

***In vitro* REGENERATION INDUCED IN LEAF EXPLANTS OF *Citrus limon* L. Burm cv. ‘Primofiore’**

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Abstract. *In vitro* organogenesis was studied using *Citrus limon* L. Burm cv. ‘Primofiore’ leaf explants. The purpose of the present study was to optimize conditions for callogenesis and organogenesis of *C. limon*. Explants of *C. limon* were cultured on 16 different media supplemented with various combinations of plant growth regulators, both auxins and cytokinins, such as 6-benzylaminopurine (BAP), naphthalene acetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), and kinetin. The best shoot induction was obtained when the leaf explants were cultured on Murashige & Tucker media supplemented with 3.5 mg·L⁻¹ BAP. Histological investigation revealed most likely the initial phase of development of leaf explants during *in vitro* regeneration of *C. limon*.

Key words: *Citrus limon*, phytohormones, explants, callus induction, auxin, cytokinin, 6-benzylaminopurine

INTRODUCTION

Lemon is a very popular fruit all over the world due to the taste, aromatic flavor and healing properties [Mukhtar et al. 2005b, Ali and Mirza 2006, Sarma et al. 2011, Gaurav and Richa 2012]. Beside apples and bananas, citrus fruits are the most important fruit crops [FAO 2001]. They are widely used to prevent flu and colds and support the immune system [Dhanavade et al. 2011]. Citrus fruits are also used for patients susceptible to health problems such as gastritis, fever and arterial sclerosis. The juice of lemon is used in the pharmaceutical industry since it contains a high quantity of citric acid (a source of ascorbic acid and citric acid) and essential oils [Bansode et al. 2012]. There are also reports about positive effects of lemon fruits against cancer of gastrointestinal and upper respiratory tracts [Foschi et al. 2010]. The genus is cultivated for its fruit and fragrance, partly due to flavonoids and limonoids [Ashok Kumar et al. 2011].

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Citrus limon L. Burm is a tree in the *Rutaceae* family originating from Asia but now it is grown commercially worldwide in regions with tropical and warm temperate climates, especially in the United States, Brazil, Spain, Italy, Egypt, Mexico, China, and South Africa [FAO 2001]. Trees bloom and bear fruit almost without a period of rest. On plantations, the seeds or rooted plantlets of propagated plants are sown or planted mainly in spring. Generally, fruit harvests are carried out in winter, because of the highest content of vitamin C in this period. Given the great importance of the representatives of the *Rutaceae* family in various branches of industry, the micropropagation of *Citrus* has always aroused great interest among scientists. There is a growing demand to develop new varieties of plants resistant to pathogens and adverse environmental conditions and characterized by high quality of fruits [Yaacob et al. 2014]. Traditional techniques for creating new species are not effective in the case of lemons due to the physiological barriers associated with sexual reproduction such as heterozygosity and polyembryony [Tusa et al. 1990, Carimi et al. 1994, Savita et al. 2010, Benabdesselam et al. 2011, Lombardo et al. 2011]. Lemon plantations face a number of problems such as pests, slow growth, susceptibility to disease, sensitivity to low temperatures, and substantial losses during storage [Mukhtar et al. 2005a, b, Savita et al. 2010, Sarma et al. 2011]. *In vitro* culture is a technique that can solve these problems. In addition, this technique can also produce crops on a relatively large scale in comparison with traditional plant breeding. Furthermore, *in vitro* cultures eliminate infections and can be faster than traditional plant cultures [Savita et al. 2011, Singh and Kaur 2011].

Production of callus and subsequent regeneration of plants are the main stages in the biotechnological manipulation *in vitro*. The compositions of the medium and culture conditions are one of the most important things that determine the effect of organogenesis [Rattanpal et al. 2011]. Therefore, in this paper, we have undertaken research on micropropagation in one of the *Citrus limon* cultivars – cv. ‘Primofiore’, which is the most popular cultivar growing in Spain. We did not find any information in the available literature concerning the micropropagation of this cultivar. We focused on optimization of the callus formation and lemon plant regeneration with the use of leaf explants. Taking into account the issues discussed above, we also performed histological analysis of these processes.

MATERIAL AND METHODS

Plant material and culture conditions. In this study, lemon tissue (*Citrus limon* L. Burm cv. ‘Primofiore’) was used as explants. The experiments were carried out using MS [Murashige and Skoog 1962] and MT [Murashige and Tucker 1969] culture medium solidified with agar. The media were prepared by dissolving previously prepared solutions of organic compounds, mineral salts, and plant growth regulators in deionized water; afterwards the pH was adjusted to 5.6–5.7 with a pH meter using 1M KOH or 1M HCL, and next the culture medium was autoclaved for 20 minutes at a temperature of 121°C.

Seeds were collected from commercial lemon fruits, dried for two weeks, and then surface sterilized for a few seconds by treatment with 70% ethyl alcohol. Next, they

were transferred to a 40% "Domestos" (commercial bleach) solution in sterile deionized water for 10 minutes and rinsed three times in sterile deionized water for 5, 10, and 15 minutes. After sterilization, the seeds were placed in jars containing the MS medium (30 g·L⁻¹ sucrose, 7.9 g·L⁻¹ agar) and germinated aseptically to obtain seedlings. Subsequently, the cultures were incubated in a chamber with a 16/8 photoperiod (16 hours of light, 8 hours of darkness, 170 μmol m⁻² s⁻¹ light intensity) and a temperature of 23°C. The leaf explants were obtained from young lemon seedlings after 45 days of culture. The explants were cultured on petri dishes containing MS (30 g·L⁻¹ sucrose, 7.9 g·L⁻¹ agar) and MT (50 g·L⁻¹ sucrose, 8.0 g·L⁻¹ agar) media supplemented with different combinations of plant growth regulators. A callus induction assay was carried out on MS and MT media supplemented with 16 combinations of plant growth regulators, which are shown in Table 1. The petri dishes were incubated for 30 days in the dark and at a temperature of 23°C for callus induction. Finally, well-regenerated shoots were transferred to the MS medium with 1 mg·L⁻¹ NAA for rooting.

Table 1. Effect of different concentrations of plant hormones on callus, shoot and root induction (%) of *Citrus limon*

Medium containing different concentration of plant hormones	Callus induction (%)	Shoot induction (%)	Root induction (%)
MS + 5 mg·L ⁻¹ BAP + 1 mg·L ⁻¹ NAA	0	0	82
MS + 1 mg·L ⁻¹ KIN + 1 mg·L ⁻¹ NAA	77	0	80
MS + 0.2 mg·L ⁻¹ KIN + 1 mg·L ⁻¹ NAA	97	7	0
MS + 0.2 mg·L ⁻¹ KIN + 1 mg·L ⁻¹ 2,4-D	70	3	0
MS + 1 mg·L ⁻¹ BAP	0	0	0
MS + 2 mg·L ⁻¹ BAP	0	0	0
MS + 2.5 mg·L ⁻¹ BAP	0	0	0
MS + 3 mg·L ⁻¹ BAP	0	0	0
MS + 3.5 mg·L ⁻¹ BAP	0	0	0
MS + 4 mg·L ⁻¹ BAP	0	0	0
MT + 1 mg·L ⁻¹ BAP	63	0	0
MT + 2 mg·L ⁻¹ BAP	82	0	0
MT + 2.5 mg·L ⁻¹ BAP	85	0	0
MT + 3 mg·L ⁻¹ BAP	85	0	0
MT + 3.5 mg·L ⁻¹ BAP	33	37	0
MT + 4 mg·L ⁻¹ BAP	0	0	0

Changes in tissue during the culture were observed with a stereoscopic binocular microscope (Nikon SMZ-2T) and photographic documentation was prepared with Nikon Coolpix 4500 and Nikon D300 with mikronikkor 60. Explants were collected at different stages of development and prepared for microscopic evaluations.

Statistical analysis. Twenty explants were used per treatment. The experiment was repeated three times. The frequency of callus, shoot, and root induction (percentage of explants forming calluses, shoots, and roots) was scored after 3 months of culture.

Microscopic analysis. Leaf explants were collected at 0 (control), 7, 8, and 17 days from initiation of the culture and prepared for light microscopy (LM). Small fragments of the explants were excised and immediately vacuum-fixed for 30 min (3×10 min) in 4% (w/v) paraformaldehyde and 0.1% (w/v) glutaraldehyde in 0.1 M phosphate buffer (PBS; pH = 7.2), then rinsed three times in phosphate buffer (PBS; pH = 7.2) followed by dehydration in a graded ethanol series. Later, the samples were infiltrated in a mixture of LR White resin (LRW) and absolute ethanol (1:3, 1:1, 3:1 v/v; 1 h in each mixture), and stored overnight (12 h) in 100% LRW and again for 8 h in 100% LRW. Finally, infiltrated fragments were embedded in gelatin capsules and polymerized for 24 h at 50°C. Semithin sections (1.5 μ m) of the tissue were cut with an ultramicrotome (Reichert Ultracut S) and stained with 1% toluidine blue. Microscopic sections were obtained using a Nikon Eclipse Ni microscope with a Nikon DS-Qi1Mc camera.

RESULTS

The regeneration capabilities of *Citrus limon* L. Burm cv. 'Primofiore' were investigated starting from dried seeds. After surface sterilization, the seeds (fig. 1A) were transferred to the MS medium (fig. 1B). After 45 days of incubation, young seedlings (fig. 1C) were obtained and used in the further stages of the *in vitro* regeneration. Leaf explants (fig. 1D) were cultured in petri dishes with the MS and MT culture media supplemented with different combinations of plant growth regulators. After 80 days of culture conducted on the MT medium supplemented with 3.5 mg·L⁻¹ BAP, cream-colored callus induction (fig. 1E) was observed on the explant surface. After 7 days, well-developed fragments of calli appeared (fig. 1F) and 7 days later (fig. 1G), the caulogenesis process was observed on their surface. Young shoots continued development until the ready-to-rooting stage (fig. 1H). Young plantlets with several leaves (fig. 2A) were transferred into a rooting medium (fig. 2B). Callus regeneration was observed in a majority of the tested BAP concentrations in the MT medium (fig. 3C) but only one concentration induced organogenesis.

After 60 days of culture of explants incubated on the MS medium supplemented with various concentrations of BAP, only processes of plant tissue degradation were observed. There were no symptoms of regeneration processes.

In explants cultured on the MS medium supplemented with the combinations of plant growth regulators: 5 mg·L⁻¹ BAP and 1 mg·L⁻¹ NAA, 1 mg·L⁻¹ kinetin and 1 mg·L⁻¹ NAA only root induction – rhizogenesis was observed after 60 days of incubation (fig. 3A).

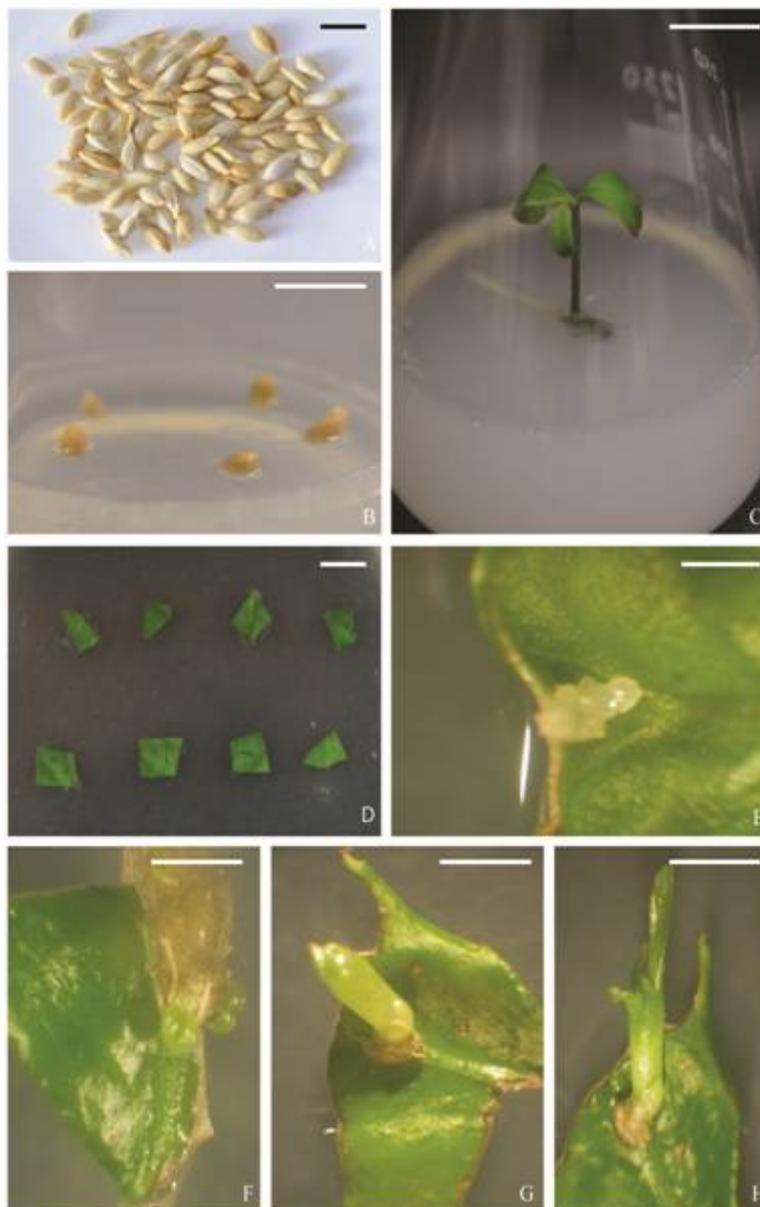


Fig. 1. *In vitro* plant regeneration of *Citrus limon* L. Burm cv. 'Primofiore'. A. Extracted and dried seeds from mature lemon fruits; B. *Citrus limon* seeds in the MS basal medium; C. Young seedling of lemon after 45-days culture; D. Leaf explants cultured on the MT medium; E–H. Developing shoot on leaf explant on the MT medium supplemented with $3.5 \text{ mg}\cdot\text{L}^{-1}$ BAP after 80 (E), 87 (F), 94 (G) and 101 (H) days of culture; (A) Bar = 1 cm, (B–C) Bar = 2 cm, (D) Bar = 5 mm, (E) Bar = 1 mm, (F–G) Bar = 2 mm, (H) Bar = 5 mm

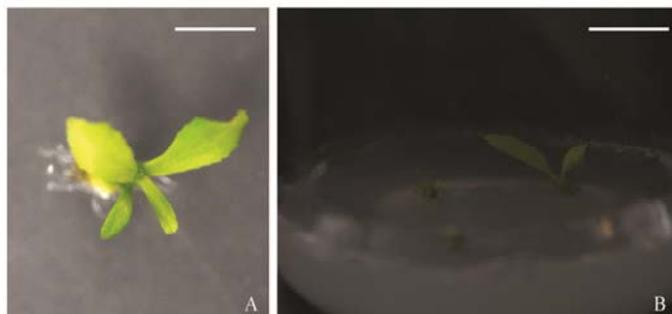


Fig. 2. *In vitro* plant regeneration of *Citrus limon* L. Burm cv. 'Primofiore'. A–B. Regenerated microshoots of lemon after transfer to the rooting medium (B); (A) Bar = 5 mm, (B) Bar = 1 cm

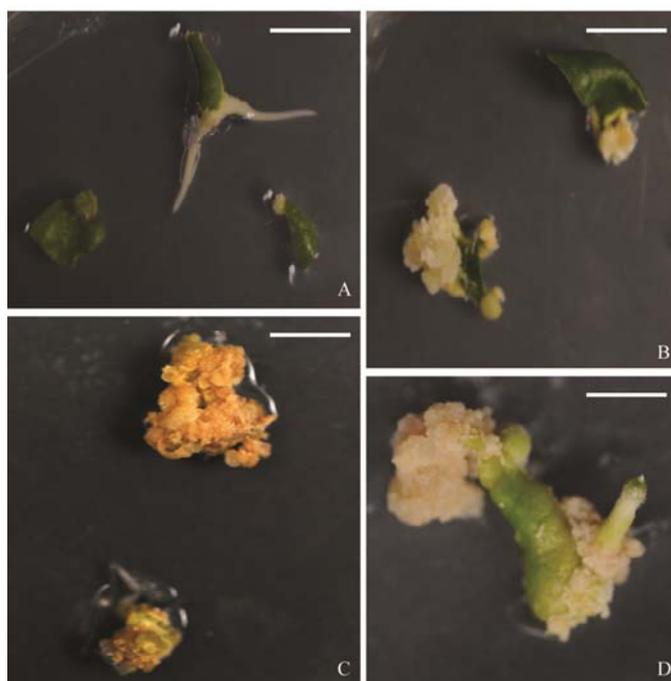


Fig. 3. *In vitro* plant regeneration of *Citrus limon* L. Burm cv. 'Primofiore'. A. Rhizogenesis on explants cultured on the MS medium supplemented with 1 mg·L⁻¹ kinetin and 1 mg·L⁻¹ NAA after 60 days of culture; B. Callus induction on explants on the MS medium supplemented with 0.2 mg L⁻¹ kinetin and 1 mg·L⁻¹ NAA after 60 days of culture; C. Callus tissue on the MT medium supplemented with 2 mg·L⁻¹ BAP after 80 days of culture; D. Indirect organogenesis on the MS medium supplemented with 1 mg·L⁻¹ NAA after 88 days of culture; (A–D) Bar = 5 mm

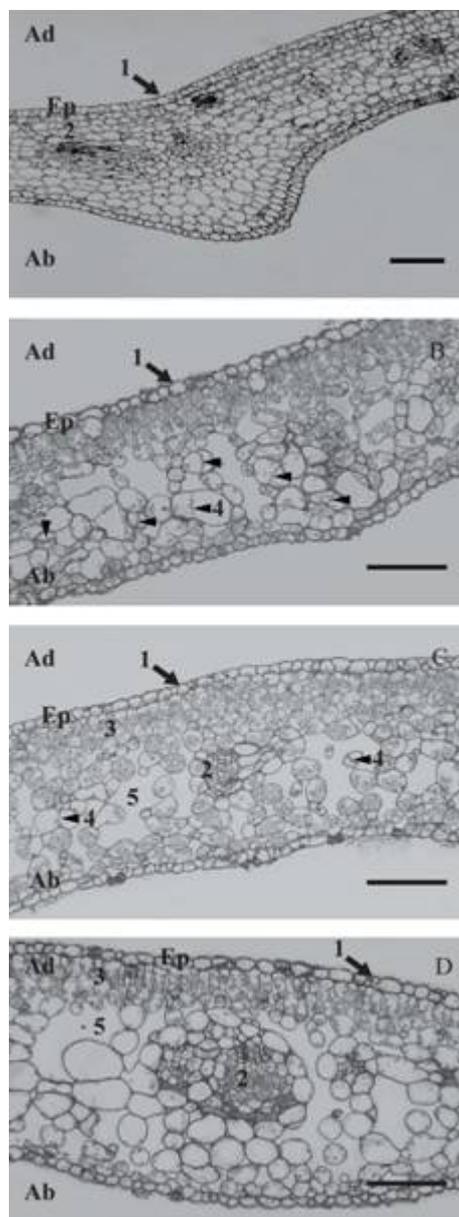


Fig. 4. Cross sections of *C. limon* leaves through tissues and differentiation regions. Sections (A-D) of leaves showing the presence of (1) cuticle, (Ep) epidermis, (2) vascular bundle, (3) palisade layer of mesophyll, (4) divided cells, (5) intercellular space. Ad = adaxial surface, Ab = abaxial surface; A. Leaf section, day 0. Bar = 100 μ m; B. Leaf explant after 7 days of culture. Bar = 60 μ m; D. Leaf explant after 8 days of culture. Bar = 60 μ m; E. Leaf explant after 17 days of culture. Bar = 60 μ m

After 60 days, explants cultured on the MS medium supplemented with other combinations of plant hormones such as $0.2 \text{ mg}\cdot\text{L}^{-1}$ kinetin and $1 \text{ mg}\cdot\text{L}^{-1}$ NAA, or $1 \text{ mg}\cdot\text{L}^{-1}$ kinetin and $1 \text{ mg}\cdot\text{L}^{-1}$ 2,4-D, creamy-yellow callus tissues were observed (fig. 3B). After subsequent 14 days of culture, newly regenerated shoots were observed on the MT medium supplemented with $0.2 \text{ mg}\cdot\text{L}^{-1}$ KIN and $1 \text{ mg}\cdot\text{L}^{-1}$ NAA (fig. 3D).

The effect of different concentrations of plant hormones on callus, shoot, and root induction of *Citrus limon* L. Burm cv. 'Primofiore' is indicated in Table 1.

Microscopic analysis of leaves with initial symptoms of regeneration of *in vitro* grown *Citrus limon*. At the time of explanting (day 0), *C. limon* leaves exhibited the typical tissue arrangement found in leaf tissue (fig. 4A). The anatomical structures of leaf showed juvenile features of the tissue. The cells were similar in size and shape and closely adhered to each other. After 7 days of culture, cross-section of the explant revealed an increased number of newly divided cells, especially in the spongy parenchyma (fig. 4B). The anatomical structure inside the leaf blade after the successive days of culture slightly changed, i.e. the differences between palisade and spongy parenchyma became less visible. As the leaf grew, the parenchyma cells of all layers became similar to each other. They became spherical, increased their size, and most of them were filled with a large amount of the cytoplasm (fig. 4C). During the next days of the culture, a significant increase in the size and shape of the cells was observed, especially in the palisade layer of the mesophyll. In parallel, this led to valid growth of tissue within the vascular bundles, in which a number of cells after division was seen. During the incubation of the leaf explants on the culture medium, substantial enlargement of tissues was observed (fig. 4D). This increase was caused by cell divisions and cell enlargement. Massive cell proliferation and growth were observed in the explants at 2 and 3 weeks on the culture medium. Most probably, the process of organogenesis started at that point.

DISCUSSION

Regeneration in *Citrus* species is a slow process [Singh and Rajam 2009]. Processes of callus induction and organogenesis in *C. limon* are long-lasting and much more difficult than in herbaceous plants [Kalinina and Brown 2007, Benabdesselam et al. 2011]. In our studies, we tried 16 various concentrations and combinations of hormones, and two different types of culture medium for callus induction. Addition of $50 \text{ g}\cdot\text{L}^{-1}$ sucrose to the MT medium seems to be inductive for regeneration of *C. limon*. In other citrus species, for example *Citrus assamensis*, the addition of $30 \text{ g}\cdot\text{L}^{-1}$ sucrose and pH 5.8 was better for *in vitro* regeneration, as recently reported by Yaacob et al. [2014].

In our studies, callus induction was observed in 8 combinations of the culture medium. The MT culture medium supplemented with $3.5 \text{ mg}\cdot\text{L}^{-1}$ BAP proved to be the best medium for shoot induction. The BAP concentration greater than $3.5 \text{ mg}\cdot\text{L}^{-1}$ induced neither callogenesis nor shoot formation. The results obtained by us are similar with those reported by Almeida et al. [2002] and confirm that high concentrations of BAP do not improve organogenesis results.

Rooting of plantlets was induced using the MS medium supplemented with $1 \text{ mg}\cdot\text{L}^{-1}$ NAA. Positive effects of NAA alone or in combinations with another auxin on rooting *in vitro* were observed by many researchers [Mukhtar et al. 2005a, Saini et al. 2010]. In other species like *C. limonia*, *C. sinensis*, and *C. jambhiri*, other auxins added to MS or MT media, IBA (indole-3-butyric acid) or IAA (indole-3-acetic acid) cause rooting [Almeida et al. 2002, Singh and Kaur 2011]. Rooting of *in vitro* regenerated *C. reticulata* and *C. limon* were also observed using a combination of IBA, NAA, and BAP [Singh et al. 1994].

The important role of BAP in shoot induction in citrus species has been well documented by many scientists [Saini et al. 2010, Rattanpal et al. 2011]. The regeneration was observed in the presence of BAP alone or in combination with other phytohormones. In the case of *C. aurantifolia*, *C. sinensis*, and *C. jambhiri*, shoot induction was obtained on MS medium supplemented with various concentrations of BAP [Rattanpal et al. 2011]. Addition of BAP to culture media is required for shoot induction in *C. clementina* [Lombardo et al. 2011]. In the case of *C. reticulata* and *C. limon*, a combination of three hormones: BAP ($1.0 \text{ mg}\cdot\text{L}^{-1}$), kinetin ($0.50 \text{ mg}\cdot\text{L}^{-1}$), and NAA ($0.50 \text{ mg}\cdot\text{L}^{-1}$) caused the highest shoot proliferation using shoot tips from 5- to 6-year old plants as explants [Singh et al. 1994]. In *C. reticulata*, shoot induction was observed on MS medium with BAP; however, improved results were observed on MT medium supplemented with various concentrations of BAP, which is in agreement with our results [Mukhtar et al. 2005 A, Ali and Mirza 2006]. In *C. limon*, shoot induction from nodal explants was observed on the MS medium with $2 \text{ mg}\cdot\text{L}^{-1}$ BAP and $2 \text{ mg}\cdot\text{L}^{-1}$ GA₃, while the highest number of shoots were obtained using $2 \text{ mg}\cdot\text{L}^{-1}$ BAP [Sarma et al. 2011].

Recently, parasites of *Citrus* trees such as fungi, bacteria, and viruses have become more resistant and increasingly widespread, which can aggravate the problems of pest control during plant growth. Undoubtedly, antifungal or antimicrobial plant protection products are not indifferent to human health. Owing to the sterile conditions during the growth of the callus and seedlings, the *in vitro* methods allow avoiding infection or eliminate parasites during the first and most crucial steps in the *Citrus* propagation. Therefore, micropropagation can be useful as a remedy for different diseases of *Citrus limon* varieties and can be useful in obtaining more resistant plants. This can also be an auxiliary method to the efforts of scientists in obtaining new varieties of *Citrus* species that fulfil the constantly increasing demand from farmers and consumers. The results obtained in this paper can be a basis for a further investigation of micropropagation in the valuable varieties of *C. limon*. They can be also used as a basis for investigation with the aim of preservation of older varieties of crops eliminated due to the new demands expressed by farmers and consumers concerning the quality of citrus fruits.

CONCLUSIONS

In the present studies, a protocol was established from leaf explants of *in vitro* grown *C. limon* seedlings. Regeneration in the *Citrus* genus can be obtained through direct and indirect organogenesis with the use of leaf explants, as clearly demonstrated in the present investigation. The highest shoot induction was obtained on the MT me-

dium supplemented with 3.5 mg·L⁻¹ BAP. It was revealed that BAP had a major influence on shoot proliferation in *C. limon*. Rooting was obtained using the MS medium with 1 mg·L⁻¹ NAA.

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REGENERACJA *in vitro* *Citrus limon* L. Burm cv. 'Primofiore' Z EKSPANTATÓW LIŚCIOWYCH

Streszczenie. Organogenezę *in vitro* badano u *Citrus limon* L. Burm cv. 'Primofiore', wykorzystując eksplantaty z liści. Celem badań była optymalizacja warunków do indukcji tkanki kalusowej oraz regeneracji roślin *C. limon*. Eksplantaty *C. limon* wykładano na 16 pożywek różnego rodzaju, które dodatkowo uzupełniano kombinacjami roślinnych regulatorów wzrostu, zarówno auksyn, jak i cytokinin, takimi jak 6-benzylaminopuryna (BAP), kwas naftalenoocetowy (NAA), kwas 2,4-dichlorofenoksyocetowy (2,4-D) i kinetyna. Regenerację pędową uzyskano, gdy eksplantaty liściowe wykładano na pożywkę Murashige and Tuckera uzupełnioną 3,5 mg·L⁻¹ BAP. Badania histologiczne pozwoliły na przedstawienie początkowej fazy rozwoju eksplantatów z liści w trakcie regeneracji *in vitro* *C. limon*.

Słowa kluczowe: *Citrus limon*, fitohormony, inicjacja tkanki kalusowej, auksyny, cytokiny, 6-benzylaminopuryna

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