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In vitro Production of *Indian citrus ringspot virus*-free Plants of Kinnow Mandarin (*Citrus nobilis* Lour×*C. deliciosa* Tenora) by Nucellar Embryo Culture

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Abstract: *Indian citrus ringspot virus* (ICRSV)-free plants of Kinnow mandarin (*Citrus nobilis* Lour× *C. deliciosa* Tenora) were produced from a virus-infected plant using nucellar embryo culture. The parent kinnow plant was tested by indirect ELISA and RT-PCR before using its explants. An amplified product of 539 bp (partial cp gene) was obtained by RT-PCR in ICRSV infected plants. The nucellar embryos obtained from seeds collected from immature fruits of the infected plant were cultured on Murashige and Skoog's (MS) basal medium supplemented with various concentrations of 2,4-D and malt extract (ME) alone or in various combinations. Maximum embryogenic callus induction (33.33%) was observed on MS medium supplemented with 2,4-D (9.02 μM) in combination with malt extract (400 mg L^{-1}). Transfer of embryogenic calli to MS medium containing different concentrations of malt extract alone or in combination with ABA resulted in somatic embryogenesis with a maximum of 56.94% cultures in MS medium supplemented with malt extract (500 mg L^{-1}) and ABA (7.56 μM). Cotyledonary shaped embryos when transfered to different strengths of MS medium supplemented with NAA (10.74 μM) developed into complete plantlets in maximum of 72.22% cultures on ½ MS medium. The plantlets were successfully acclimatizated, transferred to screen house and indexed for ICRSV employing indirect ELISA and RT-PCR and all were found negative of virus. A distinct feature of this study is the induction of somatic embryogenesis from nucellar embryos to produce virus-free plants.

Key words: Citrus, ICRSV (Indian citrus ringspot virus), Kinnow (*Citrus nobilis*×*Citrus deliciosa*), nucellar embryo culture, somatic embryogenesis, ELISA, RT-PCR

INTRODUCTION

Kinnow (*Citrus nobilis* Lour×*C. deliciosa* Tenora), a hybrid of two varieties of mandarins (king and leaf willow), is widely cultivated in India 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, 15,155 ha area is under kinnow cultivation (Thind *et al*., 2005). However, in recent years, ICRSV infection has resulted in tremendous loss in the yield and quality of this fruit crop. The existence of ICRSV in India was first documented in 1993 from Delhi (Byadgi *et al*., 1993) and is now placed in genus Mandarivirus of *Flexiviridae* family (Adams *et al*., 2004). It has been found widely distributed throughout the country with its incidence ranging from 10 to 100% (Thind *et al*., 2005; Pant and Ahlawat, 1998). ICRSV is readily transmissible through budwood but is not known to be transmitted through soil, seeds, nematodes and insect vectors like white fly, *Citrus psylla*, *Aphis citricida*, *A. gossypii*, *A. craccivora* and *Myzus persica* (Pant and Ahlawat, 1998; Thind *et al*., 1999). The leaves of infected plants exhibit chlorotic flecks, mottling, ringspots and irregular chlorotic patterns. Severely infected plants show dieback and decline type of symptoms. Thind *et al*. (2005) observed decrease in fruit weight, size, juice content, granulation, Total Soluble Solids (TSS), TSS/acid ratio, vitamin C and reducing sugars content in fruits of virus infected plants. The health of the infected trees deteriorates year after year and leads to a loss of 20-98%. So far, the only effective measure is to eradicate virus affected plants. This alarming decline in kinnow production necessitates the involvement of *in vitro* techniques for raising healthy ICRSV free kinnow orchards in the country, especially in Punjab. Three tissue culture techniques have been used to produce virus-free citrus plants: shoot tip grafting,

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ovule and nucellus culture (Navarro, 1984). The later approaches are effective because citrus viruses are not usually transmitted through seed and nucellar plants produced through somatic embryogenesis have many characteristics similar to mother plants.

According to Koltunow (1993) and Koltunow *et al.* (1996) polyembryonic seed formation in Citrus is one of many apomictic processes that occur in the ovules of angiosperm species. Polyembryonic and adventitious embryos arise *in vivo* from nucellar tissue. Most Citrus varieties are known to produce sexual as well as nucellar embryos (Dhillon *et al.*, 1993). The seedlings raised from nucellar embryos have genotype identical to the mother plant whereas the zygotic embryos produce plants genetically different from the mother plant (Dhillon *et al.*, 1993; Koltunow *et al.*, 1996). *In vitro* somatic embryogenesis from nucellar embryos can serve as an effective technique to produce virus free plants. Since most of citrus viruses are not transmitted through seed and furthermore, nucellar plants produced through somatic embryogenesis have many characteristics similar to mother plants, therefore, nucellar tissue has been used successfully in a number of cultivars of several citrus species (Rangan *et al.*, 1968; Rangan *et al.*, 1969; Button and Borman, 1971; Kochba *et al.*, 1972; Esan, 1973; Button and Bornman, 1974; Juarez *et al.*, 1976; Button and Kochba, 1977; Kochba *et al.*, 1982; Navarro, 1984; Litz *et al.*, 1985; Obukosia and Waithaka, 2000; Das *et al.*, 2000; Ricci *et al.*, 2002; Madhav *et al.*, 2002). Somatic embryogenesis from nucellar tissues has also been reported from other plants like Mango (Litz *et al.*, 1982, 1984; Ara *et al.*, 2000, Chaturvedi *et al.*, 2004), Cocoa (Sondahl *et al.*, 1993), Cashew (Ananthakrishnan *et al.*, 1999; Cardoza and D'Souza, 2002).

Keeping in view, a continuous decline in kinnow production due to ICRSV infection and potential of using nucellar embryo culture for raising virus free plants, an attempt was made to induce somatic embryogenesis from nucellar embryos for raising ICRSV-free plants. The main objective of the present study was to study the effect of different combinations of plant growth regulators on callus induction and somatic embryogenesis from nucellar embryos of virus infected plant and to index *in vitro* raised plantlets to confirm elimination of virus.

MATERIALS AND METHODS

Selection of mother plant: Kinnow plants located at Government Horticulture Nursery, Attari (Punjab) were tested for the presence of ICRSV employing indirect ELISA and RT-PCR. One of the plants tested positive for ICRSV was selected for explant collection. Immature fruits of this plant 100-120 days after pollination were collected and transported to tissue culture lab. The present study was initiated in March, 2003. The virus indexing was performed at Institute of Himalayan Bioresource Technology, C.S.I.R., Palampur (H.P) while tissue culture studies were carried out at Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar (Punjab).

Detection methods for virus infection: Indirect ELISA and RT-PCR were performed on the following: (1) Leaves from infected field trees used as a source of explant; (2) leaves from young acclimatized plantlets produced via somatic embryogenesis from nucellar embryos. The procedures adopted for indirect ELISA and RT-PCR have been described in an earlier report (Singh *et al.*, 2005).

Explant preparation: The seeds were extracted from immature fruits of virus infected kinnow plant. Seed coats were removed under aseptic conditions and seeds were surface sterilized with 0.1% (w/v) $HgCl_2$ for 1-2 min and cut in to two halves. Nucellar embryos were extracted from the micropylar end of kinnow seed which is known to contain 7-8 nucellar embryos around the zygotic embryo (Nafees *et al.*, 2001). The zygotic embryos and endosperm were discarded.

Callus induction: Excised nucellar embryos were cultured on Murashige and Skoog (1962) medium supplemented with various concentrations of 2,4-D (4.51, 9.02, 13.54, 18.05, 22.57 μM) or malt extract (200, 300, 400, 500 mg L^{-1}) either alone or in different combinations along with 3% (w/v) sucrose and 0.8% (w/v) agar (SRL, Mumbai) for induction of embryogenic callus. The pH of the medium was adjusted to 5.6 with 1N NaOH and autoclaved at 121°C and 15 lb in^{-2} pressure for 20 min. Single nucellar embryo was inoculated per tube (25×150 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°C with a luminous intensity of 40 μmole m^{-2} sec^{-1} and 16-h photoperiod. For each treatment, number of cultures showing embryogenic callus induction was recorded after 4 weeks of initial culture and their percent frequency was calculated.

Somatic embryo formation and germination: Embryogenic callus was subcultured on MS medium supplemented with different concentrations of malt extract (200-600 mg L^{-1}) alone and in combination with various concentrations of abscisic acid (3.78, 7.56, 11.34, 15.12 μM) for somatic embryogenesis. For each treatment,

number of cultures showing somatic embryo development was recorded after 4 weeks of inoculation. The somatic embryos at the cotyledonary stage were transferred to MS, ½ MS and 1/4 MS medium supplemented with NAA (10.74 μM) for germination. For each set of experiment, 24 tubes were inoculated per replicate and the experiments were repeated thrice.

Hardening and transplantation: Plantlets developed from somatic embryos 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°C, 16-h day-length (40 μmole m^{-2} sec^{-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 transferred to the screen house.

Virus indexing of plants raised from somatic embryos: The plantlets produced from somatic embryos of nucellar embryo culture were indexed by indirect ELISA and RT-PCR during acclimatization. The plants shifted to screen house were repeatedly indexed after every six months up to two years age.

Statistical analysis: Statistical computations were performed using computer software. The data pertaining to callus induction, somatic embryogenesis and somatic embryo germination were subjected to one-way analysis of variance (ANOVA) test and the differences among means were compared by high-range statistical domain (HSD) using Tukey's test (Meyers and Grossen, 1974).

RESULTS

Callus induction: Table 1 shows the effect of different concentrations and combinations of malt extract and 2,4-D on callus induction from nucellar embryos cultured on MS medium containing 0.8% agar and 3% sucrose. Among different concentrations of 2,4-D tested, maximum callus induction (27.77%) was observed with 2,4-D at 9.02 μM while malt extract at 500 mg L^{-1} showed maximum callus induction (24.99%) among different concentrations of malt extract tested. Among different combinations of malt extract and 2,4-D maximum percentage of embryogenic callus induction (33.33%) was observed when nucellar embryos were cultured on MS medium supplemented with ME at 400 mg L^{-1} and 2,4-D at 9.02 μM (Fig. 1a). A further increase in concentration of either 2,4-D or ME resulted in a decrease in percent callus induction.

Table 1: Effect of different concentrations and combinations of malt extract and 2,4-D on callus induction from nucellar embryos cultured on MS medium containing 0.8% agar and 3% sucrose. (Observations recorded after 4 weeks of culture)

MS medium supplemented with Malt extract (mg L^{-1})	2,4-D (μM)	Cultures showing callus induction (%) Mean±SE
-	4.51	19.44±1.38^{a}
-	9.02	27.77±1.38^{a}
-	13.54	26.38±2.77^{a}
-	18.05	24.99±2.40^{a}
-	22.57	23.60±2.78^{a}
		$F_{(df\ 4,\ 10)}$ = 2.039*; HSD = 9.693
200	-	18.05±2.41^{a}
300	-	19.44±1.40^{a}
400	-	23.61±1.40^{a}
500	-	24.99±2.39^{a}
600	-	20.83±2.40^{a}
		$F_{(df\ 4,\ 10)}$ = 2.389*; HSD = 8.071
200	4.51	20.83±2.41^{a}
200	9.02	29.16±2.40^{a}
200	13.54	27.77±1.38^{a}
200	18.05	23.61±1.38^{a}
200	22.57	22.22±2.79^{a}
		$F_{(df\ 4,\ 10)}$ = 2.789*; HSD = 9.319
300	4.51	22.22±1.38^{a}
300	9.02	30.55±2.78^{a}
300	13.54	26.38±3.69^{a}
300	18.05	20.83±2.41^{a}
300	22.57	18.05±1.38^{ab}
		$F_{(df\ 4,\ 10)}$ = 3.907*; HSD = 10.759
400	4.51	24.99±2.40^{a}
400	9.02	33.33±2.41^{a}
400	13.54	23.61±1.38^{ab}
400	18.05	19.44±1.38^{ab}
400	22.57	16.66±2.40^{ab}
		$F_{(df\ 4,\ 10)}$ = 9.543*; HSD = 8.921
500	4.51	23.61±1.39^{ab}
500	9.02	31.94±1.39^{a}
500	13.54	22.22±2.79^{ab}
500	18.05	18.05±1.38^{ab}
500	22.57	15.27±2.40^{ab}
		$F_{(df\ 4,\ 10)}$ = 13.113*; HSD = 7.610

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 embryogenesis: The effect of different concentrations of malt extract alone and in combination with different concentrations of absicisic acid (ABA) on somatic embryo production after 4 weeks of culture of embryogenic callus is shown in Table 2. Somatic embryo formation was observed after 3-4 weeks of culture (Fig. 1b). Different responses on somatic embryo formation was observed among calli formed on MS medium containing either 2,4-D or ME alone or in various combinations. Somatic embryos were not at all formed from the callus obtained under the influence of any

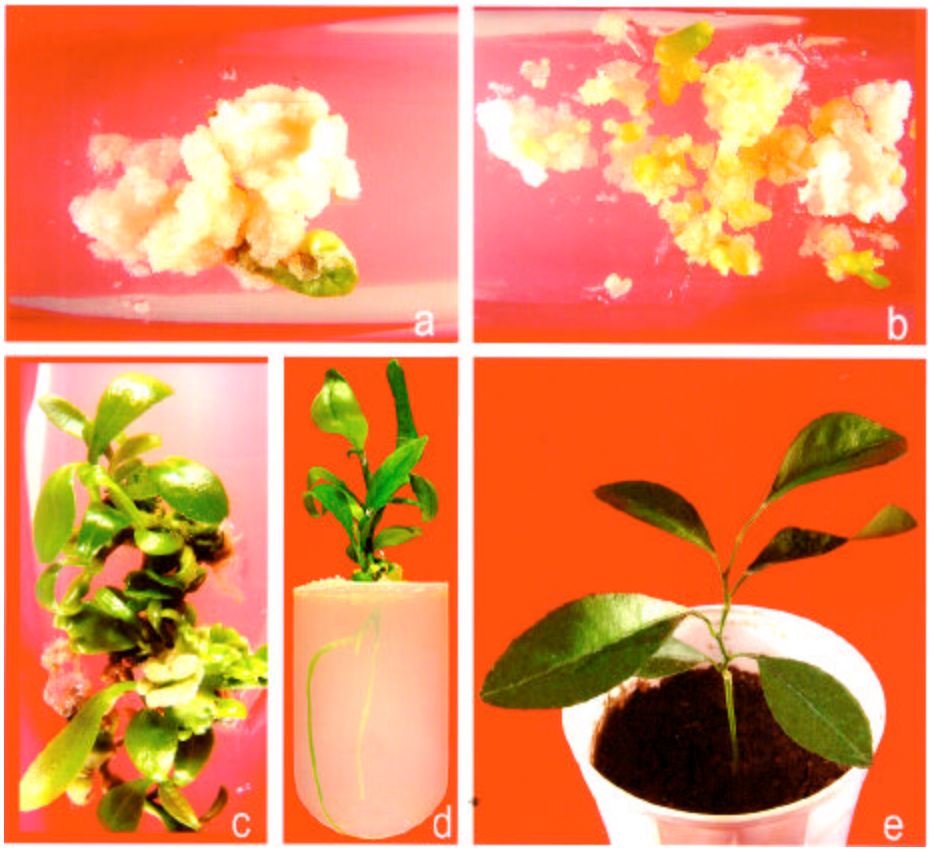

Fig. 1: Somatic embryogenesis from nucellar embryos in kinnow mandarin: (a) Embryogenic callus from nucellar embryos on MS medium supplemented with malt extract (400 mg L^{-1}) and 2,4-D (9.02 μM). (b) Somatic embryo formation on MS medium containing malt extract (500 mg L^{-1}) and ABA (7.56 μM). © Plantlet development from somatic embryos on ½ MS medium with NAA (10.74 μM). (d) A single plantlet on ½ MS medium with NAA (10.74 μM). (e) Nucellar embryo culture raised acclimatizated plantlet

concentration of 2,4-D tested. However, callus obtained under the influence of ME alone or in combinations with 2,4-D resulted in the formation of somatic embryos. Maximum percentage (56.94%) of somatic embryos were formed on MS medium supplemented with ABA (7.56 μM) along with ME (500 mg L^{-1}) (Fig. 1b). Any further increase in concentration of either ABA or malt extract does not show much effect on somatic embryo development. During development globular, torpedo, heart and cotyledonary shaped somatic embryos were observed. All the somatic embryos at the cotyledonary stage were shifted to different strengths of MS medium supplemented with NAA (10.74 μM) for germination. Maximum somatic embryo germination (72.22%) was observed on half strength MS medium (Table 3, Fig. 1c). The plantlets, thus developed (Fig. 1d), were transferred to pots containing a mixture of garden soil, sand and vermiculite (3:1:1) for acclimatization (Fig. 1e), which showed 68% 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 free of virus were used for budwood grafting on Jatti Khatti (*C. jambhiri*) root stock.

Virus indexing: 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 nucellar embryo culture were also indexed by indirect ELISA and

Table 2: Effect of different concentrations of malt extract alone or in combination with ABA on somatic embryogenesis from nucellar embryogenic callus cultured on MS medium containing 0.8% agar and 3% sucrose. (Observations recorded after 4 weeks of culture)

MS medium with Malt extract (mg L^{-1})	ABA (µM)	Cultures showing somatic embryogenesis (%) Mean±SE
200	-	33.33±2.41^{ab}
300	-	34.72±1.38^{a}
400	-	36.11±1.39^{a}
500	-	41.66±2.40^{a}
600	-	38.88±1.38^{a}
		$F_{(df\ 4, 10)}$ = 3.221*; HSD = 8.069
200	3.78	40.27±1.38^{a}
300	3.78	43.05±1.37^{a}
400	3.78	44.44±2.78^{a}
500	3.78	47.22±1.39^{a}
600	3.78	45.83±2.41^{a}
		$F_{(df\ 4, 10)}$ = 1.849*; HSD = 8.509
200	7.56	48.61±1.36^{a}
300	7.56	51.38±2.39^{a}
400	7.56	52.77±2.77^{a}
500	7.56	56.94±1.38^{a}
600	7.56	54.16±2.78^{a}
		$F_{(df\ 4, 10)}$ = 1.922*; HSD = 9.695
200	11.34	45.83±2.41^{ab}
300	11.34	47.22±1.38^{ab}
400	11.34	48.61±1.38^{a}
500	11.34	55.55±1.37^{a}
600	11.34	52.77±1.38^{a}
		$F_{(df\ 4, 10)}$ = 6.061*; HSD = 7.118
200	15.12	40.27±1.38^{ab}
300	15.12	43.05±2.77^{ab}
400	15.12	49.99±2.40^{a}
500	15.12	54.16±2.39^{a}
600	15.12	48.61±1.37^{a}
		$F_{(df\ 4, 10)}$ = 6.672*; HSD = 9.312

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

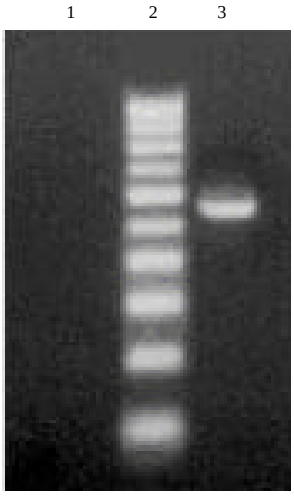

Fig. 2: Agarose gel electrophoresis of PCR product (partial coat protein gene, 539 bp) of ICRSV (Lane 1. Nucellar embryo culture raised acclimatizated plantlet at two years age growing in screen house, Lane 2. M = 100 bp ladder marker, Lane 3. ICRSV infected plant as control)

Table 3: Effect of different strengths of MS medium along with NAA (10.74 µM) on somatic embryo germination recorded after 3 weeks of culture

Medium	Somatic embryo germination (%) Mean±SE
MS+ NAA	56.94±1.38^{b}
½ MS+ NAA	72.22±1.41^{a}
¼ MS+ NAA	49.99±2.40^{c}
	$F_{(df\ 2, 6)}$ = 40.198*; HSD = 6.204

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

RT-PCR during acclimatization. These plants are now at two years age and are negative for ICRSV as tested by indirect ELISA and RT-PCR (Fig. 2).

DISCUSSION

Nucellar tissues have been used for the production of somatic embryos in woody species such as *Citrus* (Rangan *et al*., 1968), *Mango* (Litz *et al*., 1982,1984; Ara *et al*., 2000), *Cocoa* (Sondahl *et al*., 1993), *Cashew* (Cardoza and D` Souza, 2002).The present study has shown that somatic embryogenesis from nucellar embryos of kinnow mandarin (*Citrus nobilis*×*C. deliciosa*) requires different plant growth regulator supplements for different stages i.e., callus induction, somatic embryogenesis and germination of somatic embryos.

Callus induced under the influence of different concentration of either 2,4-D or malt extract alone or in various combinations when subcultured in the similar media resulted in the proliferation of callus only and somatic embryos were not formed with these treatments. Such calli were then transferred to MS medium supplemented with different concentrations of malt extract in combination with various concentrations of ABA, which resulted in somatic embryogenesis.

Although 2,4-D was more effective on callus induction at certain concentrations, such calli turned brown and necrotic after 3-4 weeks of callus initiation and never developed somatic embryos when cultured on somatic embryogenesis development medium. This is in confirmation with one of the earlier studies on *C. reticulata* where Gill *et al.* (1995) had also observed that 2,4-D induces non-embryogenic and friable callus. Cardoza and D'Souza (2002) while working on somatic embryogenesis from nucellar tissue of cashew also experienced that callus produced with 2,4-D and 2,4,5-T could not be maintained as it turned necrotic within 2 weeks in culture. Tao *et al.* (2002) while working on seedling explant of *C. grandis* reported that 2,4-D at 4.5 µM or lower concentrations resulted in two types of calli i.e., yellow friable and green compact. They also observed that only green compact callus was able to

regenerate. Higher concentarions of 2,4-D (higher than 4.5 μM) resulted in yellow friable callus only, which was non-embryogenic. Some other studies on callus induction from nucellar tissue of *Mangifera indica* (Litz *et al*., 1984); *Anacardium occidentale* (Ananthakrishnan *et al.* 1999) and *C. sinensis* (Das *et al*., 2000) have shown that 2,4-D induce embryogenic callus. Ananthakrishnan *et al.* (1999) found that 2,4-D at a concentration of 6.76 μM was required for the induction of embryos whereas its continued presence in the subculturing medium did not enhance further development after torpedo stage, which occurred on 2,4-D free medium. Other studies have shown that 2,4-D not only induces somatic embryogenesis but also enhances maturation of somatic embryos in *Picea glauca* (Tremblay, 1990) and *Helianthus annus* (Prado and Berville, 1990).

In Citrus, malt extract has been widely used for embryogenic callus induction and somatic embryogenesis (Kochba *et al*., 1972; Gmitter and Moore, 1986; Carimi *et al*., 1998; Gloria *et al*., 1999; Pérez *et al*., 1999; Ricci *et al*., 2002). The present study also demonstrates the effectiveness of malt extract in embryogenic callus induction as well as somatic embryo development. For somatic embryo development malt extract was used in combination with Abscissic acid (ABA). ABA is phytohormone and has shown to be involved in many physiological processes including seed development (Walton, 1980; Graudat *et al*., 1994). It has also been shown to promote and synchronize somatic embryogenesis (Roberts *et al*., 1990; Label and Lelu, 1994; Torres *et al*., 2001). Senger *et al.* (2001) demonstrated the role of ABA in early embryogenic events. Kuo *et al* (2002) have also shown that high frequency of somatic embryogenesis in *Corydalis yanhusuo* can be induced by ABA from epidermal cells of converted primary embryos. Cardoza and D'Souza (2002) reported that 0.5 mg L^{-1} ABA was important in the conversion of globular embryos to heart shaped and cotyledonary shaped embryos. Augustine and D'Souza (1997) in *Gnetum edule* and Raviv *et al.* (1998) in Avocado have used 10 mg L^{-1} ABA for the maturation of somatic embryos. According to Prado and Berville (1990), ABA in the presence of 2,4-D improved embryo development in *Helianthus annus*. In contrast, this regulator (ABA) inhibited embryo development in some genotypes of *Daucus carota* (Borkird *et al*., 1986). Kochba *et al.* (1978) reported that the addition of low levels of ABA to the culture medium stimulated somatic embryogenesis in ovular callus of sweet orange cv shamouti.

Removal of ABA from the culture environment is required for the germination of embryos (Merkle *et al*., 1995). This was demonstrated in the present study also. For somatic embryo germination, they were transferred to different strengths of MS medium supplemented with NAA (10.74 μM). Maximum germination was observed with ½ strength MS medium. Komai *et al.* (1996)have also shown ½ MS medium to have high competence for somatic embryogenesis. Similarly Gill *et al.* (1994) have also shown that ½ strength MS medium was best for plant regeneration from epicotyl derived callus of kinnow mandarin. NAA (10.74 μM) was found to be effective for somatic embryo germination. This is in line with previous studies of Gill *et al.* (1995) and Das *et al.* (2000) who had also reported that MS medium with NAA was effective for somatic embryo germination.

The plantlets produced by nucellar embryo culture were found negative for ICRSV as indexed by ELISA and RT-PCR. Earlier also nucelli raised plants of Tempo tango (*C. reticulata*×*C. sinensis*) were found free of exocortis viroid, tristeza and vein enation virus (Bitters *et al*., 1970). Navarro (1984) also obtained citrus plants free of viruses by nucellus culture. Obukosia and Waithaka (2000) used nucellar embryo culture of *C. sinensis* and *C. limon* to propagate healthy seedlings and to eradicate Huanglonbing (HLB) and viral diseases from nurseries. Virus diseases of Citrus are rarely seed borne (Bitters and Murashige, 1967) and are generally restricted to the host vascular tissue particularly the phloem. Button and Kochba (1977) reported that as there is no direct vascular connection between the parent plant and nucellar embryos, so most of citrus viruses get eliminated from nucellar progenies. The present study indicates that nucellar embryo culture can be used as an efficient system for production of virus-free plants.

CONCLUSIONS

Kinnow is vegetatively propagated and use of infected budwood results in widespread occurrence of ICRSV. As the virus is not transmitted through seed and nucellar plants produced through somatic embryogenesis have characteristics similar to the mother plant so this method is effective for production of virus free kinnow plants.

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