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Assessment of Genetic Diversity in Exotic Fenugreek (*Trigonella foenum – graecum*) Germplasm Using Sds-Page Analysis

Muhammad Miraj[1], Durrishahwar[1], Saifullah[2], Muhammad Mehran Anjum[2], Nawab Ali[2] and Sajid Islam[2]

[1]*Plant Breeding and Genetics, The University of Agriculture Peshawar, Pakistan*
[2]*Department of Agronomy, The University of Agriculture Peshawar, Pakistan*

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ABSTRACT

Fenugreek (*Trigonella foenum-graecum* L.) is widely used as a spice in India and the Mediterranean region and is identified to have a number of medicinal properties. In order to assess the genetic diversity in fenugreek germplasm using SDS-PAGE, 21 accessions of fenugreek were collected from gene bank of PGRI, NARC, Islamabad. The present study aimed at determining the genetic variability for total seed protein among 21 Fenugreek accessions using SDS-PAGE technique. Fenugreek accessions of different geographical origin were screened for genetic variability using 14 percent acrylamide gel concentration. Sixteen bands were found in the accessions investigated. SDS analysis of 21 fenugreek accessions indicated that two of them were monomorphic and 14 accessions were polymorphic. On the basis of cluster analysis, four clusters were identified at similarity level of 1.8.Overall on the basis of total seed protein, grouping pattern of *Trigonella Foenum graecum* was mostly compatible with their specie status. Accessions belonging to same species clustered together and vice versa. Moreover, the variation present in accessions can effectively be used by the breeders and molecular biologists for the future varietal development programs.

Key words: Fenugreek, Genetic Diversity, SDS-PAGE, Cluster Analysis

Introduction

The genus *Trigonella* is one of the biggest genera of the tribe. *Trigonella*, derived from Latin meaning 'little triangle', refers to the triangular shape of its small yellowish white flowers. The species epithet *foenum graecum* means 'Greek hay' (Rosengarten, 1969). It is also called 'ox horn' or 'goat horn' because of the two seed pods projecting in opposite directions usually from the nodes of the stem base that resemble ox or goat horns (Petropoulos, 2002). Fenugreek (*Trigonella foenum-graecum* L.) is an annual, self-pollinated plant which belongs to *Leguminosae* family having small seeds. Since old times it has been known as a medicinal herb (Slinkard, 2006). Acharya (2007) and his co-researchers reported that, although fenugreek is grown as a spice in most parts of the world, the species name "*Foenum-graecum*" means "Greek hay" indicating its use as a forage crop in the past (Acharya *et al*., 2006; Petropoulos, 2002). In this concern, Saraswat (1984) identified carbonized fenugreek seeds from Rohira village in the Sangrur district of Indian Punjab, which indicates that people of the Harappa civilization used fenugreek in trade as far back as 2000–1700 B.C. The origin of fenugreek is assumed to be in the Mediterranean region and adjacent areas (Duke *et al*., 1981).

Fenugreek is widely cultivated in different parts of the world, including India, Egypt, Ethiopia, and England. It can enrich soil, fix nitrogen and used for animal field and human consumption (Bromfield *et al*., 2001). Fenugreek seeds supplies dietary proteins for vegetarians that lack animal and fish protein in their diet. Leaves and seeds of Fenugreek are consumed in different countries around the world for different purposes including medicine (anti-diabetic, lowering blood sugar and cholesterol level, anti-cancer, minimizing symptoms of menopause, good for kidney trouble, relieves constipation, reduces cardiovascular risk, relieves sore throats, anti-microbial, etc.), ingredient of food (stew with rice in Iran, flavor cheese in Switzerland, syrup, mixed seed powder with flour for making flat bread in Egypt, curries, dyes, young seedlings eaten as a vegetable, etc.), roasted grain as coffee-substitute (in Africa), controlling insects in grain storages, perfume industries,

Corresponding Author: Muhammad Miraj, Plant Breeding and Genetics, The University of Agriculture Peshawar, Pakistan. E-mail: miraj1374@gmail.com

etc. (Erum *et al*., 2011).

Fenugreek contains a wide variety of beneficial nutrients, including iron, magnesium, manganese, and copper, as well as vitamins B_6, protein, and dietary fiber. Fenugreek also contains a number of powerful phytonutrients, including choline, trigonelline, yamogenin, gitogenin, diosgenin, tigogenin and neotigogens.

The exact number of species of fenugreek also has been debated. Petropoulos (2002) indicated that older taxonomists like Linnaeus (0000) have suggested that as many as 260 species of fenugreek exists. In contrast, about 128 species of fenugreek were reported by Vasil'chenko (1953), 97 by Fazli (1967) and 70 by Hector (1936), Hutchinson (1964) and Roukand Mangesha (1963).A total of 18 different species of fenugreek (*Trigonella*) currently are recognized in the primary literature.

Plant genetic diversity is a useful character in plants that can be transmitted genetically from parents to off spring. The sources of tremendous variation in plants support all other forms of life on land. Plant genetic diversity covers a wide range, at both the evolutionary and ecological level. Ecologically the variation ranges from the natural ecosystems and traditional low-input agriculture to modern, intensive production systems. At the crop evolutionary level it covers a wide range of diversity from wild ancestors to modern cultivars. The resulting diversity in plants has been the basis for providing food and satisfying other human needs for millennia.

Biochemical markers such as storage proteins provide more accurate assessment regarding genetic diversity in germplasm collection. A large number of lines can be characterized in a short period of time through this approach. In addition the data reflect genetic variability more precisely, as biochemical markers are direct gene products not influenced by the environment. The present study was carried out to investigate genetic diversity of exotic fenugreek accessions using SDS PAGE Analysis.

Materials and Methods

The experiment was conducted in the Evaluation Lab of Plant Genetic Resources Institute (PGRI), National Agricultural Research Center (NARC) Islamabad. Experimental material comprised 21 genotypes of fenugreek (Table I) provided by gene bank of PGRP, NARC, Islamabad.

Table I: List of fenugreek species genotypes used in the present study

Sr.no	Accessions	Sr.no	Accessions
1	4596	12	PI-143501
2	4602	13	PI-143503
3	4608	14	PI-143504
4	4617	15	PI-143505
5	4621	16	PI-164140
6	4622	17	PI-164507
7	4623	18	PI-164625
8	4624	19	PI-164762
9	021882	20	PI-165900
10	021900	21	PI-179058
11	022260		

Preparation of Seed Samples:

Ten seeds of each Genotype were taken, crushed and grinded in mortar and pestle. 10mg (0.01g) seed flour was weighed by an electronic balance and put into 1.5 ml microtube. After each sample weighing mortar and pestle were cleaned with great care so that there should not be even a single particle of last seed flour. To extract proteins from flour, 800µl of the protein extraction buffer was put into the microtube and mixed well by the test tube mixer (vortex). Then sample tubes were centrifuged at 12000 rpm for 10 minutes and extracted proteins were recovered as clear supernatant and stored in Room temperature.

Preparation of solutions:

Protein Extraction Buffer

Tris	0.6057g
Sodium Dodecylsulphate (SDS)*	0.2g
Urea*	30.3g
Distilled water	about 70ml
HCl (conc.)	Adjust to pH 8.0
2-Mercaptoethanol	1ml
Total volume of 100ml	

(0.05 M Tris-HCl pH 8.0, 0.2% SDS, 5M Urea, 1% ß-mecaptoethanol)
A little bit Bromophenol blue (BPB) was added. Buffer solution was stored in a refrigerator.

- Tris; Tris (hydroxymethyl) aminomethane
- *SDS and urea solubilize and denature proteins.

Solution A
(3.0 M Tris-HCl pH 9.0, 0.4% SDS)

Tris	36.3g
SDS	0.4g
Distilled water	About 70 ml
HCl (conc.)	Adjusted to pH 8.8
Total volume of 100ml Stored in a refrigerator	

- Stored in a refrigerator

Solution B
(0.493 M Tris-HCl pH 7.0, 0.4% SDS)

Tris	5.98g
SDS	0.4g
Distilled water	About 80 ml
HCl (conc.)	Adjusted to pH 7
Total volume of 100ml Stored in a refrigerator	

Solution C
(30% Acrylamide, Acrylamide/Bis = 30: 0.8

Acrylamide*	30g
Bis-acrylamide (Bis)*	0.8g
Distilled water	Total volume of 100 ml
Stored in refrigerator	

Acrylamide and Bis-acrylamide are highly toxic and carcinogenic. Gloves were used while preparing solution using these reagents.

10% APS

Ammonium Persulfate (APS)	0.1g
Distilled water	Total volume 1 ml

- Can be stored in a refrigerator for several days but it was prepared fresh all the times for better performance

Electrode Buffer Solution
(0.025 M Tris, 0.129 M Glycine, 0.125% SDS)

Tris	3.0g
Glycine	14.4g
SDS	1.25g
Distilled water	Total volume of 1000 ml

- Stored at room temperature

Staining Solution

Methanol	440 ml
Acetic Acid	60 ml
Distilled water	500 ml
Coomassie Brilliant Blue (CBB)* R250	2.25g
Total volume of 1litre	

- Solution was stirred for 30 minutes and then filtered, stored at room temperature. *CBB is a protein staining dye.

Destaining Solution:

Methanol	200ml
Acetic Acid	50 ml
Distilled water	750ml

- Total volume 1 litre, Stored at room temperature.

Preparation of Electrophoretic Gel

Seed protein was analyzed through slab type SDS-PAGE (Laemmli *et al*., 1970) using 14 % polyacrylamide gel. Glass plates used for electrophoresis were cleaned up from internal side with80% Ethanol and Kimwipe. Gaskets were sealed on glass plates with spacer; it was kept in mind that gaskets should not overlap with spacer of plates. Sets of glass plates were fixed with double clips and marked 2 cm from the top. To make sure that there is no leakage; glass plate set ups were filled with water and placed for some time.

Separation Gel with 1mm thickness (For two mini gels):

Separation gel 14%

Solution A	5.1ml
Solution C	9.3ml
10% APS	200µl
Distilled water	5.7ml
TEMED	50µl

TEMED (N-N-N-N-Tetramethylethylenediamine) was added at the end and shaken well.

Separation gel was prepared and incorporated into the space between a set of glass plates (up to 2cm from the top). Small amount of distilled water (120µl) was added on separation gel gently to prevent gel surface from air bubbles and promote in the developing countries. (Thanh *et al.,* 2006). The set up was left for 25 minutes so that gel was fixed. During the fixation time of separation gel, stacking gel was prepared.

Stacking Gel (For two mini gels)

Stacking gel 4.5%

Solution B	2.5ml
Solution C	1.5ml
10% APS	70µl
Distilled water	6.0ml
TEMED	50µl

TEMED was added at the end and shaken well. When separation gel was fixed, distilled water was removed from its top and stacking gel solution poured on it. Combs were fixed into the stacking gel. Combs were put with special care and it was confirmed that there was no air bubble at the bottom of the combs. The set up was left for 15 minutes so that the stacking solution became gel. Combs, clips and gaskets were removed from glass plates carefully.

Electrophoresis:

Electrode buffer solution was put into the bottom pool of the apparatus. Gel plates were placed in the apparatus, here again air bubble formation was avoided. Electrode buffer solution was also put into the top pool of the apparatus; wells formed by combs were washed by syringe. 10µl of supernatant of seed samples (Vertexed and centrifuged at 12,000 rpm for 10 minutes), was put into wells with the help of micropipette. Protein molecular weight marker was put in first well of each glass plate. The numbering of seed samples and wells were noted to avoid repetition. The apparatus was connected with + (red) and – (black) electrodes of power supply. The voltage of apparatus was kept constant at 80V for 20 minutes and then raised to 100 volt; apparatus was left until a blue line of BPB came at the bottom (2mm above bottom) of the gel plates.

When blue line reached at the bottom of the gel plates, electric supply was disconnected. Gel plates were taken out from the apparatus and separated by spatula. Stacking gel was removed with the help of same spatula. Separation gel was put in the box which contained staining solution. Box was put on the shaker for 40-60 min. Staining solution was exchanged by de-staining solution and the box was shook gently almost overnight until the background of the gel disappeared. To absorb excess CBB, a piece of Kimwipe was put in the de-staining solution. The gels were then analyzed and photographed. In order to check reproducibility of the method separate gels were run thrice under similar electrophoretic conditions.

Results and Discussion

The SDS Analysis method used in the present study have also been used as effective tools to evaluate genetic diversity and to throw light on the genetic differences among the different accessions of *Trigonella* under study. The present investigation revealed variation in the evaluated 21 accessions of *Trigonella* species collected from gene bank and to make a distinction of 21 germplasms with other genotypes through slab type SDSPAGE using 14.00% polyacrylamide gel. The SDS-PAGE gels are presented in Figure I. In total, 16 bands were detected in SDS-PAGE electro-photograms. The polymorphic bands were appeared at fourteen positions as A, B, C, D, E, F, G, H, I, J , L ,M ,N, O, P. SDS analysis of 21 fenugreek accessions indicated that two of them was monomorphic and 14 accessions were polymorphic. Accession PI-164762 shows high polymorphism among all the accessions. Little banding pattern was identified between the accessions 4596 and 4602. Considerable differences was observed in banding pattern of PI-164762,4617,4621,4622,4623,4624,21882, 21900 and 22260. Polymorphism in a given population is often due to existence of genetic variants represented by the number of bands and their frequency of distribution. Based on the results of electrophoretic band spectra, similarity index was calculated for all possible pair of electrophori grams. The similarity matrix thus generated was converted to a dissimilarity matrix and used to construct the dendrogram.

Studying genetic diversity and taxonomic relationships of plants previously scored for many plants (Bult and Kiang, 1992; Zviniene and Pank, 1996), results showed that limited interspecific diversity was found at protein level.

Cluster Analysis

All the monomorphic and polymorphic bands visible to the eye were scored and only unambiguously scored bands were used in the analysis. Each band was given score 1 for presence and 0 for absence. Dendrogram was formed on the basis of similarity coefficient. At 1.8 level of similarity four clusters were formed.

Dendrogram was shaped on the basis of similarity coefficient. At 1.8 level of resemblance four clusters were made. The first cluster is subdivided into two sub clusters first sub cluster includes following accessions of Trigonella 4596, 4624, 4602 and 21900. Accessions 4596 and 4624 showed high level of similarity with each another. The second sub-cluster comprised 4617, 4622, 4623, 21882, 22260 and 4621. The second cluster composed of PI-143501, PI-143503, PI-164507, PI-179058 and PI-143504. Accessions PI-143503 and PI-164507 were found more similar. The third cluster includes PI-143505 and PI-164140. Accessions PI-164625, PI-164762 and PI-165900 were not

include in any cluster and remained separate. Overall on the basis of total seed protein, grouping pattern of *Trigonella Foenum graecum* was mostly compatible with their specie status. Accessions belonging to same species clustered together and vice versa.

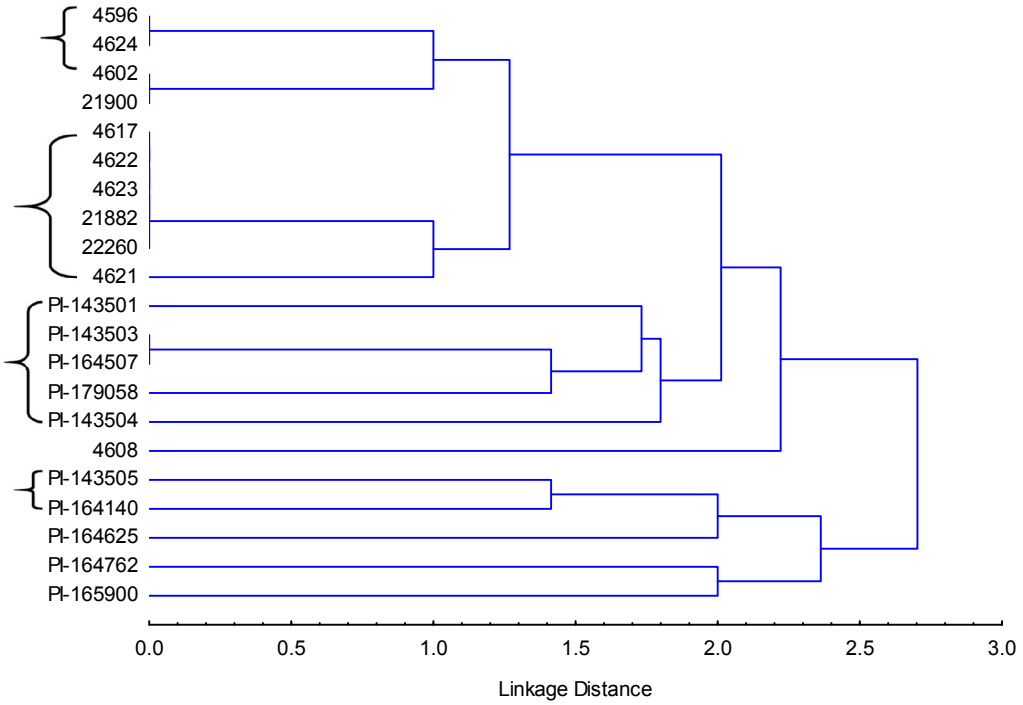
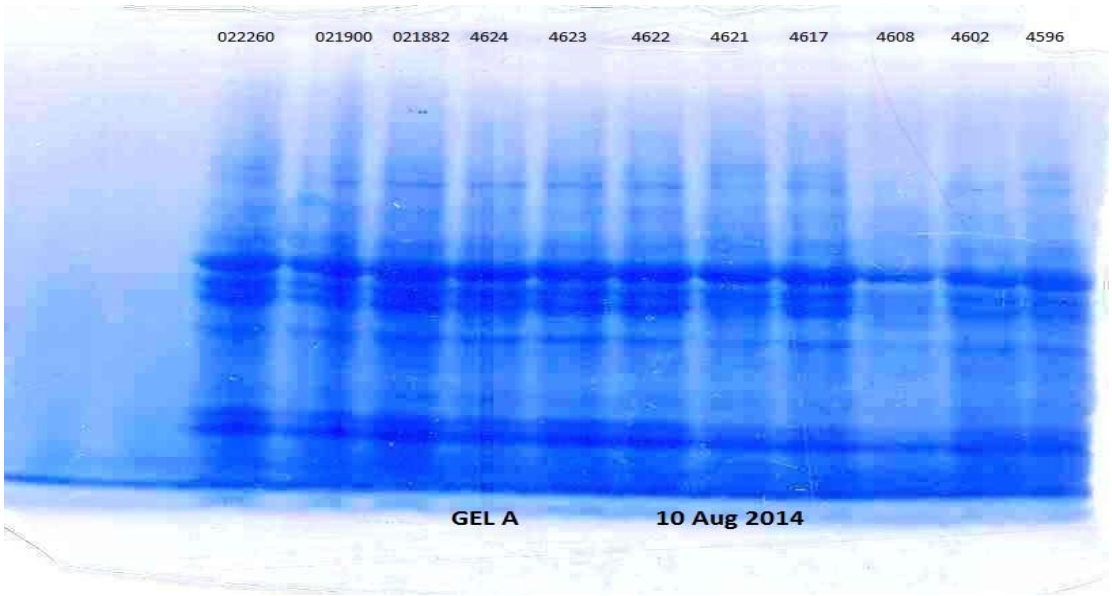


Fig: 1: Dendrogram showing the relationships among 25 genotypes of *Fenugreek based* on SDS-PAGE of seed storage proteins.



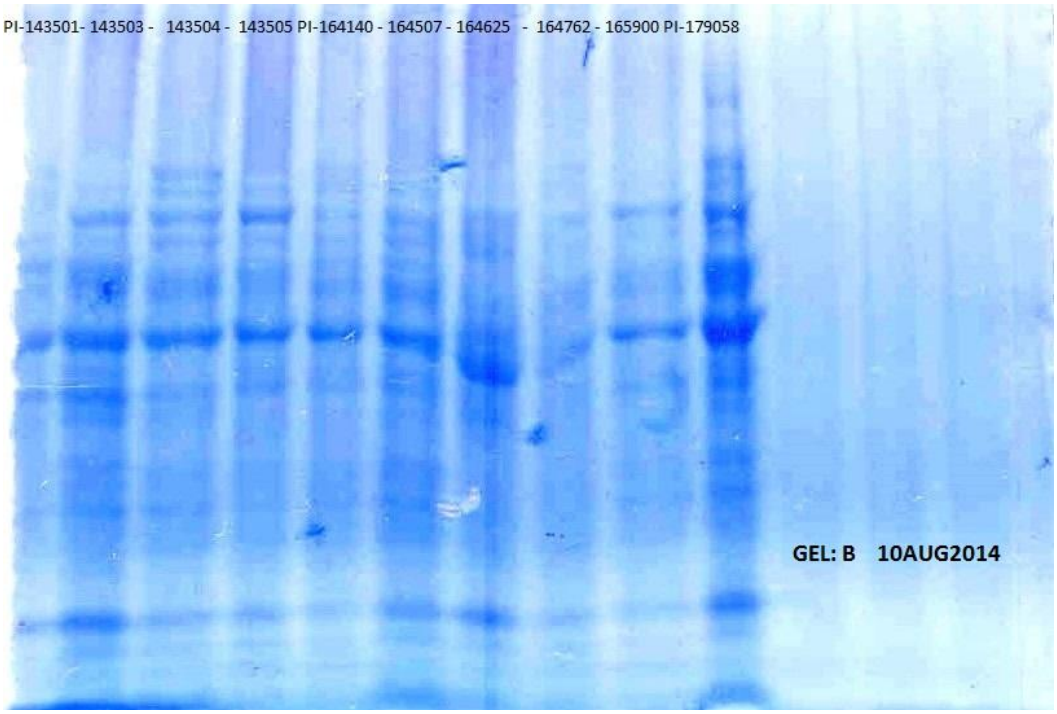


Fig. 2: Banding pattern of different fenugreek genotypes

Conclusions and Recommendations

It is concluded that biochemical analysis of SDS-PAGE revealed considerable genetic variation among all 21 accessions of fenugreek. Polymorphism was observed on the basis of seed storage peptide profiling. Using morphological, biochemical and molecular markers, considerable genetic variation was reported in exotic fenugreek germplasm, which is encouraging as it suggests broad genetic base of *fenugreek germplasm*. This genetic diversity could be utilized for the selection of new high yielding early maturing agronomical adaptive and superior genotypes. The information revealed in this study could also be used for the selection of good parental combination with maximum genetic variation. The knowledge of genetic diversity could be helpful for the development of inbred-lines with highest genetic variation which could be further used in the varietal development programs. Also future researchers are required to use more advanced biochemical techniques for protein analysis in fenugreek germplasm. Similarly, besides using other tools of molecular analysis for DNA fingerprinting, new SSR based primer systems should be employed to valuate germplasm for molecular characterization so that cultivars identification and finger printing may become possible.

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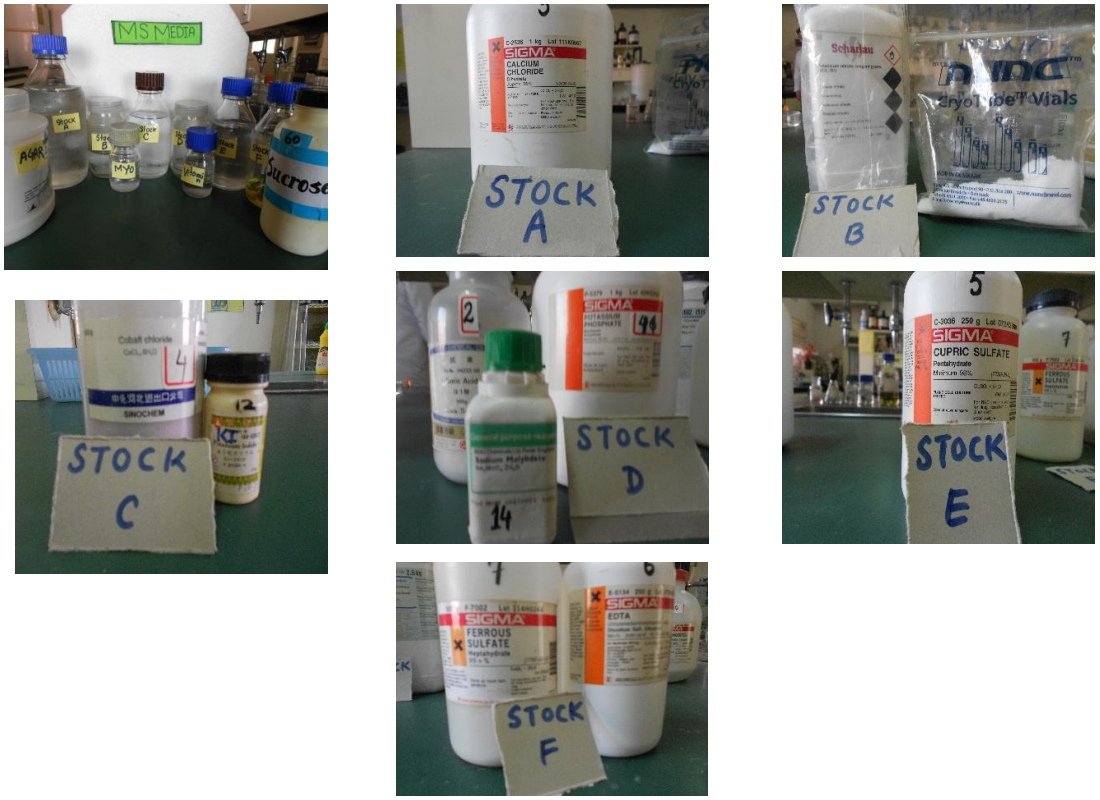
Extra Achievements

The present study was carried out in the In vitro Preservation Lab, Plant Genetic Resource Institute (PGRI), National agriculture Research Centre (NARC) Islamabad on MS media.

Methodology:

MS Basal Media Preparation



Murashige and Skoog medium (MS)

Murashige and Skoog medium (MS) was used for routine multiplication of the potato plants as it is the most suitable and commonly used basic tissue culture medium for plant regeneration. It was developed by Toshio Murashige and Folke K. Skoog in (1968).

Nutrients required by plants in large amounts e.g; carbon, hydrogen, oxygen, nitrogen, phosphorus, potassium, calcium, magnesium and sulfur are termed as macro-nutrients while nutrients required in traces e.g; iron, manganese, zinc, boron, copper, molybdenum and chlorine and these are termed as micro nutrients (Dodds and Roberts, 1985).

Table 3.2: Murashige and Skoog Media Composition

Stocks	Component	Amount/ L of Stock	Amount of Stock /1000ml of Media	Functions
A	$CaCl_2.H_2O$	44g l^{-1}	10ml	Increase resistance to outside attack and play role in producing plant tissue
B	Na_4NO_3 KNO_3	33g l^{-1} 33g l^{-1}	50ml	Form cellulose, lignin and important role in reservation of food
C	KI $CoCl.6H_2O$	166mg^{-1} 5mg l^{-1}	5ml	Improves growth of roots and callus
D	KH_2PO_4 H_3BO_3 $N_aMoO_4.2H_2O$	34g l^{-1} 1240mg l^{-1} 50mg l^{-1}	5ml	Promote root formation and maturity
E	$MgSO_4.7H_2O$ $MnSO_4.4H_2O$ $CuSO_4$-$5H_2O$ $ZnSO_4$-$7H_2O$	74g l^{-1} 4.46g l^{-1} 5mg l^{-1} 1720mg l^{-1}	5ml	Helps to develop enzyme and vitamin, and chlorophyll
F	Na_2EDTA	7.45g l^{-1}	5ml	Stimulate growth and increase leaf Area
Myo-Inositol	Myo-Inositol	2g/ 100ml	5ml	Carbohydrate, play role in storage, transport & release of auxin and calcium messenger system
Vitamins	Nicotinic acid Pyridoxine Thaimine Glycine	0.05g/100ml 0.05g/100ml 0.01g/100ml 0.2g/100ml	1ml	Essential compound for growth and plant metabolism
Sucrose	-	-	30g	Provide nutrients and is a Carbon source
Agar	-	-	7g	Aid in solidification of media and help in anchorage of plant
pH		The pH of media is adjusted to 5.8		