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*Research note*

# Propagation of mulberry variety – $S_{54}$ by synseeds of axillary bud

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## Abstract

Synseeds were produced from aseptic axillary buds of mulberry variety – $S_{54}$ (*Morus indica* L.) to investigate their *in vitro* and *ex vitro* conversion potential. The synseeds when cultured on Linsmaier and Skoog's basal medium supplemented with 6-benzyl amino purine (8.88 $\mu$M) and 2,3,5-tri iodo benzoic acid (2 $\mu$M), produced shoots after 21 days exhibiting $48.2 \pm 0.60$ *in vitro* conversion response. The synseeds *ex vitro* conversion response was found to be $45.5 \pm 0.76$ on soilrite mix containing half strength LS nutrients after 21 days of sowing. Further, synseeds stored at 4 °C for 1–4 months resulted in maximum conversion response under both *in vitro* and *ex vitro* conditions, followed by development into complete plantlets without any significant loss of conversion potential. Plants regenerated from the synseeds of axillary bud were hardened, acclimatized and established in soil with varying survival frequency.

*Abbreviations:* BAP – 6-benzyl amino purine; LSBM – Linsmaier and Skoog's basal medium; Synseed (s) – synthetic seed (s); TIBA – 2,3,5-tri iodo benzoic acid

Mulberry (*Morus* spp.) plays a significant role in the silk industry due to its foliage, which constitute the chief food for the silkworm. Conventional methods of propagating mulberry through stem cuttings or grafting or seed propagation are beset with numerous problems (Ohyama and Oka, 1987). Under these circumstances, synthetic seed technology would be a potential tool for a more efficient and cost-effective rapid clonal propagation system (Kavyashree et al., 2004). The use of synthetic seed technology for mulberry propagation is limited to a few species of *Morus indica* (Bapat and Rao, 1990). The possibility of using *in vitro* derived vegetative propagules for synseed production has been explored by researchers since somatic embryogenesis has not been documented in mulberry (Pattnaik and Chand, 2000; Kavyashree et al., 2004). Further, adequate efforts have not been made to explore the possibility of storing synseeds derived from vegetative propagules at low temperature for long duration (Fujii et al., 1987). In addition, the most important requirement for synseeds to be used for mass clonal propagation is high uniform conversion under non-sterile conditions (Fujii et al., 1993).

Hitherto, there is no report on the development of synseed system for clonal propagation of mulberry variety – $S_{54}$ using *in vitro* axillary bud. This investigation reports optimized parameters for the production and conservation of synseeds to study their conversion potential under *in vitro* and *ex vitro* conditions.

Shoot cultures were established using vegetative propagule axillary bud excised from *in vivo* ten years old mature plants of mulberry variety – $S_{54}$ on Linsmaier and Skoog's basal medium (LSBM) supplemented with 6-benzyl amino purine (BAP) (8.88 $\mu$M) and 2,3,5-tri iodo benzoic acid (TIBA)

(2 $\mu$M). Axillary buds excised from axenic shoot cultures measuring 0.5 cm long were used for encapsulation. Sodium alginate and Carboxy methyl cellulose were added in the range of 2, 4, 6 and 8% (w/v) to liquid initiation medium separately. For complexation, $25\times10^3$ $\mu$M, $5\times10^4$ $\mu$M, $75\times10^3$ $\mu$M and $10\times10^4$ $\mu$M calcium chloride solutions were prepared using distilled water. Both the gel matrix and complexing agents were autoclaved at 121 °C for 15 mins after adjusting the pH-5.8. Gel complexation was done by mixing the axillary buds with hydrogels, dropping these into different concentrations of calcium chloride solution and incubated in orbital shaker for different time intervals (20, 30, 40, 50 and 60 mins) to obtain uniform beads. The sodium alginate and carboxy methyl cellulose embedded axillary buds were collected using a sterilized tea strainer and rinsed 2–3 times in sterile water to remove traces of calcium chloride. The synseeds were tested for their conversion potential under *in vitro* and *ex vitro* conditions.

Fifty synseeds were cultured on LSBM supplemented with BAP (8.88 $\mu$M) and TIBA (2 $\mu$M) and maintained under culture conditions in order to retrieve complete plantlets (for solidification agar agar (Hi Media) was used at 0.8% concentration). A group of 50 synseeds were sown in petridishes containing horticultural grade soilrite mix – peat:perlite:vermiculate (1:1:1– v/v) supplemented with half strength Linsmaier and Skoog's (LS) nutrients under non-aseptic conditions. The petridishes were covered initially with lids and maintained in the culture room. Further, every alternate day, soilrite mix was irrigated with half strength LS nutrients until initiation of shoot. After shoot emergence from the synseeds, the lids of the petridishes were removed and maintained till the development of shoots and roots. A group of 50 synseeds were stored in sterile water at 4 °C for 1–6 months in seed modulator for *in situ* conservation. The synseeds were tested for their conversion potential every month for a period of 6 months by following two methods:

– *in vitro* culturing on LSBM supplemented with BAP (8.88 $\mu$M) and
– TIBA (2 $\mu$M) and *ex vitro* sowing in petridish containing horticultural grade soilrite mix – peat:perlite:vermiculate (1:1:1–v/v) supplemented with half strength LS nutrients under non-aseptic conditions.

All the cultures were maintained in culture room at a temperature of $25\pm2$ °C with photon flux density of 30–50 $\mu$molm$^{-2}$s$^{-1}$ under a photoperiodic regime of 16 h light and 8 h dark cycles. The experimental data was analysed statistically, in each experiment 6 replications were used and each experiment was repeated three times. The regenerated plantlets from synseeds under *in vitro, ex vitro* conditions and also from the conserved (1–6 months) seeds were transferred to pots containing horticultural grade soilrite mix. For hardening and acclimatization, these plantlets were maintained in green house at temperatures of $28\pm1$ °C during day and $22\pm1$ °C during night with irradiance of 500 $\mu$molm$^{-2}$s$^{-1}$ under a photoperiodic regime of 16 h light and 8 h dark cycles and Relative Humidity (RH) between 85–90%. The hardened and acclimatized plants were established in soil and their survival frequency was recorded.

Of the two gel matrices, carboxy methyl cellulose failed to complex with calcium chloride, producing only soft synseeds in all the concentrations tested. This is perhaps due to differential purity in the mannuronic acid:guluronic acid ratio as described by Redenbaugh et al. (1986). However, the 4% sodium alginate, upon complexation with $5\times10^4$ $\mu$M calcium chloride with ion exchange duration of 40 mins, produced firm, clear and uniform synseeds (Figure 1a). This corroborates the findings of Pattnaik and Chand (2000) who reported that axillary bud synseed formation is influenced by the concentrations of the gel matrix and complexing agent in mulberry. Lower concentration of sodium alginate (2%) was not suitable because the resultant synseeds were too soft to handle. Higher concentrations of sodium alginate (6 & 8%) were also not preferred because the resultant synseeds were very hard and took long duration to germinate as reported by Kavyashree et al. (2004). In addition, lower ($25\times10^3$ $\mu$M) and higher ($75\times10^3$ $\mu$M and $10\times10^4$ $\mu$M) concentrations of calcium chloride with short (20 and 30 mins) or long (50 and 60 mins) durations not only prolonged the ion exchange time but also affected the synseeds quality producing soft or hard beads respectively. This agrees with the findings of Mathur et al. (1989) and Pattnaik and Chand (2000).

The conversion response of the synseeds (Figures 1b, c) cultured on LSBM supplemented with BAP (8.88 $\mu$M) and TIBA (2 $\mu$M) was found to be $48.2\pm0.60$ (Table 1). Further, maximum

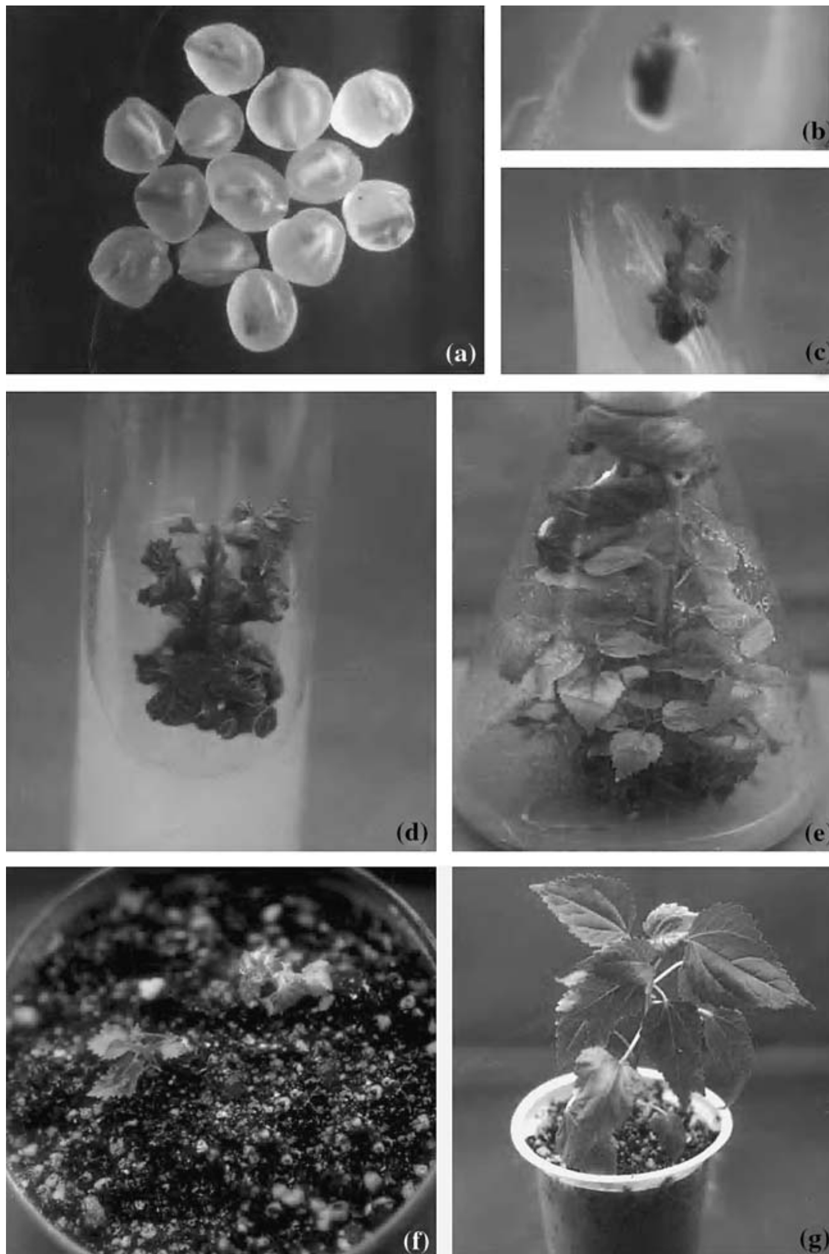


*Figure 1.* Propagation of axillary bud synseeds of mulberry variety – $S_{54}$. (*a*) Axillary bud synseeds of Mulberry variety – $S_{54.}$ (*b*) *In vitro* conversion of synseed into shoots. (*c*) Single shoot formation. (*d*) Multiple shoot formation. (*e*) Multiplication of shoots and roots. (*f*) *Ex vitro* conversion of synseed into shoot and root. (*g*) Hardened plantlet derived from synseed.

number of multiple shoots ($15.5 \pm 0.76$) with roots ($6.8 \pm 0.61$) was achieved after 40 days of subculture on the same medium (Figure 1d, e). This corroborates with the findings of Mathur et al. (1989), Sharma et al. (1994) and Kavyashree et al. (2004).

The synseeds after 21 days of sowing in horticultural culture grade soilrite mix – peat:perlite:vermiculate (1:1:1-v/v) containing half strength LS nutrients initiated shoot with $45.5 \pm 0.76$ conversion response (Table 1). After 30 days, the development of shoots with gradual emergence of roots, which penetrated into the soilrite mix was noticed (Figure 1f). Rapid development of plantlets with maximum number of

*Table 1. In vitro* and *ex vitro* conversion response of synseeds into multiple shoots, roots and survival frequency of retrieved plants of Mulberry variety – $S_{54}$

| Duration (months) | *In vitro* | | | | | *Ex vitro* | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Conversion response $\bar{X}^* \pm$ SE | Culture duration | No. of shoots $\bar{X}^* \pm$ SE | No. of roots $\bar{X}^* \pm$ SE | Survival frequency (%) | Conversion response $\bar{X}^* \pm$ SE | Culture duration | No. of shoots $\bar{X}^* \pm$ SE | No. of roots $\bar{X}^* \pm$ SE | Survival frequency (%) |
| 0 | 48.2 ± 0.60 | 15 days | 15.5 ± 0.76 | 6.8 ± 0.61 | 92 | 45.5 ± 0.76 | 21 days | 11.6 ± 0.66 | 7.7 ± 0.76 | 81 |
| 1 | 46.5 ± 0.76 | 15 days | 14.8 ± 0.60 | 4.5 ± 0.76 | 87 | 44.7 ± 0.71 | 24 days | 10.8 ± 0.60 | 6.7 ± 0.49 | 79 |
| 2 | 44.3 ± 0.88 | 20 days | 14.2 ± 0.47 | 5.2 ± 0.47 | 80 | 41.5 ± 1.26 | 28 days | 8.5 ± 0.76 | 5.1 ± 0.30 | 75 |
| 3 | 42.5 ± 1.26 | 23 days | 13.8 ± 0.47 | 4.3 ± 0.49 | 76 | 39.3 ± 0.88 | 32 days | 8.2 ± 1.01 | 4.8 ± 0.30 | 68 |
| 4 | 40.2 ± 1.17 | 28 days | 11.5 ± 0.67 | 3.5 ± 0.48 | 70 | 36.3 ± 1.28 | 40 days | 7.7 ± 0.88 | 4.1 ± 0.30 | 66 |
| 5 | 36.3 ± 1.26 | 34 days | 7.3 ± 0.49 | 3.3 ± 0.49 | 64 | 30.2 ± 0.87 | 51 days | 5.3 ± 0.80 | 3.7 ± 0.33 | 57 |
| 6 | 29.2 ± 1.36 | 41 days | 6.5 ± 0.76 | 3.1 ± 0.30 | 46 | 25.8 ± 0.83 | 63 days | 4.8 ± 0.54 | 2.8 ± 0.60 | 44 |

Note : *: Mean of six replications.
SE : Standard Error.

multiple shoots (11.6 ± 0.66) was observed after the establishment of roots (7.7 ± 0.76). This is due to the supply of half strength LS nutrients to soilrite mix every alternate day until shoot and root development as suggested by Fujii et al. (1987). This is in agreement with the findings of Bapat and Rao (1990), Sakamoto et al. (1992) and Fujii et al. (1993) who have reported that synseed conversion under non-aseptic conditions resulted in high percentage of regenerants.

The synseeds stored at 4 °C for a period of 1–4 months resulted in maximum conversion after 15–28 days of culture under *in vitro* (46.5 ± 0.76 to 40.2 ± 1.17) condition and after 24–40 days of sowing under *ex vitro* (44.7 ± 0.71 to 36.3 ± 1.28) condition (Table 1). This agrees with the findings of Pattnaik and Chand (2000). However, as storage period increased beyond 4 months, a decline in conversion response and increase in duration for conversion was recorded (Table 1). The decline in the conversion response observed in synseeds stored for a period of 5–6 months may be due to inhibited respiration of plant tissues by alginate as described by Redenbaugh et al. (1987).

The statistical analysis with respect to conversion of synseeds into multiple shoots and root formation revealed that fresh synseeds (0 month conservation) resulted in maximum number of multiple shoot and roots followed by synseeds conserved for one to six months (Table 1).

Plants retrieved from synseeds under *in vitro*, *ex vitro* conditions and also from the conserved seeds were hardened (Figure 1g) and successfully established in soil with varying percentages of survival frequency. A fair percentage of survival frequency was recorded for the period of 0–4 months under *in vitro* (92–70%) as well as *ex vitro* (81–66%) conditions. Moreover, after 4 months the frequency of plantlet formation was relatively lower under *ex vitro* conditions, when compared to *in vitro* conditions (Table 1). Perhaps the major limiting factor for the development of synseeds into plantlets under non-sterile conditions is the non-availability of nutrients as suggested by Fujii et al. (1987). The regenerated plants did not show any variations with regard to morphological characters when compared with the donor.

Conservation of synseeds upto 4 months without loss of conversion ability at low temperature offers the possibility of using this method for *ex-situ* germplasm conservation of economically important varieties of mulberry.

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