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Explant Initiation Date and BA Concentration Influence Shoot Proliferation in vitro of Two *Liatris* Interspecific Hybrids

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***Abstract*. Shoot proliferation cultures were established in vitro using flower-stem explants from two different interspecific hybrid plants of *Liatris*. Explants taken on two dates from field-grown plants were successfully established and axillary shoot growth promoted on a medium consisting of Murashige and Skoog basal salts and vitamins with 30 g·L^{-1} sucrose, 1.0 µM BA, and 7.0 g·L^{-1} agar, with a medium pH = 5.7. Initial explant contamination rates were significantly higher among explants collected later in the growing season. Addition of BA (1.0, 2.0, 4.0, 8.0, or 16.0 µM) improved shoot formation compared to the control for both plants. Proliferation rates differed between the dates of establishment, the plants, and the BA treatments. Shoots rooted readily in medium without PGRs or with 1.0, 2.0, 4.0, or 8.0 µM K-IBA. Overall rooting was 88%. About 90% of the plants rooted in the presence of 1.0 µM K-IBA were successfully established in the greenhouse. Chemical names used: 6-benzyl adenine (BA); potassium salt of indole-3-butyric acid (K-IBA).**

Liatris Schreb., commonly known as blazing star or gay feather, is a genus of the Asteraceae consisting of ≈40 species native to the eastern and central United States (Gaiser, 1946). Plants produce one to several upright flowering stems, each bearing multiple capitula with fuchsia to pink, or white florets. Individual plants typically bloom for 3 weeks and different taxa are in bloom from midsummer to late fall. Selections of *Liatris aspera* Michx., *L. pycnostachya* Michx. and *L. spicata* (L.) Willd. are commercially grown for cut-flower production (Stevens et al., 1993). These species, and to a lesser degree *L. ligulistylis* (A. Nels.) K. Schum., *L. punctata* Hook., and *L. scariosa* (L.) Willd., are grown as garden perennial plants (Armitage, 1997; Stevens et al., 1993). All *Liatris* are highly recommended plants for butterfly gardens (Clausen and Ekstrom, 1989).

Plants are grown from seed, stem cuttings, or division of the persistent woody corms (Stevens et al., 1993). Stem cuttings produce short flowering stems (Salac and Fitzgerald, 1983) and for this reason are not widely used commercially (Stevens et al., 1993). Corms can be readily divided in spring or fall; however, the number of divisions that can be obtained from each clump is limited. Seed germinates within 21 to 28 d at 20 to 21 ºC (Nau, 1996) and is likely the most widely utilized method of propagation. However, at least four species are self-incompatible (Levin, 1968; Godt and Hamrick, 1995). If this is valid for all of the taxa of commercial interest, then seed are likely being produced from outcrossed individuals, which may result in genetically and phenotypically variable progeny.

Liatris can form hybrid swarms in the wild (Gaiser, 1951; Levin, 1968), which points out the potential for developing novel interspecific forms for horticultural use. Though this information has been available for a number of years, virtually no intentionally developed interspecific hybrids have been introduced into either the commercial cut flower or garden plant trade. The reduction of fertility seen in some of the naturally occurring hybrid combinations (Levin, 1968), and the presumptive self-incompatibility of the genus, may preclude the development of uniform seed lines from hybrid combinations. Therefore, a system for the mass clonal propagation of selected interspecific hybrid plants may be needed. Tissue culture could offer a method for clonal propagation. There are, to date, only two reports on the tissue culture propagation of *Liatris,* both of *L. spicata* (Stimart and Harbage, 1989; Stimart and Mather, 1996). This current study was initiated to assess the potential for tissue culture propagation of two interspecific *Liatris* hybrids developed in a breeding program.

Materials and Methods

An individual 2-year-old, field-grown plant was selected from each of two hybrid crosses: accession #98088 (*Liatris* [*spicata* 'Kobold' × *ligulistylis*] × [*pycnostachya* 'Alba' × *spicata* 'Floristan Weiss']) and accession #98112 (*Liatris* [*pycnostachya* 'Alba' × *spicata* 'Floristan Weiss'] × [*ligulistylis* × *scariosa* var. *nieuwlandii*]). Two shoots were removed from each plant on each of two explant initiation dates (29 June and 13 July 2000, respectively). The distal portion with visible flower buds of each stem was removed and discarded. All leaves were removed from the remaining stem portions and the stems cut into 5- to 7-cm segments, which were surface-disinfested for 12 min in a stirred solution of 1.05% sodium hypochlorite with 3 drops of Tween 80 (Sigma Chemical Company, St. Louis, Mo.) per 500 mL of solution, then individually rinsed for 10 min each in a single change of sterile distilled water. Explants ≈1.0 to 1.5 cm in length with one to three nodes each were individually placed basal end down in 25 × 150-mm culture tubes containing 10 mL of initiation medium [MS (Murashige and Skoog, 1962) basal salts and vitamins, 30 g·L^{-1} sucrose, 1.0 µM BA, 7.0 g·L^{-1} Sigma A 1296 agar (Sigma Chemical Co., St. Louis, Mo.), and pH of 5.7]. Before explant placement, culture tubes were sealed with polypropylene caps and autoclaved at 121 ºC for 15 min. In total, 40 explants were cultured for each plant on the first initiation date and 32 explants per plant the second initiation date. Fewer explants were used the second initiation date as there was proportionally less stem tissue without expanding flower buds available for use. After explant placement, culture tubes were sealed with Parafilm, placed upright in 40-tube racks, and maintained at 20 ºC in the light - a 14-h photoperiod and 30 µmol·m^{-2}·s^{-1} [measured with a quantum sensor (LI-190SA; LI-COR, Inc., Lincoln, Neb.)] provided by two 40-W cool-white fluorescent lamps.

Primary explants were cultured for three to 4 weeks, at which time all noncontaminated axillary shoots (identified by a lack of visible contaminants on or in the medium) were excised and individually transferred to fresh medium as above. Due to the high levels of initial contamination, 2 mL·L^{-1} plant preservative mixture (PPM; Plant Cell Technology, Wash., D.C.) was added to the medium for one culture period. Axillary shoots one to three cm in height were subsequently excised and transferred to fresh medium without PPM every 3 to 5 weeks until a sufficient number of shoots were formed for both the proliferation and rooting studies. Due to the variable initial explant survival and different proliferation rates during the shoot increase phase, it took a total of 4 months to increase the number of shoots for accession #98088 initiated the earlier date; 11 months for accession #98088 initiated the later date; 7 months for accession #98112 initiated the earlier date; and 11 months for accession #98112 initiated the later date.

To assess the influence of BA concentration, individual, unbranched axillary shoots were aseptically excised from shoots generated in vitro then transferred to culture tubes containing MS medium with 0.0, 1.0, 2.0, 4.0, 8.0, or 16.0 µM BA. After 6 weeks of culture, the number of harvestable axillary shoots per explant was recorded. A shoot was scored as harvestable if it had three or more leaves each ≥1.0 cm in length.

The rooting study was initiated at the same time as the shoot proliferation study for each accession and culture initiation date. Individual, nonrooted axillary shoots were aseptically excised from the shoots generated

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in vitro, and cultured on MS medium with 0.0, 1.0, 2.0, 4.0, or 8.0 μM K-IBA (potassium salt of indole-3-butyric acid). After 6 weeks of culture, the number of rooted shoots per treatment was recorded. A shoot was scored as rooted if it had 1 or more roots ≥1.0 cm in length.

On 8 July 2002, 120 nonrooted shoots of each clone were transferred to fresh medium with 1.0 μM K-IBA. After four weeks of culture, rooted shoots were removed from culture, rinsed free of medium, and planted in 72-cell plug trays containing Sunshine SB 300 Universal Mix (SunGro Horticulture, Vancouver, B.C., Canada). Trays were covered with clear plastic domes, and placed in a shaded greenhouse with ≈15.25 h of sunlight daily and an average maximum/minimum temperature of 29/16 °C (averaged over the first 7 d). Domes were removed after several days. Plant survival was recorded 7 weeks after plants were removed from tissue culture.

Experimental design and statistical analysis. In total, 40 explants were randomly assigned to each shoot proliferation treatment and to each rooting treatment. After explant placement, the tubes for each treatment were placed in the same rack and the racks randomly placed in the incubator. The shoot proliferation and rooting experiments were conducted once. Data were subjected to polynomial regression (linear and quadratic level). Rooting percentages were transformed using arcsine before statistical analysis (nontransformed data presented in table). The G-statistic was used to compare explant survival between the two initiation dates for each plant and to compare shoot acclimatization in the greenhouse between the two clones. Data were analyzed using CoStat statistical software (CoHort Software, Berkeley, Calif.).

Results and Discussion

The number of visually noncontaminated explants for each plant decreased significantly from the first to the second culture initiation date [from 40% to 9.4% for plant 98088 (P = 0.0026**) and from 40.0% to 12.5% for plant 98112 (P = 0.0085**)]. Each of the surviving explants produced one to three axillary shoots. There was no callus or root production. Visible contamination was not a factor after the second culture period.

Disinfestation of field-grown plant material may be problematic due to the high bacterial and fungal load often encountered in the field. In one report, explants taken from greenhouse-grown citrus trees resulted in<5% contamination, whereas explants taken from the same cultivar that was field-grown resulted in 85% contamination (Niedz and Bausher, 2002). It is also not uncommon for contamination to increase as the field season progresses (George, 1993), as was observed in this study. One potential contributing factor was that a greater portion of the *Liatris* stem tissue was discarded the second initiation date due to flower bud expansion, thereby resulting in explants being taken from stem tissue closer to ground level. It is possible that soilborne contaminants were more prevalent on this lower stem tissue due to soil splashing on the stems during irrigation. For in vitro explant initiation from field grown plants of *Liatris*, this study suggests that explants should be taken earlier in the season to lessen contamination. Conversely, *Liatris* stock plants can be grown in a greenhouse for use as explant sources in vitro (Stimart and Harbage, 1989). This latter study does not report explant contamination rate.

For both clones of *Liatris*, all of the BA treatments increased the number of shoots formed per explant in comparison with the control treatment (Table 1). The highest regeneration rate was 8.3 shoots per explant, which was promoted by 16.0 μM BA for clone 98088 from the earlier explant initiation date. Shoot formation varied between the two clones (Table 1). Differential response to shoot promoting treatments even between closely related genotypes is a commonly reported phenomenon. For example, significant differences were observed in regeneration response between six cultivars of *Gypsophila paniculata* L. (Zuker et al., 1997). By the way of comparison, Stimart and Harbage (1989) reported a maximum shoot formation rate in *Liatris spicata* of 19.8 shoots per explant as induced by 2.7 μM BA. For optimal shoot formation in different genotypes of *Liatris*, it is therefore suggested that a range of BA concentrations be tested.

The data suggests that even higher shoot proliferation rates may have been attained if higher BA concentrations had been tested (Table 1). However, the optimization of in vitro shoot propagation systems does not necessarily rely just on the maximum number of shoots regenerated, but also on shoot quality. It was observed in this study that the shoots formed on the media with either 8.0 or 16.0 μM BA exhibited higher rates of shoot hyperhydricity (also known as vitrification; data not reported). This condition, which results in succulent and brittle-appearing shoots, invariably negatively impacts subsequent shoot regeneration and rootability. The growth regulator BA has been implicated in a number of studies as promoting hyperhydricity (George, 1996). Therefore, while a higher concentration of BA may in turn stimulate a higher rate of shoot formation, the number of usable shoots produced needs to be examined in determining the optimal concentration of BA for propagation purposes.

There was a difference in shoot formation due to explant initiation date, as fewer shoots formed on average from explants initiated on the later date (Table 1). Stems used for the later initiation date were physiologically more mature, as evident by a greater number of axils with expanded flower buds. Often mature tissue exhibits a lower shoot regenerative competence. For example, the regeneration competence was much higher in *Gypsophila paniculata* when younger rather than older leaf explants were used (Zuker et al., 1997). Conversely, cultures initiated on the later date in this study were maintained longer in vitro before the shoot induction study. A decrease in shoot formation

Table 1. Effects of culture establishment date (2000) and BA treatment on the mean number of shoots regenerated in vitro for two *Liatris* interspecific hybrid plants.

BA treatment	Shoots per explant for each plant and initiation date[z]			
	#98088		#98112	
(μM)	29 June	13 July	29 June	13 July
0.0	1.5	1.7	1.2	1.1
1.0	5.4	4.6	3.1	2.8
2.0	4.7	4.6	3.5	2.9
4.0	3.8	3.6	4.0	3.6
8.0	5.5	5.1	5.0	3.9
16.0	8.3	5.2	4.7	4.4
Linear	***	***	***	***
Quadratic	NS	***	**	NS

[z]Values represent mean number of shoots from 40 explants for each plant. Date is when plants were established in tissue culture.
NS,**,***Nonsignificant or significant at $P \leq 0.01$ or 0.001, respectively.

Table 2. Effects of culture establishment date and K-IBA treatment on rooting percentages in vitro for two *Liatris* interspecific hybrid plants.

K-IBA treatment	Rooting frequency (%) for each plant and initiation date[z]			
	#98088		#98112	
(μM)	29 June	13 July	29 June	13 July
0.0	90	63	61	98
1.0	85	100	77	100
2.0	95	100	84	98
4.0	70	100	85	100
8.0	75	98	85	100
Linear	NS	NS	**	NS
Quadratic	NS	NS	*	NS

[z]Values represent rooting percentage of 40 shoots for each plant establishment date. Rooting percentages were transformed with arcsine before regression analysis. Nontransformed data presented. Date is when plants were established in tissue culture.
NS,*,**Nonsignificant or significant at $P \leq 0.05$ or 0.01, respectively.

competence with increasing time in vitro has been also demonstrated for a number of taxa, such as for three *Morus* species in which the propagation rate declined after three to five culture periods (Pattnaik and Chand, 1997). A more controlled study of the effects of explant age and duration in culture on shoot formation in *Liatris* is warranted.

Shoots rooted readily in vitro in MS medium with or without K-IBA (Table 2). There was no significant difference in rooting between the K-IBA treatments for clone 98088 for either explant initiation date, or for clone 98112 for the second initiation date (Table 2). Overall rooting percentage was 88%, which should be adequate for commercial propagation purposes. By comparison, a higher rooting percentage (98%) was observed for in vitro generated shoots of *L. spicata* (Stimart and Harbage, 1989).

There was a difference in rooting percentage between the two explant initiation dates. 80% of the shoots from the cultures initiated the earlier date subsequently rooted, whereas 95% of the shoots from the cultures initiated the later date subsequently rooted. This is in contrast to the shoot proliferation rates, in which more shoots formed from explants initiated during the first initiation session. Cultures established on the later date were maintained in vitro much longer (4 and 7 months respectively for the two accessions) than the cultures established the earlier date before the rooting study. For many woody plants, an increased duration in vitro often promotes a juvenile growth state, which in turn can increase rootability. However, this has not been widely reported to also occur in herbaceous plants. Further research on the effect of culture duration on *Liatris* rooting in vitro is recommended.

The two clones did not differ significantly for plant acclimatization in the greenhouse ($P = 0.2729^{ns}$). Plant acclimatization was 92% (72/77 shoots) for accession #98088 and 87% (103/118) for accession #98112. These percentages appear to be acceptable for commercial production. The two published studies on tissue culture propagation of *Liatris spicata* (Stimart and Harbage, 1989; Stimart and Mather, 1996) also reported successful greenhouse acclimatization, but did not report the rates.

In summary, BA has a stimulatory effect on in vitro shoot production of two interspecific hybrid *Liatris*. The data suggests that the optimal concentration may vary for each genotype tested, and that a higher concentration than those tested in this study may be warranted. Shoot hyperhydricity needs to be monitored against BA concentration. Shoots root readily in vitro with or without K-IBA treatment and can be successfully acclimatized to greenhouse conditions. Shoot proliferation rates and rooting percentages may change with time in culture, which needs further experimentation to verify. While explants can be successfully isolated from field grown plants, high initial contamination rates suggests that stock plants should be greenhouse grown before explant initiation into culture.

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