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Biochemical Analysis of Eight Mutants of *Vicia faba* L. in M_2 & M_3 Generation

Kamble Sonali R[1], More A. D.[2]

[1]Department of Botany, BJS, ASC, College, Pune
Corresponding Author Email: *sonaliganesh02[at]gmail.com*

[2]Department of Botany, Fergusson College, Pune

Abstract: ***Vicia faba L. is a flowering plant belonging to family Fabaceae which reflects all properties of a legume crop such as soil improvement and benefit of human health.. Vicia faba seeds contains high nutritional value and opulent in protein, carbohydrates, in volute vitamins, folic acid, niacin and vitamin C, dietary fibres and macro and micro nutrients. The present research work was conducted to study morphological changes and chlorophyll mutation in leaves of Vicia faba by single and combined treatment of Gamma rays and Ethyl Methanesulphonate. Screening of mutation was carried out in M_1 generation of Vicia faba variety Wal Kokan Bhushan. Studies were planned to investigate the biochemical aspects of different promising M_2 and M_3 generation mutants of the plant like the parameters of leaf protein and seed proteins content. In the present studies eight mutants induced namely robust, branched, dark green, early flowering, late flowering, tall, dwarf, luxuriant mutants respectively.***

Keywords: *Vicia faba*, EMS, Gamma rays, biochemical, Chlorophyll, Nutrient.

1. Introduction

In leaf and seed material of plants and their nutritional improvement of legumes through mutation breeding have attracted substantial attention of the world (Milner, 1972). It has been increasingly realized that the genetic variability available in legumes can be exploited for increasing the proteins properties to generate nutritionally improved varieties (Jain, 1969; Swaminathan and Jain, 1972). The nutritional characteristics of different legumes have been thoroughly understood and the need for improvement in certain factors in plant system can be highlighted reported by (Baldev*et al*.,1988). One of the major trends in modern plant mutation breeding has been the support of traditional methods by biochemical investigations to obtain a better estimate of a variety's mutation breeding values. The economic value of economic plants was simply not limited to the number and weight of seeds produced. Proteins, oils, carbohydrates, minerals, and secondary metabolites such as tannins, alkaloids, and so on are all stored in seeds. Essentially, the leaf, seed proteins, and seed oils are important sources of nutrition for both humans and animals. Legumes are not only a major source of protein, but they are also a good source of dietary fibre, which is a type of carbohydrate. Minerals are also essential components of human nutrition. They are most important factors in maintaining physiological processes, and act as catalyst for many biological reactions. In last few decades, the aspects of genetically influenced on protein composition have been widely worked out. A new aspect in the agriculture has been the improvement of some such crucial materials, which are vital from nutrition point of view, through the mutation breeding approach. The nutrition improvement of legumes through mutation breeding program has an immense importance in the world of food crisis. Induction of mutation is an important part of breeding programme as it creates, the gene pool through creation of genetic variability. (Swamy and Reddy, 2004). Therefore, the genetic variability is the basic requirement for making improvement in crop breeding programme. Many researchers have worked in the field of mutation breeding and estimation of biochemical studies like Deshpande (1980), Kothekar (1983), Hakande (1992), More (1992).

The broad bean is drought- tolerant legume belongs to family *Fabaceae*. Pods are used as a vegetable for human consumption and the crop is grown for the cattle feed and as green manure. Studies were planned to investigate the biochemical aspects of different promising M_3 generation mutants of the plant like the parameters of leaf protein and seed proteins content. In the present studies M_3 generation eight mutants induced namely Robust, branched, dark green, early flowering, late flowering, tall, dwarf, luxuriant mutants respectively.

Using EMS, Gamma rays and Combination of both mutagens treatments and selected for their better performance. They were estimated for their chlorophyll content, leaf protein, seed protein, carbohydrates and mineral contents.

2. Material and Methods

1) Chlorophyll Estimation

Chlorophyll is a major component occurring in chloroplast and it is present in all green plants. It is a green pigment, made up of proteins and extracted in organic solvents like acetone. Each chlorophyll molecule made up of a porphyrin and magnesium present at the centre and a long hydrocarbon chain known as phytol which is attached through a carboxylic acid group. In higher plants chlorophyll a and b is present.

Principle:
Extraction of chlorophyll was done using 80% acetone. The wavelength at which it was measured at 663nm and 665 nm for chlorophyll a and b. Using the absorption coefficient, the chlorophyll amount can be estimated.

Chemicals:
Pre-chilled Acetone 80%

Procedure:
Weight 1gm of finely cut leaves into clean mortar. The sample was grind into fine pulp by adding of 20 ml of 80% acetone. Extract was centrifuge at 5000 rpm for 5 min. The supernatant was transferred to 100 ml volumetric flask. The residue was again mixed with 20ml of 80% acetone and transferred to volumetric flask. The procedure was repeated till the residue becomes colourless.

2) Estimation of leaf protein and Seed protein by Lowrey et al. (1951) method

Material Used:
Healthy leaves and dry seeds

Procedure for Extraction:
The 0.5g of the material was used and it was homogenized in hot 80% ethanol. The homogenate sample was them collected and transferred in a test tube. The extract was boiled in hot water bath. The sample was collected with 5% PCA (Perchloric acid). Again, sample was centrifuged at 5000rpm for 10 minutes, the supernatant was discarded which contains high molecular weight compounds like DNA and RNA. Residue was extracted at 30°C for 30 minutes in 5-6ml of 2% Na_2CO_3 prepared in 0.1 n NaOH. The sample was centrifuged at 5000 rpm for 10 minutes then the residue was discarded, and supernatant was saved. Supernatant was added with 10 ml of distilled water. The prepared crude protein extract was utilized for present protein estimation.

Estimation of Protein:

Principle:
The colorimetric method is used for determination of the protein content for the plant material. The method is simple and sensitive. The principle is based on the presence different amino acids and their reaction with protein, The bluish purple colour is developed by the biuret reaction of protein with alkaline cupric tartarate. The intensity of the coloured product is measured at 660 or 750nm colorimetrically or spectrophotometrically.

Procedure:
Preparation of the test tube for general BSA solution changed into carried out from 0.1 to 0.5 ml and the quantity in all 1-8 test tube changed into adjusted with 0.5 ml of distilled water. The plant extract changed into 0.1 ml was delivered to test number 7 and 8. The 5 ml of freshly prepared reagent C was added to all test tubes. The content material was mixed very well and incubated at room temperature for 20 minutes. Addition of reagent D in all test tubes is observed with incubation for 20 minutes. Absorbance of the pattern at 750nm recorded using spectrophometer. The test tube #1 used as a blank for standardization. The graph plotted for quantity of protein v/s absorbance at 750nm. The quantity of protein in the sample was calculated from the graph.

0.1ml of plant extract contains= X µg protein (from the graph)
∴ 10 ml of extract (=total volume of extract) contain=10
This 10ml extract was prepared from 0.5g of plant material
∴ 0.5g plant material contains = Yµg protein
1g plant material contains = Y/0.5 = Z µg protein
(Source: Sadasivam and Manikam, 2008)

3) Total estimation of carbohydrate byAnthrone method (Hedge and Hofreiter, 1962) Principle:
The Carbohydrates are hydrolyzed into simple sugars using a dilute hydrochloric acid. Glucose is dehydrated to hydroxymethyl furfural in an hot acidic medium which develops a green coloured compound. The green coloured product is measured at the absorbance of 630nm by Anthrone method.

Procedure:
The seed sample was cleaned and grind to fine powder. The powdered seeds was then hydrolysed The supernatant was collected and from the sample 0.5 and 1 ml aliquots are used for carbohydrate analysis. The intensity of the green colour develop is measured spectrophotometrically at 630nm. To prepare a standard curve the D-glucose is used at 100ug /ml. Using the following formula the amount of carbohydrate is calculated.

4) Estimation of Minerals

Total Nitrogen estimation by Micro-Kjeldahl method:
The total nitrogen content of the seed sample was estimated using the Micro-Kjeldahl distillation method. The dried and healthy seeds of control and viable mutants was used for estimation

Procedure:
The seed sample of 100 mg was transferred to a 30 ml of digestion conical flask. Chemicals like potassium sulphate 1.9 ±0.1g and mercuric chloride 80±10 mg was added. In the digestion flask 2 ml of H_2SO_4 was also added. By adding the boiled chips the sample was digested till it becomes colourless. The digested sample was then cooled and diluted with a small quantity of distilled ammonia-free water. Sample was then transferred to the distillation apparatus. During the titration the micro Kjeldhl flask should be rinsed with successive small quantities of water. Boric acid 5 ml was used as indicator for reaction. In the apparatus add 10 ml of sodium hydroxide-sodium thiosulphate solution. Rinse the tip of condenser. The sample solution was titrated against standard acid to observe the change from colourless to violet. The nitrogen content was estimated using the following formula.

$$\text{N g/kg} = \frac{\text{ml HCl} - \text{ml blank}) \times \text{Normality} \times 14.01 \times \text{Final volume}}{\text{Weight (g)} \times \text{Aliquot volume}}$$

(Source: Sadasivam and Manikam, 2008).

3. Results

1) Chlorophyll Content
The control plant sample shows 1.7012 mg/gm of total chlorophyll content. The highest amount of chlorophyll content 3.0306 mg/gm was observed in dark green leaf mutant followed by luxuriant mutant 3.0295 mg/gm. Lowest amount of chlorophyll 1.7533 mg/gm was recorded in dwarf mutant. The total amount of chlorophyll content in the morphological mutant was in the range 1.7533 mg/gm to

3.0306 mg/gm in M_2 generation of *Vicia faba* L. In M_3 generation the control shows 0.7915 mg/gm of chlorophyll content. The highest chlorophyll content is observed in luxuriant mutant (3.010 mg/gm) followed by dark green leaf mutant 2.993 mg/gm.

The lowest chlorophyll content was recorded in early flowering mutant 1.778 mg/gm. The range of chlorophyll content varies from 1.778 mg/gm to 3.010 mg/gm in M_3 generation of *Vicia faba* L. Fluctuations was observed in chlorophyll content which shows direct effect on physiology of plants.

Table 1.1: Effect of mutagens on Chlorophyll content of the morphological mutants of *Vicia faba* L. in M_2 generation

Morphological mutants	Chlorophyll "a" mg/gm	Chlorophyll "b" mg/gm	Total Chlorophyll mg/gm
Control	0.6052	1.0960	1.7012
Robust mutant	0.9668	1.7524	2.7192
Branched mutant	0.7029	1.2722	1.9751
Dark green mutant	1.0767	1.9539	3.0306
Early flowering mutant	0.6458	1.1690	1.8148
Late flowering mutant	0.6568	1.1887	1.8455
Tall mutant	0.8795	1.5949	2.4744
Dwarf mutant	0.6235	1.1298	1.7533
Luxuriant mutant	1.0772	1.9523	3.0295

Table 1.2: Effect of mutagens on chlorophyll content in morphological mutants in M_3 generation of *Vicia faba* L

Morphological mutants	Chlorophyll "a" mg/gm	Chlorophyll "b" mg/gm	Total Chlorophyll mg/gm
Control	0.7055	1.0860	0.7915
Robust mutant	0.9568	0.7124	2.6692
Branched mutant	0.7026	1.2721	1.9747
Dark green mutant	1.0667	1.9439	3.0106
Early flowering mutant	0.6148	1.1590	1.7738
Late flowering mutant	0.6365	1.1887	1.8252
Tall mutant	0.8192	1.5847	2.4039
Dwarf mutant	0.6635	1.1294	1.7929
Luxuriant mutant	1.0812	1.9121	2.9933

2) Protein content (Table No 1.3 and 1.4)

Leaf protein content and Seed protein content

The estimated sample of leaf protein content in the control was 4.52%. The leaf protein content in the mutant showed increase in values as compared to control. The average of leaf protein content of eight different mutants was in the range from 3.40% to 8.50%. The highest leaf protein content was observed in luxuriant mutant and lowest leaf protein content 3.40% was observed in early flowering mutant. The dwarf mutant shows 7.40% of the leaf protein content next to the luxuriant mutant in M_2 generation. The seed protein content in majority of the mutants showed increasing values as compared to control. The seed protein content in the control is 5.19%. The highest seed protein content 8.50% was observed in luxuriant mutant and 8.42% in tall mutant. The lowest seed protein was observed in branched mutant. The average of seed protein content in eight mutants was found in the range 4.71% to 8.42% respectively.

In the M_3 generation of *Vicia faba* L. the control shows 4.20% of seed protein and 4.51% of leaf protein. The highest content of seed protein is recorded in luxuriant mutant 7.80% followed by tall mutant 8.18%, the range of seed protein content varies from 4.66% to 7.80%. The highest leaf protein is observed in luxuriant mutant 8.31% followed by tall mutant 7.40%. Lowest amount of leaf protein content is observed in early flowering mutant 3.44% in M_3 generation. The range of leaf protein content varies from 3.44% to 8.31%.

3) Total Carbohydrate content (Table No. 1.3 and 1.4)

Carbohydrate content was 5.58% observed in dwarf mutant and lowest 1.79% recorded in branched mutant respectively in M_2 generation of *Vicia faba* L. In the M_3generation the carbohydrate content in control was observed to be 2.39%. The highest amount of carbohydrate content is recorded in tall mutant 5.58% followed by dwarf mutant 4.03%. The lowest amount of carbohydrate content is observed in branched mutant 1.79%. The range of carbohydrate content varies from 1.79% to 5.58% in M_3 generation of *Vicia faba* L.

Table 1.3: Effect of Mutagens on Leaf protein, Seed protein content and total carbohydrate content in *Vicia faba* L. in M_2 generation

Morphological mutants	Seed protein %	Leaf protein %	Carbohydrate content %
Control	5.19	4.52	2.33
Robust mutant	7.17	4.92	2.44
Branched mutant	4.71	4.36	1.79
Dark green mutant	5.1	5.44	2.09
Early flowering mutant	5.67	3.4	2.08
Late flowering mutant	7.46	4.44	2.13
Tall mutant	8.42	6.56	4.02
Dwarf mutant	7.44	7.4	5.58
Luxuriant mutant	8.5	7.8	3.03

Table 1.4: Effect of mutagens on leaf protein, seed protein and total carbohydrate content in M_3 generation of *Vicia faba* L.

Morphological mutants	Seed protein %	Leaf protein %	Carbohydrate content %
Control	4.2	4.51	2.39
Robust mutant	7.11	4.19	2.13
Branched mutant	4.66	4.31	1.79
Dark green mutant	5.11	5.44	2.08
Early flowering mutant	5.61	3.44	2.09
Late flowering mutant	7.44	4.23	2.13
Tall mutant	8.18	6.5	5.58
Dwarf mutant	6.44	7.4	4.03
Luxuriant mutant	7.6	7.9	3.09

4) Mineral content (Table No. 1.5)

Table 1.5: Effect of mutagens on Mineral content of the morphological mutant of *Vicia faba* L.

Morphological Mutants	Mineral content in morphological mutants of *Vicia faba* L.										
	N%	P%	K%	Ca%	Mg%	S%	Fe ppm	Mn ppm	Zn ppm	Cu ppm	Na%
Control	4.26	0.45	1.5	0.39	0.19	0.1	118	16	50.6	Nil	0.55
Robust mutant	1.26	0.59	1.7	0.35	0.42	0.15	106	7.9	57.2	Nil	0.4
Branches mutant	4.09	0.6	1.5	0.3	0.44	0.2	254	12	50.2	Nil	0.6
Dark green mutant	4.41	0.56	1.31	0.4	0.41	0.19	143	16.13	50.9	Nil	0.4
Early flowering mutant	4.2	0.54	1.5	0.56	0.38	0.15	130	14	45	Nil	0.5
Late flowering mutant	4.59	0.6	1.49	0.43	0.33	0.24	130	18	51	Nil	0.4
Tall mutant	3.86	0.5	1.4	0.47	0.32	0.17	256	18	46	Nil	0.4
Dwarf mutant	5.22	0.61	1.68	0.7	0.55	0.09	356	10	68.6	Nil	0.55
Luxuriant mutant	4.48	0.37	1.4	0.43	0.3	0.06	178	10	46.4	Nil	0.4

Macro and micronutrients was estimated from the seed sample of *Vicia faba*L.mutant. The different minerals like N, P, K, S, Mn, Fe, Mg, Zn, Cu, and Na was estimated from the given seeds sample of eight different viable mutants. The average of the mineral content in the seeds of the control ranges from 0.10% to 4.26% and in other minerals showed 16 ppm to 118 ppm. The highest 5.22% nitrogen content was observed in dwarf mutant. The highest 0.60% phosphorous minerals content was observed in branched and late flowering mutant. The highest 0.50% potassium minerals content was observed in branched and early flowering mutant. The highest 0.70% calcium mineral content was found in dwarf mutant. The other Mg, S and Na mineral content was 0.9% to 0.50% observed in varied content of minerals in seed samples. In case of Fe, Mn, Zn and Cu the mineral content was 7.90 ppm to 256 ppm was observed in dwarf mutant.

4. Discussion

For the improvement in pertaining to protein content through genetic approach can be a permanent source.

The general seed protein content and the protein pattern with relative proportions of the distinctive protein organizations influenced with the aid of using the exceptional mutant genes Gottschalk and Muller (1970). Tah (2006) pronounced improved seed protein content in types of Mungbean towards the control. Auti and Apparao (2008) expected the seed protein content in feasible mutants of Mungbean. Wani and Anis (2008) studied that the reduction in protein content of bold seeded in excessive yielding mutant of Chickpea. Pavadai*et al*. (2010) pronounced the upgrades in seed protein content of Soyabean. Sri Devi and Mullaainathan (2012) recorded that during improved seed protein content of mutant in Black gram.

Hendawey and Younes (2013) studied the biochemical evaluation of some broad beans cultivars under rainfed conditions and reported the photosynthetic pigments, seed protein content and total vicine content in the broad cultivars. A experiment is carried out by Saber (2016) to study the effect of foliar spraying with plant growth regulators treatments on some promising varieties of *Vicia faba* L. cultivars and the results had positive effect on the yield of seeds, increase in total sugar content, protein, total carbohydrates, total nitrogen content and seed yield in all the treatments. Osman and Hoda (2020) studied the karyotype variation and biochemical analysis of five *Vicia* sps and found a phylogenetic relationship between the studied species depending upon the data of protein markers.

Many researchers have made efforts for improving protein content in viable mutants of different crop plants like (Hakande, 1992) in *Psophocarpus tetragonolobus* L. (More, 1992) in *Medicago sastiva*L. (Sagade and Apparao, 2011) in *Vigna mungo* L. (Kamble and More, 2019) in *Vicia faba,* (Mukadir et al., 2020) in *Vicia faba*L. (Saber, 2016) in *Vicia faba* L. (Hendawey and Younes, 2013) in *Vicia faba* L., (Osman and Hoda, 2020) in broad beans.

From the above observations, there is a lot of scope for improving the Broad beans system through mutation breeding in regard to important features like chlorophyll content, leaf protein, seed protein, carbohydrates and mineral contents. In M_3 generation very less fluctuations were induced by the mutagens as regards to protein content. The estimation of biochemical components indicated the application of mutation breeding in the development of superior genotype carrying improved nutritional values in broad beans.

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