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Technical Note

Effects of zeatin and gibberellic acid on regeneration and *in vitro* flowering of *Phlox paniculata* L.

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Abstract: The present work reports on the regeneration and *in vitro* flowering of *Phlox paniculata* L. Different explants (leaves, petioles and stems) were cultured on Murashige and Skoog (MS) solid medium supplemented with different concentrations and combinations of α-napthaleneacetic acid (NAA) and 6-benzylaminopurine (BAP). Among the explants studied, leaves were found to be the most responsive and had the highest regeneration frequency (100%) when they were cultured in the presence of 8.0 µM NAA and 6.6 µM BAP. However, the regenerated plantlets did not produce any flower buds in successive subcultures. *In vitro* flowering was only induced after 4 weeks of culturing from shoot tip segments which were maintained on MS solid medium supplemented with zeatin (2.2-11.2 µM) or gibberellic acid (GA_3,1.4-7.2 µM). In the present study treatment with 11.2 µM zeatin showed the highest frequency of *in vitro* flowering (67.8 ±3.2%), whereas 5.6 µM GA_3 gave a lower percentage (48.8±9.3%). Although the flowers generated *in vitro* were sterile, our study would provide an important step towards future investigation on the essential factors in *in vitro* flowering in *P. paniculata* and to elucidate other developmental, physiological and environmental stimuli, which are required for promoting or inhibiting the transition of a vegetative state to a flowering state in this species.

Keywords: *Phlox paniculata* L., *in vitro* flowering, zeatin, gibberellic acid, micropropagation

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INTRODUCTION

Phlox paniculata L. is an important flowering plant that belongs to Polemoniaceae [1]. *P. paniculata* L. is a perennial ornamental plant, bears dense terminal clusters of flowers in pink, crimson and mauve, is commonly grown as borders, and is also suitable for window boxes and tubs [2-4]. The species produces sterile seeds [1, 2], and even though the traditional method of propagation is by root cuttings, the roots are often damaged by red ants and sometimes by soil-born fungi. Therefore, *in vitro* culture is an effective technique for obtaining large-scale clonal propagation of *P. paniculata*. Schnabelrauch and Sink [5] studied the clonal propagation of *P. paniculata* through axillary bud culture. Later, shoot regeneration was induced from adult leaf segments cultured by Declerck and Korban [6]. These studies revealed the potential for inducing multiple shoots in *in vitro* cultures of this species. Moreover, multiple shoot regeneration and the *in vitro* technique are more advantageous for rapidly obtaining clonal plants as well as for conservation [7-11]. In some studies rapid micropropagation of plants is achieved through synthetic seed technology and somatic embryos [12-16]. Besides *in vitro* flowering, valuable bioactive molecules that promote antidiabetic activity, antioxidation and anticancer activity [17-21], as well as provide pigments for paint and coating technology are also obtained from plants either grown *in vivo* or *in vitro* [22-28].

Flowering is a unique developmental event in plants, which involves a transition from the vegetative shoot apex to either an inflorescence or a floral meristem, followed by initiation and subsequent maturation of the floral organ [29]. The flowering process is one of the most critical stages in plant life and is vital for the completion of the life cycle and seed production. Under natural growth, the flower formation usually begins when a plant reaches maturity. The transition from the vegetative state to the floral stage is considered to be a complex process regulated by a combination of various environmental and genetic factors. Some of the important factors are plant growth regulators, carbohydrates, light and pH of the culture medium [30]. However, the mechanism of the transition from the vegetative to reproductive state is not well understood in most plants. *In vitro* flowering is an important tool for minimising the influence of environmental factors and therefore this technique clarifies the key influences on flowering by a precise control of plant growth regulators. Furthermore, *in vitro* flowering also provides an ideal experimental system for plants grown *in vivo* in order to study the biological mechanism of flowering. In the present work influences of plant growth regulators on clonal propagation with different explants and *in vitro* flowering from shoot apices of *P. paniculata* have been examined. As far as we know, *in vitro* flowering in 'Garden Phlox' has not been reported. In the current work we used various concentrations of α-napthaleneacetic acid (NAA) and 6-benzylaminopurine (BAP) to simultaneously induce direct regeneration and *in vitro* flowering from shoot tips and other explants of this species using zeatin and gibberellic acid (GA_3).

MATERIALS AND METHODS

Plant Materials and Culture Conditions for *in Vitro* Shoot Regeneration

Three types of explants from leaves, petioles and stems were collected from intact garden-raised mature plants during their vegetative stages. All explants were initially washed with tap water for 30 min., surface-sterilised with 50% commercial bleach (containing 5% sodium hypochlorite) for 1 min., then rinsed with sterile distilled water at least three times and finally dipped in 70% (v/v) ethanol for 1 min., followed by rinsing with sterile distilled water three times. These sterile explants

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were cut into segments (approx. 5-10 mm in length for both petioles and stems and around 5x5-mm^2 pieces for leaves) and cultured on Murashige and Skoog solid medium [31] (0.8% Agar Technical, No.3, Oxoid Ltd., England) supplemented without (control) or with combinations of 2.6, 5.3, 8.0 and 10.6 µM NAA (Sigma-Aldrich, USA) and 2.2, 4.4, 6.6 and 8.8 µM BAP (Sigma-Aldrich, USA). The pH of the media was adjusted to 5.8 and they were then sterilised by autoclaving at 121ºC and 103 kpa for 20 min. All the cultures were maintained at 25 ± 1ºC with a 16-h photoperiod (1000 lux).

Explant Preparation and Culture Conditions for *in Vitro* Flowering

Shoot apices were collected from 4-month-old axenic cultures (vegetative stage) of the *P. paniculata* plant. The explants were further aseptically excised into small pieces containing meristems with 3-4 true leaf primordia and cultured on the solid MS medium supplemented without (control) or with various concentrations (2.2, 4.5, 6.7, 9.0 and 11.2 µM) of zeatin (Sigma-Aldrich, USA) or GA_3 (Sigma-Aldrich, USA) at 1.4, 2.8, 4.3, 5.6 and 7.2 µM. The pH of the media was adjusted to 5.8 and they were then sterilised by autoclaving (121ºC, 103 kpa) for 20 min. All the cultures were incubated at 25 ± 1 ºC with a 16-h photoperiod (1000 lux).

Morphological Analysis

All cultures were continuously observed from one week of treatment to evaluate their development by counting the total number of shoot buds/leaves initiated by the explants, and the state of the apical meristem, either vegetative or floral, was recorded. A meristem was classified as floral when the first sepal primordium of the flower that characterises the reproductive structure was visible. It was referred to as 'vegetative' when there was no apparent reproductive morphogenesis. The presence or absence of a basal callus and roots was also noted. Each experiment was repeated at least twice with 10 explants per treatment. Pooled results of the different experiments were analysed and presented.

Statistical Analysis

All data and variables were statistically analysed using SPSS statistical package version 11. Values were presented as mean ±SE. One-way ANOVA and Multiple Range Analysis were done on all data using 95% LSD intervals method.

RESULTS AND DISCUSSION

Shoot Regeneration from Different Explants

Initially, three types of explants were obtained from mature plants; leaves, petioles and stems were sterilised and cultured on MS solid medium supplemented without (control) or with different levels of phytohormones as described in the Materials and Methods section. In the beginning of the culture direct shoot bud formation was observed in all types of explants when treated with phytohormones (NAA and BAP) but not in the control. However, none of the explants showed callus formation. Within 5 weeks of culture, vegetative buds further developed and regenerated into shoots (Figure 1a). The highest frequency (100%) of shoot regeneration was obtained from leaf explants when cultured on medium supplemented with 8.0 µM NAA and 6.6 µM BAP (Figure 1b). However, at this level of phytohormones, maximum shoot regeneration was also observed in petiole and stem explants with a rate of 67.8±3.2 and 48.8±9.3% respectively (Figure 1b). Subsequently,

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root formation was observed in all clonal shoots within 11 weeks and they all developed into individual plantlets. When acclimatised and transferred to the field, about 90% of the clonal plantlets survived under natural environmental conditions without showing any morphological variation during their development.

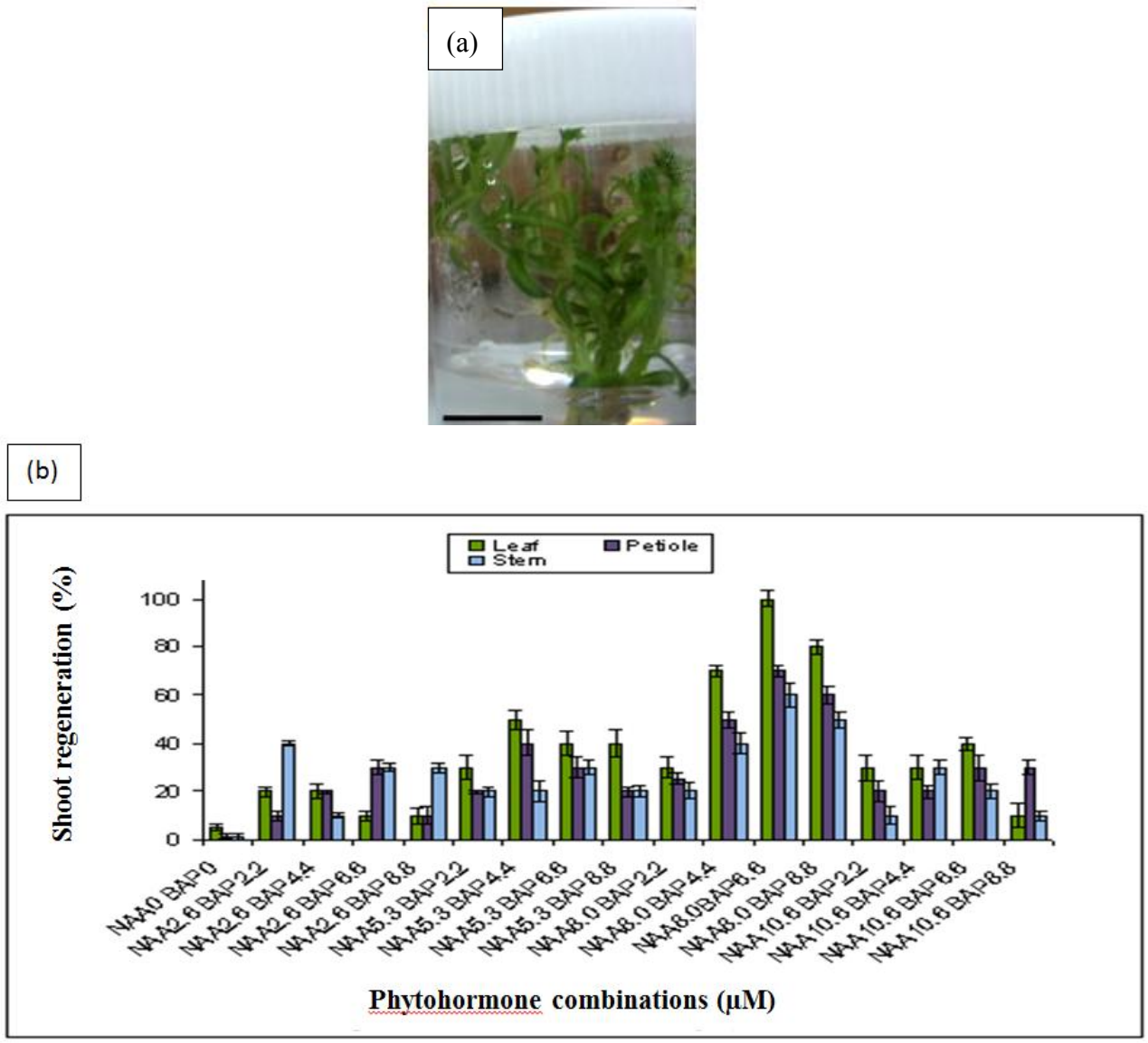

Figure 1. *In vitro* shoot regeneration of *Phlox paniculata*: (a) profuse vegetative shoot formation from mature leaf explants cultured on MS solid medium supplemented with NAA and BAP (8.0 and 6.6 µM respectively) in plastic sterile tubes after 5 weeks. The bar indicates 1 cm; (b) graph showing *in vitro* shoot regeneration pattern from different explants in response to various concentrations of NAA and BAP, after 8 weeks of culture

Cytokinin and auxin addition for the *in vitro* shoot regeneration has been studied in many plant species [32-34]. Various reports have shown that the *in vitro* shoot regeneration can be successfully induced by using a combination of BAP and NAA [35-38]. In the medicinal plant *Withania somnifera* cultured on MS medium supplemented with 8.8 µM BAP and 0.5 µM NAA, multiple shoot regeneration was observed [39]. This was also observed in *Celosia argentea* cultured on MS medium supplemented with 1.0 mg/L BAP and 0.5 or 1.0 mg/L NAA [40]. In addition, in an *in*

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vitro multiple shoot regeneration protocol of *Boerhaavia diffusa* by Roy [41], it was shown that a maximum frequency of 90% could be obtained only when it was cultured in MS medium containing BAP (6.6 μM) and NAA (2.6 μM). Consistent with those previous findings, in the present work we have also shown that NAA (8.0 μM) plus BAP (6.6 μM) treatment generates a maximum frequency of multiple shoot regeneration *in vitro* in *P. paniculata*. Even though NAA promotes rooting, there is a synergistic effect in combination with a slightly higher NAA concentration for inducing multiple shoots in *P. paniculata*. Similar findings were also reported by Elias et al. [42] and Ahmed et al. [43] in the *in vitro* shoot formation of *Echinocereus cinerascens* and *Phyla nodiflora* respectively. Thus, the success in raising plants through direct regeneration and bypassing the callogenesis phase has opened up the possibility for a large-scale clonal propagation of *P. paniculata*. When other combinations of phytohormones, like kinetin and NAA, kinetin and 3-indole acetic acid (IAA), BAP and 2,4-dichlorophenoxyacetic acid (2,4-D) or kinetin and 2,4-D, were used at different concentrations, profuse shoot regeneration was not evident in all three types of cultured segments. However, in the current work successive subculturing of the regenerated plants did not show further morphogenetic differentiation such as floral transition.

In Vitro Flowering

To induce *in vitro* flowering, shoot tip explants were further collected from 4-month-old aseptic plants and cultured on MS solid medium supplemented with various concentrations of singly applied zeatin and GA_3 (Table 1). After 4 weeks of treatment, the development of multiple shoots and initiation of flower buds were observed from cultured explants only when treated with NAA or BAP but not in the control (Table 1). Their successive subcultures generated white flowers with purple or pink stripes (Figure 2). In this experiment 3-4 flowers per plantlet developed within 7-8 weeks of culture. Treatment with zeatin at a concentration of 11.2 μM resulted in the highest frequency (67.8±3.2%), whereas 5.6 μM GA_3 gave 48.8±9.3% of *in vitro* flower induction (Table 1). However, treatment with either zeatin or GA_3 resulted in the formation of white or pink flowers occasionally. All the developed flowers were approximately 1.7-2.0 cm in width and were formed from the apical and axillary buds (Figure 2). Each bloom had 5 sepals, slightly extended and pointed at their middles with an extended and fused throat that opens into 5 distinct and overlapping lobes. However, the *in vitro* flowers failed to develop other reproductive organs such as stamens, stigmas or pistils even when they were subcultured for a long period of about 10 months

In earlier studies the cytokinins requirement for the growth and development of flower buds has been reported in both monocots [44] and dicots [45-46]. The promotion of *in vitro* flowering by cytokinins was repeatedly reported [47-53]. The influence of cytokinins on the *in vitro* flowering of *Perilla frutescens* is surprising [54]. Also, the beneficial effect of cytokinins on the induction of flowering for other plants was reported in orchids [55], *Fortunella hindsii* [56] and *Lemna* [57]. The results presented here in Table 1 are similar to those previous findings. However, high concentrations of zeatin caused inhibition of *in vitro* flowering (data not shown).

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Table 1. *In vitro* flowering of *Phlox paniculata* on MS medium supplemented with different concentrations of zeatin and GA_3

Treatment (μM)	Response	Mean % of flowering plantlets (±SE)
MS + zeatin (0.0)	No flowering response	0
MS + zeatin (2.2)	Flowering response	21.6 ± 3.7 de
MS + zeatin (4.5)	Flowering response	25.6 ± 2.9 d
MS + zeatin (6.7)	Flowering response	26.9 ± 2.8 d
MS + zeatin (9.0)	Flowering response	37.0 ± 3.3 c
MS + zeatin (11.2)	Flowering response	67.8 ± 3.2 a
MS + GA_3 (0.0)	No flowering response	0
MS + GA_3 (1.4)	Flowering response	35.1 ± 8.4 c
MS + GA_3 (2.8)	Flowering response	39.0 ± 3.6 bc
MS + GA_3 (4.3)	Flowering response	47.9 ± 3.8 b
MS + GA_3 (5.6)	Flowering response	48.8 ± 9.3 b
MS + GA_3 (7.2)	Flowering response	34.4 ± 2.3 cd

Note: Mean values followed by the same letter within a column are not significantly different at the 0.05 level according to LSD test.

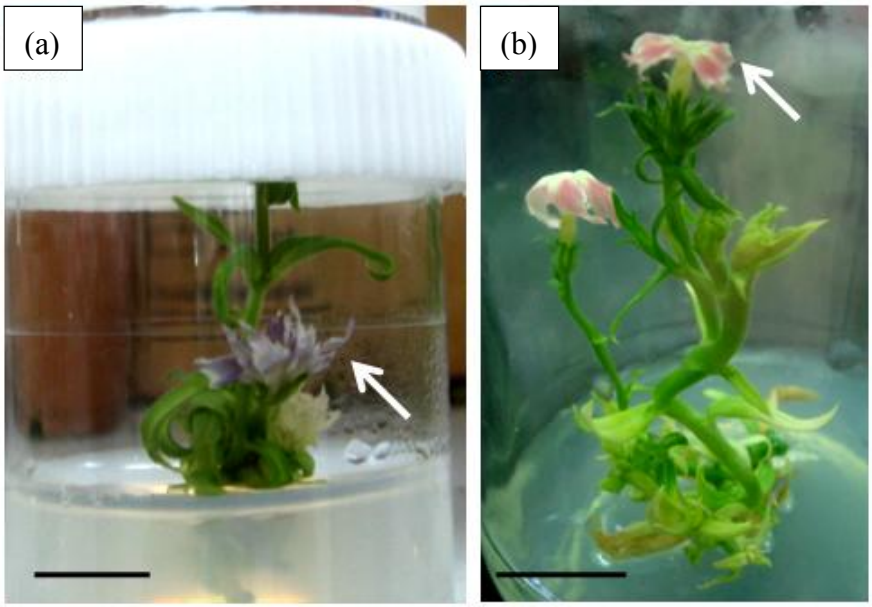

Figure 2. Flower development after 6 weeks on shoot tip explant culture of *Phlox paniculata*. The MS medium, supplemented with zeatin (11.2 μM) or GA_3 (5.6 μM), gives shoot multiplication with flowers (arrowed) of purple (a) or pink stripes (b). Bar indicates 1 cm.

In certain plants auxin has been reported to be either ineffective or inhibitory [58] in the *in vitro* flowering induction process. In this study an absence of *in vitro* flowering formation was observed when different concentrations of 2,4-D and IAA were utilised in the culture medium. In contrast, gibberellins have been reported as an inducer of the flowering process in several long-day and cold-requiring rosette plants [59]. GA_3 was even able to regenerate sporophytes from gametophyte

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explants in ferns [60]. In earlier work it has been postulated that a GA_3 promotive pathway exists in *Arabidopsis thaliana* [61], where GA_3 activates the *LEAFY* (a floral meristem identity gene) transcription [62]. Considering these findings, our results support the idea that independently, zeatin or GA_3 may form one of the key factors without which floral bud initiation and their subsequent development are not possible in *P. paniculata*. However, a detailed study on the actual mode of action of zeatin and GA_3 in the *in vitro* flowering process in *Phlox* remains to be examined. In both cases, two to four flowers were produced from each *in vitro* cultured explant (Figure 2). However, neither the flowers supplemented with zeatin nor with GA_3 showed fruiting in the subsequent culture. This might have been due to the absence of other reproductive organs. In the current study complete flowering was not observed even when they were cultured in the presence of kinetin and IAA; perhaps different conditions might be required for the induction and development of normal flowers. In previous study by Taha [63] on *Murraya paniculata*, complete plant regeneration was achieved from portions of cotyledons and shoot explants when they were cultured in MS medium supplemented with 4.44 μM BAP. On subsequent subculture on MS basal medium, 80% of flowering was obtained, while MS medium fortified with 2.69-10.74 μM NAA gave 62-72% of flowering [63]. In *Begonia x hiemalis*, the best explant for *in vitro* flowering was inflorescence cultured on MS medium supplemented with 4.44 μM BAP, 5.37 μM NAA, 4% sucrose and 40 mg/L adenine [64]. It seems that different species require different hormonal regimes for *in vitro* flowering.

Flowering is an important phase in the developmental processes of floricultural crops. In this study an attempt was made to identify the most favourable set of environmental and nutritional conditions for adventitious shoot regeneration and flower induction *in vitro*. An interesting feature of the present study is that the process of *in vitro* shoot apex flowering is responsive to only zeatin or GA_3. This is a significant phenomenon considering the fact that the explants were obtained from axenic cultures and that it is possible to avoid the maturation period spanning several months before a plant produces flowers. Further experiments should lead to a better understanding of the physiological and molecular events underlying the shift from the vegetative state to the flowering state, as well as a better understanding of factors related to overcoming flower sterility and of seed formation *in vitro*.

CONCLUSIONS

Among the explants tested, those from leaf segments were found to be the most responsive and have the highest regeneration frequency (100%) when cultured in the presence of 8.0 μM NAA and 6.6 μM BAP, whereas treatment with 11.2 μM zeatin gave the highest frequency of *in vitro* flowering (67.8 ±3.2%). This protocol may be extended to plant breeding studies for the purpose of quick flowering and fruit and seed formation under *in vitro* conditions.

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