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ORIGINAL PAPER

Regeneration and plantlet development from somatic tissues of *Aristolochia fimbriata*

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Abstract *Aristolochia fimbriata* is a small herbaceous perennial in the basal angiosperm family Aristolochiaceae. The family contains diverse floral forms ranging from radial to monosymmetric flowers with a wide variety of insect pollinators. Additionally, *Aristolochia* species contain secondary metabolites that are important natural toxins and traditional medicines, and are critical to the reproduction of swallowtail butterflies. These characteristics, in combination with the small genome size and short life cycle of *A. fimbriata*, have prompted further development of this species as a model system to study the evolution of basal angiosperms. As a prerequisite for developing a genetic transformation procedure for *Aristolochia*, we developed protocols for in vitro plant multiplication, shoot organogenesis, rooting, and acclimation of tissue culture-derived plants. Two varieties of *Aristolochia* were multiplied in vitro and rooted with 100% efficiency. Shoot regeneration was achieved within 1 month of culture initiation from whole leaf, internodal stem, and petiole explants. The highest regeneration success (97%) was recorded for stem explants. Regenerated and rooted shoots were acclimated to greenhouse conditions and developed flowers within 4 weeks of transplanting.

Keywords *Aristolochia fimbriata* · Basal angiosperm · Micropropagation · Shoot organogenesis

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Introduction

Aristolochia fimbriata is a flowering perennial plant cultivated for its interesting flowers, attraction of butterflies, and traditional medicinal properties. The flowers of *Aristolochia* species have a unipartite, monosymmetric perianth adapted for insect pollination, while other genera in Aristolochiaceae have radially symmetric flowers (e.g., *Saruma*, *Asarum*) (Gonzalez and Stevenson 2000). *Aristolochia* species have been studied for their pollination strategies associated with floral aroma and a fly-trapping perianth (Banziger et al. 2006; Hall and Brown 1993; Murugan et al. 2006; Petch 1924; Sakai 2002; Trujillo and Sersic 2006), as well as for species-specific host plant relationships with swallowtail butterfly larvae. Secondary metabolites produced by *Aristolochia* plants are critical for the defense and survival of the butterflies during their larval feeding stage (Klitzke and Brown 2000; Rausher 1981), such that the decline of particular swallowtail butterfly populations is attributed to the declining distribution of particular *Aristolochia* species (Sands et al. 1997). These secondary metabolites include aristolochic acids and aristolactams produced via alkaloid biosynthesis pathways (Kumar et al. 2003).

Aristolochia species, particularly the roots of the plants, have been used as traditional medicines (Reddy et al. 1995) and have been studied to better understand the molecular mechanisms underlying their observed human health effects

(Hranjec et al. 2005; Hwang et al. 2006; Lemos et al. 1993; Levi et al. 1998; Meinl et al. 2006; Nortier et al. 2000; Qiu et al. 2000). The potent toxins found in *Aristolochia* offer promise in specific applications, including antivenom (Abubakar et al. 2006; Jimenez-Ferrer et al. 2005; Otero et al. 2000), antibacterial (Gadhi et al. 2001a, b, 1999), antifertility (Gupta et al. 1996; Pakrashi and Chakrabarty 1978; Pakrashi and Pakrasi 1979), cytotoxic (Hinou et al. 1990; Kupchan and Doskotch 1962), antimicrobial or trypanocidal (Abe et al. 2002; Elizabeth and Raju 2006; Kumar et al. 2006), and insecticidal (Broussalis et al. 1999; Jbilou et al. 2006; Lajide et al. 1993; Nascimento et al. 2004).

In addition to its medicinal value and importance for butterfly reproduction, *Aristolochia* occupies an important phylogenetic position. As a basal angiosperm from the magnoliid clade (Jansen et al. 2007), it offers uncommon opportunities for studying the evolution of development in flowering plants. Evolutionarily conserved developmental pathways, such as those controlling floral organ identities in angiosperms, have been identified via functional studies in model plants including *Arabidopsis*, *Petunia*, *Zea*, and *Oryza* (Agrawal et al. 2005; Kramer et al. 1998; Ma and dePamphilis 2000; Whipple et al. 2004; Zahn et al. 2005). Comparative studies of these plant species have led to hypotheses about the evolution of gene function in flowering plants (Jaramillo and Kramer 2004; Kim et al. 2005). However, the monocot and eudicot lineages from which current model systems arose diverged only about 113–133 million years ago (mya), some 28–48 million years after the angiosperm divergence (~161 mya) (Bell et al. 2005; Leebens-Mack et al. 2005). If pathways found in monocots and/or core eudicots are also found to be present in a basal angiosperm such as *Aristolochia*, this would imply that the conserved components were present in angiosperms before the origin of these two major lineages of angiosperms. Comparative gene-functional analysis between basal angiosperms and derived model plants is a powerful approach to test this hypothesis. For these reasons research by the ancestral angiosperm phylogeny group (AAGP; http://ancangio.uga.edu/ancangiodb.html) has generated 30,000 capillary ESTs from pre-meiotic flowers and more than 2 million 454 whole-plant EST sequences for *A. fimbriata*. Therefore, further establishment of this plant species as basal angiosperm experimental model will be of great benefit to the study of plant evolutionary biology.

Plant genetic manipulation is essential for testing hypotheses about gene function, and *Agrobacterium*-mediated transformation offers a convenient and efficient tool for this purpose. Transformation can be used to generate both loss-of-function and gain-of-function transgenic plants. The easy-to-use and efficient *in planta* "dip" transformation method currently used with *Arabidopsis thaliana* is rare among plant systems (Bent 2000). Traditional tissue culture based transformation systems are used in other model plant species, including *Nicotiana tabacum* (An et al. 1986), *Medicago truncatula* (McKersie et al. 1997), *Lotus japonica* (Handberg and Stougaard 1992), *Petunia hybrida* (Napoli et al. 1990), *Lycopersicon esculentum* (Dan et al. 2006), *Brachypodium distachyon* (Draper et al. 2001), *Oryza* (Sallaud et al. 2003) and *Zea* (Frame et al. 2002).

Efficient tissue culture based propagation and regeneration systems for *A. fimbriata* are important prerequisites for the development of *Agrobacterium tumefaciens*-mediated transformation. Prior to this study, in vitro methods for *Aristolochia indica*, a species valued for medicinal use (Shafi et al. 2002), were developed using axillary shoot multiplication and shoot organogenesis to provide source tissue for the purification of secondary metabolites (Remashree et al. 1997; Soniya and Sujitha 2006). Here we report the development of methods for micropropagation and shoot regeneration of two genotypes of *A. fimbriata*, a species with a small genome size (Bharathan et al. 1994) and promising characteristics for use as a basal angiosperm model system, such as short generation time and small size. These methods will provide tools for fast propagation of large number of clonal plants and will support the development of genetic transformation methods.

Materials and methods

Plant material

Seeds of *A. fimbriata* were provided by Larry D. Rosen, Florida, USA and by Jardim Botanico, Departamento de Botanica, Universidade de Coimbra, Portugal. Plants were grown from the seeds and maintained in a greenhouse at The Pennsylvania State University, University Park, PA. All seeds were germinated in soil-free potting medium (Pro-Mix BX, Premier Horticulture Inc., Quakertown, PA) in shallow germination trays with drainage holes, in the greenhouse at 18–27°C (varying from night to day) and 40–70% humidity. The trays were incubated on heating mats operating at ~27°C. Natural day length was supplemented with high-pressure sodium lamps (1,000 W) from October through April to provide 12 h days. Plants received regular watering as needed. Depending on the stage of growth, regular fertilizer applications were provided, as a drench, alternating Peter's Professional 15-16-17 Peat Lite Special at 200 PPM nitrogen (once to twice weekly) with Peters Professional 21-7-7 Acid Special (Scotts Horticulture, Marysville, OH) at 200 PPM nitrogen (approximately every 6 weeks). The plants were drenched

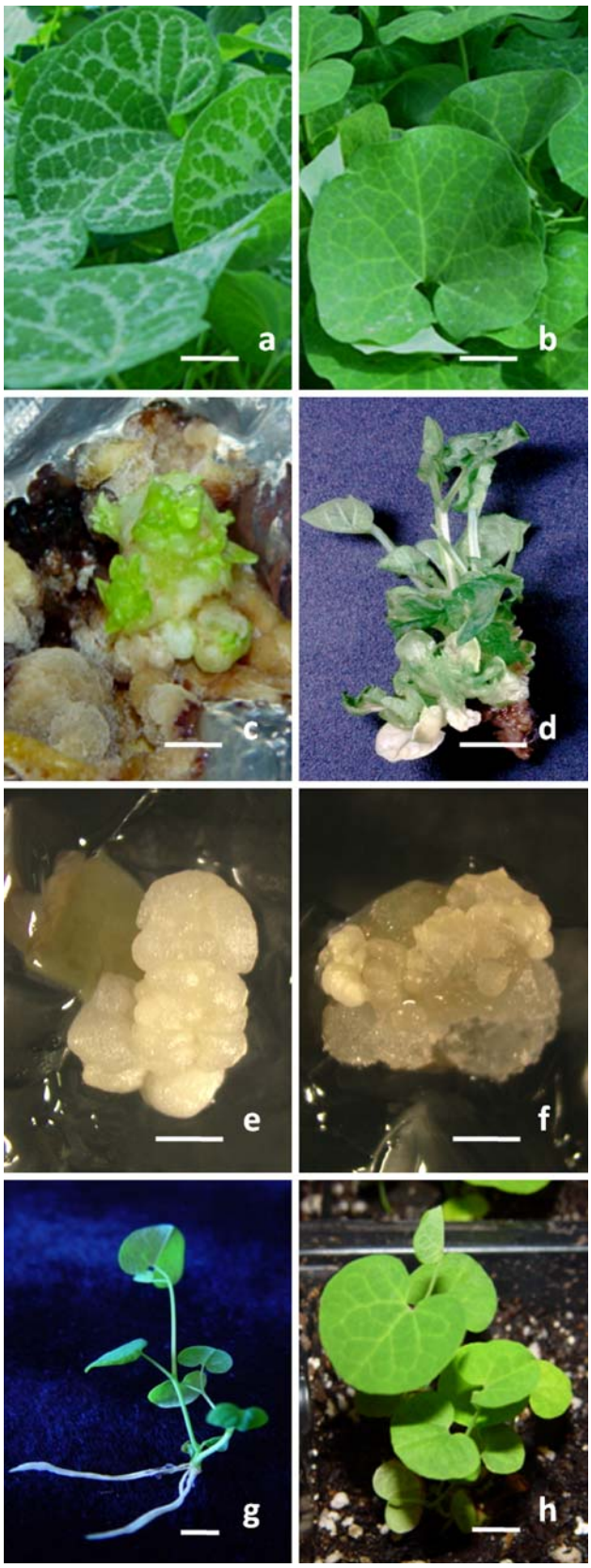

Fig. 1 *Aristolochia fimbriata*. **a** Variegated leaf (*VL*) genotype; **b** non-variegated (*NV*) genotype; **c** regenerating petiole from whole leaf explant; **d** elongating shoots; **e** regenerating stem from small section; **f** regenerating petiole from small section; **g** single shoot, separated and rooted; **h** rooted regenerant after 7 days in soil. *Scale bars* = 1 cm (**a**, **b**, **c**, **d**, **g**, **h**) or 1 mm (**e**, **f**)

once a month with 100 ppm chelated iron (Sprint 330 10% iron, RoseCare.com, Santa Barbara, CA). Plants grown from Florida seeds had variegated leaves (VL), while those from Coimbra were not variegated (NV) (Fig. 1a, b).

Micropropagation initiation and multiplication

Six month old, healthy, flowering plants of both NV and VL genotypes grown in greenhouse were selected for in vitro propagation. Green stems were collected from newly developed branch terminals. Only the three most distal (youngest) nodes per stem were used and single-node cuttings (1–2 cm long, 1.5–2.0 mm in diameter) were generated. Based on material availability total of 30 NV and 95 VL cuttings with apical or axillary shoots were generated. Fully expanded leaves were removed and the cuttings were sterilized for 20 min in 1% Chlorox solution (Commercial Solutions®; 6.15% sodium hypochlorite) with 1% Tween 20 detergent (polyoxyethylene sorbitan monolaurate; Sigma Chemical Co., St. Louis, MO, USA). Explants were rinsed three times in sterile de-ionized water and stems freshly trimmed to a 45° angle before being inserted into 60–75 ml micropropagation initiation medium (MI, Table 1) in disposable plastic food cups (Sweetheart, Owings Mills, MD, #DSD8X and #LDS58). Five cuttings were placed in each cup. Cultures were incubated in a Conviron growth chamber (Winnipeg, Canada) at 25°C with 300 micromoles m^{-2} s^{-1} light (16 h a day) for $\sim$20 days. The number of shoots produced from each greenhouse cutting was counted 21 days after introduction in culture.

The newly established tissue cultured *A. fimbriata* plants were further multiplied in vitro by single-node cuttings (1–2 cm long with a apical or axillary shoot). The cuttings were cultured on different micropropagation media (MP) (Table 1). The MP media contained combinations of 6-benzoaminopurine (6BA) (concentrations of 1.0, 2.0, 3.0, 4.0, or 5.0 mg/l) and α-naphthalene acetic acid (NAA) (concentrations of 0.0, 0.1, 0.5 or 1.0 mg/l). The number of shoots produced from each in vitro cutting was counted after 21 days and significant differences among the media were determined by the nonparametric Mann–Whitney test, using Minitab WINSV12.11 software (State College, Pennsylvania, USA).

In vitro rooting

In order to develop an in vitro rooting protocol, we evaluated root initiation (RI) media supplemented with 0.0, 0.5 or 1.0 mg/l of indole-3-butyric acid (IBA) (Table 1). Eight to 15 two-node cuttings with fully expanded leaves were excised from NV and VL plantlets propagated in vitro in the best performing MP medium. The lower leaf was removed, the stems were freshly cut at a 45° angle and inserted straight up in solid RI medium supplemented with 0.0, 0.5, or 1.0 mg/l IBA (RI, Table 1) such that node and stem below were fully immersed. Explants in RI medium were incubated in the dark for 3 days at 25°C then transferred to low light conditions (20–100 micromoles m^{-2} s^{-1}, 16 h a day) at 25°C until roots appeared. Newly rooted plantlets were transferred to individual containers with modified root elongation medium (REN1; Table 1) (Maximova et al. 1998) and cultured under high light (300 micromoles m^{-2} s^{-1}, 16 h a day). The number of rooted plants, number of roots produced per plant and total root length per plant was recorded after 30 days.

Two-way general linear ANOVA model of Minitab WINSV12.11 was used to determine if any factor or interactions among factors produced a significant difference in percentage rooting, average number of roots per plant or average root length per plant among treatments. The two-way general linear ANOVA model assumes sampling errors are normally distributed and independent.

Rooted plants maintenance

Rooted plants were established and maintained in REN1 media and used as explant source (stock plants) for in vitro rooting and shoot regeneration experiments. The stock plants were trimmed and transferred to individual containers of fresh REN1 medium every 3–4 weeks. Plant/shoot clusters of more than three shoots were divided at the crown so that individual rooted plants and non-rooted shoots were separated and transferred to fresh medium.

To increase the fitness of the stock plants and extend the period between transfers, the REN1 medium was modified by increasing the MS basal media concentration by 50% (REN2) (Table 1). The plants on REN2 medium were cultured at 25°C and reduced light intensity of 150 micromoles m^{-2} s^{-1}, and transferred to fresh medium every 4–5 weeks. Stock plants under these conditions produced longer internodes and reduced number of roots compared to the REN1 plants.

Shoot regeneration from whole leaf explants

The top two fully expanded, dark green apical leaves of stock NV and VL plants cultured on REN1 medium were excised and cut into ~1.5 cm^2 explants. The explants from the base of the leaves included 0.5–1 cm (more than one half) of the petiole. Explants were then cultured on shoot induction media (SI) containing 6BA (0.5 mg/l), thiadiazuron (TDZ) at 1 mg/l and varying concentrations of NAA (0.5, 1.0, 1.5 or 1.75 mg/l) (Table 2). SI media (50 ml aliquots) were poured in 100 × 20 mm Petri dishes (VWR cat. 25382-166, Becton Dickinson Falcon, Franklin Lakes, NJ, USA). A total of 25 explants per genotype (five explants per Petri dish) were cultured for the individual treatments evaluated. Cultures were incubated on SI medium in the dark at 25°C for 14 days. The explants from each SI medium were separated in three different groups then transferred to shoot regeneration (SR) media with three different

Table 1 Media formulations for micropropagation

Media components	Micropropagation				
	MI	MP	RI	REN1	REN2
MS basal medium (g/l)[a]	4.4	4.4	2.2	2.2	3.3
6BA (mg/l)	2.5	1.0–5.0	–	–	–
NAA (mg/l)	–	0.0–1.0	–	–	–
TDZ (mg/l)	–	–	–	–	–
IBA (mg/l)	0.25	–	0–1	–	–
Sucrose (g/l)	30.0	30.0	20.0	20.0	20.0
PhytaGel (g/l)	2.0	2.5	2.7	2.8	2.7
pH	5.5	5.7	5.5	5.7	5.7

MI micropropagation initiation; *MP* micropropagation medium; *RI* root initiation; *REN1* root elongation #1; *REN2* root elongation #2; *SI* shoot induction; *SR* shoot regeneration; *MS* Murashige and Skoog basal medium; *6BA* 6-benzoamino-purine; *NAA* α-naphthalene acetic acid; *TDZ* thidiazuron; *IBA* indole-3-butyric acid

[a] All tissues culture media contained Murashige and Skoog basal medium (Murashige and Skoog 1962) (Sigma M5519)

Table 2 Media formulations for shoot organogenesis

Media components	Shoot organogenesis	
	SI	SR
MS basal medium (g/l)[a]	4.4	4.4
6BA (mg/l)	0.0–0.5	1.5–2.0
NAA (mg/l)	0.50–1.75	1.0
TDZ (mg/l)	0.0–1.1	–
Sucrose (g/l)	30.0	30.0
PhytaGel (g/l)	2.7	2.7
pH	5.7	5.7

MI micropropagation initiation; *MP* micropropagation medium; *RI* root initiation; *REN1* root elongation #1; *REN2* root elongation #2; *SI* shoot induction; *SR* shoot regeneration; *MS* Murashige and Skoog basal medium; *6BA* 6-benzoamino-purine; *NAA* α-naphthalene acetic acid; *TDZ* thidiazuron; *IBA* indole-3-butyric acid

[a] All tissues culture media contained Murashige and Skoog basal medium (Murashige and Skoog 1962) (Sigma M5519)

concentrations of 6BA (1.5, 1.75 or 2.0 mg/l) (Table 2). Thus the total number of regeneration media/treatments (combinations of SI/SR media) was 24. The SR cultures were incubated in the dark, at 25°C for an additional 14 days. After that explants were transferred to fresh SR media every 14 days for four more times and incubated under low-light conditions. Explants producing shoot primordia were subdivided into clusters of 1–3 shoots as needed, to allow elongation and additional shoot proliferation. During the SR incubation period individual regenerated shoots with two extended nodes and leaves were selected and transferred to REN1 for rooting as described above.

A binary logistic regression model was applied to all the regeneration media treatments using Minitab WINSV12.11 to determine if plant genotype, level of NAA in the SI media, level of 6BA in the SR media, or interactions had a significant effect on the percent of regenerating explants.

Shoot regeneration from petiole and stem sections

Two sets of experiments were performed with explants that were generated by excising stem and petiole segments (2–3 mm long) from the upper three nodes of dark green, rooted *A. fimbriata* VL and NV stock plants maintained in vitro in REN1. Explants were placed horizontally on SI medium containing 0.5 mg/l 6BA, 1 mg/l NAA, and 1 mg/l TDZ and pressed lightly into the media to ensure adequate contact (up to 60 explants per plate). All cultures were first incubated on SI in the dark at 25°C for 14 days. After that all cultures were transferred and maintained under low-light at 25°C, but were separated into three different groups. Each group was incubated on SI for different periods of time including 14, 21 or 40 days. The 21 and 40-day SI cultures were transfers to fresh SI media every 14 days for up to 21 and 40 days, respectively. Following SI treatment the 14 and 21-day cultures were transferred to SR medium containing 1.75 mg/l 6BA and 1.0 mg/l NAA and incubated at 25°C and low-light. These cultures were also transferred to fresh SR media every 14 days for up to 40 days after culture initiation (including SI). The number of regenerating explants was recorded at 40 days.

Using Minitab WINSV12.11, a binary logistic regression model was applied to determine if genotype, tissue type, length of time on SI media, or interactions significantly affected regeneration.

To further optimize the system, in a third set of experiments, stem and petiole explants (2–3 mm long) were selected from rooted VL stock plants maintained on REN2 medium. The explants were cultured on SI medium (20–30 explants per plate) for 14 days in the dark, at 25°C followed by transfer to SR medium. The SI and SR media were identical to those described above, but for this experiment explants on SR media were maintained in the dark until shoot primordia were developed. At each transfer to fresh SR (every 14 days), cultures were evaluated and only those explants with visible shoots were moved to low-light for shoot elongation. When distinct leaves were visible, the base and sides of the newly regenerated shoots were trimmed to remove remaining callus tissue and the shoots were transferred from SR to REN2 medium under low-light to promote further shoot elongation. Shoots cultured on REN2 were transferred to fresh medium every 4–5 weeks until roots and shoots with fully expanded leaves were developed. Plants with two or more roots and minimum 3 cm total root length were transplanted into multi-cell plant trays with four-cell packs (Kord, Canada) filled with water-saturated soil-free potting mix consisting of one part Metro-Mix 200 (Sun Gro Horticulture. Ltd, Vancouver, BC, Canada) and four parts Miracle-Gro Potting Mix, which contains Miracle-Gro slow-release plant fertilizer (Scotts Miracle-Gro, Scotts Horticulture, Marysville, OH). Potted plants were covered with clear plastic lids and placed in the growth chamber under medium-light, shaded with one layer of white paper towels for 1 day to reduce transplant shock. Plants were acclimated for one more week by gradually opening the plastic lids to reduce the humidity. The lids were completely removed by the end of day three. The acclimated plants already in soil were then transferred to the greenhouse (conditions previously described) where they were monitored weekly and the number of healthy plants was recorded.

Results

Micropropagation and in vitro rooting

Single node green cuttings from two different genotypes of *A. fimbriata* were obtained from greenhouse stock plants and introduced into tissue culture on MI medium. All cuttings, from both genotypes in three different experiments developed at least two new axillary shoots, doubling the number of shoots in 21 days. Following MI medium the explants were transferred to different MP media to evaluate the effect of various NAA and 6BA concentrations. After 21 days on MP medium the mean multiplication rate for the treatments without NAA was significantly greater ($P < 0.05$) than for the treatments containing NAA (Table 3). The statistical analysis indicated that the response to NAA concentration was not a function of 6BA concentration (Table 3). However, our results demonstrated that explants in MP containing 1 mg/ml 6BA and no NAA had the highest mean multiplication rate of 3.06-fold ($P < 0.05$) (Table 3).

The analysis of the in vitro rooting data demonstrated that the number of roots and average root length met the

Table 3 Effect of the type and concentration of plant growth regulators on micropropagation

Combination of plant growth regulators		# New shoots harvested	# Nodal cuttings initiated	Multiplication factor
NAA mg/l	6BA mg/l			
1.0	3	36	25	1.44
0.5	3	36	20	1.80
0.1	5	50	22	2.27
0.1	4	48	25	1.92
0.1	3	46	24	1.92
0.1	2	35	20	1.75
0.0	**3**	**47**	**20**	**2.35***
0.0	**1**	**52**	**17**	**3.06***

All media contained 4.4 g/l Murashige and Skoog (MS) basal medium, 30 g/l sucrose, solidified with PhytaGel, in addition to the plant growth regulators indicated. Number of proliferated axillary shoots was recorded 21 days after culture initiation

* Mean multiplication for treatments without NAA was significantly greater ($P < 0.05$). Significantly better treatments are indicated in bold

Table 4 Effect of the concentration of indole-3-butyric acid (IBA) on rooting of shoots multiplied in vitro

IBA (mg/l)	% Rooted shoots (*n*)	Average number of roots/shoot ± SD[a]	Average total root length/plant (mm) ± SD[b]
0.0	100 (17)	2.6 ± 1.1	32.8 ± 14.6
0.5	100 (29)	2.6 ± 1.6	29.2 ± 15.8
1.0	100 (27)	2.7 ± 1.6	27.2 ± 14.2

All root initiation media evaluated contained 2.2 g/l Murashige and Skoog (MS) basal medium, 20 g/l sucrose, solidified with PhytaGel, in addition to the IBA levels indicated. Two-way ANOVA indicated no significant differences ($\alpha = 0.05$) in average number of roots/shoot or average root length among IBA levels

[a] Only the number of the primary adventitious roots was counted

[b] All primary and secondary adventitious roots were measured

normal distribution assumptions of the two-way ANOVA model, and there were no significant effects of genotype, IBA concentration, or interaction detected. All explants developed roots in 21–30 days (Table 4) therefore we concluded that it was not necessary to apply IBA for root induction. Further rooting of shoots and maintenance of the rooted plants were done on hormone-free medium (REN).

The tissue culture initiation, multiplication, and rooting protocols reported here (Fig. 2) yielded approximately six times more plants than the original number of explants introduced into culture and have been successfully implemented with both genotypes (NV, VL). We were able to generate and maintain a large number of aseptic plants as a source of explant tissue for regeneration and transformation experiments.

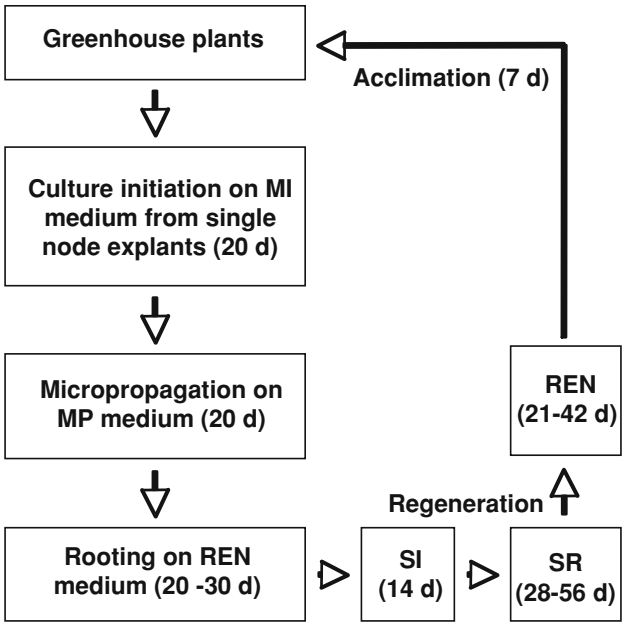

Fig. 2 Schematic representation of micropropagation and regeneration protocols of *A. fimbriata*. The number of days at each step is indicated. *Abbreviations* MI, micropropagation initiation; MP, micropropagation; REN, root elongation; SI, shoot induction; SR, shoot regeneration

Shoot regeneration from whole leaf explants

Results from our preliminary experiments, titrating TDZ and 6BA in different combinations, indicated that shoot primordia were initiated only on SI media supplemented with 0.5 mg/l 6BA and 1.0 mg/l TDZ (data not shown). Therefore, we proceeded to evaluate different concentrations of NAA in the shoot induction medium followed by different concentrations of 6BA in the shoot regeneration medium.

Shoot regeneration from whole leaf explants was observed on all SI media containing 1.0 mg/l NAA (Table 5). Regeneration from explants induced with SI media containing 1.0 mg/l NAA was not significantly different than media containing 0.5 or 1.5 mg/l NAA. However, 1.75 mg/l NAA in the SI media produced significantly fewer regenerating explants ($P < 0.05$). The SR media supplemented with 1.5 or 2.0 mg/l 6BA produced lower number of regenerating explants than 1.75 mg/l 6BA ($P < 0.05$). Overall, the greatest percentage of regeneration (36%) occurred in explants initiated on SI medium containing 1.0 mg/l NAA, followed by incubation on SR media containing 1.75 mg/l 6BA (Table 5).

The results of binary logistic regression analysis indicated that the amounts of NAA in the SI media and 6BA in

Table 5 Direct organogenesis of *A. fimbriata* from variegated (VL) and non-variegated (NV) whole leaf explants

Induction medium	Regeneration medium	% Explants regenerating		
NAA (mg/l)*	6BA (mg/l)*	VL	NV	Overall
0.50	1.50	20	4	12
0.50	1.75	0	0	0
0.50	2.00	16	4	10
1.00	1.50	20	8	14
1.00	**1.75**	**36**	**36**	**36**
1.00	2.00	12	4	8
1.50	1.50	0	0	0
1.50	1.75	8	20	14
1.50	2.00	4	16	10
1.75	1.50	0	0	0
1.75	1.75	8	0	4
1.75	2.00	0	0	0

All regeneration media evaluated contained 4.4 g/l Murashige and Skoog basal medium, 30 g/l sucrose, solidified with PhytaGel. The shoot induction media contained 0.5 mg/l 6-benzoamino-purine (6BA), 1.0 mg/l thidiazuron (TDZ) and different concentrations of the α-naphthalene acetic acid (NAA). The shoot regeneration media contained 1.0 mg/l NAA and different concentrations of 6BA

* Binary logistic regression factors with a significant effect on regeneration ($P < 0.05$). The overall best combination of media is highlighted in bold

the SR media each independently had a significant effect on regeneration. Genotype and interactions were not significant factors in the statistical model. Close observation of whole leaf explants on SR medium indicated that shoot primordia originated only from the petiole region (Fig. 1c). Shoots elongated and developed 2–3 leaves between 28 and 56 days on SR medium (Fig. 1d).

Shoot regeneration from stem and petiole explants

The effect of the time exposure of the explants to SI medium was evaluated in two separate experiments (#A and B, Table 6). For those experiments explants were collected from stock plants maintained on REN1 medium under high light conditions. The highest percentage in both experiments was recorded after incubation on SI medium for 14 days only (Table 6). Explants cultured on SI medium for 21 days produced a significantly lower percentage of regenerating explants compared to the explants cultured on SI medium for 14 days ($P < 0.05$). However, there was no significant difference observed between the 14-day and 21-day treatments in the second experiment. Explants cultured on SI medium for 40 days in both experiments produced lower percentage of regenerating explants (Table 6). Statistical analysis of the data indicated that stem explants were significantly less likely ($P < 0.05$) to regenerate than petiole explants. The tissue type and number of days on SI (14, 21, 40) were significant factors ($P < 0.05$) in the statistical model. Interactions and genotype were not significant factors. Overall, the best regeneration occurred from petiole explants induced on SI medium for 14 days.

Table 6 Shoot regeneration of petiole and stem explants from REN1 stock plants

Experiment number	Tissue type*	Number of days on SI*	Number of replicates	Total # explants per replicate	% Explants regenerating (SD)
A	Petiole	**14**	3	9–10	**57.8 (15.4)**
		21	3	9–10	21.5 (1.3)
		40	3	10	53.3 (5.8)
	Stem	14	3	14–15	16.7 (10.9)
		21	3	14–15	4.8 (8.2)
		40	3	14–15	22.5 (15.1)
B	Petiole	**14**	6	5–8	**58.9 (25.6)**
		14	6	7–11	**50.3 (43.5)**
		21	6	5–10	**49.0 (27.1)**
		21	6	7–10	**67.5 (27.4)**
		40	6	4–10	37.8 (17.5)
		40	6	4–11	49.3 (35.3)
	Stem	14	6	8–10	44.1 (44.4)
		14	6	6–12	51.6 (38.9)
		21	6	7–11	35.1 (26.6)
		21	6	6–11	36.6 (37.7)
		40	6	7–11	32.8 (22.6)
		40	6	8–12	29.4 (27.6)

Explants from source plants maintained on root elongation medium #1 (REN1) were incubated on shoot induction (SI) medium for varying periods of time and transferred to shoot regeneration (SR) medium. Data was collected at 40 days after SI culture initiation

* Binary logistic regression factors with a significant effect on regeneration ($P < 0.05$). Significantly better treatments are indicated in bold

To verify the results of the 14-day exposure to SI medium an additional experiment was conducted with petiole and stem explants taken from stock plants maintained on REN2 and under medium light conditions. As a result 60% of petiole explants and 80–97% of stem explants regenerated shoots (Table 7). Both petiole and stem explants regenerated at higher frequency than the maximum regeneration recorded for whole leaf, stem and petiole explants collected from plants maintained on REN1 medium in high light conditions (Tables 5, 6). Furthermore, on average, each stem or petiole explant in this experiment produced three shoots per explant (Fig. 1e, f), with the highest number of shoots per explant (7) recorded for stem explants (Table 7). All of the newly regenerated shoots uniformly elongated and developed roots on REN2

Table 7 Shoot regeneration, rooting and acclimation of plants regenerated from petiole and stem explants from plants maintained in root elongation medium #2 (REN2)

Experiment number	Tissue type	Total # explants	Explants regenerating (%)	Total # shoots	Rooted shoots (%)	Acclimated plant (%)
1	Petiole	10	60	21	100	100
	Stem	10	80	69	100	100
2	Stem	30	97	90	100	100
	Stem	18	94	46	100	100

medium ~4 weeks after transfer (Table 7; Fig. 1g). Acclimation to greenhouse conditions was 100% successful (Table 7) and shown in Fig. 1h.

Discussion

During our initial attempts to regenerate shoots from *A. fimbriata* leaf explants, we evaluated shoot organogenesis methods reported for *Aristolochia indica* (Manjula et al. 1997; Remashree et al. 1997; Soniya and Sujitha 2006), tobacco (Fisher and Guiltinan 1995), and apple (Maximova et al. 1998). The higher levels of cytokinin applied under these protocols caused the tissue to blacken and die in 2–3 weeks. Thus, the concentration of 6BA was reduced in the regeneration media, while the concentration of TDZ (Murthy et al. 1998) remained at 1 mg/l as applied for regeneration from apple leaf tissue (Maximova et al. 1998). Our preliminary experiments with explants from different types of tissues did not produce regenerants from root and leaf blade sections, and sections containing the base of the leaf without the petiole. Regeneration from leaf bases with the petiole attached (data not shown) was comparable to that of the whole leaf explant (36%). The results of the study described here clearly demonstrate that stem and petiole sections regenerated with the highest success. Additionally we observed minimal callus formation during the shoot organogenesis. Stem and petiole explants offered the substantial advantage of reducing the space required by the regeneration system by 80%.

Our results also indicated that the light intensity and the concentration of the basal medium applied during the micropropagation phase of *A. fimbriata* had an influence on the shoot and plant growth during micropropagation, and also on shoot organogenesis from stem and petiole explants. The initial high light and a lower nutrient medium (REN1) protocol produced plants closely resembling greenhouse plants with respect to compact form, leaf size, and root development. The plants propagated under medium light and a higher nutrient medium (REN2) developed slightly elongated stems with smaller, darker-green leaves. In addition to requiring less frequent maintenance, stem and petiole explants from stock plants cultured on REN2 regenerated more uniformly and with greater success than did explants from plants cultured on REN1. The variation observed in the shoot regeneration response could be explained with different "preconditioning" of the tissues under the different light conditions and increased basal medium concentration (Mohamed et al. 1992). Plant tissues could be developing different cell sizes or have different photosynthesis rates, or the effects could be due to changes in the nutrient metabolism or endogenous hormone production (Husaini and Abdin 2007; Molina et al. 2007; Saebo et al. 1995; Tabatabaei et al. 2008). Further analysis of the tissues from plants propagated under the different micropropagation regimes is necessary to reveal the underlying physiological differences influencing organogenesis.

Conclusions

The high frequency protocols reported here for in vitro propagation of *A. fimbriata* can provide a large quantity of greenhouse ready clonal material in a period as short as 3 months, with minimal space requirements. This manuscript describes the first successful protocols for micropropagation, regeneration, in vitro rooting and acclimation of two genotypes of *A. fimbriata*. The protocols are currently used for the development of a genetic transformation system for *A. fimbriata* VL genotype. This will allow the study of gene function in vivo in this plant and will establishment of *A. fimbriata* as a model system for basal angiosperms. The study further enhances the importance of Aristolochiaceae for research on evolution of plant development.

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