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Enhancement of papaya axillary shoot proliferation in vitro by controlling the available ethylene

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Abstract. The timing of ethylene concentration favorable for in vitro proliferation of papaya (*Carica papaya* L.) multiple shoot clusters through outgrowth of axillary buds was investigated. Exogenous ethylene in culture flasks was regulated during the first week by using a gas diffusion equilibration procedure, which used a transparent box as an indirect space for the ethylene injection and flasks sealed with 0.02 μm filters for diffusion and equilibration. There was a 36% increase in shoot number and a 50% increase of leaf number by adding 0.2 or 0.4 ppm ethylene into the box the first week, followed by aerating the flasks the following two weeks. The level of endogenous ethylene was also modified by adding the ethylene biosynthesis precursor (ACC) and the inhibitors (AVG and $CoCl_2$) to the culture medium in the flasks under sealed conditions. The greatest enhancement rate of shoot number (75%) was achieved with 2 μM ACC. Shoot proliferation rates by applying 0.5 μM AVG and 5 μM $CoCl_2$ were enhanced by 23% and 49%, respectively. The relationships between shoot number and ethylene concentration during a three-week incubation period were analyzed to reveal the most favorable ethylene concentrations. Concentrations with the best proliferation were 0.34, 0.20 and 0.15 ppm for Weeks 1, 2 and 3, respectively. The results indicated that a relatively higher ethylene level during the early incubation period, followed by low levels subsequently might be most favorable for improved rates of papaya axillary shoot proliferation.

Keywords: ACC; AVG; *Carica papaya*; Cobalt chloride; Ethylene; Gas diffusion equilibration procedure.

Abbreviations: ACC, 1-Aminocyclopropane-1-carboxylic acid; **AVG,** Aminoethoxyvinylglycine; **BAP,** 6-Benzylaminopurine; **MACC,** 1-(Malonylamino) cyclopropane; **NAA,** α-Naphthaleneacetic acid; **PP,** Polypropylene.

Introduction

Papaya (*Carica papaya* L.) is an economically important fruit crop of the tropics and subtropics that is highly susceptible to the papaya ringspot virus (PRSV). Currently, the most promising method for overcoming plant viral diseases is genetic engineering. Production of PRSV resistant papaya plants via introduction of the PRSV coat protein gene has been reported (Cheng et al., 1996). What is needed now is a rapid clonal propagation protocol to increase the virus-resistant transgenic genotypes for commercial planting. Plant multiplication in tissue culture by axillary bud proliferation has proved suitable for papaya clonal multiplication (Litz and Conover, 1978). The earlier papaya micropropagation studies established the details with respect to sexual type selection, the season for explanting (Litz and Conover, 1981), source of explants, and hormone components of culture media (Litz and Conover, 1978; Drew, 1988). Nevertheless, the shoot proliferation rates have remained generally low.

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Plant growth and development in vitro is governed not only by the composition of the culture medium, but also by constituents of the culture vessel atmosphere, such as carbon dioxide, oxygen, and especially, the plant growth regulator, ethylene (Blazková et al., 1989; Buddendorf-Joosten and Woltering, 1994). The effects of ethylene on in vitro culture have been studied in relation to flowering (Blazková et al., 1989), rooting (Pérez-Bermúdez et al., 1985), and axillary shoot development (Nour and Thorpe, 1994). The ethylene levels were controlled by either supplementing the gas exogenously (Kevers et al., 1992) or by experimenting with various types of culture vessels and closures to control the accumulation of endogenously evolving ethylene (Blazková et al., 1989). Dimasi-Theriou and Economou (1995) found that the application of 0.1 ppm ethylene during the first two weeks of culture increased the number and length of peach axillary shoots, but that excessive ethylene supplements inhibited shoot growth. A similar finding was reported by Lai et al. (1998) for axillary plant proliferation of papaya. Compared to continuously unaerated cultures, a 41% increase in the number of new shoots was obtained when endogenous ethylene was permitted to accumulate in culture vessels to 0.06 ppm during the first week of culture, followed by aerating the vessels the subsequent two weeks.

In vitro cultures are also affected by the nutrient medium addenda of substances that regulate ethylene biosynthesis or action. The substances ordinarily used have been the biosynthesis precursor ACC (Van Dijck et al., 1988) and the biosynthesis inhibitors AVG and $CoCl_2$ (Gaspar et al., 1989). Promotive (Van Dijck et al., 1988; Panizza et al., 1993), as well as inhibitory, effects on papaya noded cultures have been reported (Magdalita et al., 1997). However, the correlations between effector-modified ethylene concentrations and shoot proliferation have not yet been elucidated.

In this investigation, we studied the effects of ethylene concentrations on papaya axillary shoot multiplication through modifying the ethylene concentrations by adding ethylene to the gas phase of culture vessels or by supplementing the culture medium with ethylene effectors.

Materials and Methods

Plant Material and Culture Conditions

In vitro cultures of papaya (*Carica papaya* L. cv. Tainung No. 2) shoots, derived from axillary buds of field-grown hermaphrodite trees, were established as described previously (Yang and Ye, 1992). Clusters composed of multiple buds were stripped of shoots that were ≥0.5 cm long and were used as explants. Four clusters, each 0.5 × 0.5 cm^2, were inoculated into each 250-ml culture flask containing 50 ml medium. The culture medium contained major and minor salts of MS (Murashige and Skoog, 1962), B_5 (Gamborg et al., 1968) vitamins, 3% sucrose, 1% agar, 0.02 mg l^{-1} NAA, and 0.2 mg l^{-1} BAP. Its pH was adjusted to 5.7 ± 0.1 before autoclaving. Cultures were incubated in a growth chamber at 28 ± 1°C and illuminated 14 h daily with cool white fluorescent lamps emitting 53 μE m^{-2} s^{-1}. Data on shoot number, leaf number, leaf length/width, and petiole length were taken after incubating 3 weeks and statistically analysed by Duncan's multiple range test.

Gas Diffusion Equilibration with Aerated Flasks

To regulate ethylene levels exogenously, aeratable or gas exchangeable culture flasks were assembled by sealing each of their mouths with a Sigma® Sun Cap Closure, composed of a 120 × 120 mm transparent membrane with a 6.0 mm diameter and a 0.02 μm pore-sized filter disc at the center. The disc enabled gas exchange and had a 50 min t_{50} ethylene leakage rate (Lai et al., 1998). The flasks containing explants were placed in a transparent rectangular box (35 × 22 × 22 cm^3), sealed to prevent leakage of ethylene. These boxes were made of transparent acryl and permitted light to penetrate them well (Figure 1). The illumination of the sealed box was set to 53 μE m^{-2} s^{-1}, detected by Lambda LI-185A quantum/radiometer/photometer. There were a inlet and a outlet sealed with a serum stopper for injection of ethylene and sampling. A gas mixture containing either 1,000 ppm ethylene or ambient air was injected into the box with a hypodermic syringe to adjust the ethylene concentration inside to 0, 0.1, 0.2, 0.4, 0.6 or 0.8 ppm via the ethylene diffusion equilibration during the first week of incubation. Each treatment of flasks were placed in the same box. The ethylene levels were monitored on the1st, 4th, and 7th days subsequently in order to check the stability of ethylene levels. Such levels fluctuated less than 0.06 ppm. After 7 days, the culture flasks were removed from the box and permitted gas exchange with the ambient air for the 2nd and 3rd weeks.

For unaerated controls, cultures in unaerated flasks with mouths sealed by two layers of polypropylene membranes were established, and their internal ethylene levels were measured at 7-day intervals during the 28-day incubation period. In addition, ethylene levels were measured at 3-day intervals during the first 15-day incubation period in order to get a more accurate comparison with the 7-day intervals.

Application of Ethylene Effectors

To investigate the effects of ethylene precursor and inhibitors, aqueous solutions of ACC, AVG, and $CoCl_2$ were filter sterilized and added to autoclaved media at 60°C. The concentrations employed for ACC and AVG were 0, 0.5, 1, 2, 4 and 8 μM, and those for $CoCl_2$ were 0.1, 1, 5, 10, 50 and 100 μM. To allow endogenous ethylene to accumulate, the culture flasks were sealed with two layers of polypropylene membranes. The membranes had a 3-day t_{50} ethylene leakage rate (Lai et al., 1998). The concentration of ethylene in flasks was measured weekly during the 3-week incubation period. Data were statistically analysed by Duncan's multiple range test.

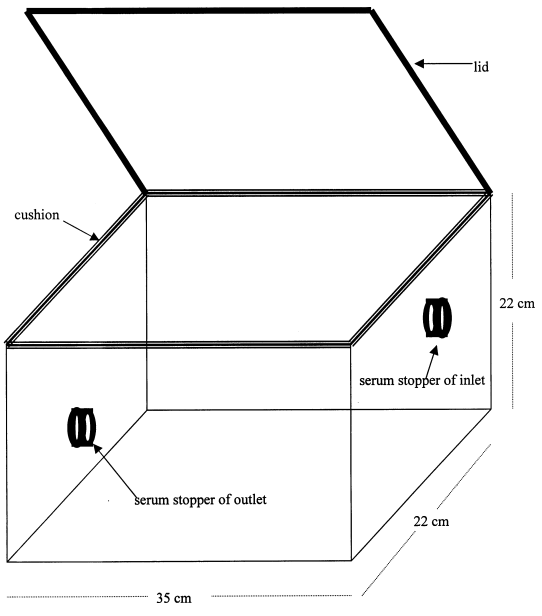

Figure 1. The diagram of the rectangular box (35 × 22 × 22 cm^3) that was applied to the gas diffusion equilibration procedure. It was made of transparent acryl and permitted light to penetrate well. The box was air-tight with a cushion and petroleum jelly at its lid. An inlet and outlet sealed with serum stopper were used for injecting and sampling ethylene.

Measurement of Ethylene Levels

The polypropylene membrane of the unaerated culture flask or the serum stopper of the sealed box in the gas diffusion equilibration procedure was penetrated by the pinpoint of a hypodermic syringe. A one ml gas sample was taken from the atmosphere of the flask bottleneck or the box 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. The standard linear curve (peak areas vs. ethylene concentrations) was established by diluting 1,000 ppm ethylene into six concentrations (0, 0.2, 0.4, 0.6, 0.8, 1.0 ppm) and then measuring each peak area for the corresponding concentration.

Results

Ethylene Accumulation in the Unaerated Control Flasks

Figure 2 shows the cubic calculation curves of ethylene accumulation and shoot proliferation in unaerated control flasks during the 15-day and 28-day incubation periods. With shorter 3-day measuring intervals, the ethylene accumulation peaked at about 0.19 ppm on the 12th day (11th - 12th day plateau), then declined subsequently. With longer 7-day measuring intervals, the ethylene accumulation peaked at about 0.11 ppm on the 15th day (12th - 17th day plateau). This ethylene peak is in the front of the peak of shoot proliferation that reached its maximum at about 14 shoots on the 22th day (21th - 22th day plateau).

Effects of Exogenous Ethylene Application

Table 1 presents the results of growth measurements of multiple shoot explants after a week of incubation with exogenous ethylene, followed by free gas exchange during subsequent weeks 2 and 3. The resultant multiple shoot clusters are shown in Figures 3A-C. Shoot and leaf numbers were significantly higher than the control at ethylene concentrations ranging from 0.1 to 0.4 ppm, with a maximum 36% increase in shoot number and 45% increase of leaf number in the 0.2 ppm ethylene treatment. There was no significant difference in shoot proliferation between the control and the higher ethylene concentrations of 0.6 and 0.8 ppm.

Effects of Ethylene Precursor ACC Application

Ethylene concentrations in the unaerated flasks containing ACC-supplemented media are presented in Table 2 and Figure 4A. The ACC supplemented flasks contained 4.1 to 7.7 times more ethylene than the ACC-free control flasks (0.075 ppm) after Week 1. During Weeks 2 and 3, only flasks supplemented with 4 and 8 μM ACC remained significantly higher than the control in ethylene levels. Data of shoot development are shown in Table 2. The corresponding shoots are shown in Figures 3D-F. The average numbers of shoots and leaves per flask of the cultures

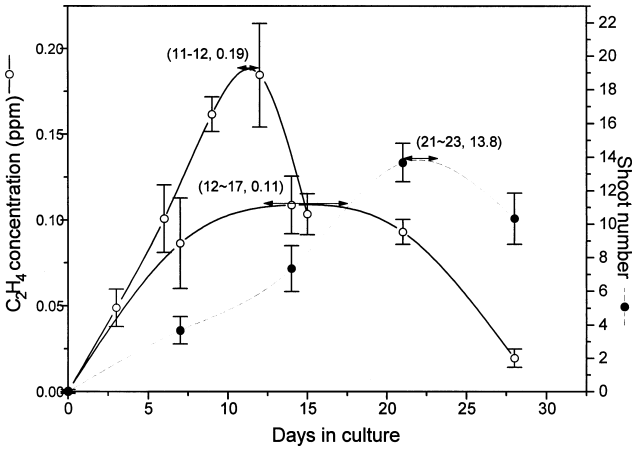

Figure 2. The ethylene accumulation pattern and the shoot proliferation in unaerated control flasks, with mouths sealed by two layers of polypropylene membranes during the 15- and 28-day incubations of papaya multiple shoots at 3- and 7-day measuring intervals. The coordinates display plateaus in parenthesis. All curves were smoothed by cubic calculation. Bars = ± S.D. (n=3)

Table 1. Effects of exogenous ethylene on papaya axillary shoot proliferation. Ethylene gas was introduced into the equilibration during Week 1. Flasks were then aerated during the subsequent 2 weeks.

C_2H_4 conc. (ppm)	Number of shoots[a] /Flask	Number of leaves /Flask	Leaf length × width (cm^2)	Petiole length (cm)
0	12.00 [b](1.00)[c] C[d]	65.33 (1.00) D	1.71 (1.00) A	0.63 (1.00) ABC
0.1	14.00 (1.17) B	86.00 (1.32) C	1.24 (0.72) B	0.60 (0.95) C
0.2	16.33 (1.36) A	94.67 (1.45) AB	1.25 (0.73) B	0.60 (0.96) BC
0.4	15.33 (1.28) AB	98.00 (1.50) AB	1.54 (0.90) A	0.66 (1.05) A
0.6	11.67 (0.97) C	73.00 (1.12) D	1.61 (0.94) A	0.65 (1.04) AB
0.8	12.00 (1.00) C	68.33 (1.05) D	1.65 (0.97) A	0.61 (0.97) ABC

[a]≥0.5 cm shoots.
[b]The values are means based on 3 flasks.
[c]The treatment of 0 ppm C_2H_4 is denoted by a value of 1.
[d]In each column data followed by the same letter are not significantly different according to Duncan's multiple range test (p = 0.05).

Table 2. Effects of ACC on papaya axillary shoot proliferation and ethylene evolution. ACC was added to the culture medium in unaerated flasks and cultures were incubated 3 weeks.

ACC conc. (μM)	Number of shoots[a]/Flask	Number of leaves/Flask	Leaf length×width (cm^2)	Petiole length (cm)	Ethylene concentration (ppm)		
					Week 1	Week 2	Week 3
0	13.60 [b](1.00)[c] D[d]	83.40 (1.00) D	1.28 (1.00) B	0.64 (1.00) D	0.073 (1.00) D	0.152 (1.00) CD	0.089 (1.00) CD
0.5	16.80 (1.24) C	112.80 (1.35) B	1.22 (0.95) B	0.72 (1.11) B	0.313 (4.27) C	0.130 (0.86) D	0.084 (0.95) CD
1	19.40 (1.43) B	115.80 (1.39) B	1.40 (1.09) A	0.67 (1.05) C	0.373 (5.08) B	0.132 (0.87) D	0.062 (0.70) D
2	23.80 (1.75) A	147.80 (1.77) A	1.40 (1.09) A	0.76 (1.18) A	0.402 (5.48) B	0.170 (1.12) C	0.114 (1.29) C
4	17.20 (1.26) C	94.20 (1.13) C	1.09 (0.85) C	0.65 (1.02) CD	0.314 (4.28) C	0.283 (1.87) B	0.210 (2.37) B
8	12.20 (0.90) D	68.60 (0.82) E	0.87 (0.68) D	0.50 (0.77) E	0.577 (7.86) A	0.358 (2.36) A	0.267 (3.02) A

[a]≥0.5 cm shoots.
[b]The values are means based on 5 flasks.
[c]The treatment of 0 μM ACC is denoted by 1.
[d]Data in each column followed by the same letter are not significantly different according to Duncan's multiple range test (p = 0.05).

Table 3. Effects of AVG on papaya axillary shoot proliferation and ethylene evolution. AVG was added to the culture medium in unaerated flasks and cultures were incubated 3 weeks.

AVG conc. (μM)	Number of shoots[a]/Flask	Number of leaves/Flask	Leaf length×width (cm^2)	Petiole length (cm)	Ethylene concentration (ppm)		
					Week 1	Week 2	Week 3
0	12.40 [b](1.00)[c] B[d]	67.20 (1.00) AB	1.01 (1.00) C	0.65 (1.00) B	0.076 (1.00) A	0.106 (1.00) A	0.099 (1.00) A
0.5	15.20 (1.23) A	69.80 (1.04) A	1.12 (1.12) BC	0.68 (1.05) B	0.078 (1.01) A	0.057 (0.54) B	0.049 (0.49) B
1	13.00 (1.05) B	63.80 (0.95) AB	1.00 (0.99) C	0.69 (1.07) B	0.050 (0.65) B	0.032 (0.30) C	0.023 (0.23) C
2	12.20 (0.98) B	59.40 (0.88) BC	1.37 (1.36) A	0.76 (1.17) A	0.035 (0.45) C	0.032 (0.30) C	0.016 (0.16) D
4	10.80 (0.87) C	55.20 (0.82) C	1.22 (1.21) B	0.81 (1.25) A	0.015 (0.20) D	0.012 (0.11) D	0.007 (0.07) E
8	7.80 (0.63) D	40.80 (0.61) D	1.15 (1.14) B	0.68 (1.06) B	0.011 (0.14) D	0.001 (0.01) E	0.000 (0.00) F

[a]≥0.5 cm shoots.
[b]The values are means based on 5 flasks.
[c]1 denotes treatment with 0 μM AVG.
[d]Data in each column followed by the same letter are not significantly different according to Duncan's multiple range test (p = 0.05).

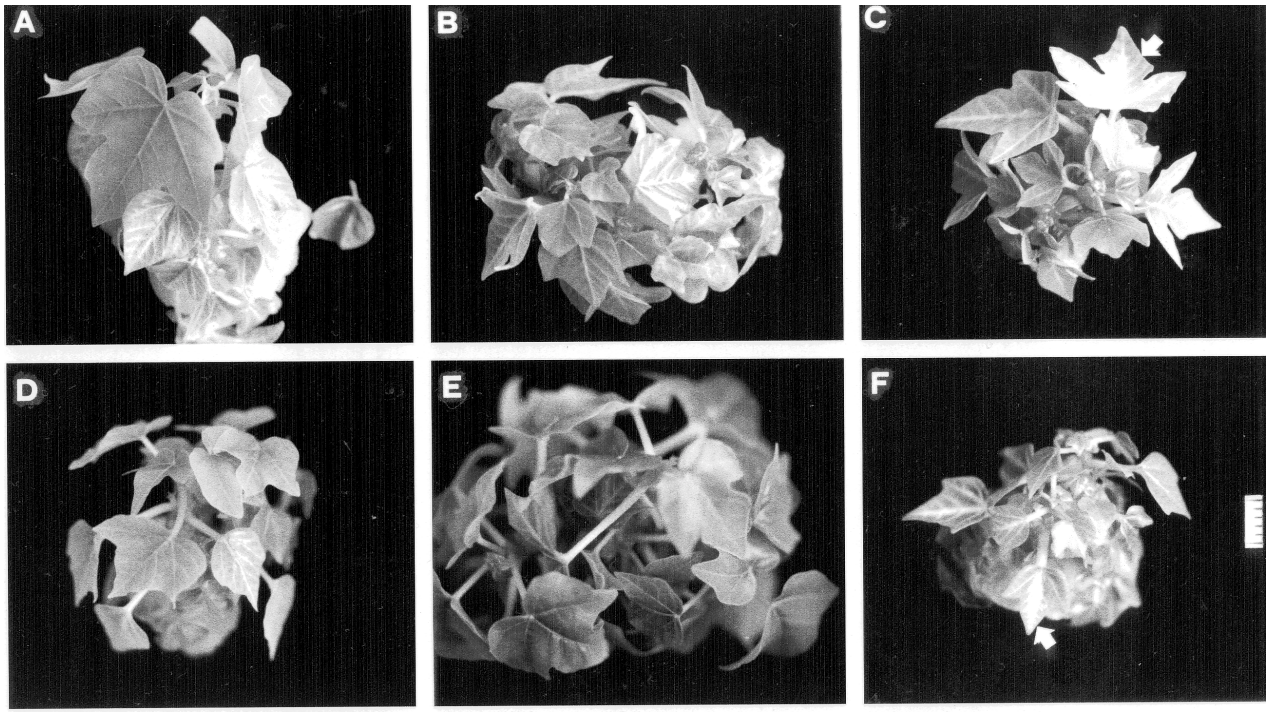

Figure 3. Papaya multiple shoot clusters developed from multiple bud explants after a 3-week incubation. Bar = 0.5 cm. A-C, Gas diffusion equilibration procedure were used to supplement 0 (A), 0.2 (B) and 0.6 ppm (C) of exogenous ethylene gas during Week 1 of culture. The flasks were aerated during the subsequent weeks, 2 and 3. D-F, Ethylene precursor ACC at 0 (D), 2 (E) and 8 μM (F) was added to culture media in the unaerated flasks. Arrows indicate the epinasty and vitrified leaves.

supplemented with 0.5 to 4.0 µM ACC were significantly higher than those of the unsupplemented controls. The maximum enhancements of shoot and leaf number by the 2 µM ACC addendum were 75% and 77%, respectively. Since the supplement of 0.5 to 4.0 µM ACC generated ethylene at levels of 0.3 and 0.4 ppm that were promotive of shoot development (see Table 1), these data are consistent with those observed for the effects of ethylene application.

Effects of Inhibitors of Ethylene Biosynthesis AVG and $CoCl_2$

In general, the AVG treatments reduced ethylene accumulation in culture vessels throughout the 3-week experimental period. Greater reductions were associated with higher concentrations of AVG (Figure 4B), the only exception being the 0.5 µM AVG treatment. This concentration of AVG did not reduce ethylene to levels below the control level in Week 1 and was correlated with a significant 23% increase in shoot number (Table 3 and Figures 5A, B). At high concentrations of AVG, 4 and 8 µM, shoot and leaf numbers decreased (Table 3) and leaves displayed chlorosis (Figure 5C).

Low $CoCl_2$ addenda, 10 µM and below, appeared to stimulate ethylene accumulation during the initial week of culture. But the stimulation disappeared during subsequent weeks, and the suppression resulted from a $CoCl_2$ concentration as low as 5 µM. Concentrations of $CoCl_2$ above 50 µM suppressed ethylene accumulation throughout the three-week experimental period (Figure 4C). The enhanced ethylene accumulation by low $CoCl_2$ concentrations, especially 5 µM during Week 1, caused significant increases in numbers of shoots and leaves, i.e. 20 and 116 per flask, respectively (Table 4 and Figure 5E).

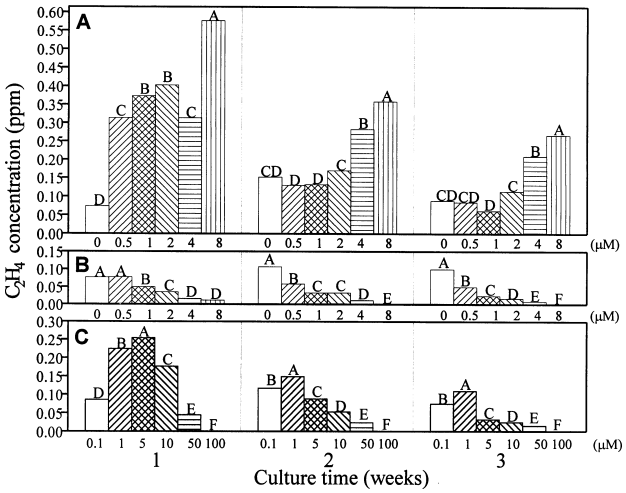

Figure 4. Effects of supplementing ethylene effectors on ethylene accumulation during the 3-week incubation periods of papaya multiple bud culture. ACC (A), AVG (B) and $CoCl_2$ (C) at various concentrations were added to the culture media using unaerated culture flasks. Ethylene concentration columns, labeled by different letters in the same block, differ significantly according to Duncan's multiple range test (p=0.05; n=5).

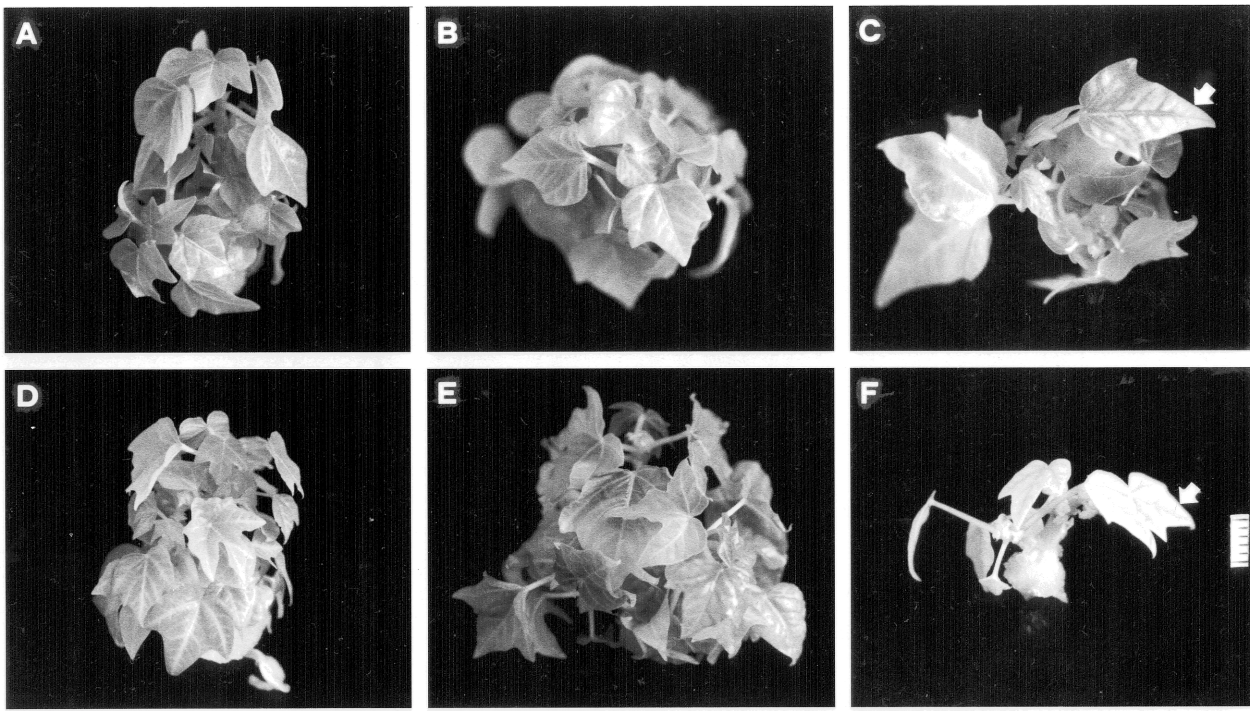

Figure 5. Effects of ethylene inhibitors on papaya multiple shoot development after the multiple bud explants were incubated in unaerated flasks for three weeks. Bar = 0.5 cm. A-C, Media were supplemented with 0 (A), 0.5 (B) and 8 µM (C) AVG. Note leaf chlorosis (arrow) in (C). D-F, Media were supplemented with 0.1 (D), 5 (E) and 50 µM (F) $CoCl_2$. Note the yellowing of leaves (arrow) in (F).

Correlation Between Ethylene Concentration and Shoot Proliferation

An analysis of the correlation between ethylene concentration and shoot number in the gas equilibrated experiment showed that a controlled ethylene concentration of 0.28 ppm during Week 1 resulted in a maximum of 17 shoots per flask (Figure 6A). To determine whether the enhancement of shoot numbers (up to 20 shoots per flask) of the ethylene-effector experiments was ethylene concentration associated, and to determine accurately the favorable ethylene profile during various stages of the 3-week incubation period, polynomial regression analyses were performed on the cumulative data of the three ethylene effector experiments (Figures 6B-D). In contrast to the exogenously regulated ethylene of the gas equilibrated procedure, the concentrations of endogenous ethylene influenced by the different ethylene effectors were limited to narrow ranges (Figure 4). The regression equations showed a significant association between ethylene concentration and average shoot number per culture flask. The coefficients for weeks 1, 2 and 3 were 0.82, 0.77 and 0.68, respectively, and these were significant at the 0.001 or 0.01 level. Based on polynomial regression analysis, the levels of available ethylene during Weeks 1, 2 and 3 of incubation for the best proliferation were determined to be 0.34, 0.20 and 0.15 ppm, respectively. In this ethylene concentration range, a projected maximum of 19 shoots per flask can be expected.

Variation of Ethylene Concentrations Resulted from Different Culture Periods

The variation of ethylene concentration at different culture times were analysed by gas chromatography. The results are summaryed in Figure 7 polynomial regression analyses indicated that a significant higher concentration (P>0.01) of controlled ethylene was found at Week 1 compared to Weeks 2 and 3 (Figure 7A). The ethylene levels of ACC effector induced in the culture medium experiments during Week 1 of incubation were also found significant (P>0.05) higher than Weeks 2 and 3 (Figure 7B), but less

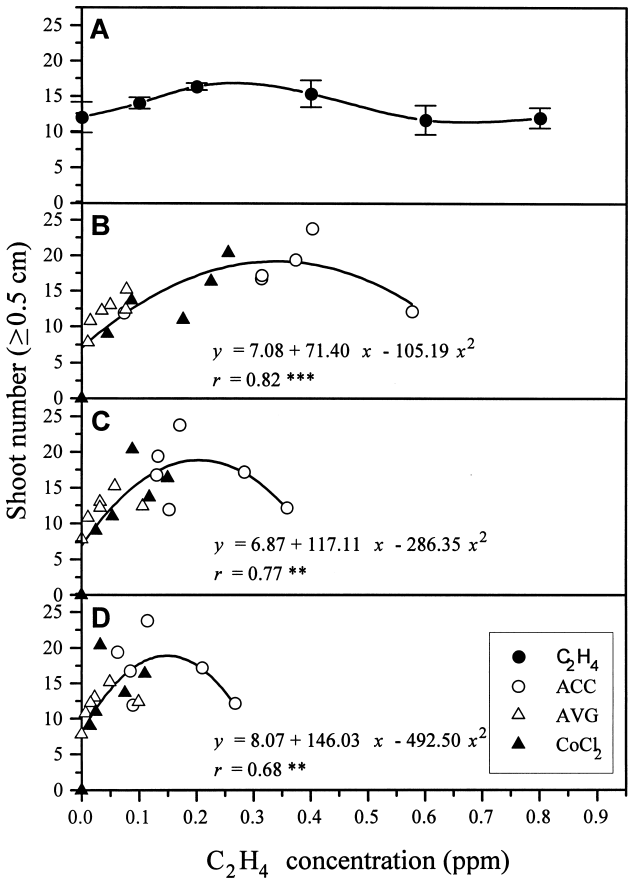

Figure 6. Correlation between ethylene concentrations and shoot numbers at the end of 3-week incubation of the papaya multiple shoot explants. A, Ethylene gas applied exogenously during Week 1 using gas diffusion equilibration procedure. Flasks were aerated during Weeks 2 and 3. Points represent the mean of three replicates with S.D. as the vertical bars. Curve was smoothed by cubic calculation. B-D, Polynomial regression curves of the combined data from the three tested ethylene effectors at Week 1 (B), Week 2 (C) and Week 3 (D). Each point represents the mean of three to five replicates. *** and **: Significant at 0.001 and 0.01 levels, respectively.

Table 4. Effects of $CoCl_2$ on papaya axillary shoot proliferation and ethylene evolution. $CoCl_2$ was added to the culture medium in unaerated flasks and cultures were incubated 3 weeks.

$CoCl_2$ conc. (μM)	Number of shoots[a]/Flask	Number of leaves/Flask	Leaf length×width (cm^2)	Petiole length (cm)	Ethylene concentration (ppm) Week 1	Week 2	Week 3
0.1	13.67 [b](1.00)[c] C[d]	81.33 (1.00) B	1.22 (1.00) AB	0.64 (1.00) D	0.086 (1.00) D	0.118 (1.00) B	0.075 (1.00) B
1	16.33 (1.19) B	107.67 (1.32) A	1.11 (0.91) AB	0.73 (1.14) CD	0.225 (2.60) B	0.150 (1.27)A	0.110 (1.46) A
5	20.33 (1.49) A	116.00 (1.43) A	0.99 (0.81) B	0.82 (1.28) BC	0.225 (2.95) A	0.088 (0.75) C	0.033 (0.43) C
10	11.00 (0.80) D	71.00 (0.87) B	1.35 (1.10) A	0.87 (1.35) B	0.177 (2.04) C	0.053 (0.46) D	0.025 (0.33) D
50	9.00 (0.66) D	47.33 (0.58) C	1.14 (0.94) AB	1.00 (1.55) A	0.045 (0.52) E	0.024 (0.21) E	0.015 (0.21) E
100	0.00 (0.00) E	0.00 (0.00) D	0.00 (0.00) C	0.00 (0.00) E	0.000 (0.00) F	0.000 (0.00) F	0.000 (0.00) F

[a]≥0.5 cm shoots.
[b]The values are means based on 3 flasks.
[c]The treatment with 0.1 μM $CoCl_2$ equals 1.
[d]Data in each column followed by the same letter are not significantly different according to Duncan's multiple range test (p = 0.05).

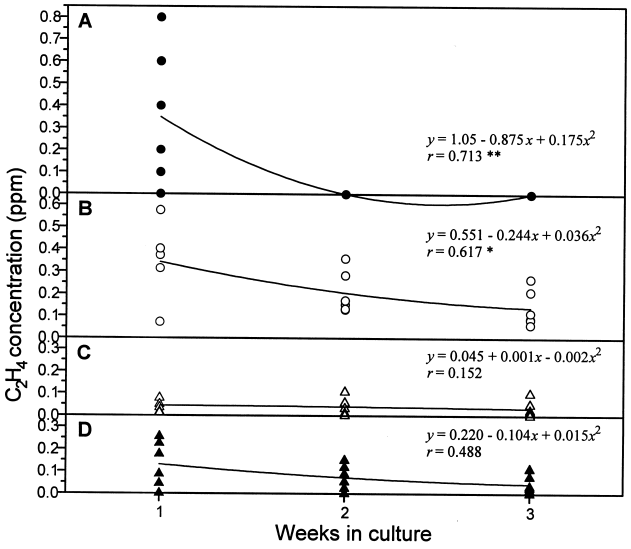

Figure 7. Variation of ethylene concentrations from different culture periods of the papaya multiple shoot explants. Polynomial regression curves of the data from ethylene gas applied exogenously during Week 1 using gas diffusion equilibration procedure (A) and the addition of different concentrations of ACC (B), AVG (C), and $CoCl_2$ (D) under sealed conditions. Each point represents the mean of three to five replicates from each condition of the effectors which are described in Figure 4. ** and *: Significant at 0.01 and 0.05 levels, respectively.

variable than the ethylene treated one (Figure 7A). However, the ethylene levels from the treatments of the AVG and $CoCl_2$ effectors resulted in nonsignificant changes in Weeks 1, 2 and 3 (Figures 7C, D).

Discussion

Methods using various types of culture vessels and closures were reported previously (Nour and Thorpe, 1994). The limitations of these methods are that ethylene accumulation can only attain a limited level, as disclosed in this study (Figure 2). Higher concentrations of ethylene have been achievable by injecting ethylene into culture vessels (Pérez-Bermúdez et al., 1985; Kevers et al., 1992; Dimasi-Theriou and Economou, 1995). However, microbial contaminations are frequently associated with such injections. In this report, we have employed a gas diffusion and equilibration procedure to regulate the ethylene concentration in the culture vessels over a potentially unlimited range, without contamination problems. This method permitted us to increase shoot production by enabling a higher ethylene concentration than is possible in sealed, air-tight vessels. The finding of an average shoot number of 12 in the 0 ppm ethylene control was in agreement with that of a 3-week continuously aerated treatment of our earlier study (Lai et al., 1998). This method can potentially regulate the ethylene level within a wide concentration range that the effector application methods can not accomplish.

ACC application in an appropriate concentration range also enhanced the proliferation of shoots. Similar results were obtained with *Aechmea victoriana* (Van Dijck et al., 1988) and *Lavandula officinalis* (Panizza et al., 1993) using different types of explants. ACC application in the appropriate concentration range resulted in ethylene accumulation during Week 1 to levels that corresponded to the shoot development stimulating concentrations of 0.3-0.4 ppm, attained by the gas diffusion equilibration procedure. However, the mechanism underlying the enhanced shoot proliferation by such an ethylene concentration range remains undetermined.

Magdalita et al. (1997) previously reported an improvement of shoot number in nodal cultures of papaya with 500-1,500 µM ACC supplements. We obtained enhanced papaya shoot numbers by using substantially lower concentrations, 0.5-4 µM, of ACC. When ACC concentrations higher than 4 µM were used, we observed a continuously high ethylene accumulation during Weeks 2 and 3. This caused undesirable leaf epinasty and vitrified shoots, as well as reductions in shoot and leaf numbers. The discrepancy between our finding and those of Magdalita et al. (1997) may have been due to different genotypic responses—or to the nodal explants used by Magdalita et al., which were possibly less sensitive to ethylene than our in vitro derived multiple bud explants. In this study, a low concentration (0.5-4 µM) of ACC stimulated ethylene evolution only during the early culture period. Also, polynomial regression analyses showed that ethylene level with ACC treatments varied with culture period but did not vary significantly with either AVG or $CoCl_2$. These results indicate that AVG and $CoCl_2$ have a more persistent effect on papaya multiple buds than ACC. This supports the findings of Kvaalen (1994), who used embryo tissues of the Norway spruce. According to Philosoph-Hadas et al. (1985), the reduced ethylene accumulation during Week 2 and 3, associated with low ACC applications, might be due to exhaustion of ACC through malonylation to MACC.

A higher ethylene concentration was observed in the ethylene effector experiments than in the gas infusion experiment. This caused greater enhancement of shoot proliferation in the former instance. Since the ethylene concentrations were determined by sampling the gas from the atmosphere surrounding the flasks, the actual ethylene levels within the target tissues were probably different from measured values for the two experimental conditions. Furthermore, in the effector experiments, ethylene was produced endogenously by the target tissues, where the morphogenesis was triggered directly by the gas before it had been released into the atmosphere surrounding the flasks and made available for concentration determinations. On the other hand, in the diffusion equilibration experiments, ethylene was introduced into the atmosphere bathing the flasks first and was, thus, available for concentration determination before it had diffused to target tissues to trigger morphogenetic actions. Thus, the time-course of ethylene action in the two experiments was probably dissimilar and might have caused the differences in shoot proliferation.

We obtained enhanced shoot proliferation by applying the ethylene biosynthesis inhibitors AVG and $CoCl_2$. Various forms of growth promotion by these compounds have been reported in diverse plant species and in vitro culture systems, for example, in *Helianthus annuus* cotyledon cultures (Chraibi et al., 1991), *Albizzia julibrissin* excised roots (Sankhla et al., 1995), *Raphanus sativus* hypocotyl explants (Pua et al., 1996), and *Carica papaya* node cultures (Magdalita et al., 1997). Our measurements of ethylene levels in culture flasks revealed that, in growth stimulating concentrations, both ethylene biosynthesis inhibitors suppressed the ethylene levels only after the first week of culture. During Week 1, ethylene synthesis was either promoted or remained the same as the control level. Thus, it appears that a normal to high initial ethylene concentration, followed by a lower concentration during subsequent culture, is favorable for culture development.

Both $CoCl_2$ and AVG were toxic at high concentrations. AVG is an analogue of Rhizobium phytotoxin and is known to inhibit pyridoxal enzymes (Yang, 1980). It is probable that at high AVG concentrations, in addition to ACC synthase, the other essential pyridoxal enzymes are also inhibited, thus causing tissue chlorosis. Toxicity of Co^{2+} at high concentrations has been previously reported in tobacco callus (Murashige and Skoog, 1962). The ethylene biosynthesis promoting effect of low $CoCl_2$, on the other hand, may have been due to its micronutrient role. It was one of the standard ingredients of the basal medium used in this study. Thus, the effects of AVG and $CoCl_2$ on proliferation of shoots may not solely depend on the effect on ethylene regulation.

Although all three ethylene effectors enhanced shoot development, their promotive effects were clearly an indirect consequence of their influence on the timing of ethylene accumulation. Polynomial regression analyses showed that for maximum shoot production, ethylene accumulation should be high during Week 1 and low during the following two weeks. A higher correlation between shoot number and ethylene concentration was observed during Week 1 than during Weeks 2 and 3.

According to the observations of Van Dijck et al. (1988), shoot proliferation through axillary bud formation after placing the multiple bud clusters on BA containing medium can be divided into two stages morphologically: (1) axillary bud swelling and (2) its outgrowth. High ethylene levels at the bud swelling stage followed by low levels at bud outgrowth possibly maximize shoot development. This conclusion is supported by the work of Kevers et al. (1992) on *Rosa hybrida*, where a supplement of 5 ppm ethylene during the first 7 to 14 days of culture resulted in an increased shoot number. Our earlier work (Lai et al., 1998) showed that greater shoot numbers under a sequence of an initial week of non-aeration followed by two weeks of aeration of the culture flasks is also consistent with this conclusion. In this study, the result for unaerated control flasks indicates that ethylene concentration peaked before the maximum shoot proliferation was reached. This effect could be inducible.

We also observed a positive relationship between leaf number and shoot number, with significant coefficients larger than 0.82 (data not shown). The effects of various treatments on leaf number coincided with their effects on shoot number. This suggests that the primary effect of the treatments was on shoot number. None of the treatments currently tested promoted leaf number increase without increasing shoot number.

In conclusion, axillary shoot proliferation in papaya can be enhanced by regulating either the exogenous ethylene concentration or the endogenous ethylene biosynthesis. The high cost and potential toxicity of ethylene effectors make them unsuitable for commercial use. On the other hand, use of a gas diffusion equilibration procedure or a method of controlling exogenous ethylene levels seems feasible in large-scale micropropagation operations.

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調控有效性乙烯以促進木瓜組織培養腋莖之繁殖

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本研究探討乙烯濃度在木瓜（*Carica papaya* L.）組織培養腋芽長成櫱生莖中之時程。在培養第一週時利用氣體平衡交換法調控培養瓶中乙烯：以透明箱作為乙烯注射的間接空間以及 0.02 μm 孔徑濾膜封住培養瓶使能擴散平衡。結果以 0.2 及 0.4 ppm 乙烯環境下先經過一週隨後通氣培養皿兩週，則分別得到最大促進效果之幼莖數提高 36% 及葉數提高 50%。本研究同時也利用外加乙烯生成前驅物 ACC、生成抑制劑 AVG 及氯化亞鈷於培養基中以改變內生乙烯，其中以 2 μM ACC 對幼莖數可達到最大的促進效果(提高 75%)。利用 0.5 μM AVG 及 5 μM 氯化亞鈷分別可提高櫱生莖增殖率 23% 及 49%。為了找出培養期間適當之乙烯濃度，本研究亦分析幼莖數與乙烯濃度之間的關係，能產生最多莖數之乙烯濃度於第一、第二及第三週分別為 0.34、0.20 及 0.15 ppm。這些結果顯示要提高木瓜腋芽生成櫱生莖的產量可能需要於培養早期施以較高濃度的乙烯及隨後降低乙烯濃度。

關鍵詞：ACC；AVG；木瓜；氯化亞鈷；乙烯；氣體平衡交換法。