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In Vitro Flowering of *Boronia megastigma* Nees. and the Effect of 6-Benzylaminopurine

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Abstract. Successful flower bud initiation and development was achieved on *Boronia megastigma* in vitro. The effect of cytokinin on flowering was investigated in environmental conditions that promote flowering as well as under conditions that stimulate vegetative growth (nonfloral promotory conditions). Flower initiation and differentiation was enhanced by cytokinin; however, many flower buds reverted when the media contained high levels of cytokinin. Anthesis occurred only on media that had no cytokinin added and under floral promotory conditions.

Boronia megastigma Nees. is a woody understorey shrub 1–2 m in height, bearing a profusion of strongly scented brown, purple, and yellow flowers in early spring. They are initiated in autumn and continue to differentiate during the winter. Floral and vegetative buds are normally initiated from uncommitted primordia located in the axils of nonexpanding leaves, on current season's node-bearing structures, under a range of environmental conditions. More flowers initiate (up to three per leaf axil) under low night temperatures (5–7°C) with short days (10 h) and 50–100% full sunlight (floral promotory conditions) (Roberts and Menary 1989a,b).

Differentiation and development of preexisting flower buds in vivo is enhanced by exogenous cytokinin under floral promoting conditions. However, when applied under long days (16 h) and moderate night temperatures (>15°C) many flower buds revert to vegetative differentiation rather than abort (Roberts 1989). Successful reversion to vegetative growth depends partly on the stage of flower bud differentiation when the cytokinin is applied. A flowering gradient exists along most laterals where the more mature flowers are usually located in the axils of the fourth leaves below the apex; flower maturity decreases both acropetally and basipetally from this point (Roberts and Menary 1990). The presence of the flowering gradient makes it difficult to carry out investigations on buds that initiate at the same time and have the same degree of sink strength; however, this can be achieved in vitro. The aim of this experiment was to investigate flower bud initiation and development in vitro using different levels of cytokinin and two environments.

Materials and Methods

Preliminary Experiments

Generally for woody species, embryos or young inflorescences must be used to regenerate shoot types or organs in vitro; hence, the explant must be in to a juvenile state (Tran Thanh van 1981). For example, in *Citrus* Altmen and Goren (1974), Garcia-Luis et al. (1989), and Tisserat et al. (1990) used explants that consisted of the whole bud, portions of bark and wood from the branchlet and the stump end of the petiole; Tsujikawa et al. (1990) used axillary leaf buds or growing shoot apices for successful flowering of Japanese pear. Trial experiments were carried out with *Boronia* using explants derived from a variety of sources, including: shoot proliferations, in vitro grown shoots, and current seasons' laterals. These preliminary experiments showed that flower buds could be initiated in vitro using current season's laterals as explants. However, it was necessary for explants to have an attached fully expanded leaf and no vegetative apex. No flowering was observed on media that had less than the normal concentration of mineral elements used for growth of shoot proliferations (Roberts 1989). Also, no flower buds were initiated on media containing less than 3% sucrose.

Explant Handling

Selected cuttings were taken from clonal plants to provide explants, these were located 3–4 nodes below the apex. Each explant consisted of a short section of stem (0.5 cm), a pair of fully expanded leaves with an uncommitted meristem in each leaf axil, and a further 0.5 cm of stem above the leaf junction. They were

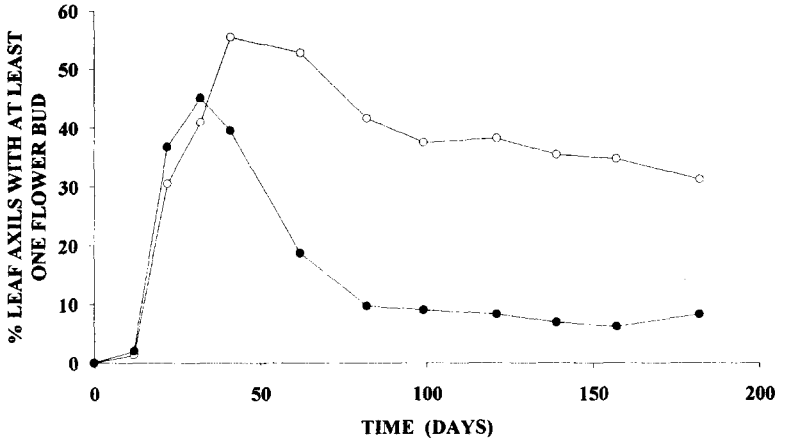

Fig. 1. The percentage of flower buds (averaged over all BAP concentrations), in vitro, in flower promoting (○) and non-promoting (●) conditions.

washed under running tap water for 30 min followed by a 20 min soak in 50% White King (commercial bleach, active ingredient sodium hypochlorite, 4%). Explants were then aseptically transferred into autoclaved water for a 20 min rinse followed by a further 10 min rinse and placed on autoclaved paper toweling in a laminar flow cabinet. Two explants were placed in 250 ml polycarbonate Bunzyl flasks containing 35 ml of media and 1.1% agar. Six flasks were used per treatment.

Media

6-Benzylaminopurine (BAP) was added at 0, 2, 4, 6, 8, and 10 mg L^{-1} to Murashige and Skoog (1962) medium as modified by Luckman (1989) and supplied with 3% (wt/vol) sucrose and 1.1% (wt/vol) agar, the pH of the media was adjusted to 5.8 before autoclaving.

Growing Conditions

Tissue culture vials were placed into two growth cabinets where the photon flux density was 165 μmol $m^{-2}s^{-1}$ at the flask height. One cabinet (flower promoting) received 10 h days at 15 ± 1°C with 14 h nights at 5 ± 1°C; the second cabinet (nonpromoting) received 16 h days at 20 ± 1°C with 8 h nights at 15 ± 1°C (Roberts and Menary 1989b).

Examination and Statistical Analysis

Explants were examined routinely by viewing through a stereo microscope. The presence or absence of floral, vegetative, transitional, aborted, and unidentified buds were recorded for each leaf axil. The percentage of leaf axils with a particular bud type was then calculated for each treatment. Since three buds can initiate from a leaf axil, it is possible to have greater than 100% of leaf axils with combined bud types.

An analysis of variance was performed on $\sqrt{(x + ½)}$ transformed data for each examination date. Transformation was necessary since the data consisted of zeros and ones. Significance was calculated at the 0.05 probability level.

Results

The percentage of leaf axils with flower buds found in both environments (averaged over all BAP concentrations) is shown in Fig. 1. This peaked after approximately 32 days in nonpromotory conditions compared with 41 days in promotory conditions (irrespective of the BAP concentration). There were significantly more flower buds in promotory conditions from day 41 to the end of the experiment. This was due to fewer flower buds reverting or aborting under these conditions.

Increasing the BAP concentration from 0–10 mg L^{-1} resulted in a general increase in flower buds except at 6 mg L^{-1} (Fig. 2). From the relatively small range of BAP concentrations trialed, it is not certain whether the responses are optimal; however, there was little difference in the response from 8–10 mg L^{-1}, which suggests that this was approaching a maximum response. The percentage of leaf axils with flower buds was significantly higher on media containing 8–10 mg BAP L^{-1} than for all other BAP concentrations from days 22–121.

The differentiation and development of flower buds was affected by the concentration of BAP and by the environment. This was reflected by the earlier exposure of small sepals and petals (with a reduced flower pedicel) at higher BAP concentrations. In nonpromotory conditions, higher BAP concentrations resulted in very short flower pedicels and the exposure of all floral parts that were immature. The effect of BAP on the speed of floral bud initiation can be seen by the comparison of the percent of leaf axils with flower buds at 0 mg L^{-1} compared with explants on media containing cytokinin (Fig. 2). The number of flower buds on media without cytokinin reached a peak at 182 days, while those with BAP peaked at 41 days. The lack of BAP in the media resulted in significantly fewer flower

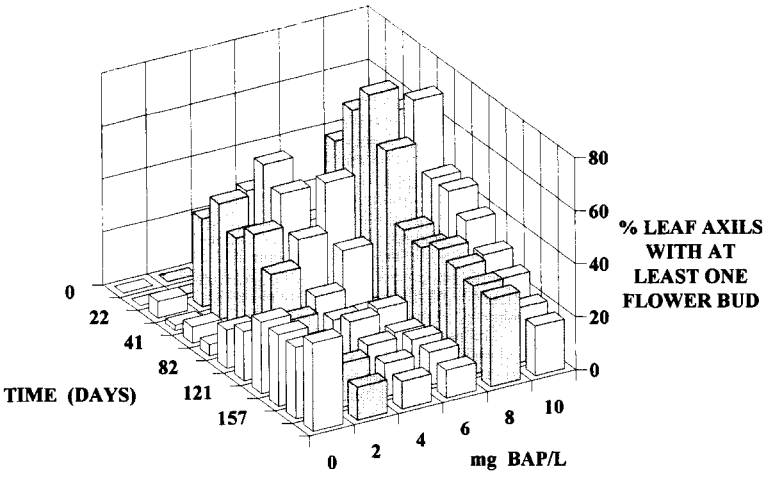

Fig. 2. The percentage of flower buds, in vitro, in flower promoting and nonpromoting conditions, on media containing 0–10 mg L^{-1} BAP.

buds from days 22–62 compared with media containing BAP. Almost no flower buds were initiated on media without BAP in nonpromotory conditions.

Many flower buds reverted to a vegetative form; however, their leaves were single-lobed compared with the normal tri-lobed and they were arranged in a floral phyllotactic pattern described by Roberts and Menary (1989a). Reversion was seen by an increase in the percentage of leaf axils with transitional buds (Fig. 3). The time at which the greatest numbers of transitional buds were recorded (62 and 82 days for nonpromotory and promotory, respectively) correlated with a decline in flower bud number in both environments. Significant differences between the environments occurred between days 41–82 and 121–139. Significantly more buds aborted under nonpromotory conditions between days 32–182 (Fig. 4). The only flowers to reach anthesis were found under promotory conditions on media containing no BAP. In promotory conditions, at 2 and 4 mg BAP L^{-1}, several flowers were approaching anthesis after 182 days. Further increases in BAP concentration in promotory conditions resulted in distorted flowers that were very compact (2–3 mm diameter) and contained chlorophyll in the petals.

The highest percentage of leaf axils with vegetative buds (averaged over all BAP concentrations) was found at 139 days in both environments (Fig. 5). There were significantly more vegetative buds under promotory conditions from days 62–182. The inclusion of BAP resulted in a significant increase in the number of vegetative buds (Fig. 6); this effect was enhanced under nonpromotory conditions. Increasing the BAP concentration resulted in a decrease in the plastochron, as a consequence many vegetative buds had short internodes (<1 mm). Also, the vegetative buds that initiated at higher BAP concentrations often had single short lobed (2–3 mm long) leaves in comparison with the more elongate (5 mm) tri-lobed leaves at lower BAP concentrations.

The slow rate of initiation and differentiation of buds at 0 mg L^{-1} eventually resulted in a high percentage of the buds aborting before their differentiation pattern was identified as floral. This was seen by the large increase in percentage of leaf axils with aborted buds at 99 days (Fig. 7). Buds on media containing BAP began aborting at 32 days; however, the rise in aborted buds increased relatively slowly, with the exception of media containing 6 mg L^{-1}.

Discussion

The addition of cytokinin to the media enhanced the number of floral and vegetative buds that initiated in vitro. This has been noted in a number of other plants—for example, leaf explants of *Streptocarpus nobilis* (Simmonds 1982), thin cell layers of *N. tabaccum* L. (Heylen and Vendrig 1988, Van der Krieken et al. 1988), *Panax ginseng* embryoids derived from mature root cells (Chang and Hsing 1980), and *Passiflora suberosa* L. (Scorza and Janick 1980). Furthermore, the optimal concentrations of both cytokinin and auxin for flower bud development on *N. tabaccum* have been determined by Van der Krieken et al. (1988) and Smulders et al. (1988). From the concentration range investigated, it was not possible to determine an optimum level of BAP for flower bud initiation.

Van der Krieken et al. (1988) were able to understand the mechanism of hormone action by relating hormonal levels to the number of flower buds produced. They were able to quantify the uptake and

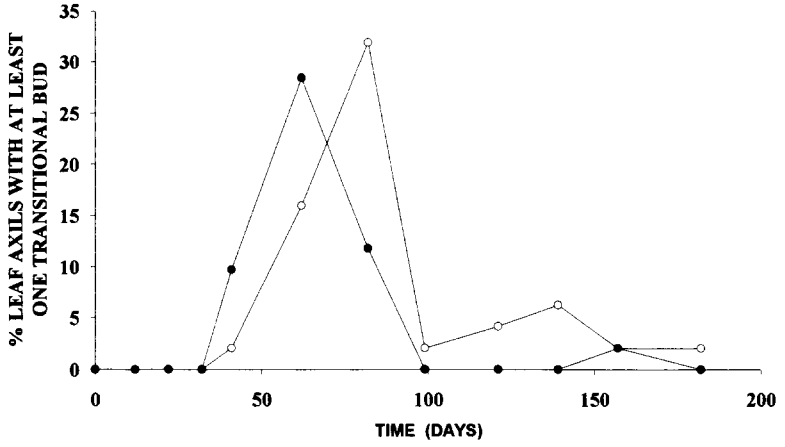

Fig. 3. The percentage of transitional buds (averaged over all BAP concentrations), in vitro, in flower promoting (○) and nonpromoting (●) conditions.

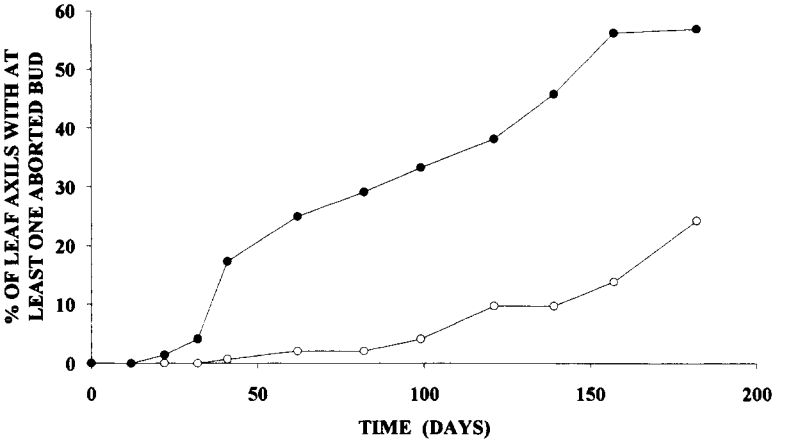

Fig. 4. The percentage of aborted buds (averaged over all BAP concentrations), in vitro, in flower promoting (○) and nonpromoting (●) conditions.

conversion of BAP and correlate its effects on flowering in tobacco pedicel tissue. Similarly, Roberts et al. (1991) found that the level of endogenous cytokinins in vivo increased only after flower bud initiation in *B. megastigma*. However, the radioimmunoassay technique used was only applicable for three forms of cytokinin and not to metabolites.

Richards (1985) found that exogenous applications of BAP in vivo did not enhance flowering in *B. heterophylla* under either inductive or noninductive conditions. Preexisting floral buds on intact *B. megastigma* plants under nonpromotory conditions showed an enhanced rate of development after exogenous BAP applications (Roberts 1989). Hence, the rate of development of predetermined differentiation patterns was enhanced by BAP, which correlates with the elevated endogenous cytokinin concentrations after flower bud initiation in situ reported by Roberts et al. (1991).

The fact that flower buds were initiated on media containing no cytokinin further suggests that enhanced levels may not be required for floral initiation. Alternatively, the explants may have been able to supply endogenous cytokinin from metabolism of conjugates. The slow development of flower buds on media without BAP is likely to reflect the limited ability of the explant to produce its own cytokinin under these conditions.

In *Boronia*, higher cytokinin levels enhanced the rate of flower bud development; and this was reflected by the earlier exposure of small sepals and petals and a reduced flower pedicel length. However, it also tended to promote reversion resulting in vegetative differentiation. The failure of most *Boronia* flowers to reach anthesis, particularly at the higher BAP concentrations, could have resulted from different hormonal requirements at various stages of floral development (Rastogi and Sawhney 1989). The importance of timing on auxin and cytokinin in the media with respect to flower bud initiation and development in *N. tabaccum* L. was demonstrated by Smulders et al. (1990a,b). Some of the floral abortion on *Boronia* explants may have been due to a decline of mineral nutrients and/or hormones.

The promotion of vegetative differentiation in the

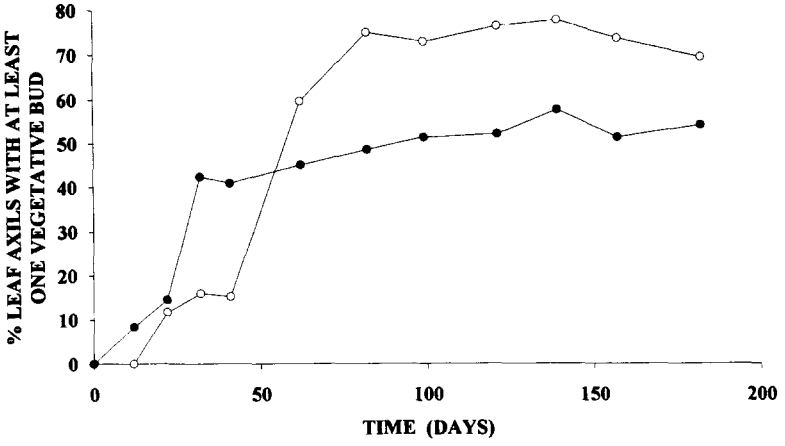

Fig. 5. The percentage of vegetative buds (averaged over all BAP concentrations), in vitro, in flower promoting (○) and nonpromoting (●) conditions.

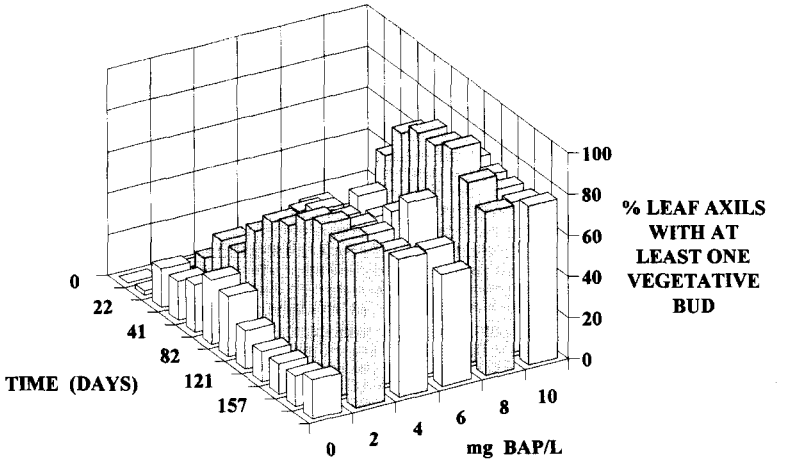

Fig. 6. The percentage of vegetative buds, in vitro, in flower promoting and nonpromoting conditions, on media containing 0–10 mg L^{-1} BAP.

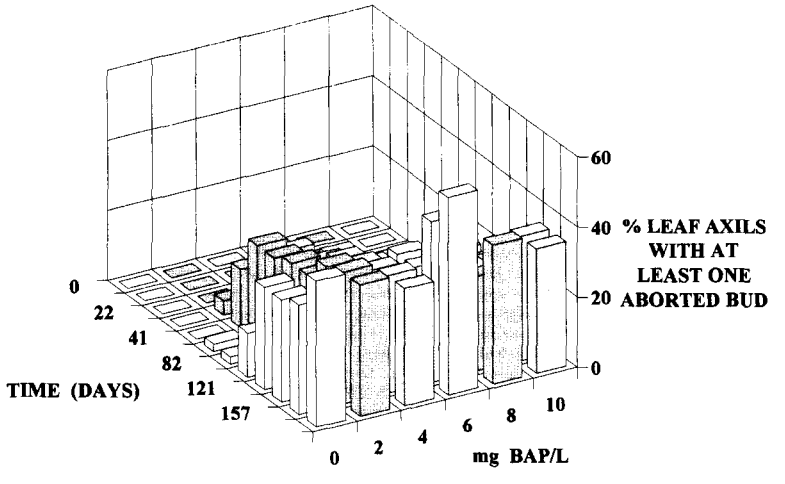

Fig. 7. The percentage of aborted buds (averaged over both flower promoting and nonpromoting conditions), in vitro, on media containing 0–10 mg L^{-1} BAP.

presence of high cytokinin concentrations is supported by the work of Richards (1985) on *Boronia heterophylla*, where the exogenous application of BAP under nonpromotory conditions increased the number of laterals that developed. Vegetative apical dominance in *B. megastigma* in vivo has been described by Roberts and Menary (1990); it is likely that the strong tendency for flower buds to revert with increased BAP concentration in vitro is the expression of apical dominance.

Boronia explants required promotory conditions for flowers to reach anthesis; thus, indicating that the development of flowers in vitro is dependent upon the appropriate environmental stimuli. Similar

results were demonstrated in *Citrus limon* (L.), where lateral buds required relatively cool conditions (14–20°C); but in this case the promotory effect could be inhibited by treatment at higher temperatures (25°C) (Tisserat et al. 1990).

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