Effects of mutagens on morphology and minerals present in Spinach

Submitted by

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To
Department of Biotechnology & Biosciences
In partial fulfillment of the requirement for award of the degree

of

MASTERS IN PHILOSOPHY IN BOTANY

Under the guidance of

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June 2015

Certificate

This is to certify that BILQUES FAROOQ doing M. Phil project entitled **Effects of mutagens on morphology and minerals present in Spinach** under my guidance and supervision. To the best of my knowledge the present work is the result of her original investigation and study. No part of the thesis has ever been submitted for any other degree or diploma at any university.

The report is fit for the submission and the partial fulfillment of the condition for the award of **Master of Philosophy in Botany.**

Date: Signature of Advisor

Dr. Leena Parihar

Declaration

I hereby declare that the thesis entitled, **Effects of mutagens on morphology and minerals present in Spinach** submitted for Master of Philosophy degree is entirely my original work and all ideas and references have been duly acknowledged. It does not contain any work for the award of any other degree or diploma at any university.

Date:

Investigator

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Acknowledgement

I feel immense pleasure in bringing out this project report for which I have to go from pillar to post to make it a reality. This project work reflects contribution of many people with whom I had long discussion and without which it would not have been possible. I must first of all, express our heartiest gratitude to my respected and learned Research Supervisor **Dr. Leena Parihar** for providing her guidance to complete the project.

It would be unfair if I do not mention the invaluable contribution and timely cooperation extended to me by other person who was behind the curtain and without them the project would be incomplete.

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Chapter 1

Introduction:

Introduction

Chemical mutagens such as Ethyl Methane Sulphonate (EMS) and Colchicine have been commonly used to induce a large number of functional variations in different plants. EMS treatments is the most efficient technique for mutagenesis, these techniques open new possibilities for improving spinach.

Aim of the project is to check the effect of mutagen on morphology and mineral presents in Spinach.

Spinach

Scientific Classification

Kingdom: Plantae
Phylum: Angiosperms
Order: Caryophyllales
Family: Chenopodiaceae
Genus: *Spinicia*
Species*: oleracea*
Bionomial name*: Spinacia oleracea*

Spinach (*Spinacia oleracea*) belongs to family Amaranthaceae, is an edible flowering plant and its native is central and southwestern Asia. It is an annual plant. It can attain height of up to 30 cm and may survive over winter in temperate regions. The leaves are variable in shape like it may be simple, alternate, triangular-based or it may be ovated. Spinach plant also variable in size, it may be about 2–30 cm long and 1–15 cm broad. Larger leaves are present at the at the base of the plant and small leaves are at the higher on the flowering stem. The flowers are inconspicuous, yellow-green in colour; they may be inconspicuous about 3–4 mm in diameter. Mature flower turns into hard, dry, a small lumpy cluster of fruit which may be 5–10 mm in diameter containing several seeds. Common spinach, *Spinacia oleracea*, was long considered to be in the Chenopodiaceae family, but in 2003, the Chenopodiaceae family was combined with the Amaranthaceae family under the family name 'Amaranthaceae' in the order Caryophyllales. Within the Amaranthaceae family, Amaranthoideae and Chenopodioideae are now subfamilies, for the amaranths and the chenopods, respectively **(Seaman 2014).**

NUTRITION VALUE: Per 100g of Spinach has a high nutritional value and fresh, quick boiled or steamed Spinach is extremely rich in antioxidants. Spinach is a rich source of vitamin A and especially high in lutein, but vitamin C, vitamin E, vitamin K, magnesium, manganese, folate, betaine, iron, vitamin B_2, calcium, potassium, vitamin B_6, folic acid, copper, protein, phosphorus, zinc, niacin, selenium and omega-3 fatty acids. Recently, opioid peptides called rubiscolins have also been found in spinach. Folate (vitamin B_9 or folic acid) is a vital constituent of cells, and spinach is a good source of folic acid. Boiling spinach reduces half of the folate content in spinach leaves but there is no effect of microwaves on the folate present in spinach. For the first time in the year 1941 Vitamin B_9 was isolated from spinach. Other contents present in spinach are: Energy: 97kj, Carbohydrates: 36g ,Sugar: 0.4g, Fat : 0.4g, Protein: 2.9g,Water: 91.4g, Beta carotene: 5626 micrograms, Iron: 2.71mg, Calcium: 99mg **(Koike *et al* 2008).**

Table 1: Types of spinach

NAME	LEAF TYPE	USES	EXAMPLE
SAVOY	Curly, deeply crinkly and dark green leaves.	Resistance to blotting.	Merco Nero, Viroflay and regiment.
FLAT LEAF SPINACH	Smooth, broad leaves easily to rinse and clean.	Used in frozen and cannned spinach baby foods, soups and processed foods.	Gaint noble.
SEMI-SAVOY SPINACH	Slightly crinkled leaves.	Used to grown for processing and for selling fresh in the market.	Tyee hybrid.

(Source: California agriculture research directory, 2006.)

Green photosynthetic pigment called as chlorophyll helps plants in photosynthesis to get energy from light. By utilizing that energy plants combine water and carbon dioxide into carbohydrate so as to sustain their life. There are many factors that may affect the process of photosynthesis; the main factors are temperature, carbon dioxide concentration and light intensity. The chlorophyll content could depend on seasonal and environmental changes. During winter low chlorophyll a was observed in phytoplankton and that is because of the light limitation **(Shibghatallah *et al* 2013).**

Xanthophylls are the yellow pigments present in leaves. They are synthesized within the plasmids and are considered as oxygenated carotenoids. Xanthophylls are independent of light. All young leaves as well as etiolated leaves are the rich source of xanthophylls **(Pratheesh *et al* 2009).**

Iron is most important element not only in plants and humans but also in bacterial species as well. It can be found in a wide variety of food sources. Iron and its compounds are widely used in industries. It is major constituent element used in steel making. Iron is also used as polishing compound, magnetic ink, paint pigment, and coatings for several magnetic tapes. The soluble salts of iron are variously used as soil conditioning, treatment of sewage, dyeing mordant, catalysts, pigments, fertilizer, feeds, and disinfectants, in tanning and industrial wastes **(Ahmed *et al* 2008).**

Carotenoids are the plant pigments play a vital role in defining the quality parameters of fruit and vegetables. They are present in photosynthetic bacteria, plants and algaes.They play important role in the photosynthetic process. Carotenoids are responsible for many of the orange, yellow, and red colour of plant leaves, fruits, and flowers, as well as the colors of some birds, insects, fish, and crustaceans **(Eldahshah *et al* 2013).**

A permanent change in the DNA sequencing that makes up a gene is called as gene mutation. Mutations can occur to a single DNA or to a large fragment of chromosome. There are two ways of Gene mutations: one mutation can occur through inheritance from the parents and another way is to acquired during a person's lifetime. Hereditary mutations or germ line are those which are passed from parent to children that is also called as germ line mutation

because they are present in the egg and sperm cells, which are also called germ cells. This type of mutation is present throughout the life of person **Shirsat *et al* 2010).**

Chemical mutagens (EMS, DEB and sodium azide) and irradiation (Gamma rays, X-rays and fast neutrons) have been widely used for mutations **(Talebi *et al* 2012).**

Colchicine is an alkaloid obtained from various species of *Colchicum.* It is phenanthrene derivative. The chemical name for Colchicine is (S)-N-(5,6,7,9-Tetrahydro-1,2,3,10-tetramethoxy-9-oxobenzo heptalen-7-yl) acetamide (molecular weight 399.4). Colchicine alters neuromuscular function, constricts blood vessels, increases sensitivity to central depressants, heightens response to sympathomimetic compounds, lowers body temperature, depresses the respiratory centre, causes hypertension by central vasomotor stimulation, and Colchicine inhibits cell division in the metaphase by interfering with the mitotic spindle **(Siiddiqi 1983).**

Looking into various effects of mutagenic compounds, the current study is aimed to analyze the effects of two compounds Ethyl methane sulfonate (EMS) and Colchicine on the growth and mineral nutrient level of Spinach.

Chapter 2

Terminology

Terminology

EMS - Ethylmethanesulfonate

Colchicine

Flame photometer

ICP- Inductively coupled plasma

Spectrophotometer

1-10 phenanthroline

Chapter 3

Review of Literature

Review of literature

Chemical mutagens such as ethyl methane sulphonate (EMS), diepoxybutane-derived (DEB), sodium azide and irradiation (Gamma rays, X-rays and fast neutrons) have been widely used to induce a large number of functional variations in rice and others crops. More than 430 new varieties have been derived as mutants of rice (*Oryza sativa*) via the application of different mutagenic agents. Chemical and physical mutagenesis has been used to increase genetic variability in crop plants. In this study, seeds of potential genotype of the popular variety, (*Oryza sativa* L. spp. *indica* cv. MR219) were treated with EMS at concentrations of 0.25%, 0.50%, 0.75%, 1%, 1.25%, 1.5% and 2%. Sensitivity to EMS was determined by various measurements on the M1 generation. Among chemical mutagens, the alkylating agent, ethyl methane sulfonate (EMS) is the most commonly used in plants as it causes a high frequency of nucleotide substitutions, as detected in different genomes. As concentration of applied EMS increased, will decrease in germination, seedling height, root length and emergence under field conditions was observed in M1 generation as compared to the non-treatment control. Plant height and root length also decreased with increases in EMS mutagenesis in an approximately linear fashion. The LD25 and LD50 values were observed based on growth reduction of seedlings after EMS treatment with 0.25% and 0.50% on the rice variety (*Oryza sativa* L. spp. *indica* cv. MR219)

(Talebi *et al* 2012).

Chlorophyll mutations are used as markers in genetic, physiological and biochemical investigations, while chlorophyll formation in plants is the last result from long chain of biochemical proce*sses where are included a lot of locuses. Chlorophyll mutations are used also as test systems for evaluation of genetic action of mutagenic factors (Gaul, 1964). They can be identified very easy in M2 generation and can give quick information **(Svetleva 2001**).

Mutation breeding is one of the most reliable techniques in improving crop plants. Physical and chemical mutagens are used to bring the variability. Among chemical mutagens, to identify the efficient mutagen that can bring broad spectrum of variability, it is therefore necessary to study the efficiency and effectiveness of various mutagens. In present study, two varieties of *horsegram*, viz., SINA (K-42) and KS-2 were treated with three concentrations of

ethyl methane sulphonate (EMS), N- nitroso N- ethyl urea (NEU) and sodium azide (SA). Identification of effective mutagen could be possible by studying different parameters. In M2 generation studies were made on the mutagenic efficiency, mutagenic effectiveness and mutagenic rate. In the present study, the results revealed that efficiency of mutagens was variable, sodium azide was most effective mutagen and showed highest mutagenic rate. **(Shirsat *et al* 2010)**

Particular study was done on the Effect of ethyl methane sulfonate (EMS) and N-nitrose-N´-ethyl urea (ENU) mutagenic on the common bean *Plovdiv*. 7 day old sterile leaf petiole was taken as explants. The explants was cultured three times ".Seeds of the common bean *Plovdiv* were pre-cultivated on MS basal medium supplemented with 1 µmM BAP. Then, both mutagens EMS and ENU were applied for different times such as 15, 30, 60 and 90 min on the explants at the concentrations of: 2.5, 10-2 M and 6.2, 10-3 M, respectively". Callus growth was effected by changing the time criteria of mutagenic chemicals. 30 min treated with both mutagens showing the highest weights. In both cases, the results were same at 90-min mutagen application. They show similar effect either on callus browning or growth inhibition. There was higher effect of subcultures on callus growth than mutagenic treatments. The results were checked by using the correlation ratio (η%) were quite low. This study concludes that ENU showed a stronger effect than EMS. **(Svetleva** 2005**).**

The study shown that the Or genes are the strong tool of genetic, which may be used to enrich the carotenoids contents in transgenic potato tubers. Main source of Or genes are orange cauliflower mutant. "Transgenic plants have facilitated our understanding of the functional roles of genes and the metabolic processes affected in plants". It was found that the Or transgene may facilitate the carotenoid bio- synthetic pathway. The Or transgenic tubers increased levels of carotenoids and three additional metabolite intermediates of phytoene, phytofluene, and z-carotene. They also observed that long-term cold storage greatly enhanced carotenoid content in the Or transgenic tubers to a level of 10-fold over controls. "Expression of the Or transgene in the transgenic plants caused no dramatic changes in the transcript levels of the endogenous carotenoid bio- synthetic genes, which is in agreement with the Or gene not directly controlling carotenoid biosynthesis. Microscope analysis revealed that the Or transgene conferred the formation of chromoplasts containing carotenoid sequestering

structures in a heterologous system. Such structures were not observed in tubers of potato cultivars that accumulate high levels of carotenoids. This study conclude that the Or gene effects the carotene accumulation (**Lopez *et al* 2007).**

Spinacia oleracea is very important green leafy vegetable. The leaves of Spinach are full of nutrients and have therapeutic use. Leaves looses their quality within few hours after the detachment from plant due to excessive respiration and transpiration,so it may be considered as the perishable vegetables. The attempts were made to make the spinach leaves shelf-stable by using the process of dehydration, maintained at different isothermal temperatures ranging from 50 to 800C with an interval of 100C in a cabinet drier. The effect of blanching treatment was studied. The leaves dehydrated under different temperatures and were crushed manually by using mixer grinder. The characterization of dehydrated leaf powder were studied out on the basis of physical and optical parameters. The studied properties were found to be governed by dehydration temperature, treatment applied and on particle size. The dehydration temperature of 60OC for unbalanced and 70OC for blanched spinach could be used for production of enhanced quality green leafy vegetable powder of wider acceptability **(Ankita *et al 2013).***

Wheat grass is very commonly known and easily available plant. It is being used for treatment of various diseases since from early time. It is very healthy to our body also. Varieties of wheat grass are available in market, but there is no proper method to detect the actual amount of minerals present in formulation. The aim of the present study was to determine minerals based on flame photometry. This method is very simple, inexpensive and less time consuming. This method is properly based on using standard chemicals and it can be applied to formulation **(Shah *et al* 2011).**

Colchicine and Para dichlorobenzene treatment was given to seed of tomato Var. Roman VF for determining the effectiveness of mutagens in the production variation in tomato. The observation revealed that there is significant variation for different parameters like germination percentage , number of leaves ,and height at maturity. Increased concentration of Colchicine treatment results in increase in leaf length , height at maturity while decrease in seedling height , number of branches and germination percentage prominently decrease was

visualized .Besides this ,Para dichlorobenzene also show decrease in some parameter as well as increase in above said parameters. Although whatever was the trait was taken in consideration there was decrease in them. However, there was increase in seedling height, leaf length, and number of branches with increased concentration. Furthermore Para dichlorobenzene showed more prominent effect on seedling height, germination percentage at different time intervals, while as Colchicine showed effect alone on number of branches .Ultimately the result revealed that 1mm and 4 mm Colchicine and Para dichlorobenzene is recommended for inducing mutation to obtain variability **(Adelanwa *et.* 2011)**

In pineapple compact nodular callus was induced from various plant parts that showed high regenerative ability. Colchicine was used to obtain some variants. In Colchicine-treated callus about 5% of the regenerates were variants which showed significantly low chlorophyll a and chlorophyll b. Shoot and root regenerating potentiality and other growth behavior were poor in variants compared to normal plants. Shoot tip culture of the variants and callus from the same source continuously produced variant progenies for a number of (4 - 5) in vitro cycles. Compared to normal, two important peroxidase bands were missing in the variant pine apple **(Mujib, A 2005).**

The present work is further extension of research of effect of such mutagens on various plants and evaluating their roles in polyploidy development and we had worked on two mutagens namely Ethyl Methyl Sulphonate and Colchicine on green iron rich vegetable, Spinach.

Chapter 4

Rationale and Scope of the study

Rationale and Scope of the study

- Mutation caused by various chemical mutagens may increase the size of leaves, which will be commercially beneficial.
- Spinach is green leafy vegetable, very less in calories and is considered as a good source of vitamins (ascorbic acid, riboflavin, niacin and folic acid), minerals (iron and calcium) and dietary fibers. Spinach is having known amount of essential nutrient elements.
- Consumption of spinach leaves decreases the risk of persistent metabolic diseases.
- A mutation creating genetically modified plant which promises to produce new qualities which can be beneficial for consumers.

Chapter 5

Objectives

Objectives

1. To check the effect of chemical mutagens (Ethyl methane sulfonate and Colchicine) on germination percentage of Spinach seeds.

2. To evaluate the effect of chemical mutagens on morphological parameters (leaves length and width, root length, shoot length and biomass) of Spinach plant

3. To check the effect of chemical mutagens on the concentration of essential elements (Ca, K, Na, Cd and Fe) in leaves.

4. To evaluate the effect of chemical mutagens on different photosynthetic pigments present in spinach by TLC analysis.

Chapter: 6

Material and Methodology

Material and Methodology

Materials required

Spinach seeds, distilled water, double distilled water, EMS, Colchicine, methanol, hexane, acetone, sodium anhydrous, 1-10 phenanthroline, hydroxylamine hydrochloride, sodium acetate and silica gel.

Methods

For mutagen treatment to seeds:

Seeds were placed in a beaker containing 100ml of distilled water and were soaked overnight at room temperature. Water was decanted and seeds were treated with different concentrations (0.25%, 0.50%, 0.75% and 1.00%) of mutagens EMS and Colchicine.

Preparation of stock solution of 10 ml of each EMS and Colchicine by dissolving 0.1g of compound in 10ml of distilled water. Seeds were treated with mutagen chemicals for 6 hours. Then washing 15 times each with distillessd water. After that seeds were sowed in propagation tray and placed in incubator at 28 ° C for three days **(Taleba *et al* 2012).**

For mineral extraction (Ca, Na, and K)

Methanolic extraction of dried Spinach leaf sample

Preparation of standard solutions

Analysis of minerals by using flame photometer **(Shah et al 2011)**

Cd estimation:

Cadmium estimation was done in Citrus Estate Hoshiarpur lab**.** By using ICP **(Rachcigi and Payne 1990)**

For iron estimation:

Iron was extracted by using 1-10-phenanthroline

Analysis was done by spectrophotometer **(Roa *et al 1987).***

For extraction of photosynthetic pigments:

Extraction was done by using acetone while grinding the fresh sample in mortar and pestle.

Qualitative analysis was done by using TLC method. Hexane and acetone in ratio of 7:3 were used as solvent

Quantitative analysis were done by taking spectrophotometer reading at three different wave lengths like 663, 645, and 470 nm

Formulas for quantitative analysis of photosynthetic pigments are:

Chlorophyll a = [(12.7x A663) – (2.6x A645) ml/mg of leaf.

Chlorophyll b = [(22,9x A645) – (4.68x A663) ml/mg of leaf.

Xan+ Car. = 1000A470 – 1.90 chl a – 63.14 chl b/214 **(Johnson 2007).**

Chapter 7

Results and discussion

Results and discussion

Fig.1 Mutagen treated seeds of spinach

Spinach seeds were treated with different concentrations of Colchicine and EMS (0.25%, 0.50%, 0.75% and 1%) for 7 hours at room temperature.

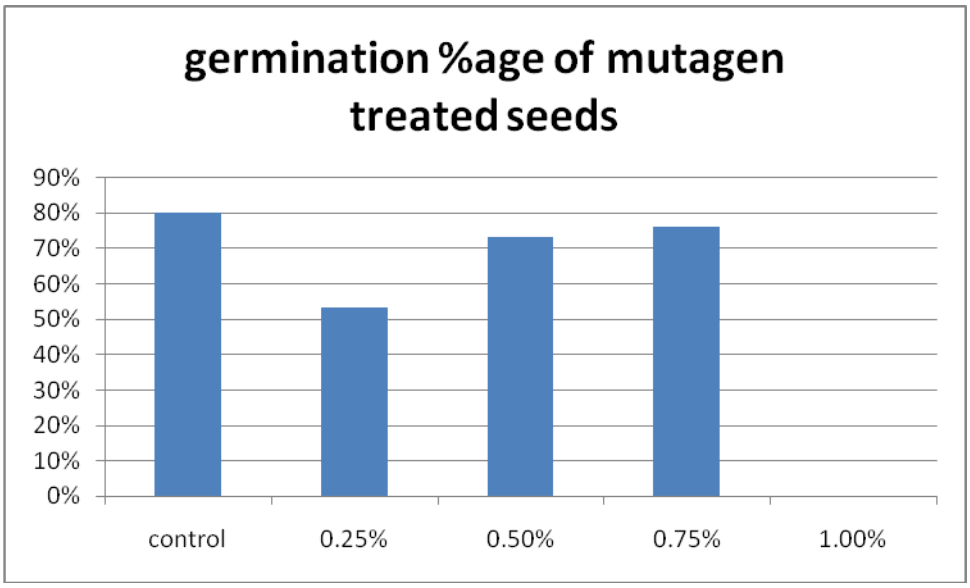
Fig. 2 Propagation trays after seed sowing

Spinach seeds were grown in propagation trays under lab conditions.

Table 2: Germination percentage of chemically treated spinach seeds

	Germination of spinach seeds			
concentrations	**0.25%**	**0.50%**	**0.75%**	**1.00%**
Colchicine	53%	73%	76%	No germination
EMS	66%	75%	77%	No germination
Control	80%			

Graph 1: Germination percentage of chemically treated spinach seeds



Germination rate of spinach seeds were found increased with increasing concentration of Colchicine and ethyl methane sulfonate while in comparison with control where the seeds were germinated without any chemical treatment the germination was recorded as 80% which is highest of all treated seeds.

Fig.3 Spinach in field

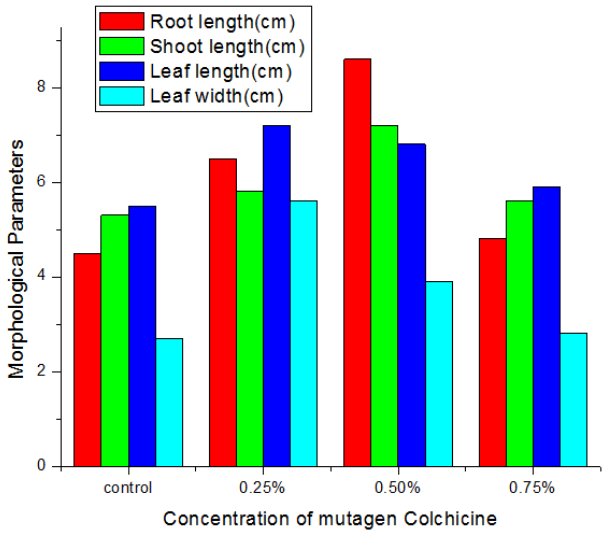
Fig.4 Effect of Colchicine on morphological parameters of spinach

Fig.5: Effect of EMS on morphological parameters of spinach

Table 3: Effect of Colchicine on morphological parameters of spinach

Different Concentrations of Colchicine	Morphological parameters				
	Root length (cm)	**Shoot length (cm)**	**Leaf length (cm)**	**Leaf width (cm)**	**RWC (%)**
Control	4.5	5.3	5.5	2.7	66
0.25%	6.5	5.8	5.7	2.8	95
0.50%	8.6	7.2	6.8	3.9	91
0.75%	4.8	5.6	5.9	2.8	88

Graph 2 Effect of Colchicine on morphological parameters of spinach

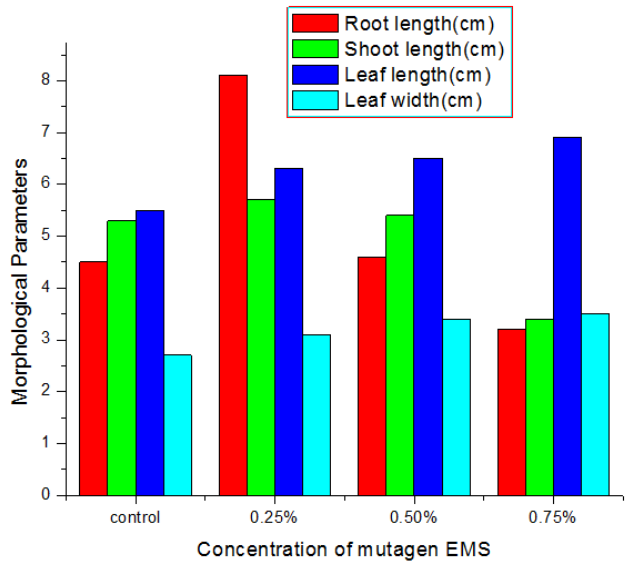


Root length, shoot length, leaf length, leaf width, and relative water content percentage increases by the dosage of Colchicine as we compare it with the control. There is maximum growth of all morphological parameter at 0.50% and less growth at 0.75%.

Table 4: Effect of EMS on morphological parameter of spinach

Different Concentrations of EMS	Morphological parameters				
	Root length (cm)	Shoot length (cm)	Leaf length (cm)	Leaf width (cm)	RWC (%)
Control	4.5	5.3	5.5	2.7	66
0.25%	8.1	5.7	6.3	3.1	70
0.50%	4.6	5.4	6.5	3.4	81
0.75%	3.2	3.4	6.9	3.5	97

Graph 3 Effect of EMS on morphological parameter of spinach



There is positive effect of 0.25% and 0.50% of EMS on the morphological parameters of spinach as we compare it with the control. But at 0.75% EMS there is a gradual decrease in the root length and shoot length of Spinach plant.

Table 5: Effect of Colchicine on Na, K, and Ca presents in spinach (Flame photometer)

Concentration of Colchicine	Elements/ppm		
Control	**Na**	**K**	**Ca**
	30	89	805
0.25%	32	82	878
0.50%	36	86	934
0.75%	45	88	1107

Graph 4: Effect of Colchicine on Na, K, and Ca presents in spinach (Flame photometer)

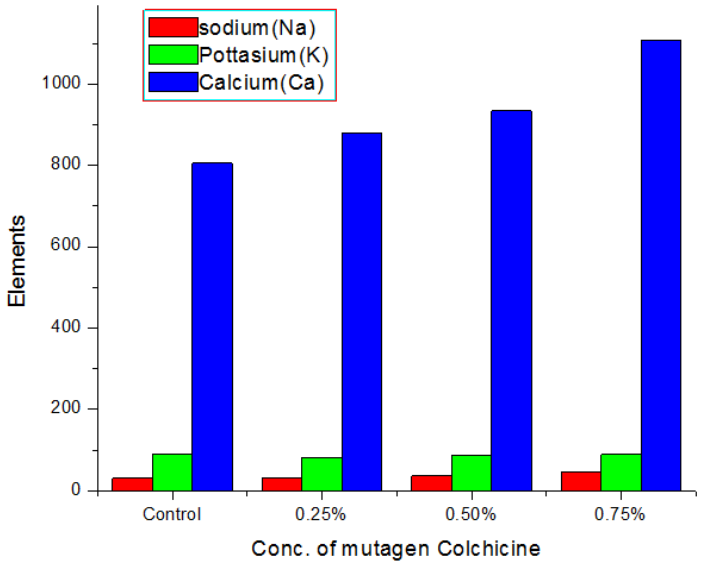


Table 6: Effect of Colchicine on Cd present in spinach (ICP analysis)

Concentration of Colchicine	Cadmium/ppm
Control	0.282
0.25%	0.342
0.50%	0.426
0.75%	0.466

Graph 5 Effect of Colchicine on Cd present in spinach (ICP analysis)

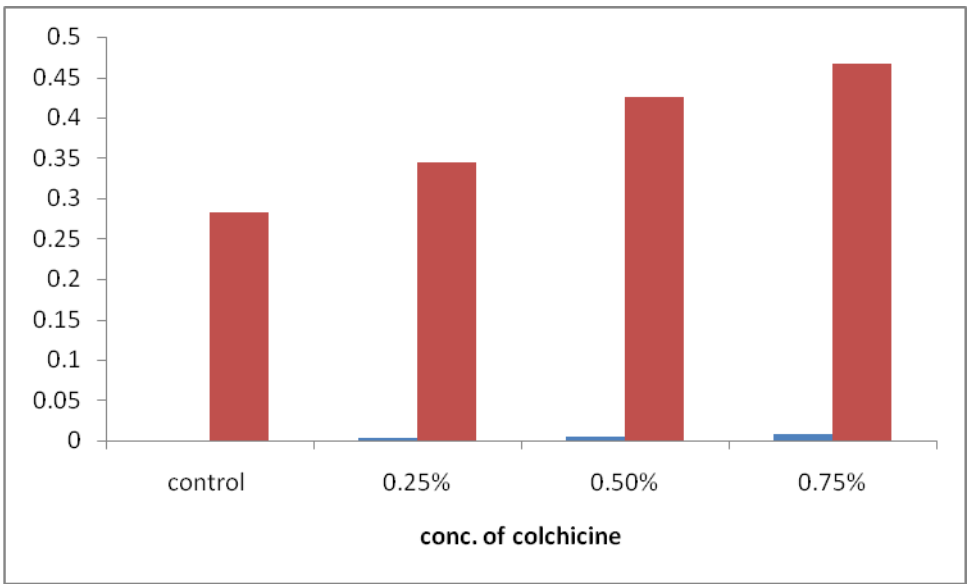


Table 7 Effect of EMS on Na, K and Ca present in Spinach (flame photometer)

Concentration of EMS	Elements /ppm		
	Na	**K**	**Ca**
Control	30	89	805
0.25%	42	58	983
0.50%	43	69	909
0.75%	55	82	1035

Graph 6: Effect of EMS on Na, K and Ca present in Spinach (flame photometer)

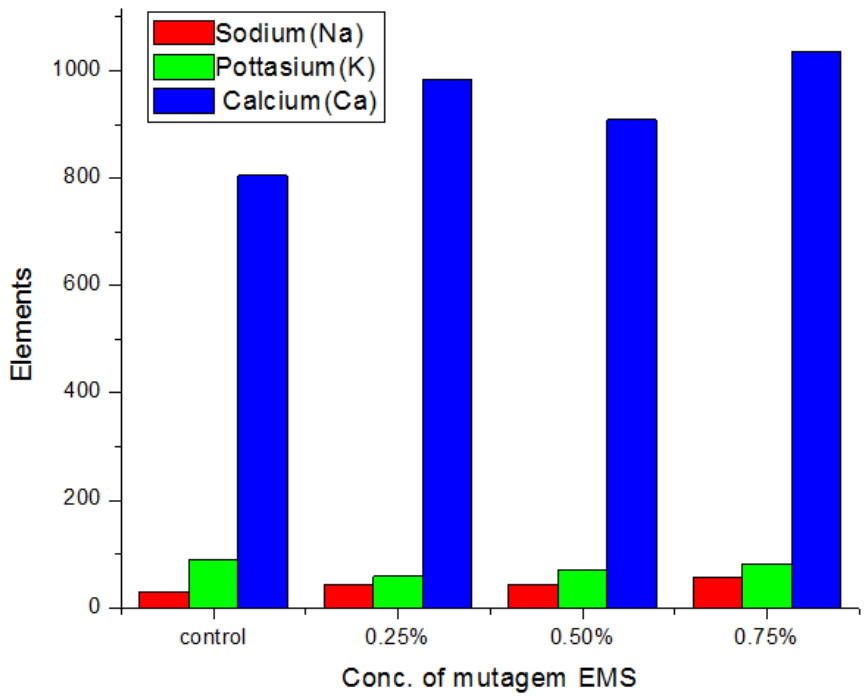
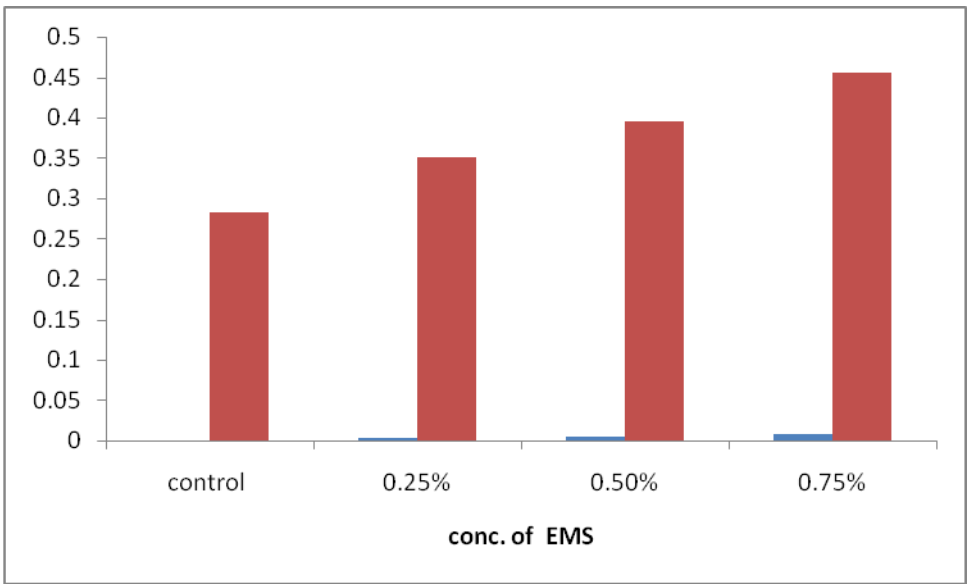


Table 8: Effect of EMS on Cd present in Spinach (ICP)

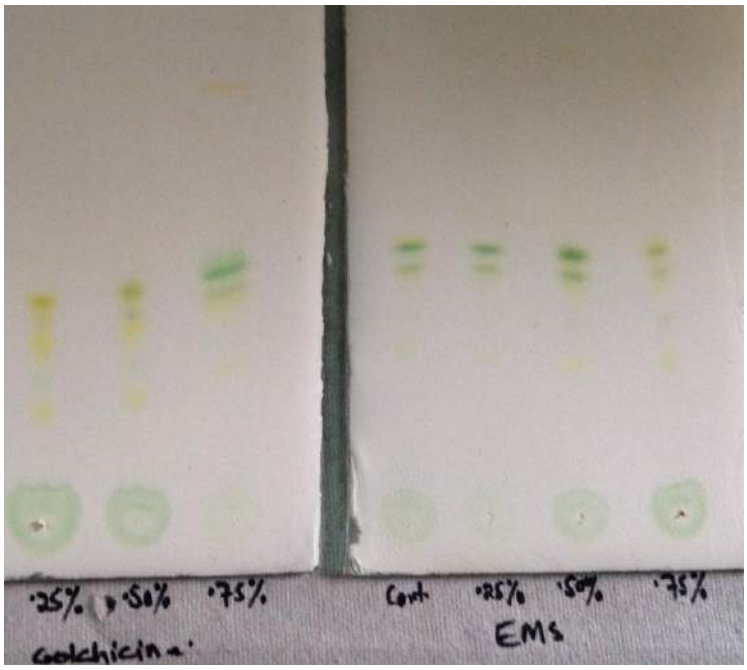
Concentration of EMS	Cadmium/ppm
Control	0.282
0.25%	0.350
0.50%	0.395
0.75%	0.456

Graph 7: Effect of EMS on Cd present in Spinach (ICP)



There is positive effect of both Colchicine and EMS on Na, Cd and Ca content present in Spinach. Concentration of all the minerals increases with the concentration of mutagens increase as we compare it with control. Only K concentration deceases as concentration of mutagens increases in comparison with control.

Figure 6: TLC analysis



TLC plates for the analysis of different photosynthetic pigments.

Table 9: TLC analysis: Effects of Colchicine on pigments present in spinach

Concentrations of Colchicine	**R*f* values**			
	Chlorophyll a (light green)	**Chlorophyll b (dark green)**	**Xanthophylls (yellow)**	**Carotene (yellow orange)**
Control	0.300	0.285	0.240	0.9
0.25%	NA	NA	0.231	0.85
0.50%	NA	0.273	0.230	0.81
0.75%	0.298	0.293	0.239	0.89

Table 10: TLC analysis: Effects of EMS on pigments present in spinach

Concentrations of EMS	**R*f* values**			
	Chlorophyll a (light green)	**Chlorophyll b(dark green)**	**Xanthophylls(yellow)**	**Carotene(yellow orange)**
Control	0.300	0.285	0.240	0.9
0.25%	0.299	0.270	0.235	0.7
0.50%	0.287	0.275	0.237	0.6
0.75%	NA	0.277	0.238	0.8

Table 11: Effect of Colchicine on the photosynthetic pigments of spinach

Concentration of Colchicine	**Effect of Colchicine on pigments present in per ml/mg of spinach leaves**		
	Chlorophyll a	**Chlorophyll b**	**Xanthophylls and carotenoids**
Control	0.246	12.353	2.764
0.25%	0.022	1.786	0.838
0.50%	0.079	10.759	0.919
0.75%	0.200	11.923	0.967

Table 12: Effects of EMS on the photosynthetic pigments of spinach

Concentration of EMS	**Effect of EMS on pigments present in per ml/mg of spinach leaves**		
	Chlorophyll a	**Chlorophyll b**	**Xanthophylls and carotenoids**
Control	0.246	12.353	2.764
0.25%	4.431	5.454	0.435
0.50%	5.206	5.949	1.213
0.75%	2.474	5.705	0.719

There were found very less effect of mutagens like Colchicine and EMS on the photosynthetic pigments of spinach leaves. In case of Colchicine (0.25%) chlorophyll a and b both are absent, and in Colchicine (50%) chlorophyll a was absent. In case of EMS (0.75%) TLC result show absence of chlorophyll a. R*f* value decreases in all cases while comparing with the control. Concentration of photosynthetic pigments per ml per mg of leaf also show decrease on the comparison with control.

Table 13: Spectrophotometer analysis: Effect of Colchicine and EMS on the Iron present in spinach

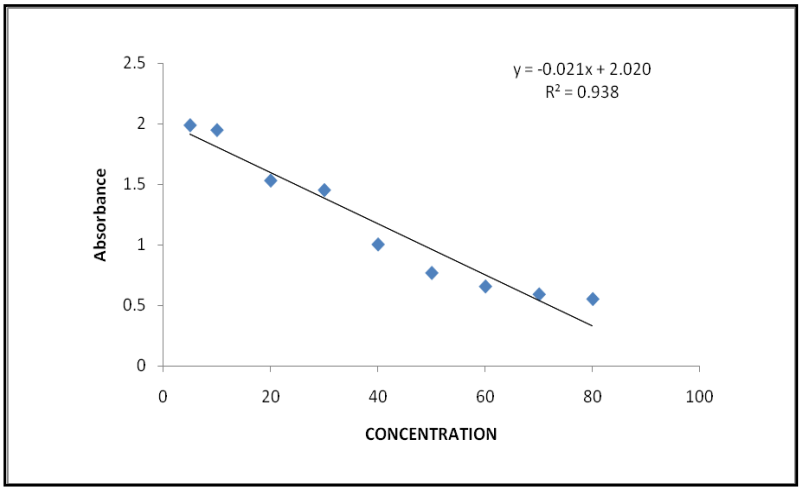
Concentration of mutagens	O.D of Colchicine (at 510nm)	O.D of EMS(at510nm)
Control	0.534	0.534
0.25%	0.550	0.561
0.50%	0.569	0.674
0.75%	0.604	0.796

Values of absorbance of iron at two different concentrations of mutagens (Colchicine and EMS) were found increasing with increase in the concentration as well as by comparing their values with control.

Table 14: Absorbance of spinach samples at various concentration of mutagens

	Absorbance at 510nm	Concentration(µg/ml)
Control	0.539	70.523
Colchicine 0.25%	0.406	76.857
Colchicine 0.50%	0.512	71.809
Colchicine 0.75%	0.604	67.428
EMS 0.25%	0.571	69
EMS 0.50%	0.574	68.857
EMS 0.75%	0.576	68.761

Graph 8: Standard curve for iron estimation



Decreasing trend of concentration was reported for both the mutagens on the concentration of iron present in spinach leaves. The quantities of Colchicine treated spinach sample were higher in comparison with the untreated control sample of spinach. The values of Colchicine treated samples at 0.25 % and 0.50% were recorded as 76.857 and 71.809 respectively where as the result recorded in case of control was 70.523.

Chapter 8

Experimental work

Experimental work

For mineral extraction

1. The seeds were initially germinated in propagation trays in laboratory conditions with optimum temperature and after proper germination seedlings were transplanted in the field directly. The seedlings were watered intermittently. Fresh leaves of spinach were collected from field for mineral extraction**.**

2. The leaves were washed under tap water then with distilled water and then allowed for drying in hot air oven at 50° C for 3 hours

3. For methanolic extraction, dried powder of fresh spinach leaves was macerated thoroughly, using mortar and pestle.

4. The crushed leaves was completely exhausted by adding small quantities of methanol and Filtering off every time in a successive manner, to yield final volume of 1 liter.

5. Calcium standard preparation – 0.62g of calcium carbonate, was dissolved in 2ml of double distilled water and mixed with 2ml of 1:1 HCl, the final volume was prepared 250ml by adding double distilled water. 5 ml of standard solution was now mixed with double distilled water. The value of Ca in standard was measured by using flame photometer. The value of unknown Ca of treated spinach leaves were observed in similar manner as taken for standard.

6 Na and K standard solutions- 0.635g of NaCl and 0.447g of KCl in 250ml of double distilled water was prepared in two volumetric flasks. The solutions obtained will have 1000 ppm. 5 ml of standard solution was now mixed with 95ml of double distilled water. The value of Na and K in standard was measured by using flame photometer. The value of unknown Na and K of treated spinach leaves were observed in similar manner as taken for standard **(Shah *et al* 2011)**

For extraction of Cd:

1. Weighing 0.1g of dried Spinach leaf sample
2. Add 6ml of nitric acid to it.
3. After filtration 50ml of double distilled water were added to dilute the solution
4. Filtrate were directly feed to ICP
5. Reading were taken by multiplying the readings with dilute factor **(Rachcigi and Payne 1990)**

For extraction of pigments

1. Fresh leaves of spinach were collected from field.

2. Washed under running tap water.

3. 0.5g of leaf was grinding in 1ml acetone with the help of mortor and pestle.

4. Poured the extract in ependroff tube. Rinse mortor and pestle with acetone so as to maximum extracted is collected.

5. 2ml hexane and 2ml distilled water were added and shaked gently.

6. Tubes were allowed to stand for some time for maximum separation of layers.

7. Lower layer separated out with pasture pipette.

8. 2ml of distilled water was added.

9. Again lower water was washed off. Some remained water was removed by adding small pinch of sodium anhydrous. It will absorb water present in tube.

10. Extract was transferred to ependroff tube for TLC analysis.

11. TLC plate was prepared by dissolving silica gel in distilled water

12. Plates were prepared by pouring silica gel on it and were allowed to dry in hot air oven for about 3 hours at 50 °C.

13. After drying, a drop of sample was inserted using capillary tube on plate and kept in 100 ml solvent (hexane: acetone in ratio of 7:3).

14. Calculation of the R*f* value was done by using formula R*f* = Distance travelled by compound / distance travelled by solvent **(Johnson 2007).**

Iron extraction:

1. The 2 g of fresh leaves of spinach were collected from field.

2. Washed first under tap water then with distilled water and then allow drying in hot air oven at 50° C.

3. 20 ml of (0.6%) phenanthroline was added to 2 g of sample and gently stirred in order to embath the sample with the extractant.

4. The sample in glass beaker were stoppered and allowed to stand for 16 hrs at room temperature.

5. The contents were filtered through whatman filter paper.

6. Iron was directly estimated in the filtrate by measuring absorbance at 510nm

Preparation of Standard curve:

1. 5, 10,20,30,40,50,60,70 and 80 ml of 100 ppm sol. of iron was added in a series of 100 ml volumetric flask.

2. Add 2 ml of 10% hydroxylamine hydrochloride was added in each flask.

3. Shake the flask and add 2 ml of o.phenantroline solution and 5ml of 1N sodium acetate in each flask and make final volume of 25 ml.

4. Bright orange red color developed.

5. Colour solution contained 0.0, 5.0, 10.0, 20.0, 30.0, 40.0, 50.0, 60.0, 70.0, and 80.0 ppm iron

6. Absorbance was recorded at 510nm and standard curve was prepared **(Roa *et al* 1987).**

Chapter 9

Conclusion and future scope

Conclusion and future scope

- Mutations are the unexpected sudden changes in the DNA. These changes can be beneficial as well as harmful for living beings.
- By using chemicals like EMS and Colchicine beneficial results were received in present study, in future that may also help us to create improved varieties of the plant resulting in great diversity and therefore greater adaptability in the environment.
- There is positive effect of 0.25% and 0.50% of EMS on the morphological parameters of spinach as we compare it with the control. But at 0.75% EMS there is a gradual decrease in the root length and shoot length of Spinach plant.
- There is positive effect of both Colchicine and EMS on Na, Cd and Ca content present in Spinach. Concentration of all minerals increases with the increasing concentration of mutagens as compare to control. Only K concentration shows major deceases as concentration of mutagens increases in comparison with control.
- There were found very less effect of mutagens like Colchicine and EMS on the photosynthetic pigments of spinach leaves. In case of Colchicine 0.25% chlorophyll a and b both are absent, and in Colchicine 50% chlorophyll a was absent. In case of EMS 0.75% TLC result show absence of chlorophyll a. R*f* value decreases in all cases while comparing with the control. Concentration of photosynthetic pigments per ml per mg of leaf also show decrease on the comparison with control.
- Values of absorbance of iron at two different concentrations of mutagens (Colchicine and EMS) were found increasing with increase in the concentration as well as by comparing their values with control
- In future new varieties of plants which will be genetically modified and may promises to produce different forms of leaves can also be created.
- Spinach is a good source of secondary metabolite carotenoids and essential elements like Fe, Ca, Na, Cd etc. The amount of these compounds will increase and that will be used in various fields of medicine and agriculture.

Chapter 10

References

References

1. Ankita & K.Prasad. Studies on Spinach Powder as Affected by Dehydration Temperature and Process of Blanching. *Int. J. Agric. food Sci. Technol.* **4,** 309–316 (2013).

2. Seaman A., Abawi G., beth K. Gugino, Helms M. Production Guide for Organic Spinach. *new york state Dep. Agric. Mark.139,* (2011).

3. Koike, S. T, Cahn.M, Cantwell .M, Fennimore .S, Lestrange .M, Natwick.E, Richard.F and Takely.E. Spinach production in california University of California. *UC Veg. Res. Inf. Cent.* (2008).

4. Shibghatallah Abdul, M., kholimah.S, Suhundodn.S ,Viridi.S, Kesuma.T. Measuring Leaf Chlorophyll Concentration from Its Color : A Way in Monitoring Environment Change to Plantations. *Nucl. Phys. Biophys. Res. Div.* 8–11 (2013).

5. Pratheesh, V. B., Benny, N. & Sujatha, C. H. Isolation , Stabilization and Characterization of Xanthophyll from Marigold Flower- *Tagetes erecta* - L. *Mod. Appl. Sci.* 3, 19–28 (2009).

6. Ahmed, M. J. & Roy, U. K. A simple spectrophotometric method for the determination of iron(II) aqueous solutions. *tubitak* 33, 709–726 (2009).

7. Eldahshan, O. A. & Singab, A. N. B. Journal of Pharmacognosy and Phytochemistry. *J. Pharmacogn. Phytochem. carotenoids* 2, 225–234 (2013).

8. Talebi, A. B., Talebi, A. B. & Shahrokhifar, B. Ethyl Methane Sulphonate (EMS) Induced Mutagenesis in Malaysian Rice (cv . MR219) for Lethal Dose Determination. *Am. J. Plant Sci.* 2012, 1661–1665 (2012).

9. siiddiqi,S.H. and khan,B.M. cytomorphological effects of colchicine on wheat (triticum aestivum). *pakistan.j. Agri.res* 4, 2 (1983).

10. Shirsat, R. K., Mohrir, M. N., Kare, M. A. & Bhuktar, A. S. Induced m utations in horsegram : mutagenic efficiency. *Recent Res. Sci. Technol.* 2, 20–23 (2010).

11. Svetleva, D. L. & Crinó, P. Effect of ethyl methanesulfonate (ems) and n-nitrose-n ´ -ethyl urea (enu) on callus growth of common bean*. Cent. Eur. A griculture* 6, 59–64 (2005).

12. Lopez, A. B.Eck.J, Conlin.B, Paolillo.D, Jennifer and Li Li . Effect of the cauliflower Or transgene on carotenoid accumulation and chromoplast formation in transgenic potato tubers. *J. Exp. Bot.* 59, 213–223 (2009).

13. Shah, K. V, Kapupara, P. K. & Desai, T. R. Pharma Science Monitor an International Journal of Pharmaceutical Sciences Determination of Sodium , Potassium , Calcium And Lithium in a Wheat Grass By Flame Photometry. *Int. J. Pharm. Sci.* 2, 745–751 (2011).

14. Adelanwa, M.A., Habeeb, M.L. and Adelanwa, E. B. Morphological studies of the effect of colchicine and paradichlorobenzene on tomato (*Lycopersicon esculentum*). 3, 122–127 (2011).

15. Mujib, A. PTC & B Colchicine Induced Morphological Variants in Pineapple. *Plant tissue cult.& Biotech* 15, 127–133 (2005).

16. Rechcigi, J.E and Payne, G.G. Determination of micronutrient of leaf by wet digestion of nitric acid and microwave. Journal of plant nutrition 13, 2085-2108 (1990).

17. Johnson, R. Identification of Leaf Pigments. *Colby J. Res. Meth.* **5,** 8–10 (2007).

18. Rao, J. K., Sahrawat, K. L. & Burford, J. R. Diagnosis o f iron deficiency in groundnut , *Arachis hypogaea* . *Int. Crop. Resarch Inst. Semi-arid Trop.* **359,** 353–359 (1987).