growth phase of the species [52]. The relative resistance of microorganisms to radiation is divided into sensitive categories, whereby bacteria with less than 1 KGy are classified as highly sensitive to ionizing radiation and those with more than 10 KGy as resistant [53].

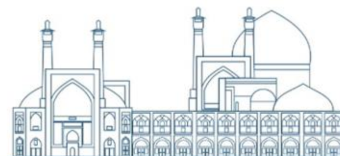


B. subtilis spores that are genetically identical but sporulate in different environments show very different resistance to radiation [53]. Most strains of microorganisms are stored in our laboratory in freeze-dried ampoules. If the cell concentration before freeze-drying is 10^6-10^{10} cells/ml and the cells survive the drying process, the viability of a strain can be maintained for more than 20 years. It has been reported that freeze-dried bacteria can survive for up to 35 years[44]. In this study, the inactivation dose of 18 kGy was calculated for Bacillus subtilis bacteria in lyophilised form.

The most important goal of ionising radiation is the destruction of the DNA molecule of microorganisms. Any alteration or damage to the DNA molecule can cause the cell to lose its ability to survive or multiply. Biological changes can be attributed to direct and indirect effects of radiation. The direct effect is caused by the withdrawal of electrons from the DNA, which damages it (double-strand or single-strand breaks, chemical alteration of bases and cross-linking with matrix proteins or associated nucleotides). The indirect effect results from the attacks of radiolytic radicals (hydrogen and hydroxyl), which are already produced during the ionisation of water molecules in the cell. The extent of the damage caused by radiolytic radicals depends on the number of DNA reactions. Any factor that can prevent reactions, such as immobilising radicals by freezing food, reduces the indirect effect of radiation[54] .

In recent decades, single cell gel electrophoresis (SCGE) has become one of the standard methods for DNA damage assessment used in genotoxicity testing, human biomonitoring, molecular epidemiology and ecotoxicology, and basic research on damage and DNA repair. Comet results can be divided into four types: Type 0, Type 1, Type 3 and Type 4 when counted manually. These categories are classified based on the extent of DNA damage. Type 0 means that no DNA damage has occurred. Type 1 means that only minor DNA damage has occurred. Type 3 indicates extensive DNA damage and type 4 indicates complete destruction of the DNA[55].

It is suggested that the frequency of DNA breaks is best expressed as a percentage of the DNA sequence, as the damage caused by the sequence is clearly visible. In the above study, the results showed that the use of the effective dose of inactivation led to the destruction of the genetic content of the Bacillus subtilis bacterial cells in the freeze-dried state. From the beginning of irradiation to the inactivation dose, the amount of tail formation in the comet index increased, so that the lowest value was observed at a dose of zero and the highest at an inactivation dose of 18 kGy.



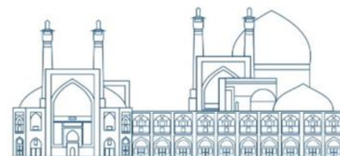
In this study, gamma rays were used to inactivate Bacillus subtilis bacteria. Common methods for inactivating bacteria include thermal inactivation, UV inactivation, formalin inactivation and ultrasound, and there is no information on inactivation by gamma rays. These methods result in the death of the microorganisms and each method has a different mode of inactivation. However, it should be noted that an inactivation method that can preserve the properties of the microorganisms for inoculation and synthesis of paraprobiotics is more appropriate[56]. Thermal inactivation can affect several components of the cell structure of microorganisms that are of particular importance: Disruption of membrane integrity, loss of nutrients and ions, ribosome accumulation, DNA strand breaks, inactivation of essential enzymes and protein coagulation.

However, the effectiveness of heat inactivation can be influenced by several factors, including the type of microorganisms, the growth medium used, the growth stage, the type of heating, whether the microorganisms are in vegetative or spore form, the pH of the medium and the water activity, to name a few. Ultraviolet inactivator falls as non-ionising radiation in the electromagnetic spectrum from 200 to 400 nm and can effectively inactivate a wide range of bacterial cells and spores [57].

UV irradiation leads to denaturation of the bacterial protein and formation of dimers between adjacent pyrimidine molecules on the same DNA strand, interrupting transcription and translation processes, resulting in mutagenesis and cell death[58]. Few studies have been conducted on UV-mediated inactivation of probiotics, using an exposure time of 2.5 hours for inactivation [7,59,60]. [٧,٥٩,٦٠]Because of their high penetration depth into the bacterial cell, gamma rays are least able to damage the wall by destroying DNA, and compared to UV rays, they are more effective in preventing microbial growth during storage. Gamma rays work better against more resistant strains that are able to form a protective spore form, and they do not regenerate them [61].

Conclusion

The results of this study suggest that the use of gamma rays as ionising radiation is effective in destroying the activity of *Bacillus subtilis* endospore-containing bacteria in freeze-dried form at a dose of 18 KGy. This dose is recommended for the production of the freeze-dried form of this strain.



Material and Methods

Bacillus subtilis strain

The *Bacillus subtilis* bacterial strain with the accession number *Bacillus subtilis* subsp. subtilis ATCC 23857 was obtained from the National Centre of Genetic and Biological Resources of Iran in lyophilised form. To prepare a uniform suspension, the freeze-dried bacteria were resuspended in LB liquid medium and the resulting suspension was linearly cultured in LB agar culture medium and maintained at 30°C for 24 hours.

Preparation of the freeze-dried form of bacteria

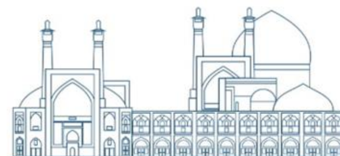
Individual colonies of the bacteria were transferred to a test tube containing 5 ml of broth and incubated at 37°C for one night. In the next step, the contents of the test tubes were transferred to 500 ml of broth medium and incubated until the logarithmic growth stage (determined by absorbance at 600 nm using a spectrophotometer). The contents of the flasks were then transferred to sterile Falcon 50 and centrifuged at 6000 rpm for 15 minutes. The bacterial cells at the end of the 50 were mixed evenly with 10% dry milk powder and then placed in a freezer at a temperature of -70 °C. In the next step, these mixtures were transferred to the freezer (Figure 5). After freeze-drying overnight, the dried samples were removed and transferred to the desired system for irradiation.

Irradiation and determination of the inactivation phase

First, the freeze-dried form of bacteria was irradiated with doses of 1, 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 kGy delivered by Co-60 at a rate of 0.92 Gy/sec through a Canadian-made Gamacell Nordian 220 device (Figure 6). 0.1 g samples of the *B.subtilis* were serially diluted with 0.6 normal saline after irradiation and the number of colonies was counted at different doses.

Selection of the inactivation dose

According to the results of the primary irradiation, the *B. subtilis* freeze-dried form was inactivated with 18 KGy. Then the prepared freeze-dried bacterial activation powder was divided into two parts without and with irradiation and filled into sterile 50 ml tubes. Irradiation was then carried



out using the Canadian-made Gamacell Nordian 220 device at a rate of 0.73 Gy/sec and the corresponding dose. To ensure that no live bacteria were present, the irradiated powder was diluted and transferred to the solid culture medium. At this stage, the results confirmed that the irradiated doses did not contain viable bacteria.

The cell survival curve is often used in radiobiology to determine the relationship between the percentage survival of the irradiated cell population and the radiation dose. The residual population at different doses represents the percentage of cells capable of reproduction in relation to the total number of cells [62].

D10 value

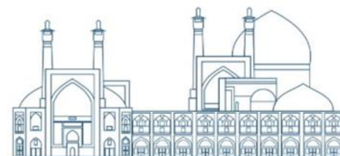
The D10 value is the radiation dose required to inactivate 90 % of the viable microbial population or to reduce the population by a factor of 10 [14,63,64]. Estimation of D10 values is important for evaluating the design of microbial population reduction procedures [64]. The D10 value is the slope of the exponential part of a survival curve. This value can also be determined using the following relationship. The remaining fractions, log10 (N/N0) of the microorganisms were calculated and used as relative changes in their actual number of viable cells [65]. The D10 values were calculated by plotting log10 (N/N0) against dose (D) according to the following equation:

$$x = \frac{\text{Radiation Dose (D)}}{Log10(\text{N0} - \text{N})}$$

Where N_0 is the prime number. N is the number of living beings after irradiation with the dose D. D is the radiation dose. The linear correlation coefficient (r2) and the regression equations were also calculated. In this study, D10 was first calculated and the inactivation dose of the bacterial cells was determined on this basis. Irradiation was carried out at the desired dose and the survival rate of the bacterial cells was calculated [66]. Based on the radiation dose, the D10 value was calculated using the above formula and the inactivation dose.

Comet assay

Evaluation of genomic changes (DNA) in the irradiated bacterium Bacillus subtilis by comet assay, measurement of DNA damage by comet assay [12,67]. In brief, the amount of 10^3 cells of lyophilized bacteria irradiated with different doses and the weight equivalent of non-irradiated bacteria were diluted in PBS buffer. A 0.5% normal melting agarose (NMA) was placed in a preparation Beaker



and heated in the microwave for less than 1 minute to boiling point and then placed in a 60°C water bath. For the first layer on the slide, 50 µL of NMA was added to the coarsely ground portion of the slide along the left edge. The slides were dried on a hot plate at 60 °C (about 5 minutes).

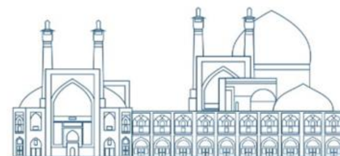
Then, in the second layer, another 85 µl NMA was spread from the center of the slide to the left and right and, in the next step, a large coverslip was slowly placed from left to right. The slide was placed in an ice box for 10 minutes (to solidify the agarose). For the next layer, 0.7 % low-melting agarose (LMA) was heated in the microwave (less than 1 minute) and then transferred to a 37 °C water bath.

The slides were labeled in pencil (right edge). A large glass slide was removed from the slide and for the next layer a quantity of 200,000 cells was well suspended in 90 µl LMA (0.7%). A total of 90 µl was spread on the slide and carefully covered with a large coverslip (24 × 70 mm). The slides were then placed in a cold ice box for 10 minutes.

To prepare the lysis stock solution, add 2.5 M sodium chloride, 100 mM disodium EDTA and 10 mM Tris-Hcl to 700 mL double-distilled water and dissolve with stirring. Then add 12 g of sodium hydroxyl to the mixture and stir again. After complete dissolution, 1 g of sodium dodecyl sulfate is added. The pH is adjusted to 10 and the final volume is brought to 890 mL with double-distilled water. The solution is filtered and stored at room temperature. Remove 108 mL of the lysis stock solution from the working solution and add 1.6 mL of 1% Triton X-100 [68].

After removing the coverslips, the slides were placed in a lysis solution. They were stored at 4°C for 60 minutes. To untangle the DNA strands, the slides were then placed in an alkaline buffer, 300 mM NaOH , 1 mM EDTA (pH > 13) for 20 minutes. The slides were then transferred to the electrophoresis tank containing alkaline buffer and electrophoresed for 20 minutes at 25 V and 300 mA at a temperature of 4°C. This was followed by three 5-minute baths in neutralization buffer (0.4 M Tris, pH 7.5). Finally, the slides were stained with a Giemsa working solution prepared from a commercially available stock solution and digital images of the slides were taken with a light microscope.

For each irradiated and non-irradiated bacterial sample, two slides, each containing approximately 100 cells randomly selected at different sites, are considered and the quantitative percentage of DNA in the comet sequence [% DNA Tail] is measured using CometScore™ software. It was



taken.

$$\%DNAT^{1} = 100 \times (DNAW^{2} - DNAH^{3}) / DNAW$$

Statistical analysis

The data was processed using Excel software. All percentages were converted to arcsine. The statistical analysis was carried out using SPSS version 2022 statistical software. A one-way analysis of variance (ANOVA) and Duncan's test for differences in means in several areas with a significance level of $P < 0.05$ were also performed to conduct additional studies and compare with the control. The charts and tables were created using Excel software version 2019.

Ethical approval. We confirm that all the experimental research, including the collection of samples, complied with relevant institutional, national (Ethics Committee of the Faculty of Sciences, University of Tehran Protocol Number = E52# 412-17), and international guidelines and legislation. All of the material is owned by the authors and/or no permissions are required.

Data availability. Some or all data of this study are available from the corresponding author upon reasonable request.

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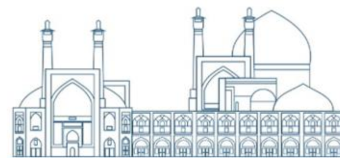
[1] Percent tail DNA
[2] Whole
[3] Head



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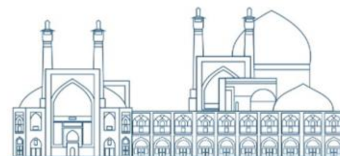

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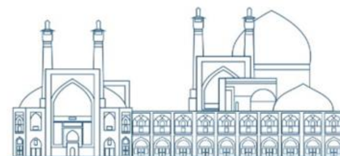

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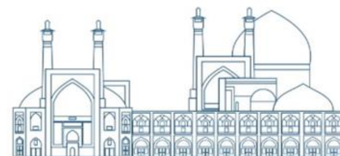

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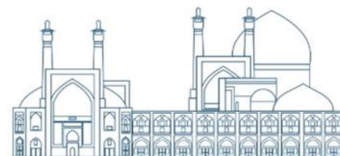

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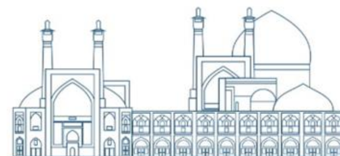

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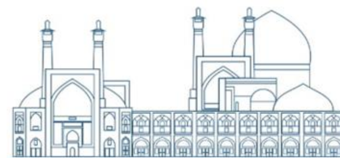

Effect of gamma irradiated bucillus subtilis as a paraprobiotic on intestial histology of Rainbow trout (Paper ID: 1623)

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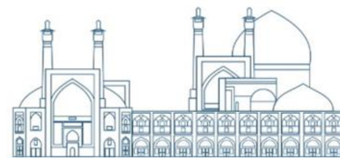
Abstract

The aim of this study was to produce gamma-irradiated *Bacillus subtilis subsp. Spizizenii* for use as a paraprobiotic in the diet of rainbow trout (*Oncorhynchus mykiss*). The live freeze-dried form (PRO) and the irradiated non-live freeze-dried form (PAR) of the bacteria were added to the fish feed at a rate of 10^8 CFU per gram of feed for the PRO and the weight equivalent for the PAR. 54 trout (74.51 ± 3.15 g) were transferred to nine 300-liter tanks and fed PRO, PAR and control diets (n=3) for 8 weeks. Kidney, liver and intestinal tissue were analyzed. The growth indices, improved in the PAR and PRO groups compared to the control group ($p<0.05$). Intestinal histology results showed that the height of intestinal folds and goblet cells increased in fish fed PRO and PAR compared to the control group ($p<0.05$). Although melatonin increased in the PAR and PRO groups, it decreased in the liver of PAR compared to PRO ($p<0.05$). The results of this study show that the paraprobiotic produced in this study is effective under culture conditions and can therefore be used as a paraprobiotic supplement in trout farming.

Keywords: histology, immunilical histology, paraprobiotic,

Introduction

Restrictions and problems in the use of pharmaceuticals and chemical substances such as antibiotics have led to probiotics, prebiotics and synbiotics being an important solution to replace these substances. Studies are being proposed that show that the use of probiotics is an extremely effective solution for combating infectious diseases in aquaculture. The usual definition of probiotics is "live microorganisms" that, in sufficient quantities, have an appropriate effect on the health of the host. According to this definition, probiotic organisms must be alive to have a positive effect on the host [1].



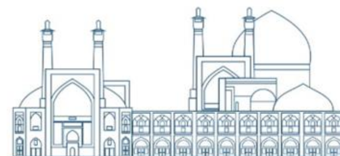
Despite numerous studies on the positive effects of probiotic microbes, there are concerns about their use. Some of these concerns relate to survival in the product/food, different colonization patterns and resistance in the gut, and even the possibility of obtaining virulence genes from pathogenic bacteria through horizontal gene transfer [2, 3]. These problems, as well as new information on the beneficial effects of non-living probiotic microbes that resemble living species, have drawn attention to the use of non-living probiotics, known as paraprobiotics [4-8]. In contrast to vertebrate models with a higher degree of evolution, the concept of using paraprobiotics in aquaculture is still in its early stages. Given the concerns regarding the consumption of live microbes on the one hand and the benefits of non-live microbial compounds on the other, the use of paraprobiotics in aquaculture has been considered [4, 5, 7, 9].

Probiotics of the genus Bacillus are used in aquaculture [10, 11]. The bacteria of this genus include Bacillus subtilis, whose positive effects on growth, strengthening of the immune system, antitumor effects and increased resistance to various diseases have been demonstrated in various fish species [10, 12-15]. On the other hand, the paraprobiotic produced by heating B. subtilis can also have positive effects on fish species [15, 16].The aim of this research is therefore the propagation of paraprobiotic bacteria from B. subtilis, inactivated by gamma irradiation, as an immunity and growth supplement in fish feed and their effect on the histology of various organs.

Material and Methods

Production of paraprobiotic powder

According to the results of the irradiation, the freeze-dried strain was inactivated at 19 KGy. Subsequently, a large amount of the freeze-dried form of the bacteria was prepared. The freeze-dried powder was divided into two parts without irradiation (PRO) and with irradiation (PAR) and filled into sterile 50 ml vials. To ensure that no live bacteria were present, the irradiated powder was diluted in the next step and transferred to a solid culture medium. At this stage, the results confirmed that no viable bacteria were present in the irradiated doses.



Fish and feed preparation

Trout weighing 74.51±3.15 g with no clinical signs of the disease were obtained from Alborz Caspian Fish Farm and, after a 1-week acclimatization period, were transferred to different 300-liter culture tanks with 6 fish per tank and 3 replicates for each treatment and fed diets containing freeze-dried bacteria without irradiation (PRO) and with irradiation (PAR) at 5% of body weight for 2 months. One group was fed as a control group without any additives added to the controlled diet.

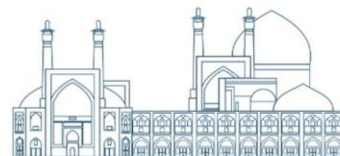
The experiment was conducted in aerated tanks with airflow stones in each tank and a central aeration pump. The trout were fed a basic diet containing the following: Protein 39.23±3.32%, fat 12±3.1%, crude fiber 3.12±0.85%, ash 9.2±2.3% and moisture 8%. For the PRO group, the amount of 108 CFU per gram of freeze-dried live Bacillus subtilis powder was considered and for the PAR group the weight equivalent of the inactive freeze-dried bacterial cells. The PAR and PRO powders were sprayed onto the feed and mixed well. Vegetable oil was then used to cover the surface of the feed and prevent the release of bacteria into the water.

Growth indices

To determine the growth indicators, all fish in the different groups were weighed at the end of the experiment and the average weight, daily growth rate, percentage weight gain (WG), specific growth rate (SGR) and feed conversion ratio (FCR) were calculated [17].

Histological studies of liver, kidney and intestine

To determine the possible negative and positive effects of the PAR and PRO diets on the liver, kidney and intestinal tissues of the tested fish, two fish from each experimental unit were randomly selected at the end of the experiment and dissected with a sterile scalpel blade after anesthesia and death; the tissues were carefully removed and fixed in 10% formalin. In general, the phases of classical histology were carried out in the following order: 1) Dehydration 2) Clarification 3) Paraffinization 4) Shaping 5) Cutting the tissue 6) Sticking the prepared tissue sections on a slide 7) Staining with hematoxylin-eosin H&E method and 8) Mounting the slide [18].



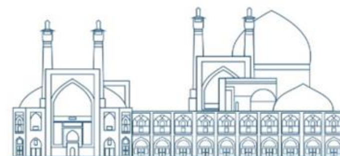
Statistical analysis

The data was processed using Excel software, with all percentages converted to arcsine. Statistical analysis was performed using SPSS version 2022 statistical software. One-way analysis of variance (ANOVA) was used to investigate the role of supplements added to the diet and their effects on different indicators. Duncan's test with a significance level of ($P<0.05$) was performed to determine the difference in means between the experimental groups. The charts and tables were created using Excel software version 2019.

Results

Trout growth indices

In the study, a strain of *Bacillus* bacteria inactivated by gamma radiation was used in the diet of rainbow trout. At the beginning of the breeding period, no significant difference was found between the weights of the different study groups (Fig. 2 a). The results showed that the growth rate in the PAR and PRO groups was higher than in the control group ($p < 0.05$) (Fig. 2 b). The amount of specific growth factor in the paraprobiotic group showed a significant increase compared to the control group ($p < 0.05$) (Fig. 2 c). Feed conversion was lowest in the PAR and PRO groups compared to the control group ($p < 0.05$) (Fig. 2 d).



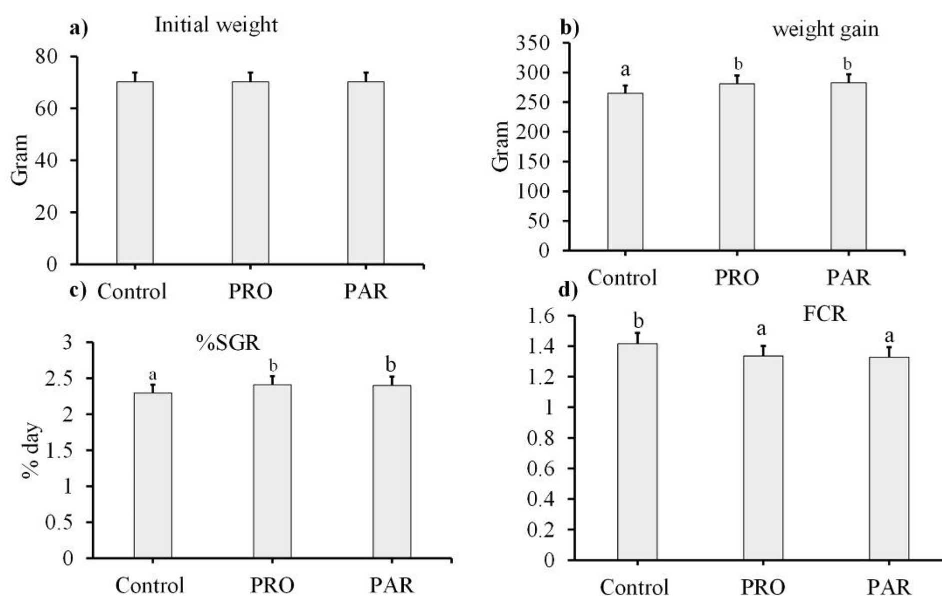
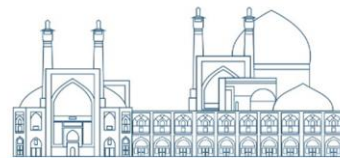


Figure 2. comparison (mean ± SD) of initial weight (a), weight gain (b), specific growth rate; SGR (c), feed conversion ratio FCR (d) in rainbow trout fed PAR and PRO supplements and C (no microbial addition) after 8 weeks of feeding

Histological analysis

The results showed that melanin accumulation was observed in the anterior renal tissue of the PRO group and the condition of the renal corpuscles and proximal and distal tubules was normal. The results showed that melanin accumulation was observed in the PAR-fed group and the condition of the renal corpuscles and proximal and distal tubules was normal (Fig 4a).

The results showed that melanin accumulation was observed in the liver tissue of the PRO-fed group. The results also showed that less melanin accumulation was observed in the PAR-fed group than in the PRO-fed group (Fig. 4 b). An increase in the height of the intestinal folds and the number of goblet cells was observed in the PAR and PRO fed groups (Fig. 4 c and d).



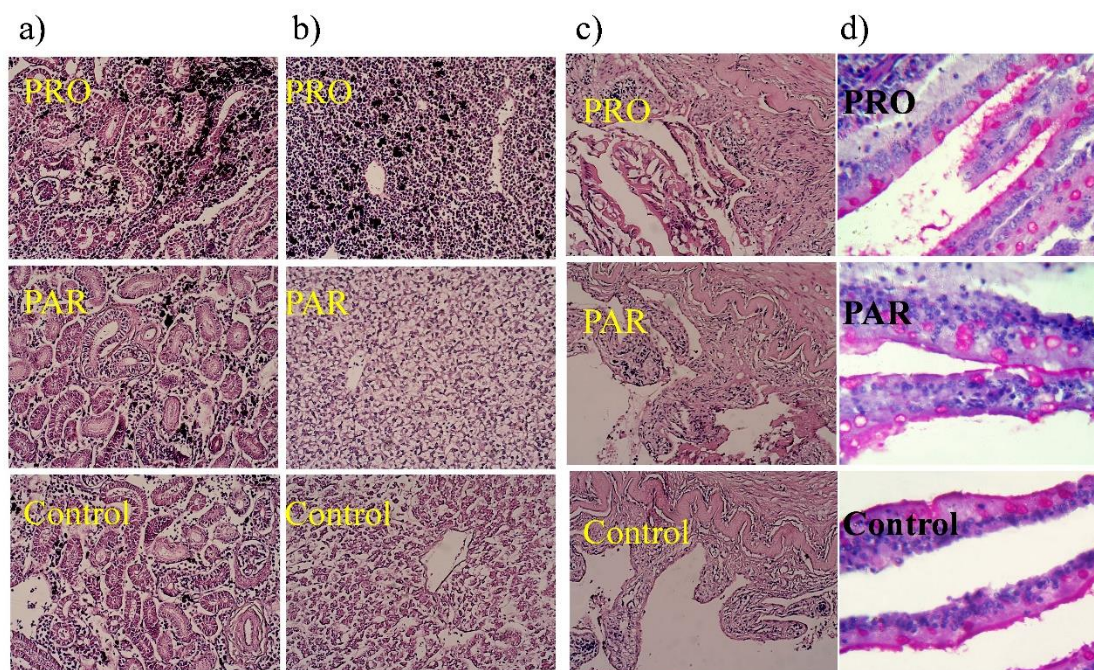
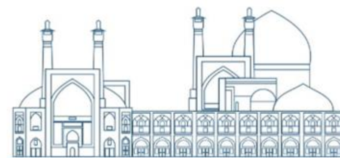


Figure 4. (a) Kidney tissue, (b) liver tissue, (c) intestinal tissue of trout fed with PAR and PRO stained with the hematoxylin and eosin method, (d) staining of intestinal tissue with the PAS stain in trout after 8 weeks of feeding with PRO, PAR and the control group

Discussion

Despite the abundant scientific evidence for the beneficial effects of probiotics, concerns have been raised about the functionality and practical benefits of these live microbes. Some of the main concerns relate to survival and persistence in the gut and the possibility of acquiring virulence genes from pathogenic bacteria through horizontal gene transfer. These issues, and recent evidence that even non-viable microbes are as beneficial to the host as their live counterparts, have led to the use of non-viable probiotic preparations, now known as paraprobiotics [9, 19, 20]. However, the effectiveness of an inactivation method can be influenced by a variety of factors, including the type of microorganism, the growth medium used, the stage of growth, the heating method, whether the microorganisms are in vegetative or spore form, the pH of the medium and the water activity, to name but a few. Ultraviolet inactivator falls within the electromagnetic spectrum of 200 to 400 nm as non-ionizing radiation and can effectively inactivate a wide range of bacterial cells and



spores [21]. Gamma radiation is more effective against more resistant strains that are able to form a protective spore shape and prevents them from regenerating [22]. In this study, a gamma radiation-inactivated bacterial strain of *B. subtilis* was used to feed rainbow trout.

Like probiotics, paraprobiotics can also improve growth performance and feed conversion in aquatic animals. For example, it has been reported that a heat-killed paraprobiotic (*L. planarum*) can significantly increase the growth performance and feed conversion of redfish (*Pagrus major*) [23]. Rodriguez-Estrada et al [24] found a significant increase in weight gain (WG), specific growth rate (SGR), feed efficiency and protein efficiency (PER) in rainbow trout (*Oncorhynchus mykis*) fed with heat-inactivated cells. *Enterococcus faecalis* were fed. Feeding heat-inactivated probiotic *Bacillus pumilus* significantly improved the final weight, WG and SGR of grouper (*Epinephelus coioides*) in the juvenile fish phase [25]. Even a diet with heat-killed *L. plantarum* did not change the growth parameters of freshwater shrimps [26].

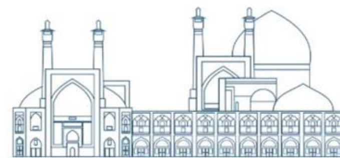
It is evident that paraprobiotics can positively influence various parameters of fish growth, but the mechanism by which this occurs is not yet clear. In the current study, the use of paraprobiotics produced from the strain *Bacillus subtilis* resulted in improved growth in rainbow trout.

Conclusion

This study shows that the production of paraprobiotics from different strains of *B. subtilis* is able to alter the biological responses of rainbow trout, leading to an improvement in growth and showing activity related to organs important for immunity. Such results are important, especially when concerns are raised about potential safety issues associated with the release of live probiotic microbes into the aquatic environment.

Acknowledgements

The authors would like to thank all colleagues at the Nuclear Science and Technology Research Institute, especially the Nuclear Agriculture Research school, who helped us carry out this research project.



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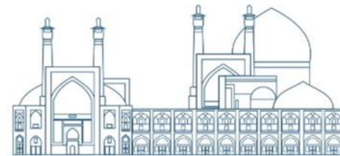

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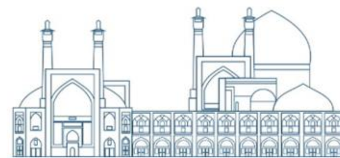

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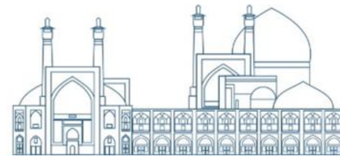

How does the nitrogen-15 technique contribute to accurate measurements? (Paper ID: 1625)

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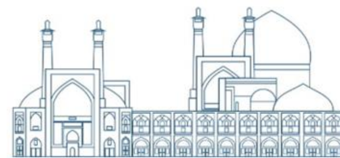
Abstract

Management of nitrogen (N) is a challenging task and several methods individually and in combination are needed to use for managing its efficiency. A field experiment was conducted to determine the effects of applying urea in combination with nitrapyrin (NP) as a nitrification inhibitor and gibberellic acid (GA_3) as a plant growth regulator on winter wheat yield, nitrogen use efficiency (NUE) and ammonia volatilization (AV) in a calcareous soil. The experiment comprised of three treatments with five replications: control treatment, urea (300 kg urea ha^{-1}), and urea in combination with NP and GA_3 (300 kg urea ha^{-1}). By using ^{15}N-labeled urea and setting up three ^{15}N microplots inside each plot, the nitrogen in the plant derived from fertilizer (Ndff) and the fate of N fertilizer were directly investigated. The grain yield and 1000-grain weight in urea+NP+GA_3 treatment were significantly increased by 15% and 13%, respectively, compared to urea. The N difference method displayed 21% increase in NUE when urea was applied with NP and GA_3 but tracking ^{15}N showed that the combination of urea+NP+GA_3 reduced NUE by about 8%. Urea coated with NP increased AV and decreased Ndff in the plant by about 22% and 15%, respectively. The use of NP is like a double-edged sword for areas with high potential for AV, so it is necessary to find an alternative solution to simultaneously improve yield and NUE.

Keywords: Calcisols, Gibberellic acid, ^{15}N-labeled urea, Nitrapyrin, Winter wheat.



1. Introduction

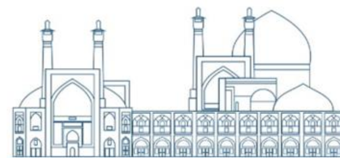
Nitrogen (N) is a crop essential nutrient that is commonly applied as fertilizer and subjected to many transformations in soil. These transformations which occur at the soil surface and within the soil, can influence nitrogen use efficiency (NUE). Leaching, runoff and volatilization are major processes for loss of N in soils (Sharma and Bali, 2018). Management of N is a challenging task and several methods individually and in combination are in use to manage its efficiency. However, only 47% of the reactive nitrogen applied globally to cropland is converted into harvested products, meaning that more than half of the nitrogen used as fertilizers is currently lost into the environment (Lassaletta et al., 2014).

Ammonia volatilization (AV) is a major pathway of nitrogen fertilizer loss, with approximately 11 Tg yr^{-1} estimated to occur from global fertilizer use (Beusen et al., 2008). Ammonia, as a reactive nitrogen type involved in global nitrogen cycle processes, may have detrimental effects on terrestrial and aquatic ecosystems, as well as climate (Liu et al., 2013). The global average losses of NH_3 from urea fertilizers are estimated to be close to 14% but nitrogen loss through AV can account for more than 30% of fertilizer nitrogen input particularly in areas with alkaline soils. The emission process is influenced by meteorological conditions (Congreves et al., 2016), soil properties and field management (Huijsmans, 2003).

Nitrous oxide (N_2O) is an important greenhouse gas and soils are the largest anthropogenic source of N_2O. Agricultural activities are responsible for about 59% of the anthropogenic N_2O emissions (Ciais et al., 2013). The Intergovernmental Panel on Climate Change (IPCC) has recommended nitrification inhibitors (NIs) to be used as a potential mitigation option for agricultural N_2O emission (IPCC, 2014).

The objective of using NIs is to control the loss of nitrate by leaching or the production of nitrous oxide (N_2O) from the topsoil by keeping N in the ammonium form longer and thus increasing NUE (Trenkel, 2010). The commonly used NIs are nitrapyrin (NP) (2-chloro-6-(tri-chloromethyl) pyridine), commercialized with the name of N-Serve, dicyandiamide (DCD) and 3, 4-dimethylpyrazole phosphate (DMPP) (Dawar et al., 2021a).

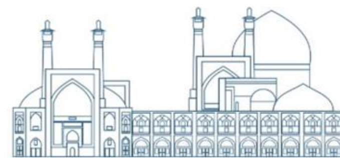
However, since NIs retain N in the form of ammonium, there have been increasing concerns about NIs' potential to increase soil NH_3 volatilization. The positive effect of NI on NH_3 volatilization



has been reported in many studies (Kim et al., 2012; Lam et al., 2017; Li et al., 2018). Qiao et al. (2015) conducted a meta-analysis including 27 observations, that showed NI increased AV by an average of 20%. Some studies have suggested that these compounds can increase the AV by up to 38% (Pan et al., 2016). A meta-analysis with 234 observations from 89 individual studies showed that, on average, NIs increased AV by 36% (Wu et al., 2020). Mirkhani et al. (2021a) examined the effect of applying urea with a nitrification inhibitor-NP on AV and they reported the positive effect of NP on AV in a calcareous soil. Although NI can increase N losses through AV, numerous studies in literature have also shown that NIs can significantly reduce N losses through NO_3^- leaching and N_2O emissions. Therefore, N fertilizers with NIs are considered being more climate-smart. In contrast, some studies showed that the application of N fertilizers with NIs did not significantly influence the yields of a variety of agricultural crops, including winter wheat. Influence of NP on winter wheat yield was investigated for instance by Mirkhani et al. (2021b) and the crop yield data showed that urea applied with NP only increases slightly grain yield (about 4%), with the increase not significant compared to without NP.

To further increase the effectivity of NI with regards to NUE and crop productivity, the question arises as to whether plant growth regulators can help. In recent decades, plant growth regulators (PGRs), including Gibberellic acid (GA), have attracted the interest of agricultural science, and are broadly used on agronomic crops (Ahmad et al., 2018). GA is an important signaling plant hormone that regulate many aspects of plant development, including seed germination, stem elongation, leaf expansion, pollen maturation, and the development of flowers, fruits and seeds (Upreti and Sharma, 2016; Gao and Chu 2020). Among the GAs produced in plants, only a few of the GAs, such as GA_1, GA_3, GA_4 and GA_7, have biological activity as regulators of plant growth and development (Hedden and Thomas 2012; Castro-Camba et al. 2022). Plant hormones have been shown to play a significant role in modifying grain filling progress and other various factors that regulate grain filling progress (Cui et al., 2020). Spraying GAs at the pre-flowering stage may help to redistribute photo assimilates towards seeds (Dawar et al., 2021b).

However, there are only a limited number of studies about the effect of applying urea together with NI and PGRs. Therefore, the objective of the present study is to evaluate the combined effect of NP and GA_3 on AV, NUE, the nitrogen in the plant derived from fertilizer (Ndff) and crop



productivity. Emphasis has been put on the NUE and the N difference method and the ^{15}N tracer method were used to evaluate the NUE in this research.

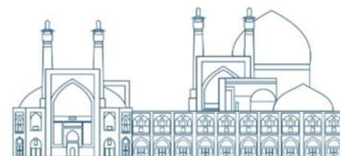
2. Materials and methods

A field experiment was conducted on an arable site (35º 56' N, 50º 58' E) on the experimental station of the University of Tehran. The soil is classified as Haplic Calcisols (WRB, 2015) with aridic soil moisture and thermic temperature regimes.

Seeds of the winter wheat variety PASTOR*2/PRL were sown at 200 kg seed ha $^{-1}$. Simultaneously with sowing, basal doses of phosphorus (P) at 60 kg P_2O_5 ha^{-1} in the form of triple superphosphate and potassium (K) at 35 kg K_2O ha^{-1} in the form of potassium sulphate were applied. Foliar spraying of micro-fertilizer was applied at stem elongation stage and forming the seeds. During the growing season, irrigation was applied by sprinklers.

The experiment comprised of three treatments with five replications: T_1: control treatment, T_2: urea (300 kg urea ha^{-1}), and T_3: urea (300 kg urea ha^{-1}) in combination with NP and GA_3. In T_2 and T_3 treatments, urea alone and urea coated with NP were applied in three split applications: one third at tillering growth stage (GS 21), one third at stem elongation stage (GS 32), and the other third at booting stage (GS 40). To reduce AV and incorporate urea into the soil, shortly after each split application irrigation was performed. GA_3 was foliar sprayed only at stem elongation stage (GS 33 to 34). Nitrapyrin and GA_3 were applied at a rate of 0.51% and 0.03% of the applied N (weight/weight), respectively.

To study the NUE, a ^{15}N-aided N balance study was performed by installing three ^{15}N microplots and ^{15}N-labeled urea replaced the unlabelled urea according to the time of fertilizer application. For these microplots, ^{15}N-labeled urea was used with enrichment of 5.19 atom% ^{15}N excess. For ^{15}N uptake by plant parts (grain and straw), 15 cm by 15 cm area from the middle of each microplot were harvested and the grain of these plants was separated from straw. The dry plant material was then powder milled and analyzed for total N and ^{15}N by isotope ratio mass spectrometer (IRMS). Calculations of ^{15}N recovery in grain and straw were carried out as described by the International Atomic Energy Agency (2008).



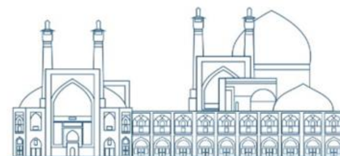
Ammonia volatilization was measured with semi-static chambers. The detailed description and validation of these chambers were reported in the studies conducted by Jantalia et al. (2012) and Martins et al. (2017 and 2021). To reduce the error, five NH_3 chambers were installed in each area (1.5 m by 1.5 m) per plot (more details about the required number of ammonia chambers can be found in Martins et al. (2021)). The chambers were relocated each day to different positions, a procedure previously outlined by Jantalia et al. (2012). The measurements of NH_3 volatilization were performed 2 weeks after each split application.

The statistical analyses were carried out based on randomized complete block design as well as the orthogonal contrasts and Tukey's HSD (honestly significant difference) test for treatment mean comparisons using SAS software.

3. Results

The total cumulative NH_3 emissions of fertilized treatments are shown in Figure 1, which is the cumulative emission for each treatment minus the background emission from the control treatment-T_1. The total cumulative NH_3 emissions of urea alone and urea+NP+GA_3 treatments were 38 kg N ha^{-1} and 45 kg N ha^{-1}, which corresponded to an emission factor of 27% and 33% of the applied N for T_2 and T_3 treatments, respectively. Addition of the NP to the urea caused a significant increase in NH_3 emissions and increased NH_3 losses by 22% of the applied urea.

The crop yield data showed that urea applied with NP and GA_3 had a significant ($p < 0.01$) effect on grain and straw yields compared to other treatments (Table 1). Urea in combination with NP and GA_3 increased grain yield by 15% and 51% compared to urea and control treatments. Grain and straw yields were higher ($p < 0.01$) when urea was applied with NP+GA_3 than urea alone but grain yield was improved further in comparison with straw yield. The percentage of N in grain and straw parts were not influenced by the application of NP and GA_3. There were no significant differences on the number of tillers, number of grains per spike, and spike length between T_2 and T_3 treatments, whereas there was a significant difference ($p < 0.01$) on 1000-grain weight (Table 2).



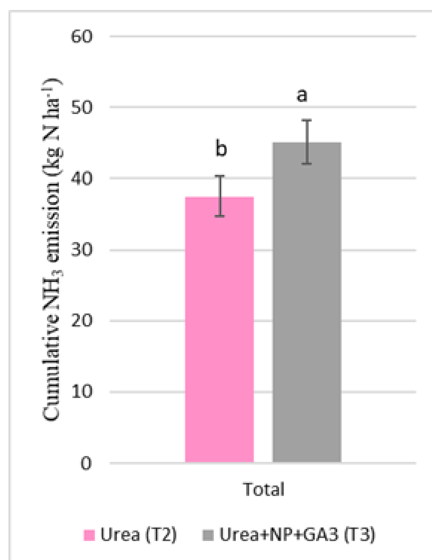


Figure 1. Total cumulative NH_3 emissions of T_2 and T_3 treatments. The vertical bars indicate the standard deviation of the mean (n=5). The letters indicate a significant difference at $p < 0.05$.

Table 1. The effect of different treatments on grain and straw yields (t ha^{-1}), %N, FNY (kg N ha^{-1}) and %NUE (mean ± SD). A mean (n=5) comparison between treatments from Tukey's HSD-test indicates a significant difference at $p < 0.01$.

Treatment	Grain yield (t ha^{-1})	Straw yield (t ha^{-1})	% N-Grain	%N-Straw	FNY-Grain (kg N ha^{-1})	FNY-Straw (kg N ha^{-1})	%NUE-^{15}N (total)	%NUE (total)
Control	6.6 ± 0.4^c	9.6 ± 0.4^c	1.2 ± 0.1^b	0.4 ± 0.0^b	***	***	***	***
Urea	8.7 ± 0.2^b	11.7 ± 0.1^b	1.7 ± 0.1^a	0.5 ± 0.0^a	39 ± 4	13 ± 2	38 ± 3	48 ± 3^b
Urea+NP+GA_3	10.0 ± 0.3^a	12.6 ± 0.1^a	1.6 ± 0.1^a	0.5 ± 0.0^a	37 ± 4	12 ± 2	35 ± 3	58 ± 4^a

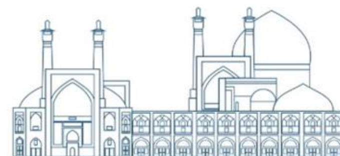
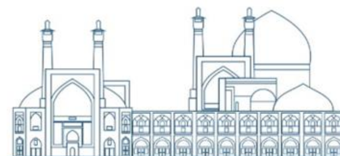


Table 2.The effect of different treatments on spike length, number of grains per spike, 1000-garin weight, and number of tillers (mean ± SD). A mean (n=5) comparison between treatments from Tukey's HSD-test indicates a significant difference at $p < 0.01$.

Treatment	Spike length (cm)	Number of grains per spike	1000-grain weight (g)	Number of tillers
Control (T_1)	8.0 ± 0.2^b	25.5 ± 0.3^b	48.3 ± 0.7^c	3.8 ± 0.4^b
Urea (T_2)	8.6 ± 0.2^a	31.0 ± 0.5^a	51.7 ± 0.7^b	4.8 ± 0.4^a
Urea+NP+GA_3 (T_3)	8.5 ± 0.1^{ab}	31.1 ± 0.7^a	58.4 ± 0.5^a	5.0 ± 0.0^a

Urea applied with NP and GA_3 significantly affected %Ndff in grain and straw parts of winter wheat (Figure 2). %Ndff in grain and straw parts decreased ($p < 0.05$) when urea was applied with NP+GA_3 by 11% and 14%, respectively compared to urea alone.

The fertilizer nitrogen yields (FNY) of grain and straw parts were 39 kg N ha^{-1} and 13 kg N ha^{-1} in urea alone treatment and 37 kg N ha^{-1} and 12 kg N ha^{-1} in urea+NP+GA_3 treatment (Table 1), which corresponded to the nitrogen use efficiency-^{15}N (%NUE-^{15}N) of 38% and 35% of the applied N fertilizer for urea alone and urea+NP+GA_3 treatments, respectively. However, the calculation of NUE based on the N difference method showed different results. According to this method, NUE was 48% in urea alone treatment and 58% in combined urea+NP+GA_3 treatment (Table 1).



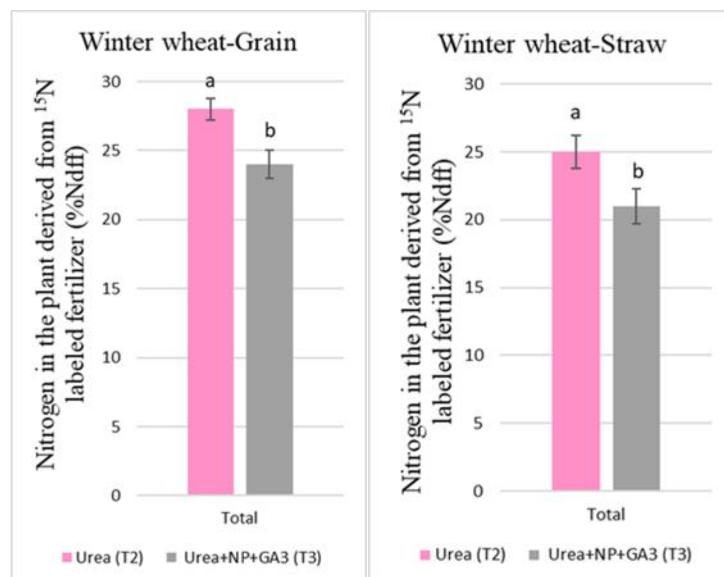
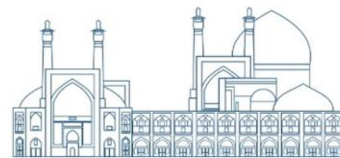


Figure 2. Total %Ndff of winter wheat in grain and straw parts. The vertical bars indicate the standard deviation of the mean (n=5). The letters indicate a significant difference at $p < 0.05$.

4. Discussion

The cumulative NH_3 emissions increased when urea was applied with NP and GA_3 (Figure 1). These results may be due to the preservation of N in the form of NH_4^+ in the soil after application of NP, which could increase AV. Also, it has been found that the application of NIs can increase the soil pH by the combined effect of reducing the nitrification rate. Soil pH affects the balance between NH_3 and NH_4^+, as soil pH increases, the balance shifts into more NH_3 emission (Qiao et al., 2015; Wu et al., 2020).

Previous studies have shown that applying GA_3 improves source-sink relation (Solaimalai et al., 2001; Rafique et al., 2021) but the rate of urea hydrolysis was relatively fast. It seems that when the soil pH is high, the plant is not able to capture this part of N and the application of GA_3 did not help. Since PGRs are known to improve plant growth by increasing N uptake and reducing biotic and abiotic stresses (Bose et al., 2013; Kurepin et al., 2014; Iftikhar et al., 2019; Dawar et al., 2021b), this significant increase of 15% in this study may be due to foliar application of GA_3. Urea in combination with NP and GA_3 increased 1000-grain weight by 13% compared to urea. This increase is probably related to foliar application of GA_3, which improved the source-sink relation



and finally increased the grain yield with the increase in the weight of 1000-grain (Pan et al., 2013; Islam et al., 2014).

Although grain and straw yields were increased by adding NP+GA_3 to urea, caution must be taken. The application of NP+GA_3 significantly reduced the %Ndff by around 15% compared with urea due to increased AV of about 22%. Therefore, the %N, nitrogen yield (NY), FNY in grain and straw and consequently %NUE-^{15}N were not influenced by the application of NP+GA_3. Our findings showed that in the combined strategy with the addition of NP+GA_3, the total %NUE-^{15}N did not increase and decreased by about 8% compared to urea. When the N difference method was used to calculate NUE, in addition to overestimating NUE, urea in combination with NP and GA_3 showed a significant increase in NUE by about 21%.

It is quite reasonable that 22% increase in AV was accompanied by 8% decrease in NUE-^{15}N. Since in the ^{15}N tracer method, NUE is calculated based on the Ndff, its results are different from the N difference method. Previous studies also showed that the N difference method overestimated NUE compared to the ^{15}N tracer method due to the priming effect of N fertilizer on soil N mineralization (Cassman et al., 2002; Gardner and Drinkwater, 2009).

5. Conclusion

Although in our study, urea in combination with NP and GA_3 was found to increase crop productivity, their use represents an additional cost for farmers. From an economic point of view, the use of these substances is only cost-effective in the case of supportive policies by the government. However, it should not be overlooked that this 15% increase in crop yield is accompanied by a 22% increase in total cumulative ammonia emissions. We need to find a management strategy that maximizes grain yield and NUE while minimizing N losses to the environment and we did not achieve this goal in this study.

Acknowledgments

The support of the International Atomic Energy Agency (IAEA) is gratefully acknowledged.

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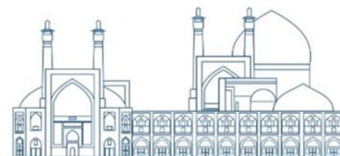

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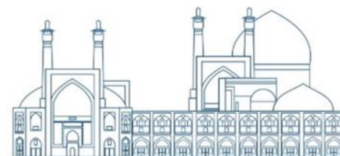

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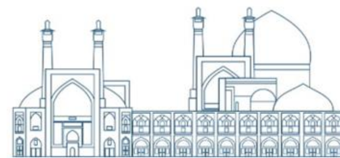

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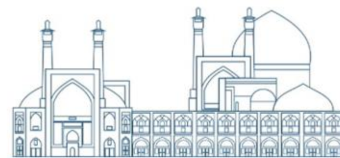

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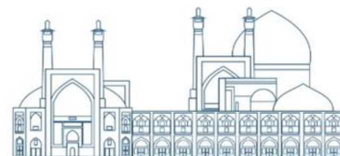

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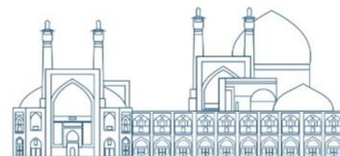


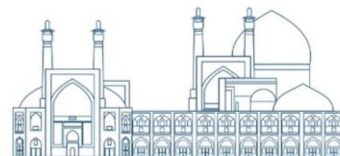
Supplementary Information

Block 1: T_3 T_1 T_2
Block 2: T_1 T_2 T_3
Block 3: T_2 T_1 T_3
Block 4: T_2 T_3 T_1
Block 5: T_3 T_2 T_1

Tx
2 m buffer zone
9 m × 8 m plot

^{15}N Subplot 3 (1m×1m)
^{15}N Subplot 2 (1m×1m)
^{15}N Subplot 1 (1m×1m)
N_2O Chamber (1m×1m)
Wheat Yield (3m×3m)
NH_3 Chambers (1.5m×1.5m)
1 m buffer zone
15 cm × 15 cm
Individual plot of 9 m × 8 m

In this section, the summary of the theoretical basis should be given, if any. Here, you can cite handbooks



Assessment of some baking and edible quality parameters of grain of mutant drought-tolerant rice lines of Tarom Mahalli (Paper ID: 1649)

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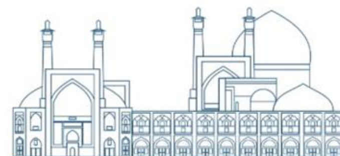
Abstract

The cooking and eating qualities of rice grains are significant issues in many rice-producing regions of Asia, including Iran. This study aimed to evaluate three critical parameters related to the baking and eating qualities of rice: amylose content, gel consistency, and gel temperature. These parameters were assessed in 29 mutant drought-tolerant Iranian rice lines in comparison with their parental landrace, Tarom Mahalli. Considering the preference of Iranian people for rice with intermediate amylose levels, this characteristic is essential in the development of new rice lines through gamma irradiation. The results revealed that gamma irradiation induced considerable variations in some mutant lines. However, the cooking and eating quality parameters remained unchanged in most of the mutant lines when compared to the popular parental landrace. Additionally, T-tests indicated significant differences in Amylose Content (AC), Gel Temperature (GT), and Gel Consistency (GC) among the genotypes studied. Ultimately, some of these mutant lines may be recommended as new elite rice varieties for cultivation in Iran, pending the final experiments for cultivar registration and introduction.

Keywords: *Oryza sativa* L., Baking and eating quality, mutant lines, Landrace Tarom Mahalli.

Introduction

Rice (*Oryza sativa* L.) plays a crucial role in the global economy and nutrition, contributing significantly to poverty alleviation [1]. It is a staple food for over half of the world's population, underlining its importance in the dietary habits of people worldwide [2]. The development and evaluation of rice genotypes with superior cooking qualities and nutritional values are pivotal in combating human malnutrition [3]. The cooking properties of rice, which include amylose content



(AC), gel consistency (GC), and gelatinization temperature (GT), are essential factors [4]. Rice with an intermediate level of AC is preferred in most rice-producing regions for its soft texture without excessive stickiness [5]. GT, a critical physical characteristic, influences cooking time, water absorption, and the temperature at which the starch irreversibly loses its crystalline structure during cooking. Varieties with low or intermediate GT are desirable for their reduced cooking times, marking them as high-quality rice [6]. Hallajian et al. (2022) assessed the impact of drought stress on the grain composition and cooking attributes of Iranian rice mutants, identifying two drought-tolerant mutant lines, TM-B-7-1 and HM-250-E-1-1, as promising candidates for final cultivar registration experiments [7]. Additionally, Ashwar et al. (2014) investigated the effects of gamma irradiation on the physicochemical properties of alkali-extracted rice starch. Their findings indicated a significant decrease in apparent amylose content, pH, swelling power, and syneresis, alongside an increase in carboxyl content, water absorption capacity, and transmittance with rising irradiation doses [8].

Experimental

Plant materials

This study evaluated the baking and edible quality parameters of 29 mutant drought-tolerant rice lines derived from the Tarom Mahalli landrace. These drought-tolerant lines were generated through gamma irradiation of Tarom Mahalli seeds and were selected from the M4 population based on spikelet fertility a few years ago [9]. All 29 lines exhibited higher yields than their parental landrace, Tarom Mahalli. Except for TM1, these lines flowered 5-11 days earlier than Tarom Mahalli (Table 1).

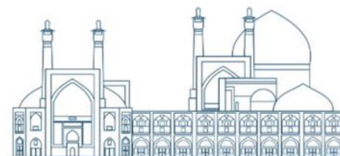
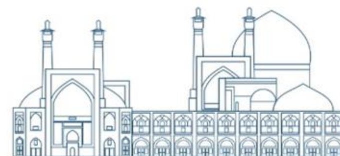


Table 1. Properties of drought-tolerant mutant rice lines of Tarom Mahalli landrace

Symbol	Genotype	Plant height (cm)	Fertile Spike	Spike Length (cm)	Flag Leaf Length (cm)	Flag Leaf Width (cm)	Yield (Kg/Hec)	Full Seeds	Total Seeds	Tolerance on spikelet fertility
TMa*	**Tarom Mahalli**	147	13	26.5	35	1.1	3600	1391	1534	1
TM1	**TM4-220-10-4**	115	19	21.5	26.5	0.8	4732	1862	2318	1
TM2	**TM4-220-13-1**	124	24	21	23.5	1	4952	2160	2256	1
TM3	**TM4-220-15-2**	113	16	28	20	1	5100	1456	1536	1
TM4	**TM4-230-VE-7-5**	124	18	24	17	0.8	5400	1458	1530	1
TM5	**TM4-230-VE-8-1**	128	18	20	26	1.1	6040	1548	1602	1
TM6	**TM4-230-1-1**	121	21	22	20	1.1	6108	1869	1995	1
TM7	**TM4-230-1-2**	120	17	26	22	1	5720	935	1037	3
TM8	**TM4-250-2-1**	112	15	20	22	0.8	4448	1275	1665	1
TM9	**TM4-250-3-5**	115	18	22	29	1	4092	1692	1890	1
TM10	**TM4-250-8-6**	103	17	22	28	0.7	4088	1105	1190	3
TM11	**TM4-250-9-6**	119	19	22	30	0.9	4728	1292	1425	1
TM12	**TM4-250-9-7**	120	16	26	27	1	4472	1104	1200	3
TM13	**TM4-250-10-5**	128	13	24	25	1	4212	1144	1209	1
TM14	**TM4-250-10-6**	121	17	22	20	1	5108	1292	1360	1
TM15	**TM4-250-10-7**	135	17	23	20	1	6000	1649	1717	1
TM16	**TM4-250-11-5**	128	13	26	27	1	5380	1495	1599	1
TM17	**TM4-250-11-6**	124	12	23	18	1	4200	1044	1104	3
TM18	**TM4-250-15-5**	120	16	19	21	0.8	4292	1152	1280	1



TM19	**TM4-250-16-5**	123	22	20	23	1	4100	2002	2244	1
TM20	**TM4-250-17-5**	126	18	26	22	1.1	4628	1440	1566	1
TM21	**TM4-250-17-6**	127	14	21	20	1.1	4624	1162	1274	1
TM22	**TM4-300-1-1**	116	11	23	22	0.9	4388	990	1067	3
TM23	**TM4-300-2-1**	130	21	22	17	0.8	4976	2016	2163	1
TM24	**TM4-300-4-1**	126	22	24	21	1.1	5024	3322	3652	1
TM25	**TM4-300-4-2**	114	14	23	20	1	5776	1610	1806	1
TM26	**TM4-300-5-1**	121	16	23	21	0.9	5852	1296	1456	1
TM27	**TM4-300-6-1**	130	19	22	18	0.8	4662	1330	1691	1
TM28	**TM4-300-6-2**	124	18	25	33	1	4000	2070	2466	1
TM29	**TM4-300-8-1**	116	21	20	19	1	4040	2079	2268	1

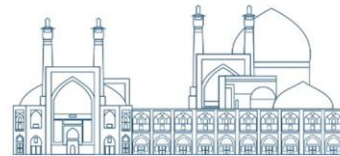
*TMa and TM are abbreviation of Tarom Mahalli and Tarom Mutant.

Evaluation of some grain physicochemical properties

We assessed several physicochemical parameters across 30 genotypes, including gelatinization temperature (GT), amylose content (AC), and gel consistency (GC).

Alkali spreading value (ASV)

To estimate the GT of milled rice, we used its alkali spreading value (ASV). We added 10 ml of 1.7% potassium hydroxide (KOH) solution to a small glass petri dish and placed two sets of six whole milled rice grains evenly within the dish, ensuring adequate space for expansion. The petri dishes were covered and left undisturbed for 23 hours at room temperature. The degree of spreading was measured on a 7-point scale: 1 (grain unaffected), 2 (grain swollen), 3 (grain swollen with an incomplete and narrow collar), 4 (grain swollen with a complete and wide collar), 5 (grain split or segmented with a complete and wide collar), 6 (grain dispersed, merging with the collar), and 7 (grain completely dispersed and intermingled) [10]. GTs were categorized as follows based



on the scale: 1-2 (high, 74.5-80°C), 3 (high intermediate), 4-5 (intermediate, 70-74°C), and 6-7 (low, <70°C).

Amylose content (AC)

The AC of milled rice was measured by determining the relative absorbance of the starch-iodine color in a solution of 100-mesh rice flour digest, according to methods outlined by Perez and Juliano (1978) [11] and Williams et al. (1958) [12]. Rice varieties were classified into five groups based on their AC: waxy (0-2%), very low (3-9%), low (10-19%), intermediate (20-25%), and high (>25%) [13].

Gel consistency (GC)

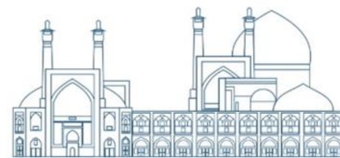
GC was assessed based on the consistency of a cold 5.0% rice paste in 0.2 N KOH [14] and measured by the length of the gel in a horizontally held test tube for 1 hour. Gel Consistency (GC) was arbitrarily classified into three categories based on gel length: hard (< 40 mm), medium (41-60 mm), and soft (> 61 mm) [15].

Statistical analysis

SAS statistical software version 9.1 was utilized for conducting an analysis of variance (ANOVA) to explore varietal differences in the baking and edible qualities of the rice genotypes. This analysis was followed by a t-test ($p < 0.05$) to determine statistical significance [16].

Results and discussion

Our findings demonstrate that gamma irradiation induced changes in the amylose content, gelatinization temperature, and gel consistency of grains in certain mutant rice lines. Mir et al. (2015) observed that gamma irradiation decreased the amylose contents in cultivars with low, intermediate, and glutinous amylose content (AAC) but did not affect cultivars with high AAC. Although no visible changes in gelatinization temperature were detected post-irradiation, the peak time was reduced at different dose levels. Gel consistency significantly increased in the tested cultivars, particularly in high AAC indica rice [17]. Similarly, Yu and Wang (2007) reported that gamma irradiation impacted apparent amylose content, gel consistency, and gelatinization temperature, with an increase in dose leading to decreased amylose content and increased gel consistency and gelatinization temperature [18].



In our study, lines TM1, TM2, TM4, TM5, TM6, TM7, TM17, TM19, TM27, TM28, and TM29 showed no significant difference in amylose content compared to their parental landrace, Tarom Mahalli, with their amylose content being approximately the same as that of the landrace. However, the amylose content of other mutant lines differed from Tarom Mahalli, suggesting that these effects are generally related to changes in starch structure [19]. Yu and Wang (2007) also noted that an increase in irradiation dose led to a decrease in AC, making rice starch more susceptible to enzymatic hydrolysis in humans [18]. Mir et al. (2015) reported that irradiation treatment reduced kernel hardness, amylose content, and pasting properties, with a slight decrease in the thermal properties of rice samples [17].

Our research found that only five lines (TM3, TM6, TM8, TM20, and TM28) had a gelatinization temperature similar to that of Tarom Mahalli. Significant differences in gelatinization temperature were observed between the other lines and the landrace. A higher gelatinization temperature slows the starch gelatinization rate, consequently increasing boiling time [20]. The gel consistency of grains from seven rice lines (TM1, TM3, TM4, TM6, TM9, TM14, TM16, TM22, and TM23) was similar, with no significant differences compared to Tarom Mahalli (Tables 2 and 3).

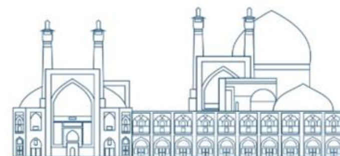


Table 2. Numerical amounts of baking and edible grain quality parameters of 30 genotypes

Symbol	Genotype	GC	GT	AC
TMa*	**Tarom Mahalli**	50.5	4.7	21.95
TM1	**TM4-220-10-4**	56	3.91	20.7
TM2	**TM4-220-13-1**	45	3.91	22
TM3	**TM4-220-15-2**	51	4.33	21.8
TM4	**TM4-230-VE7-5**	48	3.83	21.5
TM5	**TM4-230-VE8-1**	32	3.91	21.9
TM6	**TM4-230-1-1**	48.5	4.88	22.1
TM7	**TM4-230-1-2**	44	4.58	21.7
TM8	**TM4-250-2-1**	42.5	4.41	21.9
TM9	**TM4-250-3-5**	47	4.25	21.9
TM10	**TM4-250-8-6**	45	4.25	21.2
TM11	**TM4-250-9-6**	45	3.91	21.5
TM12	**TM4-250-9-7**	43	4.08	22.5
TM13	**TM4-250-10-5**	42.5	4.16	22.6
TM14	**TM4-250-10-6**	50	4.08	23.3
TM15	**TM4-250-10-7**	50	4.08	22.9
TM16	**TM4-250-11-5**	48	4.16	21.1
TM17	**TM4-250-11-6**	45	3.75	21.3
TM18	**TM4-250-15-5**	55	4.25	22.1
TM19	**TM4-250-16-5**	53.5	3.75	22.8
TM20	**TM4-250-17-5**	41	4.33	21.5
TM21	**TM4-250-17-6**	41	4.08	21.3
TM22	**TM4-300-1-1**	47.5	4.83	21.5
TM23	**TM4-300-2-1**	51	4.25	21.8
TM24	**TM4-300-4-1**	42.5	3.83	20.8
TM25	**TM4-300-4-2**	42	4	20.8
TM26	**TM4-300-5-1**	46	3.58	21.3
TM27	**TM4-300-6-1**	55	4.16	21.1
TM28	**TM4-300-6-2**	57	4.41	21.9
TM29	**TM4-300-8-1**	41	4.25	22.2

*TMa, TM, GC, GT and AC are abbreviation of Tarom Mahalli, Tarom Mutant, Gel Consistency, Gelatinization Temperature and Amylose Content.

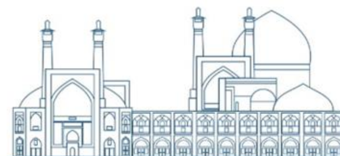
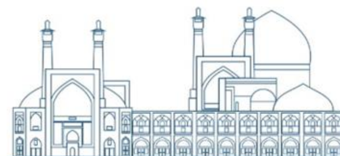


Table 3. Mean comparison of AC, GC and GT parameters of 30 genotypes by Duncan method

Symbol	Parameter		
	GT	GC	AC
TMa*	ns**	ns	ns
TM1	*	*	ns
TM2	*	*	ns
TM4	ns	ns	*
TM4	*	ns	ns
TM5	*	*	ns
TM6	ns	ns	ns
TM7	*	*	ns
TM8	ns	*	*
TM10	*	*	*
TM11	*	*	*
TM12	*	*	*
TM13	*	*	*
TM14	*	ns	*
TM15	*	*	*
TM16	*	ns	*
TM22	*	ns	*
TM17	*	*	ns
TM18	*	*	*
TM19	*	*	ns
TM20	ns	*	*
TM21	*	*	*
TM24	*	*	*
TM25	*	*	*
TM26	*	*	*
TM27	*	*	ns
TM28	ns	*	ns
TM29	*	*	ns

*TMa, TM, GC, GT and AC are abbreviation of Tarom Mahalli, Tarom Mutant, Gel Consistency, Gelatinization Temperature and Amylose Content. **ns: non-significant and *: significant in 1% level.

Research by other researchers has demonstrated that gel consistency (GC) increases with irradiation doses ranging from 0 to 10 kGy. This variation in GC may be attributed to changes in the ratio and structure of amylose and amylopectin, as well as the applied irradiation dose [21]. It



has been observed that upon cooking, rice varieties with higher GC tend to harden more quickly compared to those with lower GC, which remain softer after cooling [22].

To further classify the mutant rice lines and their parental landrace, Tarom Mahalli, based on amylose content, gel consistency, and gelatinization temperature, a t-test was conducted. The rice genotypes were divided into three categories based on the amylose content of the grains: the first group included genotypes with an amylose content of less than 21% (low amylose), the second group comprised genotypes with an amylose content between 21% and 25% (intermediate amylose), and the third group consisted of genotypes with an amylose content of more than 25% (high amylose). The first group contained three genotypes, each with three replicates, totaling nine samples. The second group included 26 genotypes, each also with three replicates, making up 78 samples in total. The third group had only one genotype with three replicates, totaling three samples. The t-test results indicated significant differences among all groups, as summarized in Table 4.

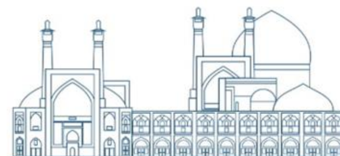
Table 4. Calculation of Mean, Standard Deviation (SD), Standard Error (SE) and Test of homogeneity of variances by T test- Amylose Content

Group	Number	Mean	SD	SE	F	t	Significance level
1	9	20.5658	0.39035	0.11268	3.207	-5.548	0.000
2	78	22.0383	0.90552	0.09390			

Group	Number	Mean	SD	SE	F	t	Significance level
1	9	20.5658	0.39035	0.11268	2.948	-21.641	0.000
3	3	25.7	0.2	0.11547			

Group	Number	Mean	SD	SE	F	t	Significance level
2	78	22.0383	0.90552	0.09390	2.073	-6.965	0.000
3	3	25.7	0.2	0.11547			

A T-test was conducted to compare the mutant rice lines and their parental landrace, Tarom Mahalli, based on amylose content, gel consistency, and gelatinization temperature. These rice



genotypes were divided into three groups according to the amylose content of their grains. The first group, characterized by low amylose content, included genotypes with less than 21% amylose. The second group, with intermediate amylose content, included genotypes with 21-25% amylose. The third group, with high amylose content, comprised genotypes with more than 25% amylose. The first group contained three genotypes with nine samples in total, the second group consisted of 26 genotypes with 78 samples, and the third group had only one genotype with three samples. The T-test indicated significant differences among all three groups (Table 4).

Furthermore, these rice genotypes were classified into two groups based on the gel consistency of their grains. The first group included genotypes with a gel consistency of less than 41%, and the second group included those with a gel consistency of 41-60%. The first group had one genotype with three samples in total, while the second group comprised 29 genotypes with 87 samples in total. The T-test revealed significant differences between the two groups at the 1% level (Table 5).

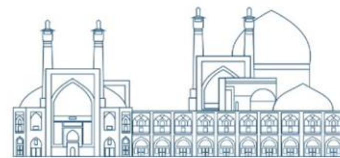
Table 5. Calculation of Mean, Standard Deviation (SD), Standard Error (SE) and Test of homogeneity of variances by T test- Gel Consistency

Group	Number	Mean	SD	SE	F	t	Significance level
1	3	32.6667	1.1547	0.66667	4.186	-4.938	0.000
2	87	47.9714	0.34116	052124			

Lastly, these rice lines were categorized based on their gelatinization temperature. The first group included those falling within scale 3-4, and the second group included those within scale 4-5. The first group consisted of 12 genotypes with 36 samples in total, and the second group included 22 genotypes with 66 samples. This categorization further highlights the variability in response to gamma irradiation among different rice genotypes, underscoring the complexity of breeding for desired cooking qualities (Table 6).

Table 6. Calculation of Mean, Standard Deviation (SD), Standard Error (SE) and Test of homogeneity of variances by T test- Gelatinization Temperature

Group	Number	Mean	SD	SE	F	t	Significance level
1	36	4.2903	0.1909	0.03182	12.727	4.018	0.000
2	54	3.9458	0.49379	0..06078			



Conclusion

Given the preference of Iranian people for rice with intermediate amylose levels, it is essential to consider this factor when developing new rice lines through gamma irradiation. Our study revealed that gamma irradiation introduced variations in the nutritional quality of some mutant lines without significantly altering the cooking and eating quality of most mutants when compared to the well-regarded parental landrace, Tarom Mahalli. Additionally, T-tests demonstrated significant differences in Amylose Content (AC), Gelatinization Temperature (GT), and Gel Consistency (GC) among the genotypes studied. Based on the results of this research, certain mutant lines emerge as promising candidates for novel elite rice varieties suitable for cultivation in Iran. These findings underscore the potential of gamma irradiation in enhancing rice breeding programs, aiming to meet the specific culinary pReferences and nutritional needs of the Iranian population.

Acknowledgements

We would like to express our sincere gratitude to our colleagues at the Nuclear Agriculture Research School and the Rice Research Institute of Iran for their invaluable contributions to this research. Additionally, We acknowledge the financial support provided by the Nuclear Science and Technology Research Institute of Iran and the Rice Research Institute of Iran, which was crucial in conducting this study.

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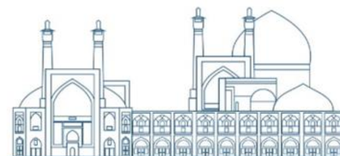

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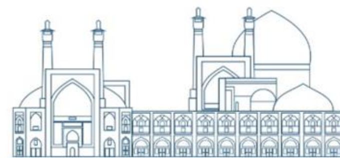

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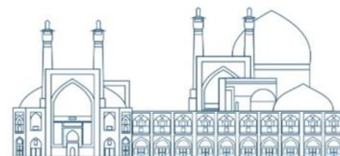

DNA stable isotope probing as a precise tool to identify the plant core microbiome under stress condition (Paper ID: 1672)

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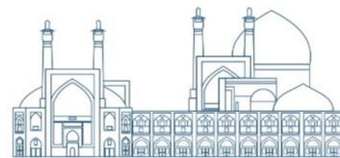
Abstract

Identification of the core microbiome in plant-microbes interaction is an obstacle to applying this approach to accelerate adaptation to climate changes. DNA and RNA stable isotope probe methods generally provide high phylogenetic separation of microbes that actively associated with plants. By combining stable isotope probing with molecular biology and genomic technique, the identification of rare species with low abundance, novel enzymes and bioactive compounds is facilitated. The development of the use of this technology in the investigation of changes in the plant rhizosphere microbiome in the stress conditions, and the relationship of its components with the plant stability, can lead to the identification of the rhizosphere core microbiome and applying its non-cultivable part. However, this progress calls for the introduction of this technology in areas such as microbiome metagenomic studies, which have received little attention. This study provides a brief overview of stable isotope probing and its conjunction with metagenomic methods for microbiome studies.

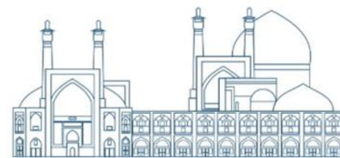
Keywords: DNA-SIP, Metagenome, Microbial community, Plant-microbe interaction, Rhizobacteria

Introduction

The mutual effect of plant and microbes in improving plant tolerance to environmental stress is an effective strategy to accelerate adaptation to climate change through non- power technologies [1,2]. Microbiome communities are closely related to plant health, productivity, and environmental



adaptation [3], and can potentially improve sustainable agricultural practices. There is growing evidence that certain interactions between plants and microbes can increase the stress tolerance of plants. This has led researchers to manipulate microbial communities to promote plant growth during times of environmental stress [4]. Identification of the core rhizosphere microbiome under stress conditions with the aim of improving stress tolerance in plants is less developed. Several studies have shown that bacterial communities are dynamically formed by various factors such as root exudates and soil, plant genotype, and chemotype [5]. Disturbances in plant metabolism, such as those caused by abiotic stress, can alter their microbiome, potentially affecting host fitness [6]. When a plant is stressed by a biotic or abiotic factor, the composition of the microbial community around the root changes, altering the secretions of the rhizosphere to cope with the stressor [7]. In this situation, microorganisms that are able to directly metabolize the plant's photosynthetic products and have a direct relationship with the plant play an important role in reducing stress and improving plant growth. Thus, accurate identification of plant-associated microbial populations in the rhizosphere is important to better understand this interaction and improve plant growth and maintain stability under stress conditions [8]. However, the lack of further molecular evidence and detailed description of microbial traits limits our ability to harness and manipulate the microbiome because of a lack of genetic and functional information about individual members of the rhizosphere community [9]. Examining soil and rhizosphere microbial community structure under stress conditions based on metagenomics and operational taxonomic unit (OTU) abundance provides useful information on the relative abundance changes of different microbial phyla. However, identification of the core and active microbiome under stress needs to be studied with more precise tools. Studying the direct effect of root exudates on rhizosphere microbial community structure and function with stable isotopes has already been established as a valuable research strategy [10]. Indeed, plants in a $^{13}CO2$-enriched atmosphere convert $^{13}CO2$ to organic carbon through photosynthesis and release some of it to the soil via root exudation. Uptake of ^{13}C-containing organic compounds used as selective markers by the rhizosphere microbiota can be assessed by separating ^{13}C-DNA and ^{12}C-DNA using a density gradient. DNA and RNA stable isotope probe methods generally provide high phylogenetic separation of microbes that actively produce root exudates and those that decompose soil organic matter [11]. The mRNA- SIP

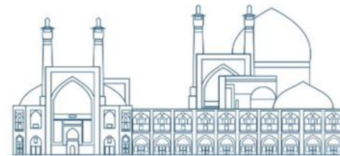


approach is also a powerful tool to study the effect of plants through root exudates on microbial gene expression [12]. The combination of deoxyribonucleic acid stable isotope probing (DNA-SIP) and metagenomics is a remarkable technique that correlates specific microbial identification and metabolic functions [13] and significantly advances the understanding of the functional properties of core microorganisms [14].

Microbial communities' structure

Microbiome refers to the total number of microorganisms, including bacteria, fungi, and viruses, present in a given area. This term is used for plants and soils mainly for the rhizosphere (thin soil around the roots). Plants alter the composition of the microbial community in the rhizosphere by excreting photosynthetic products and secondary metabolites under different environmental conditions and stresses [15]. The rhizosphere microbiome is a key factor in seed germination, seedling establishment, growth and development, nutrition, disease, environmental stress, and crop productivity. Root secretions contain primary and secondary plant metabolites that determine the uptake or inhibition of microbes from the soil. The mutual connection of roots and the rhizosphere community is usually achieved through chemical communication, and a better understanding of the chemistry of rhizodeposits and their production and distribution in spatiotemporal patterns is important to better describe the interactions between microbes and plant roots [16].

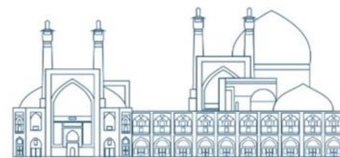
Studies of microbes in the rhizosphere have traditionally relied on culture-dependent techniques [17]. The study of the rhizosphere microbiome is greatly facilitated by molecular biology and genomic techniques. With recent advances in high-throughput sequencing and other high-resolution techniques, the composition of the rhizosphere microbial community can be studied in detail. Metagenomic studies with high-throughput sequencing provide comprehensive information on the microbial community structure in soil and rhizosphere. This is very important to identify and access the non-cultivable part of the soil microbiome, which usually account for more than 99% of them, and identify their metabolic potential, and provides the opportunity to exploit this biocatalytic potential. A current limitation of metagenome sequencing studies is the high proportion of sequences representing unknown genes from known or unknown organisms, as well as sequences for which homologs are not found in public databases [18]. However, no single



technique is able to describe the microbial diversity of the rhizosphere and provide a complete scenario of plant-microbe interactions. High-throughput sequencing should be combined with other complementary and cross-validated techniques. Different combinations of molecular biology and genomic methods may be appropriate for different purposes. For example, by combining stable isotope probing (SIP) with DNA sequencing methods, more microbes that use plant carbon and the expression of their major functional genes can be studied more efficiently. In other words, the combination of SIP and DNA sequencing and transcription can be used to identify microbes in the rhizosphere that can utilize carbonaceous root secretions and quantify the level of gene expression of microbes in the rhizosphere.

DNA-SIP for the core microbiome

Root exudation can be considered a functional trait of the plant involved in plant-microbe interactions. Studying the direct effect of root exudates on rhizosphere microbial community structure and function with stable isotopes has already been established as a valuable research strategy [10]. Indeed, in an atmosphere enriched in $^{13}CO_2$, plants convert $^{13}CO_2$ to organic carbon through photosynthesis and release some of it to the soil via root secretions. The uptake of ^{13}C-enriched organic compounds by the rhizosphere microbiota as a selective marker can be assessed by separating (^{13}C-DNA or ^{13}C-RNA) from (^{12}C-DNA or ^{12}C-RNA) by density gradient centrifugation. Stable isotope DNA and RNA probing methods generally provide high phylogenetic resolution of microbes that actively absorb root exudates or decompose soil organic matter [11]. In addition, the mRNA-SIP approach is a powerful tool for studying the effects of plant and root exudates on microbial gene expression [12]. SIP in combination with molecular biology techniques can be used to identify the composition and functions of the microbial community and to study microbial interactions and their metabolic functions in a complex community. The stable isotopes ^{13}C, ^{15}N, ^{18}O, and $^{34/36}S$ are commonly used [19]. Multiple isotopes can be labeled simultaneously, allowing their use in complex model systems and in situ environmental studies. When in situ or microcosm samples are exposed to a matrix enriched in stable isotopes, microbes can use the stable isotopes as a source of carbon or nitrogen for growth. By extracting, isolating, purifying, and analyzing isotope-labeled biomarkers, we can correlate microbial community composition and function. By tracking heavy isotope fluxes of key elements

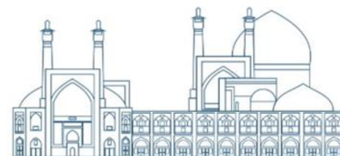


such as carbon and nitrogen, DNA-based SIP can provide clear evidence of substrate uptake by microorganisms in complex environments [20]. This technique is widely used in rhizosphere microbial ecology, metagenomics, organic pollutant degradation, etc. The rhizosphere environment is microbially active and dynamic, with the presence of newly produced carbon from root exudates and old carbon from soil organic matter (SOM). The combination of DGGE and SIP techniques was used to determine bacterial communities in the assimilation of carbon sources in the rhizosphere of four plant species [21]. Bacteria that absorb root exudates were identified by ^{13}C DNA analysis, whereas those that absorb SOM were identified by ^{12}C DNA.

The rhizosphere is the region where microorganisms exist in the soil and grow under the direct influence of the plant's root system. The rhizosphere plays an important role in the process of nutrient cycling and also protects plants from various environmental stresses such as salinity, flooding, pathogens, temperature, pollutants, etc. The interactions between plants and microorganisms and the protection of plant health and productivity in a stressful environment are not yet well understood. Strategies such as stable isotope study, metagenomics, and bioinformatics tools offer insights into integrated functional coordination between plants and microorganisms to improve plant growth in stressful environments [22]. Microbiome study of active bacteria and fungi colonizing roots and rhizosphere soil using high-throughput sequencing and stable isotope probing identifies species active in plant carbon uptake by $^{13}CO_2$ labeling and compares them to other, less active groups that do not involve plant composition. The abundance pattern of microbial groups and plant carbon uptake allows identification of active phyla in the rhizosphere core microbiome under stress conditions.

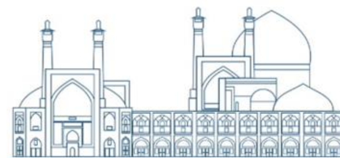
Metagenome and DNA-SIP conjunction

Metagenomic approaches are now applied in different environments, from the human gut microbiome to soil and from the deep sea to the inner atmosphere, with the aim of identifying different microorganisms living in different environments and reconstructing metabolic pathways to predict the environmental functions of uncultured microorganisms [23–25]. However, the metagenome is of relatively limited use for predicting the performance of members of a low abundance community and requires deeper sequencing to gain insight into the performance of



members of a lower abundance community. Moreover, this part of the low-abundance microbiome can play an important role in a specific function that is overlooked by this approach [13]. DNA Stable Isotope Probing (DNA-SIP) is a recently developed method that uses the stable isotope composition of a labeled substrate to identify the function of microorganisms in the environment. This technique is now being used in conjunction with metagenomics to establish links between microbial identity and specific metabolic functions [26,27]. The combination of DNA-SIP and metagenomics not only enables the identification of rare species with low abundance from metagenomic libraries, but also facilitates the identification of novel enzymes and bioactive compounds. The use of shotgun sequencing-based metagenomics to determine microbial diversity is limited by its inability to fully resolve more complex communities. Combining DNA-SIP with metagenomics as a filter to enrich DNA from microorganisms that serve a specific function can help reduce sample complexity [13,28]. The culmination of applying DNA-SIP to metagenomics is the creation of a specialized metagenome library to identify the microbial community with fewer members and determine its function [29]. Several studies examining the rhizosphere microbiome have shown that combining DNA-SIP with metagenomics is possible to fully characterize the function of microorganisms that may be present in low numbers. The study of the diversity of endophytic diazotrophs of Miscanthus sinensis root showed that bacteria of the genera Herbaspirillum, Rhizobium, Devosia, Pseudomonas, Microbacterium and Delftia are important endophytes for the growth of this plant in contaminated soils around mines. DNA-SIP-based metagenomics using the $^{15}N_2$ atom identified Pseudomonas, Rhizobium, and Exiguobacterium as diazotrophic endophytes of M. sinensis that contained essential genes for nitrogen fixation and plant growth promotion. This study showed that the heavy DNA labeling approach with $^{15}N_2$ atoms allows more accurate identification of diazotrophic bacteria [30].

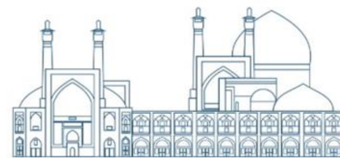
These studies show that filtering environmental DNA metagenomes with isotopic labeling leads to a better understanding of metabolic pathways and key genes, as well as identification of microbial species active in a particular function, which contributes to better management of various agricultural processes, especially in stress situations. However, the application of DNA-SIP is associated with some criticisms. The high concentration of labeled compounds required during the incubation period to generate the desired amount of labeled DNA for metagenome



library construction may result in the transfer of isotopic labeling to secondary consumers through "cross-feeding." The inhibitory effect of a high concentration of labeled substrates on the activity of some microbial groups is also among the other controversial aspects of this technique [31].

Future research needs

Linking the identity of wild microbes to their ecophysiological properties and ecological functions is a central goal for microbial ecologists. Among the many techniques that strive to achieve this goal, stable isotope probing (SIP) remains one of the most comprehensive techniques for studying entire microbial communities in situ. Metagenomics with active DNA SIP can directly relate identity to function, and by reducing complexity, it is possible to reconstruct individual genomes in less abundant species. In DNA SIP, actively growing microorganisms that ingest an isotope-rich substrate produce heavier DNA that can be fractionated and sequenced according to density. In other words, current metagenomic studies can at best infer the metabolism of microorganisms from their DNA sequences, and further experimental validation is needed. Combining DNA-SIP with metagenomics can help elucidate the function of target microorganisms at the population level (i.e., in relation to the uptake of specific substrates). To date, only a few studies combining DNA-SIP techniques with metagenomics have been published. From these studies, it appears that this approach may have advantages over traditional metagenomics. The biotechnological potential of combining DNA-SIP with metagenomics may lead to significant advances in the near future. However, several issues need to be addressed before this technique can be used on a large scale. Isolation of a sufficient amount of DNA to build a metagenomic library is a fundamental problem with this technique, and the development of high-throughput methods for DNA-SIP analysis of multiple samples is an important research priority. Another major problem is likely to be the time-consuming analysis of multiple samples simultaneously. The bioindustry uses high-throughput methods to screen multiple samples, and conventional metagenomics has been used in this process. In order to use SIP metagenomics to analyze DNA-SIP incubations and isolate ^{13}C-DNA on a large scale, a similar high-throughput method needs to be developed.



Conclusions

Metagenomic studies using high-throughput sequencing provide extensive information on soil and rhizosphere microbial community structure and metabolic potential. Data on microbial functional diversity are essential to fathom the role of the microbiome in different environments, and quantification of the microbial community is always an important task. DNA and RNA stable isotope probe methods generally provide high phylogenetic resolution of the microbiome with a given function and can be useful in studying the expression level of key genes and linking identity to function. SIP combined with molecular biology techniques, can be used to identify microbial community composition and microbial functions and to study microbial interactions and their metabolic functions in complex communities. The combination of DNA-SIP and metagenomics not only enables the identification of rare species with low abundance from metagenomics libraries, but also facilitates the identification of novel enzymes and bioactive compounds. Metagenomics with activated DNA-SIP can provide a direct link between identity and function and enable reconstruction of individual genomes in less abundant species by reducing complexity. The development of the use of this technology in the study of changes in the microbiome of the plant rhizosphere, especially under stress conditions, and the association of its various parts with the basic and effective functions in maintaining the stability of the plant requires extensive and in-depth studies that can lead to the detection of the core microbiome and the use of its non-cultivable part.

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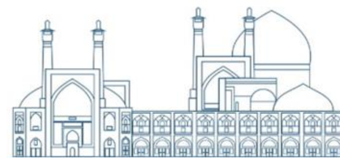

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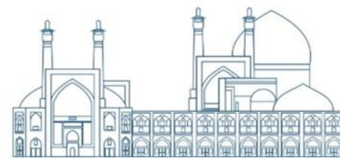

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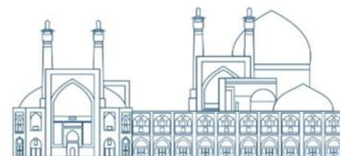

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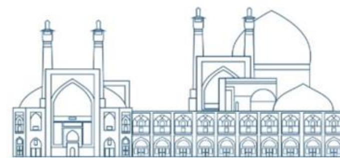

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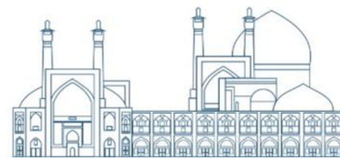

Gamma-ray induced overproduction of bio-based secondary metabolites (Paper ID: 1673)

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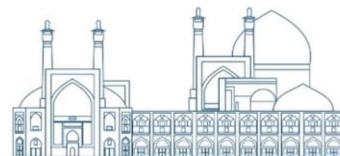
Abstract

The cost-effective production of bio-based secondary metabolites by gamma radiation is an area of interest. Valuable secondary metabolites for the food, pharmaceutical and agricultural industries can be synthesized by various biological sources. Studies have shown that treatment of cell cultures with different doses of gamma rays increases the yield of secondary metabolites. Irradiation of plant material in in vitro culture increases the accumulation of callus biomass and increases the yield of flavonoid metabolites more than fivefold. Gamma irradiation was also found to increase the output of mutant microbes more than 4-fold in the microbial production of secondary metabolites. Increasing the production of secondary metabolites using gamma radiation is pursued by various strategies, which may include enhancing growth and reproduction, accumulating biomass, altering biosynthetic pathways, increasing the ability to utilize the substrate, and improving the tolerance of environmental conditions by living organisms. This study investigates the effectiveness of gamma ray technology in increasing the production of plant and microbial secondary metabolites and attempts to outline various gamma ray performance enhancement strategies to develop the application of this technology.

Keywords: Biosynthesize, Improvement strategies, Mutation, Overproduction, Oxidative stress

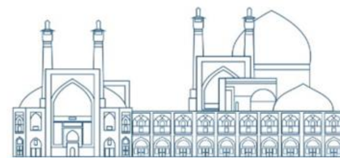
Introduction

Secondary metabolites are also referred to as specialized metabolites, toxins, secondary products or natural products. They are organic compounds produced by any life form, e.g. bacteria, fungi, animals or plants, which are not directly involved in the growth, development or reproduction of



the organism [1]. In general, a series of chemical reactions mediated by enzymes in living organisms is called metabolism. This series of coordinated metabolic reactions is consistent with the synthesis of essential molecules such as sugars, amino acids, fatty acids, etc. and is called primary metabolism. The compounds produced in primary metabolism are essential for the survival and vital processes of living organisms. In addition, there are other metabolic pathways in living organisms that lead to the production of compounds that are not necessary for the existing vital processes and are referred to as secondary metabolites. These secondary compounds are usually produced by living organisms as a defense system or for better access to nutrients and elements. The diverse structural and chemical properties of secondary metabolites make them suitable compounds for biofuels, biomaterials and the pharmaceutical industry [1]. Despite advances in the field of artifact chemistry, the extraction of secondary metabolites still depends on biological sources. Considering the speed and low production rate of secondary metabolites in biological synthesis routes, the production of these products is not economical, and the development of optimal methods for their rapid and mass production seems necessary.

The production of secondary metabolites is carried out both by plants and by using various microorganisms. The approach to increase the production of these biological molecules may include the selection of lines or strains with high production capacity, the optimization of nutrients, growth regulators and raw materials, and the optimization of environmental conditions [2]. The development of industrial biotechnology has greatly increased the search for "overproduction" lines or strains to improve production and achieve better yields. For several years, γ-radiation has been regarded as a new, rapid method for altering the quantitative and qualitative characteristics of various organisms. Low-dose ionizing radiation affects cell growth, proliferation, germination rate, enzyme activity and stress resistance. Successful examples of strain improvement in biotechnology are mostly due to the use of mutation and selection techniques which lead to the selection of the best producer strain for the desired product [3]. Gamma rays are very high-energy short-wave electromagnetic radiation emitted by certain radioactive isotopes such as cobalt 60. Exposure of plant or microbial material to gamma rays may have far-reaching effects. Direct effects of gamma radiation may occur through DNA single or double strand breaks, oxidation of bases, conformational changes of DNA cross-linking proteins. The formation of free radicals

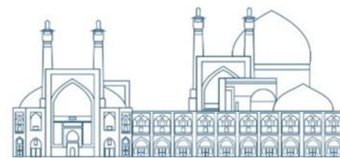


through the effect of gamma rays on intracellular water also causes indirect gene mutations. Successful enhancement of secondary metabolite production by gamma mutation in plant or microbial organs has been reported in several studies [4]. This study focuses on the enhancement of the production of various secondary metabolites in plant and microbial materials by gamma radiation and attempts to explain different strategies to increase the accumulation of secondary metabolites by this technology.

Gamma induced overproduction of secondary metabolite in plants

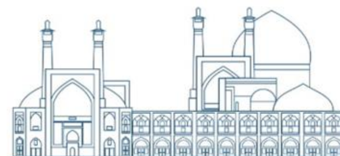
There are some unique secondary metabolites that are very specific to certain plant species and have unique properties that are beneficial to humans. Various types of secondary metabolites produced by plants have been used by humans for many purposes since ancient times. Many of them have been used as medicines, drugs, treatment agents, flavorings, anesthetics, etc [5]. Secondary metabolites are often biosynthesized from primary metabolites or share substrates derived from primary metabolites. The regulation of carbon flux between primary and secondary metabolism is controlled by a trade-off between growth and defense mechanisms, resulting in a protective adaptation to environmental stresses. Their quantity is very low (1% of dry weight) and depends largely on the physiological stages and growth of the plant [6].

Both biotic and abiotic stress factors often induce the production of secondary metabolites in plant tissue cultures. The effect of various biotic and abiotic stimuli and their role in secondary metabolite production has been reported and reviewed recently [7]. Increased production of secondary metabolite production can be induced in cell cultures using elicitors in the culture medium [8,9]. Gamma radiation is widely used in medicine and biology for the biological effects resulting from the counterintuitive shift from stimulation at low dose to inhibition at high dose. Relatively low doses of gamma radiation have been shown to accelerate cell proliferation, germination rate, cell growth, enzyme activity, stress resistance and crop yield. Earlier studies have almost all reported the same effects on cell cultures in vitro. Several studies have shown that treatment of cell cultures with relatively low doses of gamma radiation significantly increases the production of secondary metabolites. Plants exposed to gamma radiation produce various defense and antioxidant enzymes, many of which produce secondary metabolites which in turn reduce



induced oxidative stress conditions [10]. Reports on gamma irradiation of callus cultures show interesting examples where irradiated cultures showed an increase in secondary metabolites, total soluble proteins, amino acids, soluble sugars and antioxidant enzymes.

El-Beltagi et al. investigated the effect of different doses of gamma radiation on phenol and flavonoid production and antioxidant properties of 4-week-old callus cultures. Rosemary (*Rosmarinus officinalis* L.). The content of total phenolics and total flavonoids in callus irradiated with 20 Gy increased 5- and 5.6-fold, respectively. Irradiated samples showed 80% increase in phenylalanine ammonia lyase (PAL) activity [11]. Azeez et al. investigated the effect of gamma radiation on biomass formation and function of pharmacologically related secondary metabolites in callus cultures induced from different parts of the seedling (leaf, stem and root). The phytochemical screening performed on different types of extracted callus cultures shows for the first time the stimulatory effect of gamma irradiation on the production of phenolic compounds and naphthodianthrones in Hypericum *triquetrifolium Turra*. Treatment of callus cultures with 10 Gy gamma radiation was effective in stimulating the production of callus biomass and the accumulation of phytochemicals, but higher doses resulted in a decrease in these compounds, especially in the case of 40 Gy radiation [12]. Khalifa et al (2022) reported the effect of different concentrations of phenylalanine on callus from stem explants obtained from seedlings irradiated with gamma radiation (25 and 50 Gy). Callus from seed explants exposed to 25 Gy gamma radiation and treated with 4 mg/L phenylalanine had the highest phenolic content. The highest flavonoid content was found in callus cultures from seed explants irradiated with 25 Gy γ-radiation and treated with 1 mg/L phenylalanine. Similarly, HPLC quantification showed that the production of flavonoids was greatly increased in callus cultures from seed explants exposed to 25 Gy irradiation at a phenylalanine concentration of 1 mg/l. In addition, a total of 11 major flavonoids were determined in all callus cultures, with the exception of acastin-7-O-rutinoside, which was not found in the control callus culture. The gamma irradiation method can be used at different stages of secondary metabolite production to improve the performance of secondary metabolites in plants. Relatively low doses of c-irradiation (20-40 Gy) can be effective in enhancing secondary metabolite production, while higher doses (50 Gy or more) impair tissue viability and viability [13]. A detailed mechanistic understanding of secondary metabolite production in response to c-

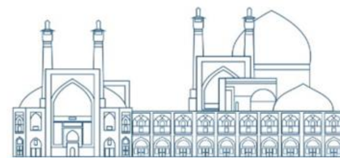


irradiation stimuli may facilitate the scaling of operations for multiple increases in secondary metabolite synthesis.

Gamma induced overproduction of microbial secondary metabolite

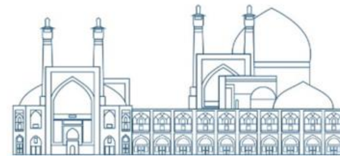
Low molecular mass products are secondary metabolites produced by microbes. Due to the technological and financial advantages, new technologies for the production of microbial products have replaced synthetic manufacturing processes. These products include drugs, organic acids, important agricultural metabolites, enzymes, flavorings and dietary supplements such as vitamins and amino acids. Organic substances known as secondary metabolites are substances that are formed during or at the end of the stationary growth phase and are not directly related to the growth, maturation or reproduction of microorganisms. These products play an important role in health care as antibacterial, antiparasitic, anticancer, enzyme inhibiting and immune system suppressing agents, etc. [14]. They are formed by bacteria during their advanced developmental phase, with their production typically suppressed during the logarithmic and stationary growth stages. These compounds are designed to confer specific advantages to the organism, yet they are not essential for its growth or reproduction. The synthesis of these compounds can be significantly influenced by the type and quantity of nutrients present in the culture medium [15]. However, challenges associated with their manufacture, such as limited productivity, costly raw materials, and high expenses for downstream processing, have hindered their widespread adoption in large-scale applications.

Exposure to gamma radiation can have profound implications for microbes, causing various mutations in genes as a result of DNA single or double strand breaks, oxidation of bases, and conformational changes in DNA cross-linking proteins [16]. The mutagenic potential of gamma rays has been observed to surpass that of chemical substances such as ethyl methane sulfonate, primarily due to the superior ability of gamma rays to penetrate cells. Notably, Elkenawy et al. showed that subjecting the bacterium *Serratia marcescens* to a radiation dose of 200 Gy had the remarkable effect of doubling the production of prodigiosin pigment when glycerol was used as the carbon source [17]. This significant enhancement was achieved by carefully optimizing conditions and manipulating the production temperature. Increasing the antagonistic activity of



Bacillus and Streptomyces against the diseases of root rot and withering of legumes caused by *Macrofomina Phaseolina* and *Fusarium oxysporum*. To increase the antagonistic effect, *Bacillus subtilis* BRBac4, *Bacillus siamensis* BRBac21, and *Streptomyces cavourensis* BRAcB10 were subjected to random mutagenesis using different doses of gamma radiation (0.5-3.0 kGy). When the mutants were compared with wild-type strains, they showed plant growth-promoting properties and hydrolytic enzyme activity. The disease suppression potential of selected mutants, *B. subtilis* BRBac4-M6, *B. siamensis* BRBac21-M10, and *S. cavourensis* BRAcB10-M2, was tested in green gram, black gram, and red gram. The combined inoculation of *B. siamensis* BRBac21-M10 and *S. cavourensis* BRAcB10-M2 reduced the incidence of root rot and wilt disease. These findings suggest that gamma-induced mutation can be effectively used to improve the biocontrol properties of Bacillus and Streptomyces. [18]

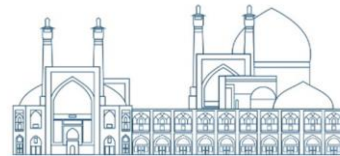
Wild-type strain Pseudomonas putida 33, subjected to gamma-ray mutagenesis and designated mutant P. putida 300-B, is a microbial rhamnolipid producer that utilises remote carbon sources (e.g. hydrocarbons, waste frying oils, 'WFOs' ', vegetable oil refinery was used molasses) in a minimal environment under shake flask conditions. The mutant strain 300-B showed its ability to grow on all substrates tested and produced rhamnolipid surfactants in varying amounts. Soybean and corn WFOs were found to be the preferred carbon sources, followed by kerosene and kerosene oils. The best cell biomass (3.5 g/l) and rhamnolipids (4.1 g/l) were obtained with soybean WFO as carbon source and glucose as growth initiator under fed-batch culture, with the optimum specific growth rate (m) 0.272 per hour being the specific product [19]. El-Housseiny et al. reported that UV- or gamma-induced random mutagenesis of *P. aeruginosa* resulted in enhanced RL production, the best of which was 15GR, which produced RL at twice the concentration of the original isolate. The rhamnolipids of this mutant showed improved activity and higher emulsifying capacity than those of the parent. Therefore, isolate P6 and its mutant 15GR are promising RL producers that have the advantage of producing RLs from glucose without induction with hydrophobic carbon sources [20].



Strategies of overproduction

It has been proven that abiotic and biotic environmental stress factors influence various reactions in organisms. As stress factors, triggers induce or enhance the biosynthesis of secondary metabolites that are supplied to a biological system (Alvarado). They are categorized into different groups based on their nature and origin: physical or chemical, biological or non-living. Early studies on the extraction of secondary metabolites were carried out on plant cells and have been extended over the years to bacteria, animal cell cultures and filamentous fungi. Starting from mutation, followed by random screening, careful fermentation experiments are then carried out and new mutants are selected. These methods use physical mutagens such as UV light and gamma rays or mutagens. The advantages of classical genetic methods include simplicity, the absence of complex equipment, a minimum of specialized technical manipulations, effectiveness (rapid increase in titer), the only disadvantage of which is the intensive work. On the face of it, isolation of mutants appears to be a time-consuming and cost-effective method for selecting superior producers of secondary products [21]. Mutants are usually expected to behave like their wild-type parents. However, this argument seems to be wrong. In general, it can be said that under the mutation treatment, secondary product production is much more affected than the ability to grow in a complex environment. Also, although mutagenesis affects metabolite production mostly in a negative direction, it can lead to superior production. We do not know the main reasons for the effect of the mutation on the secondary metabolism, but the following possibilities may be considered: 1. The mutation in the biosynthetic pathway of a primary metabolite is the precursor of the secondary product ("direct effect"). 2. The mutation in the common pathway A primary or primary metabolite branch is a branched pathway that leads to a primary product and a secondary product ("branch pathway effect"). 3. A mutation in a primary pathway that does not involve the precursor molecule, but the resulting changes in concentration Cellular metabolites affect the secondary product pathway by some form of cross-pathway regulation, i.e., control of a pathway by an end product of an unrelated biosynthetic pathway ("cross-pathway effect") [22].

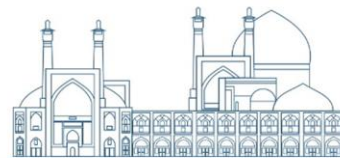
One of the effects of mutagenesis is creating a high tolerance in an organism to a toxic product. Many metabolic products are toxic to young cultures of the organism producing a specific product.



This is not a factor in production because the secondary crop is usually produced after growth is complete in the idiophase [15]. Using inhibitors to select resistant cultures will lead to more secondary metabolite production. It was reported that the parent culture of 152/52 produced 6000 units/ml and its growth was inhibited by 2000 units/ml of nystatin. A mutant producing 15,000 units/ml was resistant to 20,000 units/ml. It is a well-known fact in primary metabolism that certain analogs of end products act as false feedback agents and inhibit the growth of microorganisms. Selection of mutants resistant to such analogs often results in regulatory mutations no longer being inhibited or suppressed by the normal end product. Some of these mutants overproduce the final product to such an extent that it is excreted into the environment [23]. That such a concept can be used to produce a secondary crop, by selecting mutants resistant to structural analogs, will produce a higher yield than the original culture. Mutations can also be used to enrich the fermentation broth in a component of a mixture of related secondary products. In the sense that, the ratio of a specific metabolite compared to the total metabolites is significantly increased by mutation. In addition, mutation can be used to produce new metabolites. Mutations of streptomyces that are inactive or weak in antibiotic production result in cultures capable of producing antibiotics that are not produced by the parent culture based on antibacterial spectra. These findings show that increasing the production of secondary metabolites through gamma mutagenesis can include different strategies, and targeting only to increase production through improving the growth of the organism or even improving biosynthetic pathways due to mutation is an underestimation of the potential. This is technology. While this approach can include increasing production capacity, tolerance to environmental conditions or toxic metabolites, increasing the ratio of the target metabolite to the total metabolic products of the organism, and even creating new metabolites.

Perspective of future research

In order to fully exploit the potential of mutations in the future, more information should be obtained about the biosynthetic steps leading to the production of secondary metabolites. Once the effective factors of biosynthesis are known, knowledge about their regulation becomes very important. Only with this information can a correct understanding of the biochemical basis of the "superior" mutations be obtained. It is remarkable that after years of successful application of this



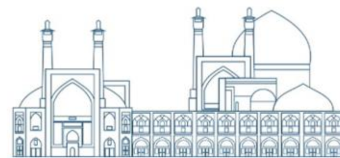
technology in industrial organisms, we are completely unaware of the fundamental changes in the biosynthetic pathways of this genetic event. This lack of information prevents the development of rational screening methods for the selection of improved cultivars. In addition, awareness of the various strategies to achieve the expected goals of this approach in the production of valuable biological products is still not at an adequate level in the country. Therefore, in addition to the detailed investigation of the impact of mutagenesis on the metabolic and biosynthetic pathways of secondary metabolites, also requires the study and investigation of various strategies to increase the production of bio-industrial metabolites through mutagenesis.

The thorough examination of mutagenesis's impact on metabolic and biosynthetic pathways of secondary metabolites as well as exploring strategies to enhance the production of bio-industrial metabolites through mutagenesis.

A comprehensive study on the impact of mutagenesis on metabolic and biosynthetic pathways of secondary metabolites is essential. Additionally, exploring strategies to enhance the production of bio-industrial metabolites through mutagenesis is crucial.

Conclusions

Plant secondary metabolites are increasingly required for the commercial and industrial production of various pharmaceutical, food and industrial formulations. The production of secondary metabolites in organisms is a response to environmental conditions. These compounds can facilitate the organism's reciprocal and antagonistic interaction with its environment to cope with changing conditions. Optimal gamma radiation facilitates the production of secondary metabolites. Gamma radiation has been shown to lead to the synthesis of higher concentrations of secondary metabolites. Gamma irradiation resulted in significantly higher accumulation compared to biotic and abiotic stimuli. The gamma irradiation method can be used at different stages of secondary metabolite production to improve the performance of biological secondary metabolites. Gamma irradiation by mutations in the biosynthetic pathway of a primary precursor metabolite ("direct effect"), mutations in the primary common pathway or the branch of the primary metabolite is a branched pathway that leads to the primary product and the secondary product ("branch pathway



effect"), and the mutation is in a primary pathway that does not involve the precursor molecule, but the resulting changes in cellular metabolite concentrations affect the secondary product pathway by a type of cross-pathway regulation ("cross-pathway effect") can produce metabolites increase the production of secondary metabolites by gamma mutagenesis through different strategies, including increasing organism growth and tolerance to environmental conditions or toxic metabolites, improving biosynthetic pathways, increasing the ratio of the target metabolite to other metabolic products and the creation of new metabolites is pursued, however, for a better understanding of the mechanisms of action as well as a more precise application of strategies towards the development of standardized washes for the profitable production of various secondary metabolites should be done.

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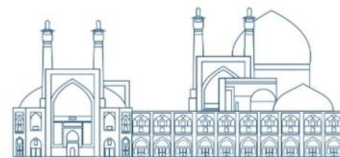

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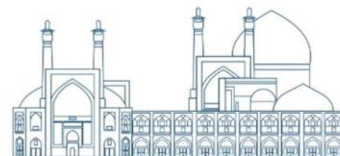

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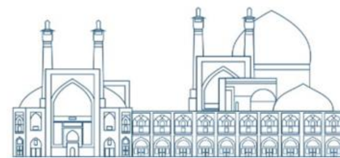

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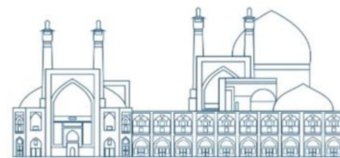

Application of Whole-Genome Sequencing in Plants Exposed to Gamma Rays for Developing Pathogen-Resistant Lines and Cultivars (Paper ID: 1675)

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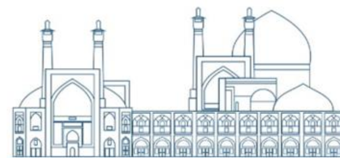
Abstract

The emergence of aggressive plant pathogens jeopardizes global food security. Traditional breeding methods for disease-resistant cultivars are often slow and limited by available resistance traits. Gamma-ray mutagenesis provides a powerful tool for generating random mutations in plant genomes that may create new resistance mechanisms. However, identifying these mutations and their effects on disease resistance is a major challenge. Whole-genome sequencing (WGS) has revolutionized the analysis of these mutations. With WGS, researchers can detect single nucleotide polymorphisms (SNPs), insertions/deletions (indels), and other variations in gamma-irradiated plants compared to non-irradiated controls. By using comparative, functional, and reverse genetic approaches, researchers can link these mutations to specific genes and pathways associated with pathogen resistance. This review explores the successful application of WGS in gamma-mutagenized plants to develop new lines and cultivars with enhanced resistance to various pathogens. We discuss case studies of resistance to rice blast, wheat powdery mildew and bacterial wilt in tomatoes and show how WGS identifies specific mutations that lead to improved disease tolerance. Finally, we explore the benefits of WGS with gamma mutagenesis, including identifying novel resistance mechanisms, discovering quantitative trait loci, and enabling marker-assisted selection for efficient breeding programs. We also acknowledge the challenges such as genome complexity and the need for functional validation, and emphasize the future potential of this approach for protecting plant health and ensuring global food security.

Keywords: Plant disease resistance, Gamma-ray mutagenesis, Whole-genome sequencing (WGS), Single nucleotide polymorphism (SNP).



Introduction

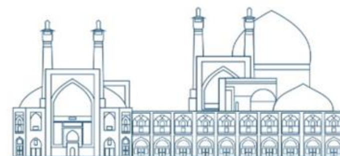
The continual rise of highly virulent plant pathogens poses an alarming menace to the stability of global food supplies. Conventional breeding strategies, essential for cultivating varieties resilient to these threats, are frequently marred by prolonged timelines and hampered by the scarcity of inherent resistance traits within the breeding stock. In this critical juncture, gamma-ray mutagenesis emerges as a transformative tool, disrupting these constraints by instigating a cascade of random mutations within plant genomes. These mutations hold immense potential, capable of spawning innovative defense mechanisms against a wide spectrum of pathogens. However, the task of pinpointing these invaluable mutations and deciphering their intricate roles in fortifying plant immunity remains a formidable challenge, occupying the forefront of research endeavors in the field (1, 2).

The Revolution of Whole-Genome Sequencing (WGS)

The advent of whole genome sequencing (WGS) marks a paradigm shift in the field of plant mutagenesis. This groundbreaking technology allows researchers to conduct in-depth studies of mutations that are present in the genomes of gamma-irradiated plants, as opposed to their non-irradiated counterparts. Using WGS, scientists can meticulously reveal a range of genetic changes, including single nucleotide polymorphisms (SNPs), insertions/deletions (indels) and various variations inherent in the DNA sequence (2).

Linking Mutations to Disease Resistance

By integrating a mix of comparative, functional and reverse genetics methods, researchers can elucidate the intricate relationship between the identified mutations and the precise genes or pathways that are central to plant defense mechanisms (Figure 1). Comparative genomics involves studying the genomes of both irradiated and non-irradiated plants to identify the distinct mutations that are exclusive to the irradiated lines. Functional genomics is concerned with unraveling the precise role of the mutated genes and their contribution to disease resistance. Finally, reverse genetics involves the manipulation of specific genes to validate their influence on the observed



resistance phenotype. This multifaceted approach provides a comprehensive understanding of the genetic landscape underlying plant immunity (3).

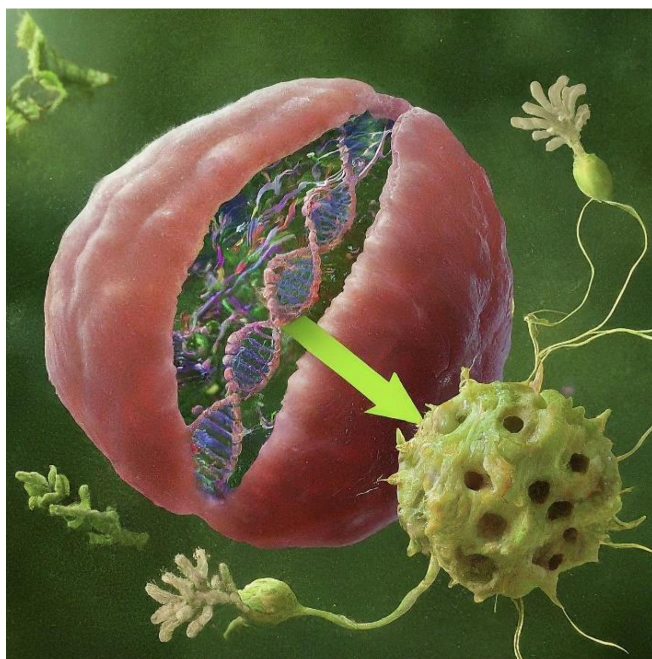
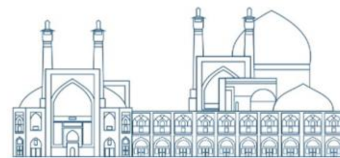


Fig.1. Unveiling Plant Defense: The Struggle Against Disease. This intricate illustration delves into the nucleus of a plant cell, the core of its genetic identity. The winding strands represent DNA, the blueprint of the plant's existence. Zooming in, a single base pair mutation is highlighted within one chromosome—a subtle change capable of reshaping gene function and, consequently, disease resistance. The image vividly portrays this critical concept: on the left, a robust plant cell fortified with the mutated gene successfully repels a fungal invasion, depicted by the intertwining threads. In stark contrast, the cell on the right, lacking the mutation, succumbs to the relentless fungal attack. This visual narrative underscores the profound impact of mutations in plant genomes on a plant's resilience against disease.

Case Studies: Success Stories of WGS in Gamma Mutagenesis

The integration of whole-genome sequencing (WGS) in gamma-mutagenized plants has yielded remarkable advancements in breeding new lines and cultivars with heightened resistance against various pathogens. Three noteworthy case studies demonstrate the efficacy of WGS in uncovering novel resistance mechanisms: Firstly, in rice blast disease, researchers identified a specific SNP mutation in a rice blast resistance gene, enhancing resistance against the fungus *Magnaporthe oryzae*. Secondly, in wheat powdery mildew, mutations in defense signaling pathways genes



improved resistance against *Blumeria graminis* f. sp. *tritici*. Lastly, in tomato bacterial wilt, mutations in genes producing antimicrobial compounds bolstered resistance against *Ralstonia solanacearum*. These case studies underscore the potential of WGS in fortifying plant defenses across diverse species (Table 1).

Table 1. Unveiling resistance mechanisms: WGS success stories in gamma-mutagenesis

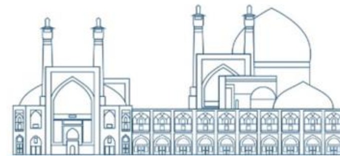
Crop	Disease	Mutation Identified	Outcome	Reference
Rice	Blast disease (*Magnaporthe oryzae*)	SNP mutation in a resistance gene	Enhanced resistance against the fungus	(4)
Wheat	Powdery mildew (*Blumeria graminis* f. sp. *tritici*)	Mutations in defense signaling pathway genes	Improved resistance against powdery mildew	(5)
Tomato	Bacterial wilt (*Ralstonia solanacearum*)	Mutations in genes responsible for antimicrobial compound production	Increased resistance against the bacterial pathogen	(6)

Advantages of Combining WGS with Gamma Mutagenesis

The combination of WGS with gamma mutagenesis presents a formidable toolkit with several distinct advantages for the development of pathogen-resistant cultivars:

Unveiling Novel Resistance Mechanisms: WGS serves as a powerful lens, enabling researchers to uncover previously unknown mutations that bestow plants with resistance against pathogens. This knowledge not only expands our comprehension of how plants interact with pathogens but also presents new opportunities to enhance crop resilience. Armed with this information, researchers can develop innovative strategies to breed crops with enhanced resistance to diseases (3). This can involve targeted breeding programs aimed at selecting and breeding plants with specific resistance-conferring mutations identified through WGS.

Illuminating Quantitative Trait Loci (QTLs): Gamma mutagenesis induces mutations in genes with subtle yet significant effects on disease resistance, known as quantitative trait loci (QTLs). WGS



serves as a compass, guiding researchers to these elusive loci, thereby facilitating targeted breeding efforts aimed at enhancing disease resistance in crops (7).

Streamlining Marker-Assisted Selection (MAS): Through the precise identification of markers linked to desirable resistance mutations, WGS streamlines the process of marker-assisted selection (MAS). This strategic approach empowers breeders to swiftly pinpoint and select plants carrying the desired mutations during breeding programs, thereby expediting the development of resilient cultivars capable of withstanding pathogenic onslaughts (3).

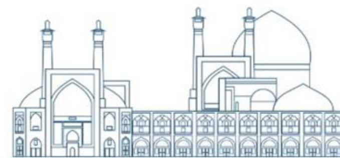
By synergizing WGS with gamma mutagenesis, researchers and breeders unlock a wealth of possibilities, revolutionizing the quest for pathogen-resistant crops and heralding a new era in agricultural sustainability and food security.

Challenges and Future Potential

While whole-genome sequencing (WGS) stands as a potent instrument for scrutinizing gamma-mutagenized plants, certain hurdles persist. The intricate nature of plant genomes demands robust bioinformatics tools to streamline data analysis effectively. Furthermore, the imperative lies in validating identified mutations functionally through targeted experiments to ascertain their role in disease resistance. Looking ahead, the trajectory of WGS technologies and bioinformatics is poised for advancement, promising heightened efficiency and precision in mutation detection. Additionally, the fusion of WGS with other mutagenesis methodologies holds the potential for broadening the spectrum of genetic variations and expediting the discovery of novel resistance mechanisms. The amalgamation of WGS and gamma mutagenesis emerges as a formidable strategy for safeguarding crop vitality and upholding global food security amidst the perpetual evolution of plant pathogens.

Conclusion

The integration of whole genome sequencing (WGS) into the field of gamma-mutagenized plants represents a groundbreaking milestone in plant breeding. This synergy has sparked a revolution that is reshaping the landscape of crop improvement. By uncovering novel resistance mechanisms



and pinpointing quantitative trait loci (QTLs), WGS provides breeders with a comprehensive toolkit for developing robust and durable disease resistance in crops. In addition, by facilitating marker-assisted selection (MAS), WGS streamlines the breeding process, accelerating the development of enhanced varieties. Undoubtedly, challenges remain, such as the intricate complexity of plant genomes and the need for robust functional validation. However, advances in technology and ongoing research efforts are steadily overcoming these obstacles and strengthening the efficacy of this approach. As WGS continues to evolve and seamlessly integrates with complementary mutagenesis techniques, the horizon is brimming with potential. This convergence promises to catalyze the discovery and deployment of novel resistance mechanisms at an unprecedented rate, underscoring its central role in strengthening our global food system and ensuring its resilience and sustainability for generations to come.

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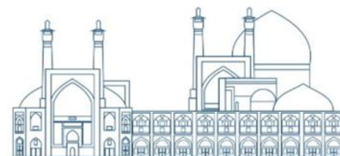

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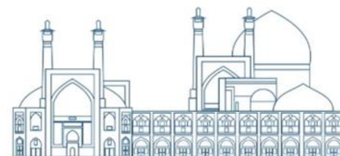

Use of RNA-Seq Technique to Find Key Genes in Plants that Were Under Gamma Ray Treatment to Increase Resistance to Pathogens (Paper ID: 1677)

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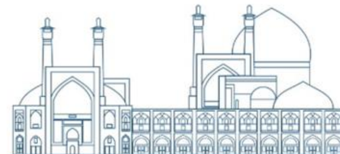
Abstract

Plant diseases caused by a variety of pathogens pose a significant threat to global food security. While breeding for disease resistance is crucial, conventional methods are reaching their limits. Recent advances in mutagenesis, such as gamma-ray treatment, offer promising alternatives as they can induce random mutations in the plant genome and thus increase resistance. RNA- RNA-sequencing technology (RNA-Seq) is a powerful tool to identify key genes involved in this acquired resistance. This review explores the application of RNA-Seq to analyze changes in gene expression in plants exposed to gamma rays to improve their resistance to pathogens. The principles of gamma-ray mutagenesis and its effectiveness in generating resistant plant lines are discussed. In addition, RNA-Seq technology is examined, highlighting its strengths in providing a comprehensive picture of gene expression. The review outlines the steps involved in using RNA-Seq for this purpose, including plant material preparation, RNA isolation, library construction, sequencing, and data analysis. Emphasis is placed on the identification of differentially expressed genes (DEGs) potentially associated with observed resistance. Successful studies using RNA-Seq to identify key genes for disease resistance following gamma irradiation will also be presented. The advantages of RNA-Seq, including its high-throughput nature, comprehensiveness, and sensitivity in detecting subtle expression changes, are discussed. However, limitations such as cost and data complexity are also acknowledged. This review serves as a valuable resource for researchers exploring the use of RNA-Seq to understand the molecular mechanisms underlying gamma-ray-induced disease resistance in plants.

Keywords: Plant disease resistance, Gamma-ray mutagenesis, RNA-Seq, Differential gene expression



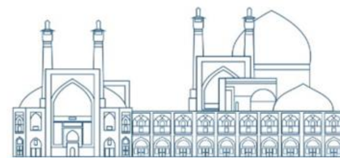
Introduction

A myriad of pathogens, spanning bacteria, fungi, and viruses, collectively pose a formidable challenge to global food security, catalyzing plant diseases that inflict substantial yield losses, thereby denting agricultural productivity and economic stability. Mitigating these losses necessitates a strategic approach, with breeding for disease resistance standing as a pivotal strategy. However, conventional breeding methods are marred by their time-consuming nature, labor-intensive processes, and constraints imposed by the availability of desirable resistance traits within existing germplasm pools. Fortunately, recent strides in mutagenesis techniques present promising alternatives, offering novel avenues for fortifying plants against diseases (1).

Gamma Ray Mutagenesis for Disease Resistance

Gamma-ray mutagenesis entails subjecting plant seeds or vegetative tissues to controlled doses of gamma radiation. This radiation instigates random mutations within the plant genome, potentially leading to alterations in gene function. Remarkably, these mutations can activate or augment plant defense mechanisms, thereby bolstering resistance against specific pathogens. The method boasts several advantages:

Broad-spectrum effects: Gamma-ray mutagenesis holds the capacity to induce mutations across the entire genome, thus influencing a wide array of traits, including disease resistance.
Efficiency: Compared to traditional breeding methods, gamma ray mutagenesis stands out for its swiftness and efficacy in generating genetic variability.
Novel traits: Notably, this approach has the potential to engender entirely novel resistance mechanisms previously absent in the original germplasm.
However, it's imperative to note that gamma-ray mutagenesis may also induce undesirable mutations that could adversely affect plant growth or yield. Hence, meticulous selection and thorough evaluation of mutagenized lines are paramount (2).



RNA-Seq: A Powerful Tool for Gene Expression Analysis

RNA-Seq technology has revolutionized our understanding of gene expression in living organisms. It allows researchers to measure the abundance of all RNA molecules (including messenger RNA, ribosomal RNA, and transfer RNA) within a cell or tissue sample. This comprehensive data provides valuable insights into which genes are actively transcribed and at what levels (Figure 1) (3).

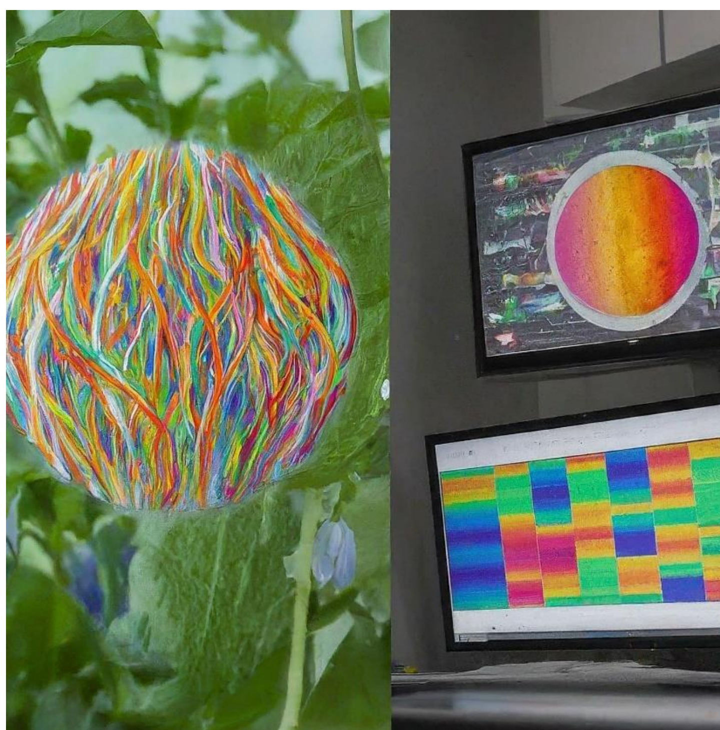
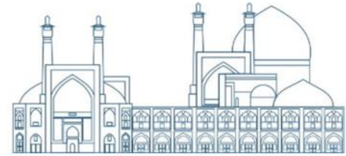


Fig.1. Heatmap visualization of gene expression: A scientist analyzes a heatmap on a computer screen, where color intensity represents gene expression levels in a plant.

In the context of studying gamma ray-induced disease resistance, RNA-Seq can be used to identify key genes whose expression is altered following gamma ray treatment and subsequent pathogen challenge. By comparing the transcriptomes of treated and untreated plants, researchers can



pinpoint differentially expressed genes (DEGs) that may be involved in the observed resistance phenotype.

Utilizing RNA-Seq to Identify Key Genes for Disease Resistance

Here's a breakdown of the steps involved in using RNA-Seq to study gamma ray-induced disease resistance:

Plant Material Preparation:

Plants from mutagenized lines displaying enhanced disease resistance are selected.
Samples like leaves or other appropriate tissues are collected from both resistant and control (non-irradiated) plants.
Timing of sample collection is crucial – it should coincide with the expected activation of defense responses during pathogen infection.

RNA Isolation:

High-quality RNA is extracted from the collected plant tissues using specialized kits or protocols.
RNA integrity is rigorously assessed to ensure reliable downstream analysis.

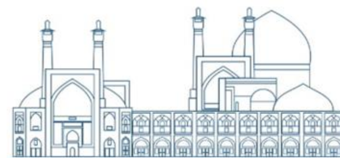
Library Construction:

Isolated RNA is converted into cDNA libraries suitable for sequencing. This involves reverse transcription to generate cDNA from the RNA templates and subsequent library preparation steps like adapter ligation and fragmentation.

Sequencing:

The prepared libraries are loaded onto next-generation sequencing (NGS) platforms for high-throughput sequencing. Sequencing generates millions of short reads representing the RNA transcripts present in the samples.

Data Analysis:



The raw sequencing data undergoes quality control and preprocessing to remove adapter sequences and filter out low-quality reads.

The cleaned reads are aligned to a reference genome of the plant species under study.

Differential expression analysis is performed to identify DEGs that are significantly upregulated or downregulated in resistant plants compared to controls.

Functional Annotation:

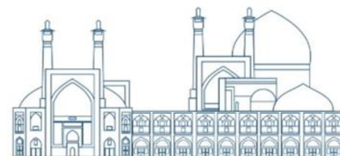
The identified DEGs are subjected to functional annotation using bioinformatics tools to predict their potential roles in plant defense pathways. Gene ontology (GO) analysis, protein domain identification, and comparison to known resistance genes in other plant species can provide valuable clues about their function.

Examples of Successful Applications

Several studies have successfully employed RNA-Seq to identify key genes associated with gamma ray-induced disease resistance in plants. For instance, research on rice exposed to gamma rays revealed the upregulation of genes involved in defense signaling pathways and pathogenesis-related (PR) protein production after a challenge with a fungal pathogen. Similarly, studies in tomatoes identified DEGs linked to the biosynthesis of secondary metabolites with antimicrobial activity following gamma ray treatment and subsequent infection (Table 1)(4, 5).

Table 1. Identification of Key Genes for Disease Resistance in Various Plant Species Through Gamma-Ray Mutagenesis: A Review of RNA-Seq Studies

Plant Species	Pathogen	Identified Genes	Putative Function	Reference
Rice	*Magnaporthe oryzae* (Fungal pathogen)	OsWRKY45, OsNPR1	Regulation of defense signaling pathways	(4)
Tomato	*Botrytis cinerea* (Fungal pathogen)	StNPR1, StGST	Activation of defense responses, Glutathione S-transferase involved in detoxification	(5)
Soybean	*Pseudomonas syringae* pv. *glycinea* (Bacterial pathogen)	GmWRKY76, GmPAL	Regulation of defense signaling pathways, Phenylalanine ammonia-lyase involved in secondary metabolite production	(6)



Barley	*Bipolaris sorokiniana* (Fungal pathogen)	HORVU3Hr 1G019920 (glycine-rich protein) and HORVU5Hr 1G120850	Pathogenesis-related protein and regulator of defense responses	(7)

Advantages and Limitations of RNA-Seq

RNA-Seq offers several advantages for studying gene expression changes in plants (3):

1- High-throughput: It allows for simultaneous analysis of thousands of genes.
2- Comprehensiveness: It provides a genome-wide view of transcriptional activity.
3- Sensitivity: It can detect subtle changes in gene expression
4- Dynamic range: It can capture a wide range of transcript abundance levels.

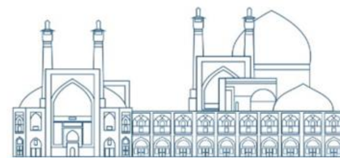
However, RNA-Seq also has some limitations to consider:

1- Cost: The cost of library preparation, sequencing, and data analysis can be high, especially for large-scale studies.
2- Data complexity: The vast amount of data generated by RNA-Seq requires specialized bioinformatics expertise for analysis and interpretation.
3- Technical challenges: Factors like RNA quality, library preparation methods, and sequencing bias can influence the results.

Future Directions

Future research directions in this field could involve:

Integrating RNA-Seq data with other omics technologies like metabolomics and proteomics to gain a more comprehensive understanding of the plant's response to gamma rays and pathogen challenges. Moreover, functional validation of candidate genes identified through RNA-Seq using techniques like gene editing (CRISPR-Cas9) to confirm their role in disease resistance. Also, Developing computational tools to streamline data analysis and facilitate the identification of key



regulatory pathways involved in the resistance response. By combining RNA-Seq with these advanced approaches, researchers can gain deeper insights into the complex mechanisms of gamma ray-induced disease resistance in plants, ultimately leading to the development of more durable and sustainable strategies for protecting our crops from devastating pathogens.

Conclusion

RNA-Seq technology is proving to be a powerful tool for unraveling the intricate molecular mechanisms underlying gamma radiation-induced disease resistance in plants. By identifying key genes whose expression is modulated following mutagenesis and pathogen attack, RNA-Seq is laying the groundwork for the development of novel disease resistance strategies. However, it's imperative to recognize the inherent limitations of this technique and to carefully employ appropriate controls and data analysis methods to ensure the generation of reliable and meaningful insights. This review highlights the enormous potential of RNA-Seq in unraveling the complex genetic framework governing plant disease resistance following gamma irradiation. As technological advances continue to drive down costs and increase accessibility, RNA-Seq is poised to play an increasingly central role in both plant breeding efforts and disease management strategies, ushering in a promising era of innovation and resilience in agricultural practices.

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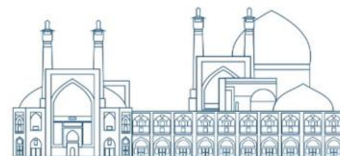

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