Origin and Development of Embryoids from Microspores in Anther Culture of Citrus

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Anthers of *Poncirus trifoliata* and *Citrus aurantium* cultured on agar media were cytologically examined at three-day intervals for microspore dedifferentiation. The results proved that plantlets obtained by anther culture of these citrus originated from microspores. Microspore development *in vitro* followed three main routes. In route A and B, microspores ultimately lost their contents. In route C, multi-nucleate-cellular structures developed into microspore embryoids. In this route C, microspores showed abnormal nuclear division; one route (C₁), produced two types of morphologically similar nuclei which were rather large or small and classified as 'vegetative-type' (C_{1a}) or 'generative-type' nuclei (C_{1b}) respectively. The other route (C₂), produced 'vegetative-type' and rather small 'generative-type' nuclei. Route C₂ diverged to two routes, C_{2a} and C_{2b}. In C_{2a}, only a 'vegetative-type' nucleus divided repeatedly whereas route C_{2b} showed independent division of both 'vegetative' and 'generative-type' nuclei. That is, repeated division of both 'vegetative' and 'generative-type' nuclei independently of each other contributed to the formation of microspore embryoids in citrus anther culture. Of these pathways, the route C₂ may be the most responsible for the embryoid formation. Nuclear fusion occurred in route C and appeared to contribute to ploidy variation. Calli which appeared from the inside of the anthers originated from both anther wall and microspores. Embryoids derived from somatic tissue of anthers were not observed.

KEY WORDS: *Citrus aurantium*, *Poncirus trifoliata*, anther culture, androgenesis, ploidy, nuclear fusion.

Introduction

Anther culture has been widely researched in many plant species and this technique has led to speculation regarding its potential in plant breeding and crop improvement. In *Citrus* and its relatives, haploid, mixoploid and diploid plantlets were produced from anthers of *Poncirus trifoliata* (L.) RAF. (HIDAKA *et al.* 1979) and *C. madurensis* LOUR. (CHEN *et al.* 1980), and only diploid plantlets from anthers of *Citrus aurantium* L. (HIDAKA *et al.* 1982) and *C. sinensis* OSBECK (HIDAKA 1984a). Haploid, mixoploid and polyploid plants have been obtained in many plant species, and furthermore, the possibility of heterozygous diploid regenerants from microspores has been raised (WENZEL *et al.* 1976; VEILLEUX *et al.* 1985).

The study of the early stage of anther culture may provide valuable information on the origin of anther-derived plantlets and the sources of their variability. Previous studies on pathways of development from microspores to entire plantlets have concentrated mainly on herbaceous species, e.g. *Nicotiana*, (HEBERLE-BORS, 1985) and two pathways have been described. One pathway has an intermediate step involving callus formation and another is a direct pathway observed in a few species such as *Nicotiana* (SUNDERLAND and WICKS, 1971) and *Datura* (GUHA and MAHESHWARI, 1967), and the former is seen in many species, e. g. *Oryza* (OONO,

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1975). However, few studies have been made on arboreal species. In anther culture of *Citrus*, nuclear divisions of microspores and multinucleate microspores have been confirmed (HIDAKA *et al.* 1982). Furthermore, direct embryoid formation was observed in anther culture of both *Poncirus* (HIDAKA *et al.* 1979) and *Citrus* (HIDAKA 1984a; HIDAKA *et al.* 1982). In *Poncirus*, haploid plantlets were obtained in addition to diploids, hyperhaploids and mixoploids. However, many of the plantlets differentiated in these studies had a diploid chromosome number of 18 (HIDAKA 1984a; HIDAKA *et al.* 1979, 1982). In recent studies on anther culture of *Hordeum* (CHEN *et al.* 1984), some different pathways on early nuclear divisions of microspores have been described.

The present paper reports the direct pathway from microspores to embryoids as revealed microscopically, and provides insight into the mechanisms of spontaneous variability in anther culture of citrus.

Materials and Methods

Immature flower buds of trifoliolate orange, strain Kan-H, (*Poncirus trifoliata* (L.) RAF. and of sour orange (*Citrus aurantium* L.) were collected from single tree of each species planted in our orchard. They were immersed for five seconds in 70% ethanol for surface sterilization. After removal of the petals, anthers, assessed visually as containing microspores at the uninucleate stage, were transferred to culture media.

Media were based on that of MURASHIGE and SKOOG (1962) supplemented with 0.15 M sucrose. Optimum concentrations growth regulators to obtain embryoids were added to the media for the two species (HIDAKA *et al.* 1979, 1982); 10^{-6} M indole-acetic acid and 10^{-5} M kinetin for trifoliolate orange and 10^{-7} M of each for sour orange. Prior to autoclaving, media were adjusted to pH 5.8 and solidified with 8 g/l agar. The cultures were incubated at $27 \pm 1^\circ\text{C}$ in the dark.

In order to follow microspore development, anthers of both species in each of 20 culture tubes were sampled at three-day intervals from three days to 66 days after culture and fixed in formalin acetic alcohol fluid (formalin 5: glacial acetic acid 5: 50% (v/v) ethanol 90). After dehydration in an ethanol and n-butanol series, the collected anthers were embedded in paraffin wax. Sections were cut at 8 to 12 μm thickness on a rotary microtome with a steel knife and heated onto slides. They were stained with safranin O and fast green FCF and observed by light microscopy.

Results

As there were no differences in the results obtained for *P. trifoliata* and *C. aurantium*, except where mentioned, they are reported here as one.

After three or six days of culture, besides microspores at the uninucleate stage (Fig. 1a) or the dividing nuclei described previously (HIDAKA *et al.* 1982), microspores with abnormal nuclear divisions were observed. That is, many of these microspores contained more than two nuclei; some with rather big 'vegetative-type' nuclei (Fig. 1b), some with small 'generative-type' nuclei (Fig. 1c), or some with both types (Fig. 1d). A few microspores developed as normal, such as found *in vivo*. They had a large, typical vegetative nucleus and

Fig. 1. Early stages of development of microspores.

- a. Microspores at the uninucleate stage after three days of culture (sour orange).
- b. A microspore with two vegetative-type nuclei (arrows). Six days after culture (sour orange).
- c. A microspore with two generative-type nuclei (arrows). Six days after culture (trifoliate orange).
- d. A microspore with two vegetative-type (V) and a generative-type (G) nuclei. Six days after culture (trifoliate orange).

one or two small condensed typical generative nuclei (Fig. 1e). However, this phenomenon was rather unusual. After nine days of culture, although microspores with more than four nuclei were observed (Fig. 1f), some microspores reduced their contents or nuclei were not stained (Fig. 1g). Microspores containing only generative-type nuclei were seldom observed after 12 days of culture. In such microspores, however, divisions of generative-type nuclei were confirmed still after 18 days of culture (Fig. 1h). Nuclear fusion among vegetative and generative-type nuclei was observed in some microspores and this phenomenon was observed directly after the first mitosis (Fig. 1i) and also after repeated division of nuclei (Fig. 1j). As time passed, the proportion of microspores without contents increased.

Many multinucleate microspores were observed after 12 days of culture (Fig. 1k). However, the number of them decreased after 21 days. After 21 days of culture, multi-nuclear-cellular structures were observed which appeared until after 42 days (Fig. 2). A proembryoidal structure were also observed after 24 days of culture (Fig. 3). Embryoids with definite polarity, the future shoot and root, were also observed after 24 days of culture (Fig. 4). The plural globular embryoids which seemed to differentiate from one microspore were confirmed and

Fig. 1. Early stages of development of microspores.

- e. A microspore with a typical vegetative (V') and two generative (G') nuclei such as found *in vivo*. Nine days after culture (sour orange).
- f. A microspore with four vegetative-type nuclei. Nine days after culture (trifoliate orange).
- g. Microspores with reduced contents. Nine days after culture (trifoliate orange).
- h. A microspore with plural generative-type nuclei. At least seven nuclei are shown (arrows). After 18 days of culture (trifoliate orange).

quintuplets were observed at 36 days of culture (Fig. 5). After about 42 days of culture, torpedo-shaped mature embryoids were observed in the anther wall (Fig. 6) and appeared from within anthers as mentioned previously (HIDAKA 1984a, HIDAKA *et al.* 1979, 1982). Some embryoids emerged from the inside of anther walls before maturing (Fig. 7).

In some cases, calli appeared from within the anthers (Fig. 8) besides from outside of anther wall as described previously (HIDAKA 1984b) after five weeks of culture. It was observed, however, that they originated from internal tissue of anther wall and microspores (Fig. 9).

Embryoids from somatic tissue of anthers were not observed in this experiment.

Discussion

The results seemed to have proved that plantlets obtained by anther culture of citrus originated from microspores.

In previous studies on anther culture of citrus, both embryoids and calli were obtained

Fig. 1. Early stages of development of microspores.

- i. A microspore showing nuclear fusion (an arrow) after the first mitosis. Six days after culture (trifoliate orange).
- j. A microspore showing nuclear fusion (an arrow) after repeated division of nuclei. 12 days after culture (trifoliate orange).
- k. A multinucleate microspore. After 12 days of culture (sour orange).

Fig. 2. A multi-nuclear-cellular structure. After 42 days of culture (trifoliate orange).

(HIDAKA 1984a; HIDAKA *et al.* 1979, 1982). Some calli appeared from within dehiscent anthers (HIDAKA, 1984b). In the present experiment, embryoids were revealed to be of the microspore origin, while calli originated mainly from somatic tissue even when they emerged from the inside of anthers. By the histological observation of the previous study of *C. aurantium* (HIDAKA *et al.* 1982), nuclear divisions of uninucleate microspores and multinucleate microspores were observed. In the present study, three main routes of microspore development *in vitro* were confirmed as shown in Fig. 10. In route A, the nucleus does not divide and microspores lose their contents. In route B, microspores show normal nuclear division producing typical one vegetative and one or two generative nuclei just as found *in vivo*. In this pathway, nuclei do not show any further division and the microspores lose their contents as in route A. Route C can be divided into two routes which show abnormal nuclear division. One of them, route C₁, produced two types of morphologically similar nuclei. In route C_{1a}, only vegetative-type nuclei were observed and divided repeatedly. In the other route, C_{1b}, only generative-type nuclei were observed and showed further divisions. In another route, C₂, a nucleus divided into two different sized nuclei, and this path way can be divided

Fig. 3. Proembryoidal structure after 24 days of culture (sour orange).

Fig. 4. An embryoid with definite polarity, the future shoot and root, after 30 days of culture; W=anther wall (trifoliate orange).

Fig. 5. Quintuplets of globular embryoids (arrows) from a microspore. After 36 days of culture (trifoliate orange).

Fig. 6. A torpedo-shaped matured embryoid in anther wall after 55 days of culture. Arrows point anther wall (trifoliate orange).

again into two routes, C_{2a} and C_{2b}. In route C_{2a}, a generative-type nucleus did not show further division and a vegetative-type nucleus divided repeatedly. The other route, C_{2b}, show independent division of both vegetative and generative-type nuclei. That is, in the four pathways of route C, microspores developed into multi-nuclear-cellular structures and formed multicellular structures which developed into microspore embryoids. Furthermore, by the frequency of appearance of multinuclear microspores in the pathways, the route C₂ seems the most responsible for embryoid formation.

In anther culture of *P. trifoliata* and *C. aurantium*, embryoids were formed by repeated division either of only the vegetative, or of the generative-type nuclei, and also by independent division both of the vegetative and generative-type nuclei as mentioned above. From anther culture of *Hordeum vulgare*, BOUHARMONT (1977) and WILSON *et al.* (1978) concluded that an equal first division or vegetative nuclear division resulted in microspore callus formation and that the generative nuclear division did not produce any multicellular structure. CHEN *et al.* (1984), however, reported that independent division of the vegetative and generative cell, and division of fusion product of the two also resulted in multicellular structure formation. In addition to the conclusion of former authors (BOUHARMONT 1977; WILSON *et al.* 1978), SUN-

- Fig. 7. A torpedo-shaped prematured embryoid emerged from the inside of anther wall after 42 days of culture; W=anther wall (sour orange).
- Fig. 8. Calli (C) appeared from within a dehiscid anther of trifoliate orange after 5 weeks of culture; F=calli from a filament, W=anther wall (dehiscid).
- Fig. 9. Calli from internal tissue of anther wall and microspores (trifoliate orange); A=calli from internal tissue of anther wall, M=calli from microspores, P=pollen, W=anther wall, X=calli seemed to be mixed with A and M.

DERLAND and his associates (SUNDERLAND *et al.* 1979; SUNDERLAND and EVANS 1980) reported that the embryo-like component might be derived from the generative cell in anther culture of same species, *Hordeum vulgare*. In an other species, an independent role of the generative cell in microspore embryogenesis was proved by RAGHAVAN (1978) on *Hyoscyamus niger*. In that experiment, only undifferentiated or generative-type nuclei were functional for microspore embryoid formation and vegetative-type nuclei did not contribute. In anther culture of *Nicotiana tabacum*, SUNDERLAND and WICKS (1971) reported that pollen embryoids were formed by repeated division of vegetative cell. MATSUBAYASHI and KURANUKI (1975), however, confirmed that the multicellular pollen grains were formed from either vegetative or generative nuclei, or from both in some species such as *Hordeum distichum*. The conclusion of our present investigation accords with that of CHEN *et al.* on *H. vulgare*. These differences could be the result of differences in genotypes of the material and/or culture conditions employed.

One of the present author and his associates (HIDAKA *et al.* 1979, 1982; HIDAKA 1984a) reported the formation of diploid plantlets in anther culture of *Poncirus trifoliata* and *Citrus*.

In this experiment, nuclear fusion among vegetative and generative nuclei throughout route C were observed. This phenomenon could be the main reason for ploidy level increase.

Fig. 10. Developmental pathways of microspores in anther culture of citrus.

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カンキツ薬培養における花粉胚様体の発達過程

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材料には、場内のカラタチとサワーオレンジのそれぞれ1本の木から採取した、1核期前後の花粉を含むと思われる若い薬を用いた。

培地は、MS培地に0.15 Mしょ糖を加え、植物生長調節物質として、カラタチには 10^{-6} M IAA 及び 10^{-5} M カイネチンを加え、サワーオレンジにはそれぞれ 10^{-7} Mを加えたものを用いた。培地の寒天濃度は0.8 %, pHは5.8に調整した。培養は27℃の暗黒下で行なった。

花粉からの胚様体形成過程の観察のために、置床後3日おきに66日目まで、管ビン20本分の薬を取り出し、FAA液に固定した。その後エタノール-n-ブタノールシリーズで脱水し、8-12 μ mのパラフィン切片とし、サフラニンO、ファーストグリーンFCFで染色して観察した。

その結果、カンキツの薬培養における花粉の発達過程は次のような経路をたどることが明らかになった。(Fig. 10)。

経路Aでは核は分裂せず、花粉は死に至る。経路Bでは、花粉は*in vivo*と同様に発達し、1個の栄養核と1、2個の生殖核を持つが、それ以上分裂せず、Aと同様に死に至る。経路Cは2つの経路、 C_1 と C_2 に分かれる。 C_1 では、核は同じ大きさの核、栄養核タイプと生殖核タイプの核としてそれぞれ分裂する。そして、 C_{1a} では比較的大きな栄養核タイプの核として分裂を続け、 C_{1b} では比較的小きな生殖核タイプの核として分裂を続ける。 C_{1a} 、 C_{1b} とも最終的には胚様体を形成する。一方の経路 C_2 は、異なる大きさの核、栄養核タイプと生殖核タイプの核に分裂し、その後、2つの経路 C_{2a} と C_{2b} に分かれる。 C_{2a} では生殖後タイプの核はそれ以上分裂せず、栄養核タイプの核のみが分裂を続ける。 C_{2b} では栄養核タイプと生殖核タイプの核それぞれが別個に分裂を続ける。また、観察の結果から、この C_2 が、胚様体を形成する主な経路と思われた。

これらのCの経路では、分裂が進むにしたがい、両タイプの核はそれぞれの特徴を失い、最終的に多核多細胞構造を作る。そしてそれらは胚へと生長し、植物体を形成する。また、Cの経路では、随時核融合が起こっており、これが植物体の倍数性に影響を与えている1つの原因と思われる。

また、薬壁などの組織からはカルスは形成されたが、植物体は誘導されないことが明らかになった。