J. Plant Biotechnology(2002) *Vol. 4(1). pp. 29~32*

Journal of Plant Biotechnology

Effect of Embryogenic Callus Conditions on Plant Regeneration in Satsuma Mandarin (*Citrus unshiu* Marc.)

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Key words: adventitious embryoid, embryogenic callus, regeneration, satsuma mandarin

Abstract

The ability to form embryiod from callus in satsuma mandarin is low and unstable. In this study, the conditions of embryogenic calli induced from nucellar tissue for promotion of plant regeneration in satsuma mandarin were investigated. The calli of I, II and III line were divided into two sizes of 0.5 mm and 1.0 mm in diameter and two weight gradients of percoll at 40% and 50% though the filter mesh. The frequency of embryo formation from ϕ 1.0mm-40% was slightly higher than callus that from others. Adventitious embryoids developed to a globular stage were transferred to regeneration medium. In 'Miyagawa Wase', the embryos from I and II line developed into a heart stage from most of ϕ 0.5 mm-40% and ϕ 1.0 mm-40% calli, but it failed in 'Sugiyama Unshu'. In the cultivar of 'Miyagawa Wase', 63% of adventitious embryos transferred to the regeneration medium developed into the heart stage from the most ϕ 1.0 mm-40% calli of I line, but of 'Sugiyama Unshu' failed in some calli condition. The embryoids from two callus lines developed further to shoots and plantlets, while the embriods from III line abnormal failed to regenerate in the cultivar. From these results, it is suggested that the plant regeneration from embryogenic callus in satsuma mandarin could be affected by callus conditions.

Introduction

Plant transformation has made it possible to modify just one or two traits, while retaining the unique characteristics of original. The characters that could potentially be mainpulated by genetic transformation of *Citrus* included pest and disease resistance, growth habit, and fruit quality (Péez-Molphe-Balch and Och-Alejo, 1998). In order to use this technology in *Citrus*, it is essential to develop a efficient genetic transformation system suitable for *Citrus*. Some research groups have developed *Agrobacterium tumefaciens*-mediated transformation systems suitable for sweet orange and some other cultivars limited (Hitaka et al., 1990; Moore et al., 1992; Peñaet al., 1995; Péez-Molphe-Balch and Och-Alejo, 1998). The technology, however, is not yet generally accepted as routine method in *Citrus* breeding. The ability to regenerate embryo from callus, single cells or protoplast in sweet orange (*Citrus sinesis*) is high (Kobayashi et al., 1983, 1985, 1988; Kochba and Spiegel-Roy, 1973; Vardi and Spiegel-Roy, 1982), but it is low and unstable in satsuma mandarin. Fortunately, Ling et al. (1990) succeeded in inducing embryogenic calli by culturing undeveloped ovules excised from mature fruits of satsuma mandarin and in regenerating whole plants from embryogenic calli. However, the regeneration efficiency of protoplast culture obtained in these studies was not enough for experiments on genetic transformation to be undertaken. Kunitake et al. (1991) described an efficient protoplast culture system for some leading cultivars of satsuma mandarin using adenine, and subsequent whole

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Received Jan. 5, 2002; accepted Feb. 15, 2002.

plant regeneration via somatic embryogenesis using lactose. The adventitious embryo development from sespension calli divided into sizes through the filter mesh: viz. 37 μm, was much better than others size callus in carrot (Sato, 1979). Han et al. (2002) reported the number of embryo a from on embryogenic calli induced from a nucellar tissue and divided into two sizes and two weights gradient of percoll was 1/8 to 1/15 of sweet orange in satsuma mandarin.

In this study, the conditions of embrogenic calli for promotion of regeneration efficiency in satsuma mandarin were investigated.

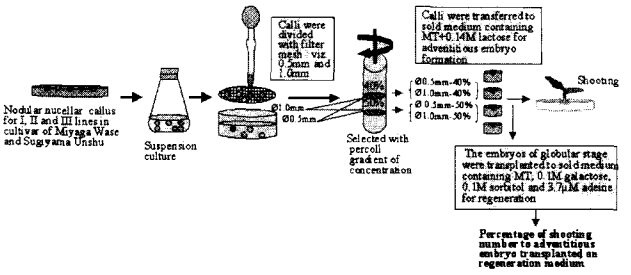

Figure 1. Methods of plant regeneration from nucellar calli in Ⅰ, Ⅱ and Ⅲ lines of satsuma mandarin divided with filter mesh: viz. 0.5 mm and 1.0 mm, percoll gradient of concentration for promotion of regeneration in satsuma mandarin.

Materials and Methods

Establishment of cell culture

Embryogenic calli in satsuma mandarin (*Citrus unshiu* Marc.), 'Miyagawa Wase' and 'Sugiyama Unshu' were induced according to the methods of Hidaka et al. (1990). Calli were obtained from aborted seeds in immature fruits on solid Murashige and Skoog's (MS) medium (1962) containing 0.2 M sucrose, 5 μM kinetin and 0.2% gellan gum. Calli were maintained on the same solid MS medium with 3-weeks interval subculture in a growth chamber at 25±2 ℃ and under 18 hr photo period at a intensity of 33 μM · m^{-2} · s^{-1}.

Selection of calli and plant regeneration

To separate the callus clump into small pieces, three calli of three dishes of each cultivar were transferred to Erlenmyer flask containing 40 ml liquid subculture medium with the same chemical composition except gellan gum, and shaked at 70 rpm at the same temperature and photo condition as mentioned above. The calli in a dish were considered as one line. The medium was changed at intervals of 2 weeks for three times. Calli were divided into two sizes through the filter mesh: viz. 0.5 and 1.0 mm. The calli also were cultured for 1 week in liquid medium containing 0.1 M galactose and 0.1 M sorbitol, the same liquid medium subculture medium. And then, calli were selected with concentration gradient of percoll (colloidal PVP coated with silica, Sigma) at 40 and 50% by centrifugation for 5 min at 4,000 rpm (Figure 1). For the induction of adventitious embryo, the selected calli were cultured on the embryo induction medium, Murashige and Tucker (MT) (1969) solid medium a 0.14 M lactose. Adventitious embryos developed to globular were transplanted to regeneration medium, MT with 0.1 M galactose, 0.1 M sorbitol , 3.7 μM adeine and 0.2% gellan gum. The plant regeneration rates were expressed as percentage of shooting number for adventitious embryos transferred on the medium (Figure 1).

Results and Discussion

Satsuma mandarin is important in *Citrus* industry in Korea and Japan. Its improvement has been based on mutations and clonal selections (Nishiura, 1965). Hybridization of satsuma mandarin with other *Citrus* cultivars and species is difficult due to polyembrony and male sterility. The isolation, culture and plant regeneration of *Citrus* protoplast can be performed in many cultivars and species (Grosser and Gmitter, 1990; Kobayashi et al., 1985; Ohgarwara et al., 1985). The plant regeneration from a single protoplast or callus is dependent on the high potential of somatic embryo formation. However, the potential of cell division, embryo formation and plant regeneration from protoplast or callus is very low in some *Citrus* species and cultivars, especially in mandarin due to the browning and poor growth of callus and embryoids. If the system for plant regeneration from the protoplast and callus of satuma cultivation is established, its biotechnological breeding may be possible. The adventitious embryo development from sespension calli divided into sizes through the filter mesh: viz. 37 μm, was much better than that from the other size in carrot (Sato, 1989).

After embrogenic calli were cultured on the embryo induction medium containing MT and 0.14 M lactose for one month, adventitious embryo formation was observed for both cultivars. The embryo formation from ϕ 1 mm-40% was slightly higher than that from others (Table 1, Figure 2). Adventitious embryoids of globular stage were transferred to the regeneration medium containing MT, 0.1 M galactose, 0.1 M sorbitol, 3.7 μM adeine and 0.2% gellan

Table 1. Effect of callus size and density gradated by percoll concentrations on the adventitious embryoid formation from embryogenic callus in satsuma mandarin.

Cultivars	Diameter (mm)	Percoll concentration (%)	The numbers of embryoid formation[a]
Sugiyama Unshu	0.5	40	6.0±0.63[b]
		50	1.6±0.41
	1.0	40	8.0±1.13
		50	6.0±1.63
Miyagawa Wase	0.5	40	2.0±0.41
		50	0.0±0.00
	1.0	40	13.3±1.13
		50	5.6±0.68

[a]the number of adventitious embryo in 0.1 mL callus volume.
[b]standard error (n=3).

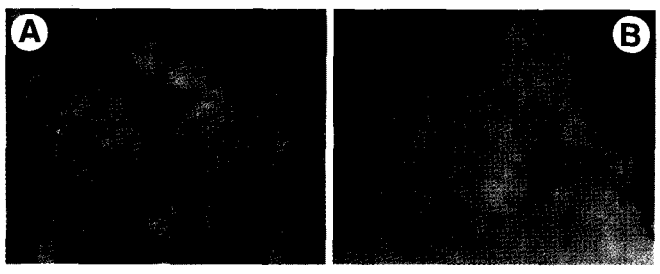

Figure 2. Adventitious embryo formation from 1.0 mm-40% nucellar calli in 'Miyagawa Wase' (A) and 'Sugiyana Unshu' (B) on MT medium containing 0.14M lactose after about one month cultivation.

gum. After transferred to the regeneration medium, adventitious embryoids at globular stage from most ϕ 1.0 mm-40% calli of I line developed into the heart stage with a rate of 63% for 'Miyagawa Wase', but did not for 'Sugiyama Unshu' (Table 2). The embryoids at heart stage further developed into torpedo stage and regenerated subsequently from the cultivar (Figure 3). All embryoids from the III line developed abnormal embryo, and failde to regenerate (Figure 4, Table 2). The callus size weight affected plant regeneration. The adventitious embryo formation and plant regeneration percentage were high from ϕ 1.0 mm-40% callus of I line in 'Miyagawa Wase'. However, the plant regeneration of 'Sugiya Unshu' was not observed under same conditions (Table 2). The calli of 'Miyagawa Wase' were inducted within two years, but the calli from 'Sugiyama Unshu' were even longer. The ϕ 1.0 mm callus may have a potential of cell division, embryo formation and plant regeneration from the I callus line of 'Miyagawa Wase' because the size of callus is same as sweet orange (Han et al., 2002). When the calli were divided more than

Table 2. Effect of cell line, callus size and dencity gradated by percoll concentrations on the plant regeneration in satsuma mandarin.

Cultivars	Cell line	Diameter (mm)	Percoll concentration (%)	Percentage of plant regeneration[a] (%)
Sugiyama Unshu	I	0.5	40	0
			50	0
		1.0	40	0
			50	0
	II	0.5	40	0
			50	0
		1.0	40	0
			50	0
	III	0.5	40	0
			50	0
		1.0	40	0
			50	0
Miyagawa Wase	I	0.5	40	36
			50	12
		1.0	40	19
			50	4
	II	0.5	40	22
			50	13
		1.0	40	63
			50	26
	III	0.5	40	0
			50	0
		1.0	40	0
			50	0

[a]percentage of shooting number to adventitious embryo trans planted on regeneration medium.

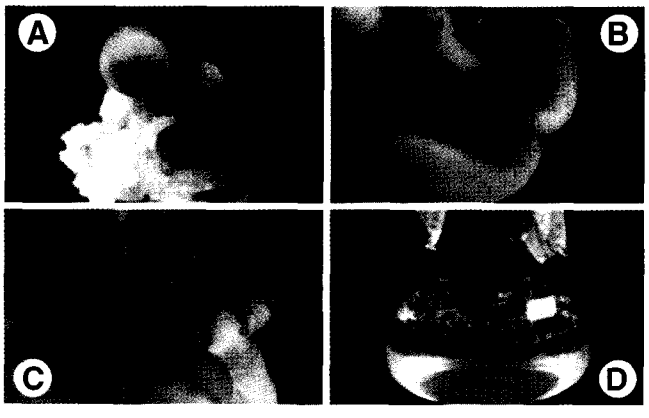

Figure 3. Development of embryoids (A; cotyledon, B; tropede, C; plantlet and D; shoot) from 4 to 10 weeks after transplanting on the regeneration medium from I callus line of ϕ 1.0 mm-40% in 'Miyagawa Wase' calli.

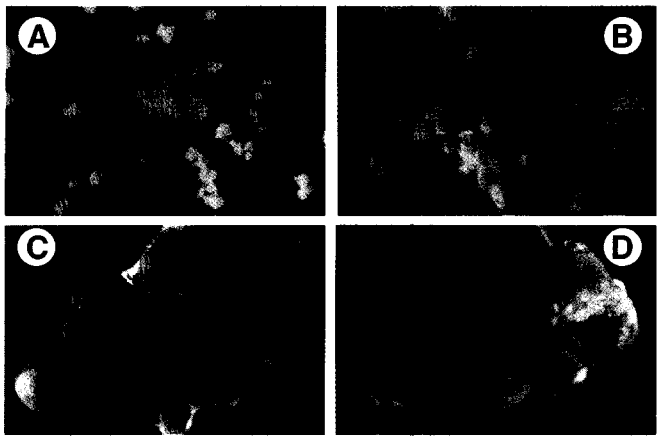

Figure 4. Development of unusual embryoids (B, C and D) on bad condition of embryo (A) from 4 to 10 weeks after trans planting to the regeneration medium in Ⅲ callus line of 'Miyagawa Wase' calli.

one time by weight gradient of percoll concentration, plant regeneration frequency of 40% - part - callus was higher than that of 50% - part callus. It is because the former may contain new callus clump generated for suspension culture. Through present studies, we suggest that the plant regeneration from embryogenic callus in satsuma mandarin is affected by callus line, ages, size and weight. The ϕ 1.0 mm-40% calli of I line from 'Miyagawa Wase' is found to be good materials for transformation and protoplast culture of satsuma mandarin because of its high potential in plant regeneration.

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