A Simple Method for Vacuum Extraction and Quantitative Determination of Internal Ethylene of Excised Apple Tissue

Tomonori Kawano* and Keishi Shimokawa
Faculty of Agriculture, Miyazaki University, Miyazaki 889 -21

Summary

A rapid and simple method for extracting internal ethylene from excised apple tissue and its quantitative determination are described. The method is suitable for correlating the ethylene concentration with the physiological status of the plant tissues. One advantage was that the time required for the tissue excision, vacuum extraction of ethylene, and injection of a gas sample into a gas chromatograph was about a minute. The method provides accurate and reproducible results, i.e. ca. 80% or more of the internal ethylene in apple tissue can be extracted and determined. Another advantage of this method is that the tissue from which ethylene was extracted is still available for further analyses.

The method was applied to the study ethylene regeneration in apple tissue after the removal of the internal ethylene. The formation of ethylene in apple tissue increased after the existing ethylene was removed led us to propose that ethylene synthesis is governed by a feedback inhibition. The inhibition (ca. 20%) of ethylene formation was significantly related to the level of internal ethylene in apple tissue.

Introduction

Ethylene is the simplest olefin which regulates various aspects of plant growth, development, and senescence. Under normal physiological conditions it exists in plant as a gaseous hormone. Even in the earlier stages of ethylene research, gas chromatography has been used for its determination because the instruments can accurately detect minute quantities of the gas. Ethylene diffusing from plant tissues when placed in a desiccator can be detected and assayed through gas chromatography, using a gastight syringe for sampling. The syringe and all the other instruments must be made of materials which do not produce or adsorb ethylene; hence, rubber stoppers must be avoided. When the concentration of ethylene is extremely low, mercuric perchloride (MP) solution can be used to trap the ethylene; the gas is liberated by treating the MP/ethylene complex with LiCl or HCl (Young et al., 1952). Whether MP is used or not, the ethylene detected is that which diffused and not internal ethylene.

It is difficult to estimate the level of ethylene existing in plant tissues from the amount of ethylene diffused from tissue. Staby and De Hertogh (1970) detected ethylene in the internal atmosphere of 5 bulb species. Vacuum extraction of ethylene from plant tissues has been conducted by Beyer and Morgan (1970), Blanpied (1971) reported a method of extracting ethylene from fresh plant tissue, but the apparatus consisted of a lot of rubber materials. We have devised a new, simple and sensitive method for withdrawing internal ethylene from tissues to determine the concentration with a high degree of sensitivity. A method like this has been needed to concurrently associate the occurring phenomena inside plants and the quantity of internal ethylene. We have applied this method to the study of the biofeedback regulation of ethylene synthesis in apple tissues.

Materials and Methods

Mature apple fruit (Malus pumila Mill. var. domestica Schreid L. Borkh. cv. Fuji) was obtained from a local market and stored at 4 °C. The fruits was incubated at 25 °C for 24 hr before the ex-
Experiments and all processes described in this report were conducted at that temperature. For replication, apple tissues (10 mm in diameter and 10 mm thick) were excised from the cortex just below the equator of each fruit, with a cork borer and a kitchen knife.

Description of apparatus and procedure

The apparatus for extracting ethylene from apple tissue and its determination consists of a 100-ml gas sampling syringe equipped with a stopcock and a needle (GL Science Ltd. Tokyo, Fig. 1-1), a 50-ml gas sampling bottle (GL Science Ltd., Fig. 1-2), a 50-μl gastight syringe (Hamilton, Nevada, Fig. 1-3), and a gas chromatograph (Shimadzu GC-8AIF, Tokyo) fitted with a glass column (200 × 0.32 cm) packed with activated alumina TR (60 ~80 mesh, GL Science Ltd.).

The procedure can be divided into 3 steps; 1) the vacuum extraction of ethylene; 2) the gas sampling; and the determination.

Step 1. Place a weighed apple tissue in the gas sampling cylinder while the piston rod is removed (Fig. 2-1). Insert the piston rod into the cylinder and close the stopcock (Fig. 2-2). To create a vacuum inside the cylinder, slide the piston rod out until vacuum in the cylinder reaches 90 mm Hg, then hold it for 15 sec (Fig. 2-3). The negative pressure forces the ethylene out from the apple tissue.

Step 2. Open the stop cock to let in 20 ml of ethylene-free air and close it immediately (Fig. 2-4). Fit a needle on the tip of the gas sampling cylinder (Fig. 2-5) and insert it through the silicone resin stopper of the gas sampling bottle aspirated to 80 mm Hg vacuum (Fig. 2-6). Open the cylinder's stopcock and let the air containing ethylene flow into the gas sampling bottle.

Step 3. Open the cock of the gas sampling bottle to restore atmospheric pressure inside (Fig. 2-7). Sample the atmosphere inside the bottle by inserting a 50-μl gastight syringe through the silicone stopper of the bottle (Fig. 2-8) and then inject the sampled gas into the gas chromatograph for analysis.

To determine pressure in the gas sampling bottle and the gas sampling cylinder, an inlet pressure gauge (A type 60 ~760 kg/cm², GL science Ltd., Fig. 1-4) was used.

The excision and preparation of a tissue prior to extracting ethylene must be done quickly to minimize loss of ethylene before measurement. Each preparation (step 1 ~ 3) in this experiment took only 20 seconds.

Exp. 1. Test of ethylene extracting efficiency

It is difficult to estimate the extraction efficiency since it is impossible to extract all ethylene from the tissue. Therefore we attempted to estimate the approximate percentage of internal ethylene that could be extracted. In this test procedure of extracting ethylene from tissue, the vacuum infiltration method by immersing the tissue in the gas sampling cylinder containing 10 ml of 10 μM CoCl₂. The piston rod was inserted into the cylinder and the stopcock closed. Ethylene was extracted according to the previously described procedure until the tissue became saturated with the CoCl₂ solution. The plugs of apple tissue was placed into vials and incubated at 25 °C for 120 min after the gas extraction. Ethylene that diffused from the tissue in the vials was sampled at 15 and 30-min intervals.
Exp. 2. Further ethylene formation after the removal of internal ethylene

Two apple plugs were excised from the cortex just below the equator of a fruit and the ethylene in one plug was removed and the sample assayed as above. The extracted plug was transferred to a 10-ml vial with a silicone cap. The second plug...
which had not undergone ethylene extraction was placed in another vial as a control. Both vials were incubated at 25 °C for 120 min. The atmosphere in both vials were sampled separating with a gastight syringe at 15- and 30-min intervals and assayed for ethylene. The amounts of the initial ethylene and that which diffused during the incubation period by the two plugs should give an estimate of the ethylene produced after the initial extraction.

Results and Discussion

Reproducible accuracy of replicate samples

Twelve tissues were excised from 3 apple fruits yielding 4 plugs per fruit which were used for extracting ethylene. The ethylene extracted from each plug was sampled four times with a syringe and injected into the gas chromatography to estimate accuracy. From excision of tissue to the completion of the ethylene extraction, it took approximately one min for each replicate. Data from each series of replicates showed that almost the same amounts of ethylene had been extracted from each tissue (Table 1), demonstrating that the method of measuring internal ethylene from apple tissue is highly reproducible and accurate.

Test of ethylene extracting efficiency

After the extraction of ethylene and CoCl2 treatment, the apple plugs were placed in vials and incubated for 120 min during which the gas in vials were sampled at 15- or 30-min intervals. The data are in Table 2. Almost all the internal ethylene remaining in the apple tissue after the internal extraction diffused out within 120 min. The data presented are the averages of quadruple injections. A small percentage of diffused ethylene in the vials was considered to be new ethylene produced after the extraction operation because ethylene synthesis was not inhibited perfectly despite CoCl2 treatment (ca. 30% inhibition of control for 120 min). The amount of diffused in the vials gradually ceased increasing within the 120 min (Table 2). Further apparent increase in ethylene concentration in the vials could not be observed throughout the few hours after the 120 min measurement (data not shown). The mean of the extracted ethylene was ca. 75 nl and that of the diffused ethylene sampled at the end of the incubation period was ca. 18 nl. Assuming all the ethylene that diffused out in the vials had already existed before extraction and that all the internal ethylene in apple tissue which had not been extracted diffused out within 120 min period, the tests indicate that more than ca. 80% of the internal ethylene was extracted from apple tissue through this procedure. This efficiency is high enough to be useful in many plant biological and horticultural studies.

Further ethylene formation after the removal of internal ethylene

The results are from a typical experiment which was replicated 18 times. Though a larger amount of ethylene diffused from the control tissue than from the ethylene-extracted tissue during the incubation period, incremental rates of ethylene diffusion after the first 15-min measurement was

Table 1. Reproducibility of extracting ethylene from 4 plugs of apple tissue excised from 3 different fruits.

| Time   | Extracted internal ethylene (nl·g⁻¹) | Diffused ethylene
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fruit 1</td>
<td>Fruit 2</td>
</tr>
<tr>
<td>0 min</td>
<td>215 ± 4.8</td>
<td>216 ± 2.2</td>
</tr>
<tr>
<td>15 min</td>
<td>231 ± 4.7</td>
<td>235 ± 1.2</td>
</tr>
<tr>
<td>30 min</td>
<td>240 ± 2.8</td>
<td>220 ± 0</td>
</tr>
<tr>
<td>45 min</td>
<td>240 ± 2.8</td>
<td>220 ± 0</td>
</tr>
<tr>
<td>60 min</td>
<td>239.3 ± 2.9</td>
<td>205 ± 2.3</td>
</tr>
</tbody>
</table>

*Data are the mean ± SE of 4 repeated injection of gas chromatographic determination.

Table 2. Time-course of cumulative ethylene evolved and evolution rate by 'Fuji' apple tissues after vacuum extraction of internal ethylene during a 2-hr incubation.

| Time   | Diffused ethylene
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(nl·g⁻¹)</td>
</tr>
<tr>
<td>Extracted</td>
<td>74.97±16.09</td>
</tr>
<tr>
<td>0 min</td>
<td>0.61 ± 0.56</td>
</tr>
<tr>
<td>15 min</td>
<td>7.27 ± 1.97</td>
</tr>
<tr>
<td>30 min</td>
<td>10.39 ± 2.95</td>
</tr>
<tr>
<td>45 min</td>
<td>13.02 ± 3.57</td>
</tr>
<tr>
<td>60 min</td>
<td>14.72 ± 4.18</td>
</tr>
<tr>
<td>90 min</td>
<td>17.04 ± 4.79</td>
</tr>
<tr>
<td>120 min</td>
<td>17.94 ± 4.30</td>
</tr>
</tbody>
</table>

*Data are expressed as the mean ± SE of 4 repeated injection for gas chromatographic determination.
higher in the vial of tissue which had undergone ethylene extraction (Table 3). The concentration of CO₂ in both vials at the end of the incubation period was less than 0.3% which is statistically negligible (Bufler, 1986). Quantitatively, very similar amounts of internal ethylene were extracted from the paired plugs after the incubation period. Comparison of the total ethylene produced from the test and the control tissues reveals that the summation of the ethylene which was diffused during incubation and that extracted after the incubation period was considered to be the total ethylene production for the ethylene-extracted tissue. The summation of the ethylene which was diffused during incubation and that extracted after the incubation period was considered to be the total ethylene production for the control tissue (Fig. 3). Apparently more ethylene was produced by the ethylene-extracted plugs than the control plugs indicates that ethylene synthesis in excised apple tissue was enhanced by the removal of internal ethylene. However in a few replicates, such enhancement of ethylene production was not detectable when the volume of internal ethylene extracted was very small. The enhancement of ethylene production was proportional to the amount of extractable ethylene in the tissue (Fig. 4). We attribute the difference in the rate of ethylene production between two tissues to the difference in residual concentrations of the internal ethylene because the amounts of ethylene at the end of the incubation periods were very similar (Fig. 3) and the rate of ethylene production gradually became closer (Table 3).

Ethylene is known to regulate its own biosynthesis, both positively and negatively. A negative phenomenon in which ethylene inhibits its own biosynthesis is known as autoinhibition. This auto-

Table 3. Cumulative ethylene evolved (C) and evolution rates (R) from vacuum extracted 'Fuji' apple plugs vs untreated control plugs.

<table>
<thead>
<tr>
<th>Time</th>
<th>Ethylene extracted</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C (nl·g⁻¹)</td>
<td>R (nl·g⁻¹·h⁻¹)</td>
</tr>
<tr>
<td>0 min.</td>
<td>0</td>
<td>4.9</td>
</tr>
<tr>
<td>15 min.</td>
<td>9.3</td>
<td>37.2</td>
</tr>
<tr>
<td>30 min.</td>
<td>15.7</td>
<td>25.6</td>
</tr>
<tr>
<td>45 min.</td>
<td>21.2</td>
<td>22.0</td>
</tr>
<tr>
<td>60 min.</td>
<td>28.1</td>
<td>27.6</td>
</tr>
<tr>
<td>90 min.</td>
<td>32.1</td>
<td>8.0</td>
</tr>
<tr>
<td>120 min.</td>
<td>35.2</td>
<td>6.2</td>
</tr>
</tbody>
</table>

![Fig. 3](image.png)

Fig. 3. Comparison of total ethylene produced between two plugs excised from the opposite sides of the same apple. The left column represent the summation of internal ethylene extracted before incubation in vial (upper); the ethylene which diffused (middle); and the internal ethylene extracted at the end of the 102 min incubation period (lower). The right column represents the total ethylene from the control apple tissues. The symbols are the same as above.
The inhibition of ethylene production has been recognized in a number of fruits and vegetative tissues (Saltveit and Dilly, 1978; Vendrell and McGlasson, 1971; Yang and Hoffman, 1984; Zauberman and Fuchs, 1973). Vendrell and McGlasson (1971) showed that ethylene treatment significantly inhibited wound ethylene production in banana pulp slices. Likewise, slicing of flavedo tissue of citrus fruit enhanced wound ethylene production, but this ethylene production was greatly inhibited by exogenous ethylene (Riov and Yang, 1982). Saltveit and Dilly (1978) demonstrated that autoinhibition of ethylene production occurred in pea segments by exposing them to exogenous ethylene or propylene. They found that the inhibition was rapidly reversed following the removal of ethylene.

In our experiments, ethylene biosynthesis in the evacuated tissue was promoted, whereas it was inhibited in the control plugs. We conclude from these results that autoinhibition of ethylene production naturally occurs in apple fruit when the concentration of internal ethylene rises.

We believe that the ethylene produced by the excision of apple tissue during the sampling period is minimal because it requires only about a minute. In the efficiency test, ethylene formation by wounding/excision was inhibited by CoCl₂, a known inhibitor of ethylene biosynthesis. However, Smith et al. (1992) reported that Co²⁺ appears to stimulate the process at concentrations above ca. 20 μM. Ethylene formation via ACC (1-aminocyclopropane-1-carboxylic acid) oxidase by excised plugs was inhibited by 10 μM Co²⁺ compared to the non-treated plugs (data not shown). In the last experiment, in order to study how ethylene is formed in tissue after the removal of internal ethylene, we did not use any inhibitor of ethylene biosynthesis. Thus, the control tissue and the treated tissue continued to produce ethylene during the incubation period. If significant amounts of ethylene were produced as a result of wounding, ethylene production should have been promoted in all samples, irrespective of the level of internal ethylene (Fig. 4).

Acknowledgement

We would like to show our gratitude to Dr. David Wildon, School of Biological Sciences, University of East Anglia, England, for critical reading of the manuscript and thank Mr. K. Nakachi.
for his cooperation in this investigation.

Literature Cited


リンゴ果実組織内エチレンの減圧抽出・定量法

河野智謙・下川敬之

宮崎大学農学部 889-21 宮崎市学園木花台西1丁目1番地

摘 要

ごく短時間で簡単に行える、正確かつ再現性のある植物果実組織内エチレン定量法を、リンゴ果実組織切片を使用し検討したので報告する。減圧によるエチレンの抽出とガスクロマトグラフによるその測定からなるこの方法では、切片の作成からガスクロマトグラフへのサンプルの注入までを約1分間で終えることができた。この方法により約80%の組織内エチレンを抽出し測定することができた。この方法の特徴は、非破壊的に経過内エチレンを定量できるものであり、1度使用した組織切片からのエチレン生成を引き続き測定できる。この方法を用いて、エチレン除去に伴う急激なエチレン濃度の減少がその後のエチレン生成に及ぼす影響を、エチレンを除去しない組織切片との比較により検討した。組織切片からエチレンを除去すると、その後のエチレン生成量は、エチレンを除去しない組織からの生成量を上回った。しかし、このようなエチレン生成量の増加は、組織内エチレン濃度が低い組織切片からエチレンを除去した場合にはみられなかった。このようなエチレンを除去した組織と除去しない組織とのエチレン生成量の差は、組織内エチレン濃度が高いになるにつれて大きくなり、差の大きさと組織内エチレン濃度との間には正の有意な相関があった。このことは、エチレンを除去しない組織において、存在するエチレン濃度が高くなるとエチレンみずからがエチレン生成を阻害する、いわゆるエチレン生成の自己阻害が起きていることを示唆している。