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Role of Ethylene in the Production of Sporophytes from *Platycerium coronarium* (Koenig) Desv. Frond and Rhizome Pieces Cultured *in Vitro*

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Abstract. The effect of ethylene on in vitro plant regeneration from frond and rhizome explants of *Platycerium coronarium* was investigated. Ethylene levels in the culture vessels increased with time, resulting in a decrease in the percentage of sporophytes produced. Addition of the ethylene action inhibitor silver thiosulfate resulted in an increase in the percentage of plants regenerated, indicating an inhibitory effect of ethylene on regeneration. However, the presence of 2,5-norbornadiene was not effective in reversing the effect of ethylene. Inhibitors of ethylene biosynthesis, such as cobalt chloride, salicylic acid, benzylisothiocyanate, and aminoethoxyvinylglycine, were also ineffective in increasing sporophyte regeneration. 1-Aminocyclopropane-1-carboxylic acid, the ethylene precursor, was ineffective in increasing the level of ethylene in the culture vessels. Therefore, the biosynthetic pathway of ethylene in the fern *P. coronarium* appears to be different from that of higher plants but similar to that of some other ferns.

The role of ethylene in various physiological responses and its effect at different stages of higher plant growth and development have been documented (Abeles et al. 1992, Lieberman 1979, Yang and Hoffman 1984). Ethylene has been shown to be produced by plant tissues grown in vitro (Kumar et al. 1987, LaRue and Gamborg 1971), and its presence has diverse influence on the growth and development in such systems (Biddington 1992). In some cases ethylene has a promotory effect (Kumar et al. 1987), whereas in others the presence of ethylene is inhibitory (Chraibi et al. 1991, Pua and Chi 1993).

Ethylene has been shown to affect spore germination (Warner and Hickok 1987), the growth of gametophytes (Miller et al. 1970), and the production of apogamous buds (Elmore and Whittier 1973) in various ferns. Studies have also been carried out using ethylene inhibitors to determine the biosynthetic pathway of ethylene in ferns. Such studies have shown that some ferns follow the same ethylene biosynthetic pathway as higher plants (Tittle 1987), whereas others do not (Osborne 1989). Despite these conflicting observations, there have been few studies examining the role of ethylene in this group of evolutionarily primitive plants.

Plant tissue culture techniques have been employed widely to propagate commercial fern species (Higuchi et al. 1987, Knauss 1976) but, to date, there is no report on the effect of ethylene in such in vitro regeneration systems. The production of sporophytes from frond strips and rhizome pieces has been reported in *Platycerium coronarium* (Wee et al. 1992). In the present study our goal was to examine the role of ethylene in the de novo regeneration of sporophytes. In addition, inhibitors of ethylene action and biosynthesis were used to try to determine if ethylene production in *P. coronarium* is via the same biosynthetic pathway as in higher plants.

Materials and Methods

Plants

Explants of rhizomes and fronds from 2-month-old sporophytes grown under aseptic conditions (Wee et al. 1992) were used. Rhizomes that were about 3 mm in diameter were cut into discs of 1-mm thickness, and each disc was then cut into 16 pieces. Fronds were cut into 2 × 7-mm strips. Twelve explants were cultured per 100-mL Erlenmeyer flask containing 25 mL of me-

Abbreviations: SA, salicylic acid; AVG, aminoethoxyvinylglycine; BITC, benzylisothiocyanate; STS, silver thiosulfate; ACC, 1-aminocyclopropane-1-carboxylic acid

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dium. There were five replicate flasks in each experiment. With rhizome pieces, only MS basal medium (Murashige and Skoog 1962) was used; with frond strips, MS medium was supplemented with 6 μM α-naphthaleneacetic acid. 2% sucrose and 0.2% Gelrite (Merck) were added to all of the media. The pH of the media was adjusted to 5.6 before autoclaving for 20 min at 121°C. Cultures were incubated at 25 ± 1°C under a 12-h photoperiod using cool white fluorescent lamps generating photosynthetically active radiation of 20 μmol m^{-2} s^{-1} at the level of the explants. The number of explants regenerating sporophytes was counted at the end of 45 days. Using this, the percentage of total number of explants forming sporophytes was calculated.

Effect of Different Seal Types

To study the effect of ethylene on regeneration of sporophytes, three types of seals were used: aluminum foil, foam bung, and serum caps. To absorb ethylene produced in the flasks during the period of culture, 1 mL of 0.25 M mercuric perchlorate was put into a presterilized glass vial placed partly embedded in the center of each flask (Kumar et al. 1987). The seals used in this case were aluminum foil and serum caps. Six Erlenmeyer flasks, each with 10 explants of either rhizome or leaf segments, were set up per series of experiments. In addition, the same number of explants was also cultured in Magenta GA-7 vessels as control. The flasks with serum caps were wrapped around the rim with Gladwrap (First Brands Corporation, USA). Flasks using aluminum foil and GA-7 vessels were sealed with a layer of Nescofilm (Bando Chemical Ind. Ltd., Kobe, Japan). All experiments were repeated twice.

Effect of Ethylene Inhibitors, Precursor, and Ethrel

Six types of ethylene inhibitors were examined. These were added to the culture medium at varying concentrations. Salicylic acid (SA, at 0, 5, 10, 25, 50, 100, 150, 200, and 250 μM) and cobalt chloride ($CoCl_2$, at 0, 10, 20, 30, 40, 50, 80, and 100 μM) were added before autoclaving. Aminoethoxyvinylglycine (AVG, at 0, 0.5, and 5 μM), benzylisothiocyanate (BITC, at 0, 2, 4, 8, 10, 15, and 25 μM), and silver thiosulfate (STS, at 0, 0.5, 5, 10, 15, 25, 50, and 100 μM) were filter sterilized and then added to autoclaved medium. The preparation of STS was according to Biddington and Dearman (1986); that of BITC was according to Patil and Tang (1974). In the case of 2,5-norbornadiene (0, 1,000, 2,000, 4,000, and 8,000 μL/L) a small vial containing a strip of filter paper placed in the center of the flask was used. The flasks were also aired in the laminar flow every 5 days for 30 min, and fresh 2,5-norbornadiene was added before resealing the flasks.

The effect of ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC, at 0, 10, and 100 μM) on the amount of ethylene produced during regeneration was also studied. The response of rhizome explants to the ethylene-releasing agent, Ethrel (2-chloroethanephosphonic acid, at 0, 20, 40, 80, and 160 μM) in the medium was also investigated. ACC and Ethrel were filter sterilized before adding to the medium.

All flasks were sealed with aluminum foil except when 2,5-norbornadiene was added. The flasks were sealed with serum caps in the latter case. Five flasks with 12 explants each were set up per concentration for all inhibitors tested except for $CoCl_2$, where six flasks with 10 explants each were set up. All experiments were repeated twice.

Ethylene Measurement

Two types of seals were used to compare their effect on ethylene production. Five flasks each with 12 explants were sealed with serum caps throughout the experiment, and another five were sealed with aluminum foil. After estimating the accumulated ethylene on every 10th day, the flasks sealed with aluminum foil were aired in the laminar flow for 15 min and resealed. Serum cap-sealed flasks containing culture medium alone were used as controls.

For experiments using inhibitors, the flasks initially sealed with aluminum foil were aired for 1 h in the laminar flow on every 9th day and resealed with serum caps. The amount of ethylene produced was measured 24 h later. After the measurement, the flasks were aired and resealed with aluminum foil.

Ethylene was estimated by gas chromatography (Kumar et al. 1987). A 1-mL gas sample was withdrawn from the headspace of each flask with a hypodermic syringe and injected into a gas chromatograph (Hewlett-Packard model 5890) equipped with a 6-ft Porapak N (80/100 mesh) column, a flame ionization detector, and integrator (Hewlett-Packard 3390A). The operating temperatures were set at 100, 150, and 200°C for the oven, injector, and detector, respectively. Nitrogen flowing at a rate of 35 mL/min was used as a carrier gas. The primary standard ethylene gas used was 4 μL/L.

Statistical Analysis

All data obtained were subjected to one-way analysis of variance followed by Duncan's multiple range test. Prior to analysis, data obtained for ethylene production in the presence of inhibitors were subjected to natural logarithm transformation; the data for regeneration of sporophytes were subjected to arcsine transformation.

Results

Both the frond strips and rhizome pieces turned dark brown after a few days in culture. Regeneration of sporophytes was observed about 15 days later when the whole explant began to turn green. In the case of rhizome segments, besides greening there was also an increase in size. Bud-like structures appeared on these green explants soon after, and these developed into sporophytes. Each frond explant produced 8–10 sporophytes; 5–6 sporophytes regenerated from each rhizome explant. The percentage of explants showing regeneration was significantly lower in flasks sealed with serum caps as compared with those sealed with aluminum foil or foam bungs (Table 1). With serum caps as seals, ethylene was found to accumulate in the flasks (Fig. 1) The level of ethylene was about 1.5- to 2-fold higher in flasks sealed with serum caps as compared with those sealed with aluminum foil. The percent-

Table 1. Effect of different seal types on the percentage of sporophyte regeneration in frond and rhizome explants after 45 days in culture. Data from three independent experiments were pooled prior to analysis. Means with the same letter in the same column do not differ significantly (p = 0.05) as indicated by one-way analysis of variance followed by Duncan's multiple range test.

Seal type	Frond explants (%)	Rhizome explants (%)
Foam bung	55.5[bc]	50.0[a]
Foil	61.7[bc]	48.3[a]
Serum cap	30.0[d]	34.5[b]
Foil + mercuric perchlorate	76.1[a]	48.9[a]
Serum cap + mercuric perchlorate	44.5[c]	55.0[a]
GA7	68.9[ab]	53.3[a]

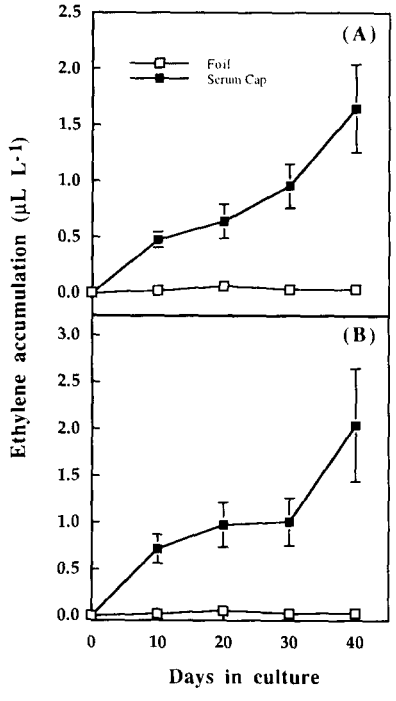

Fig. 1. Effect of two different seal types, serum cap and aluminum foil, on ethylene accumulation in frond (*A*) and rhizome (*B*) cultures of *P. coronarium*. (*Vertical bars* = S.E. of means.)

age of regeneration in flasks sealed with serum caps was increased significantly when mercuric perchlorate was used (Table 1). About 76% of the frond explants regenerated sporophytes when the usage of mercuric perchlorate was combined with aluminum foil seal.

The addition of STS to the culture medium increased the regeneration of sporophytes when compared with the STS-free control. Incorporation of STS at 25 and 15 μM resulted in the highest percentage of regeneration from frond and rhizome explants, respectively (Fig. 2*A*). About a 62% increase in regeneration from frond explants was observed in the presence of 25 μM STS. In the case of rhizome explants, the increase in regeneration was about 18% with the addition of 15 μM STS.

Addition of 2,5-norbornadiene, however, caused an overall decrease in regeneration. Only about 16% of the frond explants and 27% of the rhizome explants regenerated sporophytes when treated with 1000 μL/L 2,5-norbornadiene. At concentrations higher than 4000 μL/L the regeneration was below 10% for both types of cultures. Explants that did not regenerate turned dark brown within 10 days of culture initiation; those that showed regeneration produced only about two to three sporophytes per explant at the end of 45 days. In addition, the portion of the explant which did not regenerate sporophytes remained dark brown. A slightly higher level of ethylene was produced by frond explants in the presence of 2,000 μL/L 2,5-norbornadiene on the 10th and 20th days of culture (Fig. 3). On the 40th day the amount of ethylene produced by frond explants in the presence of this inhibitor was about half that of the control. In the case of rhizome explants, no difference in ethylene production was detected in the presence of 8,000 μL/L 2,5-norbornadiene except on the 10th day of culture when it was reduced by half as compared with the control (Fig. 3).

When the rhizome explants were treated with 40–160 μM Ethrel, more than 30% reduction in sporophyte regeneration was observed. However, at the 20 μM level, Ethrel did not have a significant effect on sporophyte regeneration from rhizome explants.

When BITC, $CoCl_2$, and SA were used as inhibitors, there was no significant increase in the percentage of regeneration as compared with the control (Fig. 2, *B*, *C*, and *D*). Higher concentrations of these inhibitors resulted in a decrease in the percentage of sporophyte regeneration. In addition, the explants that did not regenerate turned black, and the culture medium around these explants was brown. The amount of ethylene produced in the culture vessels in the presence of BITC and $CoCl_2$ did not show a significant reduction except on the 10th day of culture by the frond explants (Fig. 4 and 5). Ethylene production by these cultures was about 2-fold lower in the presence of 4 and 15 μM BITC and 100 μM $CoCl_2$ (Figs. 4 and 5). AVG did not reduce ethylene production except on the 10th day of culture when used at 0.5 and 5 μM (Table 2).

Similarly, the addition of ACC at 10 and 100 μM to the culture medium did not result in a increase in ethylene production except in two cases (Table 2). On the 30th day of culture, flasks containing frond

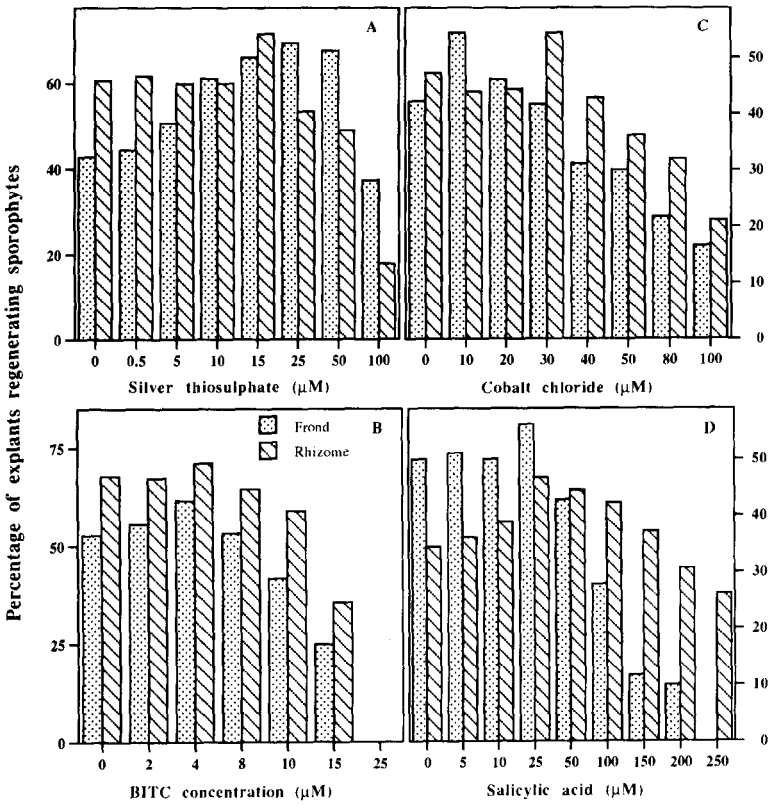

Fig. 2. Effect of STS (*A*), BITC (*B*), $CoCl_2$ (*C*), and SA (*D*) on the percentage of frond and rhizome explants regenerating sporophytes after 45 days in culture. Data from three independent experiments were pooled.

explants showed about a 3-fold increase in ethylene production in the presence of 100 μM ACC compared with the control. With rhizome explants, ACC at 100 μM caused a minor increase in ethylene production only during the 20th day (Table 2). This is contrary to the expectation of a marked increase in ethylene production in response to ACC treatment as observed in the majority of higher plants.

Discussion

The present study showed that ethylene was produced by frond and rhizome explants of *P. coronarium* throughout the culture period. The presence of this phytohormone in the culture vessels had an inhibitory effect on the percentage of sporophyte regeneration. The use of aluminum foil or foam bungs, which are not airtight, resulted in a low level of ethylene in the flasks. This might be the cause of the observed increase in regeneration. When serum caps were used to seal the flasks, ethylene accumulated (Fig. 1), and regeneration was inhibited. The inhibitory effect of ethylene was overcome by the use of mercuric perchlorate (Table 1) as has been reported in other species (Abeles et al. 1992, Kumar et al. 1987).

Treatment with STS, an inhibitor of ethylene action, increased sporophyte regeneration. This is consistent with our observation that ethylene is an inhibitor of sporophyte regeneration in *P. coronarium*. Silver ion interferes directly with ethylene action but not its production, and this has been found to be effective in a number of tissue culture systems (Avihai et al., 1988, Chraibi et al. 1991). The most important properties of silver as an antiethylene agent are its specificity, persistence, and lack of phytotoxicity at effective concentrations (Beyer 1976). In the present study, the explants could still produce ethylene in the presence of STS. However, they did not respond to the gas produced probably because of the inhibitory action of STS. Silver ions are competitive inhibitors of ethylene action; when applied, they may form complexes with ethylene receptors of the cells, preventing them from responding to ethylene (Abeles et al. 1992). Hence, the observed increase in regeneration of sporophytes in *P. coronarium* cultures, in response to treatment with STS, is likely a result of its ability to block ethylene action rather than a result of nonspecific physiological effects.

Another inhibitor of ethylene action, 2,5-norbornadiene, could reverse the effect of ethylene in a number of species (Lentini et al. 1988, Songstad et al. 1988). It was, however, not effective in the

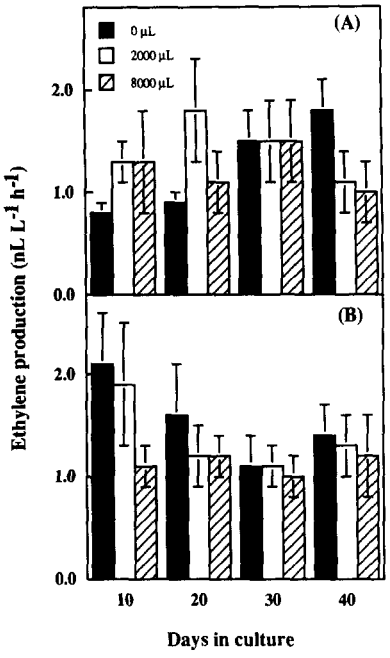

Fig. 3. Effect of different concentrations (0, 2,000, 8,000 µL/L) of 2,5-norbornadiene on ethylene production from frond (*A*) and rhizome (*B*) explants during 40 days of culture. (*Vertical bars* = S.E. of means.)

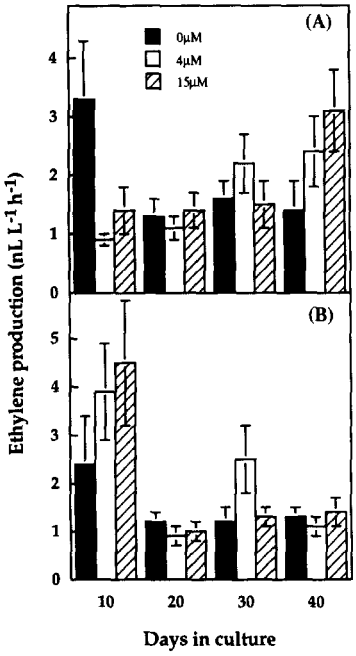

Fig. 4. Effect of different concentrations (0, 4, 15 µM) of BITC on ethylene production from frond (*A*) and rhizome (*B*) explants during 40 days of culture. (*Vertical bars* = S.E. of means.)

present study. Sisler and Yang (1984) proposed that the effective range of action of 2,5-norbornadiene is between 500 and 2,000 µL/L. Higher concentrations of this cycloolefin are toxic. In the case of

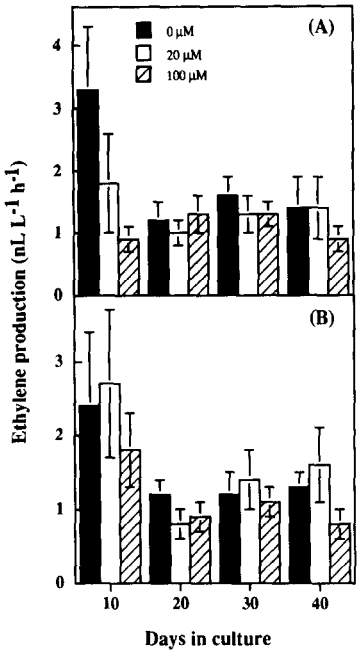

Fig. 5. Effect of different concentrations (0, 20, 100 µM) of $CoCl_2$ on ethylene production from frond (*A*) and rhizome (*B*) explants during 40 days of culture. (*Vertical bars* = S.E. of means.)

frond and rhizome explants of *P. coronarium*, 2,5-norbornadiene appeared to be toxic even at 1,000 µL/L as indicated by the dark brown appearance of the explants after about 10 days in culture. This could have resulted in the increase in ethylene production (as a response to senescence) during the first 20 days of culture. Also, frond explants may be more sensitive to 2,5-norbornadiene than the rhizome explants. Hence, we conclude that 2,5-norbornadiene is ineffective as an inhibitor in this case, and its toxic effect may be the reason for the significant reduction in regeneration of sporophytes.

Ethrel undergoes chemical decomposition in aqueous solutions at pH above 4.1, releasing ethylene. Hence, incorporating Ethrel into the culture medium will elicit physiological responses in the tissues similar to those induced by ethylene (Abeles et al. 1992). We observed significant inhibition of regeneration when the rhizome explants were cultured on medium containing Ethrel. In addition, the use of mercuric perchlorate enhanced regeneration in serum-capped flasks (Table 1). Taken together, these clearly show that ethylene is the main inhibitor of regeneration in *P. coronarium*.

The biosynthetic pathway of ethylene from methionine in higher plants has *S*-adenosylmethionine and ACC as intermediates (Adams and Yang 1979). $CoCl_2$, BITC, SA, and AVG have been demon-

Table 2. Effect of AVG and ACC on ethylene production from frond and rhizome explants during 30 days of culture. Data are means (±S.E.) of three independent experiments.

Treatment (μM)	Ethylene production (nL/L/h)		
	10 days	20 days	30 days
AVG			
Frond explants			
0	1.9 ± 0.3	2.2 ± 0.5	0.9 ± 0.2
0.5	1.3 ± 0.3	1.7 ± 0.3	1.4 ± 0.3
5.0	0.9 ± 0.2	1.6 ± 0.4	1.7 ± 0.5
Rhizome explants			
0	3.0 ± 0.8	1.3 ± 0.2	2.0 ± 0.5
0.5	1.2 ± 0.2	2.0 ± 0.8	1.6 ± 0.2
5.0	1.9 ± 0.3	1.8 ± 0.5	1.4 ± 0.3
ACC			
Frond explants			
0	1.9 ± 0.3	2.2 ± 0.5	0.9 ± 0.2
10	1.7 ± 0.3	1.5 ± 0.2	1.7 ± 0.4
100	1.2 ± 0.2	1.8 ± 0.5	3.1 ± 0.8
Rhizome explants			
0	3.0 ± 0.8	1.3 ± 0.2	2.0 ± 0.5
10	2.0 ± 0.5	1.0 ± 0.1	1.9 ± 0.3
100	1.8 ± 0.3	2.2 ± 0.5	1.7 ± 0.4

strated to be effective inhibitors of ethylene biosynthesis (Lau and Yang 1976, Leslie and Romani, 1986, Patil and Tang 1974). $CoCl_2$ and SA are capable of inhibiting the enzymatic conversion of ACC to ethylene (Meijer and Brown 1988), whereas AVG inhibits the enzyme ACC synthase, preventing it from converting *S*-adenosylmethionine to ACC (Biddington 1992). Ethylene levels in vitro could also be varied by the application of ACC (Adams and Yang 1979). In the present study, however, all of the inhibitors of ethylene biosynthesis tested were ineffective in causing a significant decrease in ethylene production. The slight decrease in the amount of ethylene produced by frond explants, only on the 10th day in the presence of BITC and $CoCl_2$, cannot be explained at present.

The inclusion of ACC to the culture medium also did not result in a significant increase in the level of ethylene produced in our cultures. However, plants that produce ethylene via the methionine pathway are expected to produce (more than an order of magnitude) higher level of ethylene in response to added ACC (Abeles et al. 1992, Songstad et al. 1988, Yang and Hoffman 1984). ACC was ineffective in promoting ethylene production in the ferns *Trichomanes speciosum, Ophioglossum reticulatum, Salvinia natans,* and *Azolla caroliniana* (Osborne 1989). However, in the ferns *Pteridium aquilinum, Matteuccia struthioptheris,* and *Polystichum munitum,* the addition of ACC increased significantly, and the presence of AVG and $CoCl_2$ decreased ethylene production (Tittle 1987). It is possible that ferns that do not produce ethylene via the same pathway as angiosperms may produce ethylene from the intermediates of the tricarboxylic acid cycle as in the case of microorganisms (Jacobsen and Wang 1968, Wang et al. 1962) or by an alternative pathway. Despite the fact that *P. coronarium* produces ethylene and responds to it, our results suggest that in this fern, production of ethylene could be by another pathway and not by the methionine pathway reported for higher plants. In view of this, more studies are needed to elucidate such a pathway for ethylene production in ferns.

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