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Full Length Research Paper

In vitro flowering in embryogenic cultures of Kinnow mandarin (*Citrus nobilis* Lour × *C. deliciosa* Tenora)

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Embryogenic cultures of Kinnow mandarin (*C. nobilis* Lour × *C. deliciosa* Tenora) were raised from unfertilized ovules dissected from unopened flower buds of this plant inoculated on MS medium supplemented with 2 mg/L kinetin (KN). *In vitro* flowering was induced in these cultures by using different concentrations of KN and sucrose as well as subjecting these cultures to different photoperiods. Maximum percentage (31.94%) of cultures producing flowers and maximum number (5.58) of flowers per culture was observed on MS medium supplemented with KN (2 mg/L) and sucrose 40 g/L at 12-h photoperiod.

Key words: Embryogenic callus, *in vitro* flowering, Kinnow (*Citrus nobilis* × *C. deliciosa*), ovule culture.

INTRODUCTION

Kinnow (*Citrus nobilis* Lour × *C. deliciosa* Tenora), a cross breed of two varieties of mandarins (king and leaf willows) is used for preserve, syrup and fresh consumption because of its special flavour and taste. The development of efficient plant tissue culture procedures for *in vitro* flowering in citrus is important for the application of these technologies for citrus improvement. The transition from vegetative state to reproductive development in plants is of great interest to botanist but is still poorly understood (Koonneef et al., 1998). *In vitro* culture provides an ideal experimental system to study molecular mechanisms of flowering (McDaniel et al., 1991). Flowering is considered to be a complex process regulated by a combination of environmental and genetic factors.

In vitro flowering has been reported in a number of plant species e.g. *Dendrocalamus strictus* (Singh et al., 2000), *Gentiana triflora* (Zhang and Leung, 2000, 2002), *Streptocarpus nobilis* (Floh and Handro, 2001), *Pharbitis nil* (Galoch et al., 2002), *Ammi majus* (Pande et al., 2002), *Hypericum brasiliense* (Abreu et al., 2003), *Bambusa edulis* (Lin et al., 2003, 2004) and *Psygmorchis pusilla* (Vaz et al., 2004). A limited number of reports on citrus and its relatives include those on *Citrus unshiu* (Garcia-Luis et al., 1989; Garcia-Luis and Kanduser, 1995), *Citrus limon* (Tisserat et al., 1990), *Murraya paniculata* (Jumin and Nito, 1995; 1996; Jumin and Ahmad, 1999) and *Fortunella hindsii* (Jumin and Nito, 1996). Important factors for *in vitro* flowering are carbohydrates, growth regulators, light and pH of the culture medium (Heylen and Vendrig, 1988). Jumin and Nito (1995, 1996) have successfully induced flowering from plantlets derived from protoplasts of orange jessamine (*Murraya paniculata)*. In the present report, an attempt has been made to induce *in vitro* flowering in embryogenic cultures of Kinnow mandarin.

MATERIALS AND METHODS

Unopened flower buds of kinnow plant were collected from the citrus orchard of Government Nursery, Attari, Amritsar, Punjab (India). Flower buds were washed with 5% (v/v) teepol solution for 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 and ovules were dissected

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Abbreviations: BA, 6-benzyladenine; KN, kinetin; ME, malt extract; MS medium, Murashige and Skoog's (1962) medium.

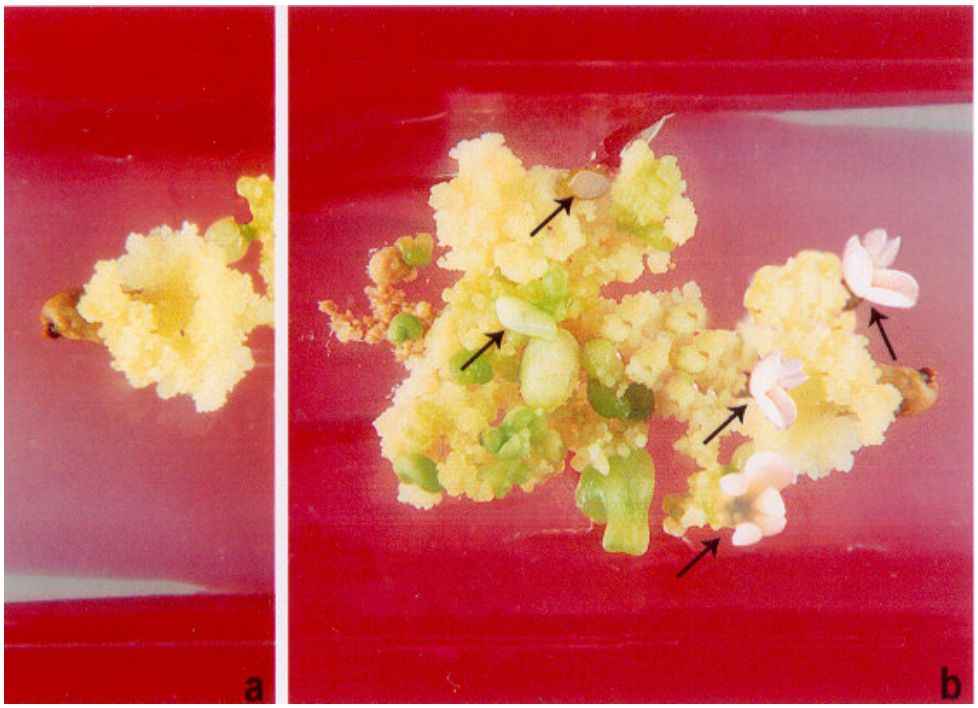

Figure 1. (**a**) Embryogenic callus induction from ovules of kinnow mandarin on MS medium supplemented with 4 mg/L Kinetin. (**b**) *In vitro* flowering in embryogenic cultures of Kinnow mandarin cultured on MS medium supplemented with 2 mg/L kinetin, 40 g/L sucrose at 12-h photoperiod.

out with scalpel. The ovules were cultured on MS medium (Murashige and Skoogs, 1962) containing 30 g/L sucrose and 8 g /L agar (SRL Mumbai) supplemented with kinetin (KN) at 4 mg/L (Singh et al., 2005). The pH was adjusted to 5.6 with 1 N NaOH before autoclaving. Twenty ml of medium was dispensed in to individual 25x150 mm glass culture tubes and finally autoclaved at 121°C and 15 lb in^{-2} pressure for 20 min. Each culture tube containing 20 ml of medium was inoculated with three-four ovules and plugged with non-absorbent cotton wrapped in two layers of cheese cloth. All cultures were maintained at 25±2°C under white fluorescent light (40 µmole m^{-2} s^{-1}) with 16-h photoperiod.

After 8 weeks of initial culture, callus was transferred to fresh MS medium containing 30 g/L sucrose and 8 g/L agar supplemented with different concentrations (1, 2, 3 and 4 mg/L) of KN or benzyladenine (BA). The cultures were maintained in a culture room at 26±1°C with a luminous intensity of 40-µ mole m^{-2} s^{-1} at 16-h photoperiod. Different concentrations of sucrose (10, 20, 30, 40, 50 g/L) and durations of photoperiod (0, 8, 10, 12, 16-h) were also tested to find out their optimum level for *in vitro* flowering. *In vitro* flowering was recorded after 4 weeks of culture for each treatment. 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 compared by high-range statistical domain (HSD) using Tukey's test (Meyers and Grossen, 1974).

RESULTS AND DISCUSSION

In a previous study (Singh et al., 2005), different concentrations of KN or malt extract (ME) were tested for their effect on callus induction from unfertilized ovules of kinnow cultured on MS medium. Initially, the callus formed was friable and creamish (Figure 1a). Maximum callus induction (31.94%) was observed on MS medium containing KN (2 mg/L). A further increase in concentration of KN resulted in a decrease in percent callus induction. However, with ME, callus induction was observed in maximum of 20.83% cultures at 800 mg/L while further increase in ME concentration (1000 mg/L) resulted in decreased response (16.67%).

After 4 weeks of culture of embryogenic callus, different stages of somatic embryos and flower buds were observed. Maximum percentage (23.61%) of cultures producing flower buds and number (5.33) of flowers per culture was observed in MS medium supplemented with 2 mg/L KN and 30 g/L sucrose at 16-h photoperiod as shown in Table 1. Embryogenic callus cultured on MS medium containing various concentrations of BA showed only somatic embryogenesis but no flower bud formation. The effect of sucrose on *in vitro* flowering is shown in Table 2. Embryogenic callus cultured on MS medium containing 2 mg/L KN without sucrose did not show flower bud formation. Maximum percentage (27.77%) of cultures producing flower buds and number (5.41) of flowers per culture was observed at 40 g/L sucrose (Table 2). Flower bud formation decreased with further increase or decrease in sucrose concentration. Effect of photoperiod on *in vitro* flowering in embryogenic cultures is shown in Table 3. It was observed that 12-h photoperiod was most

Table 1. Effect of different concentration of kinetin on *in vitro* flowering in embryogenic cultures of kinnow recorded after 4 weeks of culture on MS medium containing 8 g/L agar and 30 g/L sucrose at 16-h photoperiod.

Kinetin (mg/L)	Percentage of cultures producing flower buds	Number of flowers per culture
1	11.11 ± 1.39[b]	1.16 ± 0.30[d]
2	23.61 ± 1.40[a]	5.33 ± 0.86[a]
3	19.44 ± 1.38[a]	3.66 ± 0.81[b]
4	6.94 ± 1.38[b]	1.20 ± 0.41[c]
	$F_{(df\ 3,\ 8)}$ = 29.95*; HSD= 5.615	$F_{(df\ 3,\ 92)}$= 227.70*; HSD = 0.452

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.

Table 2. Effect of different concentration of sucrose on *in vitro* flowering in embryogenic cultures of kinnow recorded after 4 weeks of culture on MS medium containing 2 mg/L Kinetin, 8 g/L agar at 16-h photoperiod.

Sucrose (g/L)	Percentage of cultures producing flower buds	Number of flowers per culture
10	9.72 ± 1.38[b]	1.87 ± 0.67[d]
20	19.44 ± 1.40[a]	2.91 ± 0.65[c]
30	23.66 ± 1.39[a]	5.29 ± 0.80[a]
40	27.77 ± 1.40[a]	5.41 ± 0.77[a]
50	19.40 ± 1.38[a]	4.29 ± 0.85[b]
	$F_{(df4,\ 10)}$= 23.28*; HSD = 6.015	$F_{(df\ 4,\ 115)}$= 98.39*; HSD= 0.571

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.

Table 3. Effect of different photoperiods on *in vitro* flowering in embryogenic cultures of kinnow recorded after 4 weeks of culture on MS medium containing 2 mg/L Kinetin, 8 g/L agar and 40 g/L sucrose.

Photoperiod (h)	Percentage of cultures producing flower buds	Number of flowers per culture
8	1.38 ± 1.39[b]	1.12 ± 0.33[b]
10	6.94 ± 1.40[b]	1.16 ± 0.38[b]
12	31.94 ± 1.38[a]	5.58 ± 0.82[a]
16	27.77 ± 1.38[a]	5.33 ± 0.86[a]
18	9.72 ± 1.38[b]	1.16 ± 0.38[b]
	$F_{(df\ 4,10)}$= 94.218*; HSD = 6.012	$F_{(df\ 4,\ 115)}$ = 362.04; HSD= 0.457

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.

effective for flowering which showed highest percentage (31.94%) of cultures producing flower buds and maximum number (5.58) of flowers per culture on MS medium containing 2 mg/L KN with 40 g/L sucrose (Figure 1b). No flowers were observed when cultures were kept in dark.

This study has shown that kinnow mandarin unfertilized ovules excised from unopened flower buds are highly responsive for the induction of embryogenic cultures and *in vitro* flowering. The embryogenic response of unfertilized ovules observed in this study is more when compared to fertilized ovules studied by other workers (Starrantino and Russo, 1980; Carimi et al., 1998; Moore, 1985). Explants produced friable creamy white embryogenic callus which when subcultured on MS medium supplemented with KN (2 mg/L) rich medium resulted in flower bud differentiation. Strong effect of cytokinin, sucrose and photoperiod on flowering in embryogenic cultures was

observed.

Cytokinin is a common requirement for *in vitro* flowering (Scorza, 1982). A number of studies report the use of cytokinins for *in vitro* flowering in a number of species like *Citrus unshiu* (Garcia-Luis et al., 1989), *Murraya paniculata* (Jumin and Ahmad, 1999), *Fortunella hindsii* (Jumin and Nito, 1996), *Gentiana triflora* (Zhang and Leung, 2000), *Pharbitis nil* (Galoch et al., 2002) and *Ammi majus* (Pande et al., 2002). Differentiation of flower bud from undifferentiated embryogenic callus cultures in kinnow mandarin required exogenous KN at concentration of 2 mg/L. Increase in cytokinin concentration has also been reported during *in vivo* flowering of *Xanthium strumarium* (Phillips and Cleland, 1972) where it was shown that cytokinin levels in excretory products from aphids feeding on flowering plants are more when compared to those from aphids feeding on vegetative plants. Increased levels of cytokinins during flower induction have also been observed in some other species (Bernier, 1988). However, in the present study BA at any concentration did not show its effect on flowering. This means that all cytokinins do not show their effects on *in vitro* flowering. This observation that different cytokinins have different effects on *in vitro* flowering has also been made by Meeks-Wagner et al. (1989), who showed that in *Nicotiana tabacum*, KN promoted flower formation whereas zeatin promoted leafy shoot formation.

Sugars are necessary carbon source for reliable induction and development of flowers. Addition of sugar to the medium is necessary for induction of floral stimulus. Sucrose is known to be the main carbon source of choice for *in vitro* flower culture studies (Rastogi and Sawhney, 1987). In the present investigation, the effect of different concentrations of sucrose on flower induction was studied by keeping all other parameters constant. Flower bud differentiation was observed only when the sucrose concentration was at 30-60 g/L. The frequency and efficiency of flower bud differentiation was higher in the presence of 40 g/L sucrose in the medium; this result coincides with earlier reports of flowering on citrus (Tisserat et al., 1990), gentian (Zhang and Leung, 2000, 2002) and bamboo (Lin et al., 2003, 2004). Sucrose availability in aerial parts of the plant promotes flowering in *Arabidopsis thaliana* (Roldan et al., 1999). Sucrose and cytokinins interact with each other for floral induction in *Sinapis alba* by moving between shoot and root (Havelange et al., 2000).

As shown in this work, embryogenic cultures did not show flower bud formation in darkness. Prolific flowering occurred at 12-h photoperiod on MS medium containing 40 g/L sucrose and 2 mg/L KN. In continuous light, no flower bud was observed in cultures. This work also indicates that specific photoperiod with some darkness is essential for *in vitro* flowering in embryogenic cultures.

Long photoperiods above 16-h in citrus were considered to be non inductive to the flowering process (Lenz, 1969). The most predictable factor in plants to time their reproduction is light period or day length (Bernier and Perilleux, 2005). Importance of photoperiod for *in vitro* flowering has been frequently demonstrated in *Murraya paniculata* plantlets derived from protoplasts, which only flowered at 16-h photoperiod but not in continuous darkness (Jumin and Nito, 1995). The effects of photoperiod on vegetative and reproductive development in *Psygmorchis pusilla* were investigated and it was observed that plants incubated under 20-h or longer photoperiod are negatively affected for floral bud development (Vaz et al., 2004). Similarly our results suggest that photoperiod is important in flowering. Flower formation *in vitro* can provide a model system for studying molecular details of flower induction and development, means for conducting microbreeding and a source of biochemicals and pharmaceuticals (Tisserat and Galletta, 1990).

This simplicity yet plasticity of the *in vitro* system as presented in this investigation suggests that embryogenic cultures from unfertilized ovules can be promising to study *in vitro* flowering in kinnow mandarin. This is the first report on *in vitro* flowering of kinnow mandarin and is expected to have important basic and applied values to understand reproductive biology of mandarins.

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