Plant Regeneration from Protoplasts Isolated from Embryogenic Callus of Satsuma

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Abstract. Embryos were obtained from unfertilized and undeveloped seeds of satsuma (*Citrus unshiu* Marc.) cultured on a modified Murashige and Tucker (MT) medium. Embryogenic callus was induced from the hypocotyl region of the embryos. The callus was successfully maintained through subculturing on MT medium with 185 μM adenine, 2.8 μM GA₃, and 400 mg malt extract/liter, solidified with Gelrite. Somatic embryogenesis occurred from callus subculture on medium containing 50 g lactose/liter and in the absence of plant growth regulators. Somatic embryos developed into plants on medium with sucrose and GA₃. Protoplasts isolated from this callus produced somatic embryos through colony formation; subsequently, normal, entire plants were regenerated.

Systems for somatic embryogenesis and plant regeneration from protoplasts have been developed in *Citrus* (Hidaka and Kajiura, 1988; Kobayashi et al., 1983; Kochba et al., 1972; Ling et al., 1989; Vardi et al., 1975; Vardi et al., 1982) and its related genus *Microcitrus* (Vardi et al., 1986). For the successful production of somatic hybrids by protoplasm fusion, embryogenic capacity of one of the partners is a prerequisite. Fused protoplasts can regenerate embryos and plantlets even though one of the partners lacks embryogenic capacity (Grosser et al., 1988a, 1988b; Kobayashi et al., 1988; Kobayashi and Ohgawara, 1988; Ohgawara et al., 1985; Vardi et al., 1987).

Satsumas are very important for the *Citrus* industry of Japan, and their improvement has been based on mutations and clonal selections (Iwamasa, 1983). Hybridization with satsumas has been difficult to achieve due to polyembryony and male sterility. If plant regeneration from protoplasts was possible, there would be new possibilities for satsuma breeding. Although mesophyll protoplasts of satsuma were used as one of partners for somatic hybridization (Kobayashi and Ohgawara, 1988; Kobayashi et al., 1988), we found no reports of plant regeneration from satsuma protoplasts. This paper deals with the establishment of embryogenic callus and plant regeneration from callus protoplasts in satsuma cultivars.

Mature fruits from open-pollinated flowers were harvested from trees of satsuma, cultivars Kusumoto-wase, Miyamoto-wase,

Tokumori-wase, Ichifumi-wase, Kanazawa-wase, Ishizuka-wase, and Sakikubo-wase. These are very early ripening cultivars derived from the Wase (early ripening) satsumas through bud mutation (Iwamasa et al., 1981). Undeveloped seeds ≈ 2 mm long were dissected aseptically from the fruit and cultured on the medium of Murashige and Tucker (MT) (1969) with a combination of 0.02 μM 2,4-D and 0.02 μM BA, or 400 mg malt extract (ME)/liter and 185 μM adenine. Media were supplemented with 50 g sucrose/liter and solidified with 1% (w/v) agar (Ka-

tayama Chemical Co., Osaka, Japan). The pH was adjusted to 5.7 before autoclaving at 121°C for 20 min. Cultures were incubated in the dark at $25 \pm 1^\circ\text{C}$.

Somatic embryos developed from cultured seeds of all cultivars on both media 4 months after explanting (Table 1). These embryos were assumed to have arisen from very small nucellar embryos that occur in unfertilized ovules (Wakana and Uemoto, 1988). Frequency of somatic embryo occurrence in seeds was higher on medium with adenine and ME than on that with 2,4-D and BA. Four to 10 somatic embryos emerged from one seed, and in some cultivars more than 10 somatic embryos were formed. In 'Kanazawa-wase' and 'Ishizuka-wase', direct callus initiation was observed from seeds, but the callus became brown and failed to grow.

When somatic embryos were transferred to MT medium with 2.8 μM GA₃, some embryos callused in the hypocotyl region and did not germinate (Fig. 1). Other somatic embryos developed roots and shoots but produced no callus. The callus was separated from somatic embryos, grown on the same medium, and subcultured twice at 3- to 4-week intervals. The callus was friable and its growth was poor. Its appearance resembled that of embryogenic callus obtained from sweet orange (Hidaka and Kajiura, 1988; Kobayashi et al., 1983, 1984; Kochba and Spiegel-Roy, 1977a, 1977b) and Calamondin (Ling et al., 1989). When transferred to a medium with 50 g lactose/liter and without growth regulators, the callus produced somatic embryos (Fig. 2).

Prevention of browning and vigorous mul-

Fig. 1. Callus at hypocotyl region of embryos.

Fig. 2. Embryo formation in callus of 'Ishizuka-wase'.

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Table 1. Callus and somatic embryo formation from undeveloped seeds of satsuma cultivars.

Cultivars	MT + 0.02 μ M 2,4-D + 0.02 μ M BA		MT + 185 μ M adenine + 400 mg ME/liter ^y	
	No. of seeds		No. of seeds	
	Formed callus	Formed embryoids	Formed callus	Formed embryoids
Kusumoto-wase	0	6	0	14
Miyamoto-wase	0	8	0	13
Tokumori-wase	0	17	0	23
Ichifumi-wase	0	15	0	20
Kanazawa-wase	0	12	3	16
Ishizuka-wase	0	13	1	26
Sakikubo-wase	0	15	0	24

^yFifty seeds were inoculated in each medium for each cultivar.

^yMT = Murashige and Tucker medium; ME = malt extract.

Table 2. Effects of plant growth regulators and gelling agents on callus growth.^z

Plant growth regulator					Ratio of callus growth ^y	
2,4-D (μ M)	BA (μ M)	Adenine (μ M)	GA (μ M)	ME (mg·liter ⁻¹)	Agar	Gelrite
---	44.4	---	---	---	0.4	1.2
---	44.4	---	---	400.0	0.6	1.3
0.45	44.4	---	---	---	0.2	0.2
0.45	44.4	---	---	400.0	0.2	0.4
4.5	44.4	---	---	---	0.4	0.3
4.5	44.4	---	---	400.0	0.3	0.2
---	---	185.0	---	---	5.0	7.0
---	---	185.0	---	400.0	7.0	8.3
---	---	---	2.8	---	4.8	5.8
---	---	---	2.8	400.0	5.4	7.0
---	---	185.0	2.8	---	5.9	7.3
---	---	185.0	2.8	400.0	6.6	11.5

^zThe concentrations of agar and Gelrite were 1% and 0.2%, respectively.

^yRatio of callus growth was calculated by (Final weight - Initial weight)/initial weight.

Fig. 3. First protoplasm division ($\times 800$). Fig. 4. Colony formation 30 days after plating ($\times 100$).

triplication were prerequisites for the establishment and maintenance of embryogenic callus. Effects of plant growth regulators and gelling agents on callus growth were exam-

ined with the callus of 'Ishizuka-wase'. The combinations of 2,4-D, BA, adenine, GA₃, ME, agar, and Gelrite (Kelco Div., Merk & Co., San Diego, Calif.) were prepared for

the screening of suitable media (Table 2).

The concentrations of agar and Gelrite were 1% (w/v) and 0.2% (w/v), respectively. Slow and scant callus proliferation, as well as browning, occurred on media with 2,4-D and/or BA. Adenine alone or in-combination with GA₃ and ME stimulated callus proliferation. Callus grew best on the medium with 185 μ M adenine, 2.8 μ M GA₃, and 400 mg ME/liter solidified by Gelrite. Vigorous callus growth was also observed on this medium for the other satsuma cultivars.

Embryogenic capacity was retained on this medium over successive subculture, i.e., somatic embryos were formed on these long-term cultured calli following transfer to growth regulator-free medium with 50 g lactose/liter. In sweet orange, embryogenic callus is maintained for a long period on medium with a high concentration (44.4 μ M) of BA (Kobayashi et al., 1984) or lacking plant growth substances (Vardi et al., 1982). Conversely, for satsumas, adenine, GA₃, and ME instead of BA were required. Maintenance of embryogenic callus of satsumas depended on the combination of plant growth regulators and Gelrite.

Protoplasts were isolated in a maceration medium of 0.4% (w/v) Macerozyme R-10 (Yakuruto Pharmaceutical Co., Toyko), 0.2% (w/v) Cellulase Onozuka R-10 (Yakuruto), 0.1% (w/v) Driselase (Kyowa Hakko Kogyo Co., Tokyo), half-strength MT medium, and 0.7 M sorbitol, according to the methods described by Ling et al. (1989). Typically, yields in the order of 10⁷ protoplasts per gram of callus were obtained. Protoplasts were plated on 60 $\times$ 15-mm plastic dishes at a final density of 5 $\times$ 10⁴ cells/ml. The protoplasm culture medium consisted of plant regulator-free MT medium, 0.3 M sucrose, and 0.3 M sorbitol, solidified with 0.6% (w/v) Difco Bacto-agar. Cultures were maintained in an incubator at 25 $\pm$ 1°C in darkness.

The first protoplasm division (Fig. 3) and colony formation (Fig. 4) were observed 10 to 14 days and 30 days after incubation, respectively. The plating efficiency at 30 days was $\approx$ 10%. Green somatic embryos were apparent 60 days after plating and were transferred to MT medium with 50 g sucrose/liter and 1% (w/v) agar, to stimulate rooting and shoot elongation, under 35.3 μ mol·s⁻¹·m⁻² light intensity for 16 hr daily, at 25 $\pm$ 1°C. At least 20 plantlets were regenerated for each cultivar within 2 months. Plantlets that developed from the somatic

Fig. 5. Plants derived from protoplasts: left 'Ishizuka-wase'; right 'Miyamoto-wase'.

embryos were transplanted to pots in a greenhouse after acclimatization (Fig. 5). Phenotypically, they had a remarkable similarity to nucellar seedling of each cultivar from which the protoplasts were derived.

Embryogenic capacity was maintained through the protoplasts isolated from the callus that was induced from hypocotyl regions of embryos. The full sequence from isolated protoplasts into normal plants in satsuma was achieved by using such embryogenic callus.

Embryogenic callus was established from embryos of undeveloped seed and maintained by this procedure for 14 satsuma cultivars, including the very early, early, midseason, common, and late-ripening types. The protoplasts-from these embryogenic calli will be useful for establishment of somatic hybrids through cell fusion with other citrus protoplasts.

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