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RAPID AND LARGE SCALE MICROPROPAGATION OF TRUE TO TYPE CLONE OF *MUSSAENDA ERYTHOPHYLLA* SCHUM AND THOM CV. SCARLET THROUGH SOMATIC EMBRYOGENESIS

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ABSTRACT

A somatic embryogenesis was attempted for 'scarlet' variety of *Mussaenda*. MS medium containing 2mg/l BAP, 0.5mg/l NAA and 100mg/l L-glutamine produced the best amount of embryogenic callus. Transfer of differentiating calli into the medium containing lower concentrations of cytokinin and auxin and addition of 50mg/l CH and 100mg/l L-glutamine resulted into the development of cotyledonary stage embryoids. Maturation of somatic embryoids and subsequent plant regeneration was accomplished in MS medium supplemented with L-glutamine (100mg/l) and CH (50mg/l).

Isoenzyme studies on peroxidase exhibited no differences between donor and *in vitro* regenerated plants. Cytological analysis also did not reveal any marked differences either in chromosome number or structure between the donor and *in vitro* regenerated plants.

Key words: Medicinal plant, *Mussaenda,* Somatic embryogenesis.

Introduction

Mussaenda erythrophylla Schum and Thom cv. scarlet (Rubiaceae), an ornamental shrub, is valued for its gorgeous terminal trusses of flowers. It is a native plant of Africa. Three different cultivars of this species are horticulturally important. The colour of the petaloid calyx lobes varies from white, yellow, pink to red depending upon the variety. The different parts of this plant viz. leaves, roots, flowers as well as the whole plant are used in curing coughs, ulcers and leprosy possibly, due to the presence of saponin (Jasrai *et al.,*1999). Of these, the 'scarlet' has a high market demand because of its attractive red bracts. The scarlet compared to the two other cultivars, namely 'Queen sirikit' and 'Rosea', is extremely slow growing and is difficult to be propagated by conventional methods. It is usually propagated by grafting on to the rootstock *M. philippica,* or the 'Rosea' and 'Queen sirikit' cultivars of *M. erythrophylla* (Bose & Chowdhary, 1991). The development of an *in vitro* system for the propagation of this elite cultivar is desirable. Therefore, the present investigations was undertaken to establish a suitable approach for somatic embryogenesis and to detect true-to-type nature of donor plants and regenerated plants by chromosome study and isozyme analysis.

Material and Methods

Leaves of *M. erythrophylla* var. 'scarlet' were taken as explants collected from donor plants grown in the experimental garden. After excision, the explants were subjected to preliminary washing under running tap water for 10 to 30 minutes to remove the microflora

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to a substantial extent. Healthy and uniform explants were disinfected thoroughly in 5% savlon solution for 8 minutes. Explants were then rinsed under running tap water. Then they were sectioned into a suitable size (0.5-1 cm) and surface sterilized with 0.1% mercuric chloride solution for 8-10 minutes, followed by 6-8 washes in sterile distilled water. For induction, growth and maintenance of callus tissues, Murashige and Skoog's (1962) basal medium (MS) was used.

The explants were cultured in the MS medium supplemented with different combinations of growth regulators such as 6-benzyl amino purine (BAP), α-naphthalene acetic acid (NAA), indole acetic acid (IAA), indole butyric acid (IBA), L-glutamine and casein hydrolysate (CH). The MS medium also contained 3% sucrose and 0.5% (w/v) agar. The medium pH had been adjusted to 5.6 before it was autoclaved at 121°C for 15 minutes. Cultures were grown at 22-24°C with a relative humidity of 50-60% and a photoperiod for 16h per day provided by fluorescent tube (12000 lux). Each treatment had 6 replicates. Healthy rooted plantlets were taken from the rooting medium and washed several times with sterile distilled water. Plantlets were potted in sterile sand:loam:peat (in a ratio of 1:1:1) mixture, covered with a polythene bag to maintain high humidity and were kept under contrølled temperature at 22-26°C and light (12000 lux) conditions in the culture room. The bags were removed periodically for gradual hardening. After 4-6 weeks when new leaves emerged from such plantlets, they were taken outside the culture room and kept in a shady place under normal temperature and light.

Biochemical studies:

Isozyme (Peroxidase) analysis was performed by macerating 1 gm leaf material collected from mature plants and two months old tissue culture raised plants growing in the same environment. The leaves were excised and kept in -70°C for some days to avoid chlorophyll interference. The materials were crushed in an ice-cold mortar pastle with ice-cold PEB buffer. After centrifugation, the pellet was discarded and the supernatant was lyophilized for 10-12 hours as required. SDS-PAGE was performed using 12% polyacrylamide gels. After polymerization 150 µg of protein samples were loaded to each well with the help of a micropipette fitted with multiflex tips. When the run was completed, the stacking gel part was cut off and the rest was incubated in buffer and substrate solution of Isozyme. Guaicol- H_2O_2 method by Hislop and Stahmann (1971) was followed for the visualization of peroxidase isozymes.

Cytological studies:

The root tips of *M. erythrophylla* var. 'scarlet' (from donor plants and regenerated plants through callus culture) collected from mature plants were pretreated with saturated solution of PDB for 4 hours and the chromosome preparation were made using aceto-orcein method.

Observations:

The explants were inoculated in MS medium with various concentrations of BAP: (0.5- 3.0) mg/l, NAA: 0.5 and 1.0 mg/l and L-glutamine: 100 mg/l for callus initiation and subsequent differentiation. The leaf derived greenish to greenish yellow, moist and compact differentiated calli were maintained by subculturing at an interval of 2-3 weeks. After 4 weeks the differentiated calli were again subcultured on MS medium supplemented with lower concentrations of BAP-NAA along

with other growth regulators such as L-glutamine and CH for the development of the embryoid. The calli containing embryoids in the cotyledonary stage were then transferred to MS medium augmented with L-glutamine (100 mg/l) and CH (50 mg/l) for the maturation of somatic embryoids and plant regeneration. Somatic embryos developed into plantlets on growth regulator free MS medium.

The leaf explants were used for the induction of callogenesis of 'scarlet' variety of *Mussaenda*. Initially callus formation was observed from the cut ends and the epidermal surfaces of the young leaves as protuberances within 10-12 days (Fig 1). The whole explants were gradually covered by callus within 40 days of culture. BAP in the range of 0.5-3.0 mg/l, NAA 0.5-1 mg/l and L-glutamine 100 mg/l in different combinations were tested for callus induction. Of all the combinations and concentrations of growth regulators tested, MS medium supplemented with BAP (2 mg/l), NAA (0.5 mg/l) and L-glutamine (100 mg/l) proved to be the best with regard to callus formation and their further development into embryoids (Fig 2 and 3). The explants cultured in higher concentrations of BAP (3 mg/l), NAA (1 mg/l) and L-glutamine (100 mg/l) did not initiate callus and the explants began to become brown within 30 days. The globular structures appeared on the surface of the pro-embryogenic callus gradually became enlarged to a detectable size within 10 weeks of culture.

Visual identification and selection of embryogenic sectors and removal of non-embryogenic portions maintained the embryoids developed from friable callus during subcultures at two weeks interval. The maximum number of globular embryoids was recorded (62.3±0.2) on the MS medium containing 1.0 mg/l BAP+0.5 mg/l NAA+100 mg/l L-glutamine+50 mg/l CH (Fig 4) after 15 weeks of culture (Table 1).

Table – 1. Effect of BAP and NAA at different concentrations and combinations on somatic embryo multiplication in leaf derived 'scarlet' variety of *Mussaenda* cultured on MS medium containing L-glutamine (100 mg/l). Results are the mean of 6 replicates±SE.

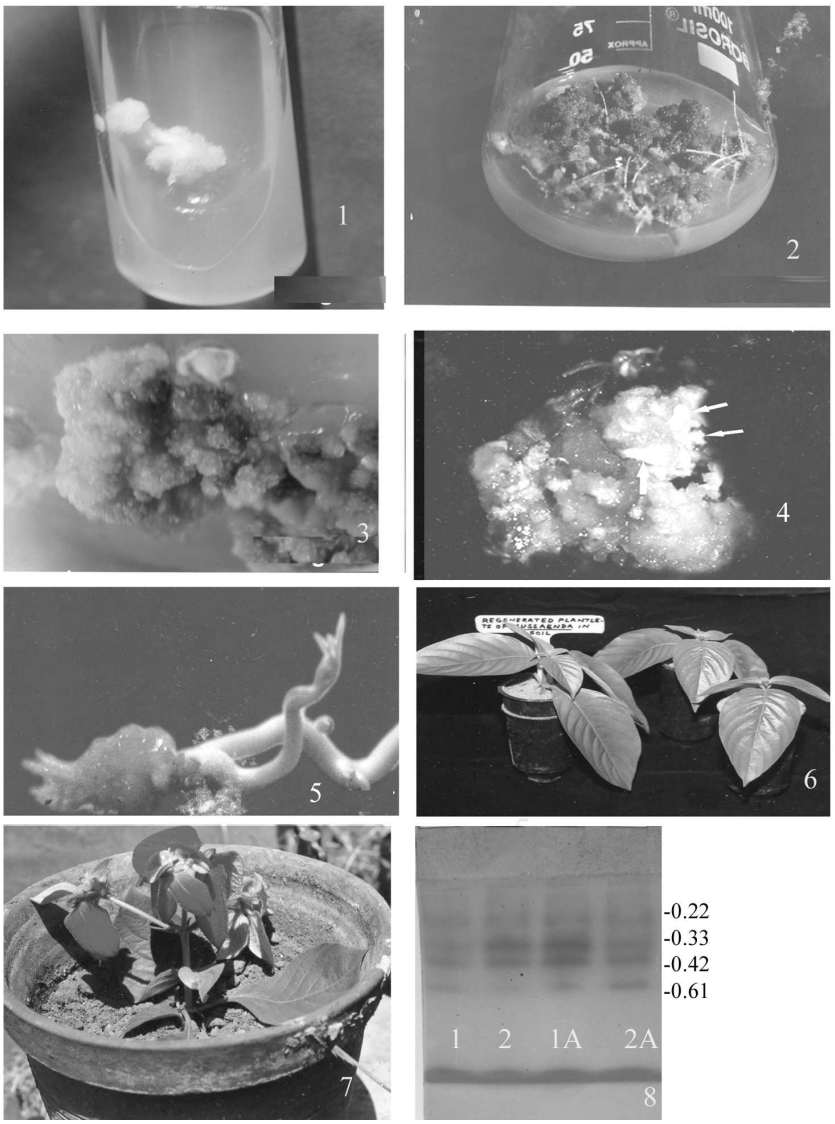
MS basal medium		Total no. of embryoids
BAP(mg/l)	NAA(mg/l)	
0.5	0.5	20.33±0.2
1.0	0.5	62.33±0.2
1.5	0.5	53.83±0.2
2.0	0.5	32.67±0.4
2.5	0.5	20.33±0.4
0.5	1.0	23.67±0.8
1.0	1.0	-

The callus containing globular embryos grew rapidly when subcultured in MS medium augmented with 1 mg/l BAP, 0.2 mg/l NAA, 100 mg/l L-glutamine and 50 mg/l CH and produced embryoids of cotyledonary stages (Fig 4).

Cotyledonary-stage somatic embryos were separated individually, and transferred to MS medium containing 100 mg/l L-glutamine and 50 mg/l CH for maturation. In this medium the cotyledonary-stage somatic embryos reached physiological maturity, they lost their green colour, and acquired a creamy yellow colour.

Somatic embryos developed into plantlets on growth regulator free MS medium (Fig 5). Germination with simultaneous development of root and shoot resulted in whole plant regeneration. The regenerated plantlets with well developed root systems were removed from the culture medium. They were subsequently hardened and acclimatized. The *in vitro* raised shoots grew vigorously and developed without any visible deformities (Fig 6). An almost 70% survival of the transplanted plantlets (Fig 7) of 'scarlet' variety of *Mussaenda* was observed in field.

In peroxidase isozyme, three bands were observed in the upper (+ve) region of the gel



Somatic embryogenesis of *Mussaenda erythrophylla* var. 'scarlet':

Fig. 1. Induction of callus from leaf segments on MS medium containing BAP (2.0 mg/l)+ NAA (0.5 mg/l)+L- glutamine (100 mg/l).

Fig. 2. Profuse growth of callus on MS medium containing BAP (1.0 mg/l)+NAA (0.2 mg/l)+L-glutamine (100 mg/l) +CH (50 mg/l) within 8 weeks of culture.

Fig. 3. Emergent globular embryos from the light yellow callus on MS medium containing BAP (1.0 mg/l)+NAA (0.5 mg/l)+L-glutamine (100 mg/l)+CH (50 mg/l) within 8 weeks of culture.

Fig. 4. Mature embryos of different shapes isolated from yellow green embryogenic cluster.

Fig. 5. Germinating embryos in growth regulator free MS medium.

Fig. 6 & 7. Acclimatized plantlet showing normal flowering.

Fig. 8. Peroxidase isozyme pattern.

1&1A=donor plants of *M. erythrophylla* var. 'scarlet'.

2&2A= *in vitro* raised plants of *M. erythrophylla* var. 'scarlet'.

(Fig 8). The Rf values of three bands were 0.22, 0.33 and 0.42 respectively. A fourth band was observed in the intermediate region with a Rf value of 0.61. No band was detected in the lower region (-ve).

Both the donor plants and regenerated plants had 2n=24 chromosomes. Difference in chromosome morphology between donor plants and tissue culture raised plants of 'scarlet' variety of *Mussaenda* were not detected.

Discussion:

Although MS medium containing 2.0 mg/l BAP, 0.5 mg/l NAA and 100 mg/l L-glutamine induced somatic embryogenesis in 'scarlet' variety of *Mussaenda* was used in the present study, the concentrations of cytokinin and auxin are also known to be critical in the induction of differentiating callus and their subsequent proliferation. Mostly auxins or substances having auxin like activity were reported to be effective for inducing somatic embryogenesis in different plants (Carman, 1990; Eapen & George, 1992; Craig *et al.,* 1997). The auxin-cytokinin combination, which was found to be effective in inducing somatic embryogenesis in the present investigation, was also used in some species including *Mussaenda philippica* var. aurorae for the induction of somatic embryogenesis (Eapen & George, 1992).

BAP, which has been used as a source of cytokinin in the present study has also been used successfully to induce somatic embryogenesis in several plants (Chand & Singh, 2001; Anand *et al.,* 2001; Prakash *et al.,* 2001).

We have observed that the low concentration of auxin (0.2 mg/l NAA) was needed for the proliferation of the differentiated calli although higher concentration (0.5 mg/l NAA) was necessary for the initiation of the callus. According to Zimmerman (1993), removal of auxin results in the inactivation of a number of genes so that the embryogenesis program cannot proceed further.

The use of L-glutamine in the embryo proliferation medium was very critical and effective at a concentration of 100 mg/l. Increase in its concentration (more than 100 mg/l) in the medium restricted embryo proliferation. The nitrogen content of the culture medium has been reported to influence the morphogenic effects of growth substances. L-glutamine that has been reported critical for embryogenesis among the amino acids used as a source of nitrogen (Shetty & McKersie, 1993; Sinha *et al.,* 2000) was also found to be effective nitrogen containing compound for somatic embryogenesis in 'scarlet' variety of *Mussaenda*.

The method of embryogenesis described in the present investigation demonstrates the potentiality of the leaf explants for generating highly proliferative, embryogenic cultures for *Mussaenda* variety. Since *Mussaenda* is a medicinal plant, this approach can be useful in maintaining the germplasm of the genus for future conservation.

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