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In vitro Propagation of Junos Orange (*Citrus junos* Sieb.) through Nucellar Polyembroid Cultures

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ABSTRACT : An *in vtro* nucellar polyembryo propagation method was established with mature seed of the *Citrus junos* Sieb. 7-8 nucellar polyembryos per seed were induced on MS basal medium without plant growth regulators. The polyembryos developed to complete plantlets on teatment with IBA. These shoots grew further in MS medium without plant growth regulators. Rooting of shoots occurred on MS medium supplemented with IBA. These plantlets were successfully transplanted to small plastic pot containing soil mixture. Somatic embryos were induced from nucellar polyembryo and maturation occurred spontaneously from proliferating cultures on MS medium without growth regulators. Random Amplified Polymorphic DNA (RAPD) marker analysis of *in vitro* and *in vivo* grown junos orange showed identical polymorphism indicative of their genetic stability. The RAPD polymorphism produced revealed same banding pattern in each regenerant. Hence, propagaton of junos orange by nucellalr polyembryos was efficient and produced in genetically stable plants under *in vitro* conditions.

Key words : genetic stability, *in vitro* propagation, polymorphism

INTRODUCTION

The genus *Citrus* is recognized as one of the economically important plant throughout the world. Among these *Citrus* family, junos orange (*Citrus junos* Sieb.) is an important fruit, its annual production exceeding 78 million tons (Ling & Iwamasa, 1997). There are more than twelve local species of junos orange grown in the coastal Korean peninsula including the Jeju islands.

C. junos is cultivated in the southern part of Korea and Japan, whose fruit is processed into juice and is often preferred to vinegar as an ingredient in sauces and salad dressings for its special flavor (Kato-Noguchi *et al.*, 2002). *C. junos* peel possessed potent allelopathic activity and the powder of the peel was also effective as a weed suppressive agent because of the growth inhibition of several weed species (Fujihara & Simizu, 1999). In addition, naringenin, a constituent of major flavanone to be included in *C. junos* had novel anti-cholinesterase activity *in vitro* and *in vivo*. (Heo *et al.*, 2003).

However, breeding of *Citrus* by recombination and selection or mutation is limited in application due to heterozygosity and prolonged juvenile period (Button & Kochba, 1977). Hybridization by conventional methods between *Citrus* and related genera is also hampered because of incompatibility, poor germination and in viability of hybrids (Germana *et al.*, 1994). Uses of *in vitro* methods for selection and propagation

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are thus of great importance in improving the *Citrus* cultivars. Micropropagation methods and morphogenesis have been described for a number of Citrus species (Duran-Vila *et al.*, 1989; Duran-Vila *et al.*, 1992; Gill *et al.*, 1995). However, there is still requirements for an efficient regeneration protocol. Similarly, junos orange is also under constant danger from disease and infections. A few *in vitro* culture methods have been attempted as an alternative method for germplasm preservation and genetic improvements of junos orange (Marin & Duran-Vila, 1991; Tao *et al.*, 2002). However, improved methods like tissue culture for junos orange are scanty. This study describes an efficient propagation system by nucellar embryonic cultures of junos orange with genetic stability *in vitro* cultures.

MATERIAL AND METHODS

Plant material

15-old year Junos orange plants were collected in Korea in 2002 from cultivation of Namhae island (Fig. 1A), the fruits were handpicked, washed with water and surface sterilized by treating with 70% (v/v) ethanol for 8 min, 6% (w/v) sodium hypochlorite (NaOCl) solution for 10 min, followed by three rinses with sterile distilled water. The fruits were cut open with a sterile knife to collect the seeds. All these operations were carried out in laminar flow hood.

Nucellar polyembryo development

For the investigation of nucellar polyembryo formation and plantlet formation based on seed maturity, fruits were collected between June 26 to October 01 in 2002 (Fig. 1B). Seeds were uncoated and incised with sterile blade to wound to being placed on MS medium.

Several basal media; White (White, 1963), LP (Quoirin & Lepoivre, 1977), B5 (Gamborg *et al.*, 1968), SH (Schenk & Hildebrandt, 1972), MS (Murashige & Skoog, 1962), 1/2 MS and 2MS with 3% (w/v) sucrose and 0.3% (w/v) gelite were tested for ability to support nucellar polyembryo development.

Shoot and root elongation of nucellar polyembryos were carried out in MS medium supplemented with 2, 4-D, IBA, IAA and NAA at various concentrations (0.0~2.0 ㎎ ℓ^{-1}).

All media pH was adjusted to 5.6±0.1 and about 20 ㎖ aliquots poured into 86 ㎜ Petri plates. Each plate was inoculated with three seeds and incubated in a growth chamber at 25±2℃ with 16/9 hr (light/dark) photo period, under cool fluorescent white light (25 ㎛ol $m^{-2}s^{-1}$).

Acclimatization of plant

Rooted plants were removed from culture flasks, washed for free of agar and transferred to small plastic pots containing sterilized soil, sand and organic material 1:1:1 (v/v) and covered with polythene bags. These were grown for 3~4 weeks in a greenhouse maintained 70% relative humidity (RH) and a temperature of 26℃.

Extraction of genomic DNA

Junos orange leaves from *in vitro* and *in vivo* plants were collected and the genomic DNA was extracted by the modified method of Murray & Thomson (1980). 0.2~0.5 g of leaf material was homogenized and transferred into a clean plastic tube containing 3.0 ㎖ of extraction buffer (2% CTAB, 100 mM Tris-HCl, 20 mM EDTA and 1.4 M NaCl) pre-heated to 65℃ and maintained for 30 min. To this 3.0 ㎖ of chloroform and isoamyl alcohol (24:1, v/v) was added and mixed thoroughly by inversion and subjected to centrifugation at 12,000 rpm for 15 min. The supernatant was transferred to a clean plastic tube to which 400 ㎕ 1 M CsCl and 800 ㎕ absolute ethanol was added and kept at -20℃ for 10 min. The DNA was pelleted by centrifugation at 12,000 rpm for 5 min washed with 70% ethanol and dried. Finally, DNA was dissolved in 50 ㎕ TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) buffer. After centrifugation, the pallet was dissolved in 100 ㎕ sterile distilled water. One ㎕ of this sample was used for a 25 ㎕ volume PCR reaction.

PCR amplification and eletrophoresis

RAPD analysis was carried out by PCR amplification using 20 commercial random ten-mer primers (Operon Tech, USA). The PCR reaction conditions

were optimized as described by Cai *et al.* (1994). Amplification reactions were carried out reaction recipes in total of 10 μℓ containing; 0.2 μℓ of Taq polymerase (TaKaRa, Biomedical INC., Japan), 1.0 μℓ of 10X Taq buffer, 0.4 μℓ of 20 mM dNTP, 0.2 μℓ of 10 pM primer (Forward), 0.2 μℓ of 10 pM primer (Reverse), 1.0 μℓ genomic DNA (100 ng) 7 μℓ of sterile H_2O. PCR was carried out for 30 cycles after an initial denaturation at 95℃ for 2 min. Thermocycler (Gene Tech Company Ltd., Japan) was programmed with a denaturation for 30 sec at 95℃, annealing for 30 sec at 34℃ and ramp for 1 min from 34 to 72℃ with and extension for 2 min at 72℃. The last cycle was followed by incubation at 72℃ for 10 min. Amplified DNA fragments were loaded onto 1.2% agarose gels containing ethidium bromide. Electrophoresis was carried out at 100 V for 1 hr. Amplified DNA products were radiated under UV light. In all cases, 1 kb DNA ladder (Fermentas, USA) served as DNA size marker.

Statistical analysis

For each set of experiments, 15 replicates were taken and the experiment was repeated twice, thus giving 30 replicates for each treatment. Duncan's Multiple Range Test (DMRT) and LSD (Least Significant Difference) were used to analyze the variations. Values were represented as mean ± standard deviation (SD).

RESULTS AND DISCUSSION

Plantlet formation of nucellar polyembryos

Junos orange seeds were uncoated and transferred onto various basal media to develop nucellar polyembryos. The nucellar embryo in each seed was developed into above 4 plantlets (Fig. 1C and D). Excised shoots, on transfer to the same medium without growth regulators, yielded a fresh crop of 8~10 shoot buds, but root not induced before transferring

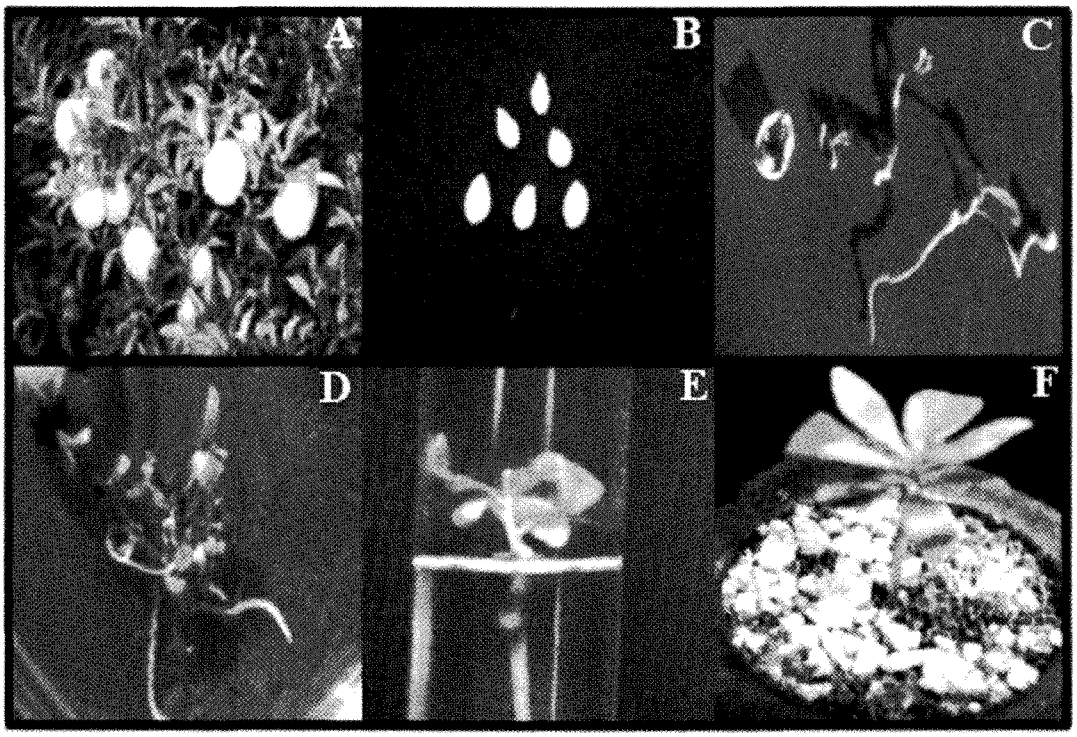

Fig. 1. *In vitro* propagation of nucellar polyembryo of junos orange. (A) Native junos orange tree growing in Namhae Island, Korea. (B) Seeds of junos orange collected from fruits. (C) Nucellar embryos generated from junos orange seeds cultured on MS medium with 0.1 mg ℓ^{-1} IBA (4 weeks after culture). (D) Germination of nucellar polyembryo on MS medium with 0.1 mg ℓ^{-1} IBA (4 weeks after culture). (E) Shoot elongation and rooting of nucellar polyembryo in a MS medium with 0.1 mg ℓ^{-1} IBA (8 weeks after culture). (F) 6-month-old generated plants in pots of greenhouse.

to rooting medium. Although the percentage of rooting and shooting decreased in later subcultures, the length of shoots or roots thus formed did not show much change. All shoots rooted well and produced complete plantlets (Fig. 1E). These plantlets could be transferred to the soil with 60% success and be maintained. The plants grew vigorously in potted soil (Fig. 1F).

Effect of seed maturity on nucellar polyembryos development and plantlet formation

The effects of seed maturity on to correct effect *nucellar polyembryos* formation and plantlet formation were determined by counting the number of embryos and plantlets formed from each seed. The experiments demonstrated that young seeds (collected early in the fruiting season) were more efficient in nucellar polyembryo formation in comparison to mature seeds (collected later in the fruiting season) (Fig. 2). On the contrary, mature seeds were better than young seeds in plantlet formation. This suggests that germination and development of nucellar polyembryo may be different physiological processes.

Nucellar polyembryo development was recorded in all seeds irrespective of seed maturity. However, the germination from the nucellar polyembryos was independent of the time of seed collection periods. Thus, intermediate seed maturity (collected in 6 August) was more efficient for good nucellar polyembryo formation as well as germination rates. The seed collection time was important in determining of the nucellar polyembryo cultures, since it is related to maturity of embryos and therefore is responsible for variation, further the ecological condition under which the plants are grown could be yet another factor (Taylor, 2001; Chaudhury *et al.*, 2001).

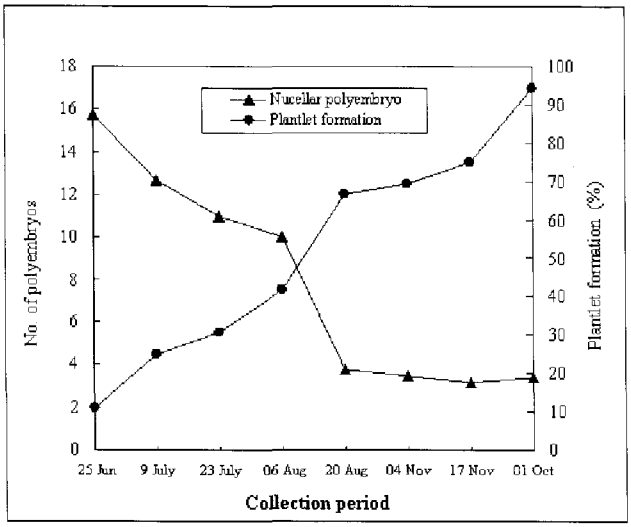

Fig. 2. Plantlet formation and polyembryos number based on maturity of junos orange seeds. Seeds collected between Jun 26 to Oct 01 in 2002 were allowed to investigate nucellar polyembryo formation on MS basal medium. The nucellar polyembryos formed were counted after 4 weeks culture.

Effects of medium and plant growth regulators on nucellar polyembryos development

On efficient culture medium for nucellar polyembryos development, induction of multiple shoots and roots of junos orange, MS medium was more efficient than others on both applications (Table 1). MS based media such as 1/2 MS, MS and 2MS were efficient for shoot induction, whereas 1/2 MS, B5 and LP medium had almost the same effect on germination of junos nucellar polyembryos. However, White and SH medium were ineffective on root and shoot induction. Based on these results, MS medium was selected as the most effective culture medium for root and shoot induction. Somatic embryo propagation of junos orange members required supplementation of

Table 1. Effects of various culture media in induction of shoots and roots from nucellar polyembryos of junos orange.

Medium	Induction of shoot (%)	Induction of root (%)
White	20.63 ± 0.58^{c}	5.26 ± 1.15^{cd}
LP	44.37 ± 0.11^{bc}	34.60 ± 0.29^{ab}
B5	44.31 ± 0.51^{bc}	39.15 ± 0.58^{ab}
SH	19.63 ± 1.10^{cd}	20.13 ± 0.58^{bc}
1/2MS	58.68 ± 2.00^{ab}	29.39 ± 0.50^{b}
MS	81.58 ± 0.70^{a}	63.61 ± 1.00^{a}
2MS	43.70 ± 0.50^{bc}	6.15 ± 1.04^{c}

The junos orange seeds were cultured on various basal culture media without growth regulators for 4 weeks. Duncan's Multiple Range Test (DMRT) and LSD (Least Significant Difference) were used to analyze the variations. All data represents mean standard deviation.

0.05 mg ℓ^{-1} 2, 4-D, 0.05 mg ℓ^{-1} benzyl adenine (BA), 400 mg ℓ^{-1} malt extract and 50 g ℓ^{-1} sucrose in the basal media (Ling & Iwamasa, 1997).

The experiments undertaken to determine the effects of growth regulators (2,4-D, IBA, IAA and NAA) on nucellar polyembryo development in MS medium, demonstrated that IBA was the most effective (Data not shown). IBA concentration of 0.1 mg ℓ^{-1} was found to be optimal for shoot and root induction (Table 2). In general, adding of growth regulators to the medium markedly enhances the embryogenic response of plants. However, we observed that nucellar polyembryo development could also occur in junos explants cultured on MS basal medium without growth regulators. It may be possible that the nucellar polyembryos have enough tissue to contain the necessary endogenous plant growth regulators required for embryogenesis (Gill *et al.*, 1995).

Table 2. Effect of IBA concentration on the shoot and root induction in nucellar polyembryo of junos orange.

IBA Con. (mg ℓ^{-1})	Induction of shoot (%)	Induction of root (%)
0.0	35.63 ± 0.58^{d}	40.26 ± 1.15^{cd}
0.1	75.37 ± 0.11^{a}	80.60 ± 0.29^{a}
0.5	59.30 ± 0.51^{bc}	51.15 ± 0.58^{bc}
1.0	45.63 ± 1.10^{cd}	42.13 ± 0.58^{c}
2.0	55.68 ± 2.00^{bc}	63.39 ± 0.50^{b}

Seeds of junos orange were cultured on MS medium with various concentrations of IBA for 4 weeks. Duncan's Multiple Range Test (DMRT) and LSD (Least Significant Difference) were used to analyze the variations. All data represents mean standard deviation.

Induction and development of somatic embryo

Somatic embryos from nucellar polyembryo of junos mature seed were induced on MS basal medium without growth regulators. The embryogenic tissues formed after three to four weeks in cultures are selected visually for embryogenic features and transferred to MS basal medium for maintenance (Fig. 3A). It swelled up and formed many tiny somatic embryo. Within 4 weeks, embryogenic tissues were developed to various developing stages (Fig. 3B). The frequency of somatic embryo formation often increased subculturing of embryoid tissues. Culturing these explants in the light led to greening and further development of embyoids. Germination of somatic embryos and development of root and shoot was achieved (Fig. 3C).

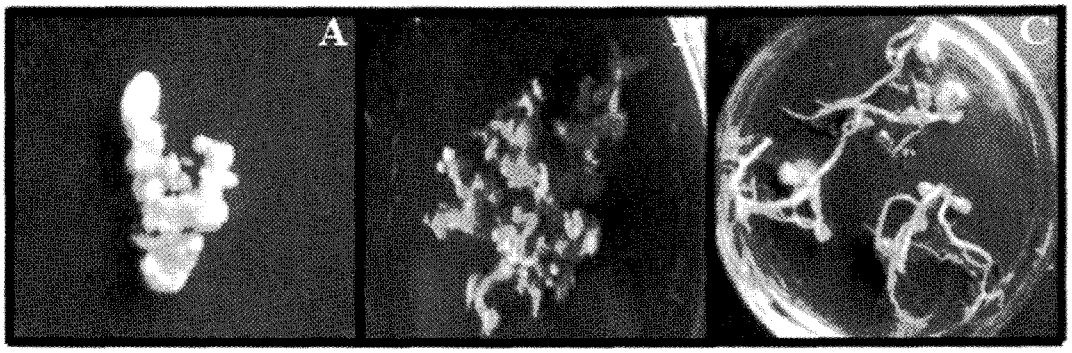

Fig. 3. Somatic embryo induction and plantlet formation from nucellar polyembryony of junos orange. (A) Induction of somatic embryo on MS medium without growth regulator (after 4 weeks culture). (B) Development of somatic embryos (after 8 weeks culture). (C) Germination of somatic embryos and plantlet formation (after 12 weeks culture).

Genotype stability of regenerated junos orange plant

The high quality of genomic DNA was obtained by extraction of junos orange leaves from *in vivo* and *in vitro* plantlets, and PCR amplified with 10 mer random primers. All of the primers, OPG (5'-AGGGCCG TCT-3') OPH (5'-CCTACGTCAG-3') and OPI (5'-ACAACGCGAG-3') OPJ (5'-AAGCCCGAGG-3') primer sets yielded electrophoresis banding pattern

specific to junos orange (Fig. 4). These primers produced above 5 bands in each regenerants. The size of amplified DNA fragments by ten primers ranged from about 700 to 4361 base pairs. The RAPD polymorphism produced revealed same banding pattern in each regenerants. Regenerated plantlets exhibited normal morphology compared to seedling and were not observed phenotypic variation among regenerants. This result indicated that *in vitro* propagation of nucellar polyembryo of junos orange was highly efficient.

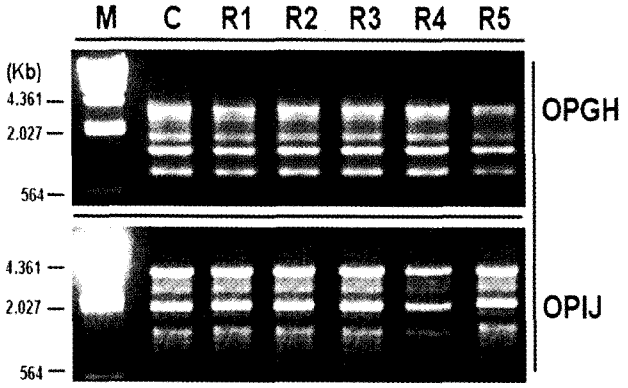

Fig. 4. Gel electrophoresis of amplication products in the nucellar polyembryo derived plants (R1-R5) and native growing plants (C) of junos orange with RAPD primers OPGH (Upper) and OPIJ (Bottom).

Although plants regenerating in *in vitro* culture may show variability (Chakravarty & Sen, 1992), the plants regenerating from citrus culture appear to be uniform and genetically stable, as also reported by others (Kabayashi *et al.*, 1984; Germana *et al.*, 1994). Chaturvedi & Sharma (1987) found no variation among plants regenerating from 9-year-old root cultures of Solanum khasianum and 5- to 6-year-old cultures of *Atropa belladona*. Nucellar adventive polyembryos are of the great significance in Citrus propagation. The adventitious embryo provide uniform seedling of parental type, as obtained through vegetative propagation by cuttings. In this study, we determined optimal culture conditions required for nucellar polyembryo development and plantlet formation. Our results demonstrate that junos orange can be efficiently mass propagated by nucellar polyembryo into plants with genetic stability.

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