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# ***De novo*** **organogenesis in** ***Citrus latipes*** **(Swingle) Yu. Tanaka:*in vitro*** **root and shoot development from cotyledonary explants**

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**Abstract**

*This study investigates the effects of different concentrations of Naphthaleneacetic Acid (NAA) and 6-Benzylaminopurine (BAP) on the morphological growth parameters of Citrus latipes (Swingle) Yu. Tanaka, with a focus on cotyledon as the potential explant. The optimal concentrations for individual application were identified as 0.4 µM for NAA and 0.3 µM for BAP. Combination of both regulators resulted in enhanced growth, with cotyledon explants demonstrating superior responsiveness and uniform morphological development. Heatmaps were utilized to visualize the effects of various concentrations, while network plots illustrated the influence of each treatment on growth parameters. Additionally, the Bray-Curtis index of dissimilarity was applied to assess correlation between treatments and morphological traits. The findings emphasize the crucial role of cotyledon explants in optimizing in vitro propagation protocols. This study provides valuable insights into the hormonal interplay governing organogenesis in C. latipes, contributing to the refinement of micropropagation strategies for conservation and agricultural applications.*

**Keywords:** Cotyledons, *Citrus latipes* (Swingle) Yu. Tanaka, *in vitro* propagation, shoot and root induction.

## Introduction

The genus *Citrus* (family: Rutaceae) comprises several commercially and ecologically significant species cultivated worldwide for their fruits, medicinal properties, and economic value (Nassarawa *et al.* 2024). *Citrus latipes* (Swingle)Yu. Tanaka, commonly known as Khasi papeda, is a lesser-known but important species endemic to Northeast India (Upadhaya *et al.* 2016). It is valued for its genetic diversity, adaptability and potential use in breeding programs for disease resistance and stress tolerance (Arlotta *et al.* 2024). However, conventional propagation methods of *C. latipes*, including seed germination and vegetative propagation, are limited by factors such as long juvenility, low seed viability and susceptibility to biotic and abiotic stresses (Conti *et al.* 2021). Therefore, the development of efficient *in vitro* propagation techniques is crucial for its conservation, mass propagation and genetic improvement.

Tissue culture-based propagation offers a promising alternative for the multiplication and conservation of elite *Citrus* germplasm (Iqbal *et al.* 2019). Among the different explants used in *in vitro* regeneration, cotyledonary explants have shown potential in several plant species due to their high regeneration capacity and responsiveness to growth regulators (Kato 1986; Jhankare *et al.* 2011; Sharma and Srivastava 2014; Gambhir *et al.* 2017; Rajput *et al.* 2022). However, the morphogenetic responses of *Citrus* species cotyledonary explants to different culture conditions remain largely unexplored. The induction of adventitious shoots and roots from cotyledonary explants can provide an effective system for rapid multiplication, genetic transformation studies and cryopreservation.

The success of organogenesis in *Citrus* is largely influenced by the choice of explant, culture medium and the auxin-to-cytokinin ratio rather than the individual concentrations of plant growth regulators (PGRs). Previous studies on *Citrus* species have demonstrated that an optimal balance between cytokinins, such as 6-benzylaminopurine (BAP) and kinetin (Kn), and auxins, such as indole-3-butyric acid (IBA) and naphthalene acetic acid (NAA), is critical for coordinated shoot and root induction (Sharma *et al.* 2021). However, the specific hormonal ratio required for efficient organogenesis in *C. latipes* remains to be optimized.

This study aims to establish an efficient *in vitro* regeneration protocol for *C. latipes* using cotyledonary explants. Specifically, it evaluates the effects of different concentrations and combinations of cytokinins and auxins on shoot and root induction. The findings of this study will contribute to the conservation and large-scale propagation of *C. latipes*, facilitating its potential application in genetic improvement programs and *ex situ* conservation efforts.

**Methodology**

Mature fruits of *C. latipes* were collected from Upper Shillong, Meghalaya, India. These fruits were then brought to the laboratory and the seeds were then excised. These seeds were then washed with labolene in a 500 mL beaker and then kept in running water for 45 min. After 45 min, the seeds were then transferred into a laminar air flow cabinet and were surface sterilized with 0.2% mercuric chloride for 2 min followed by repeated rinsing with double distilled water 3-5 times. This is then followed by treating the seeds with 70% ethanol for 5 min and repeated rinsing with double distilled water for 5-10 times. An incision was made on the seed coat and the two cotyledons were split. Cotyledon explants were initially cultured in Woody Plant Medium (WPM) supplemented with NAA or BAP individually at concentrations ranging from 0.1 to 0.5 µM to determine their individual effects. Based on the best-performing treatments, a combined treatment of 0.4 µM NAA + 0.3 µM BAP was also evaluated for potential synergistic effects. The pH of the medium was adjusted to 5.8 using 1N NaOH prior to autoclaving at 15 psi, 121°C for 15 min.

**Table 1.** Composition of Woody Plant Medium (WPM) (Lloyd & McCown, 1980).

| Component | Concentration (mg/L) |
|---|---|
| **Macronutrients** | |
| $NH_4NO_3$ (Ammonium nitrate) | 400 |
| $KNO_3$ (Potassium nitrate) | 2500 |
| $CaCl_2·2H_2O$ (Calcium chloride dihydrate) | 288 |
| $MgSO_4·7H_2O$ (Magnesium sulfate heptahydrate) | 370 |
| $KH_2PO_4$ (Potassium dihydrogen phosphate) | 170 |
| **Micronutrients** | |
| $MnSO_4·H_2O$ (Manganese sulfate monohydrate) | 22.3 |
| $ZnSO_4·7H_2O$ (Zinc sulfate heptahydrate) | 8.6 |
| $H_3BO_3$ (Boric acid) | 6.2 |
| KI (Potassium iodide) | 0.83 |
| $Na_2MoO_4·2H_2O$ (Sodium molybdate dihydrate) | 0.25 |
| $CuSO_4·5H_2O$ (Copper sulfate pentahydrate) | 0.025 |
| $CoCl_2·6H_2O$ (Cobalt chloride hexahydrate) | 0.025 |
| **Iron Source** | |
| $FeSO_4·7H_2O$ (Ferrous sulfate heptahydrate) | 27.8 |
| $Na_2EDTA·2H_2O$ (Disodium ethylenediaminetetraacetic acid dihydrate) | 37.3 |
| **Organic Additives** | |
| Myo-inositol | 100 |
| Thiamine-HCl (Vitamin B1) | 1 |
| Pyridoxine-HCl (Vitamin B6) | 1 |
| Nicotinic acid (Niacin) | 1 |
| Glycine | 2 |
| **Carbon Source** | |
| Sucrose | 20,000.00 |
| **Gelling Agent** | |
| Agar | 7,000.00 |
| **pH** | Adjusted to 5.2 – 5.5 |

The test tubes containing the cotyledons were initially kept in the dark for 7 days before transferring them to culture room conditions at 25±2°C and 16 h photoperiod at 50 µM $m^{-2}s^{-1}$ light intensity (Light intensity was measured using a Traceable™ Dual-Display Light Meter, Fisher Scientific, Mumbai, India). The regeneration rates as well as other morphological growth parameters were assessed 30 days after inoculation. On the other hand, time taken for regeneration was accessed every week. The emergence of miniature shoots or roots were taken as a sign of regeneration.Ten replicates were maintained and the experiment was repeated thrice.

The plantlets that emerged from these cotyledons were subcultured every 2 months by transferring them on to a freshly prepared WPM medium (**Table 1**) supplemented with the best combination of NAA and IAA three times.

**Statistical analysis**

All data obtained in this investigation were subjected to one-way ANOVA. Means of the data generated in this investigation were subjected to significance test employing Duncan's test of significance at significance level $p \leq 0.05$ using origin pro statistical software (PC version 8.1, Northampton, MA). All chemicals used in this study were of analytical grade and were procured from HiMedia Laboratories (**Table 2**).

**Table 2.** List of chemicals used in this study.

| Chemical Name | Catalog Number | Company Name |
|---|---|---|
| 6-Benzylaminopurine (BAP) | PCT0802 | HiMedia Laboratories |
| Naphthaleneacetic acid (NAA) | RM3986 | HiMedia Laboratories |
| Indole-3-butyric acid (IBA) | RM3987 | HiMedia Laboratories |
| Murashige & Skoog Basal Medium | PT001 | HiMedia Laboratories |
| Woody Plant Medium (WPM) | PT010 | HiMedia Laboratories |
| Sucrose | GRM077 | HiMedia Laboratories |
| Agar | RM026 | HiMedia Laboratories |
| Sodium nitroprusside | RM577 | HiMedia Laboratories |
| α-Tocopherol (Vitamin E) | RM2762 | HiMedia Laboratories |

**Results and discussion**

The results indicated that the optimal individual concentrations for shoot and root induction were 0.4 µM NAA and 0.3 µM BAP, respectively. To further assess potential synergistic effects, these concentrations were combined (0.4 µM NAA + 0.3 µM BAP), resulting in enhanced regeneration efficiency (**Table 3**). These findings align with previous studies that highlight the role of auxins and cytokinins in plant tissue culture, particularly in the regulation of cell division, elongation, and differentiation (Sosnowski*et al.* 2023; Hairuddin *et al.* 2023).

**Table 3.** Effect of varying concentration of Napthaleneacetic acid (NAA) and 6-benzylaminopurines (BAP) on several morphological growth parameters in *Citrus latipes* (Swingle) Yu. Tanaka.

| Treatments | Concentration (µM) | Number of cotyledons assessed | Regeneration Percentage (%) | Number of leaves | Shoot length (cm) | Root length (cm) | Root number | Shoot number | Time taken for regeneration (in days) |
|---|---|---|---|---|---|---|---|---|---|
| NAA | 0.1 | 30 | 41.25±0.05 | 4.21±0.22 | 5.65±0.33 | 9.63±0.24 | 4.22±0.07 | 2.24±0.08 | 17.25±0.11 |
| | 0.2 | | 43.66±0.07 | 5.14±0.02 | 5.86±0.05 | 9.88±0.08 | 4.58±0.14 | 2.36±0.06 | 16.52±0.07 |
| | 0.3 | | 47.85±0.02 | 5.36±0.07 | 6.25±0.01 | 10.85±0.02 | 4.98±0.05 | 2.48±0.08 | 16.02±0.05 |
| | 0.4 | | 52.25±0.06 | 6.02±0.04 | 5.12±0.08 | 10.56±0.22 | 5.24±0.05 | 2.85±0.14 | 15.24±0.15 |
| | 0.5 | | 49.35±0.02 | 5.56±0.01 | 5.02±0.05 | 10.14±0.06 | 5.02±0.08 | 2.74±0.10 | 15.88±0.16 |
| BAP | 0.1 | | 36.68±0.02 | 4.65±0.02 | 4.85±0.04 | 7.52±0.05 | 3.25±0.14 | 3.36±0.02 | 18.56±0.11 |
| | 0.2 | | 39.65±0.04 | 4.88±0.15 | 4.96±0.22 | 7.65±0.02 | 3.88±0.17 | 3.45±0.24 | 18.02±0.05 |
| | 0.3 | | 49.25±0.12 | 6.66±0.11 | 5.35±0.06 | 8.12±0.25 | 4.25±0.06 | 4.21±0.25 | 16.24±0.08 |
| | 0.4 | | 47.25±0.01 | 5.36±0.04 | 5.10±0.02 | 8.59±0.07 | 4.05±0.06 | 3.95±0.14 | 16.88±0.14 |
| | 0.5 | | 43.44±0.05 | 5.68±0.05 | 5.02±0.04 | 8.18±0.05 | 3.95±0.07 | 3.66±0.15 | 17.52±0.01 |
| NAA+BAP | 0.4+0.3 | | 61.36±0.04 | 7.15±0.11 | 6.88±0.14 | 11.55±0.02 | 4.98±0.02 | 4.55±0.11 | 14.45±0.22 |

Heatmaps were utilized to visually represent the effect of different molar concentrations of NAA and BAP on various growth parameters. This approach provided a comprehensive and intuitive analysis of the data, allowing for the identification of trends and interactions between the plant growth regulators. The heatmaps revealed a clear concentration-dependent response, where increasing concentrations of NAA beyond 0.4 µM and BAP beyond 0.3 µM led to diminishing returns or even inhibitory effects on growth (**Fig. 1**). This visualization highlights the importance of precise hormonal balance for optimal *in vitro* regeneration.

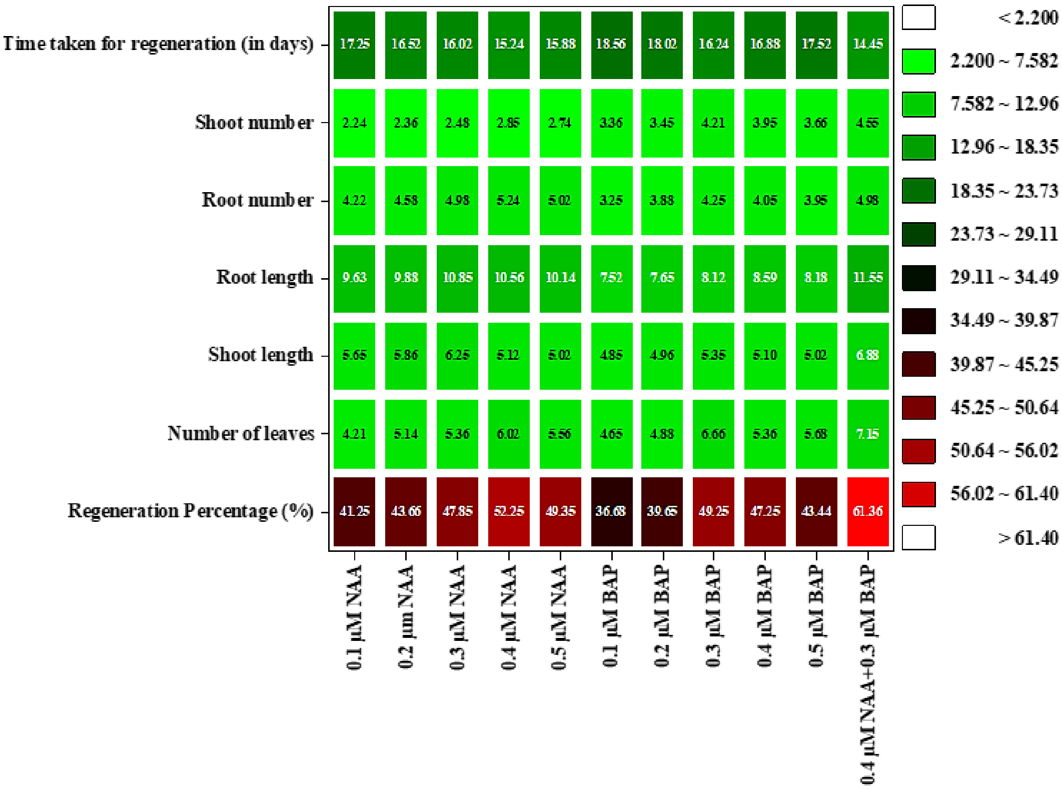


**Fig. 1.** Heatmaps showing a comparison of the effect of different molar concentration of Napthaleneacetic acid (NAA) and 6-benzylaminopurines (BAP) on several morphological growth parameters in *Citrus latipes* (Swingle) Yu. Tanaka.

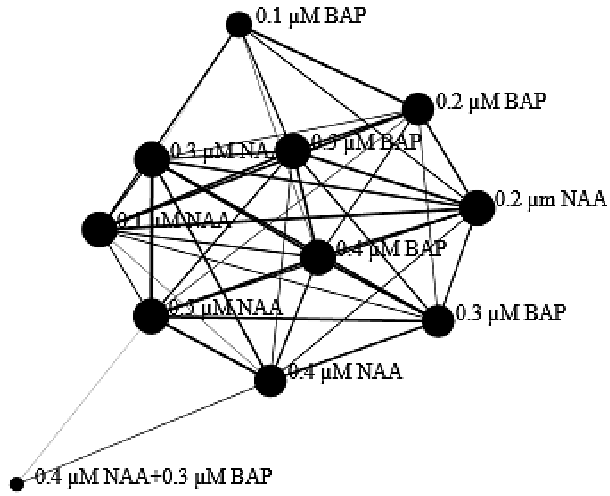


**Fig. 2.** Network plot analysis illustrating the influence and correlation between different concentrations (µM) of naphthaleneacetic acid (NAA) and 6-benzylaminopurine (BAP) on plantlet morphological growth parameters, including regeneration percentage, number of leaves, shoot and root length, shoot and root number, and time taken for regeneration in *Citrus latipes* (Swingle) Yu. Tanaka. The nodes (dots) represent specific NAA and BAP treatment concentrations. The bold lines indicate strong correlations between treatments and plant growth parameters, while the lighter lines represent weaker correlations.

In addition to heatmaps, a network plot was prepared to illustrate the level of influence of each treatment on the morphological growth parameters. This analysis helped in identifying key hormonal interactions and their relative contributions to growth responses. The network plot analysis (**Fig. 2**) revealed that irrespective of the compound used, when they are not combine, they independently often exhibit closely related pattern of growth. However, when these compounds are combined together there is a sharp increase in the overall plant development as evident from the treatment being an outlier (**Fig. 3**).


**Fig. 3.** Sequential development of *Citrus latipes* (Swingle) Yu. Tanaka plantlets in Woody Plant Medium (WPM) supplemented with 0.4 µM NAA in combination with 0.3 µM BAP. (A) 15-day-old cotyledon explant cultured in WPM. (B) Root emergence from cotyledon explants at 15 days after inoculation. (C) Shoot initiation from cotyledon explants at 30 days after inoculation. (D) 1.5-month-old plantlets developed in WPM. (E) 2-month-old plantlets showing further growth and development. (F) Well-developed roots of 2-month-old plantlets.

Furthermore, the correlational relationships between different treatments and growth parameters were analyzed using the Bray-Curtis index of dissimilarity. This analysis allowed for a quantitative assessment of the degree of similarity or divergence between treatment effects (Ricotta and Pavoine 2022), thereby highlighting the extent to which certain hormone concentrations elicit comparable or distinct morphological responses. The results revealed clusters of treatments that induced similar growth patterns, reinforcing the idea that specific NAA and BAP concentrations produce predictable and reproducible effects on plant development (**Table 4**).

**Table 4.** Correlation between different treatments using Bray-Curtis index of dissimilarity by taking all plantlets morphological growth parameters into account

| **Treatments** | **0.1 µM NAA** | **0.2 µM NAA** | **0.3 µM NAA** | **0.4 µM NAA** | **0.5 µM NAA** | **0.1 µM BAP** | **0.2 µM BAP** | **0.3 µM BAP** | **0.4 µM BAP** | **0.5 µM BAP** | **0.4 µM NAA+0.3 µM BAP** |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **0.1 µM NAA** | 1.0000 | 0.9709 | 0.9338 | 0.9014 | 0.9256 | 0.9307 | 0.9565 | 0.9145 | 0.9374 | 0.9552 | 0.8358 |
| **0.2 µM NAA** | 0.9709 | 1.0000 | 0.9626 | 0.9281 | 0.9523 | 0.9089 | 0.9373 | 0.9350 | 0.9535 | 0.9645 | 0.8640 |
| **0.3 µM NAA** | 0.9338 | 0.9626 | 1.0000 | 0.9587 | 0.9782 | 0.8740 | 0.9022 | 0.9520 | 0.9607 | 0.9319 | 0.9010 |
| **0.4 µM NAA** | 0.9014 | 0.9281 | 0.9587 | 1.0000 | 0.9746 | 0.8520 | 0.8801 | 0.9495 | 0.9386 | 0.9133 | 0.9244 |
| **0.5 µM NAA** | 0.9256 | 0.9523 | 0.9782 | 0.9746 | 1.0000 | 0.8758 | 0.9040 | 0.9673 | 0.9615 | 0.9359 | 0.9015 |
| **0.1 µM BAP** | 0.9307 | 0.9089 | 0.8740 | 0.8520 | 0.8758 | 1.0000 | 0.9709 | 0.8852 | 0.9079 | 0.9359 | 0.7878 |
| **0.2 µM BAP** | 0.9565 | 0.9373 | 0.9022 | 0.8801 | 0.9040 | 0.9709 | 1.0000 | 0.9142 | 0.9368 | 0.9649 | 0.8161 |
| **0.3 µM BAP** | 0.9145 | 0.9350 | 0.9520 | 0.9495 | 0.9673 | 0.8852 | 0.9142 | 1.0000 | 0.9724 | 0.9487 | 0.9004 |
| **0.4 µM BAP** | 0.9374 | 0.9535 | 0.9607 | 0.9386 | 0.9615 | 0.9079 | 0.9368 | 0.9724 | 1.0000 | 0.9684 | 0.8783 |
| **0.5 µM BAP** | 0.9552 | 0.9645 | 0.9319 | 0.9133 | 0.9359 | 0.9359 | 0.9649 | 0.9487 | 0.9684 | 1.0000 | 0.8507 |
| **0.4 µM NAA+0.3 µM BAP** | 0.8358 | 0.8640 | 0.9010 | 0.9244 | 0.9015 | 0.7878 | 0.8161 | 0.9004 | 0.8783 | 0.8507 | 1.0000 |

The combined application of NAA and BAP resulted in even greater enhancement of morphological traits when cotyledon explants were used. This synergistic effect can be attributed to the interplay between auxins and cytokinins in regulating organogenesis. While auxins primarily influence root formation and cell expansion, cytokinins promote shoot proliferation and delay senescence (Revathi *et al.* 2020; Benedetto *et al.* 2023). The heatmaps and network plots further demonstrated that cotyledon explants showed a more responsive and uniform growth pattern under the optimized concentrations, reinforcing their suitability for *in vitro* propagation.

The enhanced response observed with cotyledon explants suggests that this tissue type may have a higher capacity for organogenesis under optimal hormonal conditions. Cotyledons often contain high levels of endogenous growth regulators, which could contribute to their enhanced response when exogenous NAA and BAP are applied (Ċosiċ *et al.* 2015). Additionally, the physiological state of the cotyledonary tissue might be more conducive to hormonal uptake and signal transduction, thereby amplifying the growth-promoting effects of the supplemented medium.

These findings emphasize the importance of optimizing plant growth regulators for *in vitro* propagation of *C. latipes*. The use of heatmaps and network plots as data visualization tools provided an additional layer of insight, enabling the identification of optimal concentration ranges, key treatment influences, and potential threshold effects. The application of the Bray-Curtis index of dissimilarity further strengthened the statistical evaluation, offering a robust framework for understanding treatment correlations. Future studies could further explore the molecular mechanisms underlying these interactions and evaluate the long-term effects of these hormonal combinations on plantlet acclimatization and field performance. Understanding these dynamics is crucial for refining micropropagation protocols, particularly for the conservation and mass propagation of economically and ecologically significant citrus species.

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**Conflict of Interest**

The authors declare that there are no known financial or any other conflicts of interest.

**Data availability**

All data presented in this investigation will be provided upon request.

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