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Plant regeneration from petioles of *in vitro* regenerated papaya (*Carica papaya* L.) shoots

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Abstract. The leaf petioles of shoots regenerated from mature embryos of papaya (*Carica papaya* L.) germinated *in vitro* were used as major initial materials for regeneration of plantlets because of their availability and high multiplication rate. Cross sections of the petioles were induced to form callus with 62% efficiency on MS medium supplemented with 2.5 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.4 μM 6-benzyladenine (6-BA). Culture of these callus in MS medium with 5.0 μM 2,4-D produced numerous embryoids with 49% efficiency. The embryoids were able to germinate to form single shoot or multiple shoots with 94% efficiency on MS medium supplemented with 0.1 μM α-naphthaleneacetic acid (NAA) and 0.8 μM 6-BA. An efficient process was established for rooting the separated shoot with 92% of success. The regenerated plantlets were transplanted from the culture tubes to the vermiculite and further grew into plants with 80% efficiency 14 days after culturing under ambient environmental conditions. The results showed that the plants could be regenerated from the petiole-derived callus within eight months with 36% efficiency. This regenerating process is potentially applicable for transgenic papaya via Ti plasmid transformation.

Key words: Papaya; Petiole callus; Plant regeneration.

Introduction

Papaya (*Carica papaya* L.) is one of the most widely grown and economically important plants of the tropics. Effective gene transfer systems, somaclonal variants and micropropagation of character-selected parental papaya require readily available explants as well as an efficient system for plant regeneration. However, low success rate of papaya plant regeneration in earlier investigations with cotyledon (Litz *et al.*, 1983), petiole (DeBryijne and Delanghe, 1974), hypocotyl (Yie and Liaw, 1977), and root (Chen *et al.*, 1987) of seedlings were hardly useful for these applications. In addition, seasonal variations in anther (Tsay and Su, 1985), ovule (Litz and Conover, 1982) and immature embryo developments (Manshardt and Wenslaff, 1989; Fitch and Manshardt, 1990) of field-grown papaya plants affect the plant regenerability of explants and the efficiency of somatic embryogenesis. In order to overcome the inconsistency and difficulty for regeneration, we tried to establish an efficient system by initiating callus from petioles of seedlings that were germinated from mature zygotic embryos *in vitro*. Culture proliferation can be recycled and highly reproducible. This process provides a consistent and reliable source for plant regeneration of papaya.

Materials and Methods

Seedling Shoots from Zygotic Embryo Germination

Seeds of papaya, *Carica papaya* L. cv. "Sunrise" were kindly supplied by Feng-Shan Tropical Horticultural Experiment Station of Taiwan Agricultural Research Institute. They were thoroughly washed with tap water for 1 h, immersed in 70% alcohol for 3 min, and sterilized in 2% sodium hypochlorite and 0.01% Tween 20 solution for 20 min with 150 rpm gyratory

agitation. They were finally rinsed in sterilized distilled water 3 times. Culture conditions for all experiments were set at 25°C under 16 h daily fluorescent illumination at 50 $\mu Em^{-2}s^{-1}$ light intensity.

Embryos were isolated aseptically with the aid of a dissecting microscope and cultured on P1 medium composed of 1/2 basal MS (MS basal medium minus 1/2 macronutrients) supplemented with filter-sterilized 8.7 μM GA_3 (Yang, 1988). After two weeks, the embryos germinated into two-leaf seedlings, 4 to 6 cm tall. Their shoot tips were excised and cultured onto P2 medium, or MS basal medium supplemented with 0.1 μM NAA and 0.8 μM 6-BA (Chow and Liu, 1987). Three months after twice monthly subcultures on P2 medium, the shoot tips proliferated into multiple shoots (Fig. 1A). Petioles of these shoots were used as sources for regeneration experiments. The tip-excised seedling grew and finished its life cycle. Finally, this seedling explant was proved to be a female type with the common horticultural properties of the "Sunrise" papaya.

Embryogenesis

Petiole sections (2 to 3 mm long) were placed on P3 medium (MS basal medium supplemented with various concentrations of 2,4-D and 6-BA to induce callus for four weeks after subcultured once at two week intervals or not subcultured (Table 1). The four-week-old callus formed on P3 medium were transferred to P4 medium (MS basal medium supplemented with 5.0 μM 2,4-D which was selected from previous results) to induce embryoid formation (Table 2). The callus was further subcultured on the same medium and transferred at monthly intervals until embryoids appeared, which were observed by a dissecting microscope.

Plant Formation

The mature embryoids (Table 1B) were transferred from P4 to P2 medium for germination. Shoots developed within two months after transfer. Individual shoots of 1.5 to 2.0 cm in length were excised and cultured on P5 media (MS basal medium supplemented with varying levels of IBA; Table 3) for seven days for root induction, and then transferred to P6 medium (MS basal medium) for three weeks for root development to form plantlets. The other rooting procedures followed the method of Manshardt and Wenslaff's (1989): The individual shoots were severed at base, dipped in a talc powder mixed with 0.5% IBA, and planted in P7 medium (Vermiculite supplemented with 1/2 MS mineral salts) for four weeks for induction of roots to form plantlets.

Plant Established under Ambient Conditions by Hardening Treatment

Plantlets formed on P6 medium were removed from culture tubes to vermiculite in plastic pots and covered with transparent plastic bags that were opened gradually over a two-week period to adapt the plantlets to ambient conditions. A 1/2 MS basal salt solution was weekly sprayed on the surface of the growing medium to supply developing plants with mineral nutrients and water.

Table 1. *The effects of growth regulator combinations in P3 media on callus formation from petioles of papaya seedling shoots after four-week culture*

P3 media	2,4-D/6-BA (μM)	Callus formation					
				Morphological differences			
		Type of callus	Frequency[a] (%)	Browning (%)	Size (cm^2)	Cotton fiber-like substance formed on callus surface area (%)	Texture
P31	2.5/0.4	1	62 (57-67)[b]	0	1.0	0	rigid
P32	2.5/0.8	2	31 (25-37)	20	0.7	0	rigid
P33	5.0/0.4	3	20 (15-25)	60	0.5	65	spongy
P34	5.0/0.8	4	10 (7-14)	80	0.5	100	spongy

[a]Mean frequency from three replications.
[b]95% confidence limit.

Table 2. *Effects of subculture on P3 media on different type of callus to form embryoid on P4 medium*

Type of callus	Treatment	Embryoid formation (%)
1[a]	Subcultured[b]	49 (43-55)[c]
2		18 (13-23)
3		13 (9-18)
4		11 (9-16)
1	Non-subcultured	13 (9-18)
2		10 (7-14)
3		9 (6-13)
4		8 (5-12)

[a]See table 1.
[b]Two-week old call on P3 medium were subcultured once on the same medium.
[c]See table 1.

Table 3. *Effects of P5 rooting media on formation of plantlets from individual shoots and plant establishment*

P5 media		Plantlets formation	Plant establishement
Name	IBA (μM)	(%)	(%)
P51	0	0 (0-0)[a]	0 (0-0)[a]
P52	0.5	16 (9-25)	14 (4-34)
P53	1.25	47 (35-59)	41 (26-57)
P54	2.5	92 (82-97)	80 (64-91)
P55	5.0	86 (75-94)	71 (55-84)
P56	10.0	84 (70-92)	65 (49-79)
P57	2.5×10^4	54 (42-67)	14 (6-27)

[a]See table 1.

Results

Shoot Proliferation

Sets of multiple shoots were established from the shoot tips of seedlings derived from mature zygotic embryos, within three months after subculturing twice on P2 medium (Fig. 1A). The frequency of multiple shoot formation from shoot tips was above 90%. In a 17-day period, each shoot cluster yielded 30 to 40 petiole sections as explants for regeneration studies. Moreover, each shoot cluster with petioles removed could be divided and these were able to grow on P2 medium to form new shoot cluster within another 17-day period. Thus, the number of petiole explants could be increased as much as 120 times each 17 days, once the shoot cluster were established.

Embryogenesis

The petiole sections became swollen in size, seven days after culturing on P3 media. The effects of growth regulator combinations (2,4-D/6-BA in μM) in P3 medium on frequency of callus formation and morphology are shown in Table 1. The results indicated that the optimal combination (2.5/0.4 in P31 medium) could induce 62% of petiole sections to form callus, whereas combinations of 2.5/0.8 in P32 medium, 5.0/0.4 in P33 medium and 5.0/0.8 in P34 medium gave only 31%, 20% and 10%, respectively. Subculturing once on P3 media did not increase the frequency of callus formation significantly.

The pale yellow callus grew and developed globular embryoids two months after one monthly subculturing on P4 medium. In this period, the embryogenic callus showed no apparent increase in size. The globular embryoids developed into mature embryoids after another one-month subculture (Fig. 1B). There were a few cotyledons growing out of the embryoids. At the optimum growth regulator combination, the type 1 callus (Table 1) subcultured once on P31 medium, greatly increased the ability to differentiate into embryoids on P4 medium with a rate of 49% (Table 2), whereas the unsubcultured type 1 callus gave only 13%. At non-optimal growth regulator combinations the embryogenesis rates of types 2, 3 and 4 callus (Table 1), were generally low (8-18%) (Table 2).

Plantlets from Mature Embryoids

The mature embryoids produced green shoots and plantlets after culturing in P2 medium for two months (Fig. 1C). The rate of embryoid-to-shoot conversion if based on the ratios of the culture tubes was 94%. In this period, the callus resumed active growth about 2.5 times/month. There was an average of 4 shoots formed per culture. The abnormal leaves of the shoots (Fig. 1C) gradually became normal as those of shoot tips of seedlings (Fig. 1A).

Individual shoots (1.5 to 2.0 cm in height) of the shoot cluster were excised and cultured in P5 media for seven day to induce roots. Small amounts of callus grew out of the surface of the excised shoots in the media. The shoots were then transferred to P6 medium, where roots developed to generate plantlets after 21 days (Fig. 1D).

The effects of the different media on rooting efficiency of the individual shoots are summarized in

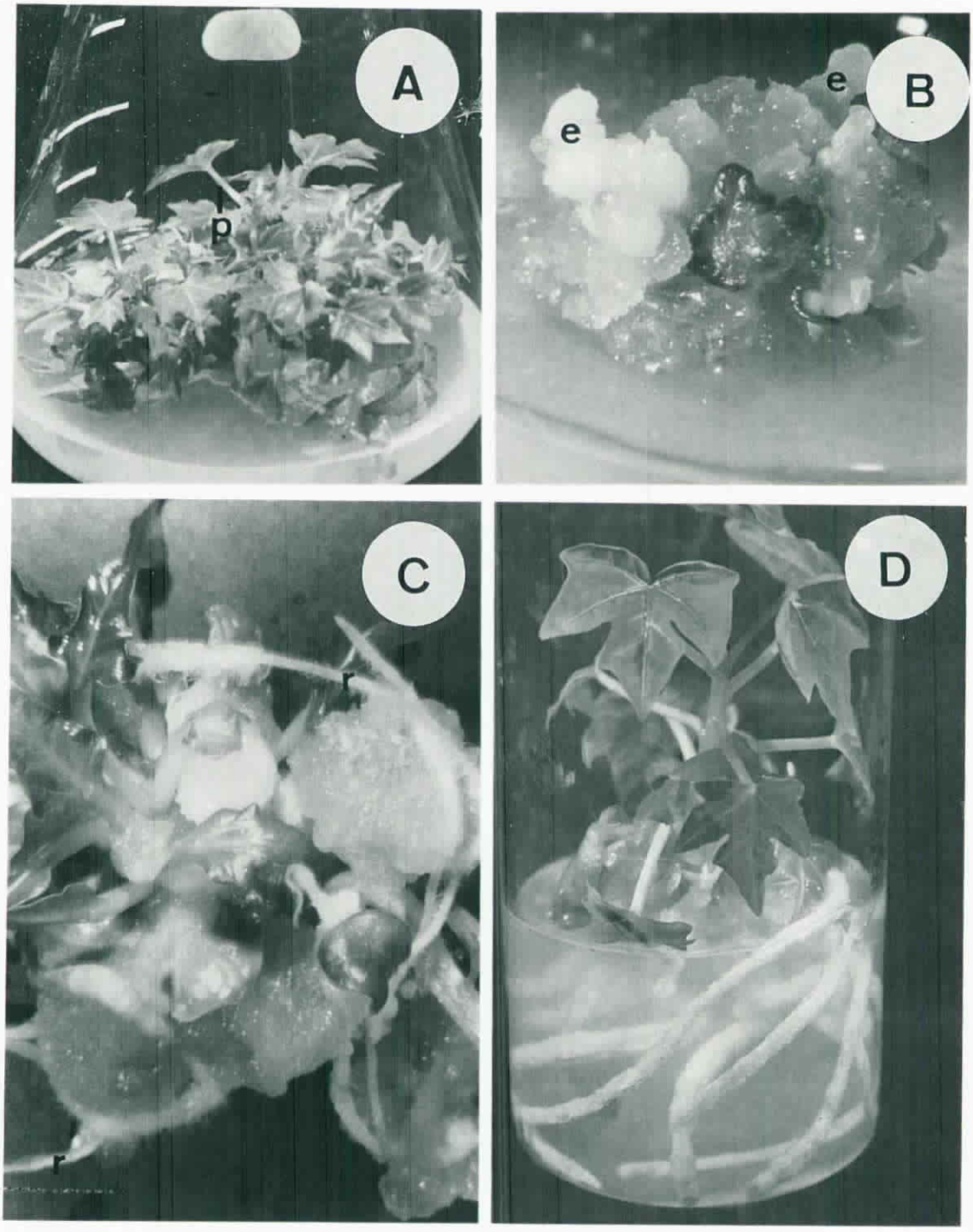

Fig. 1. The plantlets of papaya variety "Sunrise" were regenerated from explants of multiple shoot petiole tissues of papaya via callus induction, embryoid formation and rooting processes. A, The proliferative multiple shoots formed 3 months after culturing shoot tips of seedlings germinated from mature zygotic embryos on P2 medium. The picture shows three multiple shoot sets growing in 250 ml culture flask; B, The mature embryoids formed from the globular embryoids after culturing on P4 medium; C, The green shoots with roots grew out of green embryoids after the second subculture on P2 medium; D, A plantlet grew on P6 medium two week after transferring. p: petiole; e: mature embryoid; r: root.

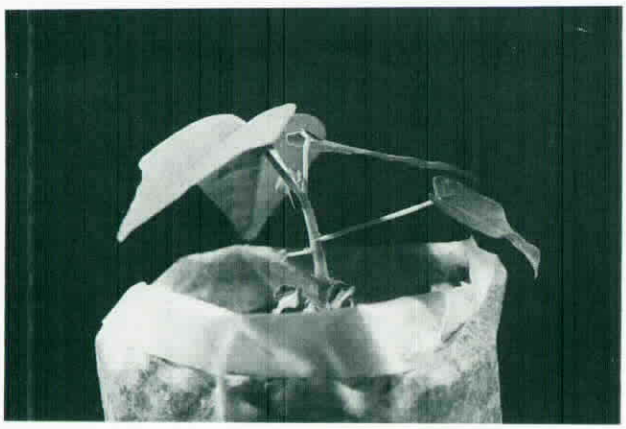

Fig. 2. The regenerated plant grew in the vermiculite in plastic bag fourteen days under the ambient environmental condition.

Table 3. The best treatment was a sequence of MS medium with 2.5 μM IBA in P54 medium for one week followed by MS medium for three weeks, with a root formation rate of 92%. Treatments of talc powder with 25 mM IBA in vermiculite resulted in a lower root formation rate of 54%. No root formation was observed when unsupplement MS medium was used. Fourteen days after hardening treatment, healthy plants established and grew well in vermiculite under natural environmental condition (Fig. 2). Plantlets rooted in MS medium with 1.25, 2.5, 5.0 and 10 μM IBA followed by MS medium gave plant establishment rates of 41, 80, 71 and 65%, respectively, whereas plantlets from talc powder were established at a low rate of 14%.

Discussion

In this report, the success rates for sequency of steps under optimal conditions were 62% for callus induction, 49% for embryoids formation, 92% for rooting of shoots, and 80% for establishment of plants in vermiculite. Thus, the efficiency of plant regeneration from petiole sections of multiple shoot was 22% within nine months, or from callus was 36% within eight months calculated by multiplying the percentage efficiency of each step. The results indicate that our procedure is more efficient and reproducible than the plant regeneration system in published work with cotyledon (Litz *et al.*, 1983), petiole (DeBrijne and Delanghe, 1974), hypocotyl (Yie and Liaw, 1977), and root (Chen *et al.*, 1987) of the seedlings. This is probably because none data in these papers showed the percentage efficiency of each step from the explant to plant regeneration.

The multiplication rate for production of petiole explants of shoots derived from mature zygotic embryos per 17 days was 30 to 40 fold. Besides, the shoot cluster forming process could be repeated and the cluster increased three times within 17 days. The process provided a consistent and abundant source of highly regenerative material. The 17-day period of prolifecation in P1 medium was three days shorter than that of Litz and Conover (1978). This is probably because the petioles from shoot tips of seed embryo was younger than those from apices of more mature field grown plants. In addition, we found that the growth rate of embryo-derived shoots was greater than those of mature plants. Therefore, use of the abundantly obtainable shoot petioles from mature zygotic embryos as explants for plant regeneration is concluded as being superior to that of using seedling organs as explants directly as done by others (Chen *et al.*, 1987; DeBrijne and Delanghe, 1974; Litz *et al.*, 1983; Yie and Liaw, 1977).

Subculturing the two-week old callus from seedling petioles of papaya dramatically increased the embryogenic frequency, from 13 to 49% (Table 2). This callus-subculturing process might have reduced factors which inhibit embryogenesis. For example, accumulating ethylene has been reported to inhibit morphogenesis *in vitro* of *Brassica campestris* (Chi *et al.*, 1991), *Helianthus annuus* (Paterson-Robinson and Adams, 1987) and *Nicotiana tabacum* (Huxter *et al.*, 1981). Subculturing might have prevented the accumulation of ethylene. The callus-subculturing process produced callus that appeared to be rigider than that of unsubcultured callus. This observation was similar to that of isolated potato stolens, which formed white callus at the base of the stolens when treated with 5 μl/l ethylene after 30 days in culture (Mingo-Castel *et al.*, 1974).

The widespread and destructive papaya ringspot virus (PRV) is a major limiting factor for commercial production of papaya (Purcifull *et al.*, 1984). PRV-resistant papaya is not as yet avaiable (Yeh *et al.*, 1988). Our efficient papaya regeneration system using petioles derived from shoots of mature zygotic embryos cultured *in vitro* can be an aid in combatting papaya ringspot disease. It can be used for the production of transgenic papaya, expressing PRV coat protein

gene for virus resistance (Fitch *et al.*, 1990) via Ti plasmid-mediated transformation, the selection of somaclonal variants tolerant to PRV, and the micropropagation of hermaphroditic plants with the best horticultural properties and virus tolerance or resistance.

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木瓜欉生芽葉柄癒合組織再生植株

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培養木瓜欉生芽葉柄段於含有 2.5 μM 2,4-dichlorophenoxyacetic acid (2,4-D) 及 0.4 μM 6-benzyladenine (6-BA) 之 MS 培養基，可誘導其中的 62% 形成癒合組織。初生癒合組織經一次繼代培養，可促進其中的 45%形成體胚。若不經繼代培養則僅有 13%的形成率。此成熟體胚培養於含有 0.1 μM α-naphthaleneacetic acid 及 0.8 μm 6-BA 之 MS 培養基，可有 94%發芽長成植株。切株扦插於含有 2.5 μM indolebutyric acid 之 MS 培養基中，可誘導 92%發根率。接著移植於管外培養，可有 80%再生植株存活。結果顯示欉生芽葉柄初期癒合組織，早期繼代培養一次後，可促進再生植株效率至 36%。這項再生植株過程，將有助於木瓜基因轉移及體細胞變異篩選之研究試驗。