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Development of an Efficient in Vitro Regeneration System for Endangered Wild Orange Citrus Chrysocarpa L.

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Abstract

A method for *in-vitro* propagation of wild type Indian orange (*Citrus chrysocarpa* L.) was developed by shoot organogenesis from seed. Mature seed embryos were used as explants and treated with different hormones and plant growth regulators on MS medium for callus, shoots and roots induction. For callus induction seed embryos were treated with different concentration of 2, 4-dichlorophenoxyacetic acid (2,4-D), 6-benzylaminopurine (BA) and a-naphthalene acetic acid (NAA) and maximum 90.90% callus was observed on MS medium supplemented with 2, 4 -D at 16.0 µM concentration.

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Approximately 85% of the callus was granular, while 15% was smooth and compact. Maximum 83.33% shoot regeneration was observed on MS medium supplemented with BA at 13µM concentration. Regenerated shoots were rooted on MS medium supplemented with different hormones and maximum 80% roots were observed in MS medium supplemented with 10 µM *Indole-3-butyric acid* (IBA). The regenerated plantlets were successfully acclimatized in *ex-situ* condition for further study.

Keywords: Citrus chrysocarpa L.; In-vitro; Callus induction; Shoots formation; Explants, Rooting; acclimatization.

1. Introduction

Citrus is one of the leading fruit crops in the world. The genus *Citrus* includes more than 162 species. This single genus *Citrus* includes grapefruits, lemons, limes, oranges, and various other types and hybrids. It is widely considered that *Citrus* originated in Australia, New Caledonia and New Guinea [1]. As of 1987, orange trees were found to be the most cultivated fruit tree in the world. Orange trees are widely grown in tropical and subtropical climates for their suitable growth and development. The fruit of the orange tree can be eaten fresh, or processed for its juice or fragrant peel. In 2013, 71.4 million metric tons of oranges were grown worldwide, production being highest in Brazil and the U.S. states of Florida and California [2]. The fruit of the *Citrus chrysocarpa* L. is considered as a wild type Indian orange and recent searches showed that the home range is confirmed in Meghalaya, where it grows in the Garo Hills. The fruit is low in calories, contains no saturated fats or cholesterol, but is rich in dietary fiber, pectin, potassium, calcium and also contains a variety of phytochemicals and flavonoids like- hesperetin, naringin, and naringenin [1]. It is an excellent source of vitamin C (provides 53.2 mg per 100 g, about 90% of DRI). Commercially grown orange trees are propagated asexually by grafting a mature cultivar onto a suitable seedling rootstock to ensure the same yield, identical fruit characteristics, and resistance to diseases throughout the year. Thus, rootstocks influence the rate of growth and have an effect on fruit yield and quality. Rootstocks must be compatible with the variety inserted into them otherwise, the tree may decline, be less productive, and even die. To overcome these problems plant tissue culture technology has been successfully used for the commercial production of microbe free plants [3] and to conserve the germplasm of rare and endangered plant species to conserve them from extinction [4]. This techniques can be applied as a helpful tool to reduce the time for improvement of orange through somaclonal variation. Further, this technique made it easy to improve citrus against different abiotic stresses, low yield and conserve important *citrus* genotypes through exploiting somaclonal variations, somatic cell hybridization [5]. By using micro-propagation method, there is a chance to establish a cell line of virus free Malta. Several workers [6].

Reference [7] Conducted research on C*itrus sinensis, Citrus aurantifolia* and others reported the tissue culture system of different orange species which is considered as an endangered species in native to India. Threats to the species have included habitat destruction caused by slash-and-burn (jhum) activity. The present *in-vitro* propagation study on *Citrus chrysocarpa* L. was taken to develop a method for multiplication of this endangered species and to generate somaclone with desired traits which in future will help to develop virus & disease free orange with improved qualities.

2. Materials and Methods

2.1. Preparation and sterilization of explants, equipment and media

Mature seeds of local orange (*Citrus chrysocarpa* L.) were used for *in vitro* propagation. The orange seeds were collected from local nursery and Citrus Research Station, a division of the Bangladesh Agricultural Research Institute, Jointapur, Sylhet. Mature orange seed embryos were used as explants for callus induction. Selected healthy seeds were manually dehusked and washed with sterile distilled water then kept in 70% ethanol for 3-5 minute, followed by washing with autoclaved distilled water for several times. Seeds were further sterilized by continuous shaking with 0.1% mercuric chloride with few drops of tween-20 for 3-5 minutes. Surface sterilized seeds were rinsed 5-6 times with sterile distilled water before inoculation. Sterile glasswares were used in every phase of this research.

2.2. Media for callus induction

The ovaries of the collected seeds were cultured on MS [8] basal medium enriched with concentration of 2, 4-D (5.0, 7.0, 9.0, 11.0, 13.0, 16.0, 19.0 µM) alone and in combination with 2, 4-D (5 µM) [24], BA (1.0, 2.5, 3.0 µM) and NAA (1.5, 3.0, 3.0 µM).

2.3. Media for shoot proliferation

Shoot regeneration was carried out from callus of *C. chrysocarpa* L. Healthy portion of the calli were taken and placed on shoot regeneration medium. MS medium supplemented with BA (6.0, 8.5, 11.0, 13.0, 15.0, 18.0 µM), Kinetin (5.0, 7.0, 10.0 µM) separately and in combination of BA (9.0, 13.0, 18.0 µM) and Kinetin (10.0, 14.0, 19.0 µM) and combination of BA (5.0, 7.0, 9.0 µM), NAA (2.5, 5.5, 8.0 µM) and Kinetin (2.5, 5.0, 7.0 µM) for shoot regeneration.

2.4. Media for Root proliferation

MS medium supplemented with different concentrations of IBA at (5.0, 5.5, 7.5, 8.8, 10, 12, 15) µM concentrations, NAA (5.5, 8.0) µM separately and in combination of IBA 5.0 µM with NAA 2.5 µM and IBA 5.0 µM with NAA 5.5 µM were used. Observations were taken in every three days and the effects of different growth regulators were quantified on the basis of percentage of calli, shoots and roots.

3. Results and discussion

3.1. Effect of different concentration of hormones on callus induction

The study was designed to find out the ideal conditions for micropropagation of local orange (*Citrus chrysocarpa* L.) because not much work has been done on this endangered species in Bangladesh. To establish an efficient *in vitro* propagation system, the experiments were conducted aseptically and then explants were cultured on MS medium supplemented with different concentrations and combinations of hormones. Reference

[6] reported that highest percent (68%) callus was obtained from 2, 4-D at 2 mg/L followed by moderate callus induction of 2, 4-D in 1.0 mg/L and 3 mg/L in *Citrus sinensis*. [9] Also reported that 2, 4-D at 4.0 mg/L and 2, 4-D at 3 mg/L showed highest percent callus induction in same species. [10] Reported that the seeds formed callus in MS medium supplemented with BA + 2, 4-D each at 1 mg/L. In this study, after 8-15 days of inoculation in culture medium high efficient calli were produced (Figure 1).

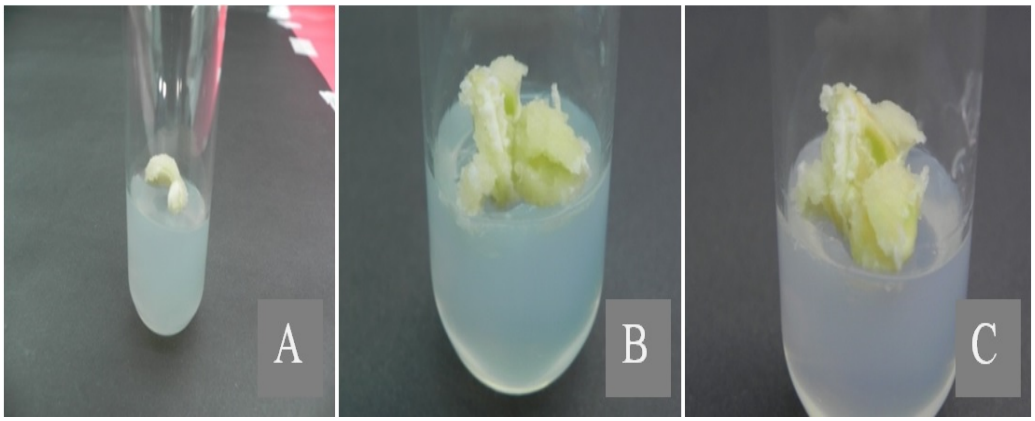

Figure 1: Calli of *Citrus chrysocarpa* L. in MS media supplemented with 2, 4-D at16.0 µM (A, after 3 days, B, after 10 days and C, after 15 days of inoculation).

Approximately 85% of the calli were granular, while 15% were smooth and compact. The color of the calli produced were light green, whitish green, yellowish green and light brown (Table 1).

Among all hormonal concentrations, the best callus response (90.9%) was observed MS medium supplemented with 2, 4-D at 16.0 µM. Around 81.81% callus response observed in concentrations of 2, 4-D at 13.0 µM while 2, 4-D at 5.0 µM in combination with BA at 2.5 µM and NAA at 3.0 µM showed 66.67% callus initiation. Around 50% callus initiation was found on 2, 4-D at 11.00 µM but 2, 4-D at 9.00 µM, 2, 4-D at 5.0 µM with BA 2.5 µM and NAA 1.5.0 µM observed reduced rate of callus initiation. In case of 2, 4-D at 5.00 µM and 2, 4-D at 7.00 µM showed no response (Table 1, Figure 2).

Reference [11] Reported callus development in sweet orange (*Citrus sinensis*) on MS medium supplemented with 1 mg/l 2, 4-D or 1 mg/l NAA. [12, 13] Observed increased callus induction percentage with increasing levels of auxins, NAA and 2, 4-D in the media. Callus induction occurred on half strength MS medium supplemented with BA at 1.0 mg/L and 2, 4-D at 5.0 mg/1 were obtained by for *Citrus macroptera* [14]. MS medium supplemented with different concentration of BA & NA was found to be best callusing in case of *Citrus grandis* by [15].

3.2. Effect of plant growth regulators for optimal shoot proliferation

For shoot regeneration, healthy calli were cut into small pieces and these pieces were cultured on MS medium supplemented with different concentration of BA and also, combination of BA, Kinetin and BA, NAA, Kinetin. After 30 days of inoculation in regeneration medium, shoot appears on some calli and extended shoot became

visible after 45 days.

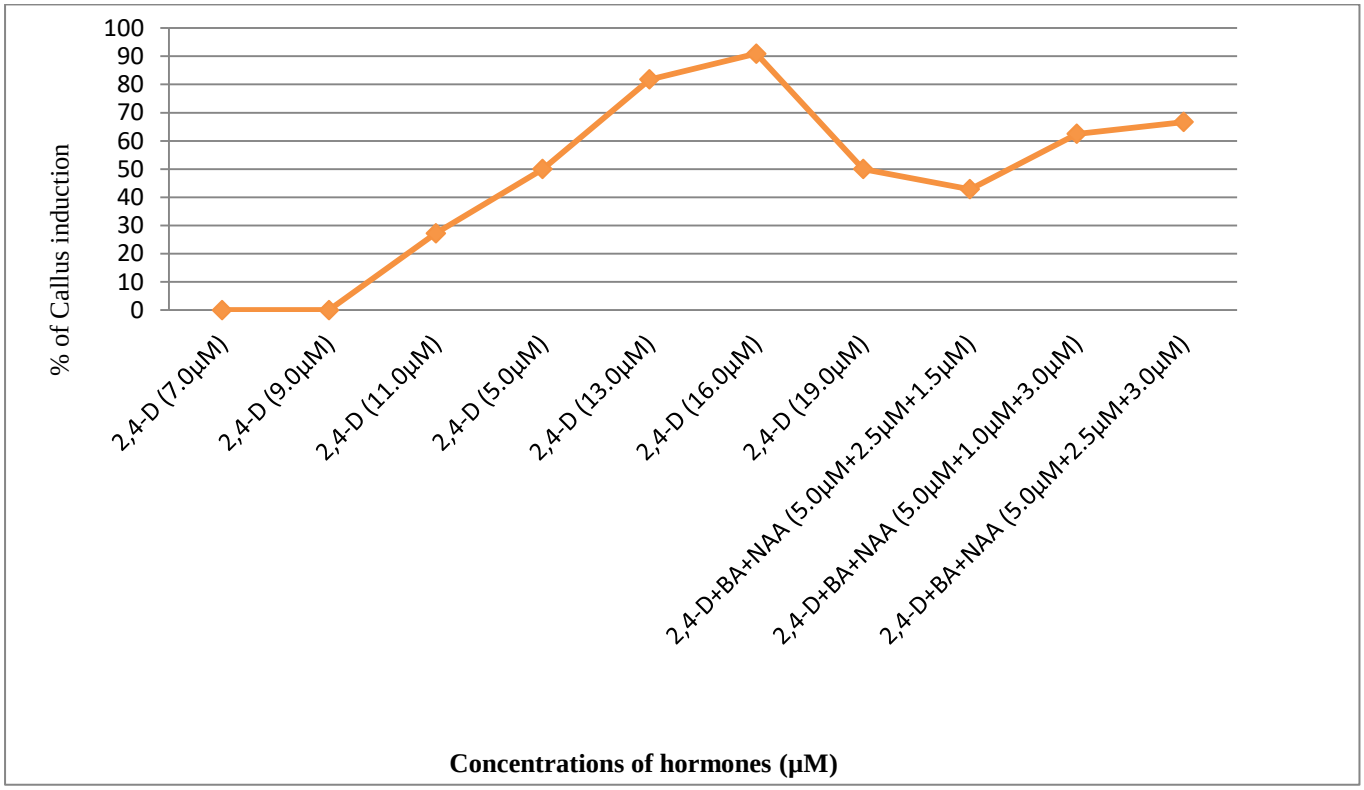

Figure 2: Effects of phytohormones on callus initiation

Figure 3: Shoots regeneration (A, B & C) of *Citrus chrysocarpa* L. in MS media treated with BA at13.0 µM.

Regeneration of different species of *Citrus* has been already investigated using MS medium supplemented with BA at 3 mg/l or with BA at 1 mg/l [16]. Earlier, combination of BA and NAA have been shown favorable for shoot regeneration from calli of different *Citrus spp*. [17]. The present study showed better regeneration response than those cited in these reports. In this study maximum shoot regeneration response (83.33%) was observed on MS medium supplemented with BA 13 µM followed by 70% with BA at 15 µM. The lowest shoot regeneration response (20%) was observed on MS medium supplemented with BA at 5 µM, Kinetin at 2.5 µM and NAA at 2.5 µM when used in combination. Although the medium with combination of BA and Kinetin showed low percentage of shoot proliferation but it gave highest number of shoots per explant which was around 11(Figure 3; Figure 4). Some studies have shown use of BA alone to be better treatment for shoot regeneration in different *Citrus spp.* [18]. [19] Reported shoot regeneration at BA from 0.5 – 4 mg/l for *Citrus*

paradise (Macf) epicotyl explants.

Table 1: Effects of different concentrations of 2, 4-D and combinations of 2, 4-D, BA and NAA for callus initiation (data were taken after 7-15 days of inoculation).

Concentration of hormones (µM)		Number of explants inoculated	Number of explants survived	Survival Rate (%)	Number of explants that give rise to callus	Percent of callus Induction (%)	Color of the callus
2, 4 - D	5	12	10	83.33	0	0	-
	7	12	9	75	0	0	-
	9	12	11	91.67	3	27.27	Whitish
	11	12	10	83.33	5	50.0	yellowish
	13	12	11	91.67	9	81.81	greenish
	16	12	11	91.67	10	90.90	greenish
	19	12	10	83.33	5	50.0	Whitish
2,4-D + BA+ NAA	5.0+2.5+1.5	10	7	70	3	42.85	Whitish
	5.0+1.0+3.0	10	8	80	5	62.50	greenish
	5.0+2.5+3.0	10	9	90	6	66.67	greenish

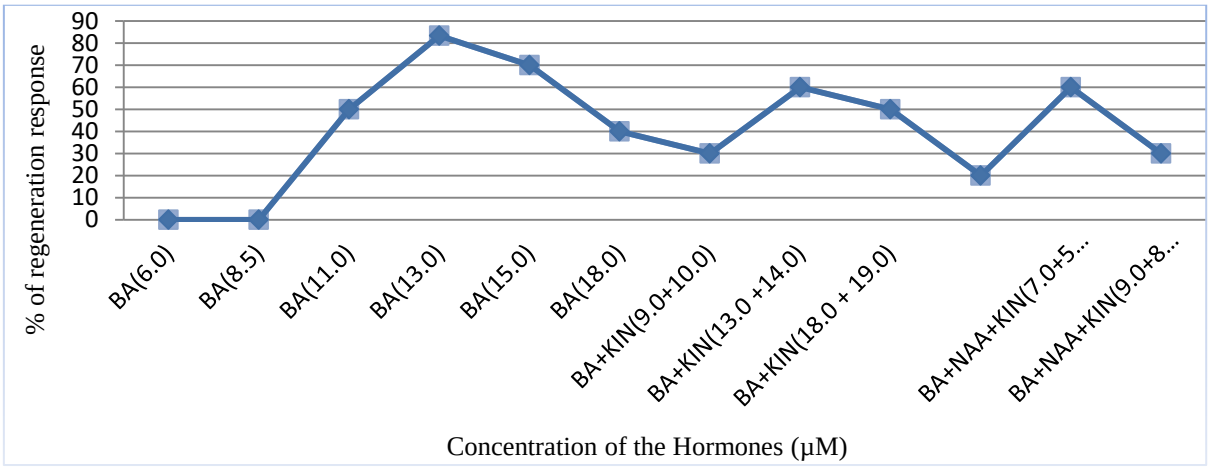

Figure 4: Effects of hormones on calli for shoot regeneration.

3.3. Effect of different plant growth regulators for root development

Shoots were cultured on rooting medium containing different concentrations of IBA, NAA and their combination. Roots appeared within 20 days after inoculation. Maximum (90%) roots were recorded when MS medium supplemented IBA at 10 µM. Media supplemented with IBA at 12 µM and NAA at 8 µM showed 70% and 62.5% root formation respectively (Figure 6; Figure 5 A). In this study IBA was found to give better response as compared to NAA. On the contrary, NAA was found to give better response in *Citrus acida* [20]. Other reports which showed NAA to be better rooting hormone for *Citrus* spp. [16, 20, 21, 22].

3.4. Acclimatization

The rooted shoots were removed from the culture tubes, washed with tap water and transferred to plastic pots containing garden soil for successful acclimatization to study their adaptability. The plantlets were placed in outside the laboratory environment (Figure 5 B). [23] Observed efficient *in-vitro* rooting and acclimatization of micro propagated shoots in citrus with very high establishment in the soil.

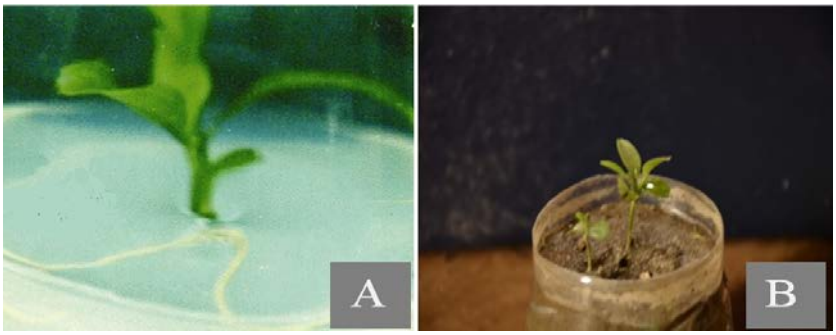

Figure 5: Root initiation on MS media treated with IBA at 10 µM (A) after 30 days of inoculation and acclimatization outside laboratory conditions (B)

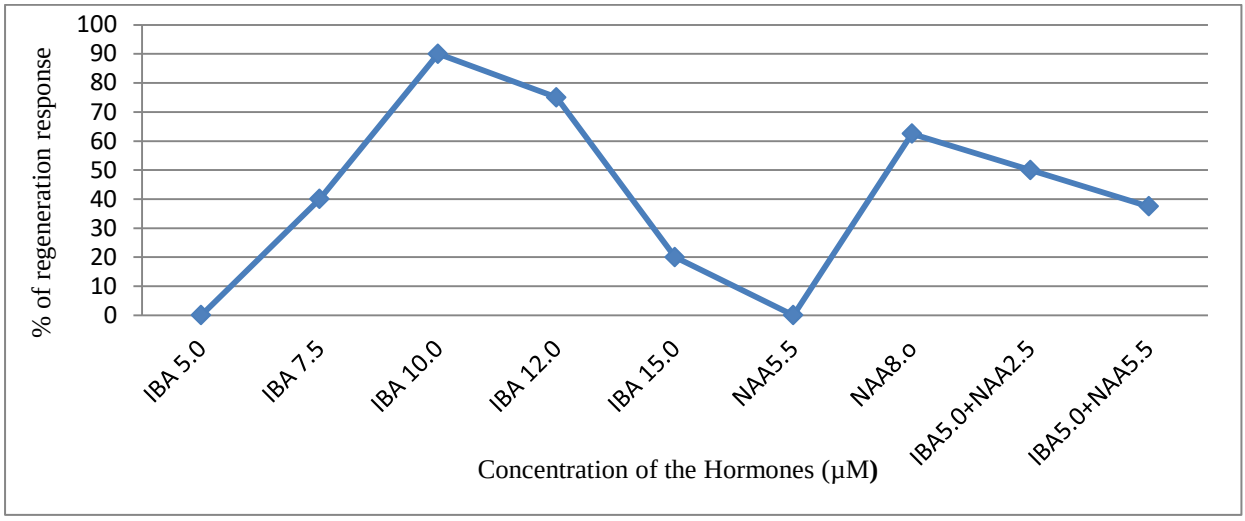

Figure 6: Effects of IBA, NAA and their combinations for root formation.

4. Conclusion

Many research works were conducted on *Citrus* sp. (cultivated) but very less works are done on wild orange *Citrus chrysocarpa* L. In this study we tried to develop efficient regeneration system of this endangered *Citrus* species. Considering the above results, 2, 4-D showed the best plant growth regulator for callus initiation, BA for shoot initiation and IBA for root initiation of wild orange *Citrus chrysocarpa* L. and the best concentration for each growth regulator were 16 µM, 13 µM and 10 µM respectively. So far we know this is the first work on *in vitro* regeneration system of *Citrus chrysocarpa* L.

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