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Uniformity of plants regenerated from orange (*Citrus sinensis* Osb.) protoplasts

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Summary. Using 25 plants (protoclones) regenerated from orange (*Citrus sinensis* Osb.) protoplasts, several characters, including leaf and flower morphology, leaf oil, isozyme patterns and chromosome number, were examined. No significant variations in each character were recorded among the protoclones. Uniformity observed among protoclones was identical to that of nucellar seedlings.

Key words: *Citrus sinensis* Osb. – Protoplast – Protoclone – Phenotypic stability

Introduction

Citrus is one of the most important fruit bearing trees in the world and citrus protoplast technology has a potential application in the genetic improvement of this economic plant. Previously, we reported regeneration of plants from 'Trovita' orange protoplasts derived from nucellar callus (Kobayashi et al. 1983; Kobayashi et al. 1985). In order to apply protoplast technology to practical citrus breeding, the investigation of various characters of plants (protoclones) regenerated from protoplasts is required. Therefore, the present article analyses protoclones with respect to leaf and flower morphology, leaf oil, isozyme patterns and chromosome number.

Materials and methods

Plant materials

Callus derived from nucellar tissues of 'Trovita' orange (*Citrus sinensis* Osb.) (Kobayashi et al. 1984) was used as a source of protoplasts. Twenty-five protoclones were regenerated from the protoplasts. Three-year old protoclones and nucellar seedlings grown for approximately the same number of years were used for analysis.

Leaf and flower morphology

Fully expanded leaves were collected from both protoclones and nucellar seedlings. Leaf shape index was calculated by the length/width of the leaf blade. Other leaf characteristics, such as wing, thickness, color, apex shape and margin serration, were also recorded. Since one of the protoclones reached the flowering stage, morphological characters of the flowers and pollen stainability were examined. Pollen stainability was checked by staining 1,000 pollen grains with acetocarmine.

Chromosomes

Root-tips of protoclones pretreated with 8-hydroxyquinoline (2 mM) for 20 h at 10 °C were fixed in ethanol: acetic acid (3 : 1) for 24 h, and then stained with lacto-propionil orcein for 3 h according to Oiyama (1981).

Gas chromatography of leaf oil

Leaf oil samples were prepared by the following sequences: fresh leaves (1 g) were cut into narrow strips (about 2 mm) and placed into a sample tube containing 10 ml methylene chloride for 16 h at 4 °C. About 0.5 g active clay was added to the sample tube, which was then filtered through Whatman paper (No. 1). The filtrate was condensed to about 100 µl in vacuo at 40 °C. Leaf oil samples prepared from all protoclones and control nucellar plants were analysed by a gas chromatograph (Shimadzu, Model GC-9A) equipped with a flame ionization detector. A column packed with 20% PEG 20M on 60–80 mesh Chromosorb W, 3.2 mm × 2.1 m, was used. The column temperature was programmed from 60 °C for 5 min to 195 °C for 15 min at a rate of 10 °C per minute. The flow rate of nitrogen gas, hydrogen gas and air were 50, 45 and 500 ml/min, respectively. A 2 µl sample was injected into the instrument, whose injection block temperature was kept at 230 °C. Peaks on the chromatograms were identified by comparing their relative retention time (Rt) with retention times of control compounds and/or GC-MS.

Isozyme analysis

Freshly isolated roots were stored at −70 °C until use. The frozen tissue (0.2 g) was ground in a small mortar with 1 ml of cold 20% sucrose solution. The homogenate was then centrifuged for 5 min at 15,000 × g. Aliquots from the supernatant (20~30 µl) were layered on the top of a vertical polyacrylamide slab gel containing 7.5% acrylamide, 0.2% BIS, 20% glycerin, 2.5% carrier ampholyte (pH 3–10), 0.05% TEMED and 0.24% ammonium persulfate. The anode vessel on the top and the cathode vessel at the bottom were filled with 40 mM DL-aspartic acid and 1 M NaOH, respectively. Voltage across the gel was constantly kept at 200 V and electrophoresis was performed in a refrigerator at 4 °C for 14 h. Following electrophoresis, the gels were washed for 20 min with distilled water and then immersed in the solution shown in Table 1.

Results

Morphological and cytological characteristics of protoclones

Leaf characteristics of protoclones were examined. The leaf shape index and a photograph of leaves are shown in Table 2 and Fig. 1, respectively. There were no significant differences in the leaf shape index among protoclones, and between protoclones and nucellar seedlings; no differences were noticed in other leaf characters such as wing shape, thickness, color, apex shape and margin serration among these plants. Since one protoclone set the flowers, observations on flower characteristics were made in those of this protoclone and of a whole plant. No significant differences were seen between the two types of plants (Fig. 2, Table 3).

All protoclones possessed diploid chromosomes (2n = 18), whose morphology was normal (Fig. 3).

Gas chromatography of leaf oil

Gas chromatograms and chemical composition of leaf oil were shown in Fig. 4 and Table 4, respectively.

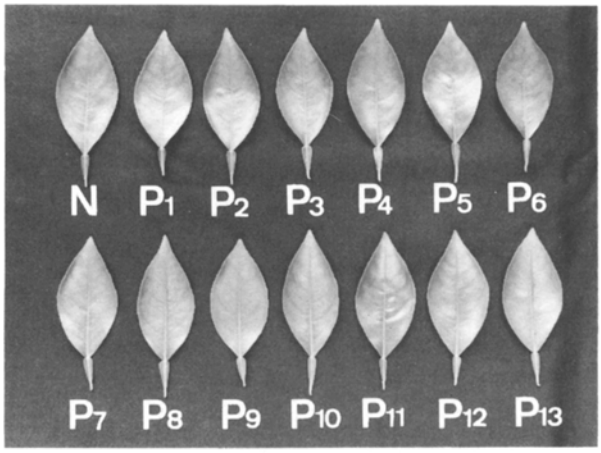

Fig. 1. Leaves of protoclones ($P_1 \sim P_{13}$) and a nucellar seedling (N)

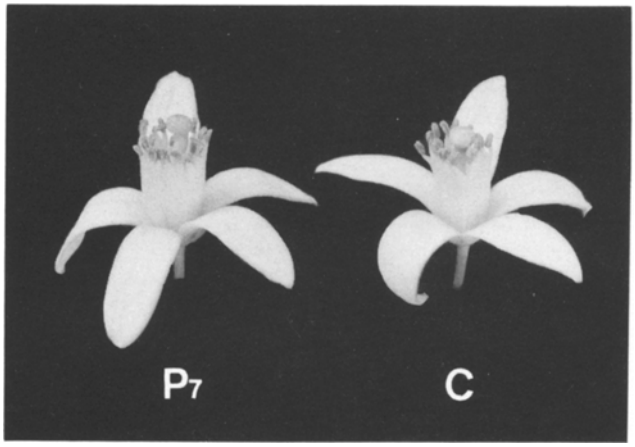

Fig. 2. Flowers of a protoclone (P_7) and a whole plant (C)

Table 1. Staining protocol of the enzymes

Enzyme	Staining			Reference
	Solution	Temp.	Time (h)	
Peroxidase	94.5 ml 2M Na-acetate buffer (pH 5.0), 50 mg 3-amino-9-ethyl calbazol (disolved in 5 ml N-N-dimethyl formamide), 0.5 ml 3% H_2O_2	room	0.5	Hoyle (1977)
Esterase	*preincubate:* 98 ml 0.1 M phosphate buffer (pH 6.3), 2 ml 1% α-naphthyl acetate in 50% acetone	37 °C	1	De Loose (1979) (modified)
	staining: 0.5% Fast Blue B salt (spraying)	room	0.5	
Phosphatase	100 ml 0.1 M Na-acetate buffer (pH 4.6), 30 mg α-naphthyl acid phosphate, 300 mg Fast Blue BB salt	room	2	Keck (1961)
Glutamate-oxaloacetate transaminase	10 ml 1 M tris-HCl buffer (pH 8.8), 115 ml H_2O, 5 mg pyridoxal-5-phosphate, 200 mg L-aspartic acid, 100 mg α-ketoglutaric acid, 150 mg Fast Blue BB salt (in the dark)	room	2	Torres (1978)

Table 2. Comparison of leaf shape index between protoclones and nucellar seedlings

Plant		Leaf shape index	Plant		Leaf shape index
Protoclone	1	2.00±0.07[a]	Protoclone	15	1.98±0.08
	2	1.97±0.08		16	1.97±0.08
	3	1.96±0.08		17	1.96±0.08
	4	1.99±0.09		18	1.97±0.07
	5	1.96±0.07		19	1.95±0.09
	6	1.99±0.09		20	1.96±0.09
	7	1.95±0.08		21	1.99±0.10
	8	1.98±0.08		22	1.96±0.08
	9	1.96±0.07		23	1.98±0.08
	10	1.97±0.10		24	1.96±0.07
	11	1.97±0.08		25	1.98±0.09
	12	1.96±0.07			
	13	1.96±0.09	nucellar	1	1.98±0.07
	14	2.01±0.08		2	2.00±0.08

[a] Each value represents the mean ± standard deviation

Table 3. Comparison of the quantitative characteristics of a flower from a protoclone and a whole plant

Plant	Petal length (mm)	Stamen no.	Filament length (mm)	Pollen stainability (%)	Ovary diameter (mm)	Ovary height (mm)
Protoclone 7	20.2±0.5[a]	23.6±2.2	11.8±0.4	76.6±3.2	3.4±0.2	3.2±0.2
A whole plant (control)	20.3±0.4	24.1±1.4	11.7±0.4	77.4±4.0	3.4±0.2	3.2±0.2

[a] Each value represents the mean ± standard deviation

Table 4. Chemical composition of leaf oil

Peak no.	Compound	Peak area percentage
1	α-pinene	0.6±0.10[a]
2	sabinene	8.0±1.50
3	Δ^3-carene + myrcene	8.9±1.19
4	limonene	34.6±6.13
5	r-terpinene	3.9±0.49
6	terpinolene	0.9±0.22
7	unidentified	1.0±0.19
8	citronellal	2.6±0.69
9	linalool	4.3±0.83
10	isopulegol	2.2±0.40
11	terpineol-4	1.5±0.17
12	caryophyllene	2.0±0.42
13	α-terpineol	12.7±2.11
14	geranial	16.8±3.09

[a] Each value represents the mean ± standard deviation

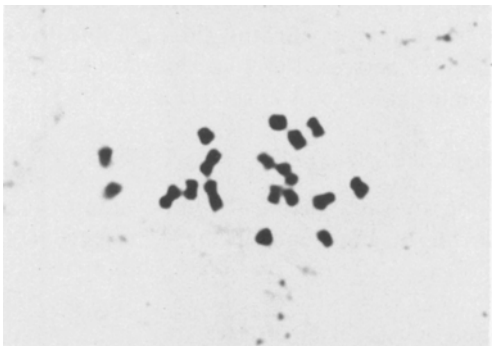

Fig. 3. Eighteen chromosomes were present in all of the protoclones

Although only gas chromatograms of 8 protoclones are shown in the figure, the other protoclones showed almost similar profiles. In the gas chromatograms, minor differences were observed among protoclones, however, such minor differences existed even between each leaf of the same plant. For this reason, it was concluded that no variation was present in the chemical composition of the leaf oil among protoclones; no differences were noticed between the protoclones and control plants.

Isoelectric focusing

Four enzymes were analysed in protoclones and control plants. Photographs of the isozyme patterns of 9 protoclones were shown in Fig. 5. The other protoclones showed similar profiles. With respect to the isozyme patterns of all enzymes analysed, no differences were

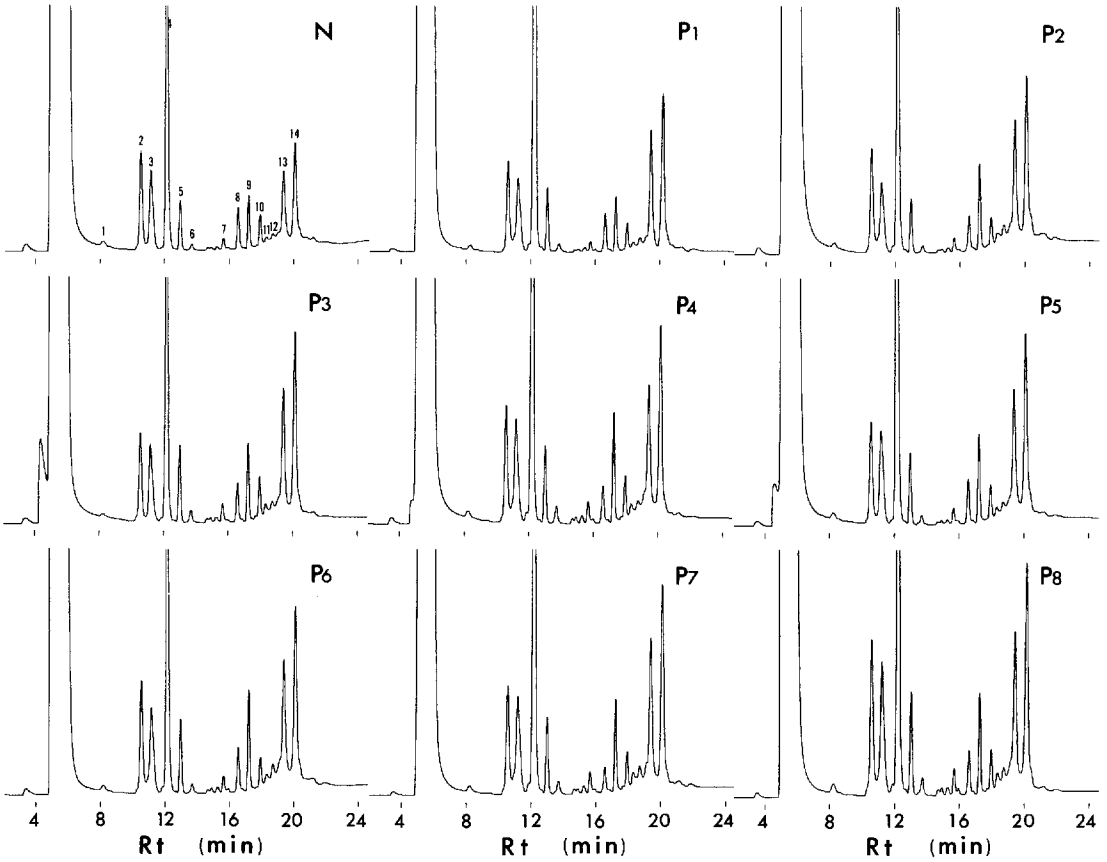

Fig. 4. Gas chromatograms of leaf oil from protoclones and nucellar seedling. N: nucellar seedling; $P_1 \sim P_8$: protoclones

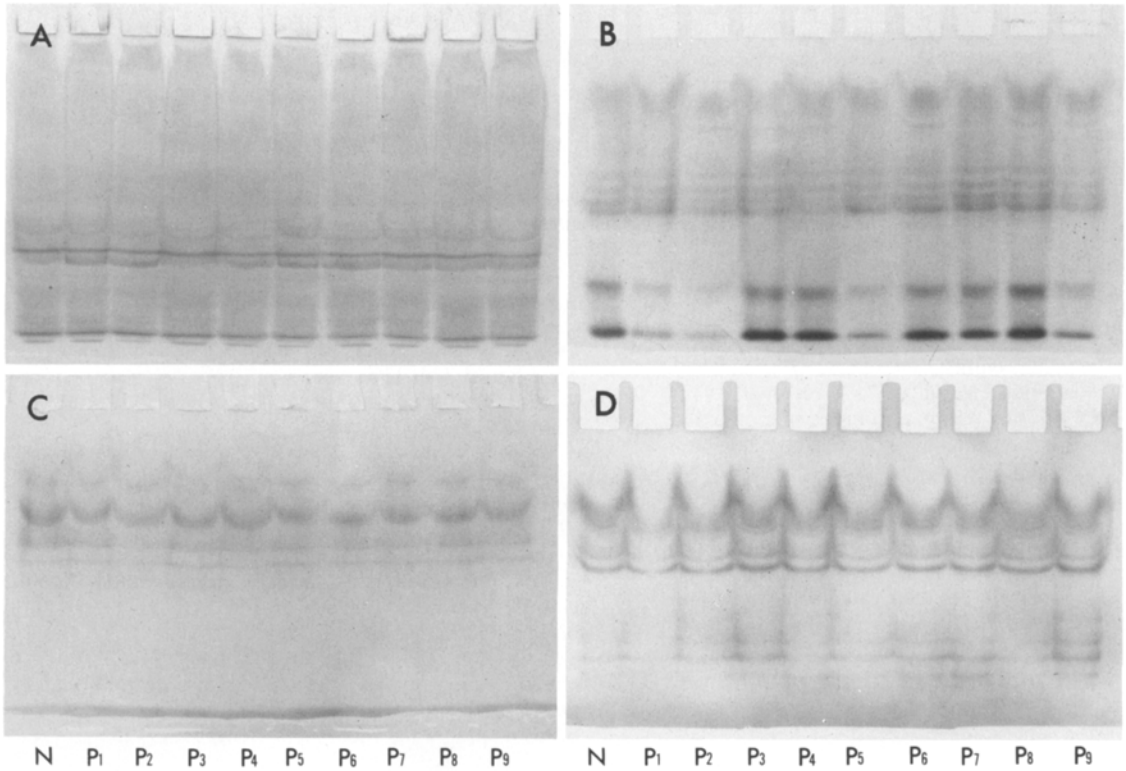

Fig. 5. Isoelectric focusing profiles of several isozymes including peroxidase (**A**), esterase (**B**), phosphatase (**C**) and glutamate-oxaloacetate transaminase (**D**) from protoclones ($P_1 \sim P_9$), and nucellar seedling (N)

seen in the relative position or number of bands among protoclones, and between protoclones and the control plants.

Discussion

In recent years, the significance of protoplasts as a new tool for the improvement of plants has been repeatedly emphasized. When crop improvement, especially improvement of only one phenotypic character, is planned by protoplast technology, the regeneration of plants possessing normal phenotypic characteristics is needed.

In many cases so far reported, however, plants (protoclones) regenerated from protoplasts display considerable phenotypic variability (Matern et al. 1978; Prat 1983; Shepard et al. 1980; Takebe et al. 1971; Thomas et al. 1982). On the other hand, genetic stability of protoclones has also been reported in some plants: asparagus, tobacco and potato (Bui Dang Ha et al. 1975; Hayashi and Nakajima 1984b; Wenzel et al. 1979). In citrus, Vardi (1977) reported that 9 out of 10 protoclones examined had the diploid number of chromosomes ($2n=18$) while the exception was tetraploid ($2n=36$). She also noted that the peroxidase profiles of 5 out of 6 protoclones examined were identical to the enzymes of nucellar seedlings while the sixth showed a different enzyme profile. Vardi et al. (1982) also reported that protoclones had normal and uniform morphological features. In the present paper, intensive analysis was made using 25 protoclones of 'Trovita' orange. The results clearly show that all protoclones are phenotypically similar to the control plants with respect to the several characteristics examined. Vasil (1983) described that genetic stability found in plants regenerated from callus or tissue of many species of cereals and grasses was considered to be the result of regeneration through somatic embryogenesis. In fact, protoclones showing phenotypic stability were regenerated via somatic embryo (Bui Dang Ha et al. 1975) or via small organized globular cell clumps (Hayashi and Nakajima 1984a). Wenzel et al. (1979) did not show this manner of plant regeneration. On the contrary, protoclones showing phenotypic variability have been regenerated via organogenesis from well-developed calli (Matern et al. 1978; Prat 1983; Shepard et al. 1980; Takebe et al. 1971; Thomas et al. 1982). Consequently, phenotypic stability found in citrus protoclones could be due to the manner of plant regeneration, namely through somatic embryogenesis (Kobayashi et al. 1983; Kobayashi et al. 1985; Vardi 1977; Vardi et al. 1982). The facts presented in this study encourage the practical application of protoplast technology to citrus breeding program.

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