International Journal for Multidisciplinary Research (IJFMR)
E-ISSN: 2582-2160 ● Website: www.ijfmr.com ● Email: editor@ijfmr.com

Effect of Explant Type and Phytohormone Concentration on Micropropagation of Citrus

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Abstract
Citrus fruits are among the most popular fruits in the world, thanks to their energizing flavor and health advantages. In this work, the ideal levels of activated charcoal and plant growth regulators were determined for micropropagation of lemon. Citrus limon (Osbeck Family: Rutaceae) has been successfully regenerated in vitro using both direct and indirect organogenesis in the current study. For indirect organogenesis, callus was stimulated and multiplied from leaf explants of in vitro grown seedlings on Murashige and Skoog (MS) medium with 6-Benzylaminopurine (BAP) alone or in conjunction with -naphthalene acetic acid (NAA). The placement of explants on MS medium containing BAP alone or in combination with NAA was done for direct organogenesis. For root multiplication, various concentrations of NAA were applied to well-developed microshoots. The frequency of callus induction with BAP(4µl) + NAA(0.2µl) was observed to be 94.5%. When the plants started to root, MS medium treated with 2.0 µl of IBA and 30 g/l of sucrose showed abundant root development. In order to quickly multiply disease-free potential material and take over wide regions in a short amount of time, it may be necessary to establish mature micropropagation methods for new elite lemon cultivars. This phenomena is therefore of fundamental economic relevance. In this study, it was shown that Microshoots were best rooted (90.8%) when IBA (2.0 µl) was treated for 48 hours with activated charcoal.

Keywords: Citrus species, Micropropagation, Invitro rooting, Nodal shoot segment

Introduction

One of the most significant commercial fruit crops in more than 100 nations is citrus fruit,[1]. It is the second largest produced fruit crop in the world after bananas is citrus (> 108 million tonnes) [3] . India ranks sixth in the production of citrus fruits and is the world's top producer of lemons and limes, according to UN data from 2007. About 30% of citrus fruits are processed to create different products, mostly juice. Similarly, the grape industry, which mostly produces wine, is the largest fruit processing industry, surpassing the citrus sector. A common fruit crop, citrus is mostly grown in tropical and subtropical regions of the world. The *Rutaceae* family includes the genus Citrus and related genera *Fortunella*,

Poncirus, Eremocitrus, and Microcitrus. Of these genera, citrus is the one that is planted the most commonly and is known for producing fruits like oranges, mandarins, lemons, limes, and grapefruits. In addition to the seeds, citrus processing also yields byproducts such as peel, pulp, rag, and seeds. These ingredients nevertheless include significant amounts of pectin, polyphenols, essential oils, fiber, and protein. Currently, citrus waste is mostly used as animal feed. Before spreading to the rest of the planet. Despite the fact that the genus Citrus has more than 100 species, citrus taxonomy is complex. The marketplaces for processed juice and fresh fruit are separate[5][7]. The vast majority of citrus species are diploids (2n = 2x = 18), which were domesticated in Southeast Asia many thousands of years ago and then dispersed around the world. Depending on the taxonomist, there are anywhere from 16 to 156 species in the Citrus genus. Orange, mandarin, lemon, and acid are the four subgroups that make up the citrus race. There are various species in each group, and each species has several strains and variants. [6][10]. Citrus is cultivated across India, however the majority of the country's output is concentrated in the states of Andhra Pradesh and Maharashtra.[8]

Citrus may be spread using both sexual and asexual means; typically, rootstocks are spread through seeds, whilst the majority of commercial types are spread using a variety of asexual techniques[2]. Citrus plants cannot be quickly adopted and replaced with new types due to conventional vegetative propagation's lengthy process, dependence on the season, and lack of plant material. Since the seeds of the majority of citrus species are polyembryonic, nucellar seedlings are utilized both for growing uniform rootstock and for direct planting. Citrus viruses are not spread through seeds, thus it also helps to grow healthy plants; however, the same method cannot be used to propagate genotypes that lack seeds. The existence of natural poly-embryony in certain Citrus species offers an effective method for mass reproduction without harming the mature tree. [12][13][11][14][4].

There are also a few commercially significant seedless Citrus clones that can only be grown in large numbers by vegetative tissues. Instead, axillary bud proliferation is frequently used for citrus in vitro propagation because it guarantees the greatest genetic consistency of the resultant plants [5]. The goal of this study was to get the highest average number of branches from axillary bud culture using tissue culture procedures on MS medium with varied hormone concentrations (auxin and cytokinin).

Material and methods

Preparation and sterilization of culture medium

Young meristem cutting explants were inoculated in sterilized MS media that had been treated with various amounts of plant growth regulators (Murashige and Skoog, 1962)[09]. Macronutrients, micronutrients, sugar, gelrite, and vitamins (thiamine and myo-inositol) were all present in the culture medium. The medium was heated in the microwave for ten minutes until transparent after being adjusted to a pH of 5.8. After being put into the bottles, the media was autoclaved at 121°C for 15 psi.

Procedure for Surface sterilization of Explant

Plant material must be first sterilized to remove any bacteria or fungal spores that are present in the explant. By peeling the leaf sheath off of six-month-old, field-grown plants, fresh plant materials (healthy young meristems) were harvested. These tender meristems under one and five centimeters in length were divided into thin pieces. In the laminar air flow cabinet, the explants were placed after being properly cleansed under running water for five minutes. The juvenile meristem explants received a minute-long treatment with 70% alcohol, 10% sodium hypochlorite treatment, and a final rinse with 0.1% mercuric chloride

(HgCl2). The immature meristem cuttings were then properly cleansed three times with sterile distilled water before being inoculated into the sterile nutrient agar media. In the laminar airflow cabinet, the aforementioned procedures were all carried out aseptically.

Incubation of cultures

- The cultures were cultured in a culture environment at a temperature of 26°±2°C with a photoperiod of 16/8 hours of light and dark.

Growing the Plants

Put the surrounding plant tubes inside the BOD. The explant's shoots will likely mature more quickly if it is placed under a fluorescent or grow lamp that provides at least 12 hours of light each day. 28°C is the preferred BOD temperature. New shoots ought to start to appear in two weeks and be fully developed by four. If any of the tubes show indications of infection, inspect them and discard them. Buds may begin to appear in three weeks. After bud creation, the medium will need the rooting hormones for the proper growth. This is transferred to the second rooting media under the same sterile conditions. The roots of the plant begin to form within the first five weeks.

Shoot Proliferation

The original nodal segments' sprouting shoots were subcultured on MS medium supplemented with various growth regulators, such as BAP at 2.0 or 4.0 mg/l with 0.2 or 0.5 mg/l NAA. The following combinations of the growth regulators BAP and NAA were used:

S.No.	Treatment No.	Concentration (mg/l)		
		BAP		NAA
1	T-1	2.0	+	0.2
2	T-2	2.0	+	0.5
3	T-3	4.0	+	0.2
4	T-4	4.0	+	0.5

Table1:Shows different concentrations of plant growth hormone for shoot proliferation.

Root Proliferation

IBA, an auxin, was introduced to the MS medium with or without activated charcoal (200 mg/l) at concentrations of 2 or 4 mg/l to promote effective and speedier root initiation. Every treatment included a steady addition of sucrose, or 30 g/l.

S.No	Treatment No.	Details	Concentration (mg/l)
1	T-1	IBA	2.0
2	T-2	IBA	4.0
3	T-3	IBA+AC	2.0+200
4	T-4	IBA+AC	4.0+200

Table 2: Shows different concentrations of auxin with activated charcoal for root proliferation

Acclimatization

This was also carried out in already developed cultures. The micro-plants were moved aseptically into a glass jar with a polythene lid and agro-peat: soilrite (1:1) moistened with MS (1/4 salts) free of calcium, organic, growth regulators, and sucrose components. Between 5.75 and 5.85, the medium's pH varied. Rubber bands were used to shut the cultured pots after they were wrapped in polythene. Two weeks were spent keeping the culture in the culture chamber. By loosening the cap or poking four to five pin holes every day, the relative humidity within the polythene cover (200 gauge) was progressively lowered.

Transfer of Plantlets to Glass house

The hardened plantlets were then placed in clay pots of a size of 12 inches, filled with a mixture of FYM, sand and garden soil (2:1:2:1), plus one full teaspoon of bone meal and 20 gms of neem cake each pot, and maintained in the field.

Result and Discussion

The experimental findings obtained in the present investigation entitled “Effect of explant type and phytohormone concentration of micropropagation of citrus species” have been presented. The results and trends obtained in the present study have been presented and discussed in this observation:

Shoot Multiplication

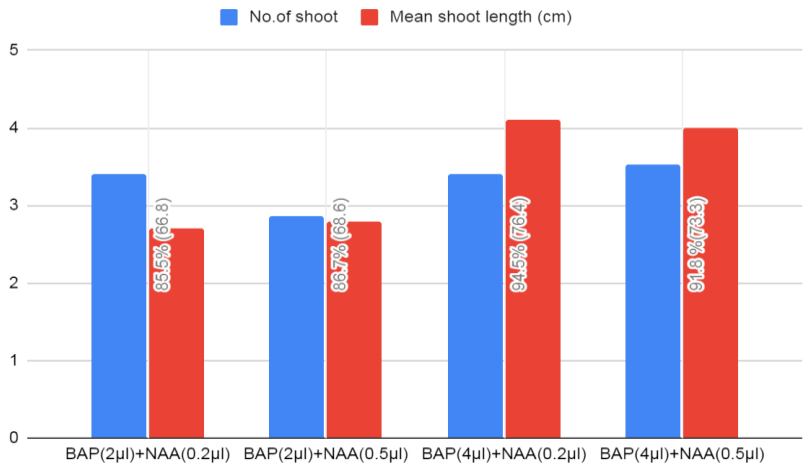

Figure 1 : Effect of growth regulators on in vitro shoot proliferation in citrus species

In the present study the direct shoot organogenesis was achieved without intention of callus. The fortification of basal medium was found essential as basal medium devoid of growth substance did not respond in any of the explant cultures of the species. The addition of cytokinin(BAP) initiated the response and different concentrations of BAP produce various results. Further the addition of auxin(NAA) significantly improved the response for different parameters. It is a well established fact that the optimal ratio of cytokinin; auxin causes the dormant meristematic zone existing on the axillary bud region of the node to initiate shoot organogenesis or differentiation. Efficiency of synthetic cytokinin was found to be better stable in the media.

As evident from data presented in the table, there was a marked difference in shoot multiplication and mean shoot length in the citrus genotypes. The highest number of shoots per culture is 3.52 shoots per explant was recorded with the treatment 4.0mg/l BAP+0.5mg/l NAA. In citrus limon, the longest shoots (4.1cm) were produced in the medium supplemented with 4.0mg/l BAP + 0.2mg/l NAA followed by 4.0 cm with 4.0 mg/l BAP + 0.5mg/l NAA.

Root Multiplication

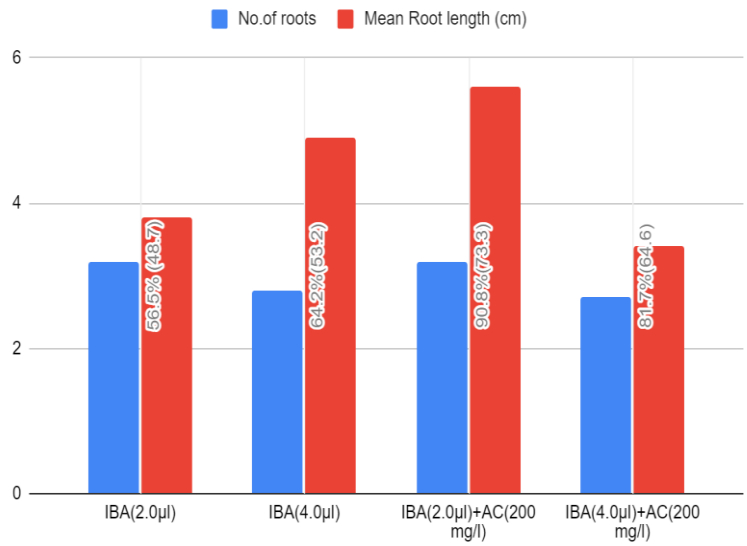

Figure 2 : Effect of growth regulators on in vitro root proliferation in citrus species

The different auxin of supplemented media with or without charcoal influence the rooting of microshoots. As evident the maximum rooting registered with IBA(2.0mg/l) + activated charcoal (200 mg/l) in citrus limon followed by 90.8%. The maximum is delayed with 4.0 mg/l IBA + 200 mg/l activated charcoal in MS medium for citrus limon.

The Ultimate success of the plant tissue culture as a commercial means of plant propagation depends on the ability to transfer the plants out of culture on a large scale with a high survival rate. The water stress caused during the acclimatization has been reported to be a major cause for the reduced survival in the hardening process.

The reduced survival may be attributed to the physiological changes that take place during the transition of Sampling from heterotrophic to autotrophic condition survival during hardening of citrus limon.

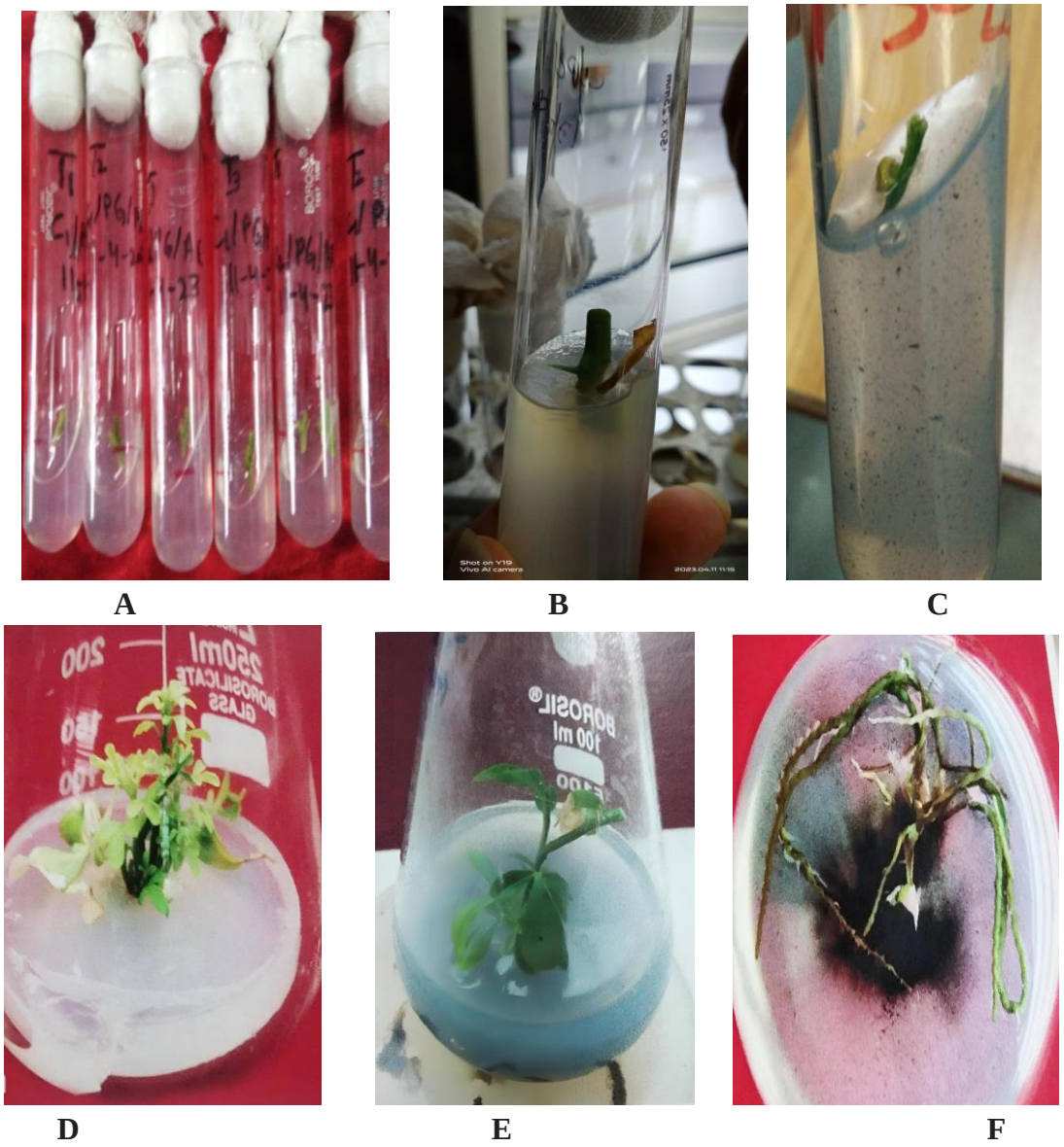

Figure 3 : Micropropagation of C.limon through nodal segment A. Inoculation of explant into the MS medium. B. Production of shoot in 15 days of C. limon on amended MS + BAP (2µl) + NAA (0.5µl). C. Transfer of explant into the activated charcoal after 30 days having plant growth hormone. D. Production of multiple shoots in 4.5 days by repeated transfer of explants amended on MS + BAP (4µl) + NAA (0.5 µl) . E. Transfer of shoots into the activated charcoal media after 8 days amended on MS + IBA (2µl)+Activated charcoal (200 mg/l). F. well developed rooted in 18.4 days of C.limon by repeated transfer of microshoots plantlets.

Conclusion

In the present study we have to achieve the direct and indirect organogenesis-based micropropagation method for citrus limon. The MS medium fortified with BAP and NAA, which may be effective for bulk multiplication of chosen elite cultivars, produced the greatest results for direct organogenesis. Similar to how explants cultivated in MS media mixed with IBA and activated charcoal showed the optimum responses for indirect organogenesis, this method may be effective in the future for the regeneration of new "Citrus limon." In in vitro produced microshoots, the IBA containing MS media was able to stimulate

90.8% root growth, however more trials are needed to authenticate this research for the survival of these in vitro raised plants in outdoor circumstances.

Abbreviations and Acronyms

MS= Murashige and skoog medium, IBA= Indolebutyric acid, NAA= Naphthalene acetic acid, BAP = 6-benzyl aminopurine, AC= Activated Charcoal.

Conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of this article.

Acknowledgement

I extend my heartiest gratitude to my assistant Dr. Anjali Rathi and Dr. Sonia Sharma for her guidance, constant encouragement and assistance during the course of preparation of my research paper.

I also thank my family, friends and teachers for giving a helping hand in the successful completion of the research paper.

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