Vegetative Propagation of Trifoliate Orange (*Poncirus trifoliate*) via Tissue Culture Technique

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Abstract

The influence of the nutrient composition of plant tissue culture media on axillary shoot proliferation from shoot tips of orange trifoliate (*Poncirus trifoliata*) was investigated. In recent years, the use of this rootstock for fruit trees has been increased dramatically. Shoot tips were cultured on the following media: Murashige and Skoog (MS) (full, half and quarter strength) and Lloyd and McCown (WPM). Media were supplemented with 2-benzyladenine (BA) in combinations with mg.l^{-1} Kintien and mg.l^{-1} GA3. The media type and half strength of the media significantly affected the multiplication number, lengths (cm) of axillary shoots and number of leaves. The WPM media and WPM containing half strength of mineral salts was the best. Highest number of shoots per explant, maximum shoot length and highest leaves number (4.2, 5.18 cm and 13) (4.8, 6 cm and 13), respectively were obtained.
Keywords: In vitro, trifoliate orange, salt strength, MS, WPM.

Introduction

The orange trifoliate (*Poncirus trifoliata*) has grown in China for thousands of years and in Japan since the 8th century. It was introduced into Australia in the latter part of the 19th century, but because of the restricted growth of many trees propagated on this stock, it became unpopular. Trifoliate orange became the preferred rootstock for heavy soils after research identification of transmission of viroids, in particular citrus exocortis viroid, via infected bud wood as the cause of the dwarfing and decline problem. Many selections of *P. trifoliate* have been made and named. The one in common use in Australia is a small leaf, small flower selection known as the Australian selection or *P. trifoliate* 22. A dwarfing selection known as 'Flying Dragon' is sometimes used as a stock for the retail trade.

P. trifoliate Rafinesque 'Rubidoux' is a rootstock widely used in Japan, Uruguay and in other countries of temperate climate. It is recommended for combinations with sweet oranges, acid limes and tangerines. *P. trifoliate* induces excellent juice quality ideal for cold and humid regions, produces low vigorous plant, thus reducing the canopy size, making the fruit harvesting easier and grove less dense, and is highly resistant to *Phytophthora* spp., nematodes, tristeza and xyloporosis. However, it is susceptible to exocortis, intolerant to blight, presents poor growth development in the nursery as well it is intolerant to drought, requires high soil fertility, and is incompatible with sweet orange 'Pêra', true limes and tangor 'Murcott' (Pompeu Junior, 1991; Castle *et al*., 993). In *citrus*, the success of the scion largely depends upon the rootstock on which it has been grafted. 'Troyer' citrange is an important rootstock used world-wide for high density planting. It is also one of the most promising rootstock for several Indian scion varieties such as 'Nagpur' mandarin, 'Kinnow' mandarin, grapefruits, acid lime, 'Satsuma' sweet orange and 'Mosambi' sweet orange. In addition, it has high resistance to tristeza virus, which causes *citrus* decline, and has good adaptability for acidic soil. Commercial rootstock cultivars are commonly propagated by seeds. However, seeds of many *citrus* varieties are moderately recalcitrant and also face loss of viability within a short period. The plants grown from seeds exhibit extended juvenility (4 to 10 years), thus making vegetative propagation and micropropagation a desirable method of propagation. Stem cuttings that are nutritionally weak and/or attacked by pests and pathogens are not easily rooted (Platt and Optiz, 1973).

Poncirus trifoliate .is a rootstock diversification alternative for the Brazilian citrus culture. The tissue culture allows obtain a large number of plants in a short period of time.

Micropropagation methods are used for rapid clonal multiplication of pear and other woody plant species. Protocols for micropropagation of pear have been published, beginning in the late 1970s, for over 20 genotypes, including the major *Pyrus communis* L. cultivars and genotypes of four other species. These studies were reviewed by Chevreau *et al*., (1992) and by Bell and Reed (2002). Empirical studies to determine optimum cultivar-specific protocols have been conducted for a few, but not all, of the major cultivars of several Pyrus species.

Most studies have used MS media without modification (Murashige and Skoog, 1962), but a few have reduced the overall ion concentrations or modified the nitrogen concentrations or nitrogen sources (Chevreau *et al*., 1992; Bell and Reed, 2002). Only few studies made comparisons among several nutrient media. Nedelcheva (1986) found that shoot proliferation of 'Bartlett' was the greatest on a media devised by Quoirin and Lepoivre (1977; QL), in comparisons with MS media. In contrast, Baviera et al., (1989) obtained better shoot proliferation of 'Conference' on MS than QL. Wang (1991) observed a higher degree of multiple shoot formation of the *P. communis* L. rootstock BP10030 on Woody Plant Medium (WPM; Lloyd and McCown, 1981) and QL than MS in a double-phase culture system consisting of a liquid media overlaid on semisolid media.

While many studies have concentrated on the influence of plant growth regulators, the influence of the nutrient media has received less attention and accordingly the objectives of this study was to verify the effects of media type and different concentrations of salt strength "*in vitro*" multiplication of orange trifoliate rootstock and to obtain uniform plants through axillary branching method of slow growing rootstock variety.

Materials and Methods

Plant material and explant sterilization:

The current experiment was conducted in Plant Tissue Culture Laboratory of the Horticulture Department, Collage of Agriculture, University of Duhok, Kurdistan Region of Iraq. Shoot tips of orange trifoliate were taken from tree grown in lath house. Shoot tips were washed in running tap water for 15 min to remove the dust or sand particles. The tips were surface sterilized by using 1% of mercuric chloride for 10 min few drops of Tween-20 were also added as a surfactant. After 20 min the plant material was washed 3 times with sterile distilled water to remove the traces of bleach with gentle shaking under sterile conditions.

Shoot multiplication:

Shoot multiplication of orange trifoliate was evaluated by varying different parameters like type of media. In the first experiment**,** the simple MS (Murashige & Skoog, 1962) and WPM (Lloyd & McCown, 1980) media were used to identify the optimum basal media for *in vitro* multiplication. Half strength salts were used for both basal media. In the second experiment**,** the effect of 3 salts concentrations of (full, 1/2 half and 1/4 quarter strength (MS) was studied.

In the third experiment, the effect of 3 concentrations of (full, half and quarter strength) Lloyd and McCown (WPM) (1980) were studied. All media were supplemented with 2 $mg.l^{-1}$ BA and 2.0 $mg.l^{-1}$ Kin and 1 $mg.l^{-1}$ GA_3.The pH of the media was adjusted to 5.7± 0.1 using 0.1 N HCl and/or 0.1 N NaOH prior to autoclaving at 121°C temperature and 15 lb pressure for 20 min before autoclaving. The cultures were maintained in the light at a photon fluency rate of 80–100 mol·m under a 16-h photoperiod with lamps (Lux TM) in a culture room at 24°C. Relative humidity was kept at 55–65%. There were 5 replicates per treatment, each including 3 micro shoots. In all experiments, the following parameters were measured: mean shoot number, mean shoot length (cm) and mean leaves numbers after 8 weeks from culturing. The results were analyzed statistically using a completely randomized design (CRD).The comparison between means was carried out according to Duncan's Multiple Range Test ($p < 0.05$) using a computerized program of SAS (SAS, 2001).

Results and discussion

Different inorganic salt concentrations (quarter, half and full salt strengths) were investigated in shooting ability of trifoliate orange. Table 1 illustrates the effect of different WPM salts concentrations on orange trifoliate shooting ability. Results showed that highest shoot number (4.8), maximum shoot length (6 cm) and high leaf number (13 leaves/explant) were observed on half salt strength WPM media which was significantly different from those on full and quarter strength media. On the other hand, shoot number and leaves number in quarter media didn't shows significant difference when compared with full strength media, but shoot length of quarter is significant deferent when compared with full strength.

Table 1: Effects of different concentrations of WPM salt strength media on proliferation and growth of axillary shoot from shoot tips. Data were recorded after 8 weeks on WPM media

WPM medium salt strength	No. of shoots/ explant	Average length of shoots (cm)	No. of leaves/explant
Quarter salt strength 3/4	3.4 *b	5.3 b	12.6 ab
Half salt strength 1/2	4.8 a	6 a	13 a
Full salt strength	3.6 b	5.4 b	11.7 b

***Means within a column followed by different letters are significantly different according to Duncan's Multiple Range Test ($p<0.05$).**

Table 2 illustrates the effect of different MS salts strength on orange trifoliate shoot multiplication. Half strength MS media was also superior when compared to other treatments, which produced highest shoot number per explants (2.6), high shoot length (3.1 cm) and highest leaves number (11.6) .This was significantly different from full and quarter strength MS media. Quarter strength MS media shows significant difference about shoot number and shoot length when compared with full strength MS media. While about leaf number on quarter MS medium didn't show significant difference when compared with full salt strength. The half salt strength MS media was the optimum when compared with other salt strength. This may be due to the increased carbohydrate ratio to nitrogen, mean increasing energy source (carbohydrate) which considered necessary for shooting (Hartmann *et al*., 2002**)**

Table 2: Effects of different concentrations of MS salt strength media on proliferation and growth of axillary shoot from shoot tips. Data were recorded after 8 weeks on MS media.

MS medium salt strength	No. of shoots/ explant	Average length of shoots (cm)	No. of leaves/explant
Quarter salt strength 3/4	2.4 *a	2.9 a	8.2 b
Half salt strength 1/2	2.6 a	3.1 a	11.6 a
Full salt strength	1.6 b	2 b	6.8 b

***Means within a column followed by different letters are significantly different according to Duncan's Multiple Range Test ($p<0.05$).**

The promoting effect of mineral concentrations of the culture media on shooting can be attained as inorganic ions, which participate in the process of regulating hormonal balance (Amzallag *et al*., 1992). This practice is used for herbaceous plants as well as woody ornamentals, fruit trees and forest species (Monocousin, 1998). The favorable effects of a diluted mineral solution on shooting can be explained by the reduction of nitrogen concentrations (Driver and Suttle, 1987 and Al-Bahir *et al*., 1999).

Table 3: Effects of media type on proliferation and growth of axillary shoot from shoot tips. Data were recorded after 8 weeks on half strength media.

Media type	No. of shoots/ Explant	Average length of shoots (cm)	No. of leaves/explant
WPM	4.2 *a	5.18 a	13 a
MS	2.8 b	4.14 b	11 b

***Means within a column followed by different letters are significantly different according to Duncan's Multiple Range Test ($p<0.05$).**

The effect of different media on orange trifoliate shooting is shown in Table 3. The results indicated that WPM was superior to MS media on shoot multiplication. A high shoot number (4.2), maximum shoot length (5.18 cm) and highest leaves number (13) was achieved in WPM, and differed significantly when compared with MS media.

In vitro propagation of woody plants has always been difficult due to problems with establishment of aseptic cultures, microbial contamination and varied nutritional media requirements (Purohit and Kukda 1994, Agrawal *et al*., 2002). Nutritional requirements for the optimal *in vitro* growth depend on an involved species. Similarly, tissues from different plant organs may have different requirements for respective growth (Murashige and Skoog, 1962). For this reason, one single medium cannot be suitable for each type of plant tissue and organ. Therefore, when starting with a new species cultivar, it is crucial to develop a medium that can fulfill the specific requirements of the tissue (Bhojwani and Razdan, 1993).

Two basal salt mixtures (high and low), Woody Plant Media (Lloyd & McCown, 1980) and MS media (Murashige & Skoog Media, 1962), were used initially to study the effect of basal media on shoot multiplication. In the light of our results, the WPM media proved to be better than MS media since it showed highest number of leaves and shoots along with the maximum length of shoot. The WPM was basically designed to overcome the chloride ion susceptibility of the woody plants (George, 1993). Since our results showed better on WPM media, thus the WPM media, which has a low salt media as compared to other media, is better for the enhanced multiplication of axillary shoots of woody plants. Micro-propagation can provide an opportunity to obtain large number of homogenous plants (Pospíšilová *et al*., 1999). The efficiency of micro-propagation is related to the *in vitro* cultural conditions as the type of media, growth regulators or media pH. Each plant species has its own characteristic elementary composition which can be used to adapt the media formulation (Nas and Read, 2004). Besides the cultural media, the present techniques involved in the tissue culture, the hormonal balance is crucial for organogenesis. The influence of hormones is expressed both individually and also by changing the ratio between the stimulators and inhibitors.

In orange, the optimum response was found on WPM media. This may be attributed to a greater demand of smoke bush for nitrogen and potassium which stimulate production of new proteins (Guru *et al*., 1999). These components are higher in WPM media as compared to MS. It is worth mentioning that, although the average number of shoots was significantly increased on WPM media, numerous studies have been carried out to optimize conditions for the *in vitro* regeneration and multiplication of woody species. Unfortunately, the effectiveness of some culture conditions appears to be highly genotype specific. There is a need for more protocols on the tissue culture of woody plants and an understanding of the processes involved. Fruit

rootstocks are traditionally propagated either by relatively slow and labor-intensive vegetative methods (division and cuttings techniques) or from seed, which often results in a non-uniform material. The application of tissue culture methods for vegetative propagation of temperate fruit rootstocks started in the mid-70s, and a considerable number of improved protocols were developed ever since. Generally, the goal of micro-propagation is obtaining rapid, large-scale and low-cost production of genetically identical, physiologically uniform and pathogen-free plants (Rathore *et al*., 2004).

Successful *in vitro* clone propagation methods are reported in many rootstocks, including plum (Morini *et al*., 1990; Nacheva *et al*., 2002; Vujović *et al*., 2007), cherry (Ružić *et al*., 2003; Sedlák *et al*., 2008) and pear rootstocks (Yeo, Reed 1995; De Paoli *et al*., 2002; Ružić *et al*., 2008). While most of the studies were focused on the influence of nutrient media, including mineral composition, carbohydrates content and type/ concentration of plant growth regulators, the influence of repeated sub-culturing on shoot multiplication and growth received less attention in literature (Grant, 1999). Khan *et al*., (2004) found that the terminal buds of pineapple plant cultured on full and half strength MS media supplemented with 5.0 $mg.l^{-1}$ BA responded better compared to other concentrations in respect to the number and lengths of shoots per explants.

The successful growth and development of axillary buds has been obtained on various basal salts media, including (Standardi and Catalano, 1985; Murashige and Skoog, 1962, Chée and Pool, 1987; Gabryszewska, 1997; Kamenická and Lanakova, 2000; Parris *et al*., 2012; Sokolov *et al*., 2014).

Increased MS-salts concentrations stimulated shoot formation in orange trifoliate (Table 1). Mineral components in the growth media are vitally important for the *in vitro* regeneration process in plants (Williams, 1993). Some mineral compounds are related to endogenous cytokinin biosynthesis. An increase in nitrate resources may induce the expression of genes responsible for the biosynthesis of cytokinins, resulting in accumulation of these hormones in plants (Wang *et al*., 2004). However, as this study verified in *orange explants*, the addition of only a cytokinin resource in the media could not ensure an organogenesis response, indicating that the concentration of salts is very important to ensure and enhance cell division, fresh and dry mass accumulation relate to nutrition (Huang *et al*., 2010). Aranda-Peres *et al*., (2009) verified that calcium plays an important role during *in vitro* growth. Furthermore, it exerts a positive influence on the absorption of other nutrients in the media.

Optimization of growth media based on mineral nutrition for micro-propagation is very challenging due to the diverse nutrition requirements of various plant species and the many interactions of the chemical nutrients. There are many approaches for improving the growth media based on mineral nutrition. Recent studies noted the effect of mineral nutrients on plant growth and development (Nas and Read, 2004; Adelberg *et al*., 2010; Halloran and Adelberg, 2011; Greenway *et al*., 2012). Changes in the concentrations of nutrient supply can also affect availability and uptake of mineral nutrients resulting in effects on growth and development (Williams, 1993). The most common way to improve growth media is modification based on changing mineral components compared to MS (Driver and Kuniyuki, 1984). In addition, optimum mineral supply for media modification was investigated or defined by adapting the concentrations of minerals to match the biological mineral components of *in vivo* plants (Morard and Henry, 1998; Monteiro *et al*., 2000) or field plants (Nas and Read, 2004).

This result was in agreement with those obtained by Pereira-Pinto *et al*., (1996), who noticed that the use of total force and half strength of growing medium salts gave proportionally higher shoot ratios of *Kielmeye racoriacea*. Sakr *et al*., (1999 b) stated that with *Magnolia grandiflora*, MS media at full-strength was more effective in increasing the number of shoots/explants than other media strengths.

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