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# Ploidy of somatic embryos and the ability to regenerate plantlets in melon (*Cucumis melo* L.)

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**Summary.** The number of chromosomes in cells of callus, somatic embryos and regenerated plantlets during somatic embryogenesis were examined in two cultivars of melon (*Cucumis melo* L.). Somatic embryos were diploid (50.0%/32.1%), tetraploid (38.5%/57.5%) and octoploid (11.5%/10.4%) whereas in callus cells diploidy (41.9%/43.3%), tetraploidy (27.9%/25.8%), octoploidy (11.6%/15.5%) and a low frequency of other types of ploidy and aneuploidy were observed. Mixoploid somatic embryos were not observed. These results suggest that the somatic embryos were selectively differentiated from diploid, tetraploid and octoploid cells, and that endopolyploidization of cultured cells occurred before the start of cell division leading to somatic embryogenesis. The ratio of diploid to tetraploid (1.30/0.55) in somatic embryos was less than that in callus cells (1.50/1.68) while ratios of diploid to octoploid (4.35/3.09) and tetraploid to octoploid (3.35/5.52) in somatic embryos were greater than those in callus cells (3.61/2.80 and 2.40/1.67). Therefore, it appears that the ability of callus cell to differentiate into somatic embryos increases in the following order: octoploid<diploid< tetraploid. Regenerated plantlets were diploid (65.5%/55.1%) and tetraploid (34.5%/44.9%). No octoploid plantlets were observed. The ratio of diploid to tetraploid in regenerated plantlets (1.72/1.23) was greater than that in somatic embryos. Therefore, it appears that the ability of somatic embryos to develop into plantlets increases in the following order: octoploid<tetraploid<diploid.

**Key words:** *Cucumis melo*; somatic embryogenesis; diploid; tetraploid; octoploid; plantlet-regeneration ability.

## Introduction

Regeneration of plants via organogenesis of adventitious shoots (Moreno *et al.*, 1985, Bouabdallah and Branchard, 1986, Kathal *et al.*, 1988, Dirks and Buggenum, 1989), somatic embryogenesis (Oridate and Oosawa, 1986, Kageyama *et al.*, 1991) and formation of shoot primordia (Nagai *et al.*, 1989, Shimonishi *et al.*, 1993) has been reported in melon (*Cucumis melo* L.). Bouabdallah and Branchard (1986) observed heterogeneity in the ploidy level of plants regenerated from adventitious shoots of melon, ranging from diploid to tetraploid. Ezura *et al.* (1992a,b) reported that a high frequency of tetraploidy (*ca.* 30%) was observed in regenerated plants obtained by adventitious shoot organogenesis and somatic embryogenesis in this species. In order to use these culture systems efficiently for further genetic manipulation, for example for micropropagation, we have to eliminate tetraploids. Therefore, it is important that we understand the process of formation of tetraploids.

In organogenesis of adventitious shoots in melon, plants were regenerated selectively from diploid and tetraploid cells (Ezura and Oosawa, 1994). We might assume, therefore, that the same phenomenon occurs during somatic embryogenesis in melon.

In this study, the numbers of chromosomes in cells of callus, somatic embryos and regenerated plantlets of melon were analyzed. Ploidy in somatic embryogenesis and its relationship to regenerative ability are discussed.

## Materials and methods

*Somatic embryogenesis*

Somatic embryogenesis was achieved by the method of Ezura *et al.* (1992a). Embryos in mature seeds of two cultivars of melon (*Cucumis melo* L.), Prince (Sakata Seed Co. Ltd., Yokohama, Japan) and Sunday Aki (Yokohama Nursery Co. Ltd., Yokohama, Japan), were used as the sources of explants. The embryos were sterilized for 15 sec in 70% (v/v) ethanol followed by 15 min in a 20% (v/v) solution of sodium hypochlorite ( the final concentration of available chlorine was 1%). They were rinsed 3 times with sterilized distilled water and soaked in sterilized distilled water for 6 hours. The hypocotyls were then excised from the embryos. These expalnts were cultured at 25 °C on Murashige and Skoog (MS) medium (1962) containing 1 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 2 mg/l 1-naphthalenacetic acid (NAA), 0.1 mg/l benzylaminopurine (BAP), 3% (w/v) sucrose and 0.8% (w/v) agar (pH 5.8) in the

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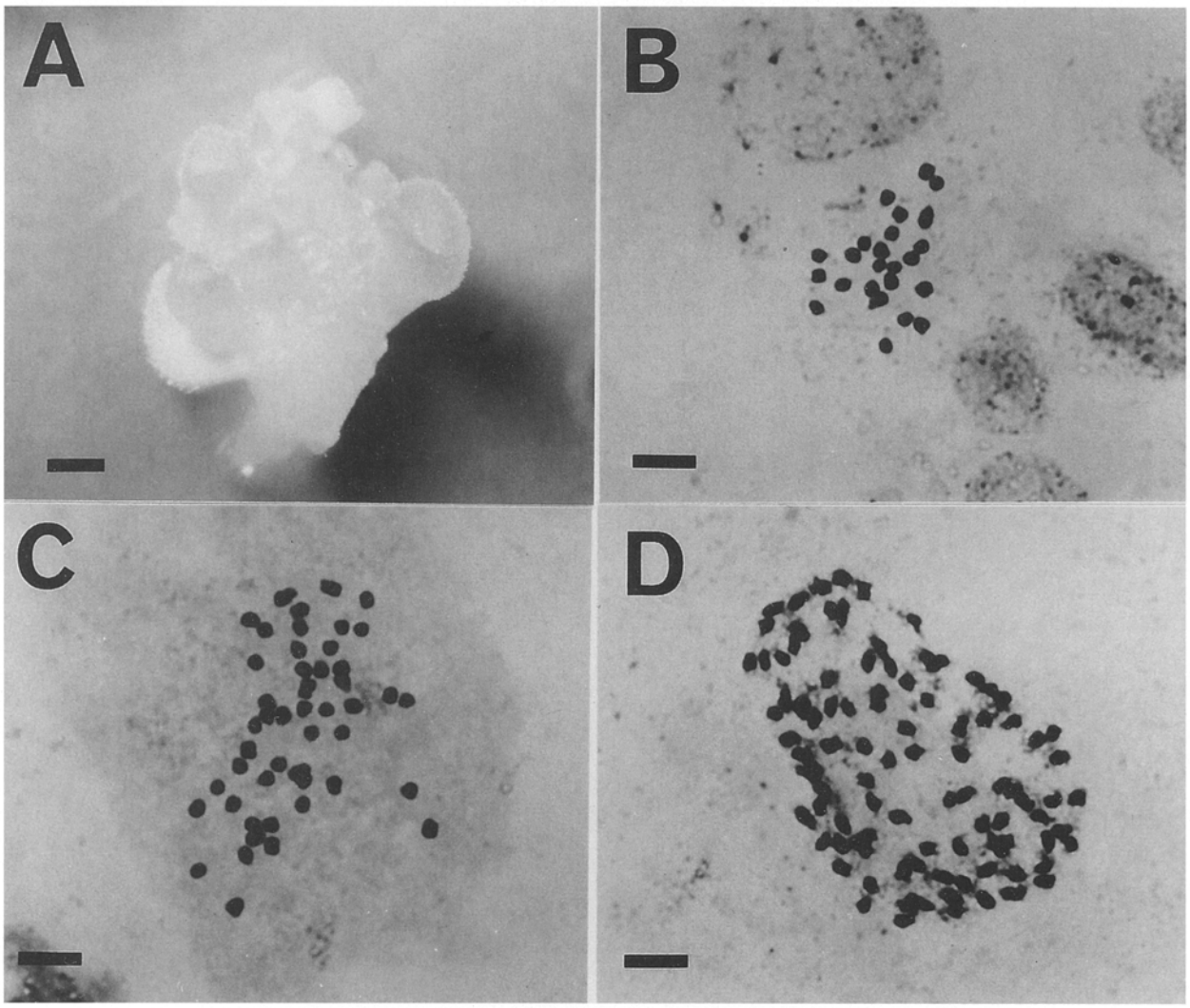


Fig. 1. Somatic embryos induced on callus of melon (*Cucumis melo* L. cv. Prince) and their chromosome contents. A, Somatic embryo; B, diploid (2x=24); C, tetraploid (4x=48); D, octoploid (8x=96). Bar in A:1 mm; Bars in B, C and D:5μm.

light (16 h light at 5,000 lux supplied by fluorescent lamps). After 4 weeks of culture, the somatic embryos on the explant callus were transferred to hormone-free MS medium with 0.4% (w/v) Gellan Gum (Wako Pure Chem. Ltd, Oosaka, Japan) for germination (pH 5.8). They were cultured at 25 °C in the light.

*Counting of chromosomes in callus cells*

After 4 weeks of culture on the somatic embryo-induction media, callus from which somatic embryos had been removed was stored for a day in distilled water at 5 °C, and then fixed 3:1 ethanol : acetic acid (v/v). After washing the callus with distilled water pieces of callus were treated with an enzyme solution that contained 4% (w/v) Cellulase "Onozuka" RS (Yakult Co., Ltd., Tokyo, Japan), 1% (w/v) Pectolyase Y-23 (Seishin Co., Ltd., Tokyo, Japan), 7.5 mM KCl and 7.5 mM EDTA (ph 4.0) for 50 min in darkness at 37 °C. The callus was then washed with distilled water and the cells spread in a drop of fixative. Chromosomes were stained with a 20 % (v/v) solution of Giemsa's solution for microscopy (Merck, Darmstadt, Germany) in 0.067 M sodium phosphate buffer (pH 6.8) for 30 min at room temperature. The chromosomes in intact metaphase plates were counted.

*Counting of chromosomes in the cells of somatic embryos*

Somatic embryos were cultured on germination medium as described above for 2 days before counting of chromosomes. Preparation for counting chromosomes was made by the methods as described above. Ten intact metaphase plates were counted per embryo.

*Counting of chromosomes in the cells of regenerated plantlets*

Root tips from regenerated plantlets were collected between 9:00 AM and 12:00 AM. Preparation for counting chromosomes was made by the methods as described above. Ten intact metaphase plates were counted per plantlet.

## Results

The chromosomal numbers in the cells of callus, somatic embryos and root tips of regenerated plantlets of the melon, cultivars Prince and Sunday Aki, were examined. Somatic embryos were formed on the surface of callus

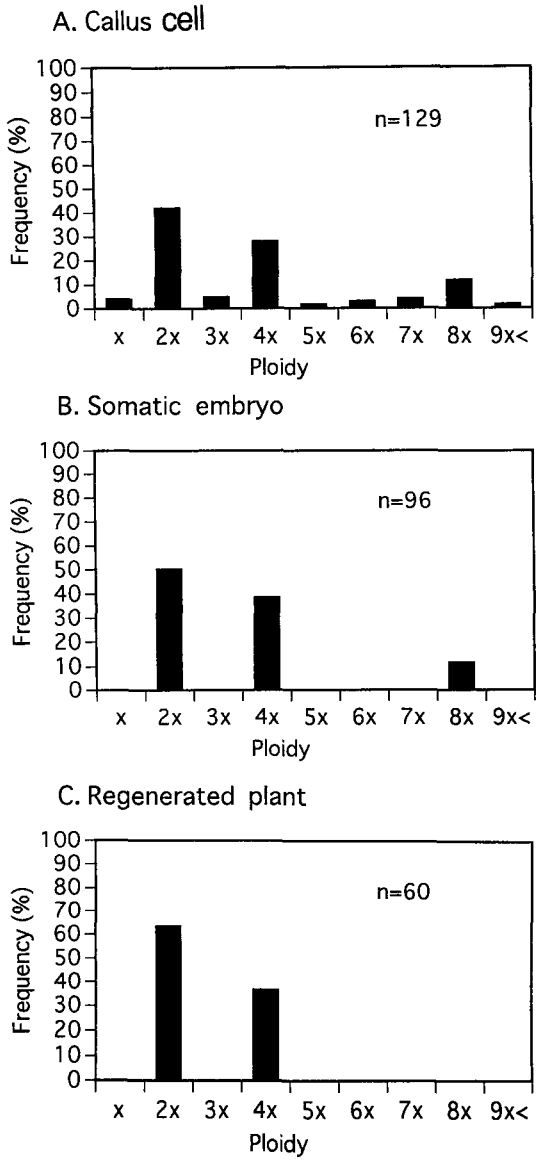


Fig. 2. Variations in the ploidy of callus cells induced on the medium used for somatic embryogesesis (A), somatic embryos (B) and root tip cells of regenerated plantlets (C) for the smooth-skinned melon cultivar, 'Prince'. The number in each panel is the number of callus cells, somatic embryos and regenerated plantlets examined, respectively.

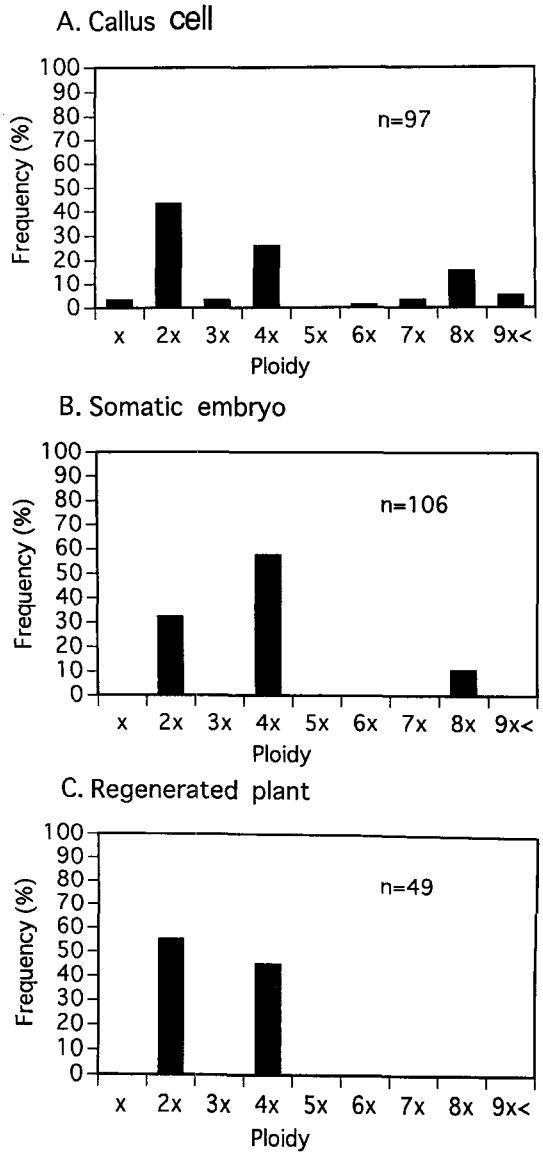


Fig. 3. Variations in the ploidy of callus cells induced on the medium used for somatic embryogenesis (A), somatic embryos (B) and root tip cells of regenerated plantlets (C) for the net-skinned melon cultivar, 'Sunday Aki'. The number in each panel is the number of callus cells, somatic embryos and regenerated plantlets examined, respectivly.

(Fig.1). Ten to 60% of the somatic embryos germinated and developed into plantlets when transferred onto hormone-free MS medium. The others failed to germinate or to develop into plantlets.

Among 129 callus cells of the cultivar Prince, a varying degree of ploidy from haploidy (x=12) to more than nonaploidy (9x=108) was observed (Fig.2-A). A small number of aneuploid cells for each level of ploidy was also observed. The frequency of aneuploid cells was added to the frequency of cells with the closest corresponding ploidy in estimating the frequency of cells of each ploidy to follow the changes in ploidy, Fig.2-A. The frequencies of diploid (2x=24), the original ploidy of this cultivar, tetraploid (4x=48) and ocotoploid (8x=96) cells were 41.9%, 27.9% and 11.6%, respectively. The frequencies of haploid, triploid, pentaploid, hexaploid and more than nonaploid cells were less than 4%. Of 96 somatic embryos examined, diploid, tetraploid and octoploid embryos were observed (Fig.1), the respective frequencies being 50.0%, 38.5% and 11.5% (Fig.2-B). Of 60 regenerated plantlets 65.5% were diploid and 34.5% were tetraploid (Fig.2-C). Mixoploidy and aneuploidy were not observed in somatic embryos or regenerated plantlets.

Among 97 callus cells of the cultivar Sunday Aki, a varying degree of ploidy from one to more than

Table 1. Ratios of cells of various ploidy levels from callus, somatic embryos and regenerated plantlets of two cultivars of melon.

| Stage | Diploid cells/ tetraploid cells | | Diploid cells/ octoploid cells | | Tetraploid cells/ octoploid cells | |
|---|---|---|---|---|---|---|
| | Cultivar | | Cultivar | | Cultivar | |
| | Prince | Sunday Aki | Prince | Sunday Aki | Prince | Sunday Aki |
| Callus | 1.50 | 1.68 | 3.61 | 2.80 | 2.40 | 1.67 |
| Somatic embryo | 1.30 | 0.55 | 4.35 | 3.09 | 3.35 | 5.52 |
| Plantlet | 1.72 | 1.23 | - | - | - | - |

nine was observed (Fig.3-A). A small number of aneuploid cells at each level of ploidy were also observed. In Fig.3-A, the frequency of aneuploid cells were added to the frequency of cells with the closest corresponding ploidy as in the cultivar Prince. The frequencies of diploid, tetraploid and octoploid cells were 43.3%, 25.8% and 15.5%, respectively. The frequencies of haploid, triploid, hexaploid, heptaploid and more than nonaploid cells were less than 4%. Of 106 somatic embryos, 32.1% were diploid, 57.5% were tetraploid and 10.4% were octoploid (Fig.3-B). Tetraploid embryos were more frequent than diploid and octoploid embryos. Of 49 regenerated plantlets, the frequencies of diploids and tetraploids were 55.1% and 44.9%, respectively, (Fig.3-C) and no other ploidy was observed. Mixoploidy and aneuploidy were not observed in either somatic embryos or regenerated plantlets as in the cultivar Prince.

## Discussion

Somatic embryos of melon were found to be diploid, tetraploid and octoploid whereas in callus cells various degrees of ploidy and aneuploidy, from haploid to nonaploid, were observed. This indicates that somatic embryos differentiate selectively from diploid, tetraploid and octoploid cells. Such selective differentiation has been observed in the regeneration of adventitious shoots of melon (Ezura and Oosawa, 1994). Plantlets regenerated from the somatic embryos of melon were diploid and tetraploid, indicating that plantlets regenerated selectively from the diploid and tetraploid somatic embryos. Similar selective development of somatic embryos induced from colchicine-treated embryogenic callus of *Citrus sinensis* has been reported. Diploid, triploid, tetraploid, hexaploid and octoploid callus cells were observed, although regenerated plantlets were exclusively diploid and tetraploid (Gmitter *et al.*, 1991). In two *Nicotiana* species, regenerating adventitious shoots showed various degrees of ploidy, aneuploidy and mixoploidy, although the mature plants were diploid and tetraploid indicating that diploid and tetraploid shoots selectively mature during shoot development (Nut Ronchi *et al.*,1981). In contrast, Smith and Street (1974) reported a low frequency of embryogenesis from hyperploid and aneuploid cells of *Daucus carota.*

Our data suggests that somatic embryos selectively differentiate from diploid, tetraploid and octoploid cells despite the variety of polyploid cells in the callus, and that plantlets selectively regenerate from diploid and tetraploid somatic embryos. Our results, and those from *C. sinensis* (Gmitter *et al.*, 1991), suggest that cells of some ploidy levels regenerate more readily than others. Changes in the frequency of ploidy during somatic embryogenesis were compared by examining the ratios of diploid, tetraploid and octoploid callus, somatic embryos and regenerated plantlets (Table 1).

In comparing the ploidy levels of callus and somatic embryos in the cultivar Prince, the ratio of diploid to tetraploid cells in somatic embryos (1.30) is less than that in callus (1.50), indicating that tetraploid cells differentiate into somatic embryos more easily than diploid cells. The ratios of diploid to octoploid (4.35) and tetraploid to octoploid (3.35) somatic embryos are both greater than those in callus cells (3.61 and 2.40), indicating that diploid and tetraploid cells are both more likely to differentiate into somatic embryos than octoploid cells. In the cultivar Sunday Aki, the ratio of diploid to tetraploid somatic embryos (0.55) was less than that in callus cells (1.68). Ratios of diploid to octoploid (3.09) and tetraploid to octoploid (5.52) somatic embryos were again greater than those in callus (2.80 and 1.67). Apparently, the ability of callus cells to differentiate into somatic embryos increases in the following order:octoploid<diploid<tetraploid, while differentiation of somatic embryos from other polyploid and aneuploid cells is infrequent.

In somatic embryos and regenerated plantlets of the cultivar Prince the ratio of diploid to tetraploid among plantlets (1.72) was greater than that among somatic embryos (1.30), indicating that diploid somatic embryos are more likely to regenerate plantlets than tetraploid somatic embryos. In the cultivar Sunday Aki, the ratio of diploid to tetraploid among plantlets (1.23) was also greater than that among somatic embryos (0.55). No octoploid plantlets were obtained in this experiment although octoploid somatic embryos were observed in both cultivars. It appears that diploid and tetraploid somatic embryos regenerate plantlets more readily than octoploid somatic embryos. The ability of somatic embryos to develop into plantlets increases in the following order: octoploid<tetraploid<diploid.

Mixoploidy was not observed in either somatic embryos or regenerated plantlets of the two cultivars. Fujimura and Komamine (1980) suggested that each

somatic embryo of *Daucus carota* develops from a single cell. If we assume that, somatic embryos develop from single cells and, that somatic embryogenesis has two developmental stages; stage I before the start of the cell division that leads to somatic embryogenesis and stage II which is subsequent somatic embryo development. Then, if endopolyploidization of cultured cells occurs only at stage I, mixoploidy of embryos might not be observed. However, if endopolyploidization occurs at stage II, then mixoploid somatic embryos might be observed. On the basis of our observation, it is likely that endopolyploidization in cultured cells of melon occurs before the start of the cell division that leads to somatic embryogenesis and not during somatic embryo development. In embryogenesis in microspore culture of *Brassica napus* and *B. oleracea*, it has been observed that karyokinesis occurred without cytokinesis in the early stages of culture (Nitta *et al.*, 1993). If it is the case that endopolyploidization occurs in stage I then it may be possible to reduce the frequency of polyploid somatic embryos by altering the conditions during the early stages of culture. However, such an approach will only be effective if specific culture condition induce endopolyploidization, or culture condition can be used to select for particular ploidy levels. The chromosome content of callus and embryos during the early stages of culturing and embryogenesis in different culture condition must be observed in detail in order to take our hypothesis.

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## References

Bouabdallah L, Branchard M (1986) Z. Pflanzenzuchtg. 96:82-85.

Dirks R, Buggenum M (1989) Plant Cell Rep., 7:626-627.

Ezura H, Amagai H, Yoshioka K, Oosawa K (1992a) Jpn. J. Breed., 42:137-144.

Ezura H, Amagai H, Yoshioka K, Oosawa K (1992b) Plant Sci., 85:209-213.

Ezura H, Oosawa K (1994) Plant Tissue Cult. Lett., 11:26-33.

Fujimura T, Komamine A (1980) Z. Pflanzenphysiol., 9:1-8.

Gmitter FG, Ling HB, Cai CY, Grosser JW (1991): Plant Sci., 74:135-141.

Kageyama K, Yabe K, Miyajima S (1991) Jpn. J. Breed., 41:273-278.

Kathal R, Bhatnagar SP, Bhojwani SS (1988) Plant Cell Rep. 7:449-451.

Murashige T, Skoog F (1962) Physiol. Plant., 15:473-479.

Moreno V, Garcia-Sogo M, Granell I, Garcia-Sogo B, Roig LA (1985) Plant Cell Tissue Org. Cult., 5:139-146.

Nagai T, Nomura Y, Oosawa K (1989) J. Jpn. Soc. Hort. Sci.,58(Suppl.1):208-209.

Nuti Ronchi V, Nozzolini M, Avanzi L (1981) Protoplasma 109:433-444.

Nitta T, Takahata Y, Kaizuma Y (1993) Jpn. J. Breed., 43 (Suppl.1):55.

Oridate T, Oosawa K (1986) Jpn. J. Breed., 36:424-428.

Shimonishi K, Nagai T, Nomura Y, Yoshioka K, Oosawa K(1993) Plant Tissue Cult. Lett. 10:17-24

Smith SM, Street HE (1974) Ann. Bot. 38:223-241.