

Callus and Embryoid Induction by Anther Culture of Apple

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Introduction

Apple (*Malus pumila* MILL. var. *domestica* SCHNEID.) is one of the fruit trees in which it is difficult to select and produce a true-breeding line for some useful agronomic characters because it is heterozygous in many loci due to self-incompatibility and because it has a long juvenile period. Since apple is very hard to propagate by cuttings, it is commonly grafted on seedlings or clonal rootstocks. Tissue culture techniques have the possibility of offering several useful means of solving these problems. Indeed shoot apex culture of apple has been used to propagate own-rooted trees of many cultivars (JONES *et al.* 1979, ZIMMERMAN and BROOME 1980). Haploid plant production by anther culture also promises the reliable selection and rapid establishment of true-breeding lines for some important characters such as disease or insect resistance.

The formation of callus from apple anthers and attempts to obtain organogenesis from this callus have been reported (NAKAYAMA *et al.* 1971, 1972). This report describes the formation of callus from pollen and from anther wall tissue, differences in callus formation among four apple cultivars, and effects of different medium composition on callus formation. In addition, embryoid induction directly from pollen is also reported.

Materials and Methods

Four apple cultivars, 'Fuji', 'Ralls Janet', 'Jonathan' and 'Megumi', were used. Flower buds were excised just before the petals turned pink. Most of the pollen grains were uninucleate at this stage of flower development when they were examined under a microscope.

Calyxes and petals were removed from the flower buds and then about 20 anthers from each flower were planted aseptically on the sterile medium in an 18×105 mm test tube. Approximately 350 anthers of each cultivar were planted on each type of medium.

The basal medium used was the revised one of MURASHIGE and SKOOG (1962) supplemented with 30 g/l of sucrose and 8 g/l of agar. Seven types of media were used for anther culture (Table 1). The basal medium was supplemented with the growth regulators, indole-3-acetic acid (IAA), α -naphthaleneacetic acid (NAA) and kinetin in various combinations. In addition, the sucrose concentration was increased to 50 g/l in media F and G. The development of embryoids derived from pollen grains was promoted with the basal medium or the basal medium supplemented with 1 mg/l of 6-benzylamino-purine (BAP). The media were adjusted to pH 5.8 before autoclaving.

The cultures were maintained at 25°C under 12 hr of illumination provided by fluo-

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rescent light.

The ploidy level of pollen callus cells was determined by counting the chromosomes stained by alcoholic hydrochloric acid-carmin (SNOW 1963).

Results and Discussion

Callus from Pollen and Anther Wall Tissue

Both pollen and anther wall tissue formed callus (Table 1). The pollen-derived callus emerged from anthers which split open or in which the walls burst. These calluses were white and friable, and easily separated from the anther wall tissue. In contrast, whole or a part of the anther sometimes thickened and formed somatic calluses that were firm, compact, and often turned green. Chromosomal examination of callus cells from pollen showed that they were predominantly haploid, but polyploid and aneuploid cells were also observed frequently. These might be originated by endomitosis and/or chromosomal irregularities during culture.

The Frequency of Callus Formation

The frequency of callus formation from both pollen and anther wall tissue varied significantly among the four cultivars tested (Table 1). Pollen of all four cultivars formed callus on basal medium and the addition of 0.1 mg/l kinetin alone increased the frequency of this callus formation only with 'Megumi'. IAA in combination with this concentration of kinetin increased the frequency of pollen callus formation only of 'Fuji' and then only at 30 g/l of sucrose. Combining NAA with kinetin improved the frequency of callus formation from pollen of three of the cultivars, but the cultivars varied in response to different concentrations of these two growth regulators. NAA also increased the frequency of callus formation from anther wall tissue in all four cul-

Table 1. Callus formation by anther culture of apple

| Medium type | Growth regulators (mg/l) | | | | Callus type* | % of anthers forming callus** | | | | |
|-------------|--------------------------|-----|---------|---------------|--------------|-------------------------------|-------------|----------|--------|--|
| | IAA | NAA | Kinetin | Sucrose (g/l) | | Fuji | Ralls Janet | Jonathan | Megumi | |
| A | — | — | — | 30 | P | 1.3 | 1.6 | 10.3 | 17.0 | |
| | | | | | S | 0 | 4.4 | 0.3 | 0 | |
| B | — | — | 0.1 | 30 | P | 1.2 | 2.2 | 9.2 | 27.0 | |
| | | | | | S | 0 | 7.1 | 0 | 0 | |
| C | 0.1 | — | 0.1 | 30 | P | 3.2 | 0.9 | 8.7 | 7.4 | |
| | | | | | S | 0 | 3.3 | 0 | 0 | |
| D | — | 0.1 | 0.1 | 30 | P | 5.2 | 21.3 | 15.4 | 9.0 | |
| | | | | | S | 17.0 | 32.7 | 0 | 8.1 | |
| E | — | 1.0 | 1.0 | 30 | P | 2.4 | 27.5 | 5.3 | 8.4 | |
| | | | | | S | 21.8 | 55.8 | 16.3 | 17.1 | |
| F | 0.1 | — | 0.1 | 50 | P | 0 | 0 | 3.7 | 12.2 | |
| | | | | | S | 0.6 | 1.5 | 0 | 0.9 | |
| G | — | 0.1 | 0.1 | 50 | P | 4.6 | 1.7 | 4.7 | 10.1 | |
| | | | | | S | 23.3 | 6.3 | 6.2 | 14.0 | |

* P, pollen callus; S, somatic callus from anther wall tissue.

** After two months of culture.

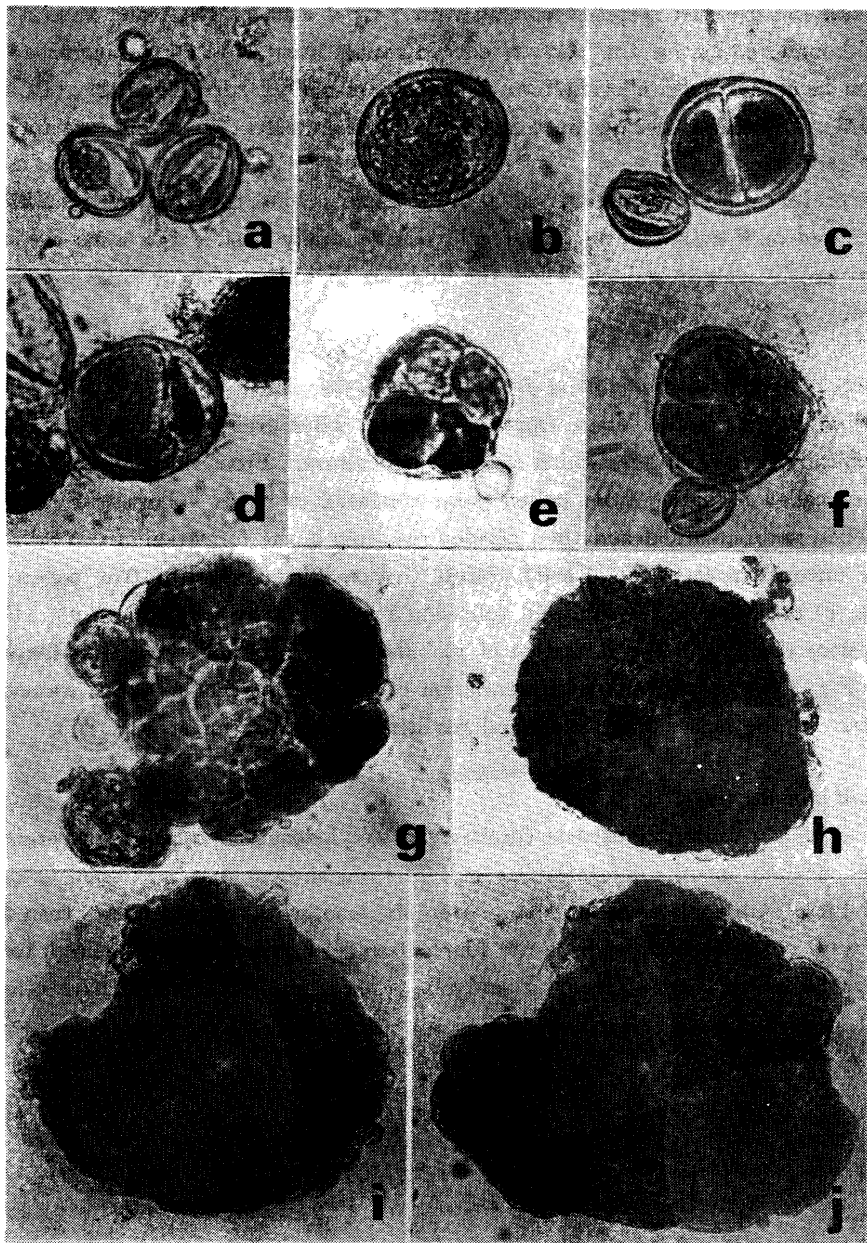


Fig. 1. The process of callus formation from pollen by anther culture of apple.

- a, Uninucleate pollen grains.
- b, Enlarged pollen grain.
- c, and d, Two equal and unequal cells in size within the pollen exine respectively, after the first cell divisions.
- e, Four cells in the exine after the second division of both cells shown in c.
- f, Three cells in the exine after the second division of the larger cell shown in d.
- g, Multicellular body released from the exine.
- h, Globular embryoid.
- i, Heart-shaped embryoid.
- j, Dedifferentiation of the heart-shaped embryoid to callus.

tivars, with the greatest response coming with 1 mg/l each of NAA and kinetin (Table 1). Although increasing the sucrose concentration from 30 to 50 g/l increased callus formation in anther culture of *Lolium multiflorum* and *Hordeum vulgare* (CLAPHAM 1971, 1973), a similar increase here had no significant effect on callus formation from pollen or anther wall tissue.

For preferential induction of callus from apple pollen, the basal medium alone or supplemented only with kinetin (0.1 mg/l) was generally best. For some cultivars in which it was difficult to induce callus, the addition of either IAA or NAA proved to be effective.

The Process of Callus Formation from Pollen

Various developmental stage from uninucleate pollen grains to callus were observed microscopically within anthers which split during culture. From these observations, the process of callus formation from pollen grain appeared to be as follows. First, uninucleate pollen grain enlarged and then divided resulting in the formation of two cells either equal or unequal in size (Fig. 1 a-d). Cell division continued within the pollen exine (Fig. 1 e, f) until the exine ruptured releasing a multicellular body (Fig. 1 g). Further cell divisions resulted in the formation of globular (Fig. 1 h) and then heart-shaped embryoid (Fig. 1 i). However, dedifferentiation occurred after that as callus formed from the surface of the heart-shaped embryoid resulting in a callus mass (Fig. 1 j).

Embryoid Formation

In addition to callus, embryoids consisting of cotyledons and hypocotyl emerged after two and one half months of continuous culture from anthers that split open (Fig. 2 a). Most of these embryoids were albino. After six months of culture, more than 20 embryoids were obtained from each of the four cultivars on different media, except for those containing 50 g/l of sucrose (media F and G). Those embryoids might have originated

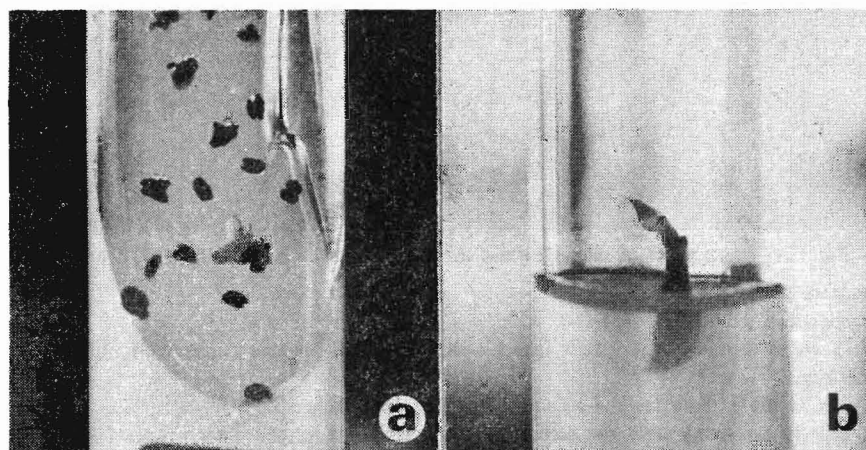


Fig. 2. Apple embryoids induced by anther culture.

- a, Embryoid with cotyledons and hypocotyl, emerging from inside an anther which has split open.
 b, Embryoid with elongated hypocotyl and expanded leaf-like structure.

from heart-shaped embryoids on which callus formation did not occur. This embryoid formation may have resulted from changes in the medium composition during the long term culture, but the exact cause could not be determined exactly.

The embryoids obtained were transferred to fresh basal medium or to basal medium supplemented with 1 mg/l of BAP. With time, most of the embryoids enlarged and turned green. The medium containing BAP was more effective in stimulating embryoid development than the basal medium. The hypocotyl of one of these transferred embryoids elongated and formed an expanded leaf-like structure (Fig. 2 b). However, the embryoids gradually developed abnormally and tended to dedifferentiate and form callus. No roots were initiated on any of the embryoids.

Despite the difficulties encountered in growing the embryoids, the fact that they were produced directly from pollen grains is an important step toward production of haploid apples, particularly since organ differentiation from apple pollen callus has not yet been possible.

Summary

In the anther culture of apple, both pollen and anther wall tissue formed callus on various medium used, although the frequency of callus formation varied among the four cultivars tested. For preferential induction of pollen callus, the basal medium, the revised one of MURASHIGE and SKOOG (1962), alone or supplemented only with 0.1 mg/l of kinetin was generally best. For some cultivars in which it was difficult to induce pollen callus, the addition of either IAA or NAA proved to be effective, while NAA also increased the callus formation from anther wall tissue.

By the microscopical observation of cultured anthers, the process of pollen callus formation in the course of culture appeared to be as follows. First, uninucleate pollen grain repeated cell division as embryogenesis and then formed heart-shaped embryoid. But after that, dedifferentiation occurred from the surface of embryoid resulting in a callus mass.

However, in some cases, perhaps without the dedifferentiation, embryoids consisting of cotyledons and hypocotyl emerged. On the fresh basal medium or the medium supplemented with 1 mg/l of BAP, most of them enlarged in size, and one of them expanded leaf-like structure, but no roots were initiated on any of them.

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