Studies on the Breeding of Triploid Plants by Diploidizing Gamete Cells.

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1. Introduction

Available information indicates that there are many natural triploids among the good varieties of cultivated plants which can be asexually propagated. The fact that these triploids have been selected and developed in spite of their sterile habit goes to show that they have certain advantages that are elsewhere not possible.

In fact, triploid mulberries rather than diploid mulberries are widely cultivated in Japan because of their higher yield of leaves (OsaWA 1916); and triploid apples, which are common both in Europe and America, are more vigorous in growth and live longer than diploid apples (HEILBORN 1932). It is well known that the seedless advantage of banana is due to the reproductive failures, characteristic in triploids (CHEESMAN 1932). Further, works with flowers such as tulips, lilacs, gladiolus and hyacinths, show triploids have larger and more showy flowers than diploids. SIMURA & INABA (1953) have pointed out that triploid varieties of tea plants are more hardy to cold than diploid varieties.

Triploids may be obtained by crossing tetraploids, which are induced from diploids, with diploids. This method had been successfully explored by Kihara & Nishiyama (1947) as method for producing seedless watermelons. Similarly, Matsumura & Mochizuki (1953) have produced triploid sugar beets, which gave a yield greater than diploid beets.

In woody plants such as tea, mulberry and the various fruit trees this method, however, is not always convenient from the practical standpoint, because even if the tetraploids are induced, it takes a long time to obtain the flowers necessary for crossing.

Hence, the author attempted a new method, using tea plants to produce triploids with out tetraploids by diploidizing gamete cells in diploids. The method is shown diagrammatically in Fig.1.

![Diagram](image_url)

Fig. 1 Comparison of former and author's methods.

2. Methods and Results

Tea plants, native to the Nagoya district, whose diploid chromosome number (2n=30) was previously ascertained, were used as the materials.

In early September of 1953, more than 340 flowering buds, ranging from 3 to 4 cm. in diameter, whose anthers were in the meiotic stage, were treated with a 0.05, 0.1 and 0.2 per cent aqueous solution of colchicine. In some buds the colchicine was injected into them by using a thin syringe needle, and in the other buds it was dropped on them which previously had been pin-holed and wrapped in a mass of cotton by means of a glass.

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eye-dropper. Using the injection method 0.2 to 0.3 cc of 0.05, 0.1 and 0.2 per cent colchicine solution respectively was administered per flowering bud per day for 1 and 2 days respectively. Using the dropping method 2 to 3 cc of 0.05, 0.1 and 0.2 per cent colchicine solution respectively was administered per flowering bud per day for 3 and 6 days respectively (see Table 2). The treatments were performed in a field covered with vinyl tents.

When the treated flowering buds began to flower in the middle of October, later than the untreated buds, it was found that some of the flowers contained giant pollen grains as well as normal ones (Fig. 2).

![Fig. 2 Normal and giant pollens and their germination.](image)

1: Normal pollen. 2: Giant pollen mixed with normal pollen. 3: Germination of normal pollen. 4: Germination of giant pollen.

Table 1 shows the giant and normal pollen sizes. In polyploids Takenaka (1943) had demonstrated that when the nuclear quantity increased N times the size of the organs were \( \sqrt[3]{N} \) times; therefore, from his theory, it was considered that the nuclear quantity of giant pollen grains would be doubled because they expressed an increased rate of pollen size similar to \( \sqrt[3]{2} \) or 1.26 times that of normal ones.

<table>
<thead>
<tr>
<th></th>
<th>Longitudinal Diameter (( \mu ))</th>
<th>Width Diameter (( \mu ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Pollen</td>
<td>42.4 ± 0.6 (1.00)</td>
<td>31.5 ± 0.5 (1.00)</td>
</tr>
<tr>
<td>Giant Pollen</td>
<td>55.5 ± 1.0 (1.30)</td>
<td>43.1 ± 1.1 (1.37)</td>
</tr>
</tbody>
</table>

Number in ( ) shows ratio index.

Of the different treatments, the best results were obtained by dropping a 0.05 per cent solution of colchicine on the buds for three days. In this treatment 37.9 per cent of the flowers contained giant pollen. Using solutions of 0.1 and 0.2 per cent, flowers having only abortive pollen instead of giant ones were produced. The results may be seen in Fig. 3 and Table 2.

![Fig. 3 Pollens derived from various treatments.](image)

1: Normal pollen from non-treatment. 2: Giant pollen mixed with normal and abortive pollens from adequate treatment. 3: Abortive pollen from stronger treatment.
Table 2. Results of the colchicine treatments of flowering buds.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% of Colchicine</th>
<th>Number of Days</th>
<th>Total of Flowering Buds Treated</th>
<th>% of Flowering Buds Killed</th>
<th>% of Flowers with Normal Pollen</th>
<th>% of Flowers with Abortive Pollen</th>
<th>% of Flowers with Giant Pollen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Injection</td>
<td>0.05</td>
<td>1</td>
<td>27</td>
<td>14.3</td>
<td>29.3</td>
<td>55.5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>28</td>
<td>14.3</td>
<td>25.0</td>
<td>39.3</td>
<td>21.4</td>
</tr>
<tr>
<td></td>
<td>0.10</td>
<td>1</td>
<td>21</td>
<td>11.1</td>
<td>29.6</td>
<td>59.3</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>25</td>
<td>20.0</td>
<td>24.0</td>
<td>56.0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.20</td>
<td>1</td>
<td>30</td>
<td>16.7</td>
<td>23.3</td>
<td>60.0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>29</td>
<td>20.7</td>
<td>13.8</td>
<td>65.5</td>
<td>0</td>
</tr>
<tr>
<td>Dropping</td>
<td>0.05</td>
<td>3</td>
<td>29</td>
<td>6.9</td>
<td>27.6</td>
<td>27.6</td>
<td>37.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>27</td>
<td>11.1</td>
<td>26.0</td>
<td>33.3</td>
<td>29.6</td>
</tr>
<tr>
<td></td>
<td>0.10</td>
<td>3</td>
<td>24</td>
<td>12.5</td>
<td>25.0</td>
<td>62.5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>30</td>
<td>16.7</td>
<td>23.3</td>
<td>56.7</td>
<td>3.3</td>
</tr>
<tr>
<td></td>
<td>0.20</td>
<td>3</td>
<td>34</td>
<td>26.5</td>
<td>23.5</td>
<td>50.0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>32</td>
<td>21.9</td>
<td>21.8</td>
<td>56.3</td>
<td>0</td>
</tr>
</tbody>
</table>

According to an investigation made by cotton blue staining giant pollens were found mixed with normal and abortive pollens forming about 10.5 per cent (Table 3), although the percentage varied with each flower.

Table 3. Proportion of giant, abortive and normal pollens in a flower.

<table>
<thead>
<tr>
<th>No. of Flowers Used</th>
<th>Total Pollens Observed</th>
<th>% of Giant Pollen</th>
<th>% of Abortive Pollen</th>
<th>% of Normal Pollen</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>626</td>
<td>10.5</td>
<td>36.7</td>
<td>52.5</td>
</tr>
</tbody>
</table>

In order to compare the germination of giant pollen and the growth of their tubes with those of normal ones, a germination test was performed in pollens cultured in a drop of water, hanging under the cover glass covering a hole-slide.

As shown in Fig. 2 and 3, giant pollen showed lower germinability than normal pollen and a retarded growth of their tubes; therefore, it was necessary to select the giant pollen for crossing purposes.

Fig. 4 Comparison of the germinability of giant and normal pollens.

Fig. 5 Comparison of the growth of giant and normal pollen tubes.
The following year, many treatments were performed by dropping a 0.05 per cent solution of colchicine on the buds for three days, this method having given the best results in the previous year. The giant pollen grains thus induced were carefully selected, using a preparation microscope magnifying 60 times, and transferred with a needle to the stigmata of diploid flowers which had been previously castrated. About 10 pollens were given to each stigma. After that the pollinated flowers were wrapped in paraffin paper. A series of photographs on the procedure may be seen in Fig. 6.

![Fig. 6. Steps in procedure.](image)


Of the 53 flowers pollinated, 7 developed successfully into fruit in the following year and each fruit formed only one seed as is common in artificially pollinated flowers of tea plants. The seeds obtained at their maturity showed no differences in outward appearance from diploid natural seeds (Fig. 7).

![Fig. 7 Seeds formed at maturity.](image)

1: Natural seeds. 2: Seeds obtained as the result of pollination with giant pollens.

Fig. 8 Seedlings of diploid (Cont.) and artificially induced triploids (Nos. 1 and 2).

Fig. 9 Stomata of diploid (Cont.) and artificially induced triploids (Nos. 1 and 2).

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Table 4. Comparison of the stomata of diploid and artificially induced triploid seedlings.

<table>
<thead>
<tr>
<th></th>
<th>Number of Stomata per 1/8 mm²</th>
<th>Stomatal Size</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Longitudinal Diameter (μ)</td>
</tr>
<tr>
<td>Diploid</td>
<td>Cont.</td>
<td>23.1±0.6 (100)</td>
</tr>
<tr>
<td></td>
<td>Experimental Triploid</td>
<td></td>
</tr>
<tr>
<td>No. 1</td>
<td>10.6±0.3 (46)</td>
<td>43.2±0.5 (142)</td>
</tr>
<tr>
<td>No. 2</td>
<td>12.7±1.1 (55)</td>
<td>41.2±0.7 (136)</td>
</tr>
</tbody>
</table>

Number in ( ) shows ratio index.

The determination of the chromosome number was made by employing the paraffin method to root tips fixed with CRAF's fluid. NEWTON's gentian violet staining schedule was used. As shown in Fig. 10, both the seedlings had the chromosome number of 45.

Adding to the series of the experiments, above mentioned, a cytological study was made on the meiosis of the flowering buds which had been treated with colchicine, using the aceto carmine smearing method. The chromosomes of the first and second metaphases were scattered at random or lumped up without being arranged regularly on the equator (Fig. 11). From these observations it was thought that giant pollen grains were produced by the use of colchicine as a result of the disturbance of the spindle functions and cell membrane formation, as previously reported regarding the effects of colchicine on meiosis of many other plants by other workers (WALKER 1938, LEVAN, SATO, SHIMAMURA, TAKENAKA 1939).

![Fig. 10 Somatic chromosomes of artificially induced triploids.](image)

![Fig. 11 Meiotic chromosomes in the flowering buds treated with colchicine.](image)

1-4 and 5-8 show the first and second metaphases respectively. 1, 5: Regularly arranged chromosomes. 2, 3, 6, 7: Lumped chromosomes. 4, 8: Chromosomes scattered at random.

3. Discussion

It was very difficult to carry out the selection of giant pollen microscopically. The reason, that the giant pollens were mixed with normal and abortive pollens, seemed to be that the anthers in a flowering bud belonged to different development stages at the
time of the treatments. Further, it was not considered favourable that most of the flowers failed to produce fruit after pollination; however, that fact is not specific to this experiment, but also is common in all normally pollinated flowers of tea plants (Simura & Osone 1956). Greater success with this experiment may depend upon solving these problems.

Although only 2 triploid tea plants were obtained from this experiment, they could be numerous propagated by cutting. Thus, in spite of the difficulty of the technique and the low probability of success this method will be promising for plants which can be asexually propagated.

It also may be possible to produce triploids by diploidizing egg cells. This method seems not to be as efficient as the method diploidizing pollen, because unreduced egg cells can not be selected before pollination. Actually, the author has found one triploid mulberry seedling among about three thousand seeds derived from flowering buds treated with a 0.05 per cent solution of colchicine; however, the frequency of emergence was too low to attribute it to the treatments. In the breeding of triploid watermelons, Kihara & Nishiyama (1947) have reported that only crosses involving the female as the tetraploid and the male as the diploid pollinator were successful, though in sugar beets (Matsumura & Mochizuki 1953) the reciprocal procedure also succeeded. In such cases, the application of this method must be made on egg cells.

It may be assumed that there are three methods in which triploids may be produced in nature: 1) triploids produced by the crossing of diploids and tetraploids; 2) triploids derived from the formation of unreduced gametes in diploids; 3) triploids produced by other triploids. The results of this experiment demonstrates the second method.

4. Summary

1) Rather recently, it has been recognized that some of the natural triploids in cultivated plants are more vigorous in growth or more hardy to cold as compared with diploids.

2) Triploids can be obtained by crossing tetraploids with diploids. In woody plants, however, this method is not so convenient because the tetraploids, if induced, require a long time to produce the flowers necessary for crossing; therefore, a new attempt was made with tea plants to produce triploids without tetraploids, by diploidizing gamete cells (Fig. 1).

3) The flowering buds at the meiotic stage were treated with colchicine. At the flowering stage some of the flowers contained giant pollen which seemed to have a diploidized chromosome number (Fig. 2, Table 1). The best results were obtained by dropping 0.05 per cent solution of colchicine on the flowering buds which previously had been pin-holed and wrapped in a mass of cotton (Table 2, Fig. 3).

4) As giant pollen mixed with normal and abortive ones and showed a lower germinability and a retarded growth of their tubes (Table 3, Fig. 4, 5), some of them were carefully selected, using a preparation microscope magnifying 60 times, and transferred with a needle to the stigma of diploid flowers (Fig. 6).

5) Among the 53 flowers pollinated 7 developed successfully into fruit, of which each fruit formed only one seed at maturity (Fig. 7). The germination test resulted in 2 seedlings having larger stomata with a lower frequency on the abaxial surface of the leaf than diploids (Fig. 8, 9, Table 4). Finally, it was determined from the somatic chromosomes in their root tips that both of the seedlings were triploids having a chromosome number of 45 (Fig. 10).

6) A cytological study on the meiosis of the flowering buds treated with colchicine showed that the chromosomes in the first and second metaphases were scattered at ran-
dom or lumped up without being arranged regularly on the equator (Fig. 11): therefore, it was thought that giant pollens were produced by the use of colchicine as a result of the disturbance in the spindle functions and cell membrane formation.

Acknowledgements

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Literature cited


生殖細胞倍加による三倍体植物の育成に関する研究

大曾根元一

最近、多くの栽培植物に於て、自然三倍体の品種が、二倍体に比べて生育が旺盛であったり、又は、耐寒性が強いなどの優れた点が認められるに至った。

三倍体は、二倍体と四倍体との交雑によって得ることができる。しかし、木本性植物に於ては、何れ四倍体を作り得ても、それが交雑に必要な花をつけるまでは長い年月を要するので、筆者はニトウを材料として、二倍体植物の生殖細胞を直接倍化させて、直ちに三倍体を育成する方法を試みた（Fig. 1）。

成熟分裂期の薬をコルヒチンで処理し、開花期に幾つかの花から、染色体数を倍化したと思われる巨大花粉を得ることが出来た（Fig. 2, Table 1）。薬を結び包んで0.05%コルヒチン水溶液を滴下したもののが、最も高い成功率を示した（Table 2, Fig. 3）。

巨大花粉は、正常花粉より不稔花粉と共存し、発芽率・花

粉管の伸長長に正常花粉より劣っていたので（Table 3, Fig. 4, 5）。針を用いて、個数毎に（x60）で観察し、二倍体植物の柱頭上に人工交配を行った（Fig. 6）。

交配された53花の内、7花が結果し、各々1誔の種子を生じた（Fig. 7）。発芽試験の結果、二倍体植物よりも大きな形を有する2誔の種子を得ることができた（Fig. 8, 9, Table 4）。根端細胞の染色体の観察から、何れも45の染色体数を持つ三倍体であることが確かめられた（Fig. 10）。

コルヒチンで処理した薬の花粉母細胞に於て、第1・第2分裂中期の染色体が、赤道板に並べないで不規則に散らばったり、又は固定され sjするもののが観察された（Fig. 11）。紡錘体の機能と細胞膜の形成が抑制された結果、巨大花粉が形成されたものと考えられた。（摘要）