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In Vitro Production of Indian Citrs Ringspot Virus-Free Plants of Kinnow Mandarin (*Citrus nobilis* Lour *X C. deliciosa* Tenora) by Ovule Culture

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Abstract

Indian citrus ringspot virus (ICRSV)-free plants of Kinnow mandarin (*Citrus nobilis* Lour *x C. deliciosa* Tenora) were raised from virus-infected plants using unfertilised ovules as explants. Plants were tested by indirect ELISA and RT-PCR before using their explant. An amplified product of 539 bp was obtained by RT- PCR in ICRSV infected plants. Unfertilized ovules were excised from unopened flower buds of plants tested postive for virus and were cultured on Murashige and Skoog's (MS) basal medium supplemented with various concentrations of kinetin (KN) or malt extract (ME). Maximum induction (31.94%) of embryogenic callus was observed on MS medium supplemented with KN (9.29 μM). Transfer of embryogenic calli to similar media composition resulted in somatic embryogenesis in all cultures, with an average number of 60.36 globular, 17.39 heart and 7.71 cotyledonary-shaped somatic embryos per culture. All cotyledonary shaped embryos developed into complete plantlets within 60 days on transfer to similar medium. Embryogenic callus induction, somatic embryo formation, maturation, germination and plantlet formation were achieved on MS medium supplemented with KN (9.29 μM) alone. The plantlets derived from somatic embryos were transferred to sterilized soil, sand and vermiculite (3:1:1) mixture. After acclimatization, the plantlets were transferred to screen house and were indexed for ICRSV employing indirect ELISA and RT-PCR and found free of virus. A distinct feature of this study is the induction of somatic embryogenesis from unfertilised ovules to produce virus-free plants.

Key words: Citrus, ICRSV (Indian citrus ringspot virus), Kinnow (*Citrus nobilis x Citrus deliciosa*), Ovule culture, Somatic embryogenesis

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Introduction

Kinnow (*Citrus nobilis* Lour *x C. deliciosa* Tenora) was first introduced in the USA (California)) from crossbreeding of two varieties of mandarins(king and leaf willow). It became popular in Punjab(India) in 1958 and is the major fruit crop. Kinnow has replaced the traditional cultivars of sweet orange due to its outstanding adaptation to agroecology of Punjab, which led to profuse vegetative growth and heavy yield with good fruit quality. In Punjab, out of 10,350 ha area of *Citrus*, 9933 ha is under kinnow cultivation. However, in recent years, ICRSV infection resulted in tremendous loss in the yield and quality of this fruit crop. In India, the existence of ICRSV was first documented in 1993 from Delhi (Byadgi et al. 1993). ICRSV is graft transmissible and is disseminated primarily by infected bud-wood. It has been found widely distributed throughout India (Byadgi et al. 1995), with its incidence ranging from 10 to 100% (Pant and Ahlawat 1998). Lamina of infected plant exhibits chlorotic flecks, leaf mottling, ringspots and irregular chlorotic patterns on mature leaves. An alarming decline in kinnow production necessitates the development of strategies for controlling viral diseases and production of healthy virus-free kinnow orchards in the country, especially in Punjab.

Three types of *in vitro* techniques, viz. nucellus culture, ovule culture and shoot tip grafting (STG), had been used to recover virus-free citrus plants by Navarro (1984). Somatic embryogenesis had been observed in *Citrus* through the culture of entire fertilized ovules, or isolated nucellar embryos from polyembryonic *Citrus* genotypes (Litz et al. 1985). Somatic embryos had also been produced from abortive (Bitters et al. 1970), unfertilised (Button and Bornman 1971) and undeveloped (Starrantino and Russo 1980; Moore 1985) ovules. Navarro (1984) used ovule culture to produce virus free *Citrus* plants. Gill et al. (1994) reported somatic embryogenesis and plantlet regeneration from callus derived from seedling explants of kinnow mandarin. Rahaman et al. (1996) obtained cotyledonary embryoids from ovules of five *Citrus* species, i.e. *C. reticulata , C. reticulata x C. sinensis, C. madurensis, C. reshni* and *C. sinensis*, using MS medium supplemented with kinetin or BAP. Carimi et al. (1998) reported somatic embryogenesis from undeveloped ovules of sweet orange (*Citrus sinensis*). Perez et al. (1999) reported callus induction in 25% cultures from ovules of kinnow. D'Onghia et al (1997) reported that somatic embryogenesis initiated from style results in virus elimination from lemon cultivars. D'Onghia et al. (2001) successfully eliminated *Citrus* psorosis virus by somatic embryogenesis using stigma and style explant of common mandarin (*C.reticulata*), sweet orange (*C. sinensis*) and Dweet tangor (*C. sinensis x C. reticulata*). Hao and Deng (2003) induced somatic embryogenesis in seed derived callus of sweet orange. Since, there is a continuous decline in kinnow production due to ICRSV infection and no report is available on its elimination from kinnow, therefore, efforts were made to develop a protocol for regeneration of plantlets through somatic embryogenesis from ovules of kinnow for the production of ICRSV-free plants.

Materials and Methods

Selection of mother plant

Kinnow plant, which bears a large number of good quality fruits but infected with ICRSV, was selected by screening for the symptoms of the disease from *Citrus* orchard of Govt. Horticulture Department, Attari, Amritsar, Punjab (India) in January 2002.

ELISA

Symptomatic leaves from field grown and leaves of *in vitro* raised plants were tested for the presence/absence of ICRSV employing indirect ELISA. Leaf samples were triturated in extraction buffer (1:10). Antibodies, positive and negative controls were procured from Dr. Y.S. Ahlawat, Indian Agriculture Research Institute (IARI), New Delhi (India). Enzyme conjugate and substrate were procured from Bangalore Genei Pvt. Ltd. (India). The procedure adopted for ELISA was as described by Clarks and Adams (1977). The absorbance at 405 nm was measured with flow ELISA microplate reader. The reaction was considered positive only if the mean absorbance value was more than three times of negative control.

RT-PCR

Symptomatic leaves (100 mg) of field grown plant and of *in vitro* raised plants were used for total RNA extraction by Tri-reagent (Sigma, USA) according to manufacturer's instruction. Reverse transcription (RT) was performed in 0.2 ml thin walled tube using the total RNA as template. Specific primers for ICRSV (Upstream (U): 5' GCCAACTGGATGAAT 3', Downstream (D): 5' GAGCCAAGCGTTCAGA 3') were designed by aligning the sequences available in the EMBL Database to amplify partial coat protein gene (539 bp) and were got synthesized from Genei, Bangalore, India. RT reaction was carried out in a total reaction volume of 50 μl at 37℃ for 1.15 h with 5x reverse transcriptase buffer (Promega, USA), 10 mM each dNTP, 200 ng down stream (D) primer, 25 units of RNase inhibitor and 200 U of M-MLV reverse transcriptase (Promega, USA). This was followed by incubation at 70℃ for 5 min and the reaction mixture was transferred to ice immediately.

PCR was carried out in thermocycler (Gene Amp PCR System 9700, Applied Biosystems, Singapore) with 50 μl of total reaction mixture containing 3 μl cDNA, 1 μl (0.2 μg) of Upstream primer (U), 1 μl (0.2 μg) of Downstream primer (D), 5 μl of 10x Taq polymerase buffer (Genei, Bangalore, India), 1 μl of 30 mM dNTPs mix and 0.5 μl of Taq DNA polymerase (3 units/μl) (Genei, Bangalore, India). The reaction mixture was heated to 94℃ for 5 min and then 30 cycles each consisting of denaturation at 94℃ for 1 min, annealing at 52℃ for 1 min and elongation at 72℃ for 5 min were performed. PCR product was run on 1% agarose gel at 80 V, stained In ethidium bromide (0.5 mg/ml) and visualized with UV transilluminator

Plant material

Unopened flower buds of kinnow plant tested positive for ICRSV in ELISA and RT-PCR were collected from the *Citrus* orchard at Govt. Horticulture Department, Attari. Flower buds were washed with 5% (v/v) teepol solution (10 min). After rinsing in tap water, the buds were surface-

sterilized aseptically in laminar cabinet with 0.05% mercuric chloride for 5 min and rinsed three times with sterilized double-distilled water. The ovaries were excised from flower buds before anthesis and unfertilised ovules were dissected out with scalpel.

Callus induction

The unfertilized ovules were cultured on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with various concentrations of kinetin (KN) (4.64, 9.29,13.94,18.59 μM) or malt extract (ME) (200, 400, 600, 800, 1000 mgl^{-1}) containing 3% (w/v) sucrose and 0.8% (w/v) agar (SRL, Mumbai) for induction of embryogenic callus. For control cultures, the explants were inoculated on MS medium only i.e without kinetin or malt extract. The pH of the medium was adjusted to 5.6 with 1 N NaOH and autoclaved at 121℃ and 15 lb in^{-2} pressure for 20 min. Three or four ovules were inoculated per tube (25x150 mm) containing 20 ml of the medium. For each set of experiment, 24 tubes were inoculated per replicate and experiment was repeated thrice. All cultures were maintained at 26±1℃ with a luminous intensity of 40 μmole m^{-2} s^{-1} and 16-h photoperiod. For each treatment, number of cultures showing embryogenic callus induction was recorded 60 days after initial culturing and their percent frequency was calculated.

Maturation and germination of somatic embryos

Embryogenic calli were subcultured onto fresh medium supplemented with different concentration of KN for somatic embryo formation. Three hundred and fifty mg of callus was inoculated per culture. The somatic embryos were allowed to mature and germinate on the same medium and the effect of different concentrations of KN on somatic embryo production was recorded after 60 days of subculturing.

Acclimatization and transplantation to the field

The well-developed plantlets were washed with water in order to remove adhering agar and transferred to autoclaved plastic pots containing a mixture of garden soil, sand and vermiculite in the ratio of 3:1:1. Hardening of potted plantlets was accomplished in a culture room set at 26± 2℃, 16-h day-length (40 μ mole m^{-2} s^{-1}) and covered with polyethylene bags to maintain high humidity. After 12-15 days, polyethylene bags were removed initially for a short duration (15-30 min) daily for about one week. Gradually, the daily exposure time was increased by 30 min for each day. Polyethylene bags were removed after 20 days, subsequently the plantlets were transferred to the earthen pots containing only garden soil and kept in the polyhouse for one month, and thereafter transferred to the screen house. The plantlets raised from somatic embryos were used for detection and identification of ICRSV employing indirect ELISA and RT-PCR.

Statistical analysis

For each treatment, 24 tubes were inoculated and the experiments were repeated thrice. The effects of treatments were tested by one-way analysis of variance (ANOVA), and the differences among means were tested by Tukey's HSD range test ($p \leq 0.05$).

Results and Discussion

Callus induction

Callus induction was observed from ovules after 60 days of incubation on MS medium containing various concentrations of kinetin (KN) or malt extract (ME). However no response was observed on MS medium without kinetin or malt extract (control). Initially, the callus formed was friable and creamish. Maximum induction of embryogenic callus (31.94%) was noticed on MS medium supplemented with KN (9.29 μM) after 60 days (Table 1, Fig. 1A). A further increase in concentration of KN resulted in a decrease in percent callus induction. Rahaman et al. (1996) have also shown that kinetin favoured the formation of cotyledonary embryoids obtained *in vitro* from ovules of five *Citrus* species, viz., *C. reticulata, C. reticulata x C. sinensis, C. madurensis, C. reshni* and *C. sinensis*. Jimenez et al. (2001) suggested the role of cytokinins to be important for somatic embryo development. They studied endogenous hormone levels in habitiuated nucellar *Citrus* callus during initial stages of regeneration. They transferred embryogenic nucellar callus cultures of different *Citrus* species and cultivars growing in hormone free medium to medium containing either sucrose or glycerol as the only carbohydrate source. They observed that glycerol favoured the continued development of somatic embryogenesis and also stimulated the accumulation of cytokinins in most of the cultivars evaluated. This is in constrast with an earlier study in which Kochba and Spiegel-Roy (1977) observed that the addition of various cytokinins to habitiuated ovular callus of sweet orange cv shamouti resulted in a reduction in embryogenesis. Oliveira et al. (1994) while studying embryo-

Table 1. Effect of different concentration of kinetin on embryogenic callus induction from ovules of kinnow after 60 days of inoculation.

Kinetin (μM)	Embryogenic callus induction(%) (Mean±SE)
Control	-
4.64	18.06 ± 1.38[b]
9.29	31.94 ± 1.38[a]
13.94	22.22 ± 1.38[b]
18.59	16.67 ± 2.40[b]
	$F_{(df\ 3,8)}$ = 16.46*, HSD = 6.868

Data shown are Mean±SE of three experiments, each experiment consisted of 24 replicates.
*Significant at $p \leq 0.05$.
Means followed by the same letter are not significantly different using HSD multiple comparison test.

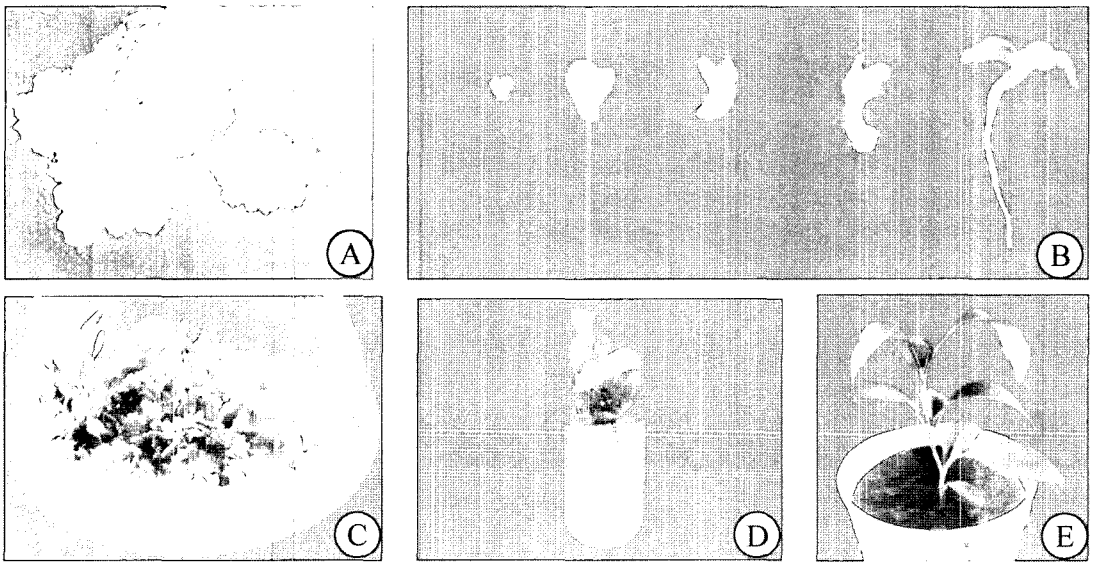

Figure 1. Somatic embryogenesis in kinnow. (A) Embryogenic callus from ovules on MS medium supplemented with KN (9.29 μM); (B) Successive stages of embryoid development on MS medium containing KN (9.29 μM); (C) Callus showing germination of somatic embryos (D) Somatic embryo-derived plantlet and (E) Transplanted plantlet in the pot

genic callus induction in *Citrus* reported that MT medium supplemented with IAA and Kinetin induced best nucellar response for embryogeneic callus formation in Cleopatra mandarin (*Citrus reticulata*. Blanco). Table 2 shows the effects of ME on embryogenic callus induction from ovules of kinnow. The increase in concentration of ME resulted in increase in percent callus induction, with a maximum frequency (20.83%) of embryogenic callus at 800 mg l^{-1} after 60 days followed by a decrease at 1000 mg l^{-1}. Earlier Perez et al (1999) reported callus induction in 25% cultures from ovules of kinnow inoculated on MS medium containing 50 g l^{-1} sucrose and 10 g l^{-1} agar along with 500 mg l^{-1} malt extract. The difference in the embryogenic response of the present study from that of study of Perez et al. (1999) might be due to different developmental stages of the explants. In the present study unfertilized ovules were used while Perez et al. (1999) used undeveloped ovules collected after 120 days of anthesis. Another reason for this might be sucrose concentration; in our study we used 30 g l^{-1} while Perez et al. (1999) used 50 g l^{-1}. In *Citrus* malt extract has been widely used for embryogenic callus induction and somatic embryogenesis (Kochba et al. 1972; Vardi et al. 1982; Moore 1985; Gmitter and Moore 1986; Carimi et al. 1998; Gloria et al. 1999; Perez et al. 1999). Carimi et al. (1998) studied somatic embryogenesis from undeveloped ovules and stigma/style explants of 11 different genotypes of sweet orange (*Citrus sinensis*) and showed that undeveloped ovules were more responsive than sigma/style explants in

Table 2. Effect of different concentration of malt extract on embryogenic callus induction from ovules of kinnow after 60 days of inoculation.

Malt extract (mg l^{-1})	Embryogenic callus induction(%) (Mean ± SE)
Control	-
200	11.11 ± 1.39[ab]
400	12.50 ± 2.41[a]
600	13.89 ± 1.39[a]
800	20.83 ± 2.40[a]
1000	16.67 ± 2.40[a]
	$F_{(df\ 4,10)}$ = 3.49*, HSD = 8.921

Data shown are Mean±SE of three experiments, each experiment consisted of 24 replicates.
*Significant at $p \leq 0.05$.
Means followed by the same letter are not significantly different using HSD multiple comparison test.

somatic embryo induction and development. They cultured the explants on MS medium containing 500 mg l^{-1} malt extract with and without kinetin (4.6 μm) or BAP (13.3 μm). They found that addition of BAP to medium (MS+malt extract 500 mgl^{-1}) improved the embryogenic response in all cultivars. However addition of KN to the above medium (MS+ME 500 mgl^{-1}) did not improve the embryogenic response significantly in most of cultivars while in some of them, a decrease in effect was observed.

Somatic embryogenesis

Table 3 shows the effects of different concentrations of KN on somatic embryo production after 60 days of subculturing of embryogenic callus. Embryogenic calli showed all stages of embryogenesis, viz., globular, heart and cotyledonary-shaped structures as well as small plantlets (Figs. 1B,C). KN at 9.29 μM showed somatic embryogenesis in all cultures (100%) with an average of 60.36 globular, 17.39 heart-shaped and 7.71 cotyledonary-shaped embryos per culture (350 mg of callus). All cotyledonary stage embryos developed into complete plantlets using similar media conditions (Fig. 1D). Higher concentrations of KN resulted in lesser number of globular somatic embryos. Patel et al. (1994) reported that higher amount of cytokinins was required for somatic embryogenesis in *Cajanus cajan*. This is in contrast to our observation which revealed that higher amount of cytokinins did not favour embryo induction.

In the present study, addition of KN was found to be better than addition of malt extract for somatic embryogenesis as well as germination of somatic embryos. Rahaman et al. (1996) have also found KN to be the best treatment for germination of embryoids into plantlets. Carimi et al. (1998) induced somatic embryogenesis in 11 different genotypes of sweet orange navel group (*C. sinensis*) from cultures of stigma/style explants and undeveloped ovules using MS medium supplemented with malt extract 500 mgl^{-1} and 13.3 (μM BAP. While in some other studies auxins

Table 3. Effect of different concentration of kinetin on somatic embryo production after 60 days of subculturing.

Kinetin (μM)	Number of embryos per culture[a]		
	Globular stage	Heart stage	Cotyledonary stage
4.64	22.84 ± 0.29[d]	8.46 ± 0.12[c]	5.36 ± 0.08[c]
9.29	60.36 ± 0.28[a]	17.39 ± 0.10[a]	7.71 ± 0.08[a]
13.94	45.28 ± 0.37[b]	12.62 ± 0.09[b]	7.05 ± 0.09[b]
18.59	24.39 ± 0.33[c]	8.49 ± 0.09[c]	4.62 ± 0.08[d]
	$F_{(df\ 3,232)}$ = 3261.03* HSD = 1.069	$F_{(df\ 3,232)}$ = 1775.34* HSD = 0.346	$F_{(df\ 3,232)}$ = 286.84* HSD = 0.285

Data shown are Mean±SE of three experiments, each experiment consisted of 24 replicates.
*Significant at $p \leq 0.05$.
Means followed by the same letter are not significantly different using HSD multiple comparison test.
[a]Culture produced from 350 mg of callus mass

have been shown to be essential for induction of somatic embryogenesis (Evans et al. 1981; Ammirato 1983). Somatic embryogenesis is potentially one of the most efficient methods for *in vitro* mass propagation, because unlimited production of single plantlets with both functional shoot and root is possible (Luttman et al. 1994). The plantlets, thus developed, were transferred to pots containing a mixture of garden soil, sand and vermiculite (3:1:1) for acclimatization (Fig. 1E), which showed 67% survival rate. After hardening in fogging and misting chamber the plants were shifted to screen house. Plants showing undesirable properties associated with juvenility were eliminated from screen house and only plants with normal character and identical to parent were used for budwood grafting on Jatti Khatti (*C. jambhiri*) root stock.

Indexing of plantlets obtained from ovule culture

The parent plant from which explant was taken was found positive for ICRSV using Indirect ELISA. RT-PCR of the same plant showed amplification of 539 bp fragment of partial coat protein gene indicating the presence of ICRSV. The plantlets produced from ovule culture were also indexed by indirect ELISA and RT-PCR during acclimatization and after every six months from the date of transfer to screen house. These plants are now at two years age and are negative for ICRSV as tested by indirect ELISA and RT-PCR (Fig. 2). Earlier also this technique has been found effective in recovering citrus plants free of exocortis, psorosis, concave gum and vein enation (Bitters et al. 1970; Navarro et al. 1979). D'Onghia et al. (1997) reported that somatic embryogenesis from style can be used as possible mean for virus elimination in *Citrus*. D'Onghia et al. (2001) had also employed somatic embryogenesis using stigma and style explants of mandarin (*C. reticulata*), sweet orange (*C. sinensis*) and Dweet tangor (*C. sinensis x C. reticulata*) to eliminate *Citrus* psorosis virus. The present study indicates that ovule culture can be used as an efficient system for production of virus-free plants.

Conclusion

Kinnow is vegetatively propagated and use of infected budwood results in widespread occurrence of ICRSV. A protocol has been developed to produce virus free plants through somatic embryogenesis with the ultimate aim of its use to release healthy bud-wood for commercial propagation. Although somatic embryogenesis is common in citrus, yet it is less commonly used to produce virus free plants. The protocol developed for the somatic embryogenesis from ovules of kinnow in the present investigation seems to be much effective as it involves the use of kinetin alone for all the steps, starting from induction of embryos till the development of plantlets.

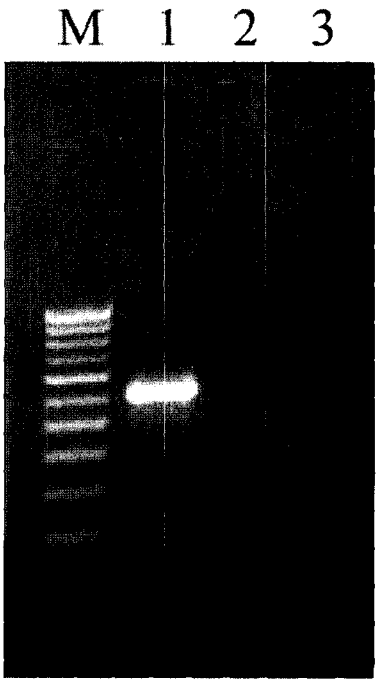

Figure 2. Agarose gel electrophoresis of PCR product (partial coat protein gene, 539 bp) of ICRSV (M=100 bp ladder as marker, 1. ICRSV infected plant selected at Attari, 2. Ovule culture raised acclimatizated plantlet, 3. *In vitro* raised plant at two years age growing in screen house)

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