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Efficient rooting for establishment of papaya plantlets by micropropagation

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Abstract

A low cost micropropagation protocol to produce high quality root systems which are easy and economical to acclimatize is essential for large-scale micropropagation of papaya (*Carica papaya* L.). In this study, individual shoots (>0.5 cm) with 2∼3 leaves from *in vitro* papaya multiple shoots were cultured on MS agar medium containing 2.5 μM IBA under dark conditions for 1 week for root induction. They were then transferred to agar or vermiculite media, containing half strength MS medium, under aerated or non-aerated conditions, for root development. Rooting percentage of shoots cultured for 2 weeks in aerated vermiculite was 94.5%, compared with 90.0% in non-aerated vermiculite, 71.1% in aerated agar, and 62.2% in non-aerated agar. Shoots with roots were acclimated in vermiculite under 100% RH for 1 week and then under ambient conditions for 2 weeks in a temperature-controlled growth chamber (28 °C). The survival rates of the plantlets were 94.5% from aerated vermiculite, 87.8% from non-aerated vermiculite, 42.2% from aerated agar, and 35.6% from non-aerated agar. Thus, root induction in low-concentration IBA agar medium followed by root development in vermiculite containing half strength MS medium under aerated conditions results in efficient rooting of *in vitro* papaya shoots.

Abbreviations: IBA – indole-3-butyric acid; NAA – α-naphthaleneacetic acid; BA – 6-benzyladenine; CPA – p-chlorophenoxyacetic acid

Introduction

Papaya shoot culture has been reported in detail (Litz and Conover, 1981; Drew and Smith, 1986; Drew, 1988). Rooting and acclimatization are critical for establishing papaya micropropagules. The auxins [indole-3- butyric acid (IBA), α-naphthaleneacetic acid (NAA), and p-chlorophenoxyacetic acid (CPA)] have been used to induce roots from *in vitro* papaya shoots. (Drew, 1987; Drew et al., 1993). IBA was superior in terms of rooting percentage and numbers of roots per shoot when the basal medium was supplemented with riboflavin (Drew, 1987; Drew et al., 1993). Rooting percentages can be increased by short exposure (3 days) of shoots grown *in vitro* to IBA (Drew et al., 1993). Also, rooting percentages above 90% (Drew, 1988, 1992; Drew et al., 1993) and high acclimatization percentages up to 100% (Drew, 1988; Manshardt and Drew, 1998) have been reported. Although highly uniform female plantings of 14,000 plants of a single clone have been reported in Australia (Manshardt and Drew, 1998), the development of an efficient and low cost micropropagation protocol to generate high quality root systems is essential for large-scale micropropagation of papaya.

Rooting *ex vitro* is an alternative method for mass propagation (Kataoka and Inoue, 1992; Reuveni and Shlesinger, 1990); however, this approach is limited by stringent rooting conditions, time, seasonal factors, and explant type (Kataoka and Inoue, 1992).

In order to develop an efficient rooting process to establish micropropagated papaya plantlets, *in vitro* shoots were used to investigate root formation in vermiculite or agar media under aerated or non-aerated

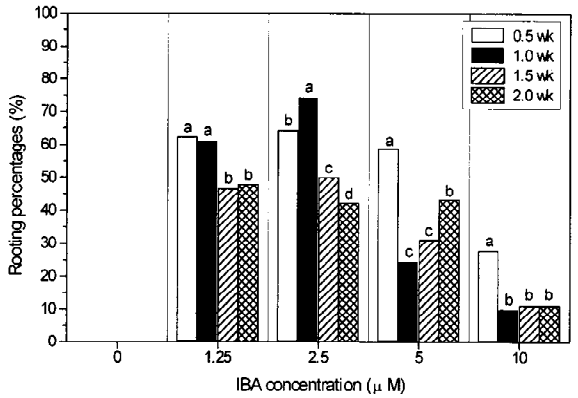

Figure 1. Effects of different IBA concentrations on rooting percentages of papaya. Individual shoots were placed on MS basal medium with different IBA concentrations under dark conditions for 0, 0.5, 1, 1.5, and 2 weeks, and then transferred to hormone-free MS medium under light conditions for 3 weeks. Values represent means from 3 replicas with a total of 60 explants for each treatment. Data in each plot followed by the same letter are not significantly different according to Duncan's multiple range test (p=0.05).

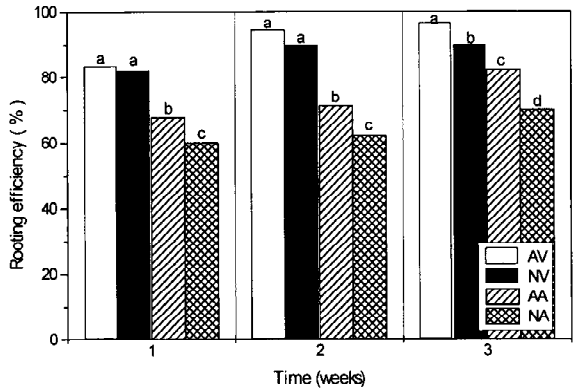

Figure 2. Effects of the different media and aeration on rooting percentages of individual shoots of papaya 1, 2, and 3 weeks after culture on 1/2 MS medium containing 2.5 μM IBA. AV: aerated vermiculite medium. NA: non-aerated vermiculite medium. AA: aerated agar medium. NA: non-aerated agar medium. Values represent means from 3 replicas with a total of 60 explants for each treatment. Data in each block followed by the same letter are not significantly different according to Duncan's multiple range test (p=0.05).

conditions. Acclimatization conditions for plant establishment were also determined.

Materials and methods

Plant materials and in vitro culture

The shoot tips (0.5 cm) of papaya (*Carica papaya* L. var. Tainung No. 2) were explanted, and proliferating shoot cultures were established using the medium and techniques described by Yang and Ye (1992). All the media consisted of MS salts (Murashige and Skoog, 1962), B_5 vitamins (Gamborg et al., 1968), and various growth regulators. The MSNB medium for culturing papaya multiple shoots contained MS salts, B_5 vitamins, 0.1 μM NAA and 0.8 μM BA, 30 g l^{-1} sucrose and 9 gl^{-1} agar. The pH of the medium was adjusted to 5.7±0.1 with 1 N KOH before autoclaving at 1.1 kg cm^{-2} and 121 °C for 20 min. Four bud clusters (buds<0.5 cm in length and 0.5×0.5 cm^2) were grown in 250 ml flasks containing 50 ml MSNB medium for 3 weeks to form multiple shoots. The plant materials were incubated in a growth chamber at 28±1 °C with cool white fluorescent lamps which provided 14-h photosynthetic radiation of 53 μmol m^{-2} s^{-1}. Individual shoots containing 2 to 3 leaves (>0.5 cm in length) were experimental units. The basal part of multiple shoot clusters were cut and transferred to fresh MSNB medium for cycling (Yang and Yu, 1992).

Root initiation

Individual shoots were cultured on 50 ml semisolid MS medium with different IBA (0, 1.25, 2.5, 5, 10 μM) in each 250 ml flask under dark conditions for 0, 0.5, 1, 1.5 and 2 weeks for root induction, and then transferred to hormone-free MS agar medium for 3 weeks under light conditions for root development.

Effect of culture media and aeration on root development

Individual shoots were cultured on root induction medium containing 2.5 μM IBA for 1 week. They were further incubated for root development, under aerated and non-aerated conditions, in glass jars (5.6 cm open mouth, 10 cm height, 350 ml volume) containing 50 ml 1/2 MS agar (9 g l^{-1}) medium or vermiculite medium [50 ml 1/2 $MS_{(aq)}$/100 ml vermiculite (No. 3)] for 1, 2 and 3 weeks. For aeration treatments, the glass jars were sealed with a Sun Cap closure (0.02 μm filter) (Sigma Chemical Co. St. Louis, MO) which allowed gases to diffuse in and out of the jar. Polypropylene (PP) membrane seals were used for non-aerated controls.

Effects of different MS concentrations on root development

Individual shoots after root induction were transferred to agar or vermiculite containing different concentrations of MS (MS, 1/2 MS, and 1/4 MS) or to sterile

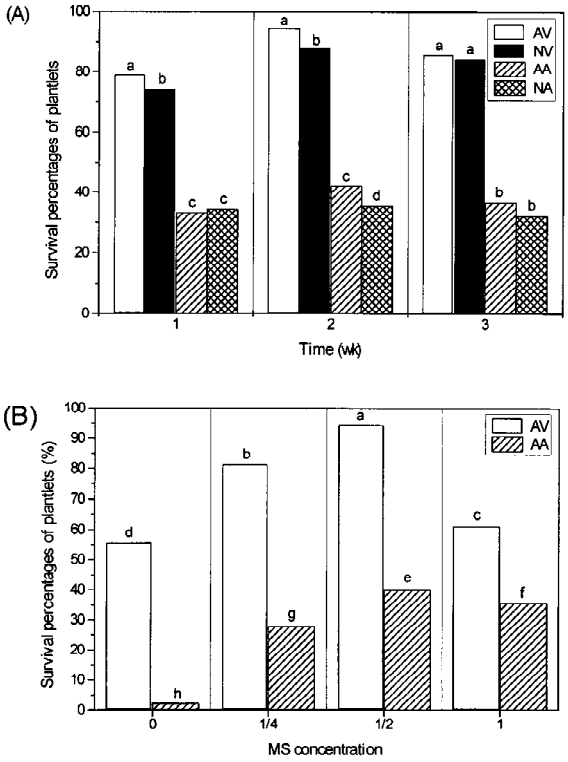

Figure 3. Plantlet survival under different rooting conditions, recorded 5 weeks after the onset of chamber acclimation. (*A*) different rooting materials for root development under aerated and non-aerated conditions for 1–3 weeks following root initiation. (*B*) root development as affected by different strengths of MS concentration under aerated conditions for 2 weeks after root initiation. AV: aerated vermiculite medium. NA: non-aerated vermiculite medium. AA: aerated agar medium. NA: non-aerated agar medium. Values represent means from 3 replicas with a total of 60 explants for each treatment. Data in each block of (*A*) or in the figure (*B*) followed by the same letter are not significantly different according to Duncan's multiple range test (p=0.05).

distilled water. Fifty ml of different strengths of MS solution was added to 100 ml vermiculite. Each jar contained 10 individual shoots and each treatment had 6 jars. Each treatment was repeated three times and a total of 180 shoots from 18 jars were analyzed. Rooting ratios, root numbers and root lengths were recorded at weekly intervals after culturing under different conditions.

Acclimatization and plantlet survival

Individual shoots with roots were planted in polyethylene (PE) bags (size 10 cm×11 cm) which were punched with 16 holes (5 mm diameter) prior to the addition of 100 ml medium (vermiculite:peat moss=3:1). The bags were placed in plastic crates (size 45 cm×35 cm×12 cm) which were covered with PP film to maintain RH at 100%. The plantlets were grown in a temperature-controlled (28 °C) growth chamber for 1 week and then exposed to ambient conditions for 2 weeks with the PP film removed. The plantlets were further placed in a temperature-controlled (28 °C) greenhouse for 2 weeks under ambient conditions without supplementary light. Finally, the plantlets were moved to a greenhouse without temperature control (22–38 °C) for further hardening. The survival percentages of the plantlets were determined 5 weeks after transfer to the acclimation chamber.

Results and discussion

Conditions for root induction

The best rooting responses were obtained when shoots were pulsed with lower IBA concentrations of 1.25–2.5 μM for 0.5 and 1 week, and in the higher IBA concentrations of 5–10 μM for 0.5 week (Figure 1). The best induction for individual shoots of papaya was obtained with 2.5 μM IBA for 1 week, with a rooting percentage of 74.4% after 21 days incubation on MS medium, compared to 61.1% from 1.25 μM IBA treatment, 24.4% from 5 μM IBA, and 10% from 10 μM IBA. Shoots on medium without IBA did not form roots. According to Drew et al. (1993), there are two phases of adventitious root formation: (1) root induction, when exposure to auxin is essential and (2) root development, when auxin is not required or is inhibitory (Went and Thimann, 1937; Went, 1939). Leaves of individual shoots became chlorotic or epinastic and the roots were thick and stumpy when higher IBA concentrations were used $\geq$1 week. These symptoms have been previously described by Drew (1987, 1988).

Effects of culture media on root development

After culturing on MS agar medium containing 2.5 μM IBA under dark conditions for 1 week, the individual shoots were transferred to different rooting media to encourage root growth. The rooting percentages in vermiculite and agar medium under non-aerated conditions are shown in Figure 2 (NV, NA). The rooting percentages of shoots cultured in the vermiculite medium for 2 or 3 weeks were 90%, and $\geq$82% for 1 week. By contrast, on agar medium the rooting percentages were only 60–70% for 1 to 3 weeks. In papaya, rooting has usually been achieved using agar-solidified medium *in vitro* (Drew, 1988; Teo and Chan,

Table 1. Effects of root development media and aeration for different time intervals on the number and length of roots from individual papaya shoots transferred to 1/2 MS medium after root induction

	Treatments[3]	Time (weeks)		
		1	2	3
Root No.[1]				
	AV	4.0 a[4]	4.9 a	6.0 a
	NV	3.5 b	4.5 ab	5.7 a
	AA	4.1 a	4.1 b	5.5 a
	NA	2.8 c	3.1 c	3.1 b
Root length[2]				
(cm)	AV	2.6 a	5.4 a	6.9 a
	NV	2.4 a	3.7 b	5.2 b
	AA	1.7 b	3.0 c	5.2 b
	NA	2.0 b	1.8 d	3.4 c

[1]Number of primary roots.
[2]Mean of the greatest root length.
[3]AV: aerated vermiculite medium. NV: non-aerated vermiculite medium. AA: aerated agar medium. NA: non-aerated agar medium.
[4]Data in each column followed by the same letter are not significantly different according to Duncan's multiple range test (p=0.05). n=30.

1994). However, in this study, we found that rooting was improved in the vermiculite medium. Teo and Chan (1994) observed that papaya roots growing on the surface of the agar were normal, but those growing into the agar were thick and stumpy. Roots growing in the vermiculite appeared to be normal, growing vigorously with root hairs and without callus formation.

Effects of aeration on root development

Rooting percentages under aerated conditions are shown in the Figure 2 (AV, AA). In vermiculite, the best rooting percentage was 96.7% under aerated conditions for 3 weeks, compared to 94.4% for 2 weeks, and 83.3% for 1 week. On agar medium, the rooting percentages were only 82–67% under aerated conditions. Our results showed that aeration increased rooting percentages significantly in agar medium in weeks 1–3, but only significantly in week 3 for vermiculite medium. With agar and vermiculite, rooting percentages were best 3 weeks after transfer. Root numbers and length under different conditions at time intervals are shown in Table 1. Root numbers were greater in the aerated treatments at 1 week than in any of the non-aerated treatments. Fewer roots developed in non-aerated agar than in aerated or non-aerated vermiculite in weeks 1 and 2. Vermiculite media for 1 week generated longer roots than agar media. Root length was significantly better after 2 and 3 weeks on aerated vermiculite than under other conditions. Root elongation was significantly better in aerated vermiculite at 2 weeks than under other conditions. Roots reached 6.9 cm when shoots were incubated in aerated vermiculite for 3 weeks. Root number and length in aerated vermiculite were superior to those under non-aerated conditions or in agar media.

Magdalita et al. (1997) observed that papaya nodal cultures were very sensitive to ethylene, and significant reduction in growth occurred at ethylene concentrations as low as 0.1 ppm, which is well below the ethylene concentration observed in 2 to 3 week-old closed nodal cultures. Under aerated conditions, the vessel ventilation was good, and no ethylene was detected (Lai et al., 1998), and this may be the reason that individual shoots cultured in the aerated vermiculite medium for root development had vigorous growth and developed healthy roots.

Effect of different MS concentrations on root development

Effects of different MS concentrations on rooting percentages are shown in Table 2. The best rooting percentage (94.4%) was in the aerated vermiculite containing half strength MS medium. In agar medium, the best rooting percentage (71.1%) occurred when 1/2 MS concentration was used. The effects of MS concentration on root numbers and root length are also shown in Table 2. The shoots had greater numbers of roots, but with shorter length, in vermiculite containing 1/4 MS than in 1/2 MS medium.

Acclimation and plantlet survival

After culturing in 2.5 μM IBA for 1 week for root initiation, individual shoots were transferred to different rooting media for root development for 2 weeks, followed by 5 weeks acclimation in the growth chamber and greenhouse. The plantlet survival was 94.4% after aeration in vermiculite for 2 weeks, which exceeded 87.7% of plantlets from non-aerated vermiculite, 42.2% from aerated agar, and 35.5% from non-aerated agar (Figure 3A). Plantlet survival from individual shoots under aerated conditions was significantly higher than under non-aerated conditions. The best rooting occurred after aeration in vermiculite for 3 weeks (Figure 2); this treatment yielded the best root numbers and root lengths (Table 1). However, the best

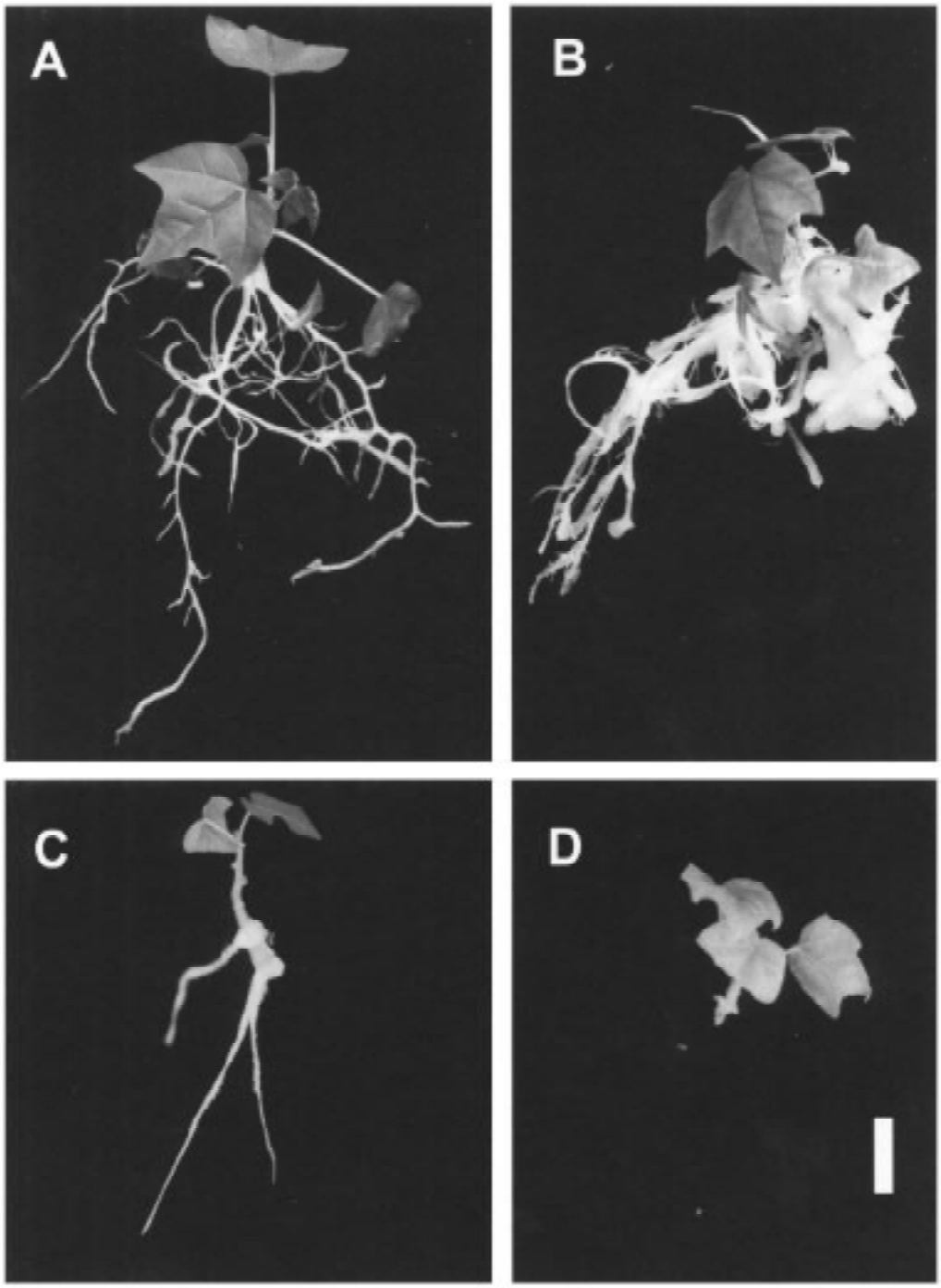

Figure 4. Root development and plantlet establishment under different conditions after 2.5 μM IBA treatment for 1 week. (*A*) a shoot with vigorous green leaves and primary roots with many lateral roots after culturing for 2 weeks in aerated vermiculite medium containing 1/2 MS medium. (*B*) a shoot with chlorotic leaves and many thick and stumpy roots after culturing for 2 weeks in agar medium containing 1/2 MS medium. (*C*) individual shoot of papaya cultured in vermiculite wetted with sterile distilled water for 2 weeks developed only primary roots. (*D*) root development of individual papaya shoots in agar with sterile distilled water. Bar=1 cm.

Figure 5. Plantlets, which can be planted in the field, grow vigorously after 2 weeks greenhouse acclimation.

Table 2. Effects of different MS concentrations (50 ml solution mixed with 100 ml vermiculite) on the percentage, number and length of roots of individual papaya shoots transferred to the root development medium under aerated conditions for 2 weeks after root induction

MS concentration	Root percentage (%)		Root number[1]		Root length[2] (cm)	
	vermiculite	agar	vermiculite	agar	vermiculite	agar
0	75.6 c[3]	17.8 d	2.2 b	1.7 c	3.6 b	1.5 c
1/4	88.9 b	42.2 c	5.6 a	2.2 b	3.2 b	3.9 a
1/2	94.4 a	71.1 a	4.9 b	4.1 a	5.4 a	3.0 b
1	77.8 c	52.2 b	4.3 c	2.1 b	2.5 c	3.7 a

[1]Number of primary roots.
[2]Mean of the greatest root length.
[3]Data in each column followed by the same letter are not significantly different according to Duncan's multiple range test (p=0.05). n=30.

plant survival occurred after aeration in vermiculite for 2 weeks (Figure 3A). The shoots from 3 weeks aeration in vermiculite had long roots which were easily damaged and tended to decline during acclimation.

Plantlet survival in MS medium at different strengths after 5 weeks acclimation are shown in Figure 3B. The best plantlet survival (94.4%) was significantly increased in aerated vermiculite containing 1/2 MS medium compared to the other treatments. The shoots in vermiculite containing 1/4 MS medium had more roots than in 1/2 MS medium, but the roots were slender and shorter, and easily damaged during hardening.

In general, large green leaves, new leaves and vigorous roots developed (Figure 4A) from the shoots expand to aerated vermiculite medium containing 1/2 MS medium for 2 weeks, while in the agar treatment there were small yellow leaves and thick, short stumpy roots (Figure 4B). Production of small yellow leaves and stumpy roots from individual papaya shoots in the agar rooting system have been described by other investigators (Drew, 1987; Katoaka and Inoue, 1987; Drew and Miller, 1989; Drew et al., 1993; Teo and Chan, 1994). It has also been observed in genera such as *Celis, Quercus, Magnolia* (McCown, 1988) and many of the conifers (Mohammed and Vidaver, 1988). Such roots are difficult to handle during transfer and the plantlets are difficult to establish (Drew, 1987; Drew and Miller, 1989). Rooting occurred when shoots were cultured in aerated vermiculite wetted with water, lateral roots developed from the primary roots, leaves were small and gradually became yellow (Figure 4C), and more time was needed for acclimatization. The plantlets declined with time. The individual

shoots in the aerated agar medium containing only water showed a rooting percentage of 17.8% (Table 2) and most shoots were stunted with chlorotic leaves (Figure 4D).

Drew et al. (1993) reported that a high proportion of shoots produced roots in the rooting medium with riboflavin, and high survival rate acclimatization was also reported (Drew, 1988; Manshardt and Drew, 1998). Nevertheless, an efficient and low cost micropropagation protocol for high quality roots is essential for large-scale micropropagation of papaya. In this investigation, we demonstrated that root initiation in agar medium with a low IBA concentration, followed by root development in vermiculite with 1/2 MS medium under aeration provided a highly efficient rooting system for *in vitro* micropropagation of papaya. Rooting in vermiculite *in vitro* was also effective for *Haworthia* spp. (Rogers, 1993) and *Castanea dentata* (Sanchez et al., 1997). Plantlets grow normally and vigorously after greenhouse acclimation (Figure 5) and transplanted well.

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