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Research note

Enhancement of *in vitro* growth of papaya multishoots by aeration

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Abstract

Efficient micropropagation of papaya (*Carica papaya* L.) has become crucial for multiplication of specific sex types of papaya or transgenic lines resistant to virus infection. In this study, aeration at different intervals with a 0.02 μm filter disc in the closure of culture flasks ensured exchange of gas components. The effect of aeration on development of multibuds to multishoots was investigated. Multibuds grown in culture flasks after one-week without aeration followed by a two-week aeration treatment caused a 41% increase in the number of shoots $\geq$0.5 cm, 42% increase in leaf expansion, and 17% increase in leaf numbers in comparison with unaerated materials. Ethylene and oxygen concentrations in the culture flasks were measured by gas chromatography and oxygen electrode at weekly intervals during the culture period. Oxygen concentrations were slightly different between aerated and unaerated culture flasks. Ethylene in the unaerated flask reached the highest level (0.11 ppm) 2 weeks after the treatment, while accumulation of ethylene in the aerated flasks was not detected. The multishoots grown for 3 weeks without aeration showed growth retardation on leaves and epinasty on petioles.

Abbreviations: PP – polypropylene; PRSV – papaya ringspot virus

Production of papaya (*Carica papaya* L.), an economically important fruit crop in the tropics and subtropics, is limited by the serious disease caused by papaya ringspot virus (PRSV) and only tolerant cultivars have been obtained by conventional breeding (Conover et al., 1986). This problem may be solved by the use of plant genetic engineering techniques. The gene transfer systems for producing transgenic papaya have been described in several reports (Cheng et al., 1996; Fitch et al., 1990; Yang et al., 1996). To propagate and maintain transgenic papaya lines for field evaluation or commercial application tissue culture techniques are crucial. Moreover, hermaphroditic or female fruits (of dioecious types) of papaya are more commercially desirable. Thus, an efficient micropropagation method to preserve the specific sex type of papaya with uniform horticultural properties is essential.

Plants established from shoot tips have been shown to preserve the integrity of the parental genotype (Drew, 1996), whereas those regenerated via callus culture often demonstrate considerable instability. The improvement of quantity and quality of shoots propagated *in vitro* is essential for papaya micropropagation because it increases the survival rates of plantlets. In early studies, sex type, season of explanting, source of explants, and plant growth regulator components in the medium were studied, but the propagation rates were generally low. Growth and development of plants *in vitro* not only depend on the composition of the culture medium, but also on the composition of the gaseous atmosphere. In order to manipulate the gaseous conditions, many different types of vessels and closures were used and their effects on differentiation of shoot buds (Kumar et al., 1987), growth of shoots, root formation, leaf expansion and flowering have been reported.

In this study, aeration treatments using closures with microfilter discs to allow gas exchange in the

culture flasks were applied at different culture intervals, and the effects on papaya shoot propagation were investigated. The results indicated that a particular combination of one-week nonaeration and two-week aeration improves the yield and quality of shoots that can be used for an efficient *in vitro* propagation of papaya.

In vitro propagated multishoots of papaya (*Carica papaya* L. cv. Tainung No. 2) were established from axillary buds of field-grown hermaphroditic trees (Yang and Ye, 1992). The MSNB medium for multibud culture of papaya consists of MS salts (Murashige and Skoog, 1962), 0.02 mg l^{-1} NAA, 0.2 mg l^{-1} BA, 3% sucrose and 1% agar. The pH of the medium was adjusted to 5.7 $\pm$ 0.1 with 1 N KOH before autoclaving at 1.1 kg cm^{-2} and 121 °C for 20 min. The four multibud clusters (buds <0.5 cm in length; size: 0.5 $\times$ 0.5 cm^2) were grown in 250 ml flasks containing 50 ml MSNB medium for 3 weeks to form multishoot clusters. All cultures were incubated in a growth chamber at 28 $\pm$ 1°C with cool white fluorescent lamps which provided a 14-h photosynthetic active radiation of 53 μmol m^{-2} s^{-1}.

For the nonaerated treatment the flasks were sealed with one inner layer of Sun Cap closure (0.02 μm filter disc; Sigma Co.) and two outer layers of polypropylene (PP) membrane which caused gases to accumulate in the flasks. For aeration the PP membranes were removed at 0, 1, 2 and 3-week intervals after culture (Figure 1).

Ethylene concentration in culture flasks was measured by gas chromatography at 0, 1, 2, 3 and 4-week intervals. One ml gas sample was removed with a hypodermic syringe and injected into a Shimadzu GC-14A equipped with an aluminum oxide column and a flame ionization detector. Column temperature was set at 60 °C and pure N_2 was used as the carrier gas. The peak areas were measured by a Hitachi D-2500 Chromato-Integrater. A standard linear curve was established by diluting 1000 ppm ethylene into 6 concentrations (0, 0.2, 0.4, 0.6, 0.8, 1.0 ppm).

Concentrations of oxygen at 0, 1, 2, 3 and 4-week intervals were measured in a leaf-disc oxygen electrode chamber (Hansatech, King's Lynn, UK). Samples were taken at 10 h after the onset of the photoperiod. A standard linear curve (millivoltage signals vs. oxygen concentrations) was established by diluting ambient air with N_2 into 5 oxygen concentrations (0, 2, 4, 6, 8 μmol ml^{-1}) and measuring each millivoltage signal for the corresponding concentration.

Ventilation of aerated or nonaerated flasks without culture with 4 ppm ethylene or pure nitrogen was determined by measuring the leakage rates of ethylene and the inflow rates of oxygen via gas chromatography or oxygen electrode, respectively, at 0, 1, 2, 4, 8, 16, 32, 64 and 128-h intervals, as described above.

After culturing at different intervals for all different combinations of treatments, the shoot ($\geq$0.5 cm in length) and leaf numbers were measured for assessing the efficiency of multishoot formation. Dimension of leaf lamina and length of petiole of the largest leaf of the individual shoot were recorded to evaluate the quality of multishoots.

The leakage rates of ethylene and oxygen in the empty flasks covered with Sun caps or polypropylene (PP) seals are shown in Figure 2. The ethylene in the aerated flasks declined rapidly to half (t_{50}) 50 min after aeration and became undetectable 4 h after aeration, while the t_{50} value of the unaerated flasks was retained for up to 3 days (Figure 2A). On the other hand, ambient oxygen diffused quickly into the aerated flasks to replace pure nitrogen and the t_{50} value was less than one hour and reached the ambient concentration 4 h later, whereas the concentration of oxygen under nonaerated conditions required 64 h to balance with ambient air (Figure 2B). These results indicated that the Sun cap seals were able to efficiently ventilate the flasks and PP membranes effectively prevented the leakage of ethylene. Furthermore, our results indicated the Sun cap sealed flasks have a shorter t_{50} (50 min) for removing ethylene than the loosely sealed vessels (305 ml polycarbonate boxes covered with polypropylene lids, t_{50} = 1–2 h) investigated by Jackson et al. (1991). Thus, our ventilating design for balancing ethylene and oxygen concentrations between the culture flask and ambient air was used for the aerated and nonaerated experiments.

Figure 3 demonstrates ethylene accumulation in the nonaerated or aerated flasks during a multiplication cycle. There was no detectable ethylene in the flasks when the multibud clusters were subcultured. Ethylene was not detected in the aerated flasks, neither in the empty flasks and the ambient air (Figure 2A). In nonaerated flasks the various accumulations of ethylene during different culture periods (Figure 3) may depend on variable ethylene biosynthesis under a constant low leakage rate (Figure 2A).

Oxygen concentration in the flasks under different aeration treatments was also measured. At week 2, the oxygen concentration in the nonaerated flasks (C_{0-2}) was significantly lower (6%) than in empty flasks (B_{0-2}). Depletion of oxygen in unaerated flasks was

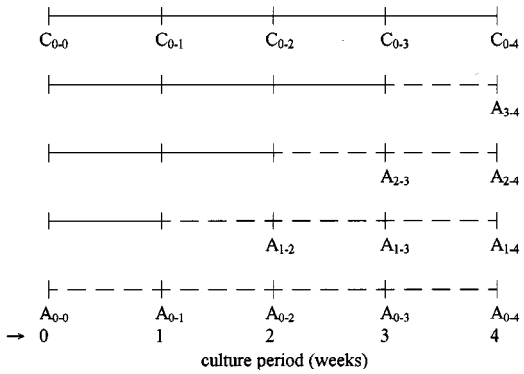

Figure 1. Aeration of papaya multibuds cultured *in vitro*. The culture flasks were sealed with one inner layer of Sun cap membrane (0.02 μm filter, Sigma Co.) and two outer layers of polypropylene. The outer polypropylene layers were removed for gas exchange. There were 4 multibud clusters (size: 0.5 × 0.5 cm^2) growing in each 250 ml flask. A (- - - - - - -), aeration treatment. C (– – – –), nonaeration control. A_{0-1}, time interval at 1 week aeration treatment. C_{0-1}, time interval at 1 week nonaeration treatment.

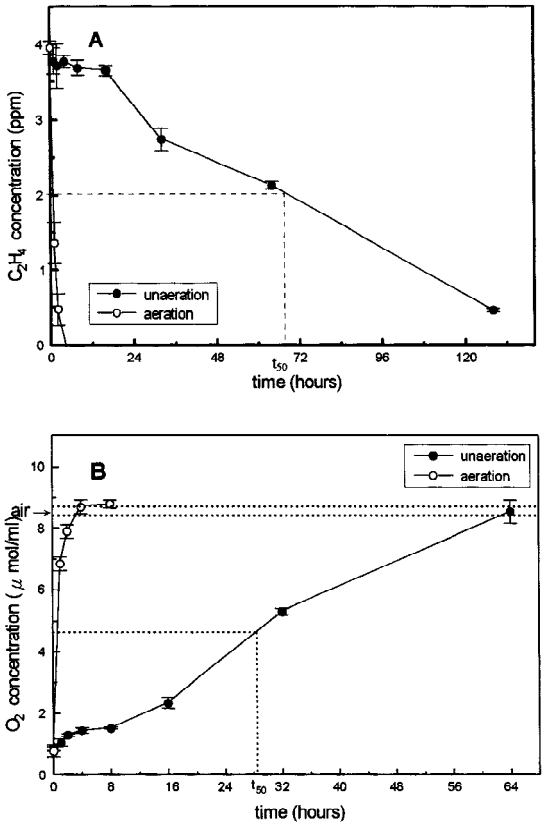

Figure 2. Ventilation of the sealed flask with the ambient air. Ventilation of the aerated and the 3 nonaerated flasks with 4 ppm ethylene or pure nitrogen was determined by measuring leakage rates of ethylene and inflow rates of oxygen via gas chromatography or oxygen electrode at 0, 1, 2, 4, 8, 16, 32, 64 and 128-h intervals. *A*, the leakage rate of ethylene in flasks originally filled with 4 ppm ethylene. *B*, the inflow rate of oxygen in flasks originally filled with pure nitrogen. (●), PP seals (unaeration). (◯), aeration seals. Bars = ±S.D. (n = 3).

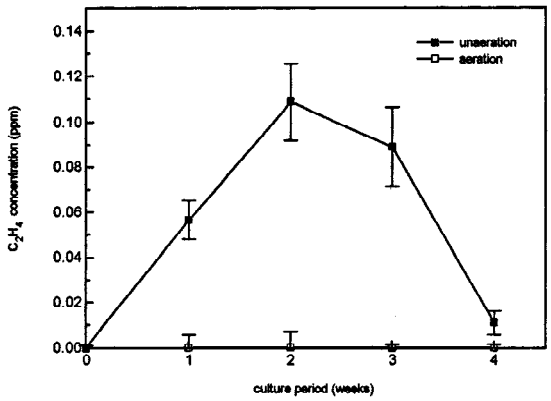

Figure 3. Ethylene accumulated in the nonaerated and aerated flasks at various periods of the papaya multibud culture. (■): nonaeration. (□): aeration. Bars = ±S.D. (n = 3).

much less than in previous studies, ca. half amount of oxygen remained at day 23 for *Ficus lyrata* shoots cultured in vessels tightly sealed with 2 layers of Nescofilm (Jackson et al., 1991). The net respiration of multibuds growing in nonaerated controls at week 2 might be the highest for the depletion of oxygen. After 1, 2 and 4 weeks, the concentration of oxygen in the aerated flasks was higher than that in the nonaerated controls, with the highest (108% as the nonaerated control) in the A_{1-4} aeration treatment. This observation implied that the multibuds in the aerated flasks might have higher net photosynthesis rates.

Table 1 shows the effects of different treatments on shoot numbers of the multishoot clusters. The multibud clusters nonaerated for 1 week and then aerated (A_{1-n}) showed a significant increase in shoot numbers in comparison with other treatments at the same week intervals. The nonaerated treatment for 1 week followed by aeration for 2 weeks (A_{1-3}) had the highest number of 17.33 shoots, which was 41% more than that of the control (C_{0-3}).

Nonaeration for 1 week (the ethylene accumulated 0.06 ppm) followed by aeration (ethylene removed treatment; not detectable) could enhance shoot proliferation. This result was similar to shoot culture of *Pinus radiata* (Kumar et al., 1987). The number of shoots in most treatments gradually decreased after culturing for 4 weeks, due to senescence and apical dominance. Shoots with larger leaves and the light yellow leaves increased, especially in the A_{1-4} treatment.

The multishoot cluster of the control (C_{0-3}) exhibited epinasty symptoms and slightly hyperhydric leaves. Epinasty was similar to symptoms caused by ethylene in tomato and arabidopsis. More and larger leaves were observed in A_{1-3}. The increased number of shoots in A_{1-3} (17.33 shoots per flask) in comparison with A_{0-3} (11.50 shoots per flask) might to be due to ethylene accumulation within the first week which might promote the formation of new axillary buds. Kevers et al. (1992) showed that exogenous ethylene (5 ppm) provided during the first 7–14 days of the culture period caused an increase in number of *Rosa hybrida* shoots.

The A_{1-3} treatment (5.78 shoots per week) had the highest proliferation capacity. Shoots which possessed more large green leaves had higher rooting and plantlet survival rates after hardening (data not shown). Multishoots in the nonaerated treatment showed epinasty and abscission after 3 weeks and the symptoms became prominent after 6 weeks; however, multishoots with one-week nonaeration followed by 5-weeks aeration exhibited vigorous growth with larger green leaves (data not shown). These results indicated that aeration maintains vigor of papaya multishoots so that subculture intervals can be delayed for 3 weeks. Increased axillary bud proliferation ($\geq$0.5 cm in length) may be a physiological responses to aeration after one-week nonaeration. Ethylene may be needed at the early period of axillary bud initiation, but inhibits subsequent bud growth.

Ethylene accumulation in the headspace of the vessel used for papaya nodal culture resulted in poor growth and leaf senescence and addition of ethylene-suppressant significantly improved nodal culture growth (Magdalita et al., 1997). In this investigation, the favorable and unfavorable effects of ethylene on multishoot culture were described. Through a simple combination of nonaeration and aeration treat-

Table 1. Effect of aeration on quality of papaya multishoots at different weekly intervals under different combinations of nonaeration–aeration.

Treatment[1]	Av. shoot No.[2] /flask	Av. leaf No. /flask	Av. leaf length × width (cm^2)	Av. petiole length (cm)
C_{0-1}	3.67[3] (1.00)[4] a[5]	3.67 (1.00) b	1.12 (1.00) a	0.55 (1.00) a
A_{0-1}	6.00 (1.63) a	6.67 (1.82) a	1.04 (0.93) a	0.50 (0.92) a
C_{0-2}	7.33 (1.00) c	25.00 (1.00) b	1.18 (1.00) b	0.58 (1.00) a
A_{1-2}	13.00 (1.77) a	41.33 (1.65) a	1.67 (1.42) a	0.60 (1.03) a
A_{0-2}	10.33 (1.41) b	35.00 (1.40) ab	1.65 (1.40) a	0.52 (0.91) a
C_{0-3}	12.33 (1.00) b	63.33 (1.00) b	1.31 (1.00) b	0.60 (1.00) c
A_{2-3}	10.00 (0.81) b	32.67 (0.52) c	1.23 (0.94) b	0.55 (0.91) c
A_{1-3}	17.33 (1.41) a	74.33 (1.17) a	1.86 (1.42) a	0.70 (1.16) b
A_{0-3}	11.50 (0.93) b	37.00 (0.58) c	1.77 (1.35) a	0.76 (1.27) a
C_{0-4}	11.83 (1.00) bc	60.33 (1.00) b	1.71 (1.00) b	0.74 (1.00) ab
A_{3-4}	10.33 (0.87) c	71.67 (1.19) a	1.33 (0.78) c	0.60 (0.82) c
A_{2-4}	12.00 (1.01) b	65.67 (1.09) ab	1.67 (0.98) b	0.62 (0.84) c
A_{1-4}	15.33 (1.30) a	64.67 (1.07) ab	2.20 (1.28) a	0.81 (1.10) a
A_{0-4}	13.00 (1.10) bc	61.00 (1.01) b	2.00 (1.17) a	0.74 (1.00) ab

[1] See Figure 1.
[2] $\geq$ 0.5 cm shoots.
[3] Values represent mean from six replicates.
[4] Ratios to the nonaerated conditions for C_{0-1}, C_{0-2}, C_{0-3} and C_{0-4} as controls.
[5] Data in each column of the block followed by the same letter are not significantly different according to Duncan's multiple range test (p = 0.05).

ments, the maximum growth of the multishoots can be achieved.

Acknowledgements

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