RESEARCH ARTICLE

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Effects of different combinations of growth regulators on the *in vitro* growth parameters of *Poncirus trifoliata* L. (Raf.)

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Abstract

In this study, an in vitro propagation protocol of *Poncirus trifoliata* L. (Raf.) rootstock, which is one of the main rootstocks used in citrus, was created. Monocular node parts of the plant, which is the source of explant, were cultured in MS nutrient media containing BAP at dissimilar concentrations (0.5-1.0-2.0 mg/l). Afterward, it was observed that shoot appearance and tillering were achieved in the node explants cultured in MS nutrient medium comprising 1.0 mg/l BAP. The shoots were removed to a new culture in MS nutrient media containing NAA and IBA for rooting. While 30% rooting was achieved in the rooting medium, 62% of the rooted plantlets were acclimated to external conditions. 2 mg/l BAP and 2 mg/l silver nitrate ($AgNO_3$) doses were supplied to the MS medium. The results of this study were obtained and a compound was found to the yellowing and leaf fall problem. As a result, in vitro propagation of *Poncirus trifoliata*, which is among the important citrus rootstocks, was tried, and its rooting ability was investigated in different nutrient media concentrations after shoots were obtained from different explants.

Keywords: *Poncirus trifoliata* L., *In vitro*, Silver nitrate, Propagation, Tissue culture

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INTRODUCTION

Micropropagation is a tissue culture technique used to produce a large number of genetically similar plants from plant parts taken from a plant that has the potential to form a whole plant, in artificial nutrient media and under sterile conditions (Kozai et al. 1997; Hatipoğlu 1999; Mansuroğlu and Gürel 2001; Ak et al. 2021).

In this study, the *in vitro* propagation of the genus *Poncirus trifoliata* L., which is used as a rootstock for citrus fruits and preferred as an indoor ornamental plant in landscaping in our country, was tried. Different concentrations have been developed for rooting conditions. It is aimed to be a reference source for *in vitro* researches on this rootstock.

MATERIALS AND METHODS

Material

This study was carried out in the laboratory of the Department of Horticulture, Faculty of Agriculture, Harran University. In the study, 5-year-old *Poncirus trifoliata* cuttings were used as material.

Methods

After cutting the leaves of the new cuttings taken from the plant and separating the leaves, approximately 15-20 mm green cuttings were procured. The cuttings, which were washed under water for 30 minutes in the laboratory environment, were kept at 70% ethyl alcohol concentration for 1 more minute and then rinsed with sterilized distilled water. After the rinsing phase, the materials are dipped in 5%, 10% and 20% commercial-gradely used bleach (0.525% NaOCl) to which 1-2 drops of Tween 20 are added for sterilization, and the surface is cleaned for 15 minutes, and then 3 times for 5 minutes. It was washed with sterile distilled water and sterilization was concluded. Shoot growth was observed in equal number of materials (10 each) sterilized at these 3 different bleach concentrations, and no contamination was observed at 10%.

After sterilization was concluded, the explants were transferred to the culture medium. The pH value of the culture medium containing 30 g/l sucrose, 7 g/l agar, 4.4 g/l MS and 1 mg/l BAP to support the growth and development of the plant was brought to 5.8 with added NaOH and HCl. After the culture medium was boiled, 0.3 g of GA_3 hormone was added to support the longitudinal growth of the plant. Then, every 7 days, the percentages of explants showing signs of contamination in the culture medium and growing healthy were affected as percentages.

Equipment such as magenta cups, blotting papers, scalpels and forceps to be used in the experiment are wrapped in aluminum foil in sufficient amounts (to be completely covered) and heated at 1 atm for 15 minutes. It was sterilized in an autoclave at 121°C under pressure. The forceps and scalpels used at every stage of the study were sterilized continuously by immersing them in 96% ethyl alcohol concentration. 15 minutes before starting work in the cabinet, the cleaning of the sterile cabinet was carried out with 70% ethyl alcohol concentration. Afterward, the sterilization of the cabin was concluded by keeping the UV lights on for 15 minutes, and then work was started in laminar flow cabinet. The nutrient media were sterilized in an autoclave at 121°C for 15 minutes under 1 atm pressure.

For the experiment, a climate cabin with a light intensity of 2500 - 3000 lux and a light intensity of 10000 lux was used. The interior of the cabin is programmed to have a photoperiod of 16 hours, with 16 hours of light and 8 hours of darkness. The temperature of the environment inside the cabinet was kept constant at 24±1°C.

After superficially sterilized cuttings were cut with at least one eye on them, they were planted in containers containing 10 ml of initial culture medium. The development of the explants was followed every 7 days until leaves and shoots were obtained in the initial culture medium. In order to reproduce the shoots, the shoot clusters with tillering were separated into individual shoots, the tillering numbers were settled and the transfer to the new culture medium was ensured. The pH value of the MS culture medium, which is preferred as the initial culture medium, containing 6 g/l agar, 1 mg/l BAP, and 20 g/l sucrose, was adjusted to 5.8 with the support of HCl and NaOH before autoclaving. After this process, the culture medium was sterilized at 121°C for 15 minutes. After sterilization, 2-4 cm long explants were taken and transferred to *in vitro* shoot culture medium without damaging the eyes on the shoots. These explants were transferred to subculture in the same shoot culture medium at intervals of 4 weeks until leaf appearance was observed in the shoot culture medium and until new shoot appearance was observed.

Each shoot of 2-5 cm long explants that formed callus was separated and transferred into different containers. Three different shoot rooting media were created.

The development of the produced materials in 3 different mediums was examined for 4 weeks and no signs of rooting were observed. Yellowing and shedding of the leaves of the plants have been observed, and the reason for this is; It has been concluded that there is ethylene production, which is called "Maturation or Aging Hormone" due to the effect of accelerating maturity and color appearance, causing fruit and leaf fall on plants (Walsh, 2003).Later, in the literature studies on this subject, studies proving that silver nitrate is a very effective inhibitor of ethylene effect were encountered. And 2 mg/l of BAP and 2 mg/l of silver nitrate ($AgNO_3$) were added to the MS medium that in order to reduce the amount of phenolic substance in the medium. The results of this study were obtained and a compound was found for the yellowing and leaf fall problem. In the rooting medium, the materials formed callus, but no rooting was observed.

In order to find an infusion to the rooting problem, a new rooting medium has been produced. 500 mL of growth hormone NAA to support rooting and growth hormone widely used in rooting of cuttings to support rooting in a medium consisting of 20 g/l sucrose, 6 g/l agarose and 1/2 MS. hormone, 500 mL of IBA was added. The pH of the rooting nutrient medium was adjusted to 5.8 with NaOH or HCl before sterilization. The environmental conditions of this rooting medium were adjusted to be compatible with the environmental conditions of the previous experiments. Results were obtained from this experiment and rooting was observed in the materials.

It was observed that the explants in the rooting medium formed a sufficient amount of roots under micropropagation conditions after about 30-35 days. After this stage, after the explants were removed from the nutrient medium. Then a mixture of 1/2 peat and ½ perlite was made provision for, this mixture was sterilized at 121°C for 15 minutes. And the explants were staggered into a sterile soil medium, which was evenly distributed in plastic containers. In order to facilitate the adaptation of the plants to the external environment, the explants were covered

with perforated transparent containers and the humidity rate was tried to be kept in balance. The pots containing the plants, which were tried to be acclimatized to the outside conditions, were opened every day more (5-10 minutes) after about a week, and the preparation of the plantlets for the external environment was supported.

Root measurements were made after the rooted plants were removed from the nutrient media and cleaned to be surprised in the soil. And the root length of each plant material was measured and the average root length was calculated. Then, they were transferred to medium containing 50% peat and 50% perlite.

The statistical evaluation of the data obtained as a result of the research in the *in vitro* technique trial for *Poncirus trifoliata* L. rootstock was taken randomly from different parts of the plant, the stages of the research were repeated several times without being dependent on each other, and each independent trial consisted of a different number of shoots. When the statistically notable transactions were detected, the differences between the mean data were subjected to the LSD test at the $P<0.05$ level. In order to settle the effects of different cytokinin, gibberellic acid and auxin densities on the parameters, PCA analysis was performed with the support of the PAST package program.

RESULTS AND DISCUSSIONS

Determination of Sterilization Protocol

The most basic issue in *in vitro* (tissue culture) studies is the effectual sterilization of plant materials. In the literature review, which is the first step of the research, our priority has been to investigate research on sterilization in detail. Again, the most important step *in vitro* studies is the surface sterilization of theplantmaterials to be used in these studies and the selection of the most appropriate sterilization method for the material to be used. Sterilization methods differ according to the media in which the plant material is grown and the parts of the plant material from which the explant is taken. The most commonly used disinfectants in the sterilization phase of explants are; sodium hypochlorite, ethanol, silver nitrate, calcium, mercury, and hydrogen peroxide chloride can be counted as (Babaoglu et al., 2002).

In general, the sterilization process of the explants at the beginning of the *in vitro* experiments is used to break the superficial resistance of these explants. rinsing in sterilized distilled water (Ainsley et al., 2001a). In addition, some researchers perform plant material sterilization in some stages. Some researchers (Gurel and Gulsen 1998; Ainsley et al. 2001b; Pruski et al. 2005) rinsed the explants under running water for different times 'tween 5 minutes and 2 hours before sterilizing the plant material to ensure rough cleaning. Muna et al. (1999), on the other hand, did not find rinsing the plant materials under running water sufficient to provide rough cleaning and washed them with one or two drops of Tween-20 for 10-15 minutes and then scourged to surface sterilization. In Jain and Babbar (2003), they first washed the plant materials that they would use in their studies with 10% Teepol and then rinsed them under running water for 30 minutes and passed to the surface sterilization stage. Espinosa et al. (2006) kept the plant materials in ethanol at 50-70% immersion for a short time between 30 seconds and 5 minutes and then sterilized the plant material with commercial-grade bleach.

In this study, no contamination was observed in the initial cultures of plant materials that had been superficially sterilized in 10% NaOCl. *In vitro* shoots did not undergo any morphological changes in the observed process and continued to develop healtihily. In this trial, 10% NaOCl disinfectant solution was settled to be the most suitable concentration for the surface sterilization of plant materials, and this concentration was continued to be used during the sterilization of the plant material throughout the research process.

Shoot Development of Explants and Proliferation

In this study, the tip of the shoots and semi-woody parts of the plant material were used as explants.

There is MS nutrient medium containing 6 g/l agar and 20 g/l sucrose in the nutrient medium primed for the shoot development of the explants taken from the shoot tips of *Poncirus trifoliata* L. rootstock. In this environment, shoot growth was observed in plants. Approximately 4 weeks after the beginning of the culture, the ratios of explants that developed enough to be subcultured and explants that did not show sufficient growth were settled.

As it was applied on the shoot tip explants, plant parts formed by separating the tilled explants after the shooting medium were also grown in the same medium. By adding 0.5, 1.0 and 2.0 mg/l BAP to the same combination, the effects of node explants taken from the plant on shoot growth were investigated and the number of leaves, shoot length and shoot number were measured in the following process. (Table 1.) Approximately 3 weeks after the start of culture, the ratio of explants that developed enough to be subcultured and explants that did not show growth were settled.

In the study, firstly, the explants taken from the plant were taken into the shooting medium at different BAP doses (0.5 mg/L, 1 mg/L and 2 mg/L) and their development was examined. The growth rates of explants were calculated in the number of leaves, shoot number and shoot length measurements made in shoot appearance media. As a result of these calculations, the explants in the medium containing 1 mg/L BAP showed more growth than the explants in the other media. The explants in this medium were 11.1% in terms of the number of leaves compared to the explants in the medium containing 0.5 mg/L BAP; It increased 20.48% more than the explants in the medium containing 2 mg/L BAP. In terms of shoot number, 5% compared to explants in medium

Table 1. Explant growth chart at different BAP concentrations

		1st Medium (0.5 mg/L BAP)	2nd Medium (1 mg/L BAP)	3rd Medium (2 mg/L BAP)	LSD (%5)
1st Measurement	Number of Leaves	1.50±1.14b	3.00±0,57a	1.68±1,52b	0.943
	Number of Shoots	1.20±0.91b	2.10±0,57a	1.30±0,57b	0.518
	Length of Shoots	1.16±0,30b	1.63±0,15a	1.84±0,35b	0.468
2nd Measurement	Number of Leaves	2.10±0,57b	4.00±1,53a	2.40±1,15b	1.079
	Number of Shoots	1.70±1,00b	2.70±0,82a	1.70±0,57b	0.668
	Length of Shoots	1.49±0,21b	2.19±0,72a	1.27±0,37b	0.469
Average	Number of Leaves	1.80±0.30b	3.50±0.50a	2.04±0.36b	1.011
	Number of Shoots	1.45±0.25b	2.40±0.30a	1.50±0,20b	0.593
	Length of Shoots	1.32±0.16b	1.91±0.28a	1.55±0.28b	0.468

The parameters that LSD values were determined and given letters were found to be statistically significant.

containing 0.5 mg/L BAP; It increased 20% more than explants in medium containing 2 mg/L BAP. In terms of shoot length, 69% compared to explants in medium containing 0.5 mg/L BAP; It showed 40% more growth than explants in medium containing 2 mg/L BAP. Thus, shoot medium containing 1 mg/L was settled as the most appropriate immersion.

In order to probe the effect of GA_3 on shoot growth at the proliferation stage, 2 different media were tried and the results are given in Table 2.

Then, to transfer the explants in MS culture medium containing 1 mg/l BAP, 30 g/l sucrose, 7 g/l agar, which was settled as the initial culture medium, transfer to 2 different mediums was tried. Half of the explants were given to 1st medium containing 20 g/l sucrose, 1/2 MS, 6gr/l agar, 1 mg/l BAP, 0.5 mg GA; the other half was taken into the second medium containing 20 gr/l sucrose, 2.2 mg/l MS, 6gr/l agar. The mean values of the explants, whose growth was observed for 2 weeks, in the media after 2 weeks were calculated. While the average shoot length of the explants in the 1st medium was 1.97 cm and the average number of leaves was 11.11, the average shoot length of the explants in the 2nd medium was 2.18 cm and the average number of leaves was 15.12.

Then, the development of the explants at 3 different Rooting concentrations prime for the explants that concluded their development in the BAP medium settled at the appropriate doses was observed and their measurements were made (Table 3). In these measurements, the number of leaves, shoot length and callus appearance of the explants were examined.

Callus appearance was observed in the explants, but rooting was not observed. Then, 500 mL of NAA and 500 mL of IBA were added as rooting hormone to support rooting in the medium consisting of 20 g/l sucrose, 6 g/l agar and 2.2 g/l MS. The pH of the rooting nutrient medium

Table 2. Effects of GA3 on shoot development

		1st Medium (20 g/l sucrose, 2.2 mg/l MS, 6gr/l agar, 1 mg/L BAP)	2nd Medium (20 g/l sucrose, 2.2 mg/l MS, 6gr/l agar, 1 mg/l BAP, 0.5 mg GA_3)	LSD (%5)
1st Measurement	Number of Leaves	12.44±2.51a	8.37±9.33b	3.816
	Length of Shoots	1.80±0.30a	1.43±0.62a	n.s.
2nd Measurement	Number of Leaves	14.56±3.05a	11.11±3.05a	n.s.
	Length of Shoots	2.10±0.43a	1.87±0.43a	n.s.
Average	Number of Leaves	13.50±1.06a	9.74±1.37b	4.236
	Length of Shoots	1.95±0.15a	1.65±0.22b	0.620

The parameters that LSD values were determined and given letters were found to be statistically significant.

Table 3. Explant growth at different rooting concentrations

		1st Medium (0,25 mL/l NAA)	2nd Medium (0,15 mL/l NAA+ 0,15 mL/l IBA)	3rd Medium (0,15 mL/l IBA)	LSD (%5)
1st Measurement	Number of Leaves	2.50±0.57a	2.70±1.00a	1.90±1.00a	n.s.
	Length of Shoots	9.43±2.08a	10.00±0.68a	8.80±6.08a	n.s.
2nd Measurement	Number of Leaves	2.75±0.57a	4.20±2.33a	2.60±0.57a	n.s.
	Length of Shoots	6.00±2.08a	11.00±8.18a	11.89±4.35a	n.s.
Average	Number of Leaves	2.62±0.12a	3.45±0.75a	2.25±0.35a	n.s.
	Length of Shoots	7.71±0.71b	10.50±0.50a	10.34±1.54a	7.506

The parameters that LSD values were determined and given letters were found to be statistically significant.

was first adjusted to 5.8. Results were obtained from this experiment and the rooting phase of the explants was realized. The rooted explants were stunned with 50% peat and 50% perlite and their roots were expected to develop well in order to acclimate them to the external environment, and after they had developed sufficiently, they were transferred to the media and continued to be observed. 62% of these plants healthily continued theiır lives (Figure 1.).

Figure 1. Images of explant development at different stages (1.Shoot-forming explant, 2. Explants in proliferation medium, 3 Explants in rooting medium, 4. Rooted explant)

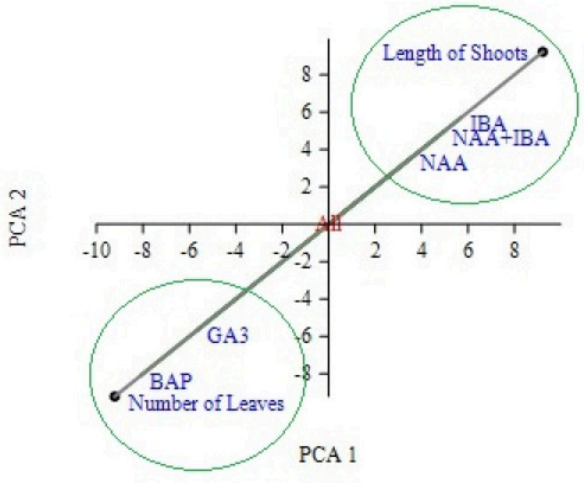

Figure 2. Effects of hormones according to PCA

In the statistical evaluation (PCA) made as a result of these measurements, it was concluded that cytokinin and gibberellin were effectual in the number of leaves and auxins were effectual in the length of shoots (Figure 2.).

Ali and Mirza (2006) have experienced that the appropriate medium to ensure callus appearance in propagation experiments from Citrus explants with tissue culture tack is MS medium with 1.5 mg/l 2,4 D added, and MS medium with 3 mg/l BA added for rooting. Amgai et al. (2016) investigated the effects of 10 different hormone levels including BAP and IAA in MS medium and 3 different culture periods of 4, 8 and 12 weeks on the reproduction of C. reticulata Blanco (mandarin) plant *in vitro*. The most successful shoot appearance was obtained in nutrient media containing 0.2 mg/l IAA and 0.5 mg/l BAP. Cengiz and Kacar (2019) carried out micropropagation and rooting experiments of citrus rootstocks named 'C-35 citrange' and 'Tuzcu 31-31 orange' under the influence of the traditionally known solid culture and temporary immersion principle in tissue culture, in comparison with the Plantform bioreactor system. In solid culture micropropagation experiments on citrus rootstocks, Murashige, Woody Plant (WPM) and Skoog (MS) were also used as a plant growth regulator with nutrient media Kinetin (KIN) (0; 0.5; 1.0 mg L-1), Isopentenyladenine (2IP) (three different concentrations: 0; 1.0; 2.0 mg L-1) and Benzylaminopurine (BAP) (0; 1.0; 2.0 mg L-1). In rooting trials; MS, ½ MS, WPM to nutrient media Indole-3-butyric acid (IBA) (0; 0.5; 1.0; 2.0 mg L-1) and Naphthaleneaceticacid (NAA) (0; 0.5; 1, 0; 2.0 mg L-1) by including different concentrations of plant growth regulators added. The more suitable micropropagation results betwitx these two genotypes were MS nutrient medium containing 2.0 mg L-1 BAP and the more suitable rooting results were ½ MS nutrient medium containing 0.5 mg L-1 NAA. As a result of these trials in terms of Plantform system plant quality, good results were obtained in the tillering medium in these two different genotypes. In the medium for rooting, the Plantform system was observed to be more valuable than the solid culture nutrient medium. In plants propagated and rooted in the plantform system; It was settled that there was no improvement as a result of scanning with SSR markers.

Chamandoosti(2017) conducted research on the shoot appearance of *C. latifolia* plant under *in vitro* conditions. In his study, he observed that the most successful shoot development was in the nutrient medium containing 0.053 µM NAA and 4.44 µM BA. The most successful medium in terms of plant height was the nutrient medium with 0.049 µM IBA and 4.44 µM BA. Again in this study, the effect of 1 mg/l NAA and 1 mg/l IBA on rooting of 9 different genotypes was examined. In rooting studies, the data of root length, root number and plant height were evaluated. In rooting studies, IBA was settled as the most effectual growth regulator for the height and length of the roots of the plant, and NAA as the most effectual growth regulator for the number of roots.

CONCLUSION

In the results of the study, 10% NaOCl disinfectant solving was settled as the most suitable assiduity for the sterilization of the plant material used in the experiment, and this concentration was continued to be used in the surface sterilization of the plant material throughout the research process. For the starting medium, the medium containing 1.0 mg/l BAP, where the plants developed best, was settled as the best engrossment and the process was continued with this concentration. While the study was going on, the problem of yellowing and shedding of leaves was encountered. The reason for this problem was thought to be ethylene production in the environment, and ethylene in the environment was tried to be evacuated, but the result was unsuccessful. Later, studies were found proving that silver nitrate is a very effective inhibitor of ethylene effect. 2 mL/l BAP, 2 mL/l and silver nitrate (AgNO3), which is thought to reduce the amount of phenolic substance in the medium, were added to the MS medium. The results of this study were obtained and a solving was found to the yellowing and leaf fall problem. And finally, various attempts were made for rooting and the result was obtained by adding 500 mL/l NAA and 500 mL/l IBA to the medium consisting of 2.2 g/l MS containing 20 g/l sucrose, 6 g/l agar.

As a result, *in vitro* propagation of *Poncirus trifoliata* L., which is one of the important citrus rootstocks, a widely known hedge plant and also a very preferred indoor landscape plant, was tried, and its rooting ability was investigated in different nutrient media concentrations after shoots were obtained from different explants. Thus, different denseness has been developed for *in vitro* rooting of three-leaf rootstock. With plant tissue culture experiments, it is possible to scan and detect different characteristics of plants more quickly and to reproduce them at the same time. In the study, it is seen that *Poncirus trifoliata* L. It can be a very suitable material for propagation by rootstock tissue culture. However, it is recommended that the appearance obtained as a result of plant tissue culture experiments be tested again under field conditions. Today, citrus cultivation is a very important issue. The fact that no citrus is grown in some countries in the world is proof of how important citrus imports and exports are. For this reason, today, due to both commercial-grade and scientific studies, breeding studies of citrus rootstocks and varieties continue without slowing down in the world.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest

The authors declared that there is no actual, potential or perceived conflict of interest in this research article.

Author contribution

The contribution of the authors to the present study is equal. All the authors verify that the text, figures, and tables are original. The authors read and approved the final manuscript.

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Ethical approval

Ethics committee approval is not required.

Data availability

Not applicable.

Consent for publication

Not applicable.

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