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BRIEF COMMUNICATION

Production of triploid plants from endosperm cultures of *Phlox drummondii*

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Abstract

Triploid plants of ornamental *Phlox drummondii* Hook. were raised from cultures of endosperm excised from immature fruits having zygotic embryo at early dicotyledonous stage. Endosperm tissue was firstly cultured with the embryo on the Murashige and Skoog's (MS) medium supplemented with 5 μM 6-benzylaminopurine (BAP) + 10 μM α-napthaleneacetic acid (NAA) for 7 d and recultured after the embryo was removed. A friable callus appeared two weeks after removal of the embryo and it became compact callus mass in another three weeks. Upon transfer of this 5-week-old callus to the MS medium with 10 μM BAP + 2.5 μM indole-3-acetic acid (IAA), maximum percentage of green nodular shoot buds appeared from which regenerated dwarf shoots. Elongation of the dwarf shoots, however, required transfer of the individual dwarf shoots excised from the callus on the fresh medium and best results achieved on medium with low concentration of IAA (0.5 μM) in presence of 10 μM BAP. The shoots were then rooted *in vitro* and plants subsequently established in pots containing soil. Over 70 % of plants were triploid with a chromosome number of 2n=3x=21. Size of stem, leaves, flowers, pollen, and stomata of these triploid plants were higher and the plants were more vigorous as compared to naturally occurring diploid plants. In particular, flowers showed bright colour with enlarged central eye adding to their ornamental value.

Additional key words: auxins, callus, cytokinins, *ex vitro* transfer, *in vitro* culture, ploidy.

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Phlox drummondii Hook. (family *Polemoniaceae*) is an ornamental plant with attractive flowers which bloom in different shades of colours like red, purple, pink, scarlet, violet, *etc*. (Razdan *et al.* 2008). They flower profusely in gardens, pots, and window boxes. Application of *in vitro* techniques for propagation of ornamental plants that are commercially important has significantly increased over the past two decades (Rout *et al.* 2006, Pinto *et al*. 2013, Smith 2013). Triploid plants are reported to show better performance (*e.g*., larger flowers and/or foliage) as compared to related diploids (Miyashita *et al.* 2009) that make them viable for commercial trade. The advantage of triploids over conventional colchicine induced tetraploids in *Phlox drummondii* is that tetraploids have abnormal chromosomal pairing which result in unstable phenotypes (Koul and Raina 1996). Triploid plants of *Phlox* do not occur in nature and attempts to produce these plants by sexual crossings have not been successful. Endosperm culture is possibly the feasible *in vitro* technique for production of triploid plants although success has been achieved only in a limited number of plant species (Razdan 2003, Thomas and Chaturvedi 2008, Hoshino *et al*. 2011).

Endosperm is a unique tissue in its origin, development, and ploidy level and serve for nutrient requirements of embryo (Bhojwani and Razdan 1996). Triploid plants raised from endosperm are generally sterile, but this trait does not affect commercial utility of endosperm-raised triploid plants, *e.g*., timber-yielding plants, edible fruit plants, or ornamentals propagated vegetatively and multiplied mainly through micro-propagation. Naturally occurring *P. drummondii* plants

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Abbreviations: BAP - 6-benzylaminopurine; CH - casein hydrolysate; 2,4-D - 2,4-dichlorophenoxy acetic acid; IAA - indole-3-acetic acid; KIN - kinetin; NAA - α-napthaleneacetic acid.
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are diploid with chromosome number 2n=2x=14 and there are no reports on the production of triploid plants of this species either through sexual crossings or application of tissue culture methods. In the present study, we successfully developed the protocol for producing endosperm-derived triploid plants of *P. drummondii* which demonstrated the enhanced trait quality as compared to natural diploids.

Immature fruits at the early dicotyledonous stage of the embryo were collected from 4 - 5 months old plants raised from seeds (*Sluis and Groot*, Enkhuizen, Netherlands) of *Phlox drummondii* Hook. growing in the botanical garden at the Department of Botany, the University of Delhi, India. Seeds were generally sown in September and immature fruits harvested next year in January - February. The harvested fruits after thorough wash in 1 % (m/v) *Savlon* and *Tween 20* solution (1:1) for 10 min were given 3 rinses in sterile distilled water. This was followed by surface sterilization of the fruits with 0.1 % (m/v) $HgCl_2$ solution for 8 min and washing thrice with sterile distilled water in a laminar air flow box under aseptic conditions. Endosperm from the immature fruits (7 - 10 d after pollination, DAP) was excised and cultured (with or without zygotic embryo) on Murashige and Skoog (1962; MS) basal medium with 3 % (m/v) sucrose in absence or presence of plant growth regulators. All media were adjusted to pH 5.8 with 1 M HCl solution, gelled with 0.8 % (m/v) agar (*Hi Media*, Mumbai, India), and autoclaved at 1.6 kg cm^{-2} and 121 °C for 18 min. Cultures in all experiments were initially incubated in dark and after callus induction transferred for further growth under a 16-h photoperiod with irradiance of 18 µmol m^{-2} s^{-1} (provided by cool day-light fluorescent tubes, 40 W, *Philips*, New Delhi, India), temperature of 25 ± 1 ºC, and relative humidity of 50 to 60 %. Four endosperm explants per plate were incubated in disposable sterile Petri plates (diameter of 50 mm, *Tarsons*, Bangalore, India), each containing 15 - 20 cm^3 of the culture medium. After 3 weeks, an endosperm derived callus was transferred into a culture tube (25 × 150 mm, *Borosil*, New Delhi, India) containing 20 cm^3 of the culture medium and plugged with non-adsorbent cotton wrapped in cheese cloth.

In the first experiment, MS media variously supplemented with 6-benzylaminopurine (BAP, 2.5 or 5 µM), 2,4-dichlorophenoxy acetic acid (2,4-D, 5 or 10 µM), α-napthaleneacetic acid (NAA, 5 or 10 µM), and casein hydrolysate (CH, 0.5 g dm^{-3}) were used for induction and growth of calli derived from 500 endosperm explants (Table 1). Further, the endosperm derived callus was cultured for shoot induction on MS medium without growth regulators or with cytokinins BAP (2.5, 5, or 10 µM) or kinetin (KIN, 2.5,5 or 10 µM) singly and in combination with auxins (NAA, 2.5, 5 µM) or indole-3-acetic acid (IAA 0.5, 2.5, 5 µM). For each treatment, 50 endosperm-derived calli were used to achieve shoot regeneration. About 300 dwarf shoots developing on all the calli were excised and transfered to fresh MS medium with various concentrations of growth regulators (as mentioned in Table 2) for shoot elongation. Rooting was induced in about 5-week-old elongated shoots on media containing full, half, or quarter strengths MS basal medium containing 3 % sucrose and NAA (1, 2.5, and 5 µM) or IAA (1, 2.5, 5, 7.5, and 10 µM) singly or in combination with BAP (2.5 or 5 µM). Rooted plants (*ca*. 80) were washed to remove the agar and transferred to plastic pots (22 × 15 cm) containing autoclaved soil (1 part of *Vermiculite* and 3 parts of garden loam) and covered with polythene bags that had small holes (2 mm) for 20 d to maintain high humidity. The potted plants were grown in a chamber with temperature of 25 - 27 ºC, relative humidity of 50 - 60 %, a 16-h photoperiod, and irradiance of 18 µmol m^{-2} s^{-1}, and after 4 weeks, they were shifted to a greenhouse.

Each experiment was repeated thrice, data subjected to analysis of variance (*ANOVA*), and means were compared using a *SAS* computer software according to Anderson-Cook (2004).

For chromosome studies, root tips (0.3 - 0.5 cm) were excised from the 4-week-old transplanted plants, washed

Table 1. Callus induction [%] on endosperm excised from immature fruits (7 - 10 DAP) of *Phlox drummondii* on MS medium with different concentrations of growth regulators after 5 weeks. Means ± SE, $n = 50$; means in the same column followed by different letters are significantly different at $P \leq 0.05$ and $F = 96.56$ (see Anderson-Cook 2004). No callus induction occurred on MS basal medium.

Growth regulators [µM]	Endosperm without embryo	Endosperm with embryo removed after 1 week
5.0 µM BAP + 5.0 µM 2,4-D	1.05 ± 0.50d	13.60 ± 0.20d
2.5 µM BAP + 10.0 µM 2,4-D	3.06 ± 0.00d	27.30 ± 0.28c
5.0 µM BAP + 10.0 µM 2,4-D	3.30 ± 0.05d	38.00 ± 0.28b
5.0 µM BAP + 5.0 µM NAA	5. 03 ± 0.05c	20.05 ± 0.25d
2.5 µM BAP + 10.0 µM NAA	12.06 ± 0.08b	44.60 ± 0.28ba
5.0 µM BAP + 10.0 µM NAA	19.06 ± 0.30a	53.00 ± 0.02a
5.0 µM BAP + 10.0 µM NAA + 0.5 g dm^{-3} CH	6.03 ± 0.02c	21.00 ± 0.28cd
2.5 µM BAP + 10.0 µM NAA + 0.5 g dm^{-3} CH	1.13 ± 0.02d	14.60 ± 0.00d
5.0 µM BAP + 10.0 µM 2,4-D + 0.5 g dm^{-3} CH	1.89 ± 0.00d	30.00 ± 0.00c
2.5 µM BAP + 10.0 µM 2,4-D + 0.5 g dm^{-3} CH	1.03 ± 0.0 d	25.30 ± 0.28c

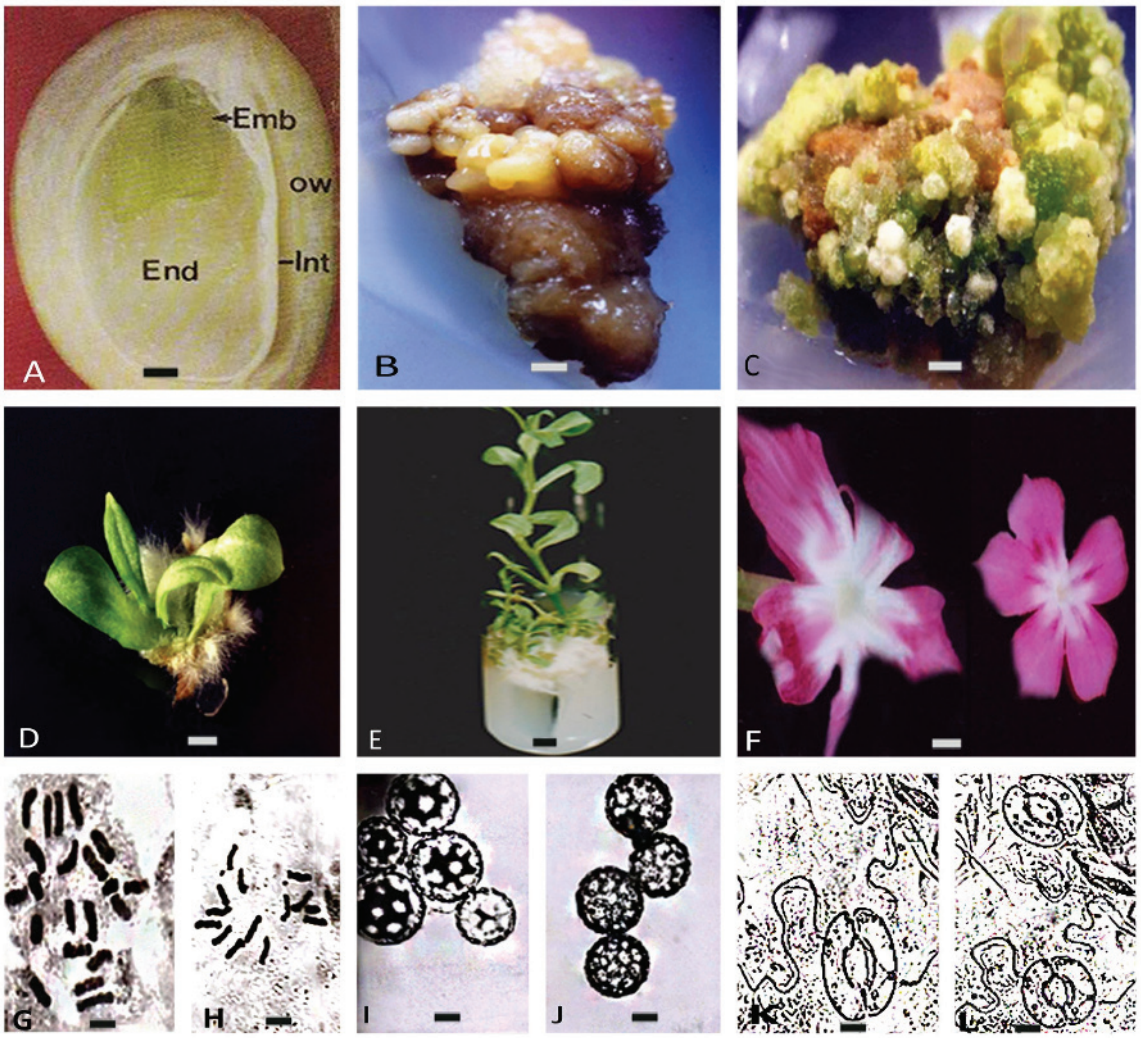

Fig. 1. Different stages of regeneration in endosperm-derived plants of *Phlox drummondii*. *A* - Milky white, thick, and gelly endosperm enclosing a zygotic embryo excised from immature fruits 7 - 10 d after pollination (Emb - embryo; End - endosperm; Int - integument; ow - ovary wall; *bar* = 0.15 cm). *B* - White friable callus mass developing from the 2-week-old endosperm culture after removing an embryo on MS medium with 5 µM BAP + 10 µM NAA (*bar* = 1.25 cm). *C* - Green nodular shoot buds appearing after transfer of a 5-week-old callus on MS medium with 10 µM BAP + 2.5 µM IAA (*bar* = 2.5 cm). *D* - A dwarf and thick leafy shoot arising from the green nodular shoot bud after 5 - 6 weeks (*bar* = 3.5 cm); *E* - Elongation of an excised dwarf thick shoot after 4 - 5 weeks on MS medium with 10 µM BAP + 0.5 µM IAA (*bar* = 7 cm). *F* - Flowers of triploid (*on the left*) and diploid plants (*bar* = 4 cm); *G, H* - Chromosomes of endosperm-derived triploid (*G*) and diploid (*H*) plants (*bar* = 0.2 mm); *I*, *J* - pollen grains of triploid and diploid plants (*bar* = 0.4 mm). *K*, *L* - Stomata of triploid and diploid plants (*bar* = 0.4 mm).

under running tap water, cooled on ice for 24 h, or pre-treated with 0.5 % (m/v) colchicine (*Sigma*, Munich, Germany) in dark for 3 h. This was followed by their fixation in 3 parts of absolute ethyl alcohol and 1 part of glacial acetic acid (v/v). The fixed root tips were subsequently stored at 4 ºC and used for analysis of the chromosome number by staining in Feulgen according to the procedure of Raja *et al*. (1992). Pollen grains were isolated from flowers and their size determined under a microscope. The size of stomata was determined by placing epidermal peel from abaxial surface of the mature leaves on a glass slide in a drop of distilled water beneath a cover glass, and 60 stomata from 7 to 10 fields were measured. The flower size was measured directly on the potted plants. For all these observations, endosperm-derived triploid and natural diploid plants were used for comparison.

In *Phlox*, the endosperm is milky-white, appearing as thick jelly in immature fruits, 7 - 10 d after pollination, enclosing the embryo at early dicotyledonous stage (Fig.1*A*). When fruits mature, endosperm converts into white solid mass, which is very difficult to excise and culture. Only the endosperm explant excised from immature fruits showed regeneration response in culture. The excised endosperm was cultured either without or with the zygotic embryo. The endosperm cultures without

Table 2. Shoot bud induction and subsequent regeneration of dwarf shoots on 5-week-old endosperm-derived callus cultures of *Phlox drummondii*, followed by culture of each dwarf shoot excised from callus for shoot elongation on MS medium supplemented with different concentrations of growth regulators. Means ± SE, n = 50; means in the same column followed by different letters are significantly different at $P \leq 0.05$ and $F = 96.56$. No shoot bud induction occurred when callus was cultured on MS basal medium or MS medium supplemented only with cytokinins.

Growth regulators	Shoot bud induction [%]	Number of shoots [callus^{-1}]	Excised shoot elongation [%]
10.0 µM BAP + 5.0 µM NAA	2.60 ± 0.28e	1.50 ± 0.28d	5.50 ± 0.10de
10.0 µM BAP + 2.5 µM NAA	10.35 ± 0.25e	2.00 ± 0.15d	10.50 ± 0.25d
10.0 µM BAP + 5.0 µM IAA	40.30 ± 0.50c	2.30 ± 1.00d	17.00 ± 1.40d
10.0 µM BAP + 2.5 µM IAA	82.60 ± 0.86a	9.00 ± 0.50a	35.60 ± 1.40c
5.0 µM BAP + 2.5 µM IAA	24.60 ± 0.28dc	4.00 ± 0.28c	20.00 ± 0.00d
10.0 µM BAP + 0.5 µM IAA	62.50 ± 0.02b	7.20 ± 0.50ba	99.50 ± 0.28a
10.0 µM KIN + 5.0 µM NAA	1.50 ± 0.28e	1.20 ± 0.05d	1.05 ± 0.25e
10.0 µM KIN + 2.5 µM NAA	6.25 ± 0.50e	1.30 ± 0.25d	2.25 ± 0.50e
10.0 µM KIN + 5.0 µM IAA	19.30 ± 0.00d	1.60 ± 2.00d	10.60 ± 0.28d
10.0 µM KIN + 2.5 µM IAA	49.50 ± 0.28c	1.20 ± 0.05d	25.00 ± 0.00cd
5.0 µM KIN + 2.5 µM IAA	43.73 ± 0.08c	4.50 ± 0.02c	12.60 ± 0.50d
10.0 µM KIN + 0.5 µM IAA	30.00 ± 0.00cd	6.70 ± 0.00b	64.60 ± 0.25b

Table 3. Rooting response in 5-week-old elongated shoots of endosperm origin in *Phlox drummondii* on full strength MS medium supplemented with different concentrations of growth regulators after two weeks of culture. Means ± SE, n = 25; means in the same column followed by different letters are significantly different at $P \leq 0.05$ and $F = 96.56$. No rooting was observed on MS basal medium or on ½ or ¼ strength MS media with growth regulators. ** - No rooting response on this medium as callus developed at the base of shoot.

Growth regulators	Rooted shoots [%]	Number of roots [shoot^{-1}]	Length of the longest root [cm]
1.0 µM IAA	8.00 ± 0.28d	1.83 ± 0.14d	0.80 ± 0.00c
2.5 µM IAA	33.30 ± 1.40c	2.73 ± 0.25cd	2.06 ± 2.10bc
5.0 µM IAA	60.30 ± 0.28ab	5.56 ± 0.02b	3.00 ± 0.00b
7.5 µM IAA	54.00 ± 0.28b	4.00 ± 0.00bc	3.96 ± 0.10a
10.0 µM IAA	37.60 ± 0.50c	3.03 ± 0.02c	3.56 ± 0.50ab
1.0 µM NAA	19.00 ± 1.44d	0.83 ± 0.14d	1.00 ± 0.28c
2.5 µM NAA	15.60 ± 0.83d	0.76 ± 0.08d	0.80 ± 0.15c
5.0 µM NAA	-**	-	-
5.0 µM BAP + 5.0 µM IAA	41.00 ± 1.40bc	4.60 ± 0.14bc	3.13 ± 0.00ab
2.5 µM BAP + 5.0 µM IAA	63.30 ± 0.28ab	6.03 ± 0.02ab	4.00 ± 0.50a
2.5 µM BAP + 5.0 µM IAA + µM 1.0 NAA	80.00 ± 0.00a	8.00 ± 0.00a	4.50 ± 0.35a

embryos showed very poor callus regeneration (1 - 19 %), whereas the endosperm with the embryo removed after a week showed better regeneration (13 - 53 %; Table 1). Further, it was observed that the callus from endosperm cultured without embryo measured maximum 10 × 5 mm and that size of the callus from endosperm cultured with embryo initially was in the range of 10 × 5 to 10 × 25 mm. Embryo seems to play a significant role in enhancing callus induction. Of all combinations of growth regulators tested, the best callus induction (53 %) and size (10 × 25 mm) occurred on the MS medium with BAP (5 µM) + NAA (10 µM). After removal of embryo, the endosperm cultured on this medium started growing into white friable mass in two weeks (Fig. 1*B*) and in another three weeks, it became a compact callus. Upon transfer of this 5-week-old callus to a medium supplemented with high concentrations of BAP or kinetin along with low concentrations of IAA or NAA, several green nodular shoot buds developed all over the callus in 3 weeks (Fig. 1*C*) from which dwarf shoots subsequently regenerated. It took overall 5 - 6 weeks to a callus in culture to develop dwarf shoots. MS medium supplemented with BAP (10 µM) and IAA (2.5 µM) was found suitable for maximum differentiation of green nodular shoot buds as in these cultures, *ca*. 82 % shoot bud regeneration could be achieved (Table 2) but developed shoots were with very thick enlarged leaves. About 1 - 9 dwarf leafy shoots developed on a callus depending on combinations of growth regulators used in the medium (Table 2). These dwarf shoots did not elongate even after 8-weeks of regeneration in the culture and, therefore, each dwarf shoot (Fig. 1*D*) growing on a callus from all the cultures was ultimately excised and cultured on a fresh MS medium supplemented with combination of the same

growth regulators as mentioned in Table 2. After 4 - 5 weeks, 99 % of shoots elongated in the MS medium with BAP (10 µM) and very low concentration of IAA (0.5 µM). The length of shoots was up to 6 - 7 cm and they had 5 - 6 internodes (Fig. 1*E*). Leaves and stem were apparently thicker than those of normal diploid plants.

It was reported that a species in which induction and multiplication of shoot requires a full-strength medium, the reduction of salt concentration in the medium to a half or one-quarter may be satisfactory for root induction (*c.f.* Razdan 2003). However, in the present investigation, no root formation occurred on half or quarter strength MS medium supplemented with various concentrations of plant growth regulators and shoots rooted only on the MS full strength medium. The best rooting occurred on the MS medium containing IAA (5 µM) and NAA (1 µM) along with BAP (2.5 µM). Interestingly, it was observed that on media supplemented only with auxin(s), shoots started drying after rooting and plants finally died. Thus, it became essential to add a small amount of cytokinin along with auxins in the medium for rooted plants to survive (Table 3). Endosperm-derived rooted plants were transplanted to soil in pots.

Roots of 80 plants derived from endosperm cultures were analyzed for the chromosome number. Of these, 70 % of plants were triploids (2n=3x=21, Fig. 1*G*) and 5 % diploids (2n=2x=14, Fig. 1*H*), whereas remaining 25 % were aneuploids. It was observed that flowers of endosperm raised (triploid) plants of *P. drummondii* were not only larger (ca. 3.95 cm) in size but more attractive with large flowers and central eye (*ca*. 2.85 cm) showing enlarged striations in comparison to flower size (*ca*. 2.75 cm) and central eye (*ca*. 1.02 cm) of naturally occurring (diploid) plants (Fig. 1F). Further, pollen analysis showed the size (*ca*. 8.60 µm) of pollen in the endosperm-derived triploid plants (Fig. 1*I*) proportionately larger as compared to pollen (*ca*. 5.35 µm) in the diploid control plants (Fig. 1*J*). Similarly, stomata length (*ca*. 7.03 µm) and width (*ca*. 6.57 µm) in triploids (Fig. 1*K*) were proportionately larger as compared to length (*ca*. 5.20 µm) and width (4.50 µm) of stomata in diploids (Fig. 1*L*).

The earliest attempt to grow endosperm tissue in cultures was made by Lampe and Mills (1933). However, the first tissue culture of immature maize endosperm was raised by La Rue (1949). Since then, immature and mature endosperm of few species regenerated in cultures (Bhojwani and Razdan 1996, Bhojwani and Dantu 2013). Although totipotency of endosperm cultures was first reported by Johri and Bhojwani (1965), the production of triploid plants from these cultures has not been easy since morphogenic response was reported only in 64 species (Thomas and Chaturvedi 2008) and shoots/triploid plants were obtained in 27 species belonging to 14 families (Razdan 2003). In the present study, we were successful in raising the triploid plants of *Phlox drummondii* from immature endosperm cultures. It was found that endosperm requires the initial association with embryo to enhance the growth of callus. Similar observations were reported in mulberry (Thomas *et al.* 2000) and neem (Chaturvedi *et al.* 2003) endosperm cultures. Induction of endosperm callus sometimes requires, in addition to auxins and cytokinins, a rich source of organic nitrogen, such as yeast extract or casein hydrolysate (CH; Nakajima 1962, Gmitter *et al.* 1990, Thomas *et al.* 2000, Chaturvedi *et al.* 2003). For *Phlox drummondii*, CH was also added along with NAA and BAP but it showed a poor response. In fact, the best induction of callus from endosperm was found on the MS medium supplemented with BAP (5 µM) and NAA (10 µM). When such calli were transferred to a medium containing higher concentration of BAP (10 µM) and lower concentration of IAA (2.5 µM), nodular shoot buds appeared all over the callus from which green dwarf shoots subsequently regenerated. Elongation of these dwarf shoots, however, required to excise them from the callus and transfer to a fresh medium with IAA (0.5 µM) and BAP (10 µM).

The growth of triploids is generally higher than respective diploids (Thomas and Chaturvedi 2008). Also, the triploid plants of *Phlox drummondii* derived from the endosperm cultures had thicker stem, thicker and larger leaves, bigger flowers, bigger pollen grains, and larger stomata than diploid plants. The presence of pubescence (small hairs) on the internodal portion of stem and large, bright flowers were characteristic features unique to the endosperm-derived triploid plants of this species. The present protocol may be exploited by floriculturists in commercial scale.

References

Anderson-Cook, C.M.: Regression and *ANOVA*: an integrated approach using *SAS* software. - Amer. Statistics **58**: 172-173, 2004.

Bhojwani, S.S., Dantu, P.K.: Plant Tissue Culture: an Introductory Text. - Springer, New Delhi**,** India 2013.

Bhojwani, S.S., Razdan, M.K.: Plant Tissue Culture: Theory and Practice. A Revised Ed. - Elsevier, Amsterdam, 1996.

Chaturvedi, R., Razdan, M.K.., Bhojwani, S.S.: An efficient protocol for the production of triploid plants from endosperm callus of neem, *Azadirachta indica* A.Juss. - J. Plant Physiol. **160**: 557-564, 2003.

Gmitter, F.G., Ling, X.B., Deng, X.X.: Induction of triploid plants from endosperm calli *in vitro*. - Theor. appl. Genet. **80**: 785-790, 1990.

Hoshino, Y., Miyashita, T., Thomas, T.D.: *In vitro* culture of endosperm and its applications in plant breeding: approaches to plant breeding. - Sci. Hort. **130**: 1-8, 2011.

Johri, B M., Bhojwani, S.S.: Growth responses of mature endosperm in cultures. - Nature **208**: 1345-1347, 1965.

Koul, K.K., Raina, S.N.: Male and female meiosis in diploid and colchitetraploid *Phlox drummondii* Hook. (*Polemoniaceae*). - Bot. J. Linn. Soc. **122**: 243-259, 1996.

La Rue, C.D.: Cultures of the endosperm maize. - Amer. J. Bot. **36**: 798, 1949.

Lampe, L., Mills, C.O.: Growth and development of isolated endosperm and embryo of maize. - Bull. Torrey bot. Club **63**: 365-382, 1936.

Miyashita, T., Ohashi, T., Shibata, F., Araki, H., Hoshino, Y.: Plant regeneration with maintenance of the endosperm ploidy level by endosperm culture in *Lonicera caerulea* var. *emphyllocalyx*. - Plant Cell Tissue Organ Cult. **98**: 291-301, 2009.

Murashige, T., Skoog, F.: A revised medium for rapid growth and bioassays with tobacco tissue cultures. - Physiol. Plant. **15**: 473-479, 1962.

Nakajima, K.: Induction of useful mutations in mulberry by gamma radiation. - JARQ **6**: 195-198, 1962.

Pinto, A.C.R., Dematê, M.E.S. P., Santos, D.M.M., Barbosa, S., Crest, S.: Growth regulators for *in vitro* propagation of *Tilandsia gardneri* (*Bromeliaceae*). - Acta Hort. **1002**: 51-57, 2013.

Raja, V.G., Koul, K.K.., Raina, S.N., Parida, A.: Ploidy dependant genomic stability in tissue cultures of ornamental *Phlox drummondii* Hook. - Plant Cell Rep. **12**: 12-17, 1992.

Razdan, M.K.: Introduction to Plant Tissue Culture. 2nd Ed. - Science Publishers, Enfield 2003.

Razdan, A., Razdan, M.K., Rajam, M.V., Raina, S.N.: Efficient protocol for *in vitro* production of androgenic haploids of *Phlox drummondii.* - Plant Cell Tissue Organ Cult. **95**: 245-250, 2008.

Rout, G.R., Mohapatra, A., Jain, S.M.: Tissue culture of ornamental pot plant: a critical review on present scenario and future prospectus. - Biotechnol. Adv. **24**: 531-560, 2006.

Smith, R.H.: Plant Tissue Culture: Techniques and Experiments. 3rd Ed. Elsevier, New Delhi 2013.

Thomas, T.D., Bhatnagar, A.K., Bhojwani, S.S.: Production of triploid plants of mulberry (*Morus alba* L.) by endosperm culture. - Plant Cell Rep. **19**: 395-399, 2000.

Thomas, T.D., Chaturvedi, R.: Endosperm culture: a novel method of plant improvement. - Plant Cell Tissue Organ Cult. **93**: 1-14, 2008.