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In vitro regeneration of Philippine lime (*Citrus microcarpa*) and yellow *citrus* fruit (*C. limon*) through immature embryo culture

Priyanshi Gadhiya, Anshul Das and Swati Patel

Abstract
"A protocol for *in vitro* regeneration of mature plants of Philippine lime and yellow *citrus* fruit using plant part as explant such as seed has been developed for multiplication from seed concentration of BAP, KIN and NAA were used from them. Highest seed germination in terms of the total number of Explant observed in half concentration of MS media containing BAP + NAA + KIN. The establishment of sterile *in vitro* regeneration of Philippine lime and yellow *citrus* fruit is a major challenge because of contamination. The use of multiple concentrations of alcohol and sodium hypochlorite along with tween-20 was considered the best treatment for the reduction of contaminants".

Keywords: Philippine lime, yellow *citrus* fruit, micro propagation, sterilization

Introduction

In order to contribute to human nutrition and economic value, *citrus* is a crucial crop that is grown extensively and commercially over the world (Ollitrault and Navarro 2012) [32]. Around the world, tropical and subtropical locations are used for *citrus* species cultivation. From the Himalayas through north-central China, India, the Philippines, Burma, Thailand, Indonesia, and New Caledonia, the native lemon is grown (Scora 1975) [33]. The productivity of lemons in India is 8.5 tone/hector8, whereas it is 10% internationally and 25% nationally (French and Bressler 1962) [3]. Trees blossom and produce fruit practically immediately after being planted (Devos, Van Landeghem, and Deschoolmeester 2012) [34].

Lemon species are categorized according to various characteristics relating to their repeatability and high heterozygosity, which provide significant challenges to breeding initiatives. Due to physiological limitations involved with sexual reproduction, such as heterozygosity and polyembryony, standard methods have little impact on creating new species when it comes to lemons. Lemon plantations deal with a variety of issues, including pests, poor growth, disease susceptibility, low-temperature sensitivity, and significant storage loss. A technique called *in vitro* culturing can be modified to get around these issues.

The calamondin tree (Cirrus *microcarpa*) is also known as Philippines Lemon, calamansi, calamansi, kalamonding, and *Limon* sito. This tree was developed in China as a result of the crossbreeding of mandarin and kumquat, and it is now widely cultivated throughout several states in India and other south Asian nations.

Table 1: Scientific Classification of Calamondin tree is

Kingdom	**Plantae**
Subphylum	Angiosperms
Class	Eudicots
(Unranked)	Rosids
Order	Sapindales
Family	Rutaceae
Genus	*Citrus*
Species	*Microcarpa*

This shrub is tall, measuring between two and seven meters. After planting, this tree needs 5 to 6 years to start producing fruit. From flowers to fruit, the entire fruit development process takes about 5 months (Xin *et al.* 2022) [13]. The nutrients calcium, phosphorus, iron, and vitamin C make up the lemon fruit's nutritional worth. From 0.75 kg to 50 kg of fruit are.

Produced for a 3 year old to a 10 year old. The fruit's adaptable climate is warm, but it can also thrive in cold, frost-free climates. The best soil for this species is well-drained, sandy, or clay loam soil that is rich in organic matter, has a pH range of 5.5 to 7.0, and receives 1500- 2000 mm of precipitation per year.

The calamondin fruits are well recognized for their enormous number of uses, including its acidic juice, which is used to flavor dishes with seafood and meat, as well as their use as ice cubes in beverages like tea, soft drinks, and cocktails (Nguyen *et al*. 2018) [14]. It also has a number of additional medical applications. For instance, natural acne treatments, cough medicines, and remedies for constipation can all relieve the swelling and itching brought on by insect bites. Additionally, as a poisonous antidote (Cheong *et al*. 2012) [15].

Similar to this, the Rutaceae family of flowering plants includes the small evergreen tree species *Citrus Limon* (Rivera *et al*. 2022) [16], which is indigenous to South Asia, primarily Northeast India. This fruit has several names (*Citrus Limon* [L.] *Burm*.) The common name for the yellow *citrus* fruit is citron, which also appears in German, Italian, French, French, and Zitrone.

Table 2: Scientific classification of *Citrus Limon*

Kingdom	Plantae
Order	Sapindales
Family	Rutaceae
Genus	*Citrus*
Species	*C. limon*

A genomic analysis of lemon revealed that it is a cross between citron and bitter orange. Lemon is a fruit that contains plenty of vitamin C, much like all other *citrus* fruits. Numerous phytochemicals, including polyphenols, terpenes, and tannins, are present in lemons. Lemon juice contains slightly more citric acid per litre (47 g/l) than lime juice, about five times as much citric acid as orange juice, and about twice as much citric acid as grapefruit. Lemon is also used in odd ways, such as in the creation of marmalade, lemon liquor, and lemon curd (Kanmani 2014) [1]. Additionally, it is employed to stop the oxidation (enzymatic browning) of some fruits, such as bananas, apples, and avocados. Tea, grilled meats, and different kinds of seafood have all been prepared using tree leaves in the past.

By applying contemporary plant tissue culture techniques, micropropagation is a successful technology for the rapid multiplication of stock plant material for the generation of offspring plants on a vast scale. This process results in the multiplication of genetically altered or conventionally bred plants. The different techniques for micropropagation, such as meristem, protoplast, callus, suspension, embryo culture, etc., are listed here. Since a few years ago, Philippine lemon has been grown in India, however, each plant has a high market value of roughly 150 rupees. To get over this problem, micropropagation from different explants like nodes, shoot tips, and leaves have been used to demonstrate in-vitro plant regeneration of the calamondin species, C. *microcarpa*. As an asexual technique that can be utilized to produce clone plants, micropropagation systems and rates of multiplication are crucial. By using tissue culture, specifically direct organogenesis, the plant was effectively regenerated *in vitro*, producing plants that possessed the desired traits for lemon or other fruit crops grown for commercial purposes.

Materials and Methods

The Department of Biotechnology, ASPEE SHAKILAM Biotechnology Institute, Surat, Navasari Agricultural University, India conducted this study from 2019 to 2020 with the title "Standardized protocol for in-vitro regeneration of Chinese Lemon (C. *microcarpa*) and *Citrus limon*." Plants' mature seeds were germinated to create young plantlets, which were then maintained in MS medium with hormone treatment.

Source of explants materials

Three Hundred seeds of Philippine lime and yellow *citrus* fruit were collected from Vrundavan nursery, Surat.

Explant Inoculation and stock solution preparation

Fresh healthy seeds of Philippine lime and yellow *citrus* fruit were employed as an explant for the *In-vitro* germination in half-strength Murashige and Skoog (MS) regeneration medium (Bose and Sarma 1975) [35]. Each explant was inoculated in a test tube and Glass bottle (250 ml) with screw caps with the help of sterile forceps inside the laminar airflow chamber. The test tubes and bottle were moved to the culture chamber after the inoculation and incubated at 25'C. (Tucker and Murashige 1968) [26]. 40 W white fluorescent tube light was used to maintain the photoperiod at 16 hours of light and 8 hours of darkness (Holcomb and Berghage 2001) [27]. For three months, data were logged once a week. The necessary amount of chemicals were dissolved in double-distilled water to create the stock solutions, which were then chilled before being stored. Growth regulators were freshly made every week, and stock solutions were prepared every 4-6 weeks (Skirvin *et al*. 1986) [28]. The medium's pH was adjusted to 5.8 using agar (8 gm /lit) that had been boiled, poured into appropriate bottles, then autoclaved 5 (Debergh 1983) [29].

Preparation of medium

On half-strength MS (murashige and Skoog, 1968) [26] basal medium enriched with hormonal concentrations of BAP + NAA, BAP + NAA + KIN, 2, 4- D + BAP + NAA, and 12 MS medium without growth regulator, freshly collected seeds of two kinds were cultivated. The media's PH was changed from 5.8 to 6.0.

Preparation of growth hormone stock

The standard procedures of preparation of growth hormone stock solutions were followed as described below. The growth hormones are added to media from stock solution at the required concentration.

Table 3: Solvent concentration for growth hormones

Growth Hormone	Stock solution concentration (mg/10ml)	Solubility in
BAP	10	0.1 N Na OH
NAA	10	0.1 N Na OH
Kinetin	10	0.1 N Na OH
2,4-D	10	0.1 N Na OH

Standardization of Multiplication Media

The trial makes use of the widely utilized MS media. To reduce contamination and try to standardize the best shoot multiplication medium for *Citrus microcarpa* and *Citrus Limon*, the mediums were treated with various amounts of growth regulators in various ways and concentrations. The treatment information is included below.

Table.4: Seed explant: Hormones were added in Multiplication Media during media preparation**.**

No	Name of Media	Concentration Of Hormones
1	N1	Half strength MS
2	N2	Half strength MS + 2,4-D: BAP: NAA (4: 2: 1)
3	N3	Half strength MS + BAP: NAA (2: 1)
4	N4	Half strength MS + BAP: NAA: KIN (1: 4: 2)

Sterilization of media, culture vessels, and instruments

The culture vessels were autoclaved for 20 minutes at a temperature of roughly 1210C under a pressure of 15 lb /inch. The autoclaved media-containing culture vessel was then moved into an air-conditioned space. They were kept for a minimum of four days before being used.

Scalpels, scissors, forceps, Petri dishes, beakers, and other tools were initially wrapped in aluminium foil before being autoclaved, then maintained in a cabinet with no dust.

Standardization of surface sterilization method for the Explants

A trial was done utilising several chemicals/sterilizing agents as listed below in order to standardize the most efficient surface sterilisation process to isolate contaminants-free seed explants of *Citrus microcarpa* and *Citrus Limon* for culture establishment. The sterilization procedures were carried out in an aseptic laminar airflow cabinet. Three to four times of sterilised double distilled water were used to remove any traces of sterilization. The explants were then injected into an MS medium that contained various hormone concentrations.

Table 5: Sterilization methods

No.	Sterilant used	Time duration
T1	0.1%Tween-20 0.1% $Hgcl_2$	15 min 10 min
T2	20% NaOCl	20 min
T3	20% NaOCl 75µl Streptomycin	20 min 7 min
T4	95% alcohol 70% alcohol 5% sodium hypochlorite 2-3 drops of tween-20	1 min 30 s 5 min in

Inoculation of seed

Each hormonal combination had two replications in a separate time frame, and approximately 10 seeds were cultivated in each combination on a separate bottle for seed germination (a gap between 15 days). The effect of the hormone combination on seed germination was measured by visual observation over a period of 7 days for each replication using 10 samples.

Culture condition

At a relative humidity of 55 + 5% and a temperature of 26 + 20C, all the cultures were incubated in the culture room. Fluorescent tubes with a 16:8 hour light/dark cycle were used to illuminate cultures, and they were kept 50 cm above the surface of the bench (3000 lux) (Sharaf El-Din *et al*. 2011) [36]. (Tucker and Murashige 1968) [26].

1.1. Observation recorded

After three weeks of inoculation, several measurements on a % scale were made, including the percentage of culture establishment, the percentage of non-responsive explants, the length of the shoot (inches), and the percentage of culture contamination. Furthermore, the contamination of explants was calculated by using the following formula.

$$\text{Contaminated explants (\%)} = \frac{\text{No. of contaminated explants}}{\text{Total no. of explants used}} \times 100$$

Results and Discussion

Standardization of protocol

Citrus microcarpa and *Citrus Limon* are the most fruiting commercially significant crops that can be reproduced *in vitro* tissue culture. There will be a number of procedures using micropropagation technology, as well as different cultural and environmental contexts. The physiological preconditioning of explants and establishment of the culture are stages 1 and 2, *in vitro* rooting is stage 3, and acclimation of the plantlets produced *in vitro* and transferred to the field are stages 4 and 5. Results of research projects titled "*In vitro* regeneration of Philippines lime and yellow *citrus limon*" carried out at the Department of Plant Biotechnology, ASPEE SHAKILAM BIOTECHNOLOGY Institute, Surat, Navsari Agricultural University, India, in 2019–20.

Micropropagation techniques

For the best results, a number of elements, including contamination issues, surface sterilization agents, explant sources, plant growth hormones, PH, sucrose, light, etc., were taken into consideration when standardizing the process of micropropagation of the Philippines lime and yellow *citrus Limon*.

Surface sterilization treatment for seed explants Philippines lime and yellow citrus *Limon*

The yellow *citrus Limon* and Philippines lime seed from the nursery were typically heavily contaminated with fungus and bacterial spores. As a sterilant to lessen contamination in the culture, sodium hypochlorite (NaOCl) surface sterilization has been described.

The highest survival rate (95%) and lowest contamination rate (5%), as well as the maximum shoot length and number of shoots per explant, were recorded for treatment T_4. Although treatment T_1 caused explant destruction, there was no reaction for explant establishment. Treatment T_2 had the largest proportion of contamination, and treatment T_3 had the lowest

survival (30%) and most contamination (70%).
In T4 treatment, where seeds are sterilized with 95% alcohol, 70% alcohol, 5% sodium hypochlorite, and 2-3 drops of tween-20, the maximum survival of yellow *citrus* lemon and Philippines lime is 68% and 62%, respectively.

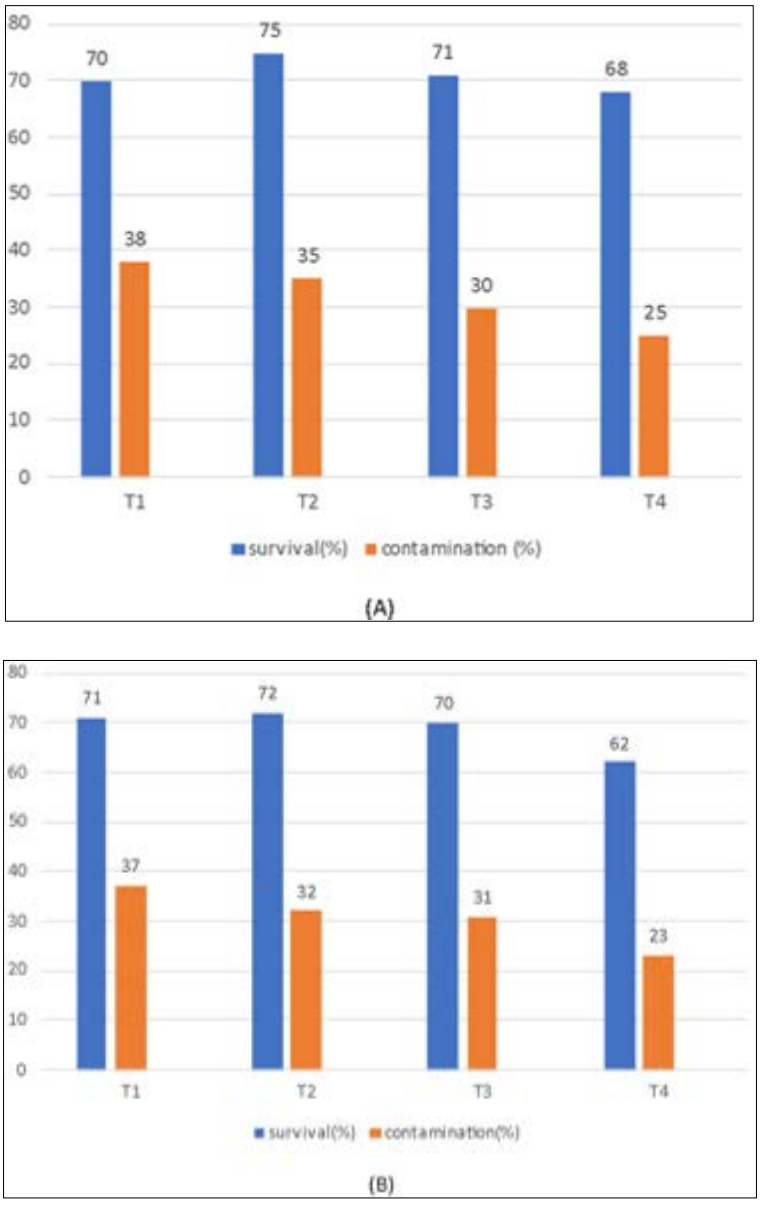

Fig 1: (A) Surface sterilization treatment for seed explants yellow *citrus Limon*, (B) sterilization treatment for seed explants Philippines lime

Standardization of Multiplication Media

Effect of medium composition on multiple shoot induction from seed

The basal medium was supplemented with two cytokinin in order to determine the best and most acceptable cytokinin concentration for the *in vitro* multiplication of the yellow *citrus Limon* and Philippines lime. *Citrus* seeds have an extremely short lifespan because they are vulnerable to drying out while being stored, which causes them to lose their viability (Johnston, 1968) [37]. This is why citron seed that had recently been extracted was used. In the instance of the sweet orange, removal of the seed coat demonstrated an early response for shoot production; the maximum (70%) shoot formation was obtained from seeds without the seed coat.

Table 6: Standardization of Multiplication Media

Species	Treatments (mg/L)	Growth rate	Shoot length (cm, Mean ± SD)	Shoot multiplication (Mean ± SD)	Seed germination Mean ± SD
Yellow *citrus limon*	MS basal 2,4-D + BAP + NAA {callus obtained} BAP +NAA (2:1) BAP +NAA +KIN (1:4:2)	Low - Average Good	0.733±0.251661 - 1.4±0.2 2.8±0.264575	0.666±0.577350269 - 1.33±0.577350269 2.66±0.577350269	14.66±1.527525232 - 19.33±0.577350269 24.33±0.57735
Philippines lime	MS basal 2,4-D +BAP +NAA {callus obtained) BAP +NAA (2:1) BAP +NAA +KIN (1:4:2)	low- Average Good	1.5±0.5 - 2.6±0.44.833±0.251661	0.666±0.577350269 - 1±0 3.33±0.577350269	15.33±2.516611478 -201 24±1

In the yellow *citrus limon*, the maximum growth rate, plant height (2.8 cm), number of shoots (2.66), and seed germination (98.66%) are present in the N4 treatment, which uses media supplemented with half strength MS + BAP, NAA, and KIN. Different outcome parameters for the two species, including the Maximum Number of Shoots (2.66) in the yellow *citrus limon* and (3.3) in the Philippines lime, the Maximum Plant Height (2.8 cm) in the yellow *citrus limon* and (4.8 cm) in the Philippines lime, and the Maximum Seed Germination (98.66%) in the yellow *citrus limon* and (96%) in the Philippines lime, were present in the N4 treatment, in which media was supplemented with Half strength MS + BAP + NAA + KIN.

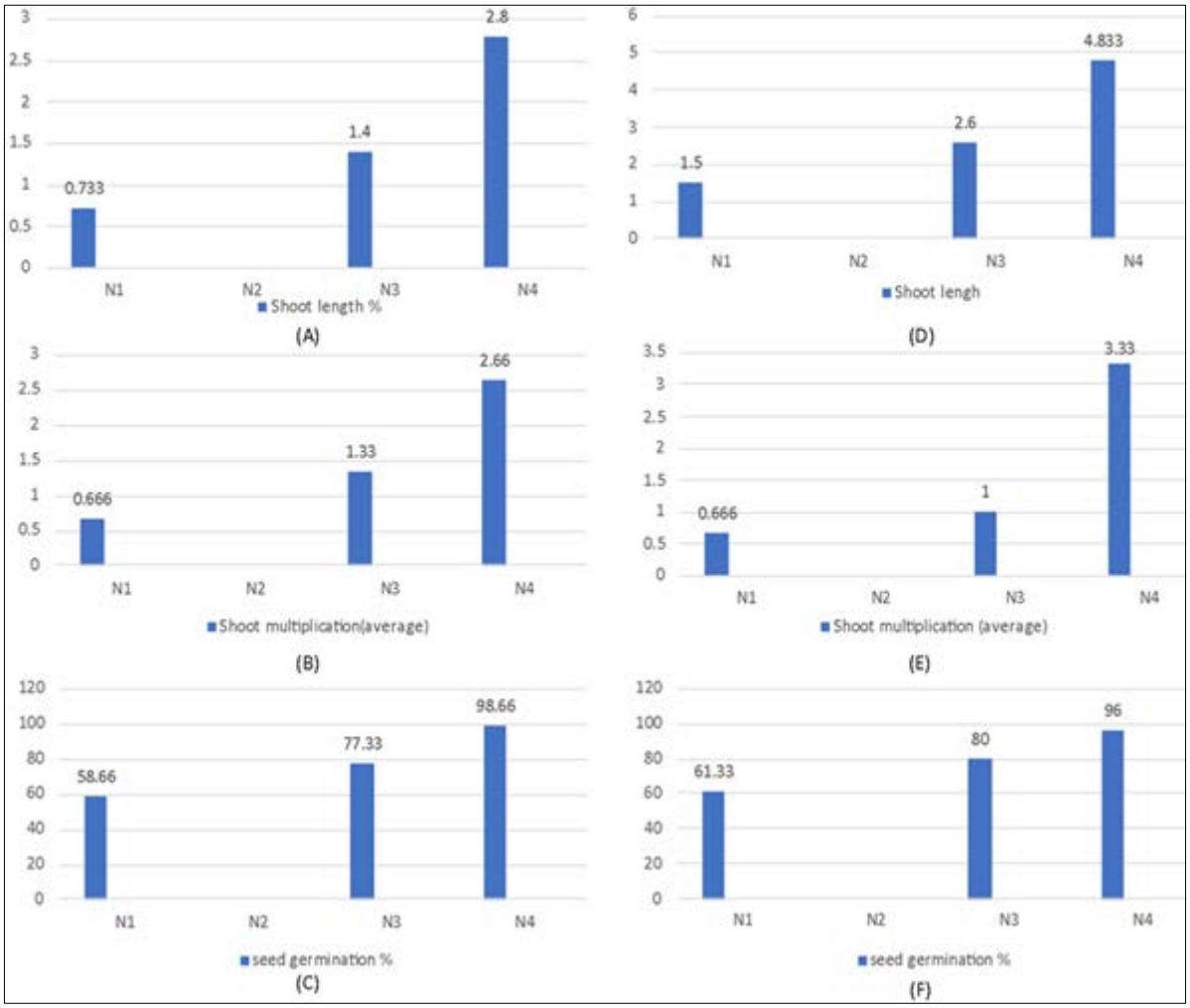

Fig 2: (A) Shoot length of yellow *citrus limon*, (B) shoot multiplication in yellow *citrus limon*, (C) seed germination in yellow *citrus limon*, (D) shoot length of Philippines lime, (E) shoot multiplication in Philippines lime, and (F) seed germination in Philippines lime

In this experiment, plantlets of the citron species that were 5 weeks old were obtained directly from seed. 98% of seeds germinate when grown on half-strength MS media supplemented with BAP, NAA, and KIN.

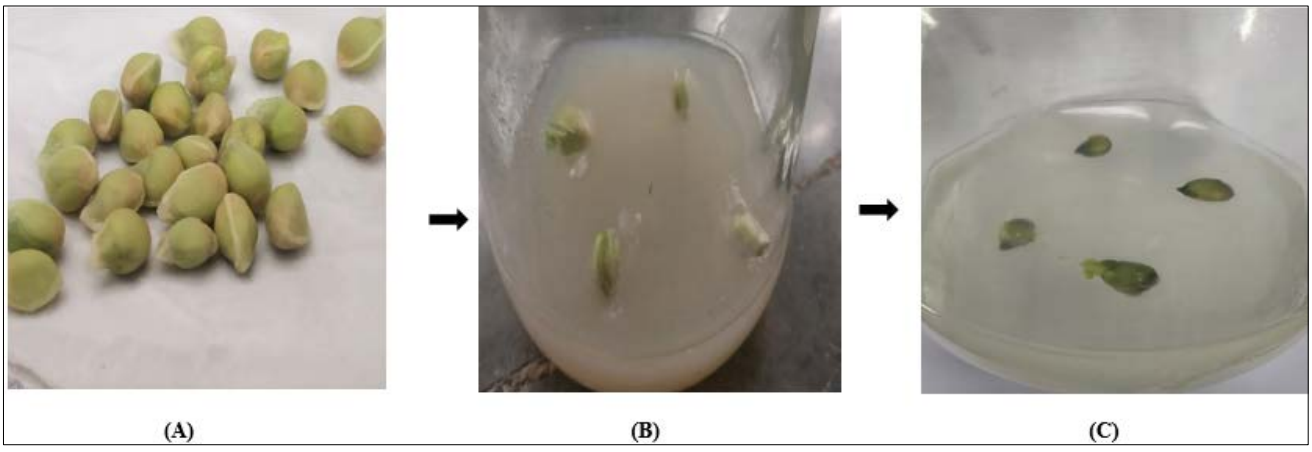

Fig 3: (A) Seed before inoculation, (B) seed after inoculation, (C) seed after 2 day

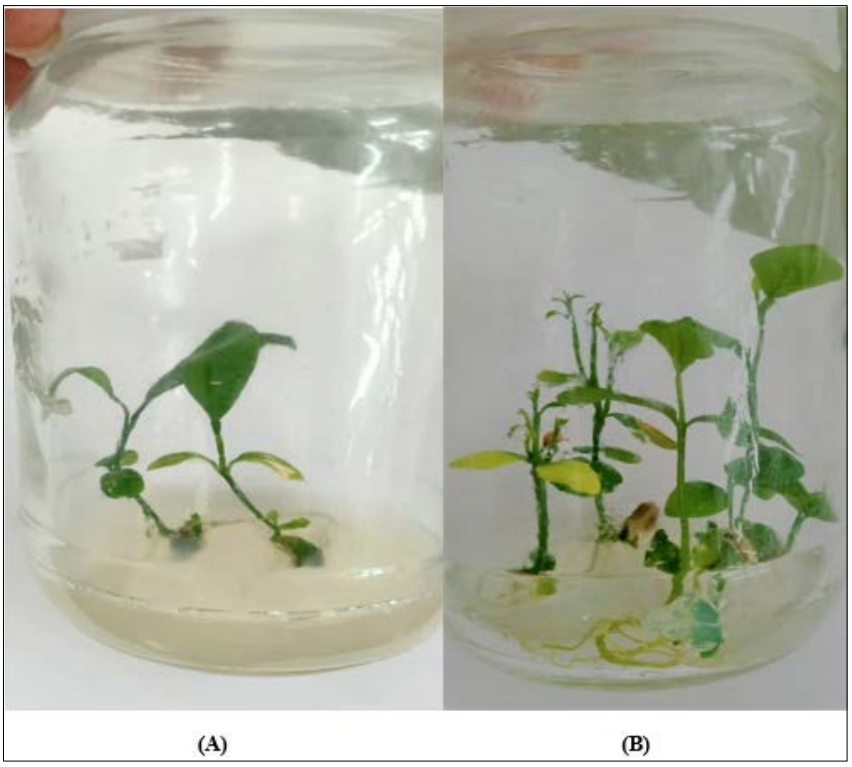

Fig 4: Plantlets were obtained using BAP, KIN AND NAA for (A) yellow *citrus Limon* (B) Philippines lime

Discussion

A protocol has been devised for the production of numerous fruit crops, and the plant tissue culture technique is currently being effectively used for the quick creation of consistent and superior quality planting material. For micro-propagation, a variety of tissues can be employed as explants from various crops. In the current study, we used an *in vitro* cultivated seed explant to produce shoots using micropropagation. Explant from mature plants displayed the highest level of contamination due to the presence of bacteria, fungus, and adherent soil and dust particles. Therefore, the explants must undergo a successful surface sterilisation procedure prior to inoculation.

Effect of surface sterilization treatment

In prior research, the survival rate for the yellow *citrus Limon* and the lime in the Philippines was around 58%; however, in my research, these rates were increased to 68% and 96%, respectively (Mohammad *et al*. 2015) [38].

Effect of BAP, KIN and NAA on seed explant response

The germination rate in the previous literature review was around 90%, but in the part of the study presented here, it was improved through a variety of parameters, including plant height (2.8 cm), number of shoots (2.66), and seed germination (98.66%) in the yellow *citrus limon* and (96% in the Philippines lime) (Mohammad *et al*. 2015) [38].

References

1. Ollitrault P, Navarro L. *Citrus*. Fruit Breeding; c2011. p. 623-662.
2. Rainer WS. On the history and origin of *citrus*. Bulletin of the Torrey Botanical Club. 1975;102(6):369-375.
3. French BC, Bressler RG. The Lemon Cycle. Journal of Farm Economics. 1962;44(4):1021.
4. Nils Urbach, Benjamin Müller (auth.), Yogesh K, Dwivedi Michael R, Wade Scott L. Schneberger (eds.) Integrated Series in Information Systems 28 Springer-Verlag New York, Year; c2012.
5. Zhang H, Langham D, Zhao Y, Khalafalla A, Miao H. Traditional Breeding in Sesame. In the Sesame Genome Springer, Cham; c2021. p. 145-158.
6. Chaubey CN, Senadhira D. Conventional plant breeding for tolerance to problem soils. In Soil Mineral Stresses. Springer, Berlin, Heidelberg; c1994. p. 11-36.
7. Heim RH, Wright IJ, Scarth P, Carnegie AJ, Taylor D, Oldeland J. Multispectral, aerial disease detection for myrtle rust (*Austropuccinia psidii*) on a lemon myrtle plantation. Drones. 2019 Mar 7;3(1):25.
8. Rappaport Jacques. *In vitro* culture of plant embryos and factors controlling their growth. The botanical review. 1954;20(4):201-225.
9. Winkelmann T, Geier T, Preil W. Commercial *in vitro* plant production in Germany in 1985–2004. Plant cell, tissue and organ culture. 2006 Sep;86(3):319-27.
10. Dang T, Tan SH, Bodaghi S, Greer G, Lavagi I, Osman F, *et al*. First report of *Citrus* Viroid V naturally infecting grapefruit and calamondin trees in California. Plant disease. 2018;102(10):2050-2050.
11. Marutani M. Calamansi (× *Citrofortunella microcarpa*) for potential *citrus* fruit production for the island of Guam. In International Symposia on Tropical and Temperate Horticulture-ISTTH2016 1205; c2016 Nov 20. p. 419-424.
12. Sharma, Anuj, Christopher M. Ference, Deepak Shantharaj, Elizabeth A. Baldwin, John A. Manthey, and Jeffrey B. Jones. Transcriptomic analysis of changes in *Citrus*× *microcarpa* gene expression post Xanthomonas citri subsp. citri infection. European Journal of Plant Pathology. 2022;162(1):163-181.

13. Xin YH, Wu YX, Qiao B, Su L, Xie SQ, Ling P. Evaluation on the phenotypic diversity of Calamansi (*Citrus microcarpa*) germplasm in Hainan island. Scientific Reports. 2022 Jan 10;12(1):1-2.
14. Nguyen TN, Huynh TN, Tran VT, Dang CH, Hoang TK, Nguyen TD. Physicochemical characterization and bioactivity evaluation of essential oils from Citrus microcarpa Bunge leaf and flower. Journal of Essential oil rEsEarch. 2018 Jul 4;30(4):285-92.
15. Cheong Mun Wai, Zhi Soon Chong, Shao Quan Liu, Weibiao Zhou, Philip Curran, Bin Yu. Characterisation of calamansi (*Citrus microcarpa*). Part I: Volatiles, aromatic profiles and phenolic acids in the peel. Food chemistry. 2012;134(2):686-695.
16. Rivera Diego, Antonio Bermúdez, Concepción Obón, Francisco Alcaraz, Segundo Ríos, Jorge Sánchez-Balibrea, *et al*, Robert Krueger. Analysis of Marrakesh *limetta*' (*Citrus*× *limon* var. *limetta* (Risso) Ollitrault, Curk & R. Krueger) horticultural history and relationships with limes and lemons. Scientia Horticulture. 2022;293:110688.
17. Kanmani P, Dhivya E, Aravind J, Kumaresan K. Extraction and analysis of pectin from *citrus* peels: augmenting the yield from *Citrus limon* using statistical experimental design. Iranian (Iranica) Journal of Energy & Environment. 2014;5(3):303-312.
18. Oğuz Hİ, Oğuz İ. Determination of Energy Usage Efficiency and Greenhouse Gas Emissions of Lemon (*Citrus limon* L.) Production in Turkey: A Case Study from Mersin Province. Erwerbs-Obstbau. 2022 Jun 22:1-9.
19. Narayanan Meyyappan, Divya Baskaran, Vasumathi Sampath. Experimental design of hydrotropic extraction for recovery of bioactive *limon* in from lemon (*Citrus limon* L.) seeds. Separation Science and Technology. 2022;57(5):707-718.
20. Mohanapriya M, Lalitha Ramaswamy, Rajendran R. " Health and medicinal properties of lemon (*Citrus limon* um). " International Journal of Ayurvedic and Herbal Medicine. 2013;3(1):1095-100.
21. Nowakowska M, Pavlović Ž, Nowicki M, Boggess SL, Trigiano RN. *In vitro* propagation of an endangered Helianthus verticillatus by axillary bud proliferation. Plants. 2020 Jun 3;9(6):712.
22. George EF, Hall MA, De Klerk GJ. Plant propagation by tissue culture. The background. Plant Propagation by Tissue Culture. 2008;1:205-26.
23. Ghosh SN. Proceedings of the International Symposium on Minor Fruits and Medicinal Plants for Health and Ecological Security (ISMF & MP), West Bengal, India, 19-22 December, 2011. In Proceedings of the International Symposium on Minor Fruits and Medicinal Plants for Health and Ecological Security (ISMF & MP), West Bengal, India, 19-22 December, 2011. Bidhan Chandra Krishi Viswandyalaya; c2012.
24. Navarro L, Juarez J. Tissue culture techniques used in Spain to recover virus-free *citrus* plants. In Symposium on Tissue Culture for Horticultural Purposes. 1977;78:425-436.
25. Müller Hermann, Md Rafiqul Islam, Rüdiger Raue. Research on infectious bursal disease—the past, the present and the future. Veterinary microbiology. 2003;97(1-2):153-165.
26. Tucker David PH, Murashige TOSHIO. High temperature growth effects on *Citrus limon* fruit tissue as studied *in vitro*. Journal of Horticultural Science. 1968;43(4):453-461.
27. Holcomb E Jay, Robert Berghage. Photoperiod, chilling, and light quality during daylight extension affect growth and flowering of tissue-cultured Easter lily plants. Hort Science. 2001;36(1):53-55.
28. Skirvin Robert M, Mel C Chu, Mary L Mann, Heather Young, Joseph Sullivan, and Thomas Germania. Stability of tissue culture medium pH as a function of autoclaving, time, and cultured plant material. Plant Cell Reports. 1986;5(4):292-294.
29. Debergh PC. Effects of agar brand and concentration on the tissue culture medium. Physiologia plant arum. 1983;59(2):270-276.
30. Mészáros Annamária, Andrea Bellon, Éva Pintér, Gábor Horváth. Micropropagation of lemon balm. Plant cell, tissue and organ culture. 1999;57(2):149-152.
31. Kamruzzaman M, Akther A, Faruq O, Pervin A, Myti S, Prodhan SH. Establishment of an efficient callus induction method from leaf and stem in kinnow mandarin (*Citrus reticulata* Blanco.) and citron (*Citrus medica* L.). African Journal of Biotechnology. 2015;14(15):1290-6.
32. Ollitrault P, Terol J, Chen C, Federici CT, Lotfy S, Hippolyte I, Ollitrault F, Bérard A, Chauveau A, Cuenca J, Costantino G. A reference genetic map of C. clementina hort. ex Tan.; citrus evolution inferences from comparative mapping. BMC genomics. 2012 Dec;13(1):1-20.
33. Scora RW. On the history and origin of Citrus. Bulletin of the Torrey Botanical Club. 1975 Nov 1:369-75.
34. Devos J, Van Landeghem H, Deschoolmeester D. SMEs and IT: Evidence for a market for lemons. Electronic Journal of Information Systems Evaluation. 2012 Jan 1;15(1):26-35.
35. Bose KS, Sarma RH. Delineation of the intimate details of the backbone conformation of pyridine nucleotide coenzymes in aqueous solution. Biochemical and biophysical research communications. 1975 Oct 27;66(4):1173-9.
36. Ibrahim F, Sharaf El-Din MK, Eid MI, Wahba ME. Validated stability indicating liquid chromatographic determination of ebastine in pharmaceuticals after pre column derivatization: application to tablets and content uniformity testing. Chemistry Central Journal. 2011 Dec;5(1):1-5.
37. Johnston JP. Some observations upon a new inhibitor of monoamine oxidase in brain tissue. Biochemical pharmacology. 1968 Jul 1;17(7):1285-97.
38. Mohammad SM, Kiritchenko S. Using hashtags to capture fine emotion categories from tweets. Computational Intelligence. 2015 May;31(2):301-26.