

DISCUSSION

In the present investigation, attempts have been made to understand the response of nodal explants and shoot apex for shoot induction. Attempts have also been made to identify somaclonal variations from directly regenerated plants, to standardize the semilethal dose/ concentrations for induction of mutations and to isolate mutants based on morphological parameters. Besides, the possibilities of obtaining variants/ mutants through long term cultures were also studied.

1. Direct regeneration:

In recent days, *in vitro* technology has been widely used not only to obtain mass propagation of crop species but also to induce genetic variabilities. The variabilities can easily be assessed by morphological and biochemical parameters of *in vitro* derived plants.

Axillary buds are reported to be the most suitable explants for micropropagation in many crop plants, since the emerging young buds being meristematic in nature, have a great potential for vigorous development of shoots (Yadav *et al.*, 1990 and Pattanaik *et al.*, 1996). This method of direct regeneration is significantly advantageous in plants like mulberry, where callus differentiation by organogenesis or embryogenesis have been found to be comparatively difficult and results are non reproducible (Seki *et al.*, 1971; Narayan *et al.*, 1989; Narayan *et al.*, 1994). x x
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The cytokinin, BAP is known to stimulate formation of adventitious buds in tissue culture especially, in fruit trees and other crops (Tisserat, 1987). BAP at low (0.5 - 1.0 mg/l) concentration was reported to be favourable for induction and proliferation of adventitious shoots also in mulberry species like *Morus alba* and *M. indica* by various workers (Oka and Ohyama, 1981; Mhatre *et al.*, 1985; yky
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Chattoopadyaya, 1990; Jain *et al.*, 1990; Raghunath *et al.*, 1992; Hossain *et al.*, 1991; Islam *et al.*, 1993). High BAP concentration (8.0 mg/l) was reported to be useful in regeneration and micropropagation of *M. laevigata* (Islam *et al.*, 1992). In the present investigations, the low concentration of BAP (1.0 mg/l) showed maximum response of shoot induction (plate 1: A and B) compared to tested concentrations employed for culture of nodal explants in mulberry var. S₃₆ (Table 1). 29

Jain *et al.* (1990) studied *in vitro* propagation of axillary buds in five (5) genotypes of mulberry and recorded differences in their response. Kathiravan *et al.* (1995) evaluated *in vitro* responses of nodal explants in eight (8) mulberry varieties and recorded differences in shoot formation. Tewary *et al.* (1996) recorded genotypic differences in *in vitro* shoot development in ten (10) selected mulberry varieties. The requirement of BAP, IBA and GA₃ was shown to vary among the genotypes as reported earlier by Mhatre *et al.* (1985) and Kim *et al.* (1985).

Another important and suitable material for mass micropropagation is shoot apex. The micropropagation of mulberry plant through shoot apex culture has the unique advantage of increased rate of multiplication (Tewary and Subba Rao, 1990; Tewary *et al.*, 1995). In the present investigation, shoot apices were subcultured up to 240 days (Tables 2 and 3) to increase the rate of multiplication. It was noticed that for each subculture, maintained up to 120 days, the number of shoots formed was found to increase in logarithmic pattern (plate 2: A and B). However, the multiplication rate after 120 days declined. Similar phenomenon was reported in other plants like guava (Amin and Jaiswal, 1988) and *Pistachio* (Barghchi and Alderson, 1985). Recently, Tewary *et al.* (1995) have reported similar results in mulberry genotypes S₃₄ and V₁ and have also remarked that, uniform increase in shoot formation could be achieved up to 100 days from a single shoot apex. The present findings on multiple shoot induction are in confirmation with above mentioned earlier reports. X

In the present study, the shoots derived from *in vitro* culture were further multiplied by selecting nodal explants from them and culturing on MS medium supplemented with different concentrations of GA₃. The maximum response of nodal explant for shoot elongation was recorded on BAP(1.0 mg/l) + GA₃ (5.0 mg/l) (Table 4). The observed result in the present study was found closer to the results obtained on ground nut (Venkatachalam *et al.*, 1996), and also on *Nicotiana* (Engelke *et al.*, 1973). The promotive effect of GA₃ on the shoot bud induction in culture was reported earlier in *Chrysanthemum* (Earle and Langhans, 1974). The present findings support the practice of selecting nodal buds from *in vitro* shoot as potential explants for improvement of micropropagation method in mulberry.

In recent days, the multiplication of several horticultural crops by adopting liquid shake culture method has been reported by several workers including Takayama,(1991). In woody plants, however, only a limited number of studies have been made (Alvard *et al.*, 1993; Hammerschlag,1982). In the present study, it is clearly shown that several multiple shoot buds could be induced (plate3 : A and B) from a single shoot tip by employing MS medium supplemented with low concentration of 4 pu (0.5 mg/l) (Table 5). The present study also shows that not only multiple shoots but adequate number of roots also can be induced in the same liquid medium. Therefore, the liquid shake culture method employed could be more stable and easy to multiply the mulberry variety S₃₆ presently studied. The results are in conformation with the earlier reports on eucalyptus (Ito *et al.*,1996) and on other mulberry genotypes (Oka,1995 ; Tewary and Oka,1999).

2. Indirect regeneration (through callus):

In the present investigation, for selection of suitable medium for obtaining callus and subsequent regeneration, two media viz., MS and B5 media were tested. MS medium favoured better growth of callus than B5 medium (Table 21). MS medium has been successfully used for direct micropropagation and also callus regeneration in mulberry by several workers (Oka and Ohyama , 1974, 1975; Kim *et al.*, 1985; Narayan *et al.*, 1989; Rao and Raghunath, 1993; Raghunath *et al.*, 1992; Jain and Datta, 1992; Tewary *et al.*, 1995).

In majority of *Morus* species investigated, the auxin viz., 2,4-D was found necessary for the induction and establishment of callus from various explants (Plate 9: A). Addition of low concentration of cytokinin (BAP) to auxin containing medium is not obligatory to induce callus formation (Oka and Ohyama, 1973; Tewary, *et al.*, 1989; Narayan *et al.*, 1989 and Satyanarayana, 1999). 2,4-D has been widely used to initiate regenerative callus in 70 % of the crop species. Depending upon the species, 2,4-D in the range of 1.0 - 2.0 mg/l was found optimal for induction of callus (Evans *et al.*, 1981). In the present study on var. S₃₆ however, addition of cytokinin (BAP) at lower concentration (0.5 mg/l) could enhance the callus initiation and proliferation from internodal segment and also from leaf explants.

Internodal segments and young leaf explants of mulberry variety S₃₆ when cultured on MS medium supplemented with different concentrations and combinations of 2,4-D, BAP, NAA and IAA, exhibited varied responses after 2 - 3 weeks of culture (Tables 21 and 22). Profuse nodular calli were produced from both the explants on the above medium. But low concentrations of cytokinin BAP (1.0 mg/l) and auxin IAA (0.5 mg/l) could facilitate medium callus formation from leaf explant (plate 9: C). This hormonal combination (BAP + IAA) was also found to be equally potent, as 2,4-D, for induction of nodular callus and also for regeneration of adventitious shoot buds.

BAP is known to enhance the morphogenic potentialities of callus cultures in inducing shoot bud formation in *Nicotiana tabaccum* (Skoog and Miller, 1957; Gupta *et al.*, 1966). The stimulatory effect of BAP on shoot initiation has also been reported earlier in *Nicotiana* (Prabhudesai and Narayaswamy, 1973), *Crotalaria burhia* (Rajahansali *et al.*, 1978) and also in *Dalbargia latifolia* (Sudhadevi and Nataraj, 1984). In addition, BAP is considered as an active cytokinin and it could release axillary buds from apical dominance in tobacco (Schaeffer and Sharpe, 1969, 1970). Similarly, addition of an auxin IAA at low concentration to the BAP medium could enhance the formation of shoot buds (Mehra and Mehara, 1971; Phillips and Padikkala, 1989; Swarankar and Bohra, 1989; Yong *et al.*, 1996).

In the present investigation too, addition of low level of IAA (0.5 mg/l) and BAP (1.0 mg/l) to MS medium could induce adventitious shoot buds in leaf callus (plate 9 : C). Similar requirement of low level of IAA was reported by Phillips and Padikkala (1989) to transform root meristem to shoot meristem in *Vanilla planifolia*. Higher callusing frequency and regeneration of adventitious shoot buds were obtained on low concentrations of IAA and BAP supplemented media in mulberry (Kathiravan *et al.*, 1997 and Rao *et al.*, 1989). In the present study, adventitious shoot buds when subcultured on BAP (1.0 mg/l) + CH (100 mg/l) medium resulted in shoot formation (plate 10 : A, B). These results are concurrent with earlier reports on other mulberry varieties (Srinivasa, *et al.*, 1997).

Tewary *et al.* (1996) have remarked that shoot differentiation from callus tissue in mulberry was not studied well so far and repeatability of protocols developed are therefore not assured. From the present studies on mulberry var. S₃₆ it has however become clear that the regeneration of an adventitious shoot from leaf callus is not only a genotype dependent problem, but could also be influenced by hormones and culture conditions. This observation is also in conformation with the findings of

Mhatre *et al.* (1985), Rao *et al.* (1989) and Narasimhulu and Chopra (1988) on mulberry and other crops. There is still further scope to enhance the frequency of regeneration of shoots from such leaf callus tissue devoid of meristem towards achieving a healthy reproducible system.

Wide variety of natural extracts have been used to supplement the culture media by several workers. Vanverbeck *et al.* (1941) for the first time used coconut milk (CM) for the successful culture of young *Datura* embryos. Requirement of CM for profuse callusing and its growth in cultures of *Dalbargia latifolia* and *Leucaena leucocephala* was reported by Sudhadevi (1984). The composition of the coconut milk involving aminoacids, vitamins, growth regulating substances *etc.*, has been reported by several workers (Steward and Shantz 1958; Pollard *et al.*, 1961; Tulecko *et al.*, 1961; Van staden and Drews, 1975 a; Dix and Vastaden, 1982). In the present work, supplementation of coconut milk (CM) 20 - 150 % (v/v) led to the moderate callus formation. An optimum concentration of CM at 5 - 10 % (v/v) could induce callus and its growth. However, it was noted that addition of CM was not obligatory to enhance the formation of callus and its growth as compared to the effect of 2,4-D.

It was recorded in the present work that the callus initiation and growth was dependent on type of explants used and the auxin supplemented either singly or in combination with cytokinin (BAP) and also the adjuvants (CM and CH). The leaf explant was proved to be better for indirect organogenesis compared to internodal segment (Table 21).

3. In vitro mutagenesis (involving explants and callus):

The efficiency of mutation breeding *per se* is restricted to some extent by the phenomena of diplontic selection and subsequent chimera formation due to multicellular origin of plants. Such undesirable results can be avoided by *in vitro* mutagenesis. This is because plants obtained adventitiously from cultured explants will usually be of single - cell origin and hence give rise to a pure mutant type avoiding chimerism (Broertjes and Keen, 1980).

Induction of *in vitro* mutants of plants through tissue culture has been reviewed by several authors including Dullieu (1972), Devreux (1973), Street (1974) and recently by Maluszynski *et al.* (1995), Ahloowalia *et al.* (1996). In majority of the cases, the mutants obtained are nonchimeral and it is, therefore obvious that only one cell must have been involved in the formation of plantlet (Skirvin, 1978; Broertjes and Keen, 1980). In *Saintapaulia*, Sparrow *et al.* (1960) have shown that the plantlets formed from irradiated leaves in many of the cases, were either solid mutants or completely normal. Ahloowalia *et al.* (1996) have strongly advocated *in vitro* mutagenesis compared to conventional breeding in crop improvement programmes. Mutagenesis *in vitro* has been investigated for the first time in the mulberry var. S₃₆ with an to induce (possible) beneficent mutants. year?

3.1. Identification of LD₅₀ and LC₅₀ dosages :

In mutation research, parameters such as germination / sprouting and survival percentages would help in determining LD₅₀ values (Kamala and Rao, 1982). In the present investigation, LC₅₀ and LD₅₀ dosages were determined as indices for mutagen efficiency of EMS and gamma rays, respectively for mulberry var. S₃₆ (Tables 6,7 and 8). It was noted that LD₅₀ of gamma rays was 3 kR and 2 kR for nodal explants and shoot apex explants, respectively and similarly, LC₅₀ of EMS was 0.2% for 4 hours treatment on the nodal explants. year??

Although most of the researchers use LD_{50} as an index of mutagen efficiency, Gaul (1960) has suggested the application of the highest possible doses of mutagen to enhance frequency of mutations and also to save the time. Therefore, in the present investigation, shoot apex and nodal explants were treated at LD_{50}/LC_{50} levels of mutagens besides using lower or higher dosages/ concentrations for explant treatments and to recover mutants.

In the present investigation, the combination treatments of gamma rays and EMS were also tried to obtain high frequency of mutation (Table 11). Decrease in the mutation rate was noticed in the combination treatment compared to individual treatment of each mutagen. Chopra and Swaminathan (1966) have also reported reduction in the frequency of viable mutations in the combined treatments. But Favert (1963) and Gaul (1980) have remarked that, though LD_{50} or LC_{50} can be precisely determined for individual mutagenic treatment, it is rather difficult to apply this technique for combined mutagenic treatments. The effect of each of the mutagens in combination treatments can neither be determined nor regulated. Therefore, the effects of each mutagen was studied separately and results are discussed. Swaminathan *et al.* (1970), Mikaelson *et al.* (1971), Prasad (1972), Hussain *et al.* (1974) and Sadanandan (1981) have reported that EMS was found to be highly superior to gamma rays.

3.2. Response of growth in gamma and EMS treated explants (nodal explants and shoot apex) :

In the present study, stimulatory effect of mutagens on the growth of the *in vitro* shoots was recorded both in the nodal and shoot apex cultures at 1.0 kR gamma and 0.2 % of EMS (Tables 9 and 10). The stimulatory effect of lower doses or concentrations of these mutagens was reported earlier by several workers. Saric

et al. (1961) reported that radiation at low doses stimulated growth in some wheat cultivars. Similar observations were made by Khanna and Meharchandani (1981) and Rao (1980) in *Okra and Cyamopsis*. The stimulated growth of carnation plants in low doses of EMS was reported by Singh *et al.* (2000). It was also proved that the low exposure to mutagens cause increased oxygen uptake and organic and inorganic peroxy radicals (Sax, 1955). Therefore, in the present investigation, the enhanced sprouting, survivability and growth rate of variant shoots could be due to the formation of peroxy radicals caused by mutagens.

The higher doses/ concentrations of gamma rays / EMS inhibited the growth of the *in vitro* shoots. Maximum inhibition was observed at 3 kR and above doses. Similarly, treatments with doses of 0.2 % and above of EMS inhibited the growth in both the explants studied.

The downwardly curved relationship between the higher dosage of gamma rays and EMS treatments on the sprouting percentage of M_1 plants (variants) in the present study could be due to an increase in the production of the active radicals, responsible for lethality or gross physiological changes as shown earlier by Brock, (1965). Similarly, Gaul (1964) has opined that this type of retardation of growth at higher doses / concentrations of mutagens could be due to damage of genetic (chromosomal) and non-genetic (physiological) targets. Sinha and Godward (1972) explained that the low survival rate and germination percentage might be due to disturbances caused at physiochemical levels of the cells or acute chromosomal damage or due to the combined effect of both. The results obtained in the present study on retardation of growth in variant plants (of S_{36}) are in agreement with earlier reports by Kaicker and Swarup (1972) and KolontaeV (1973 b, 1974 a,c), who have reported influence of mutagens like DES, NMU, EI, X- rays and gamma rays on growth. They found that the leaf primordia and buds were the source of auxins and

therefore destruction of these cells would cause either lower endogenous auxin level or loss of apical dominance that results in poor growth.

3.3. Response of encapsulated axenic nodal explants:

Encapsulation offers a fairly good system for achieving regeneration in crop species. In the present investigation, encapsulation studies were carried out using young nodal explants derived from *in vitro* shoots of M_1 generation (*i.e.*, gamma and EMS treated ones). These were cultured on MS medium supplemented with optimal concentration of BAP (1.0 mg/l). It was noted clearly that sodium alginate provides a superior matrix for encapsulation compared to agar. This could be due to better permeability of sodium alginate than agar (Molle *et al.*, 1993; Sharief, 1994). It was recorded in the present work that the recovery of new sprouted shoots (M_2) from encapsulated buds was high compared to that from the control (Table 12). It was noted further that the ability to produce shoots and roots was not affected even after three consecutive (3) generations (M_2 to M_4 generations) (Tables 19 and 20). Thus, it is clear from the above study that it is feasible to use *in vitro* encapsulated buds not only for recovery of mutant plants (M_2) but also for significantly enhanced mass propagation. The present results are also in concurrence with the observations of Shen *et al.* (1990). m n y

3.4. Rooting in variant shoots :

Rooting of *in vitro* raised shoots is the most important process for hardening and acclimatization of the tissue cultured plants. In the present study, rooting was induced in both control and variant plants using 1/4 MS basal (hormone free) medium. It was found that this medium was optimal for inducing maximum roots in shoots derived from normal plants (control) (Tables 13 and 14), whereas, the hormonal supplementation was very much essential for induction of rhizogenesis in gamma

and EMS treated shoots (variant shoots) (Tables 15,16,17,18,19 and 20). The latter were actually cultured on 1/4 MS medium supplemented with IBA/ NAA (0.5 - 1.0 mg/l).

Variations in the *in vitro* rooting in mulberry var. S₃₆ on IBA, NAA and also on hormonal free media could be attributed to differential response of induced genotypic variations (Tables 13,14,15,16,17,18, 19 and 20). The results are in agreement with reports of Kathiravan *et al.* (1995) on genotypic variation in *in vitro* root formation among different cultivars of mulberry. Berguran *et al.* (1985) reported that rooting ability is dependent on the genotype, the type of shoot and the number of previous subculture. In the case of micropropagation of different genotypes of mulberry, it was observed that difference in rooting was akin to hormonal requirement among micropropagated plants (Jain *et al.*, 1990; Tewary *et al.*, 1996). Therefore, this kind of differential response of *in vitro* shoots (plants) / variants could be attributed to the different genetic constituents. The results obtained on rooting behaviour in variants (plate 4 and 5 : D) compared to the control (plates 1: C; 2: C) are in concurrence with the above said earlier reports. spelling

The *in vitro* shoots possessing adequate number of roots both in control and variant plants were subjected to hardening under polytunnel. The hardened plants (plate 8 : A,B and C) were then transferred to experimental pots and kept under natural condition for acclimatization and further evaluation.

3.5. Effect of mutagens on callus:

In the present investigation, it is clearly shown that 2 kR gamma and 0.2 % EMS treatment could influence the callus induction to a lesser degree compared to the control. Effect of low dose of gamma treatment in callus cultures have been reported earlier by Bajaj *et al.*, (1970) ; Degani and Pickholz, (1973) ; Rao and

Narayanaswamy, (1977); Werry and Stoffelsen, (1981); Sharma *et al.*, (1983); Novak *et al.*, (1985) and Sehaik *et al.*, (1996). It is apparent that the green shoot bud formation in the calli of internode segment and leaf (plate 7 : C) was due to the effect of lower dosages (1 kR and 0.1%) of mutagenic treatments (Tables 23 and 25). Variation in callus inducing ability and regenerating capacity between the control and mutagenic induced callus was reported in *Triticale* (Reddy and Reddy, 1983). According to them, variability may be due to increased endogenous GA₃ level. Similarly, Moustafa *et al.*, (1989) have reported that the callus growth and regeneration capacity decreased with increasing levels of gamma and EMS. In the present study also, the callus growth was inhibited at higher dosages or concentrations of mutagens thereby affecting regeneration capacity.

4. Isolation and screening of variants in M₁ and M₂ generations:

In the present study, a viable morphological somaclonal variant at M₂ generation from long term shoot apex culture and five (5) mutants from nodal explant treatments (with EMS and gamma rays) were isolated (Tables 27 and 28).

4.1. Isolation and screening of somaclonal variant:

Somaclonal variation is a variation among regenerated plants that occurs as a result of tissue culture of any type and it may arise from pre-existing or induced variation (Evans *et al.*, 1984; Skirvin *et al.*, 1994). In the present study the somaclonal variation (M₂ plants) has been recovered from long term shoot apex cultures. The culture was first raised on high cytokinin medium (BAP 8.0mg/l) and later maintained by series of subcultures on low cytokinin (BAP 1.0 mg/l) supplemented media, up to 24 weeks (Table 3). These *in vitro* shoots were then rooted after 12 weeks of the initial culture.

Several mechanisms have been proposed to account for variability generated under *in vitro* culture system (Karp and Bright, 1985; Larkin and Scowcroft, 1981; Evans and sharp, 1986). In the present work, the somaclonal variation obtained in shoot apex culture could be due to the effect of high concentration of BAP accompanied by long term cultures.

Several workers have reported that the somaclonal variation could be due to various reasons like residual culture conditions (Swartz *et al.*, 1976; Orshinky and Tomes 1984), prolonged culture condition (Bush *et al.*, 1976; Molina and Gracia, 1998) and effect of high or low concentrations of hormonal condition in the medium that could alter the frequency of polidy level verses point mutations (Graybosch *et al.*, 1987; Jain *et al.*, 1989; Mc Clintock, 1984; Evans and Bravo, 1980; Griesbach and Semenluk, 1987; Ashalatha and Seo, 1995 and Pramanik and Datta, 1986).

In the present study, the long term shoot apex culture influenced by high concentration of cytokinin could led to the direct formation of a somaclonal variant in mulberry var. S₃₈. The present findings conform to the earlier reports (Skirvin and Janick 1976; Dhawan and Bhojwani, 1987; Vuylsteke and Swennen, 1990; Vuylsteke *et al.*, 1991, and Skirvin *et al.*, 1994).

The somaclonal variant isolated in the present work was found superior to the parent plant in agronomical traits such as high sprouting, height of the plant, number of branches and rooting parameters (Tables 26 and 28) as well as the biochemical components such as total sugar, phenol and chlorophyll contents (Table 29). These results are in concurrence with the earlier reports in other crops (Orton, 1984; Scowcroft *et al.*, 1985). Shepard (1979) has stated that somaclonal variant of potato was superior to the control plant in terms of number of tubers,

shape, vigour and yield under field condition. Beneficial somaclonal variants as to their increased yield or yield components have been reported in Indian mustard (Jain *et al.*, 1989), sugarcane (Heitz *et al.*, 1977; Liu and Chen, 1978; Krishnamurthy, 1981), potato (Secor and Shepard, 1981), rice (Schaefer *et al.*, 1984) and also in other mulberry varieties (Chakraborty *et al.*, 1999).

4.2. Isolation and screening of mutants:

In the present investigation, details of quantitative traits were recorded in M_1 and M_2 generations of mutant plants pertaining to their morphological traits viz., sprouting %, height of plant, number of branches, internodal distance, leaf area, moisture content of leaf, root induction percentage, number of roots and length of roots. Mean shift in these morphological parameters to positive and negative directions was observed. The results obtained are presented in Tables 27 and 28.

The mutant plants obtained in the present work have expressed high sprouting percentage and more shoot length compared to the control (Table 27). The results of the present study are in concurrence with the results reported in *Plantago ovata* (Sareen, 1999) and also in mulberry (Ramesh, 1997). Some abnormal behaviour was recorded in the present study as to the morphological structures of the stem such as expression of more lateral branches. These abnormalities obtained in *M. indica* var. S_{36} indicate the dichotomy or bifurcation in mutant population probably due to the splitting of the apical meristem or caused by disturbance in auxin contents, resulting in abnormal branching pattern noted in M_1 and M_2 plants compared to control plants. Such abnormal reports in morphological structures are not uncommon in crop plants. In x-ray irradiated vegetatively propagated variety of *Zinnia*, the profuse branching was observed due to active proliferation of axillary shoot buds (Johnson, 1948). Increase in number of lateral branches when treated at lower doses

of gamma rays was also reported in *Capsicum* (Sadanandam, 1981; Rajam, 1982; Christopher, 1989). Further, the forking of stem was noticed in x-ray irradiated apple scions (Bishop and Alders, 1955). The latter also noted that death of group of cells in the critical location in the apical shoot meristem may cause change in polarity resulting in bifurcation. This view was further supported by Subhash and Prolaram (1987) who reported induction of multiple shoots in cotyledon cultures of *C. annuum* using low doses of gamma irradiation.

In the present study it was noted that, mutagens employed could also influence internodal distance (Table 27) in M_1 and M_2 plants of mulberry var. S_{36} . Reddy *et al.* (1975) reported variation in internodal distance following EMS treatment in earlier reports. This feature is well documented in number of other crops like rice (Kuwai, *et al.*, 1961; Guevarra and Chang, 1965; Kawai and Narahari, 1971; Narahari, 1972) and barley (Blongestern and Gale, 1984). Shortened internode in plants (mutants) recovered from physical and chemical mutagen treatments have also been reported in mulberry var. Mysore local and M5 (Jayaramaiah and Munirajappa, 1987; Ramesh, 1997).

The effect of physical and chemical mutagens on leaf parameters such as leaf area, leaf size and shape were reported from time to time by several investigators in various mulberry genotypes (Hazama *et al.*, 1968; Katagiri, 1970; Kuchkarov and Ogurtsov, 1987). Such alteration in leaf characteristics is also recorded in the mutants recovered in the present study. Dandin *et al.* (1996) registered the existence of several spontaneous leaf mutants in mulberry viz., venosa, crinkled leaf, round tipped leaves, glossy leaves, variegated leaves etc. Karpate and Choudhary (1975) noted the occurrence of induced leaf mutants showing curly leaf, irregular shaped leaf and leaves with two or more incisions in *Linum usitatissimum*. In the present study, variation recorded in the leaf area, size and shape in M_1 and M_2 plants in mulberry var. S_{36} were caused by the mutagens employed.

Rooting ability of a variety is purely a genetic character and plays a very important role in the cultivation of vegetatively propagated crops. One of the fundamental considerations in vegetatively cultivated crop is the rooting ability and root initiation (Hartman and Kester, 1959). In the present study, it was observed that rooting parameters such as root induction percentage, number of roots and length of roots increased drastically (Plate 16 : A and B) in M_1 and M_2 plants compared to control plant (Table 28). The results on rooting was in agreement with earlier reports by Abifarin and Rutger (1982) who have described the enhanced growth of rice plant at low dose of gamma ray treatment that triggered root development and also number of secondary roots per seedling. Fugita and Wada (1982) have also reported the possibilities of obtaining useful mutants with high rooting ability in other mulberry varieties following gamma irradiation.

Based on quantitative traits, five mutants in mulberry S_{36} were isolated at M_2 generation in the present study. Mutation frequency in crops is known to be dependent on the acute dose/ concentration of mutagen employed. Sree Ramulu (1970) and Nair (1971) reported that the frequency of mutation was directly related to the concentration of the mutagen employed. In *Capsicum*, a number of mutants were screened following individual physical and chemical mutagenic treatment and also of combination treatments (Sadanandam, 1981; Rajam, 1982; Qasim, 1984 and Christopher, 1989). Such high frequency mutations have also been reported recently in *Vigna mungo* (Raisinghani and Mahna, 1994), *Arachis hypogea* (Venkatachalam and Jayabalan, 1997) and also in *Morus* (Ramesh, 1997; Satyanarayana, 1999). The results obtained on mutants isolated in the present study following gamma and EMS treatments from nodal explants concur with the reports of earlier workers. A notable observation made in the present study is that EMS induced higher mutation frequency than gamma rays when treated individually.

Such higher mutation frequency of EMS treatment was reported in number of plants like legumes (Blixt and Gottschalk, 1975 a), Chillies (Sadanandam, 1981 and Rajam, 1982), *Lycoersicum* (Majid, 1975), wheat (Prasad, 1972) and also in finger millet (Bindokumar *et al.*, 1996).

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Variations noted especially in isolated M₂ plants as to their plant growth and branching pattern following mutagenic treatments are important and significant observations in the present study. Vargheese and Swaminathan (1968) reported a number of viable mutants with altered plant height and branching pattern. Tall, dwarf, high yeilding and vigorous mutants have also been recovered from chemical treatments in *Capsicum* (Christopher, 1989). Raisinghani and Mahna (1994) have reported mutation affecting plant height and number of branches of *Vigna mungo* and also in *Arachis hypogea* (Venkatachalam and Jayabalan, 1997). Similar observations have also been made in other mulberry varieties (Jayapal Rao *et al.*, 1984; Ramesh, 1997; Satyanarayana, 1999).

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Changes/ alterations in the leaf characters like leaf area, shape and size recorded in mutant plants isolated (at M₂ geneeration) following mutagenic treatments (Plate 15 : A and B) are significant. The abnormalities observed by earlier workers in the leaves of different taxa following mutagenic treatments have been attributed to various other causes such as disruption of phytochrome systems, chromosomal aberration, mitotic inhibition, disrupted auxin synthesis, enlargement of mesophyll cells, disturbances in DNA synthesis, *etc.*, (Irvine, 1940; Decmerc, 1954; Gunckel and Sparrow, 1954; Beibl, 1956; Mutsumura and Fujii, 1957; Love, 1960; Meiseloman *et al.*, 1961; Moh, 1962; Haber and Ford, 1962; Mikaelson *et al.*, 1968; Abraham and Ninan, 1968). The results observed are in concurrence with the earlier reports in other mulberry varieties where alternations

in leaf area, shape and size viz., rolled, biforked, mottled leaves etc., have been recorded (Hazama *et al.*, 1968 ; Katagiri., 1970 ; Kuchkarov and Ogustsov, 1987 and JayapalRao *et al.*, 1984).

Rooting ability of a variety is purely a genetic character and plays a very important role in plant survivability and also for cultivation and productivity. One of the fundamental considerations in vegetatively cultivated crop is the rooting ability and root initiation (Hartman and Kester, 1959). In the present study, it was observed that, the rooting ability measured in terms of root induction percentage, number of roots and length of roots has increased drastically in mutants (Plate 16: A and B) over the control plant (Table 28). The results recorded in the present study are in agreement with earlier reports of Abifarin and Rutger (1982), who have also described the enhanced growth of rice plant at low dose of gamma ray treatment that triggered root development including increase in number of secondary roots per seedling. Fugita and Wada (1982) have also reported the possibilities of obtaining useful mutants with high rooting ability in mulberry following gamma irradiation. Therefore, based on the quantitative traits expressed following mutagenic treatments, five (5) solid mutants have been isolated at M₂ generation in the presently worked out mulberry var.S₃₆. These five mutants have been described briefly in earlier chapters (Plates 12: B (ii); 13: A (ii), C (ii); 14: A (i) and C).

5. Evaluation of somaclonal variant and mutants:

During the course of present investigations, one somaclonal variant and five (5) solid mutants that were isolated/ selected were evaluated as to their morphology, ultra structural aspects of leaves, cytology and also biochemical parameters.

5.1. Morphological variations:

The somaclonal variant plant selected in mulberry variety S₃₆ has exhibited certain improved morphological characters like better sprouting percentage, increase in height of the plant, number of branches, improved leaf characters and better rooting ability (Plate 11: A, B; Plate 16: A) compared to that in control (Table 26 and 28). The results obtained in the presently isolated somaclonal variant of the mulberry var. S₃₆ agree with earlier reports on somaclones in several crop species (Orton, 1984; Scowcroft *et al.*, 1985; Jain *et al.*, 1989; Heinz *et al.*, 1977; Krishnamurthy, 1981, Schaeffer *et al.*, 1984 and Chakraborty *et al.*, 1999). 77
spelling

The five mutants selected in the present study were screened (Plates 12: B (ii); 13: A (ii), C (ii); 14: A (i) and C). It was recorded that each mutant was different from the other one in their morphological characters (Table 27 and 28). The increase in the shoot height, number of branches, leaf area and rooting ability were recorded in all the five mutants compared to the control. Similarly, reduction in internodal distance was also noted. The results obtained in present investigation concurr with the earlier reports on mutants obtained in *Capsicum* (Christopher, 1989), *Vigna mungo* (Raishinghani and Mehna, 1984), *Arachis hypogea* (Venkatachalam and Jayabalan, 1997) and also mutants obtained in other mulberry varieties (Hazama *et al.*, 1968; Katagiri, 1970; Fugita and Wada, 1982, Jayapal Rao *et al.*, 1984; Ramesh, 1997 and Satyanarayana, 1999). ✓

5.2. Ultra structural variations:

In the present study, some ultra structural changes in leaves of somaclonal variant and mutant plants were recorded (plates 17a and 17 b: A,B,C,D,E,F,G,H,I,J,K,L,M and N). Attempts have been made earlier to differentiate mulberry species based on the shape of idioblasts, the length and density of trichome

(Hotta, 1951; Katsumata, 1972; Koidzumi, 1917 and Fugita and Uchikawa, 1986). In the present investigation, the idioblasts were found distributed only on abaxial surface of leaves and also exhibited multiformity. Clear differences of idioblasts in control, somaclonal variant and mutant plants were recorded. The present result is in conformity with the results of Minamizawa (1976). But Fugita and Uchikawa (1986) have reported idioblasts on both the surfaces of leaves. The presence of more trichomes with varying size and shape on the leaf surface in the somaclonal variant and in all the five mutants of mulberry var. S₃₆ could be due to the effect of mutagens inducing such changes.

5.3. Cytological variations:

Cytological investigation in somaclonal variant and mutants of S-36 variety presently showed no change in their chromosome number as exhibited by the diploid chromosome number ($2n=28$) (plates 18a and 18b : B,C,D,E,F and G) in all the cases as compared to the control plant (plate 18a : A). Similar results have been reported in some crops such as lotus (Orshinsky and Tomes, 1985), rice (D'Amato, 1978) and also in *Hordeum* (Bayliss, 197; Sacristan, 1971) and Sacristan, 1967). In *Sorghum*, callus regenerants (somaclones) were reported to have normal chromosome complement (Gamborg *et al.*, 1977). No deviation from the normal chromosome number was also detected in potato protoclones (Shepard *et al.*, 1980). It is also reported that chromosome loss or addition was not evident as the primary cause of the variation (Larkin *et al.*, 1984; Ashalatha and Seo, 1995). Further, Konzak (1984) have advocated the use of chemical mutagens like EMS and many other compounds as they should induce (relatively) more point mutations and a few chromosome aberrations. Therefore, the phenotypic variation in the somaclone and mutants obtained in the present study could be linked to a possible change due to recessive or non - recessive mutation of nuclear genes. Such explanations were

also offered by Orshinky and Tomes (1985). Hence, further detailed genetic studies at the molecular level is necessary to arrive at definite conclusions.

5.4. Bio-chemical variations:

A somaclonal variant and five induced mutants of mulberry variety S₃₆ exhibited a marginal increase or decrease in the mean value of some biochemical traits such as nitrogen, sugar, phenol and chlorophyll contents as compared to the control (Table 29 and 30).

It is an established fact that the biochemical constituents of mulberry leaves viz., proteins, total sugars (carbohydrates), free amino acids, chlorophyll, minerals, vitamins and sterols are important from the nutritional point of view to the silkworm (Ito and Arai, 1963; Ito and Nimura, 1966 and Machii and Katagiri, 1991). Protein is the main constituent of mulberry leaf which plays a vital role in the development of silk gland (Anfinson *et al.*, 1958; Fukuda *et al.*, 1959; Takeuchi, 1960 and Qader, 1987). It has been proved that the silkworm derives over 70% of the proteins from the mulberry leaves in the biosynthesis of silk (Fukuda *et al.*, 1959; Kawase, 1975). Free amino acids and total soluble sugars are also required for the synthesis of fibroin, Serecin and fatty acids. Moisture content of leaves determines overall nutritive quality of leaves and plays an important role in the food assimilation, healthy growth and development of the silkworm larvae (Narayan *et al.*, 1967; Parpiev, 1968; Krishnaswami *et al.*, 1970; Koul *et al.*, 1980; Chaluvachari, 1995).

(a) Leaf moisture content:

In the present investigation, marginal decrease has been recorded in the moisture content of leaves (71.39 %) in somaclonal variant compared to that of control (72.09%), while the moisture content was noted to decrease further in mutants

the range being 65.03 - 70.05 % compared to that in control (71.81%). Roarke and Quisenberry (1977) reported that inheritance of water retention capacity in F1 and F2 generations of cotton is under the influence of additive and dominant genes with an estimated narrow gene heritability of 25%. The result of present investigation is in agreement with the report of earlier workers in other mulberry varieties (Dwivedi *et al.*, 1987 ; Bose, 1989 ; Sikdar, 1990; Susheelamma *et al.*, 1991; Chakraborty *et al.*, 1999).

(b) Other bio-chemical parameters:

The other bio- chemical constituents investigated in the present study such as total nitrogen , sugar and chlorophyll showed marginal increase in the somaclonal variant and also in mutant -1, whereas, the rest of the mutants showed decrease in nitrogen content compared to the control (Table 29 and 30). The variation in the biochemical parameters mentioned above was due to the effects of mutagens employed in the present study. There was also an increase in chlorophyll content both in the somaclonal variant and mutants, except mutant -3 which showed decrease in chlorophyll content. The increase in chlorophyll contents in the variants can be directly correlated to the enhanced photosynthetic activity which in turn results in the improved leaf yield. The increased chlorophyll content in variants obtained in the present investigation are in agreement with the earlier report on the somaclonal variant of another mulberry variety S₁ (Chakraborty *et al.*, 1999).

Phenolic contents in the leaves of crop plants play an important role in inducing resistance against disease (Lily and Ramadasan, 1979; Sharma *et al.*, 1983). The phenols may involve in the metabolism of plant either with their oxidative products toxic to pathogens (Kosuge, 1969) or by their direct influence on other metabolic processes (Zenk and Muller, 1963). So far, however, there was no report

on phenols from mutant plants in mulberry. The present findings are the first reports on phenolic content in the induced mutants. In the present study an increase in the phenolic content in somaclonal variant was recorded. Similarly, among all the five induced mutants, only the mutant-4 showed considerable increase in phenolic content (Tables 29 and 30). This enhanced phenol content is reported to increase tolerance to fungal disease in crop plants (Chattopadhyay, 1989). Similarly, Reddy *et al.* (1987) have reported the increase in phenolic contents in castor plants due to gamma irradiation. The increase in phenolic contents in plant system could lead to the production of disease resistant plants. However, probe as to the effect of phenols, need further investigation to ascertain disease resistance in mulberry.

(c) Pattern of protein bands variability :

Variability in banding pattern obtained after SDS - PAGE of leaf protein was used as marker to demonstrate induced variation among the somaclones and also in mutants [plate 19 : M1, M2, M3, M4 and SV (somaclonal variant)] (Table 31). It is well known that, electrophoretic banding pattern of buffer soluble protein showing presence or absence of specific soluble protein bands can be used to establish induced variabilities in plants (Hirano, 1980; Chandel *et al.*, 1998; Nayeem *et al.*, 1999).

In mulberry, the leaf and its biochemical contents have a considerable influence on the nutrition and growth of the silkworm. Improvement of the leaf quality, which has been a breeding objective is probably determined mainly by the protein quantity and quality (Hirano, 1980). However, the available literature shows that the studies on leaf protein profile is poorly understood in mulberry crop (Iizuka and Arakawa, 1979; Hirano and Naganuma, 1979; Hirano, 1982; Absar, 1995). Attempts have been made in the induced variants of mulberry in the present study to understand the banding pattern of protein for the first time. A few notable differences in the

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frequency of banding patterns are recorded in the somaclonal variant and also in all the five mutants of mulberry variety S₃₆. Based on presence or absence of specific bands in the said induced variants compared to the control, it is possible to identify the protein profile in leaf. Similar work on differences in protein profile especially as to the stress tolerant proteins have been reported in albino plants derived from Indica rice varieties by Chandel *et al.* (1998) and also differences in seed proteins caused by recurrent radiation in wheat (Nayeem *et al.*, 1999). However, further probe is needed to identify specific protein bands in the induced mulberry variants.