Embryoid and Callus Formation from Pollen Grains of Eggplant and Pepper by Anther Culture

Sachiko Matsubara, Kailin Hu and Kenji Murakami
Department of Agriculture, Okayama University, Tsushima, Okayama 700

Summary

Effective methods for the production of haploid plants by culturing anthers of eggplant (Solanum melongena L.) and pepper (Capsicum annuum L.) were studied. Anthers containing pollen grains at the uninucleate stage were plated on MS medium supplied with various concentrations of 2,4-D and kinetin and then exposed to high and low temperatures. Embryoids from pollen grain and adventitious shoots from calli were obtained, and they grew to plantlets upon transferring to a MS medium.

Results obtained for eggplant were as follows:

1. Anthers cultured on MS medium supplied with 0.1 mg liter\(^{-1}\) of both 2,4-D and kinetin and treated with 35\(^\circ\)C for 24 hr formed calli and embryos at the highest frequency, but low temperature treatment (5\(^\circ\)C for 24 or 48 hr) was ineffective or rather inhibitory.

2. Regeneration of embryoid and adventitious buds from calli was induced by transferring calli to MS medium without phytohormones or with 1 mg liter\(^{-1}\) kinetin plus 0 or 0.05 mg liter\(^{-1}\) NAA.

3. Plantlets on MS media supplied with 0.8% agar and 5~8% sucrose grew normally and vigorously.

Results for pepper were as follows:

4. Calli were formed at high frequency on MS media supplied with 0.2 mg liter\(^{-1}\) 2,4-D plus 0.1 mg liter\(^{-1}\) kinetin or 0.1 mg liter\(^{-1}\) both of 2,4-D and kinetin. On the other hand, embryos were directly formed at high frequency on MS media supplied with 0.02 mg liter\(^{-1}\) kinetin or 0.004 mg liter\(^{-1}\) 2,4-D plus 0.1 mg liter\(^{-1}\) kinetin.

Introduction

Anther culture is usually used for production of haploid plants from pollen grains and is utilized for a rapid large scale production of homozygous lines from which superior lines for hybrid seed production might be selected. The majority of studies have been conducted on Solanaceae and Cruciferae families (Bajaj, 1983), but the haploid producing rate of these plants in Solanaceae family was rather low except for tobacco. The most important factor for haploid production was the thermal treatment (Bajaj, 1983).

The present study was designed to investigate the conditions necessary to stimulate high yield of embryoids from eggplant and pepper anthers and to regenerate embryoids and adventitious shoots from calli obtained from cultured anthers. The investigation concentrated on the following aspects of the technique, a) the high and low temperature treatments during incubation, b) supplement of phytohormones to the media for embryoids formation and c) regeneration of adventitious buds from callus and kinds of gelling agents and sugar concentrations in the media for plantlet growth.

Materials and Methods

Anthers of eggplant (Solanum melongena L.) ‘Wase Shinkuro’ were obtained from plants which flowered in the field of Okayama University from September to November, 1988 and 1989. Length of anthers used were 1/3~1/2 of petal length. Anthers of pepper (Capsicum annuum L.) ‘California Wonder’ were obtained from plants which flowered in the field of Okayama University from September to November, 1990. They were enclosed in
about 2 mm of petals. Pollen grains in both eggplant and pepper anthers were at an uninucleate stage. A basal medium was composed of Murashige and Skoog inorganic salts (Murashige and Skoog, 1962) plus (all in mg•liter\(^{-1}\)) 100 myo-inositol, 0.5 both of nicotinic acid and pyridoxine•HCl, 0.1 thiamine•HCl, 2.0 glycine, 3% sucrose and a gelling agent, pH was adjusted to 5.7. Kinetin and 2,4-D were supplied at various concentrations to a basal medium. Culture was carried out at 25°C under 16 hr daylength supplemented with a light intensity of 20 \(\mu\text{mol•sec}^{-1}•\text{m}^{-2}\) from a fluorescent light. Thirty flasks were used for each treatment. Various treatments for anther culture were tried to promote the rate of embryoid formation and adventitious shoot regeneration.

For obtaining normal plant growth from embryoids and adventitious shoots, shoot apices were imbedded in a basal medium of gelrite or agar, and supplemented with sucrose at four concentrations, and studied for plant growth and vitrification. Each treatment used ten test-tubes.

1. Phytohormones and high temperature treatments for anther culture

Five to seven pepper anthers were planted in a 100 ml flask containing 30 ml basal medium of 0.2% gelrite and supplemented with sixteen combinations of 0 to 0.1 mg•liter\(^{-1}\) both of 2,4-D and kinetin. Anthers of eggplant were also plated by the above method on the media supplemented with six combinations of 0 to 0.5 mg•liter\(^{-1}\) both of 2,4-D and kinetin. They were heated to 35°C for 24 hr and then transferred to 25°C, or incubated at 25°C without high temperature treatment.

2. Temperature treatments for anther culture

Five to seven eggplant anthers were plated in a 100 ml flask containing 30 ml basal medium of 0.2% gelrite, and supplemented with 0.1 mg•liter\(^{-1}\) both of 2,4-D and kinetin. Cultures were incubated at 35°C for 24 or 48 hr, 5°C for 24 or 48 hr, or 35°C for 24 or 48 hr after the treatment at 5°C for 24 or 48 hr treatment, then they were transferred to 25°C. Control culture was incubated at 25°C without low or high temperature treatment. Numbers of anthers that formed calli and embryoids were counted after 40 days.

3. Regeneration of embryoids and adventitious shoots from callus

Calli were obtained from eggplant anthers cultured on a basal medium supplied 0.1 mg•liter\(^{-1}\) both of 2,4-D and kinetin for 40 days. One callus block (2~5 mm in diameter) was plated in a test-tube containing 10 ml basal medium supplied with 0.2% gelrite and phytohormones at eight combinations of kinetin at 1 and 4 mg•liter\(^{-1}\) and NAA at 0.05, 0.5, 1 and 4 mg•liter\(^{-1}\) and without phytohormones. Numbers of anthers which regenerated embryoids and adventitious shoots were observed after 15 days and 2 months, respectively.

4. Gelling agents and sucrose concentrations for plantlet growth

Eggplant seedlings were used to obtain basal data, since enough embryoids and adventitious buds were not obtained. Seeds were sown on a basal medium supplied 0.8% agar and 3% sucrose without phytohormones. Shoot apices, 1 cm length, were dissected from 10-day-old seedlings. Each shoot was imbedded in a test-tube containing 10 ml of a basal medium supplied with 0.2% gelrite or 0.8% agar, and 2, 3, 5 or 8% sucrose. Ten seedlings were used for each treatment. Growth rates of top and root were observed after one month.

5. Chromosome numbers and morphogenesis of plants from embryoids

Embryoids and adventitious shoots derived from eggplant and pepper anthers were transplanted to a modified basal medium plus 5% sucrose and 0.8% agar and cultured for one month until roots appeared. They were transplanted in pots filled with vermiculite and acclimatized for about 1 month. Root tips were stored in 3°C water for 24 hr, then fixed in 50% FAA, and stained with Schiff’s reagent. Chromosome numbers were counted under microscope.

**Results**

1. Phytohormones and high temperature treatments for anther culture

Pepper anthers formed calli on the media containing 0.02 mg•liter\(^{-1}\) 2,4-D and 0.02 or
0.1 mg·liter⁻¹ kinetin, or 0.1 mg·liter⁻¹ 2,4-D and 0.1 or 0.02 mg·liter⁻¹ kinetin. Embryoids were directly regenerated from anthers on eleven media, and 21.0 or 27.6% of the anthers regenerated embryoids on the media containing 0.02 mg·liter⁻¹ kinetin or 0.004 mg·liter⁻¹ 2,4-D and 0.1 mg·liter⁻¹ kinetin, respectively (Table 1, Fig. 1).

Eggplant anthers on all media held at 35°C for 24 hr formed calli at higher percentage than did that of control without high temperature treatment. Heat-treated anthers produced embryoids at the frequency of 0.6 to 4.9% (Table 2), but those kept at 25°C did not. Anthers on the medium supplied 0.1 mg·liter⁻¹ both 2,4-D and kinetin formed calli and embryoids at the highest frequency (Table 2).

2. Temperature treatments for anther culture

Exposure of eggplant anthers to 35°C was very effective for callus and embryoid formation (Table 3, Fig. 2). In the control treatment, only 5.6% of anthers formed calli but they did not form embryoids, whereas, 23 to 33% of heat-treated anthers for 24 or 48 hr, or 5°C for 24 hr and then transferred to 35°C for 48 hr, formed calli, and 4.8% anthers treated at 35°C for 24 hr formed embryoids. The number of embryoids was 9.6 per 100 anthers plated. Low temperature treatment after planting was ineffective or inhibitory to embryoid formation.

3. Regeneration of embryoids and adventitious shoots from callus

Calli cultured on the media supplied 1 mg·liter⁻¹ kinetin plus 0 and 0.05 mg·liter⁻¹ NAA, or on the phytohormone-free medium regenerated 7.1, 5.9 or 9.2 embryoids per 100 callus blocks, respectively. Callus on media supplied auxin at higher concen-
trations did not regenerate embryoids (Table 4), but those on the former two media and the medium with 4 mg liter⁻¹ kinetin and 1 mg liter⁻¹ NAA regenerated adventitious shoots (Table 4).

4. Gelling agents and sucrose concentrations for plantlet growth

Top and root growth differed depending on kinds of gelling agents and concentrations of sucrose. Shoot length and fresh weight were largest on the medium supplemented with 5% sucrose on Gelrite medium, and shoot length and fresh weight on Gelrite medium were not significantly different from those on 0.8% agar plus 5% sucrose medium, but root density and fresh weight were about a half of those on 0.8% agar plus 5% sucrose medium (Table 5). On the agar medium, root growth was lower at higher sucrose concentrations, but were more numerous and heavier at higher sucrose concentrations. Shoot length and fresh weight were largest on the medium supplemented with 5% sucrose on Gelrite medium, and shoot length and fresh weight on Gelrite medium were not significantly different from those on 0.8% agar plus 5% sucrose medium, but root density and fresh weight were about a half of those on 0.8% agar plus 5% sucrose medium (Table 5). On the agar medium, root growth was lower at higher sucrose concentrations, but were more numerous and heavier at higher sucrose concentrations.

5. Chromosome number and morphogenesis of plants from embryoids

Chromosome numbers in root tips of diploid eggplant and pepper were 24 (Fig. 3), and in those of haploid plants from embryoids, 12 (Fig. 4). In eggplant, 35 plants were haploid, and others were diploid or aneuploids. On the other hand, 90% of plants from pepper embryoids was haploid.
Table 3. Effect of temperature treatments during anther culture on formation of callus and embryoid in eggplant.

<table>
<thead>
<tr>
<th>Temperature treatment</th>
<th>No. of anthers cultured</th>
<th>No. of anthers producing calli</th>
<th>Frequency\textsuperscript{a} (%)</th>
<th>No. of anthers producing embryoids</th>
<th>Frequency\textsuperscript{a} (%)</th>
<th>No. of embryoids per 100 anthers</th>
</tr>
</thead>
<tbody>
<tr>
<td>35°C 24 hr → 35°C 24 hr</td>
<td>167</td>
<td>76</td>
<td>33.5</td>
<td>8</td>
<td>4.8</td>
<td>9.6</td>
</tr>
<tr>
<td>35°C 24 hr → 35°C 48 hr</td>
<td>163</td>
<td>38</td>
<td>23.3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5°C 24 hr → 35°C 24 hr</td>
<td>164</td>
<td>6</td>
<td>3.7</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5°C 48 hr → 35°C 48 hr</td>
<td>170</td>
<td>10</td>
<td>5.9</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Control\textsuperscript{x}</td>
<td>144</td>
<td>8</td>
<td>5.6</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Number of anthers producing calli
\textsuperscript{b} Number of anthers cultured × 100.

Table 4. Effect of concentrations of phytohormones on regeneration of embryoids and adventitious buds from calli in eggplant.

<table>
<thead>
<tr>
<th>Phytohormone</th>
<th>Kinetin (mg·liter(^{-1}))</th>
<th>NAA</th>
<th>No. of anthers cultured</th>
<th>No. of calli regenerating embryoids</th>
<th>Frequency\textsuperscript{a} (%)</th>
<th>No. of embryoids per 100 calli</th>
<th>No. of calli regenerating adventitious buds</th>
<th>Frequency\textsuperscript{a} (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>0</td>
<td>65</td>
<td>4</td>
<td>6.1</td>
<td>9.2</td>
<td>0</td>
<td>0</td>
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<tr>
<td></td>
<td>0</td>
<td>1.00</td>
<td>19</td>
<td>0</td>
<td>0.0</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>0</td>
<td>42</td>
<td>2</td>
<td>4.8</td>
<td>7.1</td>
<td>2</td>
<td>4.8</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>0.05</td>
<td>40</td>
<td>2</td>
<td>5.0</td>
<td>5.0</td>
<td>3</td>
<td>7.5</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>0.50</td>
<td>40</td>
<td>0</td>
<td>0.0</td>
<td>0.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>1.00</td>
<td>40</td>
<td>0</td>
<td>0.0</td>
<td>0.0</td>
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<tr>
<td></td>
<td>1.00</td>
<td>4.00</td>
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<tr>
<td></td>
<td>4.00</td>
<td>1.00</td>
<td>37</td>
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<td>40</td>
<td>0</td>
<td>0.0</td>
<td>0.0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Number of anthers regenerating embryoids
\textsuperscript{b} Number of anthers cultured × 100.

Fig. 3. Chromosomes in a root tip of a diploid eggplant.

Fig. 4. Chromosomes in a root tip of a haploid eggplant.
rest was diploid. Growth of haploid plants in both eggplant and pepper was not vigorous and the plants were small compared with diploid plants (Fig. 5). The leaf of the haploid eggplant characteristically lacked the proximal half. Flowers of haploid eggplant and pepper plants were smaller than were those of diploid flowers and they were male sterile (Fig. 6); therefore, some haploid plants produced parthenocarpic fruits (Fig. 7).

Table 5. Effect of sucrose concentration and gelling agents on growth of plantlets in eggplant.

<table>
<thead>
<tr>
<th>Gelling agent (%)</th>
<th>Sucrose (%)</th>
<th>Plantlet growth</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Root length (cm)</td>
<td>Root fresh weight (mg)</td>
</tr>
<tr>
<td>Agar (0.8)</td>
<td>2</td>
<td>8.4 a</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>6.4 b</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>4.3 c</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>3.6 c</td>
</tr>
<tr>
<td>Gelrite (0.2)</td>
<td>2</td>
<td>4.3 c</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>4.2 c</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>3.9 c</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>0 d</td>
</tr>
</tbody>
</table>

* Within each comparison, values in one column followed by the same letter are not significantly different (P = 0.05).

Fig. 5. Leaves of haploid (upper) and diploid plants (lower) of eggplant and pepper, respectively.

Fig. 6. Flowers of diploid (right) and haploid plants (left) of eggplant and pepper, respectively.
Discussion

Several researchers have reported on the production of haploid plants from anthers of eggplant (De Vaulx and Chambonnet, 1982; Isouard et al., 1979) and pepper (De Vaulx et al., 1981; Kuo et al., 1973). The most important factor for formation of callus or embryoid according to these reports was the effectiveness of the thermal treatments. The high temperature (35°C) in the dark during the first 8 days of in vitro culture of eggplant (De Vaulx and Chambonnet, 1982) and pepper anthers (De Vaulx, 1981) was effective for promoting plant regeneration. On the other hand, low temperature treatment was effective in other species such as Datura (Nitsch and Norreel, 1973), tomato (Debergh and Nitsch, 1973) and tobacco (Radish and Reinert, 1981). In the present study on eggplant, although anthers were treated with high (35°C) and low (5°C) temperatures under the dark condition during the first 24 hr, only high temperature was effective, whereas, low temperature was ineffective or inhibitory. The stimulatory effect of thermal shocks combined with centrifugation (Sangwan-Norreel, 1977) or charcoal treatment (Bajaj and Reinert, 1977) has also been observed. As early as 1922, Blakeslee et al. obtained haploid Datura stramonium plantlets from plants exposed to low temperatures at the time of fertilization. These stimulatory thermal shocks may activate cell division of pollen grains at uninucleate stage. Thermal shocks given to plants could alter the mode of division of the microspore nucleus (Sax, 1935; Stow, 1930), by causing the dissolution of microtubules and dislodging the spindle which, in turn, cause abnormal division of the microspore nucleus (Helper and Palevitz, 1974). The period of treatment also differed among the studies. The period was very short in the present study in comparison with 8 days in other studies (De Vaulx, 1981; De Vaulx and Chambonnet, 1982), but both periods showed the same effectiveness as follows; 12 and 18 plants were produced from 100 anthers by the 8-day treatment in eggplant and pepper, respectively (De Vaulx et al., 1981; De Vaulx and Chambonnet, 1982), and 12.6 plants including 9.6 plants from direct embryogenesis and 3 plants from callus were produced from 100 eggplant anthers by a 24-hr treatment and 41 plants from 100 pepper anthers without high temperature treatment, in the present study. Therefore, the effectiveness of thermal treatment might vary depending on cultivars, species, temperatures, and duration.

The compositions of the media and the phytohormone concentrations in the media are important factors in determining plant production from anthers and the subsequent development of the plant. Pollen embryogenesis can be induced on a simple mineral-sucrose medium in plants like tobacco (Nitsch, 1969) and Hyoscyamus (Raghavan, 1975), for androgenesis to be completed, a supplement of certain phytohormones is required. For instance, cereal anthers require both auxins and cytokinins (Clapham, 1977), and eggplant and pepper anthers also require 2,4-D and kinetin. The high temperature treatment combined with a supplement of 0.01 mg·liter⁻¹ both of 2,4-D and kinetin were necessary for plant regeneration in eggplant (De Vaulx and Chambonnet, 1982), but in the present study, 0.1 mg·liter⁻¹ both of 2,4-D and kinetin were at the same level also effective. Although lower concentrations of phytohormones such as 0.01 mg·liter⁻¹ were effective for plant regeneration at 25°C (De Vaulx and Chambonnet, 1982), only callus formation was observed in other present study. Effective concentrations of phytohormones were, however, different for callus formation, and for direct formation of embryoid, and for eggplant and pepper. Complex media enriched with auxins such as 2,4-D encourage the formation of callus should be avoided. Simple media with low levels of auxins are advisable to promote direct embryogenesis. Pepper formed embryoids more readily than did eggplants.

Regeneration of embryoids and adventitious shoots from eggplant calli needed supplemental addition of 1 mg·liter⁻¹ kinetin or 1 mg·liter⁻¹ kine-

Fig. 7. Normal fruit of a diploid plant (right) and parthenocarpic fruit of a haploid plant (left) of pepper, respectively.
tin plus 0.05 mg·liter⁻¹ NAA. The addition of auxin, especially 2,4-D, to the medium appeared to influence the ploidy of the regenerated plants (Gleddie et al., 1986); 15–50% of the regenerated plants were diploid. In the present study with 2,4-D, 35 and 17% of the regenerated plants were haploid and diploid, respectively. It was not clear whether they were derived as dihaploids from pollen grains or diploids from anther walls.

Haploid plants were smaller than diploid plants in both eggplant and pepper, and were male sterile. These morphological characteristics were common in many species. The proximal half of leaf was observed to be lacking in haploid eggplants. It is not clear if this characteristic is specific to ‘Wase Shinkuro’, or not.

**Literature Cited**


ナスおよびピーマンやく培養による花粉からの半数体とカルス形成

松原幸子・胡 関林・村上賢治
岡山大学農学部 700 岡山市総合

摘 要

ナスおよびピーマンのやく培養による半数体の生産率を高めるための処理を試みた。1 核期の花粉を含んだやくを種々の 2, 4-D とカイネチンを含む MS 培地に植え付け、培養物は高・低温処理をした。胚様体、カルスからの不育胚が得られ、それらを MS 培地に移植し、次回の結果を得た。ナスについては次の結果を得た。

1. 培地については、0.1 mg·liter⁻¹の 2, 4-D とカイネチンを添加した培地に植え付けたやくで最も高率にカルスや胚様体を形成した。温度処理については、培地に植え付けたやくを 35℃で 24 時間処理するものがカルスや胚様体形成に最も効果的で、低温処理は効果がないか、むしろ抑制的であった。

2. カルスからの胚様体や不定芽再生は、ホルモン無添加、1 mg·liter⁻¹のカイネチン、または 1 mg·liter⁻¹のカイネチンと 0.05 mg·liter⁻¹の NAA 添加培地にカルスを移植することにより効果的に得られた。

3. 小植物体は 0.8 % 寒天と 5 〜 8 % のショ糖、または 0.2 % ゲルライトと 3 〜 5 % ショ糖を添加した培地に移植したとき正常に生育した。

ピーマンについては次の結果を得た。

4. カルスは培地に 0.1 mg·liter⁻¹ 2, 4-D と 0.1 mg·liter⁻¹カイネチン、または 0.1 mg·liter⁻¹の 2, 4-D とカイネチンを添加した培地で高率に得られた。一方、花粉から直接胚様体を得るためには、0.02 mg·liter⁻¹カイネチン添加、または 0.004 mg·liter⁻¹ 2, 4-D と 0.1 mg·liter⁻¹カイネチン添加培地に植え付けるが有効であった。