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Influence of ammonium bromide and picloram in *Syngonium* micropropagation

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ABSTRACT

Micropropagation is a promising way to overcome difficulties in plant propagation such as time consumption, maintenance of plant uniformity and disease infection. The increased demand for the ornamental plant, *Syngonium* can be aptly met by the large scale multiplication through tissue cultures. The present study was carried out during rainy (*kharif*) season 2020 to winter (*rabi*) season 2023 at Anbil Dharmalingam Agricultural College and Research Institute, Tiruchirapalli, Tamil Nadu reports optimized protocol for the micropropagation of *Syngonium* through direct organogenesis and somatic embryogenesis pathways in two important species, *Syngonium podophyllum* and *Syngonium auritum*, respectively. Randomized block design (RBD) and single-factor ANOVA was used to analyze the effects of organogenesis and embryogenesis Direct organogenesis was achieved through the formation of axillary buds in *Syngonium podophyllum* using shoot tip and nodal explants in modified Murashige and Skoog (MS) media with 1 mg/L BAP and 0.1 mg/L NaH_2PO_4. In addition, the presence of 0.1 mg/L ammonium bromide consistently enhanced the proliferation rate up to five subculture cycles of shoot proliferation. Plant regeneration through somatic embryogenesis was achieved in *Syngonium auritum* using MS medium supplemented with 10 mg/L picloram and 1 mg/L 2,4-D. Embryogenic calli formed in the stem explant was devoid of nodes in *Syngonium auritum* and could be regenerated into full plants when subcultured in modified MS medium with 2 mg/L NAA and 1 mg/L IAA. The *in vitro* rooted plantlets were successfully acclimatized in portray containing 1:1 ratio vermicompost and coir pith compost soil medium and later transferred to the larger pots containing a mixture of red earth and vermicompost in 3:1 ratio. Secondary hardening was done by transferring plants to polythene bags and maintained under shade net house for six weeks until it is ready for planting in indoor or outdoor conditions.

Keywords: Embryogenic calli, Micropropagation, Organogenesis, Somatic embryogenesis, *Syngonium*

Syngonium, also known as arrowhead vine is primarily used for interior decoration and environmental protection (Li *et al.* 2008, Jang *et al.* 2010, He *et al.* 2015, Yang and Deng 2017, Chao *et al.* 2019). The plants can be grown indoors either by train up in six to eight foot thin stakes or grow in a hanging basket (Alippi *et al.* 1994, Jouen *et al.* 2008, Kumar *et al.* 2014, Yang and Deng 2017). *In vitro* micropropagation will be a viable strategy for the large-scale multiplication of *Syngonium*, and *in vitro* regeneration of these plants with shoot bud organogenesis and leaf somatic embryogenesis has been reported (Wang *et al.* 2007, Davies *et al.* 2017, Hasnain *et al.* 2022). However, a region-specific optimized protocol for *in vitro* regeneration and acclimatization is necessary for subtropical regions (Preece and Sutter 1991, Posposilova *et al.* 1999, Kozai and Zobayed 2000, Hazarika 2003). *Syngonium* is a notable example in the ornamental plant propagation through *in vitro* culture, where about 22 new somaclonal variants are selected in the commercial greenhouses (Dogra 2023). There are few *in vitro* regeneration protocols available in *Syngonium* through organogenesis (Kalimuthu and Prabakaran 2014). The production of *Syngonium* through *in vitro* micropropagation possesses additional advantages, including shortening the production time, producing multiple branched vigorous plantlets and eliminating diseases and pathogens, which cannot be possible through traditional propagation because cuttings can carry and spread diseases (Zuzarte *et al.* 2024).

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Direct somatic embryogenesis in *Syngonium* has been reported by inoculation of leaf bit and petiole explants in Murashige and Skoog (MS) medium supplemented with N-(2-chloro-4-pyridyl)-N′-phenylurea (CPPU) or N-phenyl-N′-1,2,3-thiadiazol-5-ylurea (TDZ) with either α-naphthalene acetic acid (NAA) or 2,4-dichlorophenoxyacetic acid (2,4-D) (Zhang *et al.* 2006). The roles of BAP, NAA, TDZ and NaH_2PO_4 supplementation in microshoot initiation, proliferation of microshoots and rooting have been compared (Kalimuthu and Prabakaran 2014). The present investigation reports the increased proliferation of microshoots by ammonium bromide in modified MS medium for *Syngonium podophyllum*. In addition, Picloram plus 2,4-D-mediated micropropagation by somatic embryogenesis has been demonstrated in ornamental and fruit crops (Chaudhary and Prakash 2019).

MATERIALS AND METHODS

Plant materials: The present study was carried out during rainy (*kharif*) season 2020 to winter (*rabi*) season 2023 at Anbil Dharmalingam Agricultural College and Research Institute, Tiruchirapalli, Tamil Nadu. *Syngonium podophyllum* and *Syngonium auritum* plants were obtained from Horticulture College and Research Institute for Women of Tamil Nadu Agricultural University. Prior to the excision of explants, the plant samples were washed thoroughly in tap water to remove soil and any debris present over the plant. The stem was separated from the leaves for nodal and shoot tip explants were excised for organogenesis studies. The leaves, petiole and stem segment devoid of nodes were used for embryogenic calli induction. Experiment was carried out with four explants in each treatment with six replicates subjected to RBD analysis. Average number of explants responding to organogenesis is given in Table 1.

Explants collection and sterilization: For nodal or shoot tip culture, the intact node with 0.5 cm stem on both side was excised. The excised explants were pretreated by immersion in 0.1% bavistin solution and kept in a shaker for 60 min. After bavistin pre-treatment, the explants were sterilized in 70% ethanol for 30 sec and washed thrice with sterile distilled water. Ethanol-washed explants were immersed in 20% sodium hypochloride (bleach) for 5 min. followed by washing with sterile distilled water for three times. For embryogenic callus induction experiment, 0.5–1 cm of stem without nodes, petioles and leaf bits were excised and subjected to disinfection. Under laminar air hood chamber the disinfected leaves were cut into one square centimeter pieces, nodes were cut into 0.5 cm pieces and petioles were cut into 0.5–1 cm long pieces in sterile petridishes. These explants were inoculated and cultured in the primary growth room maintained at 25°C temperature and 24 h dark for first three weeks. At the end of three weeks, the 24 h dark was altered to a photoperiod cycle of 16 h light and 8 h dark.

Culture initiation and multiplication: MS and WPM media were prepared by following the composition and protocol as mentioned previously with the addition of agar and necessary elements (Murashige and Skoog 1962, McCown and Lloyd 1981). For direct organogenesis, the explants were inoculated in different combinations of MS media with BAP, NaH_2PO_4 and NH_4Br (Table 1). The inoculated explants were maintained in primary growth room under 16 h light and 8 h dark cycle. Subculture was performed once in every three or four weeks in fresh medium. The formation of multiple microshoots and the proliferation rate during each subculture were observed regularly. For somatic embryogenesis, different concentrations of picloram and 2,4-D were added to MS or WPM basal media.

Table 1 Effect of BAP, NaH_2PO_4 and NH_4Br on organogenesis of *Syngonium podophyllum*

S. No	Medium composition (MS medium) mg/L			Explantsrespond to organogenesis (out of 4)
	BAP	NaH_2PO_4	NH_4Br	
1	1.0	0.0	0.0	0.28[a]
2	2.0	0.0	0.0	0.28 [a]
3	5.0	0.0	0.0	0.28 [a]
4	1 .0	0.1	0.0	3.42 [b]
5	5 .0	0.1	0.0	0.28 [a]
6	5 .0	0.0	1.0	0.56 [a]
7	0.0	0.0	0.0	0.28 [a]
8	1.0	0.1	1.0	3.51 [b]
SED				0.3619
CD (0.05)				0.7276

[b] has the best treatment groups, and Group [a] are the poorest treatment groups.

Acclimatization and hardening: After *in vitro* root formation, the well-developed plants after successful regeneration were gently removed from the *in vitro* culture bottles for acclimatization and hardening. The roots were gently washed in running tap water to remove the bound nutrient from the agar medium. Then, the plants were planted into the wells of a grow-tray containing 1:1 ratio mixture of vermicompost and coir pith compost soil medium. Initial acclimatization was achieved by growing plants in a growth room at 25°C with regular watering for 2 weeks. Then, the plants were removed from the growth tray and planted into large pots filled with red earth and vermicompost 3:1 ratio. The plants were moved to shade net house and maintained with regular irrigation for 6 weeks. Then, individual plants were planted in polythene bags containing a 2:1:1 ratio of red earth, sand and farmyard manure and maintained with normal agronomic practices for six or more weeks prior to distribution for outdoor or indoor planting.

Statistical analysis: Statistical analysis was carried out in AGRES analysis software (Panse *et al.* 1967). RBD and single-factor ANOVA was used to analyze the effects of organogenesis and embryogenesis. The resulting data were represented as mean ± Standard error (SE). The experiment was carried out in 4–10 replications with 4–10 plants in each replicate.

RESULTS AND DISCUSSION

Direct organogenesis of Syngonium podophyllum *is promoted by NaH_2PO_4 and ammonium bromide*: In order to regenerate *Syngonium* by direct organogenesis, disinfected nodal or shoot tip explants were inoculated in modified MS medium with 6-Benzylaminopurine (BAP), NaH_2PO_4 and ammonium bromide (Table 1). Nodal explants were inoculated and cultured in modified MS medium containing 1 mg/L BAP, 0.1 mg/L NaH_2PO_4 and 0.1 mg/L ammonium bromide for 15 weeks with continuous subculture once every three weeks. After 18 weeks, the microshoots were subcultured in modified MS medium devoid of ammonium bromide. Stages of organogenesis at 1 week, 6 weeks, 12 weeks, 15 weeks and 18–24 weeks are displayed in the Fig. 1. The formation of axillary buds and microshoots was observed on a regular basis. At 15-weeks post inoculation microshoots were observed (Fig. 1). Among the media combinations, modified MS medium containing 1 mg/L BAP, 0.1 mg/L NaH_2PO_4 in the presence or absence of 1.0 mg/L NH_4Br responded well for initial establishment and auxiliary bud formation (Table 1). BAP is most widely used cytokinin for *in vitro* regeneration of *Syngonium podophyllum* (Rajeevan *et al.* 2002, Hassanein 2004, Rajesh *et al.* 2011). The results of the present study showed that BAP and NaH_2PO_4 in the modified MS medium promote direct organogenesis and it is similar to the previous report in other crops and trees (Zhang *et al.* 2006, Massot *et al.* 2000, Raziq *et al.* 2023). Reports showed that the MS media contains 170 mg/L of KH_2PO_4 as source of phosphorous supply in culture medium, and addition of NaH_2PO_4 may provide excess phosphorous supply to explants for effective initial establishment and axillary bud formation (Bonetti *et al.* 2016). In specific, the addition of two fold NaH_2PO_4 increase the *in vitro* culture efficiency in ornamental plants like Dieffenbachia (Elsheikh *et al.* 2013).

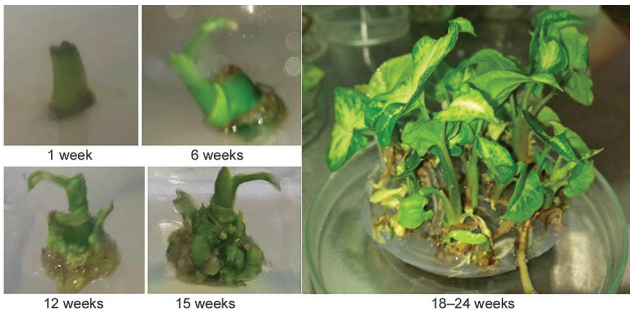

Fig. 1 Direct organogenesis in *Syngonium podophyllum*.

Ammonium bromide increases the shoot proliferation rate. In a period of 18-weeks of the initial inoculation, the explants underwent six sub cultures. After six subcultures, the microshoots responded for multiple shoot induction and proliferation. In order to assess the proliferation rate, the microshoots routed through six subculture were inoculated into modified MS medium containing BAP and NaH_2PO_4 in the presence or absence of ammonium bromide. The explants inoculated in MS with 1 mg/L BAP, 0.1 mg/L NaH_2PO_4 and 1.0 mg/L NH_4Br showed a high rate of microshoot proliferation than MS with 1 mg/L BAP and 0.1 mg/L NaH_2PO_4 alone (Table 2). When proliferated microshoots were subsequently separated and subjected to subculture in modified MS media with BAP, NaH_2PO_4 and NH_4Br, a high number of shoot proliferation up to five subculture cycles were observed (Table 2). Possibly the ammonium bromide present in MS medium provides ammoniacal form of nitrogen effectively taken by the plant cells in the early days (Gamborg and Shyluk 1970, Zhang *et al.* 2019). The excess availability of phosphorous in NaH_2PO_4 and ammoniacal nitrogen in ammonium bromide may increase the proliferation rate in *Syngonium*. Once a sufficient number of microshoots were produced, the microshoots were transferred into modified MS medium with BAP and NaH_2PO_4 devoid of ammonium bromide for rooting. Root induction occurred in 2–3 weeks, and the *in vitro* regenerated plantlets were ready for hardening.

Picloram and 2,4-D promote somatic embryogenesis in Syngonium auritum: To study the somatic embryogenesis potential, the explants from *Syngonium podophyllum* and *Syngonium auritum* were sterilized and cultured in modified MS or modified WPM media. Among the two species, *Syngonium auritum* only responded to embryogenic calli formation, and only *Syngonium auritum* was proceeded further. The explants excised from leaves, petiole and stem segments devoid of nodes were studied for embryogenic calli induction, and shoot explant devoid of node produced embryogenic calli structures after nine weeks (Fig. 2).

Table 2 Shoot proliferation rate in the repeated subculture phase of micropropagation

Treatment	Mean rate of proliferation per explant during five continuous subculture				
	1	2	3	4	5
MS + 1.0 mg/L BAP	1.29 [a]	1.14 [a]	1.14 [a]	1.00 [a]	1.14 [a]
MS + 1.0 mg/L BAP + 0.1 mg/L NaH_2PO_4	2.28 [b]	2.14 [b]	2.29 [b]	2.14 [b]	1.85 [b]
MS + 1.0 mg/L BAP + 0.1 mg/L NaH_2PO_4 + 1.0 mg/L NH_4Br	8.29 [c]	7.71 [c]	7.43 [c]	8.71 [c]	7.14 [c]
SED	0.2608	0.3367	0.3086	0.6389	0.3364
CD (0.05)	0.5480**	0.7074**	0.6484**	1.3422**	0.7074**

[c] has the best treatment groups

Experiment was carried out with ten explants in each treatment with six replicates subjected to RBD analysis. Average number of explants proliferation is given in table.

Table 3 Effect of Picloram and 2,4-D on somatic embryogenesis of *Syngonium auritum*

Media composition (mg/L)			Embryogenic callus*
Medium	Picloram	2,4-D	
MS	0.5	0.0	0 .00±0.00
MS	5.0	0.0	0.43±0.43
MS	5.0	0.5	1.14±0.74
MS	5.0	1.0	2.00±1.31
WPM	0.5	0.0	0.00±0.00
WPM	5.0	0.0	0.57±0.57
WPM	0.5	1.0	0.42±0.43
WPM	5.0	1.0	1.71±1.26
WPM	10	1.0	2.42±1.66
MS	10	1.0	7.71±0.78**
SED			1.2278
CD (0.05)			2.4560

*Mean values ±SEmof 7 replicates

Experiment was carried out with ten explants in each treatment with seven replicates and subjected to RBD analysis. Average number of explants proliferation is given in table.

Modified MS with 10 mg/L picloram and 1 mg/L 2,4-D showed the highest embryogenic calli induction (Table 3). The embryogenic calli produced by *S. auritum* were divided in to 10 pieces of visually equal size, and subcultured in half strength MS medium with 2 mg/L kinetin and 1 mg/L IAA for plant regeneration. Three weeks after subculture, the formation of plantlets from the somatic embryo was observed (Fig. 3). To know the possible of number of clones developed from embryogenic calli, the number of regenerated plants was counted, and it was observed that each excised portion of the explant produced an average of 9.07 plants. Complete recovery of somatic embryogenesis regenerated plants was noticed during acclimatization and hardening. Somatic embryo formation from petiole explants with TDZ and CPPU compounds has been reported previously (Zhang *et al.* 2006, Qian and Richard 2006). In the present study it was found that the stem explants devoid of nodes produced embryogenic calli in *Syngonium auritum* (Table 3). Additionally, stem explants possess advantages in easy handling during disinfection process compared to tender explants such as leaves. Also the induction of embryogenic calli was increased in the presence of picloram and 2,4-D (Table 3). The presence of 2,4-D increase the cell division, proliferation and leads to formation of embryogenic calli cells (Raghavan 2004). The combination of 2,4-D and stress in the induction of somatic embryogenesis has been documented in ornamental plants (Cueva *et al.* 2015). Picloram possibly triggers stress to the explant, and this will trigger the embryogenic calli process. Notably Picloram used in this study has been reported for promoting somatic embryogenesis in several crop plants (Takamori *et al.* 2015, Gantait and Mahanta 2021, Hassan *et al.* 2021, Khatri and Joshee 2024). The combination of 2,4-D and Picloram have been reported to enhance somatic embryogenesis in Papaya (Chaudhary and Prakash 2019). In addition, the chemicals 2,4-D and Picloram are cost effective when compared to chemicals such as TDZ. The regeneration potential of one fractionated piece can produce nearly nine plants, and remaining nine pieces can produce similar number of plants, a total of ninety plants can be regenerated from the embryogenic calli obtained from single explant (Supplementary Table 1).

This investigation unveils an efficient and reproducible protocol for the micropropagation of *Syngonium* through direct organogenesis and somatic embryogenesis. It is found that BAP, NaH_2PO_4 and NH_4Br are the ideal components to be included in the modified MS nutrient medium to enhance organogenesis and picloram and 2,4-D are the essential components to trigger somatic embryogenesis. Microshoot

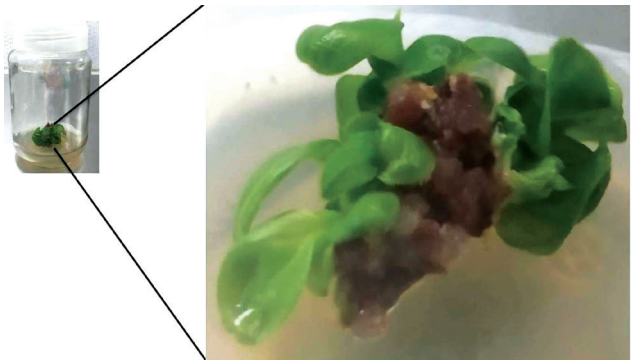

Fig. 3 Regeneration of *Syngonium* from somatic embryo.

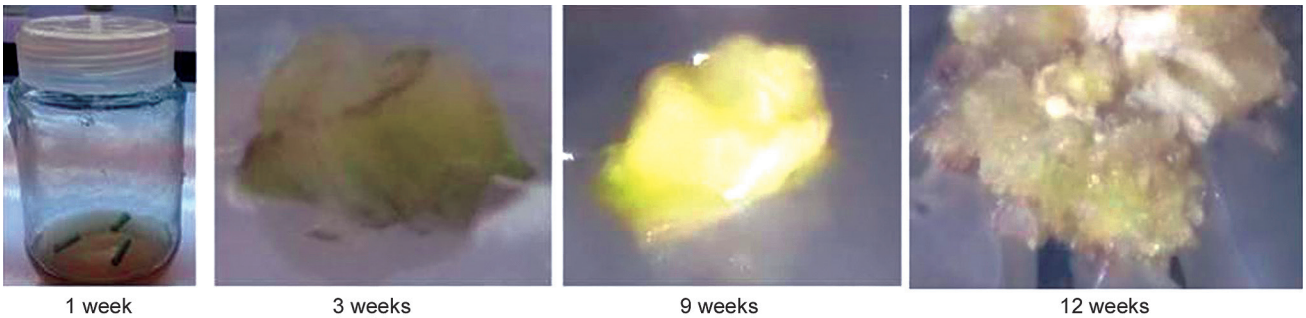

Fig. 2 The stages of somatic embryogenesis at 1 week, 3 weeks, 9 weeks and 12 weeks.

induction in the proliferation medium takes approximately eighteen weeks, and after that the microshoots capable of proliferate into 6–8 microshoots in each subculture. Rooting of elongated shoots takes approximately 2–4 weeks and the final acclimatization procedure will take four weeks. Considering the time and duration of subculture and following the optimized protocol demonstrated in this report, approximately 30000 plantlets can be envisaged from a single explant by organogenesis in a year.

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