Ethylene Production by Growing and Senescing Pear Fruit Cell Suspensions in Response to Gibberellin\textsuperscript{1,2}

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ABSTRACT

A pear (\textit{Pyrus communis} L. cv Passe Crassane) cell suspension was used as a model system to study the influence of gibberellin on processes related to fruit ripening. Growth of the cell cultures was inhibited and their loss of viability was accelerated when 0.5 millimolar gibberellic acid (GA\textsubscript{3}) was added to suspensions at two stages of cell development, namely, growth and quiescence. Cell respiration rate was unaffected up to 2 millimolar GA\textsubscript{3} but ethylene production, both basal and 1-aminocyclopropane-1-carboxylic acid-induced, was inhibited in all stages of cell development. However, the degree of inhibition decreased as the cell cultures aged. The site of ethylene inhibition by GA\textsubscript{3} appeared to be related to the ethylene-forming enzyme. The coincident acceleration of cell senescence and inhibition of ethylene production indicate that the pear cell suspension cannot serve as an analogous model for studying the mode of action of gibberellin in delaying ripening and senescence of fruits in its entirety, although certain specific effects might be relevant.

Gibberellins are used in commercial fruit production to modify fruit set (13) and to delay maturation and ripening (1, 5, 8). The gibberellins slow ripening by delaying Chl degradation (1, 12), carotenoid synthesis (12, 14), climacteric respiration (1, 2), ethylene evolution (6), and softening (2, 5, 17). The physiological mechanisms responsible for gibberellin action in fruit ripening are unknown, as is the relationship between the response to applied gibberellins and to endogenous gibberellin action in fruit. It is not known whether the inhibitory effects result from an overall restraint on the penultimate step to ripening and senescence, or if each of the steps can be individually affected by the hormone.

Inasmuch as gibberellin treatments are being successfully to control ripening of fruit such as persimmons (5), there is interest in gaining an understanding of the underlying mechanism. Rather than using whole fruit, we have studied the effects of GA\textsubscript{3} on suspension-cultured pear fruit cells during growth and ageing. It has been shown that such cells respond in ways that are often similar to the responses of tissues during senescence or fruit ripening (4, 19). Because of the effects of gibberellin on growth, ethylene production, and respiration of whole fruit (1, 2, 5, 8), we investigated growth and associated changes in protein synthesis, viability, ethylene evolution, and respiration of pear cell suspensions.

Cultured cell suspensions have been used to study the effects of gibberellins on cell expansion and growth, which are enhanced by addition of up to $10^{-6}$ M GA\textsubscript{3} (11). At higher concentrations, cell growth has sometimes been inhibited, and as these are the concentrations which are required to inhibit fruit ripening (1, 2, 5, 8), we applied a similar range of concentrations to the pear fruit cell suspensions.

MATERIALS AND METHODS

Cell culture

Pear cells (\textit{Pyrus communis} L. cv. “Passe Crassane"), initially derived from fruit, were maintained in liquid culture in a GM\textsuperscript{3} with 4.5 $\mu$M 2,4-D and no cytokinins (18). Cells were subcultured weekly either into fresh growth medium or into an AM which contained 0.4 mM mannitol and no 2,4-D. The latter was a modification of the AM of Codron et al. (9), in that we did not first subculture the cells into a 2,4-D free medium before subculturing again into a one-fourth strength nutrient medium containing mannitol. Our modified procedure was sufficient to maintain cells in a state of reduced growth without rapid mortality for 14 d and still responsive to ethylene stimulation (see below). Fifty milliliters of culture solution were placed in 250 mL flasks and adjusted to 0.5 mM GA\textsubscript{3} at the time of subculturing. Each experiment was composed of three replicate flasks per treatment and was repeated at least twice. Cells were grown at 27°C under low light on a rotary shaker (100 rpm).

Cell Measurements

Fresh and dry weights were determined on 10 mL aliquots of cells after filtration on glass fiber paper (Whatman GF/A). Viability was measured using Evan’s blue, with cells being counted in a hemocytometer. For soluble protein measurement, the cells collected for fresh weight determination were resuspended in 2 to 3 mL 0.1 M phosphate buffer (pH 7.2) and sonicated for 30 min. The cell debris was precipitated by

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\textsuperscript{3} Abbreviations: GM, growth medium; AM, aging medium; ACC, 1-aminocyclopropane-1-carboxylic acid; EFE, ethylene-forming enzyme; AOA, \textalpha-aminoxyacetic acid; AVG, aminoethoxyvinylglycine.
Figure 1. Ethylene production by pear cells growing 10 d in GM. Log phase cells (5 mL) were placed in 25 mL flasks with or without additions of 0.5 mM ACC and 1 mM GA3, sealed for 1 h at the given incubation times; ethylene was measured in the flask head space. Bars represent SE.

Hormone Treatments

The effect of GA3 on ACC-induced ethylene evolution by cell cultures at different stages of the growth cycle was assayed by placing 5 mL aliquots of a cell suspension into 25 mL conical flasks containing ACC and GA3 at various concentrations (total volume generally = 5.2 mL). The flasks were placed in a shaking water bath at 25°C and, after periods ranging from 1 to 24 h, sealed for 1 h with silicone rubber caps. Head space analyses of ethylene and CO2 were done by gas chromatography on 1 mL samples from a total head space volume of 32.8 mL. Controls contained fresh medium or cell-free filtrated medium instead of the 5 mL cell suspension.

Statistical Analysis

All data were subjected to analysis of various and significant differences were determined at the 95% level of probability.

RESULTS

Ethylene produced by cells growing in GM was inhibited by GA3 both in the presence and absence of ACC (Fig. 1). The basal level of ethylene production (−ACC) was reduced by ca. 33% with 1 mM GA3 after 3 h incubation at 25°C. With the addition of 0.1 mM ACC, the more than 10-fold increase in ethylene production, which occurred within 5 to 7 h, was inhibited by 44%. Maximum ethylene production and GA3-induced inhibition occurred after 7 h incubation.

When pear cell suspensions were cultured in the presence of 0.5 mM GA3, growth rates were noticeably reduced (Fig. 2). This was the case under both the actively growing (GM) and the aging (AM) conditions. However, the increase in dry weight was not as strongly inhibited by GA3 in the GM as the increase in fresh weight. There was very little increase in cell dry weight in the AM and the addition of GA3 inhibited growth completely (Fig. 2B). Cell viability decreased in both media during growth, and GA3 increased cell death in the AM after 5 d (Fig. 3). No effects of GA3 on viability in the GM were found until 14 d after addition of the hormone. The GA3-treated cells were smaller, more regular in shape, and showed less tendency to form clumps than untreated cells. Initially, cell growth in GA3 was accompanied by a reduction in soluble protein concentration in GM and AM. However, when inhibition of growth by the gibberellin became more apparent, the protein content of the treated cells increased above that of the control in both media: after 6 d
in AM, 39.8 ± 1.9 versus 16.5 ± 5.0 g/mg dry weight, and after 9 d in GM, 141.3 ± 8.6 versus 58.5 ± 5.2 g/mg dry weight, with and without GA3, respectively.

Ethylene evolution by cells cultured in GM was approximately 2 to 5 nL/g fresh weight/h throughout the growth cycle, whereas cells in AM did not produce any measureable amount (<0.1 nL/g fresh weight/h—almost equivalent to the controls). However, upon addition of 0.1 mM ACC, both cultures were capable of enhancing their ethylene production indicating their EFE potential (Figs. 1 and 4). The pattern of ethylene evolution resembled the climacteric pattern of the ripening fruit (Fig. 4) with an earlier peak in quiescent cells (AM). However, ethylene production could also be loosely related the growth rate of the cells (Fig. 2). The maximum values of ethylene production were attained close to the peak of cellular growth in GM, just prior to the beginning of the senescent or static phase. Moreover, the more vigorously growing cells in GM attained higher levels of ethylene production than the cells cultured in AM.

Inhibition of ethylene production by cells cultured in both media was significant at concentrations as low as 0.5 mM GA3 (Fig. 5). At a concentration of 0.1 mM GA3, ethylene evolution was inhibited by ca. 10% and no effect was observed at lower concentrations. The relative response of cells grown in GM and AM to different GA3 concentrations was similar for cultures of different physiological ages producing different amounts of ethylene (Fig. 5). However, GA3 was more effective in inhibiting ethylene production in young cultures, with the extent of inhibition declining linearly with increasing culture age (Fig. 6). As expressed on an age basis, inhibition was always greater in cells on GM than on AM.

Respiration rates of the cells were unaffected by GA3 at concentrations which heavily reduced ethylene production, i.e., up to 2 mM (data not shown). Initial rates, measured at 1 h after addition of GA3, were between 0.13 and 0.18 mg CO2/g/h. A 5 mM GA3 concentration, which caused a 90% inhibition of ethylene production, immediately inhibited respiration by ca. 50%.

Other inhibitors of ACC synthesis, AVG and AOA, evidently acted independent of GA3. When applied together with GA3, in the absence of ACC, their effect was additive (Table 1). In the presence of ACC in excess (1 mM), AOA at 100 mM and AVG at 50 mM showed no effect on ethylene production. When GA3 was added together with either inhibitor, there was little or no additional inhibition beyond that provided by GA3 alone. Cobalt (Co2+) at 0.5 mM inhibited ethylene production to the same extent as 1 mM GA3, with or without added ACC. When applied together with GA3, Co2+ had no additive effect.

**DISCUSSION**

Gibberellin is generally regarded as a stimulant of vegetative growth, yet it can have the reverse effect on reproductive
Table 1. GA3 Inhibition of Ethylene Evolution by Pear Cell Suspensions in Presence of 1 mM ACC and Other Inhibitors

<table>
<thead>
<tr>
<th>Inhibitor (mM)</th>
<th>Ethylene Inhibition</th>
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<tbody>
<tr>
<td></td>
<td>−ACC</td>
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<tr>
<td>GA3 (1)</td>
<td>33</td>
</tr>
<tr>
<td>AOA (0.1)</td>
<td>18</td>
</tr>
<tr>
<td>GA3 + AOA</td>
<td>52</td>
</tr>
<tr>
<td>GA3 (1)</td>
<td>45</td>
</tr>
<tr>
<td>AVG (0.05)</td>
<td>73</td>
</tr>
<tr>
<td>GA3 + AVG</td>
<td>82</td>
</tr>
<tr>
<td>GA3 (1)</td>
<td>42</td>
</tr>
<tr>
<td>CO2 (0.5)</td>
<td>43</td>
</tr>
<tr>
<td>GA3 + CO2</td>
<td>45</td>
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</tbody>
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* Two separate experiments were conducted to compare each inhibitor with GA3. Cells were from 10 d suspension cultures on GM.

tissue and it may even inhibit fruit growth (5). This, however, could be reconciled with its effect of delaying fruit senescence (8), thereby maintaining fruit at a more juvenile stage. In suspension cultures, growth of cells of both vegetative (spinach leaves) and reproductive (rose petals) origin was stimulated by GA3, at concentrations ranging from 10−10 to 10−4 M (11). However, at a higher concentration (10−4 M) growth of rose cells was inhibited, and it was concluded that GA3 might become inhibitory under conditions of slow growth. The GA3 concentration used in our study was 0.5 mM, and its effect on growth of both actively growing and more quiescent pear fruit cells was inhibitory (Fig. 2). This might be the result of reduced cell size, resulting from promotion of cell division by GA3, with little subsequent cell expansion. Digby et al. (10) showed that similar GA3 concentrations induced an increase of approximately 100% in cell number in suspensions of Acer pseudoplatanus without any increase in the overall fresh weight, due to a decline in mean cell size. However, the increase in the number of nonviable pear cells with GA3 (Fig. 3), especially in the ageing medium, suggests that GA3 might be accelerating senescence prior to death. Moreover, the GA3-induced increase in protein content of the cells after 6 d in AM or 9 d in GM, may also indicate the onset of cell death. Pech and Romani (19) showed that death of cultured pear fruit cells was preceded by a burst in protein synthesis, which in their case was evidently induced by auxin deprivation (3, 15).

If on the other hand increased ethylene production can be regarded as an expression of senescence, and inhibition of production as its deferral, GA3 may indeed be retarding cell senescence as it retards fruit ripening. Inhibition by GA3 of ACC-induced ethylene production was greater in the more actively growing suspensions and, as the cultures aged in either medium, the effect decreased (Fig. 6), suggesting that GA3 is delaying senescence, it does so only when the cells are either dividing or expanding and aged cells are less responsive, similar to the response of IAA-treated cells to added ACC (20). However, the GA3 response is the reverse of the pear cell response to auxins, where stimulation of ethylene production is much more pronounced in senescent cells that in growing cells (20). Both phenomena indicate a similarity in behavior of cell suspensions and plant systems, in response to the phytohormones. Conversely, it is also possible that the extent of inhibition of ethylene production by GA3 is simply directly related to the absolute rate of ACC-induced ethylene production (Fig. 4), as with inhibition by salicylic acid (16), or to the viability of the cells (Fig. 3). The changing rate of ethylene evolution in aging cultures did not show a continuous decline, though the general tendency was in this direction (Fig. 4). Therefore, the increasing mortality of the cells might be, at least partially, responsible for the decline in both ethylene production and its inhibition by GA3. A 10-fold increase in GA3 concentration (5 mM) appeared to have toxic effects irrespective of culture age.

A comparison of GA3 with AVG and AOA, which are known to inhibit ACC synthase activity, suggests different sites of inhibition. When applied together, the effects were additive with AOA or enhanced with AVG. When compared with CO2, the extent of inhibition by GA3 was similar, but not additive when the two were applied together. This indicates that GA3 may be acting similarly to CO2, inhibiting EFE function, though not necessarily in the same manner (21). As EFE is a membrane-bound enzyme, requiring the maintenance of cell integrity, a decline in ethylene production could also be interpreted as a stimulation of cell death, rather than a delay in the onset of senescence. If such is the case, the inhibition of ACC-induced production by GA3 is here the result, but not the cause, of GA3-induced cell senescence and death. In conclusion, though GA inhibition of ethylene production by suspension-cultured pear cells might coincide with its juvenility effects on intact fruits, the overriding effect of GA3 on the pear cells appeared to be an acceleration of senescence. In this regard, such cells, while providing a system for studying specific effects related to gibberellins, are not a satisfactory system for studying the mode of action of GA3 in delaying fruit ripening.

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Literature Cited