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# Sex and ploidy of anther culture derived papaya (*Carica papaya* L.) plants

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**Summary**

To improve the efficiency of papaya anther culture, we investigated (1) hormonal medium conditions for inducing haploids or dihaploids; (2) identified the sex of established plantlets using a sex-specific DNA molecular marker and (3) estimated their ploidy by flow cytometry analysis of DNA content. Anthers with a mixture of uninucleate, mitotic, and binucleate microspores were collected from a male plant, and cultured on MS agar medium with different concentrations of CPPU and NAA. An embryo induction rate of 13.8% was attained on MS agar medium with 0.01 mg $l^{-1}$ CPPU and 0.1 mg $l^{-1}$ NAA. The induced embryos were subcultured on medium with 0.0025 mg $l^{-1}$ CPPU. Rooting of the developed shoots was promoted by treating their basal parts with 1500 mg $l^{-1}$ IBA in a 50% ethanol solution for about 10 seconds. All the embryo-derived plantlets (27 plants) were identified as female, implying that they were derived from microspores. In addition, 26 plants were determined to be triploids and one to be tetraploids. We also observed a wide range of morphological variation (e.g., in tree height and fruit size) among the established plants. Based on the results, we discussed a potential value of anther culture techniques for the breeding of papaya.

*Abbreviations:* N-(2-chloro-4-pyridyl)-N′-phenylurea (CPPU), ∂-naphthalene acetic acid (NAA), sequence-characterized amplified region (SCAR)

## Introduction

Papaya is a popular fruit crop in the tropics and subtropics. It exists in three sex forms, male, female and hermaphrodite. As reviewed by Manshardt (1992), all conventional breeding methods useful in the improvement of self pollinating species are applicable to the breeding of gynodioecious papaya. Population improvement methods are also useful in both dioecious and gynodioecious populations. Haploid production via pollen or anther culture will contribute beneficially to both breeding system of papaya, by virtue of shortening the breeding cycle and efficiently developing $F_1$ hybrids.

Successful in vitro androgenesis depends on various factors, e.g., genotype and physiological state of the donor plant, microspore developmental stages, culture medium, and pre-culture treatments (Zhang & Lespinasse, 1992; Palmer & Keller, 1997). Growth regulators in culture media are one of the most important components for pollen embryogenesis via anther culture (Zhang & Lespinasse, 1992; Sopory & Munshi, 1996). In some plant species, an auxin or a cytokinin alone is required for inducing pollen embryogenesis (Sopory & Munshi, 1996), while in others, such as cereals and fruit crops, a combination of both auxins and cytokinins are necessary (Bajaj, 1990; Zhang & Lespinasse, 1992; Ochatt & Zhang, 1996).

In papaya, Litz and Conover (1979) produced haploids from anther culture in MS (Murashige & Skoog, 1962) liquid basal medium with 1% activated charcoal, 3% sucrose, 0.1–0.5 mg $l^{-1}$ ∂-naphthalene

acetic acid (NAA) and 1–2 mg $l^{-1}$ 6-benzyladenine (BA). Tsay and Su (1985) reported a 0.7% embryo induction rate on MS agar medium without growth regulators.

We previously reported that the embryo induction rate (rate of anthers forming embryos) was at the most 4.2% when anthers were cultured on agar medium with 0.1 mg $l^{-1}$ BA and 0.1 mg $l^{-1}$ NAA after pre-treating anthers in water for 1 day at 35 °C (Rimberia et al., 2005). In this study, we further investigated the effect of N-(2-chloro-4-pyridyl)-N′-phenylurea (CPPU) and NAA on androgenesis, without examining the effect of pre-treatments. In addition, we analyzed the sex of plantlets derived from anther culture by the sex diagnostic PCR technique of papaya as developed by Urasaki et al. (2002). We also analyzed their ploidy by a flow cytometric assay. Based on the results obtained, we discussed the usefulness of anther culture techniques for papaya breeding.

## Materials and methods

### *Plant materials*

The $F_1$ male plants (18 months old) between a female cultivar 'Wonder Blight' (a dwarf type) and an unknown male strain were used in this study.

### *Effects of exogenous hormones on embryo induction*

Florets (10–14 mm long) were collected from the inflorescences of plants in November 2002. Anthers were dissected from the florets and used in the experiment. Before initiating the experiment, we confirmed that the anthers contained a mixture of 48.4% uninucleate, 23.3% mitotic, and 28.4% binucleate microspores, which was analyzed by staining the nuclei of microspores with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI).

The florets were surface sterilized in 1% sodium hypochlorite solution for 10 minutes. Anthers were aseptically removed and transferred into 100 ml Erlenmeyer flasks, each containing 30 ml MS medium supplemented with various combinations of CPPU (0.005, 0.01, 0.1, or 0.5 mg $l^{-1}$) and NAA (0.01, 0.1, 0.5 or 1.0 mg $l^{-1}$). Sucrose (3.0%) was added to the medium and the pH was adjusted to 5.8. The medium was solidified with 0.8% agar (Nakalai Tesque Inc., Kyoto, Japan) and autoclaved at 1.6 kPa for 15 minutes. Cultured anthers were maintained at about 25 °C with a day length of 18 h, supplemented with 27 $\mu$ mol $m^{-2}$ $s^{-1}$ from a fluorescent light. Each flask was inoculated with 20 anthers and the plates were replicated four times per hormonal plot.

Embryos formed on anthers were transferred to MS agar medium with 0.0025 mg $l^{-1}$ CPPU and subcultured for 6 weeks on the medium. To promote the growth of shoots, they were subsequently transferred to MS liquid medium with 0.0025 mg $l^{-1}$ CPPU, and subcultured for 3 cycles at monthly intervals in fresh medium of same CPPU concentration. Subsequently, the basal parts of shoots were treated with 1500 mg $l^{-1}$ indolebutyric acid (IBA) in a 50% ethanol solution for about 10 seconds to promote rooting (Kataoka & Inoue, 1991).

Established plantlets were transferred to pots half-filled with vermiculite medium supplemented with full strength hyponex solution (N: 7, P: 6, K: 19). They were then acclimatized in an acrylic resin boxes (28 × 37 × 19 cm) with gradually decreasing humidity under the ambient light and temperature conditions described above (Rimberia et al., 2005). After a month of acclimatization, they were transplanted to the field.

### *Sex determination of plantlets generated from embryos*

Twenty-seven established plants and one male anther donor plant were used for the sex diagnostic assay. Out of the established plants, 10 strains were derived from the previous study (Rimberia, et al., 2005) and 17 strains from this study. Genomic DNA was extracted from young leaves (about 0.3 g) using the standard CTAB method with minor modifications (Rimberia et al., 2005).

Multiplex polymerase chain reaction (PCR) for co-amplification of the papain gene and the sex-specific SCAR marker was carried out according to Urasaki et al. (2002). The papain gene was used as an internal control and the SCAR marker as specific for male or hermaphrodite plants. The P5′ and P3′ primers were used to amplify a partial sequence of the papain gene. The SDP-2 and SDP-3 primers were used for the sex-specific SCAR marker. PCR was carried out in a 20 $\mu$l reaction mixture containing genomic DNA (10 ng), P5′ and P3′ primers (each 0.5 $\mu$M), SDP-2 and SDP-3 primers (each 1.0 $\mu$M), Ex *Taq* DNA polymerase (0.2U) (TaKaRa Co., Ltd., Shiga, Japan), dNTPs (250$\mu$M), Tris-HCl (10 mM, pH 8.3), KCl (50 mM), and $MgCl_2$ (1.5 mM).

Amplification was performed in a thermo-cycler (Mastercycler Personal 5332, Eppendorf, Hamburg, Germany) programmed for 30 cycles of 1 min at 92 °C, 1 min at 55 °C, 1 min at 72 °C, followed by a final extension of 5 min at 72 °C. Amplification products were electrophoresed in 1.5% agarose gels, and detected by staining with ethidium bromide. Standard size markers (New England Biolabs Inc., Beverly, MA, USA) were used in electrophoresis. UV trans illuminated gels were photographed on Polaroid film.

*Ploidy determination of embryo-derived plants*

The strains used in the experiment were the same ones that were used in the sex diagnostic PCR assay. The female diploid papaya cultivar 'Wonder Blight' served as an internal standard. The ploidy of these plants was analyzed using a Partec PA (Partec GmbH, Munster, Germany) flow cytometer.

An approximately 33 $mm^2$ leaf disk from a mature leaf was sampled with a cork borer (diameter 6.5 mm) and cut in half (about 16 $mm^2$). A halved leaf disk from each strain was chopped with a razor blade in 0.5 ml Partec HR-A solution. An equivalent piece of control leaf disk was processed simultaneously (co-chopped) with a leaf sample from each strain. The digested samples were then filtered through Partec CellTrics (30 $\mu$m nylon mesh) into the sample tube. Five minutes after filtration, about 2 ml Partec HR-B solution was added to the nuclear suspension. Following staining at room temperature, the relative nuclear DNA content of the nuclear suspension was measured using the PA flow cytometer. The ploidy of the embryo derived strains was estimated from a ratio of relative fluorescence intensity (RFI) of the leaf disk sample to that of the control for every set analyzed. The 27 histograms obtained from all strains revealed a single peak with a CV range of less than 5.0.

## Results

*Effects of CPPU and NAA on embryo induction*

The highest embryo induction rate, 13.8 %, was attained at 0.01 mg $l^{-1}$ CPPU and 0.1 mg $l^{-1}$ NAA (Table 1). No embryos were induced in the combinations of 0.005-0.5 mg $l^{-1}$ CPPU with 0.01 mg $l^{-1}$ NAA. In the combinations of 0.005–0.5 mg $l^{-1}$ CPPU with 0.1-1.0 mg $l^{-1}$ NAA, the embryo induction rates ranged from 0.0 to 3.8%. Callus was also induced in the combinations of 0.1–0.5 mg $l^{-1}$ CPPU with 0.1–1.0 mg $l^{-1}$ NAA. However, all these calli were non-regenerative and died after a few months of subculture.

*Table 1.* Effects of CPPU and NAA on embryo and callus induction from cultured anthers

| CPPU (mg $l^{-1}$) | NAA | No. of anthers with embryos (%) | No. of anthers with callus (%) | No. of anthers used |
|---|---|---|---|---|
| 0.005 | 0.01 | 0 | 0 | 80 |
| | 0.1 | 1 (1.3) | 0 | 80 |
| | 0.5 | 0 | 0 | 80 |
| | 1.0 | 2 (2.5) | 0 | 80 |
| 0.01 | 0.01 | 0 | 0 | 80 |
| | 0.1 | 11 (13.8) | 0 | 80 |
| | 0.5 | 0 | 0 | 80 |
| | 1.0 | 1 (1. 3) | 0 | 80 |
| 0.1 | 0.01 | 0 | 0 | 80 |
| | 0.1 | 3 (3.8) | 0 | 80 |
| | 0.5 | 3 (3.8) | 1 (1.3) | 80 |
| | 1.0 | 0 | 3 (3.8) | 80 |
| 0.5 | 0.01 | 0 | 0 | 80 |
| | 0.1 | 2 (2.5) | 10 (12.5) | 80 |
| | 0.5 | 1 (1.3) | 5 (6.3) | 80 |
| | 1.0 | 0 | 0 | 80 |

Embryos appeared to be formed directly on anthers (Figure 1A). A few of these embryos developed into normal shoots with a pair or a triplet of cotyledons on a hypocotyl (Figure 1B). Most of the embryos grew into fasciated shoots with multiple buds during culture on agar medium with 0.0025 mg $l^{-1}$ CPPU (Figure 1C). After subculturing into the liquid medium with 0.0025 mg $l^{-1}$ CPPU, the multiple buds formed on the fasciated shoots developed into normal shoots (Figure 1D). However, the rooting ability of the shoots cultured in the liquid medium tended to be lower than that of the shoots in the agar medium (data not shown). The rate of shoots that were enforced to root by the IBA treatment was 52%. Established plantlets were well acclimatized and survived in the field.

*Sex identification of plantlets derived from anther culture generated embryos*

Figure 2 shows the results of the sex diagnostic PCR assay for 12 out of 27 strains together with the anther-donor male plant as a control. In the male plant, PCR

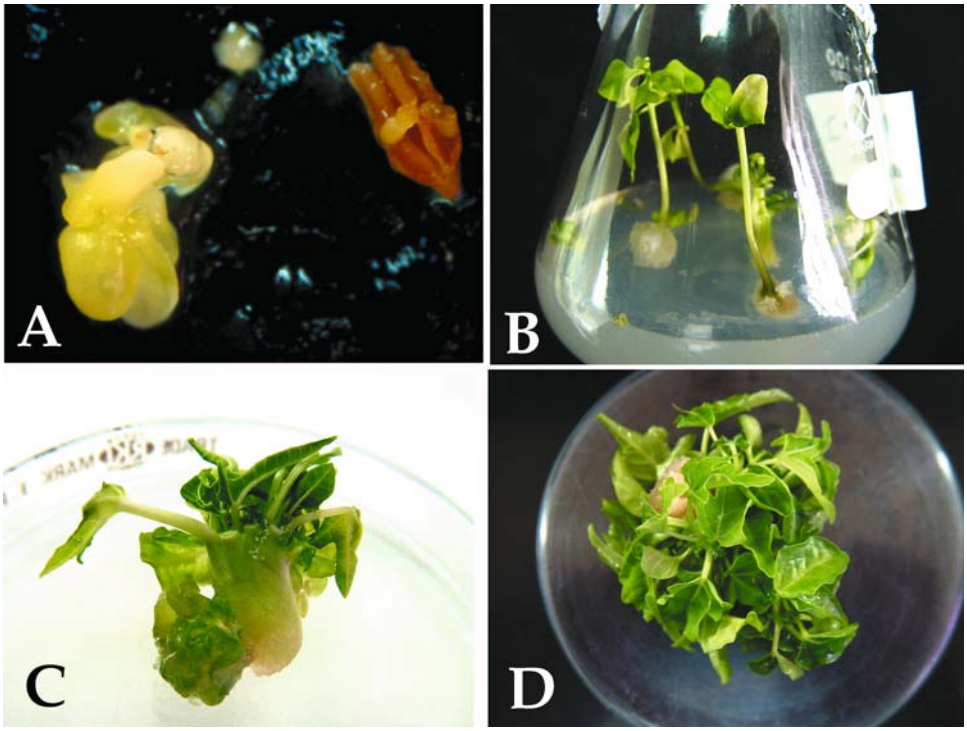


*Figure 1*. Embryos from an anther (A), shoots with a pair or a triplet of cotyledons on a hypocotyl (B), a fasciated shoot with multiple buds (C) on agar medium with 0.0025 mg $l^{-1}$ CPPU, and multiple shoots growing normally in liquid medium with 0.0025 mg $l^{-1}$ CPPU (D).


*Figure 2*. Sex diagnostic PCR assay for papaya plantlets established through anther culture. The papain gene marker detected at 221 bp was used as an internal control, and the sex-specific SCAR marker at 347 bp distinguishes male or hermaphrodite from female plants. M: 100 bp ladder size markers (New England Biolabs Inc.), m: male papaya, C: no template control (water), lanes 1-12: strains from anther-derived embryos.

products for both the papain gene and the SCAR marker were detected at 221 and 347 bp, respectively. In all 12 embryo-derived strains, the papain gene was detected, indicating that the PCR reactions were successful; but the SCAR marker was not detected in any of the strains (Figure 2). The same was true in the remaining 15 strains. These results indicated that all the established strains were female. In the field to date, 18 strains have flowered and been confirmed as female from their flower types.

*Ploidy determination of embryo-derived plants*

When the peak of RFI for the internal control (diploid) was set at approximately channel 100, the peaks for 26 different embryo-derived strains were concentrated at channel 150 and one strain concentrated at channel 200. An index was defined as the ratio of mean RFI of a test strain to that of the internal control. When the index of the internal control was set at 1, those of 26 strains ranged from 1.465 to 1.505 and these were estimated to be triploid (Figure 3A). The index for one strain was 1.99 and it was estimated to be tetraploid (Figure 3B). Three out of the 26 strains were determined to have chromosome number $2n = 27$ in a root tip cell using standard Feulgen methods (data not shown).

These triploid plants showed a wide range of morphological variation, e.g., dwarf plants with short internodes (Figure 4A) versus tall plants with long ones (Figure 4B), and large fruits (Figure 4C) versus small ones (Figure 4D). The variation in parthenocarpic ability was also found among the plants. Moreover, flesh thickness of fruits in the triploid plants was greater than that in the parental diploid plants (data not shown).

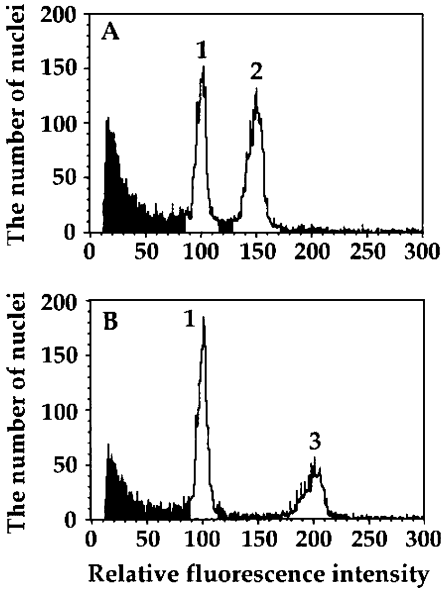


*Figure 3*. Histograms from flow cytometric analysis of nuclear DNA content in papaya. Peak 1 is from the diploid control (A and B). Peaks 2 (A) and 3 (B) are from embryo-derived strains, triploid and tetraploid, respectively.

## Discussion

Litz and Conover (1979) induced haploids at a success rate of 0.4%. Tsay and Su (1985) also reported a 0.7% embryo induction rate. Rimberia et al. (2005) obtained a 4.2% embryo induction rate. In this study, we attained a 13.8% of embryo induction rate. This rate is the highest reported for papaya anther culture thus far.

Out of the 27 plants established via anther culture, 26 (96.3%) plants were estimated to be triploid and one plant (3.7%) was tetraploid as determined by the flow cytometric analysis. No haploids or dihaploids were obtained in this study.

Plants with different ploidy levels induced via anther culture have been reported in various plant species, e.g., 6% haploids, 70% diploids and 24% triploids in *Datura metel* L (Narayanaswamy & Chandy, 1971); 34% haploids, 65% diploids and 0.8% triploids in *Capsicum annum* L. (Dolcet-Sanjuan et al., 1997); 7% haploids, 43% diploids, 29% triploids, and 21% tetraploids in apple (De Witte et al., 1999); and 14.3% diploid, 71.4% triploids and 14.3% tetraploids in *Petunia axillaris* (Engvild, 1973). Sunderland et al. (1974) demonstrated the origin of triploids and tetraploids from anther culture in *Datura innoxia* Mill. In an embryogenic grain where a single nucleus divides asymmetrically into a generative and a vegetative nucleus, an endo-reduplicated generative nucleus fuses with a vegetative nucleus to form a triploid embryo, whereas the fusion of an endo-reduplicated generative nucleus and two vegetative daughter nuclei leads to formation of a tetraploid embryo. Similarly, Gupta (1982) proposed that in *Petunia parodii*, triploids originated through a pathway where a vegetative nucleus (1C DNA) fused with an endo-reduplicated generative nucleus (2C DNA) at the binucleate stage.

Such occurrence of polyploids via anther culture has also been reported to be related to microspore developmental stages. Engvild et al. (1972) reported that anthers with young uninucleate microspores gave rise to haploid plants, anthers with older uninucleate


*Figure 4*. Morphology of triploid plants derived from anther culture. A dwarf plant with short internodes (A) and large fruits (C). A tall plant with long internodes (B) and small fruits (D). Plants and fruits were photographed 4 and 6.5 months after transplanting to the greenhouse, respectively. Each vertical bar is 50 cm.

microspores gave rise to diploid plants, and anthers with binucleate microspores gave rise to triploid plants. Engvild (1973) produced only triploid plants of *Petunia axillaris* from anthers with binucleate microspores, and proposed the feasibility of producing haploid plants if cultivation of anthers with uninucleate microspores is possible. Sunderland et al. (1974) also suggested that triploids were predominantly induced in later developmental stages of microspores, from first pollen mitosis to older pollen grains lacking a vacuole and free of starch for the most part.

In a previous study (Rimberia et al., 2005), we cultured anthers with uninucleate (80%) and binucleate (20%) microspores. In this study, we used anthers containing uninucleate (48.4%), mitotic (23.3%), and binucleate (28.4%) microspores. Litz and Conover (1978) obtained haploids by culturing anthers with microspores at uninucleate stages. Tsay and Su (1985) induced haploids using microspores at the uninucleate stage. The microspores used in this study seem to be maturer, as compared with the microspores in the other three reports. However, out of 26 established plants, 9 plants from the previous study as well as the 17 plants from this study were triploids. Thus, in this study, we could not conclude that the occurrence of triploids was related to the developmental stages of microspores. To induce the haploids or dihaploids of papaya, we need further investigations on factors such as the developmental stages and physiological state of microspores, and in vitro cultural and hormonal conditions.

All the established plants were identified as female using the sex specific SCAR marker for papaya (Urasaki et al., 2002). These results indicate that all the plants were derived from microspores, not from staminate anther wall tissues.

Based on the sex segregation ratios which resulted from cross and self pollination studies of the three basic sex forms, i.e., male ($M_1m$), hermaphrodite ($M_2m$), and female (mm), Storey (1938, 1953) and Hofmyer (1938) suggested that dominant homozygotes ($M_1M_1$, $M_1M_2$, and $M_2M_2$) were lethal. Hofmyer (1967) hypothesized that $M_1$ and $M_2$ contain inert regions from which essential genes are missing, which may account for the lethality of dominant homozygotes. Given the hypothesis by Storey (1938, 1953) and Hofmyer (1938), we propose that the absence of males among the established plantlets is due to the lethality of dominant homozygotes. Pollen grains with "$M_1$" may be impotent for embryo differentiation or differentiated embryos might have died at an early stage of development. Similarly, in the case of hermaphrodites ($M_2m$), only female plants would be established through anther culture. Therefore, anther culture techniques do not seem to be directly applicable to the breeding of male or hermaphrodite papaya. Moreover, it will be impossible to achieve an efficient seed production system for hermaphrodites by a cross between homozygous "mm" and "$M_2M_2$" dihaploids.

In subtropical regions, female cultivars usually have higher productivity compared to hermaphrodite cultivars (Manshardt, 1992; Nakasone & Paull, 1998). Thus, the breeding of female cultivars seems to be more important than that of hermaphrodites in subtropics. Aquilizan (1987) reported the Yarwun (Queensland) breeding system of papaya in which sexually ambivalent males were used to breed female inbred lines. In this breeding system, anther culture techniques possibly contribute directly to the production of female pure lines.

In this study, almost all the induced plants were triploids. Among these triploid plants, we observed a wide range of variation in plant morphology (especially in plant height and fruit size and shape) (Figure 4) and parthenocarpic ability (data not shown). From the observation of plant morphology, we found that the triploids were superior to the diploids ('Wonder dwarf') with respect to a certain morphological characteristics. For example, the fruits of triploids (which were seedless) tended to have a thicker flesh than the pollinated fruits of female diploids (data not shown). Moreover, a dwarf type of papaya, such as the plant detected in this study (Figure 4), will be important for the cultivation of papaya in a greenhouse. The variations in plant morphology and parthenocarpic ability among the triploid plants will be reported in detail elsewhere.

In papaya production using only females, one of the most important issues is how to propagate selected female genotypes. In this respect, it will be possible to propagate the female genotypes by in vitro techniques (Drew & Smith, 1986; Magdalita et al., 1997; Lai, et al., 1998). Another problem is that the triploids can not be used for the cross-breeding of papaya. In order to advance the breeding of papaya further, it will be necessary to produce dihaploid female papaya with normal fertility. The efficient seed production of female cultivars may be realized by crossing female dihaploid lines with male lines whose nuclei have been replaced by those of female lines through recurrent backcrossing. The optimal developmental stages of microspores and in vitro conditions for inducing haploids or dihaploids are under investigation.

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