Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture and FAO/IAEA Agriculture and Biotechnology Laboratory, Seibersdorf

Plant Mutation Reports

Vol. 2, No. 3 April 2011

http://www-naweb.iaea.org/nafa/index.html
http://www.fao.org/ag/portal/index_en.html

ISSN 1011-260X

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Induced mutations in improvement of crops - For more information, see content

To Our Readers

Enhancing crop yields, improving food quality and value in an environmentally friendly manner, and sustaining crop biodiversity continue to be key goals for improving agricultural production. Mutation induction techniques are undergoing a renaissance in crop improvement because of advancements in modern efficiency enhancing biotechnologies – irreplaceable tools in the tool box of the breeder. In the context of climate change and variability, mutation induction is a proven way to generate diversity in existing crop varieties, to widen the extent of adaptability and enhance productivity of crop biomass. We are encouraged by the contributions from our Member States to this journal. In many countries, we see that a broad variety of plant species and target traits are addressed using mutation induction.

In this issue, the technical papers highlight studies on induced mutagenesis using either physical or chemical mutagens in a range of food and industrial crops. Dr Tulmann Neto gives an extensive review of 40 years of induced mutations in plant breeding in

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Brazil. A number of mutant derived varieties ranging from cereals, legumes, fruits and spices were developed together with commercial breeding companies and have significant economic value in Latin America. We trust that these reports will reignite a drive in experienced users of induced mutants in plant breeding and stir an interest in younger scientists.

On a more personal note, our friend and colleague, Dr Qingyao Shu, has returned to his home institution, the Zhejiang University China. I have recently taken responsibility as editor of our biannual publication, the Plant Mutation Reports. My name is Yvonne Lokko, and I am a plant breeder coming from the International Institute of Tropical Agriculture (IITA), Nigeria. I received my PhD in crop science and my scientific interest focuses on the use of biotechnology in improving crop productivity. I will do my best to continue steering Plant Mutation Reports with the spirit of Dr Shu. You may contact me at y.lokko@iaea.org.

Yvonne Lokko

Contents

Research Article

Alteration of Leaf Anatomy of Mangosteen (*Garcinia mangostana* L.) Regenerants *In Vitro* by Gamma Irradiation

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Abstract

The mangosteen plant has a low rate of photosynthesis which is known indirectly through its leaf anatomy. We conducted a study on the effect of gamma irradiation on the alteration of leaf anatomy of 21 mutant regenerants obtained from irradiated nodular calli and compared it with a non-irradiated regenerant. Whole mount leaf stained with 1% saffranin showed an increased stomatal width, density, and index as well as epidermal cells density in most mutant regenerants compared with the controls. Transverse sections (10 µm) of leaf of mutant regenerants obtained by staining with 1% safranin and 0.5% fast-green and sliced with microtome also revealed a generally thinner upper and lower cuticle than the controls. Transverse section of mutant lines R-10/4, R-15/1, R-15/2, R-15/3 had thicker palisade, spongy parenchyma and leaf lamina than the control regenerant. Also, the number of vascular bundles was higher than control. A thinner upper cuticle coupled with high stomatal density and index may enhance penetration of incident light as well as easy diffusion of carbon dioxide into the leaf. Similarly, much thicker palisade and spongy parenchyma may increase the number of chloroplasts in the mutants and therefore indirectly increase photosynthetic efficiency.

Keywords: Mangosteen, gamma irradiation, leaf anatomy, rate of photosynthesis

Introduction

Mangosteen (*Garcinia mangostana* L.) is a tropical fruit tree species known for its delicate aesthetic appeal; hence the tree is referred to as '*Queen of tropical fruit*' (Wieble, 1993). Mangosteen fruits have high economic value and thus have good prospects to be developed into an excellent export commodity. Recently, the Indonesian government has accorded high priority to develop mangosteen for export. Available statistical data shows that the production of mangosteen fruit was 62,117 metric tons in 2004 and rose to 78,674 metric tons in 2008, an increase of almost 17%. In 2009, the volume of mangosteen fruits exported was 9,466 metric tons (Directorate General of Horticulture, 2009).

The mangosteen fruit can be consumed fresh or as processed food. Besides being used as a food, mangosteen also has medicinal properties. In South-east Asia, the pericarp of mangosteen fruits have been used traditionally as medicine to treat inflammation, diarrhea, dysentery, wounds and skin infection (Obolskiy et al., 2009). The pericarp of the mangosteen fruit contains the secondary metabolite xanthones and more than 80 xanthones have been isolated and characterized from the various parts of the *G. mangostana* plant. The major constituents of xanthones isolated from mangosteen are α-mangostin and γ-mangostin (Jung et al., 2006). According to Han et al., (2009), the biological effects of mangosteen xanthones are diverse and include antioxidant, antibacterial, antifungal, anti-malarial, anti-inflammatory, cytotoxic and HIV-1 inhibitory activities.

Mangosteen is cultivated mainly in South and South-east Asia. However, its cultivation is hindered by slow growth, poor rooting system, low intake of water and minerals, low photosynthesis rate and a long dormancy phase (Wiebel, 1993). Besides, commercialization of mangosteen trees are constrained by: (1) long juvenile phase, production of first fruits occurs only 7-15 years after planting (Downton et al., 1990); (2) low growth rate, seedlings more than 2 years old may not reach even 15 cm in height (Lim et al., 1984); (3) unpredictable bearing behavior; (4) inadequate knowledge of agronomic practices and (5) lack of genetic variability (Wiebel, 1993).

Genetically, the seeds of mangosteen are obligate apomicts and are believed to be genetically identical (Richards, 1990) or homogenous. Thus there is limited genetic variability among mangosteen plants. Consequently, conventional breeding via hybridization and selection is quite difficult. The genetic limitation is also worsened by lack of fertile pollen grains (Wiebel, 1993). Thus, the alternative approach to increase genetic variability in this perennial and apomict crop is via induced mutation (Harten, 1998).

Gamma irradiation has contributed greatly to mutation frequency as well as creation of genetic variability in many plant species. This mutation technique has successfully been used to produce mutants in banana, grapevine, pear, citrus, peach, avocado and mango (Harten, 1998) and may be useful for creating genetic variability in mangosteen. Gamma irradiation can be used not only to increase genetic variability in mangosteen but also to pro-

duce mutants with high photosynthetic efficiency through the alteration of leaf anatomy. Qosim et al., (2007) stated that gamma irradiation dose could influence plant regeneration and the LD50 that could promote nodular calli into shoot formation was 25 Gy.

Breeding for increased growth rate and yield of perennial crops such as mangosteen is time consuming, labour intensive and expensive. Therefore, indirect measurement of photosynthetic rate at the juvenile stage could be used to measure these parameters. The photosynthesis rate of mangosteen leaf in the field is very low and varies typically between 1.5 and 4.0 µmol CO_2 $m^{-2}s^{-1}$, but values up to 5 µmol CO_2 $m^{-2}s^{-1}$ have been recorded under controlled environmental conditions (Downton et al., 1990). In contrast, the routinely measured photosynthesis rate for other tropical fruit tree is 10-20 µmol CO_2 $m^{-2}.s^{-1}$ (Wiebel 1993). Photosynthesis rate can be measured directly through leaf area index, leaf angle, leaf orientation and stomatal density and it determines the growth and yield of crops; it is also species dependent (Rasmusson and Gengenbach, 1984). Furthermore, photosynthesis rate is closely related to the anatomy and structure of the leaf. Hence, the alteration of the leaf anatomy or structures such as stomata, cuticle, epidermal cells, palisade, spongy parenchyma, or the vascular bundles can affect the photosynthetic rate (Willmer, 1983). The palisade parenchyma is located directly under the upper epidermis and contains chloroplasts. The palisade parenchyma is the main photosynthetic tissue of the leaf (Trigiano and Gray, 2005). The spongy parenchyma has an open and net-like structure with large inter-cellular spaces that facilitate gas diffusion. The spongy parenchyma also contains chloroplasts and contributes to photosynthesis.

This research was therefore conducted to study the effect of gamma irradiation on the leaf anatomy of mangosteen regenerated *in vitro*. It is envisaged that this study would also give an indication of indirect selection of growth rate of mangosteen.

Material and methods

Nodular calli were induced from leaf explants of mangosteen on MS basal salts (Murashige and Skoog, 1962) supplemented with 2.2µM BAP and 2.27µM TDZ, sucrose 30gL^{-1} and pure agar 8gL^{-1}. The nodular calli were irradiated by gamma rays (^{60}Co) at a dose of 0 (control), 5, 10, 15, 20, 25, 30, 35 and 40 Gy at the Center for Research and Development for Isotope Technology and Radiation, Jakarta, Indonesia. The irradiated nodular calli were regenerated on Woody Plant Medium (WPM) supplemented with 2.2µM BAP, sucrose 30gL^{-1} and pure agar 8gL^{-1} (Fig. 1). A list of regenerant mutants and control from several gamma irradiation treatments is presented in Table 1. Age of leaf shoot *in vitro* was 12 months. Each sample was observed five times on the microscope.

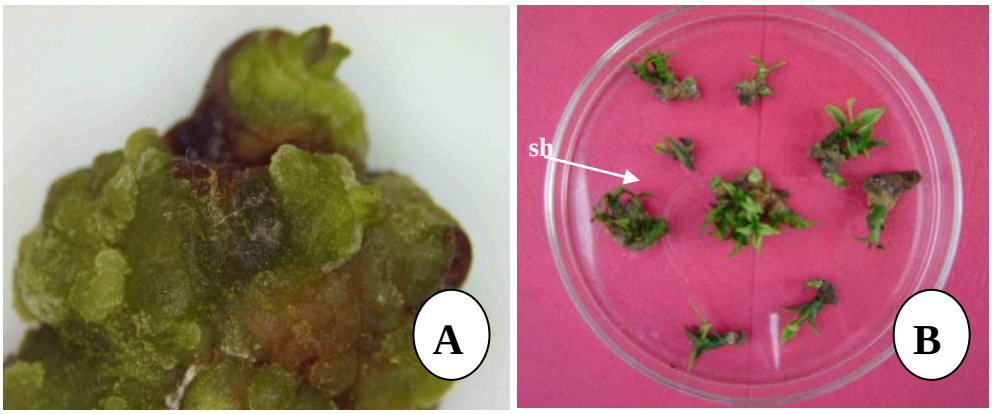

Fig. 1. Nodular calli was regenerated formed shoot (A); multiple shoot from treatment WPM medium + 2,2 µM BAP (B); sh (multiple shoot).

The leaf anatomy of mangosteen was investigated using paradermal and transverse sections. The paradermal section was studied using *whole mount methods.* The leaf segment has been fixed in an alcohol and HNO_3 solution and then colored with 1% safranin (Johansen, 1940). The stomatal width, stomatal density, epidermal cells density and stomatal index were measured during the paradermal studies using a microscope (Nikon HFX-DX). The stomatal density and index were calculated as:

$$\text{Stomatal density} = \frac{\text{No. of stomata}}{\pi r^2 \text{ or } 3.14 \times (0.219)^2}$$

$$\text{Stomatal index} = \frac{\text{(No. of stomata per unit leaf area)}}{\text{(No. of stomata per unit leaf area) + (No. of epidermal cells per unit leaf area)}} \times 100$$

Table 1.List of 21 mutant regenerants and the single control regenerant

No.	Regenerants	Background selected
1.	Control	Control
2.	R-5/1	Regenerant No. 1 of dose 5 Gy
3.	R-5/2	Regenerant No. 2 of dose 5 Gy
4.	R-5/3	Regenerant No. 3 of dose 5 Gy
5.	R-5/4	Regenerant No. 4 of dose 5 Gy
6.	R-10/1	Regenerant No. 1 of dose 10 Gy
7.	R-10/2	Regenerant No. 2 of dose 10 Gy
8.	R-10/3	Regenerant No. 3 of dose 10 Gy
9.	R-10/4	Regenerant No. 4 of dose 10 Gy
10.	R-15/1	Regenerant No. 1 of dose 15 Gy
11.	R-15/2	Regenerant No. 2 of dose 15 Gy
12.	R-15/3	Regenerant No. 3 of dose 15 Gy
13.	R-20/1	Regenerant No. 1 of dose 20 Gy
14.	R-20/2	Regenerant No. 2 of dose 20 Gy
15.	R-20/3	Regenerant No. 3 of dose 20 Gy
16.	R-25/1	Regenerant No. 1 of dose 25 Gy
17.	R-25/2	Regenerant No. 2 of dose 25 Gy
18.	R-30/1	Regenerant No. 1 of dose 30 Gy
19.	R-30/2	Regenerant No. 2 of dose 30 Gy
20.	R-35/1	Regenerant No. 1 of dose 35 Gy
21.	R-35/2	Regenerant No. 2 of dose 35 Gy
22.	R-40/1	Regenerant No. 1 of dose 40 Gy

The transverse sections of the leaves were studied using a paraffin method (Johansen, 1940). A leaf segment (0.5 cm^2) was fixed in FAA (formaldehyde, acetic acid and alcohol) solution for 24h and dehydrated in Johansen series solution with graded series alcohol (30%, 50%, 75% and 95%) transitioning to tertiary butyl alcohol (TBA) 50% and paraffin resin 50% for 2-24 h. It was infiltrated with tertiary butyl alcohol and paraffin resin into blocks and incubated at 58^{o}C for 12h. A 10 µm thick transverse section of leaf shoot was sliced by rotary microtome (Yamato RV-240) and then colored in an alcoholic-xylol series with 1% safranin and 0.5% fastgreen (Sass, 1951). The thickness of the leaf lamina, cuticle, epidermal cells, palisade, spongy parenchyma and vascular bundles were measured using an ocular micrometer in the microscope (Nikon HFX-DX).

Results and discussion

Microscopic observation revealed that the epidermal tissue of mangosteen leaf has a stomata bordered by guard cell, subsidiary cell and epidermal cell. Stomata was found on both the adaxial and abaxial epidermal tissues and thus could be classified as amphystomatic type followed by guard cells of different same sizes classified as parasitic type.

The stomatal width among the regenerants from irradiated calli ranged from 221.7-427.3/mm^2 with a mean value of 339.5 ± 21.9 /mm^2, while the control regenerant mean was 304.8 /mm^2 (Table 2). The smallest stomatal width (221.7 /mm^2) of regenerants was obtained from an irradiated nodular calli R-25/1 while the widest (432.5 /mm^2) stomata width was in R-10/3. These mutant regenerants were generally bigger than the control regenerant. The *T-test* analysis showed that seven mutant regenerants (R-10/1, R-10/2, R-10/3, R-15/2, R-20/1, R-20/3, and R-35/1) had significantly higher stomatal width (Table 2). Stomatal width influences exchange of CO_2, hence the bigger the stomatal width, the higher the exchange of CO_2. Consequently, the rate of photosynthesis is efficient (Willmer, 1983).

The stomatal density among regenerants varied, between 54.0 and 122.4 /mm^2 with a mean of 94.1 ± 7.2 /mm^2 (Table 2). The highest stomata density (122.4/mm^2) was observed in regenerants R-10/4 while stomata density of the control regenerant was 100.0/mm^2 (Fig. 2). Although

the stomata density of regenerant R-10/4 was high, it had a low epidermal cell density (1246.2/mm^2). Comparatively, the regenerant R-10/3 had a lower stomatal density of 54.0/mm^2 and stomatal width of 432.5 /mm^2, but it had the highest epidermal cell density (1587.8/mm^2). Downton et al., (1990) stated that stomatal density for field-grown mangosteen leaves was between 100 and 180/mm^2. These values are low compared to subtropical and tropical fruit and nut trees, such as 250-460/mm^2 in apple (Friedrich et al., 1986), 450/mm^2 in citrus (Kriedemenn, 1986), 360-500/mm^2 in macadamia (Stephenson, 1989), 474/mm^2 in cashew cv. Guntur and 631 /mm^2 in mango cv. Kensington (Wiebel, 1993). In this present study, the mean stomatal density of mangosteen leaf was 94.1/mm^2 and it is far lower than the examples given, thus confirming Wiebel's (1993) assertion that mangosteen leaf has very low stomata density.

Table 2. The effect of gamma irradiation on paradermal section of 21 regenerant mutant lines and one regenerant control of mangosteen *in vitro*

Regenerants	Stomata width (per mm^2) ± S.E	Epidermis density (per mm^2) ± S.E	Stomata density (per mm^2) ± S.E	Stomata index
Control	304.8 ± 13.1 ns	1310.1 ± 25.9 ns	100.0 ± 4.3 ns	7.6 ± 0.42 ns
R-5/1	304.5 ± 12.7 ns	1324.6 ± 17.8 ns	94.8 ± 5.1 ns	7.2 ± 0.53 ns
R-5/2	268.7 ± 21.7 ns	1127.2 ± 12.9 ns	119.8 ± 9.1 **	9.1 ± 0.65 **
R-5/3	351.5 ± 11.9 ns	1239.7 ± 17.9 ns	117.1 ± 8.1 **	8.9 ± 0.71 **
R-5/4	286.9 ± 21.0 ns	1258.8 ± 23.7 ns	109.2 ± 4.9 **	8.3 ± 0.67 **
R-10/1	419.3 ± 19.8 **	1456.8 ± 25.9 *	75.0 ± 8.5 ns	5.7 ± 0.84 ns
R-10/2	411.1 ± 16.7 **	1575.2 ± 31.7 *	59.2 ± 9.7 ns	4.5 ± 0.66 ns
R-10/3	432.5 ± 15.6 **	1587.8 ± 25.9 *	54.0 ± 6.8 ns	4.1 ± 0.72 ns
R-10/4	290.5 ± 17.1 ns	1246.2 ± 37.8 ns	122.4 ± 7.4 **	9.3 ± 0.64 **
R-15/1	351.4 ± 18.2 ns	1338.4 ± 28.9 ns	85.5 ± 6.7 ns	6.5 ± 0.71 ns
R-15/2	389.9 ± 11.1 **	1417.3 ± 19.7 ns	76.3 ± 8.3 ns	5.8 ± 0.84 ns
R-15/3	283.4 ± 14.1 ns	1311.4 ± 21.7 ns	109.2 ± 9.3 **	8.3 ± 0.54 **
R-20/1	442.6 ± 13.1 **	1471.9 ± 18.4 *	80.3 ± 7.4 ns	6.1 ± 0.85 ns
R-20/2	365.2 ± 9.1 ns	1377.2 ± 21.9 ns	90.8 ± 9.3 ns	6.9 ± 0.34 ns
R-20/3	427.3 ± 17.6 **	1568.0 ± 28.8 *	67.1 ± 8.9 ns	5.1 ± 0.21 ns
R-25/1	221.7 ± 13.4 ns	1258.8 ± 17.5 ns	119.8 ± 6.4**	9.1 ± 0.45 **
R-25/2	272.3 ± 14.5 ns	1384.4 ± 16.5 ns	100.0 ± 5.9 **	7.6 ± 0.18 ns
R-30/1	330.0 ± 18.1 ns	1304.8 ± 11.3 ns	106.6 ± 4.2 ns	8.1 ± 0.77 *
R-30/2	334.9 ± 17.9 ns	1272.6 ± 17.6 ns	114.5 ± 3.8 **	8.7 ± 0.84 **
R-35/1	399.8 ± 32.2 **	1516.7 ± 14.7 *	79.0 ± 8.4 ns	6.0 ± 0.72 ns
R-35/2	349.5 ± 12.4 ns	1400.2 ± 15.9 ns	86.9 ± 6.8 ns	6.6 ± 0.69 ns
R-40/1	323.5 ± 17.2 ns	1345.6 ± 23.5 ns	102.6 ± 7.4 *	7.8 ± 0.43 ns
means	339.5 ± 21.9	1367.9 ± 21.9	94.1 ± 7.2	7.2 ± 0.56

Explanation: ns = non significant; * = significant; ** = highly significant based on t-test on 5% level; SE = standard error

Of the 21 mutant regenerant lines, 15 had an epidermal cell density more than that of the control regenerant which had an average of 1310.1/mm^2 (Table 2). Six regenerants had lower stomatal density than the controls.

Generally, there was an inverse relationship between stomatal density and stomatal width, high stomatal density correlates with small stomatal width, and vice versa. Also, epidermal cell density is related to stomatal density (Willmer, 1983). Plants with high stomata density usually has a low epidermal cell density, such as in regenerant R-10/4 and a low stomata density has a high epidermal cell density such as in regenerant R-10/3

The stomatal index of mutant lines regenerants of mangosteen also ranged from 4.1 - 9.3 with a mean of 7.2 ± 0.56. The lowest stomatal index was observed in regenerant R-10/3 (4.1), while the highest was in regenerant R-10/4 (9.3) (Table 2). The stomatal index is the ratio of the total number of stomata to the epidermal cell density. Plants with low stomatal density compared to epidermal cell density will result in low stomatal index. Conversely, high stomatal density compared to epidermal cell density will result in high stomatal index. The t-*test* analysis showed that the stomatal index of seven regenerants (R-5/2, R-5/3, R-5/4, R-10/4, R-15/3, R-25/1 and R-30/2) was significantly different from the controls. These changes in the stomatal index possible genetically caused by gamma irradiation and could be inherited to offspring. Genetic variation exists for number, size and time of opening and closing of stomata. These factors influence the entry of CO_2 into the plant and has implications for the efficiency.of photosynthesis (Rasmusson and Gengenbach, 1984). Willmer (1983) has stated that stomatal index is an anatomical feature that is not influenced by the environment (stable) compare to stomatal width and the stomatal density.

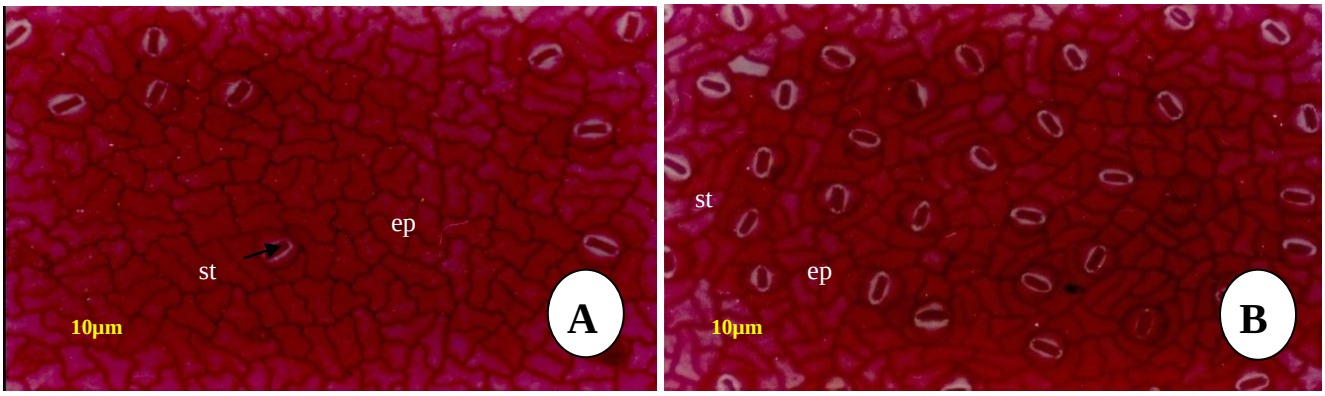

Fig. 2. Paradermal section of leaves of mangosteen regenerants in; (A) control regenerant and (B) regenrant of mutant line R-10/4. st (stomata); ep (epidermal cells).

Irradiation by gamma rays can alter the leaf anatomy. Irradiating mangosteen nodular calli with 5Gy and 25Gy clearly altered the stomatal index, while 10Gy and 35Gy had an effect on stomatal width and epidermal cell density. Other factors which are known to influence the leaf anatomy are water supply, light intensity, concentration of CO_2, and temperature (Willmer, 1983). Taiz and Zeiger (2006) have reported that stomata function as a gateway of CO_2 for photosynthesis and as a passage for water loss from the leaf via transpiration. Therefore most land plants open their stomata in the morning to enable diffusion of CO_2 to the leaf for photosynthesis.

The transverse section of the mangosteen leaf shows an upper cuticle, upper epidermal cells, palisade, spongy parenchyma, lower epidermal cells and lower cuticle. Both epidermal cell layers of a mangosteen leaf are covered with cuticle and sandwich a palisade parenchyma cell layer, hence the leaf is said to be dorsiventral. The upper cuticle is generally thicker (4 µm) while the lower cuticle is about 2 µm. In the present study, the thickness of the upper cuticle of regenerant mutant lines ranged between (1.0 µm -4.0 µm) with a mean of 2.1 ± 0.4 µm, while the lower cuticle was just 1.0 – 2.0 µm and a mean of 1.4 ± 0.3 µm. In the control regnerants, the thickness of the upper cuticle was 3.0 µm (Table 3). Only one mutant line (R-5/1) had comparatively thicker upper cuticle (4.0 µm) than the control regenerant. While for the lower cuticle, six mutant regenerant lines (R-5/1, R-5/2, R-10/1, R-20/3, R-30/2, R-35/1) had thicker cuticle than the controls. The presence of thicker cuticle suggests that these mutant regenerants could be tolerant to drought, as a thicker cuticle reduces water loss by transpiration and also reflects incident sun rays. Furthermore, a thicker, slippery cuticle can protect the plant from pests and disease attack by reducing stickiness and growth of spores on the leaf surface (Mauseth, 1988). The thickness of the lower cuticle was comparatively higher than the upper cuticle suggesting a very good adaptation for control of water loss by transpiration.

The results showed that control regenerants had epidermal cell, palisade, and spongy parenchyma developing normally (Table 3). This was different from regenerant R-15/3 which had the thickest leaf lamina (115.4 µm) compared to other regenerants, the highest number of

vascular bundles (37) and many *idioblasts* spread on palisade and spongy parenchyma. All the regenerant mutant lines developed thicker spongy parenchyma cells than the controls (Table 3). Both R-10/2 and R-35/2 showed changes in in that they had irregular shaped spongy parenchyma arranged lengthwise to horizontal which could be a possible cause for the dwarf appearance of the regenerant (Fig. 3).

The thickness of the upper and lower epidermal cells (both control & regenerants) were almost the same except in R-10/4, R-20/1 and R-30/ where both had a thickness of more than 6.0 µm. Most of the regenerants had lower and upper epidermis thickness greater than the controls. Epidermal cells are generally bigger in size with usually a large vacuole. The vacuoles of the epidermal cells contain much water and are known to absorb ultraviolet infra red radiation thereby reducing heat (Wilmer, 1983).

Table 3. Analysis of transverse section of 21 regenerant mutant lines and one regenerant control of mangosteen *in vitro*

Regenerants	Thickness							Number of vascular bundle ± S.E
	Upper cuticle (µm) ± S.E	Upper epidermal cells (µm) ± S.E	Palisade parenchyma (µm) ± S.E	Spongy parenchyma (µm) ± S.E	Lower epidermal cells (µm) ± S.E	Lower cuticle (µm) ± S.E	Leaf lamina (µm) ± S.E	
Control	3.0 ± 0.3 **	4.8 ± 0.3 ns	20.9 ± 0.8 **	33.2 ± 1.1 ns	4.2 ± 0.3 ns	1.5 ± 0,2 ns	63.1 ± 0.8 ns	11.0 ± 0.2 ns
R-5/1	4.0 ± 0.4 **	4.7 ± 0.3 ns	17.4 ± 0.9 ns	48.6 ± 0.8 ns	4.4 ± 0.2 ns	2.1 ± 0,3 **	75.1 ± 1.3 ns	24.0 ± 0.2 ns
R-5/2	2.5 ± 0.3 *	4.6 ± 0.4 ns	13.9 ± 0.5 ns	50.7 ± 1.3 *	4.9 ± 0.3 ns	2.0 ± 0,6 **	74.0 ± 1.7 ns	14.0 ± 0.3 ns
R-5/3	2.0 ± 0.2 ns	4.1 ± 0.5 ns	22.0 ± 0.7 **	49.3 ± 1.7 ns	4.0 ± 0.4 ns	1.1 ± 0,5 ns	79.4 ± 1.1 *	11.0 ± 0.4 ns
R-5/4	2.5 ± 0.2 *	3.2 ± 0.3 ns	16.7 ± 0.5 ns	47.0 ± 1.1 ns	3.4 ± 0.4 ns	1.5 ± 0,1 ns	70.3 ± 1.2 ns	17.0 ± 0.6 ns
R-10/1	1.5 ± 0.3 ns	3.4 ± 0.6 ns	12.6 ± 0.5 ns	42.6 ± 1.3 ns	3.4 ± 0.2 ns	1.7 ± 0,3 **	62.0 ± 1.4 ns	15.0 ± 0.5 ns
R-10/2	2.0 ± 0.4 ns	4.8 ± 0.3 ns	15.6 ± 0.6 ns	40.8 ± 1.4 ns	4.3 ± 0.5 ns	1.5 ± 0,4 ns	65.2 ± 1.4 ns	21.0 ± 0.4 ns
R-10/3	2.5 ± 0.5 *	5.8 ± 0.4 **	15.2 ± 0.6 **	52.6 ± 1.4 *	5.2 ± 0.4 ns	1.2 ± 0,5 ns	79.3 ± 1.4 *	20.0 ± 0.5 ns
R-10/4	1.8 ± 0.6 ns	6.2 ± 0.4 **	23.6 ± 0.5 **	47.0 ± 1.4 ns	5.2 ± 0.5 ns	1.3 ± 0,2 ns	82.0 ± 1.3 *	29.0 ± 0.6 **
R-15/1	1.5 ± 0.6 ns	5.0 ± 0.2 ns	21.2 ± 0.5 **	56.6 ± 1.2 **	5.6 ± 0.4 *	1.0 ± 0,4 ns	99.8 ± 1.2 **	23.0 ± 0.7 ns
R-15/2	1.0 ± 0.7 ns	4.8 ± 0.7 ns	19.0 ± 0.6 *	52.0 ± 1.1 **	4.8 ± 0.4 ns	1.1 ± 0,2 ns	89.7 ± 1.1 **	29.0 ± 0.6 **
R-15/3	1.5 ± 0.9 ns	5.4 ± 0.8 ns	20.2 ± 0.9 **	64.4 ± 1.0 **	4.4 ± 0.3 ns	1.0 ± 0,2 ns	115.4 ± 1.0 **	37.0 ± 0.7 **
R-20/1	3.0 ± 0.1 *	6.6 ± 0.4 **	18.6 ± 0.8 ns	56.0 ± 1.3 **	5.0 ± 0.5 ns	1.2 ± 0,4 ns	88.2 ± 1.5 **	17.0 ± 0.7 ns
R-20/2	2.0 ± 0.4 ns	4.8 ± 0.5 ns	17.8 ± 0.4 ns	42.3 ± 1.9 ns	4.7 ± 0.7 ns	1.5 ± 0,5 ns	69.7 ± 1.4 ns	25.0 ± 0.8 *
R-20/3	2.7 ± 0.2 *	5.0 ± 0.3 ns	21.0 ± 0.3 **	43.5 ± 1.8 ns	4.3 ± 0.6 ns	1.7 ± 0,3 **	73.8 ± 1.3 ns	16.0 ± 0.6 ns
R-25/1	1.5 ± 0.3 ns	4.8 ± 0.3 ns	16.2 ± 0.5 ns	41.2 ± 1.8 ns	5.6 ± 0.4 **	1.0 ± 0,4 ns	67.8 ± 1.4 ns	20.2 ± 0.8 ns
R-25/2	2.0 ± 0.2 ns	5.2 ± 0.3 ns	18.8 ± 0.6 ns	39.6 ± 1.3 ns	5.0 ± 0.8 **	1.5 ± 0,6 ns	70.6 ± 2.3 ns	17.0 ± 0.8 ns
R-30/1	2.5 ± 0.5 *	5.2 ± 0.4 ns	21.2 ± 0.5 **	53.2 ± 1.4 *	6.6 ± 0.3 **	1.3 ± 0,1 ns	86.2 ± 1.9 **	24.0 ± 0.7 ns
R-30/2	2.4 ± 0.2 ns	4.2 ± 0.3 ns	19.8 ± 0.5 *	45.5 ± 1.3 ns	4.6 ± 0.4 ns	2.0 ± 0,2 **	74.2 ± 1.7 ns	20.1 ± 0.8 ns
R-35/1	2.5 ± 0.4 *	5.0 ± 0.9 ns	20.4 ± 0.6 **	40.2 ± 0.9 ns	4.8 ± 0.3 ns	2.0 ± 0,2 **	70.4 ± 1.8 ns	33.0 ± 0.9 **
R-35/2	1.5 ± 0.2 ns	5.2 ± 0.3 ns	18.3 ± 0.4 ns	36.0 ± 1.7 ns	5.5 ± 0.7 *	1.0 ± 0,3 ns	65.0 ± 1.5 ns	20.0 ± 0.8 ns
R-40/1	1.0 ± 0.2 ns	4.0 ± 0.5 ns	16.5 ± 0.4 ns	35.5 ± 1.8 ns	3.8 ± 0.5 ns	1.0 ± 0,4 ns	59.8 ± 1.7 ns	33.0 ± 0.5 **
Means	2.1 ± 0.4	4.9 ± 0.4	18.5 ± 0.5	46.3 ± 1.4	4.9 ± 0.4	1.4 ± 0,3	74.5 ± 1.7	20.7 ± 0.6

Explanation: ns = non significant; * = significant; ** = higly significant based on t-test on 5% SE = standard error

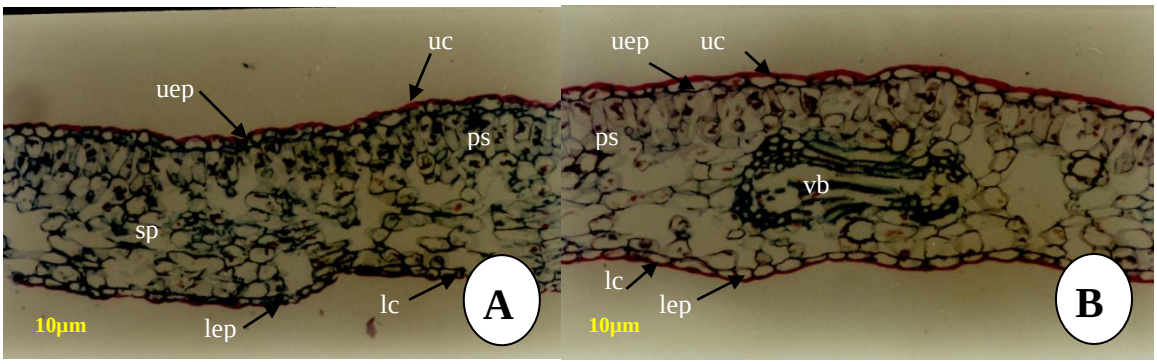

Fig. 3. Leaf of transverse section of mangosteen control regenerant (A); R-10/2 regenerant of mutant line (B). uc (upper cuticle); uep (upper epidermal cell); ps (palisade) sp (spongy parenchyma); lep (lower epidermal cell); vb (vascular bandles) (x 400).

The thickness of the leaf lamina of mutant regenerants also varied between 59.8 – 115 µm with a mean of 74.5 ± 1.7 µm, while that of the control regenerant was 63.1 µm. The t-test analysis showed that eight mutant regenerants differed significantly from the controls with a thickness of more than 79.4 µm; these mutants were R-5/3, R-10/3, R-10/4, R-15/1, R-15/2, R-15/3, R-20/1 and R-30/1. The mutant regenerant line R-15/3 thickest lamina (115.1 µm) and it contained many idioblasts. Fahn (1990) has stated that the idioblasts function by storing protein crystals. The thick leaf lamina leads to a higher volume to surface ratio, indicating that the plant is xerophytic and thus may be adapted to dry environments.

The palisade parenchyma is oriented vertical to the leaf surface and its thickness varied between 12.6 – 23.6 µm with a mean of 18.5 ± 0.5 µm. The mean thickness of the palisade parenchyma of control regenerants was 20.9 ± 0.8 µm. Only three mutant regenerants (R-5/3, R-10/4 and R-20/3) had thicker palisade parenchyma than the controls. This high value indicates that they may have efficient photosynthesis compared to any other regenerants because palisade parenchyma contains more chloroplast pigment. The thickness of the spongy parenchyma of mangosteen leaf ranged between 33.2 – 64.4 µm with an average of 46.3 ± 1.4 µm while the control regenerant had an average thickness of 33.2 µm. Comparatively, all the mutant regenerants had thicker spongy parenchyma than the control regenerant. Spongy parenchyma cells consist of irregular shape parenchyma thereby providing wider air spaces for exchange of gases (Mauseth, 1988). Therefore, the mutant regenerants with thick spongy parenchyma will enable them to store more CO_2 for photosynthesis, and thereby indirectly improving the rate of photosynthesis.

The vascular bundles which consist of the phloem and the xylem separated by the cambium are located in the midrib of the leaf in the spongy parenchyma. The number of vascular bundles of the regenerants varied between 11.0 and 33.0 with an average of 20.7 ± 0.6, while the control regenerants had and average of 11 vascular bundles. Generally, the mutant regenerants had more vascular bundles than the controls except, R-5/3 where the number of vascular bundles was same as the control (Table 3). The vascular bundle is very important for transport of water, mineral salts and assimilates to all part of the plant and thus plays an important role in the photosynthesis (Taiz and Zeiger, 2006).

Conclusion

Irradiating nodular calli of mangosteen with gamma rays between 5 and 40 Gy led to the production of regenerants with altered leaf anatomy or structure which may indirectly enhance the photosynthetic efficiency in the crop. The regenerant mutant lines have higher stomatal density and stomatal index than the controls, these very important to entry CO_2 for photosynthesis process. They have thick palisade parenchyma with high chloroplast density and store CO_2 in spongy parenchyma. The photosynthesis rates of regenerant mutant lines correlate well with the growth rate.

Acknowledgements

The authors wish thank to the Center for Tropical Fruit Studies, Bogor Agricultural University and the Directorate of Higher Education through Research Grant Competition XII ('Hibah Bersaing XII') Contract number: 027/SPPP/PP/DP3M/2005 for financial support and Head of Anatomy Laboratory, Department of Biology, Bogor Agricultural University for Laboratory facilities.

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Research Article

Induced Floral Variations in Cotton (*Gossypium hirsutum* L.)

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Abstract

Two varieties of cotton (cv. MCU 5 and MCU 11) were treated with gamma rays, ethyl methane sulphonate and sodium azide with the objective of determining the most efficient mutagens and most responsive genotype for induction of mutations affecting predominantly reproductive parts. Frequency and spectrum of mutations affecting floral traits was computed on M_2 plant population that was screened for variations in chlorophyll, flower colour and floral parts. Among the floral variations, elongated petals, colour changes and reduced petals were the dominant ones. The differences in mutations observed in the two cultivars have been attributed to the variations in the microgenomic architecture.

Key words: cotton, mutagens, mutants, changes in flower structure

Introduction

Cotton is a major commercial crop and leading natural fibre. It has played a significant role in agriculture and the textile industry in generating employment and uplifting the socio-economic status of the farming and labour communities in India and the rest of the world (Kairon et al., 1998). Cotton is also important as a major source of edible oil and protein and other by-products such as linters, absorbent cotton, hull, crude oil, oil cake and meal and cotton stalks useful in several industrial applications (Lusas and Jividen, 1987). Crop improvement for yield and quality of fibre has been accomplished till date by genetic and cytoplasmic manipulations. Efforts are being made to improve the genetic makeup of the cotton crop for higher yield and better fibre quality, besides resistance to biotic stress. Mutations are potential sources of genetic variability in the plant breeding programme. Induced mutations have been instrumental in the genetic improvement of several auto- and allogamous crops; a large number of improved varieties have been developed through induced mutations in different crops. As genetic variability is essential for any crop improvement programme, the creation and management of genetic variability is central to crop breeding. The practical utility of induced mutations for the improvement of quantitatively inherited characters in cotton is well recognized. Induced mutations have played a significant role in breeding for earliness, dwarfness and compactness, large bolls, high ginning outturn and fibre length, high yield, high seed oil content, diseases and insect resistance, drought and salinity resistance besides induction of male sterility and creating vast genetic variability for various economic and morphological characters of cotton (Basu et al., 1984). There are reports about floral variations in *Curcuma* (Abdullah et al., 2009), ginger (Kuehny et al., 2002), African violets (Seneviratne et al., 2004) and *Chrysanthemum* (Aisyah and Marwoto, 2001; Datta and Chakrabarty, 2009) on the efficacy of various mutagens for induction of mutations affecting the general reproductive system its development, floral part function and selective interference with male fertility (Singh and Ikehashi, 1979; Yamashita and Ukai, 1979; Ko and Yamagata, 1980; Mese et al., 1984; Maruyama et al., 1990 a,b). In order to induce variability and utilize useful mutations for efficient plant breeding, the systematic study of viable morphological mutations in M_2 and M_3 generations is the most dependable index. The present investigation was undertaken to study the mutagenic effects of gamma rays, EMS and SA on floral variations in M_2 and M_3 generations. In this paper we described and discussed the data for induced floral variations and it frequency in M_3 generation in cotton.

Materials and methods

The two tetraploid varieties of cotton (*Gossypium hirsutum* L.) var. MCU 5 and MCU 11 were obtained from Cotton Breeding Centre, Tamil Nadu Agricultural University, and Coimbatore, India. Dry, healthy and uniform seeds (delinted) with moisture content of about 14% were exposed to 100, 200, 300, 400 and 500 Gy of gamma rays from ^{60}Co source at Sugarcane Breeding Centre (ICAR), Coimbatore. The concentration of ethyl methane sulphonate (EMS) and sodium azide (SA) with 10, 20, 30, 40 and 50 mM were prepared in sodium phosphate buffer solution of pH 7.0 and 3.0 for EMS and SA respectively. For EMS and SA treatments, seeds were pre-soaked in distilled water for 8 h and then the seeds were treated with chemical mutagens for 4 h. The treated seeds were then washed in running water for 30 minutes and were sown in the pre-irrigated field along with control with three replications in randomised block design. The plants in the M_1 generation were bagged to ensure self-fertilisation and the seeds were collected separately from

group of plants were selected and carried forward on bulk basis to raise M_2 population. Twenty five M_2 plants form each dose/concentration were randomly selected based on morphological and agronomic characters by comparing with control and advanced to the M_3 generation. Flower structure, colour and sterile flower were noted from each plant in the M_3, the same procedure was repeated, plants selected and promoted to M_4 generation. The chlorophyll mutants (albino, xantha, chlorina, viridis and maculata type) were scored from the fifth to the fifteenth day of sowing and were classified according to Gustaffson (1940) and the mutation frequency was calculated. From three mutagenic treatments, 62 319 and 67 673 seedlings were scored for chlorophyll mutations in M_2 in the cultivars MCU 5 and MCU 11 respectively. The length of petals and thickness was measured (cm) on cross sections of the petals by ocular micrometer in the microscope and compared with petals from control plants. The colour of the flowers in M_2 and M_3 progenies were observed with reference to the control plants. The fertile and sterile flowers were also observed. Pollen fertility was assessed by staining the pollen grains in 1:1 glycerine: acetocarmine mixture and an average of five slides were scored from each plant. Data were analyzed by analysis of variance (ANOVA) and compared using Duncan's Multiple Range Test (DMRT).

Results and discussion

Mutant types are often characterised by variations in several floral characters as compared with the control plants. Mutants affecting flower structure, days to flowering and fertility were obtained in this investigation. M_2 population from M_1 seeds were studied for both structural and economic characters and advanced to M_3 generation. In this paper we describe the mutagenic effects on floral variations and its frequency. Plants treated with chemical mutagenic agents and gamma rays exhibited different responses, depending on the cultivar, concentration of chemical mutagens and duration of exposure/treatment (Tables 1 and 2). In the M_1 generation, no differences were noted in exposed/treated plants with reference to control, whereas in M_2 and M_3 generations different putative mutations were observed such as i) chlorophyll deficiency, ii) plant height, iii) early flowering (data not shown) and iv) floral variations (increased petal length, thickness, colour, sterility). The control plants did not show any variation. The number of flower petals increased in the range 4-7 (Fig. 1 a-h). Among the different floral variations, only 3 characters were dominant (petal thickness, elongated petals and reduced petals) and have been studied separately. Of the three distinguishing characters, the elongated petal thickness was observed at a lower dose concentration of the mutagens whereas reduced petals were observed at a higher dose concentration of the mutagens. The length of the petals in plants with elongated petals was 5.5 cm in MCU 5 was and 5.8 cm in MCU 11. The reduced petal length observed in plants treated with higher doses of mutagens was 2.5 cm and 2.8 cm in MCU 5 and MCU 11 respectively. The thickness of the petals was higher (2.2 mm) in both MCU 5 and MCU 11 whereas it there were minor variations in other groups when compared to control plants. Our observations are in conformity with results observed by Gridharan and Balakrishna (1992), Mehetre et al., (1992), Saxena et al., (1992), Kloth (1995), Marks (1997), Saxena et al., (1998) and Zhang et al., (2000). Comparative higher efficiency of sodium azide in inducing mutations affecting floral traits is in conformity with reports on its efficiency to induce male sterile mutations in *Zinnia* (Venkatachalam and Jayabalan, 1994a). Similar results were noted in *Gladioli* (Misra, 1998), in chickpea (Kharkwal, 2000), in rice (Jauhar and Siddiq, 1999), in *Ipomoea* (Bhate, 1999). During the observations, number of morphological changes was noted (Muthusamy and Jayabalan, 2001) and especially in flowers, such as increase or decrease in the number and size of floral parts were observed in both the cultivars (Muthusamy, 2001). We also observed induced twining and boll abnormalities in cotton and the twining characters were inherited to further generations (Muthusamy et al., 2004). In our earlier reports (Muthusamy and Jayabalan, 2002), we described the effects of mutagens on pollen fertility in cotton and observed that pollen fertility is decreasing with increasing dose/concentration of mutagens. Ahuja and Dhayal (2006) have observed the petal spotted mutant in upland cotton with desirable morphological and technological characters. Thangapandian and Thiyagarajan (2004) have reported the inheritance of floral traits in mutant rice and Seema et al., (2001) observed the floral androcarpel organ (*aco*) mutant with improved alkaloids yield in opium poppy.

The frequency and spectrum of floral variations are presented in Table 2. In general, the floral variations were increased with increasing dose/concentration of mutagens. Gamma rays induced highest number of variants in floral modification followed by SA and EMS in MCU 5 and MCU 11 (Fig. 1 a-h). The parent of the two cultivars had cream coloured petals, which later changed into yellow with elongated petals, and light rose as the petals beacame (Fig. 1 f, g). Two flowers with yellow colour petals were also observed in variety MCU 11 (Table 1). The flowers of this mutant plant in M_2 and M_3 generations were very unusual as they were very small with rudimentary and malformed organs. There was no boll formation in the flower with malformed organ due to sterile nature of the pollen and pistil. Changes in flower colour in mutants have also been obtained by gamma rays, EMS treatment in *Zinnia* (Venkatachalam and Jayabalan, 1994b), mustard (Anand and Mishra, 1985), in urdbeen

(Singh et al., 2000), and *Lathyrus* (Shanti and Shilpa, 2000). Induction of colour variations in carnation following EMS treatment has also been reported earlier (Hentrich and Glawe, 1982). Floral abnormalities were attributed to the physiological or biochemical disturbances caused by irradiation during differentiation of different floral organs (Thombre and Mehetre, 1980). Stebbins (1967) postulated that the difference between separate and united parts probably depends upon a series of co-ordinated differences in developmental pattern involving the production and distribution of various growth substances.

Table 1. Induced variations in flowers of cotton in M_3 generation

Characters	Length (cm)*	Thickness (mm)*	Petal colour	Pollen colour
MCU 5				
Control	3.0a	1.0a	Cream	Cream
Petal thickness	4.5b	2.2b	Cream	Cream
Elongated petals	5.5c	1.3c	Cream	Cream
Reduced petals	2.5cd	1.2d	Cream	Cream
MCU 11				
Control	3.2e	1.0de	Cream	Cream
Petal thickness	5.0f	2.2f	Cream	Cream
Elongated petals	5.8g	1.2g	Yellow	Cream
Reduced petals	2.8h	1.2g	Yellow	Cream

*Means within column with different letters are significant at 1% level according to DNMRT

Table 2. Frequency and spectrum of floral variations in M_2 generation in the cultivar MCU 5 and MCU 11

Mutants	Gamma rays (Gy)					Ethyl methane sulphonate (mM)					Sodium azide (mM)				
	100	200	300	400	500	10	20	30	40	50	10	20	30	40	50
MCU 5															
Structure	-	2	1	5	7	-	3	-	4	2	-	1	2	3	4
		(0.47)	(0.35)	(2.35)	(4.66)		(0.73)		(1.05)	(0.57)		(0.23)	(0.50)	(0.85)	(1.32)
Colour	-	1	-	1	2	1	2	1	3	2	-	1	-	2	2
		(0.23)		(0.47)	(1.33)	(0.23)	(0.47)	(0.24)	(0.78)	(0.57)		(0.23)		(0.57)	(0.66)
Sterile	-	-	-	4	6	-	1	1	3	3	1	-	-	5	5
				(1.88)	(4.00)		(0.24)	(0.24)	(0.78)	(0.85)	(0.21)			(1.42)	(1.66)
Total Variants	-	3	1	10	15	1	6	2	10	7	1	2	2	10	11
		(0.21)	(0.35)	(4.71)	(10.0)	(0.23)	(1.46)	(0.49)	(2.63)	(2.00)	(0.20)	(0.46)	(0.50)	(2.85)	(3.65)
M_2 Populations	410	421	285	212	150	425	410	403	380	350	486	432	400	350	301
MCU 11															
Structure	-	1	-	2	1	-	-	-	1	4	-	-	1	2	3
		(0.24)		(0.87)	(0.58)				(0.25)	(1.12)			(0.24)	(0.55)	(0.96)
Colour	-	1	-	2	4	2	1	-	3	1	-	-	1	2	4
		(0.24)		(0.87)	(2.35)	(0.45)	(0.23)		(0.75)	(0.27)			(0.24)	(0.55)	(1.28)
Sterile	-	-	1	3	5	-	-	-	3	2	-	-	1	2	1
			(0.32)	(1.31)	(2.94)				(0.75)	(0.55)			(0.24)	(0.55)	(0.32)
Total Variants	-	2	1	7	10	2	1	-	7	7	-	-	3	6	8
		(0.48)	(0.32)	(3.07)	(5.88)	(0.45)	(0.23)		(1.75)	(1.95)			(0.74)	(1.66)	(2.57)
M_2 Populations	420	415	312	228	170	440	426	417	398	358	480	425	404	360	311

Numbers in parenthesis refer to the mutation frequency

Floral modifications in cotton in MCU 5 & MCU 11

Fig. 1. a) control flower of MCU 5. b) flower showing increased thickness. c) flower showing elongated petals. d) Flower showing reduced petals. e) control flower of MCU 11. f) flower showing elongated petals with yellow colour. g) flower showing increase edpetal thicknes. h) flower showing reduced petals with changed colour.

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Research Article

Mutagenic Effects of Some Chemical Agents in Wheat (*Triticum aestivum* L. em Thell)

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Abstract

Water soaked wheat cv. NW 1014 seeds were treated with aqueous solutions (1 per cent) of 6 chemicals, namely, acid slurry, sodium carbonate, tri-sodium phosphate, sodium tri-polyphosphate, carboxy methylcellulose and sodium sulphate for 22h. Out of these chemicals, tri-sodium phosphate and carboxy methylcellulose recorded the highest mutation frequency (1.15 per cent), followed by sodium sulphate (1.00 per cent), sodium carbonate (0.85 per cent), sodium tri-poly phosphate (0.65 per cent) and acid slurry (0.0 per cent) in the M_2 generation. The highest number of mutants was observed for late heading (47), followed by dwarf stature (36), white spike (7) and high tillering (6) in the M_2 generation. On the basis of the number of high yielding mutants (M_5), the mutagenic efficiency of sodium carbonate may be placed at the top rank, followed by sodium sulphate, tri-sodium phosphate, sodium tri-poly phosphate and carboxy methyl cellulose. One type of macro-mutant (white spiked) was induced by tri-sodium phosphate whereas sodium carbonate generated 3 types of macro-mutants (large flag leaf, club shape spike and small grain). The studies reveal that all the chemicals, except acid slurry, may be used as mutagenic agents in wheat.

Key words: Wheat, mutagenic effects, chemical mutagens

Introduction

Mutation is a very effective, easy and common approach, adopted by several earlier workers (Nayeem and Syed, 1996; Nayeem et al., 1999; Kulkarni et al., 2008) to generate variants in wheat. The mutagens which are in common use today are costly and not easily available. Hence, there is a need to identify an alternative to these mutagens which may be comparatively cheaper and easily available, especially for poorer laboratories. To this end, a study was undertaken by the author to investigate mutagenic effects of synthetic detergent in wheat. To identify the chemical components responsible for these effects, the present studies were conducted with six different chemical components found in synthetic detergents (Anonymous, 1993). In previous reports some workers have observed mitotic inhibition (Kumar and Kamr, 1990; Kumar, 1991) and male gametocidal effect (Singh, 1999) by synthetic detergents in other crops.

Materials and methods

Water soaked (4.0 h) seeds of wheat (*Triticum aestivum* L. em Thell) cv. NW 1014 were treated with 1.0 per cent aqueous solutions of 6 chemical components of synthetic detergent (Anonymous, 1996), namely acid slurry ($H_2S_2O_7$), sodium carbonate (Na_2CO_3), tri-sodium phosphate (Na_3PO_4), sodium tri-poly phosphate [$(Na_3 (PO_3)_3$], carboxy methyl cellulose [$C_6H_7O(COOCH_3)_5$] and sodium sulphate (Na_2SO_4), separately for 22 h. These chemicals were coded as AS, SC, TSP, STPP, CMC and SS, respectively for the present studies. NW1014 is a variety, recommended for cultivation in Bihar and other parts of the North Eastern Plain Zone of India under the irrigated late sown situation. Three hundred fifty seeds were used for each treatment. After treatment, the seeds were rinsed in running tap water to remove the residual chemicals. Water soaked untreated parent seeds were used as the control material. One hundred fifty out of 350 treated and control seeds were used for germination studies in laboratory conditions and the remaining 200 seeds were used for field studies in the M_1 generation. The germination studies were conducted under CRD with three replications, of each of 50 seeds of each treatment. The M_1 to M_5 – generations were raised during the winter season, 2003-04 to 2007-08, under irrigated late sown conditions, adopting the recommended package and practices for Bihar, India. The M_1 to M_4 – generations were raised with the treated and untreated control (parent) materials, having 3 m row length with inter and intra-row distances of 18 and 10 cm, respectively. Single seed was sown per hill. In the M_1 generation, 100 plants from each treatment were randomly selected to raise the M_2 generation. The M_2 to M_4 generations were raised with the seeds of plants, selected in the previous generations, by adopting plant to row method. A yield trial was constituted during the M_5 generation with seeds of homozygous materials in the M_4 generation, having a plot size of 3.0 × 0.72 m (4 rows, 18 cm apart) with 3 replications under RBD to evaluate grain yield and other attributes. Twenty four mutant lines along with the untreated control (NW 1014) and 3 other commercial checks (PWB 373, DBW 14 and NW 2036) were evaluated under the trial. Ten plants in each plot were randomly selected to record observations on plant height (cm), spike length (cm), spikelets per spike, grains per spike and 1000-grain weight (g) and the remaining traits were observed on a ploy by plot basis. Statistical analyses were performed by using standard methods.

Results and discussion

M_1 – generation

Observations on the seed germination in the M_1 – generation revealed the different effects for the various treatments, ranging from inhibitory (CMC) to the stimulatory (TSP) effects (Table 1). No mutant was observed in the M_1 generation. These findings may hint at non-mutagenic effects of CMC and TSP as earlier workers (Caldecott et al., 1954; Sinha et al., 1997) have also observed similar effects. The inhibitory effect may be explained due to an inactivation of auxin (Skoog, 1935).

Table 1. Effect of treatment on seed germination (%) in M_1 generation and percentage of families segregating for morphological changes in M_2 generation

Treatment* / Control	M_1		M_2	
	No. of seed treated	Germination (%)	No. of families screened	Segregating families (%)
NW 1014 (c)	350	85	100	--
AS	350	82	100	--
SC	350	87	100	13
TSP	350	91	100	13
STPP	350	86	100	7
CMC	350	62	100	13
SS	350	86	100	10
GM		82.7		
CD (5%)		3.6		
CV (%)		2.5		

*AS – Acid slurry, SC – Sodium carbonate, TSP – Tri-sodium phosphate, STPP – Sodium tri-poly phosphate, CMC – Carboxy methyl cellulose, SS – Sodium sulphate

M_2 and M_3 generations

The number of families, showing at least one morphologically detectable mutant, for the different treatments is given in Table 1. Segregating families were observed under all the treatments, except AS. Three treatments, namely, SC, TSP and CMC, had the highest score for the segregating families (13.0 per cent), followed by SS (10.0 per cent) and STPP (7.0 per cent). These findings indicate that all the treatments, except AS, had ability to induce mutational modifications in the material under the studies. This ability for 3 treatments, viz., SC, TSP and CMC, was the strongest whereasfor SS this was medium.

The highest score for mutation frequency (1.15 per cent) was recorded for TSP and CMC, followed by SS (1.00 per cent), SC (0.85 per cent), STPP (0.65 per cent) and AS (0.0 per cent) (Table 2), inferring that TSP and CMCwere the strong mutagens; while, SS and SC were medium type and STPP was the weak type of mutagen and AS had no mutagenic ability.

Mutation spectrum (Table 2) indicates that only 4 types of mutants -dwarf, high tillering, late heading and white spiked mutants were isolated in the M_2 generation. Mutants for late heading recorded the highest number of events (47), followed by dwarf plant (36), white spike (7) and high tillering (6). These findings indicate that the 4 traits were highly sensitive to the mutagenic effects of the treatments. The number of mutants was highly influenced by the nature of the treatment and the trait. There are several similar reports (Swaminathan et al., 1962; Kalia et al., 2001), showing high frequency and wide spectrum of mutations in the M_2 generation, induced by various other mutagens. Appearanceof small numbers of mutants in the M_2 generation and the homozygosity of families in the M_3 generation suggested that these mutants were governed by recessive genes.

Table 2. Spectrum and frequency of morphological mutations induced by different treatment in M_2 generation

Treatment*	Total plants	Total morphological mutant	Mutation frequency (%)	Mutation spectrum (No. of events)			
				Dwarf plant	High tillered	Late heading	White spiked
NW 1014 (c)	2000	--	--	--	--	--	--
AS	2000	--	--	--	--	--	--
SC	2000	17	0.85	10	--	7	--
TSP	2000	23	1.15	--	3	13	7
STPP	2000	13	0.65	3	3	7	--
CMC	2000	23	1.15	10	--	13	--
SS	2000	20	1.00	13	--	7	--
Total				36	6	47	7

*AS – Acid slurry, SC – Sodium carbonate, TSP – Tri-sodium phosphate, STPP – Sodium tri-poly phosphate, CMC – Carboxy methyl cellulose, SS – Sodium sulphate

Yield trial (M_5)

A yield trial was constituted with 24 mutant lines, homozygous in the M_3 and M_4 generations, and the untreated parent (NW 1014) and 3 other commercial checks, namely, PBW 373, DBW 14 and NW 2036, to evaluate grain yield and other associated attributes in the M_5 generation. The analysis of variance shows that the experimental materials were significantly different among themselves for all the traits, except spikes per metre row. This was also substantiated by wide ranges of variation for majority of the traits (Table 3).

Table 3. Range, mean (X) and M.S. (mean square) for 9 traits of mutants in yield trial (M_5)

Trait	Range	X	S.Em.	M.S.*
Grain yield (kg/ha)	1667.00-3889.00	2508.2	± 68.67	S
Days to heading (75%)	63.00-67.67	65.8	± 0.22	S
Plant height (cm)	76.87-97.00	86.8	± 1.27	S
Leaf blight	4.67-44.67	23.5	± 7.29	S
Spikes/m row	52.00-90.00	67.4	± 7.99	NS
Spike length (cm)	7.33-9.30	8.5	± 0.15	S
Spikelets/spike	16.93-19.40	18.4	± 0.46	S
Grains/spike	31.73-41.73	36.8	± 1.71	S
1000-grain weight (g)	32.40-38.43	36.0	± 0.79	S

*S = Significant at 5% level, NS = Non-significant

Table 4 displays superior mutants of the yield trial for the various traits. On the basis of the number of significantly out yielding mutants over the highest yielding check PBW 373 (2222 kg/ha), the mutagenic efficiency of SC may be placed at the top rank, followed by SS, TSP, STPP and CMC; while, on the basis of SOP (superiority of the average yield of superior mutants over the parent variety), the treatments may be arranged in an order of SS>SC>CMC>TSP>STPP for mutagenic efficiency. The ranking of the mutagenic efficiency of the treatments varied, depending on the nature of the treatment and the character. Mutants, significantly out yielding the highest yielding control PBW 373 (2222 kg/ha; Table 4), are under further testing identification as high yielding varieties. The remaining mutants may be used under the various breeding programme to improve the different attributes of wheat varieties.

Table 4. Superior mutants in yield trial (M_5)

Treatment [x]	Trait	No. of mutants	Mutant
1	2	3	4
SC	Grain yield (kg/ha)	9	M(CCSD)(2003-04)3/2-1-1, 3/2-2-2*, 3/2-2-3*, 3/4 -1-1*, 3/4-1-2*, 3/4-1-3, 3/8-1-1, 3/8-1-2*, 3/12-1-2*(Range: 1944-3333, Av.: 2654.8, Parent: 1747,SOP: 52.0%)
	Plant height (cm)	7	M(CCSD)(2003-04)3/2-2-3, 3/4-1-1, 3/4-1-2, 3/4-1-3, 3/8-1-1, 3/8-1-2, 3/12-1-2 (Range: 83.9-97.0, Av.: 88.7, Parent: 80.2,SOP: 10.6%)
	Leaf blight	5	M(CCSD)(2003-04)3/2-2-3, 3/4-1-1, 3/4-1-2, 3/8-1-2, 3/12-1-2 (Range: 05.33-18.0, Av.: 13.3, Parent: 38.3, SOP: -65.3%)
	1000-grain weight (g)	9	M(CCSD)(2003-04)3/2-2-1, 3/2-2-2, 3/2-2-3, 3/4-1-1, 3/4-1-2, 3/4-1-3, 3/8-1-1, 3/8-1-2, 3/12-1-2 (Range: 35.0-38.2, Av.: 36.6, Parent: 32.4,SOP: 13.0%)
	Spikes/m row	1	M(CCSD)(2003-04)3/12-1-2 (86.7, Parent: 59.0, SOP: 46.9%)
TSP	Grain yield (kg/ha)	5	M(CCSD)(2003-04) 4/11-1-1*, 4/16-1-1, 4/19-1-1, 4/19-1-2*, 4/19-1-3*, (Range: 1952-2778, Av.: 2434.8, Parent: 1744.0, SOP: 39.6%)
	Plant height (cm)	5	M(CCSD)(2003-04) 4/11-1-1, 4/16-1-1, 4/19-1-1, 4/19-1-2, 4/19-1-3, (Range: 88.9-92.3, Av.: 90.6, Parent: 80.2, SOP: 13.0%)
	Leaf blight	2	M(CCSD)(2003-04)4/16-1-1, 4/19-1-3 (Range: 05.7-10.7, Av.: 8.2, Parent: 38.3, SOP: -78.6%)
	1000-grain weight (g)	3	M(CCSD)(2003-04)4/11-1-1, 4/16-1-1, 4/19-1-3 (Range: 35.1-38.3, Av.:36.4, Parent: 32.4, SOP: 12.3%)
	Spikes/m row	1	M(CCSD)(2003-04)4/19-1-2 (90.0, Parent: 59.0, SOP: 52.5%)
STPP	Grain yield (kg/ha)	2	M(CCSD)(2003-04) 5/1-1-1*, 5/1-2-2, (Range: 2222.0-2500.0, Av.: 2361.0, Parent: 1747.0, SOP: 35.1%)
	Plant height (cm)	2	M(CCSD)(2003-04) 5/1-1-1, 5/1-2-2, (Range: 87.0-88.3, Av.: 87.7, Parent: 80.2, SOP: 9.4%)
	Leaf blight	2	M(CCSD)(2003-04) 5/1-1-1, 5/1-2-2, (Range: 04.7-17.0, Av.: 10.8, Parent: 38.3, SOP: -71.8%)
	1000-grain weight (g)	2	M(CCSD)(2003-04) 5/1-1-1, 5/1-2-2, (Range: 34.7-36.0, Av.: 35.4, Parent: 32.4, SOP: 9.3%)
CMC	Grain yield (kg/ha)	1	M(CCSD)(2003-04) 6/5-1-2*, (2500.0, Parent: 1747.0, SOP: 43.1%)
1	2	3	4
	Plant height (cm)	1	M(CCSD)(2003-04) 6/5-1-2, (90.4, Parent: 80.2, SOP: 12.7%)
SS	Grain yield (kg/ha)	7	M(CCSD)(2003-04) 7/14-1-1*, 7/14-1-2*, 7/14-1-3, 7/14-2-3, 7/20-1-1*, 7/20-1-2*,7/20-1-3*, (Range: 2222.0-3889.0, Av.: 2687.3, Parent: 1747.0, SOP: 53.8%)
	Plant height (cm)	4	M(CCSD)(2003-04) 7/14-1-1, 7/14-1-2, 7/14-1-3, 7/20-1-2, (Range: 87.1-89.9, Av. 88.9, Parent: 80.2, SOP: 10.8%)
	Leaf blight	2	M(CCSD)(2003-04) 7/14-1-2, 7/14-1-3, (Range: 16.0-17.0, Av. 16.5, Parent: 38.3, SOP: -56.9%)

Treatment [x]	Trait	No. of mutants	Mutant
	1000-grain weight (g)	7	M(CCSD)(2003-04) 7/14-1-1, 7/14-1-2, 7/14-1-3, 7/14-2-3, 7/20-1-1, 7/20-1-2,7/20-1-3, (Range: 35.2-38.4, Av. 36.5, Parent: 32.4, SOP: 12.7%)
	Spikes/m row	1	M(CCSD)(2003-04) 7/14-1-1, (82.0, Parent: 59.0. SOP: 39.0%).

*Significantly superior over the best control PBW 373 (2222 kg/ha). SOP: Superiority over parent / untreated control (NW 1014).

[x]SC – Sodium carbonate, TSP – Tri-sodium phosphate, STPP – Sodium tri-poly phosphate, CMC – Carboxy methyl cellulose, SS – Sodium sulphate

Mutants with some special features

Mutant with large flag leaf (Fig. 1)

One mutant M (CCSD) (2003-04) 3/4-1-2 recorded an average flag leaf length of 20.9 cm and width of 1.7 cm (control NW 1014: length 15.9 cm and width 1.4 cm). This mutant was isolated in the M_3 – population of SC treatment.

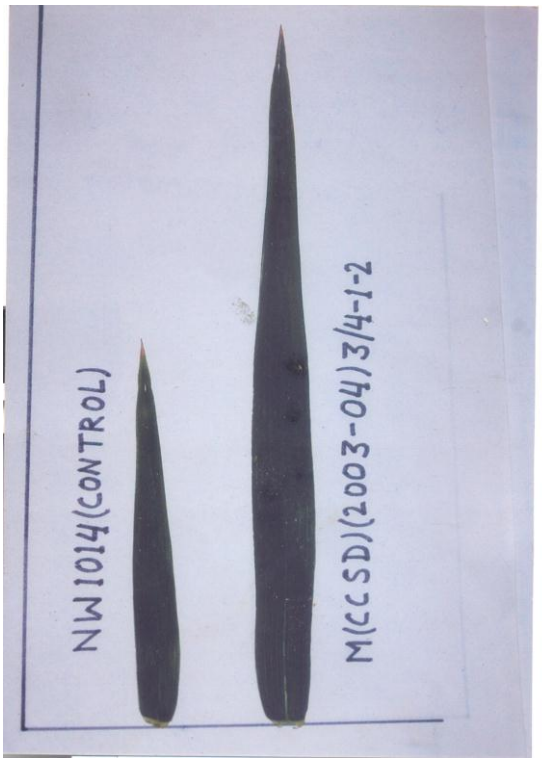

Fig. 1. Mutant with large flag leaf and parent (NW 1014).

Club shape spiked mutant (Fig. 2)

One mutant M (CCSD) (2003-04) 3/4-1-1, with a club shaped spike, was obtained after treatment SC in the M_3 – generation. The spike of the control NW 1014 was tapering type.

Fig. 2. Club shaped mutant and parent (NW 1014).

Mutant with small grain (Fig. 3)

One mutant M (CCSD) (2003-04)3/2-2-3, having grain length 6.2 mm, grain width 3.6 mm and length/width ratio 1.7, was induced by the SC in the M_3 population. Control NW 1014 had 6.8 mm grain length, 3.3 mm grain width and length/width ratio 2.1.

All these mutants seem to be governed by polygenic inheritance as they were isolated in the M_3 generation.

On the basis of over-all findings, it may be concluded that all the chemical agents under the studies, except acid slurry, had ability to induce mutants in wheat.

Fig. 3. Small grained mutant and parent (NW 1014).

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Review

Genetic Improvement of Crops by Mutation Techniques in Brazil

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Abstract

Agriculture in Brazil has a prominent position in the country´s economy due to its large spread under diverse but suitable climatic conditions which are indispensable for higher performance of many crops. Therefore, constant efforts in plant breeding by traditional methods have led Brazil to be internationally recognized as a tropical agricultural country. Spontaneous mutations constitute the source of genetic variability and have been long utilized by breeders. In Brazil, various cultivars of seed propagated, as well as vegetatively propagated plants, were obtained from spontaneous mutation, such as cultivar 'Carioca' (*Phaseolus vulgaris* L.) and cultivar 'Bahianinha' *(Citrus sinensis* L.). After introduction of mutation induction in Brazil, several breeding programmes using mutagens, such as nuclear radiation, alkylating and non-alkylating chemical compounds, have been carried out to increase crop production. As will be reported, various mutants were selected or are in the process of selection.

Introduction

In Brazil, the use of mutation induction of crop plants was introduced in 1960 by Dr. Akihiko Ando at the School of Agriculture ('Escola Superior de Agricultura 'Luiz de Queiroz' - ESALQ) of the 'Universidade de São Paulo (USP) in Piracicaba, state of São Paulo. Later, the 'Centro de Energia Nuclear na Agricultura' (CENA) was established in the campus of ESALQ, and all activities related to mutation induction were transferred and carried out at CENA. The International Agency of Atomic Energy (IAEA) played an important role in the establishment and development of CENA by supplying equipment, training personnel abroad and technical assistance by invited experts through various projects. The Laboratory of Plant Breeding in CENA, after the joining of Dr. A.Tulmann Neto as a staff member in 1972 has carried out various successful research projects to date, in collaboration with ESALQ and other Brazilian institutions, which will be presented in this review.

Two ^{60}Co sources have been installed at CENA to be utilized for mutation induction. In a second moment, chemical mutagens, such as MMS (methyl methane sulphonate), EMS (ethyl methane sulphonate), EI (ethylene imine), DES (diethyl sulphate) and SA (sodium azide) were also adopted for the same purpose. The results obtained from these studies have been considered in practical mutagen treatments in crop plants. Seeds of tobacco, rice, corn, beans, wheat and soybean were treated with gamma radiation, and in some cases with chemical mutagens, for mutation induction *in vivo*. Later, vegetatively propagated plants such as black-pepper, citrus, fig, apple and ornamental plants were included in mutation breeding programmes. After introduction of *in vitro* techniques, associated with mutation induction, banana, pineapple tobacco, black-pepper and some ornamental species have been studied. Moreover, recent projects have been supported by molecular biology. In these projects, amplification of genetic variability, caused by mutagen treatment both *in vivo* and *in vitro*, enables selection of mutants with various improved agronomic characteristics *in vivo* and *in vitro* which are used and will be used commercially and/or in basic study as well.

Tobacco (*Nicotiana tabacum*)

The first research programme in the laboratory of Plant Breeding at CENA was to study the mutagenic effect of gamma rays on aged seeds of plants. Dry seeds of *Nicotiana tabacum*, stored during 2, 3 and 5 years in desiccators under laboratory conditions, were gamma-irradiated with various doses from ^{60}Co source. Gamma ray sensitivity in the M_1 and polygenic inheritance in corolla length estimated by genetic variance in the M_2 and M_3 were evaluated. Results showed that sensitivity in the M_1 and broad sense heritability in the M_2 and M_3 tended to increase with gamma irradiation dose and storing age, suggesting that selection work of polygenic character of plants may be more efficient in aged seeds (Ando and

Vencovsky, 1967). Later, in collaboration with a private company, tobacco was used as a model system to investigate breaking sexual incompatibility between distant species by treating pollen or ovules with mutagens. Latado et al., (1998) obtained hybrids between *Nicotiana tabacum* and *N. repanda*, two sexually incompatible species, only when crosses were conducted with *N. repanda* ovaries and ovules irradiated with 10 and 15 Gy of gamma rays before pollination with *N. tabacum.* Besides, immature embryos were rescued and cultivated *in vitro*. Among the various hybrids obtained, one was fertile because of chromosome duplication, which allowed backcrossing to both parental species and selfing (Araujo et al., 2003). Lima et al., (2002) also obtained hybrid plants between *Nicotiana tabacum* and *N. repanda* using a different approach, asymmetric hybridization by protoplast fusion. After protoplast isolation from leaves of both species, cells isolated from one species were irradiated with 5 or 10 Gy of gamma rays before chemical fusion of protoplasts using polyethyleneglycol (PEG). Approximately 550 plants were obtained from the various experiments, and among those, two plants (#57 and 154) from the treatment *N. tabacum* X *N. repanda* irradiated with 10 Gy were confirmed as hybrids by molecular markers (AFLP) and flow cytometry. These two hybrids allowed the introgression of a source of resistance to the nematode *Meloidogyne javanica* in the breeding programme of tobacco, as *N. repanda* is immune to this pathogen (Stavely et al., 1973). A method was also established to irradiate leaf disks with gamma rays (24 Gy) followed by *in vitro* culture and selection of plants regenerated in MS media containing 1.7% NaCl. Selected plants were multiplied to be evaluated under high salinity field conditions (Correa et al., 2009).

Tobacco vein banding mosaic virus (TVBMV) disease is caused by a distinct *Potato Virus Y* (PVY) virus and has been reported to in many tobacco growing areas around world, in some cases with losses greater than 30% in yield, and serious damage to the tobacco quality. Globally, 3 different strains of PVY, the PVY^{o}, PVY^{n} and PVY^{c} are known. Variants within these groups like PVY^{ntn}, the very aggressive variant of PVY^{n} have been reported in the south of Brazil affecting tobacco and potato crops. Resistant varieties to this disease has been developed using a mutant line 'Virgin A Mutant' obtained through irradiation with X Rays (Koelle, 1961) as the main resistance source, which shows resistance to PVY^{n} but is susceptible to any other variant from this group. The aim of this project therefore was to obtain resistant lines to the PVY^{ntn} variant, since there are no known sources (Pulcinelli et al., 2009). Seeds from the variety K 326 were irradiated with gamma rays in an irradiator type gammacell, using the doses of 125 Gy, and immediately sown to obtain the M_1 generation. A population as large as 40 thousands plants was grown in the field in October 2007 and the plants were self pollinated to produce seeds for the M_2 generation, which after harvesting in bulk were sown in polystyrene trays with 200 cells. A population of 60 thousands plants, at 3 to 4 leaf stage (approximately 40 days after germination) was inoculated with the variant PVY^{ntn}. After the incubation period an evaluation was done and 421 seedlings were selected without symptoms of the disease. These seedlings were inoculated again and 23 seedlings, which did not show symptoms after the second inoculation, were selected. These plants were transplanted into pots and re-inoculated from which one mutant plant without any symptom of the disease was identified. This plant was self pollinated to obtain the M3 lines and a crossed with the original variety K 326 to produce hybrid seeds. On the next step backcrosses will be done to the mutant line and to the original variety. All generation will be either inoculated with the pathogen to confirm the resistance to the disease, as well as to study the inheritance of this trait, and to establish the relation between the virus (PVY^{ntn}) and the plant as tolerant or immune.

Rice (*Oryza sativa*)

Experiments on mutation induction in rice were started in 1964. The first experiment was aimed to increase mutation frequency in rice seeds irradiated with gamma rays. It is well known that some reducing compounds, such as SH-compounds, scavenge free radicals formed in biological materials after irradiation. If free radicals are eliminated immediately after irradiation, higher survival rate can be expected, resulting in increase of mutation frequency and also in amplification of mutation spectrum. For this purpose, rice seeds pre-treated with cysteine (1 mM) for various hours at room temperature were irradiated with several doses of gamma rays. Germination, seedling height and survival rate of the M_1, chlorophyll mutation frequency and spectrum in the M_2 seedlings were measured and compared to the control. To obtain higher mutation frequency, assessed through chlorophyll mutation, pre-treated seeds with cysteine were irradiated with gamma rays, and then treated with various concentrations of chemical alkylating compounds, such as EI, EMS and DS. Later, sodium azide (SA) was also used. In some combining treatments of cysteine, gamma irradiation and chemical alkylating compounds, significant increase of chlorophyll mutation frequency and changes of mutation spectrum were observed. Mutagenic effect of SA was widely studied. Dry rice seeds were treated with various concentrations of sodium azide (pH=7). Some treatments showed more chlorophyll mutation frequency, never observed for any other mutagen treatments, indicating that sodium azide, a non-alkylating chemical, can be one of the most efficient and effective mutagens used (Ando1970). An international comparative experiment

was carried out in 1968, with the selected 15 mutant lines of upland rice sent from IRRI (International Rice Research Institute), and with a local variety used as control (Ando et al., 1971). The results showed that some mutants' lines were superior to the best local variety for some agronomic characters. Mutation breeding programmes in rice were put in practise by various Brazilian institutions through collaboration with CENA. In one of them, a mutant variety (SCS114 Andosan) was selected and released in 2005 from an Experimental Station in Itajai, Santa Catarina state, Brazil. This mutant variety is characterized by reduced grain length, higher milling yield and higher grain yield and more suitable for parboiling, compared with the traditional local varieties, such as 'IR841' and 'EPAGRI 108' (Ishiy et al., 2006).

Maize (*Zea mays*)

An experiment on the effect of gamma-irradiation on heterosis in maize was carried out in 1972. Seeds of two inbred lines of dent and flint maize were irradiated with 3700 R of gamma ray. Hybrid seeds obtained from various combinations of crossing between them were sown and several characteristics were measured. The results show that gamma-irradiation modified the combining ability of inbred lines, suggesting that low dose of gamma rays may change heterosis between inbred lines of corn (Tulmann Neto, and Ando, 1980).

Wheat *(Triticum aestivum)*

A pioneer work on mutation induction in wheat breeding in Brazil was conducted in 1960 (Gomes, 1972), with seeds from the cultivar 'IAS' irradiated with gamma rays at the irradiator of the 'Instituto de Pesquisas Energéticas e Nucleares' (IPEN) from the 'Universidade de São Paulo'. From this work, lines resistant to stem rust were obtained, which were incorporated in the breeding programme, giving origin to the cultivar 'BR4' (IAEA, 1985; Table 1). After the establishment of the irradiator at CENA in 1968, seeds from various cultivars were irradiated with gamma rays, resulting in mutants with many characteristics, such as solid stem, earliness, and spikes without sterility at basal spikelet (Osorio 1972). In 1974, a collaboration between CENA and the wheat breeding programme of the 'Instituto Agronômico de Campinas – IAC' to develop mutants using the gamma rays and chemical mutagenic was established. In general, mutation induction was used to correct defects from superior cultivars from the state of São Paulo. In some cases, one spike from plants of the M_1 generation was collected and mutants were selected in the M_2 generation, but often the bulk method was adopted. The first results included the selection of shorter and earlier mutants obtained from cultivar 'IAC-5', and mutants resistant to stem rust from cultivar 'BH-1146' (Tulmann Neto et al., 1977b). Field experiments demonstrated that, despite the shorter and earlier plants, a reduction in yield was observed for the mutants (Veiga et al.,1982a), but with the mutant TICE-NA-4, derived from 'BH1146', the resistance to stem rust was maintained at many sites and yield did not decrease (Veiga et al., 1982b). These encouraging results stimulated the development of new wheat mutants, and by gamma ray treatment of seeds, with the development of various mutants for traits such as reduction in plant height (Tulmann Neto et al., 1995a, 1995b), resistance to leaf rust (Tulmann Neto et al., 1996a), and tolerance to Aluminun toxicity (Camargo et al., 1995a; Tulmann et al., 2001), which appeared to be controlled by a dominant gene (Camargo et al., 2000). In another project, seeds from a F_4 generation obtained from a cross between common wheat (6X) and Durum wheat (4X) were irradiated and selected at F_5M_2, and lines with high yielding ability were obtained (Camargo et al., 1995b, 1995c). Despite the high genetic variability obtained for various important agronomic traits (Table 2), and the high yielding potential for certain cases, none of these mutants have been released for cultivation in Brazil. One possible reason for this derives from the fact that the wheat breeding programme from IAC is highly dynamic, incorporating hundreds of new crosses each year, generating new cultivars to substitute previous material cultivated by growers. As the mutation induction programme aimed to correct small defects in current cultivars, the mutants might have not been able to compete with the new cultivars generated.

Common beans *(Phaseolus vulgaris* L.)

Common bean is a staple food in Brazil, representing an important source of protein in the Brazilian diet. The crop is cultivated under various environmental conditions, and besides the various biotic and abiotic stresses limiting production, there are many regional consumer preferences, that demand a constant effort from breeders to release new cultivars. In Brazil, mutation induction of common bean had a great emphasis with the establishment of the CENA in Piracicaba, in 1966, whose major project ('Projeto Feijão'), involved many sections of the organization, funded by the United Nation Development Programme (UNDP) and managed by the International` Atomic Energy Agency - IAEA (Project BRA/71/556).

Mutation induction involved many objectives. The main mutagenic agents employed were gamma rays and EMS, and in general, various doses and concentrations were used after preliminary experiments to determine sensitivity, and the number of seeds treated varied according with the objective and harvest method (Tulmann Neto et al., 1988; Tulmann Neto, 1990). By treating common bean seeds from a cultivar with indeterminate growth habit with 32 krad of gamma rays, a seven-day earlier and determined growth mutant was selected, which was distributed to growers in the northern Parana state under the

name of 'CAP-1070' (Tulmann Neto et al., 1989). Later, because of the earlier production and determinate growth habit, this mutant was introduced into the breeding programme of the 'FT-Pesquisa e Sementes', a private company, which developed the original cultivar 'FT-Paulistinha' (CIAT, 2000), cultivated in Parana state. Other mutants presenting important agronomic characteristics, such as increased protein content but with a reduction in yield (Crocomo et al., 1978, 1979) were obtained from the same mutation induction programme. Some of these mutants were used for basic investigations because of certain biochemical characteristics (Carvalho et al., 1989). Other mutants identified included those with higher basal height obtained with gamma irradiation (Tulmann Neto et al., 1994), or those with altered seed color from a black-seeded bean, while maintaining good agronomical traits from the original cultivars (Tulmann Neto et al., 1988).

Table 1. Mutant cultivars bred in Brazil

Crops	Mutant cultivars	Year of release	References
Rice	Andosan	2005	Ishiy et al.,2006
Beans	IAPAR 57	1992	Ciat, 2000
	IAPAR 65	1993	Ciat,2000
	CAP 1070	1987	Tulmann Neto et al., 1989
	FT-Paulistinha	1989	Ciat, 2000
	Campeiro	2003	Embrapa,2003
Chrysanthemum	Cristiane, Ingrid	1996	Tulmann Neto & Latado, 1996
	Magali	1996	Adames *et al.*, 1999
Wheat	BR4	1979	IAEA,1985

One of the most important objectives of this programme was to obtain cultivars resistant or tolerant to bean golden mosaic virus (BGMV), as no source of resistance was available at that time, and large losses were imposed by this pathogen to bean growers. Thousands of seeds were treated with gamma rays or Ethyl Methane Sulfonate, with mutant selection in an insect resistant screenhouse or field conditions. A mutant with tolerance to Common Bean Golden-Mosaic Virus obtained from EMS treatment was selected and denominated TMD-1 (Tulmann Neto et al., 1977a). Field tests with the TMD-1 mutant demonstrated that despite the good tolerance to the virus, yield was very low in comparison to the original cultivar 'Carioca'. In collaboration with the 'Instituto Agronômico do Paraná (IAPAR)', TMD-1 was included in the breeding programme, and gave origin to two new cultivars resistant to the Golden-Mosaic Virus: 'IAPAR 57' (Tulmann Neto et al., 1993) and 'IAPAR 65'. Centro Internacional de Agricultura Tropical, CIATCali, Colombia) in a publication about the impact of common bean cultivars obtained in Latin America between 1930 a 1999, reported that 'IAPAR 57' was one of the most successful cultivars from IAPAR, with a large acceptance (CIAT, 2000). CENA collaborated through irradiation services and development of methodologies for common bean mutation induction for other breeding programmes, including the Brazilian Agriculture Research System EMBRAPA, who developed a new cultivar denominated 'Campeiro', characterized by high yield and good plant architecture (EMBRAPA, 2003). In conclusion, using either gamma-irradiation or EMS for mutation induction, it was possible to obtain mutants for various important agronomic traits (Table 2), with many of the mutants being directly multiplied and adopted in culture, while others were included in breeding programmes, originating cultivars with great acceptance by growers (Table 1) in Brazil.

Soybean (*Glycine max*)

The initial steps and justifications for mutation breeding of soybean were presented in detail by Menten et al., (1984). For soybean mutation, gamma rays and EMS were used to treat a large number of seeds (from 3500 to 33 000) from four cultivars to obtain resistance to rust, virus (Brazilian bud blight) or earliness (Tulmann Neto et al., 1988). Following treatment of seeds from cultivar 'IAC-8' treated with EMS (0.05%, 8 h with 5 h of pre-imbibition in water), mutants that were 5 to 10 earlier in maturity than the controls were identified, with four mutants with similar yielding ability compared to the control (Tulmann Neto et al., 1995c). For cultivar 'Paraná', in the M_3 generation obtained from a 23 krad gamma rays treatment, eight mutants were selected with earlier maturation than the control, with two mutants presenting a 10-

day maturity earliness without affecting other agronomic traits, such as yield, plant height and growth architecture, flower color, pubescence and type of seeds (Tulmann Neto et al., 1997).

More recently, in cooperation with TMG, a private soybean breeding company located in the state of Mato Grosso, mutation induction has been used to obtain earlier maturity from the drought resistant cultivar 'Saara', which has a long cycle. Large amounts of seeds were irradiated with various doses of gamma rays, and the M_1 generation was harvested in bulk. The large amount of M_2 seeds were also used to select for herbicide and Asian rust resistances. Early maturing plants were identified and further tested.

Table 2. Some agronomic characteristics in crops, obtained by mutation induction in Brazil

Crops	Characteristics
Tobacco	Resistance to virus; break down of barrier of incompatibility in interespecific crossing between *Nicotiana repanda* and *Nicotiana tabacum*
Citrus	Compact type; seedless; alteration in maturity;
Common beans	Higher protein content; seed coat color; larger basal length; earliness; growth habit; tolerance to Bean g*olden mosaic virus*
Banana	Reduced plant height, salt resistance
Chrysanthemum	Flower color; reduced plant height; increased petal number
Wheat	Resistance to stem rust (*Puccinia graminis* f.sp. *tritici*); resistance to leaf rust (*Puccinia recondite* f.sp. *tritici);* soft culm; earliness; spikes without sterile basal spikelet; reduced plant height; tolerance to aluminium toxicity
Rice	Reduced ripening time; earliness; reduced plant height; improved grain quality for parboiling
Soybean	Reduced ripening time
Pineapple	Narrow leaf; broad leaf; variegation; large thorn; small thorn; reduction of thorn number; absence of thorn
Tomato	Ovate (elongated fruits); jointless (absence of abscission zone in fruit pedicels); dark green fruit (fruits with increased chloroplast number); never-ripening (fruits unable to develop full color and softness); reduced plant size; shortened pedicels and small fruits. higher and lower Brix value (total soluble solids in fruit)
Black-pepper	Tolerance to *Fusarium solani* f.sp.*piperis*
Apple	Fruit color
Fig	Longer peduncle; closer and smaller ostíole

Black-pepper (*Piper nigrum* L.)

The project for obtaining resistance or tolerance to *Fusarium solani* f.sp.*piperis*, in black-pepper (*Piper nigrum* L.), cultivar 'Cingapura' through mutation induction was planned and started in 1978, in collaboration with EMBRAPA (Empresa Brasileira de Pesquisa Agropecuária) in Belem and INATAM (Instituto Experimental Agricola Tropical da Amazonia) in Tome-Açu, both at Pará State About 500 budsticks were irradiated with 2 and 3 krad of gamma rays. After irradiation, the sticks were planted in a recently reclaimed *Fusarium*-free experimental field in Tome-Açu. All M_1V_1 young shoots were rooted separately in bucket with fertile soil, inoculated with high concentration of *Fusarium*. All surviving young plants were then transplanted in a *Fusarium*-occurring experimental field. After almost two years of growth, three M_1V_2 plants survived. Various cuttings with M_1V_3 buds from them were rooted and grown in another *Fusarium*-occurring experimental field for the preliminary evaluation of agronomic characters. The M_1V_4 population consisted of cuttings obtained in bulk from the surviving M_1V_3. Both M_1V_3 and M_1V_4 populations were grown in *Fusarium*-occurring fields of local black-pepper producers, and resistance or tolerance to the disease and seed productivity were continuously evaluated and selected. To date, the M_1V_4 population has shown significant tolerance to *Fusarium* disease (Ando et al., 1984, 1996).

Another mutation breeding project of black-pepper with the same objective was started to establish a useful meth-

odology of gamma-irradiation of buds in association with *in vitro* techniques (Filgueiras et al., 2010). For this methodology, the following procedure has been established: (1) a dose of 20 Gy of gamma rays is applied to buds of young plants propagated *in vitro*; (2) Advancement of generation is carried out through at least three subcultures to eliminate chimerism; (3*) in vitro* selection is carried out with culture filtrate of *Fusarium solani* f.sp. *piperis* after 28 days of inoculation on the Czapek-Dox medium; (4) micropropagation of surviving and *in vivo* selection are conducted by inoculation with 2×10^5 spores/ml of the fungus on the plants; (5) Finally, agronomic characters of the selected plants are evaluated in a *Fusarium*-occurring field of black-pepper growers.

Banana (*Musa* spp.)

Banana is an important crop in Brazil, where various problems associated with biotic and abiotic stresses and restrictions in the use of crossing schemes due to sterility for breeding, the use of mutation induction is attractive, especially because of the straightforward *in vitro* culture methods available for this species. In Brazil, the first work with banana mutation induction was irradiation of shoot apex with gamma rays (Domingues et al., 1994). When the phenotypic variation for many characteristics of the M_1V_4 generation was compared with the control, a rate of 0.85% of somaclonal variants were detected in non-treated plants, while for the various doses of radiation, the frequency of variability ranged from 2 to 13.9%. During this work, it was established that it was necessary to advance mutated lines up to the M_1V_4 generation before initiating selection, as this was the stage at which the rate of foliar variegation indicating the mutagenic effects of the applied doses stopped in comparison to the non-treated control. A total of 2765 plantlets were inoculated to screen for resistance against *Fusarium* wilt (Panama disease), but no favorable mutant was identified. At a later stage after irradiation of shoot apices with gamma rays and followed by vegetative generations, an *in vitro* selection step was included to screen for salt stress tolerance and resistance against to *Fusarium* wilt. Various mutants tolerant to up to 1.5% NaCl *in vitro* was identified (Houllou-Kido et al., 2001), from which one mutant line was later confirmed as tolerant under nutrient solution conditions (Houllou-Kido et al., 2002). In terms of *Fusarium* wilt resistance, many mutants were selected *in vitro* using filtrates from *Fusarium oxysporum* f. sp. *cubense* cultures, which were then multiplied and sent to field evaluation, where one mutant line survived under high infestation field conditions, and continue to be evaluated. Shoot apex irradiation with gamma rays followed by generation advance under *in vitro* conditions was also used to develop shorter plants to reduce losses by wind using two commercial cultivars. These mutants are currently under field evaluation (Resende et al., 2006a; 2006b).

Pineapple *(Ananas comosus* L. Merril)

Pineapple is an import fruit crop in Brazil, mainly used to produce fresh or industrial juices or as dessert. Due to the long generation time, mutation induction benefit from the availability of *in vitro* culture to produce and maintain plants, including *in vitro* selection. A mutation breeding programme was conducted to increase the genetic diversity of two pineapple cultivars using gamma ray irradiation of *in vitro* explants (Carvalho et al., 2009; Tulmann Neto et al., 2009). Shoot apexes were aseptically cultivated *in vitro* on basic MS liquid media, followed by irradiation of axillary buds at 45, 50, 60, 70 and 80 Gy. After treatment, four subcultures were conducted every 30 days to obtain generation M_1V_4. After that, ten randomly chosen plants were hardened and transfer to the field at 'Ilha Solteira', São Paulo, Brazil, while another lot was kept under greenhouse conditions at CENA. It was concluded that the 45 Gy doses was insufficient to increase the diversity. For the other doses, variegated mutants were obtained, with changes in leaf morphology (narrower or wider leaves; distinct leaf insertion angle; wrinkled; or at higher number); spines structure (smaller; absent or at lower frequency) and in fruits. The doses of 50, 60, 70 and 80 Gy of gamma rays used to treat pineapple *in vitro* plantlets increased the variability at generation M_1V_4.

Orange (*Citrus sinensis*)

Brazil is the largest world producer and consumer of sweet oranges (*Citrus sinensis* Osbeck), producing 30% of the world fruit production and 59% of manufactured orange juice (Neves & Lopes, 2005). Despite this leadership, the crop in Brazil is under great vulnerability because of the predominance of commercial groves composed by a limited number of cultivars, with low genetic diversity, while occupying large area surface. The combination of these factors increases the probability of disease and pests outbreaks, which can increase production costs and limit cultivation. Orange trees have a long vegetative cycle when propagated from seeds. Furthermore, other biological barriers (polyembriony; incompatibility and sterility) make *Citrus* breeding long and time consuming (Frost & Soost, 1968). Therefore, the majority of canopy cultivars adopted commercially originated from massal selection of seedlings derived from natural crosses or from spontaneous mutants selected from branches (sports) from commercial cultivars (Grosser and Gmitter Jr., 1990). 'Washington Navel' and low acidity sweet oranges 'Lima verde' are good examples of cultivars selected from spontaneous mutations in Brazil (Domingues et al., 1995). Spontaneous mutations are important for the development of new *Citrus* cultivars, but they appear at

very low frequency. On the other hand, induced mutation, using radiation or chemicals, can increase many folds the rate of mutation, increasing the genetic variability to be used for selection (Tulmann Neto et al., 1991). Thus, mutation induction programme in *Citrus* started in 1983, with the objective to obtain mutants from sweet orange 'Pêra', the cultivar with the largest commercial interest in Brazil, for traits of agronomic importance, such as shorter plants, absence or reduced number of seeds, change in the period of fruit maturation season, to increase harvesting season, and disease and pest resistance (Tulmann Neto et al., 1996b). After previous experiments that evaluated radiosensitivity, approximately 2300 orange buds from 'Pêra' were irradiated with 40 Gy of gamma rays at CENA, which were then grafted onto 'Rangpur lime' rootstocks. Using the method of repeated pruning, 7600 M_1V_3 plants were obtained to form the population for mutant selection (Tulmann Neto et al., 1996b). After two cycles of selection, 29 mutant clones were selected, from which 24 were seedless (Latado et al., 2001) and two were more tolerant to *Xanthomonas axonopodis* pv. *citri*, the causal agent of Citrus canker, under field conditions (Latado et al., 2006) and by artificial inoculation (Belasque Jr. et al., 2009), besides mutants with shorter fruit maturation period (Latado et al., 2005). All these mutant clones are in final stage of evaluation before commercial release.

In another study, Latado et al., (2004) demonstrated that the lower number of seeds per fruit in these mutants from 'Pêra' was correlated with a lower viability of pollen grains, possibly resulting from the mutations. More recently, due to the success from previous work with mutation induction in *Citrus*, other irradiated populations were established using *in vivo* or *in vitro* mutagenic treatment based on gamma rays. For mutation induction in 'Rangpur' lime, the main rootstock used in Brazil, the objectives are to obtain shorter plants or tolerant to a Sudden death of Citrus. Two mandarin cultivars ('Thomas' and 'Fremont') resistant to 'Alternaria brown spot' are being mutated to obtain trees bearing seedless fruits. Finally, 2000 irradiated plants of 'Tobias' sweet Orange are currently under field evaluation for mutants resistant to Huanglongbing, a bacteria disease with a high dispersion rate and large destruction potential (Latado, 2010-personal communication)

Apple (*Malus* sp)

In apple, as in other fruit trees, spontaneous somatic mutations, as well as induced mutations resulted in the release of several important cultivars (Donini, 1977, Donini & Micke 1984; Spiegel Roy, 1990; Maluszynski et al., 2000). 'Fuji' is now one of the most important apple cultivar in the entire World. 'Fuji' ranks second in production in Brazil, and it is responsible for more than 35% of the total apple produced. It is a top quality fruit, but cultivation is limited to the coldest areas in Southern Brazil. Even so, regularly it is necessary to make use of dormancy breaking agents for commercial production. 'Fuji Fry' is a natural low chill 'Fuji' mutant found in 1995 (Hauagge and Cummins, 2000), and it grows and fruits naturally well in locations that accumulate at least 450 chill units during winter. Fruit color is similar to 'Fuji', but it is worsened in subtropical warm areas, where it could be grown. As a consequence it has little commercial value as it is. A 'Fuji Frey' irradiation induced mutation breeding programme for color improvement was conducted by 'Clone Nurseries' in Araucária, Parana state, in cooperation with CENA and started in the winter of 1999. Ten centimeters virus-free cuttings of this cultivar were irradiated at CENA with 35, 45 and 55 Gy of gamma rays. These cuttings were then grafted onto 'MM.106' and produced 1172, 1257 and 1411 plants respectively by the winter of 2000. Plants originated from the 35 Gy irradiated cuttings were eliminated after one-year observation, because of the low incidence of physiological/genetic modification of the plants. Six to seven basal grafts collected from plants derived from both 45 and 55 Gy treatments were used to produce 17 000 new plants in the winter of 2000. They were grafted in 'Maruba Kaido' rootstock with 'M.9' as a 40-cm interstock. These trees were planted at density of 10 000 per ha at 'clone nurseries' in December of 2001. The plants were conducted as a regular apple orchard, and they were trained as a central axe. Fruiting and other evaluation started in 2003 and extended till 2006. A total of 250 putative fruit color mutations were initially numbered. During the winter of 2004, 102 of the selections were propagated into a new orchard at Araucária, Paraná, Brazil, replicated between 2 to 20 plants for the various mutation clones, directly grafted onto one-year-old already established rootstocks. In the winter of 2005, a new orchard with six of the best selections, with 20 replications was established at the same location, and conducted in the same manner. Finally, in the winter of 2007, four of the best clones, each mutant clone was planted in a 0.75 ha area for commercial evaluation in Araucaria. The tree density used was 2500 per ha, and grafting was conducted onto one-year old 'M.9/Maruba Kaydo' established rootstocks. One entire third row was grafted with Eva at the same time to assure pollination. The first crop was harvested in 2010, and two mutants appear to be promising as new cultivars, which we expect to define after the 2011 harvest. The potential use of this cultivar is to be grown at higher elevations, where 100 to 550 chill units accumulates. This will provide conditions for early harvest of 'Fuji', probably between January and March. The main crop for Fuji in Brazil starts in April. The selected clones have shown good stability. Even so, some new mutants continue to appear and they will be evaluated. Observed mutations varied widely in relation to leaf

shape and size, growth habit, tree vigor, fruiting habit, fruit shape, fruit size, fruit color and productivity. However, we have attained the evaluations based on productivity and fruit quality.

Fig (*Ficus carica* L.)

Fig has a large economic importance and 'Roxo-de-Valinhos' is the main cultivar in Brazil. The genetic variability of the crop is limited because it is vegetatively propagated since its introduction in the country. Furthermore, it is difficult to obtain plants derived from gametic fusion in Brazil, which hamper traditional breeding. Mutation induction represents an attractive approach to obtain genetic improvement of the cultivar, which present problems, such as susceptibility to nematodes and to fig fly (*Zaprionus indianus* Gupta). Therefore, a fig breeding programme was started in 1998. Initially, a radiossensitivity test was conducted using 30 cm long budsticks, which were submitted to increasing doses of irradiation of gamma rays, from 0 to 75 Gy at 15 Gy intervals, at CENA. Based on the number of developed leaves, the 30 Gy appeared as the ideal doses (Santos et al., 1997). The irradiated budwood from the radiosensitivity test were grown in the field, followed by repetitive pruning up to the M_1V_3 generation, when the mutants were selected for physical characteristics of the fruits. In parallel, cuttings from this mutant generation were used to establish an orchard with 450 plants on a site infested with nematodes (*M. incognita*). Root and soil samples were analyzed by the 'Laboratório de Nematologia' from the 'Agência Paulista de Tecnologia', Bauru, SP and later for identification of the species at the 'Faculdade de Ciências Agrárias e Veterinárias', UNESP, Jaboticabal, SP, which showed the presence of *Meloidogyne incognita* and *Rotylenchulus reniformes* in the area. In terms of fruit morphology, five mutants were selected, with one with longer fruits, another with longer peduncle, another with a closer and smaller ostiole. These five mutant selections were compared with another five commercial cultivars ('Pingo de mel', 'White Genova', 'White Adriatic', 'Palestino' and 'Roxo-de-Valinhos'). In this trial, the selection 'PI 189' with closer and smaller ostiole, was more productive, bearing 30 fruits per plant, in comparison to 22 from 'Roxo-de-Valinhos', and more fresh weight (44 and 38 g), and yield (3.76 × 2.27 kg/ha) (Rodrigues et al., 2009). This mutant did not differ from the original cultivar for other traits, such as fruit length, titratable acidity and soluble solids content. For the other selected mutants, other field trials are being conducted to evaluate yield and organoleptic attributes in comparison to the original cultivar 'Roxo de Valinhos'.

Tomato (*Lycopersicum esculentum)*

Tomato is a good example of a successful use of mutations affecting major genes for plant breeding (Rick, 1986), which are also valuable for gene discovery (Emmanuel & Levi, 2002) and physiological studies (Campos et al., 2009; Gratão et al., 2009; Lima et al., 2009; Zsögön et al., 2008). However, mutant alleles are only currently known for an insignificant fraction of the about 35 000 genes in the tomato genome. Large scale mutagenesis and introgression of natural genetic variation from the wild *Lycopersicon* species into a genetic model system, such as Micro-Tom (MT), can be useful to fill this gap (Meissner et al., 1997). MT has the advantages of little space requirements and rapid cycle (Meissner et al., 1997). Here, we present the reassessment of the methodology for large scale tomato mutagenesis based on different agents (chemical and physical) and targets (seeds and pollen).

The best parameter for dose adjustment of mutagen was the fertility of M_1 plants, whereas the germination of treated-seed and that of pollen were found to be poor indicators of mutagenic efficiency. Using seeds treated with 0.7% EMS and pollen treated with 80 Gy gamma rays, it was possible to isolate a large number of mutants, including some dominant non-chimeric ones in the M_1 generation derived from mutagenized pollen. Among the new mutants found in greenhouse or field screenings, some appear to be allelic to mutations already known in tomato, such as *ovate* (elongated fruits), *jointless* (absence of abscission zone in fruit pedicels) *high pigment1* or *dark green* (fruits with augmented chloroplasts and their derived pigmentation) and *Never ripe* (fruits unable to develop a full color and softness). We are currently performing allelism tests with these mutants. We also found, among others, putative novel mutants with a much reduced plant size, shortened pedicels and small fruits. Variations in both extremes were found (Pino Nunes et al., 2009) for the agronomically relevant trait brix (total soluble solids in the fruit). The occurrence of mutants with high brix in both M_1 and M_2 indicates that this complex agronomic trait could be also controlled by major genes, some of them dominant.

Besides induced mutations, natural genetic variation is a valuable resource for functional genomics in tomato, especially for the loci where cultivated tomatoes already harbor knock-out (i.e. non-functional) versions of the genes. Thus, natural genetic variations affecting diverse developmental process (e.g. augmented fruits per cluster, reduced time for flowering) and metabolic pathways (altered carotenoid and anthocyanin contents in fruits) were introgressed into MT from wild species. MT was a suitable model for a fast an inexpensive screening and characterization of advantageous mutations and natural genetic variation, which could be further transferred to elite cultivars or hybrids.

Chrysantemum (*Dendranthema grandiflora*)

The first work using mutation induction in ornamental plants in Brazil was conducted for chrysanthemum in 1993, to obtain inflorescence color mutants from cultivar 'Repin', originally bearing pink flowers, by *in vitro* mutation (Latado et al., 1996). 'Repin' was chosen because of its good agronomic characteristics, including resistance to pest and diseases, and the light pink inflorescence. Mutagenesis was targeted to floral pedicels from immature flower buds, followed by *in vitro* culture. The doses used of gamma rays were 8 Gy, established in a preliminary radiossensitivity assay. Around 600 explants were irradiated with the same dose, resulting in 690 regenerated plants, which were then planted in the field. During blossoming, 46 mutants (6.7%) were selected for inflorescence color, all with a single color, indicating that floral pedicles regenerated from one or few cells, produced solid or periclinal mutants.

Tulmann Neto & Latado (1997) also induced mutants from the same chrysanthemum cultivar, but inducing mutants *in vivo* from rooted cuttings. After irradiation with 20 Gy, successive pruning (cutting back method) were conducted, and during blossoming, 7764 plants were evaluated, and 450 (5.8%) mutant plants for inflorescence color and 14 mutants (0.18%) for inflorescence shape were observed, with sectorial or periclinal mutants. The mutants displayed bronze, salmon, dark pink and variegated colors at higher frequencies, but only the dark pink and white were launched commercially in Brazil (Tulmann Neto & Latado, 1996). The dark-pink 'Repin' (named 'Ingrid') still continued to be commercialized in Brazil, 14 years after it's launching (R. Latado, 2010, personnal communication).

The work on mutation induction in chrysanthemum continued using the dark pink 'Ingrid' cultivar as initial plant material. The work started using *in vivo* irradiation of rooted cutting to investigate the effect of pruning at distinct positions at branches of M_1V_1 plants (1^{st} to 6^{th} axillary buds) in the frequency of mericlinal mutants and size of mutated sectors (Adames et al., 1999; Latado et al., 1999). The results indicated that there was no effect of pruning height at M_1V_1 branches. However, mutants with new inflorescence color were obtained, including a brown-colored inflorescence mutant, commercially launched as 'Magali'. It is interesting to point that, as observed by other programmes; irradiation of a mutant can result in a family of interesting commercial mutants (Broertjes et al., 1980).

In a second project, Latado et al., (2004) used the *in vitro* induction method by applying chemical mutagen EMS to immature floral pedicel of the 'Ingrid' mutant. Explants were treated with 75 mM EMS solution for 1 h and 45 min, which allowed obtaining 48 (5.2%) color inflorescence mutants, mainly (89.6%) with uniform color. The color spectrum was similar to the mutants previously obtained with gamma irradiation. These results with chrysanthemum in Brazil indicated that the choice of the inflorescence color of the initial cultivar was important for the success of the breeding programme if the objective is color. Latado & Tulmann Neto (1998) working with a white inflorescence cultivar were able only to obtain yellow mutants.

Other projects with mutation induction of ornamental species have been conducted in Brazil, including *Amaryllis*, *Gladiolus*, *Calathea*, *Stromanthae* and *Aster*, which generated various mutants, but none was commercially launched as a newly developed cultivar

Conclusion

As pointed by various authors, it is evident to observe the great evolution that has occurred with plant breeding, that currently is not limited to simple selection, exploring the variability within populations, but also it concerns in creating, selecting, evaluating and multiplying desirable genotypes. For that matter, to increase the efficiency and shorten the process, the breeder has adopted and combined various tools, such as *in vitro* culture, molecular markers, and the use of mutagenesis to increase the genetic variability. The use of mutagenic itself has benefited from these new techniques, which led to the recent significant great increase in cultivar release derived from this working tool. As demonstrated via many examples in work conducted in Brazil, such efficiency can even be larger when various experts from distinct institutions interact. The many success stories obtained indicated the usefulness for breeding either seed-propagated or vegetatively-propagated plants, but it is necessary to recognize that, in comparison to other countries, in Brazil, the potential of mutation inductions has not been fully exploited.

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Research Article

Use of Gamma Irradiation for Prolonging Shelf Life of Garden Pea (*Pisum sativum* L.)

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Abstract

Garden pea pods of variety 'Arkel' were irradiated with 5 doses of ^{60}Co gamma rays ranging from 0.5-3.0 kGy and stored at ambient temperature up to 9 days along with control to study the effect of radiation in prolonging shelf life of pea pods and stabilizing its market demand. Physiological weight loss (%) decreased as the doses of gamma radiation increased. Minimum weight loss was noted in pods irradiated with 3.0 kGy gamma rays compared to control. Decay loss % showed an inverse relationship with dose of gamma radiation. Minimum decay loss was recorded with 3.0 kGy gamma ray dose. Pea pods irradiated with 1.0 kGy gamma rays recorded maximum sugar content. Pods irradiated with 0.5 kGy and 1.0 kGy gamma rays retained their green colour for a longer period. Based on a 9 point hedonic scale the highest acceptability for appearance, taste and texture was observed in pods treated with the 1.0 kGy dose.

Keywords: Garden pea, irradiation, gamma rays, prolonging shelf life

Introduction

One of the peaceful uses of atomic energy that holds great promise for agriculture in the new millennium is the storage and preservation of food and food products by improving their keeping quality, preventing early ripening and senescence, controlling losses in storage due to insect-pest infestation and microbial contamination. The first documented proposal to use ionizing radiation, to bring about an improvement in the condition of foodstuffs and in their general keeping quality was made over 95 years ago in British patent no. 1609 of 1905. In 1947 Arno Brach and Wolfgang Huber reported that meat and some food stuffs could be sterilized by high energy electron pulses. The first commercial use of food irradiation was made in 1957 in Germany by a spice manufacturer. The FAO/IAEA/WHO joint expert committee on Irradiated Food concluded in 1980 that 'The gamma irradiation of any food commodity up to an overall average dose of 10 kGy presents no toxicological hazard'; hence, toxological testing of food so treated is no longer required. Garden pea (*Pisum sativum* L., 2n=24) is a very common nutritious leguminous vegetable grown in the winter season throughout the world. It is ranked third in the worldwide production amongst the grain legumes. In Central India it is grown in about 50 000 ha area with an annual production of 125 000 tonnes. Jabalpur division is the major contributor. The majority of the produce is marketed locally due to its perishable nature and short shelf life, which fetches lower return to the growers. A very small part of the produce is sent to distant markets in the state/ country. At the time of glut farmers are forced to sell their produce at lower prices due to lack of suitable storage facilities and technical know how on how to prolong the shelf life of pea. About 25-30% post harvest losses occur in garden pea due to its perishable nature. Radiation techniques have now opened up a new avenue to increase the shelf life of vegetable because irradiation slows down the rate of metabolism, delays senescence, inactivates enzymes responsible for spoilage and eliminates pathogenic organisms through surface pasteurization without appreciably rising the temperature of the produce (Kojima and Buddenhagen, 1967). Keeping the above facts in view garden pea pods were subjected to ^{60}Co gamma radiation treatment for prolonging its shelf life.

Materials and methods

Fresh fully matured and well filled pods of garden pea var. 'Arkel' were irradiated with ^{60}Co gamma rays at the Department of Horticulture, Jawaharlal Nehru Krishi Vishwa Vidyalaya, Jabalpur (M.P.) during 2004-2006. The experiment was carried out using a completely randomized design (CRD) with 4 replications. Hundred pea pods were irradiated with 5 doses of gamma rays (0.5, 1.0, 1.5, 2.0, and 3.0 kGy) and stored at ambient temperature for up to 9 days along with control. On the 3rd, 6th and 9th day after treatment, following observations were recorded viz., physical parameters (physiological weight loss %, decay loss %), biochemical parameters (protein content %, sugar content %) and sensory attributes (colour, appearance, taste, texture and overall acceptability). Protein content % was determined by using conventional micro-kjeldahl digestion and distillation procedure by A.O.A.C. (1980). Free soluble sugar content % was determined by extraction of 80% ethyl alcohol according to the procedure of Dubois et al., (1951). Sensory quality evaluation was done by a panel of 5 judges as described by Amerine et al., (1967) on a 9-point hedonic scale. The

data were analyzed statistically using standard procedures.

Results

Pooled analysis of data of two years indicated that except for protein content percentage significant differences due to radiation treatment were observed for all the other parameters under study. With respect to physiological weight loss % (Table 1) the data revealed that as the doses of gamma irradiation increased, physiological weight loss percentage decreased. Results revealed that maximum physiological weight loss of 12.32%, 19.51% and 23.49% was recorded in untreated pea pods (control) followed by 0.5 kGy gamma irradiation (9.82%, 12.87% and 16.46%) on the 3rd, 6th and 9th day after irradiation respectively. Minimum weight loss of 5.37%, 7.53% and 10.16% was noted in pods treated with 3.0 kGy gamma irradiation at 3rd, 6th and 9th day after treatment.

With respect to decay loss %, results revealed that as the doses of irradiation increased the decay loss decreased (Table 1). Minimum decay loss of 0.65%, 2.50% and 2.64% was noted with dose of 3.0 kGy gamma irradiation at 3rd, 6th and 9th day after treatment respectively. Correlation between decay loss and storage period was positive for all the treatments. The decay loss was high in untreated pods as compared to irradiated pods.

Table 1. Effect of gamma irradiation on physiological weight loss percentage and decay loss percentage of pods

Days after treatment	Treatments	Physiological weight loss %			Decay loss %		
		2004-2005	2005-2006	Mean	2004-2005	2005-2006	Mean
3rd	Control	11.22	13.42	12.32	4.04	6.31	5.17
	0.5 kGy	9.40	10.25	9.82	2.27	4.89	3.58
	1.0 kGy	9.29	9.81	9.55	2.47	3.38	2.92
	1.5 kGy	7.02	8.10	7.56	1.47	3.01	2.24
	2.0 kGy	5.61	7.05	6.33	0.74	1.51	1.12
	3.0 kGy	4.12	6.63	5.37	0.42	0.89	0.65
	SE ±	0.49	0.281		0.25	0.136	
	C.D. 5%	1.49	0.834		0.77	0.404	
6th	Control	18.50	20.53	19.51	10.96	12.58	11.77
	0.5 kGy	11.93	13.81	12.87	6.38	8.83	7.60
	1.0 kGy	11.53	13.20	12.36	7.71	7.02	7.36
	1.5 kGy	9.96	11.88	10.92	5.29	6.55	5.92
	2.0 kGy	8.60	10.21	9.40	3.30	4.02	3.66
	3.0 kGy	6.71	8.35	7.53	1.50	3.51	2.50
	SE ±	0.23	0.246		0.65	0.195	
	C.D. 5%	0.70	0.730		1.96	0.578	
9th	Control	22.33	24.65	23.49	11.20	14.67	12.93
	0.5 kGy	15.80	17.13	16.46	7.43	10.15	8.79
	1.0 kGy	14.51	16.95	15.73	7.92	8.86	8.39
	1.5 kGy	12.01	14.28	13.14	6.52	7.20	6.86
	2.0 kGy	9.16	13.83	11.49	3.35	4.80	4.07
	3.0 kGy	8.16	12.17	10.16	1.18	4.10	2.64
	SE ±	1.01	0.177		0.43	0.138	
	C.D. 5%	3.05	0.525		1.31	0.412	

Analysis of data presented in Table 2 revealed that gamma irradiation had a significant effect on total sugar content which showed a declining trend with the increase in storage period irrespective of the treatment. It is also clear from the results that garden pea pods irradiated with 1.0 kGy gamma rays recorded higher sugar content (2.99%, 2.90% and 2.71% at 3rd, 6th and 9th day after treatment respectively) during storage. Other doses lesser and greater than 1.0 kGy showed low total sugar content and the minimum total sugar was less than the control.

In case of pod colour, variation was noted due to different doses of irradiation and duration of storage (Table 3). Garden pea pods irradiated with 0.5 kGy and 1.0 kGy gamma rays retained their green colour up to the 9th day of storage whereas pods treated with gamma rays ≥1.5 kGy turn dull green 3rd day after treatment and became yellow on 9th day of storage. The non-irradiated pods remained green up to 3rd day and then turned dull green on the 6th day of storage (Figs. 1, 2 and 3).

Table 2. Effect of gamma irradiation on protein content percentage and sugar content percentage of pods.

Days after treatment	Treatments	Protein content %			Sugar content %		
		2004-2005	2005-2006	Mean	2004-2005	2005-2006	Mean
3rd	Control	6.21	7.50	6.85	2.72	2.65	2.69
	0.5 kGy	6.82	6.81	6.81	2.91	2.85	2.88
	1.0 kGy	7.70	6.67	7.18	3.02	2.97	2.99
	1.5 kGy	6.47	6.40	6.43	2.97	2.92	2.95
	2.0 kGy	6.30	6.18	6.24	2.84	2.78	2.81
	3.0 kGy	5.90	5.98	5.94	2.74	2.61	2.68
	SE ±	0.90	0.374		0.042	0.068	
	C.D. 5%	N.S.	N.S.		0.06	0.202	
6th	Control	6.73	7.56	7.14	2.43	2.38	2.41
	0.5 kGy	7.00	7.02	7.01	2.87	2.78	2.83
	1.0 kGy	7.88	6.88	7.38	2.96	2.85	2.91
	1.5 kGy	6.90	6.53	6.71	2.89	2.80	2.85
	2.0 kGy	6.50	6.51	6.50	2.79	2.64	2.57
	3.0 kGy	6.00	6.12	6.06	2.65	2.48	
	SE ±	0.95	0.339		0.019	0.064	
	C.D. 5%	N.S.	N.S.		0.040	0.190	
9th	Control	7.10	7.73	7.41	1.88	1.78	1.83
	0.5 kGy	7.35	7.21	7.28	2.69	2.61	2.65
	1.0 kGy	7.96	7.15	7.55	2.76	2.66	2.71
	1.5 kGy	7.20	6.85	7.02	2.74	2.58	2.66
	2.0 kGy	6.82	6.64	6.73	2.61	2.53	2.57
	3.0 kGy	6.38	6.25	6.31	2.50	2.63	2.43
	SE ±	0.87	0.360		0.013	0.059	
	C.D. 5%	N.S.	N.S.		0.041	0.175	

Table 3. Effect of gamma irradiation on colour of pods

Treatments	Colour					
	3rd Day		6th Day		9th Day	
	2004-2005	2005-2006	2004-2005	2005-2006	2004-2005	2005-2006
Control	Green	Green	Dull Green	Dull Green	Dull Green	Dull Green
0.5 kGy	Green	Green	Green	Green	Green	Green
1.0 kGy	Green	Green	Green	Green	Green	Green
1.5 kGy	Dull Green	Dull Green	Dull Green	Dull Green	Yellow	Yellow
2.0 kGy	Dull Green	Dull Green	Dull Green	Dull Green	Yellow	Yellow
3.0 kGy	Dull Green	Dull Green	Yellow	Yellow	Yellow	Yellow

Fig. 1. Effect of gamma rays three days after treatment.

Fig. 2. Effect of gamma rays six days after treatment.

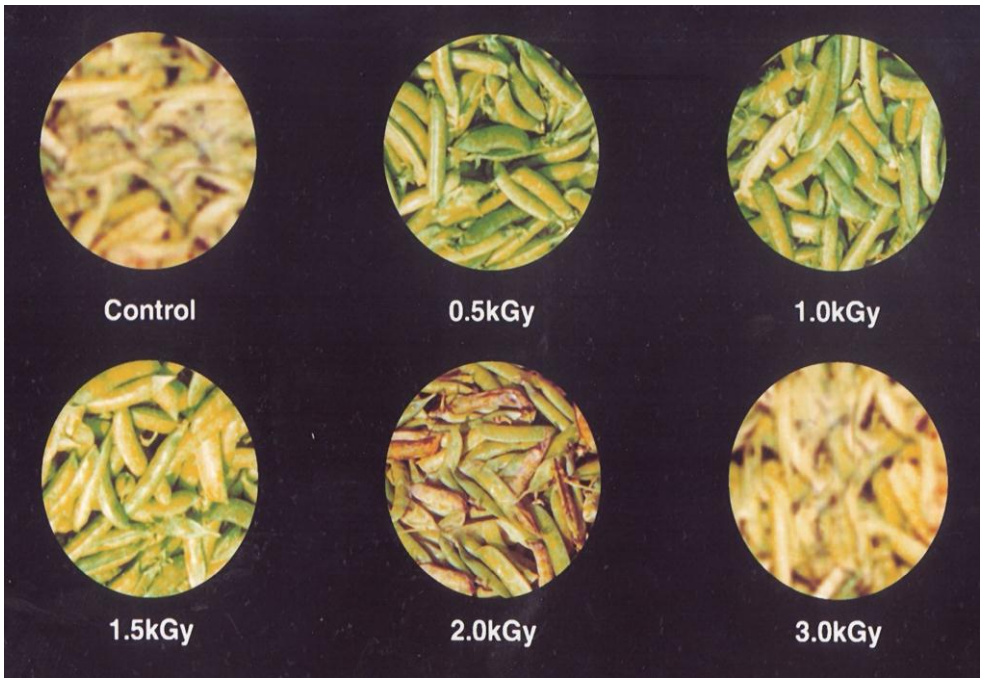

Fig. 3. Effect of gamma rays nine days after treatment.

Data on consumers' acceptability in terms of appearance showed significant difference in garden pea pods under different treatments. Pods irradiated with 1.0 kGy gamma radiation showed highest organoleptic score of 7.00 even on the 9th day after treatment followed by 0.5 kGy and 1.5 kGy gamma radiation. Garden pea pods treated at 2.0 kGy and 3.0 kGy gamma radiation doses and control were found to be unacceptable at 9th day after irradiation.

With respect to taste (Table 4) data revealed that pea pods irradiated with 1.0 kGy gamma radiation were most acceptable up to the 9th day followed by 0.5 kGy gamma rays treated pods. Garden pea pods under other treatments were not acceptable at the 9th day of storage.

On the basis of pooled data of two years, the sensory evaluation of texture showed maximum values of 8.37, 7.12 and 6.37 in pea pods treated with 1.0 kGy gamma radiation at 3rd, 6th and 9th day of storage respectively. Minimum values were noted in garden pea pods irradiated at 3.0 kGy dose (Table 5).

Overall consumer acceptability of garden pea pods exhibited maximum values of 8.62, 7.50 and 6.75 at 3rd, 6th and 9th day of storage at 1.0 kGy gamma irradiation. In general, overall acceptability rating declined with increasing storage period with all the treatments. At 3rd day of storage garden pea pods with all the treatments were acceptable. On the 6th day, pea pods treated with 0.5 kGy, 1.0 kGy and 1.5 kGy gamma radiation were found acceptable. The acceptability rating further declined by the 9th day. The pea pods irradiated only with 0.5 kGy and 1.0 kGy gamma rays were acceptable (Table 5).

Discussion

Maximum physiological weight loss was recorded in untreated pea pods while it was minimum in pods treated with 3.0 kGy gamma radiation at 3rd, 6th and 9th day after treatment respectively. This might be due to retardation of senescence and inhibition of metabolic activities that moisture is retained in irradiated pods as compared to control. These results are in propinquity with that of Lee and Kim (1972) in potato and Iglesias and Fraga (2000) in garlic.

Minimum decay loss percentage was noted in 3.0 kGy while, it was maximum in untreated pea pods (control). It might be due to effective elimination of pathogens (by gamma radiation) present on the surface of garden pea as well as internal pathogens. Organisms identified on the surface of the pods are *Alternaria* and *Cladosporium species* of fungi. A positive correlation between decay loss and storage period was noted for all the treatments. Similar results have been obtained by Menniti (1979) who reported in his findings that irradiation reduced the incidence of rots due to *Fusarium spp., Aspergillus niger* and *Penicillium spp.* in onion.

The total sugar content declined with the increase in storage period in all the treatments. Garden pea pods irradiated with 1.0 kGy gamma rays recorded higher sugar content during storage. Maximum total sugar was observed under control that might be directly correlated to the minimum sugar to starch conversion throughout the storage period. On contrary to the above findings Liu et al., (1990) reported that sugar increases during storage

(7 months) of irradiated potatoes (cv. Cardinal, Kennebec and Wu-Foon). Whereas, Lu (1988) reported that the total sugar tended to be higher at 1.0 kGy gamma irradiation, similar to the present findings. Even though protein is one of the principle constituent of garden pea but it was found to be non-significant due to irradiation.

Garden pea pods irradiated with 0.5 kGy and 1.0 kGy gamma rays retained their green colour up to 9th day of storage. Retention of colour under doses 0.5 kGy and 1.0 kGy is due to delay in metabolic activities as well as enzymatic reactions, which leads to slow down the senescence process. These findings on colour retention are in agreement with Shirodkar et al., (2002) in capsicum at 0.5 kGy.

On the basis of appearance pods irradiated with 1.0 kGy gamma radiation showed highest acceptability at 9th day after treatment because of more colour retention as well as firmness of pods. While, unirradiated pods along with those treated with 2.0 kGy and 3.0 kGy showed rapid deterioration of appearance due to degradation and tissue damage or radiation injury. El-Adawy (2000) is also in agreement with the present finding.

Table 4. Sensory attributes (appearance and taste) of pea pods as affected by gamma radiation

Days after treatment	Treatments	Appearance			Taste		
		2004-2005	2005-2006	Mean	2004-2005	2005-2006	Mean
3rd	Control	7.75	7.50	7.62	6.75	7.00	6.87
	0.5 kGy	8.25	8.50	8.37	7.75	7.50	7.62
	1.0 kGy	8.00	8.50	8.25	8.25	8.00	8.12
	1.5 kGy	7.75	8.00	7.87	7.50	7.00	7.25
	2.0 kGy	6.75	6.50	6.62	6.50	6.00	6.25
	3.0 kGy	6.00	6.00	6.00	6.00	5.50	5.75
	SE ±	0.240	0.623		0.370	0.556	
	C.D. 5%	0.720	1.860		1.130	1.653	
6th	Control	6.00	5.50		5.75	5.50	5.62
	0.5 kGy	7.50	7.75	5.75	7.00	7.25	7.12
	1.0 kGy	8.00	8.00	7.62	7.50	7.50	7.50
	1.5 kGy	6.75	6.75	8.12	6.75	6.00	6.37
	2.0 kGy	6.00	6.00	6.87	5.50	5.25	5.37
	3.0 kGy	5.00	5.00	5.70	5.00	4.50	4.75
	SE ±	0.340	0.340	5.00	0.400	0.700	
	C.D. 5%	1.040	1.040		1.06	2.080	
9th	Control	4.75	4.50	4.62	3.25	4.75	4.00
	0.5 kGy	7.00	6.25	6.62	6.25	6.25	6.25
	1.0 kGy	7.50	6.50	7.00	6.75	6.75	6.75
	1.5 kGy	6.00	6.00	6.00	6.00	5.50	5.75
	2.0 kGy	5.00	5.00	5.00	4.50	5.00	4.75
	3.0 kGy	4.25	4.25	4.25	3.75	4.00	3.87
	SE ±	0.360	0.574		0.350	0.600	
	C.D. 5%	1.110	1.700		1.060	1.790	

Table 5. Sensory attributes (appearance and taste) of pea pods as affected by gamma radiation

Days after treatment	Treatments	Texture			Overall Acceptability		
		2004-2005	2005-2006	Mean	2004-2005	2005-2006	Mean
3rd	Control	6.25	6.50	6.37	6.00	6.25	6.12
	0.5 kGy	8.00	8.25	8.12	8.25	8.50	8.37
	1.0 kGy	8.25	8.50	8.37	8.50	8.75	8.62
	1.5 kGy	7.75	7.50	7.62	8.00	7.50	7.75
	2.0 kGy	7.25	7.00	7.12	7.00	6.50	6.75
	3.0 kGy	6.25	6.50	6.37	6.50	6.00	6.25
	SE ±	0.22	0.514		1.140	0.708	
	C.D. 5%	0.66	1.528		2.540	2.100	
6th	Control	4.25	5.00	4.62	7.75	5.50	5.12
	0.5 kGy	6.75	6.50	6.62	7.25	7.00	7.12
	1.0 kGy	7.25	7.00	7.12	7.50	7.50	7.50
	1.5 kGy	6.25	6.00	6.12	7.25	6.00	6.62
	2.0 kGy	5.00	5.50	5.25	6.00	5.75	5.87
	3.0 kGy	4.25	4.75	4.50	5.00	5.00	5.00
	SE ±	0.32	0.519		1.06	0.567	
	C.D. 5%	0.97	1.540		2.24	1.679	
9th	Control	3.00	4.50	3.75	3.50	4.50	4.00
	0.5 kGy	6.00	6.00	6.00	6.25	6.25	6.25
	1.0 kGy	6.50	6.25	6.37	7.00	6.50	6.75
	1.5 kGy	5.75	5.00	5.37	6.25	5.50	5.87
	2.0 kGy	4.00	4.25	4.12	4.75	5.00	4.87
	3.0 kGy	3.25	4.00	3.62	3.75	4.00	3.87
	SE ±	0.34	0.559		1.10	0.587	
	C.D. 5%	1.02	1.659		2.35	1.737	

Maximum consumer acceptability in terms of taste was observed under 1.0 kGy gamma rays treated pods up to the 9th day followed by 0.5 kGy gamma rays.It may be due to more sugar retention and slow sugar to starch conversion which ultimately tends to slow down the deterioration of taste and maintain sweetness of pea. These results are similar to the findings of Zea and Vejerano (1971) in potato. The most important factor determining the texture of vegetables is its turgidity. In the present investigation sensory evaluation of texture showed maximum value in pea pods treated with 1.0 kGy gamma radiation throughout the storage period. Minimum value of texture in pods irradiated with 3.0 kGy gamma rays might be due to radiation injury caused by higher doses. The findings of Lu (1988) are in consonance with the present finding.

Overall acceptability ratings of garden pea pods declined with the increase in storage period under all the treatment including control. It was further observed that pods irradiated with 1.0 kGy gamma irradiated exhibited maximum value of overall consumer acceptability up to 9th day that is due to slow senescence rate. These results corroborated with the finding of Gautam et al., (1998) in button mushrooms. It is concluded that irradiation treat-

ment can be effectively used to protect decay losses and enhance shelf life of green pods. Hence, this process shows great promise in agriculture for the storage and preservation of food and food products.

Acknowledgement

The authors thank Babha Atomic Research Centre (BARC) and Dr. A.K. Sharma, Principal Scientist, BARC for providing necessary facilities to carry out this experiment.

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Research Article

Gamma Radio Sensitivity Determination for Lablab (*Lablab purpureus*) Bean

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Abstract

Mutagens like gamma rays are known to influence plant growth and development by inducing genetic, biochemical, and morpho-genetic changes in cells and tissues. The selection of efficient and effective mutagens and mutagenic doses is important for the success of a mutation breeding programme. While high doses are known to produce very drastic effects in the cell leading to death of the organism, effective mutagenic doses vary even among closely related plant species. In this study, the radio-sensitivity of a *Lablab purpureus* selection from Kenya, DL002, was assessed by exposing the dry seeds to 150, 200, 250, 300 and 350 Gy of gamma rays. Dose sensitivity was determined by measurements of physiological and morphological parameters on the M_1 generation and on the basis of frequency of various types of chlorophyll mutations obtained in the M_2 generation. With the increase in radiation dose a decrease in germination, seedling height, leaf size, number of leaves, emergence and survival rate under field conditions was observed in the M_1 generation. Chlorotic spots increased with increase in doses at the M_1 generation but no stimulatory effect in any of the traits studied was recorded. Seedling height decreased with increase in gamma radiation dose in an approximately linear fashion. The lethal dose 50 (LD50) value for seedling height was 231 Gy. The effectiveness of the dose in inducing genetic changes was estimated by counting the number of chlorophyll and morphological mutations in the M_2 generation. The frequency of mutations increased with the radiation dosage upto 250 Gy and then sharply decreased thereafter. The chlorophyll mutants observered included xantha and albina where the xantha type was more frequent than albino. The morphological mutants observed included early maturity, wrinkled leaves and seed coat colour. Based on the findings, it is proposed that the optimum dose for irradiating lablab bean using gamma rays is 200 to 250 Gy.

Introduction

Lablab (*Lablab purpureus* (L.) Sweet) is an ancient crop widely distributed in the tropics where it is being used as a grain legume and vegetable as well as for animal fodder and green manure in mixed crop-livestock systems (Smartt 1985; Shivashankar and Kulkarni 1989).

Lablab grains and pods are rich in proteins (20-28%) and vitamins (Khan et al., 2005). The protein in lablab has high levels of amino acids like lysine (6.2%) which is low in cereals grains. It can therefore play a major role in improving the diets of vulnerable rural communities in developing countries especially in sub-Saharan countries who mainly rely on starch-based diets with minimal animal protein. However, the crop is constrained by susceptibility to biotic and abiotic stresses like drought, aphids and pod borers.

Mutation breeding has become increasingly popular in recent times as an effective tool for crop improvement (Acharya et al., 2007) through supplementing the existing germplasm by creating genetic variability. Mutation is a sudden heritable change in the genetic material at the gene or chromosome level. They are caused by error during cell division or by exposure of the DNA to damaging agents or mutagens in the environment. During the past seventy years, more than 2600 mutants varieties have been officially released (Kang et al., 2007) with a wide range of characters improved (Bhatia et al., 2001; Osumanu Haruna et al., 2007). These varieties have been developed using various mutagens such as X rays, gamma rays and neutrons with gamma rays alone accounting for the development of 60% of the varieties. Most of the grain legume mutant varieties developed include those of *Glycine max, Arachis hypogaea, Phaseolus vulgaris, Pisum sativum and Vigna radiate* (Bhatia et al., 2001). There are only a few mutation breeding studies on lablab despite it being a multipurpose crop widely grown in Africa and Asia.

Mutagens like gamma rays are known to influence plant growth and development by inducing cytological, genetical, biochemical, physiological and morpho-genetical changes in cells and tissues (Girija and Dhanavel, 2009). Genetic variability is useful for selection of desired traits, while selection of efficient and effective mutagen and mutagenic doses is important for the success of a mutation breeding programme (Solanki and Sharma, 1994). High doses are known to produce very drastic effect in the cell leading to death of organism. The effective mutagenic doses vary even among closely related plant species. For example, Manjaya and Nandanwar (2007) reported gamma rays doses of 250 Gy as effective to induce genetic variability in soya beans, while Badigannavar and Murty, (2002), reported 150-200Gy in groundnuts. The important step in initiating a mutation

breeding programme would therefore be to determine the effective mutagenic dose (Praparat et al., 2001).

The present investigation was therefore undertaken to estimate the gamma irradiation doses effective and efficient for induction of genetic variability in lablab genotype DL002.

Materials and methods

Seeds of lablab (*Lablab purpureus*) variety DL002 were obtained from National Dry land Research Centre (NDRC) of the Kenya Agricultural Research Institute (KARI), Katumani, Machakos, Kenya. This variety was selected because of its wide adaptability in the major lablab growing districts in Kenya. The variety is an indeterminate semi-climber, with purple flowers. It is medium maturing and has black seed.

Two thousand four hundred uniform, dry and dormant seeds of the lablab variety DL002 were sent to the International Atomic Energy Agency (IAEA) laboratory at Vienna, Austria for irradiation with gamma rays. The seeds lot was divided into six portions of 400 seeds each. Portions were subjected to 0, 150, 200, 250, 300 or 350 Gy gamma rays respectively from a ^{60}Co gamma cell 220 source.

After the treatment, the M_1 seed were planted in Kenya for evaluation. The germination test was carried out in November 2008 in a seed germinator (model CR-20L by Fuji Electric) at 23^{o}C. The experiment was laid out in a completely radomised design (CRD) with three replications. Thirty seeds (M_1 seeds) of each of the treatment doses were placed on Petri dishes with moistened filter papers. The Petri dishes were then placed in the seed germinator. The filter papers were kept moist with tap water. The number of seeds germinated per Petri dish was recorded after five days. Seeds were recorded as germinated when the radical emerged from the side of the hilum. The results of the test were expressed as percentages of the planted seeds.

The effect of gamma rays treatment on seedling traits was determined in a greenhouse experiment at National Horticultural Research centre (NHRC) of KARI in a CRD experimental design with three replications. Planting was done in polythene bags measuring 20 × 20 × 18 cm containing a mixture of forest soil and river-bed sand mixed in the ratio of 2:1. The pots were arranged at 50 cm by 50 cm within and between the rows. Planting holes in the pots were made 2 cm deep using a wooden marker to ensure a uniform planting depth. Each experimental unit consisted of a polythene bag planted with a single seed with each treatment having 30 pots. Watering was done as required for 30 days.

Data collection commenced seven days after sowing (DAS). Data collection on leaf spots, leaf size (width and length), number of trifoliate leaves and plant height was done on all the test plants. Scoring for leaf spots was done seven days after sowing using a scale of 1-4 where; 1 = leaf green without any chlorotic spots, 2 = 10% of leaf with chlorotic spots, 3 = 30% of the leaf with chlorotic spots, 4 = >50% of the leaf with chlorotic spots. The leaf size of the primary leaves was taken 10 DAS. The leaf width was determined by taking the measurement of the leaves at the middle portion of the leaf blade while the leaf length measurement was done from the attachment of the leaf stalk to the tip. The number of trifoliate leaves was determined 30 DAS. This was done by counting the number of the already fully unfolded trifoliate leaves. The seedling heights were measured from the surface of the planting media to the tip of the top-most shoot of the plant. This was done on all seedlings 10, 20 and 30 DAS.

The effect of irradiation of M_1 seeds on plant emergence, flowering and plant survival was evaluated in the field at NHRC Thika between November 2008 and April 2009. The experiment was arranged in a randomized completely block design (RCBD) with three replications. Planting was done on a 3 × 2 m plots at a spacing of 50 cm between rows and 50 cm within rows. Each plot had 24 plants. All the recommended cultural measures like pest control, irrigation and weeding were carried out during the growth period of the crop.

Data was collected on all the plants per plot. The number of seedlings emerging in each plot was recorded at 14 DAS. Germination was considered to have occurred when the hypocotyls had emerged 1cm above the ground. The number of surviving plants per plot was counted at harvest time. A plant was considered to have survived to maturity if it produced at least one pod as suggested by IAEA, (1977). The number of plants with 50% flowering at 75 DAS was also recorded.

The data collected were analysed using Genstat release 8.2. The ANOVA for each parameter % germination, leaf width and length, seedlings leaf colour score, seedling height, % emergence in the field, % number of plants flowering at 75 DAS and survival rate in the field of the M_1 plants was done using Genstat computer programme and means separated by Fishers' protected LSD at the 5% probability level. The means of the germination and the seedling height at 30 DAS was expressed as a percentage of the non- irradiated control and a linear regression analysis of this data on the irradiation dose was carried using Genstat computer programme. The dose reducing germination and seedling height by 50% was determined using the linear regression.

To study the effect of gamma irradiation on the M_2 generation, M_2 seeds were harvested from the M_1 plants and planted in a field at KARI Thika. Samples of 1000 seeds

per treatment dose were raised in planting blocks measuring 50m by 6m between April and August 2009. The non-treated control was also planted after every two blocks for better comparison with the normal variation. Different kinds of chlorophyll and morphological mutants noted were recorded through the plant growth period. The mutation frequency was calculated as percentage of mutated M_2 progenies for both chlorophyll and morphological mutations in each treatment. The mutagenic effectiveness and efficiency was calculated on the basis of formulae suggested by Konzak et al., (1965).

Mutagenic effectiveness = M × 100 / Dose M = Total plants segregated into mutants at M_2 / Total plants studied at M_2 × 100.

Results

M_1 generation

The germination in the laboratory was not significantly (at 5% probability level) affected by the gamma rays irradiation except at 200 Gy and 350 Gy (Table 1). The highest germination reduction due to gamma rays irradiation was recorded at the highest dose (350 Gy).

Table 1. Mean germination rate (%) and other seedling traits in an M_1 population of lablab bean variety DL002 treated with different levels gamma rays doses

Treatment dose (Gy)	Mean germinate on in the laboratory (%)	Mean emergence in the field (%)	Height at 10 DAS (cm)	Height at 20 DAS (cm)	Height at 30 DAS (cm)	Leaf width (cm)	Leaf length (cm)	No. of leaves	Leaf chlorotic spots score
0 (control)	100 c	86.7a	4.9b	15.13d	36.27c	3.90b	4.07b	14.33d	1.0a
150	91.67 bc	85.5a	5.0b	11.37c	26.97b	4.23b	4.37b	12.1c	2.0b
200	88.89ab	83.5a	5.1b	10.70bc	22.13b	3.57b	3.87b	11.33bc	2.2b
250	91.98bc	78.0a	4.7b	10.50bc	13.13a	3.83b	4.47b	11.83c	2.4c
300	91.89bc	81.4a	4.7b	8.27b	11.16a	3.63b	3.93b	10.17b	3.0d
350	80.56a	80.4a	3.7a	5.67a	6.70a	2.7a	3.00a	6.03a	4.0e
CV (%)	6.1	6.9	18.8	28	43	13.3	12	6.8	7.1
LSD	9.89	10.37	0.44	1.48	4.3	0.86	0.84	1.33	0.3

Means followed by the same letters are not significantly different using LSD at 5% probability level of significance

According to this study, the emergence in the field was not significantly affected by the irradiation treatment at 5% probability level. The results however showed that the emergence percentage decreased with increasing treatment dose.

Significant seedling height reduction at 10 DAS was only detected at 350 Gy (Table 1). When the seedling height was taken at 20 DAS, all the treatment doses had significantly lower height than the non-irradiated control. The seedling height decreased from 11.37 cm at 150 Gy to 5.6 cm at 350 Gy. Like in 20 DAS, the highest seedling height at 30 DAS was recorded on the non-irradiated control. This was followed by 150Gy (26.97 cm) and 200 Gy (22.13 cm) with the lowest height (6.7 cm) obtained at 350 Gy. It was noted that the seedling height decreased with increasing dose when taken at 10, 20 and 30 DAS.

The effect of irradiation on seedling leaf size was determined by measuring leaflet width and length. All the treatment doses produced both leaf width and length which were not significantly (at 5% level of probability) different from the non irradiated control except for 350 Gy. The highest leaf width of 4.23 cm and leaf length of 4.47 cm were obtained at 150 Gy and 250 Gy respectively.

All the doses produced significantly (at 5% level of probability) lower number of leaves than the non irradiated control (Table 1). The lowest number of leaves (six) was recorded at the 350 Gy. It was further noted that the number of leaves decreased with increase of the irradiation dose.

The number of chlorotic leaf spots on primary leaves increased with the increase in dose. At lower doses (150 and 200 Gy), only 10% of the leaf area had chlorotic spots. At the highest dose evaluated in this study (350 Gy), it was observed that seedlings produced leaves with more than 50% chlorotic spots.

The effect of irradiation on the days to flowering was determined on 75 DAS when most (96.7%) of the non-irradiated control had at least 50% of the flowers opened. The highest number of plants with 50% flowering was recorded on the non-irradiated control (96.7%) followed by 150Gy (86.3%) (Fig. 1). However, these two treatments were not significantly different at 5% probability level. The rate of flowering decreased with increase in dose with only less than 25% of the plants flowering at 350Gy dose. This study indicated that the number of plants surviving up to maturity was not significantly different amongst the various treatment doses.

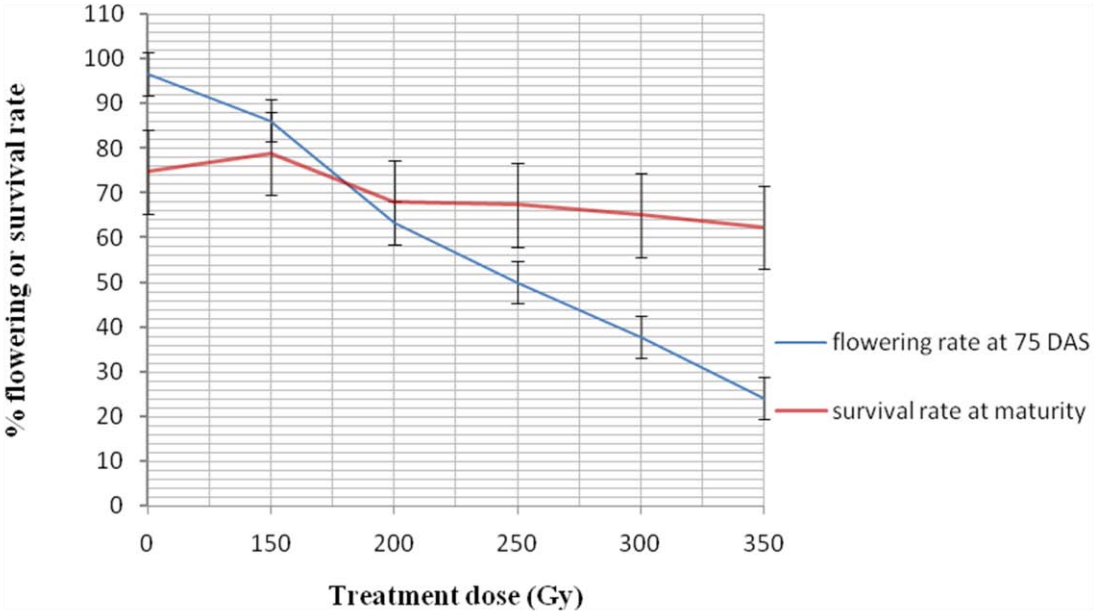

Fig. 1. Means flowering at 75 DAS and survival rate at maturity of first mutant generation of DL002 treated with different gamma irradiation doses. Error bar represents the standard error differences of means at 95% confidence intervals.

It was not possible to determine the lethal dose 50 (LD50) on the basis plant survival rate because of non significant survival rate in all treatments. The regression analysis indicated that there was no significant (5% probability level) linear relationship between the dose and the seed germination reduction. The determination of LD50 was therefore based on seedling height because a significant relationship between the dose and the seedling height reduction was detected by regression analysis (Table 2). The linear regression model accounted for 94.6% of the total variation and was therefore used to predict the LD50 for seedling height.

Table 2. Simple Regression analysis of seed germination and seedling height on gamma rays treatment doses of lablab variety DL002

Trait	Source of variation	Degree of freedom	Sum of squares	Mean square	F probability
Seed germination	Regression	1	36.86	36.86	0.259
	Error	3	57.24	19.08	
	Total	4	94.11	23.53	
Seedling height	Regression	1	2066.11	2066.11	0.003
	Error	3	81.88	27.29	
	Total	4	2147.99	537.00	

The LD50 value for seedling height estimated by the model (Y = -2.875 X + 116.49) was found to be 231.3 Gy. The graphical representation of the dose response relationships for seedling height is shown in Fig. 2. All the gamma irradiation doses studied showed reduction in seedling.

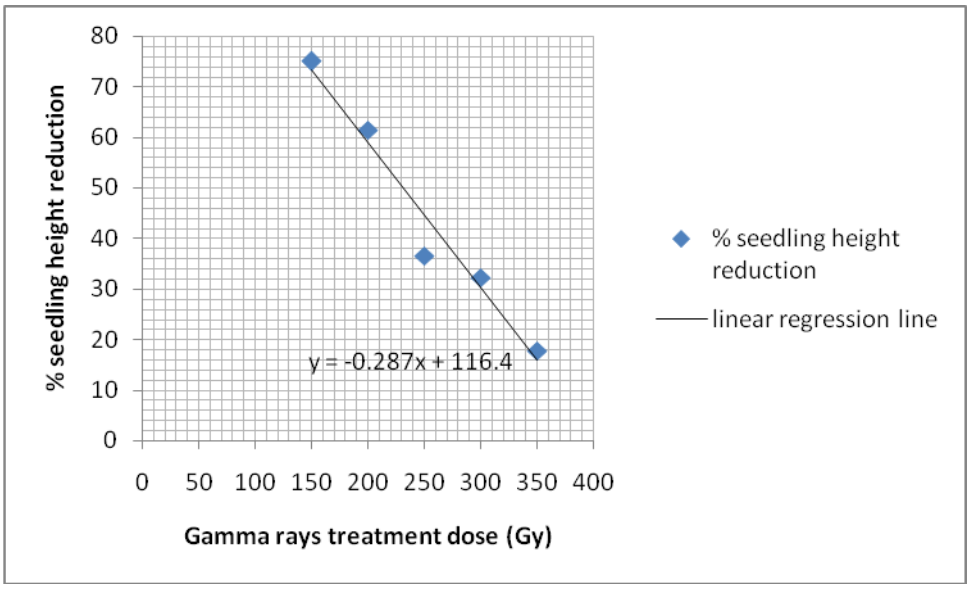

Fig. 2. Dose response relationship for seedling height in lablab genotype DL002 after treatment with different doses of gamma irradiation.

M_2 generation

The gamma radiation doses used induced a broad spectrum of mutations in the M_2 plants. The changes observed were both morphological and physiological. Both desirable and non-desirable effects were observed. Plants with changed leaf and flower colour, early maturity, seed colour and aphid resistance were recognized across the different treatments. Most of these observed mutants were observed in the 250 Gy populations (Table 3). The leaf chlorophyll mutants observed were xantha and albino. Xantha were orange yellow to light yellowish in colour while albinos were entirely white in colour. Xantha leaves were observed in all treatments except 150 Gy irradiation dose while albino leaves were only observed in one plant in the 300 Gy M_2 population. However, these mutants survived for only 2-3 weeks after emergence.

Table 3. Amount of M_2 lablab seed sown, number of seedlings germinated, and number of mutants found in populations of variety DL002 treated with 150, 200, 250, 300 and 350 Gy of gamma irradiation

Populations	# of M_2 seed sown	Seedling germinated	Mutant type					Total segregated
			Chlorophyll	Leaf type	Early flowering	Flower colour	Seed coat	
150 Gy	1000	838 (84%)	0	0	8	0	0	8
200 Gy	1000	823 (82%)	2	1	22	0	0	27
250 Gy	1000	894 (89%)	1	0	74	0	1	76
300 Gy	1000	813 (81%)	7	0	54	1	0	62
350 Gy	1000	578 (58%)	4	2	11	0	0	17
Total	5000	3946	14	3	169	1	1	188

The only leaf mutation observed was the wrinkled leaf mutation, which was observed in the 200 and 350 Gy populations. The plants were dwarfed and had all their leaves wrinkled. Of the three wrinkled mutants, only one produced pods (fertile). A single white flower mutant was observed in the 300 Gy populations against the characteristic purple flowers. Although this plant showed normal growth, it was low yielding. The mutant produced black coloured seeds which were smaller in size than the normal seeds. Flowering of DL002 in Thika area starts between 50-55 DAS. Therefore, plants that flowered 40-45 DAS, were considered to be early flowering mutants. These early flowering mutants were observed in all the five populations with the highest number obtained at 250 Gy populations. A single brown coloured seed mutant was obtained at 250 Gy populations. The mother variety (DL002) produces black seeds. This mutant did not show any change in morphological characteristics such as height, leaf, flower and pod. The brown seed was only observed in one of the three bearing branches suggesting it could be a chimera.

The mutation frequency showed an increase with increase in the dose up to 250 Gy but decreased with increase in dose thereafter (Table 4). The maximum mutation frequency (8.5%) was observed at 250 Gy while the minimum was obtained at 150 Gy. The mutation effectiveness increased with increase in dose but decreased with increase in dose from 250 Gy. The mutagenic efficiency in this study was only based on seedling height injury i.e. height reduction. The results indicated that the highest mutagenic efficiency was observed at 250 Gy.

Table 4. Gamma rays mutagenic frequency - effectiveness and efficiency in M_2 generation

Treatment dose (Gy)	Seedling height reduction Injury (%)*	Mutation frequency**	Effectiveness $\frac{M \times 100}{\text{dose}}$	Efficiency $\frac{M \times 100}{I}$
150	25	0.95	0.63	3.80
200	39	3.23	1.62	8.28
250	63	8.50	3.40	13.49
300	68	7.60	2.53	11.17
350	82	2.94	0.84	3.50

*I = seedling height reduction injury; **M = mutation frequency

Discussion

In this study, the germination percentage decreased with the increasing doses. The decrease in germination at higher doses of the mutagens may be attributed to disturbances at cellular level (caused either at physiological level or at physical level) including chromosomal damages. Disturbance in the formation of enzymes involved in the germination process may be one of the physiological effects caused by the mutagenic treatment leading to decrease in germination. Reduced growth due to higher doses was also explained differently by different workers. It may be attributed to one or more of the following reasons (i) the increase in destruction of growth inhibitors (ii) the increase in growth promoters (iii) the sudden increase in metabolic status of seeds at certain levels of dose or (iv) it may be due to the induced chromosomal aberrations. These findings are in close agreement with the earlier reports of Wang and Yu (1988), Solanki and Sharma (1999), Solanki and Sharma (2002), Kumar and Selvaraj (2003) Solanki and Phogat (2005). A decrease in seed germination with increasing radiation doses has also been reported in Chick pea (*Cicer arietinum* L.); Mung bean (*Vigna radiata* L.); Pigeon pea (*Cajanus cajan* L. Millsp.) and in Soyabean (*Glycin max* L. Merrill) (Shaikh et al., 1980; Venkateswarlu et al., 1981; Sharma and Sharma, 1986; Ignacimuthu and Babu, 1988; Muhammad, 1990; Badigannavar and Murty, 2002). Muhammad (1990), treated dry seeds of chickpea accession ILC 3279 with 11 different doses of gamma irradiation and found that the germination percentage decreased with increasing doses from 10-110 kR. Badigannavar and Murty (2002) showed a reduction in germination percentage of groundnut seeds in a laboratory experiment with increase of gamma rays irradiation dose from 150-350 Gy.

The current study indicated that the seedling height decreased linearly with increasing doses of gamma radiation. Reduction in plant height may be attributed to a drop in the auxin level, inhibition of auxin synthesis or due to decline in assimilation mechanisms (Girija and Dhanavel, 2009). The height reduction caused by the doses was consistent with the findings of Kharkwal (2000) who reported that reduction of plant heights belongs to the most frequently arising types in mutation experiment. The reduction of leaf size and the number of

leaves on plants treated with high gamma irradiation (in this case 350 Gy) could have also contributed to the reduced plant height. Plant leaves are responsible for photosynthesis processes that manufacture assimilates for plant growth. Similar reports of reduced leaf size have been reported in soya beans by Karthika and Subbalakshmi (2006). Leaf spots on the primary leaves increased with increasing radiation dose. The percentage of chlorotic spots in this study rose from 10% (score 2) at 150 Gy to more than 50% (score 4) at 350 Gy. Chlorotic spots or streaks developing on the leaves of irradiated seeds are usually correlated to gene mutations (IAEA, 1977). The presence of spots on the leaves of the irradiated seeds reported here, suggests that gamma irradiation doses used in this study were successful in inducing genetic changes on the lablab genotype.

The choice of irradiation doses to use is critical for the success of mutation breeding programmes (Owoseni et al., 2006). The LD50 is of great importance to know the sensitivity of genotypes to the critical dose of mutagens causing 50% mortality. Several studies have been carried out in many leguminous crops to determine the LD50 for growth (Manyanja and Nandanwar, 2007). Different parameters such as germination, seedling height and survival rate at harvest in M_1 plants have been used to measure the LD50 (Mehetre et al., 1994). In this study, the LD50 for survival rate could not be determined because of no significant difference in treatments effect. This observation was in agreement with the work of Ciftci et al., (2004) who could not determine LD50 for germination on faba beans because of no significant difference in treatments effect. The present investigation exhibited that the germination percentage of lablab decreased significantly with the increase in the dose from 150 Gy to 350 Gy. However, the LD50 for germination could not be determined because the regression analysis indicated a non significant relationship between the germination reduction and the dose. A non significant relationship meant that the decrease in the germination was not proportional to the increase in dosage and did not follow a definite pattern and therefore could not be effectively used to predict the LD50. Similar results have been reported in rice by Akbar and Atta (2003).

The frequency of chlorophyll and viable (morphological) mutants observed in M_2 generation is mainly used as a dependable measure of genetic effect of mutagen (Gautam and Mittal, 1998). In this study both chlorophyll and viable mutants were observed. The chlorophyll mutants included xantha and albino. Treatment of soya beans with gamma rays produced *chrolina* and *virids* type of chlorophyll mutants besides the *xantha* and *albino* mutants (Karthika and Subbalakshmi, 2006). This study indicated that the number of *xantha* mutants was more than *albino*. This result contrasts with the report in soybean by Karthika and Subbalakshmi, (2006) and in rice by Singh et al., (2001) who reported higher frequency of *albino* than *xantha* on gamma treated populations. Xing et al., (1997) explained that *albino* mutants resulted from delayed development of chloroplast due to lack of internal thylakoid system in leaves.

The morphological mutants observed here included early maturity, wrinkled leaves and seed coat. In their study with soybean, Karthika and Subbalakshmi, (2006) observed only broad and narrow leaves type of leaf mutants. Seed colour mutants have also been reported in cow peas (Girija and Dhanavel, 2009); in soybean (Pavadai, 2006), in sesame (Ganesan, 1992) and in Indian mustard (Verma and Raj, 1980). Early maturity mutants have also been reported by Girija and Dhanavel (2009) in cowpea.

In order to obtain high effectiveness and efficiency, the mutation effect must greatly surpass other effects in the cell, which reduce cell survival and eliminate the mutation (Girija and Dhanavel, 2009). Mutagen effectiveness refers to the rate of mutation induction as dependent upon the mutagenic doses while mutagenic efficiency is the mutation rate in relation to the various biological effects such as lethality, sterility or injuries like seedling height reduction (Konzak et al., 1965). In general, the mutagen effectiveness and efficiency increased with increasing dosage up to a certain limit, beyond which it exhibited a decline. This shows that a saturation point was reached at 250 Gy. Akbar and Atta (2003) attributed this decline at higher dose level to the rigor of diplontic and haplontic selections in the irradiated materials. Similar results were recorded by Girija and Dhanavel (2009) in cowpeas.

For breeding purpose mutagenic treatments with low physiological effects and strong genetic effects are desirable. The ultimate aim of a mutagenic treatment is to induce mutations leading to genetic improvement of a specific trait. In practice, for radiation treatments often a seedling height reduction of 50% (LD50) for M_1 plants in comparison with the control plants is taken as a criterion for a promising treatment. In the present studies, LD50 for seedling height ranged between 200 to 250 Gy (precisely 231 Gy). In addition, 250 Gy produced maximum frequency of mutations in the basis M_2 population. We therefore conclude that in lablab DL002, 200 and 250 Gy of gamma treatments can be used safely for practical breeding purposes with low physiological effects and high frequency of mutations.

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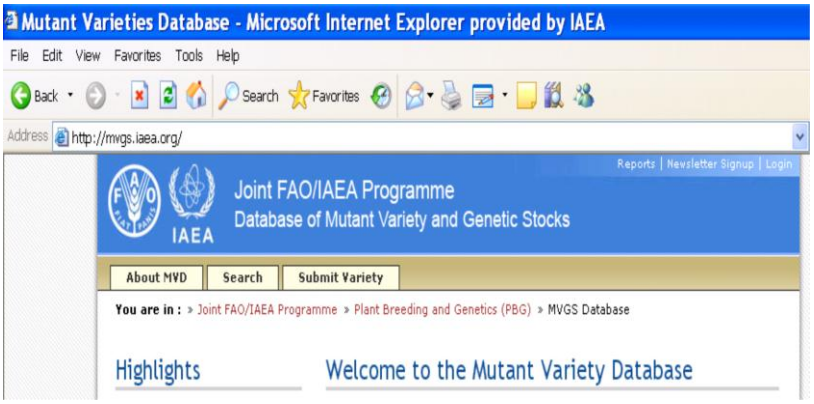

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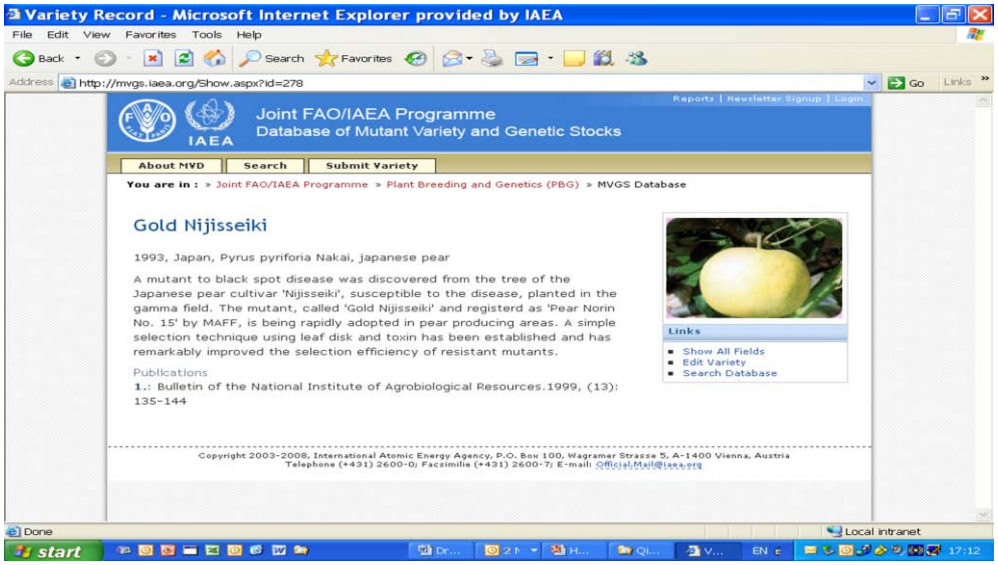

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Joint FAO/IAEA Programme
Nuclear Techniques in Food and Agriculture

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Plant Mutation Reports
Vol. 2, No. 3

The Plant Mutation Reports is prepared twice a year by the Plant Breeding and Genetics Section, Joint FAO/IAEA Programme of Nuclear Techniques in Food and Agriculture and FAO/IAEA Agriculture and Biotechnology Laboratory, Seibersdorf.

International Atomic Energy Agency, Vienna International Centre, PO Box 100, 1400 Vienna, Austria
Printed by the IAEA in Austria, April 2011.

11-08931