Proliferation of *Citrus aurantifolia* by In Vitro Epicotyl Segment Culture

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Abstract— *It is clear that, beneficial species of Citrus need to improvement especially by new methods due to limitations of conventional methods. New methods like biotechnology and gene transfer need to establishment of regeneration plants by tissue culture. Shoot proliferation in Citrus is easy but rooting of proliferated micro shoots has been discussed in various articles. The goal of this study is presentation of a proper method for rooting of micro shoots in Citrus by manipulation of media content. Hypocotyl segments of Citrus aurantifolia from 45 day old seedlings and 0.5 – 0.7 cm in length were cultured on MS media supplemented with different kinds and concentrations of plant hormones suitable for shooting and rooting such as BA, IBA and NAA alone or together. 1 mg/l BA and 1.5 mg/l NAA on MS media was the best treatment for shooting and rooting respectively. In this study we can overcome one of the most important problems of establishing regeneration system in Citrus and opening the way for biotechnology and gene transfer for this important and economic plant*.

Keywords— Citrus aurantifolia, gene transfer, rooting, shooting.

I. INTRODUCTION

Citrus fruits duo to extremely pleasant aroma and taste also effect on human health for its useful substances, which is one of the most important fruits in Iran like all over the world. There are many biotic and abiotic stresses that have negative and harmful effects on cultivation of these valuable horticultural products. For example fungal diseases that cause significant problems in the production of *Citrus* fruits, such as postbloom fruit drop, caused by *Colletotrichum acutatum*; Alternaria brown spot, caused by *Alternaria alternata*; scab diseases, caused by *Elsinoe fawsettii* and *E. australis*; melanose, caused by *Diaporthe citri*; and greasy spot caused by *Mycosphaerella citri*[1][1] and bacterial diseases such as *Citrus* canker, *Citrus* variegated chlorosis, and Huanglongbing (HLB) that caused by members of the bacterial species *Xanthomonas citri*, *Xylella fastidiosa* and '*Candidatus Liberibacter*' respectively[2] and Virus and Virus – Like diseases as *Citrus Tristeza Virus* (CTV), *Citrus Psorosis* (CPV), *Citrus Exocortis Viroid* (CEVd), and also some of the agents pathogen such as Candidatus *Phytoplasma aurantifolia* that causes WBDL (Witches Broom Disease) [3 and 4]. Some of these diseases are very important in Iran. So it is very clear that *Citrus* industry mainly need to a strong program for improvement for supply of new cultivars with desirable characteristics like resistance against diseases.

Conventional breeding methods have demonstrated limitations with respect to *Citrus* improvement due to some of the biological characteristics of woody plants such as nucellar polyembryony, high heterozygosity, long juvenile period, and auto incompatibility [5 and 6].Genetic engineering of *Citrus* presents the possibility to produce *Citrus* plants with resistance to different diseases. Genetic engineering needs to viable shoots which can be regenerated via organogenesis firstly.

Regeneration of plants from single cells and complex explants is therefore the key process in every genetic manipulation work; unless this can be achieved consistently and efficiently, no genetic improvement by somatic methods is possible. To accomplish this, a source of genetically homogeneous cells and tissues is necessary. Micropropagation cultures are an ideal source of homogeneous cells and tissues and this system is useful for the propagation of plants emanating from experiments of genetic variation [7]. So we need to a proper regeneration system via regeneration of shoots and roots and whole regenerated plants finally for gene transfer protocols.

There are several reports indicative problems of rooting of regenerated shoots in *Citrus* [8; 9 and 10]. It is clear that, this seemingly insignificant problem complicates the transformation process. Therefore optimizing as much as possible of regeneration systems is necessary. The goal of this study is presentation of simple and repeatable proliferation system for one of the most important species of *Citrus* (*Citrus aurantifolia*).

II. MATERIAL AND METHODS

These experiments were conducted in Iranian Research Institute of Plant Protection, Agricultural Research, Education and Extension Organization (AREEO), Tehran, Iran during April to October 2019.

2.1 Preparation of sterile seedlings and explants

Seeds of [*Citrus aurantifolia* (lime)] were sterilized with commercial hypochlorite sodium for 60 min. Then in order to complete removal of harmful effects of disinfectant, seed were rinsed with sterile distilled water 4 – 5 times. Seeds were incubated in dark and in sterile distilled water for 72 h. Afterwards seeds were cultured on MS (Murashige and Skoog 1962) [11] medium without Plants Growth Regulators for 30 days in dark and 10 days in room light and 25 ± 2ºC respectively. All steps were done in a laminar airflow cabinet. For preparation of explants, epicotyls of sterile seedlings were cut (0.5 – 0.7 cm in length). A figure of sterile seedling is presented in Fig. 1.

FIGURE 1: Seedling of *Citrus Aurantifolia*

2.2 Media and other conditions of experiments

Explants were cultured on MS basal media supplemented with different kinds of Plant Growth Regulators such as BA alone or with NAA for shooting and NAA,IBA and BA for rooting. Also all of media contained 30 g/l sucrose and 7/5 g/l bacteriological agar that were added to media after adjusting their pH on 5/8 media autoclaved for 20 min at 121ºC.

2.3 Experimental design and data analysis

Experiments were conducted in a completely randomized design. Data were analyzed by Dunkan Multiple Range Test.

III. RESULTS AND DISCUSSION

3.1 Shoot regeneration

The results of Table 1 showed significant effect of different concentrations of BA alone or plus NAA (especially alone) on adventitious buds induction, growth of them and proliferation of micro shoots on epicotyl explants. A lot of adventitious bud and micro shoots formed at both ends of the explant on MS media supplemented with different concentration of BA directly or with very small amount of callus (Fig. 2).

TABLE 1
EFFECT OF DIFFERENT CONCENTRATIONS OF BA AND NAA ON SHOOT PROLIFERATION PERCENTAGE, NUMBER OF SHOOT PER EXPLANT AND AVERAGE OF SHOOT LENGTH

PGRs (mg/l)		Explants with shoot proliferated	Number of shoot/explant	Average of shoot length
BA	NAA			
1	0	99[a]	6.453[a]	3.667[a]
2	0	91[b]	5.194[b]	3.541[a]
3	0	95[ab]	6.001[ab]	3.332[a]
1	0.01	97[ab]	5.219[b]	3.441[ab]
2	0.01	95[ab]	6.476[a]	2.996[b]
3	0.01	97[ab]	5.553[a]	3.001a[b]

Mean followed by same letter(s) are not significantly different

So we can result that gradient endogenous plant hormonal of this plant had no effect or had low effect on shoot organogenesis. The advantage of using the conjugate system of BA as PGRs (Plant Growth Regulators) or plant hormone and epicotyl as explant has been mentioned in tissue culture and biotechnology (gene transfer) program in *Citrus* [12 ; 13; 14 ; 15 ; and 16]. Also the results showed that there is not a direct relationship between the concentration of BA and quantity and quality of shooting. The results were reported by Dejam *et al*., [17] are similar to this study (0 – 2 mg/l) and opposed (2 – 4 mg/l) for another species of *Citru*s genius, Bakrai (*Citrus reticulata* Blanco × *C. limetta* Swing). Apparently it seems that the concentrations of BA more than 2 mg/l have preventive effects on the majority of *Citrus* species. Sometimes and few instance it seemed that on media with 2 mg/l BA or more the number of regenerated shoots were more but with very small leaves (Fig. 3) and also the best results of the number of proliferated shoots, dimension of leaves and plantlet regeneration were resulted on media with 1 mg/l BA (Fig. 4). One of the best results of our experiments was the effect of media supplemented with BA on proliferation of micro shoots and longitudinal length of them (Fig.5). Therefore we did not need to subculture of explants on media proper for elongation of micro shoots.

3.2 Root regeneration

Different kind and concentrations of plant hormones were tested for rooting of regenerated micro shoots in this study and micro shoots were cultured on the mentioned media. The results of Table 2 showed that NAA is the one of the best of these hormones for rooting. On MS media with 1.5 mg/l NAA 99% micro shoots rooted after 14 – 20 days in culture practically (Fig.6). Also on other media that were contained other plant hormones such as IBA and BA (asauxin), the rooting was observed also. These roots was long, white and thick, but versus the roots on media with 1.5 mg/l, NAA they were fewer in number (Fig. 7). And the best and the most complete whole plantlets were obtained on this media (that they contained NAA as auxin) extremity (Fig. 8). The important point here is facility of rooting of micro shoots. There are many reports that showed rooting of micro shoots in *Citrus* species is not easy [18; 19 and 20]. Soheilivand *et al*., in order to rescue rootles micro shoots of sour lime (*Citrus aurantifolia* L.) used microgragfting method. It is clear that rooting of micro shoots on tissue culture media and subculture of explants from shoot induction media to root induction media is very easier method with microgragfting method. This method need to evaluate many factors for achievement such as size and type of scions, grafting type, micro grated plantlets and usage of nutrient media [20].

TABLE 2
EFFECT OF DIFFERENT CONCENTRATIONS OF BA AND NAA ON ROOT PROLIFERATION PERCENTAGE, NUMBER OF ROOT PER EXPLANT AND AVERAGE OF ROOT LENGTH

PGRs (mg/l)			Explants with root proliferated	Number of root/explants	Average of root length
NAA	IBA	BA			
1	0	0	99[a]	3.75[ab]	2075[b]
1.5	0	0	99[a]	4.2[a]	3.23[a]
2	0	0	93[b]	3.33[b]	2.74[b]
0	1	0	74[c]	1.29[d]	2.55[b]
0	1.5	0	44[ed]	1.88[cd]	1.99[c]
0	2	0	38[e]	0.77[e]	1.84[c]
0	1	0.2	55[d]	2.46[bc]	1[cd]
0	1.5	0.3	51[d]	2.51[bc]	1.33[c]
0	2	0.3	29[f]	0.84[e]	0.92[d]

Mean followed by same letter(s) are not significantly different

3.3 Whole plantlet regeneration

Rooted micro shoots or whole plantlets after initial hardening in sterile perlite were transfer to disposable glasses containing of sterile pot soil. The glasses were covered with another glass in order to moisture retention and placed in lab for a few days. The covers were perforated daily and step by step. Maximum survival was 85% and plants grew well and acquired new leaves.

IV. CONCLUSION

Biotechnology programs and gene transfer studies need to establishment of a proper, repeatable and easy plant regeneration method by tissue culture systems. Proliferation of shoots in *Citrus* species is feasible but rooting of proliferated shoots and whole plantlets regeneration need to optimizing of method as it mentioned in discussion sections. In this study we present a simple and repeatable method for rooting of regenerated micro shoots in *Citrus* so it was prepared an introduction of gene transfer program in this very important species in order to attaining new cultivars.

FIGURE 2-8: Plantlet regeneration of *Citrus aurantifolia* on MS media with different kinds and concentrations of PGRs. 2. Shoot proliferation on MS media supplemented by 1 mg/l BA. 3. Shoot proliferation on MS media supplemented by 2 mg/l BA. 4 and 5. Growth and foliation of micro shoots on MS media supplemented by 1 mg/l BA. 6. Rooting of micros hoots on MS media supplemented by 1.5 mg/l NAA. 7. Weak rooting of micro shoots on MS media with 1 mg/l IBA and 0.3 mg/l BA (the arrows show only 2 roots). Whole regenerated plantlet of *Citrus aurantifolia*).

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