Aust. J. Bot., 1996, **44**, 343–353

An Improved Embryo-rescue Protocol for a *Carica* Interspecific Hybrid

Pablito M. Magdalita[AB], *Stephen W. Adkins*[A], *Ian D. Godwin*[A] *and Roderick A. Drew*[CD]

[A]Department of Agriculture, The University of Queensland, Brisbane, Qld 4072, Australia.
[B]Institute of Plant Breeding, College of Agriculture, University of the Philippines at Los Baños, College, Laguna 4031, Philippines.
[C]Queensland Department of Primary Industries, Redlands Research Station, Horticulture Centre, P O Box 327, Cleveland, Qld 4163, Australia.
[D]Corresponding author; drewra@dpi.qld.gov.au

Abstract

An improved embryo-rescue protocol was developed for embryos (90 days old) of *Carica papaya* L. (Clone 2001), and subsequently was utilised for efficient production of interspecific hybrids of *C. papaya* × *C. cauliflora* Jacq. from 90- to 120-day-old embryos. Pre-incubation of *C. papaya* embryos for 7 days on a germination medium containing half-strength De Fossard nutrients supplemented with gibberellic acid (10 μM), 6-benzylamino-purine (0.25 μM), α-naphthalene-acetic acid (0.25 μM), sucrose (58 mM) and agar (8 g L^{-1}) supported 100% germination. Subsequent transfer of germinated embryos to a nutrient medium that was identical, except that it was free of plant growth regulator, allowed good growth but induced shoot etiolation and callus production. Reducing the pre-incubation of *C. papaya* embryos on this medium to 5 days before transfer to the medium free of plant growth regulator produced similarly high germination (96%), but allowed for the production of good quality seedlings that were unetiolated and free of unwanted callus. For interspecific hybrids, a 5-day pre-incubation of the embryos on a liquid formulation was better than the solid formulation as it promoted better growth and vigour of the normally abortive interspecific hybrid embryos. Using the improved protocol, 1981 of 2100 (94%) interspecific hybrid embryos consisting of single and multiple forms were germinated. In all cases, the germinating multiple embryos underwent further embryogenesis that allowed for the production of 485 (25%) morphologically normal hybrid plants grown in soil in the glasshouse.

Introduction

Papaya (*Carica papaya* L.) is a popular tree crop producing flavoursome fruit rich in vitamins A and C. The green fruit is a source of papain, an enzyme used in medical and industrial preparations. In Australia, the *C. papaya* industry is valued at $A10–12 million annually with Queensland producing 95% of the national total (National Farmers Federation 1993).

The world production of *C. papaya* has been greatly reduced by papaya ringspot virus type-P (PRSV-P*). This disease was first detected in Australia in 1991 and has spread to several production areas in Queensland (Thomas and Dodman 1993). Symptoms of the disease include ringspotting on the fruit, chlorosis and distortion of leaves, and water-soaked lesions on stems and petioles (Conover 1964). This disease causes yield reduction and

*Abbreviations used: BAP, 6-benzylamino-purine; DF, De Fossard medium; GM, germination medium; GA_3, gibberellic acid; LGM, liquid germination meduim; MS, Murashige and Skoog medium; NAA, α-naphthalene-acetic acid; PRSV-P, papaya ringspot virus-type P; PPFD, photosynthetic photon flux density; PGRF-PGM, plant growth regulator-free-plantlet growth medium; SGM, solid germination medium.

0067-1924/96/030343$05.00

ultimately plant death (Manshardt 1992). At present, tolerant *C. papaya* cultivars (Conover *et al.* 1986; Huang 1988) with average yield in the presence of PRSV-P are being grown as a short-term control measure. Cross-protection (Yeh *et al.* 1988), sanitation and quarantine (Thomas and Dodman 1993) are also being implemented to prevent losses to this disease.

The most practical and reliable solution to PRSV-P control is the development of genetically resistant *C. papaya* cultivars. A good source of resistance is not available within the species (Cook and Zettler 1970). However, several wild relatives within the genus *Carica* carry genetic resistance to PRSV-P. For example, *C. cauliflora* is resistant to a wide range of PRSV-P isolates occurring worldwide (Alvizo and Rojkind 1987; Magdalita *et al.* 1988). Transfer of this resistance from *C. cauliflora* to *C. papaya* has been prevented by incompatibility barriers that bring about early abortion of hybrid embryos. Embryo rescue has been attempted to help overcome this problem (Manshardt and Wenslaff 1989; Chen *et al.* 1991). However, in all published protocols, production of hybrid plants was inefficient and difficult, regenerant hybrids were of low quality, and no PRSV-P resistant cultivars have been produced. The present study was undertaken to: (1) develop an embryo-rescue protocol first using *C. papaya* embryos then applying it to interspecific hybrid embryos of *C. papaya* × *C. cauliflora*, and (2) to utilise the protocol for the production of *C. papaya* × *C. cauliflora* interspecific hybrid plants growing in soil.

Materials and Methods

Plant Material

Embryos used for rescue or culture were from sib-pollinated *C. papaya* (Clone 2001, PRSV-P-susceptible but high-yielding) or interspecific hybridisations between *C. papaya* and *C. cauliflora* (PRSV-P resistant). All donor plants were grown in field plots at the Redlands Research Station in south-east Queensland (27°S, 153°E).

Interspecific Hybridisation and Embryo Rescue

Pistillate flowers were bagged (bags were 12 cm long, 6 cm wide, Bunzyl Distribution, Brisbane, Australia) and 3 days later pollinated with viable pollen taken from staminate flowers. Pollen viability was checked with an aceto-carmine technique (Cohen *et al.* 1989). The pollen was transferred to the stigmatic surface of the pistillate flower with a fine-hair brush. Successfully pollinated fruits of *C. papaya* (90 days old) and *C. papaya* × *C. cauliflora* (90–120 days old) were harvested and embryos were used for embryo-rescue experiments. Further hybridisations yielded embryos for the production of interspecific hybrids. The fruits were washed in a solution of 7X detergent (5% v/v, ICN Biomedical Australasia, NSW, Australia), rinsed with distilled water and surface-sterilised with ethanol (70% v/v) before being bisected in a laminar air-flow cabinet. The seeds were aseptically removed from the central fruit cavity and embryos were isolated with the aid of forceps and a binocular stereo-microscope. The isolated embryos were germinated on one of several germination media.

Development of Embryo-rescue Media

Three experiments were undertaken to optimise a medium for rescue of *C. papaya* embryos and then to apply the technique to *C. papaya* × *C. cauliflora* interspecific hybrid embryos. In the first experiment, 90-day-old *C. papaya* embryos (isolated as described above) were incubated for 7 days on various germination media (GM) containing half-strength DF (De Fossard *et al.* 1974) basal nutrients supplemented with gibberellic acid (GA_3, 0 or 10 μM), 6-benzylamino-purine (BAP, 0 or 0.25 μM) and α-naphthalene-acetic acid (NAA, 0 or 0.25 μM). The medium of Manshardt and Wenslaff (1989) containing half-strength MS (Murashige and Skoog 1962) nutrients plus BAP (0.89 μM) and NAA (2.67 μM) was used as a standard with which these treatments were compared. After incubation, the percentage of germinated embryos on each medium was assessed. The plants were removed from the culture vessels and after 2 months of further incubation on a plant growth regulator-free-plantlet growth medium (PGRF-PGM) containing full-strength DF plus sucrose (58 mM) and agar (8 g L^{-1}). They were assessed in terms of dry weight, height, number of leaves and root length. Shoot etiolation and formation of callus on the base of the plants were assessed with an index of 0–5 (0 = no callus–unetiolated, 5 = profuse callus–etiolated).

A second experiment was undertaken to reduce shoot etiolation and callus formation of germinated seedlings. Embryos were pre-incubated on a GM containing GA_3 (10 μM), BAP (0.25 μM) and NAA (0.25 μM) for 0–7 days before transfer to the PGRF-PGM, and percentage germination was assessed. After a further 2 months of incubation on the PGRF-PGM, dry weight, height, number of leaves, root length, shoot etiolation and callus formation were assessed.

In the third experiment, interspecific hybrid embryos were cultured in liquid germination medium (LGM) on a roller drum (Bellco Glass, NJ, USA) at 3 rpm or in solid germination medium (SGM) (8 g agar L^{-1}) (5 mL) in glass tubes (2.5 cm diameter, 15 cm tall) capped with polycarbonate lids. Percentage germination was assessed after 5–10 days of incubation. The vigour and hyperhydricity (vitrification) of germinated embryos were checked against an index of 0 to 5 (0 = weak–unhyperhydric, 5 = vigorous–hyperhydric). The germinated seedlings were grown for a further 2 months on PGRF-PGM before dry weight, height, number of leaves and root length were measured.

Interspecific Hybrid Production

A batch of 2100 embryos (single and multiple forms) was germinated in LGM for 5 days before being transferred to PGRF-PGM (full strength DF) and incubated for a further 2 months. Subsequently, the hybrid plants were removed from the flasks, washed with tap water to remove the agar, and grown in trays (70 × 19 × 8 cm, length × width × depth) containing steam-pasteurised peat:perlite:polystyrene bead mix (1:1:1). They were acclimatised following the procedures of Drew (1988). The hybrids were grown for 1 month then transferred into pots (10 cm diameter, 12 cm tall) containing a peat:perlite (1:1) mix.

General Media Preparation and Culture Conditions

All media used in all experiments contained sucrose (58 mM) and Bacto-agar (8 g L^{-1}, Difco Laboratories, MI, USA), unless otherwise stated. Media used for the first and second experiments were dispensed (20 mL) into plastic petri dishes (9 cm diameter, 1 cm tall) or polycarbonate tubes (4 cm diameter, 11 cm tall, Disposable Products, Qld, Australia) while those in the third experiment were dispensed (5 mL) into glass tubes (2.5 cm diameter, 15 cm tall, Selby Scientific and Medical, Qld, Australia). The pH of all media was adjusted to 5.7 and the media were then autoclaved at 121°C for 15 min. Closed or capped culture vessels were sealed around the edges with two layers of polyethylene film (Glad Wrap, Glad Products, NSW, Australia). All cultures were incubated at 25 ± 1°C under a constant photosynthetic photon flux density (PPFD, *c.* 80 μmol m^{-2} s^{-1}, Sylvania GRO-Lux wide spectrum fluorescent tubes) which provided a 16 h photoperiod with an 8 h dark period.

Experimental Design and Statistical Analysis

All experiments were conducted in completely randomised design. In the first and second experiments, 10 replicate plates each with 20 embryos were used to determine percentage germination in the various treatments, while 20 replicate plants were used in the analysis of growth characters. In the third experiment, 12 replicate hybrid embryos per tube were used to determine percentage germination, and these plants (normally 12) were used in the assessment of the growth characteristics. Significant differences among treatments were tested with protected least significant difference (Gomez and Gomez 1984) in the first and second experiments. Student's *t*-test was used to compare the two treatment means from two separate experimental set-ups in the third experiment.

Results

Development of Embryo-rescue Media

In the first experiment, half-strength DF nutrients supplemented with GA_3 (10 μM), BAP (0.25 μM) and NAA (0.25 μM) supported 100% germination of 90-day-old embryos (Table 1). Resultant plants were significantly ($P < 0.05$) different from those produced on other treatments in terms of dry weight, height, number of leaves and root length. However, the plants were etiolated (index 3) and they produced a small amount of callus (index 1, Table 1). Good germination (94%) occurred on half-strength MS nutrients supplemented with BAP (0.89 μM) and NAA (2.67 μM), but the regenerated plants produced large

Table 1. Germination and growth of plants produced from 90-day-old embryos of *Carica papaya* L. and pre-incubated for 7 days on different germination media before culture for 2 months on a plant growth regulator-free plantlet growth medium

Values are means of 10 replicates for percentage germination data and 20 replicate plants for growth data. Means followed by the same letters are not significantly different at $P < 0.05$. Etiolation and callus indices: 0 = no callus–unetiolated, 5 = profuse callus–etiolated

Plant growth regulators				Culture germination and growth						
Medium	GA_3 (μM)	BAP (μM)	NAA (μM)	Germination (%)	Dry weight (mg)	Height (mm)	Leaves (no.)	Root length (mm)	Etiolation index	Callus index
½ DF	0	0	0	71 b	58 d	24 e	5 e	52 e	0 c	1 c
½ DF	0	0.25	0.25	74 b	60 c	29 d	6 d	65 d	0 c	3 b
½ DF	10	0	0	96 a	63 b	55 b	8 b	77 b	4 a	0 d
½ DF	10	0.25	0.25	100 a	65 a	91 a	10 a	85 a	3 b	1 c
½ MS	0	0.89	2.67	94 a	62 c	34 c	7 c	73 c	0 c	4 a

quantities of callus (index 4, Table 1). On half-strength DF nutrients containing BAP (0.25 μM) and NAA (0.25 μM) but without GA_3 (0 μM), percentage germination, dry weight, height, number of leaves, root length, etiolation and callus production were all significantly ($P < 0.05$) reduced, but callus index was higher compared with the same treatment containing GA_3. Poor germination (71%) and growth were obtained on half-strength DF nutrients without GA_3, BAP or NAA.

In the second experiment, percentage germination, dry weight, number of leaves and root length were all significantly ($P < 0.05$) highest after 5–7 days pre-incubation on GM (Fig. 1). Plants were tallest after 6 or 7 days of pre-incubation, but the plants were etiolated and

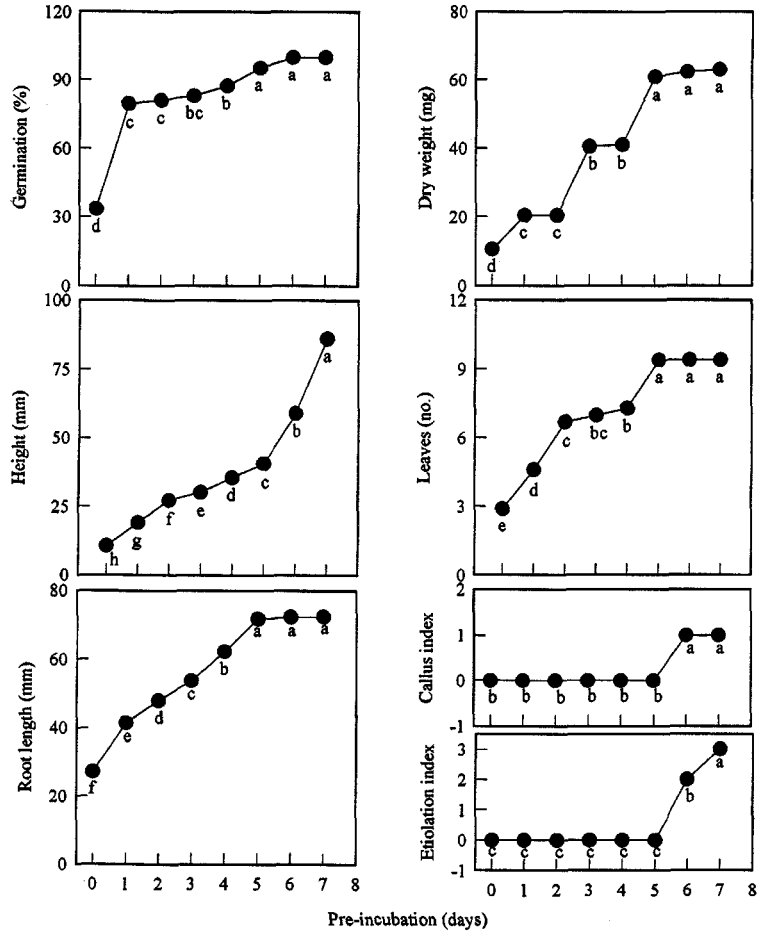

Fig. 1. Germination of *Carica papaya* embryos following pre-incubation for different periods on a germination medium before transfer to a plant growth regulator-free plantlet growth medium, and the dry weight, height, number of leaves, root length, etiolation and callus indices of the plants produced. The germination results represent the mean from 10 replicate plates containing 20 embryos, while the results of dry weight, height, number of leaves, root length, etiolation and callus indices represent the mean from 20 replicate plants produced from the embryos. Data points marked by the same letters are not significantly different at $P < 0.05$. Etiolation and callus indices run from 0 = no callus–unetiolated to 5 = profuse callus–etiolated.

Table 2. Germination and growth of plants produced from 90- to 120-day-old interspecific hybrid embryos of *C. papaya* × *C. cauliflora*
The embryos were pre–incubated for 5 or 10 days on liquid or solid germination medium supplemented with gibberellic acid, 6-benzylamino-purine and α-naphthalene-acetic acid before culture for 2 months on plant growth regulator-free plantlet growth medium. Values are means of 12 replicate embryos for percent germination data and 20 replicate plants for growth data. Means followed by the same letters are not significantly different at $P < 0.05$. Vigour and hyperhydricity indices: 0 = weak–unhyperhydric to 5 = vigorous–hyperhydric

Medium	Pre–incubation	Culture germination and growth						
		Germination (%)	Dry weight (mg)	Height (mm)	Leaves (number)	Root length (mm)	Vigour index	Hyperhydricity index
Liquid	5	100 a	65 a	62 a	9 a	80 a	4 a	0 a
Solid	5	100 a	48 b	35 b	5 b	53 b	2 b	0 a
Liquid	10	100 a	65 a	62 a	9 a	81 a	4 a	4 b
Solid	10	100 a	48 b	35 b	5 b	54 b	2 b	0 a

produced some callus. Percentage germination and growth declined as the pre-incubation time was decreased. Callus production and shoot etiolation (index 0) were avoided after 0–5 days pre-incubation.

The third experiment showed that LGM and SGM induced 100% germination of embryos but the seedlings produced on LGM had significantly ($P < 0.05$) higher dry weight, number of leaves, root length and vigour than those produced on SGM (Table 2). The embryos germinated on LGM produced seedlings that were taller and had greener and healthier cotyledonary leaves than the seedlings produced on SGM (Fig. 2*c*, *d*). No improvement in dry weight, height, number of leaves, root length or vigour was achieved by pre-incubation for 10 days compared to 5 days (Table 2). However, embryos pre-incubated for 5 days on LGM resulted in healthy seedlings while those embryos pre-incubated for 10 days on the same medium resulted to hyperhydric seedlings.

Production of Interspecific Hybrids

A batch of 2100 interspecific hybrid embryos was recovered after dissection of 43736 seeds (*c.* 5%) of 338 successful crosses of *C. papaya* × *C. cauliflora*. Seventy-nine percent of these were multiple embryo forms comprising 2–5 torpedo-shaped embryos (Fig. 2*b*). Maximum percentage germination (94%) of the interspecific hybrid embryos was attained after 5 days pre-incubation on LGM. Single embryos (Fig. 2*a*) produced single plants; however, multiple embryos underwent further somatic embryogenesis (Fig. 2*e*) and regenerated multiple whole plants (Fig. 2*f*). A total of 485 interspecific hybrid plants was successfully grown in soil in the glasshouse (Fig. 2*g*).

Discussion

Germination of *C. papaya* embryos and subsequent development into whole plants was influenced by the embryo-rescue medium (Table 1). In a preliminary study, increasing NAA concentration from 0.25 μM to 2.5 μM significantly ($P < 0.05$) decreased germination percentage; however, the presence of some NAA (0.25 μM), improved root quality (unpublished data). In the present study, maximum germination and growth ($P < 0.05$) of plantlets was achieved on half-strength DF nutrients supplemented with GA_3 (10 μM) alone, or in combination with BAP (0.25 μM) and NAA (0.25 μM). However, prolonged exposure to these media caused etiolated plants. Furthermore, unwanted callus formed at the base of the stem of the plants produced on half-strength DF nutrients plus BAP (0.25 μM) and NAA (0.25 μM). Likewise, half-strength MS nutrients containing BAP (0.89 μM) and NAA (2.67 μM) (a previously published protocol for *C. papaya* × *C. cauliflora* interspecific hybrids: Manshardt and Wenslaff 1989) effectively promoted germination but induced a significant ($P < 0.05$) amount of unwanted callus (index 4), and resulted in poor quality plants of shorter stature and roots, with less leaves. Overall, these results clearly demonstrate that the combination of GA_3, BAP and NAA added to half-strength DF nutrients optimised embryo germination and growth of the resultant plants. Similarly, GA_3, BAP and indole-3-acetic acid promoted the growth of *C. papaya* embryos (Phadnis *et al.* 1970). Although plant growth regulators were essential for maximum germination and plantlet development, they also produced undesirable side-effects. This was consistent with the findings of Manshardt and Wenslaff (1989), who reported callused roots on interspecific hybrid plantlets. Tissue-cultured *C. papaya* plants with excess basal callus are very susceptible to root rot during establishment in a non-sterile environment (Drew 1988).

To reduce the unwanted effects of the plant growth regulators while maintaining 100% germination and plantlet quality, embryos were exposed to a GM for short periods (0–7 days) before transfer to PGRF-PGM. The result suggests that 5 days pre-incubation was the shortest optimal time that is suitable for promoting germination of embryos and growth of the plants produced. This is a significant finding as it has not been reported in previous

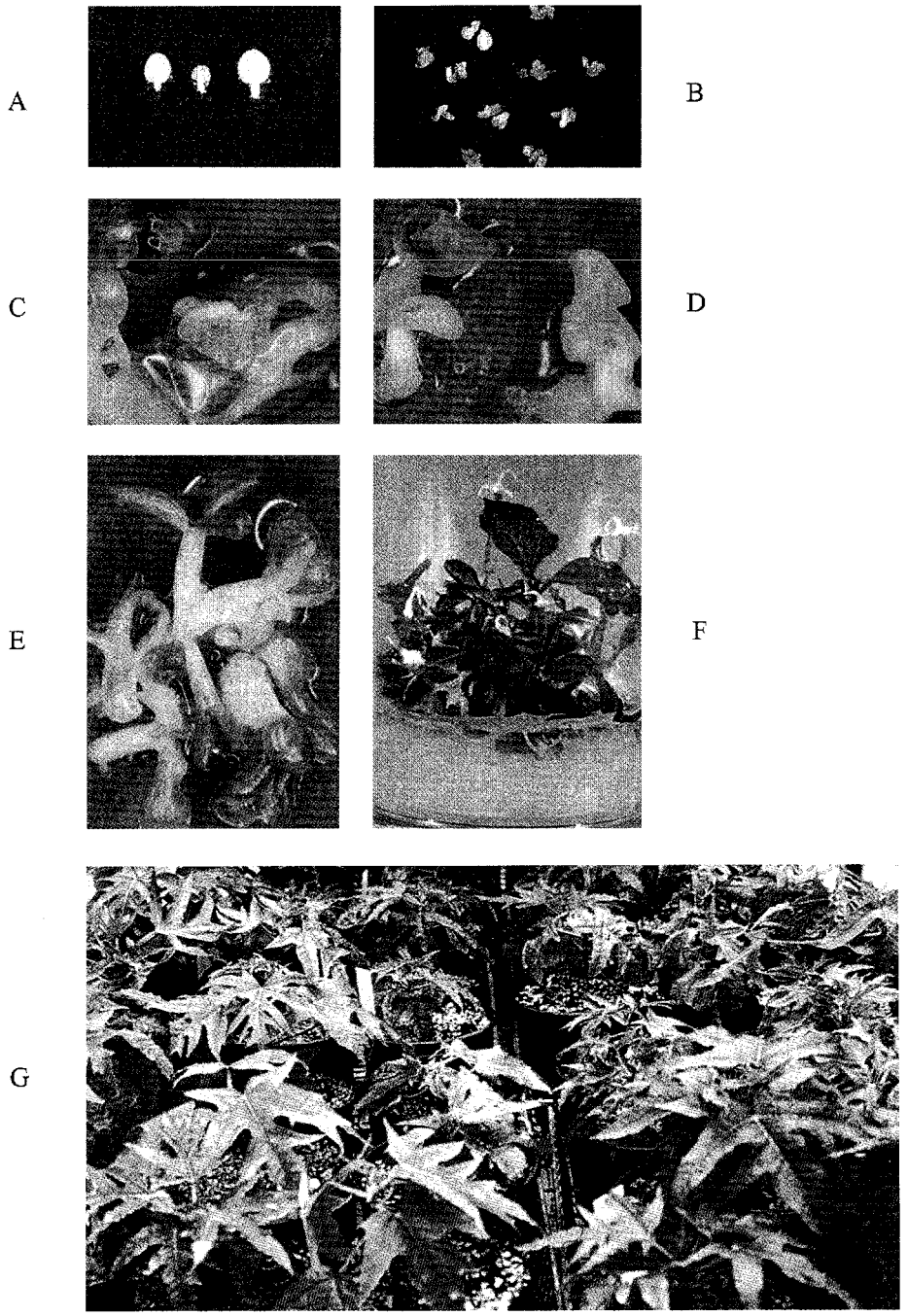

Fig. 2. (*A*) Single hybrid embryos of *C. papaya* × *C. cauliflora* (centre), *C. papaya* (left) and *C. cauliflora* (right); (*B*) multiple hybrid embryos; (*C*) hybrid embryos germinated on liquid medium; (*D*) hybrid embryos germinated on solid medium; (*E*) somatic embryogenesis of multiple embryos; (*G*) interspecific hybrid plants grown in soil in the glasshouse.

embryo-rescue studies of *C. papaya* (Phadnis *et al.* 1970; Rojkind *et al.* 1982). However, in nodal shoot cultures of *C. papaya*, a similar short pre-incubation time (i.e. 3 days) promoted root growth (Drew 1988). Decreasing the pre-incubation time from 0–5 days resulted in significant ($P < 0.05$) reduction in germination and growth of the plants, and extending the pre-incubation time to 7 days did not improve it. Furthermore, with increasing pre-incubation time, height increased correspondingly. The result demonstrates that more than 5 days of pre-incubation was inappropriate as plants that were undesirably tall were produced. Etiolation of the plants was probably caused by excessive uptake of plant growth regulators, particularly GA_3, a known potent chemical for cell elongation. More importantly, the result demonstrates that pre-incubation for 5 days or less (rather than 7 days) can prevent etiolation and callus production (Fig. 1).

Interspecific hybrid embryos of *C. papaya* and *C. cauliflora* are known to abort 90–120 days after pollination (Manshardt and Wenslaff 1989). Consequently, rescued embryos are weak and difficult to establish *in vitro*. A protocol for regeneration of high numbers of these hybrids and cultural conditions for embryo rescue is necessary for the production of PRSV-P resistant cultivars. There are no published reports of embryo-rescue of *Carica* interspecific hybrids that have been cultured in liquid medium to improve the growth of weak hybrid embryos. In the present study, although LGM and SGM promoted maximum germination (100%), LGM improved the growth of weak embryos by more than 50% compared with growth on SGM in terms of all growth parameters measured (Table 2). Embryos that were weak at the time of germination on SGM continued to lack vigour at all stages *in vitro*, and went on to produce weak plants that were difficult to acclimatise. By contrast, initial production of well-developed roots and healthy cotyledonary leaves on LGM contributed to the later good growth of the interspecific hybrids. Presumably, absorption of nutrients by the hybrid embryos was faster when cultured in LGM than on SGM. In oil palm (*Elaeis guineensis* Jacq.), liquid medium induced rapid growth of young embryos compared with those on solid medium (Rabechault *et al.* 1968). Similarly, the growth of weak *C. papaya* shoots was enhanced by liquid medium when compared with solid medium (Drew 1988). The present results also demonstrate that 5 days pre-incubation on LGM before transfer to PGRF-PGM promoted good growth of plantlets. This promotion of good growth is consistent with the present findings for the non-hybrid embryos of *C. papaya*. Exposure of embryos for 10 days produced a similar growth response but the resultant plants were of inferior quality due to hyperhydricity (Table 2). Similarly, *C. papaya* shoots exposed on a liquid medium for more than 14 days became hyperhydric and had poor growth (Drew 1988). In the present study, the pre-incubation time of hybrid embryos on a liquid medium is critical as the influence of LGM is a balance of both promotive and harmful effects on germination and growth of the hybrid plantlets. Pre-incubation of hybrid embryos on SGM avoided hyperhydricity; however, plantlet growth and vigour were reduced.

Multiple embryos were produced after pollination of *C. papaya* × *C. cauliflora* (Manshardt and Wenslaff 1989; Chen *et al.* 1991). However, in the present study, both single and multiple embryo forms were produced (Fig. 2*a*, *b*). This result suggests that the single embryos are genetic hybrids of *C. papaya* × *C. cauliflora* while the multiple embryos may be putative hybrids. Multiple embryos of some crosses of kiwifruit species (*Actinidia* spp.) regenerated only into typical interspecific hybrid plants (Mu *et al.* 1990). The confirmation of hybridity by random amplified polymorphic DNA analysis, cytology and morphology of the putative interspecific hybrids produced by this protocol is the subject of a succeeding paper. Rescuing both embryo forms with this improved protocol indicated that consistently high (94%) germination can be achieved. The result suggests that LGM consistently promoted direct regeneration of single embryos into whole plants while germinated multiple embryos underwent embryogenesis and produced multiple plants. Multiple hybrid embryos of the same cross have been shown previously to produce numerous secondary embryos

(Chen *et al.* 1991). Moreover, the present result indicates that efficient (25%) production of interspecific hybrids that developed into morphologically normal plants (485) can be achieved with this improved protocol. In previous studies, a limited number of interspecific hybrids have been produced and these had many abnormal morphological features (polycotyledony, leaf fasciation and distortion, and roots covered with callus: Manshardt and Wenslaff 1989; Chen *et al.* 1991).

The protocol developed in this study is being used for the production of interspecific hybrids of *C. papaya* × *C. goudotiana* (Tr. et Planch.) Solms, *C. parviflora* (A. DC.) Solms, *C. pubescens* Lenné et Koch., and *C. stipulata* Badillo. In addition, this protocol may also have application for the production of progenies from succeeding filial or backcross generations of these *Carica* interspecific hybrids to develop PRSV-P resistance.

Acknowledgments

The authors thank the Australian Agency for International Development (AusAID) for providing P. M. Magdalita with a scholarship, and the Australian Centre for International Agriculture Research (ACIAR) for funding this research. We are grateful for the technical assistance of Ms J. Vogler, Ms L. O'Brien, Ms R. Kerridge and Mr B. Anderson.

References

Alvizo, V. H. F., and Rojkind, M. C. (1987). Resistencia al virus mancha anular del papaya en *Carica cauliflora*. *Revista Mexicana de Fitopatologia* **5**, 61–62.

Chen, M. H., Chen, C. C., Wang, D. N., and Chen, F. C. (1991). Somatic embryogenesis and plant regeneration from immature embryos of *Carica papaya* × *C. cauliflora* cultured *in vitro*. *Canadian Journal of Botany* **69**, 1913–1918.

Cohen, E., Lavi, U., and Spiegel-Roy, P. (1989). Papaya pollen viability and storage. *Scientia Horticulturae* **40**, 317–324.

Conover, R. A. (1964). Distortion ringspot, a severe virus disease of papaya in Florida. *Proceedings of Florida State Horticultural Society* **77**, 447–448.

Conover, R. A., Litz, R. E., and Malo, S. E. (1986). *Cariflora*—a papaya ringspot virus tolerant papaya for South Florida and the Caribbean. *HortScience* **21**, 1072.

Cook, A. A., and Zettler, F. M. (1970). Susceptibility of papaya cultivars to papaya ringspot and mosaic virus. *Plant Disease Reporter* **54**, 893–895.

De Fossard, R. A., Myint, A., and Lee, E. C. M. (1974). A broad spectrum tissue culture experiment with tobacco (*Nicotiana tabacum* L.) pith tissue callus. *Physiologia Plantarum* **31**, 125–130.

Drew, R. A. (1988). Rapid clonal propagation of papaya *in vitro* from mature field-grown trees. *HortScience* **23**, 609–611.

Gomez, A. K., and Gomez, A. A. (1984). Comparison between treatment means. In 'Statistical Procedures for Agricultural Research'. (Eds A. K. Gomez and A. A. Gomez.) pp. 7–84. (John Wiley & Sons: New York.)

Huang, C. H. (1988). Superior crop varieties in Taiwan. Food and Fertilizer Technology Centre Bulletin, Taiwan.

Magdalita, P. M., Villegas, V. N., Pimentel, R. B., and Bayot, R. G. (1988). Reaction of papaya (*Carica papaya* L.) and related *Carica* species to ringspot virus. *Philippine Journal of Crop Science* **13**, 129–132.

Manshardt, R. M. (1992). Papaya. In 'Biotechnology of Perennial Fruit Crops'. (Eds F. A. Hammerschlag and R. E. Litz.) pp. 489–511. (Cambridge University Press: Oxford.)

Manshardt, R. M., and Wenslaff, T. F. (1989). Zygotic polyembryony in interspecific hybrids of *Carica papaya* and *C. cauliflora*. *Journal of American Society for Horticultural Science* **114**, 684–689.

Mu, S. K., Fraser, L. G., and Harvey, C. F. (1990). Rescue of hybrid embryos of *Actinidia* species. *Scientia Horticulturae* **44**, 97–106.

Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* **15**, 473–497.

National Farmers Federation (1993). Other horticultural crops. In 'Australian Agriculture'. (Ed. B. Pestana.) pp. 347–354. (Morescope Publishing: Victoria.)

Phadnis, N. A., Budrukkar, N. D., and Kaulgud, S. N. (1970). Embryo culture technique in papaya (*Carica papaya* L.). *Poona Agricultural College Magazine* **60**, 101–104.

Rabechault, H., Ahee, J., and Guenin, G. (1968). Recherches sur la culture *in vitro* des embryons de palmier a huile (*Elaeis guineensis* Jacq.) IV. Effets de la teneur en eau des noix et de la duree de leur stockage. *Oleagineux* **23**,233–237.

Rojkind, C., Quezada, N., and Gutierrez, G. (1982). Embryo culture of *Carica papaya*, *C. cauliflora* and its interspecific hybrids *in vitro*. In 'Plant Tissue Culture'. (Ed. A. Fujiwara.) pp. 763–764. (Japanese Association of Plant Tissue Culture: Tokyo.)

Thomas, J. E., and Dodman, R. L. (1993). The first record of papaya ringspot virus-type P from Australia. *Australasian Plant Pathology* **22**, 1–7.

Yeh, S. D., Gonzalves, D., Wang, H. L., Namba, R., and Chiu, J. R. (1988). Control of papaya ringspot virus by cross protection. *Plant Disease* **72**, 375–380.

Manuscript received 18 December 1995, accepted 12 April 1996