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Full Length Research Paper

In vitro regeneration of plantlets from unpollinated ovary culture in sweet orange (*Citrus sinensis* L. Osbeck)

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Callogenesis and organogenesis of ovary of sweet orange (*Citrus sinensis*) cv. Blood red was carried on Murashige and Skoog (MS) medium supplemented with different concentrations and combinations of N^6 benzyl adenine (BA), 1-napthaleneacetic acid (NAA) and 2,4-D. 1 mg/l BA + 0.5 mg/l NAA on MS medium was the most effective in callus induction and proliferation. Maximum number of shoots (11) was recorded on the medium with 2 mg/l NAA + 3 mg/l BA. The best medium for root induction was MS together with 2.5 mg/l indole-3-acetic acid (IAA) + 2 mg/l indole–3-butyric acid (IBA), where maximum (16) plantlets were rooted. The regenerated plantlets were successfully acclimatized in jiffy pots containing sterilized soil mixture of sand, silt and clay in 1:1:1 ratio to study their response to *in vivo* conditions.

Key words: Citrus, blood red, ovary culture, callus induction, regeneration, plant growth regulators.

INTRODUCTION

Citrus is a prize fruit of Pakistan, ranking first among all fruits both in area, (199.9 thousand hectare) and production (2132.2 thousand tones) (Anonymous, 2009). The province of Punjab is the key share holder (97.02%) in national *Citrus* production and area (94.64%) under *Citrus* grooves (Altaf and Khan, 2007). Citrus are generally propagated by traditional methods like budding and grafting. Therefore, there is possibility of virus transmission from mother plant to the propagated plants. Similarly, conventional propagation methods are cumbersome and time consuming. Recently, tissue culture techniques have been adopted for consistent commercial production of economically important plants (Honda et al., 2001). The possible method to protect the and endangered plant species to conserve them from extinction (Milkulik, 1999; Chang et al., 2000; Jaime and endangered plant species is to multiply and conserve the plants by in vitro cultures. Plant tissue culture technology has been successfully used for the commercial production of microbe free plants (Parmessur et al., 2002; Liao et al., 2004) and to conserve the germplasm of rareDa Silva, 2003). This technique involves callus induction from explants, morphogenesis, shoot development and finally root development to regenerate into a complete somaclone. All these steps require different sets of hormones and growth medium for developing somaclonal variants successfully. Embryogenic callus was successfully induced in six relatives of *Citrus* with combination of 5.0 mg/l BA, 2.5 mg 2,4-D/l and 600 mg/l malt extract in Murashige and Tucker (MT) medium (Jumin, 1995). Mukhtar et al. (2005) successfully rege-nerated plantlets from nodal segments and leaf discs of sweet orange (*Citrus sinensis* L.) cv. Musambi and Lime (*Citrus aurantifolia*) cv. Kaghzi Nimbu.

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Table 1. Effect of concentrations and combinations of BA, NAA and 2,4-D on MS medium for callus induction and growth after five weeks of incubation.

Supplement (mg/l)	Ovary that cultured	Callus response (%)	Colour of callus	Type of callus	Proliferation
BA					
1.0	40	68	Light brown	Nodular compact	++
1.5	40	70	Light green	Nodular compact	++
2.0	40	65	Light brown	Smooth compact	+
BA + NAA					
1.0 + 0.5	40	98	Yellowish green	Smooth compact	++++
1.5 + 1.0	40	95	Yellowish brown	Smooth compact	++++
2.0 + 1.5	40	90	Yellowish green	Nodular compact	+++
BA + 2,4-D					
1.0 + 0.5	40	80	Light green	Nodular compact	++
1.5 + 1.0	40	75	Brownish yellow	Nodular compact	+++
2.0 + 1.5	40	77	Brownish yellow	Nodular compact	++
BA + NAA + 2,4-D					
1.0+0.5+0.5	40	92	Whitish green	Nodular compact	++++
1.5+1.0+1.0	40	89	Yellowish green	Nodular compact	+++
2.0+1.5+1.5	40	85	Yellowish green	Smooth	++

+, Poor; ++, fair; +++, good; ++++, excellent.

Tapati et al. (1995) suggested that the best growth regulator combination for regeneration of shoots was 0.1 mg/l NAA + 0.5 mg/l BA. Somatic embryogenesis and plant regeneration from undeveloped ovules, styles/stigma of sweet orange and naval group has been reported (Carimi et al., 1998). Very slow growth of callus from different explants like ovary, anther, nuceollus and embryonic basal node of sweet orange have been reported (Altaf et al., 2000). Plants have been regenerated from callus of *Citrus* species and related genera (Ling and Iwamasa, 1997; Gmitter and Moore, 1986). These investigations were undertaken to determine the response of different concentrations and combinations of growth regulators on callus, shoot and root induction in sweet orange.

MATERIALS AND METHODS

Unopened floral buds were taken from trees growing at Tarnab Research Farm, Peshawar (Pakistan). They were surface disinfected briefly with 95% ethanol for 3 min. The buds were dissected in sterile Petri dish with the help of forcep and scalpel. Then, the ovaries were collected and cultured on MS (Murashige and Skoog, 1962) basal medium enriched with constant concentrations of BA (1.0, 1.5 and 2.0 mg/l) alone and in combination with NAA (0.5, 1.0 and 1.5 mg/l) and 2,4-D (0.5, 1.0 and 1.5 mg/l) and also in combination of BA + NAA + 2,4-D mg/l for callus induction. For shoot regeneration, MS media was supplemented with NAA (0.5, 1.0, 1.5, 2.0 and 3.0 mg/l) + BA (1.0, 1.5, 2.5, 3.0 and 4.0 mg/l). Similarly, for root induction, IBA (2.0, 2.5 mg/l), IAA (2.0 and 2.5 mg/l) alone and in combination were used. The pH of the media was adjusted to 5.8 before solidified with 1% agar and autoclaved for 15 min at 121°C. The cultures were kept in growth chamber at 25 ± 2°C under a 16-h photoperiod with a light intensity of 2500 lux provided by cool fluorescent light to initiate their growth. These cultures were regularly subcultured on fresh medium in glass tubes or baby food jars at four-week intervals. Callus initiated at the cut end of some explants after ten days of culturing. After callusing, it was shifted to regeneration medium. At four leaf stage, the shoot was transferred to rooting medium. The rooted plantlets were acclimatized in pots containing sterilized soil mixture of sand, silt and clay in 1:1:1 ratio to study their response to *in vivo* conditions.

RESULTS

Callus Induction

Differences among various treatments were observed when constant concentrations of BA (1.0, 1.5 and 2.0 mg/l) alone and in combination with NAA (0.5, 1.0 and 1.5 mg/l) and 2,4-D (0.5, 1.0 and 1.5 mg/l) and also in combination of BA + NAA + 2,4-D were added to MS medium for the induction and proliferation of callus (Table 1). The best and maximum callus response was found

Table 2. Effect of concentrations and combinations of NAA and BA on MS medium for shoot proliferation.

Supplement (mg/l)	Number of explants that cultured	Shoot regeneration (%)	Number of shoots per explant	Days to shoot initiation
NAA + BA				
0.5 + 1.0	18	88	9	31
1.0 + 1.5	26	90	10	35
1.5 + 2.5	16	81	8	37
2.0 + 3.0	19	100	11	33
.0 + 4.0	22	78	7	38

with 1 mg/l BA + 0. 5 mg/l NAA followed by either 1.5 mg/l BA + 1 mg/l NAA and/or 1 mg/l BA + 0.5 mg/l NAA + 0.5 mg/l 2,4-D with 98, 95 and 92%, respectively. The colour of callus produced was yellowish green, yellowish brown and whitish green. Approximately 80% callus was nodular and compact, while 20% was smooth and compact. The highest response of callus proliferation was observed on the treatments of 1 mg/l BA + 0.5 mg/l NAA, 1.5 mg/l BA + 1 mg/l NAA and 1 mg/l BA + 0.5 mg/l NAA + 0.5 mg/l 2,4-D while the least response of callus proliferation was observed on the medium supplemented with 2 mg/l BA alone (Table 1).

Shoot proliferation

Morphogenetical responses of explants to various concentrations of NAA and BA on the MS medium are shown in Table 2. After 30 days, shoot appeared on some cultures, and expanded shoots become visible after 42 to 57 days (Figure 1C). The combination of 2 mg/l NAA + 3 mg/l BA on MS medium was found to be optimum for the establishment of cultures and sub-sequent shoot formation. In this medium, 100% explants responded to produced shoots at the average of 11 shoots per explant. Similarly, NAA 1 mg/l + BA 1.5 mg/l produced 90% shoots with 10 shoots per explant in 35 days. The medium with 1.0 mg/l NAA + 1.5 mg/l BA favored shoot formation (90%) but the number of shoots per explants (10) was low.

Root induction

The data related to effect of different concentrations and combinations of auxins on rooting of sweet orange showed that the number of plants rooted were minimum when auxin were used alone (Table 3). Maximum rooted plantlets were obtained when IAA was combined with IBA. Maximum rooting (16) was recorded when IAA 2.5 mg/l was combined with IBA 2.0 mg/l. However, IAA + IBA, 2.0 mg/l each gave (12) rooted plantlets. The lowest results were given by IAA 2.0 mg/l where only seven plants were rooted followed by IBA 2.0 mg/l with eight plants rooted. IAA and IBA combinations were also effective in the case of average numbers of roots per plantlet. When IAA 2.5 mg/l combined with IBA 2.0 mg/l, it gave maximum (3.8) roots per plantlet followed by IBA 2.5 mg/l, where (3.4) roots per plantlet were obtained. In this study, the combination of IAA 2.5 + IBA 2.0 mg/l proved better for rooting of sweet orange plantlets.

DISCUSSION

Callogenesis and organogenesis of ovary of sweet orange (*C. sinensis*) cv. Blood red was carried out using different concentrations and combinations of plant growth regulators. In the study, best callus induction were obtained with 1 mg/l BA + 0. 5 mg/l NAA followed by either 1.5 mg/l BA + 1 mg/l NAA or 1 mg/l BA + 0.5 mg/l NAA + 0.5 mg/l 2,4-D with 98, 95 and 92% respectively. Gitarani et al. (2003) and Haoa et al. (2004) got increased callus induction percentage with increasing levels of auxins, NAA and 2,4-D in the media. Our results are also in line with that of Hassan et al. (2000) who observed copious leaf calli on MS medium with higher concentration of 2,4-D alone or in combination with either 0.25 or 0.5 mg/l BAP from leaf explants of kiwifruit variety ‘matua’.

The combined effect of different concentrations of BA and NAA was studied on shoot proliferation. The combination of 2 mg/l NAA + 3 mg/l BA on MS medium was found to be optimum for the establishment of cultures and subsequent shoot formation. In this medium, 100% explants responded to produce shoots at an average of 11 shoots per explant. Similarly, NAA 1 mg/l + BA 1.5 mg/l produced 90% shoots with 10 shoots per explant in 35 days. Inclusion of auxin in the medium has been found to be beneficial for shoot production in some cases (Chaturvedi and Mitra, 1974; Bhansali and Arya, 1978). A medium containing 22 µM BA with or without 5.4 µM NAA was optimum for shoot initiation in all the three *Citrus* rootstocks (Moore, 1986).

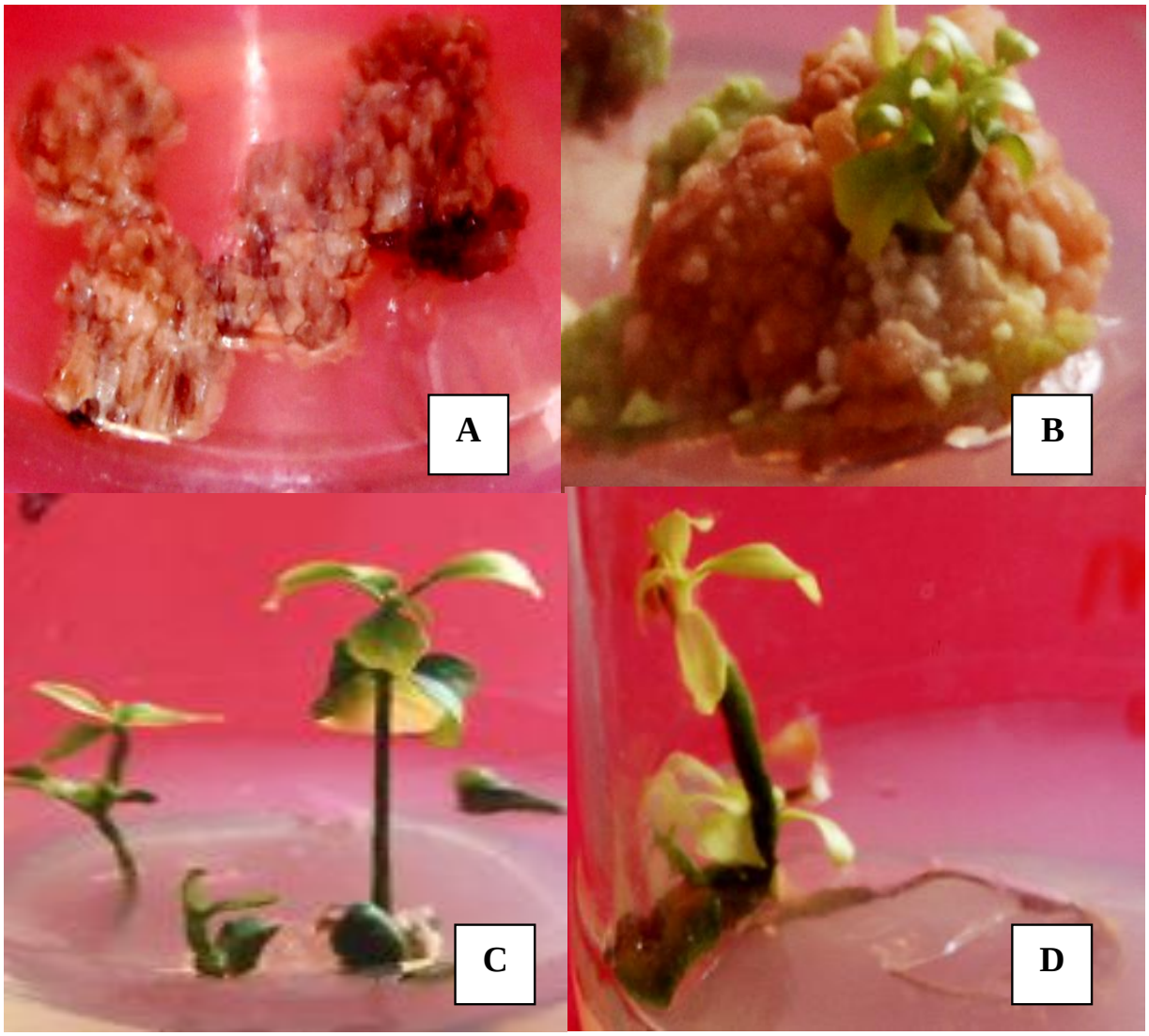

Figure 1. Callus induction from unpollinated ovary culture (A); plantlets arising from callus (B); plantlets regeneration and elongation (C) and root induction (D).

Table 3. Effect of different concentrations and combinations of auxins on MS medium for root induction.

Supplement (mg/l)	Number of plantlets that cultured	Number of plants that failed to root	Number of plants rooted	Average number of roots/plantlet
IBA				
2.0	20	12	8	2.4
2.5	20	10	10	3.4
IAA				
2.0	20	13	7	2.3
2.5	20	9	11	2.6
IAA + IBA				
2.0 + 2.0	20	8	12	3.1
2.5 + 2.0	20	4	16	3.8

Micro shoots shifted to rooting media containing different concentration of IBA and IAA alone and in combinations. Maximum rooted plantlets were obtained when IAA was combined with IBA. Maximum rooting (16) was recorded when IAA 2.5 mg/l was combined with IBA 2.0 mg/l. These results are in line with the findings of Zamir et al. (2007), who reported maximum root formation in guava when cultured on IBA 2.5 mg/l + IAA 2.5 mg/l. Hasan et al. 2000 got maximum (66.6%) rooted plantlets in kiwi fruit when IAA alone was used (5.0 mg/l). He also got maximum number of roots per plantlet (12) when IAA was combined with NAA, both at 2.5 mg/l. Here, the maximum percentage of rooted plantlets on IAA alone may be due to the higher concentration (5.0 mg/L).

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