

MINISTRY OF EDUCATION AND TRAINING
HANOI PEDAGOGICAL UNIVERSITY 2

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**STUDY ON GENETIC DIVERSITY AND THE EFFECTS
OF SELECTED MICROBIAL STRAINS ON THE
GROWTH OF *NANHAIA SPECIOSA* IN SEVERAL
NORTHERN PROVINCES OF VIETNAM**

Ngành: Plant Physiology

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SUMMARY DOCTORAL DISSERTATION IN BIOLOGY

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INTRODUCTION

Nanhaia speciosa, commonly known as Cát sâm, belongs to the Fabaceae family and is a valuable medicinal plant with numerous applications in both traditional and modern medicine. It is used to replenish the body, enhance immunity, and treat anemia, arthritis, and menstrual disorders. The plant contains a rich variety of chemical compounds, especially flavonoids and polysaccharides, which have great potential for application in functional foods. Taxonomically, this species was previously classified under different genera before being confirmed as a member of the genus *Nanhaia* based on morphological and genetic data. In Vietnam, *Nanhaia speciosa* is distributed across various mountainous and midland provinces; however, studies on its morphological and genetic diversity remain limited, underscoring the need for systematic research to support conservation and sustainable development of this medicinal resource.

In addition, soil factors such as organic matter, macronutrients, and particularly phosphorus (P) play important roles in the growth of medicinal plants. Indigenous rhizosphere fungi (such as *Aspergillus*, *Penicillium*, *Trichoderma*, *Chaetomium*, and *Mucor*) have been shown to possess phosphate- and cellulose-degrading abilities, supporting plant growth and reducing the need for chemical fertilizers. Several international studies have demonstrated the effectiveness of fungal strains in improving soil conditions and legume productivity. However, research on rhizosphere microorganisms associated with *Nanhaia speciosa* in Vietnam is still limited. Therefore, it is essential to isolate, select, and apply indigenous microbial strains to develop biofertilizer products that contribute to conservation, enhance cultivation efficiency, and promote sustainable development of this medicinal species.

Based on the aforementioned rationale, we conducted the study titled: “Study on Genetic Diversity and the Effects of Selected Microbial Strains on the Growth of *Nanhaia speciosa* in Several Northern Provinces of Vietnam”.

Research objectives: (1) To determine the genetic diversity of *Nanhaia speciosa* cultivated in Vietnam using selected molecular markers. (2) To isolate and select microbial strains from the rhizosphere of *Nanhaia speciosa* capable of solubilizing insoluble phosphate and decomposing organic matter, in order to develop indigenous microbial inoculants. (3) To evaluate the effects of microbial inoculants derived from selected strains on the growth of *Nanhaia speciosa*.

Scientific contributions: This study provides data on the genetic diversity of *Nanhaia speciosa* in Vietnam, serving as a foundation for conservation and breeding efforts. It also identifies the diversity of rhizospheric fungi and their ability to solubilize phosphate and decompose organic matter, thereby assessing their influence on plant growth. Practical implications: The results will help clarify the genetic relationships among *Nanhaia speciosa* samples, supporting breeding programs. The selection of beneficial microorganisms forms the basis for developing biofertilizer products that enhance the growth of *Nanhaia speciosa* and potentially other crops.

Research subjects: *Nanhaia speciosa* and its rhizosphere microorganisms.

Research scope: Conducted in Lạng Sơn, Bắc Giang, and Vĩnh Phúc provinces (2022–2025),

including sampling, genetic analysis, microbial isolation, and field evaluation of microbial inoculants.

Novel contributions of the study: Application of ISSR and RAPD markers to analyze the genetic diversity of *Nanhaia speciosa*. Selection of indigenous fungal strains with soil-improving and plant-growth-promoting abilities. Development and field testing of microbial inoculants aimed at establishing a sustainable cultivation model for *Nanhaia speciosa*.

Chapter 1. LITERATURE REVIEW

1.1. Overview of *Nanhaia speciosa*

The currently accepted scientific name of Cát sâm is *Nanhaia speciosa* (Champ. ex Benth.) J. Compton & Schrire, belonging to the Fabaceae family and the order Fabales. Previously, this species had been classified in the genera *Millettia* and later *Callerya*. Based on morphological characteristics and genetic analyses (including nuclear and chloroplast DNA), it was reclassified into the genus *Nanhaia*, tribe Wisterieae. Chloroplast genome studies indicate that *N. speciosa* is closely related to *Wisteriopsis reticulata*, further supporting its current taxonomic position.

Nanhaia speciosa is a perennial woody climber that can reach up to 6 meters in height. It develops tuberous roots that store reserves. The plant has imparipinnate compound leaves with dense hairs. Its flowers are aromatic, pale pink to white, and arranged in terminal racemes. The flowers have 10 stamens (9 fused, 1 free). The fruit is a hairy pod, 9–13 cm long, containing 1–10 black seeds. Each plant typically produces 2–3 kg of tuberous roots.

This species is naturally distributed in China and several northern provinces of Vietnam, such as Tuyên Quang, Lạng Sơn, and Quảng Ninh. It typically grows in secondary forests and along hillsides, particularly thriving on limestone mountains at elevations up to 1000 meters. The plant has high seed regeneration ability, with a flowering season from May to September and a fruiting season from September to December.

The tuberous roots of *N. speciosa* are rich in flavonoids, alkaloids, triterpenoids, and polysaccharides. These compounds have been shown to exhibit tonic effects, anti-fatigue properties, pain relief, liver protection, and support for respiratory treatment. Notable compounds such as maackiain, formononetin, and glycyrrhizic acid have demonstrated antioxidant and anti-inflammatory activities. Due to overharvesting, *N. speciosa* was once listed in the Vietnam Red Data Book. Currently, the species is being considered for development as a medicinal raw material, functional food, and health care product.

1.2. Genetic Diversity and the Application of ISSR and RAPD Markers in Plant Genetic Diversity Research

Genetic diversity research plays a crucial role in plant breeding and crop improvement by providing a rich gene pool for the development of high-yielding, high-quality, and stress-tolerant varieties. Additionally, genetic diversity is fundamental for germplasm conservation, helping to maintain genetic structure, prevent genetic erosion, and enhance population resilience. This diversity reflects the level of variation within and among populations, originating from mutations and genetic recombination. Factors affecting genetic diversity include mutation, selection, gene flow, genetic drift, and non-random mating, with natural

and artificial selection being the primary forces driving allele frequency changes over time. However, intense selection for superior genotypes can reduce genetic diversity, underscoring the need to exploit wild gene pools to ensure sustainability.

Among molecular biology techniques used in genetic diversity studies, ISSR (Inter-Simple Sequence Repeat) and RAPD (Random Amplified Polymorphic DNA) markers are widely applied. ISSR markers offer high polymorphism, do not require prior DNA sequence information, and are suitable for genetic analysis, conservation, and evolutionary studies. However, they are dominant markers and dependent on PCR conditions, which can limit their resolution. RAPD, on the other hand, is a simple and cost-effective technique that does not require purified DNA, but it has low reproducibility and limited ability to distinguish genotypes. Combining ISSR and RAPD techniques can enhance the accuracy of genetic diversity assessments and provide valuable support for conservation, breeding, and plant taxonomy studies.

1.3. Research on the Role of Microorganisms and Their Applications in Agriculture

Microorganisms, particularly fungi, play an essential role in soil ecosystems and have profound effects on plant growth, development, and productivity through various biological mechanisms. They form symbiotic relationships with plant roots, contributing to improved nutrient uptake, enhanced photosynthetic capacity, increased stress resistance, and regulation of plant physiological responses to environmental challenges.

Microbial groups such as nitrogen-fixing bacteria, phosphate-solubilizing bacteria, endophytic mycorrhizal fungi, and antagonistic fungi like *Trichoderma* are widely applied in agriculture due to their abilities to improve soil structure, transform nutrients, synthesize phytohormones (such as auxins, gibberellins, and cytokinins), mineralize phosphorus, and protect plants from pathogens through biological antagonism. Beyond hormone production, many fungal species—such as *Aspergillus*, *Penicillium*, *Trichoderma*, and *Fusarium*—are known not only to stimulate root and shoot development but also to enhance flowering, fruiting, and overall crop quality.

In Vietnam, numerous studies have successfully applied fungal strains and microbial formulations to process agricultural by-products, produce organic fertilizers, and develop biofertilizers for crops such as tea, coffee, cabbage, and mung bean. These applications have proven highly effective in both greenhouse and field conditions. International studies also indicate that combining beneficial microorganisms with chemical fertilizers significantly improves crop yield and quality. Thus, microorganisms are increasingly recognized as key players in modern agriculture, contributing to sustainable farming practices, reducing chemical dependency, and enhancing soil health and environmental quality.

Chapter 2. MATERIALS AND METHODS

2.1. Materials, Chemicals, and Research Equipment

2.1.1. Collection of Plant Samples

Plant samples were collected for genetic diversity analysis and related studies (details to be presented in relevant subsections).

2.1.2. Collection of Soil Samples

Soil samples were collected from the rhizosphere of *Nanhaia speciosa* plants aged 1 to 4 years for fungal isolation. The soil was taken from a depth of 0–20 cm, with approximately 0.5 kg per sample placed in polyethylene ziplock bags. Samples were stored in a cool place and processed as soon as possible upon arrival at the laboratory.

2.1.3. Chemicals and Media

- Chemicals for morphological and anatomical analysis: Javel (sodium hypochlorite), acetic acid, glycerin, iodine-potassium iodide.

- Molecular biology reagents: MyTaq 2X Mix, 70% ethanol, deionized water, 2% agarose gel, primers, etc.

- Media for enzyme activity assays: CBM, PVK, CMA, lipase, xylan, starch, and PDA media. Media compositions were adjusted according to the objectives of detecting cellulase, amylase, hemicellulase, protease, lipase, and phosphate solubilizing activities.

2.1.4. Research Equipment

The study employed standard laboratory equipment for biology, molecular biology, microbiology, and plant physiology, including: Microscope (Carl Zeiss, Germany), Autoclave sterilizer (Hirayama, Japan), Laminar flow cabinet (Esco, Singapore), Nanodrop One spectrophotometer (Thermo Scientific, USA), PCR thermal cycler Mastercycler X50s (Eppendorf, Germany), Centrifuge (Hettich, Germany), Vortex mixer (USA), Gel documentation system (Bio-Rad, USA), Electrophoresis unit (Bio-Rad, USA), SPAD chlorophyll meter (Minolta, Japan), Chlorophyll fluorometer OS-30 (ADC, UK)

2.2. Research Methods

2.2.1. Morphological and Anatomical Analysis of *Nanhaia speciosa*

Stem, leaf, and flower samples were collected, preserved in newspaper, and described morphologically following the method of Nguyễn Nghĩa Thìn. Five mature leaves were randomly selected and sectioned at the petiole, mid-leaf, and apex. The sections were soaked in distilled water and double-stained with carmin-aluné and iodine green (vert d'iode) according to Mondolot et al. (2001). Hand-cut sections were prepared as temporary slides and photographed under a microscope.

2.2.2. Genetic Diversity Analysis Using ISSR and RAPD Markers

DNA Extraction: Total genomic DNA was extracted using the CTAB method (Rogers & Bendich, 1989).

PCR with ISSR and RAPD Primers: Ten ISSR primers and five RAPD primers were used for genetic diversity analysis. DNA banding patterns were converted into binary data, and genetic polymorphism and relationships among samples were analyzed using specialized software.

2.2.4. Isolation, Selection of Microorganisms, and Bioformulation Development

2.2.4.1. Isolation and Selection of Microorganisms

Three methods were used: fungal trapping, serial dilution, and single-spore isolation.

2.2.4.2. Microbial Identification Based on ITS Gene Region

Fungal DNA was extracted from cultures grown on media using lysis solution and phenol-chloroform treatment. The ITS region was amplified using ITS1/ITS4 primers via PCR. The PCR product was electrophoresed, purified, and sequenced. Sequences were compared with

GenBank using BLAST, and phylogenetic trees were constructed using MEGA software for species identification.

2.2.4.3. Evaluation of Microbial Activities

Each fungal strain was tested for its ability to produce cellulase, xylanase, amylase, protease, lipase, and phosphate solubilization by observing clear zones on specific media. Activity was evaluated by measuring the diameter of degradation zones after indicator staining and standard processing.

2.2.5. Bioformulation Development Methods

2.2.5.1. Assessment of Compatibility Between Fungal Strains

2.2.5.2. Effects of Environmental and Nutritional Conditions on Spore Biomass Production

2.2.5.3. Effects of Carrier Ratios on Spore Viability

2.2.6. Evaluation of the Effects of Microbial Formulations on *Nanhaia speciosa*

2.2.6.1. Laboratory Evaluation of Formulation Efficiency

In nursery conditions: One-month-old plants were treated with five dosage levels (0.5–2.5 g/plant) compared to a control. Growth and chlorophyll fluorescence were monitored at 1, 3, and 6 months.

In field conditions: Four-year-old plants were treated with four dosage levels (2–5 kg/ha), applied twice. Plant growth and photosynthetic parameters were monitored after 3 months.

2.2.6.3. Evaluation of Formulation Effects on Soil Fertility

Determination of total microbial density in nursery soil before and three months after treatment with microbial formulations.

Analysis of soil parameters including pH, total nitrogen, total phosphorus and potassium, and organic matter content, according to current Vietnamese standards (TCVN).

2.2.7. Statistical Analysis

Quantitative data were analyzed using appropriate statistical parameters, with results expressed as means and standard deviations. Differences among treatments were considered statistically significant at $p \leq 0.05$.

Chapter 3. RESULTS AND DISCUSSION

3.1. Morphological, Anatomical Characteristics and Genetic Diversity of *Nanhaia speciosa*

3.1.1. Growth Form and Stem Morphology of *Nanhaia speciosa*



Figure 3.1. Branch and Leaf Morphology of *Nanhaia speciosa* from Different Regions
Caption: *a*: ND sample from Tân Yên District (Bắc Giang), *b*: CSD sample from Sơn Động District (Bắc Giang), *c*: CSS sample from Lộc Bình District (Lạng Sơn)

Table 3.1. Stem Morphological Characteristics of *Nanhaia speciosa* from Sampled Regions (4-Year-Old Cultivation Plots)

Parameter \ Sampling Location	Tanyen District	Sondong District	Locbinh District
Basal stem diameter (cm)	1,40 ± 0,06 ^{c (*)}	2,18 ± 0,04 ^b	2,39 ± 0,08 ^a
Branching height (cm)	2,87 ± 0,07 ^c	7,16 ± 0,04 ^b	6,82 ± 0,08 ^a
Number of primary branches	3,14 ± 0,04 ^c	2,45 ± 0,04 ^b	2,61 ± 0,05 ^a
Stem form	Small woody stem, climbing from base	Small woody stem, climbing from base	Small woody stem, climbing from base
Stem color	Gray-brown stem; young shoots green and covered with fine hairs	Gray-brown stem; young shoots green and covered with fine hairs	Gray-brown stem; young shoots green and covered with fine hairs
Young branches	Fewer hairs	Densely hairy	Densely hairy

Note: (*) In the same row, different letters (a, b, c) indicate statistically significant differences at $P < 0.05$

3.1.2. Morphological and Anatomical Characteristics of Leaves in *Nanhaia speciosa* Species

Table 3.2. Morphological Characteristics of *Nanhaia speciosa* Leaflets Collected from Different Regions




Parameter \ Sampling Location	Tanyen District	Sondong District	Locbinh District
Adaxial leaf surface			
Leaf apex shape	Acuminate	Bilobed apex	Bilobed apex
Leaf base shape	Rounded	Oblique	Oblique
Leaf color	Dark green	Yellowish green	Yellowish green
Other features (e.g., hair, margin...)	Odd-pinnate leaflets	Odd-pinnate leaflets	Odd-pinnate leaflets

Table 3.3. Leaflet size of *Nanhaia speciosa* collected from different regions

Parameter \ Sampling Location	Tanyen District	Sondong District	Locbinh District
Length of compound leaf (cm)	18,52 ± 0,08 ^{c, (*)}	29,70 ± 0,40 ^b	31,61 ± 0,43 ^a
Length of petiole (cm)	11,38 ± 0,08 ^c	20,66 ± 0,33 ^b	22,74 ± 0,43 ^a
Number of leaflets (no.)	5,84 ± 0,08 ^c	13,29 ± 0,74 ^b	14,30 ± 0,06 ^a
Length of leaflet (cm)	7,64 ± 0,19 ^c	10,78 ± 0,63 ^a	9,34 ± 0,09 ^b
Width of leaflet (cm)	4,16 ± 0,06 ^a	2,59 ± 0,07 ^c	4,49 ± 0,06 ^a

Note: (*) In the same row, different following letters (a, b, c, ...) indicate statistically significant differences at $P < 0.05$

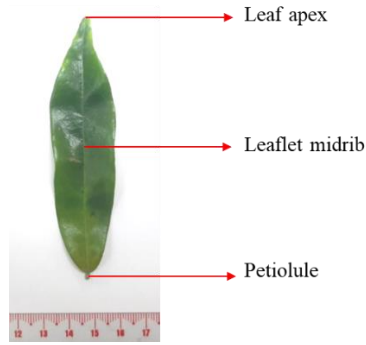


Figure 3.2. Anatomical position of *Nanhaia speciosa* leaf
 1. Epidermis; 2. Cortex parenchyma; 3. Sclerenchyma; 4. Phloem; 5. Xylem

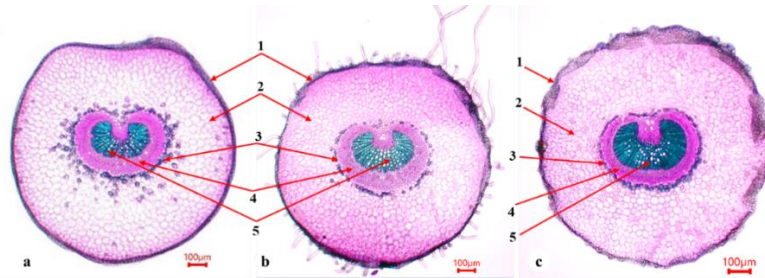


Figure 3.3. Anatomical characteristics of the leaflet petiole of *Nanhaia speciosa*
 Legend: a. Tanyen; b. Sondong; c. Locbinh

1. Epidermis; 2. Cortex parenchyma; 3. Sclerenchyma; 4. Phloem; 5. Xylem

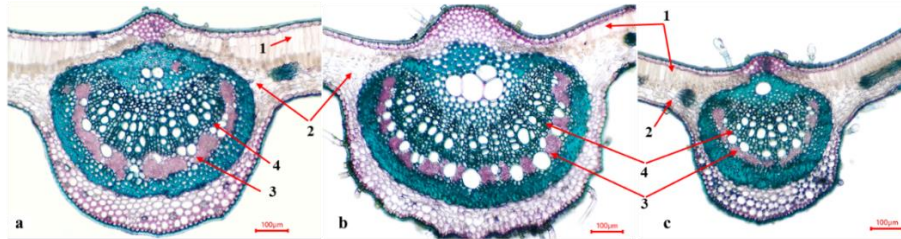


Figure 3.4. Anatomical characteristics of the midrib of *Nanhaia speciosa* leaflet
 Legend: a. Tanyen; b. Sondong; c. Locbinh

1. Palisade mesophyll; 2. Spongy mesophyll; 3. Phloem; 4. Xylem

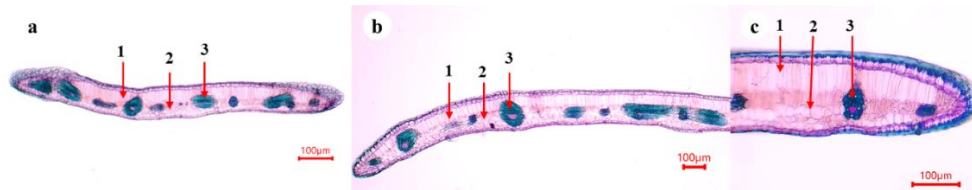








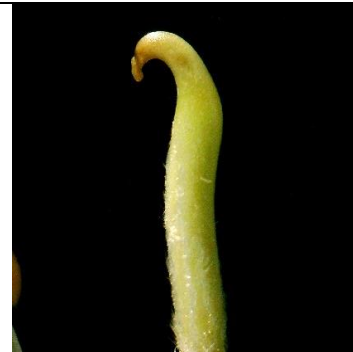




















Figure 3.5. Anatomical characteristics of the leaflet apex of *Nanhaia speciosa*
 Legend: a. Tanyen; b. Sondong; c. Locbinh

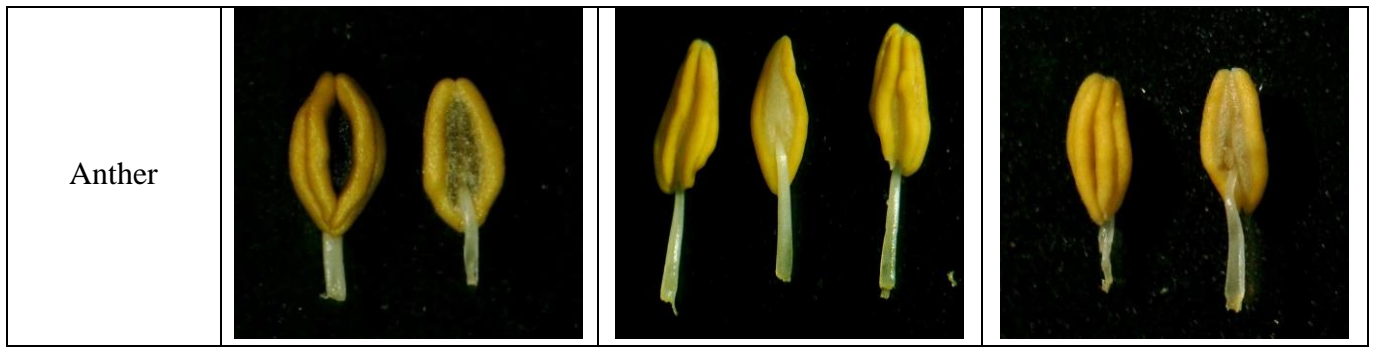
1. Palisade mesophyll; 2. Spongy mesophyll; 3. Vascular bundle (phloem and xylem)

Table 3.4. Morphological and anatomical characteristics of the flower of *Nanhaia speciosa*

Sampling Location Parameter	Tanyen District	Sondong District	Locbinh District

Inflorescence	 A photograph of a green, curved inflorescence specimen next to a ruler for scale. The inflorescence is thin and arches upwards.
 A photograph of a green, curved inflorescence specimen next to a ruler for scale. The inflorescence is thin and arches upwards.	
 A photograph of a green, curved inflorescence specimen next to a ruler for scale. The inflorescence is thin and arches upwards.	
Stigma	 A close-up photograph of a stigma, showing a yellowish-green, lobed structure.
 A close-up photograph of a stigma, showing a yellowish-green, lobed structure.	
 A close-up photograph of a stigma, showing a yellowish-green, lobed structure.	
Style morphology / Shape of style	 A photograph of a style, showing a curved, yellowish-green structure.
 A photograph of a style, showing a curved, yellowish-green structure.	
 A photograph of a style, showing a curved, yellowish-green structure.	
Floral aestivation	 A cross-section of a floral aestivation, showing a circular, layered structure.
 A cross-section of a floral aestivation, showing a circular, layered structure.	
 A cross-section of a floral aestivation, showing a circular, layered structure.	
Epicalyx	 Two photographs of epicalyx, showing small, yellowish-green, lobed structures.
 Two photographs of epicalyx, showing small, yellowish-green, lobed structures.	
 Two photographs of epicalyx, showing small, yellowish-green, lobed structures.	

Calyx	 A pale yellow, cup-shaped calyx with five lobes, viewed from a slightly elevated angle.	 A pale yellow, cup-shaped calyx with five lobes, viewed from a slightly elevated angle, similar to specimen 1.	 A pale yellow, cup-shaped calyx with five lobes, viewed from a slightly elevated angle, similar to specimen 1.
Stamen	 A stamen with a long, slender white filament and a yellow, elongated anther.	 A stamen with a long, slender white filament and a yellow, elongated anther, viewed from a different angle.	 A stamen with a long, slender white filament and a yellow, elongated anther, viewed from a different angle.
Petal / Corolla lobe	 A row of five small, pale yellow, pointed petals or corolla lobes.	 A row of five small, pale yellow, pointed petals or corolla lobes.	 A row of five small, pale yellow, pointed petals or corolla lobes.
Ovule	 A cross-section of an ovule showing a central ovule and surrounding tissue.	 A cross-section of an ovule showing a central ovule and surrounding tissue.	 A cross-section of an ovule showing a central ovule and surrounding tissue.



3.1.3. Polymorphism of ISSR and RAPD markers

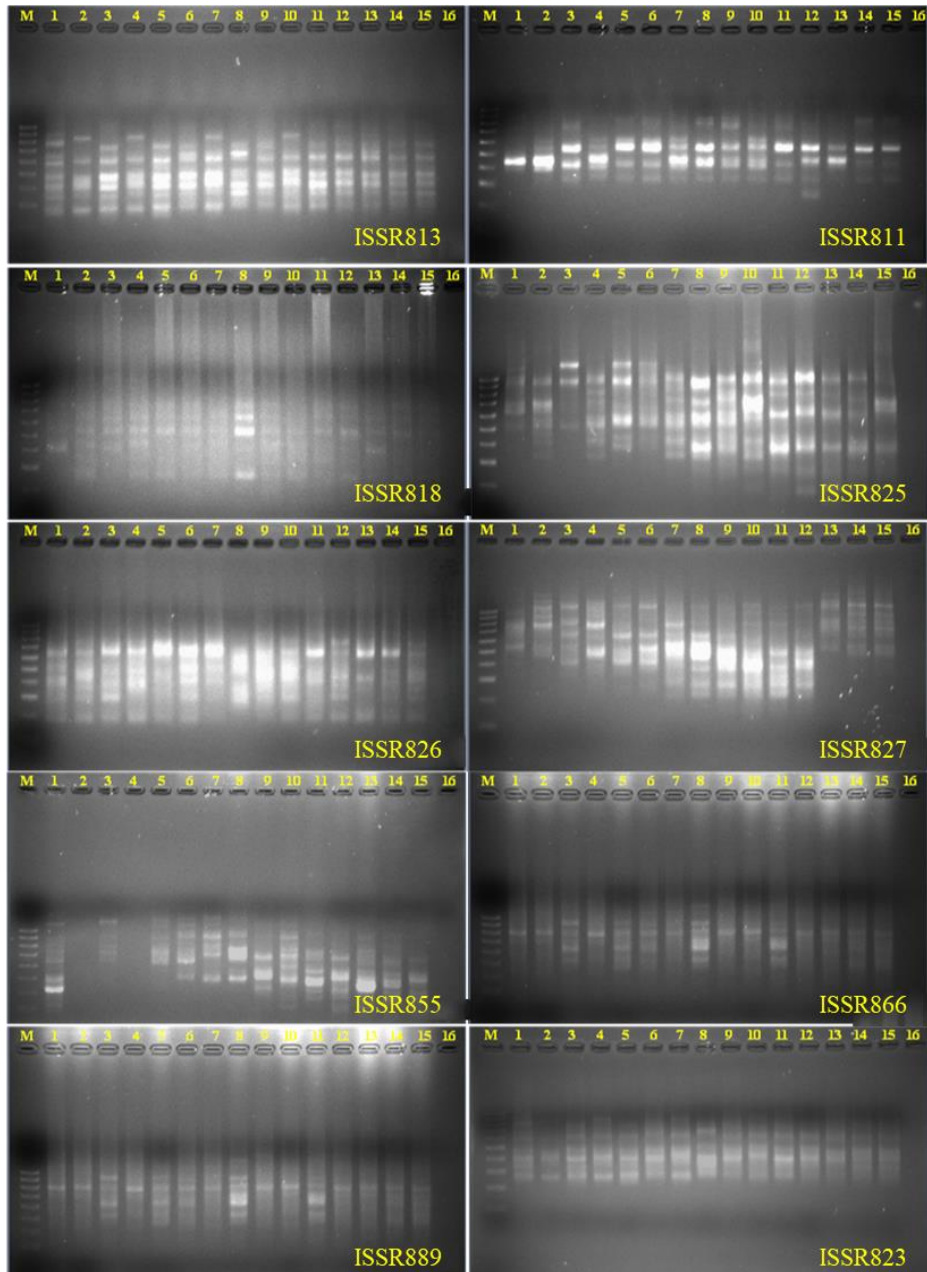


Figure 3.6. Electrophoresis results of PCR products using 10 ISSR markers on 2% agarose gel

Legend: DNA ladder M – 100 bp (Nippon Genetics, Europe);

1 – ND01, 2 – ND02, 3 – ND05, 4 – ND07, 5 – ND09, 6 – CSD03, 7 – CSD06, 8 – CSD08, 9 – CSD10, 10 – CSS06, 11 – CSS08, 12 – CSS10, 13 – CSS14, 14 – CSS15, 15 – CSS16, 16 – control

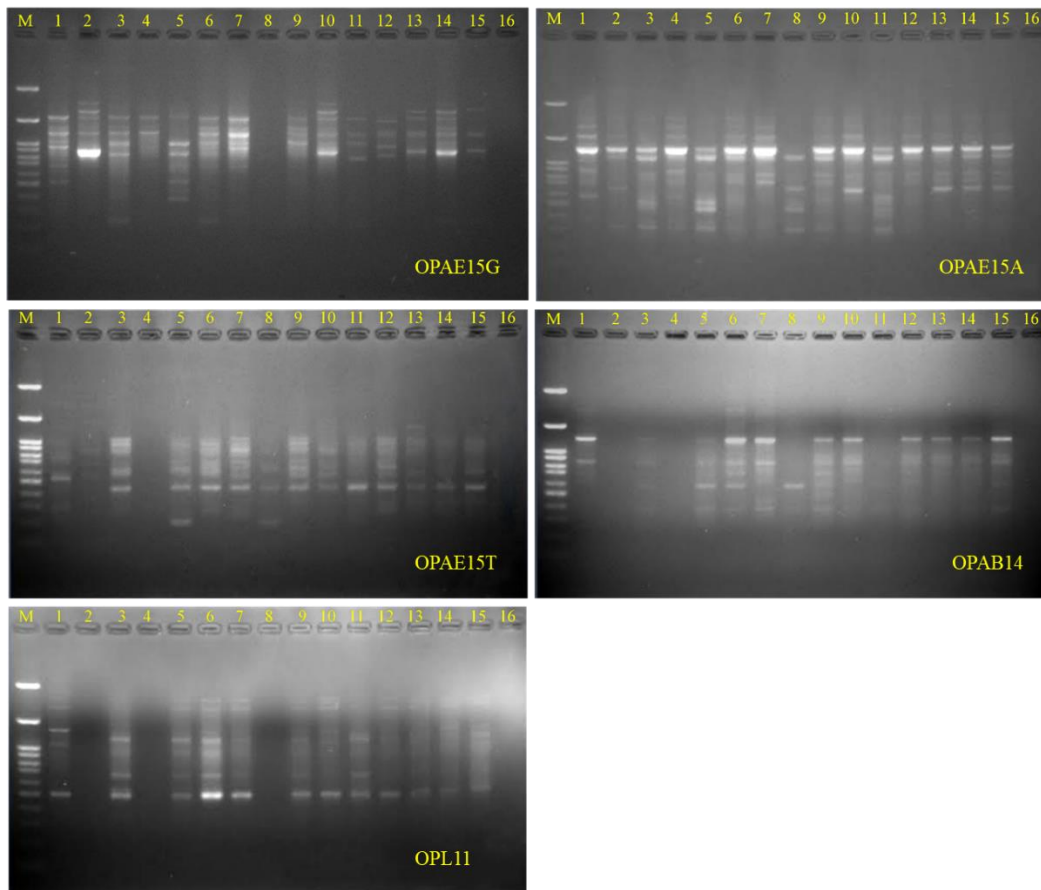


Figure 3.7. Electrophoresis results of PCR products using 5 RAPD markers on 2% agarose gel.

Legend: DNA ladder M – 100 bp (Nippon Genetics, Europe);

1 – ND01, 2 – ND02, 3 – ND05, 4 – ND07, 5 – ND09, 6 – CSD03, 7 – CSD06, 8 – CSD08, 9 – CSD10, 10 – CSS06, 11 – CSS08, 12 – CSS10, 13 – CSS14, 14 – CSS15, 15 – CSS16, 16 – control.

3.1.3.1. Polymorphism of ISSR and RAPD markers

Table 3.5. Polymorphism indices of 10 ISSR markers

Primer	Band size	Total band	Polymorphic band	% Polymorphic	PIC	E	MI	Rp
ISSR813	250-900	9	7	77,78	0,37	3,86	0,015	2,00
ISSR811	250-900	8	6	75	0,36	4,93	0,02	4,14
ISSR826	200-800	6	3	50	0,23	5,07	0,016	1,86
ISSR818	250-800	6	5	83,33	0,32	4,28	0,02	2,00
ISSR825	200-800	12	10	83,33	0,37	6,57	0,019	8,00
ISSR827	200-800	13	13	100	0,37	5,79	0,016	7,29
ISSR823	250-900	9	4	44,44	0,34	6,21	0,02	1,86
ISSR855	200-800	11	11	100	0,37	4,57	0,014	7,43
ISSR866	450-900	6	5	83,33	0,37	3,07	0,018	4,14
ISSR889	350-1000	6	5	83,33	0,37	3,50	0,02	2,71
		86	69					

Note (): PIC – Polymorphic Information Content; E – Effective Multiplex Ratio; MI – Marker Index; Rp – Resolving Power.*

Table 3.6. Polymorphism indices of 5 RAPD markers

Primer	Band size (bp)	Total band	Polymorphic band	% Polymorphic	PIC	E	MI	Rp
OPAE15G	200-2000	10	10	100	0,32	3,07	0,01	3,86
OPAE15A	300-2500	12	10	83,33	0,37	4,93	0,01	5,57
OPAE15T	300-1200	12	12	100	0,36	4,64	0,01	5,86
OPAB14	400-1600	6	6	100	0,36	2,21	0,01	3,57
OPL11	500-2500	7	7	100	0,37	3,29	0,02	5,43
		47	45	95,7				

Note (*): PIC – Polymorphic Information Content; E – Effective Multiplex Ratio; MI – Marker Index; Rp – Resolving Power.

3.1.3.2. Cluster analysis

ISSR marker:

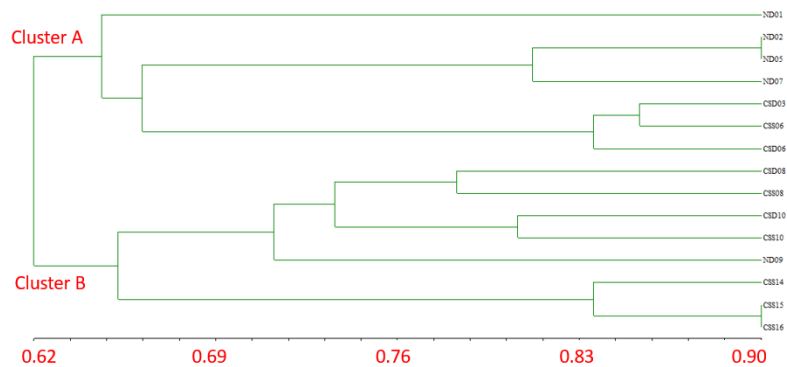


Figure 3.8. Dendrogram showing the genetic relationships among 15 *Nanhaia speciosa* accessions based on 10 ISSR markers.

Red values on the horizontal axis indicate the genetic similarity levels among the groups.

RAPD marker:

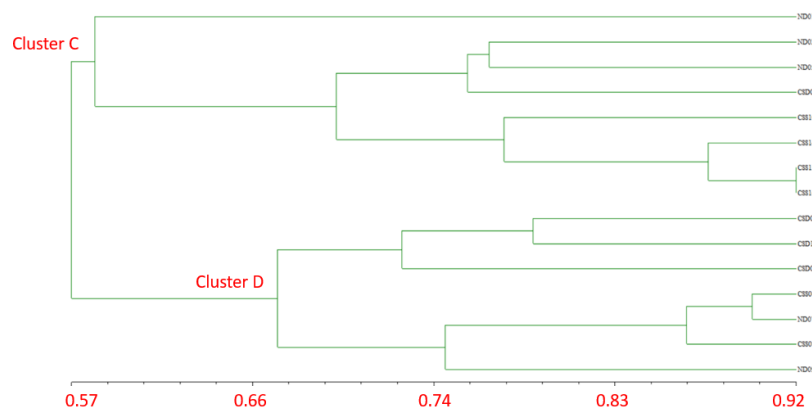


Figure 3.9. Dendrogram showing the genetic relationships among 15 *Nanhaia speciosa* accessions based on 5 RAPD markers

Red values on the horizontal axis indicate the genetic similarity levels among the groups.

Based on two molecular markers, ISSR and RAPD, it was demonstrated that ND individuals possess genetic characteristics distinct from the CSS and CSD genotypes. Specifically, 80% of ND individuals revealed ISSR profiles and 60% revealed RAPD profiles that clustered into a separate branch compared with the other genotypes.

3.2. Isolation and selection of microbial strains from the rhizosphere of *Nanhaia speciosa* with the ability to degrade organic matter and solubilize insoluble phosphorus

3.2.1. Isolation and screening of microorganism

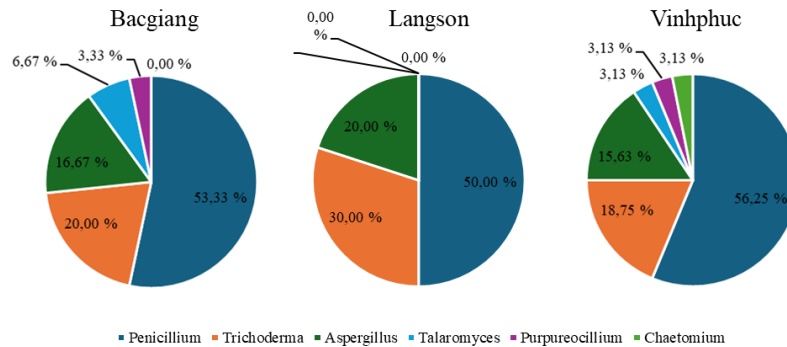


Figure 3.10. Percentage (%) of fungal genera isolated from the rhizosphere of *Nanhaia speciosa* at different sampling sites.

3.2.2. The scientific name of the selected fungal isolate was determined by sequencing and analyzing the ITS region

3.2.2.1. Amplification of the ITS region

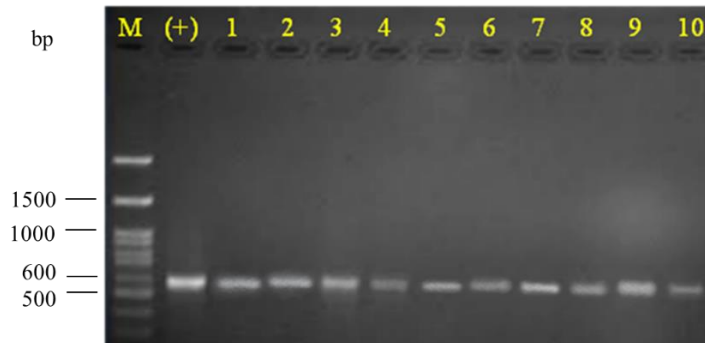


Figure 3.11. Electrophoresis results of PCR products on 1.5% (w/v) agarose gel.

Legend: M: Marker (Bio-Helix) 100–3,000 bp; (+): positive control. Lanes 1–10 correspond to the strain numbers listed in Table 3.7.

3.2.2.2. Scientific identification

Table 3.7. Scientific identification of 10 fungal isolates based on ITS sequence length and BLAST analysis

Ord.	Fungal Isolates	Accession number	Length	Identity	Scientific name	Strain code
1	M24	MW113607.1	535 bp	100%	<i>Penicillium janthinellum</i>	<i>Pj</i> -LHOP2
2	NT9.3	KX766390.1	446 bp	99,78%	<i>Penicillium simplicissimum</i>	<i>Ps</i> -LHOP1
3	T3X	OM372777.1	490 bp	99,59%	<i>Talaromyces pinophilus</i>	<i>Tp</i> -LHOP1

4	MS5	KU729029.1	536 bp	100%	<i>Trichoderma virens</i>	Tv-LHOP1
5	MS9	KY022748.1	538 bp	100%	<i>Aspergillus carbonarius</i>	Ac-LHOP1
6	MS4	MT176459.1	539 bp	100%	<i>Trichoderma virens</i>	Tv-LHOP2
7	M26	PP385632.1	535 bp	100%	<i>Purpureocillium lilacinum</i>	Pl-LHOP1
8	X4	MF992201.1	535 bp	100%	<i>Trichoderma koningiopsis</i>	Tk-LHOP1
9	X0	MT176459.1	533 bp	100%	<i>Trichoderma virens</i>	Tv-LHOP3
10	MS43	KF305757.1	523 bp	100%	<i>Chaetomium cupereum</i>	Cc-LHOP1

Phylogenetic tree

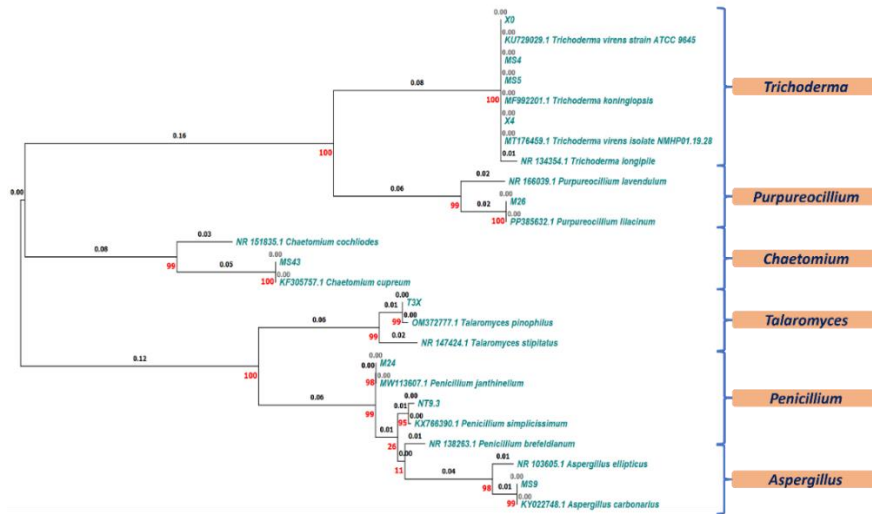


Figure 3.12. The phylogeny tree of 10 selected isolates and closely related species was recovered from GenBank based on ITS sequences.

The expressed value on each branch was the Bootstrap of 1000 replications.

3.2.3. Study on the biological characteristics of the selected microbial strain

3.2.3.1. Phosphate-solubilizing activity

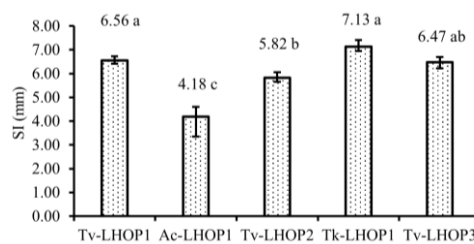


Figure 3.13. Phosphate solubilization index (PSI) of five positive fungal strains in PVK medium on day 5

Note: Different letters (a, b, c) above the columns indicate statistically significant differences at $P < 0.05$.

3.2.3.2. Cellulose-degrading activity

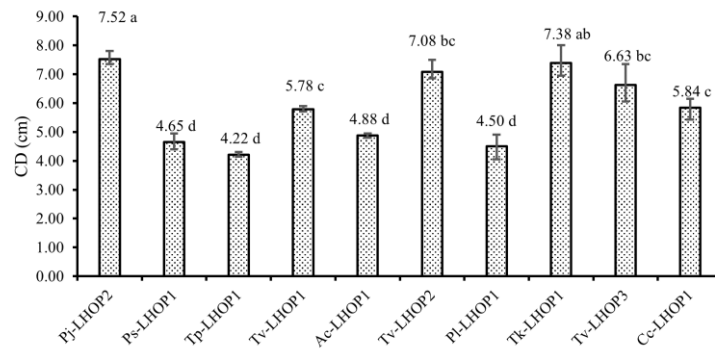


Figure 3.14. Cellulose-degrading ability of 10 fungal strains isolated from soil.

Note: Different letters (a, b, c, d) above the columns indicate statistically significant differences at $p < 0.05$

3.2.3.3. Hemicellulose-degrading activity

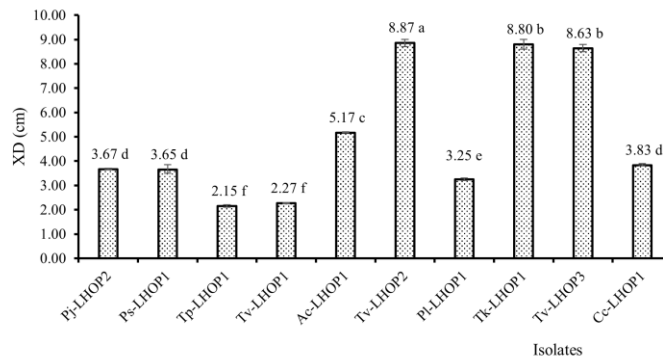


Figure 3.15. Xylan-degrading ability of 10 fungal strains isolated from soil.

Note: Different letters (a, b, c, d, e, f) above the columns indicate statistically significant differences at $p < 0.05$

3.2.3.4. Starch- and protein-degrading activities of selected fungal strains

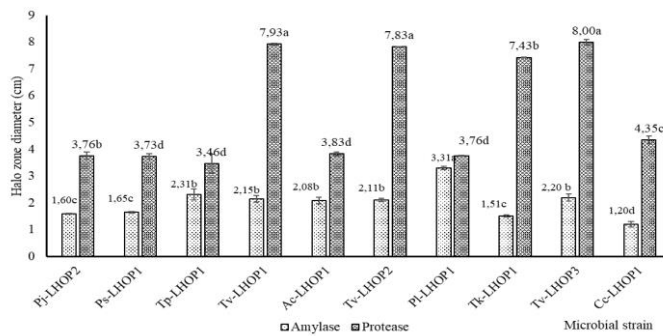


Figure 3.16. Starch- and protein-degrading activities of 10 fungal strains isolated from soil

Note: Different letters (a, b, c, d) above the columns indicate statistically significant differences at $p < 0.05$

3.2.4. Study on fungal spore biomass production for bioformulation development

3.2.4.1. Effect of culture medium type

Table 3.8. Spore yield of selected fungal strains on different culture media (after 30 days of incubation)

Fungal strain	Medium					CV(%)	LSD _{0,05}
	PDA (10 ⁸ /g)	PDB (10 ⁸ /g)	CMA (10 ⁸ /g)	Rhichard (10 ⁸ /g)			
Pj-LHOP2	19,20 ^a	17,62 ^a	19,40 ^a	9,70 ^b	10,42	2,18	
Ps-LHOP1	18,75 ^{ab}	18,20 ^b	19,00 ^a	9,20 ^c	3,42	0,70	

<i>Tp</i> -LHOP1	18,35 ^a	17,16 ^a	18,50 ^a	10,55 ^b	9,83	2,04
<i>Tv</i> -LHOP1	21,90 ^a	20,15 ^a	20,22 ^a	3,18 ^b	11,08	2,21
<i>Ac</i> -LHOP1	29,50 ^a	26,74 ^b	24,50 ^c	1,18 ^d	6,96	1,73
<i>Tv</i> -LHOP2	22,30 ^a	21,48 ^a	21,13 ^a	3,91 ^b	8,11	1,69
<i>Pl</i> -LHOP1	3,52 ^{ab}	3,22 ^b	3,86 ^a	1,23 ^c	10,48	0,37
<i>Tk</i> -LHOP1	23,74 ^a	22,28 ^b	23,15 ^{ab}	4,28 ^c	4,19	0,94
<i>Tv</i> -LHOP3	22,64 ^a	21,05 ^b	20,72 ^b	3,88 ^c	6,31	1,31
<i>Cc</i> -LHOP1	8,08 ^a	7,10 ^b	7,45 ^b	5,86 ^c	9,43	0,83

Note: Different letters (a, b, c, d) in the same row indicate statistically significant differences at $P < 0.05$

3.2.4.2. Effect of incubation temperature

Table 3.9. Effect of incubation temperature on spore yield of fungal strains used for microbial bioformulation

Fungal strain	Temp				CV (%)	LSD _{0,05}
	20 °C (10 ⁸ /g)	25 °C (10 ⁸ /g)	30 °C (10 ⁸ /g)	35 °C (10 ⁸ /g)		
<i>Pj</i> -LHOP2	9,50 ^c	20,50 ^a	19,68 ^a	16,75 ^b	7,96	1,99
<i>Ps</i> -LHOP1	8,92 ^c	19,55 ^a	19,05 ^a	15,35 ^b	5,28	1,25
<i>Tp</i> -LHOP1	8,06 ^c	19,02 ^a	19,00 ^a	14,20 ^b	5,04	1,14
<i>Tv</i> -LHOP1	22,23 ^d	28,34 ^b	30,52 ^a	26,15 ^c	4,36	1,76
<i>Ac</i> -LHOP1	21,47 ^c	29,47 ^b	33,47 ^a	29,77 ^b	4,40	1,89
<i>Tv</i> -LHOP2	20,32 ^c	27,42 ^b	29,91 ^a	27,05 ^b	4,22	1,66
<i>Pl</i> -LHOP1	2,01 ^b	3,67 ^a	3,84 ^a	2,15 ^b	13,36	1,33
<i>Tk</i> -LHOP1	24,55 ^d	32,00 ^b	34,25 ^a	27,22 ^c	4,15	1,84
<i>Tv</i> -LHOP3	23,00 ^d	29,12 ^b	32,88 ^a	26,81 ^c	5,39	2,27
<i>Cc</i> -LHOP1	4,12 ^b	7,92 ^a	8,12 ^a	8,42 ^a	8,78	0,87

Note: Different letters in the same row indicate statistically significant differences at $P < 0.05$.

3.2.4.3. Effect of culture medium pH

Table 3.10. Effect of culture medium pH on spore yield (10⁸ CFU/g) of selected fungal strains for bioformulation

Fungal strain	pH				CV (%)	LSD _{0,05}
	4	5	6	7		
<i>Pj</i> -LHOP2	15,52 ^{bc}	19,50 ^a	18,52 ^{ab}	14,29 ^c	12,49	3,19
<i>Ps</i> -LHOP1	16,62 ^b	20,34 ^a	20,42 ^a	15,55 ^b	13,25	3,64
<i>Tp</i> -LHOP1	14,75 ^b	19,05 ^a	18,72 ^a	14,84 ^b	12,17	3,08
<i>Tv</i> -LHOP1	20,23 ^c	24,23 ^b	28,53 ^a	18,58 ^c	8,64	2,98
<i>Ac</i> -LHOP1	20,23 ^b	23,47 ^a	35,21 ^a	35,98 ^b	9,54	4,12
<i>Tv</i> -LHOP2	21,15 ^b	25,22 ^a	24,64 ^a	18,02 ^c	11,89	2,98
<i>Pl</i> -LHOP1	1,25 ^b	4,57 ^a	4,85 ^a	5,12 ^a	23,71	1,41
<i>Tk</i> -LHOP1	22,10 ^a	25,42 ^a	24,87 ^a	17,55 ^b	11,10	3,78
<i>Tv</i> -LHOP3	21,18 ^b	24,72 ^a	25,05 ^a	18,72 ^c	17,93	2,29
<i>Cc</i> -LHOP1	3,12 ^{ab}	6,12 ^a	6,23 ^a	5,27 ^b	29,96	2,34

Note: Different letters (a, b, c) in the same row indicate statistically significant differences at $P < 0.05$

3.2.4.4. Effect of incubation time

Table 3.11. Effect of incubation time on spore yield of the studied fungal strains

Day \ Fungal strain	10 (10 ⁸ /g)	15 (10 ⁸ /g)	20 (10 ⁸ /g)	25 (10 ⁸ /g)	30 (10 ⁸ /g)	CV (%)	LSD _{0,05}
<i>Pj</i> -LHOP2	6,24 ^b	13,05 ^a	13,25 ^a	13,28 ^a	12,75 ^a	5,80	1,03
<i>Ps</i> -LHOP1	5,72 ^b	12,77 ^a	12,85 ^a	12,80 ^a	12,00 ^a	5,07	0,86
<i>Tp</i> -LHOP1	5,21 ^b	12,00 ^a	12,65 ^a	12,55 ^a	12,34 ^a	4,53	0,15
<i>Tv</i> -LHOP1	20,30 ^b	23,89 ^a	23,96 ^a	23,64 ^a	22,05 ^{ab}	6,48	2,22
<i>Ac</i> -LHOP1	17,50 ^b	20,50 ^a	20,62 ^a	20,63 ^a	20,61 ^a	4,74	1,43
<i>Tv</i> -LHOP2	19,66 ^b	22,71 ^a	24,05 ^a	23,34 ^a	20,36 ^b	4,76	1,59
<i>Pl</i> -LHOP1	0,05 ^c	0,85 ^b	1,26 ^b	2,67 ^a	2,86 ^a	23,00	0,53
<i>Tk</i> -LHOP1	20,80 ^b	24,05 ^{ab}	25,72 ^a	25,54 ^a	24,30 ^{ab}	3,67	1,34
<i>Tv</i> -LHOP3	20,13 ^b	23,92 ^a	24,70 ^a	23,77 ^a	22,86 ^{ab}	4,79	1,67
<i>Cc</i> -LHOP1	0,15 ^c	2,20 ^b	3,10 ^b	6,97 ^a	7,12 ^a	15,34	0,90

Note: Different letters (a, b, c) in the same row indicate statistically significant differences at $P < 0.05$

3.3. Preliminary formulation of bio-inoculants and evaluation of their effects on *Nanhaia speciosa*

3.3.1. Preliminary formulation of microbial bio-inoculants

3.3.1.1. Interaction among selected fungal strains

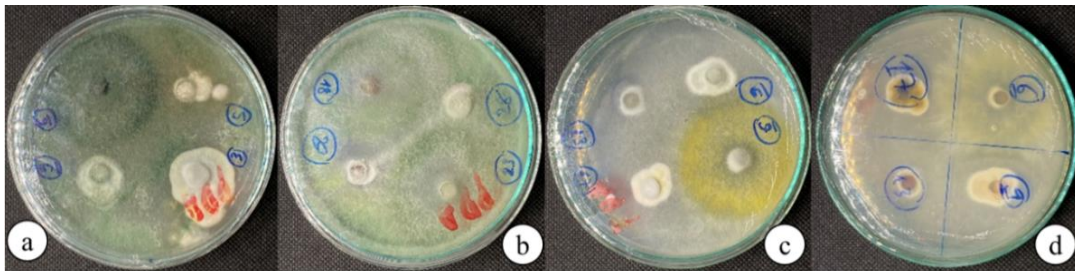


Figure 3.17. Interaction assay of fungal strains for bioformulation development

Legend: a. 9 (*Ps*-LHOP1), 6 (*Tk*-LHOP1), 3 (*Ps*-LHOP1), 5 (*Pl*-LHOP1), b. 18 (*Tv*-LHOP3), 20 (*Ac*-LHOP1), 28 (*Cc*-LHOP1), 23 (*Tv*-LHOP1), c. 13 (*Tp*-LHOP1), 17 (*Tv*-LHOP2) 6 (*Tk*-LHOP1), 9 (*Ps*-LHOP1), d. 6 (*Tk*-LHOP1), 9 (*Ps*-LHOP1), 13 (*Tp*-LHOP1), 17 (*Tv*-LHOP2)

3.3.1.2. Determination of adjuvants for bioformulation blending

Table 3.12. Spore germination ability of fungal strains in the bioformulation

Time \ Formulation	1 month (%)	3 month (%)	6 month (%)	9 month (%)
CT1	100	95,87 ^a	93,34 ^a	86,75 ^b
CT2	100	97,65 ^a	95,17 ^a	89,62 ^a
CT3	100	93,47 ^b	90,15 ^b	81,64 ^c
CV (%)	-	9,34	13,18	12,27
LSD_{0,05}	-	1,82	2,37	2,83

Note: Different letters (a, b, c) in the same column indicate statistically significant differences at $p < 0.05$

3.3.1.3. Preparation of experimental bioformulation

Table 3.13. Key technical parameters in spore production of fungal strains

No	Fungal strain	Optimal pH	Optimal temperature (°C)	Optimal incubation time (days)

1	<i>Pj</i> -LHOP2	5-6	25-30	15-20
2	<i>Ps</i> -LHOP1	5-6	25-30	15-20
3	<i>Tp</i> -LHOP1	5-6	25-30	15-20
4	<i>Tv</i> -LHOP1	5-6	25-35	15-20
5	<i>Ac</i> -LHOP1	5-6	25-35	15-20
6	<i>Tv</i> -LHOP2	5-6	25-35	15-20
7	<i>Pl</i> -LHOP1	5-6	25-30	25-30
8	<i>Tk</i> -LHOP1	4-6	25-35	15-20
9	<i>Tv</i> -LHOP3	5-6	25-35	15-20
10	<i>Cc</i> -LHOP1	4-6	25-35	25-30

After evaluating the influencing factors, the formulation was finalized as follows: mixing at the CT2 ratio (70% kaolin + 20% potassium humate + 10% fungal spores).

3.3.2. Effect of microbial bioformulation on *Nanhaia speciosa* at the nursery stage

Selected growth parameters of *Nanhaia speciosa*

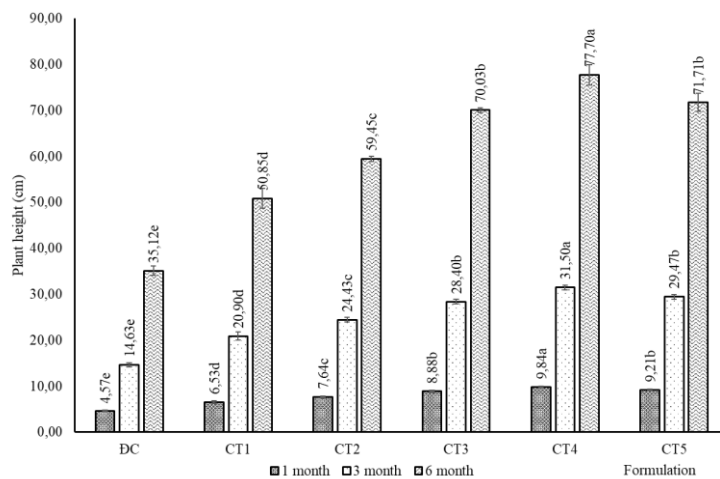


Figure 3.18. Effect of bioformulation dosage on the plant height of *Nanhaia speciosa*
Note: Different letters (a, b, c, d, e) above the columns indicate statistically significant differences at $p < 0.05$

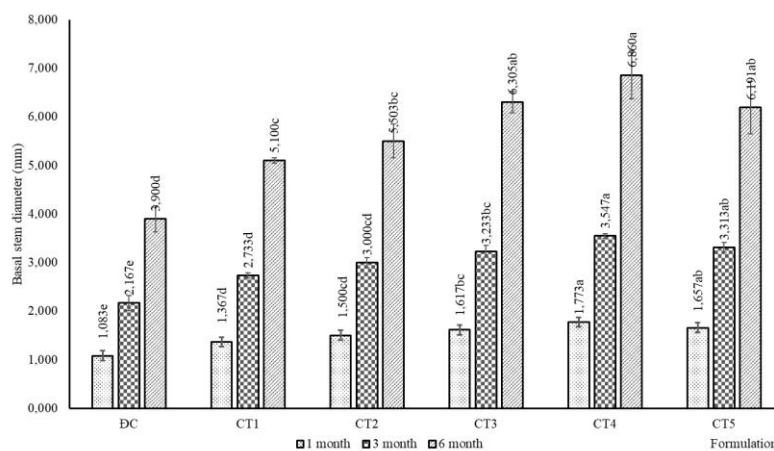


Figure 3.19. Effect of bioformulation dosage on the stem diameter of *Nanhaia speciosa*
Note: Different letters (a, b, c, d, e) above the columns indicate statistically significant differences at $p < 0.05$

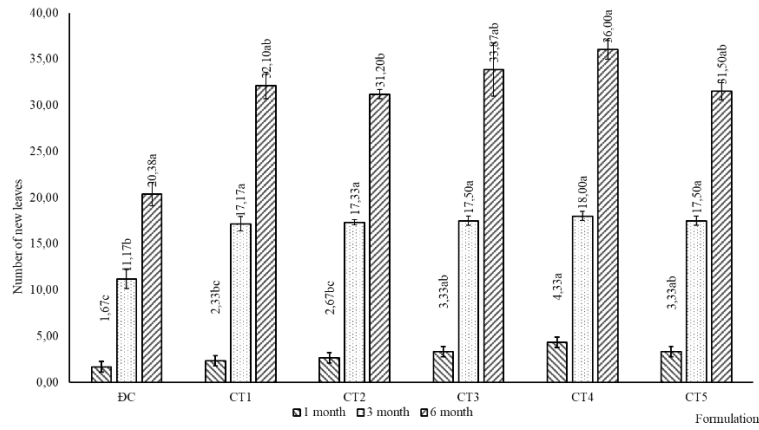


Figure 3.20. Effect of bioformulation dosage on the number of new leaves in *Nanhaia speciosa*
 Note: Different letters (a, b, c, d, e) above the columns indicate statistically significant differences at $p < 0.05$

Total chlorophyll content of *Nanhaia speciosa*

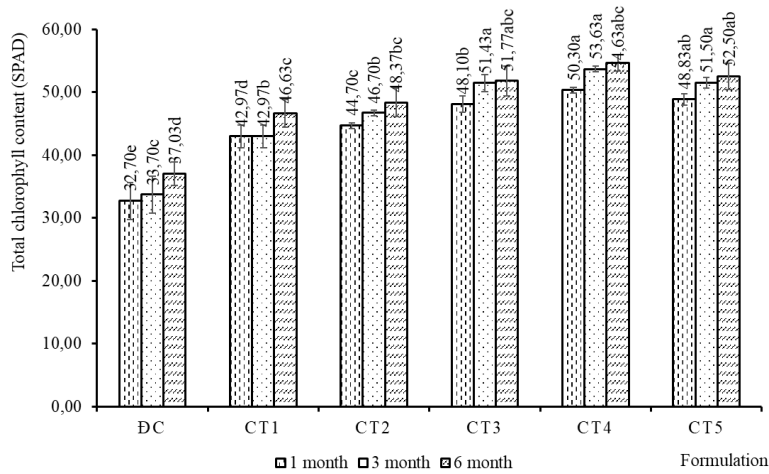


Figure 3.21. Chlorophyll content in the leaves of *Nanhaia speciosa*
 Note: Different letters (a, b, c, d, e) above the columns indicate statistically significant differences at $p < 0.05$

Chlorophyll fluorescence of *Nanhaia speciosa*

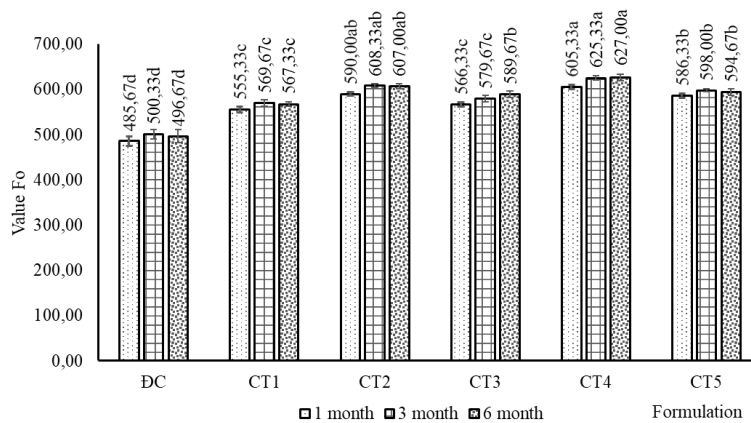


Figure 3.22. Fo chlorophyll fluorescence value of *Nanhaia speciosa*
 Note: Different letters (a, b, c, d, e) above the columns indicate statistically significant differences at $p < 0.05$

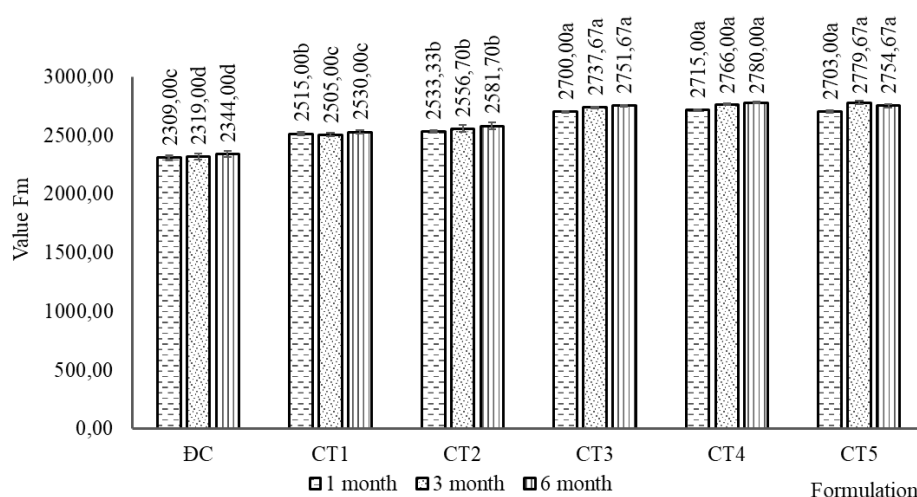


Figure 3.23. Fm chlorophyll fluorescence value of *Nanhaia speciosa*

Note: Different letters (a, b, c, d, e) above the columns indicate statistically significant differences at $p < 0.05$

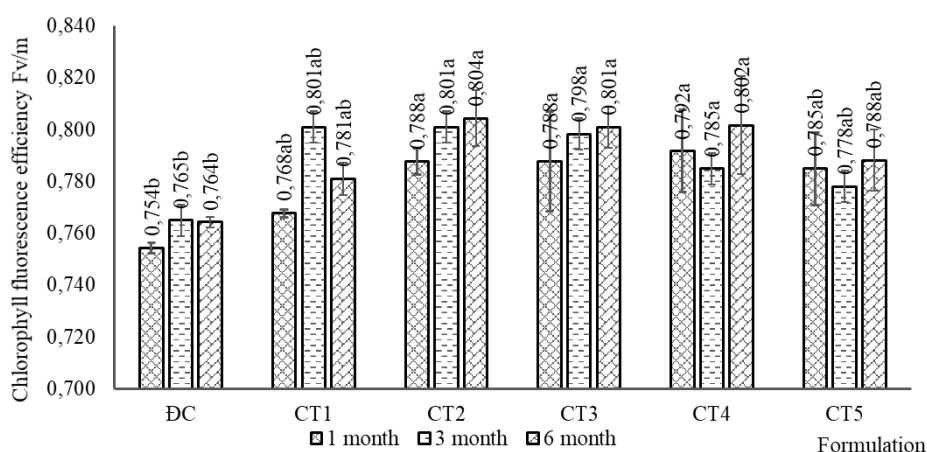


Figure 3.24. Fv/m chlorophyll fluorescence efficiency value

Note: Different letters (a, b) above the columns indicate statistically significant differences at $p < 0.05$

3.3.3. Evaluation of the effects of microbial bioformulation on the growth of *Nanhaia speciosa* under field conditions

Selected growth parameters of *Nanhaia speciosa*

Table 3.14. Effect of bioformulation dosage on the growth of 4-year-old *Nanhaia speciosa* under field conditions in Ngoc Thanh, Vinh Phuc (3 months after application)

Parameter	Plant height (cm)	Basal stem diameter (cm)	Canopy width (cm)	Plant condition
Control: no application	145,0 ^c	1,9 ^b	94,7 ^c	Slightly yellowing leaves
CT1. Treatment 2kg/ha	156,3 ^{bc}	2,1 ^{ab}	106,0 ^b	Healthy plant with green leaves
CT2. Treatment 3kg/ha	167,3 ^b	2,2 ^{ab}	111,7 ^b	Healthy plant with green leaves
CT3. Treatment 4kg/ha	180,0 ^a	2,3 ^a	126,7 ^a	Healthy plant with green leaves
CT4. Treatment 5kg/ha	178,6 ^a	2,0 ^b	123,2 ^a	Healthy plant with green leaves

<i>CV (%)</i>	4,81	10,39	5,41	
<i>LSD_{0,05}</i>	11,75	0,33	8,95	

Note: Different letters (a, b, c, ...) in the same column indicate statistically significant differences at $p < 0.05$

Total chlorophyll content of *Nanhaia speciosa*

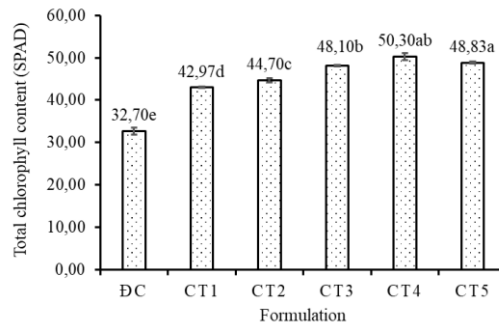


Figure 3.25. Chlorophyll content in the leaves of *Nanhaia speciosa* under field conditions

Note: Different letters (a, b, c, d) above the columns indicate statistically significant differences at $p < 0.05$

Chlorophyll fluorescence of *Nanhaia speciosa*

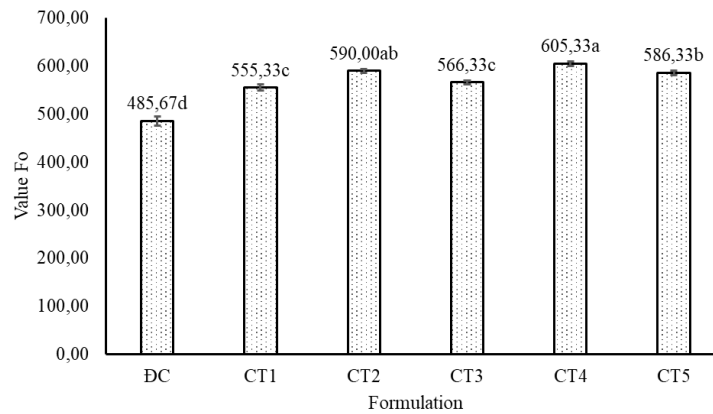


Figure 3.26. Fo chlorophyll fluorescence value of *Nanhaia speciosa* under field conditions

Note: Different letters (a, b, c) above the columns indicate statistically significant differences at $p < 0.05$

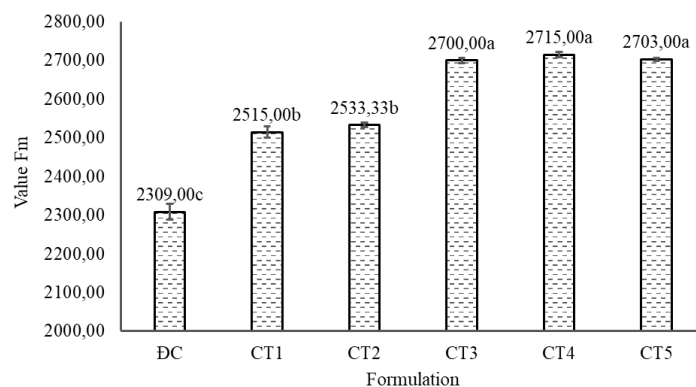


Figure 3.27. Fm chlorophyll fluorescence value of *Nanhaia speciosa* under field conditions

Note: Different letters (a, b, c) above the columns indicate statistically significant differences at $p < 0.05$

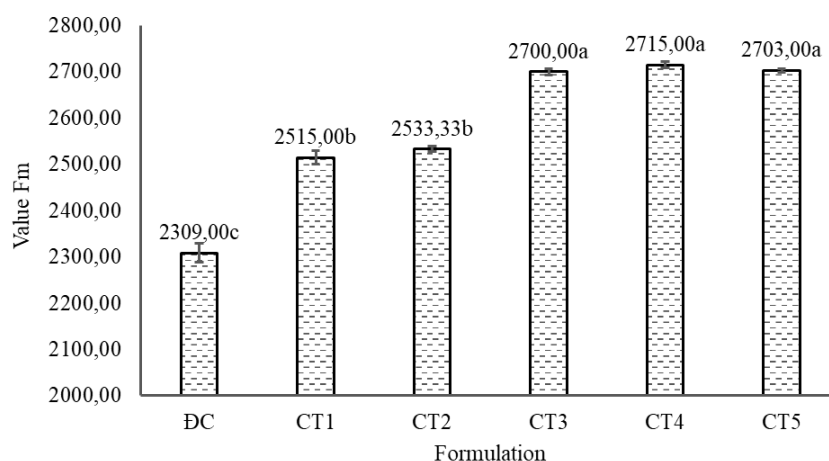


Figure 3.28. Fv/m chlorophyll fluorescence value of *Nanhaia speciosa* under field conditions

Note: Different letters (a, b, c, d, e) above the columns indicate statistically significant differences at $p < 0.05$

3.3.4. Effect of microbial bioformulation on microbial diversity and soil fertility in *Nanhaia speciosa* cultivation

Microbial diversity

Table 3.15. Effect of microbial bioformulation on microbial population density in *Nanhaia speciosa* nursery soil after 3 months of treatment

Treatment	Before application	After 3 months
Control	$1,02 \times 10^{4a}$	$3,32 \times 10^{4d}$
CT1. 0,5 g/plant	$1,01 \times 10^{4a}$	$17,18 \times 10^{4c}$
CT2. 1,0 g/plant	$0,9 \times 10^{4a}$	$20,70 \times 10^{4b}$
CT3. 1,5 g/plant	$1,03 \times 10^{4a}$	$25,36 \times 10^{4a}$
CT4. 2,0 g/plant	$1,04 \times 10^{4a}$	$27,66 \times 10^{4a}$
CT5. 2,5 g/plant	$0,94 \times 10^{4a}$	$27,83 \times 10^{4a}$
CV(%)	12,64	15,32
LSD ₀₅	0,96	2,34

Note: Different letters (a, b, c, d) above the columns indicate statistically significant differences at $p < 0.05$

Soil fertility

Table 3.16. Soil nutrient content in experimental plots after 6 months of treatment

Treatment	pH	Organic matter (%)	Total nitrogen (%)	Total phosphorus (%)
Control	4,56 ^d	3,17 ^b	0,18 ^e	0,08 ^c
CT1	4,78 ^c	4,54 ^a	0,33 ^d	0,13 ^b
CT2	4,87 ^b	4,61 ^a	0,35 ^c	0,15 ^a
CT3	4,95 ^a	4,56 ^a	0,37 ^b	0,15 ^a
CT4	4,96 ^a	4,57 ^a	0,38 ^a	0,15 ^a

Note: Different letters (a, b, c, d, e) above the columns indicate statistically significant differences at $p < 0.05$

The application of the microbial formulation contributed to improving the growth rate of *Nanhaia speciosa*. Physiological indicators such as chlorophyll fluorescence and total chlorophyll content also changed in a positive manner, beneficial to plant development. In addition, soil fertility and certain biochemical components of the soil were enhanced, leading to improved nutrient availability.

Chapter 4. CONCLUSIONS AND RECOMMENDATIONS

4.1. Conclusions

There were distinct morphological and genetic differences among the population groups of *Codonopsis javanica*. The sample collected from Tan Yen exhibited fewer leaflets, unique apex and base leaf shapes, and a different density of trichomes on the petiole compared to samples from Son Dong and Loc Binh. Floral morphological traits showed little variation among populations, indicating high genetic stability of reproductive organs. Genetic diversity analysis using ISSR and RAPD markers revealed high polymorphism rates (ISSR: 80.23%; RAPD: 95.7%), confirming the rich genetic diversity within *Codonopsis javanica* populations. Cluster analysis based on both methods showed a clear separation of the Tan Yen sample from the Son Dong and Loc Binh groups, indicating that it is a valuable genetic resource for breeding studies.

A total of 72 fungal strains belonging to six genera-*Penicillium*, *Trichoderma*, *Aspergillus*, *Talaromyces*, *Purpureocillium*, and *Chaetomium*-were isolated and morphologically characterized from rhizospheric soils of *Codonopsis javanica* in Vinh Phuc, Bac Giang, and Lang Son provinces. The genus *Penicillium* was the most diverse, accounting for over 50% of the isolates. Molecular identification based on ITS gene sequencing was used to determine the scientific names of 10 promising strains for microbial inoculant development. Their abilities to solubilize poorly soluble phosphate and decompose organic substances such as cellulose, xylan, starch, protein, and lipid were evaluated. Five strains - Tv-LHOP2, Tv-LHOP3, Tk-LHOP1, Ac-LHOP1, and Pj-LHOP2-showed strong phosphate solubilization activity. All tested strains exhibited varying levels of organic matter degradation, demonstrating potential for application in microbial bioformulations.

Culture conditions (nutrient media, temperature, incubation time, pH, etc.) for spore production and substrate mixing ratios were determined to optimize spore viability in the microbial product. Field and nursery trials demonstrated significant effectiveness: plant growth parameters such as plant height, stem diameter, and number of new leaves increased by 1.7 to 2 times compared to controls, with optimal doses of 2 g/plant (nursery) and 4 kg/ha (field). In addition, physiological indicators such as chlorophyll content and chlorophyll fluorescence efficiency (Fv/m) improved, indicating more efficient photosynthesis. The bioformulation also enhanced soil chemical parameters (humus, N, P content, and pH), contributing to improved fertility and sustainability of the soil ecosystem.

4.2. Recommendations

Further studies should focus on selecting and developing high-quality lines of *Codonopsis javanica* for medicinal plant production based on the populations studied in this research.

Expand investigations on the enzymatic activity of the isolated fungal strains and their effects on the biochemical composition of *Codonopsis javanica* roots. Additionally, pilot-scale production and application of the microbial bioformulation in *Codonopsis javanica* cultivation areas should be conducted.

LIST OF SCIENTIFIC PUBLICATIONS BY THE AUTHOR RELATED TO THE DISSERTATION

1. Ong, P. X., Cao, C. B., Khang, D. T., Huy, T. G., Cao, P. B., Chu, H. D., et al. (2024). Morphological, anatomical and genetic diversity of *Nanhaia speciosa* (Champ. ex Benth.) J. Compton & Schrire from Northern Vietnam. *Biodiversitas Journal of Biological Diversity*, 25(3). <https://doi.org/10.13057/biodiv/d250333>
2. Ong, X. P., Nguyen, T. K. L., Pham, T. T., & Nguyen, T. M. N. (2024). Characteristics of several *Penicillium* strains isolated from soils cultivated with *Nanhaia speciosa* (Champ. ex Benth.) in Northern Vietnam. *Journal of Science and Technology – Thai Nguyen University*, 229(09), 413–421. <https://doi.org/10.34238/tnu-jst.9898>
3. Ong, X. P., Nguyen, H. T., Nguyen, V. T., Kieu, T. H. M., Duong, T. V., Bui, T. L., & La, V. H. (2024, July). Production and evaluation of the effectiveness of microbial formulations on the growth of *Nanhaia speciosa* (Champ. ex Benth.) J. Compton & Schrire (2019) in Vinh Phuc province. *Journal of Agriculture and Rural Development*, Special Issue on Plant Protection.
4. Ong, P. X., Nguyen, T. V., Bui, L. T., Nguyen, T. M. N., Do, K. T., Tran, G. H., et al. (2024). Diversity of rhizospheric fungi from *Nanhaia speciosa* and their capacity to degrade insoluble phosphate and organic matters. *Biodiversitas Journal of Biological Diversity*, 25(10). <https://doi.org/10.13057/biodiv/d251020>
5. Ong, X. P., Bui, T. L., Pham, B. N., Nguyen, V. D., & La, V. H. (2025). Effects of microbial formulations on growth parameters, chlorophyll content, and chlorophyll fluorescence of *Nanhaia speciosa* (Champ. ex Benth.) J. Compton & Schrire (2019). *Proceedings of the 3rd National Conference on Plant Physiology*.