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Efficient organogenesis of an Australian passionfruit hybrid (*Passiflora edulis* × *Passiflora edulis* var. *flavicarpa*) suitable for gene delivery

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Abstract. An efficient regeneration protocol based on organogenesis from cotyledon explants and suitable for gene delivery has been developed for an Australian passionfruit hybrid. Multiple shoots were regenerated from 30-day-old cotyledon explants on Murashige and Skoog (MS) medium containing 6-benzylvaminopurine (BAP) and coconut water. Media pulsing experiments were conducted to investigate the effect on organogenesis of exposure time of the explants to MS containing 10 µM BAP and 10% (v/v) coconut water, i.e. passionfruit regeneration medium (PRM). Continuous exposure of these explants to PRM maximised the number of shoots produced to 12.1 per explant. However, periods on hormone-free medium improved the appearance of the shoots and increased the number of explants with shoots from 75 to 84.6%. Further, shoots exposed for 7 days to half-strength MS supplemented with 10 µM NAA (1-napthalene acetic acid) produced twice as many plantlets than those on half-strength MS alone. Transient GUS histochemical assays indicated delivery of the *uid*A gene via *Agrobacterium tumefaciens*.

Introduction

Passiflora is the most important genus in Passifloraceae. It contains over 400 species, including the passionfruits which are popular subtropical and tropical fruit crops. Passionfruit pulp can be eaten fresh or used for cooking, in desserts, drinks and fruit juices. Some species are valued as ornamental plants for their distinctive flowers (Martin and Nakasone 1970). The passionfruit industry in Australia is based on hybrids of *P. edulis* (purple fruit) and *P. edulis* var. *flavicarpa* (yellow fruit). These hybrids are capable of high fruit production for most of the year. They also show resistance against fusarium wilt, once a major problem of the Australian industry.

Passionfruits are susceptible to a range of fungal and viral pathogens. *Alternaria alternata* can be a problem in some seasons, particularly since the emergence of strains resistant to commercial fungicide treatments (Hutton 1988; Fitzell *et al.* 1991). The industry in Australia is threatened by cucumber mosaic virus (CMV) and severe strains of passionfruit woodiness virus (PWV), which reduce yield and quality of fruit, particularly during the cooler seasons of the year (Winks *et al.* 1988; Loebel *et al.* 1993). Vines infected with mild strains of PWV may show synergistic disease symptoms when also infected by CMV (Pares *et al.* 1985). Despite efforts to identify PWV-tolerant cultivars, the numbers of commercial hybrids available to Australian passionfruit growers has continually declined as severe strains of PWV have overcome natural resistance genes (Gough and Shukla 1992). Mild strain protection strategies have not proven effective (Simmons 1959; Peasley and Fitzell 1981). Furthermore, interspecific hybridisation using the germplasm of wild species can be difficult, with some new passionfruit hybrids demonstrating problems of infertility (Ruberte-Torres and Martin 1974) or poor fruit quality (Loebel *et al.* 1993).

Development of a gene transfer system for the Australian hybrid cultivar Supersweet would provide a method for regenerating disease-resistant plants using pathogen-mediated resistance strategies. Callus and root formation in culture has been reported for several *Passiflora* species including *P. edulis* and *P. edulis* var. *flavicarpa* (Drew 1996). Regeneration of *P. edulis* and *P. edulis* var. *flavicarpa* plantlets via organogenesis has been reported (Manders *et al.* 1994; Kawata *et al.* 1995). However, there have been no reports for *P. edulis* × *P. edulis* var. *flavicarpa* hybrids. Efficient regeneration of passionfruit hybrids is a prerequisite for a transformation system that would benefit the Australian passionfruit industry. Dornelas and Vieira (1994)

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reported highly efficient organogenesis from *P. edulis* var. *flavicarpa* with 96% of 60-day cotyledon explants producing about 57 shoots each on Murashige Skoog (MS, Murashige and Skoog 1962) medium containing *c*. 8.8 μM BAP (6-benzylamino purine). Manders *et al*. (1994) described organogenesis from immature leaves of *P. edulis* var. *flavicarpa* on MS medium containing 4.4 μM BAP. *Passiflora edulis* var. *flavicarpa* plants have also been regenerated from protoplasts isolated from leaves and seedling cotyledons (Dornelas and Vieira 1993; d'Utra Vaz *et al*. 1993). Immature leaf explants of *P. edulis* were cultured on MS medium supplemented with different combinations of NAA and BAP and produced callus and root initials after 4 weeks, but no shoots (Kantharajah and Dodds 1990). Kawata *et al*. (1995) regenerated multiple plants from *P. edulis* leaf explants. Shoot primordia were initiated on MS medium containing 1 M BAP and 1 M IBA (indole-3-butyric acid) within 4 weeks and then subcultured onto MS medium containing 10 μM BAP. Shoots developed only after shoot primordia were transferred to a lower concentration of BAP (0.1–1.0 μM). As the first step towards developing a transformation system for the commercially important hybrid *P. edulis* × *P. edulis* var. *flavicarpa*, we describe the development of a reliable system for regenerating plants from cells amenable to gene delivery by *Agrobacterium tumefaciens*.

Materials and methods

Passiflora edulis × *P. edulis* var. *flavicarpa* cv. Supersweet was used in all experiments. Seed was collected from the fruit of F1 hybrid vines at a commercial plantation at Redland Bay, in south-east Queensland. Seedlings were germinated in a 1 : 1 : 1 peat : perlite : polystyrene bead (by volume) mixture under glasshouse conditions and grown for 30–90 days.

Explants

Both leaf and cotyledon explants were used for plant regeneration experiments. Leaf explants were either excised under sterile conditions from plantlets grown *in vitro* for 1–2 years as described by Drew (1996), or from glasshouse-grown seedlings, depending on the experiment. Cotyledon explants were excised from glasshouse-grown seedlings. Shoots regenerated from the organogenesis experiments were used in the rooting trial.

Culture media, growth vessels and growth conditions

All culture media contained 30 g L^{-1} sucrose and 8 g L^{-1} Difco bacto agar, and were adjusted to pH 5.6–5.7 after the addition of growth regulators. Growth media were autoclaved at 121°C for 15 min. All cultures were maintained at 25°C, with cool white flurorescent tubes providing a photon flux density of 20–55 μmol m^{-2} s^{-1} for a 16-h photoperiod. Polycarbonate vessels, 75 mm in height × 60 mm (bottom)–80 mm (top) in diameter, containing 30 mL of medium and five explants per vessel were used for regeneration from leaf explants derived from *in-vitro* grown tissue culture plantlets. Soda glass vessels, 100 mm in height × 90 mm in diameter, containing 100 mL medium and 2–4 explants were used in all other experiments, unless otherwise described. Polycarbonate vessels 110 mm in height × 40 mm in diameter, were used in the rooting experiment with one shoot per vessel.

Comparison of disinfestation treatments

Leaf and cotyledon tissue derived from 40–60-day-old glasshouse-grown seedlings were rinsed under running tap water for 10–15 min before disinfestation. Explants were disinfested with one or a combination of 2% (v/v) chlorine for 10 or 20 min, 10% (w/v) dichlorisocyanuric acid for 10 min and 0.1 or 0.05% (v/v) benzalokium chloride for 10 min (Table 1). After disinfestation, the plant material was rinsed three times with sterile distilled water before removing the petioles and edge borders. The remaining lamina was dissected into segments 0.5 × 0.2 cm perpendicular to the midrib. Explants were then cultured for 7–21 days on MS medium supplemented with 10 μM BAP and 10% (v/v) coconut water (SIGMA, Cat. no. 5915), i.e. passionfruit regeneration medium (PRM). Explants that showed no signs of visual contamination were scored as being contamination-free.

Table 1. Comparison of disinfestation treatments for cotyledons and leaf explants from seedlings grown in the glasshouse
Columns 2, 3, 4 and 5 are from the same experiment. Column 6 and 7 represent different experiments. DICA, dichlorisocynuric acid; BC, benzalokium chloride; -, treatment is not included in this experiment

Treatment	Leaf explants free of contamination (%)		Cotyledon explants free of contamination (%)			
	7 days	21 days	7 days	21 days	7 days	7 days
2.0% chlorine (20 min)	100	100	0	0	35	8
1.0% DICA (10 min)	100	100	55	55	—	—
0.1% BC (10 min)	60	60	50	35	—	—
1.0% DICA (10 min) + 2% chlorine (10 min)	100	100	95	95	60	66
0.05% BC (10 min) + 2% chlorine (10 min)	—	—	—	—	30	—
0.1% BC (10 min) + 2% chlorine (10 min)	100	100	65	65	—	—
0.05% BC (10 min) + 1% DICA (10 min)	—	—	—	—	20	—
0.05% BC (10 min) + 1% DICA (10 min) + 2% chlorine (10 mins)	—	—	—	—	40	—
0.1 % BC (10 min) + 1% DICA (10 min) + 2% chlorine (10 min)	—	—	—	—	—	37
n	20	20	12–20	12–20	20	20

Optimisation of media components for plant regeneration

Leaf explants (*c.* 50 per treatment) derived from *in vitro* grown plantlets were placed five per vessel and cultured for a period of 46 days in a factorial array on MS medium containing 0, 5 or 10 μM BAP with or without 10% (v/v) coconut water. In a separate experiment, explants derived from 40–60-day-old seedlings were cultured in a factorial array of MS medium containing 0, 1, 3.2, 10, 32 or 100 μM BAP with or without 10% (v/v) coconut water. Explants were cultured individually in polycarbonate vessels 8 cm in height × 2.5 cm in diameter. After 12 days, groups of four explants were transferred to the same medium in soda glass jars and cultured for a total of 12 weeks (Table 2).

Effect of cotyledon and leaf age on organogenesis

Thirty-, 60- and 90-day-old glasshouse-grown explants were placed on passionfruit regeneration medium passionfruit regeneration medium (PRM) for 4 weeks and then transferred to MS medium. After 8 weeks in culture, explants were returned to PRM for another 4 weeks. An analysis of variance (ANOVA) with a Microsoft Excel statistical package, was performed to compare the mean number of shoots produced per explant, from 30-, 60- and 90-day-old cotyledons.

Comparison of media regimes for optimal organogenesis (media pulsing)

Thirty- to 40-day-old cotyledons were placed on PRM for four weeks and were then transferred to either MS or PRM according to the regimes described in Table 3. Chi-square proportion tests by GENSTAT 5 for windows-version 3.2 statistics package were performed to compare per treatment, the percentage of explants surviving and the proportion of these explants with shoots (Table 3). The method of residual maximum likelihood was used to analyse the mean height of shoots per treatment (Table 3).

Table 2. Effect of supplementation of Murashige and Skoog(MS) media with 6-benzylaminopurine (BAP) and coconut water (CW) on organogenesis of *Passiflora edulis* × *P. edulis* var. *flavicarpa* hybrids

Shoots greater than or equal to 2.5 mm were scored. Percentage values of surviving explants with shoots were calculated by dividing the number of explants with shoots by the number of explants alive at Week 4 for column 5 or Week 7 for columns 6–8. Means followed by the same letter are not significantly different at $P = 0.05$; survival rates of uncontaminated explants from 0 to 7 weeks, $n = 12–16$ for each treatment

Treatment		Explant survival (%)		Explants with shoots (%)				Number of shoots per explant at	Shoot height (cm) at
BAP (μM)	CW (%)	Weeks 0–4	Weeks 0–7	Week 4	Week 7	Week 10	Week 12	Week 12	Week 12
0	0	0	0	0	0	0	0	0	0
1	0	63	13	0	0	0	0	0	0
3.2	0	94	19	0	33	66	66a	2.0 ± 1a	0.41 ± 0.11a
10	0	100	50	0	17	17	17a	2.0 ± 0a	0.33 ± 0.08a
32	0	75	33	0	0	0	0	0	0
100	0	25	0	0	0	0	0	0	0
0	10	56	0	0	0	0	0	0	0
1	10	92	58	0	0	0	0	0	0
3.2	10	100	63	13	30	30	60a	2 ± 0.58a	0.50 ± 0.12a
10	10	100	83	25	50	70	80a	2.63 ± 1.4a	0.69 ± 0.07a
32	10	100	83	0	0	20	20a	2.5 ± 0.5a	0.25 ± 0a
100	10	44	32	0	0	0	0	0	0

Table 3. Media pulsing: effect of exposure time of explants to passionfruit regeneration medium (i.e. Murashige and Skoog medium + 6-benzylaminopurine and coconut water) on organogenesis

Means followed by the same letter are not significantly different at $P = 0.05$. At Week 8, there were four distinct treatments, at Week 12, there were seven distinct treatments. MS, Murashige and Skoog medium; PRM, passionfruit regeneration medium

Media regime Weeks 1–4	Weeks 5–6	Weeks 7–8	Weeks 9–10	Weeks 11–12	Explants alive at Week 8 (%)	Explants with shoots at Week 8 (%)	No. shoots per explant at Week 8	Explants with shoots (%) at Week 12	No. shoots per explant at Week 12	Shoot height (cm) at Week 12
MS	MS	MS	MS	MS	0	0	0	0	0	0
PRM	PRM	PRM	PRM	PRM	93.5b	65.5b	3.8a	75a	12.1a	0.41a
PRM	PRM	PRM	MS	MS			3.8a	84.6a	11.5a	0.42a
PRM	MS	MS	MS	MS	71.9a	43.5a	4.5a	70a	8.1a	0.32ab
PRM	MS	MS	PRM	PRM			4.5a	92.3a	5.4a	0.27b
PRM	MS	PRM	PRM	MS	96.0b	75.0b	4.3a	64.3a	9.2a	0.42a
PRM	MS	PRM	PRM	PRM			4.3a	100a	5.2a	0.41a
n					23–24	23–24	23–24	10–16	10–16	10–16

Rooting trial

Shoots were cultured on half-strength MS medium with or without 10 μM NAA for 7 days, before being transferred to half-strength MS medium supplemented with 10 μM riboflavin, for a total of 53 days. An ANOVA was carried out at Day 0 and Day 60 to compare the mean height of shoots or plantlets cultured with or without NAA.

Agrobacterium tumefaciens *strains*

Thirty- to 40-day-old cotyledons were inoculated with disarmed *A. tumefaciens* strains LBA 4404 and AGL1 carrying the plasmid pBI121 (Bevan 1984). Bacterial cultures were grown for 24–26 h at 28°C in YEP or LB medium (Gartland 1995) containing 50 mg L^{-1} of kanamycin, to an OD_{600} of 0.8–1.6. The bacterial cells were pelleted by centrifugation at 1500 *g* for 10–20 min at 4°C. The pellet was resuspended in five times the original volume of PRM to achieve an OD_{600} of 0.2–0.3. The plasmid pBI121 (Bevan 1984; Jefferson *et al*. 1987) carries the *uid*A gene encoding the reporter protein β-glucuronidase (GUS) driven by the cauliflower mosaic virus 35S promoter, and the *npt*II gene encoding resistance to kanamycin driven by a nopaline synthase promoter.

Inoculation and cocultivation of explants with A. tumefaciens

To determine the effect of inoculation time on gene delivery, ten 30-day-old explants were dissected while soaking in the *Agrobacterium* solution for 0, 5, 10, 15, 20, 25 or 30 min. After cocultivation on PRM for 3 days, they were assayed for transient GUS expression. To compare cocultivation times, 10 explants were cocultivated with *A. tumefaciens* for 1, 2, 3, 4 or 7 days on PRM after they were inoculated with LBA4044 for 30 minutes or AGL1 for 15 minutes.

Transient GUS assays by using a histochemical stain were used to evaluate the extent of gene transfer (Jefferson and Wilson 1991). Explants were washed three times with 300 mg L^{-1} of cefotaxime, and rinsed twice with autoclaved distilled water before being placed into a solution of X-gluc containing 0.1 M potassium ferri- and ferro-cyanide, pH 8.0 (Jefferson *et al*. 1987). All explants were incubated overnight at 37°C in the dark.

Results

Comparison of disinfestation treatments

The use of chlorine, DICA and benzalokium chloride as disinfestation agents for glasshouse-grown tissue was investigated. In general, microbial contamination was easier to remove from leaves (Table 1) than from cotyledons. Treatment of leaf explants with 2% chlorine for 20 min or 1% DICA for 10 min was sufficient to destroy any leaf contamination. A combination of chlorine and DICA or chlorine and benzalokium chloride was also effective. The only contaminated leaf explants observed (40%) were after treatments with 0.1% benzalokium chloride for 10 min. In contrast, decontamination of cotyledon explants required treatment with 1% DICA for 10 min followed by 2% chlorine for 10 min. With this treatment, 60–95% of explants were free of contamination for up to 21 days. The addition of benzalokium chloride (0.05 or 0.1%) to the combined treatment of DICA and chlorine did not decrease contamination in these explants (Table 1).

Optimisation of regeneration medium

MS media supplemented with different concentrations of BAP and coconut water were tested for their effectiveness in initiating organogenesis from cotyledons. Cotyledon explants initiated shoots on media containing BAP in the concentration range of 3.2–32 μM (Table 2) although only with the addition of 10% coconut water were shoots observed on MS medium containing 32 μM BAP. The addition of coconut water promoted rapid development of shoots and, in combination with 10 μM BAP, increased the percentage of explants with shoots from 17 to 80% by Week 12. Shoot height was also improved by the addition of coconut water. By Week 12, only treatments containing coconut water produced shoots >0.5 cm tall. Explants on MS medium containing 10 μM BAP and 10% coconut water produced the highest percentage of explant survival in culture, the highest percentage of explants with shoots (Table 2) as well as superior shoot growth and shoot quality, i.e. appearance (Fig. 1). Eighty per cent of the explants in this treatment produced healthy shoots (>0.5 cm) within 12 weeks of culture.

Comparison of explants

Leaf explants derived from *in-vitro* grown tissue culture plantlets, were cultured for 46 days on MS medium containing 0, 5 or 10 μM BAP and 10% coconut water. After this time, 66–88% of explants survived on MS media with BAP compared to only 4% on medium without BAP (data not shown). However, shoots were produced on one treatment only: 2% of explants placed on MS medium containing 10 μM BAP and 10% coconut water (PRM) initiated shoots (data not shown).

The ability of 30-, 60- and 90-day-old explants to initiate shoots was compared. By Week 12, shoots had been initiated from cotyledon explants of all ages but only from 60-day-old leaf explants (Fig. 2*A*, *B*). Of the cotyledon explants, 30-day-old explants initiated the greatest number of shoots (86%,) compared with 60- (38%) and 90-day-old (37%) explants (Fig. 2*A*). The shoots predominantly occurred along the cut edge of these explants. Futhermore, 30-day-old cotyledon explants had the highest number of shoots per explant at 9.3 ± 1.9 shoots compared with 2.2 ± 2.0 and 8.4 ± 3.4 for 60- and 90-day-old cotyledon explants, respectively (Fig. 2*B*). Generally, leaf explants survived to produce callus with no shoots. However, two 60-day-old explants gave rise to one shoot each.

Comparison of media regimes for optimal organogenesis (media pulsing)

We investigated the effect of the duration of exposure to PRM on organogenesis, i.e. to determine whether removing the growth regulators BAP and coconut water at particular stages of regeneration would improve the efficiency of organogenesis. As observed previously (Table 2), explants on MS medium without growth regulators did not undergo organogenesis (Table 3). In this study, a minimum of 6 weeks on PRM appeared to be necessary for optimal survival and shoot development. Removing BAP and coconut water after 4 weeks on PRM, reduced the survival rate of the explants from 93.5–96.0% to 71.9%, but only if the explants were not

Fig. 1. Organogenesis of passionfruit cotyledon explants after 4 weeks on passionfruit regeneration medium.

returned to PRM within 2 weeks. The percentage of explants producing shoots was also reduced from 65.5–75.0% to 43.5% (Table 3).

At Week 12, the only treatments that yielded more than 10 shoots per explant were those where the explants remained on MS medium containing the growth regulators for a minimum of 8 weeks (Table 3). Continuous exposure to PRM resulted in 12.1 shoots per explant, while replacement of PRM with MS at Week 9 yielded 11.5 shoots per explant. Treatments where explants were transferred to hormone-free medium (MS) after only 4 weeks resulted in the lowest number of shoots per explant (5.2–9.2). When such explants were returned to media containing growth regulators at Week 7 or 9, they showed no major improvement at Week 12, in the number of shoots per explant (5.2, 5.4 and 9.2 shoots per explant compared with 8.1 in explants kept on hormone-free medium). However, explants from these treatments that were returned to BAP and coconut water for the remainder of the study did show an improvement in the percentage of explants with shoots (92.3–100% compared with 64.3%). At Week 12, significant differences ($P < 0.05$) in the mean height of shoots (0.27 cm compared with *c.* 0.4 cm) occurred between treatments where explants were transferred to MS after 4 weeks and were not returned to PRM within 2 weeks, and all the other treatments (Table 3). While continuous exposure to BAP appeared to give the highest number of shoots (12.1) per explant, the quality or appearance of shoots was improved by periods on hormone-free medium. Overall, the best quality shoots were observed following culturing on PRM for 8 weeks followed by hormone-free medium for 4 weeks (Table 3). This treatment combined the benefit of good shoot quality and initial long-term exposure to growth regulators in terms of a high mean number of shoots per explant (11.5), and a high proportion of explants producing shoots (84.6%).

Rooting trial

Shoots regenerated in the experiments described above were used to provide material for an experiment on root initiation and development. All shoots continued to grow on half-strength MS medium in the presence or absence of 10 M NAA (Table 4). However, 51% of shoots initiated roots in the presence of NAA compared with only 25% in the absence of this hormone. Plantlets cultured on medium containing NAA were also significantly taller than those generated on half-strength MS (Table 4).

Preliminary investigations of the effect of inoculation and cocultivation times with A. tumefaciens *on gene transfer to passionfruit explants*

Cotyledons inoculated with PRM only showed no intrinsic GUS activity (data not shown), thus any blue staining of the tissue could be attributed to expression of the *uid*A gene from pBI121. From repeated observations, following 3-day cocultivation, AGL1-mediated gene delivery yielded an intense

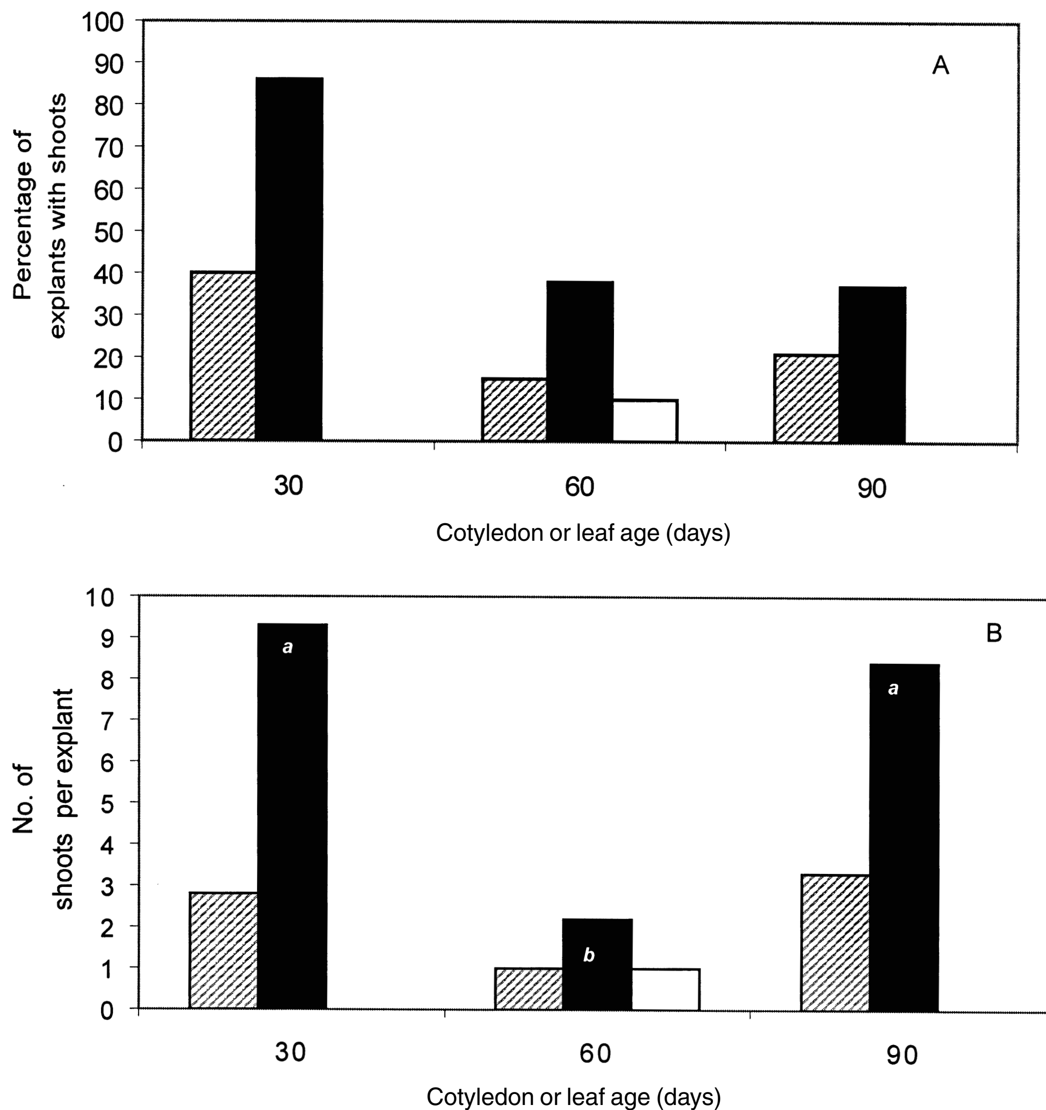

Fig. 2. Effect of cotyledon and leaf age on (*A*) percentage of explants producing shoots and (*B*) mean number of shoots per explant on passionfruit regeneration medium. Means ($n = 20$) indicated by the same letter are not significantly different at $P = 0.05$. Cotyledons after 8 weeks (hatched bars) and 12 weeks (black bars), and leaf explants after 12 weeks (open bars).

blue colour that appeared randomly within the cut edge of the explants following inoculation for at least 15 min. LBA4404-mediated gene delivery, on the other hand, showed an intense blue staining consistently along the cut edge of the explants following inoculation for at least 10 min. GUS staining appeared to be due to *uid*A expression in plant cells, based on observations of stained explants, some explants being clearly negative. However, some staining due to residual *Agrobacterium* cannot be excluded because the *uid*A gene in pBI121 does not contain an intron preventing prokaryotic expression. Low level GUS expression in passionfruit cells was also observed by using pCAMBIA 1301 (data not shown).

For both bacterial strains, at least 70% of explants showed transient GUS activity following inoculation for between 10 and 30 min (data not shown). Explants were cocultivated with each *Agrobacterium* strain for up to 7 days. Cocultivation for one day with either strain of *Agrobacterium* showed no detectable GUS activity in any of the replicates. However, cocultivation for 2–7 days resulted in 90–100% of explants showing GUS positive regions. Explants inoculated with AGL1 for 15 min and cocultivated for 7 days had the largest area of GUS-positive regions per explant (data not shown). For LBA4404, most positive regions were observed with cocultivation periods of 3–4 days.

Table 4. Rooting trial of *in-vitro* grown shoots
Means followed by the same letter are not significantly different at $P = 0.05$

Treatment		Mean height of		n
	Shoots Day 0	Shoots Day 60	Plantlets Day 60	
Half-strength MS	0.495 ± 0.05a	0.94 ± 0.14b	1.8 ± 0.37c	20
Half-strength MS + 10 μM NAA	0.634 ± 0.03a	1.21 ± 0.14b	3.98 ± 0.47d	49

Discussion

We have developed a protocol for regenerating a passionfruit hybrid via organogenesis from 30-day-old cotyledon explants. This is the first report of an efficient regeneration system for a commercially important Australian passionfruit hybrid. The explants used in this system are potentially suitable for *Agrobacterium*-mediated gene delivery. Ultimately, an efficient gene transfer and regeneration system for passionfruit hybrids would have enormous benefits for the industry with the development of disease-resistant germplasm and improvement of other traits.

The use of seedlings grown in the glasshouse, as a source of cotyledon explants, resulted in considerable bacterial contamination, which could not be eliminated by 2% chlorine alone. We did not succeed in germinating passionfruit seeds in tissue culture by using any of the published protocols (Kantharajah and Dodds 1990; d'Utra Vaz *et al*. 1993). Total removal of the seed coat, rather than scarification may work but is technically difficult as mature passionfruit seeds are small and hard. As an alternative, we improved the poor rate of disinfestation of plant materials grown in the glasshouse through use of different disinfestation agents. Although chlorine and most other agents eliminated leaf contamination, a combination of DICA and chlorine was required for treatment of cotyledon explants. The reason for this difference may have been that cotyledons grow through the soil, whereas leaves develop above the soil minimising direct contact with soil-borne bacteria.

Cotyledons were found to be a more suitable explant for regeneration of *P. edulis* × *P. edulis* var. flavicarpa than leaves. Leaves and cotyledons have both been used to regenerate *P. edulis* var. *flavicarpa* but for *P. edulis* only leaves have regenerated shoots. Our findings parallel those of Kantharajah and Dodds (1990) who showed that immature leaf explants on MS medium with NAA and BAP could form callus and root initials but not develop shoots. We did occasionally observe shoot development from hybrid leaf tissue when cultured on MS medium with BAP and coconut water. However, the frequency of this occurrence was too low for such explants to form the basis of an efficient regeneration system. Cotyledons, on the other hand, formed shoots efficiently and reliably.

Comparison of cotyledon ages showed that 30-day-old explants were the best. These explants developed the greatest number of high quality shoots on the greatest number of explants. Initial experiments where 40–60-day-old cotyledon explants were cultured on MS with containing varying BAP concentrations with and without coconut water suggested the mean number of shoots was too low for a practical gene transfer system (Table 2). However, by decreasing the age of the explants to 30 days, and optimising the plant growth regulator concentrations and exposure times, the number of shoots produced per explant was successfully increased about six fold. The optimised shoot regeneration protocol includes culture of 30-day-old cotyledon explants on PRM for 8 weeks, followed by 4 weeks on hormone-free MS medium. Finally, for root initiation, shoots 0.5–0.6 cm in length are cultured for 7 days on half-strength MS medium supplemented with 10 μM NAA, and are subsequently transferred to half-strength MS medium containing 10 μM riboflavin.

It appears that for some plant species, including passionfruit, coconut water may contain growth substances essential for optimal regeneration. In the tissue culture of spinach leaf explants, coconut water improved shoot vigour and promoted rapid development of shoots (Al-Khayri *et al*. 1992). For passionfruit regeneration we found that coconut water had a similar effect by promoting rapid development of shoots and increasing shoot height. The number of explants with shoots was also improved in combination with BAP. Significant improvements in the cellular proliferation of other *Passiflora* species has also been observed when growth media were supplemented with coconut water (Kantharajah and Dodds 1990; Dornelas and Vieira 1994). Surprisingly, the success of rooting in this study was not as high as described by Drew (1995) who obtained 90% after pulsing passionfruit shoots on 10 μM NAA for 7 days. Drew (1995) also showed that NAA was more effective than IBA for root development. It is possible that the organogenic shoots we generated had significant levels of endogenous auxins and cytokinins already present, which may have impacted on the rooting frequencies. If this was the case, reducing the length of exposure to 10 M NAA to 5 days, or reducing the concentration of NAA may promote a higher frequency of rooted shoots.

In our regeneration protocol, about 90% of explants will give rise to an average of 12 shoots per explant. Of these, about half are expected to form roots. This system yields plantlets about 4 cm tall in 5 months. Cotyledon explants

appeared to be an appropriate target tissue for *Agrobacterium*-mediated transformation since, in tissue culture, the untransformed explants regenerated shoots mainly from the cut edges of the cotyledons and in preliminary experiments gene delivery by *A. tumefaciens* appeared to occur to the same area. This work forms the basis for the development of a transformation system for hybrid passionfruit, which can then be used to deliver useful genes leading to the development of improved passionfruit cultivars.

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