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# Optimization of an *in vitro* Regeneration Protocol for Rough Lemon Rootstock (*Citrus jambhiri* L.) *via* Direct Organogenesis

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## Abstract

Standardization of a reproducible protocol for *in vitro* rough lemon rootstock mass propagation was conducted at Tigray Biotechnology Center Plc., Plant Tissue Culture Laboratory, Mekelle, Ethiopia in 2015/2016 cropping season. Rough lemon is the frequently used rootstock both in the world and Ethiopia citrus fruit production, particularly in the Tigray region due to its superior performance over other rootstocks. However, seedlings produced through conventional ways are not recommended to be used in orchards due to variability problems caused by its polyembrony nature. To overcome such variations, *in vitro* regeneration of rough lemon rootstocks was performed using nodal segments and shoot tips as explant types. The explants were inoculated on MS medium supplemented with 5% scurose and 250 mg/L streptomycin followed to surface sterilization. The most effective and reproducible auxin (NAA), cytokinin (BA) and gebrillenllic acid (GA3) for *in vitro* shoot and root induction in rough lemon rootstocks were determined. Almost all IBA and BA treatments resulted in almost 100% shoot induction except for at 0.0 and 0.1 mg/L IBA and at 1.5 and 2.0 BA mg/L. Nodal segments induced a higher percentage of explant response with longer shoots in a shorter period of time than shoot tips, which produced more shoots and leaves than nodal segments. The effect different BA and IBA concentrations on various parameters of proliferation were studied. Full strength medium produced more regenerated shoots and leaves per shoot than half-strength MS medium. In addition, longer shoots formed with 0.1 mg/L GA3 than culture medium without this plant growth regulator. Root length decreased with higher concentration of NAA and the longest root (2.5 ± 0.22 cm) was found in the 1.0 mg/L NAA and followed by (1.95 ± 0.22 cm) at 0.5 mg/L of NAA. The rooted plants were successfully established in the greenhouse on the substrate called coco-peat and sand, and their survival rate was found to be 98%. These results suggest that standardization of these factors can help in development of a commercially viable tissue culture system for rough lemon. Moreover, it signifies the need of plant variety based *in vitro* protocol development and optimization across citrus species.

**Keywords:** BA; IBA; Nodal segment; Shoot tip; Shoot and root induction

## Introduction

Citrus (Citrus spp.) is grown throughout tropical and sub-tropical regions of the world and it is grown in nearly 49 countries around the world. Citrus fruits are very important fruits, ranking first with respect to fruit production in the world [1]. In Ethiopia it is one of the most economically important fruit crops grown by smallholders and commercial farmers [2-4]. The total area coverage and the annual production of citrus were estimated 5,947 ha and 77,087 tons, respectively [5,6]. Besides, Ethiopia is the second most populated nation in Africa where agricultural sector is the leading national income supporting more than 80% of the population Abraham [7]. However, Ethiopian agricultural system is predominantly characterized by high level of subsistence production and low improvement of traditional farming practices resulting in declining of agricultural productivity. In fact, persistent dependency on rainfall and frequent occurrence of drought and other natural calamities add up for low agricultural production, and the same is true for citrus fruit production.

Added to the above heated issue, citrus species are infected with systemic diseases caused by fungi, viruses, bacteria, mycoplasma etc. Attack of pathogens does not always lead to the death of the plant but very often the infection caused by pathogens considerably reduces the yield and quality of the plant. While pathogens are nearly always transferred in plants through vegetative propagation, viral diseases occur in virtually all seed propagated as well as vegetative propagated crop species [8]. Elimination of pathogens is highly desirable to optimize the yields and also to facilitate the movement of materials across the international boundaries. Hence, application of tissue culture biotechnology in the field of agriculture seems very crucial so as to increase agricultural productions including citrus for the purposes of feeding the population with no need of international aids.

Although tissue culture of citrus species is well studied [9-11], several publications report strong genotype dependence, moreover citrus tissue culture is mostly confined to more common species like *C. reticulata*, *C. aurantifolia*, *C. aurantium* etc. There have been few reports on micro-propagation of *C. grandis* [9]. However, adopting a repeatable and reproducible *in vitro* regeneration protocol for rough lemon rootstock remains difficulty. Sometimes, plants obtained from secondary organogenesis from disorganized tissues such as callus may not be true to type due to somaclonal variation. Rapid cloning of elite genotype through *in vitro* adventitious shoot propagation is extensively employed for many fruit species [12]. Attempts to propagate pummelo from shoot tip explants of *in vitro* grown seedling have been made [13].

Regardless of the previous attempts made, most of the existing citrus collections are propagated conventionally and conserved in field gene banks in different citrus growing countries [14]. Such collections are vulnerable to biotic and abiotic hazards [15]. Ageing seeds of Citrus species are recalcitrant and lose viability within a short time. Therefore, bearing in mind the problems associated with conventional propagation and the need to develop *in vitro* regeneration protocols for specific cultivars, the aim of the present study was to develop an efficient protocol for *in vitro* clonal mass propagation and conservation of germplasm of this elite citrus cultivar by inducing multiple shoots on shoot tip and nodal segment explants. Specifically, special attention was given for the determining of the concentration of BA, NAA, IBA and GA3 for *in vitro* shoot and root induction of rough lemon rootstock.

## Materials and Methods

### Explant collection

About ten-year-old field grown rough lemon trees were collected and used as the source of nodal segments and shoot tip explants for the present investigation. Then, nearly 60 days old new shoots measuring 25-35 cm in length with 5-7 nodes were collected in polythene bags and brought to the laboratory for further treatments.

### Preparation and surface sterilization of explants

Once the associated debris dissociated from the explants collected from the intended filed, the leaves and thrones were removed, and then shoot tips and nodal segments were treated by 0.25 g of antifungal chemicals (Kocide, Bayleton and Redimol/100 ml water for 15 and 20 minute respectively). Then after, 100 ml of 5% of sodium hypochlorite were prepared and the shoot tips and nodal segments were immersed into it for nearly 7 and 10 minutes accordingly. Followed by a continuous shaking and were washed twice through sterile distilled water to remove the chemical residues. Eventually, the shoot tips and nodal segments 2.5-3 cm in length were excised under the laminar air flow (LAF) and the explants finally treated by 0.25 g of HgCl2/100 ml of water and 2 drop of Tween 20 for 5 min (shoot tips) and 10 min (nodal segments) inside the LAF. After, treatments both explants were washed again three times for 1-2 min with distilled water and inoculated on the various initiation treatment culture mediums and incubated in the growth room (at 25 ± 2°C and at a 16/8 h light/dark photoperiod) based on their treatments.

### Culture media and incubation conditions

The nutrients in the medium consisted of full and half strength MS salts and medium was solidified with 5 g/l agar-agar, supplemented with 30 g/l sugar and NAA at 0, 0.2 and 0.5 mg/L alone and in combination with BA at 0.25, 0.5 and 1.0 mg/L for multiplication, and NAA at 0.0, 0.5, 1.0, 1.5 and 2.0 mg/L for root induction. The pH of the medium was adjusted to 5.8 gelling with agar-agar with 1 NaOH and 1 NHCl. The full and half strength MS medium was then dispensed into 40 ml culture bottles and autoclaved for 20 min at 121°C and 15 Ibs psi.

### Experimental design and data analysis

Experiments were executed in a factorial completely randomized design with three replications, each with 10 explants per replicate. The data were subjected to one way analysis of variance (ANOVA) and means were evaluated at (P=0.05) level of significance using the Duncan's multiple range test.

## Results and Discussion

So far studies on the regeneration systems of citrus species reported had shown the critical effect of cytokinin concentration and type or cytokinin-auxin ratios in regeneration from various types of explants [9,16,17]. We studied the morphogenetic response of shoot tip and nodal segment explant types of Citrus jambhiri L. cultured on MS medium supplemented with different concentrations of BA alone, and in combination with NAA. As it is depicted in (Table 1) the shoot induction response was significantly affected by the choice of explant. Nodal segments showed higher shoot induction (98.44%) than shoot tips (96.9%). NAA at 0.5 mg/L or BA at 0.25 and 0.5 mg/L resulted in the highest shoot induction. The highest shoot induction response of both explant types is attributed to cytokinins, which break bud dormancy by activating meristems and causing shoots to proliferate [18]. The outgrowth of axillary buds is in general related with cytokinin level in the buds. Moreover, the number of days to shoot initiation was significant on explant types (Table 1). Nodal segments lasted fewer days to initiate shoots than shoot tips. MS medium without NAA needed less time (9.05 days) to initiate shoots than MS media with NAA (9.35 days). BA needed fewer days (8.7) to initiate shoots than other concentrations.

| Treatment | Response of explants (%) k (Mean) i | No. of days to shoot inductionk (Mean) i | No. of shoots/explant k (Mean) i | No. of leaves/explant k (Mean) i | Shoot length (cm) k (Mean)* |
|---|---|---|---|---|---|
| **Explants (A)** | | | | | |
| Nodal segment | 98.44a | 9.05b | 2.55b | 13.75b | 1.27a |
| Shoot tip | 96.9b | 9.35a | 3.01a | 16.07a | 1.05b |
| **NAA concentration (mg/L) (B)** | | | | | |
| 0 | 98.33b | 7.99b | 2.88a | 13.78a | 1.13b |
| 0.1 | 97.68b | 8.50b | 2.98a | 13.25a | 1.26a |
| 0.2 | 99.9a | 8.26a | 2.99a | 12.58a | 1.08b |

| **BA concentration (mg/L) (C)** | | | | | |
|---|---|---|---|---|---|
| 0.5 | 99.9a | 8.7c | 3.17a | 16.4a | 1.28a |
| 1 | 99.9a | 9.19b | 3.13a | 14.57b | 1.24a |
| 1.5 | 98.89a | 9.57a | 2.53b | 11.77c | 1.05b |
| 2 | 95.0b | 9.57a | 2.47b | 10.60d | 1.03b |
| **Interaction among factors** | | | | | |
| A × B | * | * | NS | * | * |
| A × C | * | NS | NS | NS | * |
| B × C | * | * | NS | * | * |
| A × B × C | * | NS | NS | * | * |

**Table 1:** NAA and BA on shoot proliferation of *Citrus jambhiri* L in MS medium. [i]simliar letters indicate means which are not significantly different (LSD, P=0.5), comparisons are made in each column within A, B and C, values represent as means. [k]Data were recorded at 4 weeks after culturing explants onto shooting medium. [*]Indicates significant difference. NS non-significant difference.

Likewise, a significantly higher number of shoots was obtained with shoot tips than with nodal segments. However, no significant different in the number of shoots was observed among the different concentration of NAA. However, among the different concentration of BA, 0.5 and 1.0 mg/L produced significantly more shoots than 1.5 and 2.0 mg/L. No significant differences were noticed among the various interactions (Table 1). Similarly, Saini and Gill reported maximum number of shoots with 0.5 mg/L BA in *Citrus jambhiri* cv. 'Rough lemon explants. More leaves were obtained with 0.5 mg/L BA than at other concentrations. In a similar fashion, longer shoots were formed from nodal segments than shoot tips (Figure 1). This may be attributed to active growth of almost all characters tested in nodal segments, particularly early shoot initiation. NAA at 0.1 mg/L and BA at 0.5 mg/L formed longer shoots than at other concentrations (Table 1). El-Wasel [19] reported a maximum shoot elongation *Poncirus trifoliata* in MS medium containing 0.1 mg/L BA alone or with 0.1 mg/L NAA.

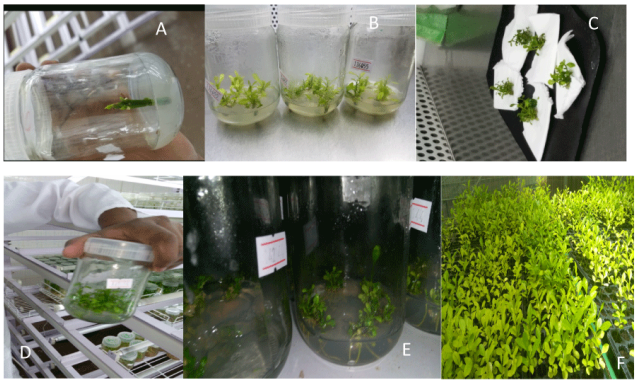


**Figure 1:** *In vitro* regeneration in rough lemon rootstock (*Citrus jambhiri* L). A) Early shoot induction from shoot tip; B) Shoot proliferation from nodal segment; C) cultures during sub culturing and measuring parameters after 4 weeks in the full strength MS medium; D) Cultures at inspection for any possible contaminates; E) Early root induction on half strength MS medium; F) Cultures at primary acclimatization stage.

MS medium with 0.1 mg/L GA3 generated no significant number of generating shoots derived from nodal segments or shoot tips in compare to MS medium without GA3 (Table 2). On the other hand, full-strength MS medium regenerated significantly more shoots than half-strength MS medium whereas MS medium with 0.1 mg/L GA3 regenerated more shoots than medium without it.

| **Treatment** | **No. of regenerated shoot k (Mean) i** | **No. of leaves/shoot k (Mean) i** | **Shoot length k (cm) (Mean) i** |
|---|---|---|---|
| **Micro-shoot (A)** | | | |
| Nodal segment | 3.5a | 11.00a | 2.79a |

| | | | |
|---|---|---|---|
| Shoot tip | 3.5a | 10.45a | 2.62b |
| **MS medium strength (B)** | | | |
| Full strength | 2.75a | 10.00a | 2.67a |
| Half strength | 1.75b | 9.35b | 2.75a |
| **GA3 concentration mg/L (C)** | | | |
| 0 | 1.95b | 8.2b | 2.65a |
| 0.1 | 2.95a | 12.25a | 2.77a |
| **Interaction among factors** | | | |
| A × B | NS | NS | NS |
| A × C | * | * | NS |
| B × C | NS | * | NS |
| A × B × C | NS | NS | NS |

**Table 2:** Effect of MS medium with or without GA3 on shoot multiplication of rough lemon. [i]simliar letters indicate means which are not significantly different (LSD, P=0.5), comparisons are made in each column within A, B and C, values represent as means. [k]Data were recorded at 4 weeks after culturing explants onto shooting medium. *Indicates significant difference. NS non-significant difference.

In regard to, the impact of MS medium with or without 0.1 mg/L GA3 on number of leaves, no significant difference was observed between shoots derived from nodal segments or shoot tips explants between full and half-MS strength medium with respect to the number of leaves (Table 2). However, MS media containing 0.1 mg/L GA3 formed more leaves per shoot than MS medium without it. Symal et al. [20] also reported a maximum number of leaves in Citrus aurantifolia Swingle shoot tip explants in MS medium containing 0.1 mg/L GA3 and 0.1 mg/L NAA. The explant BA concentration and NAA and BA concentrations interactions were significant in terms of number of leaves per shoot.

Rooting of the regenerated shoot buds was achieved in MS medium containing 0.5, 1.0 and 2.0 mg/L of NAA whereas; there was no root induction at 0.0 mg/L NAA. The highest number of roots was produced with 2 mg/L NAA (Table 3). A maximum of 5.25 roots was obtained for explants cultured on the half strength medium. Similarly, the longest root (2.5 ± 0.22 mm) was found at the 0.5 mg/L NAA and followed by (1.95 ± 0.22 cm) at 0.5 mg/L of NAA (Table 3). However, root length decreased with higher concentration of NAA. Moreover, half strength MS medium was found to be effective full strength for rooting of *Citrus jambhiri* than full strength MS medium. A study with Citrus grandis showed similar results with maximal (number) rooting at 2 mg/L NAA, and a decrease in the frequency of rooting below of NAA concentration 2 mg/L [9]. However, in the study by Parthasarathy and Nagarju, MS medium supplemented with 0.05 mg/L NAA was found to be the best for rooting in many Citrus species, excepting Musambi for which the best concentration was 0.2 mg/L NAA.

| Treatment | Root number/micro shoot K (Mean) i | Root length K (Mean) i |
|---|---|---|
| **Micro shoot (A)** | | |
| Nodal segment | 4.52a | 2.5a |
| Shoot tip | 3.8b | 1.95b |
| **MS-medium strength (B)** | | |
| Full strength | 3.1b | 1.65b |
| Half strength | 5.2a | 2.49a |
| **NAA concentration (C)** | | |
| 0 | 0.0c | 0.0d |
| 0.5 | 3.5b | 1.89b |
| 1 | 3.5b | 2.5a |

| | | |
|---|---|---|
| 1.5 | 4.75a | 1.3c |
| 2 | 5.25a | 1.27c |
| **Interaction among factors (D)** | | |
| A × B | * | NS |
| A × C | * | * |
| B × C | * | * |
| A × B × C | * | * |

**Table 3:** Effect of NAA concentration on root number and length of shoot buds of Citrus jambhiri . [i]simliar letters indicate means which are not significantly different (LSD, P=0.5), comparisons are made in each column within A, B and C, values represent as means. [k]Data were recorded at 4 weeks after culturing explants onto shooting medium. [*]Indicates significant difference. NS non-significant difference.

The rooted plantlets elongated in the rooting media and maximum elongation of 2.62 to 2.79 cm of the regenerated shoots was achieved in half MS strength medium containing 0.5 and 1 mg/L NAA respectively after four weeks of the culture.

After four weeks of culture in rooting medium, the elongated plantlets were decapitated and the effect on the growth of axillary shoots was studied. The decapitated plantlets showed the development of two to five axillary shoots within two weeks of culture. Induction of axillary shoots by decapitation of *in vitro* generated plantlets has been reported in many plant species. However, so far there have been no reports of micro propagation of Citrus species from axillary shoot tip induced by decapitation. On culturing the axillary shoot tips on bud-induction medium, they proliferated to produce multiple shoot buds (three to six) on the medium supplemented with 0.25 mg/L BA, 0.25 mg/L BA, with 0.5 mg/L NAA (Table 3). These shoot buds showed rooting and elongation in medium containing 2 mg/L NAA. The rooted plantlets were decapitated again and used for further induction of axillary shoot or they were transplanted after hardening and acclimatization. The regenerated plantlets showed 70% survival when transplanted. The success rate was recorded by emergence of two or three new leaves. Morphologically, these plants were slightly yellowish when compared to the parent plant.

## Conclusion

In this study, among the different concentrations of BA alone or in combination with NAA, BA at 0.5 and 1.0 mg/L were most suitable for shoot induction and longer shoots. Highest multiple shoot induction and longest shoots formed when shoot tips were used as explants although nodal segments were best for reducing the number of days to shoot initiation and increasing the percentage of shoot induction. A higher number of regenerated shoots, leaves, and longer shoots were observed with the treatment combination containing full-strength MS medium supplemented with 0.1 mg/L of GA3 in shoots derived from nodal segments. Between the auxins, IBA was less effective for rooting of *Citrus jambhiri* than NAA. Root length decreased with higher concentration of NAA and the longest root (2.5 ± 0.22 cm) was found in the 1.0 mg/L NAA supplemented with half MS strength. Thus, the induction of multiple shoot buds from shoot tip explants provided a novel protocol for propagation of *Citrus jambhiri* in tissue culture. It resulted in the regeneration of a large number of plantlets from single explants. This technique, therefore, is an efficient system for rough lemon rootstock mass propagation and *in vitro* germplasm conservation of this important fruit plant, as compared to propagation by seed.

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