An Efficient Regeneration System for Native Orange (*Citrus reticulata*) through *In-Vitro* Culture Technique

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How to cite this paper: Khan, Md.F., Hoque, H., Islam, Md.Q., Ashrafuzzaman, Md. and Prodhan, S.H. (2019) An Efficient Regeneration System for Native Orange (*Citrus reticulata*) through *In-Vitro* Culture Technique. *Agricultural Sciences*, **10**, 975-984.
https://doi.org/10.4236/as.2019.107074

Received: June 27, 2019
Accepted: July 22, 2019
Published: July 25, 2019

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Abstract

Citrus reticulata (Mandarin Orange), commonly known as "Sweet Orange", is one of the most difficult plants to improve through traditional breeding approaches as it poses various biological limitations that greatly hinder the cultivar improvement. In the present study, using the fresh seed of native orange as explant, an efficient, reproducible, regeneration method was developed through *in vitro* organogenesis. Mature, healthy and dehusked seeds were treated with Murashige and Skoog, (MS) media containing 3% sucrose, 0.7% agar supplemented with different concentrations and combinations of phytohormones. The highest calli initiation (93.3% ± 0.5%) responses were observed on MS medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) at 3.0 mg/L followed by 2,4-D at 3.5 mg/L (86.7% ± 1.75%) in this experiment. Maximum shoot regeneration (86.7% ± 3.35%) responses were reported using MS medium supplemented with the combination of 6-benzylaminopurine (BAP) at 3.0 mg/L and 1-naphthaleneacetic acid (NAA) at 2.0 mg/L. MS medium supplemented with NAA at 1.0 mg/L showed the best rooting (80% ± 2.89%) response in comparison to (70% ± 5.20%) indole-3-butyric acid (IBA) at 1.0 mg/L. The regenerated plantlets were acclimatized in pots containing sterile garden soil mixture to examine their response in natural conditions.

Keywords

Citrus reticulata, 2,4-D, Callus, Regeneration, Acclimatization

1. Introduction

Citrus is one of the world's leading horticultural crops which is cultivated world-

wide for its nutritive values and is mainly consumed as fresh fruit and juice [1] [2]. The mandarin orange (*Citrus reticulata*) has been cultivated in a vast tropical and sub-tropical area since the early period [3] [4]. It is a scientifically established fact that *citrus* fruits, especially oranges, by virtue of their abundance in vitamins, antioxidants, and minerals, have many proven health benefits [5]. Commercial cultivation of orange in Bangladesh is gaining popularity at different Upazilas of the districts Sylhet, Sunamganj, Hobiganj, Moulovibazar, Panchagarh, Gaibandha, Mymensingh and Thakurgaon. But recent studies showed that citrus production declining due to several factors like diseases and abiotic stresses. Despite the excessive cultivation, *citrus* plantation still has some major problems such as long juvenility, slow growth and insects, pests, diseases, alternate bearing, pre and post-harvest losses, the large number of seeds per fruit, the short season of supply and short storage life etc. By using traditional plant-breeding methods, genetic improvements of this perennial woody plant often take many years [6]. Thus, advancement in biotechnology has generated new opportunities. *In vitro* propagation has therefore been a great potential tool to overcome problems related to the field culture for such species [7]. *In vitro* culture technique made it easy to improve *citrus* against different abiotic stresses, low yield and conserve important *citrus* genotypes through exploiting somaclonal variations [8], somatic cell hybridization [9] [10], transformation of high yielding cultivars [11], disease-free plants [12]. *Citrus* is also subjected to various biotic stresses, especially caused by virus and viroids which limit the vigor, yield, and quality of the plant. So, it is imperative to apply the *in vitro* culture technique to produce more disease-free plants of *Citrus* [13]. Though much work has already been done on *Citrus* improvement and development, very little work has been carried out on the genetic improvement of *C. reticulata* species [14] [15] [16]. Besides developing the existing cultivars, micropropagation methods have the potential for mass scale generation of novel plants in a relatively short time span when compared to traditional breeding. Callus production and its subsequent regeneration are the prime steps in crop plants to be manipulated by biotechnological means and to generate somaclonal variations. Tissue culture and micropropagation protocols have been described for a number of *Citrus* sp. using a wide range of explant sources, viz. leaf explants [17] [18], seed explants [19] [20], internodal explants [21]. Successful shoot proliferation has been achieved in 33 *citrus* types belonging to 19 species [22]. Successful micropropagated plantlets were produced using 10 mg/l of BAP for shoot proliferation and the same concentration of NAA for rooting in the case of Carrizo citrange, trifoliate orange, Cleopatra mandarin, Rangpur lime and sweet orange [23]. But all these highly sophisticated techniques require the presence of a reliable, responsive and reproducible regeneration protocol [24]. In this experiment, we studied the effects of different auxins and cytokinins on callus initiation from mature seeds of *C. reticulata* and regeneration of plantlets from callus. Current study was designed to develop an efficient, reproducible regeneration method of *Citrus reticulata* using mature seed

derived callus. Very less work is done on this native orange of Sylhet region in Bangladesh. So, the present study was conducted to develop an efficient callus initiation system of *C. reticulata* through tissue culture which might be used in an effective, suitable and moreover a reproducible regeneration protocol of sweet orange in near future.

2. Materials and Methods

This work was carried out in the Plant Genetic Engineering Laboratory of Department of Genetic Engineering and Biotechnology, Shahjalal University of Science and Technology (SUST), Sylhet, 3114, Bangladesh. The orange fruits belonging to the species *Citrus reticulata* were collected from *Citrus* Research Station, Jaintiapur, Sylhet and mature healthy seeds were used as explants. After dehusking, the explants were sterilized with 70% alcohol for 4 min and washed with sterile distilled water several times. Followed by washing, the explants were further treated using 0.1% $HgCl_2$ with 1 - 2 drops of tween-20 for 6 min. After washing with sterile distilled water several times, the explants were dried on sterilized filter paper. The sterilized explants were inoculated in testtubes containing 10 - 12 ml Murashige and Skoog (MS) basal medium (containing 3% sucrose, 0.7% agar and pH 5.8 - 6.0) supplemented with different concentrations of 2,4-D, combinations of BAP, NAA and the combinations of 2,4-D, BAP, NAA (Table 1). The explants were incubated at 25˚C ± 3˚C temperature with 16 h photoperiod under 2000 lux light intensity. Visual observations were taken in every three days and the effects of different treatments were quantified on the basis of the percentage of callus induction. In every 3 weeks of inoculation, the explants were subcultured in the same media. The healthy, friable calli were divided into small pieces and cultured on MS medium supplemented with Kinetin (KIN), BAP, and the combinations of BAP, NAA and also the combinations of BAP, KIN and NAA for shoot regeneration. The cultures were maintained under same culture conditions for 4 weeks. For rooting, the regenerated shoots were cultured on MS medium enriched with different concentrations of IBA, NAA and combinations of IBA, NAA. The cultures were then incubated under same culture conditions. Visual observations were taken in every three days and the effects of different treatments on the percentage of explants showing response for root induction were also measured after 5 weeks of culture. Regenerated plantlets were washed with tap water in order to remove any adhering medium and transferred to plastic pots containing a mixture of garden soil. Hardening of potted plantlets was accomplished in culture room set at 25˚C ± 3˚C, 16 hour-day-length by covering them with polyethylene bags to maintain high humidity. After 12 - 15 days, polyethylene bags were removed initially for a short duration (15 - 30 minutes) daily for about one week.

Gradually, the daily exposure time was increased by 30 minutes for each day. Polyethylene bags were completely removed after 20 days. The regenerated plantlets were subsequently transferred to earthen pots containing only garden soil.

Table 1. Effects of different concentrations and combinations of plant growth regulators for callus induction and proliferation from seeds of native orange (*Citrus reticulata*).

Plant growth regulators (PGRs)	Concentrations (mg/L)	Number of explants inoculated	Number of explants survived	Survival rate (%)	Number of explants give rise to callus	Percentage (%) of callus response (±SD)	Color of callus
2,4-D	0.5	15	14	93.3	0	0	N/A
	1.0	15	12	80	02	16.7 ± 1.20	Whitish
	1.5	15	15	100	04	26.7 ± 2.05	Yellowish
	2.0	15	14	93.3	06	42.9 ± 2.27	Greenish
	2.5	15	13	86.7	03	23.1 ± 1.80	Yellowish
	3.0	15	15	100	14	93.3 ± 0.50	Whitish
	3.5	15	15	100	13	86.7 ± 1.75	Whitish
	4.0	15	07	46.7	01	14.3 ± 4.05	Yellowish
BAP + NAA	0.5 + 0.5	10	08	80	02	25 ± 3.21	Greenish
	1.0 + 0.5	10	09	90	01	11.1 ± 1.60	Yellowish
	1.0 + 1.0	10	07	70	03	42.9 ± 3.63	Greenish
2,4-D + BAP + NAA	1.0 + 0.5 + 0.5	05	05	100	01	20 ± 2.89	Brownish
	1.0 + 1.0 + 0.5	05	04	80	01	25 ± 2.89	Whitish
	0.5 + 0.5 + 0.5	05	04	80	0	0	N/A

*N/A = Not Applicable.

Finally, the plantlets were placed outside of the laboratory environment under appropriate sunlight. Experimental data analysis was performed using computer software (MS Excel, 2013). For each observation, this experiment was replicated for at least three times and the results were expressed as Arithmetic Mean (AM) ± Standard Deviation (SD).

3. Results and Discussions

Explants were cultured on MS medium supplemented with different concentrations of plant growth regulators. Different treatments resulted in different callus induction frequencies. In the same medium, callus induction frequencies changed with the variation of hormonal concentrations. The explants appeared as light green color after 7 days of inoculation on MS medium. After 21 days of inoculation, the callus induction was begun (Figure 1). Approximately 87% of the calli were granular, while 13% were smooth and compact. The color of the callus produced was light green, whitish green, yellowish green and light brown. Differences between various treatment were observed when MS medium supplemented with different concentrations of 2,4-D were used. Among the various concentrations of plant growth regulators, 2,4-D at 3.0 mg/L showed the highest 93.3% ± 0.50% result of callus initiation compared to 86.7% ± 1.75% at 3.5 mg/L 2,4-D (Table 1). For regeneration of shoot, green healthy calli were cut into small pieces and cultured on MS medium supplemented with different concen-

trations of PGRs. After 4 weeks of incubation in the culture room, shoot appeared on some culture, and extended shoot became visible after 45 days (Figure 2). During this experiment maximum shoot regeneration 86.7% ± 3.35% was observed on MS medium supplemented with the combinations of 3.0 mg/l BAP and 2.0 mg/l NAA. For the development of roots, proliferated shoots were cultured on MS medium supplemented with different concentrations of IBA, NAA and their combinations. After five weeks of incubation period root appeared in some of the culture vessels (Figure 3). Better rooting but very little ex-vitro survival was achieved using this protocol (Figure 4). The shoot regeneration frequencies changed with the variation of hormonal concentrations used in the same medium in this experiment (Figure 5). NAA at 1.0 mg/L in MS media

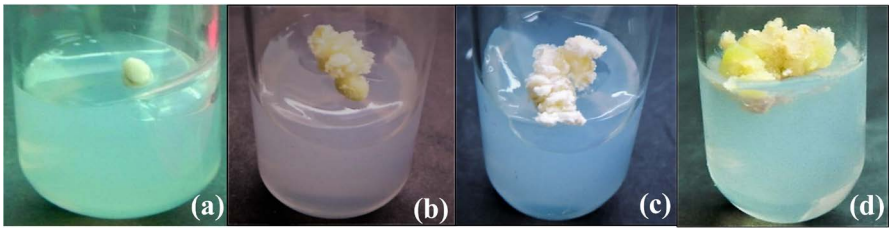

Figure 1. Callus induction from seed of native orange ((a) = initial stage of seed inoculation; (b) = initiation of callus; (c) = formation of callus; (d) = proliferation of callus).

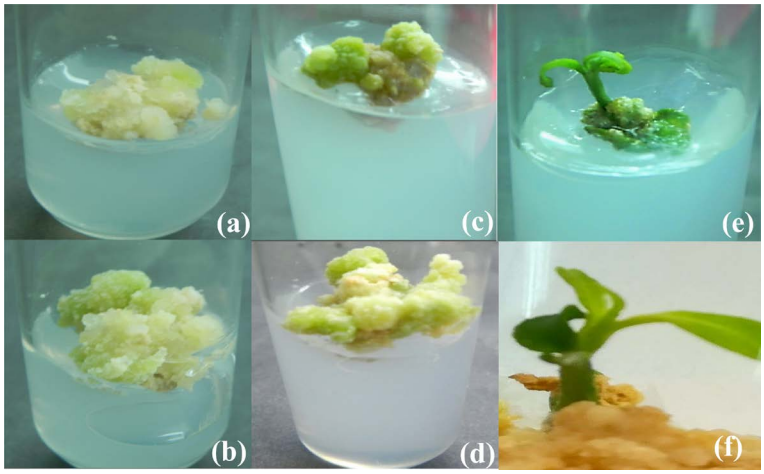

Figure 2. Regeneration of shoot from callus of native orange (((a), (b)) = initial stage of explant inoculation; ((c), (d)) = initiation of shoot; ((e), (f)) = elongation of shoot).

Figure 3. Regeneration of root from initiated shoot of native orange after 45 days of inoculation.

Figure 4. Acclimatization in a pot containing sterile soil mixture with vermi-compost.

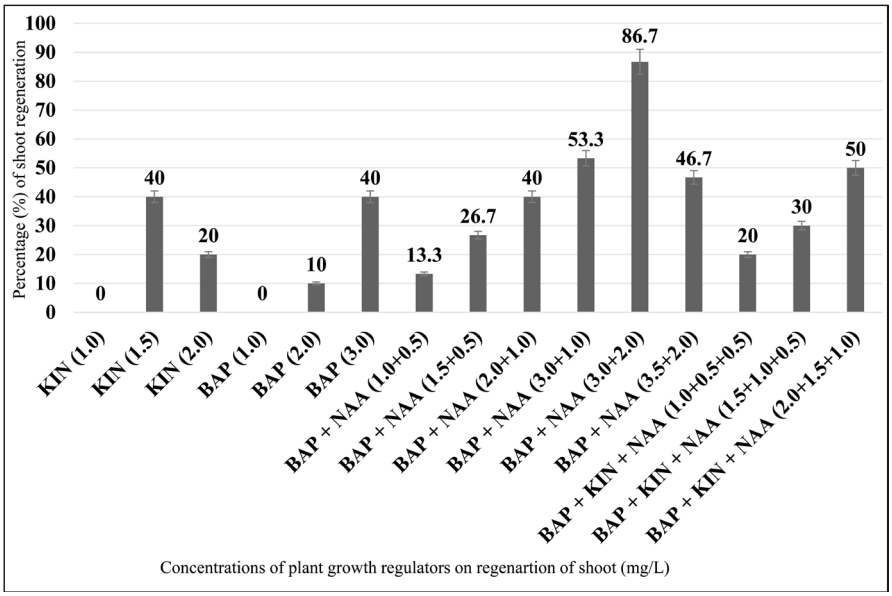

Figure 5. Effects of KIN, BAP, NAA and their combinations on shoot proliferation from seed of native orange.

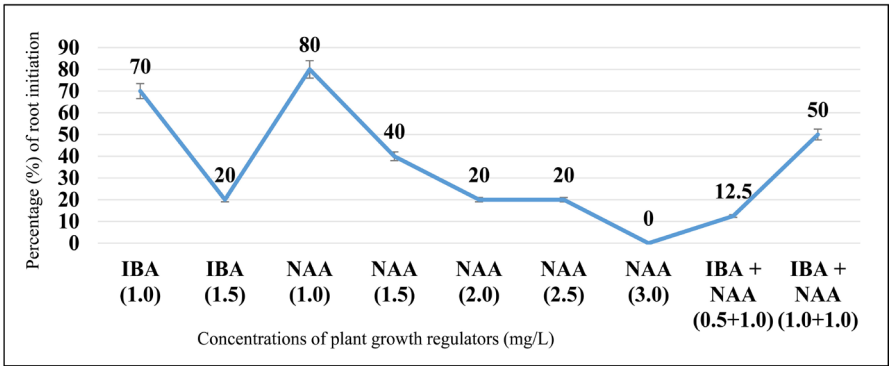

Figure 6. Effects of IBA, and NAA on regeneration of root from seed of native orange.

showed the highest 80% ± 2.89% root initiation compared to IBA 70% ± 5.20% at the concentration of 1.0 mg/L (Figure 6). The increasing concentrations of

IBA and NAA used in this experiment showed lower root initiation response (**Figure 6**). In the present study, an efficient regeneration method for native orange (*Citrus reticulata*) was developed through *in vitro* culture technique. Very less work is done on this species specially in Bangladesh and that's why a standard protocol for micropropagation is necessary to generate more disease-free plants of this species. Seed is an excellent source of explant to produce the fungus resistant plant because pathogen can't reach to the seed level even after severe infection to the plant [25]. Although regeneration in *Citrus sp*. is a very slow process [26], one of the major goals of the current study was to observe the effect of various plant growth regulators at different stages of regeneration from mature seed of *Citrus reticulata*. For callus induction of the native orange (*Citrus reticulata*), 2,4-D was used as growth promoting hormone and seeds were used as explants in the current study. The result showed that 2,4-D was the most efficient plant growth regulator in callus induction. The absence of 2,4-D and presence of BAP, KIN in the culture medium initiated shoot induction from the callus as reported earlier in other species too [14]. According to the report of [27], regeneration of shoot was found at various concentrations of BA from 0.5 - 4.0 mg/L for *Citrus paradise* (Macf) epicotyl explants. BAP 0.5 mg/l gave the best results (75%) of shooting response in *Citrus reticulata* Blanco from different explants of *in vitro* raised seedlings according to the report of [28]. The present study showed better regeneration response than those cited in these reports. NAA at 1.0 mg/L was determined as the major phytohormone for root initiation (80% ± 2.89%) in this experiment. A very similar result was observed in the earlier studies of [17]. In the present investigation, we have established an efficient and simple protocol for the plant regeneration of *C. reticulata* using callus cultures induced from mature healthy seed explants. This protocol may further be applied for genetic transformation in *C. reticulata*.

4. Conclusion

In the present investigation, a reliable and efficient regeneration method from seed explants of *Citrus reticulata* species was developed through *in vitro* culture approach. The highest 93.3% ± 0.5% callus initiation was obtained using the MS medium supplemented with 2,4-D at 3.0 mg/L. Maximum 86.7±3.35% shoot regeneration was found, when the MS medium supplemented with the combination of BAP at 3.0 mg/L and NAA at 2.0 mg/L and maximum root initiation 80% ± 2.89% was confirmed using the MS medium with NAA at 1.0 mg/L. The regenerated plantlets, thus, produced, were then successfully acclimatized inside the laboratory conditions but their survival rate in the *ex-vitro* conditions was lower than normal. After all of these experiments now we can conclude that specific concentrations of 2,4-D, BAP and NAA have a major influence on regeneration of local orange.

Acknowledgements

We are grateful to Ministry of Science and Technology (MOST), Bangladesh for

their continuous financial support to successfully conduct this research. We also thank Department of Genetic Engineering and Biotechnology, Shahjalal University of Science and Technology, Sylhet for providing us the opportunity to conduct the research.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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