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Variations in seed protein content of cotton (*Gossypium hirsutum* L.) mutant lines by *in vivo* and *in vitro* mutagenesis

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Abstract

The present work describes the influence of gamma irradiation (GR), ethyl methane sulphonate (EMS) and sodium azide (SA) treatment on yield and protein content of selected mutant lines of cotton. Seeds of MCU 5 and MCU 11 were exposed to gamma rays (GR), ethyl methane sulphonate (EMS) and sodium azide (SA). Lower dose of gamma irradiation (100-500 Gy), 10-50 mM EMS and SA at lower concentration effectively influences in improving the yield and protein content. Significant increase in yield (258.9 g plant^{-1}) and protein content (18.63 mg g^{-1} d. wt.) as compared to parental lines was noted in M_2 generations. During the subsequent field trials, number of mutant lines varied morphologically in terms of yield as well as biochemical characters such as protein. The selected mutant lines were bred true to their characters in M_3 and M_4 generations. The significant increase in protein content and profiles of the mutant lines with range of 10.21-18.63 mg g^{-1}. The SDS-PAGE analysis of mutant lines revealed 9 distinct bands of different intensities with range of 26-81 kDa. The difference in intensity of bands was more (41, 50 and 58 kDa) in the mutant lines obtained from *in vitro* mutation than *in vivo* mutation. Significance of such stimulation in protein content correlated with yielding ability of the mutant lines of cotton in terms of seed weight per plant. The results confirm that in cotton it is possible to enhance the both yield and biochemical characters by *in vivo* and *in vitro* mutagenic treatments.

Key words

Gamma irradiation, Mutagenic treatments, *Gossypium*, Mutant lines

Introduction

Mutation techniques have been widely applied to improve crop yield, quality, disease and pest resistance and they have produced much germplasm with novel, desired traits (Maluszynski *et al*., 1995). Plant cultivars derived from induced mutations have contributed billions of dollars to the economies of many countries. The main beneficiaries have been not only developing countries (e.g. India, China and Pakistan) but also North American and European countries have gained from the release of mutant cultivars (Ahloowalia *et al*., 2001). Mutations are known to enhance the genetic variability of crop plants and the role of induced mutations in crop improvements is evident from a large number of improved high yielding varieties of several crops developed through mutation breeding and released for commercial cultivation in India (Kharkwal, 1998). In cotton, various breeding procedures *viz*. mass selection, pedigree method, backcross and mutation breeding have been used in the past for genetic improvement of seed oil content and protein in cotton and especially mutation breeding was successfully used to improve seed oil and protein content (Singh and Narayanan, 1991). Variation in seed protein composition could be the reflection of genotypic variation and genetic variants showing differences in composition of seed protein were reported in many species and genera of higher plants (Stegemann and Shah, 1990; Ignacimuthu and Arockiadass, 1993; Kang and Kameya, 1995; Karthika and Subba Lakshmi, 2006; Pavadai *et al*., 2010; Mehta and Nair,

2011). Seed storage proteins, an important factor in biochemical analysis, are abundantly found in cotton. SDS-PAGE analysis of protein and isozymes aides in the systematic study of polymorphism, phylogenetic relationship between as well as within the species and analysis of seed storage protein. In SDS-PAGE induced changes in the form of difference in number and intensity of bands for different proteins may be used to identify and differentiate the species, in variety description and in assessment of the cultivars (Osanyinpeju and Odeigah, 1998; Nayeem, 1999; Singh, 2011). The present study was conducted to analyse the variations in the seed protein content and its profiles of the selected mutant lines of cotton developed from *in vivo* and *in vitro* mutagenic treatments/ irradiation.

Materials and Methods

Plant materials: The seeds of cotton (*Gossypium hirsutum* L.) varieties MCU 5 and MCU 11 used in present investigation were obtained from Cotton Research Station, Tamil Nadu Agricultural University, Coimbatore, India.

Gamma irradiation/chemical mutagenic treatment of seeds: The gamma irradiation was performed at room temperature (25-30° C) using a gamma cell equipped with ^{60}Co source at the Sugarcane Breeding Institute, Coimbatore, India. The uniform healthy seeds were irradiated with 100, 200, 300, 400 and 500 Gy gamma rays (^{60}Co gamma source) and another sets of seeds treated with 10, 20, 30, 40 and 50 mM of ethyl methane sulphonate (EMS) and sodium azide (SA) for 4 hr. The chemical mutagens (EMS) and (SA) were prepared in phosphate buffer with pH 7 and 3 for EMS and SA, respectively. The irradiated/treated seeds were sown in pre-irrigated field along with the control seeds, and the plants were observed thoroughly for the variations in terms of yield and protein content.

Ovule culture and seedling development with gamma irradiation and mutagenic treatment: Ovule culture, gamma rays, EMS, SA treatments was conducted based on the procedure standardized and described in our earlier reports (Muthusamy and Jayabalan, 2007). Briefly, bolls [15-day-post anthesis (dpa)] were excised from field grown plants and surface sterilized. Ovules were dissected out from the boll and were placed on agar-solidified MS (Murashige and Skoog, 1962) medium with B_5 vitamins (Gamborg *et al*., 1968), 0.3 % (w/v) sucrose, 0.8 % (w/v) agar and 2,4-D (1.5 mg l^{-1}), IAA (1.5 mg l^{-1}) and kinetin (0.5 mg l^{-1}) with casein hydrolysate. The pH of the medium was adjusted to 5.8 prior to autoclaving. The cultures were incubated at 24 ± 2° C under cool-white fluorescent light at 40 μE m^{-2} s^{-1} with a 16 h photoperiod. Ovules were placed on MS medium in culture bottles and exposed to 10, 20, 30, 40 and 50 Gy gamma rays (GR) and ovules were treated with 1, 2, 3, 4 and 5 mM of EMS and SA for 30 min. The untreated ovules either with Gy or EMS and SA were considered as control. The exposed/treated ovules place was on MS medium supplemented with 1.5 mg l^{-1} IAA and/or 2, 4-D and 0.5 mg l^{-1} kinetin for initiation of seedling regeneration. The procedure for elongation and rooting as previously standardized and described (Muthusamy and Jayabalan, 2007). After the initiation of seedlings, the ovules were transferred to MS basal medium for elongation. The elongated seedlings were transferred to MS basal medium for rooting. Seedlings with well-developed roots were transferred to plastic cups containing a 1:1:1 mixture of sand, red soil and manure for hardening. Well-established seedlings from cups were transferred to the field and they were grown to maturity that produced normal flowers and set seeds in bolls. The *in vitro* developed plantlets from immature ovules were transferred to the field for further observations of morphological, yield and biochemical characters.

Field evaluation of mutant lines: The hardened M1 seedlings from each treatment were planted on the pre-irrigated rows (75´45cm) in the experimental field at Bharathidasan University, Tiruchirappalli, India along with control seedlings with three replications. The plants were selfed and the seeds were collected from the bolls of each plant and their characters were recorded. The M2 and the subsequent generations of plants derived from irradiated/ irradiated ovules were grown to analyze their performance with reference to control. In M1 generation, groups of plants were selected and carried forward on bulk basis, while in advanced generations (M2, M3 and M4) single plant were selected. Twenty five M2 plants from each dose/ concentration were randomly selected from each replicates out of a larger group with good fibre yield, earliness and other economic characters and advanced to the M3 generation where the same procedure was repeated as in M2 generation and promoted to M4 generation. Finally 20 M4 mutant lines (11 and 9 mutant lines from *in vivo* and *in vitro* respectively) were selected for different agronomical characters. The *in vivo* and *in vitro* raised control plantlets from ovules were planted separately in triplicates, maintained along with the plantlets from irradiated/treated ovules and selected by the similar method was adopted for both control and plantlets which was developed from irradiated/treated from ovules.

Estimation of protein content and SDS-PAGE analysis: Total buffer soluble protein was extracted from the seeds of control and mutant lines. The soluble protein was estimated by comparing with standard protein (BSA) following method of Lowry *et al*. (1951). Based on the colorimetric estimation, only the mutant lines (both *in vivo* and *in vitro)* that showed significant higher amounts of protein than control were taken

for SDS-PAGE analysis. The SDS-PAGE analysis was performed according to Laemmli (1970) and the value of relative migration (Rm) was calculated compared with standard marker. Briefly, 100µg of the protein sample was added into each well of SDS-PAGE and the mini SDS-PAGE gel (5% stacking; 10% separating) was run on discontinuous electrophoretic system using Tris-HCl buffer (pH 8.0) with an initial voltage of 50 V for stacking the gel, and later 100 V for separating it. The gel was removed and stained in coomassie brilliant blue stain for overnight and destained with ethanol: acetic acid: distilled water (23:7:70).

Statistical analysis: The experiments were repeated thrice with triplicate samples and statistical computations were performed using computer software and the experimental design was random. The data of seed weight and protein content were subjected to analysis of variance (ANOVA) and mean separation was carried out adopting Duncan's new multiple range test (DNMRT).

Results and Discussion

The induction and selection of mutant lines provide a simple, efficient, rapid and cheap method by which to alter the genetic make-up and obtain desired genotypes with increased yield and biochemical characters (Lee *et al*., 2003). 20 mutant lines obtained from both *in vivo* and *in vitro* mutation breeding were analyzed for seed weight, protein content and its profiles. The seed weight of the mutant lines showed significant improvement over the control lines and the seed weight was reflected in seed protein content as well. Among the 20 mutant lines, TM 5 showed the higher seed weight of 258.9 g plant^{-1} and 245.1 g plant^{-1} by *in vitro* and *in vivo* mutagenesis respectively and showed significant enhancement in seed weight over control lines which showed the range of 183-208 g plant^{-1}. Similar findings have also been reported by Shinde and Deshmuk (1985) in cotton, Kajjidoni *et al*. (2006) in urdbean, Tawfik *et al.* (2011) in *Senna occidentalis* and Srivastava *et al*. (2011) in wheat.

The protein content of all mutant lines increased significantly in range of 09.25 to 18.20 mg g^{-1} d.wt. and 10.23 to 18.96 mg g^{-1} d.wt. in *in vivo* and *in vitro* mutagenesis respectively. Out of the 11 mutant lines (7 from MCU 5 and 4 from MCU 11) obtained from *in-vivo* mutation breeding, mutant line 3 (with height and early flowering character) had highest seed protein content (18.20 mg g^{-1}) and all mutant lines showed higher protein contents as compared to control lines (9.25 and 10.2 mg g^{-1} d.wt. in MCU 5 and MCU 11, respectively. Among the 9 mutant lines (4 from MCU 5 and 5 from MCU 11) selected from *in vitro* mutation breeding, TM 5 showed highest protein contents (19.86 mg g^{-1} d.wt. with higher seed index and number of bolls) and other mutant lines also showed significant increase as compared to control lines (Table 1). The estimation of soluble seed protein content revealed that the selected mutant lines showed significant improvement in protein content of the mutant seed over control lines. Our results are similar to earlier reports where significant increase in high seed protein mutants in sunflower (Elangovan and Selvaraj, 1994), rice (Shen *et al*., 1995), soybean (Imsande *et al*., 2001), cotton (Muthusamy *et al*., 2002) wheat (Yanagisawa *et al*., 2004), green gram (Samiullah and Wani, 2006) and blackgram (Arulbalachandran and Mullainathan, 2009) was observed. Thus, the high seed protein containing mutant lines observed here in cotton can be of immense potential in breeding for value addition and enhanced utility of oil and cake with high protein content. Schaeffer and Sharpe (1990) observed high-protein mutants containing high lysine levels in rice and recently increased protein and free amino acid contents have been reported in 5MT-resistant MR lines of the M_3 generation (Kim *et al*., 2004; Li *et al*., 2005).

SDS-PAGE analysis was conducted to identify changes in total buffer soluble protein and to determine the

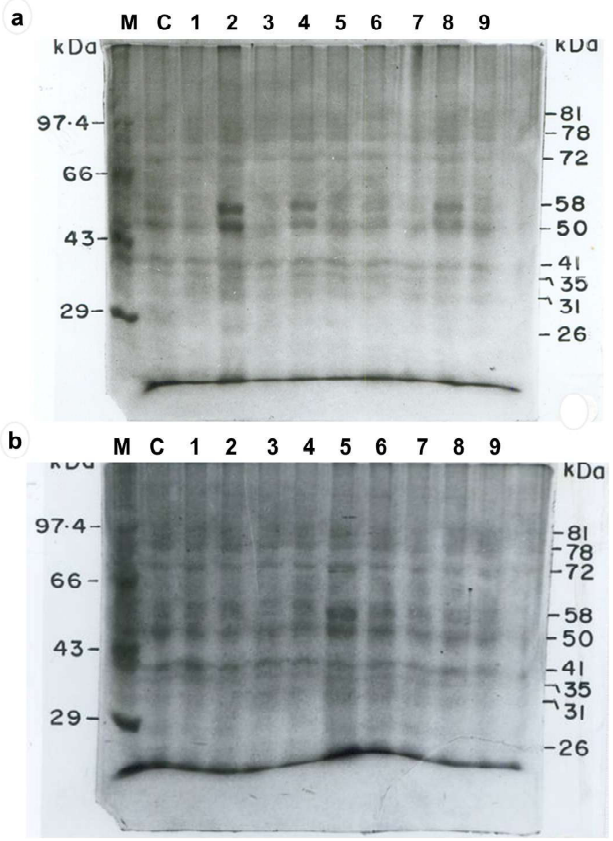

Fig. 1 : SDS-PAGE analysis of total buffer soluble seed proteins in cotton mutant lines. **(a) *in vivo* mutants**; M-marker; C-control; lane 1, mutant-2; lane 2, mutant-3; lane 3 mutant-4; lane 4, mutant-6; lane 5, mutant-7; lane 6, mutant-8; lane 7, mutant-9; lane 8, mutant-10; lane 9, mutant-11. **(b) *in vitro* mutants**; M-marker; C-control; lane 1, TM-1; lane 2, TM-2; lane 3, TM-3; lane 4, TM-4; lane 5, TM-5; lane 6, TM-6; lane 7, TM -7; lane 8, TM-8; lane 9, TM-9

profiles of mutant lines. SDS-PAGE of total proteins extracted revealed protein profile differences between the control and mutant lines (Fig. 1). Out of the 15 polypeptide bands observed, only 9 bands with a molecular mass of 26, 31, 35, 41, 50, 58, 72, 78 and 81 kDa and Rm value of 0.74, 0.69, 0.65, 0.64, 0.55, 0.47, 0.33, 0.27 and 0.24, respectively were visible and clear. Although the mutant lines varied widely in protein content, increase over the control lines were observed at the molecular weights of 72, 58, 50 and 41 kDa in mutant lines. Though the intensity of bands varied in different mutant lines, the numbers of bands in all mutants are same. Interestingly 58 and 50 kDa protein were present at higher concentration in three mutant lines, M2, M4 and M8 obtained from *in vivo* mutation breeding (Fig. 1a) whereas in *in vitro* mutated lines four mutant lines (TM4, TM5, TM6 and TM8) showed higher concentration of protein (Fig. 1b). The bands of 50 and 58 kDa proteins with the Rm value of 0.55 and 0.47 were more intense and varied from each of the selected mutant. The maximum peak area (based on the intensity of the bands) was noted in lane 3, 6 and 10 for 50 and 58 kDa proteins (data are shown). Between the two different mutated lines (*in vivo* and *in vitro*), the *in vitro* mutated lines showed more intensity in bands than *in vivo* mutated lines. These findings are consistent with those of Kim *et al*. (2004) who noted two typical mutant protein bands at 24 kDa. They also observed two distinct bands of different intensities at ca. 22 and 18 kDa in SDS-PAGE profiles of mutant prolamines and number of new protein spots found in S-(2-aminoethyl)-cysteine (AEC) resistant rice mutant lines. Recently, enhanced accumulation of the storage proteins was noted (Kim *et al*., 2005a) in four rice mutant lines suggests important differences in protein processing occurring between the mutant and the control plants and 17–28.5% increases in protein synthesis in the mutant lines was observed relative to that of the control seeds (Kim *et al*., 2005b). Electrophoretic separation and subsequent isolation of the storage proteins showed several minor changes in the mutant lines. Increased density in glutelin

Table 1 : Yield characters and its selected dose of mutagens and protein content of selected mutant lines (M_4 generation) of cotton

Mutant lines	Treatments	Characters of selected mutants	Seed weight (g plant^{-1})	Protein content (mg g^{-1})
In vivo				
MCU 5				
Control	--	--	183.0^{k}	09.25^{j}
Mutant 1	30 mM EMS	Early flowering	198.0^{i}	10.95^{gh}
Mutant 2	100 Gy GR	Twin bolls	224.2^{ef}	14.65^{e}
Mutant 3	200 Gy GR	Height, early flowering	245.1^{a}	18.20^{a}
Mutant 4	400 Gy GR	Branches, height	225.0^{e}	14.65^{e}
Mutant 5	40 mM SA	Bolls, yield, ginning percent	205.0^{h}	11.40^{g}
Mutant 6	30 mM EMS	Lengthy branches, seed index	237.8^{b}	16.80^{b}
Mutant 7	500 Gy GR	Number of seeds, boll weight	225.0^{e}	15.20^{de}
MCU 11				
Control	--	--	195.0^{ij}	10.21i
Mutant 8	40 mM EMS	Bolls, early flowering,	230.0^{cd}	15.20^{de}
Mutant 9	200 Gy GR	Branches, height	216.3^{g}	14.50^{ef}
Mutant 10	40 mM SA	Ginning percent,	235.0^{bc}	16.57^{bc}
Mutant 11	20 mM EMS	Fiber quality, yield	233.9^{c}	15.65^{d}
In vitro				
MCU 5				
Control	--	--	190.2^{h}	10.23^{i}
TM 1	4 mM SA	Early flowering	248.9^{bc}	17.80^{bc}
TM 2	30 Gy GR	Number of bolls	238.4^{d}	16.89^{d}
TM 3	2 mM EMS	Ginning percent, seed index	213.5^{f}	14.56^{fg}
TM 4	4 mM EMS	Higher yield	254.6^{b}	17.85^{b}
MCU 11				
Control	--	--	208.9^{gh}	11.25^{h}
TM 5	4 mM SA	Ginning percent	258.9^{a}	18.96^{a}
TM 6	30 Gy GR	Seed index, number of bolls	258.0^{ab}	18.63^{ab}
TM 7	5 mM SA	Fiber quality, harvest index	235.6^{e}	14.98^{f}
TM 8	40 Gy GR	Height, yield	236.3^{de}	16.52^{de}
TM 9	3 mM EMS	Number of bolls, seed index	235.6^{e}	15.87^{e}

Mean values within a column having the same alphabet are not significantly different (p = 0.05) according to New Duncan's Multiple Range Test. Gy-Gray; GR-gamma rays; EMS-ethyl methane sulphonate; SA-sodium azide

and prolamin fractions in the mutant lines probably up regulated protein levels and amino acid content (Schaeffer and Sharpe, 1990) and further confirmed the changes in protein processing in the developing grain of an enhanced lysine/protein rice mutant by analyzing ^{3}H-lysine incorporation pattern. SDS-PAGE and Western blot experiments revealed that, in the seeds of homozygous MB14 plants, the expression of the 1Bx14 subunit was specifically blocked whereas the remaining four subunits (1Ax1, 1By15, 1Dx2, 1Dy12) accumulated to levels comparable to those in the wild type plants (Zhu *et al*., 2005). Our results are also conformity with those of Asmahan (2006) and Azza *et al*. (2011) who found variations in number, intensity and or density of SDS electrophoretic bands of proteins from wheat and maize after gamma irradiation and sodium azide treatments. In the present study, a potentially useful mutant line of cotton with significantly increased proteins was noted. These mutants should prove to be very useful for improving the nutritional quality of the cottonseed.

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