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Efficient *in vitro* plant regeneration from cotyledonary explants of *Citrus reticulata* L. Blanco

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ABSTRACT

The cultivation of Khasi Mandarin (Citrus reticulata L. Blanco), an important commercial fruit, is faced with the problem of insects, pests and diseases. In order to overcome the hurdles of field cultivation of this species, in vitro approaches became necessary for selection of genotypes. The present study was undertaken to study the amenability of cotyledonary explants of C. reticulata for direct shoot regeneration without an intervening callus phase. A highly efficient, rapid and reproducible protocol was developed for in vitro shoot regeneration of C. reticulata from cotyledons of 12 day old germinated seeds. Explants were cultured on Murashige and Skoog medium (MS) supplemented with different concentrations and combinations of 6-Benzylaminopurine (BAP), α-naphthaleneacetic acid (NAA) and kinetin. The best response for multiple shoot formation after four weeks of culture was observed on media supplemented with 5 mg/l BAP where 84% of the explants responded producing an average of 6.5 buds per explant. In vitro rooting of the microshoots was tried on Murashige and Skoog medium incorporated with various concentrations and combinations of BAP, NAA and indole-3-butyric acid (IBA). IBA was found to be superior than other auxins for in vitro root induction. Eighty eight percent of the regenerated shoots developed roots when transferred to MS medium with 2 mg/l IBA. Thereafter, the in vitro regenerated plantlets were successfully acclimatized and established in soil with a survival percentage of 93.33%.

Key words: Cotyledons, Cytokinins, Auxins, Multiple shoots, Rooting.

INTRODUCTION

Citrus fruits are cultivated in more than 100 countries, making them as one of the most important commercial fruit crops of the world [1]. Mandarins (*Citrus reticulata* L. Blanco), one of the ancestral species of citrus, are widely grown in the tropical and subtropical areas. They are a good source of vitamin C [2] and are usually eaten plain or in fruit salads. Among the citrus

fruits, mandarin has gained high popularity and is commercially cultivated for its processing quality, fresh consumption and aromatic flavor. The peel of *C. reticulata* exhibits antimutagenic [3], anti-inflammatory [4; 5], antioxidant [6; 7], antitumor [8; 9] and antiatherosclerosis [10; 11] functions.

However, the cultivation of mandarins is faced with problems of slow growth, long juvenility, insect, pests, diseases, alternate bearing, pre and post harvest losses, short season of supply and short storage life [12]. *In vitro* propagation can be a viable alternative to overcome problems related to field culture of such species [13]. *In vitro* shoot regeneration systems are not only useful for large scale production and commercialization of clonal plants but also present a first step for introduction of genetic variation by genetic transformation. Therefore, *in vitro* multiple shoot proliferation is potentially useful for the genetic improvement of fruit crops [14]. The cultures derived from micropropagation systems are an ideal source of homogenous cells and tissues and are be able to regenerate viable shoots which can be efficiently propagated either by organogenesis or somatic embryogenesis. The regeneration of plants from cells or tissues is the key process in genetic transformation work without which no genetic improvement of any species is possible.

Studies on *in vitro* shoot regeneration of *C. reticulata* is rare as compared to most of the other *Citrus* species. Somatic embryogenesis has been reported from from leaf, epicotyl, cotyledon and root segments of *in vitro* grown nucellar seedlings of *C. reticulata* [15]. But though most of the somatic embryos germinated, only some produced shoots. Multiple shoot induction from shoot tips and nodal explants excised from mature and young shoots of *C. reticulata* has been reported [12]. Successful plant regeneration from epicotyl segments of *C. reticulata* was also obtained earlier [16]. Among other citrus species, *in vitro* studies from cotyledonary explants has so far been applied in *Citrus sinensis* [17] and *Citrus jambhiri* [18]. In *Citrus sinensis*, cotyledons proved to be a highly potential explant for development of somatic embryos and regeneration of plantlets. However, in *Citrus jambhiri*, though somatic embryogenesis was achieved, but the shoot bud induction frequency was very poor.

The aim of this paper was to study the efficacy of cotyledonary explants, cultured *in vitro* for direct shoot organogenesis, rooting and acclimatization of plantlets. The *in vitro* regeneration system developed in this study can be used for effective propagation of selected genotypes and can be an ideal source of homogenous material for regeneration of genetically modified plants.

MATERIALS AND METHODS

Source of tissue and preparation of explants

Mature fruits of Khasi Mandarin were peeled and the pulp removed to extract the seeds. Seeds from mature fruits of Khasi Mandarin were washed with running tap water and then treated with a few drops of tween 80, a soft detergent, to remove all pulp adhering to the surface. Thereafter, the seeds were rinsed three times in distilled water. They were dipped for 30 sec in 70% ethanol, followed by surface sterilization in 1% (w/v) sodium hypochloride for 10 min with gentle stirring. The seeds were then rinsed three times in sterile distilled water and cultured.

Culture medium

The basic nutrient medium consisted of MS [19] medium with 3% sucrose and vitamins, adjusted to pH 5.8 with 1 N NaOH and solidified with 0.8% agar (Himedia, India). The medium was sterilized by autoclaving for 20 min 121º C for 20 min, 105 kPa. Cultures were maintained at 25±2ºC under 12 h photoperiod provided by cool white fluorescent light at photosynthetic photon flux density of 3000 lux and subcultured every two weeks.

Germination of seeds and effect of (PGRs) plant growth regulators for optimal proliferation of shoots

The seeds were germinated aseptically on MS basal medium without growth regulators. Cotyledonary explants were excised from 12 day old seedlings and cultured on medium containing 6-benzylaminopurine (BAP) (0, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0 mg/l) singly or in combination with α-naphthaleneacetic acid (NAA) (0.25 mg/l) and kinetin (0.25 mg/l). One control without plant growth regulators was also used. Each treatment was tried with 20 explants. The proliferation was evaluated 4 weeks after the beginning of the experiment and the number of shoots per explant and the number of explants producing shoots were recorded.

Effect of different auxins and their concentrations on in vitro rooting of the microshoots

Actively growing shoots from the second subculture with four or five nodes were used for rooting. The composition of the rooting medium consisted of MS macro- and micronutrients fortified with indole-3-butyric acid (IBA) at concentrations of 0.5, 1.0, 1.5, 2.0 or 2.5 mg/l solely or combined with BAP (0.25 mg/l) and NAA (0.5 mg/l). Medium sterilization and culture conditions were as described above. Twenty microshoots were used for each treatment. After 3 weeks of culture, the rooting percentage, number of roots and length of roots per rooted shoot were evaluated.

Hardening and pot establishment of the plantlets

Complete plantlets with shoot and root systems were removed from culture tubes and washed with tap water to remove all agar adhering to the roots. The plantlets were transferred to small earthen pots filled with a mixture of cowdung, soil and perlite (1:1:1). The plantlets were covered with polythelene bags to ensure high humidity in the air surrounding the plantlets. The polythene bags were gradually removed after two weeks. The established plantlets were then transferred to the field and watered regularly.

Experimental design and statistical analysis

All experiments were conducted in a completely randomized block design (CRD) with twenty explants per treatment. Data on percentage of explants regenerating shoots and number of shoots per explants were recorded at 4 week interval in shoot proliferation experiments. Rooting experiments were evaluated after three week intervals and data were recorded on number of rooted microshoots and the number of roots per shoot. The data were subjected to one-way analysis of variance (ANOVA) with 5% significance level to determine the variation within the treatments. Mean comparisons were carried out by Duncan's Multiple Range Test [20]. The LSD test was used to study differences between different treatments.

RESULTS AND DISCUSSION

Germination of seeds

The seeds germinated within one week of culture and a germination percentage of 70% was obtained (Fig. 1a). After 12 days, the cotyledons were excised from the germinating seeds and cultured in shoot proliferation media.

Effect of plant growth regulators for optimal shoot proliferation

The use of plant growth regulators for shoot proliferation in tissue culture is of fundamental importance. Studies have been conducted to identify the optimal concentrations of different growth regulators on shoot multiplication and root differentiation in Citrus [21; 22]. Cytokinins and auxins are the two most important plant growth regulators used in plant tissue culture [23; 24]. BAP, at different concentrations, has been the most commonly used cytokinin for multiplication of citrus shoots [24]. In our study, no shoots were recorded in MS medium without growth regulators. The number of shoots per explants and the percentage of response of explants for shoot proliferation increased when BAP was included in the medium. The percentage of explants regenerating shoots as well as the number of shoots per explant was affected significantly when different concentrations of BAP were used. The best results occurred with with 5 mg/l BAP with an average of 6.45 shoots per explant and 84% percent of the explants responded to multiple shoot proliferation (Fig. 1b) in this medium after 4 weeks of culture ($P<0.05$). An average shoot length of 6 cms was observed in this medium.The percentage of response lowered considerably when the concentration of BAP was increased to 6 mg/l (34% with 2.27 shoots per explant). No increase in shoot proliferation was obtained when NAA and kinetin were included with the BAP treatments (Table1). An average number of 7.8 shoots per explant was obtained with an optimal concentration of 2mg/l BAP from epicotyl segments of *C. reticulata* when incubated under 16-h photoperiod [16] but the shoot regeneration frequency was less (72.5%) as compared to our studies.The presence of only 5 mg/l BAP was found to be the optimal concentration which induced 3.1 shoots per explant from shoot tips of Carrizo Citrange (*Citrus sinensis* [L.] Osb. × *Citrus trifoliata* [L.] Raf.) [25]. In internodal segments of Carrizo Citrange, 88% of the explants responded with an average of 7.5 shoots per explants and in Cleopatra Mandarin (*Citrus reshni*), 18% of the intermodal segments produced an average of 3.6 shoots per explant in the presence of 22 µM BAP [26]. The greatest number of shoots (2.5) were produced from shoot tips and intermodal segments of *C. limon* on medium fortified with 2mg/l BAP [23]. Shoot tip explants of *Citrus grandis* showed a response of 5.2 shoots per explant on 1.8µM supplemented medium [27]. It was reported to have obtained an average number of 3.96 from 68% of the epicotyl segments of *Citrus paradisi* 2mg/l supplemented medium [28]. Thus, application of only BAP appeared to be most effective in many citrus species which agree with our findings. On the contrary, 84% of the shoot tips of *C. reticulata* produced 7.99 shoots per explants in the presence of 1 mg/l BAP and 1.5 mg/l kinetin [12] which differ from the optimal concentrations of hormones required in our studies. Significantly higher (83.0%) somatic embryogenesis was exhibited from epicotyl-derived callus of *C. reticulata* on MS medium containing 10 mg/l NAA and 1 mg/l kinetin [15]. Mature stem segments of *C. sinensis* showed a response of 54% with 3.1 shoots per explant on medium supplemented with 1.8µM BAP and 0.7 µM GA_3 [29]. The best results for productivity of shoots from nodal explants of *Citrus limon* were obtained with 2 mg/l BAP and 2 mg/l GA_3 [30]. The requirement for exogenous plant

growth regulators varies with the explant type and species and apparently depends on the endogenous level of plant growth hormone [31].

Table 1. Effect of different plant growth regulators on multiple shoot induction from cotyledonary explants of *C. reticulata* after 4 weeks of culture

PGR concentration (mg/l)			MS		
BAP	NAA	Kinetin	Percentage of explants regenerating shoots (mean ±SE)	Average number of shoots per explant (mean ±SE)	Average shoot length (cm)
-	-	-	-	-	-
0.5	0.25	0.25	22.0 ± 10.0^{f}	2.4 ±0.2^{cde}	2.0 ± 0.2^{d}
1.0	0.25	0.25	30.0 ± 5.0^{def}	2.5 ± 0.2^{bcde}	2.1 ± 0.1^{d}
2.0	0.25	0.25	36.0 ± 10.0def	2.6 ± 0.2^{bcde}	3.1 ± 0.2^{c}
3.0	0.25	0.25	38.0 ±10.0^{def}	2.8 ± 0.2^{bcde}	3.3 ± 0.1^{bc}
4.0	0.25	0.25	42.0 ± 10.0^{cde}	2.9 ± 0.3^{bcd}	4.0 ± 1.0^{b}
5.0	0.25	0.25	26.0 ± 10.0^{ef}	3.0 ± 0.2^{bc}	2.0 ± 0.2^{d}
6.0	0.25	0.25	48.0 ± 10.0^{bcd}	3.1 ±0.3^{b}	3.3 ± 0.1^{bc}
0.5	-	-	38.0 ± 10.0^{def}	2.2 ± 0.1^{c}	3.3 ± 0.1^{bc}
1.0	-	-	40.0 ± 5.0^{cdef}	2.3±0.1^{de}	4.0 ± 0.1^{b}
2.0	-	-	44.0 ± 10.0^{cde}	2.4 ±0.2^{cde}	4.0 ± 0.1^{b}
3.0	-	-	56.0 ± 10.0^{bc}	2.5 ± 0.2^{bcde}	4.0 ± 0.1^{b}
4.0	-	-	62.0 ± 10.0^{b}	2.8 ± 0.2^{bcde}	4.0 ± 0.3^{b}
5.0	-	-	84.0 ± 10.0^{a}	6.5 ± 0.3^{a}	6.0 ± 1.0^{a}
6.0	-	-	34.0 ± 10.0^{def}	2.3 ± 0.1^{de}	2.2 ± 0.1^{d}

Explants were cultured on MS media supplemeted with various concentrations of BAP, NAA and kinetin. Data was evaluated after 4 weeks of culture. Values represent mean ± SE of three replicated experiments of 20 explants each. P<0.05

Table 2. Effect of different plant growth regulator on development of root after 3 weeks of culture

Composition of growth regulator (mg/l)			MS		
BAP	NAA	IBA	Percentage of shoots inducing root	Average no. of roots developed per shoot after 3 weeks of culture (Mean±SE)	Average length (cm) of roots after 3 weeks of culture (Mean±SE)
-	-	-	-	-	-
0.25	0.5	0.5	-	-	-
0.25	1.0	0.5	12.0 ± 5.0^{e}	1.500±0.224^{bc}	1.0±0.099^{e}
0.25	1.5	0.5	16.0 ± 8.0^{de}	1.833±0.307^{bc}	1.0±0.106^{e}
0.25	2.0	0.5	30.0 ± 5.0^{bcd}	2.000±0.258^{b}	1.30±0.048^{de}
0.25	2.5	0.5	22.0 ± 10.0^{cde}	1.333±0.211^{bc}	6.60±0.082^{cd}
-	0.5	-	32.0 ± 10.0^{bcd}	1.667±0.333^{bc}	2.00±0.117^{b}
-	1.0	-	34.0 ± 10.0^{bc}	1.833±0.307^{bc}	1.75±0.072^{bc}
-	1.5	-	42.0 ± 10.0^{b}	2.000±0.258^{b}	2.00±0.121^{b}
-	2.0	-	88.0 ± 10.0^{a}	3.000±0.365^{a}	5.50±0.266^{a}
-	2.5	-	46.0 ± 10.0^{b}	1.167±0.167^{c}	2.00±0.099^{b}

Explants were cultured on MS media supplemeted with various concentrations of BAP, IBA and NAA. Data was evaluated after 3 weeks of culture. Values represent mean ±SE of three replicated experiments of 20 explants each. P<0.05

Effect of different plant growth regulators and their concentrations on rooting of shoots

Regenerated shoots with 2-3 leaves after six weeks of culture were transferred to rooting medium. Root initiation was observed within 1 week after transfer to rooting medium. The best rooting percentage (P<0.05) of 88% with 3 roots per explant recorded after 3 weeks of culture

(Fig 1c) were found to be highest in MS medium with 2 mg/l IBA with an average shoot length of 5.50 cms and six roots per shoot (Table 2). Results were not very encouraging when IBA was combined with 0.25 mg/l BAP and 0.5 mg/l NAA. The incubation of shoots on a NAA or IBA-supplemented medium induces the formation of roots in many different Citrus species [24]. The longest roots in lemon was recorded with 1 mg/l IBA [23]. But in 'Carrizo citrange' NAA was observed to be more efficient in the enhancement of rooting compared with IBA or IAA [25]. The highest percentage of rooting (87%) for regenerated shoots of *C. reticulata* was obtained in media supplemented with 2 mg/l NAA [12]. A rooting percentage of 88% with an average of 2 roots per explants was achieved when basal portion of the regenerated shoots were dipped in 1000 mg/l IBA solution before placement on a filter-paper bridge [16]. Thus the presence of an auxin is generally necessary to promote rooting in *C. reticulata*.

Establishment of the plantlets in the soil

The percentage of success during acclimatization was 93.3% and the plantlets exhibited normal growth in soil with 3-4 leaves per plant.

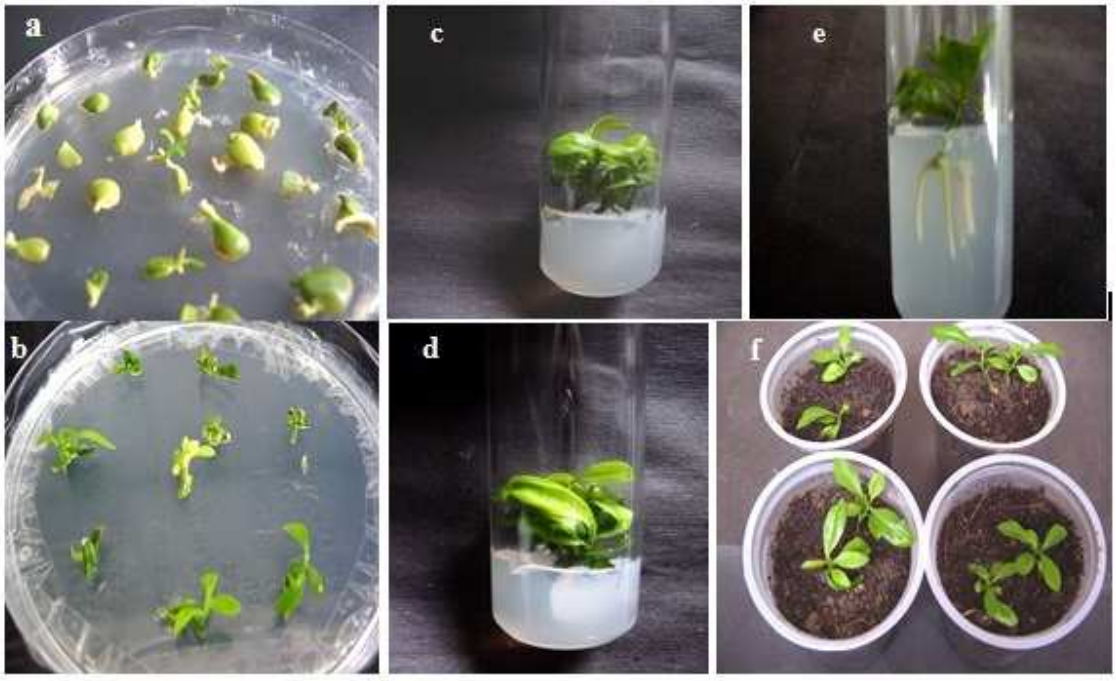

Figure 1. *In vitro* plant regeneration of *Citrus reticulata*
(a) & (b) In vitro germination of Citrus reticulata seeds in MS basal media
(c) & (d) Multiple shoot proliferation from cotyledonary segments cultured in MS+ 5.0 mg/l BAP
(e) In vitro rooting on MS + 2 mg/l IBA
(f) Plantlets established in soil and perlite during hardening

CONCLUSION

Studies on *in vitro* shoot regeneration of *Citrus reticulata* are rare. In the present investigation, an efficient and rapid protocol was established from cotyledonary segments of *in vitro grown* seedlings. The *in vitro* regeneration system was developed by manipulating different

concentrations of plant growth hormones. BAP had great influence in the shoot proliferation stage, In the rooting stage IBA produced the best results. Regenerated plantlets could be easily established in soil. The *in vitro* derived explants can be an ideal source of homogenous material for transformation experiments. The *in vitro* regeneration system can also be applied for mass propagation and commercialization of selected genotypes.

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