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Tel: (84-28)37245670
Email: jad@hcmuaf.edu.vn

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CONTENT

Sustainable livelihood development and cultural preservation through agricultural tourism: local community perspectives from Dak Lak province, Vietnam

Hong T. A. Phan*
Tourism and Hospitality Management Faculty, Vietnam Women's Academy Ho Chi Minh City, Vietnam

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***Corresponding author**

Phan Thi Anh Hong
Email:
PTAnhHong@hvpnvn.edu.vn

ABSTRACT

This study conducted in collaboration with the local community focused on sustainable agricultural tourism development in Dak Lak, Vietnam. Using a qualitative research method, we conducted in-depth interviews with 29 households and agricultural cooperatives, combined with case studies. The results showed that agricultural tourism, a product of our joint efforts, played a significant role in diversifying livelihood sources and contributing to the preservation of ethnic and cultural identity. However, the sustainable development of this sector was hindered by limitations in infrastructure, lack of investment and uneven management capacity. Based on these findings, we propose specific policies and solutions to promote sustainable agricultural tourism development and support rural communities in the context of economic transition.

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1. Introduction

Agricultural tourism, a harmonious combination of agriculture and tourism, has become an effective model for sustainable rural development globally (Barbieri et al., 2019; Phillips et al., 2021). This model helps diversify income sources for farming households and contributes to the preservation of local culture the protection of the natural environment (Ghaderi et al., 2022; Ruiz-Labrador et al., 2023). In the context of developing countries, agricultural tourism offers attractive opportunities for socio-economic transformation, particularly in regions with rich agricultural heritage and biodiversity (Despotović et al., 2017).

With its diverse terrain and long-standing agricultural tradition, Vietnam offers great potential for agricultural tourism development (Pham, 2023). Remarkably, the Central Highlands region, known for being the largest

coffee-producing area in the country and its cultural diversity among ethnic minorities, is gaining attention as a potential destination for agricultural tourism (Bui & Nguyen, 2014). Dak Lak province, located in the heart of the Central Highlands, with its extensive coffee cultivation and ethnic diversity, faces both opportunities and challenges in developing this tourism model.

Although there has been considerable research on agricultural tourism globally (Kline et al., 2016; Ospanova et al., 2022), studies within the context of Vietnam, particularly in the Central Highlands with its unique cultural diversity among ethnic minorities, remain limited. Existing research mainly focuses on the economic aspects of agricultural tourism (Nguyen, 2022), while the reciprocal relationship between agricultural tourism and the preservation and enhancement of ethnic minority cultural identity has not been fully explored. Specifically, the role of local communities in integrating traditional cultural values into agricultural tourism activities, as well as the impact of tourism on cultural change and adaptation among ethnic minority communities, are areas that require further investigation.

Findings from previous studies highlight the need for a more comprehensive and multidimensional approach to researching agricultural tourism development in Dak Lak. This approach should consider the multifaceted nature of sustainability within the specific local context, particularly the relationship between agricultural tourism, community livelihoods and the preservation of ethnic minority cultures. This study aims of this study is to provide insights into the current situation, challenges and solutions for sustainable agricultural tourism development in Dak Lak, from the perspective of the local community.

This study focuses on three main aspects: (1) How is agricultural tourism in Dak Lak impacting the livelihoods and culture of the local community? (2) What challenges and barriers do the local community face in developing agricultural tourism in Dak Lak? (3) What policies and solutions need to be implemented to support sustainable agricultural tourism development in Dak Lak?

This study is significant both for Dak Lak province and for contributing to the literature on sustainable tourism in developing countries (Sharpley, 2020) and addresses the need for more specific research on the impacts of agricultural tourism (Gomez-Arroyo et al., 2013). The research findings will provide additional information for policymakers and practitioners in similar global contexts and contribute to the theory of sustainable rural development within transitional economies.

2. Literature Review

2.1. Agricultural tourism and sustainable development

In the broader picture of sustainable rural development, agricultural tourism emerges as an indispensable piece. In recent years, there has been a surge in research on this field, ranging from a global perspective to analyses within the context of Vietnam.

Kline et al. (2016) initiated research on the multifaceted impacts of agricultural tourism, highlighting its role in generating income and preserving agricultural heritage. Barbieri et al. (2019) found that communities engaged in agricultural tourism are more resilient to economic and environmental shocks. Ospanova et al. (2022) linked agricultural tourism to food security, viewing it as a bridge between production and sustainable consumption. Phillip et al. (2021) introduced a new framework for assessing the sustainability of agricultural

tourism under climate change, emphasizing adaptability as a key factor for future success.

In the context of Vietnam, the wave of research on agricultural tourism is equally dynamic. Agricultural tourism has become an effective tool for preserving and promoting the traditional cultural values of ethnic minority communities (Do et al., 2022; Duong et al., 2023). Nguyen et al. (2022) emphasized that despite being heavily impacted by Covid-19, agri-tourism in Dak Lak province still plays an important role in the local economy, accounting for a significant proportion of production value. Duong et al. (2023) analyzed the potential for developing various types of ecotourism in Dak Lak, including the integration of agri-tourism and ecotourism, by building branded tourism products that create unique experiences, combining environmental protection with sustainable development.

2.2. The role of local communities in sustainable agricultural tourism development

Local communities play a crucial role in sustainable agricultural tourism. Wannaprasert & Choenkwan (2021) argue that active community participation improves tourism experiences and enhances resilience against economic and environmental challenges. Karim et al. (2021) highlight the importance of social capital, noting that strong social networks and community trust are vital for sustainable development. Ciolac et al. (2022) add that digital technology can support this by connecting farmers with tourists and optimizing resource management through online platforms.

In Vietnam, many studies found that community participation in planning and management enhances project effectiveness and ensures equitable distribution of benefits (Le, 2020; Bui, 2021). The involvement of local communities in decision-making, implementation and sharing of tourism benefits is considered a key factor in sustainable tourism development in that area (Bui, 2021). The community's indigenous knowledge is crucial for creating unique and attractive tourism products (Nguyen & Truong, 2019). Le (2020) emphasized that local community involvement in agri-tourism planning in Dak Lak is essential to balance economic growth and environmental protection, enhancing their understanding of sustainable resource use.

2.3. Sustainable livelihoods and cultural preservation in agricultural tourism

In the broader context of rural development, agricultural tourism emerges as a versatile tool that b enhances livelihoods and preserves the cultural identity of local communities. Gao & Wu (2017) assert that agricultural tourism is not merely an economic activity but also a catalyst for comprehensive rural development and poverty reduction. Similarly, Ghaderi et al. (2022) found that agri-tourism in Iran has provided an economic safety net for farmers, enhancing their livelihoods by stabilizing incomes and strengthening community resilience to economic shocks. Garrido-Pérez et al. (2022) noted its role in preserving agricultural heritage, while Ruiz-Labrador et al. (2023) emphasized its value in maintaining biodiversity and transmitting indigenous knowledge.

In Vietnam, the wave of research on agricultural tourism is equally dynamic. Thieu (2021) emphasizing that agricultural tourism has become a "two-in-on" tool, improving income while also serving as an effective means for preserving and promoting local culture. Vu (2023) argued that agricultural tourism has created a new "stage" for traditional crafts, helping them survive and thrive in the modern context. Dao (2021) suggested that agricultural tourism has transformed cuisine from an "intangible" cultural aspect into a "tangible"

tourism product, contributing to both the preservation and widespread promotion of local culinary values. Nguyen (2022) emphasized that agri-tourism contributes both to the preservation of indigenous culture and to the diversification of livelihoods for local residents. The integration of agricultural activities with tourism creates economic opportunities while facilitating the preservation and promotion of the community's traditional cultural values.

Overall, despite numerous studies on sustainable agricultural tourism, some important aspects remain underexplored, especially in the context of Dak Lak province. Additionally, long-term research on the impact of agricultural tourism on the livelihoods and culture of ethnic minority communities in Dak Lak is limited, with a lack of in-depth studies on the local communities involved in agricultural tourism and the challenges and barriers they face. Focusing on the voices of these communities helps address these research gaps and ensures that policies and practices in agricultural tourism development accurately reflect the needs and perspectives of local residents, contributing to the creating of a truly sustainable and equitable agrarian tourism model in Dak Lak.

3. Research Methodology

This study employs a qualitative approach and a case study methodology, to explore sustainable agricultural tourism development in Dak Lak province, Vietnam. This method is chosen for its ability to provide a comprehensive view of the experiences, perspectives and challenges faced by local communities in developing agricultural tourism (Creswell & Poth, 2016).

3.1. Research objectives and sampling method

This study employs a purposive sampling method combined with snowball sampling to ensure the inclusion of diverse and relevant participants aligned with the research objectives (Patton, 2015). The data collection was conducted from January to June 2024. Table 1 presents a summary of the specialized agricultural cultivation areas and the distribution of samples across five distinct subregions in Dak Lak province. The selection of participants was conducted carefully, based on the characteristics of each subregion, and focused on individuals actively involved in agricultural production.

Table 1. Summary of the area of specialized agricultural cultivation and the number of samples

No.	Code	Sub-region	Area (%)	Specialized crops	Sample
1	SR1	Krong Ana - Serepok River Delta	14.51	Food production such as rice and corn, cocoa...	10
2	SR2	Chu Yang Sin high-lands	3.98	Forestry and agroforestry	2
3	SR3	Ea Sup Plain Subre-gion	28.43	Cashew, wet rice, fruit trees and me-dicinal plants, industrial crops	4
4	SR4	Buon Ma Thuot – Ea H'leo Plateau	16.17	Long-term industrial crops for com-mercial production, such as coffee, rubber, pepper and durian...	11
5	SR5	M'Drak Highland and Mountainous	15.82	Industrial crops such as cocoa, fruit trees and plantation forests	2

The study ultimately included 29 participants with diverse occupations in the field of agricultural tourism, including individuals and households involved in agricultural tourism, representatives from cooperatives and local tourism managers currently engaged in agricultural tourism activities. Table 2 provides a summary of the occupations of the participants.

Table 2. Summary of surveyed participants

No.	Code	Tourism - related Job	Sub-region	Location	Gender	Age
1	LP001	Coffee plantation and processing facility	SR4	Buon Ma Thuot	Male	45
2	LP002	Coffee plantation owner	SR4	Buon Ma Thuot	Male	33
3	LP003	Coffee plantation owner	SR4	Buon Ma Thuot	Male	51
4	LP004	Coffee plantation owner	SR4	Cu M'gar	Male	38
5	LP005	Coffee plantation owner	SR4	Cu M'gar	Male	60
6	LP006	Coffee plantation owner	SR5	M'Drak	Male	36
7	LP007	Cocoa farm owner	SR4	Buon Ma Thuot	Male	45
8	LP008	Cocoa farm owner	SR1	Krong Ana	Female	37
9	LP009	Pepper plantation owner	SR4	Buon Ma Thuot	Male	43
10	LP010	Pepper plantation owner	SR1	Krong Ana	Male	35
11	LP011	Pepper plantation owner	SR1	Buon Don	Female	65
12	LP012	The fruit orchard owner	SR5	M'Drak	Female	46
13	LP013	The fruit orchard owner	SR1	Buon Don	Female	58
14	LP014	The fruit orchard owner	SR3	Ea Sup	Male	55
15	LP015	The fruit orchard owner	SR1	Krong Ana	Male	37
16	LP016	Vegetable garden owner	SR3	Ea Sup	Female	45
17	LP017	Vegetable garden owner	SR4	Cu M'gar	Female	58
18	LP018	Herb garden owner	SR4	Buon Ma Thuot	Female	42
19	LP019	Herb garden owner	SR1	Buon Don	Female	65
20	LP020	Goat farm owner	SR1	Buon Don	Male	45
21	LP021	Fish farm owner	SR3	Ea Sup	Male	33
22	LP022	Fish farm owner	SR2	Lak	Male	60
23	LP023	Agricultural cooperative	SR1	Buon Don	Male	46
24	LP024	Agricultural cooperative	SR4	Cu M'gar	Male	41
25	LP025	Local tourism management	SR3	Ea Sup	Male	50
26	LP026	Local tourism management	SR1	Krong Ana	Male	55
27	LP027	Homestay owner	SR2	Lak	Female	47
28	LP028	Homestay owner	SR1	Krong Ana	Female	49
29	LP029	Farm product store	SR4	Buon Ma Thuot	Female	60

Among the 29 interviewees, 18 were male (62.1%) and 11 were female (37.9%). The average age of the participants was approximately 46.3 years. Their occupations were diverse within the agricultural tourism sector, including coffee plantation owners (6), cacao growers (2), pepper plantation owners (3), fruit orchard managers (4), vegetable and medicinal herb garden owners (4), goat and fish farmers (3), agricultural cooperative members (2), local tourism managers (2), homestay operators (2) and farm product retailers (1).

The sample size was determined based on the principle of data saturation, which means that data collection continued until no new information or themes emerged (Guest et al., 2020).

3.2. Data collection method

In-depth interview method

In-depth interviews were conducted with all survey participants (n = 29). Each interview lasted between 60 to 90 min and was recorded with the participants' consent. The main topics covered in the in-depth interviews are presented in Table 3.

Table 3. Main topics in In-depth interviews

Code	Theme	Description
TH1	Agricultural tourism activities	Current activities and plans
TH2	Impact of agricultural tourism	Effects on local livelihoods and culture
TH3	Opportunities and challenges	Development potential and encountered difficulties
TH4	Cultural preservation measures	Efforts and methods for preserving culture during tourism development
TH5	Proposals and expectations	Opinions on the support needed from government and relevant organizations

Non-participant observation method: Non-participant observation was conducted at key agritourism sites in Dak Lak, including coffee plantations, pepper gardens, livestock farms, fruit orchards and agritourism tours. Observations were meticulously recorded in field notes.

Secondary data analysis method: Secondary data analysis involved collecting and analyzing secondary documents published between 2018 and 2023, including socio-economic development reports, tourism and agriculture statistics and local agritourism policies and development plans.

3.3. Data analysis

The collected data were analyzed using the thematic analysis method outlined by Braun and Clarke (2006). The analysis process included six steps: (1) becoming familiar with the data, (2) generating initial codes, (3) searching for themes, (4) reviewing themes, (5) defining and naming themes and (6) writing the report. To ensure the reliability and validity of the study, we applied data triangulation, comparing results from various data sources (Denzin, 2017). Additionally, the analysis results were sent back to some of the research participants for verification and confirmation (member checking).

4. Study Site and Context

Dak Lak, located in the Central Highlands of Vietnam, has significant potential for agricultural tourism development due to its unique combination of diverse cultural heritage and rich agricultural resources. With over 650,000 ha of fertile land, primarily basalt soil, the province specializes in economic crops such as coffee, rubber, black pepper, and durian, with coffee being the main product, accounting for about 30% of the country's coffee-growing area (DLSD, 2023). Additionally, Dak Lak has 237 OCOP (One Commune One Product) products with export potential, providing favorable conditions for developing agricultural tourism tied to local produce (Nguyen et al., 2022). Moreover, the distinctive culture of 47 ethnic minorities, notably the E De and M'Nong communities, along with traditional farming practices and cultural festivals, has become a unique tourism resource, attracting visitors for agricultural tourism combined with cultural exploration (DLDCST, 2022). However, this development faces infrastructure and environmental conservation challenges, requiring a balance between agricultural production and natural resource protection (Nguyen, 2022). Nevertheless, Dak Lak remains a promising hotspot for sustainable agricultural tourism, contributing to economic development and the preservation of local culture.

5. Findings

5.1. Current status of Agri-Tourism development in Dak Lak

5.1.1. Livelihood diversification through Agri-Tourism

Agri-tourism in Dak Lak has developed into a multi-layered system, reflecting a transitioning from traditional tourism models to an integrated form combining agriculture, culture and services. Based on the community participation framework in tourism proposed by Tosun (1999) and the agri-tourism development model by Phillip et al. (2010), agri-tourism activities in Dak Lak can be categorized into three primary levels of participation: passive observation, interactive experiences and value chain integration. The classification of agri-tourism activities according to the levels of participation is summarized in Table 4, which presents the key activities corresponding to each level of involvement.

Table 4. Levels and percentage of Agri-Tourism activities in Dak Lak province

No.	Level of participation	Main activities	Quantity (n = 29)	Percentages (%)
1	Passive observation	Opening for visitors; Selling entrance tickets; Direct sales of products to visitors	19	65.52
2	Interactive experience	Organizing experiential activities (from harvesting, processing, finished products); Workshops and short courses; On-site dining services	7	24.14
3	Value chain integration	Building a complete value chain (from production to consumption); Organizing specialized courses; Combining tourism with related sectors (e.g., healthcare, therapy)	3	10.34

Level 1: Passive observation - Initial connection between Agriculture and Tourism

At the most basic level, agri-tourism in Dak Lak is manifested through passive observation, where farms and gardens open their doors to tourists primarily for observation and learning. Although simple, this form plays a crucial role in establishing an initial connection between farmers and visitors and opens up income diversification opportunities for rural communities. As shown in Table 5, the comparative price analysis of key agricultural products in Dak Lak reveals a significant increase in income through tourism channels.

Table 5. Comparative price analysis of key agricultural products in Dak Lak province

Product type	Price for traders (VND/kg)	Price for tourists (VND/kg)	Increase in income through tourism (%)	Average entrance fee (VND/person)
Coffee	116,200 - 121,700	135,000 - 150,000	16.2 - 23.3	60,000 - 90,000
Durian (Thai variety)	90,000 - 98,000	120,000 - 135,000	33.3 - 37.7	75,000 - 100,000
Pepper	147,000 - 149,000	160,000 - 180,000	8.8 - 20.8	40,000 - 60,000
Avocado	45,000 - 52,000	65,000 - 80,000	55.5 - 63.4	30,000 - 50,000
Cocoa	55,000 - 60,000	80,000 - 95,000	45.5 - 58.3	50,000 - 70,000

Since we started welcoming visitors, my family's income has increased by about 20%. Tourists pay for entry tickets and also buy coffee as souvenirs. This will give us have an additional sales channel for our products (LP002). These remarks indicate that agritourism generates direct income from ticket sales and expands the distribution channels for local products. Visitors' ability to purchase products directly from the farm provides additional income for the residents and offers an opportunity to sell products at better prices compared to selling to traders.

We organize tours to visit avocado orchards during the harvest season. Tourists really enjoy seeing how we care for and harvest the avocados. Many people order avocados directly from the orchard, which helps us get a better price than selling to traders (LP012, LP013). Through tourism activities, residents also have the chance to interact directly with customers, allowing them to take orders and build long-term relationships, which contributes to diversifying and stabilizing income sources for the local community.

The current status of agri-tourism in Dak Lak shows that passive observation is predominant (LP025, LP026). According to practical surveys, 65.52% of agri-tourism services in the province are purely sightseeing tours. This reflects two critical aspects of the current passive observation model: (1) generating direct income from entrance fees and (2) expanding the distribution channels for agricultural products.

However, despite its prevalence, the passive observation model still has limitations. It is "*… limited in creating added value and profound experiences for visitors*" (LP023) and "*has not fully exploited the potential of combining agriculture with tourism*" (LP026). Agri-tourism needs to move beyond the simple concept of "*farm visits*" to create more prosperous and more meaningful experiences for visitors (LP022).

Level 2: Interactive experiences - Enhancing engagement and adding value

A prominent development in the growth of agri-tourism in Dak Lak is the emergence of interactive experience models, accounting for 24.14% of the surveyed entities, as shown in Table 4. At this level, tourists observe and actively participate in agricultural activities and product processing. This approach enhances the experience for tourists and adds considerable value to agricultural products. This model marks a significant step forward in the local tourism industry and opens up many opportunities for sustainable development in rural communities.

Interactive experiences in agri-tourism in Dak Lak have evolved beyond mere "*farm visits*". Instead, tourists engage directly in agricultural activities, from harvesting to processing products (LP001). This enhances the tourist experience and adds significant value to agricultural products and benefits the local community (LP021).

We have a program called "A day as a coffee farmer". Tourists experience the entire process, from picking coffee, drying, roasting, to tasting. This not only increases income for the cooperative but also helps promote our coffee brand (LP005). Currently, we host around 500 domestic and international visitors each month who come to explore and learn about the coffee cultivation, care and processing process (LP001).

Notably, the interactive experience model extends beyond pure agricultural activities. At Yok Don Community Tourism Village, tourists have the opportunity to participate in traditional cooking classes using ingredients from the garden: "*Tourists experience both the culinary culture and learn how to prepare local specialties*" (LP016). This creative integration of agriculture, culture and tourism creates a multi-dimensional and unique tourism product that offers memorable experiences for tourists.

Agri-tourism in Dak Lak is undergoing a notable transformation through a strategy of diversifying experiences. The cooperative at Serepok 3 hydropower reservoir leverages the natural advantages and connects local farming households to create unique tourism activities. This approach contributes to preserving and promoting local natural resources and supports sustainable tourism development, harmonizing economic benefits with environmental protection.

With the advantage of the 2,000-ha water surface and five large and small islands of the Serepok 3 reservoir, the cooperative utilizes this resource for agri-tourism. The cooperative collaborates with local residents to develop various agricultural activities: homestays, eco-tourism, clean pepper and coffee production, aquaculture, livestock raising, etc. The cooperative has invested in building stilt houses, pavilions, accommodations and resorts to create a tourism ecosystem for agri-experiences. This ecosystem helps all members share and increase income by extending guest stays and providing a more comprehensive range of services (LP023).

The development of the agri-tourism model in Dak Lak also reflects trends toward sustainable and responsible tourism. A farm owner shares: With the goal of sustainable agriculture, we have focused on developing naturally friendly coffee gardens, without using any chemicals in

production and processing. This is also why our farm attracts visitors interested in agri-tourism (LP004). This indicates that interactive agri-tourism brings economic benefits and promotes sustainable farming practices, contributing to environmental protection.

From the above analysis, it can be observed that diversifying agri-tourism activities in Dak Lak provides three main benefits: (1) Enhances the value of experiences for tourists, potentially allowing for increased service prices. (2) Creates educational opportunities and raises awareness about agricultural production processes. (3) Builds emotional connections between consumers and products, contributing to long-term marketing strategies. Interactive agri-tourism has demonstrated significant potential in creating multi-dimensional value for local communities. By combining unique experiences, education and emotional connections, this model enhances economic value and contributes to the sustainable development of the tourism and agriculture sectors.

Level 3: Value chain integration – Towards Sustainable Development

Agri-tourism in Dak Lak is evolving through interactive experiences and transitioning into an integrated value chain model, which currently accounts for 10.34% (3 out of 29 respondents) of the total surveyed agri-tourism activities (Table 4). This value chain integration covers the entire production process, starting from the initial cultivation stage to the final consumption stage, forming a cohesive tourism experience. Although this model constitutes a smaller proportion compared to other levels of participation, its impact is substantial as it focuses on high-value-added products and specialized services.

An owner of coffee plantation and processing facility shared: "*We have established a complete value chain for coffee, from the nursery to the final cup. Tourists experience the production process and participate in professional coffee tasting courses* (LP001)". Moreover, this integration extends to related fields: We not only grow and process medicinal herbs but also organize health tourism tours, combining visits to herbal gardens with traditional health treatments (LP019).

This demonstrates the tremendous potential of combining agriculture with other industries to create unique and high-value tourism products. Professional coffee-tasting courses and health tours that combine herbal medicine enhance the tourist experience and generate new income streams for businesses and local communities.

The combination of high-quality agricultural products and unique tourism experiences has contributed to building a solid brand for Dak Lak's agricultural products: The cooperative has organized for members and local farmers to change cultivation practices, produce products meeting international standards, obtain certification and affix traceability labels… to sell products at higher prices. At the same time, we have linked with export businesses to gradually form a value chain and build a brand for the coffee region (LP025, LP029).

This integrated agri-tourism model has positively impacted on the local socio-economic development. In Cu M'gar district, thanks to the formation of specialized cultivation areas and brand development for agricultural products, the

poverty rate has decreased to about 5.1% and the near-poverty rate is 7.6% (LP024).

Agri-tourism in Dak Lak is moving towards sustainable development, harmoniously combining agriculture, tourism and local cultural preservation. This provides economic benefits and plays a vital role in enhancing the community's quality of life and protecting the Central Highlands' unique ecological environment, specifically Dak Lak.

5.1.2. Agri-Tourism's contribution to local cultural preservation

The survey process shows that agri-tourism in Dak Lak has actively integrated local cultural elements into the tourism experience. An E De farmer shared: We introduce coffee and allow tourists to experience the traditional coffee brewing method of the Ê Đê people. This helps us preserve and share our culture (LP006).

Agri-tourism has significantly contributed to the revival and development of traditional crafts. An M'Nong person stated: By incorporating our traditional crafts into tourism activities, tourists visit and purchase local handicrafts, allowing us to revive traditional weaving techniques. This creates additional income and helps preserve the weaving skills of our ancestors (LP018).

Through agri-tourism activities, a platform is also created to preserve and share indigenous agricultural knowledge. A long-term Kinh farmer in Dak Lak shared: "*We introduce tourists to the cultivation of coffee combined with native plants, a method learned from ethnic minorities. This is beneficial for the crops helps protect the environment*" (LP005, LP015).

Agri-tourism also facilitates cultural exchanges between tourists and local communities. An Ê Đê homestay owner shared: When tourists stay with us, we organize cultural exchange events, introducing them to the culture of the local ethnic minorities. Many tourists are very interested and want to learn more about our culture, promoting positive cultural interaction between tourists and the local community (LP027).

This study shows that agritourism in Dak Lak has made significant contributions to preserving local culture by integrating cultural elements into the tourism experience, reviving traditional crafts, preserving indigenous agricultural knowledge, and enhancing cultural exchange. However, attention must also be paid to challenges such as the risk of cultural commercialization.

5.2. Barriers and challenges faced by the community in developing agricultural tourism in Dak Lak province

Agri-tourism in Dak Lak is evolving with promising potential, yet it encounters several barriers that hinder its growth. The analysis and synthesis from interviews and reports highlight the primary challenges as summarized in Table 6, which outlines the key factors affecting the development of agricultural tourism in Dak Lak.

Table 6. Factors affecting the development of agricultural tourism in Dak Lak

No.	Factor	Description	Households respondings (n = 29)	Response rate (%)
1	Poor infrastructure	The transportation system and tourism facilities do not meet demand	22	78
2	Lack of investment capital	Difficulty in accessing investment capital and preferential loans	19	65
3	Lack of management and service skills	Farmers lack management experience and tourism service skills	17	59
4	Language barriers	Barriers in communicating with international tourists	12	43
5	Seasonality	Agricultural tourism mainly takes place during the harvest season	16	55

(1) Infrastructure and amenities limitations: One of the biggest challenges farmers in Dak Lak face in developing agri-tourism is the limited infrastructure and amenities. According to survey data, 78% of the interviewed households identified poor infrastructure as a critical factor affecting their capacity to attract tourists. A coffee farm owner in Cu M'gar district shared: "*The road to our farm is still difficult, especially during the rainy season. We want to attract more visitors, but the difficult travel conditions make many people hesitant*" (LP004). Many homestays and agri-tourism sites lack basic amenities expected by tourists, especially international guests. A homestay owner in Krong Ana district stated: "*We want to maintain traditional aspects of our stilt house, but we also understand that tourists need modern amenities such as internet and hot water. Balancing these two factors is not easy*" (LP025).

(2) Lack of investment capital: Another major constraint, as highlighted by 65% of respondents, is the lack of investment capital. Most agri-tourism initiatives are small-scale and self-funded, making it difficult for farmers to expand or upgrade their facilities. Farmers often find it challenging to access bank loans due to stringent requirements and high interest rates. Farmers expressed: "*We have ideas to develop our farm into a tourist attraction, but lack the capital to invest in facilities and build guest reception areas. Bank loans have high interest rates and we are concerned about the risks*" (LP008, LP004, LP020).

(3) Lack of management and tourism service skills: Despite having a rich agricultural base, 59% of the surveyed households acknowledged that they have experience in agriculture but lack skills in tourism management and service (LP010, LP014, LP028). A pepper farm owner combining tourism shared: "*Initially, we faced many difficulties in welcoming and guiding tourists. We know how to grow and care for peppers, but explaining this process to tourists in an engaging way is a challenge*" (LP009, LP011).

(4) Language barriers: For 43% of respondents, language barriers are a significant challenge, especially for attracting international tourists

(LP007, LP024, LP027). "*We are very eager to welcome international guests, but most people in our community cannot speak English*" (LP006, LP007). These shortcomings limit the ability to communicate and share culture with foreign tourists (LP021, LP025).

(5) Seasonality of agricultural activities: Agri-tourism in Dak Lak heavily depends on seasons, particularly for critical crops such as coffee and pepper, as indicated by 55% of survey respondents. A coffee farm owner shared: "*The coffee harvest season from November to January is when we receive the most visitors*" (LP003). Coffee and other crops like pepper, avocado, vegetables and fruits also have seasonal patterns. During off-seasons, the number of visitors significantly decreases, "*…impacting the income of local residents*" (LP007, LP009, LP017).

5.3 Solutions for promoting sustainable agricultural tourism development in Dak Lak

Based on the research results and the analysis of the current state of agri-tourism in Dak Lak, this section proposes several solutions to promote sustainable development. These include both short-term and long-term measures, with a focus on balancing economic development, cultural preservation and environmental protection, as follows:

First, improving infrastructure and facilities: To address the issue of inadequate infrastructure and facilities, collaboration between local authorities and tourism businesses is essential. Local governments and the Department of Transport should prioritize upgrading roads to agri-tourism sites, especially rural roads. Additionally, farm owners and agri-tourism businesses should improve facilities at tourist sites, focusing on providing basic amenities such as clean restrooms, drinking water and resting areas for visitors, ensuring a harmonious blend of local cultural characteristics and tourism service standards.

Second, investment support: To tackle the issue of insufficient investment capital, local authorities should collaborate with relevant departments such as the Department of Planning and Investment and the State Bank of Vietnam, Dak Lak Branch, to develop a special investment support program for agri-tourism with favorable interest rates. Furthermore, the provincial Tourism Association can act as an intermediary to connect large tourism enterprises with small businesses, creating opportunities for investment collaboration. Regular consultations on accessing funding and financial management should also be organized to support agri-tourism businesses.

Third, enhancing management and service skills: To improve management and service skills, the Department of Tourism and the Department of Labor, Invalids and Social Affairs should collaborate with local management boards to organize short-term training courses on tourism management, marketing, guest reception and customer care for farm owners and staff. Universities and colleges in the province should develop short-term training programs on agri-tourism, combining theory with practical experience at farms. A mentoring program connecting tourism experts with agri-tourism businesses is also a feasible proposal for long-term benefits.

Fourth, enhancing language skills for community: The Department of Education and Training should organize free basic English classes for local communities involved in agri-tourism. The Department of Tourism could collaborate with technology companies

to develop multilingual mobile applications introducing agri-tourism sites and local culture. The provincial Tourism Association could connect and create a network of volunteers who know foreign languages (students, retirees) to assist with translation at agri-tourism sites when necessary.

Fifth, diversifying seasonal tourism activities: To address the seasonality of agricultural activities, farm owners and agri-tourism businesses should develop additional tourism activities that are not dependent on the season, such as crafts, traditional cooking classes and local cultural experiences. The Department of Tourism and the Department of Agriculture and Rural Development should coordinate to create a year-round agri-tourism calendar, incorporating various crops and livestock to provide diverse experiences for visitors.

Last, enhancing branding and marketing: To improve branding and marketing effectiveness, the Department of Tourism and the Department of Information and Communications should develop a comprehensive branding strategy for Dak Lak agri-tourism, including creating a unified online platform to promote all agri-tourism sites in the province. The Dak Lak Tourism Association and travel companies should organize familiarization tours for media and travel bloggers and develop travel packages combining agri-tourism with other popular tourist attractions in the province to increase appeal and diversify visitor experiences.

6. Discussion

This study has explored the current state, potential and challenges of agritourism in Dak Lak, Vietnam. The findings indicate that agritourism is playing an increasingly important role in diversifying rural livelihoods, preserving local culture and promoting sustainable development. However, the sector also faces significant challenges that need to be addressed.

The research shows that agri-tourism in Dak Lak is not merely an economic activity but an ecosystem where economic, cultural and environmental factors interact closely. This reflects the global trend towards sustainable rural tourism development (Gao & Bryan, 2017) but it also has the unique characteristics of Dak Lak. In particular, combining coffee cultivation, ethnic minority cultures and tourism in Dak Lak creates a unique model that differs from traditional agri-tourism models. While Barbieri's (2013) study in the U.S. focused on the economic aspects of agri-tourism, the model in Dak Lak demonstrates a profound intersection between agriculture, culture and tourism.

Another finding of this study is the role of agri-tourism in preserving and promoting local culture, especially the culture of indigenous ethnic minorities. This is evident through the integrating of E De and M'Nong cultural elements into the agri-tourism experience. Unlike many traditional cultural tourism models that face the risk of excessive commercialization (Rana et al., 2021), agri-tourism in Dak Lak provides a natural platform for preserving and sharing culture.

The challenges faced by agri-tourism in Dak Lak, such as infrastructure limitations, lack of investment capital and management skills, reflect common issues in rural tourism development in many developing countries (Lack, 1997; Rambodagedara et al., 2015; Mahmoodi et al., 2022). However, the specific context of Dak Lak creates unique challenges and opportunities. Language barriers, rather than

merely a challenge, can be seen as an opportunity to develop new forms of communication and cultural experiences. Some communities have started using virtual reality technology and mobile applications to overcome this barrier, creating unique tourism experiences. Similarly, the seasonality of coffee and other crops has been turned into an opportunity to develop unique events and festivals that attract visitors year-round. This demonstrates the potential of local communities to transform challenges into opportunities for development.

7. Conclusions

The research indicates that agricultural tourism in Dak Lak has made significant contributions to the diversification of rural livelihoods, the preservation of local culture and the promotion of sustainable development. However, this model still faces several limitations, including inadequate infrastructure, lack of investment capital and insufficient management skills. Additionally, language barriers and seasonality pose challenges to the long-term development of the tourism sector. Nonetheless, the active and innovative engagement of the local community has turned these obstacles into opportunities by organizing cultural events, enhancing tourism experiences and utilizing technology to attract visitors. This highlights Dak Lak's substantial potential to become a leading model of sustainable agricultural tourism development, harmoniously integrating economic growth, cultural preservation and environmental conservation.

Limitations and Further Research

Although this study provides insights into agricultural tourism in Dak Lak, it still has several limitations. The research primarily focuses on the perspectives of the local community, without incorporating the viewpoints of other stakeholders. Additionally, the number of surveyed households and cooperatives remains limited, necessitating a broader sample size to achieve a more comprehensive assessment. Furthermore, the reliance on qualitative data has restricted the capacity for quantitative analysis of the specific economic impacts of agricultural tourism.

To overcome these limitations and expand the understanding of the topic, future research should focus on evaluating the long-term socio-economic impacts of agricultural tourism in Dak Lak, comparing this model with other regions in Vietnam and Southeast Asia and analyzing tourist experiences and satisfaction. These efforts would provide valuable insights for policy-making and sustainable development of agricultural tourism in the region.

Conflict of interest

The author confirms that there are no conflicts of interest regarding this study. The research was carried out independently and there were no financial or personal influences impacting the results or conclusions.

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Effects of different potassium doses on yield and quality of two sweet corn hybrid combinations (*Zea mays* var. *saccharata*) in Ho Chi Minh City

Phuong Nguyen[1*], & Thanh D. Huynh[1,2]

[1]Faculty of Agronomy, Nong Lam University, Ho Chi Minh City, Vietnam

[2]Dong Nai - Kratie Rubber Development Company Limited in Cambodia, Kratie, Cambodia

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***Corresponding author**

Nguyen Phuong
Email:
nguyenphuong@hcmuaf.edu.vn

ABSTRACT

The study aimed to determine the appropriate amount of potassium for two newly selected sweet corn hybrid combinations of the Department of Genetics and Plant Breeding, Nong Lam University, Ho Chi Minh City. The experiment was conducted in the winter-spring crop of 2023 - 2024 in Thu Duc, Ho Chi Minh City with 4 potassium levels (70, 90, 110, and 130 kg K_2O/ha) and 2 combinations of sweet corn hybrids BN191, BN211 and control variety (Golden Cob). The results showed that different rates of potassium affected the yield, quality and resistance to pests and diseases of sweet corn hybrid combinations. The potassium dosage of 130 kg K_2O/ha gave the highest fresh ear yield, low pests, and infection rate and highest Brix for the two selected hybrid combinations and the Golden Cob variety.

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1. Introduction

Besides selecting good varieties, determining appropriate cultivation practices for the new varieties is an important solution to maximize the yield potential. Nguyen (2013) stated that fertilizers contributed 40% to crop yield, varieties contributed 30%, and plant protection products contributed 20%. Each corn variety has different growth characteristics and yield, so the fertilizer regime is also different. The growth and yield of corn depend significantly on nutrient supply, irrigation intensity, soil and environment.

Potassium is one of the essential macronutrients for plants. Potassium is required for the activity of enzymes, controlling stomatal opening, enhancing resistance to pests, drought and low temperatures. Potassium promotes photosynthesis, transports photosynthetic

products to accumulate in seeds. Potassium is necessary as an activator for over 60 enzymes in the meristematic tissue. The important factor in cell division is the effect of potassium on the extension of cells. With sufficient potassium, cell walls are thicker and cell tissue is more stable. Because of this effect, cells grow normally, strengthen resistance and resist pests (Beringer & Nothdurft, 1985). Based on this reality, determining the appropriate dose of potassium helps inhance productivity, quality and pest resistance for the two newly selected sweet corn hybrids BN191 and BN211.

2. Materials and Methods

2.1. Materials

The materials studied include two hybrid sweet corn varieties (BN191, BN211) researched and hybridized at the Department of Genetic and Plant Breeding, Nong Lam University of Ho Chi Minh City, with the control variety Golden Cob (which is the most commonly grown in Vietnam. This variety is imported from Thailand and distributed by East - West Seed Company. The Golden Cob sweet corn has a short growth period of 68 to 72 days, an average plant height, and an average weight of 490 to 520 g per ear. The kernels are aligned, bright yellow, and have a soft and sweet taste and chloride Potassium fertilizer (Phu My potassium: 61% K_2O) from Petrovietnam Fertilizer and Chemicals Corporation.

2.2. Methods

2.2.1. Field experiment set up

The experiment involved two factors arranged in a split-plot design with 12 treatments, 3 replications. The subplot factor included 4 levels of potassium (K1: 70 kg K_2O/ha, K2: 90 kg K_2O/ha (control), K3: 110 kg K_2O/ha, K4: 130 kg K_2O/ha) and the main plot factor included 2 hybrid sweet corn varieties and the Golden Cob variety (G1: BN191, G2: BN211, G3: Golden Cob (control). The total number of base plots was 36; base plot area: 2.8 m × 5 m = 14 m^2; planting distance: 70 cm × 25 cm; total experimental area: 600 m^2.

Amount of fertilizer (kg/ha): The amounts of nitrogen, phosphorus, and cow manure are the same across the treatments, specifically: 150 kg N - 80 kg P_2O_5 and 10 tons of decomposed cow manure. Urea (46% N) and DAP (18% N - 46% P_2O_5) were used. Amount of potassium: applied according to each experimental treatment. KCl (61% K_2O) was used.

Fertilization method: All cow manure and phosphorus fertilizer and 1/4 of urea were applied together at the time of sowing. There were 4 top dressing fertilizer applications in which the first application used 1/4 urea at 10 - 15 days after sowing (when the plant had 3 - 4 leaves), the second application used 1/4 urea + 1/3 potassium at 25 - 30 days after sowing (when the plant had 7 - 8 leaves), the third application used 1/4 urea + 1/3 potassium at 40 - 45 days after sowing (when the plant had 10 - 11 leaves), and the fourth application used 1/3 potassium at 50 - 55 days after sowing (when the plant had over 11 leaves).

The experiment was conducted during the winter-spring 2023 - 2024 season at the research station of the Faculty of Agronomy, Nong Lam University, Ho Chi Minh City on sandy soil (sand:silt:clay ratio of 62:28:10), slightly acidic soil (with pH values of 6.5 and 5.3 for H_2O and KCl, respectively); low organic matter content in the soil (1.7%); poor in total nitrogen and total potassium.

2.2.2. Criteria and monitoring methods

Monitoring criteria include agronomic traits, pest damage status, yield parameters and quality of sweet corn varieties. The monitoring criteria and data collection methods comply with the National Standards for Agricultural Crop Varieties - Experimental value of cultivation and utilization of corn varieties (TCVN, 2021).

Leaf area (dm^2) measured once at the tasseling stage (50 days after sowing). Leaf length (dm) measured from the leaf collar to the leaf tip, leaf width (dm) measured at the widest point of the leaf Blade, measuring all green leaves on the plant. Leaf area (S) calculated using the formula:

$S\ (dm^2) = \Sigma\ (D \times R \times 0.7)$.

Fresh ear yield (tons/ha): $FY = (EW \times 10^{-3})/(S_o \times 10^{-4})$. Where: EW is the weight (kg) of fresh ears in the middle of 2 rows; S_o is the area of the middle of 2 rows (m^2).

2.2.3. Methods for statistical data processing

The collected data were statistically processed using SAS 9.1 software.

3. Results and Discussion

3.1. Growth parameters

Table 1. Effect of potassium dose on tasseling, silking and harvesting stage of sweet corn hybrid combinations

Agronomic Trait	Potassium levels (kg K_2O/ha) (B)	Hybrid combinations (A)			Mean B
		BN191	BN211	Golden Cob	
Days to tassel (DAS)	70	47.0	48.7	48.0	47.9
	90	47.0	48.7	47.3	47.7
	110	46.7	48.3	47.3	47.4
	130	47.3	48.7	48.3	48.1
	Mean A	47.0^{b}	48.6^{a}	47.8^{ab}	
	CV (%) = 1.3	$F_A = 19.83^{**}$	$F_B = 1.95^{ns}$	$F_{AB} = 0.41^{ns}$	
Days to silking (DAS)	70	50.3	51.7	52.7	51.6
	90	51.0	52.3	52.3	51.9
	110	50.3	52.3	52.7	51.8
	130	50.7	51.7	53.0	51.8
	Mean A	50.6^{b}	52.0^{a}	52.7^{a}	
	CV (%) = 1.0	$F_A = 50.59^{**}$	$F_B = 0.66^{ns}$	$F_{AB} = 1.48^{ns}$	
Day to harvest (DAS)	70	68.3	68.3	71.3	69.3
	90	68.3	68.7	71.0	69.3
	110	68.7	68.3	71.7	69.6
	130	68.3	68.7	72.0	69.7
	Mean A	68.4^{b}	68.5^{b}	71.5^{a}	
	CV (%) = 0.8	$F_A = 111.08^{**}$	$F_B = 0.75^{ns}$	$F_{AB} = 0.75^{ns}$	

*In the same group of mean values, numbers with the same accompanying characters indicate statistically insignificant differences; ns: no difference; **: significant difference at the level of α = 0.01; DAS: days after sowing.*

The data evaluation results in Table 1 showed that the flowering and pollen shedding stage occurs in a relatively short period but is a crucial period for determining the productivity of corn plants as it affects the fertilization process, determining the number of seeds on the ear. During this stage, nutrients from the stemp and leaves directed towards to the reproductive organs and organic compounds start accumulating towards to the seeds (Tran, 2004). The experimental results showed that in the winter-spring crop of 2023-2024, the varieties affected the growth period of sweet corn; There were significant statistical differences in days to tasseling, silking and harvesting among the experimental hybrid combinations; different levels of potassium did not affect the growth period of the experimental hybrid combinations. The days to tassel of the hybrid combinations ranged from 46.7 to 48.7 days after sowing (DAS), the days to silking time ranged from 50.3 to 53 DAS and the fresh ear harvesting time ranged from 68.3 to 72 DAS. Hybrid combination, BN191, has an earlier flowering, pollen shedding and harvesting time than hybrid combinations BN211 and Golden Cob variety.

3.2. Plant parameters

Table 2 showed that: Plant heights ranging from 214.7 to 228.8 cm. Plant height tended to increase proportionally with potassium doses, with the lowest plant height at 70 kg K_2O/ha and the highest at 130 kg K_2O/ha. However, this influence on plant height was not significantly different; the differences are not statistically meaningful. The average plant heights of different varieties was not significantly different.

Varieties with low ear-setting height show good resistance to lodging and high mechanization capabilities, but affect the pollination process and are easily damaged by pests and diseases (Tran, 2004). The results in Table 2 showed significant differences in ear-setting height among experimental corn varieties, with the BN211 hybrid reaching the tallest ear-setting height at 109.2 cm and the Golden Cob variety having the shortest ear-setting height (85.3 cm). The potassium dosage did not affect the ear-setting height of the experimental hybrid combinations and the differences in ear-setting height among experimental treatments are not statistically significant.

Table 2. Effect of potassium doses on plant characteristics of sweet corn hybrid combinations

Agronomic Trait	Potassium levels (kg K_2O/ha) (B)	Hybrid combinations (A)			Mean B
		BN191	BN211	Golden Cob	
Plant height (cm)	70	214.7	228.8	222.0	221.8
	90	226.4	221.9	219.9	222.7
	110	221.5	225.4	222.3	223.1
	130	221.1	227.3	221.8	223.4
	Mean A	220.9	225.8	221.5	
	CV (%) = 3.5	$F_A = 1.44^{ns}$	$F_B = 0.07^{ns}$	$F_{AB} = 0.80^{ns}$	
Ear-setting height (cm)	70	88.2	108.5	85.3	94.0
	90	93.8	110.5	85.1	96.5
	110	89.5	107.2	86.1	94.3
	130	92.6	110.5	84.8	96.0
	Mean A	91.0	109.2	85.3	
	CV (%) = 5.5	$F_A = 67.81^{**}$	$F_B = 0.50^{ns}$	$F_{AB} = 0.29^{ns}$	
Plant diameter (cm)	70	2.9	2.7	2.7	2.8
	90	2.7	3.1	3.0	2.9
	110	2.7	3.0	2.9	2.9
	130	3.1	3.1	3.1	3.1
	Mean A	2.8	2.9	2.9	
	CV (%) = 9.9	$F_A = 0.37^{ns}$	$F_B = 1.61^{ns}$	$F_{AB} = 0.65^{ns}$	

*In the same group of mean values, numbers with the same symbol indicate a non-statistically significant difference; ns: no statistically significant difference; **: difference at a significance level of α = 0.01.*

The stem diameter reflects the growth status of the plant and was directly relate to the plant's ability to resist falling. The large stem diameter indicates good growth, nutrient absorption, root development and high resistance to falling for corn plants, whereas the small stem diameter indicates the opposite. In the winter-spring crop of 2023 - 2024, experimental hybrid combinations had stem diameters that ranged from 2.7 - 3.1 cm. with no differences in stem diameter between two hybrid combinations and the Golden Cob variety. Stem diameter tended to increase with increasing levels of potassium. with the smallest stem diameter of 2.8 cm at a potassium dose of 70 kg K_2O/ha. and the largest stem diameter of 3.1 cm at a potassium dose of 130 kg K_2O/ha.

3.3. Leaf morphology traits

Table 3 showed that the number of leaves per plant ranged from 17.5 to 18.4. In overall, which is consistent with previous studies by the author Tran (1993); 2 hybrid combinations and the check variety had similar leaf numbers in the experiment, which also matched the similar growth periods of these varieties.

Table 3. Effect of potassium dose on leaf morphological characteristics of sweet corn hybrids

Agronomic Trait	Potassium levels (kg K_2O/ha) (B)	Hybrid combinations (A)			Mean B
		BN191	BN211	Golden Cob	
Number of leaf (leaf)	70	17.9	17.8	18.5	18.1
	90	18.1	16.7	18.2	17.7
	110	18.5	17.8	19.3	18.5
	130	17.3	17.9	17.5	17.5
	Mean A	18.0	17.5	18.4	
	CV (%) = 5.2	$F_A = 2.42^{ns}$	$F_B = 2.11^{ns}$	$F_{AB} = 1.06^{ns}$	
Leaf area index	70	3.18	3.29	3.38	3.28^{c}
	90	3.33	3.31	3.45	3.36^{bc}
	110	3.46	3.38	3.50	3.42^{b}
	130	3.69	3.53	3.55	3.59^{a}
	Mean A	3.40	3.38	3.46	
	CV (%) = 2.9	$F_A = 2.26^{ns}$	$F_B = 16.23^{**}$	$F_{AB} = 1.57^{ns}$	

*In the same group of average values, numbers with the same characters indicate no statistically significant difference. ns: no statistically significant difference; **: difference at α = 0.01 significance level.*

The experiment results showed that the dosage of potassium significantly affects the leaf area index, which is directly proportional to the amount of potassium fertilizer; the minimum leaf area index is when applying 70 kg K_2O/ha (3.28) and the highest leaf area index is achieved at a potassium dosage of 130 kg K_2O/ha (3.59). Considering the factor of variety. there is no difference in leaf area index between the two hybrid combinations and the Golden Cob variety.

3.4. Pest infestation and lodging

Due to the hot and humid tropical climate, sweet corn plants are often attacked by various harmful pests and diseases. Some of the main harmful pests and diseases on corn include: fall armyworm, corn borers, ear borers, corn stalk rot. Damage caused by pests and diseases can reduce corn yield by 10 - 30%. Especially during the tasseling and silking stages, if heavily affected by pests and diseases, it can severely impact harvest yield. The results of monitoring the effects of potassium doses on the resistance to certain harmful pests, lodging and drought tolerance of sweet corn hybrids are presented in Table 4.

Fall armyworm (*Spodoptera frugiperda*) is a polyphagous species originating from America, causing frequent and severe damage to corn plants. Experimental results showed that the infestation rate of fall armyworm on corn plants varied significantly with different doses of potassium. The infestation rate significantly decreased and differed significantly when the potassium dose increases from 70 kg K_2O/ha to 130 kg K_2O/ha. The research findings indicated that potassium dosage significantly influenced the resistance to fall armyworm of sweet corn hybrids. The density of fall armyworm, the level of damage and the lowest infestation rate occur when applying potassium at a dose of 130 kg K_2O/ha (9.1%) and the highest at the 70 kg K_2O/ha level (17.7%). Considering the genetic factor, there seems to be no difference in the infestation rate of fall armyworm between the two hybrid combinations and Golden Cob variety.

Corn borer (*Ostrinia nubilalis*) is a particularly dangerous pest, capable of causing the most severe damage and directly affecting the yield and quality of corn varieties. Experimental results showed that the amount of potassium significantly affected the infestation rate of European corn borer in corn hybrids. The

lowest damage rate was observed when applying potassium at a dose of 130 kg K_2O/ha (8.2%), while the highest damage rate was recorded at 70 kg K_2O/ha (12.1%). The potassium dose of 110 kg K_2O/ha and 130 kg K_2O/ha resulted in low infestation rates with no significant difference between them, but there was a statistically significant difference compared to the rates of 70 kg K_2O/ha and 90 kg K_2O/ha. Regarding the factor of sweet corn variety, there was no significant difference in the infestation rate of European corn borer between the two hybrids and the Golden Cob variety.

Table 4. Effect of potassium dose on pest infestation and lodging in the experiment

Subject	Potassium levels (kg K_2O/ha)	Hybrid combinations (A)			Mean B
		BN191	BN211	Golden Cob	
Fall armyworm (%)	70	18.9	16.6	18.1	17.9^{a}
	90	13.9	15.3	14.1	14.5^{ab}
	110	11.7	9.4	14.0	11.7^{bc}
	130	7.5	8.1	11.6	9.1^{c}
	Mean A	13.0	12.4	14.5	
	CV (%) = 19.6	$F_A = 2.30^{ns}$	$F_B = 19.21^{**}$	$F_{AB} = 1.03^{ns}$	
Corn borer (%)	70	12.3	9.7	14.3	12.1^{a}
	90	10.6	11.0	11.9	11.1^{ab}
	110	9.3	8.0	9.0	8.8^{b}
	130	8.0	8.7	8.0	8.2^{b}
	Mean A	10.1	9.3	10.8	
	CV (%) = 28.9	$F_A = 0.79^{ns}$	$F_B = 3.73^{*}$	$F_{AB} = 0.51^{ns}$	
Ear borer (%)	70	12.0	15.0	11.7	12.9
	90	18.7	13.3	10.0	14.0
	110	12.7	11.7	12.3	12.2
	130	5.7	14.0	10.3	10.0
	Mean A	12.3	13.5	11.1	
	CV (%) = 33.7	$F_A = 1.02^{ns}$	$F_B = 1.49^{ns}$	$F_{AB} = 2.00^{ns}$	
Corn stalk rot (%)	70	6.0	10.3	9.0	8.4
	90	5.0	8.0	7.3	6.8
	110	8.7	4.0	8.3	7.0
	130	8.3	4.7	5.3	6.1
	Mean A	7.0	6.8	7.5	
	CV (%) = 55.4	$F_A = 0.11^{ns}$	$F_B = 0.56^{ns}$	$F_{AB} = 1.13^{ns}$	
Root lodging (Point 1-5)	70	1.0	2.0	1.0	1.3
	90	1.0	1.0	2.0	1.3
	110	1.0	1.0	1.0	1.0
	130	1.0	1.0	1.0	1.0
	TB A	1.0	1.3	1.3	

*In the same group of mean values, numbers with the same letter accompanying them indicate a non-statistically significant difference. ns: no statistically significant difference; *: difference at significance level α = 0.05; **: difference at significance level α = 0.01.*

The ear borer (*Heliothis armigera*) is a harmful factor that directly affects corn pollination ability, as well as productivity, quality and aesthetics of corn. The data in Table 4 showed that the rate of ear borer infestation in the experimental plots was low, belonging to level 2 (5% - 19%) on a scale of 5 levels, with no significant differences among the experimental plots.

Corn stalk rot caused by the fungus (*Rhizoctonia solani)* is harmful throughout the growth of corn plants but is most severe during the corn tasseling stage. Initially, the damage occurs on the lower leaves and under favorable conditions, the disease develops into streaks on the leaves. Experimental results indicated a low rate of stalk rot disease infection, with no significant differences among the experimental plots.

Root lodging is among the causes that reduce the productivity of corn because lodging can slow down or disrupt the plant's development. If the plant is lodged, the roots may break, affecting photosynthesis, limiting the transport of nutrients for plant growth and reducing productivity. The experiment was conducted in the Winter-Spring crop with favorable external conditions, therefore the rates of root lodging was low. The results from Table 4 showed that the rates of root lodging in corn was low. The lodging resistance of hybrid combinations and Golden Cob variety were good, not affecting the yield and ear quality.

From the results, it can be seen that potassium dosage significantly affects the resistance to pests (fall armyworm (7.5 - 14.6%); corn borer (9.3 - 10.8%); ear borer (11.1 - 13.5%) and lodging (point 1-2) of sweet corn varieties). The research results were consistent with the study findings of Ha et al. (2022).

3.5. Yield parameters

Ear length and ear diameter are factors related to yield. The longer the ear, the more kernels per row and vice versa. The more rows of kernels per ear, the larger the ear diameter and vice versa. These are also criteria that affect ear shape (Tran, 2004). The research results showed that the variety significantly affected ear length and ear diameter, while the potassium doses had unclear influence on ear diameter. Ear lengths of the varieties ranged from 18.7 to 20.9 cm, with the Golden Cob variety having the longest ear at 20.9 cm, statistically significantly different from the BN211 hybrid (19.1 cm). Ear diameters of the varieties ranged from 4.2 to 5.2 cm, with the BN191 hybrid having the largest ear diameter at 5.2 cm, statistically significantly different from the BN211 hybrid at 4.5 cm.

The number of rows of kernels on the ear is one of the genetic factors that greatly influences productivity. In addition, this factor is also influenced by external conditions as it affects the pollination and fertilization process (pollen shedding, silk emergence) under unfavorable weather conditions (heat, storms) causing unsuccessful fertilization of flowers, decrease in kernel rows, resulting in low productivity and vice versa. The results in Table 5 showed that the number of rows/ear of the varieties ranged from 14.7 to 16.1 rows; the variety factor significantly affected the number of kernel rows/ ear, the hybrid combination BN191 had the highest number of kernel rows/ear at 15.9 rows, which was statistically significant compared to the Golden Cob variety at 15.1 rows; the dosage factor of potassium did not significantly affect on the kernel row indicators.

Table 5. Effect of potassium dose on ear morphology traits of sweet corn hybrids

Trait	Potassium levels (kg K_2O/ha) (B)	Hybrid combinations (A)			Mean B
		BN191	BN211	Golden Cob	
Ear length (cm)	70	19.6	19.3	20.9	19.9
	90	19.1	19.0	20.9	19.6
	110	20.0	19.4	20.8	20.1
	130	19.7	18.7	21.0	19.8
	Mean A	19.6^{ab}	19.1^{b}	20.9^{a}	
	CV (%) = 3.1	$F_A = 27.54^{**}$	$F_B = 0.86^{ns}$	$F_{AB} = 0.62^{ns}$	
Ear diameter (cm)	70	5.1	4.6	4.9	4.9
	90	5.0	4.2	5.2	4.8
	110	5.2	4.4	5.1	4.9
	130	5.2	4.6	5.2	5.0
	Mean A	5.1^{a}	4.5^{b}	5.1^{a}	
	CV (%) = 4.1	$F_A = 40.61^{**}$	$F_B = 1.41^{ns}$	$F_{AB} = 1.29^{ns}$	
Kernel row/ ear (row)	70	15.6	15.6	14.7	15.3
	90	16.0	15.5	14.9	15.4
	110	16.0	15.3	15.5	15.6
	130	16.1	15.4	15.3	15.6
	Mean A	15.9^{a}	15.5^{ab}	15.1^{b}	
	CV (%) = 3.4	$F_A = 7.7^{**}$	$F_B = 0.75^{ns}$	$F_{AB} = 0.79^{ns}$	
Fresh Ear weight without husk (g)	70	362.5	311.9	342.4	339.0^{b}
	90	407.1	323.5	342.7	357.9^{ab}
	110	386.3	349.s5	405.1	380.6^{a}
	130	407.1	354.7	384.3	382.2^{a}
	Mean A	391.0^{a}	334.8^{b}	368.8^{ab}	
	CV(%) = 9.3	$F_A = 8.38^{**}$	$F_B = 3.33^{*}$	$F_{AB} = 0.78^{ns}$	
Fresh ear yield (Tons/ ha)	70	17.7	16.8	16.9	17.1^{b}
	90	18.2	16.5	17.7	17.5^{b}
	110	18.7	17.7	18.6	18.4^{a}
	130	18.8	17.9	18.9	18.5^{a}
	TB A	18.4^{a}	17.2^{b}	18.0^{ab}	
	CV (%) = 2,8	$F_A = 18.24^{**}$	$F_B = 17.34^{**}$	$F_{AB} = 0.98^{ns}$	

*Within the same group of mean values, numbers with the same letter indicate no statistically significant difference. ns: no statistically significant difference; *: difference at the significance level of α = 0.05; **: difference at the significance level of α = 0.01.*

The data in Table 5 showed that both varieties and potassium dosage influenced the earr weight without husks; the hybrid combination BN191 had the highest average weight of huskless ears at 391.1 g, significantly different from that of the BN211 hybrid with 224.8 g and the Golden Cob variety at 368.8 g. The huskless ear weight tends to increase when fertilized with a dosage increasing from 70 kg K_2O/ha to 130 kg K_2O/ha. with the weight being 333.9 g at 70 kg K_2O/ha and 382.2 g at 130 kg K_2O/ha.

The fresh ear yield is the yield obtained from the field and it is an important for producers. Experimental results showed that yields ranged from 16.5 to 18.9 tons/ha; both varieties and potassium dosages affected the fresh ear yield. The BN191 hybrid combination had the highest yield at 18.4 tons/ha, which was statistically different from the BN211 hybrid at 17.2 tons/ha and the Golden Cob variety at 18.0 tons/ha. Ear yields tended to increase with potassium doses, with yields reaching 17.1 tons/ha at 70 kg K_2O/ha. There was a statistical difference between potassium dosage of 110 kg K_2O/ha and 130 kg K_2O/ha, with the highest ear yield achieved at 130 kg K_2O/ha reaching 18.5 tons/ha.

3.6. Quality parameters

Table 6. Effect of potassium dose on the quality of sweet corn combination

Trait	Potassium levels (kg K_2O/ha)(B)	Hybrid combinations (A)			Mean B
		BN191	BN211	Golden Cob	
Brix level (%)	70	12.6	12.4	12.3	12.1^c
	90	12.8	13.3	12.7	12.8^b
	110	13.5	13.5	13.2	13.1^b
	130	14.0	14.0	13.6	14.0^a
	Mean A	13.2	13.3	13.0	
	CV (%) = 2.5	$F_A = 3.39^{ns}$	$F_B = 32.10^{**}$	$F_{AB} = 0.73^{ns}$	
Fragrance (point: 1-5)	70s	2.5	2.0	2.5	2.3
	90	2.0	2.5	2.0	2.2
	110	2.0	2.5	2.5	2.3
	130	2.0	2.5	2.5	2.3
	Mean A	2.1	2.4	2.4	
Flavor (point: 1-5)	70	2.0	1.8	2.0	1.9
	90	1.8	1.8	1.8	1.8
	110	1.8	2.0	2.0	1.9
	130	2.0	1.8	2.0	1.9
	Mean A	1.9	1.9	2.0	

*ns: no statistical significance; **: significance at α =0.01. For values within the same group, numbers with the same letter are not statistically different.*

The results in Table 6 showed that the Brix content of sweet corn ranged from 12.3 to 14.0%. The dosage of potassium significantly affected the Brix content of sweet corn varieties. Brix content increased with an increase in potassium dosage from 70 kg K_2O/ha to 130 kg K_2O/ha, with the highest Brix content achieved at 130 kg K_2O/ha which was statistically significant compared to that of other potassium doses. In terms of varieties, the Brix content of three hybrid combinations was not significantly different among the treatments.

4. Conclusions

Different potassium doses did not affect the growth period, but they significantly impacted the yield, quality and pest resistance of the hybrid combinations BN119, BN211 and Golden Cob variety. The potassium dosage of 130 kg K_2O/ha resulted in the best yield, quality and pest resistance and the BN191 hybrid combination yielded significantly higher than that of the BN211. There were no sighificant difference yields of the other treatments.

Conflict of interest

The authors have no conflicts of interest to declare.

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Effects of indole-3-butyric acid and 1-naphthaleneacetic acid on *in vitro* rooting and the substrate mixing ratio on growth during the nursery stage of Mai vang (*Ochna integerrima*) HD01 line

Kiet C. Nguyen*, Duy M. Pham, Van H. Phan, Tri M. Bui, & Le T. Hau
Faculty of Agronomy, Nong Lam University, Ho Chi Minh City, Vietnam

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***Corresponding author**

Nguyen Cao Kiet
Email:
kiet.nguyencao@hcmuaf.edu.vn

ABSTRACT

Identifying suitable plant growth regulators for the rooting stage and substrate mixing ratio for seedlings in the nursery remains a significant challenge, particularly in relation to Mai vang. This plant existed in culture and tradition for a long time and was considered a symbol of the traditional Tet. The experiments were conducted on Mai vang HD01 line, which was selected from Huu Duc Mai garden in Binh Loi apricot village, known for its exceptional characteristics. The objective of this study was to determine the optimal Indole-3-butyric acid (IBA) and 1-naphthaleneacetic acid (NAA) concentrations appropriate for root formation from shoot samples in in vitro condition and the optimal substrate mixing ratio appropriate for the growth of Mai vang HD01 line during the nursery stage. The study including two experiments were arranged in completely randomized design with one-factor and two-factor. For rooting induction, the culture medium supplemented with concentrations of IBA combined with concentrations of NAA was used, while to grow, Mai vang HD01 plants were planted in a substrate of coconut fiber, sand, rice husk ash, and vermicompost with different mixing ratios. The results showed that Mai vang HD01 shoots were cultured on Murashige and Skoog medium supplemented with 0.5 mg/L IBA combined with 2 mg/L NAA suitable for rooting and creating complete plants. The number of roots, root length, plant height and number of leaves were 6.9 roots; 3.5 cm; 2.3 cm and 5.9 leaves, respectively on day 60. Mai vang HD01 plants in the nursery stage were suitable for planting on a substrate with a mixing ratio of 1 coconut fiber:1 sand:1 rice husk ash:1 vermicompost with a 100% survival rate. They grew quickly to a height of 5.1 cm which was higher than that of plants planted on other substrate mix ratios on day 40.

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1. Introduction

Each flower has its own color and beauty. Among them, Mai vang plant is a wild plant, growing in the mountains and forests with a natural and attractive appearance. Over time, Mai vang plant was discovered and domesticated by humans. The plant is considered a symbol of the traditional Tet, representing spring. In Vietnam, Mai vang is a popular ornamental plant in the South and Central regions. Among them, the 'HD01' line selected at Huu Duc Mai garden in Binh Loi Mai Village has many outstanding characteristics, such as large flower diameter (up to 4 - 6 cm), extended flowering period, and darker petal and stamen color than other lines.

In vitro propagation techniques have the advantage of high multiplication as well as high uniformity of seedlings, so they have been applied to propagate many plant species with different economic values (Tran, 2005). Up to now, both domestically and internationally, there have been a few studies on in vitro propagation of Mai vang flowers, but there have been few studies on root formation as well as the seedling stage outside the nursery. Furthermore, although there has been some success in in vitro propagation of Mai vang flowers, each stage needs to be adjusted, optimizing the environment as well as adjusting the appropriate substrate mixing ratio for seedlings to grow well.

Among the plant growth regulators, auxin plays an important role in plant *in vitro* morphogenesis, especially root formation. The two most commonly used auxins were indole-3-butyric acid (IBA) and 1-naphthaleneacetic acid (NAA) which had been used individually or in combination in many studies on rooting in tissue culture (Phan et al., 2014; Irshad et al., 2018). For the seedling stage outside the nursery, commonly used pseudosynthetics are coconut fiber, sand, rice husk ash and vermicompost, which were known to grow well and have a high survival rate (Huynh, 2007; Phan et al., 2017). Therefore, in this study, we focused on studying the most suitable combination to form roots from Mai vang HD01 shoots and the most suitable substrate mixing ratio for growing plants in the nursery stage.

2. Materials and Methods

2.1. Time and place of the study

The study was carried out from July to October 2023, at the tissue culture lab and the plant nursery of the Department of Physiology and Biochemistry, Faculty of Agronomy, Nong Lam University, Ho Chi Minh City, Vietnam.

2.2. Experimental design

2.2.1. Experiment 1: Effects of IBA and NAA concentrations on the rooting process of Mai vang HD01 plant *in vitro*

Experiment 1 had two factors: IBA concentrations: 0 mg/L; 0.5 mg/L; 1 mg/L; 1.5 mg/L, and NAA concentrations: 0 mg/L; 1 mg/L; 2 mg/L; 3 mg/L. The experiment consisted of 16 treatments, with 3 replications, arranged in a completely randomized design. Each treatment in a replication had 5 flasks. Each flask had 3 samples.

2.2.2. Experiment 2: Effect of substrate mixing ratio on the growth of Mai vang HD01 plant in the nursery stage

Experiment 2 had single factor: only coconut fiber (G1-control), 1 sand:1 coconut fiber (G2), 1 sand:1 coconut fiber:1 rice husk ash (G3), 1 sand:1 coconut fiber:1 rice husk ash:1 earthworm compost (G4). The experiment consisted of 4 treatments, with 3 replications, arranged in a completely randomized design. Each treatment in a replication had 15 pots. Each pot had 1 plant.

2.3. Experimental method

The base medium in the experiment 1 was Murashige and Skoog (MS) medium (Murashige & Skoog, 1962), supplemented with 7 g/L agar, 30 g/L sucrose, and plant growth regulators according to treatments.

Figure 1. Mai vang HD01 shoot sample used in experiment 1.

In experiment 1, the shoot samples used were the samples from the best shoot multiplication treatment at the department's tissue culture room. Shoot samples were selected with a size of 0.8 to 1.0 cm and then all leaves and roots (if any) were cut off (Figure 1). Then, shoot samples were transplanted into MS medium supplemented with IBA and NAA at different concentrations and put under light 2500 ± 500 lux for 10 h per day and then recorded over a period of 60 days.

Figure 2. Mai vang HD01 plant sample used in experiment 2.

In experiment 2, the shoots were produced in large quantity following the best treatment in experiment 1 and these were cultured in MS medium for 3 weeks. Then, the plants that were 1.8 to 2.0 cm heigh, have 2 to 3 leaves and 3 to 4 roots, were chosen (Figure 2). The plants were grown on substrates with different mixing ratios and put under light intensity 3.755 ± 500 lux for 12 h per day. The plant parameters were recorded over a period of 40 days.

2.4. Parameters

2.4.1. Experiment 1

The number of roots was recorded every 10 days and these roots were counted when they reached a length of 0.5 cm. The plant height and number of leaves was also monitored every 10 days for 60 days. On day 60 of culture, the root length and stem diameter of 10 random shoots were measured.

2.4.2. Experiment 2

The monitoring indicators were recorded periodically every 10 days within 60 days. The survival rate of plants (%) was calculated by dividing the total number of living plants by the total number of plants grown in each treatment. The plant height was recorded from the root collar to the shoot tip. The number of leaves was recorded for leaves reaching a length of 1 cm or more. Five plants in each base plot were measured for the plant height and the number of leaves.

2.5. Data processing and analyses

Data were processed with Excel 2010 (Microsoft, USA). The R software (version 4.2.3) was used to perform ANOVA and post-hoc analysis with Duncan's multiple range test at a = 0.01 or a = 0.05. The number of roots and the root length in experiment 1 were transformed using the formula before statistical analysis (Gomez & Gomez, 1984).

3. Results and Discussion

3.1. Effects of IBA and NAA concentrations on the rooting process of Mai vang HD01 plant *in vitro*

3.1.1. Effect of IBA and NAA concentrations on the number root of Mai vang HD01 plant *in vitro*

The results in Table 1 showed that as early as day 40, when the root were distinguishable, significant differences among treatments due to the effects of each factor and the interaction between two factors could be observed, and these differences maintained until the end of the culture period (day 60). The trend in root number differences between treatments was also largely similar throughout the culture period. When BA and NAA were not used, no roots was formed, this proved the important role of auxin in root formation in Mai vang HD01 plant.

Table 1. Effects of indole-3-butyric acid (IBA) and 1-naphthaleneacetic acid (NAA) concentrations on the number root of Mai vang HD01 plant *in vitro*

Days of culture	IBA concentration (mg/L)	NAA concentration (mg/L)				Average (I)
		0	1	2	3	
Day 40	0	0^{g}	1.1^{f}	1.8^{bcd}	1.2^{ef}	1.0^{C}
	0.5	1.5^{c-f}	1.9^{bcd}	3.6^{a}	2.11^{bc}	2.3^{A}
	1	1.6^{b-f}	1.5^{c-f}	1.7^{b-e}	2.13^{b}	1.7^{B}
	1.5	2.0^{bc}	1.4^{def}	1.9^{bcd}	1.3^{d-f}	1.7^{B}
Average (N)		1.3^{C}	1.5^{B}	2.3^{A}	1.7^{B}	
CV (%) = 5.6; $F_I = 62.6^{**}$; $F_N = 39.9^{**}$; $F_{I*N} = 18.9^{**}$						
Day 50	0	0^{f}	1.4^{e}	2.8^{bc}	1.5^{e}	1.4^{C}
	0.5	2.1^{b-e}	2.9^{b}	4.8^{a}	2.8^{bc}	3.2^{A}
	1	2.0^{cde}	2.1^{b-e}	1.9^{de}	2.8^{bc}	2.2^{B}
	1.5	2.7^{bc}	2.1^{b-e}	2.6^{bcd}	1.9^{de}	2.3^{B}
Average (N)		1.7^{C}	2.1^{B}	3.0^{A}	2.3^{B}	
CV (%) = 5.8; $F_I = 72.0^{**}$; $F_N = 42.5^{**}$; $F_{I*N} = 22.8^{**}$						
Day 60	0	0^{f}	2.1^{e}	3.9^{bc}	2.0^{e}	2.0^{C}
	0.5	3.1^{cd}	4.1^{b}	6.9^{a}	4.1^{b}	4.6^{A}
	1	2.9^{d}	3.1^{cd}	3.0^{cd}	4.1^{b}	3.3^{B}
	1.5	3.9^{bc}	2.9^{d}	3.9^{bc}	2.8^{de}	3.4^{B}
Average (N)		2.5^{C}	3.1^{B}	4.4^{A}	3.3^{B}	
CV (%) = 4.9; $F_I = 124.0^{**}$; $F_N = 72.6^{**}$; $F_{I*N} = 33.6^{**}$						

Within the same group, mean values followed by the same letter indicate not significantly different; **: significantly different at 1% level.

On day 60, in terms of IBA concentration, Mai vang HD01 plant cultured in the medium supplemented with 0.5 mg/L IBA gave the highest number of roots, reaching 4.6 roots, a statistically significant difference compared to the remaining concentrations. Meanwhile, in terms of NAA concentration, Mai vang HD01 plant cultured in the medium supplemented with 2 mg/L NAA gave the highest number of roots, reaching 4.4 roots, a statistically significant difference compared to the remaining concentrations. In terms of the interaction between the two factors, the highest number of roots was 6.9 roots when Mai vang HD01 plant was cultured in the medium supplemented with 0.5 mg/L IBA combined with 2 mg/L NAA, a statistically significant difference compared to the number of roots of Mai vang plants cultured in the medium supplemented with other concentrations of growth regulators (Figure 3).

Figure 3. Mai vang HD01 plant of the treatments after 60 days of culture. I1-4: indole-3-butyric acid concentration corresponding to 0, 1, 2 or 3 mg/L; N1-3: 1-naphthaleneacetic acid concentration corresponding to 0; 0.5; 1 or 1.5 mg/L.

The results obtained in this research were consistent with the findings of Mai & Lam (2013), which indicated that Mai vang plants cultured on a medium with a combination of IBA and NAA effectively promoted root formation. However, the maximum number of roots produced by Mai vang plant reached only 1.9 roots after 8 weeks of culture, which was lower than the 6.9 roots observed in this study after 60 days. This demonstrates that auxin played a significant role in enhancing root formation in plant tissue and cell cultures (Nguyen, 2000), particularly in the in vitro study of Mai vang HD01 compared to conditions where auxin was not added to the culture medium. In contrast, the study by Ho et al. (2019) showed that in vitro Mai vang shoots did not depend entirely on growth regulators but were also affected by mineral concentration, shoot age, and other conditions such as the number of subcultures.

3.1.2. Effect of IBA and NAA concentrations on root length and stem diameter of Mai vang HD01 plant *in vitro*

On day 60, regarding IBA concentration, Mai vang HD01 plants cultured in the medium supplemented with 1.5 mg/L IBA exhibited the longest root length of 3.6 cm, which was statistically significantly different from the other concentrations. Meanwhile, concerning NAA concentration, Mai vang HD01 plants cultured in the medium supplemented with 2 mg/L NAA had the highest number of roots at 2.9 cm; however, this difference was not significant compared to the concentration of 3 mg/L NAA (2.8 cm), although it was statistically significant compared to the other concentrations. In terms of the interaction between these two factors, the longest root length of 4.0 cm was observed when culturing Mai vang HD01 plants on a medium supplemented with 1.5 mg/L IBA combined with 3 mg/L NAA. Although this difference was not significant compared to the root lengths of Mai vang HD01 plants cultured on media supplemented with 1 mg/L IBA combined with 3 mg/L NAA (3.6 cm), 0.5 mg/L IBA combined with 2 mg/L NAA (3.5 cm), and 1.5 mg/L IBA combined with 1 mg/L NAA (3.5 cm), as well

as 1.5 mg/L IBA (3.4 cm) and 1.5 mg/L IBA combined with 2 mg/L NAA (3.3 cm), it was statistically significant when compared to the root lengths of Mai vang HD01 plants cultured on media supplemented with other concentrations of growth regulators (Table 2). Thus, the results of this study further confirm the effectiveness of auxin on rooting in plants, demonstrating clear effects on root length (Nguyen et al., 2021).

Table 2. Effects of indole-3-butyric acid (IBA) and 1-naphthaleneacetic acid (NAA) concentrations on root length and stem diameter of Mai vang HD01 plant *in vitro*

	IBA concentration (mg/L)	NAA concentration (mg/L)				Average (I)
		0	1	2	3	
Root length (cm)	0	0^{h}	1.1^{fg}	2.2^{de}	0.9^{g}	1.1^{C}
	0.5	2.6^{bcd}	2.4^{cd}	3.5^{abc}	2.5^{cd}	2.8^{B}
	1	1.8^{def}	1.6^{efg}	2.7^{bcd}	3.6^{ab}	2.4^{B}
	1.5	3.4^{abc}	3.5^{abc}	3.3^{abc}	4.0^{a}	3.6^{A}
Average (N)		2.0^{B}	2.2^{B}	2.9^{A}	2.8^{A}	
CV (%) = 6.7; $F_I = 109.4^{**}$; $F_N = 23.6^{**}$; $F_{I*N} = 11.2^{**}$						
Stem diameter (mm)	0	1.2	1.2	1.0	1.0	1.1
	0.5	1.2	1.1	1.3	1.2	1.2
	1	1.3	0.9	0.8	1.0	1.0
	1.5	1.0	1.2	1.1	1.1	1.1
Average (N)		1.2	1.1	1.1	1.1	
CV (%) = 16.4; $F_I = 2.8^{ns}$; $F_N = 0.9^{ns}$; $F_{I*N} = 1.7^{ns}$						

*Within the same group, mean values followed by the same letter indicate not significantly different; ns: not significantly different; **: significantly different at 1% level.*

Meanwhile, on day 60, Mai vang HD01 plant cultured in medium supplemented with different concentrations of IBA and NAA had stem diameters ranging from 0.8 mm to 1.3 mm, the difference was not statistically significant.

3.1.3. Effect of IBA and NAA concentrations on the height of Mai vang HD01 plant *in vitro*

The height of plant, which represents the growth of plant, showed gradual increase in some treatments, specifically treatments with 1.5 mg/L IBA combined with 2 mg/L NAA; 1.5 mg/L IBA; 1.5 mg/L IBA combined with 3 mg/L NAA; 0.5 mg/L IBA combined with 3 mg/L NAA; 1 mg/L IBA combined with 3 mg/L NAA; 0.5 mg/L IBA; 1 mg/L NAA (1.5 cm); 1.5 mg/L IBA combined with 1 mg/L NAA over the culture period from day 40 to day 60 (Table 3). In the rest of the treatments, plant height did not increase significantly, indicating that little plant growth occurred in these treatments.

Table 3. Effects of indole-3-butyric acid (IBA) and 1-naphthaleneacetic acid (NAA) concentrations on the height of Mai vang HD01 plant *in vitro*

Days of culture	IBA concentration (mg/L)	NAA concentration (mg/L)				Average (I)
		0	1	2	3	
10	0	0.9	0.9	1.0	1.0	1.0
	0.9	1.0	0.9	1.0	1.0	1.0
	0.9	1.0	0.9	1.0	1.0	1.0
	1.0	1.0	1.0	1.0	1.0	1.0
Average (N)		0.9	1.0	1.0	1.0	
CV (%) = 14.9; $F_I = 0.5^{ns}$; $F_N = 0.8^{ns}$; $F_{I*N} = 0.3^{ns}$						
20	0	0.9	0.9	1.1	1.2	1.0
	0.5	1.0	1.1	1.1	1.2	1.0
	1	1.1	1.2	1.0	1.1	1.0
	1.5	1.2	1.1	1.1	1.0	1.0
Average (N)		1.1	1.1	1.1	1.1	
CV (%) = 14.2; $F_I = 0.9^{ns}$; $F_N = 0.7^{ns}$; $F_{I*N} = 1.2^{ns}$						
30	0	1.0	1.3	1.2	1.3	1.2^{B}
	0.5	1.2	1.2	1.4	1.3	1.3^{AB}
	1	1.2	1.2	1.1	1.2	1.2^{B}
	1.5	1.4	1.2	1.5	1.4	1.4^{A}
Average (N)		1.2	1.2	1.3	1.3	
CV (%) = 12.9; $F_I = 3.4^{*}$; $F_N = 1.0^{ns}$; $F_{I*N} = 1.3^{ns}$						
40	0	1.1^{g}	1.5^{a-g}	1.29^{d-g}	1.27^{efg}	1.3^{C}
	0,5	1.59^{a-f}	1.38^{b-g}	1.68^{a-d}	1.31^{c-g}	1.5^{AB}
	1	1.21^{fg}	1.32^{b-g}	1.22^{efg}	1.61^{a-e}	1.3^{BC}
	1.5	1.71^{ab}	1.41^{a-g}	1.8^{a}	1.69^{abc}	1.7^{A}
Average (N)		1.4	1.4	1.5		1.5
CV (%) = 10.7; $F_I = 13.5^{**}$; $F_N = 1.2^{ns}$; $F_{I*N} = 4.5^{**}$						
50	0	1.3^{d}	1.9^{ab}	1.6^{bcd}	1.4^{cd}	1.6^{B}
	0,5	2.0^{a}	1.8^{abc}	2.0^{a}	1.6^{bcd}	1.9^{A}
	1	1.5^{cd}	1.6^{bcd}	1.3^{d}	1.9^{ab}	1.6^{B}
	1.5	2.0^{a}	1.6^{bcd}	2.1^{a}	1.9^{ab}	1.9^{A}
Average (N)		1.7	1.7	1.8	1.7	
CV (%) = 8.7; $F_I = 20.1^{**}$; $F_N = 0.5^{ns}$; $F_{I*N} = 9.3^{**}$						
60	0	1.4^{g}	2.0^{a-d}	1.7^{c-g}	1.6^{efg}	1.7^{B}
	0,5	2.2^{a}	1.9^{a-e}	2.3^{a}	1.8^{b-f}	2.1^{A}
	1	1.69^{d-g}	1.7^{c-g}	1.5^{fg}	2.0^{a-d}	1.7^{B}
	1.5	2.13^{ab}	1.8^{b-f}	2.2^{a}	2.05^{abc}	2.1^{A}
Average (N)		1.9	1.9	1.9	1.9	
CV (%) = 7.4; $F_I = 24.9^{**}$; $F_N = 0.5^{ns}$; $F_{I*N} = 10.2^{**}$						

*Within the same group, mean values followed by the same letter indicate not significantly different; ns: not significantly different; *: significantly different at 5% level ;**: significantly different at 1% level.*

Despite the gradual increase in plant height throughout the culture period, the trends in height differences among Mai vang HD01 plants cultured on media supplemented with various concentrations of growth regulators remained consistent from day 40 to day 60. The height of Mai vang HD01 plants reached its maximum in the medium supplemented with 1.5 mg/L IBA. Meanwhile, the average height of Mai vang HD01 plants in relation to NAA concentration was not significantly different. Regarding the interaction between these two factors, the maximum height of Mai vang HD01 plants was observed in the medium supplemented with 0.5 mg/L IBA combined with 2 mg/L NAA.

On day 60, regarding IBA concentration, the height of Mai vang HD01 plants reached its highest level of 2.1 cm in the medium supplemented with 1.5 mg/L IBA. Although this height was not significantly different from that of Mai vang HD01 plants in the medium supplemented with 0.5 mg/L IBA (which was also 2.1 cm), it was significantly different compared to the heights of Mai vang HD01 plants grown in media supplemented with other concentrations of IBA. Meanwhile, in terms of NAA concentration, the average height of Mai vang HD01 plant in the treatments reached 1.9 cm, the difference was not statistically significant. In terms of the interaction between the two factors above, the maximum height of Mai vang HD01 plant was 2.3 cm, cultured on a medium supplemented with 0.5 mg/L IBA combined with 2 mg/L NAA. Although the difference was not significant compared to the height of Mai vang HD01 plants grown on medium supplemented with 0.5 mg/L IBA (2.2 cm), 1.5 mg/L IBA combined with 2 mg/L NAA (2.2 cm), 1.5 mg/L IBA (2.13 cm), 1.5 mg/L IBA combined with 3 mg/L NAA (2.05 cm), 1 mg/L NAA (2.0 cm), 1 mg/L IBA combined with 3 mg/L NAA (2.0 cm), 0.5 mg/L IBA combined with 1 mg/L NAA (1.9 cm), the difference was significant compared to the height of Mai vang HD01 plants cultured on media supplemented with other concentrations of growth regulators.

3.1.4. Effect of IBA and NAA concentrations on the number of leaves of Mai vang HD01 plant *in vitro*

The number of leaves (Table 4) also showed that there were few changes in the differences among treatment from day 10 to 60, indicating that the effects of the growth regulator combinations were largely determined early in the culture period. This is despite the fact that the number of leaves still increased substantially from day 10 to day 60.

On day 60, in terms of IBA concentration, the number of leaves was highest, reaching 3.6 leaves, in the medium supplemented with 0.5 mg/L IBA, a statistically significant difference compared to the other three concentrations. Meanwhile, in terms of NAA concentration, Mai vang HD01 plant cultured in the medium supplemented with 2 mg/L NAA had the highest number of leaves, reaching 3.5 leaves, a statistically significant difference compared to the other three concentrations. In terms of the interaction between the two factors above, Mai vang HD01 plant cultured in the medium supplemented with 0.5 mg/L IBA combined with 2 mg/L NAA had the highest number of leaves, reaching 5.9 leaves, a statistically significant difference compared to the number of leaves of the Mai vang HD01 plants cultured in the medium supplemented with other concentrations of growth regulators.

Table 4. Effects of indole-3-butyric acid (IBA) and 1-naphthaleneacetic acid (NAA) concentrations on the number of leaves of Mai vang HD01 plant *in vitro*

Days of culture	IBA concentration (mg/L)	NAA concentration (mg/L)				Average (I)
		0	1	2	3	
10	0	0.7^{bcd}	0.4^{e}	0.9^{ab}	0.3^{e}	0.6
	0.5	0.4^{e}	0.9	1.1^{a}	0.8^{b}	0,7
	1	0.7^{bcd}	0.9	0.4^{e}	0.8^{b}	0,7
	1.5	0.8^{b}	0.9^{ab}	0.5^{cde}	0.4^{e}	0,7
Average (N)		0.7^{AB}	0.6^{AB}	0.73^{A}	0.6^{B}	
CV (%) = 16.6; $F_I = 2.6^{ns}$; $F_N = 3.0^{*}$; $F_{I*N} = 21.0^{**}$						
20	0	1.0^{fg}	0.8^{gh}	1.3^{cde}	0.9^{fgh}	1.0^{B}
	0.5	0.8^{gh}	1.8^{a}	1.8^{a}	1.4^{cd}	1.2^{A}
	1	1.1^{ef}	0.7^{h}	0.7^{h}	1.1^{ef}	1.1^{AB}
	1.5	1.2^{de}	0.8^{gh}	0.8^{gh}	0.7^{h}	1.1^{AB}
Average (N)		1.0^{B}	1.2^{A}	1.2^{A}	1.0^{B}	
CV (%) = 9.3; $F_I = 6.3^{**}$; $F_N = 9.0^{**}$; $F_{I*N} = 52.9^{**}$						
30	0	1.51^{cde}	1.2^{ef}	1.7^{bcd}	1.3^{ef}	1.4^{B}
	0.5	1.1^{f}	1.2^{ef}	2.6^{a}	1.8^{bc}	1.7^{A}
	1	1.49^{de}	1.9^{b}	1.1^{f}	1.4^{def}	1.5^{B}
	1.5	1.47^{de}	2.4^{a}	1.3^{ef}	1.1^{f}	1.6^{AB}
Average (N)		1.4^{B}	1.7^{A}	1.7^{A}	1.4^{B}	
CV (%) = 8.1; $F_I = 9.1^{**}$; $F_N = 21.1^{**}$; $F_{I*N} = 54.0^{**}$						
40	0	2.0^{c}	1.4^{d}	2.6^{b}	1.5^{d}	1.9^{C}
	0,5	1.4^{d}	1.5^{d}	3.8^{a}	2.7^{b}	2.3^{A}
	1	2.1^{c}	2.8^{b}	1.3^{d}	2.1^{c}	2.1^{B}
	1.5	2.2^{c}	3.6^{a}	1.5^{d}	1.6^{d}	2.2^{A}
Average (N)		1.9^{B}	2.3^{A}	2.3^{A}	2.0^{B}	
CV (%) = 6.2; $F_I = 27.0^{**}$; $F_N = 29.7^{**}$; $F_{I*N} = 156.3^{**}$						
50	0	2.7^{d}	1.6^{e}	3.3^{c}	1.7^{e}	2.3^{B}
	0,5	1.6^{e}	1.7^{e}	5.0^{a}	3.5^{c}	3.0^{A}
	1	2.6^{d}	3.4^{c}	1.6^{e}	2.6^{d}	2.6^{B}
	1.5	2.7^{d}	4.1^{b}	1.7^{e}	1.8^{e}	2.5^{B}
Average (N)		2.4^{B}	2.7^{A}	2.9^{A}	2.4^{B}	
CV (%) = 9.6; $F_I = 13.0^{**}$; $F_N = 11.1^{**}$; $F_{I*N} = 78.3^{**}$						
60	0	3.1^{d}	2.1^{e}	4.0^{c}	2.0^{e}	2.8^{C}
	0,5	2.1^{e}	2.1^{e}	5.9^{a}	4.1^{c}	3.6^{A}
	1	3.1^{d}	4.0^{c}	2.0^{e}	3.2^{d}	3.1^{B}
	1.5	3.2^{d}	5.0^{b}	2.0^{e}	2.1^{e}	3.1^{B}
Average (N)		2.9^{C}	3.3^{B}	3.5^{A}	2.9^{C}	
CV (%) = 4.8; $F_I = 50.1^{**}$; $F_N = 48.4^{**}$; $F_{I*N} = 283.2^{**}$						

Within the same group, mean values followed by the same letter indicate not significantly different; *ns: not significantly different; *: significantly different at 5% level; **: significantly different at 1% level.*

The above results showed that Mai vang HD01 plant cultured on MS medium supplemented with 0.5 mg/L IBA combined with 2 mg/L NAA are suitable for root formation and complete plant formation.

3.2. Effect of substrate mixing ratio on the growth of Mai vang HD01 plant in the nursery stage

When bringing plants to the nursery, the substrate mixing ratio is one of the factors that directly affects the survival and growth rates of the plants. Therefore, a suitable substrate mixing ratio is decisive for the success of the in vitro propagation process (Lam & Nguyen, 2005).

3.2.1. Effect of substrate mixing ratio on the survival rate of Mai vang HD01 plant in the nursery stage

The results presented in Table 5 indicate that, on day 40, the survival rate of Mai vang plant grown on four types of substrates, namely, only coconut fiber (control), 1 sand:1 coconut fiber, 1 sand:1 coconut fiber:1 rice husk ash, 1 sand:1 coconut fiber:1 rice husk ash:1 vermicompost, reached an impressive survival rate of 100%.

Table 5. Effect of substrate mixing ratio on the survival rate (%) of Mai vang HD01 plant in the nursery stage on day 40

Type of substrate	Survival rate (%)
Only coconut fiber (control) (G1)	100
1 sand: 1 coconut fiber (G2)	100
1 sand: 1 coconut fiber: 1 rice husk ash (G3)	100
1 sand: 1 coconut fiber: 1 rice husk ash: 1 vermicompost (G4)	100

The findings of the above research align with the studies conducted by Stefanello et al. (2009) and Nguyen (2019), which demonstrated that plants grown on coconut fiber in combination with various other substrates significantly enhance survival rates during the nursery stage. This result was much higher than that reported by Ho et al. (2019) when growing Mai vang plant on a substrate containing sand, soil, coconut fiber, and rice husks in a ratio of 30:50:10:10, which resulted in a survival rate of 78%. This suggests that the choice of substrate plays a crucial role in promoting the successful establishment and growth of plants in vitro in the nursery stage.

3.2.2. Effect of substrate mixing ratio on the height of Mai vang HD01 plant in the nursery stage

Table 6 showed that the height of Mai vang HD01 plants increased; however, there was no change in trend from 10 to 40 days after planting. The tallest height was consistently observed in plants grown on a substrate with a mixture ratio of 1 sand:1 coconut fiber:1 rice husk ash:1 vermicompost.

Table 6. Effect of substrate mixing ratio on the height of Mai vang HD01 plant in the nursery stage

Type of substrate	Day 10	Day 20	Day 30	Day 40
G1	1.9^{b}	2.0^{b}	2.2^{b}	2.5^{b}
G2	2.0^{bs}	2.1^{b}	2.3^{b}	2.6^{b}
G3	2.0^{b}	2.1^{b}	2.2^{b}	2.6^{b}
G4	2.4^{a}	2.9^{as}	4.1^{a}	5.1^{a}
	CV (%) = 8.7 $F_{tính} = 4.4^{*}$	CV (%) = 8.0 $F_{tính} = 16.2^{**}$	CV (%) = 8.3 $F_{tính} = 50.5^{**}$	CV (%) = 10.4 $F_{tính} = 44.6^{**}$

*In the same column, mean values followed by the same letter indicate statistically insignificant differences; *: significantly different at 5% level; **: significantly different at 1% level.*

On day 40, Mai vang HD01 plant was grown on a substrate with a mixture ratio of 1 sand:1 coconut fiber:1 rice husk ash:1 vermicompost, giving the largest height of 5.1 cm, which was a statistically significant difference compared to the height of Mai vang HD01 plants grown on other mixture ratios (Figure 4).

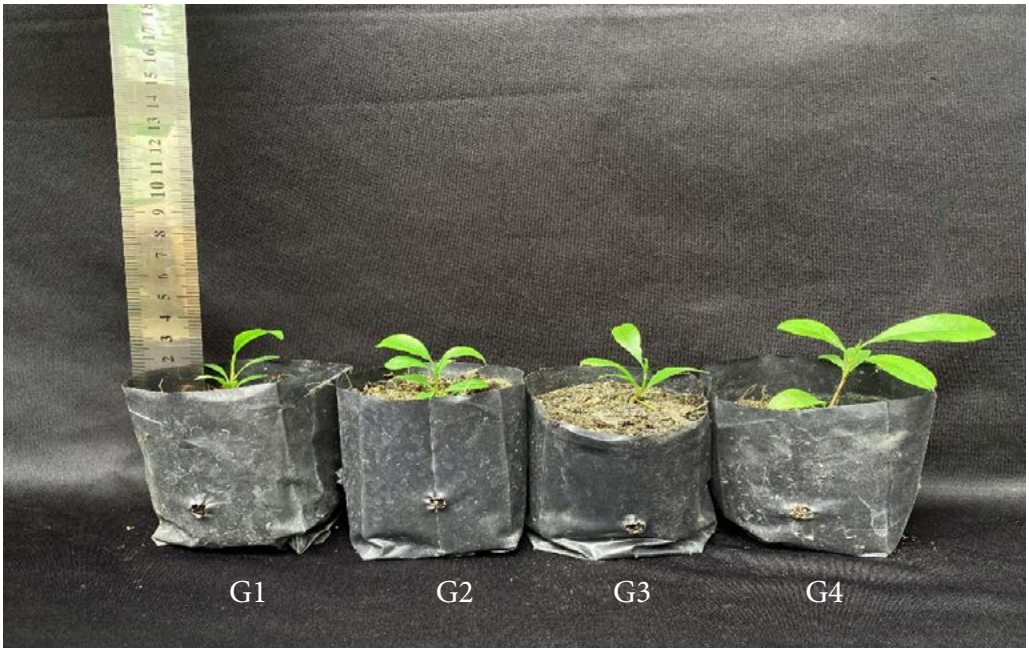

Figure 4. Mai vang HD01 plant of the treatments on day 40. G1: only coconut fiber; G2: 1 sand:1 coconut fiber; G3: 1 sand:1 coconut fiber:1 rice husk ash; G4: 1 sand:1 coconut fiber:1 rice husk ash:1 earthworm compost.

3.2.3. Effect of substrate mixing ratio on the number of leaves of Mai vang HD01 plant in the nursery stage

Contrary to height, the number of leaves of Mai vang HD01 plants grown on different mixture ratios of substrates varied from 10 to 40 days. However, the difference was not statistically significant (Table 7).

Table 7. Effect of substrate mixing ratio on number of leaves of Mai vang HD01 plant in the nursery stage

Type of substrate	Day 10	Day 20	Day 30	Day 40
G1	2.1	3.9	4.9	5.3
G2	2.9	4.1	5.0	5.8
G3	3.1	3.3	3.6	4.2
G4	2.9	4.0	5.1	6.1
	CV (%) = 14.2	CV (%) = 11.3	CV (%) = 17.2	CV (%) = 14.2
	$F_{tính} = 3.7^{ns}$	$F_{tính} = 2.3^{ns}$	$F_{tính} = 2.5^{ns}$	$F_{tính} = 3.6^{ns}$

ns: not significantly different.

The results presented above suggest that during the nursery stage, Mai vang HD01 plants are optimally suited for cultivation on a substrate with a mix ratio of 1 sand:1 coconut fiber:1 rice husk ash:1 vermicompost. This combination facilitates enhanced growth rates and height compared to plants grown with other substrate mixing ratios. The observed effects can be attributed to the addition of nutrient-rich vermicompost (Ramnarain et al., 2019). Vermicompost enhances microbial activity in the soil, increases oxygen availability, maintains optimal soil temperature, improves soil porosity and water permeability, and enriches nutrient content. These factors collectively contribute to enhanced plant growth, yield, and quality (Ansari & Ismail, 2012).

4. Conclusions

Mai vang HD01 plants were cultured on MS medium supplemented with 0.5 mg/L IBA combined with 2 mg/L NAA, which is suitable for root formation and overall plant development. The results indicated that the number of roots, root length, plant height, and number of leaves were 6.9 roots, 3.5 cm, 2.3 cm, and 5.9 leaves, respectively.

During the nursery stage, Mai vang HD01 plants thrived when grown on a substrate with a mixing ratio of 1 sand:1 coconut fiber:1 rice husk ash:1 vermicompost. This combination achieved a survival rate of 100% and promoted rapid growth, resulting in an average height of 5.1 cm, which was higher than that observed with other substrate mixing ratios.

Conflict of interest

I certify that this is my own research work. The data and results presented in the study are genuine and have never been published in any other journal.

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Effects of microbial organic fertilizer on glycoalkaloid content and yield of *Solanum procumbens* Lour.

Lieu T. T. Nguyen*, Thuy T. Dao, Dan T. Vo, & Tri M. Bui
Faculty of Agronomy, Nong Lam University, Ho Chi Minh City, Vietnam

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***Corresponding author**

Nguyen Thi Thuy Lieu
Email: nguyenlieuqn@hcmuaf.edu.vn

ABSTRACT

Solanum procumbens Lour. is a traditional medicinal plant known for its rich glycoalkaloid content. This research aimed to determine the suitable types and application rates of microbial organic fertilizers to enhance yield and glycoalkaloid content in *Solanum procumbens* Lour. A two-factor experiment was conducted using a Split-Plot Design (SPD) with three replications. The main plots included four types of microbial organic fertilizers (HD301, HD302, Komix-BL2, and HCMK7), while the sub-plots involved three application rates (2, 4, and 6 tonnes/ha per crop). Various parameters were measured, including fresh and dry biomass per plant, fresh and dry yield per ha, glycoalkaloid content, and glycoalkaloid yield across two cropping cycles. The results indicated that applying HCMK7 at a rate of 6 tonnes/ha per crop produced the highest outcomes: in the initial crop, a fresh weight of 255.2 g and dry weight of 111.1 g per plant, fresh yield of 15.77 tonnes/ha, dry yield of 6.99 tonnes/ha, glycoalkaloid content of 0.70%, and glycoalkaloid yield of 48.97 kg/ha. In the ratoon crop, the same application rate yielded a fresh weight of 282.5 g and dry weight of 134.6 g/plant, fresh yield of 17.62 tonnes/ha, dry yield of 9.21 tonnes/ha, glycoalkaloid content of 0.76%, and glycoalkaloid yield of 70.39 kg/ha. The highest average glycoalkaloid content (0.73%) and total glycoalkaloid yield (119.36 kg/ha) across both crops were also recorded at this application rate.

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1. Introduction

Solanum procumbens Lour. is a traditional medicinal plant in Vietnam, known for its rich content of glycoalkaloids and other secondary metabolites, including cholesterol, β-sitosterol, lanosterol, dihydrolanosterol, and solasodine, which offer various health benefits. This plant has been shown to have properties that prevent cirrhosis, reduce inflammation, and protect the liver (Nguyen, 2002; Huynh et al., 2021). *Solanum procumbens* Lour. thrives in areas with adequate sunlight (Do, 2004), making the Southeast of Southern Vietnam an ideal region for its cultivation, where it also shows an increase in glycoalkaloid content.

In agricultural production, chemical fertilizers are commonly used due to their ability to quickly increase productivity at a low cost. However, the use of chemical fertilizers in long term can lead to imbalances soil pH, reduced bacterial diversity and major changes in the composition of the bacterial population (Wu et al., 2020). On the other hand, microbial organic fertilizers improve soil fertility by increasing humus and organic matter content, and by optimizing the function of soil microbial populations (Zhu et al., 2021). For grey degraded soils, which have lower clay and organic matter content, microbial organic fertilizers should be added to improve soil fertility and physical properties. Research has shown that microbial organic fertilizers can increase herb yield, seed weight, and essential oil content in medicinal plants, while also enhancing their resistance to diseases and pests (Wang et al., 2019; Huang et al., 2022). Furthermore, these fertilizers have been demonstrated to boost the synthesis and accumulation of active secondary metabolites such as organic acids, saponins, alkaloids, sesquiterpenes, and lactones (Waqas et al., 2015). Therefore, the application of microbial organic fertilizers could positively impact the yield and glycoalkaloid content of *Solanum procumbens* Lour.

2. Materials and Methods

2.1. Materials

Solanum procumbens Lour. seeds (sample code QN9) were collected from Duc Pho district, Quang Ngai province, Vietnam, and subsequently planted at the Agronomy Research Station of Nong Lam University, Ho Chi Minh City, Vietnam. The seeds were sown in a nursery using a growth medium composed of 60% grey soil, 29% cow manure, 1% superphosphate, and 10% rice husk ash, and were allowed to grow for 60 days. Once the seedlings reached a height of 5 - 6 cm, developed 5 - 6 leaves, and were observed to be free of diseases and pests, they were transplanted into the field.

The microbial organic fertilizers used in this experiment were as follows:

HD301: Containing 0.5% nitrogen, 0.5% P_2O_5, 0.5% K_2O, 15% organic matter, and *Trichoderma* sp. at a density of 1.2 x 10^6 CFU/g.

HD302: Containing 1% nitrogen, 1% P_2O_5, 1% K_2O, ≥ 15% organic matter, and *Hirsutella* sp. at a density of 10^6 CFU/g.

Komix-BL2: Containing 1% nitrogen, 3% P_2O_5, 1% K_2O, 1% Mg, 100 ppm Zn, 200 ppm Mn, 16% organic matter, 30% humidity, and phosphate-solubilizing microorganisms at a density of ≥ 1 x 10^6 CFU/g.

HCMK7: Containing 18% organic matter, 2% nitrogen, 2% P_2O_5, 1% K_2O, trace elements (CaO, MgO, B, Cu, Zn), and *Trichoderma* sp. at a density of 10^6 CFU/g.

Solasodine: made by Chengdu Biopurify Phytochemicals Ltd.

2.2. Experiment design

A two-factor experiment was conducted using a Split-Plot Design (SPD) with three replications. The main plots were assigned four types of microbial organic fertilizers (HD301, HD302, Komix-BL2, HCMK 7), while the subplots consisted of three fertilizer doses (2 tonnes/ha per crop, 4 tonnes/ha per crop, and 6 tonnes/ha per crop). The experiment was carried out over two cropping cycles, with the same fertilizer doses applied in both cycles. *Solanum procumbens* Lour. planted at a spacing of 40 cm × 30 cm, resulting in a plant density of 83,333 plants/ha. Each treatment was applied to a plot size of 9.0 m^2 (6.0 m × 1.5 m). Microbial organic fertilizers were applied 3 days before planting for the initial crop and 7 days after harvesting for the initial crop.

The experiment was conducted at Agronomy Research Station in Nong Lam University, Ho Chi Minh City, Vietnam from June 2021 to March 2022

Solanum procumbens Lour. were transplanted during the rainy season in 2021, a period favorable for the growth and development of the plant. However, the high rainfall during the harvesting period posed challenges for drying the fresh herbs. The ratoon crop was cultivated during the sunny season, from November 2021 to March 2022. During this period, irrigation was necessary to support the plants' growth, reproduction, and fruiting.

The data in Table 1 showed that cultivated soil was a silty clay, moderately acidic, low organic matter and total nitrogen but high total phophorus and potassium (Rayment & Lyons, 2011). Available P_2O_5 and K_2O were low.

Table 1. Physical and chemical properties of the experimental soil

Texture (%)			pH_{KCl}	Organic matter (%)	Total N (%)	Total P_2O_5 (%)	Total K_2O (%)
Clay	Silt	Sand					
36.98	12.88	50.14	5.78	0.81	0.065	0.059	0.058

Source: Research Institute for Biotechnology and Environment, Nong Lam University (2021).

2.3. Data collection and statistical analysis

Fresh herb weight (g/plant): The fresh weight of the herb was measured as the average stems, leaves, flowers and fruits of 5 target plants at 135 days after transplantation for the initial crop and 124 days after cutting for the ratoon crop.

Dried herb weight (g): The dried weight of the herb (g/plant) was calculated the average stems, leaves, flowers and fruits of 5 target plants after drying at 70°C until weight stability was achieved.

Actual fresh yield (tonnes/ha): This was calculated using the formula: Actual fresh yield (tonnes/ha) = The fresh herb weight of each base plot (kg) x 10^{-3} × 10,000]/Area of the base plot (m^2).

Actual dry yield (tonnes/ha): This was calculated at 8% herb moisture content using the formula: Actual dry yield (tonnes/ha) = The dry herb weight of each base plot (kg) x 10^{-3} × 10,000]/ Area of the base plot (m^2) (was calculated at the moisture content of herbs 8%).

For the analysis of th glycoalkaloid content of *Solanum procumbens*: 2 g herbal powder in each treatment was extracted with 50 mL methanol-acetic acid solution 5% in 3 h. Filtering and drying residue at 50°C. Dissolving residue with methanol, filtering and transfering to a 10 mL graduated cylinder, adding methanol in enough to make 10 mL of solution. This was test solution.

Table 2. Chemicals and amount of chemicals in samples

Reagents	Standard sample	Test sampsle	White sample
Buffer solution pH8	5.0	5.0	5.0
Bromothymol blue solution 2%	0.5	0.5	0.5
Standard solasodine solution (0.5 mg/mL)	0.5	0.0	0.0
Test solution	0.0	0.5	0.0
Methanol	0.0	0.0	0.5
Chloroform	10.0	10.0	10.0

To shake for 5 min

To transfer chloroform into other tube after 30 min

To shake chloroform with 10 mL NaOH 0.05 N solution

To transfer blue solution into other tube after 30 min

To measure the absorbance of blue solution at a wavelength of 616 nm

Glycoalkaloid content was calculated using the following formula:

X (%) = [DT x 50]/[Dc x a x (100 - A]

Where:

Dc: absorbance value of the standard sample at a wavelength of 616 nm

DT: absorbance value of the test sample at a wavelength of 616 nm

a: dried herb weight

A: Herb moisture content (Nguyen & Pham, 2000)

Glycoalkaloid yield (kg/ha): This was calculated using the formula:

The glycoalkaloid yield (kg/ha) = Actual harvested dried yield (tonnes/ha) x glycoalkaloid content (%)] x 1000/100

The total glycoalkaloid yield (kg/ha) was calculated as the sum of the glycoalkaloid yields from both the initial crop and the ratoon crop.

Economic analysis:

+ Total cost (VND/ha per crop): This was calculated by summing general costs (seeds, labor, other materials) and microbial organic fertilizer costs.

+ Production cost (VND/kg glycoalkaloid): This was calculated using the formula:Production cost (VND/kg glycoalkaloid) = The glycoalkaloid yield (kg/ha)/Total cost (VND/ha per crop)

Data analysis: Data were analyzed using DSAASTAT software. Differences between treatments were tested using Duncan's Multiple Range Test at a significance level of 0.05 or 0.01.

3. Results and Discussion

3.1. Effect of microbial organic fertilizers on yield components, herb yield, glycoalkaloid content, and glycoalkaloid yield of *Solanum procumbens* Lour.

The types of microbial organic fertilizers applied had a statistically significant effect on the fresh herb (Table 3). When HCMK7 was used in the initial crop, the fresh herb weight of *Solanum procumbens* was highest, showing a statistically significant difference compared to the fresh herb weight when HD301 and HD302 ($P < 0.01$) applications. In the ratoon crop, the differences in fresh herb weight between treatments were not statistically significant ($P > 0.05$).

Regarding the amount of microbial organic fertilizer applied, both cropping cycles showed that applying 6 tonnes/ ha per crop resulted in a statistically significantly higher fresh herb weight compared to the 2 and 4 tonnes/ha per crop treatments ($P < 0.01$). The HCMK7 fertilizer, which contains 2% nitrogen, positively impacted the growth of *Solanum procumbens* Lour. and enhanced fresh herb weight. This finding aligns with results from Pham et al. (2019) and Le et al. (2020), who reported that the fresh herb weight of *Solanum procumbens* Lour. was highest with the application of 250 kg N/ha per year or 125 kg N/ha per crop. It is also consistent with Hoang et al. (2016), who observed increased fresh herb weight in *Solanum procumbens* Lour. with the application of 250 kg N + 200 kg P_2O_5 + 150 kg K_2O compared to other fertilizer rates.

Significant differences in dried herb weight were also observed between treatments in both the initial and ratoon crops. The highest dried herb weight was noted with HCMK7 application in the initial crop ($P < 0.05$), while higher dried herb weights were found with Komix-BL2 and HCMK7 application in the ratoon crop ($P < 0.05$). Additionally, the highest dried herb weight was achieved with the highest dose of microbial organic fertilizer (6 tonnes/ha per crop, $P < 0.01$).

The microbial organic fertilizers contain organic matter, N, P_2O_5, K_2O, and effective microorganisms. These fertilizers supply essential nutrients and beneficial microorganisms that enhance soil health and crop growth (Wei et al., 2024). Therefore, applying microbial organic fertilizers with higher nutrient content and in larger amounts increased the fresh and dried herb weight of *Solanum procumbens* Lour.

Data in Table 4 showed that the actual fresh yields in both the initial and ratoon crops were significantly influenced by the amounts of microbial organic fertilizers applied, with statistically significant differences observed. The highest actual fresh yields were obtained with the application of 6 tonnes/ha per crop, reaching 13.88 tonnes/ha in the initial crop and 16.48 tonnes/ha in the ratoon crop. The differences in the effects of the types of microbial organic fertilizers on actual fresh yield in the ratoon crop were not statistically significant. Both the type and amount of microbial organic fertilizers applied had a significant impact on the actual dry yield in both cropping cycles. The highest actual dry yield was achieved with the application of HCMK7 fertilizer, yielding 5.98 tonnes/ha in the initial crop and 7.72 tonnes/ha in the ratoon crop. Significant differences in actual dry yield were also observed across different fertilizer application rates. The application of 6 tonnes/ha per crop resulted in the highest actual dry yield, with 6.06 tonnes/ha in the initial crop and 8.27 tonnes/ha in the ratoon crop, both of which were statistically significant ($P < 0.01$). In contrast, the application of 2 and 4 tonnes/ha per crop resulted in lower actual dry yields in both crops

Table 3. Effect of types and amounts of microbial organic fertilizers application on fresh herb weight (g/plant) of *Solanum procumbens* Lour.

Parameters	Dose (tonnes/ha) (L)	Types of microbial organic fertilizers (P)				Average (L)
		HD301	HD302	Komix-BL2	HCMK7	
Fresh herb weight (g/plant) in initial crop	2	170.1	187.9	200.3	202.3	190.2^{c}
	4	180.9	201.5	226.7	233.8	210.7^{b}
	6	199.2	211.1	249.9	255.2	228.8^{a}
	Average (P)	183.4^{c}	200.2^{bc}	225.7^{ab}	230.4^{a}	
	CV (%) = 6.91; F_P =18.22^{**}; F_L = 21.32^{**}; F_{P*L} = 0.84^{ns}					
Fresh herb weight (g/plant) in ratoon crops	2	185.5	162.8	162.5	189.4	175.0^{c}
	4	205.0	189.8	225.0	232.5	213.1^{b}
	6	242.0	238.7	247.3	282.5	252.6^{a}
	Average (P)	210.8	197.1	211.6	234.8	
	CV (%) = 6.00; F_P =4.62^{ns}; F_L = 110.12^{**}; F_{P*L} = 2.37^{ns}					
Dried herb weight (g/plant) in plant crop	2	70.3	79.0	77.6	85.3	78.1^{c}
	4	81.4	84.0	95.1	90.7	87.8^{b}
	6	86.7	90.3	108.7	111.1	99.2^{a}
	Average (P)	79.4^{b}	84.4^{ab}	93.8^{ab}	95.7^{a}	
	CV (%) = 7.73; F_P = 9.81^{**}; F_L = 28.80^{**}; F_{P*L} = 1.75^{ns}					
Dried herb weight (g/plant) in ratoon crop	2	79.3	67.2	75.4	91.5	78.3^{c}
	4	90.6	84.1	101.7	109.3	96.4^{b}
	6	104.0	114.8	114.2	134.6	116.9^{a}
	Average (P)	91.3^{b}	88.7^{b}	97.1^{ab}	111.8^{a}	
	CV (%) = 8.52; F_P = 10.76^{**}; F_L = 65.10^{**}; F_{P*L} = 1.58^{ns}					

*Values within the same group followed by the same letters are not significantly different at $P \leq 0.05$ according to the Duncan's multiple range test; * and ** are significantly different at the 5% and 1% levels, respectively.*

Table 4. Effect of types and amounts of microbial organic fertilizers application on actual herb yield (tonnes/ha) of *Solanum procumbens* Lour.

Parameters	Dose (tonnes/ha) (L)	Types of microbial organic fertilizers (P)				Avesrage (L)
		HD301	HD302	Komix-BL2	HCMK7	
Actual fresh yield (tonnes/ ha) in initial crop	2	9.89	10.56	11.83	11.82	11.03^{c}
	4	10.35	11.93	13.48	14.29	12.51^{b}
	6	11.79	12.94	15.06	15.73	13.88^{a}
	Average (P)	10.68^{c}	11.81^{bc}	13.46^{ab}	13.95^{a}	
	CV (%) = 7.70; $F_P = 16.34^{**}$; $F_L = 26.52^{**}$; $F_{P*L} = 0.84^{nss}$					
Actual fresh yield (tonnes/ ha) in ratoon crop	2	11.48	11.09	11.84	12.95	11.84^{c}
	4	12.95	12.83	15.03	14.69	13.88^{b}
	6	15.49	15.84	16.97	17.62	16.48^{a}
	Average (P)	13.31	13.25	14.62	15.09	
	CV (%) = 4.68; FP = 4.49^{ns}; $F_L = 149.93^{**}$; $F_{P*L} = 1.23^{ns}$					
Actual dry yield (tonnes/ ha) in initial crop	2	4.11	4.87	5.05	5.07	4.78^{c}
	4	4.65	5.03	5.93	5.86	5.37^{b}
	6	5.30	5.67	6.28	6.99	6.06^{a}
	Average (P)	4.69^{c}	5.19^{bc}	5.75^{ab}	5.98^{a}	
	CV (%) = 7.30; $F_P = 18.46^{**}$; $F_L = 31.90^{**}$; $F_{P*L} = 1.37^{ns}$					
Actual dry yield (tonnes/ ha) in ratoon crop	2	5.46	5.09	5.82	6.62	5.75^{c}
	4	6.09	6.19	7.21	7.33	6.70^{b}
	6	7.61	7.98	8.26	9.21	8.27^{a}
	Average (P)	6.39^{b}	6.42^{b}	7.0^{ab}	7.72^{a}	
	CV (%) = 4.56; $F_P = 14.07^{**}$; $F_L = 136.26^{**}$; $F_{P*L} = 1.28^{ns}$					

*Values within the same group followed by the same letters are not significantly different at P ≤ 0.05 according to the Duncan's multiple range test; * and ** are significantly different at the 5% and 1% levels, respectively.*

The experiment results demonstrated that the amounts of microbial organic fertilizers had a strong impact on the glycoalkaloid content in the plants. As the amount of microbial organic fertilizer increased, the glycoalkaloid content also improved. With a fertilizer rate of 6 tonnes/ha per crop, glycoalkaloid contents reached 0.70% and 0.72% in the initial and ratoon crops, respectively. These were statistically significant differences compared to the 4 tonnes/ha per crop and 2 tonnes/ha per crop application ($P < 0.05$ in initial crop and $P < 0.01$ in ratoon crop). Regarding the effect of different types of microbial organic fertilizers, there were no statistically significant differences in glycoalkaloid content among the applications of HD301, HD302, Komix-BL2, and HCMK7 in the initial crop. However, the highest glycoalkaloid content (0.73%) was obtained with the application of HCMK7 in the ratoon crop (Table 5). The average glycoalkaloid content was influenced by the types and amounts of microbial organic fertilizers, as well as their interaction. Higher glycoalkaloid content was observed with increased microbial organic fertilizer application. The application of 6 tonnes/ha per crop significantly enhanced the glycoalkaloid content of *Solanum procumbens* Lour. In contrast, the average glycoalkaloid content was lower when HD301 fertilizer was applied. The application of 6 tonnes/ha per crop of HD302, Komix-BL2, and HCMK7 fertilizers resulted in higher average glycoalkaloid contents and showed significant differences compared to average glycoalkaloid contents in other treatments.

Table 5. Effect of types and amounts of microbial organic fertilizers application on glycoalkaloid content (%) of *Solanum procumbens* Lour.

Parameters	Dose (tonnes/ha) (L)	Types of microbial organic fertilizers (P)				Average (L)
		HD301	HD302	Komix-BL2	HCMK7	
Glycoalkaloid content (%) in initial crop	2	0.53	0.57	0.54	0.60	0.56^{c}
	4	0.56	0.61	0.72	0.66	0.64^{b}
	6	0.64	0.74	0.73	0.70	0.70^{a}
	Average (P)	0.58	0.64	0.67	0.65	
	CV (%) = 6.81; $F_P = 3.99^{ns}$; $F_L = 33.17^{*}$; $F_{P*L} = 2.38^{ns}$					
Glycoalkaloid content (%) in ratoon crop	2	0.61	0.60	0.64	0.68	0.63^{c}
	4	0.61	0.66	0.69	0.73	0.67^{b}
	6	0.69	0.70	0.72	0.76	0.72^{a}
	Average (P)	0.64^{B}	$0,65^{B}$	$0,68^{AB}$	0.73^{A}	
	CV (%) = 3.24; FP = 21.35 **; $F_L = 45.33^{**}$; $F_{P*L} = 1.61^{ns}$					
Average glycoalkaloid content (%)	2	0.57^{d}	0.58^{d}	0.59^{d}	$0.64c^{d}$	0.60^{c}
	4	0.58^{d}	0.64^{c}	0.70^{ab}	0.69^{ab}	0.65^{b}
	6	0.67^{bc}	0.72^{a}	0.73^{a}	0.73^{a}	0.71^{a}
	Average (P)	0.61^{b}	0.65^{ab}	0.67^{a}	0.69^{a}	
	CV (%) = 3.26; $F_P = 18.74^{*}$; $F_L = 85.83^{**}$; $F_{P*L} = 3.63^{*}$					

*Values within the same group followed by the same letters are not significantly different at P ≤ 0.05 according to the Duncan's multiple range test; * and ** are significantly different at the 5% and 1% levels, respectively.*

As shown in Table 6, among the four types of fertilizers, the lowest glycoalkaloid yield (27.30 kg/ha) was observed with the application of HD301, which had statistically significant differences compared to glycoalkaloid yield in treatments using Komix-BL2 (38.77 kg/ha) or HCMK7 (39.28 kg/ha) fertilizers, though the difference was not statistically significant when compared to glycoalkaloid yield when applied HD302 (33.51 kg/ha) in the initial crop. However, in the ratoon crop, HCMK7 produced the highest glycoalkaloid yield (56.37 kg/ha) compared to glycoalkaloid yield in other treatments. In both cropping cycles, the application of 6 tonnes/ha per crop resulted in the highest glycoalkaloid yield, with 47.25 kg/ha in the initial crop and 59.56 kg/ha in the ratoon crop. Conversely, the lowest glycoalkaloid yields were observed with the application of 2 tonnes/ha per crop of microbial organic fertilizer, at 26.89 kg/ha and 36.60 kg/ha, respectively.

The total glycoalkaloid yield reached its highest value with the application of 6 tonnes/ha per crop of microbial organic fertilizer, showing statistically significant differences compared to the lower rate applications ($P < 0.01$). While the application of Komix-BL2 and HCMK7 fertilizers clearly improved the glycoalkaloid yield of *Solanum procumbens*, the applications of HD301 and HD302 did not result in significant enhancement. The experiment also demonstrated that the application of HCMK7 fertilizer at 6 tonnes/ha per crop resulted in a total glycoalkaloid yield of 119.36 kg/ha across both the initial and ratoon crops, significantly higher than the yields achieved with other types and amounts of fertilizer.

The application of organic fertilizers had beneficial effects on crop growth and yield by improving the biological and physical properties of the soil (Zheljazkov & Warman, 2004). Organic fertilizers and microbial organic fertilizers have been proven to increase the yield of *Solanum procumbens* Lour. and other species in the *Solanum* genus (Nguyen et al., 2018; Nguyen & Ha, 2019). Additionally, the application of organic or microbial organic fertilizers has been reported to enhance secondary metabolite contents in the Solanaceae family. For instance, the use of organic fertilizers has been shown to increase polyphenols, phenols, flavonoids, t-ferulic acid, cyanidin, and caffeic acid contents in eggplants, tomatoes, and peppers (Faller & Fialho, 2010; Basay et al., 2021). The partial or complete replacement of chemical fertilizers with organic or microbial organic fertilizers has been observed to increase vitamin A, vitamin C, lipids, acidity, nitrogen, total sucrose content, brix, phenolics, and antioxidants in tomatoes (Oliveira et al., 2013; Dabire et al., 2016). In our study, the application of microbial organic fertilizers was confirmed to improve the yield and glycoalkaloid content of *Solanum procumbens*, and the experimental results are consistent with previous reports.

Economic efficiency analysis

The economic analysis revealed that the application of 6 tonnes/ha per crop of microbial organic fertilizer resulted in the highest total cost, whereas the applications at 4 and 2 tonnes/ha per crop were the least expensive (Table 7). Despite the higher total cost, the production cost per unit of glycoalkaloid yield was lowest with the 6 tonnes/ha per crop application, owning to the significant improvement in glycoalkaloid yield. Among the different fertilizers, HCMK7 achieved the lowest production cost compared to production cost wthen used of HD301, HD302, and Komix-BL2.

Table 6. Effect of types and amounts of microbial organic fertilizers application on glycoalkaloid yield (kg/ha) of *Solanum procumbens* Lour.

Parameters	Dose (tonnes/ha) (L)	Types of microbial organic fertilizers (P)				Average (L)
		HD301	HD302	Komix-BL2	HCMK7	
Glycoalkaloid yield (kg/ha) in initial crop	2	21.94	27.69	27.57	30.35	26.89^{c}
	4	25.99	30.87	42.64	38.52	34.51^{b}
	6	33.96	41.98	46.09	48.97	42.75^{a}
	Average (P)	27.30^{b}	33.51^{ab}	38.77^{a}	39.28^{a}	
	CV (%) = 10.88; $F_P = 10.49^{**}$; $F_L = 52.92^{**}$; $F_{P*L} = 1.92^{ns}$					
Glycoalkaloid yield (kg/ha) in ratoon crop	2	33.55	30.40	37.21	45.23	36.60^{c}
	4	37.17	41.00	49.46	53.50	45.28^{b}
	6	52.26	56.14	59.45	70.39	59.56^{a}
	Average (P)	40.99^{c}	42.51^{c}	48.70^{b}	56.37^{a}	
	CV (%) = 6.11; FP = 41.25 **; $F_L = 166.16^{**}$; $F_{P*L} = 1.72^{ns}$					
Total glycoalkaloid yield (kg/ha)	2	55.48^{f}	58.10^{f}	64.78^{ef}	75.57^{de}	
	4	63.15^{ef}	71.86^{e}	92.10^{c}	92.02^{c}	
	6	86.23^{cd}	98.12^{bc}	105.53^{b}	119.36^{a}	
	Average (P)	68.29^{c}	76.03^{bc}	87.47^{ab}	95.65^{a}	
	CV (%) = 6.08; $F_P = 26.59^{**}$; $F_L = 183.90^{**}$; $F_{P*L} = 2.81^{*}$					

*Values within the same group followed by the same letters are not significantly different at P ≤ 0.05 according to the Duncan's multiple range test; * and ** are significantly different at the 5% and 1% levels, respectively.*

Table 7. Economic efficiency of *Solanum procumbens* Lour. crop

Parameters	Dose (tonnes/ha) (L)	Types of microbial organic fertilizers (P)			
		HD301	HD302	Komix-BL2	HCMK7
Total cost (VND/ha per 2 crops)	2	233,171,159	233,421,283	243,216,715	244,193,814
	4	274,888,814	276,182,270	295,522,888	295,934,246
	6	318,432,579	319,755,974	346,648,011	347,822,270
Production cost (VND per kg of glycoalkaloid)	2	4,202,497	4,017,832	3,754,717	3,231,200
	4	4,352,625	3,843,169	3,208,546	3,216,007
	6	3,692,920	3,258,956	3,284,768	2,913,965

4. Conclusions

The application of HCMK7 fertilizer at a rate of 6 tonnes/ha per crop on *Solanum procumbens* Lour. grown in grey soils of Ho Chi Minh City resulted in notable outcomes. The fresh herb weights achieved were 255.2 g/plant and 282.5 g/plant, and the dried herb weights were 134.6 g/plant and 111.1 g/plant, in the initial and ratoon crops, respectively. The actual fresh yields were 15.73 tonnes/ha per crop and 17.62 tonnes/ha per crop, while the actual dry yields were 6.99 tonnes/ha per crop and 9.21 tonnes/ha per crop. The glycoalkaloid content was 0.70% and 0.76%, and the glycoalkaloid yields were 48.97 kg/ha and 70.39 kg/ha, respectively, in the initial and ratoon crops.

The experiment also noted the highest average glycoalkaloid content (0.73%) and total glycoalkaloid yield (119.36 kg/ha) when HCMK7 fertilizer was applied at 6 tonnes/ha per crop. This application also resulted in the lowest production cost of 2,913,965 VND/kg of glycoalkaloid.

Conflicts of interest

All authors declare no conflict of interest.

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Dietary *Saccharomyces cerevisiae* supplementation improves feed intake and milk quality of dairy cows

Hai T. Nguyen[1], Loi V. Le[1,2], Anh N. T. Dang[1], & Cuong K. Nguyen[2*]
[1]Department of Animal Production, Nong Lam University, Ho Chi Minh City, Vietnam
[2]Department of Veterinary Biosciences, Nong Lam University, Ho Chi Minh City, Vietnam

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***Corresponding author**

Nguyen Kien Cuong
Email:
cuong.nguyenkien@hcmuaf.edu.vn

ABSTRACT

The objective of this study was to evaluate the effects of daily dietary supplementation of *Saccharomyces cerevisiae*-contained product (SCP) on feed consumption, milk yield and quality of milking cows under Vietnam weather condition from November 2022 to January 2023 at the dairy farm of ANOVA Binh Duong. The study was conducted using a total of 94 Holstein Friesian (HF) crossbred cows with at least 3/4 HF blood, with days in milk (DIM) at days, and lasted 30 days (the first 15 days for the control without SCP supplementation (control) and the next 15 days for the SCP treatment with SCP addition at 5 g/cow per day (SCP). Results showed that the average feed intake (as fed) of cows in the control period was significantly lower than that of cows in the SCP period ($P < 0.01$). The SCP supplementation did not affect ($P > 0.05$) the milk productivity and milk fat, while milk protein, lactose, and solids not fat (SNF) from the milk of cows in the SCP group were significantly improved ($P < 0.01$). The SCP supplementation also significantly enhanced ($P < 0.05$) the body condition score (BCS) of dairy cows. Briefly, these results suggest that the dietary SCP addition of 5 g/cow per day seems to significantly improve the feed intake, BCS and milk quality parameters of lactating cows.

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1. Introduction

The dairy industry provides significant nutritional and economic benefits to humans and society, contributing significantly to the development of global agriculture. In developing countries, dairy production systems are affected by many factors including genetics, nutrition, infectious, parasitic diseases, or heat stress caused by high temperature and humidity (Das et al., 2016; Gauly & Ammer, 2020; Nguyen et al., 2021). Dairy production in Vietnam has experienced significant growth and development over the past decade. Currently, the milk consumption in Vietnam is still low at 27 liters/head per year (35 liters/head per year and 45 liters/head per year in Thailand and Singapore in 2021, respectively) and there will be a strong increase in demand for humans in milk consumption to reach about 40 liters/head per year by 2030 (equivalent to the growth rate of about 4% annually) (Nhat, 2023). The development of the dairy system in Vietnam requires the enhancement of knowledge and skills of farmers related to the general husbandry including genetics, nutrition, and heat stress management (Nguyen et al., 2022). Nutritional factors signigicantly affect milk yield and compositions in addition to breed selection as a primary step to improve milk productivity and feed efficiency (Hristov et al., 2004; Lee et al., 2014; Olika, 2021). Therefore, nutrient balance in daily diets plays a significant role in dairy production.

In recent years, there has been a lot of interest in using products containing yeast, *Saccharomyces cerevisiae*, as potential supplements (Majdoub-Mathlouthi et al., 2009; Julien et al., 2018; Oh et al., 2019). In fact, it is shown that there are widely used these products in diets for high-yielding dairy cows. *Saccharomyces cerevisiae* is known as a probiotic, which can positively influence the gut health and metabolic processes of dairy cows. It has been shown to improve feed efficiency, increase milk yield, and improve milk quality by modulating rumen fermentation and promoting beneficial microbes (Chaucheyras-Durand et al., 2008; Desnoyers et al., 2009). In Vietnam, however, the practical benefits of a *Saccharomyces cerevisiae*-contained product in dairy production at local climate conditions are still limited and this study is needed to clarify this point.

Therefore, the objective of the current study was to determine the effects of one product that contained the *Saccharomyces cerevisiae* on feed consumption, milk yield, and quality of lactating cows under Vietnam weather conditions.

2. Materials and Methods

2.1. Location

The study was conducted at the dairy farm of ANOVA Binh Duong from November 2022 to January 2023.

2.2. Experimental design, animals, and housing

The study was arranged into a randomized complete design with two treatments of rations, including (1) control with cows fed the current farm-based ration and (2) Saccharomyces cerevisiae-contained product (SCP) with cows fed the control-like diet supplemented at 5 g/cow per day of a *Saccharomyces cerevisiae*-contained product (Biotic-Cattle at 10^{10} *Saccharomyces cerevisiae* per g of product, SCP) (Table 1). Based on the practical conditions of the farm, the study was designed as one-group trial of cows before (control) and after (SCP treatment) the SCP addition into the daily diets of lactating cows. Cows were housed in the same cubicle shed

containing rubber mats with continual access to water (*ad libitum*). The study was conducted on a total of 94 Holstein Friesian (HF) crossbred cows with at least 3/4 HF blood, with days in milk (DIM) from 31 to 128 days (73.5 ± 25.9 days) and lasted 30 days (the first 15 days for the control treatment without SCP supplementation and the next 15 days for the SCP treatment with SCP addition) (Table 1).

Table 1. Experimental design

Treatment	Control (No dietary SCP addition)	SCP (Dietary SCP addition)
Cows (n)	94	94
Trial period	The first 15 days	The next 15 days
Daily SCP addition (g/cow per day)	0	5

SCP: Saccharomyces cerevisiae-contained product.

2.3. Daily ration of cows

All cows were fed twice a day (7:30 and 14:00, *ad libitum*) as total mixed ration method (TMR) as the current farm-based ration, including king grass (28 kg/cow per day), alfalfa hay (2 kg/cow per day), rice straw (0.5 kg/cow per day), corn silage (4 kg/cow per day) complete feed (6 kg/cow per day), molasses (0.5 kg/cow per day), brewers grain (4 kg/cow per day), and other feed additives. The *Saccharomyces cerevisiae*-contained product (SCP) was mixed with new corn powder and mixed well with TMR for the SCP treatment after the trial period of the control. The TMR feed was available at all positions of the feeding trough for the same consumption per cow.

2.4. Sample collection and measurements

Milk yield (kg/cow per day): All cows were milked by milking system into specialized container two times a day (5:30 and 15:00), using the recording machine in the milking system, and then merging two times into the average milk yield.

Milk quality: About 50 mL of milk was taken in the morning milking time to determine concentrations of milk fat, protein, solids not fat (SNF) and lactose, stored between 2 - 6°C condition and transported quickly to an analytical laboratory. Milk quality was analyzed by Ekomilk M machine (BULTEH 2000, Bulgaria) about 60 sec/sample for the testing result. Before putting the sample into the machine for analysis, the sample must be shaken well and pour about 10 mL of milk sample into the cup then press OK and wait for the machine to measure for 90 sec.

Feed intake as fed (kg/cow per day): The total amount of feed for each treatment was recorded before feeding and refusal feed was collected in the early morning for calculation of the feed consumption as fed per day.

Temperature-humidity index (THI): Use a specialized barn microclimate meter to measure temperature and humidity at a height of 1.5m from the house floor. Then, apply the equation to calculate the THI = T (°F) - 0,55 * (100 - RH%)/100 * (T - 58) (Ingraham et al., 1974; Nguyen et al., 2018).

Body condition score (BCS): Individual cow was evaluated for BCS ranging from 1 to 5 according to the official method described by Wildman et al. (1982) and Nguyen et al. (2022) on the days of 15 and 30 of the experimental periods.

2.5. Statistical analysis

Data were analyzed a Paired t-test using the Minitab Software 16.2. The percentages were compared with the χ2 test. The differences were considered significant at $P < 0.05$.

3. Results and Discussion

3.1. Feed intake

Table 2. Effect of dietary SCP supplementation on feed consumption

Parameters	Replication	Control	SCP	*P*
Average THI	15 days	81.5 ± 0.9	81.3 ± 1.6	> 0.05
Average feed intake (kg/cow per day as fed)	94 cows	36.2^{b} ± 1.3	40.3^{a} ± 1.5	< 0.01

[ab]Means in the same row without common letters are different at P < 0.01; SCP: Saccharomyces cerevisiae-contained product; THI: Temperature-humidity index.

The average THI in control within trial period of cows fed the current farm-based ration without SCP addition was 81.5 and not different from that of SCP within the trial period of cows fed diet supplemented SCP product at 81.3 ($P > 0.05$; Table 2). However, the average feed intake (as fed) of cows fed the current farm-based ration was 36.2 kg/cow per day and was significantly lower than that of cows fed diet supplemented SCP product of 40.3 kg/cow per day ($P < 0.01$). Chaucheyras-Durand et al. (2008) reported that the yeast product, *Saccharomyces cerevisiae*, contains some potential factors that stimulate the growth of rumen microorganisms, especially for lactate-utilizing species. A stable and well-developed rumen microflora will help cows increase their feed intake (Olagaray et al., 2019). In the rumen, SCP stimulates cellulolytic bacteria to break down complex fibers in the cow's diet, thus making improvements in fiber digestion, feed consumption and metabolism efficiency (Desnoyers et al., 2009).

3.2. Milk yield and quality

The average milk yield of the control was 31.72 kg/cow per day and not different from that of the SCP of 31.21 kg/cow per day ($P > 0.05$; Figure 1). The results in the current study showed that SCP supplementation into the daily diets for dairy cows did not affect the milk productivity, which is suitable with the previous findings of no difference in milk yield between cow groups with and without supplementation of *Saccharomyces cerevisiae* fermentation product (Olagaray et al., 2019). It is possible that the addition level (5 g/cow per day) in this study is not enough to improve the milk yield and milk yield is greatly affected by many potential factors besides nutrition, such as housing, heat stress, disease, etc.

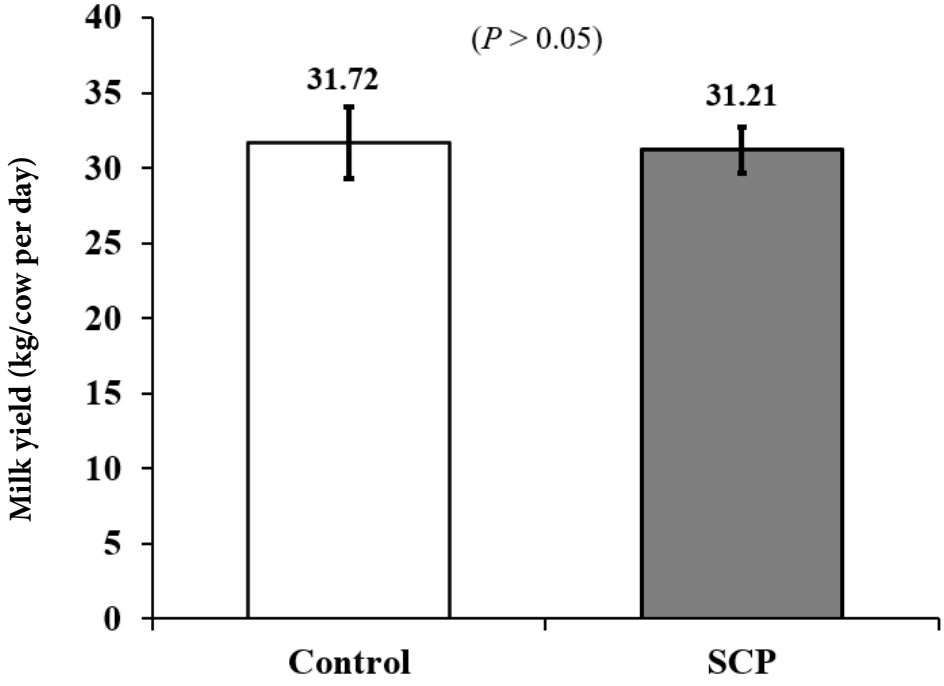

Figure 1. Effect of SCP addition on milk yield during experiment (n = 94 cows). SCP: *Saccharomyces cerevisiae*-contained product.

Table 3. Effect of dietary SCP supplementation on milk quality parameters (n = 94 cows)

Milk quality	Control	SCP	*P*
Milk fat (%)	3.90 ± 0.57	3.68 ± 0.30	0.327
Milk protein (%)	3.02^{b} ± 0.08	3.10^{a} ± 0.04	< 0.01
Milk lactose (%)	4.39^{b} ± 0.14	4.51^{a} ± 0.07	< 0.01
SNF (%)	8.00^{b} ± 0.24	8.23^{a} ± 0.10	< 0.01

^{ab}Means in the same row without common letters are different at P < 0.01; SCP: Saccharomyces cerevisiae-contained product; SNF: solids not fat.

The average milk protein, lactose and SNF from the milk of cows in the SCP group (3.10, 4.51 and 8.23, respectively) were significantly higher than those of cows in the control group (3.02, 4.39 and 8.00, respectively) ($P < 0.01$; Table 3), while there was no significant difference in the average milk fat between the two groups ($P > 0.05$). It has been reported that *Saccharomyces cerevisiae* enhances the efficiency of nutrient utilization in dairy cows by stabilizing ruminal pH and promoting beneficial microbes (Chaucheyras-Durand et al., 2008; Desnoyers et al., 2009). Therefore, this improvement in nutrient absorption often results in better milk composition, especially higher levels of milk protein and SNF, without increasing the overall milk productivity (Desnoyers et al., 2009). Meanwhile, Olagaray et al. (2019) mentioned that milk fat content increased by about 13% in the milk of cows supplemented with *Saccharomyces cerevisiae*.

3.3. Body condition score (BCS)

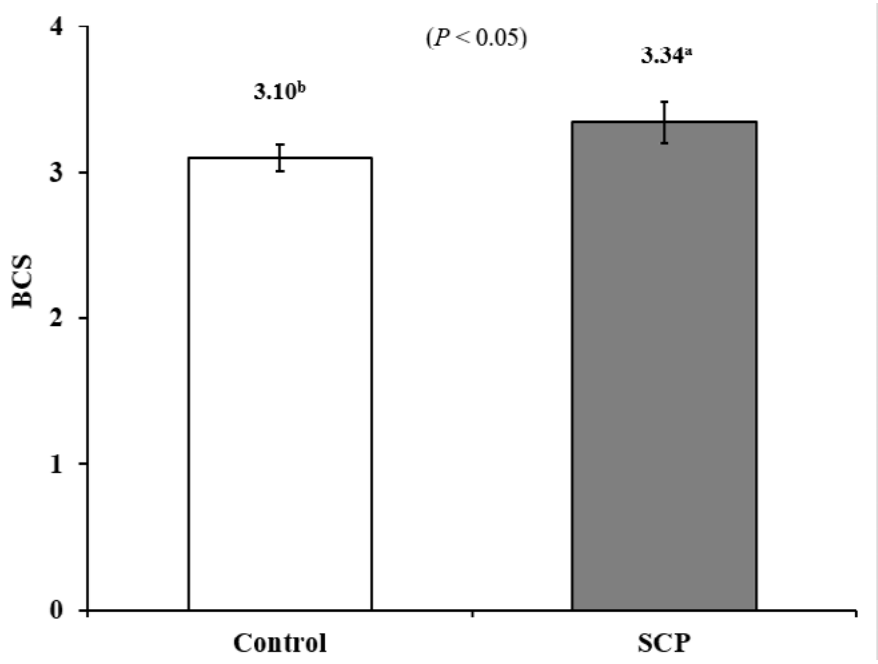

Figure 2. Effect of dietary SCP supplementation on BCS (n = 94 cows). BCS: The body condition score; SCP: *Saccharomyces cerevisiae*-contained product.

The average BCS in the control within the trial period of cows fed without SCP addition was 3.10 and significantly different from that of SCP within the trial period of cows fed with SCP product supplementation at 3.34 ($P < 0.05$; Figure 2). BCS in the trial period with SCP were higher than in the pre-trial period without SCP possibly due to increased feed intake. It has been demonstrated that *Saccharomyces cerevisiae* supplementation can stimulate appetite and increase dry matter intake, thus better supply of the energy and nutrient as animal's requirements, leading to the improved BCS (Poppy et al., 2012).

4. Conclusions

The results of the current study suggest that dietary SCP supplementation in daily diets increased feed intake and improved BSC and milk composition (solids not fat, protein and lactose) of dairy cows as compared to daily diets without SCP.

Conflict of interest

The authors declare no conflict of interest.

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Assessment of the immunity gap of two vaccination programs against Gumboro disease in Luong Phuong chickens

Lan T. H. Huynh, Truc L. T. Nguyen, Ho M. Nguyen, & Anh T. Quach*
Faculty of Animal Science and Veterinary Medicine, Nong Lam University, Ho Chi Minh City, Vietnam

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M.B strain
228E strain

***Corresponding author**

Quach Tuyet Anh
Email:
anh.quachtuyet@hcmuaf.edu.vn

ABSTRACT

Maternal-derived antibody (MDA) is the priority protection against environmental Infectious Bursal Disease Virus (IBDV) in the first weeks. The passive immunity decreases, but the active immunity is not enough to protect chicks, so shortening the high-risk period is crucial to IBD control. The objective of this study was to evaluate the immunity gap between 2 vaccination programs against infectious bursal disease (IBD) in Luong Phuong chickens. A total of 34,600 chicks were administered by subcutaneous injection of IBD vaccine at 0.1 mL/dose at the hatchery. At 12 days old, 18,000 chicks were vaccinated with the M.B strain vaccine and 16,600 chicks were vaccinated with the 228E strain vaccine by drinking water. The IBD and Newcastle disease (ND) antibody evaluations were based on the Enzyme-linked immunosorbent assay (ELISA) technique. Parameters were recorded until slaughter including body weight, average daily gain, feed conversion rate, and mortality. The IBD MDA at 1 day old was medium and uniform (3809 and 45.3%), which could protect against IBD virus from 1 to 2 weeks old. At 28 days old, the IBD antibody titer of the MB vaccine was higher than that of the 228E vaccine, various proportions of samples in the M.B group exceeding 1,000 titers (40% vs. 0%), and it was a statistically significant difference (1,133 vs. 161) ($P < 0.01$). Besides, the M.B vaccine created a faster and stronger immune response than the 228E vaccine, shortening the immune gap and protecting chicks earlier. The humoral immune response to the ND vaccine was good, with no difference between 2 groups, which proved that the M.B virus did not cause immunosuppression. The production parameters of chickens between the 2 groups were the same. In summary, the M.B vaccine made a short immune gap and did not cause immunodeficiency in chickens.

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1. Introduce

The poultry industry is facing many serious challenges, including Gumboro disease caused by Infectious Bursal Disease Virus (IBDV) which is the aetiological agent of an acute, highly contagious, and immunosuppressive disease particularly affecting chicks of 3 to 6 weeks of age. This infection transmits via the fecal-oral route and two serotypes of IBDV are identified: serotype 1 is pathogenic, while serotype 2 is non-pathogenic. Serotype 1 is classified into classical, intermediate, and very virulent strains (Jayasundara et al., 2017). Following oral infection, IBDV enters the bloodstream, replicates in the macrophages of the gut-associated tissues and lymphoid cells before it attains the bursa of fabricius (BF) (Xu et al., 2024). In the fully susceptible chicken flocks, the clinical disease includes dullness, depression, ruffled feathers, anorexia, whitish loose diarrhoea and severe dehydration (Islam & Samad, 2004). The chickens less than 3 weeks of age do not clearly exhibit clinical signs (Dahshan & Hussien, 2011). Recovery from disease and subclinical infection causes immunosuppression, principally directs towards the B lymphocytes, influences cell-mediated immunity, and leads to vaccination failures (Ingrao et al., 2013). Control of infectious bursal disease (IBD) depends on poultry health management, especially appropriate immunization schedules and maintenance of good hygienic conditions in farms (Farooq et al., 2003). However, IBDV infection is mainly controlled by live attenuated virus vaccines which are classified into a mild, intermediate, intermediate plus, or hot based on their residual virulence (Courtillon et al., 2022). The parent stocks are administered with an emulsion oil vaccine to boost an immune response and Maternal-derived antibody (MDA) in unvaccinated chickens persists up to 3 weeks old and completely decays by 4 to 5 weeks of age (Ahmed & Akhter, 2003). Moreover, the interference of MDA becomes a serious problem at the proper time of vaccination against IBD with a live vaccine (Berg, 2000). When the young chickens are vaccinated with attenuated vaccines too early that may lead to the neutralization of vaccine by MDA, and otherwise, the chicken flocks are poorly protected if applied too late due to the low level of MDA and the active immunity is not enough to prevent the field challenges (Dey et al., 2019), so shortening the high-risk period is crucial to IBD control.

Recently, the M.B strain vaccine has been used quite commonly in chicken farms and walks through the MDA levels of 800 Enzyme-linked immunosorbent assay (ELISA) Idexx while intermediate and intermediate plus vaccines break through the levels of MDA titers are 125 and 500, respectively (De Wit, 2001). Besides, the 228E strain vaccine is capable of walking through the MDA levels of 500 ELISA Idexx (De Wit, 2001). Intermediate and intermediate plus vaccines create better protection than mild vaccines, but they can cause severe bursal lesions and induce corresponding levels of immunosuppression (Rautenschlein et al., 2005). Therefore, the level of live attenuated vaccine influences the humoral immune response, and especially the M.B strain breaks through a higher MDA level. The objectives of this study were to compare the immunity gap of 2 vaccination programs and simultaneously to check whether the M.B vaccine caused immunodeficiency like other hot strain vaccines.

2. Materials and Methods

2.1. Experimental design

The study was carried out on a total of 34,600 Luong Phuong chickens, which were kept in 2 broiler houses of one commercial operation farm with the same management procedures from November 2023 to January 2024 in Binh

Duong Province, Vietnam. All day-one chickens (DOC) of the experiment were bought from the same breeder company and therefore, they were assumed to have the same MDA. All of them were administered by subcutaneous injection (SC) with an IBD immune complex vaccine dose of 0.1 mL at the hatchery. At 12 days old, house 1, 16,600 chicks, were vaccinated with the live attenuated 228E strain vaccine containing at least 10^2 embryo infective dose of 50% per dose by drinking water that was used in this farm for a long time and was suitable for the epidemical condition. Hence, house 1 was used as the control group. Besides, house 2, 18,000 chicks, were vaccinated with the M.B strain vaccine containing at least $10^{2.5}$ - 10^3 embryo infective dose of 50% per dose by drinking water at the same time. Other vaccines in the study were applied according to the below immunization schedule (Table 1).

Table 1. Immunization schedule of the current study

228E group (house 1)		M.B group (house 2)		Application
Age (days)	Vaccine	Age (days)	Vaccine	
1 (hatchery)	ND killed	1 (hatchery)	ND killed	SC with a dose of 0.1 mL/chick
	IBD		IBD	SC with a dose of 0.1 mL/chick
	IB + ND		IB + ND	Spray
7	IB + ND	10	IB + ND	Drop eye
12	228E strain	12	M.B strain	Drinking water
14	AI	15	AI	SC with a dose of 0.3 mL/chick
21	IB + ND	28	IB + ND	Drinking water
35	IB + ND	35	ND	Drinking water

2.2. Serology

Blood samples from 20 chicks were randomly collected for the determination of the IBD and ND MDA at 1 day old. After the second IBD vaccination, at 21, 24, 28, and 34 days of age for the determination of IBD antibodies and at 21, 28, and 42 days of age for the determination of ND antibodies (Figure 1). Randomly selected 15 chicks per house were taken vein blood samples at 21 days old and they were taken the leg mark ring to follow the individual antibody chicks. Then, these chicks were raised with the same environmental conditions as others in the house and continued to record their level of antibodies at other times. All blood samples were let clot naturally, were stored 2 - 8°C, and were sent to the An Phu Tien laboratory in Dong Nai Province, Vietnam. They were centrifuged at 3,000 rpm for 5 minutes to extract serum. Two types of commercial enzyme-linked immunosorbent assay kits (Idexx, Maine, USA) were used as described by the manufacturer to detect the antibodies against IBD and ND in chicken serum. As a result, the serum sample with an S/P ratio ≤ 0.2 (titer ≤ 396) is negative and an S/P ratio > 0.2 (titer > 396) is positive.

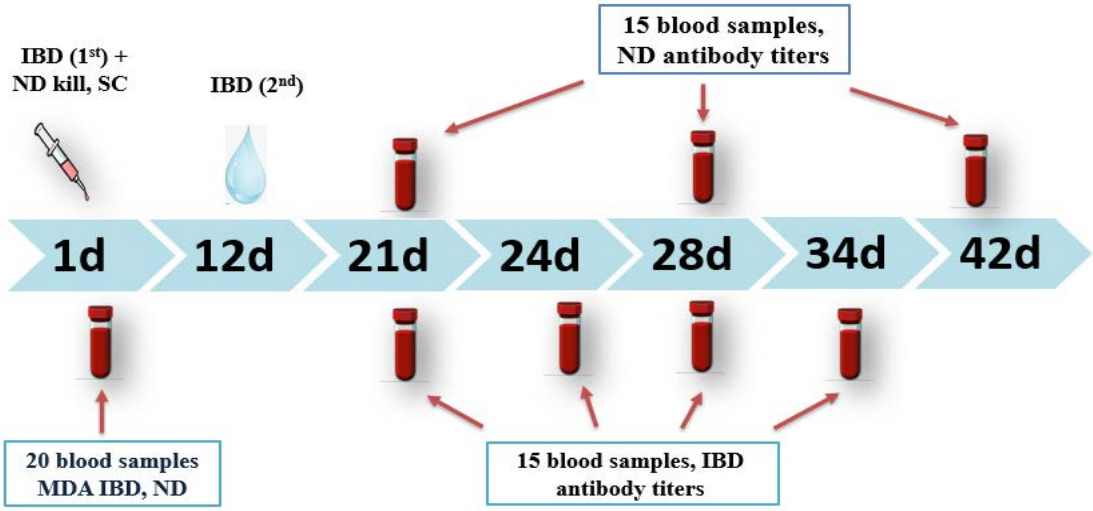

Figure 1. The experimental design per house.

2.3. Performance

The survey was conducted to compare the performance indicators of the broiler chicken flocks when they were vaccinated with two immunization schedules. The performance parameters were followed until slaughter, including body weight, average daily gain, feed conversion ratio, and mortality. The total chicken flock weight was only recorded at the time of sale and the amount of feed was monitored throughout the implementation period. The productivity norms were calculated according to the formula.

Average body weight (kg/chicken) = total weight of chickens/total number of chickens

Average daily gain (g/day) = (total of final weight - total of beginning weight)/total number of survival chicken days)

Feed conversion rate = total amount of consumed feed/total weight of chickens

Mortality (%) = total of dead chickens/total of beginning chickens *100

2.4. Statistical analysis

The data were collected and analyzed by Microsoft Excel 2016 and Minitab 16 software. Using a one-way ANOVA model and the T-test to compare the average level of antibodies between 2 groups. The differences were considered statistically significant with $P < 0.05$. The coefficient variation CV (%) is interpreted as < 30% excellent, 30 - 50% good, 51 - 80% fair, and > 90% poor response to vaccine.

3. Results and Discussion

3.1. Maternally derived IBD antibodies

The MDA is key to protecting chicks against virulent field IBDV strains during the first weeks of age. According to Kreider et al. (1991), the MDA is divided into 3 levels: low level (< 3,000), medium level (3,000 - 5,000) and high level (> 6,000). Collecting randomly 20 serum samples at 1 day old to determine their IBD MDA titers based on the ELISA technique. The titers ranged from 841 to 7,039 and the average titer was medium and uniform (3,809 and 45.3%). The

chicks of 2 houses were bought from the same breeder company. The half-life time of MDA is 3.8 days for Luong Phuong chickens (De Wit, 2001), so these titers can protect the young chickens against field viruses from 1 to 2 weeks old. The MDA can potentially neutralize the vaccine if done on very younger progeny chickens (Ahmed & Akhter, 2003). The interference of MDA is a major problem for the best time to vaccinate, serological monitoring is necessary to evaluate the level of MDA and decide the appropriate timing for vaccination (Berg, 2000). According to the Deventer formula to determine the age of vaccination application (De Wit, 2001). The M.B. strain was able to break through the MDA level of 800 ELISA Idexx and therefore a suitable time could be vaccinated at 13 days old. On the other hand, the available vaccination procedure of the farm was applied by the 228E vaccine at 12 days old, which was considered a standard program, and suitable for epidemical conditions. Hence, the second vaccination against IBD used for 2 programs was at 12 days of age.

3.2. IBD antibodies post second vaccination

Table 2. Infectious bursal disease (IBD) antibody titers

Age	M.B group			228E group			*P*
	Mean titer	CV (%)	N	Mean titer	CV (%)	N	
IBD 21 days old	186	55.7	15	168	83.4	14	0.696
IBD 24 (25) days old	146	41.9	14	117	80.4	14	0.328
IBD 28 days old	1,133[a]	88.4	15	161[b]	97.1	14	0.001
IBD 34 days old	2,215	41.1	15	1,991	56.6	15	0.554

[a- b]*Mean values of traits in rows, marked with different letters, differ statistically significantly between groups (P < 0.01).*

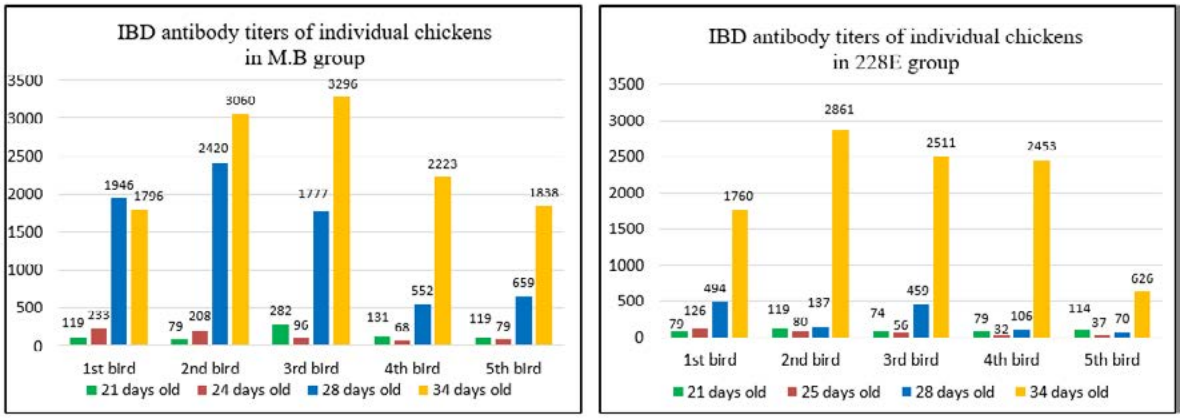

Figure 2. Infectious bursal disease (IBD) antibody titers of individual chickens.

At 21 days old, blood samples of 15 chicks per house were randomly taken and simultaneously were made the leg mark ring to evaluate the change of individual antibody chicks and could skip antibody titers of abnormal samples in the following times (Figure 2). Circulating ELISA IBD antibodies ranged from 52 to 414 in the M.B group (mean titer: 186, CV: 55.7%) and from 52 to 590 in the 228E group (mean titer: 168, CV: 83.4%). The MDA titers of both groups decreased greatly. There was no statistically significant difference between 2 groups ($P > 0.05$) (Table 2).

At 24 days old, circulating IBD antibodies ranged from 68 to 233 in the M.B group (mean titer: 146, CV: 41.9%). The passive immunity continued to reduce and the active antibodies began to create (28.6% samples) with good uniformity in the M.B group. Samples of the control group were collected at 25 days old, delaying one day due to a few objective reasons. At 25 days old, IBD antibodies in the 228E group ranged from 32 to 402 (mean titer: 117, CV: 80.4%). The difference was not statistically significant between 2 groups ($P > 0.05$) (Table 2). The same as the M.B group, the passive immunity continued to reduce greatly in the 228E group and the active antibodies also began to create (14.3% samples). Although the 228E group was one more day compared to the M.B group, but the percentage of active antibodies of samples in this group was still smaller (14.3% versus 28.6%).

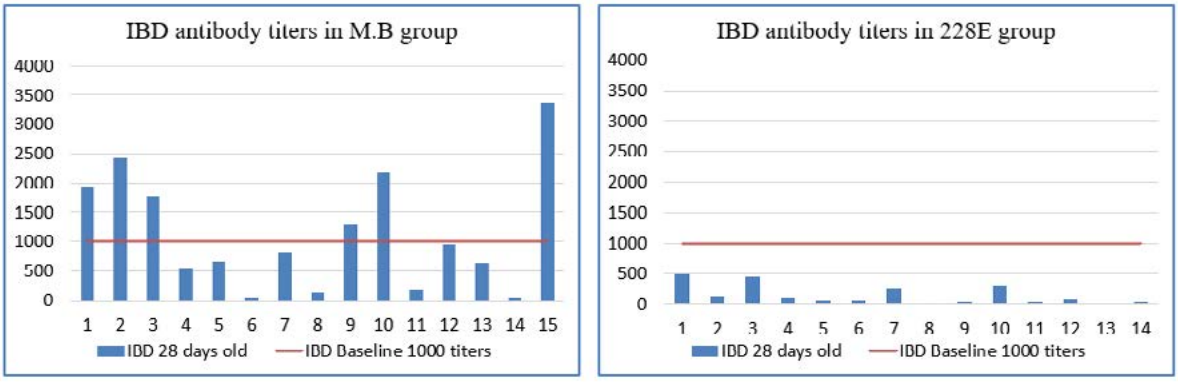

Figure 3. Infectious bursal disease (IBD) antibody titers at 28 days old of 2 vaccination programs.

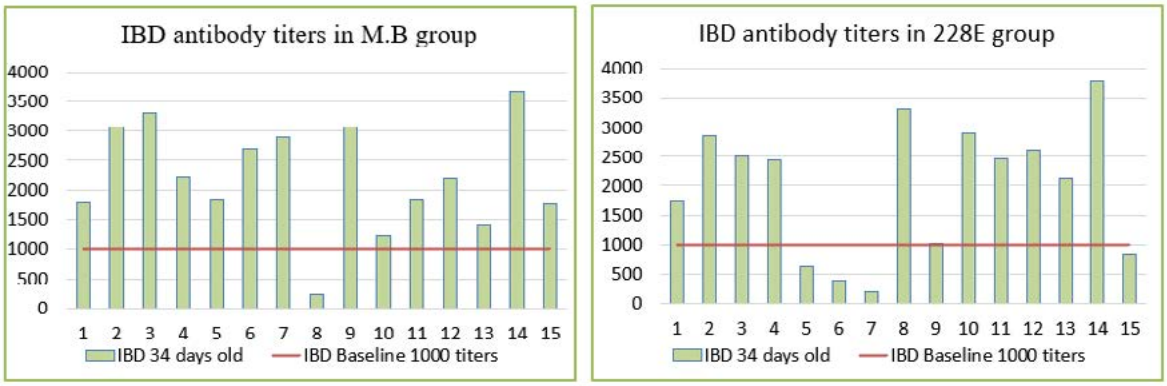

Figure 4. Infectious bursal disease (IBD) antibody titers at 34 days old of 2 vaccination programs.

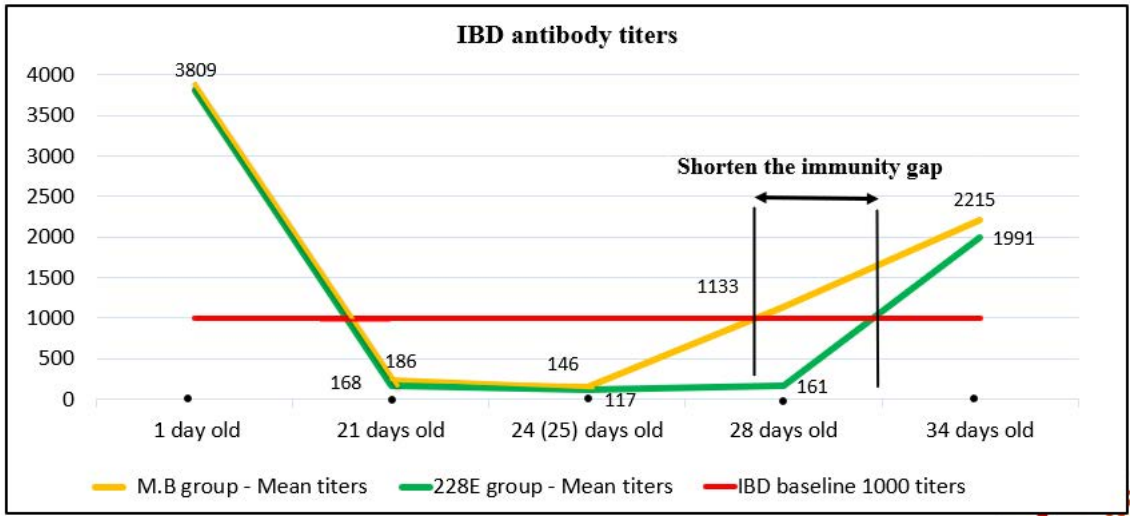

Figure 5. The average level of infectious bursal disease (IBD) antibody titers of 2 vaccination programs.

At 28 days old, IBD antibody titers ranged from 41 to 3,362 in the M.B group (mean titer: 1,133, CV: 88.4%) and from 18 to 494 in the 228E group (mean titer: 161, CV: 97.1%) (Figure 3). The active immunity in the M.B group was higher and faster than the active immunity in 228E group had recently appeared, the statistically significant difference was between 2 groups ($P < 0.01$) (Table 2). According to the Idexx recommendation, the protective antibody titers against IBD were 1,000 for the broiler chickens. The proportions of samples exceeding 1,000 titers in the M.B and the 228E vaccines were 40% and 0%, respectively. According to Smith (2019), herd immunity occurs when a sufficient proportion of the group has immunity against contagion of the pathogen to susceptible animals and even helps to maintain the stable existence of the pathogen in a population instead of completely removing field viruses in the farm. The target organ of IBDV is the bursal Fabricius at its maximum development and the acute disease is directly related to the susceptible B lymphocyte cells, so the most sensitive age is from 3 to 6 weeks old (Berg, 2000). In addition, young chickens are the most susceptible to IBDV around 30 to 35 days old (Ahmed & Akhter, 2003). Therefore, the administered M.B vaccine chickens were better protected and concurrently the herd immunity was able to reduce partially the risk of field challenges for the susceptible chickens. Furthermore, the average level of IBD antibody titers in M.B group started to overcome 1,000 titers ELISA Idexx at 27 days old, which meant the active immunity could be enough to protect chickens during the sensitive period.

At 34 days old, IBD antibodies ranged from 238 to 3,661 in the M.B group (mean titer: 2,215, CV: 41.1%), from 179 to 3,788 in the 228E group (mean titer: 1,991, CV: 56.6%) (Figure 4). The result showed that the active immunity of 2 groups was increased. There was no significant difference ($P > 0.05$). The ratios of samples exceeding 1,000 titers in the M.B and 228E groups were 93.3% and 73.3% respectively, which proved that the ability of community immunity in vaccinated M.B vaccine chickens was better to suffer from the environmental challenges.

Besides, the average level of IBD antibody titers in the 228E group started to overcome 1,000 titers at about 30 days old. As a result, the vaccination program using the M.B strain affected shortening the window of susceptibility no protection about 3 days when compared between 2 vaccination programs (Figure 5).

Another strategy to control IBD is based on a uniform active immune response post-vaccination in all individuals of the flock, environmental viruses have no chance to attach, or replicate in any chickens, and therefore, a type of optimal vaccine will generate better uniformity (CV is lower) (Nguyen et al., 2018). The result revealed that the uniformity of both groups was clearly improved respectively from 28 days to 34 days of age: the M.B group was 88.4% and 41.1% and the 228E group was 97.1% and 56.6%. Additionally, the value of CV immunity response in the M.B group was lower than the 228E group at 4 time points (Figure 6). This result indicated the better ability of individual protection of vaccination program using the M.B strain and reduced the infectious risk to other chickens in the flock.

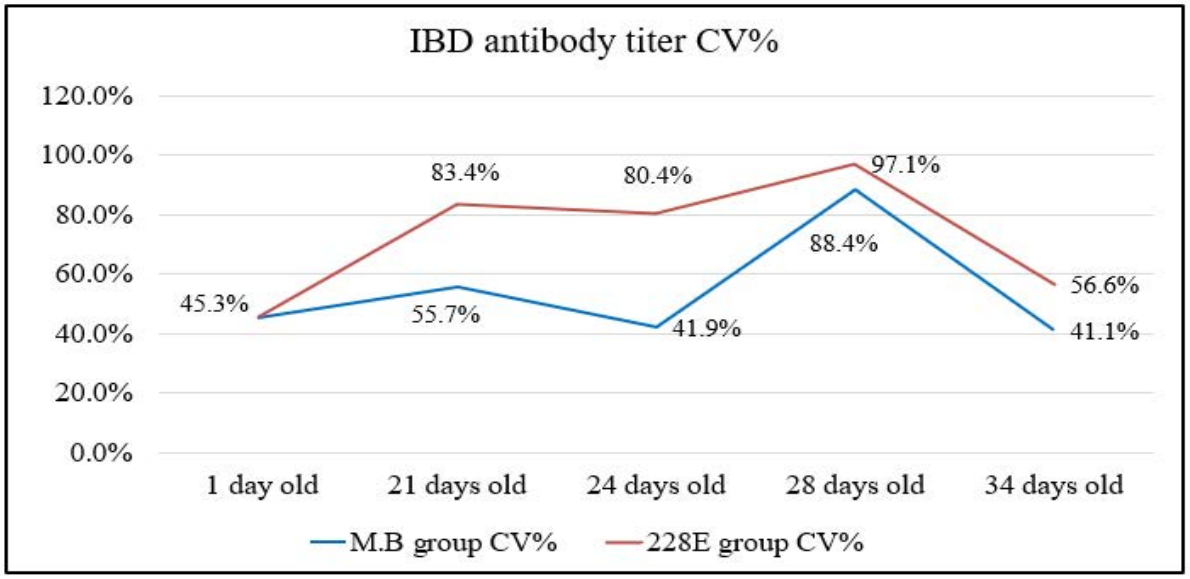

Figure 6. The uniformity of infectious bursal disease (IBD) antibody titers of 2 vaccination programs.

3.3. Humoral immune response to inactivated ND vaccine

Table 3. Newcastle disease (ND) antibody titers

Age	M.B group			228E group			*P*
	Mean titer	CV (%)	N	Mean titer	CV (%)	N	
ND 21 days old	444	183.2	15	292	116.6	15	0.509
ND 28 days old	1,199	64.4	15	1,129	102.4	15	0.848
ND 42 days old	3,029	68.5	15	1,885	50.0	15	0.062

The intermediate plus or hot live attenuated IBD vaccine can be used in complex epidemics, but these vaccines can cause B cell depletion in the bursa and immunosuppression (Courtillon et al., 2022). Immunosuppressive chicken flock is a significant concern because of losing the ability of pathogen resistance and failing other vaccination programs. In this study, both groups were assessed the humoral immune response to other antigens, such as the inactivated ND vaccine. According to the IDL (2015), the protective antibody titers against ND are divided into 3 levels: low level (1,000 - 5,000), medium level (7,000 - 12,000) and high level (16,000 - 25,000). At 1 day old, the average ND antibody titers were low and uniform (3,701 and 41.7%). All the chicks were bought from a breeder company, so the same MDA against ND. At 21, 28, and 42 days old, ND antibody titers were not statistically different between 2 groups ($P > 0.05$) (Table 3). Therefore, the M.B strain virus did not affect the ability of the humoral immune response when compared to a standard immunization schedule on the farm (Figure 7). According to Lazarus et al. (2008), a dosage of the M.B strain in the range of 10^2 to 10^4 embryo infective dose of 50% is safe and protective for commercial chicks. Another research took place in commercial broiler chickens to evaluate the efficacy of M.B, LIBDV, and Winterfield 2512 strain vaccines against IBD, this study proved that the M.B strain vaccine did not cause immunosuppression (Nguyen et al., 2018). According to Nguyen et al. (2022), the M.B strain virus was located early in the BF to field virus competition, but it did not cause serious damage, showed recovery signs, and did not affect immunodeficiency.

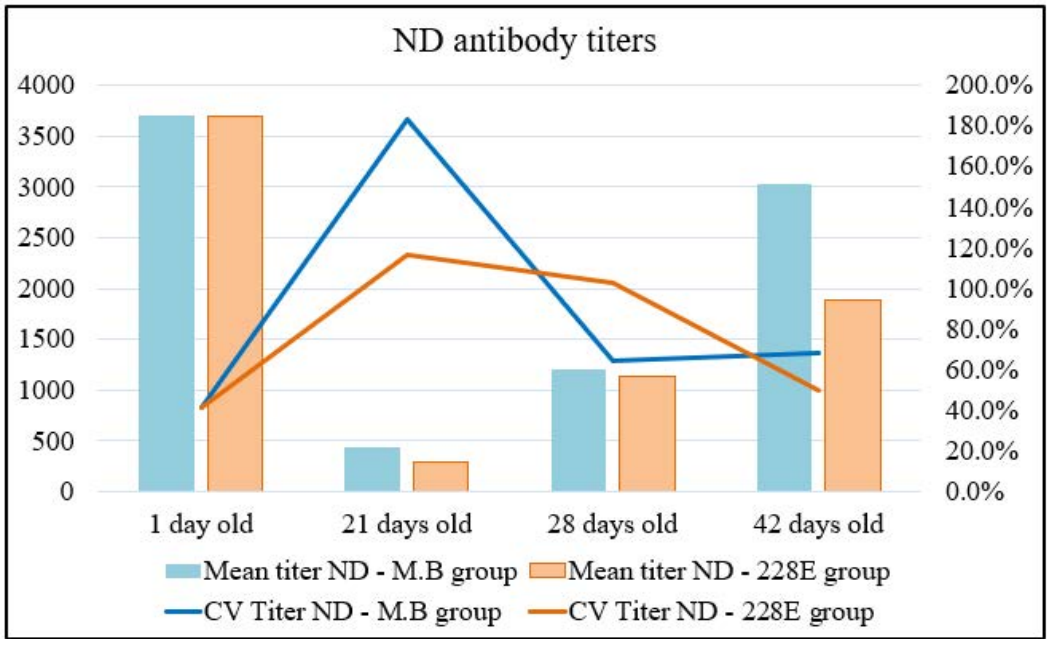

Figure 7. Newcastle disease (ND) antibody titers.

3.4. Production performance

The broiler chicken performance can be evaluated during the rearing phase, control of IBD-induced immunosuppression results in a decrease in lesions caused by bacterial secondary infections and a decrease in carcass condemnation at slaughterhouses (Lemiere, 2013). During the trial period, a total of chickens were recorded with no IBD and ND symptoms. An optimal vaccine does not only stimulate a good immune response, but also the productivity reaches the target. The body weight and feed intake were recorded for the entire trial to assess the growth performance at market slaughter age. Overall, the production parameters of 2 groups were fairly good and no significant difference (Table 4). A part of the experimental chickens should be challenged with the field virus and recorded the productivity data to obviously evaluate the difference between 2 vaccination programs, but the limitations of this study do not allow this to be done.

Table 4. Broiler performance

No.	Parameters	M.B group	228E group
1	Growing period (days)	55	54
2	Mortality (%)	3.63	4.07
3	Average body weight (kg)	1.491	1.494
4	Feed conversion ratio	2.316	2.318
5	Average daily gain (g/day)	26.42	27.06

4. Conclusions

In summary, the M.B strain vaccine generated the immune response against IBD earlier than the 228E strain and shortened the high-risk period in the susceptible chicken flock. In addition, the M.B strain virus did not cause immunodeficiency through the ability of humoral immune response with the inactivated ND vaccine and did not affect negatively the production performance. These findings support using the M.B strain vaccine as an effective strategy for preventing Gumboro disease in Luong Phuong chickens.

Conflict of interest

We guarantee that the article is done by the author's team and there are no conflicts among the authors.

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Presence of metal-resistance and antibiotic-resistance genes in *Salmonella* spp. isolated from broiler chicken farms in Vinh Long province, Vietnam

Luan M. Huynh[1,2], Thuan K. Nguyen[2*], Duy D. Nguyen[2], & Khai T. L. Ly[2]

[1]Faculty of Applied Biological Sciences, Vinh Long University of Technology Education, Vinh Long, Vietnam

[2]Faculty of Veterinary Medicine, College of Agriculture, Can Tho University, Can Tho, Vietnam

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***Corresponding author**

Nguyen Khanh Thuan
Email:
nkthuan@ctu.edu.vn

ABSTRACT

Salmonella can carry multiple antibiotic-resistant and metal-resistant genes and transmit these genes among strains worldwide. This study examined seventy-five *Salmonella* isolates from small-scale chicken farms (chicken feces, bedding, feed, wild animals) in Vinh Long province for the presence and relation of antibiotic and metal-resistance genes in these strains. The single PCR method was applied to detect seven antibiotic-resistance genes (*blaampC, blaTEM, dfrA1, tetA, strA, sul2, mcr1*) and four metal-resistance genes (*pcoR, czcD, cnrA, silE*). The results indicated that those *Salmonella* isolates harbored several patterns of antibiotic-resistance genes. Genes *blaampC* and *tetA* were the most prevalent (48.00%), while genes *mcr1* and *dfrA* were the most minor (1.33%). Of those *Salmonella* isolates, 92.00% harbored one to five antibiotic-resistance genes, and the *blaampC* + *strA* pattern was frequently obtained (12.00%). Moreover, 30.67% of *Salmonella* isolates showed multidrug resistance to three or four antibiotic categories. Among metal-resistance genes, gene *pcoR* encoding for copper resistance was the most predominant (53.33%), and gene *cnrA* encoding for cobalt-nickel resistance was the lowest (5.33%). There were diverse patterns of metal-resistance genes, and one *Salmonella* isolate carried four examined genes (1.33%). Furthermore, these *Salmonella* isolates had several combined patterns of metal-resistance and antibiotic-resistance genes. Among them, *pcoR, czcD,* and *silE* genes had a significant coefficient relation to the examined antibiotic-resistance genes. It indicated the correlation between metal resistance and antibiotic resistance genes and revealed the potential risk of increasing antibiotic resistance in *Salmonella* isolates in chicken farms in Vinh Long province.

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1. Introduction

Salmonella is one of the major foodborne pathogens that can pose a significant threat to public health, mainly through the consumption of contaminated poultry products (Chuanchuen et al., 2008; Dantas et al., 2020). *Salmonella* also causes infection in poultry, and contamination in the poultry environment facilitates the transmission of *Salmonella* through both vertical and horizontal pathways (Singh et al., 2010). In a previous report, *Salmonella* was isolated from chicken feces (7.67%), pest animals (5.98%), such as geckos, ants, cockroaches, and environmental samples (4.33%) in the chicken farms in the Mekong Delta, Vietnam (Nguyen et al., 2021). It indicated that *Salmonella* is a potential risk-causing disease for chickens and transmission in the husbandry environment.

On the other hand, the emergence of antibiotic-resistant *Salmonella* strains in food animals, including chickens, is a growing concern (Nair et al., 2018). Most *Salmonella* isolates have developed resistance to multiple drugs because of farmers' indiscriminate and repeated misuse of these antibiotics. The extensive use of antimicrobials in food animal production has been a critical driver of this trend, as it can promote the development and dissemination of resistant strains (Kulwichit et al., 2007). Zhu et al. (2017) reported that *Salmonella* isolated from broiler chickens in slaughterhouses in China exhibited multidrug resistance and harbored several antibiotic-resistance genes, such as *blaTEM, blaCTX-M, tetA, sul2, floR, aadA1, drfA1*, etc. Thus, screening for antimicrobial resistance in *Salmonella* is crucial for managing and treating *Salmonella* infections in poultry.

The extensive application of heavy metals as feed additives in livestock production has resulted in insufficient focus on pathogenic bacteria's resistance to these metals. The occurrence of heavy metal resistance genes in *Salmonella* showed the relationship between these genes and antibiotic-resistance genes (Yang et al., 2020). It has been demonstrated that the co-selection of antibiotic-resistance genes resulting from the presence of heavy metals significantly contributed to the observed rise in antibiotic-resistance genes abundance (Stepanauskas et al., 2006; Mazhar et al., 2021) and acted as a selective factor in their proliferation (Allen et al., 2010). Yang et al. (2020) reported that the presence of metal-resistance genes (*zntA, arsB, merA, pcoR, pcoA, pcoC*, and *chrA*) was found to be significantly associated with one or more antibiotic-resistance genes (*sul1, sul2, sul3, tetA, tetB, tetC, blaTEM, blaSHV*, and *blaCTX*). The interaction of these genes has increased the antibiotic resistance in bacteria, including *Salmonella*, in chicken farms. Moreover, disinfectants are essential in controlling the growth and transmission of pathogens. Nonetheless, the selective pressure imposed by disinfectants and heavy metals on microbial pathogens is increasingly recognized as a significant factor that drives the selection and dissemination of antimicrobial resistance within the food chain of humans and animals (Capita & Alonso-Calleja, 2013; Tezel & Pavlostathis, 2015).

In Vinh Long province, chickens were raised frequently; however, most farms were small-scale. The hygiene in these small-scale farms was not managed well; the pathogens could survive and spread via chickens or the environment (Alali et al., 2010; Nguyen et al., 2021). The prevalence and antibiotic resistance of *Salmonella* was recorded in several previous reports. However, few studies have been published on the prevalence of antibiotic-resistant genes. In contrast, no studies have been published on metal-resistance genes in

Salmonella isolated from chickens or husbandry environments in the Mekong Delta, including Vinh Long province. Therefore, this study aims to clarify the prevalence of antibiotic-resistance and metal-resistance genes in *Salmonella* originating from chickens and the surrounding environment. This research could provide valuable insights into the potential risk of those antibiotic-resistant *Salmonella* strains and inform strategies for mitigating poultry health risks in those farms.

2. Materials and Methods

2.1. The origin of *Salmonella* isolates

This study used 75 *Salmonella* isolates, which were isolated from broiler chicken feces (n = 15), husbandry environment samples: bedding samples (n = 6) and feed (n = 4), pests: geckos (n = 38), rats (n = 8), and ants (n = 4) in four different small-scale farms in Tam Binh and Mang Thit districts, Vinh Long province. These positive *Salmonella* strains were detected from 1,265 samples (chickens' feces, pests, and husbandry environment) from February 2022 to December 2022. The isolation and identification of *Salmonella* isolates were performed according to the instructions of Barrow & Feltham (2003). These *Salmonella* isolates were kept in Tryptic Soy Broth (TSB, Merck, Germany) supplied with 15% glycerol (Merck, Germany) at -80°C freezer in the Veterinary Food Hygiene Laboratory, Faculty of Veterinary Medicine, College of Agriculture, Can Tho University, Vietnam. One positive *Salmonella* isolate, representative of one positive sample, was selected for use in this study.

2.2. Identification of antibiotic-resistance genes in *Salmonella* isolates

The DNA of 75 *Salmonella* isolates was extracted using the heat-shock method (Ahmed & Dablool, 2017) and stored at -20°C for use in this experiment. The single PCR assay was applied to detect seven antibiotic-resistance genes, including β-lactam (*blaampC*, *blaTEM*), aminoglycoside (*strA*), tetracycline (*tetA*), polypeptide (colistin-*mcr1*), sulfonamide (*sul2*), and diaminopyrimidine (*dfrA1*) (Table 1). The PCR conditions followed the description of references in Table 1, respectively. These genes were often detected in *Salmonella* and *E. coli* isolated from chickens in previous studies in the Mekong Delta (Nguyen et al., 2015; Nguyen et al., 2021) and represented antibiotic types used frequently in our surveys in the small-scale chicken farms.

The MyTaq Mix 2X (BIO25042, Bioline, Meridian Bioscience, USA) was in the PCR reaction as a master mix. One reaction consists of a total of 25.0 µL, including Mastermix 2X (12.5 µL), forward primer (0.5 µL), reverse primer (0.5 µL), distillation water (9.5 µL), and DNA template (2.0 µL). Thermal cycle was modified as follows: 94°C - 5 min; 30 cycles: 94°C - 1 min, 58°C - 45 sec, 72°C - 1 min; 72°C - 10 min. The *Salmonella* isolates harbored those genes, previously isolated from domestic animals in the Mekong Delta, were used as a control. The PCR products were electrophoresed in 1.5% agarose gel at 50V for 60 min. Then, the gels were dyed in ethidium bromide (0.001 mg/L) before capturing the image under UV.

2.3. Identification of metal-resistance genes in *Salmonella* isolates

This study also used the DNA of seventy-five *Salmonella* isolates to detect the presence of metal-resistance genes. The single PCR (25.0 µL/reaction) and electrophoresis procedures were conducted like those used to detect antibiotic-resistance genes.

Four metal-resistance genes were examined for genes encoding resistance to copper (*pcoR*), cobalt-zinc-cadmium (*czcD*), cobalt-nickel (*cnrA*), and silver (*silE*) (Table 1). These metal-resistance genes were reported in several previous studies in *Salmonella* and *E. coli*, and these heavy metals were commonly used in disinfectant products (Woods et al., 2009; Yang et al., 2020; Mustafa et al., 2021).

The PCR conditions and primer sequences (*pcoR, czcD, cnrA*, and *silE*) followed the descriptions of references in Table 1, respectively. The *Salmonella* isolates harboring these metal-resistance genes, previously isolated from domestic animals (pigs, chickens) in our pilot studies in the Mekong Delta, were used as a control. The PCR products were electrophoresed in 1.5% agarose gel at 50V for 60 min. Then, the gels were dyed in ethidium bromide (0,001 mg/L) before capturing the image under UV.

Table 1. The nucleotide sequence of antibiotic-resistance and metal-resistance primers used in this study

Genes	Sequence 5'-3'	Size (bp)	References
Antibiotic-resistance genes			
blaampC	AATGGGTTTTCTACGGTCTG GGGCAGCAAATGTGGAGCAA	191	Caroff et al. (1999)
blaTEM	ATTCTTGAAGACGAAAGGGC ACGCTCAGTGGAACGAAAAC	1.150	Jouini et al. (2007)
strA	CCTGGTGATAACGGCAATTC CCAATCGCAGATAGAAGGC	546	Carattoli et al. (2002)
tetA	GGTTCACTCGAACGACGTCA CTGTCCGACAAGTTGCATGA	577	Randall et al. (2004)
mcr-1	CGGTCAGTCCGTTTGTTC CTTGGTCGGTCTGTAGGG	309	Elnahriry et al. (2016)
sul2	CGGCATCGTCAACATAACC GTGTGCGGATGAAGTCAG	722	Sáenz et al. (2010)
dfrA1	GGAGTGCCAAAGGTGAACAGC GAGGCGAAGTCTTGGGTAAAAAC	367	Peirano et al. (2006)
Metal-resistance genes			
pcoR	CAGGTCGTTACCTGCAGCAG CTCTGATCTCCAGGACATATC	636	Yang et al. (2020)
czcD	CAGGTCACTGACACGACCAT CATGCTGATGAGATTGATGATC	398	Anton et al. (2004)
cnrA	CCTACGATCTCGCAGGTGAC GCAGTGTCACGGAAACAACC	422	Mustafa et al. (2021)
silE	AGGGGAAACGGTCTGACTTC ATATCCATGAGCGGGTCAAC	432	Woods et al. (2009)

2.4. Statistical analysis

The statistical analysis was used to clarify the difference in the presence of antibiotic-resistant and metal-resistant genes among those *Salmonella* isolates. The Chi-square method was used to define the significant difference in the presence of antibiotic-resistant and metal-resistant genes at a confidence level of 95%. Spearman's correlation coefficient was used to determine the relation between antibiotic-resistant and metal-resistant genes. These analyses were performed in Minitab software version 17.0 (Minitab LLC, USA).

3. Results and Discussions

3.1. The presence of antibiotic-resistance genes in *Salmonella* isolates

The results (Table 2, Figure 1) indicated that *blaampC* and *tetA* genes were detected at the highest rate (48.00%), followed by *strA* (42.67%), and the most minor ones were genes *dfrA1* and *mcr-1* (1.33%). Those genes encode resistance to favored antibiotic groups (β-lactam, cycline, and aminoglycoside) frequently used to treat salmonellosis in poultry and used in small-scale farms in Vinh Long province, according to our previous studies and other reports (Nguyen et al., 2017; Nguyen et al., 2021). This indicated that *Salmonella* isolated from small-scale farms could resist antibiotics currently used, causing challenges in choosing suitable antibiotics for treating diseases there. Yildirim et al. (2011) stated that the variations in resistance were also linked to the specific serovar of *Salmonella*, the type of poultry (broilers or layers), individual farms, and the specific antimicrobial agents used.

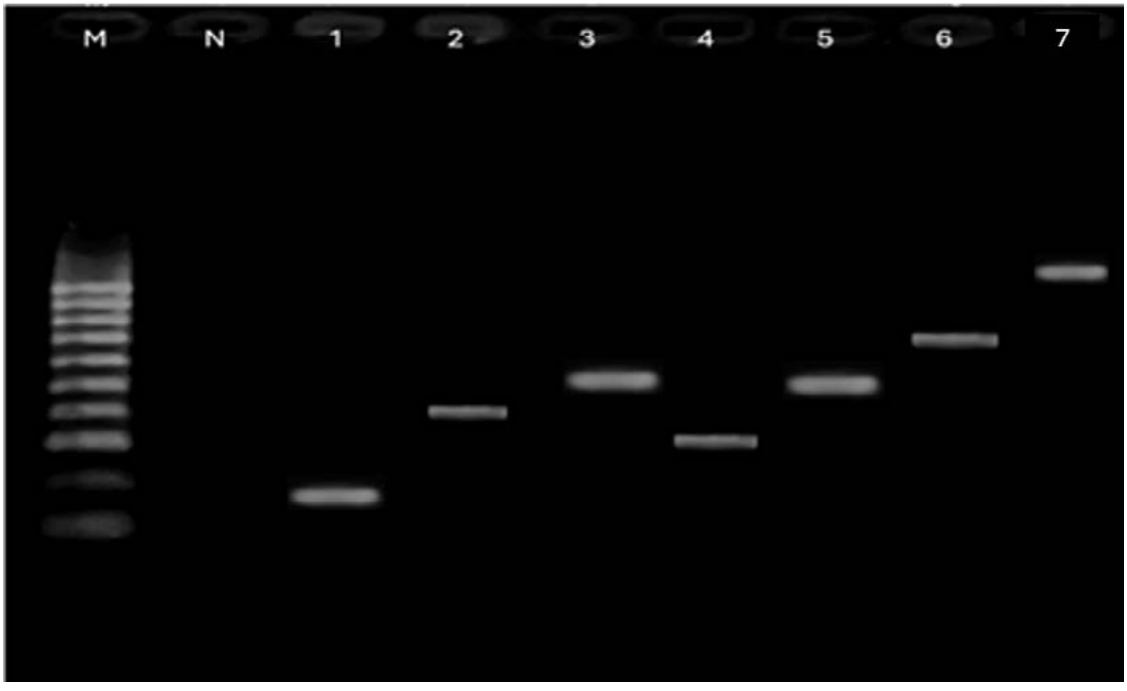

Figure 1. The electrophoresis image of PCR products in detecting antibiotic-resistance genes in *Salmonella* spp. isolates. M: ladder (100 bp), N: negative control (Distilled water), 1: *blaampC* (191 bp), 2: *dfrA* (367 bp), 3: *tetA* (577 bp), 4: *mcr-1* (309 bp), 5: *strA* (546 bp), 6: *sul2* (722 bp), 7: *blaTEM* (1.150bp).

Table 2. The prevalence of antibiotic-resistance genes in *Salmonella* isolates in the small-scale farms in Vinh Long province

Genes	Feces (n = 15)		Husbandry environment (n = 10)		Pests (n = 50)		Total* (n = 75)	
	No. of positive isolates	Percentage (%)	No. of positive isolates	Percentage (%)	No. of positive isolates	Percentage (%)	No. of positive isolates	Percentage (%)
blaampC	3	20.00	1	10.00	32	64.00	36	48.00
blaTEM	2	13.33	2	20.00	8	16.00	12	16.00
strA	5	33.33	1	10.00	26	52.00	32	42.67
tetA	11	73.33	6	60.00	19	38.00	36	48.00
mcr-1	0	0.00	1	10.00	0	0.00	1	1.33
sul2	4	26.67	2	20.00	16	32.00	22	29.33
dfrA1	0	0.00	1	10.00	0	0.00	1	1.33

**There was no significant in the prevalence of antibiotic-resistance genes among those samples (P > 0.05).*

In the current study, most genes could be detected from chickens' feces and pests; however, genes *dfrA1* and *mcr-1* were not found. These two genes were found in *Salmonella* isolates originating from the husbandry environment (bedding and feed) (Table 2). The widespread presence of *Salmonella* spp. can be attributed to their ability to adapt to hosts, their resilience to adverse conditions, and their increased capability to form biofilms. These factors lead to persistent contamination of the environment, animal feed, and livestock. Furthermore, the emergence and proliferation of antimicrobial resistance in *Salmonella* spp. presents further challenges (Velhner et al., 2018). In the Mekong Delta, there were a few large-scale chicken farms. Thus, the antibiotic resistance of *Salmonella* isolated from chickens and the environment in these farms was limited compared to this study. In other published research, Ramatla et al. (2019) reported that *Salmonella* spp. isolated from chickens and rats in poultry houses in South Africa, exhibited significant antibiotic resistance, and ultimately highlighted the importance of rats as carriers and transmitters of antibiotic-resistant bacteria to chickens and humans. In India, genes *tetA*, *tetB*, *blaTEM*, and *CTX-M* were found at a relatively high rate in *Salmonella* isolated from chickens and the environment and indicated selective pressure for adopting resistance against the tetracycline antibiotic group in *Salmonella* (Waghamare et al., 2018). In Bangladesh, Das et al. (2021) also reported that 94% of *Salmonella* isolates from broiler chickens were multidrug-resistant, 81.4% of the isolates carrying the *tetA* gene, while genes *blaTEM* and s*ul-I* were at 95.4% and 37.2 %, respectively. Thus, the husbandry environment could be a contaminated source from which antibiotic-resistant *Salmonella* isolates survived and spread out.

Table 3. The antibiotic-resistance patterns of *Salmonella* isolates in Vinh Long province (n = 75)

No. of antibiotic-resistance genes	Patterns	No. of positive isolates	Percentage (%)
1	*blaampC*	11	14.67
	strA	7	9.33
	tetA	11	14.67
2	*blaampC + strA*	9	12.00
	blaTEM + dfrA1	1	1.33
	blaTEM + strA	1	1.33
	tetA + mcr-1	1	1.33
	tetA + strA	3	4.00
	tetA + sul2	2	2.67
3	*blaampC + strA + sul2*	1	1.33
	blaampC + tetA + strA	3	4.00
	blaampC + tetA + sul2	6	8.00
	blaTEM + strA + sul2	1	1.33
	blaTEM + tetA +sul2	4	5.33
	tetA + strA + sul2	1	1.33
4	*blaampC + blaTEM + tetA + sul2*	3	4.00
	blaampC + tetA + strA + sul2	2	2.67
	blaTEM + tetA + strA + sul2	1	1.33
5	*blaampC + blaTEM + tetA + strA + sul2*	1	1.33
Total		69	92.00

Moreover, of those *Salmonella* isolates, 92.00% harbored one to five antibiotic-resistance genes (Table 3). The *blaampC + strA* pattern was frequently obtained (12.00%), and one *Salmonella* isolates (1.33%) harbored five antibiotic-resistance genes: *blaampC + blaTEM + tetA + strA + sul2*. This showed that *Salmonella* isolated in those small-scale farms in Vinh Long province could have been diverse antibiotic resistance and caused essential challenges in selecting and combining antibiotics for treating diseases in poultry. It might be due to the indiscriminate use of prescribed antibiotics, horizontal transfer, and clonal spread of resistance genes (Ngoi & Thong, 2013; Fardsanei et al., 2016). Furthermore, 23/75 (20.67%) *Salmonella* isolates showed multidrug resistance to three or four antimicrobial categories in this study, including β-lactam, tetracycline, aminoglycoside, sulfonamide, etc. The multiresistance of *Salmonella* isolates could cause difficulties in selecting antibiotics for treating chickens' diseases in these farms and a potential risk to public health. The findings of Hai et al. (2020) in China in 80% of the *Salmonella* isolated from chicken farms in Nanjing, China, isolates were resistant to three or more antibiotics, suggesting that the percentage of *Salmonella* strains resistant to antimicrobials had also increased over time. These results indicate that antibiotic-resistant *Salmonella* isolates in chicken farms in Vinh Long should be managed and controlled strictly to protect chickens and public health.

3.2. The presence of metal-resistance genes in *Salmonella* isolates

According to previous studies, this study defined four common metal-resistance genes cooperating in antibiotic resistance (Allen et al., 2010; Yang et al., 2020; Mazhar et al., 2021). The results in Table 4 and Figure 2 exhibited that gene *pcoR* encoding for copper resistance was at the highest rate (53.33%), followed by *silE* (32.00%), *czcD* (18.67%), and *cnrA* (5.33%). Most genes were detected in *Salmonella* isolates from different samples; however, gene *czcD* was not found in *Salmonella* originating from husbandry environments in this study. It is recognized that heavy metals are resistant to degradation, thereby posing a persistent selection pressure that may play a significant role in the development and sustenance of heavy metal-resistant genotypes (Baker-Austin et al., 2006). In addition, Hobman & Crossman (2015) stated that copper is also widely used as a feed additive to promote growth and to treat diarrhea, and the prevalence of *pcoR* encoding for copper resistance could decrease the preventive methods in treatment for animals. Deng et al. (2018) researched *Salmonella* isolated from retail meat (pork, chicken) and stated that the prevalence of metal-resistance genes in the *Salmonella* strains might create conditions that favor the co-selection of strains exhibiting acquired resistance to other antimicrobial agents when the application of disinfectants for decontamination or the use of metals in livestock. The research of Yang et al. (2020) also reported the prevalence of heavy metal resistance genes in *Salmonella* isolates from broiler farms and retail meat harbored several genes, such as *zntA* and *zntB* confer resistance to zinc (Zn), *pcoR, pcoC, and pcoA* confer resistance to copper (Cu), *arsB* confers resistance to mercury (Hg), *merA* confers resistance to arsenic (As), and *chrA* confers resistance to chromium (Cr).

Table 4. The prevalence of antibiotic-resistance genes in *Salmonella* isolates in the small-scale farms in Vinh Long province

Genes	Feces (n = 15)		Husbandry environment (n = 10)		Pests (n = 50)		Total* (n = 75)	
	No. of positive isolates	Percentage (%)	No. of positive isolates	Percentage (%)	No. of positive isolates	Percentage (%)	No. of positive isolates	Percentage (%)
pcoR	8	53.33	7	70.00	25	50.00	40	53.33
czcD	2	13.33	0	0.00	12	24.00	14	18.67
cnrA	1	6.67	1	10.00	2	4.00	4	5.33
silE	4	26.67	2	20.00	18	36.00	24	32.00

**There was no significant in the prevalence of antibiotic-resistance genes among those samples (P > 0.05).*

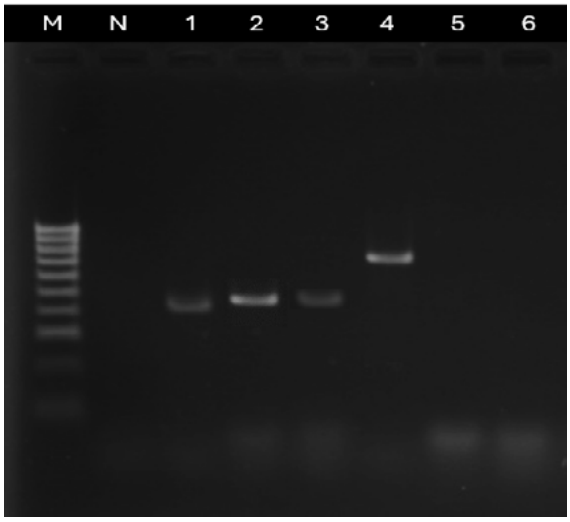

Figure 2. The electrophoresis image of PCR products in detecting metal-resistance genes in *Salmonella* spp. Isolates. M: ladder (100 bp), N: negative control (Distilled water), 1: *pcoR* (636 bp), 2: *czcD* (398 bp), 3: *cnrA* (422 bp), 4: *silE* (432 bp), 5-6: negative samples.

Table 5 presents the results of the patterns of metal-resistance and antibiotic-resistance genes. It shows that several patterns were obtained, and those genes were accompanied. In this study, Spearman's correlation coefficient analysis revealed that *pcoR*, *czcD*, and *silE* were related to all antibiotic-resistance genes ($P < 0.01$). In contrast, gene *cnrA* did not show a relation. It demonstrated that metal-resistance genes could enhance antibiotic resistance or resist disinfectant products in these *Salmonella* isolates isolated from chickens and environments in small-scale farms. Other research has shown that sub-inhibitory levels of heavy metals due to metal-resistance genes can facilitate the horizontal transfer of plasmid-mediated antibiotic resistance among bacterial populations (Zhang et al., 2018; Lu et al., 2020). In China, antibiotic resistance was highly associated with specific heavy metal resistance genes, such as the association among Cu-resistance genes (*pcoC, pcoR*) and tetracycline and sulfonamide resistance genes (*tet, sul*) (Deng et al., 2018). Ji et al. (2012) also reported that various environmental mechanisms have facilitated the co-selection of metal-resistance genes alongside antibiotic-resistance genes.

Besides, Mustafa et al. (2021) stated that the introduction of the Cr-Zn-Cd-resistance gene *czcD*, the Cu-resistance gene *pcoC*, and the Co-Ni-resistance gene *cnrA* into *E. coli* and the enhanced Cu-resistance observed in the transconjugants suggest that these resistance genes are situated on conjugative plasmids. Consequently, the overuse of metals and disinfectants as feed additives and in animal husbandry could potentially encourage the development of antibiotic resistance through co-selection, thereby sustaining and even enhancing antibiotic resistance in environments devoid of antibiotics. Thus, *Salmonella* isolated in the small-scale farms in Vinh Long province showed a severe risk of increased antibiotic resistance due to relationships with antibiotic-resistance genes.

Table 5. The patterns of metal-resistance and antibiotic-resistance genes of *Salmonella* isolated in the small-scale farms in Vinh Long province (n = 75)

Patterns	No. of positive isolates	Percentage (%)
blaampC + czcD	1	1.33
blaampC + pcoR	3	4.00
blaampC + strA	3	4.00
strA + czcD	2	2.67
strA + pcoR	2	2.67
tetA + pcoR	6	8.00
blaampC + pcoR + silE	2	2.67
blaampC + strA + czcD	2	2.67
blaampC + strA + pcoR	3	4.00
blaTEM + strA + silE	1	1.33
strA + pcoR + czcD	1	1.33
tetA + mcr1 + pcoR	1	1.33
tetA + pcoR + czcD	1	1.33
tetA + strA + pcoR	1	1.33
blaampC + strA + pcoR + silE	1	1.33
blaampC + strA + sul2 + silE	1	1.33
blaampC + tetA + strA + czcD	1	1.33
blaampC + tetA + strA + silE	1	1.33
blaampC + tetA + sul2 + silE	2	2.67
blaTEM + tetA + sul2 + pcoR	1	1.33
tetA + sul2 + pcoR + silE	1	1.33
blaampC + tetA + strA + sul2 + silE	1	1.33
blaampC + tetA + sul2 + pcoR + silE	2	2.67
tetA + strA + sul2 + pcoR + silE	1	1.33
blaampC + blaTEM + tetA + strA + sul2 + silE	1	1.33
blaampC + blaTEM + tetA + sul2 + pcoR + silE	2	2.67
blaampC + tetA + strA + pcoR + czcD + silE	1	1.33
blaampC + tetA + strA + sul2 + pcoR + silE	1	1.33
blaTEM + tetA + sul2 + pcoR + cnrA + silE	2	2.67
blaTEM + tetA + sul2 + pcoR + czcD + silE	1	1.33
blaTEM + strA + sul2 + pcoR + czcD + cnrA + silE	1	1.33
blaTEM + tetA + strA + sul2 + pcoR + cnrA + silE	1	1.33
Total	51	68.00

4. Conclusions

Salmonella isolated from broiler chickens, husbandry environments, and pests in small-scale farms in Vinh Long province harbored several antibiotic-resistance and metal-resistance genes, especially genes *blaampC* and *pcoR*. Moreover, the genes accompanied in those *Salmonella* strains exhibited a potential increase in antibiotic resistance, especially the presence of *pcoR*, *czcD*, and *silE* genes along with antibiotic-resistance genes. Thus, managing the prevalence of antibiotic-resistant *Salmonella* in poultry farms is an essential issue to protect poultry health and ensure the effectiveness of treatment.

Conflict of interest

The authors have no conflicts of interest to declare.

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Effects of dietary supplementation with probiotics on growth performance, gut health and disease resistance of striped catfish (*Pangasianodon hypophthalmus*)

Can T. Vo, Binh T.T. Vo, Dang H. Mai, Binh A. Bach, Hung T. Le, & Tuan V. Vo*
Faculty of Fisheries, Nong Lam University, Ho Chi Minh City, Vietnam

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***Corresponding author**

Vo Van Tuan
Email:
vovantuan@hcmuaf.edu.vn

ABSTRACT

This study was conducted to investigate the effects of the probiotic (*Bacillus* Liq-Vtech)-supplemented diets on growth performance, intestinal microbe, morphology of the intestine, and resistance to *Edwarsiella ictaluri* infection in striped catfish (*Pangasianodon hypophthalmus*). A total of 800 healthy striped catfish (12 ± 2 g) were randomly divided into four experimental groups with four replicates each, and were fed diets supplemented with different levels of *Bacillus* Liq-Vtech (T_1: 0 cfu/g; T_2: 0.5×10^6 cfu/g; T_3: 1.0×10^6 cfu/g; and T_4: 1.5×10^6 cfu/g) for 8 weeks. At the end of the trial, a challenge test with *Edwardsiella ictaluri* was conducted for 2 weeks. The results showed no statistically significant improvement in the growth performance and survival rate of fish with *Bacillus* Liq-Vtech supplementation ($P > 0.05$). However, there was a tendency for the improved growth performance in treatments supplemented with *Bacillus* Liq-Vtech. Additionally, the density of *Bacillus* spp. increased in the intestinal tract of fish fed probiotic-supplemented diets compared with the control. All probiotic treatments exhibited positive effects for different histomorphological features of the intestine. Mucus secreting goblet cells and villi increased in probiotic-supplemented groups. Mortality rates in treatments supplemented with *Bacillus* Liq-Vtech were lower than those of the control. These results suggested that the *Bacillus* Liq-Vtech supplementation in diets has the potential to improve growth performance, increase beneficial bacteria in the intestinal tract of the fish, and reduce the mortality of striped catfish after being challenged with *Edwardsiella ictaluri.*

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1. Introduction

Striped catfish, also known as tra catfish (*Pangasianodon hypophthalmus,* Sauvage, 1878) is a freshwater species and considered as an important commercial aquaculture fish in Vietnam. According to MARD (2024), the production of striped catfish reached 1.61 million tons and an export income of approximately 1.8 billion dollars in 2023. Striped catfish has been intensively cultured in ponds with high stocking density so that the disease outbreak is a main risk for the fish farming. Bacterial infectious diseases are the main agents that caused huge economic loss for striped catfish industry in Vietnam (Ferguson et al., 2001; Tu et al., 2008; Dang & Truong, 2022). Several approaches such as chemotherapeutics and antibiotics have been applied to control the infectious diseases in striped catfish culture, but the application of these antibiotics and chemotherapeutics is strictly regulated due to their negative impacts (Lim et al., 2013; Ramos et al., 2018).

In recent years, there is an increasing interest in the use of functional feeds that contain natural supplements such as prebiotics, probiotics, synbiotics, herbal extracts to improve fish growth and health, in which probiotics are considered as a promising solution in preventing and managing aquatic pathogenic agents (Ringø & Song, 2016; Bui et al., 2022; Liaqat et al., 2024). Application of probiotics in aqua-farming is evident in enhancing growth performance through ensuring better digestion with the proliferation of useful bacterial colony in intestine of fish (Ringø, 2020). A dietary with probiotics supplementation has been studied in many aquatic species, such as striped catfish, channel catfish, tilapia (Nguyen et al., 2022; Liaqat et al., 2024; Youssef et al., 2024).

The probiotics *Bacillus* Liq-Vtech (provided by UV company, Vietnam) is composed of *Bacillus subtilis* and *Bacillus licheniformis* with a density of 10^9 cfu/mL. A preliminary experiment has been proved that it plays an important role in inhibiting intestinal harmful bacteria and stimulating immune responses, leading to prevent diseases and enhance aquatic animal survival rate. Therefore, the objective of the present study was to evaluate effects of the probiotics *Bacillus* Liq-Vtech-supplemented diets on growth performance, intestinal microbe and morphology of the intestine, and resistance of striped catfish (*Pangasianodon hypophthalmus*).

2. Materials and Methods

2.1. Experimental diets

Four experimental diets including a basal diet were formulated to contain different levels of probiotics *Bacillus* Liq-Vtech (T_1: 0 cfu/g; T_2: 0.5 $\times$ 10^6 cfu/g; T_3: 1.0 $\times$ 10^6 cfu/g; and T_4: 1.5 $\times$ 10^6 cfu/g). The basal diet contained approximately 28% crude protein and 5% lipid, formulated from different ingredients. All feed ingredients were thoroughly mixed, extruded and pelleted to produce a floating feed at Godaco feed mill. The feed processing involved several stages as grinding, mixing, conditioning, extrusion and drying in which the extrusion lasted 5 - 10 min at 120°C and followed by drying at high temperature of 120 - 150°C. The ingredients and proximate analyses of the experimental diets was illustrated in Table 1.

The analyses of proximate composition of feed ingredients were determined by standard methods (AOAC, 1995). Crude protein content was determined by the Kjeldahl method. Crude lipid content was extracted by n-hexane using the Soxhlet method. Ash content was determined by combustion method. Moisture content was determined by the drying method using an oven at 105°C.

Table 1. Feed formulation of the basal diet and its estimated nutrient analysis

Ingredients	Percentage (%)
Rice bran	33.20
Fishmeal 60%	4.00
Soybean meal	47.09
Cassava	14.00
L-Lysine	0.03
DCP	0.63
DL Methionine	0.36
Choline Chloride	0.15
Stay Vitamin C	0.04
Premix	0.50
Nutrient level	% Dry matter
Crude protein (%)	28.00
Crude fat (%)	5.60
Fiber (%)	4.02
Ash (%)	8.90
Lysine (%)	1.55
Methionine (%)	0.70
Gross energy	4200 Kcal/kg

From the basal diet, three probiotics *Bacillus* Liq-Vtech treatment diets were produced in the laboratory by coating with probiotics at different dosages. Then all four diets were finally coated with 0.5% soy oil to prevent the probiotics leaching. All prepared feed were stored at 20°C and labelled as T_1, T_2, T_3 and T_4.

2.2. Experimental animals

Striped catfish fingerlings were purchased from private fish farm at An Giang province and transported to the Experimental Farm of Nong Lam University, Ho Chi Minh City, Vietnam. The fish were acclimated and cultured for 2 weeks and fed on a basal diet twice daily to apparent satiation.

Fish with initial weight of 12 ± 2 g were selected for the trial. The trial was carried out in 16 hapas that contained 50 fish per hapa (1.0 m × 2.0 m × 1.5 m). Fish were fed two times a day (8 am and 16 pm) to apparent satiation for a period of 8 weeks. One hour after feeding, the unconsumed feed was collected and dried to calculate the daily consumed feed for each hapa. Feed intake was recorded daily to compare the feeding intake (consumptions) of four diets at the end of the experiment.

During the feeding trial, water quality was monitored in order to evaluate the water quality. Water temperature, dissolved oxygen (DO) and pH were daily monitored using a multi-parameters photome (Hanna, Italy). Total ammonium nitrogen was weekly checked by TAN meter (Hanna, Italy).

At the end of the trial, consumed feed and survival rate of fish were measured to determine the effect of *Bacillus* Liq-Vtech supplementation on fish growth and feed utilization. Besides, intestinal microbial and histology of striped catfish intestine were also measured. Parameters were analyzed as follows:

- SR (Survival rates): %

SR (%) = (Number of survival fish/Number of initial fish) × 100

- DWG (Daily weight gain): g/fish per day

DWG = (Final weigh - Initial weight)/ Cultured days

- WG (Weight gain): %

WG = (Final weight - Initial weight)/Initial weight

- Specific growth rate (SGR).

SGR (%.day^{1}) = [(Ln(final weight) - Ln(Initial weight))/Cultured days)] × 100

- Feed conversion ratio (FCR)

FCR = Consumed feed/Weight gain

- Feed intake (FI)

- FI (g/fish per day) = (Consumed feed in tank/Number fish of tank)/Cultured days

2.3. Bacterial challenge

To evaluate the effect of *Bacillus* Liq-Vtech supplements on fish health status, fish were subjected to an immersion challenge with pathogenic bacteria *E. ictaluri*. At the end of the feeding period, 20 fish in each hapa were randomly selected and transferred to 100 L plastic tanks. Fish were subjected to a two-hour immersion challenge with *E. ictaluri* at a dosage of 2.6×10^6 cfu/mL. The concentration of bacteria in the suspension was determined through spectrophotometry at an absorbance of 600 nm and serial plate count method. Fish were housed in plastic tanks supplied with aeration and mortality of fish was monitored. At the end of the trial, survival rate and intestinal microbial (total bacteria counts and *Bacillus* sp. counts) were recorded.

2.4. Analysis of bacteria in fish intestine

At the end of the trial, two fish per hapa were randomly sampled and anaesthetized with tricaine methanesulfonate (MS - 222) with a dosage of 80 mg/L. Approximately 6 - 8 cm of fish hindgut was aseptically excised, then the inside of the intestine was gently removed and washed two times with sterile distilled saline water. The intestine samples were then homogenized, suspended in 9 mL of sterile distilled saline (0.9%), and serially diluted of suspension to 10^{-5} in 9 mL of sterile distilled saline. 0.1 mL aliquot from each dilution was spread onto duplicate PCA (plate count agar, Himedia) and HiCrome *Bacillus* Agar Base (Himedia). Plates were incubated at 28°C for 24 h. Plates containing between 30 and 300 colony-forming units (cfu) were used to enumerate the number of total cultivable bacteria and *Bacillus* sp. The results were expressed as cfu/g sample.

2.5. Histological analysis

To analyze the histo-morphological structure of the stripped catfish intestine, fragments of the middle portion of the intestine were removed with sterilized surgical instruments and fixed in 10% buffered formalin solution for 48 h, then

dehydrated in graded ethanol concentrations, cleared in xylene, and embedded in paraffin wax. Sections of 5 μM thickness were stained with Alcian blue (AB) and periodic-acid stain (PAS), and analyzed under light microscope.

To determine the height of the intestinal epithelium, five random villi per histological section were measured according to the structural characteristics. The number of goblet cells was counted and expressed in number per μM^2 of villi. The images were obtained with digital camera attached to a photomicroscope and analyzed by the program Image J.

2.6. Statistical analysis

Results were presented as means ± standard deviation (SD). All data were firstly examined for homogeneity of variance using SPSS statistic 20.0 software (IBM, New York, USA). One-way ANOVA was used to test the main effect of different diets to fish growth. The Duncan test was used to determine the significant differences among treatment groups. The probability values of $P < 0.05$ were applied to confirm the statistical difference.

3. Results

3.1. Water quality

Water quality which directly affect to reproduction, growth, and survival of aquatic organisms are considered as the most important factors in aquaculture. Aquatic organisms are susceptible to suffer stress when ecological conditions are not adequate. High stress levels generate low feeding and low growing rates as well, resulting in the appearing of sickness in the organisms (Chainark & Boyd, 2010). Striped catfish are also aquatic species but can use their swim bladder to breath air, so the demand of dissolved oxygen is not strictly required high like other fish.

During the trial, water quality parameters such as temperature, pH, DO, total ammonia nitrogen (TAN) were sampled and the results were illustrated in Table 4.

Table 4. Water quality parameters of the trial

Water quality parameters						
Temperature (°C)		pH		Dissolved oxygen (mg/L)		TAN (mg/L)
Morning	*Afternoon*	*Morning*	*Afternoon*	*Morning*	*Afternoon*	
29.7 ± 0.7	31.5 ± 0.9	7.1 ± 0.3	8.3 ± 1.1	2.6 ± 0.5	4.0 ± 1.6	0.10 ± 0.21

Results are mean of 8 weeks; TAN: total ammonia nitrogen.

The results showed that the water temperature in the morning was lower, but not significant difference in compared to the one in the afternoon ($P > 0.05$). pH and dissolved oxygen (DO) are the most important factors affecting the growth and the health status of aquatic animals. They are also important limiting factors for cultivation activities of all aquatic organisms to survive and grow (Dandruff & Dean, 1967; Boyd, 2001). During the culture season, pH should be

controlled in the suitable range for each aquatic animal. If the pH is too low or too high, it will cause negative effects to cultured species such as slow growth, stunting, and reduce ability to resist pathogens from water environment. The results of the current study showed that the average pH value was varied from 7.1 to 8.3, and was within the appropriate limits for freshwater fish growth and development (Boyd, 2001). The dissolved oxygen (DO) in the morning was 2.6 ± 0.5 mg/L and 4.0 ± 1.6 mg/L in the afternoon. The mean TAN was 0.10 ± 0.2 mg/L. In general, the values of water parameters during the trial time exhibited slight fluctuations, but still remained within the appropriate range of fish.

3.2. Growth and feed utilization

Growth performance of striped catfish expressed as final body weight (FBW), specific growth rate (SGR) and weight gain (WG) were presented in Table 5.

Table 5. Effect of *Bacillus* Liq-Vtech supplement on the growth performance of striped catfish

Treatment	IBW (g/fish)	FBW (g/fish)	DWG (g/fish per day)	WG (%)	SGR (%/day)	Survival (%)
T_1	13.44 ± 0.03	164.56 ± 14.93	2.52 ± 0.24	11.25 ± 1.10	4.17 ± 0.14	99.50 ± 1.00
T_2	13.42 ± 0.02	164.78 ± 13.22	2.52 ± 0.22	11.28 ± 0.97	4.18 ± 0.13	97.50 ± 3.76
T_3	13.39 ± 0.04	170.93 ± 9.85	2.63 ± 0.16	11.76 ± 0.72	4.24 ± 0.09	97.00 ± 2.58
T_4	13.38 ± 0.03	181.39 ± 7.71	2.80 ± 0.12	12.55 ± 0.55	4.34 ± 0.07	99.50 ± 1.00
P	0.093	0.297	0.295	0.263	0.260	0.280

Results are mean ± standard deviation (n = 4). IBW: initial body weight. FBW: final body weight. DWG: daily weight gain. WG: weight gain. SGR: specific growth rates.

The results show that the survival rates of striped catfish were quite high across all four treatments (ranging from 97% to 99.5%), but no significant difference was recorded among treatments. It means that the supplementation of *Bacillus* Liq-Vtech did not affect the survival rates of fish in normal living conditions.

The growth performance of striped catfish tended to increase from T_1 to T_4 treatments. After 8 weeks of the feeding trial, the best values in terms of final body weight, daily weight gain, weight gain percent and specific growth rate were observed in the treatment T_4 supplement compared with the control treatment. No significant difference was recorded between the fish fed probiotics supplemented diets and the control diet. However, there was a clear tendency for improved the growth performance with supplemented *Bacillus* Liq-Vtech in its diet (Figure 1).

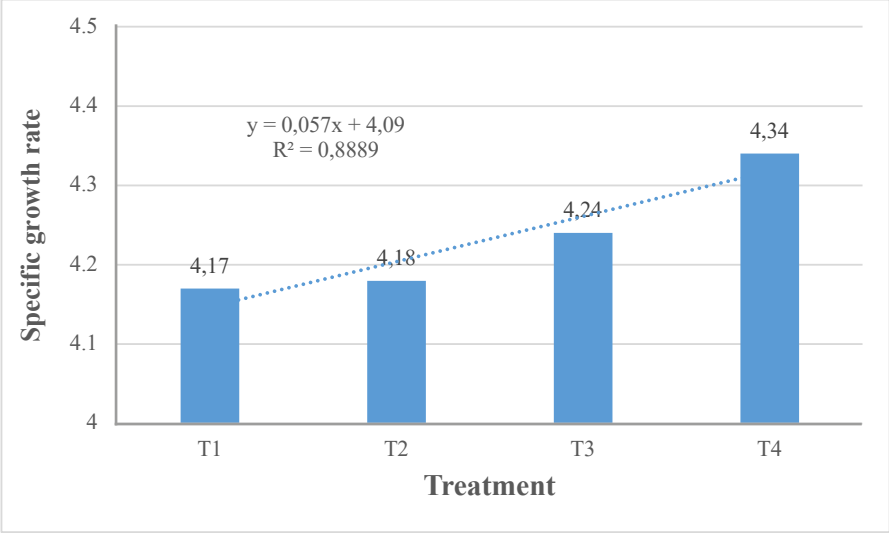

Figure 1. Specific growth rates of fish fed four experimental diets with trend line.

The feed utilization of the fish is expressed as feeding intake (FI) and feed conversion ratio (FCR). Results are presented in Table 6.

Table 6. Feeding intake and Feed conversion ratio of fish fed four experimental diets

Treatment	FI (g/fish per day)	FCR
T_1	$3.33^{a} \pm 0.23$	1.26 ± 0.01
T_2	$3.31^{ab} \pm 0.08$	1.26 ± 0.01
T_3	$3.33^{b} \pm 0.14$	1.27 ± 0.07
T_4	$3.03^{b} \pm 0.16$	1.18 ± 0.05
P	0.06	0.263

Results are mean ± standard deviation (n = 4). FI: feed intake. FCR: feed conversion ratio. Values in the same columns having the same superscript letter are not significantly different (P > 0.05) by Duncan test.

The results demonstrated that the feed intake of fish was lowest in *Bacillus* Liq-Vtech supplemented diets and significantly different from the control diet ($P < 0.05$). It indicated that *Bacillus* Liq-Vtech supplementation significantly improved the feeding intake of striped catfish ($P < 0.05$). The FCR was not significantly different among treatments; however, the T_4 treatment gave the lowest value (1.18) compared to the control diet (1.26). Considering the feed intake and FCR, it seems that the T_4 diet gave the highest efficiency in feed use. Combined growth performances and feed use efficiency (Tables 5 and 6), we can conclude that the application of probiotics *Bacillus* Liq-Vtech in the striped catfish might induce the growth rates and feeding intake.

3.3. Effects of *Bacillus* Liq-Vtech supplement on intestine morphology of striped catfish

At the end of feeding trial, intestine of fish (hind gut) was collected to determine the intestinal microbial (total bacteria and *Bacillus* sp. counts), and the structure of intestinal histo-morphology. The results are demonstrated in Table 7 and Figure 6.

Table 7. Effect of *Bacillus* Liq-Vtech supplement on intestinal bacteria and histology of the striped catfish

Parameters	Experimental diets			
	T_1	T_2	T_3	T_4
TBC ($\times 10^7$ cfu/g)	2.77 ± 1.67[a]	2.59 ± 2.03[a]	2.00 ± 1.52[a]	2.23 ± 1.20[a]
Bacillus sp. ($\times 10^7$ cfu/g)	1.27 ± 0.81[a]	2.54 ± 1.54[a]	1.73 ± 2.37[a]	2.00 ± 1.31[a]
GCs ($\times 10^{-4}$ cells/ μM^2)	5.49 ± 2.51[a]	7.83 ± 4.18[a]	6.29 ± 2.44[a]	8.35 ± 4.72[a]
Villi height (µM)	361.3 ± 96.7[a]	326.5 ± 38.6[a]	335.3 ± 54.6[a]	368.4 ± 49.8[a]
Villi width (µM)	67.8 ± 6.4[a]	75.6 ± 2.3[a]	73.8 ± 6.7[a]	74.4 ± 9.3[a]

Results are mean ± standard deviation (n = 4). TBC: total bacterial counts. GCs: goblet cells. The row with the same superscripts is not significant differences (P > 0.05).

The results showed that the total bacteria count had a tendency to decrease from the control diet to the probiotics *Bacillus* Liq-Vtech supplemented diets, while the total number of *Bacillus* sp. increased. The number of goblet cells, villi height and villi width in fish intestine showed a slightly better result in the probiotics *Bacillus* Liq-Vtech supplemented diets, but was not significantly different when compared with the control diet (Figure 2).

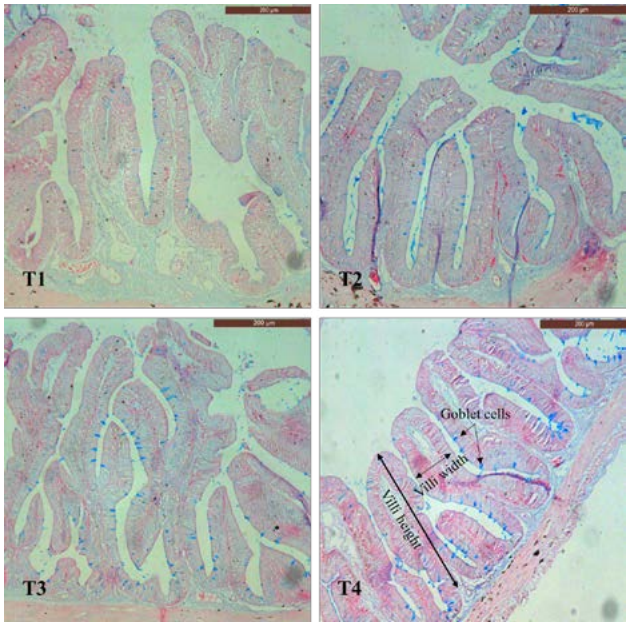

Figure 2. Histology of the striped catfish mid-intestine fed probiotics *Bacillus* Liq-Vtech supplemented diets.

3.4. Cumulative mortality of striped catfish after bacterial challenge

At the end of the feeding trial, the striped catfish were subjected to the immersion challenge with *Edwardsiella ictaluri*, and the results are illustrated in Figure 3.

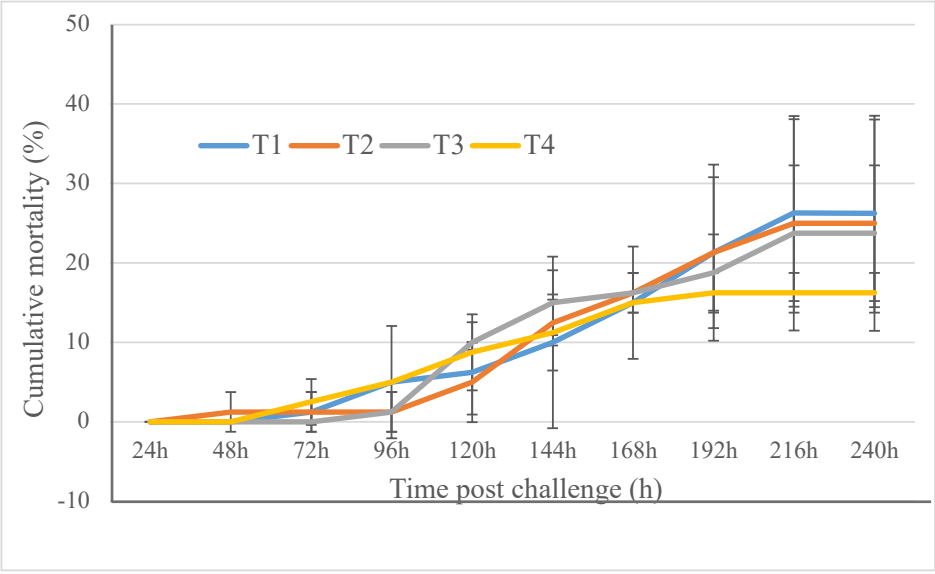

Figure 3. Cumulative mortality of the striped catfish after bacterial challenge.

The death of fish in the *E. ictaluri* challenge was initially recorded on the third day post infection (72 h post infection) in all groups. The cumulative mortality of fish in the probiotics *Bacillus* Liq-Vtech supplemented groups, especially in T_4 (1.5×10^6 cfu/g of feed) was reduced after 192 h post challenge, while it occurred continuously until 216 h post challenge in the control group. Although no significant difference in survival rate of the striped catfish challenged with pathogenic bacteria *E. ictaluri* was recorded, fish in the probiotics *Bacillus* Liq-Vtech treatments showed higher survival rate.

To evaluate the effect of *Bacillus* Liq-Vtech supplement on gut health status of the striped catfish, fish intestine was sampled at the end of bacterial challenge. Total bacterial counts and *Bacillus* sp. were determined, and the result was illustrated in Table 8.

Table 8. Effect of *Bacillus* Liq-Vtech supplement on intestinal microbial of fish

Parameters	Experimental diets			
	T_1	T_2	T_3	T_4
TBC ($\times 10^7$ cfu/g)	5.69 ± 5.41^a	4.46 ± 2.46^a	4.70 ± 3.74^a	2.73 ± 0.31^a
Bacillus sp. ($\times 10^7$ cfu/g)	3.84 ± 1.86^a	3.05 ± 1.62^a	2.75 ± 1.28^a	2.58 ± 0.91^a

Results are mean ± standard deviation (n = 4). TBC: total bacterial counts. The row with the same superscripts is not significant differences (P > 0.05).

Bacillus Liq-Vtech is a probiotic product that contains high levels of beneficial populations of bacteria. It plays an important role in supporting gut health status of fish. The current study showed that lower counts of bacteria (total bacterial counts and total *Bacillus* sp. counts) were observed in the probiotics *Bacillus* Liq-Vtech supplemented diets in comparison to the control diet. However, no significant differences of the total bacteria populations between diets were reported.

4. Discussion

The current study was carried out to evaluate the effect of the probiotics *Bacillus* Liq-Vtech supplemented diets on the growth performance, intestinal microbe, morphology of the intestine, and resistance to infection with *E. ictaluri* of the striped catfish (*Pangasianodon hypophthalmus*). The results of the current study showed that the growth performance and feed utilization of fish were not significantly different between the control diet and the probiotics *Bacillus* Liq-Vtech supplemented diets. However, fish fed diet with a dose of *Bacillus* Liq-Vtech of 1.5×10^6 cfu (T_4) had a better results in terms of growth performance, feed utilization, and survival rate compared to the control diet. After feeding in 8 weeks, there was a tendency to have an increased the growth performance of striped catfish. Dietary with 1.5×10^6 cfu/g (T_4) *Bacillus* Liq-Vtech supplement had a lower FCR than other diets (1.18 versus 1.26), but no difference was recorded between diets. The result seems not to meet the expectation of probiotics used in the striped catfish feed. A possible explanation is that the dosage of probiotics *Bacillus* Liq-Vtech was not sufficient to significantly enhance the growth performance of striped catfish. Our results align with the results of Yazici et al. (2016) in rainbow trout, Niamphithak et al. (2017) in bocourti catfish, and Nguyen et al. (2022) in channel catfish. Besides, results of the current study are in line with the results of Shelby et al. (2006) and Sirbu et al. (2022) in tilapia. These authors also concluded that supplementation of probiotics in fish diets did not produce significant effect on growth parameters; however, fish fed with probiotics diets showed potential for improved the growth performance and survival rate.

Total bacterial flora in fish intestine is considered as a key indicator to evaluate fish health status. It was found in our study that higher count of bacteria was observed in the control diet in comparison to the probiotics *Bacillus* Liq-Vtech supplemented diets; however, no significant differences of the total bacterial counts among diets was reported. Besides, the *Bacillus* sp. levels in the treatment diets were higher and showed a tendency to be increased in diets supplemented with probiotics *Bacillus* Liq-Vtech. It means that *Bacillus* sp. in the probiotics *Bacillus* Liq-Vtech has a potential to colonize and survival in the intestine of striped catfish. Our results are in line with the results of Chang & Liu (2002). These authors demonstrated that higher number of probiotic bacterial survival in the intestine of European eel (*Anguilla anguilla*).

Goblet cells or intestinal mucus-secreting goblet cells, are very important for the nutrition of the fish and its health. They have both absorptive and secretory functions in fish (Pereira et al., 2020). In the current study, the goblet cells and the size of villi have a tendency to be increased in diets supplemented with probiotics *Bacillus* Liq-Vtech. Linked to the modulate of the microbial gut communities, it might responsible for the increased goblet cells numbers. Increased goblet cells had also reported in Artic charr (*Salvelinus alpinus* L.) and tilapia (*Oreochromis niloticus*) (Lødemel et al., 2001; Liu et al., 2016).

Probiotics can be applied in aqua-farming for several purposes such as promoting growth performance, enhancing feed digestion, maintaining normal intestinal microbial communities. Besides, it has ability to control

the infectious diseases. The challenge test in this study showed that supplementation of the probiotics *Bacillus* Liq-Vtech in feed of striped catfish tended to reduced mortality caused by *E. ictaluri*. There are two possible direct mechanisms that may be involved in the process. First is the competitive ability of *Bacillus* sp. against other harmful bacteria in the intestine of fish. The other could be the ability to produce antimicrobial substances that inhibited and suppressed bacterial infection.

5. Conclusions

Supplementation of the probiotics *Bacillus* Liq-Vtech in striped catfish diets shows a potential to increase the growth performance and feed utilization. Dietary with 1.5×10^6 cfu/g feed (T_4) *Bacillus* Liq-Vtech supplement resulted a lower FCR than other diets.

Probiotics used in diets is expected to improve fish health status, and the results of the current study have showed that a tendency to increase the *Bacillus* sp. counts, goblet cells, villi height and villi width of fish intestine.

In the bacterial challenge test, higher survival rate of striped catfish was observed in the *Bacillus* Liq-Vtech supplemented diets, especially in the treatment T_4 (1.5×10^6 cfu/g feed). These findings suggest that the *Bacillus* Liq-Vtech has a positive effect on improving fish health.

Conflict of interest

The authors (corresponding and co-authors) declare no personal conflicts of interest in the present research.

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Efficacy of 17β-estradiol on survival rate, sex reversal, and growth performance of climbing perch (*Anabas testudineus*) using the immersion method

Tuan Q. Nguyen, Tu C. P. Nguyen, Loi N. Nguyen, Minh V. Tran, Hue N. D. Truyen, & Tam T. Nguyen*
Faculty of Fisheries, Nong Lam University, Ho Chi Minh City, Vietnam

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***Corresponding author**

Nguyen Thanh Tam
Email:
nthanhtam@hcmuaf.edu.vn

ABSTRACT

This study aimed to evaluate the efficacy of estradiol on the survival rate, sex reversal ratio, and growth performance of climbing perch (*Anabas testudineus*) using the immersion method. A completely randomized design was applied, involving three estradiol (E2) treatment groups at concentrations of 1.0 mg/L (1E2), 1.5 mg/L (1.5E2), and 2 mg/L (2E2), along with a control group triplications. Seven-day-old fingerlings were exposed to the E2 solution for 2 h before being transferred to nurseries in hapas placed in earthen ponds at a density of 200 fish/m² for 60 days. After the hormone treatment and 60 days of rearing, the highest survival rate was observed in the control group (86.7%). The female ratios in the 17β-estradiol (E2) treatments ranged from 72.0% to 90.0%, which were significantly higher than the ratio of the control group (55.6%) ($P < 0.05$). The 2E2 treatment demonstrated the highest female percentage, which was statistically greater than that observed in the 1E2 and 1.5E2 treatments ($P < 0.05$). The mean weight and length of fish in the E2 treatments were greater than those in the control treatment, although the differences were not statistically significant ($P > 0.05$). Additionally, the study revealed a direct proportionality between the average weight of experimental fish and the hormone concentration. Based on these findings, the recommended dose for achieving maximum mono-sex female climbing perch is 2 mg/L of 17β-estradiol.

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1. Introduction

The study of sex reversal in fish has gained significant attention due to its implications for aquaculture and conservation. Understanding and manipulating sex differentiation can have profound effects on population management and productivity (Pandian & Sheela, 1995). Sex reversal, where an individual fish changes its sex from male to female or vice versa, is a naturally occurring phenomenon in several fish species (Devlin & Nagahama, 2002). This process can be influenced by environmental factors, social cues, and hormonal interventions (Yamamoto, 1969). Globally, species such as groupers, wrasses, and clownfish are well-known for their sex-changing capabilities (Shapiro, 1987). In Vietnam, sex reversal is particularly relevant for species like the Asian sea bass (*Lates calcarifer*) and tilapia species (*Oreochromis* spp.), which are vital to the local aquaculture industry (FAO, 2010). The ability to control sex differentiation through hormonal treatments has opened new avenues for optimizing fish production and maintaining ecological balance (Hunter & Donaldson, 1983).

Climbing perch (*Anabas testudineus*) is an important food fish that is considered to be of high quality, contributing to local people's livelihoods and well-being in the Mekong Delta (Anh et al., 2003; Klemick & Lichtenberg, 2008). In growth-out farming, the use of female climbing perch is particularly important due to their superior growth performance compared to males. Female climbing perch typically exhibit faster growth rates, greater size, and improved feed conversion efficiency, making them more economically viable for large-scale production. By focusing on female fish in farming operations, farmers can optimize yield and enhance overall productivity, contributing to more sustainable and profitable aquaculture practices.

The manipulation of sex in fish populations is essential for several reasons. In hatchery production, achieving a desired sex ratio can enhance reproductive efficiency and yield (Beardmore et al., 2001). For instance, in species where one sex grows faster or reaches market size more quickly, producing monosex populations can significantly improve economic returns (Mair et al., 1997). In fish farming, controlling the sex ratio can prevent unwanted breeding, thereby reducing competition for resources and promoting uniform growth (Guerrero & Guerrero, 1988). Additionally, in conservation efforts, sex reversal can help restore endangered populations by ensuring a balanced sex ratio crucial for successful breeding programs (Rubin, 1985).

Various methods have been developed to induce sex reversal in fish, including environmental manipulation, genetic techniques, and hormonal treatments (Piferrer, 2001). Hormonal treatments are the most widely used approach due to their effectiveness and relative ease of application (Guerrero, 1975). Common hormones include androgens like methyltestosterone, which induce masculinization, and estrogens such as estradiol, which promote feminization (Yamamoto, 1969). These hormones can be administered through feed, injections, or immersion baths, depending on the species and desired outcome (Pandian & Sheela, 1995). Each method of sex reversal has its advantages and disadvantages. Environmental manipulation, such as temperature changes, is non-invasive and sustainable but can be less precise and effective (Yamamoto, 1969). Genetic techniques offer long-term solutions and are highly specific but involve complex and costly procedures (Beardmore et al., 2001). Hormonal treatments are cost-effective, easy to implement, and provide rapid results (Pandian & Sheela, 1995).

Estradiol, a primary estrogen hormone, is widely used to induce feminization in fish, playing a pivotal role in sex differentiation (Guerrero,

1975). The use of estradiol in fish sex reversal offers several advantages. It is a potent and reliable method for achieving desired sex ratios, particularly in species where female individuals are preferred for their growth rates, market value, or reproductive characteristics (Piferrer, 2001). The dosage and method of administration vary depending on the species and desired sex ratio (Pandian & Sheela, 1995). Typically, estradiol is administered through feed during the early developmental stages when gonadal differentiation is most responsive (Hunter & Donaldson, 1983). The feeding method for administering hormones in fish sex reversal has several disadvantages: (i) individual fish may consume varying amounts of food, leading to inconsistent hormone dosages and affecting sex conversion results; (ii) the processing time is lengthy, typically around 21 days; (iii) the age of the fish must be precise, usually 1-day old after the yolk is fully consumed; and (iv) it incurs high production costs (Guerrero & Guerrero, 1988). In contrast, the immersion bath method helps overcome these disadvantages: (i) it provides rapid and uniform hormone distribution, ensuring consistent sex reversal results; (ii) it is ideal for early developmental stages and hatchery environments where large numbers of fish can be treated simultaneously; and (iii) it has lower production costs (Rubin, 1985). Therefore, the research was carried out to investigate the effects of estradiol on sex reversal, survival, and growth of climbing perch (*Anabas testudineus*) using the immersion bath method (Guerrero, 1975).

2. Materials and Methods

This experimental study was designed to evaluate the effectiveness of producing female monosex climbing perch by immersion method. The experiment was conducted over a period of six months, from January to June 2024.

2.1. Test animals

The experimental fish used in the sex reversal treatment were obtained from five pairs of climbing perch broodstock (female: 13 ± 1.2 cm and 98 ± 0.6 g; male: 11 ± 1.4 cm and 79 ± 0.4 g) through artificial reproduction using hormone induction at the experimental farm of the Faculty of Fisheries, Nong Lam University, Ho Chi Minh City. Fertilized eggs obtained from these broodstocks were pooled and incubated in three separate 50-liter plastic tanks, and maintained with gentle aeration. Post-hatching, the larvae were kept in the same tanks, with the water maintained at a temperature of 29 - 32°C, dissolved oxygen (DO) level at 2 - 4 mg/L, ammonia (NH_3) levels below 0.25 ppm, nitrite levels under 1.0 ppm, and a pH range of 7.0 - 7.5. Starting at 3 - 4 days post-hatch (DPH), the larvae were fed *Moina* four times daily in adequate quantities. Upon reaching 6 days of age, feeding was ceased for one day to facilitate the experiment.

2.2. Preparation of hormone treated solutions

The 17β-estradiol hormone utilized in this study was purchased from Sigma Aldrich Ltd., Germany. A stock solution was prepared by dissolving 400 mg of the hormone in 1 L of 96% ethanol, yielding a nominal concentration of 0.4 mg/mL. To achieve the treatment concentrations, appropriate amounts of the stock solution were dissolved in a 20 L glass flask containing 10 L of water, followed by gently aeration to facilitate ethanol evaporation.

2.3. Experimental design

The experiment was carried out in 20 L glass tanks using completely randomized design.

The experiment involved three 17β-estradiol (E2) concentration treatments: 1 mg/L (1E2), 1.5 mg/L (1.5E2), and 2 mg/L (2E2), along with a control treatment (C). Each treatment was replicated three times. 7-day-old fry were randomly selected from three nursery tanks and placed into experimental glass tanks at a density of 500 fish/L for 2 h. Post hormone treatment, the fry were transferred to separate nursery hapas (1 m x 1 m x 0.5 m) at a density of 200 fish/ m^2 for a duration of 60 days. During this period, the fish were fed commercial floating pellets with varying crude protein levels: 40% from days 1 to 15, at a feeding rate of 10% of body weight, administered four times daily; 35% from days 16 to 30, at a feeding rate of 7% of body weight, administered three times daily; and 30% from days 31 to 60, at a feeding rate of 5% of body weight, administered twice daily.

2.4. Sampling and data collection

a) Sex reversal

At the end of experiment, 30 fish were randomly collected in each experimental replicate to identify the sex of the fishes (total 90 fish/treatment). Morphology of the gonads were examined and recorded. Sexing determination was done by standard aceto-carmine gonad squashing technique (Guerrero & Shelton, 1974).

b) Growth performance

Every 30 days, 10 fish were randomly collected in each experimental replicate to measure the mean growth (average weight and length), using digital calipers and an electronic balance, respectively, then released the fish back into experimental hapas. Survival rates were recorded at two hours after immersion of fish in E2 solutions and at the end of the growth experiment (day 60).

c) Statistical analysis

The data were statistically analyzed by statistical package SPSS version 16.0 in which data were subjected to one-way ANOVA and Duncan's multiple range test (DMRT) was used to determine the significant differences between the means at 5% level of significance.

3. Results

The water quality parameters maintained during the experiment are summarized in Table 1. The temperature ranged from 29 to 32°C, DO levels varied between 2.0 and 4.0 mg/L, pH levels ranged from 7.0 to 7.5, and NH_3 levels were kept below 0.25 mg/L. These parameters were within the optimal range for the growth and survival of climbing perch (*Anabas testudineus*), ensuring a stable environment throughout the study (Boyd, 1998).

Table 1. Water quality parameters during experiment

Temperature (°C)	Dissolved oxygen (mg/L)	pH	NH_3 (mg/L)
29.0 - 32.0	2.0 - 4.0	7.0 - 7.5	< 0.25

The survival rates and mean percent females for each estradiol-17β treatment group and the control group are shown in Table 2. All groups exhibited a 100% survival rate after 2 h of treatment, indicating that the initial exposure to 17β-Estradiol did not negatively impact the immediate survival of the fish. However, the survival rate at day 60 showed slight variations among these groups. The control group had a survival rate of 86.7%, whereas the treated groups showed lower survival rates: 82% for 1E2, 81.3% for 1.5E2, and 80.7% for 2E2.

Histological analysis of gonadal tissues revealed no intersex individuals across all estradiol treatments and control groups (Table 2; Figure 1). The effectiveness of 17β-Estradiol in sex reversal was evident in the increase in the percentage of females in the treated groups compared to the control. The control group exhibited a normal sex ratio of 1 female to 0.80 male. In contrast, the female ratios in the E2 treatments varied from 72% to 90%, which were significantly higher than the 55.6% observed in the control group ($P < 0.05$). The experimental results demonstrated that the highest feminization rate of 90% occurred in the 2E2 treatment (2 mg/L E2 concentration), a statistically significant difference compared to other E2 treatments ($P > 0.05$). This indicates that a concentration of 2 mg/L E2 is an effective dose for feminizing climbing perch via immersion. Consequently, the 2E2 treatment (2 mg/L E2) is identified as the most suitable concentration for sex reversal in climbing perch fry using the immersion method.

Table 2. Effects of 17β-Estradiol on survival, sex reversal, and growth in climbing perch (*Anabas testudineus*)

Treatment	Number of analyzed fish	Survival rate after 2 h (%)	Survival rate at day 60 (%)	Female (%)	Male (%)	Sex ratio Female:Male
Control	90	100	86.67 ± 1.15	55.56 ± 5.09[a]	44.4	01:00.8
1E2	90	100	82.00 ± 2.00	72.00 ± 4.00[b]	28.0	01:00.4
1.5E2	90	100	81.33 ± 3.06	72.92 ± 3.61[b]	27.1	01:00.4
2E2	90	100	80.67 ± 3.06	90.0 ± 3.33[c]	10.0	01:00.1

Values (mean ± standard deviation of data for triplicate groups) with different superscripts in the same column are significantly different (one-way ANOVA and Tukey test, P < 0.05).

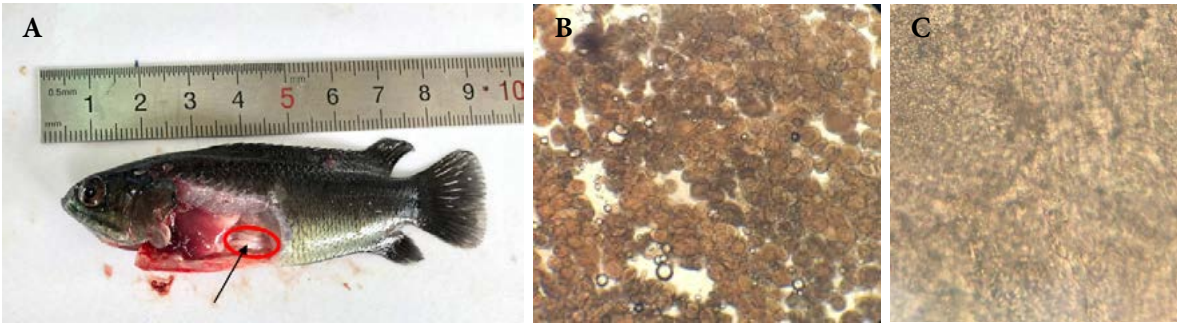

Figure 1. The observed gonad of male and female climbing perch after 60 days of 17β-Estradiol treatment. (A) Gonad removal (B) Eggs, and (C) Testis under light microscope.

The growth performance of the experimental fish over 60 days is detailed in Table 3. The control group showed a final weight of 8.112 ± 0.203 g and a specific growth rate (SGR) of 14.564 ± 0.042 %/day. The group treated with 2 mg/L estradiol-17β had the highest final weight (8.494 ± 0.214 g) and SGR (14.641 ± 0.042 %/day), indicating that higher doses of estradiol-17β may positively influence growth performance (Pandian & Sheela, 1995).

Table 3. Growth performance of experimental fish

Treatment	Initial Weight (g)	Initial Length (cm)	Final Weight (g)	Final Length (cm)	WG (g)	SGR (%/day)	DWG (g/day)
Control	0.001	0.48	8.112 ± 0.203	7.530 ± 0.129	8.111 ± 0.203	14.564 ± 0.042	0.135 ± 0.003
1E2	0.001	0.48	7.027 ± 0.436	7.173 ± 0.319	7.026 ± 0.436	14.323 ± 0.102	0.117 ± 0.007
1.5E2	0.001	0.48	7.203 ± 0.485	7.208 ± 0.239	7.202 ± 0.485	14.364 ± 0.110	0.120 ± 0.008
2E2	0.001	0.48	8.494 ± 0.214	7.643 ± 0.083	8.493 ± 0.214	14.641 ± 0.042	0.142 ± 0.004

Values (mean ± standard deviation of data for triplicate groups). WG: weight gain; SGR: specific growth rate; DWG: daily weight gain.

4. Discussion

The climbing perch (*Anabas testudineus*) is an exceptionally resilient species, capable of thriving in challenging environmental conditions, including low dissolved oxygen levels and poor water quality. This resilience was further evidenced by the high survival rates (> 80%) observed across all treatments in this study's experimental conditions.

The results of this study demonstrate that 17β-Estradiol significantly influences the sex reversal process in climbing perch. Fish treated with the hormone exhibited a higher proportion of females compared to the control group, suggesting that 17β-Estradiol effectively directs gonadal differentiation toward feminization. This finding aligns with previous research on other fish species, where 17β-Estradiol has been shown to promote female sex differentiation when administered during the critical period of sexual development (Tayamen & Shelton, 1978; Pandian & Sheela, 1995). Similarly, studies by Piferrer (2001) have shown the effectiveness of estradiol-17β in inducing feminization across different species, confirming the hormone's broad applicability. The observed sex ratios are consistent with previous studies on the effects of estrogenic compounds in fish. The ability to manipulate sex ratios through hormonal treatments can be beneficial for aquaculture practices, particularly in species where one sex is more desirable for production purposes (Hunter & Donaldson, 1983). Moreover, the immersion method appears to be an effective means of hormone delivery, ensuring consistent and adequate exposure to 17β-Estradiol.

Survival rates varied slightly among the treatment groups, with the highest survival observed in the control group (86.7%) and the lowest in the group treated with 2 mg/L estradiol-17β (80.7%). These results suggest that while higher doses of estradiol-17β can increase the feminization rate, they may also have a marginal impact on survival (Johnstone, 1985). The slight reduction in survival rates at higher doses could be due to the physiological stress induced by the hormone, as noted by Devlin & Nagahama (2002) in their comprehensive review of sex determination and differentiation in fish.

Growth performance, as indicated by final weight and specific growth rate (SGR), was also positively influenced by higher doses of estradiol-17β. The group treated with 2 mg/L exhibited the highest final weight (8.494 ± 0.214 g) and SGR

(14.641 ± 0.042 %/day). These findings align with the observations of (Pandian & Sheela, 1995), who noted enhanced growth in hormone-treated fish. Furthermore, Gale et al. (1999) observed that estradiol-17β not only influences sex differentiation but also positively affects growth rates by promoting anabolic processes in fish.

Comparative studies on the use of estradiol-17β in other fish species, such as Nile tilapia (*Oreochromis niloticus*), have shown similar patterns of feminization and growth enhancement. For instance, Mair et al. (1997) reported that higher doses of estradiol-17β resulted in a higher proportion of females and improved growth performance. This cross-species consistency underscores the potential of estradiol-17β as a universal agent for sex reversal and growth enhancement in aquaculture.

The implications of these findings for aquaculture are significant. Furthermore, the findings align with the theory that estrogen plays a crucial role in sex differentiation in teleost fish. The high efficacy of 17β-Estradiol in inducing female sex differentiation suggests its potential application in the aquaculture industry to control sex ratios and improve production efficiency (Li & Wang, 2019). By optimizing the dose of estradiol-17β, it is possible to achieve high rates of feminization, which is desirable in species where females grow faster or have better market value. The optimal dose identified in this study (2 mg/L) balances high feminization rates with acceptable growth performance and survival, making it a practical choice for commercial applications. Future studies should aim to further optimize these parameters and investigate the long-term effects of hormone treatment on fish health and reproductive capabilities, as indicated by Hunter & Donaldson (1983), who highlighted the importance of evaluating the long-term sustainability of hormone treatments in aquaculture.

5. Conclusions

The use of estradiol-17β at a dose of 2 mg/L is effective for inducing sex reversal in climbing perch (*Anabas testudineus*) through the immersion method. This dose achieves a high feminization rate, enhances growth performance, and maintains acceptable survival rates. These findings support the use of estradiol-17β as a valuable tool in aquaculture for optimizing the production of female fish. Further research is recommended to refine these parameters and assess the long-term impacts on fish health and reproductive success.

Conflict of interest

The authors declare no conflict of interest.

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Effects of dietary supplementation of shrimp head and shell by-products on growth performance and incidence of diarhea in fattening pigs from 96 to 164 days of age

Anh T. N. Dang[1], Hai T. Nguyen[1], Cuong K. Nguyen[2], Duong T. Do[3], Mi T. T Bui[3], & Khang N. Duong[4*]

[1]Department of Animal Production, Nong Lam University, Ho Chi Minh City, Vietnam

[2]Department of Veterinary Biosciences, Nong Lam University, Ho Chi Minh City, Vietnam

[3]Department of Genetics and Animal Breeding, Nong Lam University, Ho Chi Minh City, Vietnam

[4]The Research and Technology Transfer Center, Nong Lam University, Ho Chi Minh City, Vietnam

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***Corresponding author**

Duong Nguyen Khang

Email:

duongnguyenkhang@hcmuaf.edu.vn

ABSTRACT

The objective of this study was to evaluate effects of dietary supplementation of shrimp head and shell by-products on growth performance and incidence of diarhea in fattening pigs from August 2023 to October 2023 at the pig farm in Thoai Son district, An Giang province. A total of 90 crossbred pigs [(Duroc x Pietrain) x (Landrace x Yorkshire); 96 days old] were assigned to 1 of 3 treatments with 5 replicate pens of 6 pigs each according to sex, litter origin, and weight in a randomized complete block design (RCBD). The 3 dietary treatments included (1) Basal diet (control), (2) Basal diet + 3 g/kg shrimp hydrolysate powder (SH) and (3) Basal diet + 10 g/kg shrimp heads and shells meal (SM). The results showed that there were no significant differences in the average body weight (BW) and average daily gain (ADG) among the 3 treatments ($P > 0.05$). Similarly, pigs fed the SH diet (2.99) and SM diet had the same feed conversion ratio (FCR) of pigs ($P = 0.767$) as those fed the control diet (3.04) during the whole period from 96 to 164 days of age. Furthermore, no significant differences ($P > 0.05$) were found in the average daily feed intake (ADFI) and the diarrhea incidence among the 3 treatments. Briefly, the results in the current study suggest that dietary supplementation of shrimp head and shell by-products (shrimp hydrolysate powder, or shrimp heads and shell meal) seem to have no positive effects on growth productivity (BW, ADG, ADFI and FCR) and incidence of diarrhea in fattening pigs during 96 - 164 days of age.

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1. Introduction

Shrimp processing for export releases many by-products during the production process; waste from the shrimp processing industry mainly includes the head, chest, carapace, and tail, accounting for 50% of the whole shrimp (Thiago et al., 2012). Taking advantage of available by-products from the shrimp production industry to become a source of protein-rich feed ingredients is a direction in line with the circular farming trend that is being interested in the livestock farming practice to solve the problem of shortage of feed ingredients and reduce negative impacts on the ecosystem. Chitin is the primary component in shrimp shells, making up 15 - 40% of their composition (Abuzar et al., 2023). This substance can be extracted through a process known as the chitin recovery method, resulting in a product commonly used in livestock farming called chitosan; after hydrolyzing chitosan with enzymes (Pham et al., 2017) to create shrimp-derived oligosaccharide higher the biological value. Recently, it has been demonstrated that the feed conversion ratio (FCR) (Han et al., 2007) and the average daily gain (ADG) (Chatchai et al., 2019) of fattening pigs were significantly improved when they fed daily diets contained chitosan at 1 g/kg and 0.3 g/kg, respectively.

Furthermore, it has been reported that shrimp hydrolysate powder produced from shrimp heads and shells by the chemical hydrolysis method is being studied and proven to be effective in livestock farming (Liu et al., 2021; Ngo et al., 2021). Increasing the number of short-chain peptides in daily diets supported easier and better absorption in the digestive tract of livestock (Ngo et al., 2021); as well as the appearance of many amino acids that stimulate the appetite of livestock (taurine, glycine, alanine) (Liu et al., 2021). In Vietnam, however, the practical benefits of by-products from the shrimp industry in pig production are still limited in both academic research and feasible applications.

Therefore, the objective of the current study was to determine the effects of one product that contained by-products from shrimp (head and shells) on growth performance, feed consumption and diarrhea disease in pig production at the fattening stage.

2. Materials and Methods

2.1. Location

The study was conducted at the pig farm of Thoai Son district, An Giang province from August, 2023 to October, 2023.

2.2. Experimental design, animals and housing

A total of 90 crossbred pigs [(Duroc x Pietrain) x (Yorkshire - Landrace)] at 96 days of age with an initial average body weight (BW) of 43.67 1.1 kg were assigned to a randomized complete design with three dietary treatments (five replicate pens per treatment, three barrows and three gilts per pen). The average BW ($P > 0.05$), pigs' sex and litter were almost similar among the 3 treatments. Three dietary treatments consisted of (1) the basal diet (control), (2) basal diet supplemented with 3 g/kg shrimp hydrolysate powder (SH), and (3) basal diet supplemented with 10 g/kg shrimp head and shell meal (SM) (Both SH and SM provided by a commercial company) (Table 1).

Table 1. Experimental design

Treatment	Control	SH	SM
Pigs per pen, n	6	6	6
Replicate, pen	5	5	5
Total pigs	30	30	30
Duration, days	68	68	68
Pigs' dietary by-products shrimp addition, g/kg feed	0	3	10

SH: Shrimp hydrolysate powder; SM: Shrimp heads and shells meal.

Pigs were housed in an environmentally controlled building. Each pen measured 2 x 3 m in size with a slatted floor and had two nipple waterers. The chemical compositions of shrimp hydrolysate powder was shown in Table 2.

Table 2. Chemical and amino acid compositions of shrimp hydrolysate powder

Nutrients	Levels	Essential amino acids	Percentage (%)	Non-essential amino acids	Percentage (%)
Dry matter	86%	Arginine	1.22	Glutamic acid	2.33
Crude protein	30%	Histidine	0.56	Aspartic acid	1.47
Astaxanthin	11.45 ppm	Lysine	0.95	Glycine	1.43
		Leucine	1.43	Proline	1.01
		Methionine	0.46	Cystine	0.23
		Threonine	0.83	Valine	1.14
		Isoleucine	0.90		
		Phenyl alanine	1.04		

2.3. Experimental condition

2.3.1. Experimental diets and animal feeding

The daily mash feed as a basal diet was formulated with chemical compositions as nutrient requirements for the fattening pigs recommended by the NRC (2012) from common ingredients (Table 3) (produced by An Giang Agricultural Import-Export Joint Stock Company). Although the additional shrimp-derived protein was added to the basal diet, which was consistent with protein in the basal diet.

All pigs were fed twice a day (7:30 and 14:00, *ad libitum*) with distinguishing diets for each treatment. The experimental condition was set up in a temperature-controlled room and had free access to water and feed throughout the trial.

Table 3. Ingredients and chemical compositions of the basal diets

	Items	Percentage
Ingredients	Corn bran	35.58
	Rice bran	10
	Casava root meal	12
	Soybean waste	20
	Soybean meal	20
	Minerals	1.1
	Premix	1.1
Chemical compositions	ME, Kcal/kg	3,050
	CP, %	16.4
	Lysine, %	0.82
	Met + Cys, %	0.48
	Threonine, %	0.58
	Tryptophan, %	0.19

2.4. Sample collection and measurements

The BW and feed consumption were measured at the initial and final times of this experiment. Then, the ADG, average daily feed intake (ADFI), and FCR were calculated.

The incidence of diarrhea was recorded on a daily basis and based on fecal scores were based on 1 to 5 scale: 1 = normal; 2 = moist feces; 3 = mild diarrhea; 4 = severe diarrhea; 5 = watery diarrhea. The incidence of diarrhea was calculated by counting pig days with diarrhea score of 3 or greater during the whole experimental duration.

2.5. Statistical analysis

Data were analysed as a randomized complete block design by ANOVA using the GLM procedure (Minitab 16.2). The pen was considered the experimental unit. The incidence of diarrhea was compared by $\chi 2$ analysis. Treatment effects were considered significant at $P < 0.05$.

3. Results and Discussion

3.1. Growth performance

Table 4. Effect of dietary Shrimp hydrolysate powder (SH) and Shrimp heads and shells meal (SM) supplementation on body weight (kg/pig) of fattening pigs

Days of age	Treatments[1]			SEM	*P*
	Control	SH	SM		
96	42.41	44.41	44.20	0.095	0.602
135	69.93	71.75	72.94	0.131	0.695
164	91.84	96.95	95.28	0.225	0.494

[1]*5 pens/treatment and 6 pigs/pen.*

There were no significant differences in BW among the 3 treatments at 96 days of age (P = 0.602; Table 4). Similarly, dietary supplementation at 3 g/kg SH or 10 g/kg SM did not significantly increase (P = 0.695) the BW at 135 days of age (71.75 and 72.94 kg, respectively) compared with that of the control (69.93 kg/pig). The BW of the SH treatment was also similar (96.95 kg/pig) to those of the SM (95.28 kg/pig) and control treatments (91.84 kg) at the end of the experimental period (P = 0.494). Therefore, our current results indicated that dietary inclusion of SH at 3 g/kg or SM at 10 g/kg in the fattening period seems to have no positive impact on the BW of the fattening pigs. In contrast, Liu et al. (2021) demonstrated that shrimp heads are a source of unsaturated fatty acids, crude protein, essential amino acids, macro and micro minerals. It also reported that hydrolyzed shrimp protein contains many amino acids and di/tripeptides that help animals absorb directly, save energy for digestion, and at the same time stimulate appetite and bring a delicious feeling (Ngo et al., 2021).

There was no significant difference in the ADG among the 3 treatments in the period from 96 to 134 days of age (P = 0.792; Table 5), although pigs fed diets added the SH had numerically greater ADG (0.90 kg/day) than those of the SM (0.80 kg/day) and control treatments (0.78 kg/day) in the period of 135 - 164 days of age (P = 0.160). Besides, the ADG of pigs was not significantly different among the three treatments (control, SH, and SM treatments at 0.72, 0.77 and 0.75 kg/day, respectively) in the period of 96 - 164 days of age. Therefore, dietary supplementation of SH at 3 g/kg or SM at 10 g/kg has no positive impact on the ADG of the fattening pigs in the fattening period.

Table 5. Effect of dietary Shrimp hydrolysate powder (SH) and Shrimp heads and shells meal (SM) supplementation on average daily gain (kg/day) of fattening pigs

The period (days of age)	Treatments[1]			SEM	*P*
	Control	SH	SM		
96 - 134	0.68	0.68	0.72	0.002	0.792
135 - 164	0.78	0.90	0.80	0.006	0.160
96 - 164	0.72	0.77	0.75	0.002	0.606

[1]*5pens/treatment and 6 pigs/pen.*

3.2. Average daily feed intake (ADFI)

There were no significant differences (P > 0.05; Figure 1) in the ADFI among the 3 treatments in the period from 96 to 134 days of age (control, SH or SM at 1.87, 1.92 or 1.89 kg/day, respectively) and from 135 to 164 days of age (control, SH and SM at 2.70, 2.79 or 2.70 kg/day, respectively). Besides, during the whole experimental period, pigs fed diets supplemented the SH or the SM did not significantly increase the ADFI (2.30 or 2.24 kg/day, respectively) (P = 0.157) compared with that of the control (2.19 kg/day). Therefore, dietary supplementation of SH at 3 g/kg or SM at 10 g/kg has no beneficial effect on the ADFI in the fattening period period. On the contrary, previous studies indicated that shrimp heads contain about ten types of distinguishing proteases, including myofibril-associated serine proteinase, chymotrypsin, cathepsin B, pepsin, serine protease, trypsin, metalloprotease, cathepsin L, collagenase, and calpain (Xu et al., 2012) and contain many amino acids that stimulate the appetite of pigs, especially accounted for 10% glutamic acid (Liu et al., 2021), leading to increase the average daily feed intake of pig fed dietary SH supplementation (Xu et al., 2012; Liu et al., 2021).

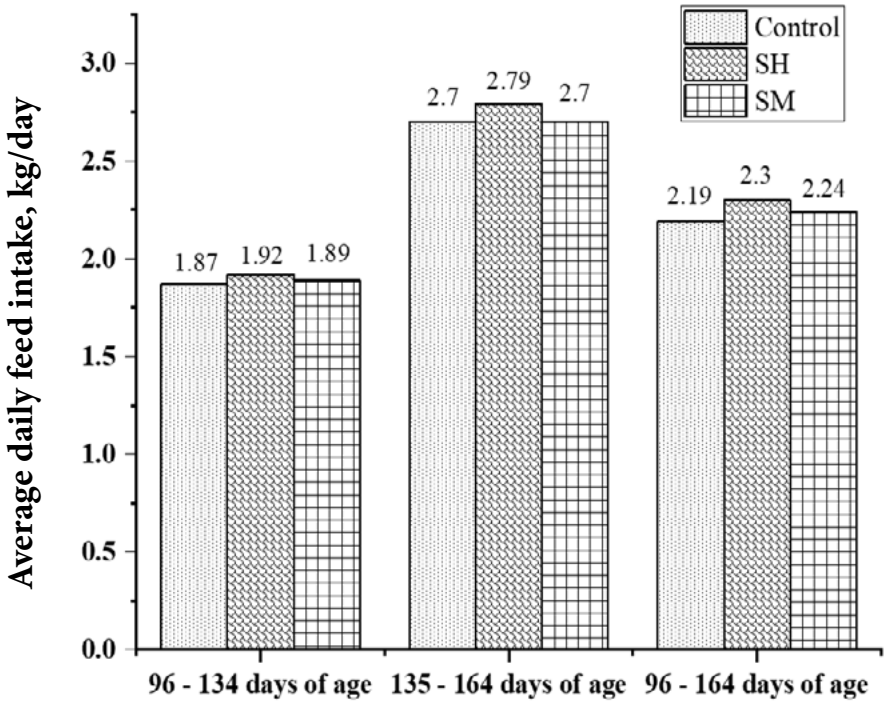

Figure 1. Effect of dietary Shrimp hydrolysate powder (SH) and Shrimp heads and shells meal (SM) supplementation on average daily feed intake of fattening pigs (kg/day).

3.3. Feed conversion ratio (FCR)

The FCR of pigs in the control treatment (2.77) in the period from 96 to 134 days of age was significantly higher than that of the SM treatment (2.63) ($P < 0.01$; Table 6). In addition, the control treatment witnessed an upward trend on the FCR (3.45) and significantly higher than that of the SH treatments (3.05) ($P < 0.01$) in the period of 135 - 164 days of age. It has been indicated that shrimp hydrolysate powder contains several amino acids such as glutamic acid, alanine, arginine and glycine, known as feed palatability (Tantikitti, 2014), stimulating appetite and feed efficiency (Martinez-Alvarez et al., 2015). However, the FCR of pigs in the whole period from 96 to 164 days of age was not improved by the inclusion of the SM or SM (2.99) into the daily diet as compared with that of the control (3.04) ($P = 0.767$).

Table 6. Effect of dietary Shrimp hydrolysate powder (SH) or Shrimp heads and shells meal (SM) supplementation on the feed conversion ratio of fattening pigs

The period (days of age)	Treatments[1]			SEM	*P*
	Control	SH	SM		
96 - 134	2.77[a]	2.84[a]	2.63[b]	0.027	0.001
135 - 164	3.45[a]	3.05[b]	3.38[a]	0.052	0.001
96 - 164	3.04	2.99	2.99	0.051	0.767

[1]*5pens/treatment and 6 pigs/pen.*

[a-b]Within a row, means with different letters differ significantly (P < 0.05).

3.4. Diarrhea incidence

Table 7. Effect of dietary Shrimp hydrolysate powder (SH) and Shrimp heads and shells meal (SM) supplementation on feed conversion ratio (FCR) supplementation on the incidence of diarrhea during the experimental period

Parameter	Control	SH	SM	*P*
Diarrhea incidence[1], %	3.15	2.14	2.58	0.446

[1]Diarrhea incidence: Diarrhea x 100/pig days; Diarrhea: number of pig days with diarrhea; Pig days: number of pigs x the number of days of diarrhea observation.

In the period of 96 - 164 days of age, the incidence of diarrhea disease of control treatment was 3.15% and not different from those of the SM and SH treatments (2.14% and 2.58%, respectively) ($P > 0.05$, Table 7). The results in the current study showed that by-products shrimp supplementation into the daily diets for fattening pigs does not affect the diarrhea incidence. In contrast, the hydrolysis reaction of protein from shrimp heads creates bioactive peptides, which perform biological functions in the animal body (Duong et al., 2018). These peptides enhance the activity of the immune system, as recorded in experimental pigs fed dietary SH supplementation (Yin et al., 2008), thereby improving health status and reducing diarrhea in pigs (Yang et al., 2012; Chatchai et al., 2019).

4. Conclusions

The dietary supplementation of shrimp head or shell by-products (shrimp hydrolysate powder, or shrimp heads and shell meal) had no significant improvements in growth performance (BW, ADG, ADFI and FCR) and incidence of diarrhea in the fattening pigs from 96 to 164 days of age.

Conflict of interest

The authors have no conflicts of interest to declare.

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Antifungal activity of mangosteen pericarp and cashew leaf extract against *Fusarium oxysporum in vitro*

Nhi N. Y. Nguyen[2], Vinh D. H Nguyen[1,2], Dong N. T. Le[1], & Ly T. P. Trinh[1,2*]

[1]Research Institute for Biotechnology and Environment, Nong Lam University, Ho Chi Minh City, Vietnam

[2]Faculty of Biological Sciences, Nong Lam University, Ho Chi Minh City, Vietnam.

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***Corresponding author**

Trinh Thi Phi Ly

Email: phily@hcmuaf.edu.vn

ABSTRACT

Polyphenols are secondary compounds that occur widely in plants and are highly effective in controlling plant pathogenic microorganisms. This study aimed to screen polyphenolic-rich plant extracts for their antifungal potential against *Fusarium oxysporum*. Several plant materials including cashew leaves, castor fruits, castor leaves, coffee husks, giant milkweed leaves, mangosteen pericarps and soapberry fruits were investigated for their total phenolic content. The results showed that cashew leaves and mangosteen pericarps contained high level of polyphenols at 108.23 and 124.14 mg GAE/g, respectively. The main phenolic compounds found in cashew leaves were gallic acid and protocatechuic acid at 377.29 mg/100 g and 56.44 mg/100 g, respectively. Mangosteen pericarps contained 16.22 mg/100 g protocatechuic acid and 55.75 mg/100 g of chlorogenic acid. The antifungal activity of cashew leaf and mangosteen pericarp extracts against *F. oxysporum* was 32.92 - 77.08% and 68.33 - 83.75%, respectively at the extract concentration from 2% to 10%. The combined use of cashew leaf and mangosteen pericarp extracts exhibited an additive inhibition against *F. oxysporum*. Cashew leaves and mangosteen pericarp are potential materials for producing bio-fungicides, which are not only effective but also safe for human and the environment.

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1. Introduction

Fusarium oxysporum is a soil-borne pathogen which causes *Fusarium* wilt disease on many plant species such as banana, tomato, peas and seriously affects crop yield by up to 80 - 90% (Ma et al., 2013; Edel-Hermann & Lecomte, 2019). Management of *Fusarium* wilt disease has been a challenge for many years. Controlling plant fungal diseases using chemical pesticides is not a sustainable solution because of high cost, increasing resistant strains, residual toxicity in the food chains and the environment. Biological control agents have gained much attention as alternatives to synthetic chemicals due to their effectiveness and safety (Muller-Riebau et al., 1995). The use of crude plant extracts rich in bioactive compounds has been approached and applied widely in agricultural processes. Polyphenols are secondary metabolites occurring abundant in plants with antioxidant, antibacterial and antifungal activities, which exhibit effective inhibition against fungal pathogens (Yuan et al., 2018). Phenolic compounds affect mycelial growth by inhibiting spore reproduction, disrupting cell membranes, and suppressing the synthesis of important proteins or enzymes (Oufensou et al., 2020). In our previous study, antifungal activity was directly proportional to the content of phenolic compounds in the plant extracts (Nguyen et al., 2024). Among locally available plant materials, cashew leaf and mangosteen pericarp emerged as prominent phenolic-rich sources, which were investigated for their main constituents and inhibitory effect on the growth of *Fusarium oxysporum*. The study provided useful information for further research and development of polyphenol-derived bioproducts for effective control of *Fusarium oxysporum*.

2. Materials and Methods

2.1. Plant materials, chemicals and reagents

Raw materials including cashew leaves (*Anacardium occidentale* L.), castor fruits and leaves (*Ricinus communis* L.), coffee husks (*Coffea arabica* L.), giant milkweed leaves (*Calotropis gigantea* L.), mangosteen pericarps (*Garcinia mangostana* L.) and soapberry fruits (*Sapindus saponaria* L.) were collected in Ho Chi Minh City, Binh Phuoc and Lam Dong province. The sample was washed, drained naturally at room temperature then dried at 50°C until the moisture content reached below 13%. Then, they were pulverized into fine powder, sieved through a sifter with a pore size of 0.1 mm and stored in zipper bags in desiccators at room temperature. The fungal pathogen *F. oxysporum* f. sp. *lycopersici* was provided by the Research Institute for Biotechnology and Environment, Nong Lam University, Ho Chi Minh City.

Gallic acid, chlorogenic acid and protocatechuic acid were purchased from Sigma-Aldrich. Acetonitrile and acetic acid (HPLC grade) were supplied by Thermo Fisher Scientific. Folin-Ciocalteau's reagent was provided by Supelco. All other chemicals were of analytical grade.

2.2. Determination total phenolic content

Plant extracts were prepared by mixing 1 g of plant material with 70% aqueous ethanol with a ratio of raw material to solvent of 1:10. The extraction was conducted using ultrasonication with a frequency of 20 kHz and power of 500 W for 15 min. Then, the extract was obtained by filtering through filter papers (Whatman No.1). The residual solid was extracted with 70% aqueous ethanol two more times. The extracts were combined and made up to a total volume of 50 mL.

Total phenolic content (TPC) was determined by spectrophotometry according to the reaction with Folin-Ciocalteu reagent (Trinh et al., 2018). Briefly, 100 µL of the prepared extract was mixed with 100 µL of Folin-Ciocalteu reagent. After 5 min, 300 µL of 20% Na_2CO_3 solution was added to the mixture to stop the reaction prior to making up to 5 mL with distilled water. The mixture was incubated for 60 min in darkness at room temperature. Its absorbance was measured at 730 nm. Gallic acid was used as the standard with a concentration range of 100 - 500 mg/L. TPC was expressed as milligram gallic acid equivalent per gram of dry matter (mg GAE/g).

2.3. Quantification of phenolic compounds in plant extracts

Gallic acid (GA), protocatechuic acid (PCA) and chlorogenic acid (CGA) in the extracts were determined by high performance liquid chromatography (HPLC) with a diode array detector (Agilent 1260 Infinity II, Santa Clara, United States). A binary pump was set up with mobile phase A (1% acetic acid in water) and mobile phase B (1% acetic acid in acetonitrile). A Poroshell 120-EC C18 reversed-phase column (100 mm × 4.6 mm, particle size 2.7 µm) was used for separation at a column temperature of 35°C. The mobile phase and elution program are presented in Table 1. The sample injection volume was 5 µL and the mobile phase was injected at a flow rate of 1 mL/min. Gallic acid and protocatechuic acid were detected at 270 nm; chlorogenic acid was detected at 320 nm (Trinh et al., 2018). Results were expressed in mg/100 g of dry material.

Table 1. Elution program for separation of phenolic compounds

Time (min)	% A	% B
2	95	5
17	60	40
20	60	40
21	95	5
25	95	5

2.4. Inhibitory activity of plant extracts against *F. oxysporum in vitro*

Fusarium oxysporum was inoculated on potato dextrose agar (PDA) at 28 ± 2°C for seven days. Then, a piece of mycelium with diameter 8 mm was placed on petri dishes (90 × 15 mm) containing PDA medium and plant extracts. Plant extracts at concentrations of 4, 8, 12, 16, 20% were prepared by extracting 4, 8, 12, 16, 20 g of dried plant material, respectively with 70% ethanol using the procedure mentioned earlier (section 2.2), then ethanol was removed by an evaporator followed by the addition of distilled water to make a total volume of 100 mL. The plant extracts were mixed with 2X PDA medium at the same volume to obtain a medium containing 2, 4, 6, 8, 10% plant extracts, respectively. The extract combination of cashew leaves and mangosteen pericarps at 4% and 8% was prepared using 2% and 4% of each, respectively by the same procedure used for the individual extracts.

Post-inoculation plates were incubated at 28 ± 2°C. Mycelium growth was observed at day 1, 3, 5, and 7 after inoculation. Each treatment was conducted in triplicate. The ability to inhibit fungi was evaluated by the following formula (Chang et al., 2000):

$$I = \frac{(D_c - \mathrm{D})}{D_c} \times 100$$

Whereas I is antifungal index, D is diameter of mycelium treated with the extracts, D_c is mycelial diameter of the control without treated.

2.5. Observation of mycelial morphology

In this study, the morphological characteristics of *F. oxysporum* mycelium were observed by microscopic examination. A small piece of the *F. oxysporum* was carefully collected and placed on a clean microscope slide. Then, a drop of sterile saline solution was added to the specimen prior to covering it with a coverslip. Observations were conducted using a microscope (Evident CX23) equipped with 40X objectives (Buffi et al., 2023).

2.6. Data analysis

Experimental results were expressed as mean ± standard error. One-way analysis of variance and Tukey's tests were used to determine significant differences ($P < 0.05$) between the means using the MiniTab 16.0 program.

3. Results and Discussion

3.1. Determination of total phenolic content

This study collected and screened locally valuable plant materials with the aim to search phenolic-rich sources, which are believed to perform antifungal capacity. The total phenolic content of collected samples was found in a wide range of 7.45 - 124.14 mg GAE/g, indicating the differences in the quantity of bioactive compounds in plants (Table 2). Most notably, cashew leaf and mangosteen pericarp contained the highest phenolic levels at 108.23 and 124.14 mg GAE/g, respectively, which were much higher than other samples.

Table 2. Total phenolic content in plant extracts

Plant materials	Total phenolic content (mg GAE/g)
Coffee husks	10.06 ± 0.78
Soapberry fruits	7.45 ± 0.40
Mangosteen pericarps	124.14 ± 0.49
Castor fruits	14.78 ± 0.51
Castor leaves	14.02 ± 0.10
Giant milkweed leaves	10.08 ± 0.22
Cashew leaves	108.23 ± 0.85

Mangosteen pericarps have long been used as a traditional folk medicine for sprains, typhoid, diarrhea and skin infections. It has attracted much attentions due to exhibiting anti-inflammatory, antioxidant, antibacterial and antifungal activity (Kaur et al., 2020). Mangosteen pericarp accounts for about 65% of the total fresh weight of the fruit, with a TPC of 140.66 mg GAE/g containing many substances such as xanthones, phenolic acids, flavonoids, and benzophenones (Rizaldy et al., 2021). Vo & Nguyen (2023) reported that mangosteen pericarp collected in Vietnam had

high polyphenol content at 195.05 mg GAE/g and showed antioxidant activity, inhibitory effect on α-glucosidase enzyme and antibacterial capacity against *Propionibacterium acnes*. Do et al. (2011) isolated α-mangostin and γ-mangostin from the alcohol extract of mangosteen pericarp, which exhibited antioxidant activity and antibacterial effect on *Edwardsiella tarda*. Cashew leaves have shown the ability to inhibit pathogenic microorganisms such as *Staphylococcus aureus, Streptococcus mutans, Escherichia coli, Candida albicans,* and *Aspergillus niger* (Shiekh et al., 2022). Duangjan et al. (2019) reported TPC of cashew leaves of 160.35 mg GAE/g with the presence of flavonoids, tannins and anthocyanins. In this study, mangosteen pericarps and cashew leaves, which were the most abundant phenolic sources, were investigated for their antifungal activity and specific phenolic compounds.

3.2. Antifungal activity

Phenolic compounds are the most diverse phytochemicals presenting in plants that have been reported to show biological effects, such as antibacterial, antifungal, antiviral, antioxidant, and anti-inflammatory activities (Nguyen et al., 2019, Soyel et al., 2022; Wang et al., 2022). A strong correlation between total phenolic content and antimicrobial activity has been demonstrated (Bouslamti et al., 2022). In our previous study, antifungal activity of plant extracts against *Fusarium oxysporum* was observed to enhance with an increase in the content of phenolic compounds (Nguyen et al., 2024). Figure 1 and Figure 2 show the effect of cashew leaf and mangosteen pericarp extracts on the growth of *F. oxysporum*. The average diameter of fungal colonies ranging from 53.67 to 18.33 mm corresponding to antifungal index ranging from 32.92 to 77.08% was observed on PDA medium supplemented with cashew leaf extracts at 2 - 10% (material weight/total volume). The mycelium didn't spread normally in the medium containing the cashew leaf extracts as compared to the control. A decrease in fungal biomass was observed at all extract concentrations and purple color completely disappeared at 8% cashew leaf extract. The antifungal activity of mangosteen pericarp extract against *F. oxysporum* was higher than that of cashew leaf extract with values ranging from 68.33 to 83.75% at concentrations of 2 - 10% (w/v). Mycelia didn't spread onto the medium supplemented with mangosteen pericarp extracts and the particular purple color wasn't observed at all mangosteen pericarp extract levels investigated.

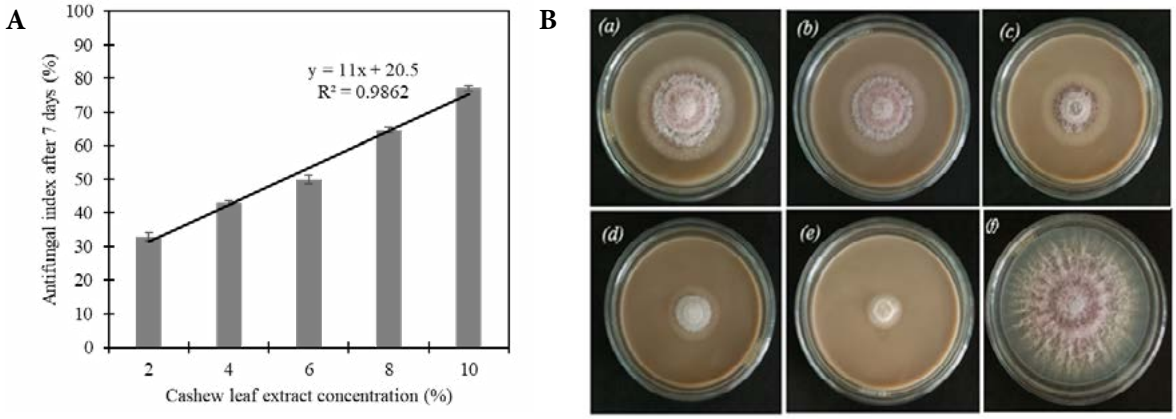

Figure 1. Effect of cashew leaf extracts on *F. oxysporum*. A: Antifungal index; B: Mycelial growth on the media supplemented with the extract at different concentrations (a-f) 2, 4, 6, 8, 10% and control, respectively.

Yenjit et al. (2007) reported that the crude extract of mangosteen pericarp at a concentration of 1000 µg/mL showed high inhibitory effects against *Pythium aphanidermatum, Puccinia psidii, Colletotrichum gloeosporioides,* and *F. oxysporum,* with an antifungal index of 45%, 56%, 34.45%, 34.38%, and 26.88%, respectively. The substances isolated from mangosteen pericarps, such as xanthones, flavonoids, benzophenones, lactones and phenolic acids have been shown to inhibit certain fungal desease in plants. Previous research showed that the aqueous and ethanolic extract of cashew leaf had inhibitory effect on *Aspergillus niger* and the antifungal activity of the extracts increased with increasing extract concentration from 20 - 100 mg/mL. The aqueous extract had a higher fungal inhibition efficacy than the ethanolic extract which might be due to the high concentration of tannins in the aqueous extract (Tafinta et al., 2020).

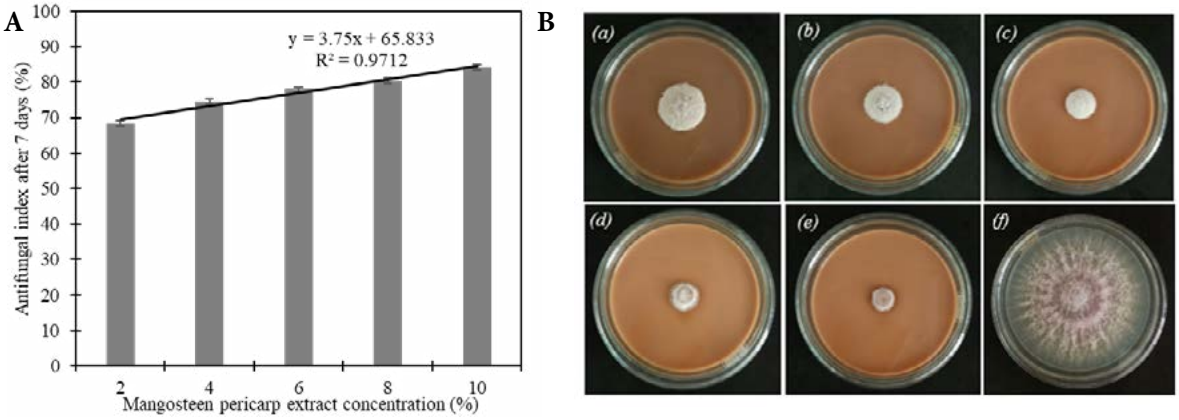

Figure 2. Effect of mangosteen pericarp extracts on *F. oxysporum.* A: Antifungal index; B: Mycelial growth on the media supplemented with the extract at different concentrations (a-f) 2, 4, 6, 8, 10% and control, respectively.

Table 3. Antifungal activity and phenolic content of individual and combined treatment

Treatment	Phenolic content (mg/disc)	Antifungal index (%)
C2	$43.29^{f} \pm 0.34$	$32.92^{e} \pm 1.44$
M2	$49.65^{e} \pm 0.20$	$68.33^{c} \pm 0.72$
C2 + M2	$92.95^{c} \pm 0.52$	$75.00^{b} \pm 0.00$
C4	$86.59^{d} \pm 0.68$	$42.92^{d} \pm 0.72$
M4	$99.31^{b} \pm 0.39$	$74.58^{b} \pm 0.72$
C4 + M4	$185.90^{a} \pm 1.03$	$81.25^{a} \pm 0.00$

C2, C4: Cashew leaf 2%, 4%; M2, M4: Mangosteen pericarp 2%, 4%.

An significant improvement in the inhibitory effect on *F. oxysporum* was reported in our previous study when using a combination of phenolic-rich extracts (Nguyen et al., 2024). In this study, a mixture of cashew leaf and mangosteen pericarp extract was prepared to investigate its effect on *F. oxysporum* in comparison to the individual extract. As displayed in the Table 3, the antifungal activity of the combination of 2% of each extract reached 75%, which was

2.3 and 1.1 folds higher than that of individual cashew leaf and mangosteen pericarp extract, respectively. Similarly, the antifungal activity reached 81.25% when the two extracts were combined at 4% of each, while individual cashew leaf and mangosteen pericarp extract inhibited the mycelial growth by only 42.92% and 74.58%, respectively. It is attributed to the higher phenolic content in the combined treatment compared to the single extract. However, mangosteen pericarp at 2% (M2) showed higher inhibitory effect than cashew leaf 4% (C4) even though it had lower phenolic content, indicating the antifungal activity was not completely propotional to the phenolic content. The combined extract (C2 + M2) showed antifungal activity comparable to a 4% mangosteen pericarp extract (M4), despite having a lower phenolic level, which indeed suggests the role of specific bioactive compounds in the mangosteen pericarp (Table 3). Briefly, mangosteen pericarp demonstrated higher antifungal activity than cashew leaf, and their combination showed an additive effect on mycelial growth of *F. oxysporum*. Our results were in agreement with the publised data. The additive or synergistic effect of extract combination appeared due to the presence of diverse phenolic compounds, such as phenolic acids and flavonoids from different plant species, and each molecule affected *F. oxysporum* by different mechanism of action (Mirghani, 2022; Nguyen et al., 2024).

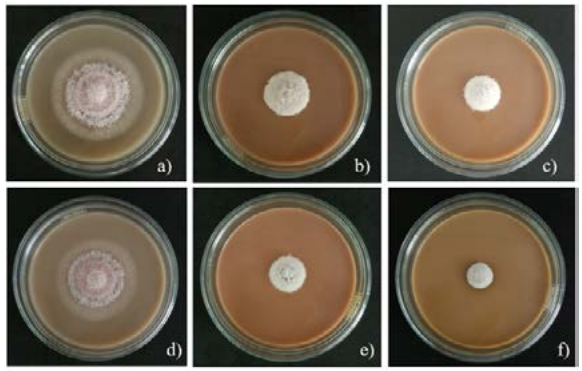

Figure 3. Mycelial growth on the media supplemented with the combined extract at different concentrations: a) C2, b) M2, c) M2 + C2 , d) C4, e) M4 and f) M4 +C4.

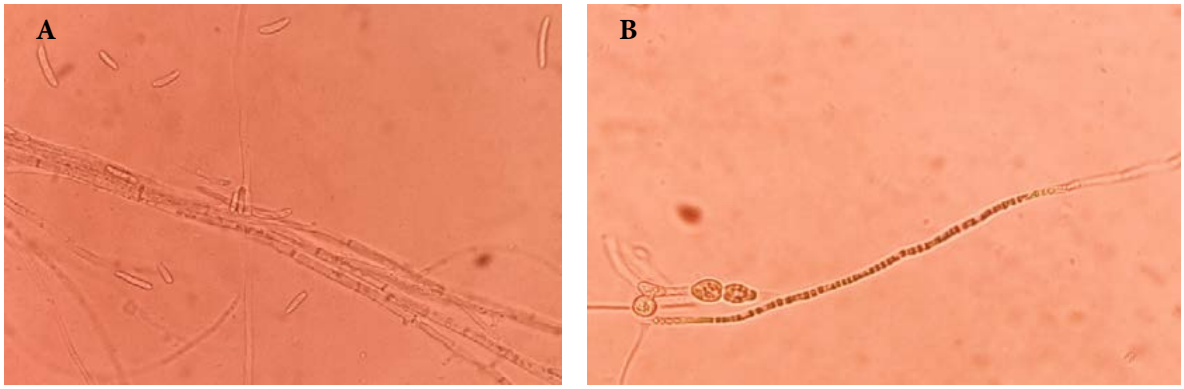

Figure 4. Mycelial morphology under the microscope (40X). A: Without treated; B: Treated with the mixture of 2% cashew leaf and 2% mangosteen pericarp.

The use of extract combination to inhibit fungal growth has been reported in the literature. The mixture of *Lawsonia alba* leaf extract and *Acacia catechu* stem extract showed an increase in inhibitory effect on the *Fusarium solani* growth up to 78.64% compared to that of the individual extracts of 62.07% and 54.69%, respectively (Bhardwaj, 2012). This is attributed to the higher levels of functional compounds in the extract combination than that of individual extracts; the greater diversity of different groups in the mixture and the synergistic effect of specific compounds in the extract combination (Nguyen et al., 2024).

Mycelia on PDA medium supplemented with the combined extract did not spread, underdeveloped, and lost purple pigment (Figure 3). Microscopic mycelial morphology observed on the medium supplemented with the combined extract showed fungal unusual growth including poor formation of hyphal septa and spores compared to the control mycelia. In addition, the intracellular components of mycelial hyphae appeared condensed and different from the control (Figure 4). The inhibitory effect of plant extracts on conidial germination of *F. oxysporum* has been well documented (Mohamed et al., 20217; Alotibi et al., 2020). Moreover, phenolic compounds such as gallic, cinnamic, vanillic, coumaric, ferulic and salicylic acids were demonstrated to induce the destruction and shrunken of fungal hyphae (Wu et al., 2014). In a earlier study, *F. oxysporum* hyphae-treated with *Rumex* sp. extract displayed significant ultrastructural changes, including cell wall deformation and damage, as well as the appearance of electron-dense material along the hyphae (Alotibi et al., 2020). Phenolic compounds with many hydroxyl groups in their structure that are able to bind to adhesives and proteins on membranes, inactivating enzymes, breaking cell membranes, and leaking intracellular substances (Cowan, 1999). Additionally, polyphenols have inhibitory effects on DNA/RNA/protein synthesis and mitochondrial dysfunction (Khanzada et al., 2021). Mode of action includes induction of programmed cell death, suppression of biofilm formation and mycelial growth, and inhibition of soluble protein synthesis that causes increased permeability membrane and disrupts membrane integrity (Acheuk et al., 2022). Therefore, particular phenolic compounds in the mangosteen pericarp and cashew leaf extract have been determined.

3.3. Quantification of specific polyphenols in the extracts

Gallic acid, protocatechuic acid and chlorogenic acid are common phenolic compounds identified in the mangosteen pericarp and cashew leaf. As shown in Figure 5 and Table 4, mangosteen pericarp contained chlorogenic acid and protocatechuic acid at 55.75 mg/100 g and 16.22 mg/100 g, respectively. Cashew leaf was a rich source of gallic acid at 377.29 mg/100 g and protocatechuic acid at 56.44 mg/100 g. Chlorogenic acid is the ester of caffeic acid and quinic acid, a hydroxycinnamic acid synthesized by plants through the phenylpropanoid pathway. According to previous research, chlorogenic acid inhibited spore reproduction and limited mycelial growth of *Fusarium solani, Verticillium dahliae, Botrytis cinerea* and *Cercospora sojina* (Martínez et al., 2017). Gallic acid and its derivatives including pyrogallic acid and syringic acid inhibited the mycelial radial growth of *Alternaria solani* and efficiently suppressed the development of early blight disease without any phytotoxic symptoms on treated tomato plants (El-Nagar et al., 2020). Protocatechuic acid is a widely distributed bioactive compound

found in many plant species. Protocatechuic acid isolated from *Paenibacillus elgii* displayed potent antifungal activity against *Botrytis cinerea* and *Rhizoctonia solani*. Moreover, gray mold formation on strawberry fruit was almost inhibited by protocatechuic acid after 7 days infected with *B. cinerea* conidia (Nguyen et al., 2015). Using the extract mixture containing abundantly diverse phenolic substances to enhance the antifungal activity could be an efficient and sustainable strategy to control plant pathogens and maintain the ecosystem as well.

Table 4. Quantitative analysis of phenolic compounds in plant extracts

Compounds	Gallic acid (mg/100 g)	Protocatechuic acid (mg/100 g)	Chlorogenic acid (mg/100 g)
Mangosteen pericarps	-	16.22 ± 0.39	55.75 ± 0.93
Cashew leaves	377.29 ± 2.05	56.44 ± 1.23	-

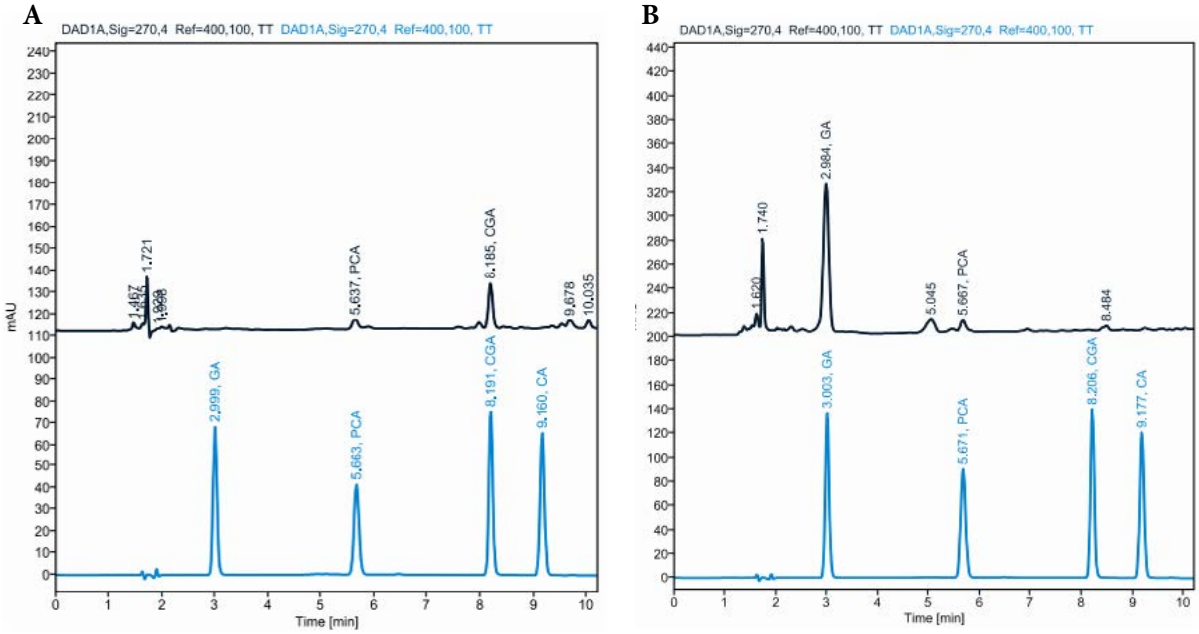

Figure 5. HPLC chromatogram A: Cashew leaves; B: Mangosteen pericarps.
GA: Gallic acid; PCA: Protocatechuic acid; CGA: Chlorogenic acid; CA: caffeic acid
Blue line: chromatogram of the standard mixture.

4. Conclusions

Cashew leaves and mangosteen pericarps contained prominent phenolic levels at 108.23 mg GAE/g and 124.14 mg GAE/g, respectively. Inhibition effects of cashew leaf and mangosteen pericarp extract on the mycelial growth of *F. oxysporum* were 32.92 - 77.08% and 68.33 - 83.75%, respectively at extract concentrations from 2% to 10%. Mangosteen pericarp exhibited greater antifungal activity than cashew leaf, and their combination yielded an additive effect on *F. oxysporum*. Cashew leaf contained 377.29 mg/100 g gallic acid and 56.44 mg/100 g protocatechuic acid; while chlorogenic acid and protocatechuic acid were determined in the mangosteen pericarp at 55.75 mg/100 g and 16.22 mg/100 g, respectively.

Conflict of interest

None of the authors of this study have any financial interest or conflict with industries or parties.

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Improving propagation of the rare plant *Huperzia squarrosa* using cuttings and *in vitro* techniques

Vuong D. Nguyen, Giang K. T. Le, Vy T. H. Dang, & Phong V. Nguyen*
Faculty of Biological Sciences, Nong Lam University, Ho Chi Minh City, Vietnam

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***Corresponding author**

Nguyen Vu Phong
Email:
nvphong@hcmuaf.edu.vn

ABSTRACT

The tassel fern, *Huperzia squarrosa*, is a rare and medicinally valuable plant known for containing Huperzine A. It propagates naturally through spores, rhizomes, cuttings, and clump division, but with a slow multiplication rate. This study aimed to optimize propagation conditions for *H. squarrosa* using stem cuttings and *in vitro* culture techniques to support its preservation and development. Apical and stem cuttings were treated with varying concentrations of indole-3-butyric acid (IBA) and naphthaleneacetic acid (NAA) before being planted in a substrate of coir dust, charcoal dust, and burnt rice husk (3:2:2). Apical cuttings treated with 1500 ppm IBA for 30 min showed the highest rooting success, identifying this method as optimal for propagation. Additionally, surface sterilization with a 40% bleach solution, followed by antibiotic treatment, achieved a 73.8% clean sample rate. *In vitro* culturing on ¼ MS (Murashige and Skoog) medium resulted in 70% survival and 55% rooting after 60 days. The highest callus formation rate (13.3%) was achieved with 0.01 mg/L IBA and 0.3 mg/L Kinetin, while the addition of 3 mg/L Glutamine did not significantly enhance callus induction. Ongoing research focuses on enhancing complete plant regeneration and improving the efficiency of *in vitro* propagation for *H. squarrosa*.

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1. Introduction

Huperzia squarrosa (G. Forst.) Trevis is a valuable ornamental and medicinal plant known for its therapeutic properties. In India, it is collected in winter, dried, and ground into powder for use as a supplement to improve memory and to treat sleep disorders and epilepsy (Yumkham & Singh, 2012). The primary active ingredient, huperzine A (HupA), is particularly effective in treating memory disorders such as Alzheimer's disease (Ferreira et al., 2016). Other alkaloids in *H. squarrosa*, such as Lycoposquarrosamin-A, Acetylaposerratinin, 8-α-hydroxyfawcettimin, 8-β-hydroxyfawcettimin, 8-β-acetoxyfawcettimin, acetyllycoposerramin-U, and lycoflexin N-oxide, are bioactive compounds with various biological activities. Though their specific functions are not fully understood, many *Huperzia* alkaloids are known for their neuroprotective effects, particularly through acetylcholinesterase inhibition, which may aid in treating neurodegenerative diseases like Alzheimer's (Katakawa et al., 2011).

Huperzia squarrosa, a valuable medicinal plant, is native to Vietnam and can be found in highland regions such as Lao Cai, Lam Dong, Tam Dao (Vinh Phuc), Nghe An, Tay Nguyen, and Quang Tri (Nguyen, 2020). This plant plays a significant role in traditional medicine due to its bioactive compound HupA, which has been associated with cognitive-enhancing and neuroprotective properties (Upadhyay et al., 2020). Despite its importance, *H. squarrosa* faces significant challenges in cultivation. The plant reproduces through unicellular spores encased in a thick, pale yellow spore wall, with germination occurring typically between 3 to 8 years post-release. Stem propagation involves laying apical sections of 5 - 15 cm long horizontally on a propagation medium and keeping them moist and warm until new growth emerges after 6 - 15 months. The method can be inconvenient due to the lengthy time required for new growth and the slow production of large specimens, which can take several years (Yumkham & Singh, 2011). Overexploitation, coupled with its slow development and limited natural reproduction rate, has led to a notable decline in this important genetic resource.

Plant tissue culture presents a viable solution for the large-scale propagation of *Huperzia* sp. while maintaining genetic uniformity. This technique not only aids in conserving this genetic resource but also facilitates the production of HupA for potential medical applications (Yang et al., 2021a). Despite its benefits, research on the micropropagation of *H. squarrosa* remains sparse. Recent studies, such as those by Tran et al. (2019), have demonstrated that MS (Murashige and Skoog) medium (Murashige and Skoog, 1962) supplemented with specific growth regulators can enhance regeneration rates and shoot multiplication. Additionally, ½ MS medium with indole-3-butyric acid (IBA) has been effective in promoting rooting.

Given the medicinal significance of *H. squarrosa* and the challenges associated with its propagation, developing efficient in vitro propagation methods is crucial for its conservation and sustainable cultivation. This study aims to address the gaps in current research by focusing on optimizing propagation techniques for *Huperzia squarrosa* using cuttings and in vitro culture methods, with the objective of enhancing callus formation and plant regeneration to support both conservation and medical application efforts.

2. Materials and Methods

2.1. Plant material

Huperzia squarrosa plants were collected from Tuyen Quang province and identified based on morphological characteristics described by Pham (1999). The plants were cultivated in the greenhouse at the Faculty of Biological Sciences and used as research materials.

2.2. Investigating propagation through cutting

This experiment investigates how two factors, including the type of cutting and the plant growth regulator (PGR) treatments, influence the propagation of *H. squarrosa* through cuttings. Healthy, pest- and disease-free *H. squarrosa* plants were cut into 8 cm segments with a bevelled end to maximize the exposed surface area with the substrate. Two types of cuttings were prepared: apical cuttings (V1) and stem cuttings (V2) taken 17 cm from the base (Figure 1a). The 10 cm-long cuttings were treated with different concentrations of Indole-3-Butyric Acid (IBA, Biobasic) at 500, 1000, and 1500 ppm for 30 min, and Naphthaleneacetic Acid (NAA, Biobasic) at 10, 20, and 30 ppm for 5 min. After treatment, the cuttings were planted in pots (7 x 7 cm) containing a substrate mixture of coconut fibre, shredded charcoal, and burnt rice husks in a 3:2:2 ratio. The pots were kept in a greenhouse with 70% light coverage and watered regularly.

The experiment was conducted three times, with each treatment including 10 apical cuttings (V1) or 30 stem cuttings (V2). After 60 days, the percentage of surviving explants, dead explants, rooted explants, and the morphological characteristics of the explants were recorded.

2.3. *In vitro* propagation of *Huperzia squarrosa*

Explant sterilization

A 3 cm shoot segment was initially washed under clean tap water, soaked in diluted soap solution for 20 min, and then rinsed under running water. The segment was soaked in a fungicide solution (Mancozeb, India) for 40 min, then briefly immersed in 70% ethanol for 30 sec. The segments were then treated with a Javel solution (NaOCl, 5%) at varying concentrations (20%, 30%, and 40% v/v) with 2 - 3 drops of Tween-80 for durations of 20, 30, and 40 min. Following this, explants were immersed in an antibiotic solution (ampicillin 2.5 mg/mL and tetracycline 2.5 mg/mL) for 30 min. Both ends of the shoots were trimmed to 2 cm, and the explants were cultured in MS medium supplemented with 2% (w/v) sucrose. The medium was adjusted to pH 5.8 and sterilized by autoclaving at 121°C and 1 atm for 20 min. The efficiency of sterilization was evaluated after 10 days of culture.

Influence of mineral salts on shoot growth

Huperzia squarrosa shoots were cut into 5 mm segments and cultured on MS or ¼ MS medium. Each treatment was performed in triplicate. After 8 weeks of culture, the percentage of survival rate (%), rooting rate (%), number of root (roots/explant), and morphology of shoot were recorded.

Effects of combinations of plant growth regulators and glutamine on callus formation

This experiment evaluated how specific combinations of IBA, kinetin, and glutamine influence callus formation in *Huperzia squarrosa*. Shoots were cut into 5 mm segments and cultured on ¼ MS medium, with or without IBA (0.01 or 0.015 mg/L), kinetin (0.3 mg/L, Biobasic), and glutamine (0.3 mg/L, Biobasic). Each treatment was replicated three times to ensure reliability.

After 8 weeks of culture, data were collected on the percentage of callus induction and the morphology of the callus.

***In vitro* culture conditions**

The *in vitro* culture conditions were maintained at a temperature of 25 ± 2°C under cool-white fluorescent lighting with a 16-h light/8-h darkness photoperiod, a light intensity of 2000 -3000 lux, and humidity levels of 60 - 70%.

2.4. Statistical analysis

All experiments were conducted using a completely randomized design (CRD). The collected data were analysed using one-way or multi-way analysis of variance (ANOVA) with Minitab 16. T-tests or Tukey's tests were used to compare mean values at a 5% significance level. Prior to analysis, data were transformed to ensure a standard normal distribution. Results are presented as M ± SD, where M is the mean and SD is the standard deviation.

3. Results and Discussion

3.1. Propagation for *Huperzia squarrosa* using cuttings

The results indicated a significant difference in survival rates across the treatments ($P < 0.05$) (Table 1). For V1 cuttings (apical cuttings), a 100% survival rate was observed in treatments B1 and B3, where cuttings were exposed to IBA at 500 ppm and 1500 ppm for 30 min, respectively. In contrast, the lowest survival rate for V1 cuttings was 20% in treatment B6, where NAA was applied at 30 ppm for 5 min. For V2 cuttings (stem cuttings), the highest survival rate of 100% was recorded in treatment B14, which did not involve any growth regulator. The lowest survival rate for V2 cuttings was observed in treatment B13 (NAA at 30 ppm for 5 min).

Overall, treatments involving NAA resulted in lower survival rates compared to those involving IBA. Specifically, V1 cuttings treated with IBA at concentrations of 500 ppm or 1500 ppm for 30 min achieved up to 100% survival, whereas V2 cuttings under the same IBA conditions had a maximum survival rate of 80%. In contrast, treatments with NAA for 5 min showed survival rates ranging from 20% to 90% for V1 cuttings and 43.3% to 63.7% for V2 cuttings, with survival rates decreasing as NAA concentrations increased from 10 to 30 ppm.

The rooting results demonstrated that V1 cuttings (apical cuttings) achieved superior rooting compared to V2 cuttings (stem cuttings). V1 cuttings reached a highest rooting rate of 100%, with the lowest at 20%, while V2 cuttings failed to form roots in all samples (Figure 1d, 1e). Treatment B3, involving V1 cuttings treated with 1500 ppm IBA for 30 min, yielded the best rooting performance. This treatment resulted in a 100% rooting rate, an average root length of 11.9 mm, and 6.1 roots per sample. The cuttings from this treatment remained green, elongated, and rooted after 2 months (Figure 1b, 1c). These results were significantly better than those from treatments using NAA at 10 ppm, which only achieved a 90% rooting rate, 3.4 roots per sample, and an average root length of 7.4 mm, with rooting performance declining at higher NAA concentrations.

Table 1. Effects of type of cutting and the plant growth regulator treatments on survival and mortality rates of *Huperzia squarrosa* cuttings after 2 months

Treatment	Cutting site	PGRs	Concentration (ppm)	Death rate (%)	Survival rate (%)	Rate of root induction (%)	Root length (cm)	No. of root (roots/ shoot)
B1	V1	IBA	500	0.00 ± 0.00^{i}	100.0 ± 0.00^{a}	100.0 ± 0.00^{a}	9.8 ± 0.4^{b}	5.6 ± 0.2^{a}
B2	V1	IBA	1000	20.0 ± 5.00^{f}	80.0 ± 5.00^{b}	80.0 ± 5.00^{b}	8.2 ± 0.2^{c}	4.2 ± 0.2^{b}
B3	V1	IBA	1500	0.0 ± 0.00^{i}	100.0 ± 0.00^{a}	100.0 ± 0.00^{a}	11.9 ± 0.1^{a}	6.1 ± 0.1^{a}
B4	V1	NAA	10	10.0 ± 5.00^{h}	90.0 ± 5.00^{b}	90.0 ± 5.00^{ab}	7.4 ± 0.2^{d}	3.4 ± 0.2^{c}
B5	V1	NAA	20	40.0 ± 5.00^{d}	60.0 ± 5.00^{c}	60.0 ± 5.00^{c}	6.9 ± 0.2^{e}	2.7 ± 0.2^{d}
B6	V1	NAA	30	80.0 ± 5.00^{a}	20.0 ± 5.00^{d}	20.0 ± 5.00^{d}	1.5 ± 0.2^{f}	0.7 ± 0.2^{e}
B7	V1	0	0	20.0 ± 5.00^{f}	80.0 ± 5.00^{b}	80.0 ± 5.00^{b}	9.6 ± 0.1^{b}	3.6 ± 0.2^{c}
B8	V2	IBA	500	10.0 ± 2.00^{h}	90.0 ± 2.00^{b}	0.0	0.0	0.0
B9	V2	IBA	1000	20.0 ± 2.00^{f}	80.0 ± 2.00^{b}	0.0	0.0	0.0
B10	V2	IBA	1500	13.3 ± 0.57^{g}	86.7 ± 0.57^{b}	0.0	0.0	0.0
B11	V2	NAA	10	36.7 ± 5.77^{e}	63.3 ± 5.77^{c}	0.0	0.0	0.0
B12	V2	NAA	20	46.7 ± 2.89^{c}	53.3 ± 2.89^{c}	0.0	0.0	0.0
B13	V2	NAA	30	56.7 ± 2.89^{b}	43.3 ± 2.89^{d}	0.0	0.0	0.0
B14	V2	0	0	0.00 ± 0.00^{i}	100 ± 0.00^{a}	0.0	0.0	0.0

In the same column, means with distinct letters indicate significant differences (Tukey HSD test, α=0.05). PGR: plant growth regulator; IBA: indole-3-butyric acid; NAA: naphthaleneacetic acid.

Figure 1. Stages of *Huperzia squarrosa* cuttings treated with IBA (indole-3-butyric acid). (a) Initial cuttings; (b) - (c) V1 cuttings (apical cuttings) treated with 1500 ppm IBA for 30 min, showing rooting and growth after 2 months; (d) - (e) V2 cuttings (stem cuttings) treated with 1500 ppm IBA for 30 min, showing no growth after 2 months.

Overall, treatments with NAA generally produced lower rooting rates and poorer root quality compared to IBA. Based on these findings, treatment B3 (V1 cuttings treated with 1500 ppm IBA for 30 min) was identified as the most effective method for propagating *Huperzia squarrosa* through cuttings. This conclusion aligns with the findings of Le et al. (2019), who demonstrated that treating 6 cm long *H. serrata* cuttings with 1000 ppm IBA for 30 min was effective for propagation. Previous studies also support the efficacy of IBA in root induction (Zhang et al., 2009; Zuo et al., 2010), whereas NAA has shown varying results for *H. serrata* (Long et al., 2014). The use of V1 cuttings (shoot segments) for propagation of *Huperzia squarrosa* represents a novel approach compared to earlier studies, contributing to the preservation of this species through asexual propagation.

3.2. In vitro propagation for Huperzia squarrosa

Effects of duration and concentration of javel solution on sterilization efficiency

The effects of different concentrations and durations of Javel solution on sterilization efficiency were evaluated by disinfecting 3 cm shoots with 5% sodium hypochlorite at concentrations of 20%, 30%, and 40% (v/v) for 20 - 40 min, with results monitored after 10 days (Table 2, Figure 2). A two-way ANOVA revealed that both the concentration of Javel (%) and the duration of exposure (min) significantly affected infection and disinfection rates, with a notable interaction between them ($P = 0.041$). Sterilization efficiency varied significantly among treatments ($P < 0.05$). The proportion of disinfected samples increased from 2.38% at 20% Javel for 20 min to 54.76% at 30% Javel for 40 min, indicating improved disinfection efficiency with higher Javel concentrations and longer

exposure times. However, when using 40% Javel, disinfection efficiency initially decreased from 67.70% at 20 min to 61.90% at 30 min, followed by an increase to 73.80% at 40 min. ANOVA results indicated that treatment duration did not significantly affect disinfection efficiency ($P > 0.05$), while increasing Javel concentration from 20% to 40% significantly improved sterilization outcomes ($P < 0.05$).

Table 2. Effects of Javel solution concentration and duration on surface sterilization efficiency of *Huperzia squarrosa* shoots after 10 days

Treatments	Concentration of Javel (%)	Duration (min)	Infection rate (%)	Disinfection rate (%)
A1	20	20	$97.62^{a} \pm 4.12$	$2.38^{c} \pm 4.13$
A2	20	30	$76.20^{ab} \pm 18.00$	$23.80^{bc} \pm 18.10$
A3	20	40	$71.43^{abc} \pm 14.29$	$28.57^{abc} \pm 14.30$
A4	30	20	$61.90^{bc} \pm 14.87$	$38.10^{ab} \pm 14.87$
A5	30	30	$54.76^{bc} \pm 10.91$	$45.24^{ab} \pm 10.91$
A6	30	40	$45.24^{bc} \pm 10.91$	$54.76^{ab} \pm 10.91$
A7	40	20	$33.30^{bc} \pm 23.00$	$67.70^{ab} \pm 23.00$
A8	40	30	$38.10^{bc} \pm 16.50$	$61.90^{ab} \pm 16.50$
A9	40	40	$26.20^{c} \pm 18.00$	$73.80^{a} \pm 18.00$
		F (Concentration)	12.35^{**}	15.23^{**}
		F (Duration)	9.54^{**}	10.67^{**}
		F (Concentration x Duration)	3.47^{*}	4.12^{*}

In the same column, means with distinct letters indicate significant differences (Tukey HSD test; (*) $P \leq 0.05$; (**) $P \leq 0.01$).

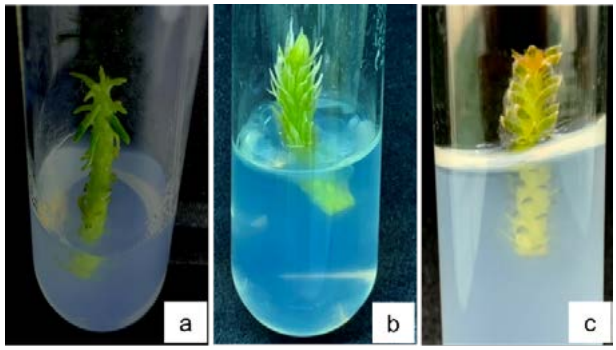

Figure 2. *Huperzia squarrosa* after sterilization.
(a) sterilized shoot; (b) - (c) bacterium and fungal-contaminated explants.

The lowest sterilization efficiency observed was 2.38% when using a 20% Javel solution for 20 min. In contrast, soaking shoot segments in a 40% Javel solution for 40 min achieved the highest sterilization rate of 73.80%. Thus, the most effective sterilization method in this study was treating the samples with 40% Javel for 40 min, followed by immersion in an antimicrobial solution (Ampicillin 2.5 mg/mL and Tetracycline 2.5 mg/mL) for 30 min.

Previous research on the *Huperzia* genus has used $HgCl_2$ for sterilizing sporangia and shoot tips, stem segments (Zhou et al., 2009), or combined $HgCl_2$ with H_2O_2 for shoot tip sterilization (Yang et al., 2008). While $HgCl_2$ is effective, it poses toxicity and handling challenges. In contrast, Javel has proven effective and safer for sterilization (Szypula et al., 2005). This study's approach of using 40% Javel for 40 min represents a novel and suitable method for sterilizing *Huperzia* cultures.

Influence of mineral salt on the growth of in vitro shoots

After 60 days of culture, significant differences between MS and ¼ MS mineral salt concentrations were observed ($P < 0.05$) in terms of survival rate, rooting rate, and shoot characteristics. Detailed data on these effects are presented in Table 3.

Table 3. Influence of mineral salt on the growth of *in vitro H. squarrosa* shoots

Mineral salt	Survival rate (%)	Rooting rate (%)	Number of root (roots/explant)	Morphological characters
MS	$15.00^{a} \pm 7.07$	$0.00^{a} \pm 0.00$	0.00	No growth, yellow leaves and death
¼ MS	$70.00^{b} \pm 14.10$	$55.00^{b} \pm 7.07$	2.09	Growing well, thick and strong stems, dark green leaves

In the same column, means with distinct letters indicate significant differences ($P \leq 0.05$). MS: Murashige & Skoog.

After 2 months of culture on ¼ MS mineral salt medium, shoots exhibited a rooting rate of 55% and a survival rate of 70%, with high-quality growth characterized by dark green stems and leaves (Figure 3.C1). In contrast, shoots cultured on MS mineral salt medium showed no growth, with explants gradually turning yellow and dying. Only 15% of the explants survived, and none developed roots (Figure 3.C2). Consequently, ¼ MS medium was selected as the optimal medium for the in vitro propagation of *Huperzia squarrosa*. These findings are consistent with previous studies indicating that low-nutrient mineral media are suitable for the micropropagation of species within the *Huperzia* genus (Szypuła et al., 2005; Yang et al., 2021b; Ho et al., 2022).

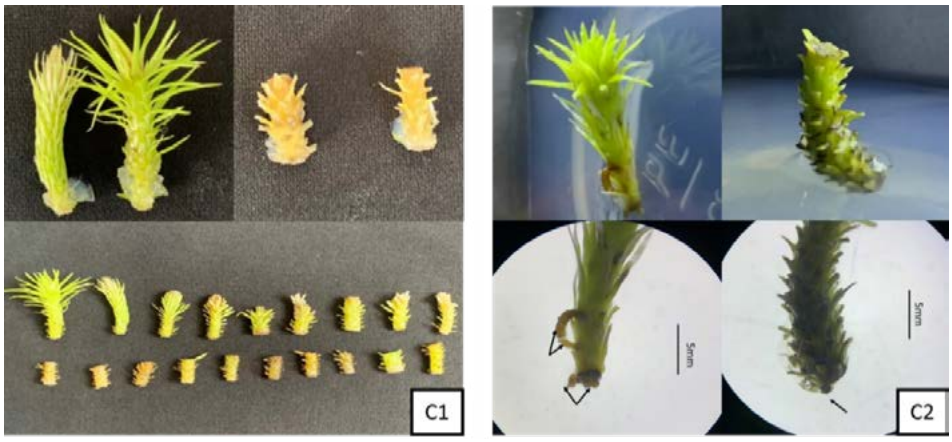

Figure 3. *Huperzia squarrosa* shoots after 60 days of culturing on MS (Murashige & Skoog) medium and ¼ MS mineral salt media. (C1) Shoots cultured on MS medium, showing no growth and yellowing; (C2) Shoots cultured on ¼ MS medium, displaying healthy growth and root development (arrows indicate root development).

Effects of plant growth regulators and glutamine concentrations on callus formation from in vitro shoot of Huperzia squarrosa

The results in Table 4 show that adding IBA significantly affects callus formation in *Huperzia squarrosa* shoots after two months of culture. The highest callus induction rate (13.3%) was achieved with Treatment S2, which included 0.01 mg/L IBA and 0.3 mg/L Kinetin. This suggests that this combination is the most effective for promoting callogenesis (Figure 4). Treatment S3, with 0.015 mg/L IBA and 0.3 mg/L Kinetin, had a slightly lower callus induction rate of 10.0%, similar to Treatment S5, which also included 3 mg/L Glutamine. The addition of Glutamine did not significantly improve callus formation compared to IBA and Kinetin alone. Treatment S4, with 0.01 mg/L IBA, 0.3 mg/L Kinetin, and 3 mg/L Glutamine, showed a lower callogenesis rate of 6.67%, indicating that this combination is less effective. Overall, the results highlight the influence of IBA on callogenesis and indicate potential interactions with other factors, although not all interactions are statistically significant. Further analysis might be necessary to explore these interactions in more detail.

Table 4. The percentage of callus induction after two months of culture

Treatment	IBA (mg/L)	Kinetin (mg/L)	Glutamine (mg/L)	Callogenesis rate (%)
S1	0	0	0	$0.00^{b} \pm 0.00$
S2	0.01	0.3	0	$13.3^{a} \pm 5.77$
S3	0.015	0.3	0	$10.0^{a} \pm 0.00$
S4	0.01	0.3	3	$6.67^{ab} \pm 5.77$
S5	0.015	0.3	3	$10.0^{a} \pm 0.00$
S6	0	0	3	$0.00^{b} \pm 0.00$
			CV (%)	3.33
			F-value	8.40^{**}

In the same column, means with distinct letters indicate significant differences (Tukey HSD test, α=0.01. IBA: indole-3-butyric acid.

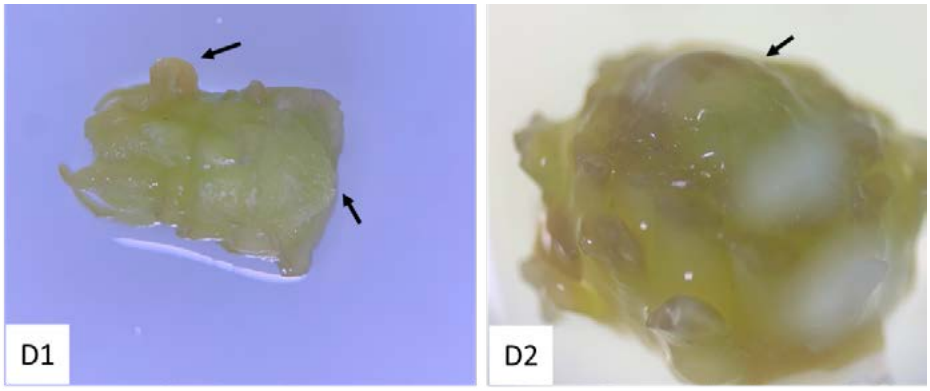

Figure 4. Callus induction after two months of culture. (D1) ¼ MS (Murashige & Skoog) medium supplemented with 0.01 mg/L IBA (indole-3-butyric acid) and 0.3 mg/L Kinetin, and (D2) ¼ MS medium supplemented with 0.015 mg/L IBA, 0.3 mg/L Kinetin, and 3 mg/L Glutamine. Arrows indicate callus development.

The callus formation process of *H. squarrosa* in this study only required a small amount of plant growth regulator, similar to the experiment conducted by Szypula et al. (2005). When 3 mg/L Glutamine was added to the culture medium, the callus formation rate for *H. squarrosa* decreased significantly to 6.67% in the S4 treatment and 10.0% in the S5 treatment (Figure 4). This result contrasts sharply with previous research on *H. serrata*, which reported a callus formation rate of 75.56% on ¼ MS medium supplemented with 0.015 mg/L IBA, 0.3 mg/L Kinetin, and 3 mg/L Glutamine (Le, 2021). This discrepancy could be due to genetic differences between *H. serrata* and *H. squarrosa*. Further research on the callogenesis of this medicinal plant is needed to optimize conditions for callus induction and growth, thereby enhancing the production of valuable medicinal metabolites. Understanding the effects of growth regulators, nutrient composition, and environmental factors can help improve the yield and quality of these bioactive compounds.

4. Conclusions

This study established effective methods for the propagation of *Huperzia squarrosa*. For cuttings, V1 shoot-tip segments treated with 1500 ppm IBA for 30 min achieved optimal rooting (100% rate, 6.1 roots per explant, 11.9 mm root length). *In vitro* conditions, the best sterilization method was 40% Javel for 40 min, followed by an antimicrobial solution, achieving a 73.8% disinfection rate. The ¼ MS medium supported shoot growth with a 70% survival rate and 55% rooting rate, and callus formation was highest with 0.01 mg/L IBA and 0.3 mg/L Kinetin. These methods provide a foundation for conserving *H. squarrosa*.

Conflict of interest

The authors declare no conflict of interest.

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Evaluating and predicting the impact of land use and land cover change on land surface temperature in Lac Duong district, Lam Dong province, Vietnam

Phuong T. Nguyen*, Vuong K. Trinh, Hien T. D. Tran, Trinh N. T. Ton, Lan T. T. Nguyen, & Anh N. Nguyen

Faculty of Land Resource and Agricultural Environment, University of Agriculture and Forestry, Hue University, Hue, Vietnam

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***Corresponding author**

Nguyen Thuy Phuong

Email: ntphuong.huaf@hueuni.edu.vn

ABSTRACT

Land use and land cover (LULC) change is a key factor influencing land surface temperature (LST) dynamics. This change reflects partly global warming and climate change at local and regional scales. This study aimed to evaluate the effect of LULC on LST change in Lac Duong mountainous district, Lam Dong province in the past 10 years (2013 - 2023), and predict the LST change in 2030. The study used satellite image data from Landsat 8 and 9 OLI to build LULC and LST maps and used the CA-ANN model to predict the LST map. The results showed that the forest land had the LST below 25°C, with the below 20°C LST area correlated negatively with the forest land area, while 20 - 25°C LST correlated positively, especially at the temperature of 22 - 25°C (R^2 = 0.97). The 22 - 25°C and 30 - 35°C temperature levels (R^2 = 0.76 and R^2 = 0.86) correlated sharply with the crop land area. The LST levels between 30 - 40°C reflected the built-up land and bare land with the highest correlation of R^2 = 0.68 and 0.88, respectively. The LST level 20 - 22°C represented the water body area (R^2 = 0.87). The LULC changes had an impact on the LST change in the past 10 years in Lac Duong district. While the forest land area decreased slightly by 0.5%, the cool LST area fell considerably by 10.5% compared to 10 years ago. An almost doubling of the cropland area also led to a doubling in the 25 - 35°C LST areas. In addition, the 35 - 40°C LST level started to happen in several regions. The LST change was predicted to keep increasing in 2030. The temperature was predicted to increase by 2 - 3°C with a maximum temperature of 42°C.

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1. Introduction

Changes in land use and land cover (LULC) impact directly on the surface biomass - the largest source and sink of terrestrial carbon (Pan et al., 2011), which helps to balance atmospheric CO_2. Therefore, LULC is one of the main drivers for limiting global warming and other aspects of climate change (Sudhakar & Kameshwara, 2010). Land surface temperature (LST) is the skin temperature of the ground derived from solar radiation (Li et al., 2023). The LST measures the radiative temperature of the vegetation canopy and the ground (Weng et al., 2004; Carrillo-Niquete et al., 2022). The LST is a crucial geophysical parameter of climate and biosphere related to surface energy, ecosystem health, and agricultural production (Bhunia et al., 2021; Li et al., 2023). Global temperature varies from -25 to 45°C (NASA, 2024). Monitoring LST dynamics helps assess atmosphere-land surface exchange processes in models and provides valuable surface condition data when combined with other physical properties such as vegetation and soil moisture (ESA, 2024). Land surface temperature is directly affected by LULC.

The LULC change is an inevitable activity of urbanization and socio-economic development. It has been happening worldwide (Rahman et al., 2017; Chang et al., 2018; Baig et al., 2022) and in Vietnam (Trinh & Cao, 2014; Hoang, 2016; Lai & Pham, 2018), leading to an increase in LST at the regional and global scales. Nyatuame et al. (2023) assessed an increase in settlement and cropland in the past and predicted a decrease in crop land and vegetation cover in Ghana in 2030 and 2050. Selmy et al. (2023) used Landsat images and CA-Markov Hybrid to analyze and predict LULC changes in arid regions. The results indicated that the accuracy of LULC categories was quite high with Kappa coefficients > 0.7. The simulation of the future LULC trends to 2050 was increasing urban and crop land.

Increasing temperature occurs not only in urban areas but also in rural and mountainous areas. However, research on LST changes in these areas is still limited. Assessing the impacts of LULC changes on LST changes helps to predict the increasing trend of LST. Thus, managers can evaluate past decisions, and better understand the impacts of current decisions before implementation (NOAA, 2024). It helps managers develop strategies to balance conservation, and handling conflicts between usage and development pressures. LULC and LST maps can be produced by satellite imagery data. Seyam et al. (2023) identified LULC using remote sensing and GIS approach in Mymensingh, Bangladesh with good accuracy from 87.2% to 89.6%.

Lam Dong is located in the Central Highlands and has favorable climate and land conditions for agricultural and forestry development and tourism development. However, Lam Dong is facing some environmental problems related to urban planning and development, deforestation, and landscape destruction. As a mountainous district in the north of Lam Dong, Lac Duong has forest land accounting for 89% of the total natural area, thus, it plays a major role in regulating the climate and creating landscapes for the whole province. The massive use of greenhouse systems on agricultural and forestry lands, and land use change are becoming more and more complicated in Lac Duong district. Therefore, to provide a more scientific basis and properly assess the ongoing negative environmental impacts, this study aims to assess the impact of LULC changes on LST in Lac Duong district during the 2013 - 2023 period and forecast LST changes in 2030.

2. Materials and Methods

2.1. Study site and data

Lac Duong district has administrative boundaries of 5 communes and one town including Lac Duong town, Lat commune, Da Sar commune, Da Nhim commune, Da Chais commune, and Dung K'No commune. It has a temperate climate with temperatures ranging from 11 - 27°C and an average total rainfall of 1,700 - 1,800 mm, so it is mild and cool all year round. High mountain terrain (> 1,600 m) accounts for about 80 - 85% of the natural area of the whole district (PCLDD, 2023). The position of Lac Duong district in Lam Dong province is shown in Figure 1.

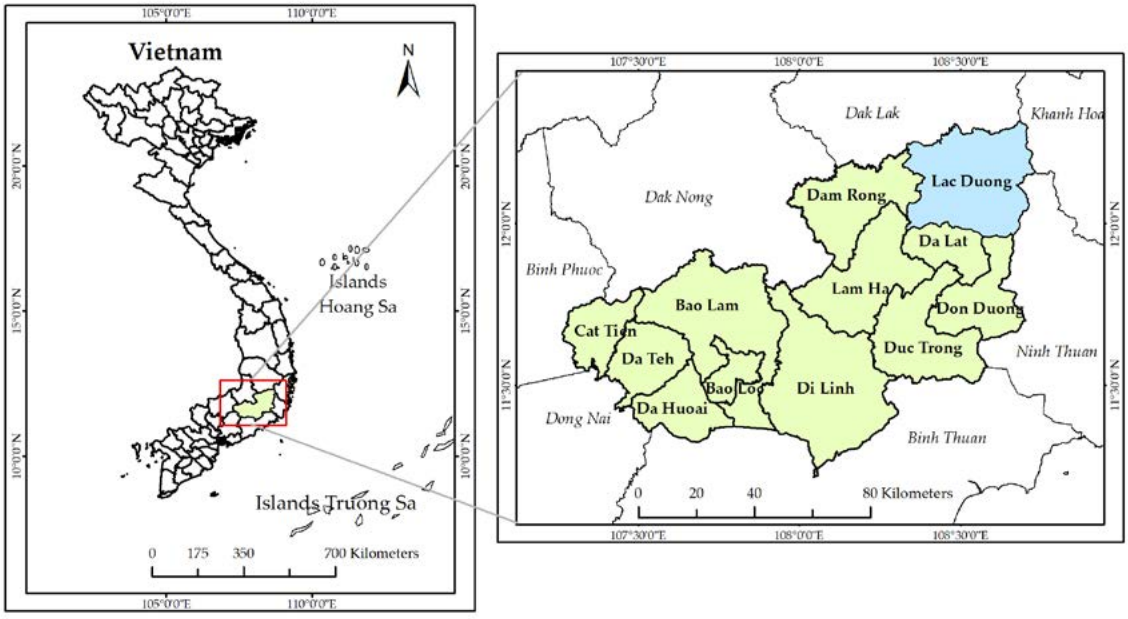


Figure 1. The geographical location of Lac Duong district.

Landsat 8 and 9 OLI satellite imagery data were downloaded from the United States Geological Survey - USGS website. Two satellite imageries (124/051 and 124/052) captured in May 2013 and in March 2015, 2017, 2019, 2021, and 2023 are used to build LULC and LST maps in this study. The satellite imageries meet cloudy conditions (< 10%) and have a spatial resolution of 30 x 30 m.

2.2. LULC and LST maps

2.2.1. LULC map

This study classifies land use and land cover into five types: Forest land, Crop land, Built-up land, Bare land, and Water body. LULC maps are constructed from satellite image data sources combined with a supervised classification method - Maximum Likelihood Classification (MLC) algorithm. The MLC method constructs probability density functions for each class. Each class is characterized by two features including mean vector and covariance matrix, from which the statistical likelihood is calculated for each class. The algorithm will then identify each remaining pixel and will be assigned to the class that it is most likely to be a member of according to the Bayesian formula (Sun et al., 2013).

$$M_k(x) = \ln \mathrm{P}(G_k) + \ln \frac{|S_k^{-1}|^{1/2}}{2\pi^{m/2}} - \frac{1}{2}(x-\mu_k)^T S_k^{-1}(x-\mu_k) \quad (1)$$

Where, is the vector of each pixel; is the likelihood function of x belonging to class k; and are the vector and covariance matrix of class k.

2.2.2. LST map

The LST is calculated using spectral reflectance and correction formulas depending on the image type. The process includes six computation steps: Top of Atmospheric (TOA) spectral radiance, TOA to Brightness Temperature conversion, NDVI, the proportion of vegetation, Emissivity, and Land Surface Temperature. Its formula can be shown as follows (Sajib et al., 2020):

$$LST = \frac{C_2}{\lambda_{eff,TIR10} \cdot ln\left(\frac{C_1 \cdot \tau_{TIR10} \cdot LSE_{TIR10}}{\lambda_{eff}^5 \cdot (L_{TOA10} - L_{up} - \tau_{TIR10}(1-LSE_{TIR10}) \cdot L_{down})} + 1\right)} \quad (2)$$

Where, is the effective wavelength of band Thermal Infrared (TIR); is the Top-of-Atmosphere thermal radiance; is the band TIR average atmospheric transmittance; is the emissivity of the band TIR; C_1 and C_2 are Planck's first and second radiation constants (C_1 = 1.19104 x 10^8 W mm^4 m^{-2} sr^{-1} and C_2 = 1.43877 x 10^4 mm K); L_{up} and L_{down} are the upwelling and downwelling radiance in the atmosphere obtained in band TIR.

2.2.3. Map accuracy assessment

The accuracy of the LULC maps was evaluated using the Kappa coefficient (K) according to the formula 3 (Cohen, 1960). This coefficient presents the measurement of rater reliability, which is computed based on the error matrix of the class identified at 500 randomly selected plots, which were created by Create Random Points in ArcToolbox in ArcMap.

$$K = \frac{N\sum_{i=1}^{r} x_{ii} - \sum_{i=1}^{r}(x_{i+} \cdot x_{+i})}{N^2 - \sum_{i=1}^{r}(x_{i+} \cdot x_{+i})} \quad (3)$$

Where, r - the number of rows in matrix; x_{kk} - the number of observations in row i and column i respectively; x_{i+} and x_{+i} - the total number of samples in row i (positive error) and total number of samples in column i (negative error), respectively; N - the total number of observations.

The coefficient K < 0.40 is low accuracy, 0.41 - 0.60 is moderate accuracy; 0.61 - 0.80 is substantial accuracy, and > 0.81 is perfect accuracy (Li, 2010).

For LULC maps, the study compared the classification results using the MLC method and real classification, which is examined based on the high-resolution images from Google Earth (Islami et al., 2022; Mehra & Swain, 2024).

The accuracy of LST maps built on remote sensing data can be assessed by three methods: temperature-based (T-based), radiance-based (R-based), and cross-validation (Li et al., 2013). Nevertheless, due to the restrictions of time and research finance, this study could not assess the accuracy using the three methods mentioned. Therefore, the reliability of the LST maps is based on the high accuracy of several published studies (Kafy et al., 2021; Onačillová et al., 2022; Nugraha et al., 2024).

2.3. LST prediction

The study uses the Cellular Automata - Artificial Neural Network (CA-ANN) model to predict LST changes by analyzing the trends of land surface temperature changes. The CA-ANN model is a combination of the spatial operation of the cellular automata model and the artificial neural network system. The prediction of LST changes uses three types of input data: (1) NDVI fluctuations over the years; (2) LST data transfer matrix; and (3) land use planning in 2030. This model is run on the MOLUSCE plugin software in QGIS 2.8.9.

The analysis results of LST changes in the period 2015 - 2023 are a basis for predicting LST map in 2030. To ensure the reliability of predictive modelling, the study was first used the CA-ANN model to estimate the LST map in 2023, and then evaluated the accuracy of this map by comparing it with the LST map calculated from remote sensing images through the Kappa coefficient.

3. Results and Discussion

3.1. LULC changes

The LULC maps from 2013 to 2023 are shown in Figure 2. The LULC maps built had high and very high accuracy with the Kappa coefficients ranging from 0.76 to 0.85 (Table 1). The Kappa coefficients of the LULC maps were approximately the same as some published studies using different satellite image data sources. Specifically, the study by Islami et al. (2022) constructed LULC maps in Sadar Watershed, Mojokerto Regency, Indonesia using Landsat 5 and Sentinel-2 images, with Kappa coefficients ranging from 0.74 - 0.80. Therefore, they are suitable and reliable for assessing the LULC changes in Lac Duong district.

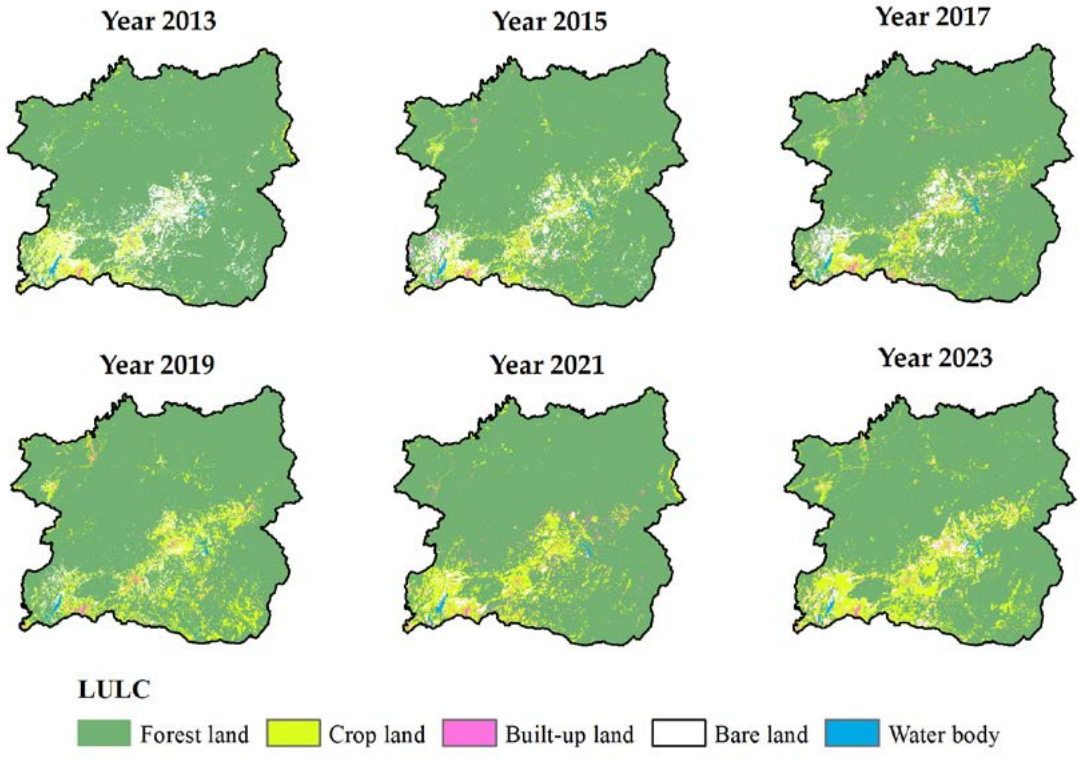


Figure 2. Land use and land cover (LULC) maps from 2013 to 2023.

Table 1. Error matrix and accuracy of land use and land cover classification

Year	ClassValue	C_1	C_2	C_3	C_4	C_5	Total	U_Accuracy	Kappa
2013	C_1	432	0	0	0	0	432	1	0
	C_2	16	33	1	4	0	54	0.61	0
	C_3	2	1	5	0	0	8	0.63	0
	C_4	0	0	0	5	0	5	1	0
	C_5	0	0	0	0	1	1	1	0
	Total	450	34	6	9	1	500	0	0
	P_Accuracy	0.96	0.97	0.83	0.56	1	0	0.95	0
	Kappa	0	0	0	0	0	0	0	0.78
2015	C_1	404	4	0	1	0	409	0.99	0
	C_2	3	32	0	2	0	37	0.86	0
	C_3	0	0	1	0	0	1	1	0
	C_4	4	9	3	36	0	52	0.69	0
	C_5	0	0	0	0	1	1	1	0
	Total	411	45	4	39	1	500	0	0
	P_Accuracy	0.98	0.71	0.25	0.92	1	0	0.95	0
	Kappa	0	0	0	0	0	0	0	0.83
2017	C_1	426	0	0	0	0	426	1	0
	C_2	7	26	1	4	0	38	0.68	0
	C_3	1	0	2	0	0	3	0.67	0
	C_4	1	5	0	27	0	33	0.82	0
	C_5	0	0	0	0	0	0	0	0
	Total	435	31	3	31	0	500	0	0
	P_Accuracy	0.98	0.84	0.67	0.87	0	0	0.96	0
	Kappa	0	0	0	0	0	0	0	0.85
2019	C_1	406	4	2	12	0	424	0.96	0
	C_2	8	34	0	4	0	46	0.74	0
	C_3	0	0	4	1	0	5	0.80	0
	C_4	0	3	0	22	0	25	0.88	0
	C_5	0	0	0	0	0	0	0	0
	Total	414	41	6	39	0	500	0	0
	P_Accuracy	0.98	0.83	0.67	0.56	0	0	0.93	0
	Kappa	0	0	0	0	0	0	0	0.76

2021	C_1	374	9	0	5	0	388	0.96	0
	C_2	10	64	2	7	0	83	0.77	0
	C_3	0	1	4	0	0	5	0.80	0
	C_4	0	0	0	23	0	23	1	0
	C_5	0	0	0	0	1	1	1	0
	Total	384	74	6	35	1	500	0	0
	P_Accuracy	0.97	0.86	0.67	0.66	1	0	0.93	0
	Kappa	0	0	0	0	0	0	0	0.82
2023	C_1	406	10	0	7	0	423	0.96	0
	C_2	4	37	1	5	0	47	0.79	0
	C_3	0	0	2	1	0	3	0.67	0
	C_4	0	3	0	23	0	26	0.88	0
	C_5	0	0	0	0	1	1	1	0
	Total	410	50	3	36	1	500	0	0
	P_Accuracy	0.99	0.74	0.67	0.64	1	0	0.94	0
	Kappa	0	0	0	0	0	0	0	0.79

C_1: Forest land, C_2: Crop land, C_3: Built-up land, C_4: Bare land, C_5: Water bodies.

Table 2. The areas of land use and land cover (LULC) types from 2013 to 2023

Index	LULC	2013	2015	2017	2019	2021	2023
Area (km^2)	Forest land	1085.31	1087.00	1084.51	1080.64	1080.10	1080.41
	Crop land	65.29	72.70	75.84	96.05	109.81	122.95
	Built-up land	23.36	24.06	24.94	25.41	26.90	26.27
	Bare land	89.68	81.57	79.80	62.51	47.67	35.10
	Water body	4.78	3.11	3.34	3.81	3.95	3.70
	Total	1268.43	1268.43	1268.43	1268.43	1268.43	1268.43

The areas of five land uses/land cover types are presented in Table 2. The total area of Lac Duong district was 1,268.4 km^2, of which forest land accounted for the majority, with about 85%. From 2013 to 2019, the forest land area decreased by about 6.4 km^2 (about 0.6% of the forest area) from 1,087.0 km^2 to 1,080.64 km^2 before remaining stable until 2023. The decrease in forest land area might be due to land-hiring companies and enterprises building a large number of greenhouses on the forest land. These areas are still forest land, yet remote sensing image interpretation and the MLC model identify them as croplands. In the following years, local authorities proceeded to dismantle greenhouses built on forest land, so the forest land area began to gradually increase again. The changing trends of crop land and bare land were quite evident in the past 10 years. The area of crop land nearly doubled from 65.29 to 122.95 km^2. In contrast,

the area of bare land decreased considerably by 61% from 89.68 km^2 to 35.10 km^2. The results of the change in the built-up land area indicated that the urbanization rate has slightly increased in Lac Duong district. After 10 years, the area of built-up land increased by about 3 km^2 (about 12.5% of this land use) to 26.67 km^2. Another land use type that was also relatively stable over time was the water body area. The slight fluctuation of this cover could be mainly due to rainfall at the time of taking the images because there was no water surface area leveling for construction and other purposes.

In short, the crop land and built-up land areas tended to rise gradually, meanwhile, the bare land area decreased in the period 2013 - 2023.

3.2. LST changes

The LST maps of years are shown in Figure 3. The surface temperature was classified into 8 levels and their areas are listed in Figure 4. The research results indicated that the low-temperature levels below 25°C were distributed in forest land, accounting for most of the district's area, ranging from 78.1% to 88.9%. This area peaked in 2013 (88.9%) and reached its lowest points in 2023 and 2015 at 79.2% and 78.1%, respectively. The areas of LST levels above 25°C presented mainly cropland, built-up land, and bare land. This could be observed on the LST maps. The temperature levels above 25°C occupied 11.2 - 21.1% of the total area of the district in the past 10 years.

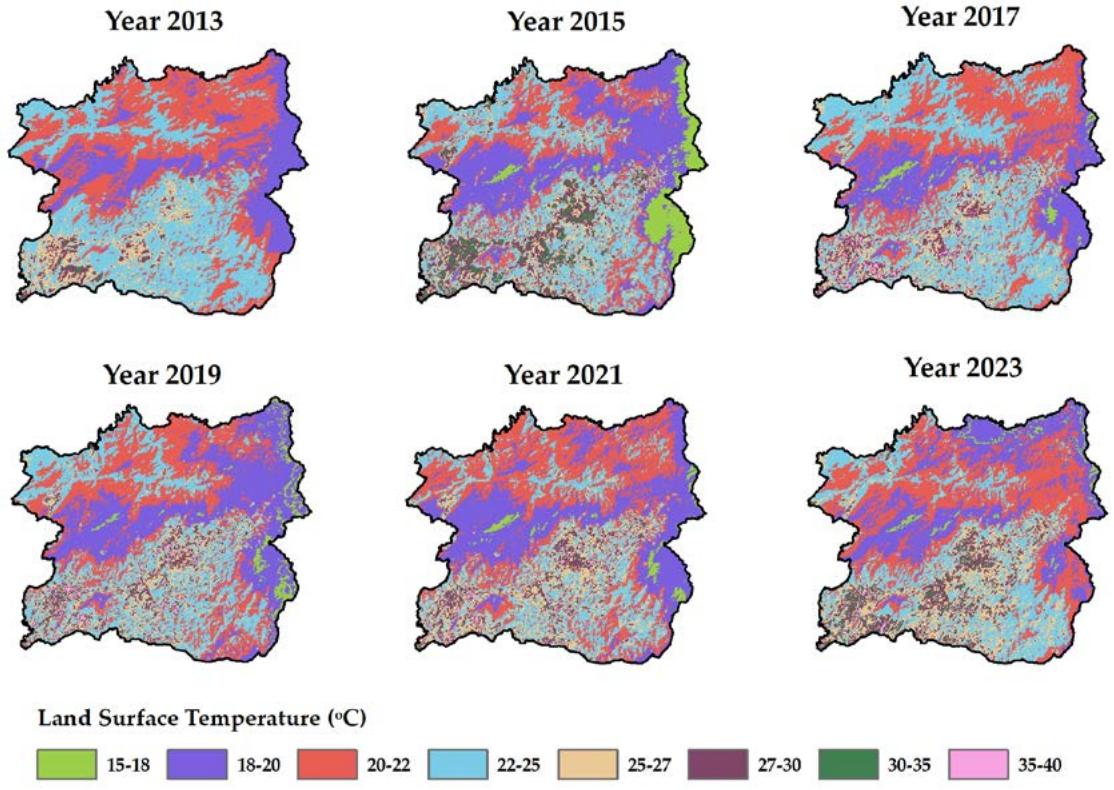


Figure 3. Land surface temperature maps from 2013 to 2023.

The LST change shows the trends over time: First, the LST difference in the district was from 15 to 40°C since 2015, higher than in 2013 (18 - 35°C). Second, the LST level above 30°C generally had a considerable increase from 3.2 km^2 to 33.1 km^2, a 7-fold increase after ten years. Third, the LST distribution in 2015 was different from other years. The areas with LST levels 15 - 18°C and 27 - 40°C were significantly larger. It could be directly related to the normalized difference vegetation index (NDVI). In 2015, the area with a low NDVI (0.15 < NDVI < 0.23) was significantly higher than those in other years, leading to an increase in the hot temperature area (27 - 40°C). In contrast, the area with a high NDVI index (0.34 < NDVI < 0.5) in this year was significantly lower than those in other years, leading to a decrease in the cool temperature area (15 - 18°C). LST changes can be influenced by factors such as land use and land cover, natural biogeography, background climate, radiation intensity, thermal resistance, evapotranspiration, soil heat flux and air temperature (Kummari et al., 2022; Patel et al., 2024). In this case study, satellite images were caught very close to each in the studied years, so the climate difference was minimized. Therefore, the LST changes over the years were mainly related to land use, land cover changes. The lowest temperature could be influenced by the NDVI index and the highest temperature was influenced by the built-up land.

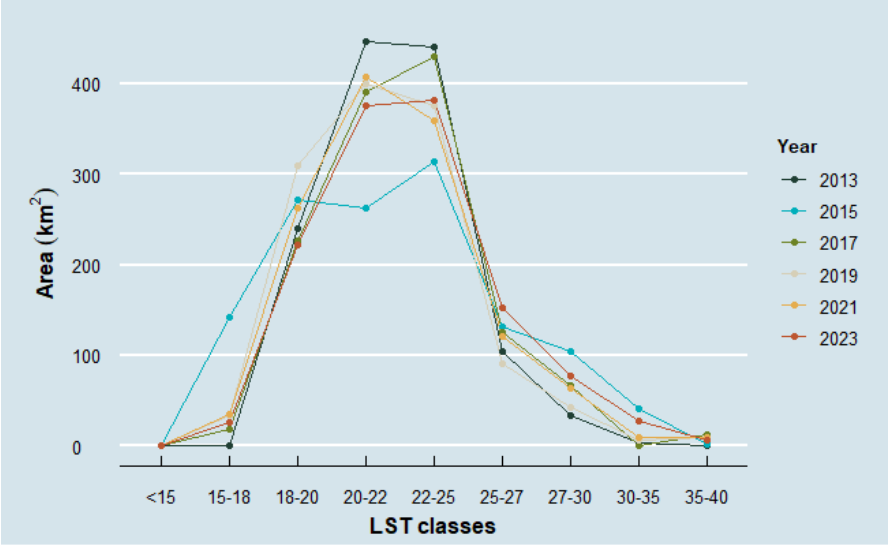


Figure 4. The areas of land surface temperature (LST) classes in 2013 - 2023.

This LST increase was also consistent with the ongoing global warming trend in the world. The LST increasing trend in Lac Duong was similar to that of the global. The global average surface temperature increased by 1.36°C, warmer than the pre-industrial standard (1850 - 1900) used to measure global warming (Nasa, 2023). A study by Tian et al. (2023) in Nanjing City, China also gave similar results but with a lower rate of LST increase than Luoyang County, within 30 years (1990 - 2020), the average LST increased by 2.24°C.

The LST distribution has been tending to go up to temperature and increase the area of higher temperatures over the past 10 years.

3.3. Effects of LULC on LST

Land surface temperature reflects the solar radiation energy of the land surface, so land surface characteristics such as vegetation index, topographic elements, soil moisture, water, land use type, land cover are very important factors affecting LST. The study assessed the impact of LULC changes on LST by evaluating the correlation between the area of a LULC type and the areas of eight LST levels (15 - 18°C, 18 - 20°C, 20 - 22°C, 22 - 25°C, 25 - 30°C, 30 - 35°C, and 35 - 40°C). The area of the temperature level that shows a sharp correlation with a LULC type, then that LST level reflects the characteristics of corresponding LULC.

Forest land exhibited LST levels below 25°C, and agricultural land, built-up land, and bare land represented LST levels ranging from 25 to 40°C. Thus, the impact of LULC changes on LST changes was rated accordingly (Figure 5). In addition, due to a considerable difference in NDVI, the data in 2015 was not included in the correlation assessment.

The results indicated that forest land area had an obvious negative correlation with LST levels 15 - 18°C (R^2 = 0.81) and 22 - 25°C (R^2 = 0.97). Meanwhile, the areas with LST levels 18 - 22°C had an unobvious correlation with the forest land. The forest quality like forest health and biomass density is a key factor affecting the lower temperature levels (15 - 18°C, 18 - 20°C, and 20 - 22°C). The denser the forest biomass is, the higher and denser the forest biomass is, the lower the LST is. Thus, the total forest area did not show a clear positive correlation with the lower-temperature levels (from 15°C to 22°C), even showing a negative correlation. The very good negative correlation of forest land area with the 15 - 18°C temperature zone (R^2 = 0.81) may be due to the fact that the total forest land area increases but the area with very dense biomass decreases. On the other hand, the forest land with moderate biomass could reflect the temperature of 22 - 25°C, so the LST levels had a fairly good correlation with the total forest area. Forests have dense vegetation which has the great adsorption capacity of solar radiation. Vegetation has the ability to 80% of incoming visible radiation, reflecting 10% and transmitting 10% (Shahidan et al., 2006). Thus, the forest land reflected the lowest LST. The result indicated that the forest area has decreased by only 0.5%, yet the LST level below 25°C has decreased by 10.5% over the past 10 years.

For crop land, the areas with LST levels of 22 - 35°C increased significantly when this land use type increased. This land use type correlates better with the 22 - 25°C and 30 - 35°C temperature levels (R^2 = 0.76 and R^2 = 0.86). Although the agricultural land was covered good vegetation (just behind the forest), the use of a large number of greenhouses in recent years in Lac Duong district has increased the surface temperature for this land cover. According to local government's report, Lac Duong has 1,648 ha of greenhouses in 2023, an increase of 182 ha compared to 2022. The materials of greenhouses increased the reflection of incoming solar radiation. An approximate doubling of the area of this LULC type also nearly doubled the 25 - 35°C LST area. A study on cropland in Yayo district, in Ethiopia also showed that the average LST on this land use type increased from 22.8°C in 1986 to 27 °C in 2003 by Moisa et al. (2023), which was identified as due to climate change.

The built-up land indicated a better correlation with the high-temperature area. The LST levels 27 - 40°C reflected built-up land. In particular, the

35 - 40°C level correlated the best with the built-up land (R^2 = 0.68). This land use type had high temperatures due to properties of construction materials (concrete, stone, asphalt...) which has high thermal conductivity and absorbs heat well and quickly, thus heating the surface quickly. In addition, due to poor water permeability and limited water evaporation, heat energy is retained on the surface of the material much higher and longer than in areas with green trees or wet soil. Zhang et al. (2023) also evaluated that the urban lands had the LST > 30°C. From the analysis of satellite data from 1992 to 2020, Jagtap et al. (2024) found that expanding construction areas caused a significant temperature increase of 4.3°C in built-up areas in Pakistan.

Meanwhile, the water body area indicated a sharp correlation with the LST level 20 - 22°C (R^2 = 0.87). For bare land, this land cover type had a very sharp correlation with the LST level 30 - 35°C (R^2 = 0.88), and lower correlations with the LST levels 27 - 30°C and 35 - 40°C (R^2 = 0.49 and 0.43, respectively). The water surfaces, despite their low thermal response, are known to be the best absorbers of radiation and provide evaporative cooling where water evaporates as solar radiation reaches the water surface and removes heat, cooling surrounding features. Increasing water accessibility increased evaporation, providing additional cooling during the day.

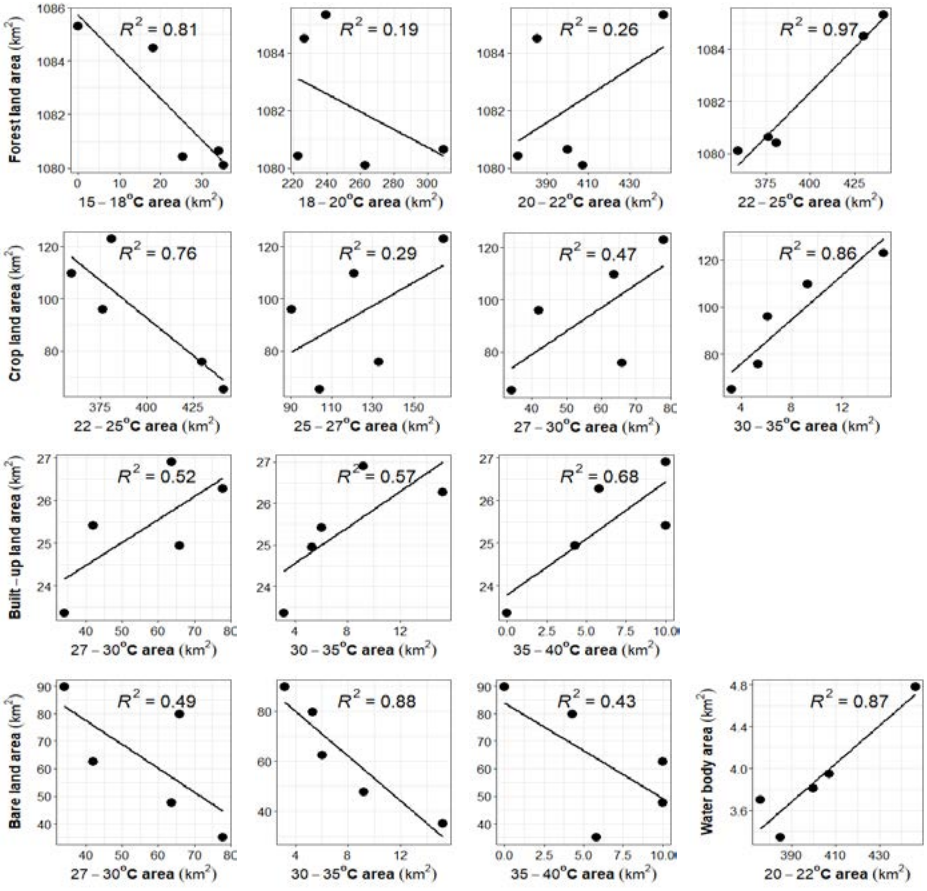


Figure 5. Correlation between areas of land use and land cover type and area of land surface temperature class.

In summary, the LST level 30 - 35°C had a better correlation with cropland area than with built-up land ($R^2 = 0.86$ and $R^2 = 0.57$). This result reflected the significant increase in the number of greenhouses in agricultural production, leading to the surface temperature increase of cropland in Lac Duong district over the past 10 years. The cropland increased 1.1 times in 2023 compared to 2021, and nearly 2 times compared to 10 years ago. Meanwhile, the LST level 30 - 35°C increased 1.7 times in 2023 compared to 2021, about 2.5 times compared to 2019, and about 5 times after 10 years. According to the local government report, Lac Duong district had nearly 0.24 km^2 of greenhouses in 2022. Although the total area of built-up land and bare land decreased considerably to one-third, from 178.3 km^2 to 61.3 km^2, the areas with high-temperature (30 - 40°C) still increased about 6.5 times, from 3.19 km^2 to 21.07 km^2 after 10 years.

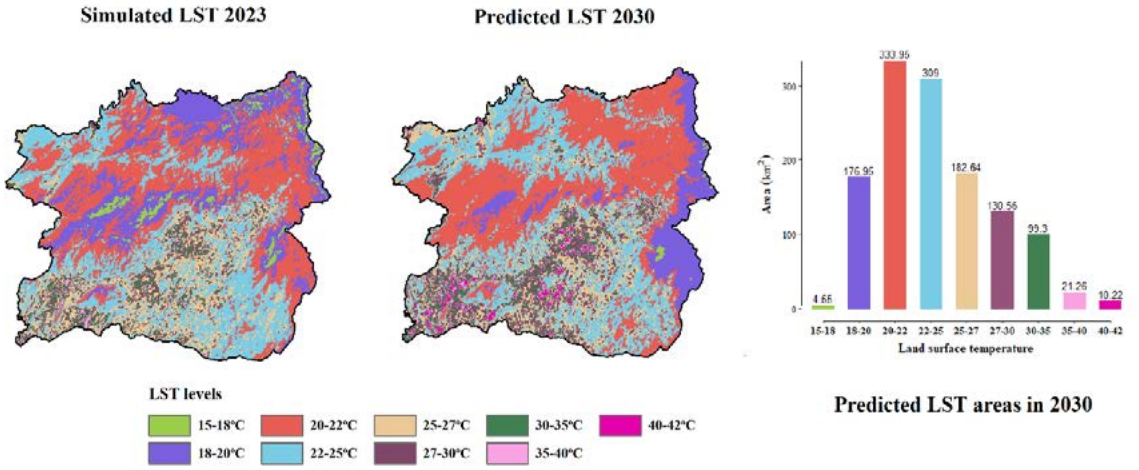
3.4. LST prediction

The mentioned assessment indicated that the LST change in Lac Duong was affected by three main factors, which are the basis for predicting the LST map in 2030. Firstly, forest biomass (represented by the NDVI index) was an important factor affecting the LST change in low-temperature areas. Except for 2015, the NDVI on forest land remained relatively stable. Therefore, the study used the fluctuations of the NDVI map for the remaining years to create a LST forecast map. Secondly, using greenhouses in agricultural production was one of the causes of the increase in LST level 30 - 35°C. However, the local government is gradually dismantling 19 hectares of greenhouses on forestry land, and restricting farmers from using greenhouses the next time. Third, the study is based on the Land Use Planning map to 2030 of Lac Duong district according to Decision No. 1869/QD-UBND as the basis for predicting the 2030 LST map (PCLDD, 2023).

To ensure the reliability of the predicted LST map, the study evaluated the accuracy of the simulated 2023 LST map. The 2023 LST map was estimated by the CA-ANN model shown in Figure 6. The accuracy of this simulated map when compared with the 2023 LST map calculated by remote sensing images gave a Kappa coefficient of 0.73. Therefore, this algorithm met the reliability for predicting the 2030 LST map.

The predicted 2030 LST map and the area statistics of the LST levels are shown in Figure 6. The forecast results indicated that LST was expected to continue to increase by 2 - 3°C, ranging from 15°C to 42°C. The low-temperature area was predicted to decrease gradually and the high-temperature area continued to increase. The LST level of 15 - 25°C tended to decrease compared to 2023, especially the area of 15 - 18°C decreased to only one-fifth of its area in 2023. The LST levels from 25 to 40°C were forecasted to increase considerably. In addition, the area with LST level 40 - 42°C was predicted to appear.

Although the limitation of greenhouse use in local agricultural production has a positive impact on LST, land use change could continue to increase the temperature in the future. Therefore, in addition to measures to limit the use of greenhouses and reforestation local authorities should combine measures to improve forest quality (increase biomass and forest health) and restrict bare land. In addition, urban planning should focus on increasing green spaces in urban



areas to reduce the increase in surface temperature on built-up land.

Figure 6. Simulated and predicted land surface temperature (LST) maps in 2023 and 2030, and LST statistics in 2030.

4. Conclusions

Forest land reflected the temperature below 25°C. Forest land area correlated with the LST 22 - 25°C. Water land mainly reflected temperature levels of 20 - 22°C. The cropland ranged from 25 to 35°C. Built-up and bare lands reflected the temperature level of 30 - 40°C, in which the highest temperature area represented built-up land. The LULC changes significantly affected the LST changes in the past 10 years in Lac Duong district. Even though the forest area decreased slightly by only 0.5%, the low-LST area below 25°C reduced considerably by 10.5% due to decreased forest quality. An almost doubling of the cropland area also led to doubling the 25 - 35°C area doubling. The area of 30 - 40°C LST levels increased over ten times due to increasing the built-up land area by 12.5%. In addition, a high temperature of 35 - 40°C appeared in this period.

The study predicted the LST change trend in 2030. The results revealed that LST would continue to increase in 2030 with temperature fluctuations ranging from 15 - 42°C. The lower temperature areas (below 25°C) was predicted to decrease and the higher temperature areas (35 - 42°C) to increase. Some factors like satellite image sources may improve the accuracy of LST maps that this study has not evaluated. Therefore, future studies should focus on the application of more different satellite image sources and other prediction models.

Conflict of interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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Effects of water quality parameters on growth performance of intensive shrimp pond *(Litopenaeus vannamei)*

Dung D. Do[1*], Anh H. Le[1], Van V. Vu[2], Duong P. T. Nguyen[3], & Hung V. Can[1]
[1]Institute of Environmental Science, Engineering and Management, Industrial University of Ho Chi Minh City, Ho Chi Minh City, Vietnam
[2]NTT Hi-Tech Institute, Nguyen Tat Thanh University, Ho Chi Minh City, Vietnam
[3]Office of Science Management and International Affairs, Industrial University of Ho Chi Minh City, Ho Chi Minh City, Vietnam

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***Corresponding author**

Do Doan Dung
Email:
dodoandung@iuh.edu.vn

ABSTRACT

Currently, to monitor water quality, farmers in Vietnam need to analyze various indicators which increase production costs. In addition, the limitation of analytical facilities and techniques is a challenge. The objective of this experiment was to evaluate the influence of water quality parameters on shrimp growth rates and the seasonal fluctuation in water quality. A total of 4 modules were randomly selected and analyzed daily for 8 critical parameters during rainy and dry seasons. The SPSS ver.26 was used to evaluate the correlation between multi-parameters and their impact on the performance of shrimp ponds. The results showed that shrimp growth was influenced by salinity, nitrite (NO_2^-), alkalinity and pH about 80.4%, 75.6%, 67.8%, and 55.7%, respectively. Moreover, water quality fluctuated more during the rainy season than during the dry season. Some parameters that exhibited high fluctuation in ponds were dissolved oxygen (DO) and nitrite.

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1. Introduction

Water quality is an essential natural indicator for assessing the life cycle of shrimp pond ecosystems (Mahmudi et al., 2022; Ariadi et al., 2023). As the most sensitive spice Shrimp health is negatively impacted when the values of physical, chemical, and mineral parameters exceed defined limits (Boyd, 2017; Ariadi et al., 2019). Moreover, fluctuations in these parameters can adversely affect shrimp performance (Hukom et al., 2020). That's why, increasing understanding of the information on contamination and limiting its effect can reduce the death rate of shrimp by expressing the daily water quality data (Ma et al., 2013).

Along with water quality monitoring, shrimp growth rates are a crucial indicator of effective farm management. Early research showed that water quality parameters had different influences on the length of the shrimp culture period. (Ariadi et al., 2019; Chen et al., 2019) concluded that enhancing the water recirculation rate can lead to better water quality and stimulate shrimp growth. In detail, dissolved oxygen (DO) is an essential abiotic parameter for evaluating water quality in intensive whiteleg shrimp (*Litopenaeus vannamei*) farming systems and needs to be measured periodically (Madenjian, 1990; Supriatna et al., 2017; Osaka et al., 2022). The ideal average dissolved oxygen concentration for this process falls between 4 and 6 mg/L (Ferreira et al., 2011), and its solubility is greatly affected by salinity and water temperature (Boyd, 2017). When DO levels are low, the toxicity of ammonia gas can increase, making aquatic organisms more susceptible to stress (Sriyasak et al., 2015). Furthermore, physical parameters, such as temperature and salinity, have significant impacts on shrimp growth (Ponce-Palafox et al., 1997; Ren et al., 2021; Atikah & Hasibuan, 2023). In some studies, the fluctuation of alkalinity and hardness concentration affects shrimp production (Boyd, 2016; Boyd et al., 2016; Ge et al., 2023). Thus, farmers strive to avoid this in aquaculture ponds. Additionally, the value decreases could prevent shrimp growth in intensive farming ponds. Meanwhile, Phan et al. (2022) and Valencia-Castañeda et al. (2019) showed that high level of ammonia, nitrate and nitrite (NO_2^-) are toxics the survival of whiteleg shrimp. In general, poor water quality reduces shrimp growth and increases mortality rates due to ecosystem fluctuations (Anand et al., 2019; Kumar, 2023; Srinivas & Venkatrayulu, 2023). To evaluate immediately and more accurately the farming environment, several parameters should be monitored in shrimp cultivation. This can be a result of high cost and reduction in price competition in the new normal period (Nikolik et al., 2024). Furthermore, in the past, some researchers have conducted an investigation of water quality impacts on the shrimp culture system using the initial weight or final weight or simulation conditions in some experimental ponds. Thus, it cannot demonstrate the daily effectiveness of water characteristics on shrimp growth in real conditions.

In this study, the size of shrimp was collected more frequently than in previous studies. Together with daily water collection, 8 significant water parameters were selected. The purpose of determining the relationship between variations in dissolved oxygen parameters and water quality and their impact on shrimp growth rates in intensive farming cycles can be achieved. This helps farmers determine the most important indicator for a successful farming cycle and supports in making decisions.

2. Materials and Methods

2.1. Study area

This research collected actual farming activities data at Minh Phu - Loc An Aquaculture Ltd, a 300-ha intensive shrimp farm in Vietnam, situated at Ba Ria- Vung Tau province, the southeast delta of Vietnam (Figure 1). Seawater is directly collected by a 20 km seawater pipeline supply for shrimp ponds. Each module includes 10 post-larvae ponds, 20 growing ponds and individual water treatment systems. Each post-larvae pond had 232 m^2 and the growing pond had 834 m^2 with 1.0 m water depths in both types. To ensure the required dissolved oxygen levels, paddlewheel aerators and bottom air diffusers were installed in each tank. Before stocking, pH and alkalinity were tested at the settlement pond, and it was pumped into post-larvae ponds. Each module is controlled separately following the module manager. A two-stage farming method was applied. The nursery phase typically lasts 12 - 15 days, followed by the grow-out phase. Normally, there is no water exchange in post-larvae ponds. The water exchange rate in grow-out ponds is 10 - 12% depending on the module manager's decision.

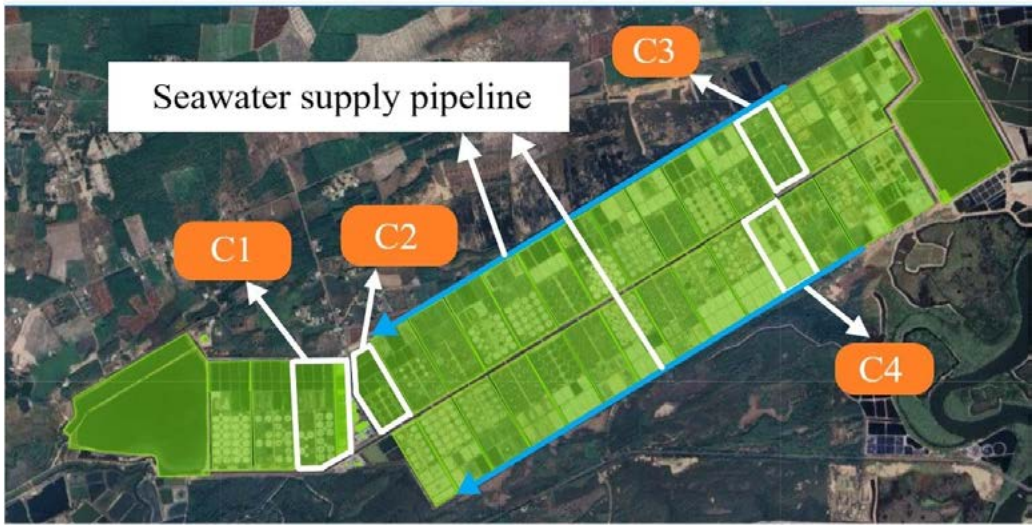

Figure 1. Map of the study area and sampling site at Minh Phu-Loc An Aquaculture Ltd, Dat do district, Ba Ria-Vung Tau province, Vietnam. C1 - C4: are denoted for shrimp modules taking water samples.

2.2. Experimental set-up

The method applied is an ex-post facto causal design, which implies analyzing the research objectives based on the existing natural phenomena in the field, over a period of a shrimp yield, with total a 8 ponds in 4 shrimp modules (2 ponds for each module) located downstream (C1 & C2) and upstream (C3 & C4) of seawater pipeline as Figure 1. In each preselected module, 2 ponds were randomly chosen. The quality parameters in the farming operations, including DO, pH, temperature, salinity, alkalinity, nitrite, phosphate, and total ammonia nitrogen (TAN), are determined on-site daily at 8 am. Because seasonal variation is considered an important natural factor influencing water quality, water samples were taken from the stocking day until

harvesting day during dry and rainy seasons. Additionally, to examine how water quality affects the growth of shrimp, the size of shrimp (g/pcs) was monitored by request from post-larvae phase to grow-out phase. The module manager determined the optimal period for measuring shrimp size.

2.3. Water sampling and analysis

Based on the experimental set-up, a Van Dorn water sampler was used to take water samples daily. A total of 506 water samples were taken from 8 crops during the rainy and dry season. Water samples were taken in the 30 - 50 cm from the surface layer. Some physical parameters, such as DO, pH, temperature and salinity were measured directly using Aqua TROLL 500 Multiparameter Sonde (In-Situ Inc, Fort Collins, USA). An on-site laboratory was set up for the analysis of chemical parameters, such as alkalinity, nitrite, phosphate, and TAN. These parameters were determined as SMEWW by using HI801-02 (iris Visible Spectrophotometer, Hanna Instruments Inc, Woonsocket, RI, USA).

Normally, shrimp size is the most suitable indicator in a batch. Previously, these assessments were only done manually on the harvesting day (Smith et al., 2002; Chen et al., 2019; Amalia et al., 2022). In this article, the artificial intelligence sizing machine S3 (Otanics Technology Jsc, Vietnam) was used to determine the shrimp body weight. With S3, we collected more data of the growth rate in a crop than in other studies because the growth rate of post-larvae phase can be easily measured by S3 than before. Furthermore, using S3 helps to keep the shrimp alive after sizing and protects the survival rate of the shrimp.

2.4. Statistical analysis

The collected data are analyzed by using SPSS ver.26 software to analyze the impact of observed parameters on shrimp growth rates. The seasonal fluctuations in water quality throughout the year are also described to understand the reflection of climate change.

The energy consumption required to maintain the desired DO levels is significant in a farming cycle. Therefore, assessing the dynamics of water quality parameters with DO levels is extremely important. The mentioned software was also used to demonstrate this relation.

3. Results and Discussion

3.1. The dynamics of water quality parameters with DO

The fluctuating values of DO concentration in a whiteleg shrimp farming cycle are presented in Figure 2. The lowest concentration point occurs at 60 days of farming on the harvesting day of module C4 at 4.73 mg/L, while the highest concentration value is observed on the first day (7.41 mg/L). The DO decrease may occur due to the retaining feeding rate after dusk. The food surplus can potentially increase synthesis and cause changes in DO. Similarly, DO consumption levels decrease along with the increase in feed input, due to the biosynthesis process of waste and other organic materials, align with Ma et al. (2013), Mirzaei et al. (2019) and Wafi et al. (2021). Our results are consistent with Ullman et al. (2019) and Weldon et al. (2021), who reported that higher feed amounts used generally lead to higher DO consumption in the water. Other reasons for the decrease at certain times include abiotic factors such as water temperature, pH, and salinity. This was reported by Boyd & Tucker (2012), Cao et al. (2019) and Rozario & Devarajan (2021).

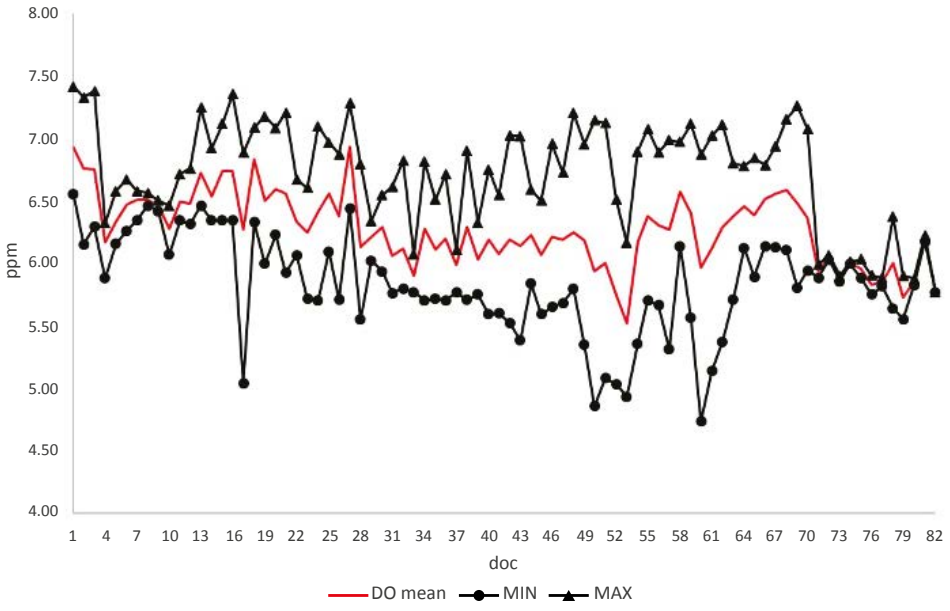

Figure 2. The fluctuation of dissolved oxygen levels during the shrimp farming period. MIN: minimum value; DO: dissolved oxygen; MAX: maximum value; doc: days of culture.

The average DO concentration is presented in Table 1. The average DO concentration is 6.33 ± 0.39 mg/L, with a minimum of 4.73 mg/L and a maximum of 7.41 mg/L. This satisfied the requirement of maintaining a DO level above 4.5 mg/L in shrimp farming during culture time, as mentioned in studies of Simbeye & Yang (2014) and Adetunji et al. (2022). The normal DO level in our study was higher than the optimal value for shrimp farming ranging between 4 - 5 mg/L as reported by Islam et al. (2004). The optimal value is crucial for changes in pond water quality and is an important parameter for aerobic respiration and redox processes in water and pond sediments (Boyd, 2017).

The data on water quality parameters for each module are presented in Table 1. The pH values of C1, C2, C3, and C4 are 7.65 ± 0.34, 7.62 ± 0.31, 7.99 ± 0.14 and 7.96 ± 0.12, respectively. Daily pH measurements for 4 modules yielded an average value of 7.8 ± 0.23. The difference of the salinity of the water in downstream (C1 & C2) modules and upstream (C3 & C4) modules was observed in Table 1. The average value of this parameter in this study area is 30.29 ppt. The distinction of module manager's decision about daily water exchange rate may be the reason of salinity change. The DO level has an average value of 6.33 mg/L. The optimal pH range for whiteleg shrimp farming is 7.5 - 8.5, with a fluctuation range of 0.5, algin with Reddy & Mounika (2018). The obtained salinity value in this study is 30.29 ppt. Meanwhile, the optimal range is approximately 20 - 25 ppt (Ferreira et al., 2011). On the other hand, shrimp can naturally thrive within a salinity range of 0 to 40 ppt due to their osmoregulatory system (Ponce-Palafox et al., 1997; Jaffer et al., 2020; Khanjani et al., 2020). The average temperature obtained in the study is 27.53°C, optimal for shrimp development. This was reported by Wyban et al. (1995) and Madusari et al. (2022), where the optimal range is 27 - 30°C. It can be affirmed that DO concentration, pH, salinity, and temperature during the shrimp farming period all fall within optimal ranges.

Similar to salinity, there are discrepancies between downstream and upstream modules of TAN, NO_2^- and PO_4^{3-} value (in Table 1). The average values of these three parameters in C1 and C2 are higher than in C3 and C4. The interaction of stocking density and farming condition influenced the variation of water quality was also proposed in studies of Biao et al. (2004) and Esparza-Leal et al. (2020). In fact, each region employs different aquaculture practices and the farmers rely on their individual experience to make decision. This was mentioned early by Kautsky et al. (2000). Minh Phu-Loc An Aquaculture Ltd, each module is operated by different groups of farmers so that each module works as separate farm. Consequently, the water characteristics were dramatically changed. Especially, extremely high nitrite values were detected in C1 and C2. While Samocha (2019) declared that a good survival of shrimp has been demonstrated when exposed to high nitrite concentration in 8 days, this study found that the tolerance threshold of shrimp survival can be up to than 50 mg/L. The effect of unusually peak level of NO_2^- was not evaluated because this situation happened when there was no water exchange on the last days of the crops to rise the color of shrimp and the feeding rate still remained.

Table 1. Average values of water quality parameters during the shrimp farming period

Quality parameter		Module				Average value of study area
		C1	C2	C3	C4	
DO (mg/L)	Mean	5.99 ± 0.41	5.95 ± 0.44	6.70 ± 0.38	6.67 ± 0.34	6.33 ± 0.39
	Range	4.86 - 7.08	4.73 - 7.28	5.55 - 738	5.77 - 7.41	
pH	Mean	7.65 ± 0.34	7.62 ± 0.31	7.99 ± 0.14	7.96 ± 0.12	7.8 ± 0.23
	Range	7.10 - 8.38	6.97 - 8.31	7.73 - 8.52	7.81 - 8.49	
Sal (ppt)	Mean	24.64 ± 4.13	24.87 ± 4.5	36.66 ± 0.53	36.83 ± 0.35	30.29 ± 0.39
	Range	16.51 - 31.04	16.37 - 30.96	34,71 - 37,38	35,89 - 37.61	
T (^{0}C)	Mean	28.53 ± 0.84	28.44 ± 0.78	26.42 ± 0.72	26.1 ± 0.82	27.53 ± 0.09
	Range	26.65 - 30.98	26.79 - 30.49	24.88 - 27.81	24.88 - 27.81	
Alk (mg/L)	Mean	144.95 ± 28.62	145.67 ± 33.86	167.04 ± 23.2	159.66 ± 23.99	153.64 ± 1.69
	Range	79 - 193	57 - 193	123 - 215	125 - 210	
TAN (mg/L)	Mean	4.03 ± 4.38	5.93 ± 4,8	2.61 ± 1.51	2.3 ± 1.32	3.67 ± 0.21
	Range	0.15 - 17.5	0.18 - 22.1	0 - 7.68	0 - 5.8	
NO_2^- (mg/L)	Mean	9.62 ± 1.335	7.81 ± 11.25	0.74 ± 0.64	0.91 ± 1.06	4.70 ± 0.58
	Range	0 - 57.6	0 - 41.6	0 - 2.90	0 - 6.65	
PO_4^{3-} (mg/L)	Mean	3.71 ± 3.76	4.10 ± 4.5	0.74 ± 0.64	0.91 ± 1.06	2.48 ± 0.20
	Range	0 - 17.7	0 - 20.4	0 - 2.90	0 - 6.65	

Note: DO - dissolved oxygen; Sal - salinity; T - temperature; Alk - alkalinity; TAN - total ammonia nitrogen.

Based on the correlation test results, the DO concentration in aquaculture ponds is related to water quality parameters, and its correlation with the physico-chemical data is presented in Table 3. The data indicates an inverse relationship with the physico-chemical parameters including phosphate, temperature, nitrite, and TAN, while there was a positive relationship with pH, salinity, and alkalinity. This finding was consistent with the results in the research of Vinatea et al. (2009) and Esparza-Leal et al. (2020). This finding also highlights the issue that high DO concentration not only wastes energy but also changes the stability of pH level in tank.

Table 2. The correlation between DO and the physico-chemical parameters of water

	Parameter	pH	Sal	T	Alk	PO_4^{3-}	NO_2^-	TAN
DO	Pearson correlation	0.729**	0.396**	-0.525**	0.300**	-0.457**	-0.365**	-0.371**
	Sig (2-tailed)	0.000	0.000	0.000	0.006	0.000	0.001	0.001
	N	82	82	82	82	82	82	82

*Note: * - correlation is significant at the 0.05 level; ** - correlation is significant at the 0.01 level; DO - dissolved oxygen; Sal - salinity; T - temperature; Alk - alkalinity; TAN - total ammonia nitrogen.*

3.2. Effects of water quality on shrimp growth rate

Based on correlation tests and descriptive analysis, nitrite concentration fluctuates at values higher than the water quality standards for intensive shrimp farming. The variation in water quality parameters is closely related and indirectly affects shrimp growth rate and aquaculture productivity (Chen et al., 2019). The impact of DO, nitrite, salinity, alkalinity, and other parameters on shrimp growth rate can be partly seen in Figure 3. The effect of DO fluctuations on shrimp growth rate in ponds can be described by the regression formula with the following equation:

$$y = -26.49 - 5.854x \ (R^2 = 0.037)$$

This means that fluctuations in DO content in the pond affect the shrimp growth rate by approximately 3.7%, while the rest is influenced by other factors. Although, it was recommended that the DO content surveyed in the shrimp ponds is always at the optimal level, and the farmers usually keep this level higher than required, it can increase the operation cost. Moreover, when analyzing the correlation between DO and shrimp growth, it does not show a strong interaction

The impact of nitrite (NO_2^-) on shrimp growth rate during the farming period can be described by the following equation:

$$y = 3.099 + 0.874x \ (R^2 = 0.756)$$

Nitrite affects the shrimp growth rate by up to 75.6%. High nitrite levels adversely affect water quality parameters and shrimp development (Ma et al., 2018; Valencia-Castañeda et al., 2019; Phan et al., 2022). High nitrite concentrations (NO_2^-) also affect feed intake, increase oxygen consumption, interfere with the ammonia excretion system, and cause moderate mortality (Valencia-Castañeda et al., 2019). Overall,

increased nitrite levels in ponds are caused by environmental fluctuations in the aquaculture ecosystem (Soares et al., 2020).

The impact of alkalinity (Alk) on shrimp growth rate during the farming period can be described by the following equation:

$y = 32.302 - 0.159x$ ($R^2 = 0.678$)

This means that fluctuations in alkalinity content in the pond affect the shrimp growth rate by approximately 67.8%, while the rest is influenced by other factors.

The impact of salinity (Sal) on shrimp growth rate during the farming period can be described by the following equation:

$y = 35.792 - 0.94x$ ($R^2 = 0.804$)

This means that fluctuations in salinity content in the pond affect the shrimp growth rate by approximately 80.4%, while the rest is influenced by other factors.

The impact of temperature (T) on shrimp growth rate during the farming period can be described by the following equation:

$y = -79.925 + 3.216x$ ($R^2 = 0.166$)

The impact of pH on shrimp growth rate during the farming period can be described by the following equation:

$y = 241.811 - 30.413x$ ($R^2 = 0.557$)

The impact of total ammonia nitrogen (TAN) on shrimp growth rate during the farming period can be described by the following equation:

$y = 8.199 + 0.257x$ ($R^2 = 0.018$)

The impact of phosphate (PO_4^{3-}) on shrimp growth rate during the farming period can be described by the following equation:

$y = 7.628 + 0.485x$ ($R^2 = 0.143$)

This means the change of temperature, pH, TAN, and PO_4^{3-} level in the pond affect the shrimp growth rate by approximately 16,6%, 55,7%, 1,8%, and 14,3% respectively while the rest is influenced by other factors.

In this study, salinity was identified as the most influential parameter affecting the growth rate of shrimp. This factor significantly influences the productivity variations in shrimp farming ponds was also proposed in the study of Prapaiwong & Boyd (2012). Salinity plays a crucial role in shrimp development, directly impacting their physiological functions and nutrient absorption capabilities. Our study has shown that shrimp thrive best at salinity levels between 15 and 25 parts per thousand (ppt). Under optimal salinity conditions, shrimp not only grows faster but also exhibit better resistance to stress and diseases. Conversely, abrupt or unsuitable changes in salinity can reduce growth rates and overall health of the shrimp.

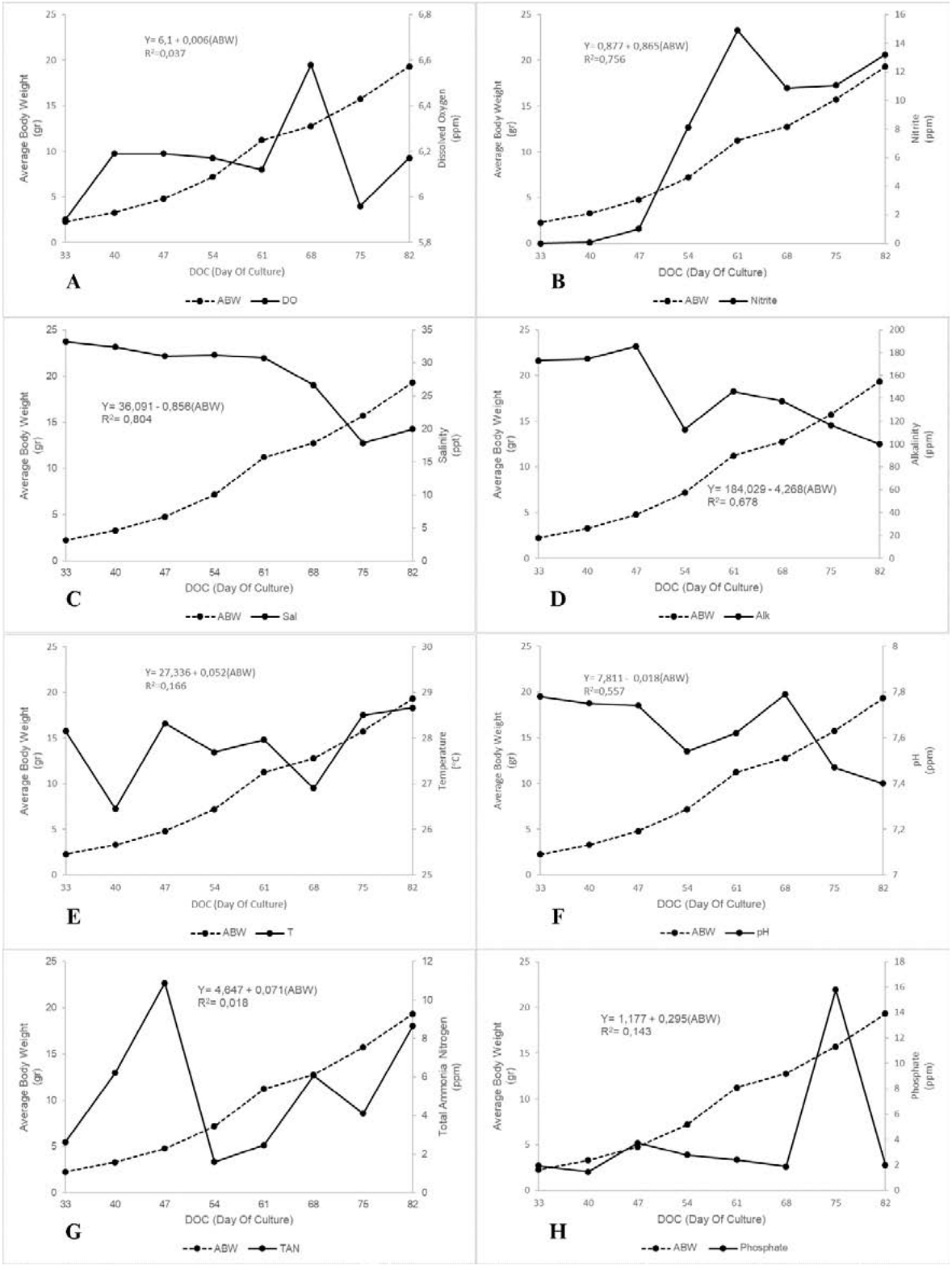

Figure 3. Effects of water quality on shrimp growth rate. ABW: average body weight; A: dissolved oxygen; B: nitrites; C: Salinity; D: Alkalinity; E: Temperature; F: pH; G: TAN; H: Phosphate.

3.3. Water quality assessment in season

In general, water quality tends to fluctuate significantly as shown in Figure 4. During the rainy season, water quality tended to fluctuate dynamically. Similar observations have been discussed in studies of Ariadi et al. (2023) that the main water quality parameters were stable in the dry season, and the rainy season has a greater negative impact on water quality source than droughts condition.

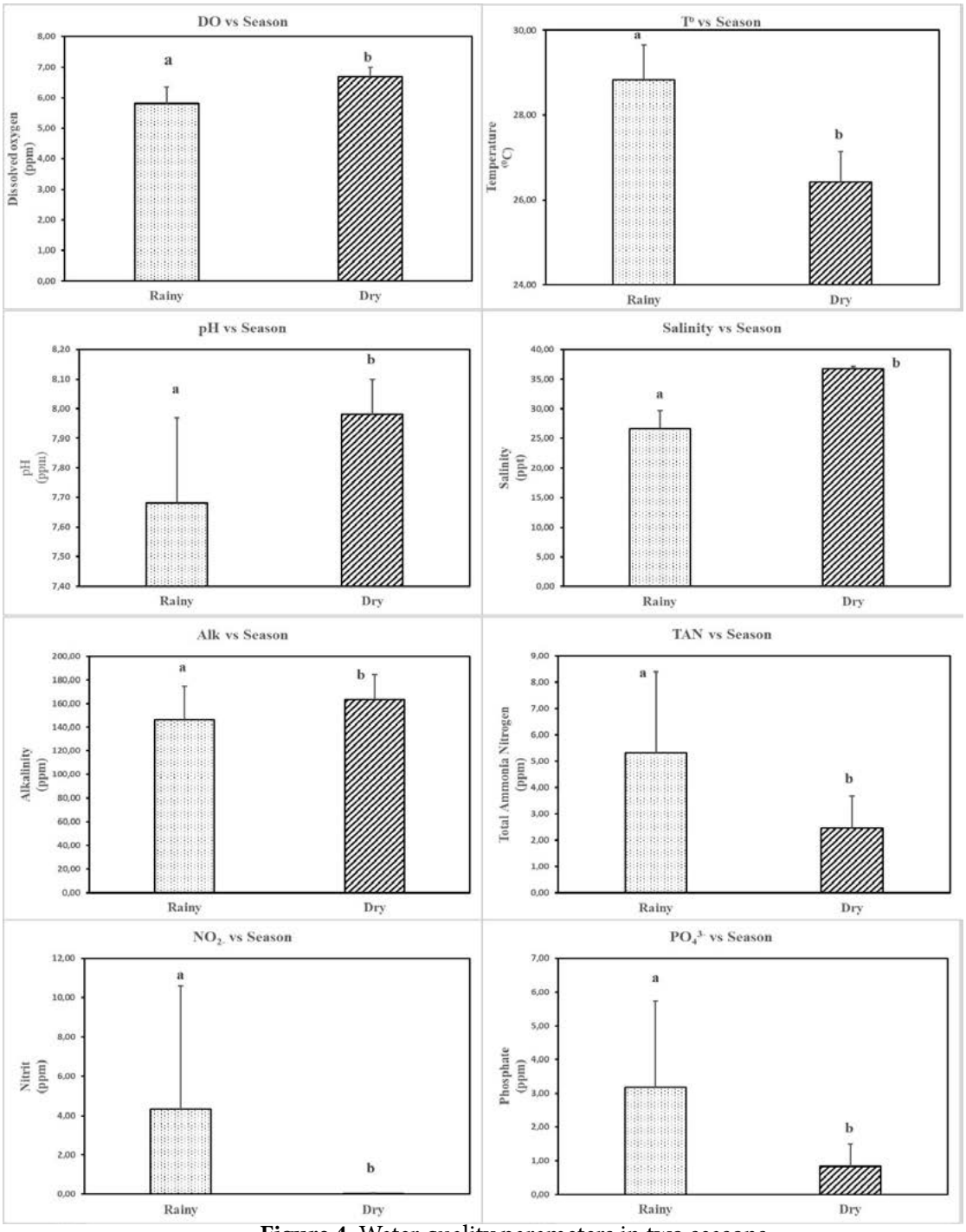

Figure 4. Water quality parameters in two seasons.

As observed in Figure 4, water parameters during the rainy season, generally have lower levels of dissolved oxygen, pH, salinity, and alkalinity compared to the dry season. Similarly, studies of Teichert-Coddington et al. (1996), Guerrero-galván et al. (1998) and Abdul et al. (2021) found the same situation of water quality change in a year. This is due to the large volume of rainwater diluting the substances in the water and reducing the concentration of mineral ions.

Conversely, the water temperature, and the levels of ammonia, nitrite, and phosphate are higher during the rainy season. This is because rainwater carries organic materials from the land, increasing decomposition processes and releasing these compounds into the water. In the dry season, the absence of diluting rainwater typically results in higher salinity and alkalinity, while the concentrations of organic materials and nitrogen-containing compounds such as ammonia and nitrite are generally lower. The evaporation process in the dry season further increases the concentration of mineral ions in the water, leading to more stable pH and salinity levels.

4. Conclusions

Previous studies have shown that pollutants stress shrimp, making them more susceptible to disease. Following this idea, on-time interpretation of water quality is an essential solution for farming environment control. Meanwhile, the more parameters are measured, the more operation costs will be paid. This study identifies the key indicators that impact shrimp growth. Thus, it helps farmers to customize the plan of water quality monitoring. From that, the cost–saving can be easily done.

As recent reports highlighted decreasing production cost is a must-be action for shrimp farmers. Table 3 shows the relationship between DO and other common quality parameters, thus the farm manager could predict the reduction of DO when some important pollutants as indicators of water quality are present. This opens up opportunities to reduce energy costs for maintaining DO when it is not necessary. Based on the results, the fluctuations in dissolved oxygen concentration during the shrimp farming period were characterized by normal levels of variation, with the highest concentration being 7.41 mg/L at 1 day old and the lowest concentration being 4.73 mg/L on day 60. Furthermore, dissolved oxygen (DO) in ponds generally correlates positively with salinity, pH, and alkalinity, and inversely with temperature, nitrite levels, photoperiod, and TAN.

There is a pronounced difference in water quality between the dry and rainy seasons. Water quality parameters during the rainy season tend to be more variable and less stable compared to the dry season. Therefore, a water quality control strategy is needed during the rainy season to minimize fluctuations in water quality.

Conflict of interest

The authors declare that they have no conflict of interest.

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Enhancing the oil extraction process and exploring phytochemical composition and bioactivities of bitter melon seeds (*Momordica charantia* L.)

Anh T. V. Nguyen[1], Thu T. A. Pham[1], Hien T. Dang[1], Linh T. Pham[1], & Hong V. C. Phung[2*]

[1]Faculty of Biological Sciences, Nong Lam University, Ho Chi Minh City, Vietnam

[2]Research Institute for Biotechnology and Environment, Nong Lam University, Ho Chi Minh City, Vietnam

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***Corresponding author**

Phung Vo Cam Hong

Email: hongpvc@hcmuaf.edu.vn

ABSTRACT

This study was conducted to determine the phytochemical composition of bitter melon seeds (*Momordica charantia* L.) grown in Long An province (Vietnam), to investigate optimal conditions for lipid extraction, and to evaluate the extracted lipid's quality. The seeds had a moisture content of 5.27%, total ash of 1.85%, total flavonoid content of 91.10 mg/100 g, and total polyphenol content of 478.95 mg/100 g. The seeds were also free of highly toxic metals such as lead and cadmium. Using the Soxhlet method, optimal lipid extraction was achieved with a material-to-solvent ratio of 1:80 (w/v) over 4 hours, resulting in a lipid extraction efficiency of 13.74%. The acid, saponification, ester, and peroxide values were 1.01 mg KOH/g, 355.60 mg KOH/g, 354.59 mg KOH/g, and 3.82 meq O_2/kg, respectively, in compliance with the quality requirements of Vietnam and Codex standards. The extracted lipids had antioxidant activity at an IC_{50} value of 119 mg/mL and inhibited the growth of two microbial strains *Staphylococcus aureus* and *Bacillus subtilis* subsp. *spizizenii*. These findings suggest that bitter melon oil has potential applications in the food, pharmaceutical, and cosmetic industries

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1. Introduction

Momordica charantia Linn (*M. charantia* L.), commonly known as bitter melon, is an annual climbing vine from the Cucurbitaceae family. Besides its widespread use as a food in many countries, bitter melon has long been used in traditional medicine throughout Asia, Africa, and Latin America due to its rich content of over 60 bioactive phytochemicals, including glycosides, saponins, alkaloids, fixed oils, triterpenes, proteins, and steroids (Behera et al., 2020). Notably, bitter melon seed oil has been applied in treating various diseases such as diabetes, inflammation, and cancer, and is also utilized in cosmetics, poultry, aquaculture feed, and biodiesel production (Horax et al., 2010; Ajuru et al., 2017; Singh et al., 2019). The oil is composed of monounsaturated fatty acids (MUFA), saturated fatty acids (SFA), and polyunsaturated fatty acids (PUFA), with conjugated linoleic acid (CLnA) as the predominant component (Liu et al., 2010; Yoshime et al., 2016).

Traditional extraction techniques for plant-derived bioactive compounds, such as pressing, maceration, and shaking water bath extraction, are widely used for their simplicity and cost-effectiveness. However, these methods often require long extraction times and involve the use of harmful solvents (Pitipanapong et al., 2007; Zaini et al., 2018; Sasongko et al., 2019). On the other hand, advanced techniques like Soxhlet extraction, ultrasonics, supercritical CO_2 (SC-CO_2) extraction, and enzyme-assisted methods offer greater efficiency, reduced extraction time, lower solvent consumption, and better selectivity (Nyam et al., 2009; Xu et al., 2014; Naik et al., 2021), though they come with higher costs and more complex procedures.

The choice of extraction method depends on various factors such as the chemical structure and physicochemical properties of the sample, as well as the research objectives. Thus, the objective of the study aimed to develop a simplified extraction method for bitter melon seeds to produce oil that complies with safety standards, retains the seeds' bioactive components, extends shelf life, and is suitable for multiple applications (Figure 1).

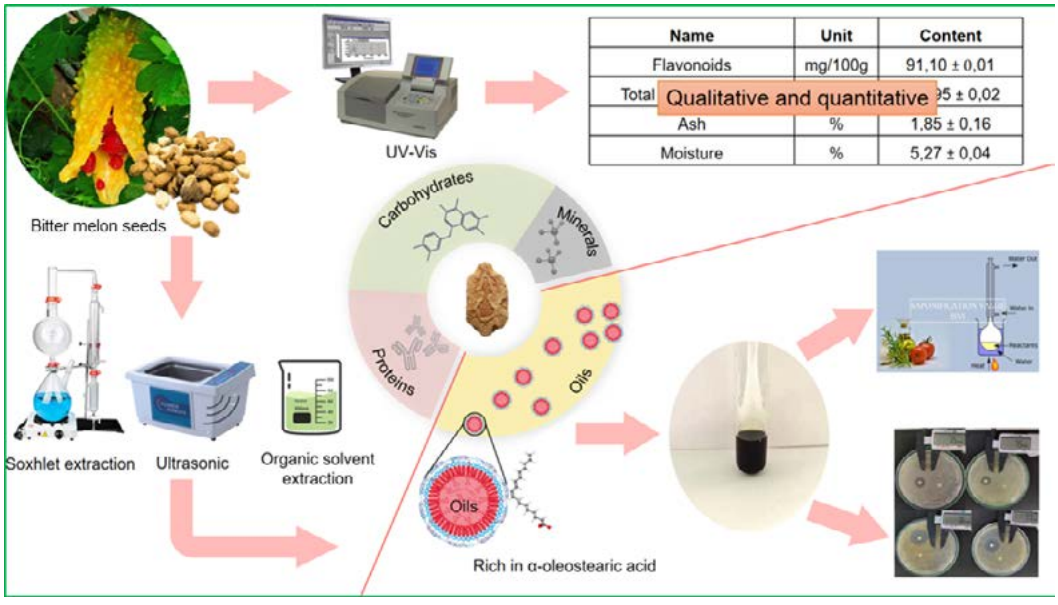

Figure 1. Graphic abstract.

2. Materials and Methods

2.1. Materials

The fruit of *M. charantia* L. was purchased from a garden in Long An province, Vietnam. After removing impurities and separating the seeds from the flesh, they were dried at 50 ± 5°C until their moisture content was below 13%. The dried seeds were then crushed and sieved through a 1 mm diameter sieve. Raw powder was stored in zip-lock bags at room temperature until further use.

The equipment used in this study included a reflux extraction system (Isolab, Germany), a Soxhlet extraction system, an ultrasonic cleaner (WUC-32, Jiayuanda, China), an evaporator (Heidolph, Germany), a drying oven (Memmert, Germany), and an ultraviolet-visible spectrophotometer (Model 752N, Jenway, England).

Chemicals used in the study included Dragendorff (Cas#39775-75-2) and Wagner reagents (Cas#39775-75-2), NaOH (Cas#1310-73-2, Xilong), HCl (Cas#D1128, Duksan), $FeCl_3$ (Cas#7705-08-0, Xilong), hexane (Cas#110-54-3, Xilong), Folin-Ciocalteu reagent (Cas#1090010100, Merck), Na_2CO_3 (Cas#1063920500, Merck), phenolphthalein (Cas#77-09-8), KOH (Cas#1310-58-3, Xilong), $Na_2S_2O_3.5H_2O$ (Cas#10102-17-7), and KI (Cas#7681-11-0, Xilong).

2.2. Methods

2.2.1. Phytochemical analysis of bitter melon seeds

Determination of moisture content

The moisture content was determined according to TCVN 7975:2008 (VS, 2008). Initially, the raw material was accurately weighed to 5 ± 0.0001 g into the moisture dish (which was dried at 105°C, cooled, and recorded its mass). The dishes containing the samples were placed in a drying oven and dried at 105 ± 2°C for 4 - 6 h. Subsequently, they were transferred to a desiccator until reaching room temperature, and their mass was re-recorded. This process was repeated multiple times until the difference in weight between two consecutive weighings did not exceed 5 mg. The moisture content of the raw material was calculated using the equation (1):

$$W = \frac{m_1 - m_2}{m_1 - m_o} \times 100 \quad (1)$$

where m_o, m_1, and m_2 was the mass of the moisture dish, the mass of the moisture dish and the sample before drying, and the mass of the moisture dish and the sample after drying (g), respectively.

Qualitative analysis

Seeds extract preparation: Two types of solvents (70% ethanol and distilled water) were used for extraction. Five g of seed powder were soaked in 30 mL of each solvent. Then, coupled with 20 mL of the same solvents, the residues were extracted by ultrasound-assisted extraction for 15 min. The extract was filtered through a Whatman No.1 filter paper and stored at 4°C for further use.

The biochemical compositions of the seed extracts, including alkaloids, flavonoids, phenolic acids, saponins, and tannins, were qualitatively determined as previously described by Pham et al. (1998) (Table 1).

Table 1. Phytochemical screening methods of seed extracts

Compounds	Reagents	Observations (Indicating Positive Test)
Alkaloids	Wagner	Formation of brown to reddish brown precipitate
	Dragendroff	Formation of red-orange to red precipitate
Flavonoids	NaOH 10%/HCl 10%	The color changed to red-orange or orange and became lighter or discolored when HCl was added.
	Metal magnesium/HCl conc.	The solution was pink to red.
Phenolic acids	$FeCl_3$ 5%	The solution was moss-green to bluish-black
	Iodine	The solution was red
Saponins	Foam test	Persistent foam for 15 min
Tannins	$FeCl_3$ 5%	The solution was moss-green to bluish-black
	Lead acetate 10%	Formation of white precipitate

Quantitative analysis

Based on the qualitative results, the main biological components of the seed extracts were quantified, as presented in Table 2.

Table 2. Quantitative analysis methods of seed extracts

Compounds	Methods
Flavonoids	Flavonoids were measured using the aluminum chloride colorimetric assay. Flavonoids in the sample were extracted using ethanol and mixed with aluminum chloride ($AlCl_3$) and potassium acetate (CH_3COOK), leading to the formation of a yellow-colored complex. The reaction mixture was incubated for 30 minutes, after which the absorbance was measured using a UV-Vis spectrophotometer at 415 - 430 nm. The total flavonoid content was quantified using a standard curve generated with quercetin as the reference compound (Chang et al., 2002).
Polyphenols	The total polyphenol content was quantified by the Folin-Ciocalteu (FC) method (Waterman & Mole, 1994). The FC reagent was used to quantify total polyphenol content through a redox reaction, where phenolic compounds were oxidized by donating electrons to molybdenum (Mo) and tungsten (W) complexes in the reagent, reducing them from Mo(VI) and W(VI) to Mo(V) and W(V). This reduction produced a blue color, with the intensity proportional to the polyphenol concentration. The reaction required an alkaline medium, typically provided by sodium carbonate, to enhance phenolic reactivity by deprotonating them into phenoxide ions. The blue complex's absorbance was measured at 765 nm, and the results were expressed as gallic acid equivalents (GAE). Gallic acid was used as a control.

Compounds	Methods
Tannins	Tannins was quantified according to AOAC 955.35. The extract was prepared with hot distilled water, and then impurities were removed via filter paper. Five milliliters of the extracted solution were taken into a 250 mL Erlenmeyer flask, followed by the addition of 150 mL of distilled water and 5 mL of 0.25% Indigo carmine, and the mixture was shaken well. The mixture was then titrated with 0.1 N $KMnO_4$ solution until it turned yellow.
Saponins	Saponins was quantified according to TCCS 231:2017/TTKNII with some adjustments for suitability (DAH, 2017). The powder material was extracted using 80% methanol. The methanol was removed, and the residue was dissolved in hot water. This solution was then shaken sequentially with diethyl ether and saturated n-butanol. The n-butanol layer was separated from the mixture and concentrated using rotary evaporation. The resulting residue was dried at 80°C until the mass was constant and weighed to determine the saponin content in the material.

2.2.2. Investigating the oil extraction process of bitter melon seeds

Maceration

One g of the sample was measured and placed in a 100 mL beaker (Schott-Duran, Germany). Hexane solvent was then added, and the sample was allowed to macerate for different durations (12, 24, and 36 h) at 70°C under various material-to-solvent ratios (1:60, 1:80, 1:100 w/v), corresponding to each experimental condition. After extraction, the sample was filtered through Newstar 101 filter paper, and the solvent was evaporated using a rotary vacuum evaporator (Heidolph, Hei-VAP Core ML/G3 XL, Germany). The oil was dried at 70°C for 6 - 8 h and cooled in a desiccator and re-weighed the flask. Repeated the experiment three times and calculated the oil yield using the equation (2):

$$W(\%)= \frac{m_1-m_0}{m \times(1-h)} \times 100 \qquad (2)$$

where W was the oil yield (%), m was the mass of the sample used (g), m_0 was the mass of the rotary flask (g), m_1 was the total mass of the rotary flask and the oil after drying (g), and h was the moisture of the sample (%).

Soxhlet extraction

One gram of the sample was placed into a Soxhlet extraction thimble (Isolab, Germany). Reflux extraction was performed for 4, 6, and 8 h using material-to-solvent ratios of 1:60, 1:80, and 1:100 (w/v). Hexane remained the solvent used in this method. After extraction, the solvent was removed by a rotary evaporator and the results were calculated according to formula (2).

Ultrasonic extraction

One gram of the sample was weighed into a 100 mL beaker (Schott-Duran, Germany), and hexane solvent was added at material-to-solvent ratios of 1:60, 1:80, and 1:100 (w/v). The sample was then extracted using an ultrasonic bath (Hwashin, South Korea, 500 W power) for 5, 10, and 15 min at 30°C. After the extraction process, the sample was filtered through Newstar 101 filter paper, and the results were calculated based on equation (2).

2.2.3. Evaluating the quality of oil extracted from bitter melon seeds

Minerals

The mineral element content of oil was quantified using atomic absorption spectrometry according to TCVN 6496:2009 (VS, 2009) with minor modifications. Approximately 1 g of the sample was accurately weighed and placed into a reaction tube. Initially, 10 mL of a 1:1 mixture of HNO_3 and H_2O was added, mixed well, and heated at 95 ± 5°C for 15 min, then cooled to room temperature. Subsequently, 5 mL of 65% HNO_3 was added and heated at 95 ± 5°C for 30 min; this step was repeated if brown-red fumes were observed until they ceased. The sample was then heated for an additional 2 h, ensuring it did not dry out, and cooled again. Next, 2 mL of deionized water and 3 mL of 30% H_2O_2 were added, heated until bubbling decreased, and cooled. Additional 1 mL increments of 30% H_2O_2 were added, heating until bubbling stopped, not exceeding 10 mL in total, followed by another 2-hour heating period. After cooling, 10 mL of 37% HCl was added and the sample was heated for 15 min and cooled. The extract and remaining solid were transferred to a 50 mL volumetric flask, filled to the mark with deionized water, filtered through Whatman No.1 filter paper, and analyzed using flame atomic absorption spectrometry. The mineral content in the sample was calculated using the following formula (3).

$$W=(C \times f \times V) / m \quad (3)$$

where W was the minerals content in the sample (mg/kg); C was concentration of minerals in the test sample according to the standard curve (mg/L); f was dilution factor of the test sample; V was volume of the test sample (mL) and m was the mass of the sample (g).

Acid value (AV)

The sample was weighed into a 250 mL Erlenmeyer flask and dissolved in a neutralized solvent (a 1:1 ratio of ethanol and diethyl ether) by gently heating. After adding the phenolphthalein indicator, the solution was titrated with standardized 0.1 mol/L KOH while continuously shaking. The titration was considered complete when adding a drop of 0.1 mol/L KOH produced a faint but stable color change lasting for at least 15 sec. The AV was determined according to TCVN 6127:2010 (VS, 2010a):

$$W_{AV} = (A \times f \times 5,6)/m \quad (4)$$

where W_{AV} was the acid value, A was the volume of the 0.1 mol/L KOH used (mL), f was the concentration of KOH used (mol/L), and m was the mass of the sample (g).

Saponification value (SV)

The saponification value was determined according to TCVN 6126:2015 (VS, 2015). Specifically, 2 g of the sample was accurately weighed and added to 25 mL of 0.5 mol/L KOH solution in ethanol. It was refluxed for 2 h for oils with high melting points and difficult saponification. About 0.5 - 1 mL of phenolphthalein solution was added to the hot mixture and titrated with standardized 0.5 mol/L HCl solution until the pink color of the indicator disappears. The blank sample was prepared using 25 mL of KOH solution in ethanol, but without the test sample. The SV value was determined using the equation (5):

$$W_{sv} = \frac{(V_0 - V_1) \times 28}{m} \quad (5)$$

where W_{SV} was the saponification value, V_0 was the volume of the standardized HCl for blank (mL), V_1 was the volume of the standardized HCl for sample (mL), and m was the mass of the sample (g).

Peroxide value (PV)

The peroxide value was determined according to TCVN 6121:2010 (VS, 2010b). Two grams of the oil were placed into a 250 mL Erlenmeyer flask, followed by the addition of 20 mL of a mixture of CH_3COOH in a 2:1 (v/v) ratio and 5 mL of saturated KI solution. The mixture was shaken well, sealed, and kept in the dark for 10 min. Afterward, 30 mL of distilled water and a few drops of 5% starch solution were added, and the liberated iodine was titrated with 0.002 N $Na_2S_2O_3$ solution until the blue color disappeared. The blank was processed using the same steps as the test sample, but without the addition of the test sample. The PV was determined using the equation (6):

$$W_{PV} = \frac{(V - V_0) \times C_{thio} \times 0.0002538 \times 1000}{m} \quad (6)$$

where W_{PV} was the peroxide value, V was the volume of the $Na_2S_2O_3$ for sample (mL), V_0 was the volume of the $Na_2S_2O_3$ for blank (mL), C_{thio} was the concentration of the $Na_2S_2O_3$ (mol/L), m was the mass of the sample (g), and 0.0002538 g iodine corresponded to 1 mL 0.002 N $Na_2S_2O_3$ solution.

Antibacterial activity

Antibacterial activity was determined by the Kirby-Bauer method (Bauer et al., 1959). The oil was tested for antibacterial activity on *Staphylococcus aureus* (*S. aureus*) (ATCC® 6538™) and *Salmonella* sp. (ATCC®700623™). A volume of 0.1 mL of the bacterial suspension was spread across the surface of LB agar plates. Sterile filter paper discs, soaked in oil, were arranged in a triangular pattern on the agar surface. The plates were then incubated at room temperature for observation. Inhibition zones were measured after 24 h for *Salmonella* and 48 - 72 h for *S. aureus*. Ampicillin 50 ppm was used as positive control and all experiments were conducted in triplicate.

Antioxidant activity

The antioxidant activity of the oil was estimated by DPPH assay (Chanda et al., 2009). The oil was filtered and diluted to different concentrations. Each 0.5 mL of sample was added to the test tube content of 3 mL 96% ethanol, and 1 mL of 0.5 mM DPPH. The mixture was incubated in darkness at room temperature for 30 min. The absorbance was measured at 517 nm. A control experiment was conducted using 70% ethanol instead of the sample. Ascorbic acid was used as a reference at 10 - 50 mg/L. The antioxidant activity was determined according to the equation (7):

$$\%AA = \frac{OD_c - OD_s}{OD_c} \times 100 \quad (7)$$

where %AA was the antioxidant activity (%), OD_c was the absorbance of the control, and OD_s was the absorbance of the sample.

A correlation was established between sample concentration and antioxidant capacity based on the linear regression equation y = ax + b, from which the IC_{50} value (the concentration at which 50% of free radicals were captured) was determined to be a basis for comparing the antioxidant capacity between experiments. The lower the IC_{50} value, the higher the antioxidant activity.

2.2.4. Data analysis

The data was expressed as mean ± standard deviation (SD) of triplicate measurements. Two-way ANOVA followed by Tukey's test was conducted using GraphPad Prism version 9 to assess significant differences among treatments ($P < 0.05$).

3. Results and Discussion

3.1. Chemical composition of bitter melon seeds

3.1.1. Phytochemical analysis of bitter melon seeds

Qualitative analyses were performed to determine the presence of biologically active compound groups within bitter melon seed extract to explain the metabolic mechanism when used as a product. The results were shown in Table 3.

Table 3. Qualitative results of some secondary compounds in bitter melon seed powder

Compounds	Reagents	Result
Alkaloids	Wagner	No red-orange precipitate was observed (-)
	Dragendroff	No brown to dark brown precipitate was observed (-)
Flavonoids	NaOH 10%/HCl 10%	Suspicious
	Metal magnesium/HCl conc.	Suspicious
Phenolic acids	$FeCl_3$ 5%	No white fluffy precipitate was observed (-)
	Iodine	No blue-black precipitate was observed (-)
Saponins	Foam test	Foam column persists for 30 min (+)
Tannins	$FeCl_3$ 5%	No blue-black precipitate appeared.
	Lead acetate 10%	No red-orange precipitate was observed (-)

+: Presence; –: Absence.

According to the results from Table 3, bitter melon seeds contain saponin, corroborating the research conducted by Zahan (Zahan et al., 2020). In addition to saponin, which showed positive results, alkaloids and tannins showed negative results, and compounds such as polyphenols and flavonoids yielded inconclusive outcomes. Hence, the project continued to conduct quantification to accurately evaluate the bioactive compounds content of seeds.

3.1.2. Quantitative results

Following the qualitative results, the study continued to conduct accurate surveys of the compound content to establish a database for subsequent experimentation. Quantitative results, such as moisture, total ash (minerals), flavonoids, and total polyphenol, were shown in Table 4.

Table 4. Quantitative results on the phytochemical composition of material

Compound	Unit	Content
Flavonoids	mgQE/100 g	91.10 ± 0.01
Total polyphenols	mgGA/100 g	478.95 ± 0.02
Ash (minerals)	%	1.85 ± 0.16
Moisture	%	5.27 ± 0.04

The results are expressed as the mean ± standard deviation of three replicates and were calculated based on absolute dry samples.

Maintaining an appropriate moisture level during storage was imperative. If the moisture level was too low or too high, it compromised the quality and integrity of medicinal herbs. High moisture fostered an environment for bacteria, mold, and insects to grow, causing spoilage of medicinal materials. The moisture content indicated in Table 4 was 5.27%, which was in accordance with the requirements of the Vietnam Pharmacopoeia (< 13%).

The investigated bitter melon seed powder's measured flavonoids content was 0.91 mgQE/g, and the TPC at 4.79 mgGA/g. These outcomes were equivalent to those of Horax *et al.* (2010), who studied the amount of total polyphenol at about 4.76 mgGA/g.

The research also investigated the quantity of minerals in bitter melon seed extract and recorded the absence of highly toxic metals such as lead (Pb) and cadmium (Cd). In addition, the extract contained some trace elements known for their nutritional and immunity benefits, including iron (Fe), zinc (Zn), manganese (Mn), and small quantities of copper (Cu) and nickel (Ni) (Table 5). These findings contributed to confirming that the quality of bitter melon seed oil conformed with Vietnam Pharmacopoeia V, where heavy metals were not detected exceeding 20 parts per million, equating to 20 mg/kg (MOH, 2011a).

Table 5. Analysis results of some metals in bitter melon seed extract

Element	Unit	Content
Cd	mg/kg	No detection (LOD = 1 mg/kg)
Pb	mg/kg	No detection (LOD = 2 mg/kg)
Cu	mg/kg	11.02 ± 0.05
Zn	mg/kg	48.26 ± 1.26
Fe	mg/kg	87.71 ± 3.20
Mn	mg/kg	66.23 ± 2.58
Ni	mg/kg	4.14 ± 0.06

3.2. The oil extraction process from bitter melon seeds

3.2.1. The oil extraction efficiency of various methods

The oil was extracted as described in section 2.2.2, and the extraction efficiency was shown in Figures 2, 3, and 4. The experiment selected for comparison featured short extraction times and low solvent consumption, yet demonstrated the highest efficiency of each method. The optimal process was then proposed by comparing it with other methods.

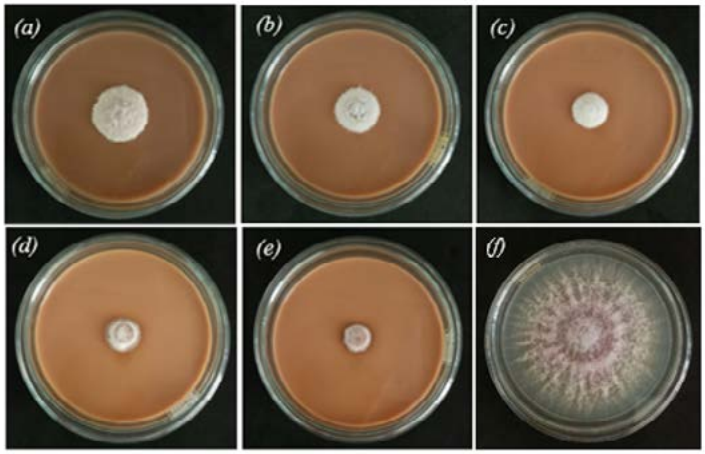

Figure 2. The lipid content was extracted by the maceration method.
ns: non-significant; *: $P < 0.05$; **: $P < 0.01$; and ***: $P < 0.001$.

The maceration method was simple and easy to execute but it was time-consuming. This method involved a static extraction mechanism where the solvent gradually penetrated the seed cells and dissolved the oil externally. As a result, the extraction efficiency was low and significantly dependent on the amount of solvent used and the lipid concentration within each plant. Investigating the impact of the material to solvent ratio and extraction duration on oil content revealed that the 12-h extraction experiment, using a ratio of 1:100, achieved the highest oil extraction efficiency. Therefore, this experiment was chosen as the optimal condition for the organic solvent extraction method.

Soxhlet extraction, using recirculated extraction solvent, was a technique that was easy to perform and required minimal equipment. However, it posed a challenge to apply on an industry scale.

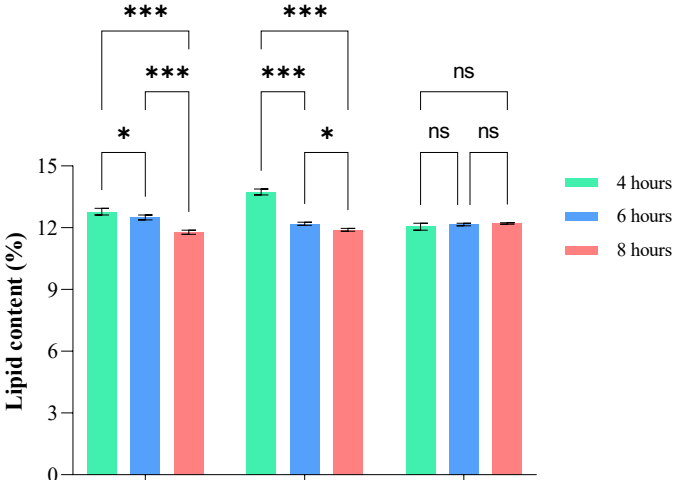

Figure 3. The lipid content was extracted by the Soxhlet method.
ns: non-significant; *: $P < 0.05$; **: $P < 0.01$; and ***: $P < 0.001$.

As shown in Figure 3, the lipid content obtained from the 4-h experiment with the ratio material: solvent of 1:60 and 1:80 was highest at 12.79% and 13.74%, respectively. These results demonstrated that increasing the amount of raw material necessitated more solvent and time extraction. However, once the saturation was reached (as observed in the experiment with a ratio of 1:100), the extraction took too long, proving to be inefficient and wasteful of solvents, which had negative implications for the environment and human health. Therefore, it was necessary to survey and select optimized extraction processes to save time, labor, and financial resources. For this method, the optimal condition was determined to be 4-h extraction time with a material to solvent ratio of 1:80.

The ultrasound-assisted extraction method had been a modern approach that accelerated the process with simple procedures but was expensive due to the need for specialized equipment.

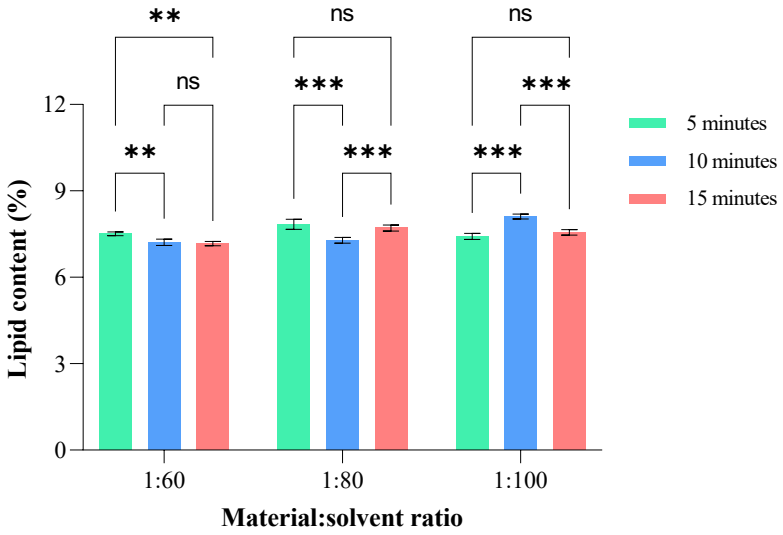

Figure 4. The lipid content was extracted by ultrasound-assisted method.
ns: non-significant; *: $P < 0.05$; **: $P < 0.01$; and ***: $P < 0.001$.

According to the test results, the 10-min extraction with a ratio of 1:100 achieved the highest oil extraction efficiencies (8.10%). Simultaneously, using low temperatures in the ultrasound-assisted method helped preserve the biological activity of secondary compounds, alongside the primary objective of maximizing lipid recovery.

3.2.2. Comparison of oil content obtained between three extraction methods

The experiments that had yielded the highest oil extraction efficiency for each method were subjected to one-way ANOVA analysis using GraphPad Prism version 9. Figure 5 showed that the average efficiency of the Soxhlet method (SL-1:80-4 h, with 13.74%) surpassed that of the maceration method using an organic solvent (M-1:100-12 h, with 11.79%) and the ultrasound-assisted method (UA-1:100-10 min, with 8.10%). These results were lower than the research by Ali et al. (2008), which reported the oil extraction efficiency of bitter melon seeds using the Soxhlet method was 26.00%. However, Ali's experiment utilized petroleum ether, a flammable solvent (boiling temperature 30 - 150°C), which could

irritate the skin, eyes, and mucous membranes and cause severe health issues such as drowsiness, dizziness, lightheadedness, nausea, unconsciousness, and coma when inhaled in high concentrations (Tociu et al., 2021). Thus, this study chose hexane as the solvent because it was not as flammable as petroleum ether (boiling temperature 68.70°C) and lower toxicity, though the extraction efficiency was lower (Herskowitz et al., 1971). Moreover, the differences in crop varieties, geographical conditions, and farming practices also influenced oil extraction efficiency.

The lower oil extraction efficiency of the ultrasound-assisted method was attributed to the short extraction time, limited power (500 W), and the presence of thick cell walls in bitter melon seeds, which led to poor separation efficiency and low dispersion within the cells. In addition, the probe's temperature could increase when the extraction time lasts too long, potentially denaturing biologically active compounds in oil. The maceration method using an organic solvent required an extended duration, ranging from 1 to 3 days, and achieved only 85% efficiency compared to the Soxhlet method. Although the extraction efficiency increased at elevated temperatures, the short circulation time of the solvent resulted in excessively prolonged extraction periods needed to completely extract the oil from the bitter melon seed samples.

Despite these challenges, the Soxhlet method consistently demonstrated the highest extraction efficiency with statistically significant differences among the methods examined. The Soxhlet technique had the potential to be scaled up for industrial use, accommodating volume up to 120 L per extraction process, making it suitable for a wide range of medicinal herbs. Thus, the practical application of this method was feasible.

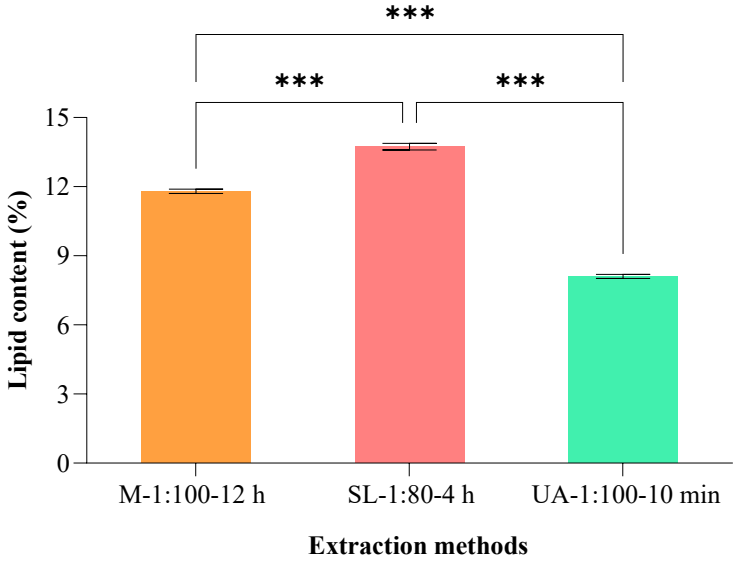

Figure 5. Compare oil extraction efficiency using different methods
ns: non-significant; *: $P < 0.05$; **: $P < 0.01$; and ***: $P < 0.001$.

3.2.3. Evaluating the quality of bitter melon seed oil

Bitter melon seed oil, extracted by the Soxhlet method as selected in the previous section, was evaluated for quality indices such as acid value, saponification value, peroxide value, and ester value (Table 6).

Table 6. Results of quality indices of bitter melon seed oil

Property	Unit	Content
Acid value (AV)	mgKOH/g	1.01 ± 0.01
Saponification value (SV)	mgKOH/g	355.60 ± 1.62
Peroxide value (PV)	meqO_2/kg	3.82 ± 0.02
Ester value	mgKOH/g	354.06 ± 1.32

FFAs, which was hydrolysis products of triglycerides (TGs) found in vegetable oils, primarily occurring during the production and preservation of oils. When fat was damaged, the amount of free fatty acids underwent degradation. Short-chain free fatty acids might be formed from the secondary oxidation of unsaturated aldehydes or other oxidation products originating from the cleavage of lipid hydroperoxides (Skiera et al., 2014; Alexandri et al., 2017). The concentration of FFA in edible oils depends on factors such as quality and variety of raw material, harvesting conditions, processing, storage, and deterioration status (Skiera et al., 2012). The acid value of bitter melon seed oil in this study was 1.01 ± 0.91 mg KOH/g, lower than the results reported by Tran et al. (2021) and Samba et al. (2022), which were 2.93 ± 0.25 mg KOH/g và 3.89 ± 0.37 mg KOH/g, respectively. Additionally, these values were higher than the research of Nkafamiya et al. (2007), which was 0.33 mg KOH/g. The lower AV, the more durable and higher the quality. Acid indices served as a crucial parameter for assessing the edibility of oil, and oil with a low acid number (< 4 mg/g) was suitable for human consumption (Moodley et al., 2007). The bitter melon seed oil had an acid value less than the limit for virgin edible oils (Codex, 1999), so it should be listed as an edible oil.

Determining the PV of edible oil was essential because PV was one of the most commonly used quality parameters to oversee lipid oxidation and control oil quality. The oil was easily oxidized during the processing and storage phases, which negatively affects oil quality and human health. Peroxide value shows the oxidation degree of the unsaturated fatty acids in products. When the peroxide value (PV) surpasses the critical threshold, edible oils could taste rancid and might even cause food poisoning (Gotoh et al., 2006). The peroxide number of bitter melon seed oil was 3.82 ± 0.81 meq O_2/kg, within the allowable standard range (< 10 meq O_2/kg) according to Codex-Stan 210-1999 (Codex, 1999). The peroxide value in this study was much lower than the PV of palm oil (16.08 meq/kg) and sorrel (Hibiscus sabdariffa) (5.00 ± 0.01 meq/kg) (Birnin-Yauri et al., 2011; Betiku et al., 2013).

The ester indices represented the amount of milligrams KOH required to saponify the esters contained in 1 g of oil, equal to the difference between the saponification index and the acid index. The ester value of bitter melon seed oil was 355.06 ± 1.77 mg KOH/g. Oil with a high saponification value was important for soap making and the cosmetic industry (Akanni et al.,

2005). The saponification value in this study was higher than argan oil (190.88 mg KOH/g) and olive oil (97.94 mg KOH/g) (Borchani et al., 2010). The solubility of soap in water depends on the quality of fatty acids, denoted by the saponification value. When this value was high, soap made from oil would be more soluble (Nyakudya et al., 2015). For that reason, bitter melon seed oil fulfills the requirement for a high SV and could be exploited as a material for making bath soap, lather shaving cream, and hair shampoo.

3.2.4. Antibacterial activity

The bitter melon seed oil was assessed for its antibacterial activity against two organisms: *S. aureus* and *Salmonella* sp. (Figure 6). These bacteria, commonly found in food and cosmetics, can cause some diseases such as skin infections, intoxication, and diarrhea. The results of the antibacterial activity of the bitter melon seed were presented in Table 7, with tetracycline 0.20% as the positive control and distilled water as the negative control.

Table 7. Results of measuring the diameter of the zones of inhibition

Organism	The diameter of the zones of inhibition (mm)	
	Bitter melon seed oil	Positive control
S. aureus	3.65 ± 0.05	20.50 ± 0.30
Salmonella sp.	3.50 ± 0.01	11.20 ± 0.05

According to Table 7, the oil extracted from bitter melon seeds demonstrated antimicrobial activity against both *Staphylococcus aureus* (3.65 ± 0.65 mm) and *Salmonella* sp. (3.50 ± 0.01 mm). The result was lower than the finding of Anjum et al. (2013), which reported inhibition zones of 22.80 ± 1.20 mm and 24.80 ± 1.30 mm, respectively, for two bitter melon varieties in Pakistan. However, the outcome surpassed the results reported by Tian et al. (2010) regarding to the antibacterial activity against *Salmonella* sp. of *Camellia oleifera* oil (2.50 mm). These differences could attributed to variations in bitter melon variety, solvent extraction, climate, agricultural practices, the growing season of each area, and the concentration of oil used in the experiments.

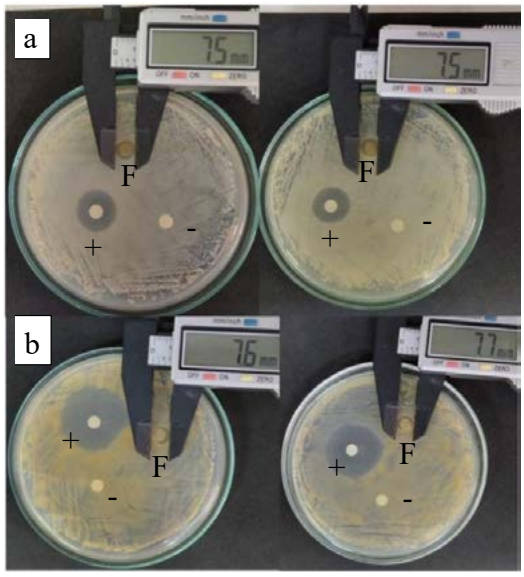

Figure 6. Testing antibacterial activity of bitter melon seed oil.
a) *Salmonella sp*.; b) *Staphylococcus aureus*; (F) Bitter melon seed oil; (+) Positive control: tetracycline 0.20%; (-) Negative control: distilled water.

3.2.5. Antioxidant activity

Based on the experimental results, the linear regression equation showing the correlation between the antioxidant activities and ascorbic acid concentrations was established as y = 1.7072x + 11.8380, with an estimated IC_{50} value of 0.03 mg/mL. Similarly, the linear regression formula showing the correlation between the antioxidant activities and the oil concentrations was established y = 0.2806x - 10.885 with an estimated IC_{50} value of 119.88 mg/mL (Table 8).

Table 8. IC_{50} of bitter melon seed oil

Sample	Linear regression equation	IC_{50} (mg/mL)
Ascorbic acid	y = 1.7072x + 11.8380	0.03
Bitter melon seed oil	y = 0.2806x + 10.885	119.88

The IC_{50} value of the oil was higher than that of ascorbic acid, indicating its weaker antioxidant capacity. However, bitter melon seed oil demonstrated a much better ability to scavenge free radicals compared to the finding on two bitter melon varieties in Pakistan (157.42 mg/mL and 143.59 mg/mL) (Anjum et al., 2013).

4. Conclusions

The qualitative analysis had shown that bitter melon seed contained various compounds, and the metal content in the seed met the standards set by the Ministry of Health in 2011. The best method for extracting oil from bitter melon seed was the Soxhlet method, which involved a 4-h extraction time and a 1:80 ratio of material to solvent, resulting in a 13.74% yield. The quality of the oil, including its acid, saponification, ester, and peroxide values, met the requirements of Vietnam standards for vegetable oils. The bitter melon seed oil also demonstrated antioxidant activity with an IC_{50} value of 119.88 mg/mL. Moreover, the oil showed inhibitory effects on the bacteria *Staphylococcus aureus* and *Salmonella* sp., with inhibition zone diameters of 3.65 mm and 3.50 mm, respectively. These results suggested that the seeds collected from bitter melon by-products were potential sources for oils and their applications in various fields.

Conflict of interest

The authors have declared no conflict of interest.

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Modeling of fresh paddy aeration on transporting barges in Mekong Delta, Vietnam

Hieu V. Nguyen[1], Duc A. Le[2], & Nghi T. Nguyen[2*]

[1]Faculty of Engineering Technology, Tien Giang University, Vietnam

[2]Faculty of Engineering and Technology, Nong Lam University, Ho Chi Minh City, Vietnam

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***Corresponding author**

Nguyen Thanh Nghi
Email:
ntnghi@hcmuaf.edu.vn

ABSTRACT

The Mekong Delta is regarded as the granary of Vietnam, with an annual production of 24.1 million tons (2023). Currently, fresh paddy is transported directly from farmers' fields to drying facilities or rice mills. Given the extensive river and canal system in the Mekong Delta, the majority of paddy (up to 92%) is transported by barges. While barge transportation is more cost-effective (91,000 VND/ton for a 100 km distance) compared to truck transportation (269,000 VND/ton), it has significant drawbacks, such as a longer transportation time of 3 to 5 days. Moreover, because the paddy is wet during transport, it is prone to discoloration (yellowing), which reduces its quality. To address this issue, this research developed an experimental paddy aeration model as a foundation for implementing aeration on barges during transportation. The model was tested with 1.4 tons of fresh paddy, specifically the DT80 variety, in Cai Lay, Tien Giang province. Key parameters monitored and analyzed included paddy temperature, moisture content, ambient conditions, and grain quality. The model was tested with a specific airflow rate of 129 ± 23 m^3/h per ton. The paddy temperature in the aeration model was maintained at 28.8°C, similar to the ambient temperature of 28.3°C. As a result, the whiteness of the aerated paddy was preserved at 3.6% after three days, whereas it decreased from 3.6% to 3.2% in paddy transported without aeration. The application of aeration helped reduce quality losses, particularly discoloration, caused by the high temperatures of fresh paddy during transportation.

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1. Introduction

Rice (*Oryza sativa* L.) is a major staple food crop for half of the world's population. With an annual production of about 24.1 million tons in 2023, the Mekong Delta is considered the granary of Vietnam. Unlike traditional practices, farmers now sell their fresh paddy to traders immediately after harvesting. The traders then transport the paddy to rice mills for drying and processing. Due to the Mekong Delta's extensive river system, paddy and other agricultural products are primarily transported by barges (Nguyen et al., 2015). In Long An province, for instance, 90% of fresh paddy is transported by barge (Nguyen et al., 2019). However, this method has disadvantages, including long transportation times, which can lead to losses, particularly in grain quality, due to discoloration caused by the high temperature within the grain pile on barges. Discoloration is triggered by fungi, bacteria, and environmental conditions such as high humidity and temperature (Gummert et al., 2020). The higher the temperature of grains during storage or transportation, the more intense the browning reaction (Fatharani et al., 2022).

Aeration involves the forced movement of ambient air through a grain bulk using fan power to improve grain storability. This technique is widely used in stored grain management programs in the United States to modify the microclimate within the grain bulk, reducing or eliminating the development of harmful organisms by lowering and stabilizing grain temperatures (Navarro et al., 2012).

While aeration technology has been applied to dry paddy in storage in Vietnam and other parts of the world, there has been limited research on its application to fresh paddy, particularly during barge transportation after harvesting. To address these research gaps, this study was conducted to identify the parameters affecting grain quality during barge transportation, complemented by experiments using a model.

2. Materials and Methods

2.1. Survey on the status of paddy transport in Mekong Delta

A survey was conducted in Tien Giang province, where the experimental model was installed, to assess the status of paddy transportation. Data were collected using a questionnaire, which included the ratio of paddy transported by different types of transportation (barges and trucks), transport costs, transport duration, and the advantages and disadvantages of each method. The sample size was determined using the following formula (Yamane, 1967; Israel, 1992):

$$n = \frac{N}{1+Ne^2} \quad (1)$$

Where: n is the number of samples (paddy transport barges); N is the estimated total number of paddy transport barges; and e is the estimated error level. With the annual rice production of 24.1 million tons/year, the annual transport capacity of barges of 1,000 tons/year, and the expected accuracy level of 0.1, the number of samples was computed at 99.6 (≈ 100) samples for survey in Mekong Delta. For the first stage of this study, a survey was conducted with 24 samples in the case of Tien Giang province with a rice production of 765,000 ton/year.

2.2. Experimental model design

Based on the practical size of a barge used for paddy transport, a model was designed for initial experiments with the dimensions of the aeration chamber of 2.5 m height (similar to the height of the paddy pile on barges), 1 m width and 1 m length (Figure 1).

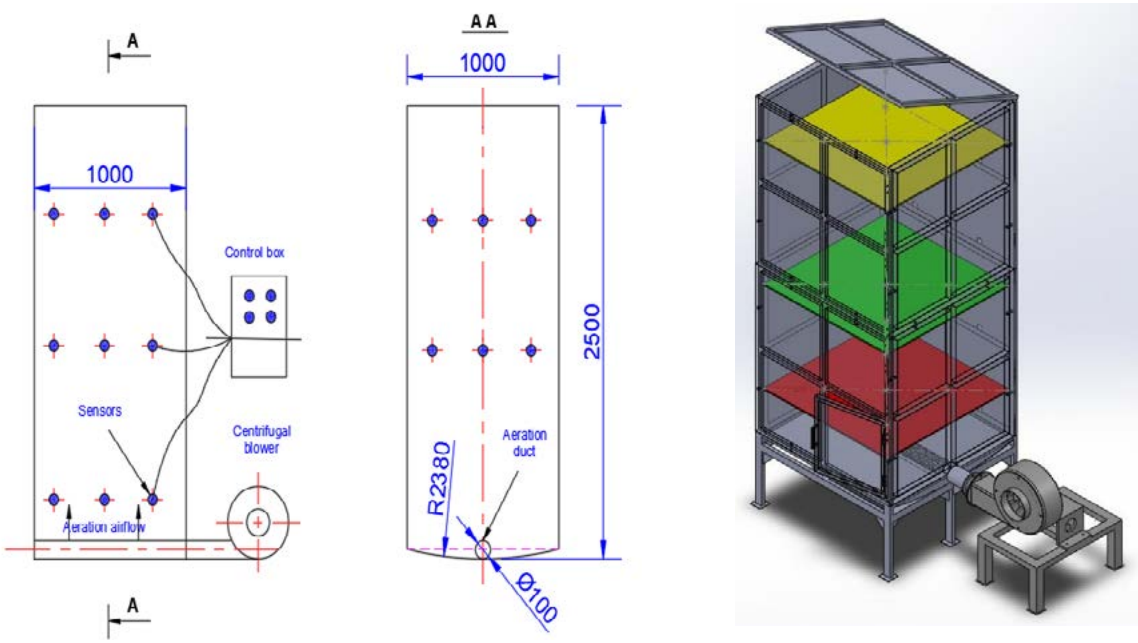

Figure 1. Experimental model (+ 3D) for fresh paddy aeration.

The model was loaded with 1.4 tons of fresh paddy, a DT80 variety, in Cai Lay, Tien Giang province. Inside the model, a set of sensors was installed in layers to monitor the temperature and humidity of the airflow, as well as the temperature of the grains. During the test, the parameters monitored included moisture content (MC), temperature, and relative humidity (RH) of the ambient air at the study site. The moisture content of the grain was measured using a moisture meter (Kett PM-600), and a digital scale with an accuracy of 0.01 g was used for weighing the paddy samples. Additionally, the static pressure of the airflow through the grain layer was measured and computed based on the following equation.

$$P = P_{1m} * h \quad (1)$$

Where: ΔP is the static pressure (Pa); h is the height of the grain layer (m); and ΔP1 m is the static pressure for each 1 m height of the grain layer (Pa/m), which is computed as follows:

$$P_{1\,m} = \frac{a*V^2}{ln\,(1+b*V)} \quad (2)$$

Where: V is the exit velocity of airflow (m/s); a and b are coefficients (a = 25700 and b = 13.2 for paddy).

2.3. Grain quality tests

Grain quality assessment was conducted based on criteria, such as head rice recovery (HRR), discoloration, whiteness percentage and amylose content. HRR was measured on milled rice and calculated using the following equation:

$$HRR, \% = \frac{Weight\ of\ whole\ grains}{Weight\ of\ paddy\ samples} x100 \quad (2)$$

Discoloration: This parameter was assessed based on the transparency which was measured using a milling meter (MM1D), with a range of 0.01 - 8.00%. The meter was also used to measure the whiteness and milling degree.

3. Results and Discussion

Figure 2. Barges for paddy transportation in Mekong Delta.

Transportation of paddy by barges was normally from 3 to 5 days for a distance of 100 - 200 km. It may take more time when the river is stuck by a dense layer of water hyacinth. The long time of transporting, together with the wet paddy being transported, the paddy is often yellowed, reducing its quality, and causing a loss in grain quality. Although transportation by truck takes less time, its cost is much higher, and it is unable to be applied in areas with a diverse river system in Mekong Delta.

As a result, the costs were 91,000 and 269,000 VND/ton for 100 km transportation by barges and trucks, respectively (Figure 3). Similarly, in Mekong Delta, rice straw bales are also mainly transported by barges and trucks. At the same distance, the costs are 470,000 VND/ton/km and 564,000 VND/ton for 100 km transporting straw bales by barges and trucks, respectively (Nguyen et al., 2015).

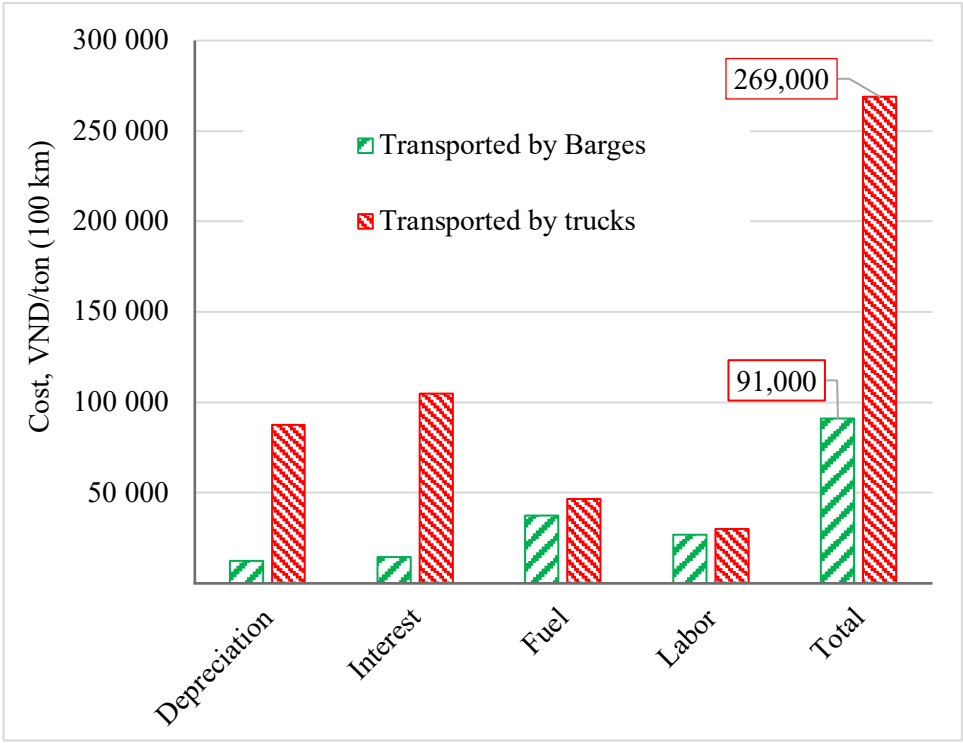

Figure 3. Components of transportation cost.

3.2. Testing results on the aeration model

Airflow rate for aeration: The airflow for aeration was supplied using a centrifugal blower with a specific airflow rate of 129 m^3/h per ton, corresponding to 2.2 m^3/min per ton, for the paddy layer of 2.5 m height in the model and the exit velocity of 3.2 m/s. According to Ranalli et al. (2002), a specific airflow rate for aerating with a 7 m height was tested at 1.3 m^3/min per ton, corresponding to an exit velocity of 4.3 m/s. However, it was 0.1 m^3/min per ton for the aeration of maize in a bin with a capacity of 597 tons (Maier, 1994). It meets the requirement for uniform airflow distribution with the exit velocity of less than 9 m/min (Navarro et al., 2012).

Temperature: The temperature of paddy in the model with aeration is 27.8°C, which is lower than the ambient temperature of 30.4°C on average. As shown in Figure 4, the bigger difference in temperature between the grains and ambient air was at noon. The reason for the lower temperature of the grains was due to the high temperature at noon while it is rather stable for the grains which were cooled at night and in the early morning.

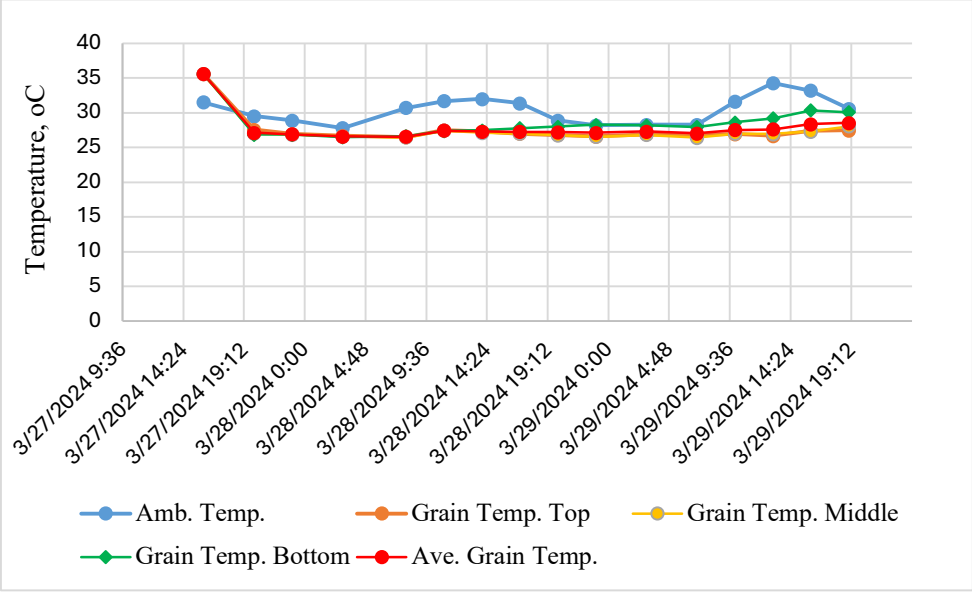

Figure 4. Fluctuation of aeration air and grain temperature.

Grain moisture content: A small but significant drying effect (moisture loss per aeration cooling cycle) was typically experienced. Although the main purpose of aeration was to cool the bulk grain, the moisture content of the grain descreased by 3%, from 25.5 to 22.7%, after the 49 h of aeration. It is explained that the moisture content was reduced due to the fundamentals of the moisture (H_2O) was produced from the respiratory process of the grain, which was removed from the grain pile during areation process. The data analysis resulted in a regression equation as shown in Figure 5. In practice, the reduction rate depends on the conditions of the ambient air around the model. As mentioned, the primary purpose of aeration is to remove heat from the paddy pile and control the grain temperature to prevent discoloration during transportation. Thus, the grain pile is aerated in both sunny and rainny conditions of the wearther.

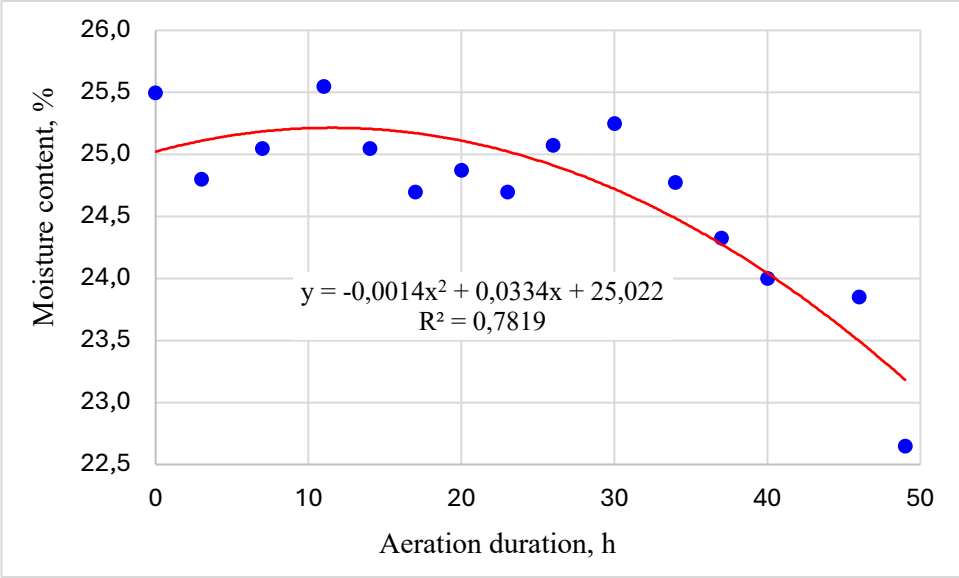

Figure 5. Grain moisture content reduction in aeration duration.

Grain quality: As a result, the whiteness of the paddy with aeration is maintained at 3.6% after three-day aeration, while it reduced from 3.6 to 3.2% for the paddy on a barge without aeration (Figure 6). This result indicates that the application of aeration helps reduce quality losses by preventing the paddy on the barges from turning yellow due to the high temperatures fresh paddy can reach during transportation.

There was no significant difference in whiteness percentage and amylose content between the paddy with aeration in the model and the paddy without aeration on barges. However, the head rice recovery was 1.1% lower for the treatment without aeration, compared to that of the treatment with areation.

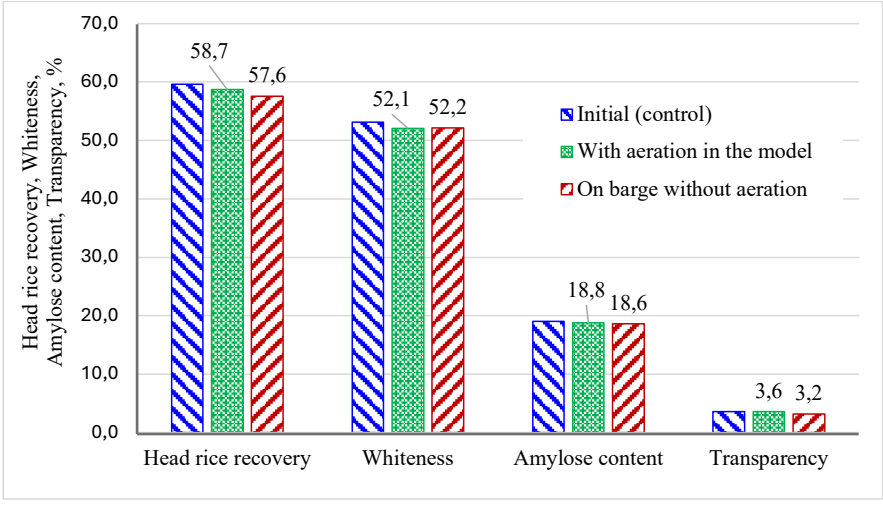

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Figure 6. Grain quality.

3.3. Designed aeration system for transporting barges

Based on the test results with the model, an aeration system was designed to be installed on a barge with a transport capacity of 60 tons, as shown in Figure 7. The system consists of the following main components: a diesel engine, a centrifugal blower, aeration ducts, sensors and instruments. The centrifugal blower with an airflow rate of 2 m^3/s, corresponding to a specific airflow rate of 120 m^3/h per ton, was driven by a separate 12-hp diesel engine installed on the barge. With a grain layer height of 2.5 m, the required static pressure corresponds to 116 mm H_2O.

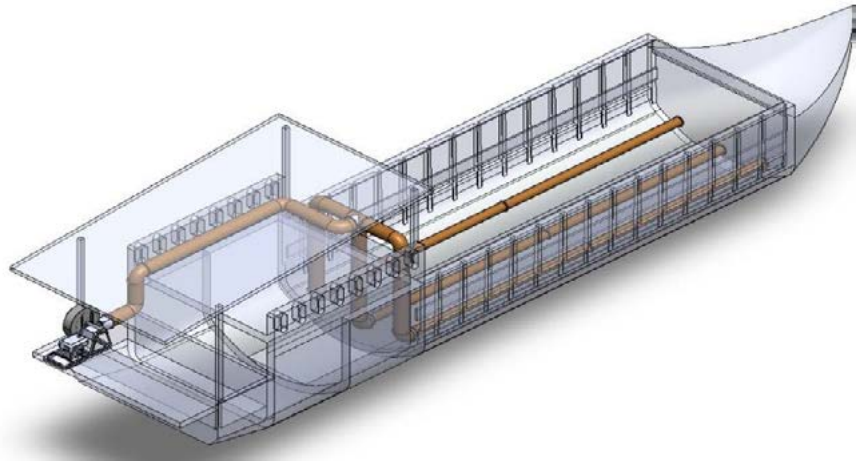

Figure 7. Aeration system designed for barges.

3.4. Environmental impact assessment

By reducing losses, the application of this technology indirectly contributes to a reduction in greenhouse gas emissions (GHGE). The higher the grain losses lead to the higher GHGE. For the stage of transporting paddy, GHGE coefficient was 0.41 kg CO_2-eq/ton per km, mainly due to fuel consumption (Gummert et al., 2020). In addition, the loss of grain quality means the loss of input materials in rice production, which contributes to GHGE during the production process. Based on the GHGE coefficient of 0.22 - 0.37 kg CO_2-eq/kg of paddy (Gummert et al., 2020; Win et al., 2020; Nguyen & Nguyen, 2023), the rice production of 24.1 million tons/year, and the estimated reduction in grain quality losses of 1%, due to on-barge aeration application, the total GHGE reduction was computed as 53,300 - 88,800 tons/year in Mekong Delta, Vietnam.

4. Conclusions

Based on the survey results, most of fresh paddy in Mekong Delta was transported to rice mills using barges after harvesting. The total cost of transportation was computed for each type of transportation (barges and trucks). It was found that the cost of transporting paddy by barges was one-third lower than that by trucks. To establish the basic parameters for designing an aeration system to be installed on barges, a model was designed and tested in Cai Lay district, Tien Giang province. The results showed that the grain temperature with aeration was controlled to be equal to or lower (especially at noon) than the ambient air temperature around the model. In terms of grain quality, transparency was maintained in samples with aeration, whereas it was lower in samples from barges

without aeration. This indicates that aeration could prevent discoloration, primarily caused by the high temperature of fresh paddy during transportation. Additionally, the reduction in losses contributed to a decrease in GHGE from inputs used in rice production.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgements

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Effects of partial precipitation and freeze-drying on morphology and physicochemical properties of rice starch hydrolysates

Ha V. Do*, Anh T. Vu, & Phuong T. Nguyen

Faculty of Chemical Engineering and Food Technology, Nong Lam University, Ho Chi Minh City, Vietnam

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***Corresponding author**

Do Viet Ha

Email:

dovietha@hcmuaf.edu.vn

ABSTRACT

Agricultural bio-catalysis is of immense scientific interest due to its increasing importance in the efforts for more sustainable agriculture while optimizing environmental impacts. In our studies, native rice starch was hydrolyzed with various alpha-amylase concentrations (0, 0.1, 0.2, and 0.3% w/w of starch) at 50°C for 20 min; then purified by partial precipitation (PP) with organic solvents, or freeze-drying (FD) without further purification. The rice starch hydrolysates (RSH) produced by different methods (PP or FD) were determined for dextrose equivalent (DE), morphology, and some physicochemical properties including bulk density, moisture content, hygroscopicity, and water solubility. The results showed that at the same alpha-amylase treatment conditions, the RSH obtained by the PP method had lower DE values and production yields than those of RSH obtained by FD method. The FD-RSH had higher DE values, lower bulk densities and moisture contents, higher hygroscopicity and water solubility. In morphology, the PP-RSH (DE 10.2) had a larger particle size and more condensed microstructure compared to the FD-RSH of almost similar DE 13.5. These findings showed that the PP method resulted in lower-DE RSH with different morphological and physicochemical properties compared to those obtained by the FD method.

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1. Introduction

Maltodextrins and glucose syrups are starch hydrolysis products (SHP) have found wide applications in the food, cosmetic and pharmaceutical industries (Castro et al., 2016). They are commercially produced from native starch through partial hydrolysis, purification, and spray-drying (Takeiti et al., 2010). They are usually classified by their values of Dextrose Equivalent (DE), a quantity that indicates the number of dextrose molecules released from the hydrolysis of starch and expressed as a percentage of the dextrose on a dry-weight basis (Dokic et al., 2004; Yunianta et al., 2015). Starch has a DE value of zero, while glucose has a DE value of 100. Maltodextrins are low convert starch products with DE values lower than 20, while high convert starch products with values equal and higher than 20 are known as corn syrup solids and glucose syrups (Klinkesorn et al., 2004; Saavedra-Leos et al., 2015; Balto et al., 2016). Their physicochemical and functional properties are influenced and controlled by the type (acid or enzymatic) and extend of hydrolysis, amylose to amylopectin ratio, source of starch, etc. (Dokic et al., 2004).

Starches from various botanical sources such as corn, cassava, manioc, wheat, oatmeal, sago, canna, maize, potato, and rice can be used for production of SHP (Klinkesorn et al., 2004; Moore et al., 2005; Takeiti et al., 2010). Traditional methods of acid hydrolysis resulted in products which are not completely soluble, colored and have a starchy taste, while enzymatic methods have been used to prepare soluble, non-hazy low DE-value maltodextrins (Dokic et al., 1998). Besides, the purification process such as partial precipitation of hydrolysates with polar organic solvents or drying methods (spray-drying/freeze-drying) could affect the hydrolysates' molecular mass, DE values, degree of polymerization (DP) range, and physicochemical properties (Kalac et al., 1984; Balto et al., 2016; Wang et al., 2020). This paper aims to study the effects of partial precipitation (PP) and freeze-drying (FD) methods on DE values, morphological and physicochemical properties of rice starch hydrolysates to have a better understanding of the properties of SHP produced from different botanical source, different purification and drying methods.

2. Materials and Methods

2.1. Materials and chemicals

Native normal rice starch (NRS) (Tai Ky Food Co., Vietnam) was purchased from a supermarket in Ho Chi Minh City, Vietnam. *Aspergillus oryzae* alpha-amylase (AAM) and commercial dextrin GLUCIDEX-12 were purchased from HiMedia Laboratories (India) and Roquette Freres (France). The organic solvents, acetone 100% and ethanol 96°, and chemical reagents, glucose ($C_6H_{12}O_6$), iodine (I_2), potassium iodine (KI), hydrochloride (HCl 37%), sodium chloride (NaCl), sodium hydroxide (NaOH), sodium dihydrogen phosphate dihydrate ($NaH_2PO_4.2H_2O$), disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4.12H_2O$), 3,5-dinitrosalicylic acid (DNS) ($C_7H_4N_2O_7$), and sodium potassium tartrate tetrahydrate ($KNaC_4H_4O_6.4H_2O$) were provided by Xilong Scientific (China).

2.2. Alpha-amylase hydrolysis of rice starch

The NRS was hydrolyzed with a fungal alpha-amylase following the method of Do et al. (2023) with minor modifications. NRS slurries containing 20% of NRS (w/v) in 0.1 M sodium phosphate

buffer of pH 6.0 were gelatinized at 95°C for 30 min and hydrolyzed with various concentrations of AAM (0, 0.1, 0.2, and 0.3% w/w of NRS) at 50°C for 20 min. The reactions were terminated by heating the mixtures at 95°C for 30 min.

2.3. Rice starch hydrolysates (RSH) purification and drying

After enzyme termination, each hydrolyzed mixture was cooled to room temperature and divided into two equal weight portions: the rice starch hydrolysates (RSH) in the first portion was purified by PP with organic solvents: hydrolysate precipitation using 3-fold volume of ethanol 96° following purification using ethanol 96° and acetone, then oven-drying at 45°C for 24 h; while the RSH in the remaining portion was obtained by FD method without purification. Yield of RSH was calculated as the percent weight of hydrolyzed starches to the initial weight of rice starch used for hydrolysis (Gunawan et al., 2023).

2.4. Dextrose Equivalent (DE) determination

DE values of NRS, commercial dextrin, and RSH were determined according to the method of Yunianta et al. (2015) with some modifications. Approximately 35 mg of sample was dissolved in 5 mL of distilled water, mixed with 15 mL of DNS solution, made up to 50 mL with distilled water, boiled for 45 min, cooled to room temperature, and then measured for absorbance at a wavelength of 540 nm. The dextrose or reducing sugar content in the sample was compared with glucose standard and the DE value of the sample was calculated according to the following formula, where C is reducing sugar content (mg/mL), V is volume of sample solution (mL), and m is sample weight (mg).

$$DE = \frac{C \times V}{m} \times 100\%$$

2.5. Scanning electron microscopy (SEM) observation

Morphological characteristics of the starch samples were observed using a Scanning Electron Microscope (S-4800, Hitachi, Japan) according to Do et al. (2023).

2.6. Physicochemical properties

Bulk density was obtained by gravimetric method according to Takeiti et al. (2010), weighing a sample powder poured into a 25 mL graduated cylinder. Bulk density was calculated as the material weight divided by the bulk volume.

Moisture content of the samples was determined by gravimetric method using an oven, measured as the percent moisture loss after drying to the initial wet weight of the sample (Duong et al., 2024).

Hygroscopicity was analyzed using 1 g of sample that was put in an aluminum cup and dried with the oven over the past 24 h, then dried sample was conditioned at relative humidity 96% in a closed saturated K_2SO_4 solution-containing chamber and weight was performed daily until equilibrium reached according the method of Hartiningsih et al. (2020). Percent moisture reabsorption of the sample was calculated concerning its initial dried weight.

Solubility of samples was analyzed according to Hartiningsih et al. (2020). Weighed 0.5 g of sample, dissolved into 50 mL of distilled water and stirred at 4000 rpm for 2 min using a homogenizer (T25, IKA, Germany). The suspension was centrifuged at 4000 rpm for 15 min, and 25 mL of the supernatant was taken and dried in the oven at 105°C for 48 h and obtained dry weight. Solubility was calculated as percent dry weight dissolved in the supernatant to the sample weight.

2.7. Statistical analysis

All measurements were performed in triplicate and results were expressed as means ± standard deviation. The analysis of variance (ANOVA) and the least significant difference (LSD) were performed at a value of $P < 0.05$.

3. Results and Discussion

3.1. Effects of hydrolysis degree, recovery and purification method on DE values

Table 1 showed measured DE values and yields of RSH obtained by PP and FD method. All the results were statistically significant differences. The PP method produced RSH with lower DE values (1.3 - 10.2) and yields (31.2 - 66.2%) compared to those (1.1 - 37.6 and 82.5 - 89.2%) obtained by the FD method. These results showed that the RSH produced by PP method were more purified with higher DP and higher molecular mass molecules, while those obtained by the FD method had higher yields of lower DP and lower molecular mass molecules. Fractional precipitation of aqueous solutions of partly hydrolyzed starches using organic solvents was performed to obtain different molecular mass fractions (Kalac et al., 1984). Ethanol was used for precipitation of starch and SHP in aqueous solutions and high ratio of ethanol (70% ethanol) narrowed ranges of maltooligosaccharides and preferentially removed glucose and maltose from SHP (Balto et al., 2016; Gunawan et al., 2023). Therefore, the PP method used in this study could narrow ranges of SHP to higher DP range and remove glucose and maltose which resulted in lower yields of RSH. The FD method without purification resulted in RSH with higher DE values and higher yields. Based on DE values, RSH could be classified into maltodextrins (DE 2-20) including PP-2001 (DE 3.9), PP-2002 (DE 7.2), PP-2003 (DE 10.2), and FD-2001 (DE 13.4), and glucose syrups were FD-2002 (DE 20.7) and FD-2003 (DE 37.6).

Table 1. Measured dextrose equivalent (DE) values of different types of rice starch hydrolysates (RSH)

Sample	C_{AAM} (%)	Yield (%)	DE
PP-control[1]	0	66.2 ± 1.4^{d}	1.30 ± 0.06^{b}
FD-control[2]	0	88.8 ± 1.3^{g}	1.09 ± 0.02^{a}
PP-2001[3]	0.1	51.2 ± 3.3^{c}	3.88 ± 0.04^{c}
FD-2001[4]	0.1	86.5 ± 1.1^{f}	13.45 ± 0.06^{f}
PP-2002[5]	0.2	31.2 ± 3.9^{a}	7.15 ± 0.01^{d}
FD-2002[6]	0.2	82.5 ± 0.4^{e}	20.72 ± 0.10^{g}
PP-2003[7]	0.3	39.7 ± 5.1^{b}	10.22 ± 0.02^{e}
FD-2003[8]	0.3	89.2 ± 2.1^{h}	37.64 ± 0.06^{h}

The data within a column followed by the different superscript letter are statistically significant difference ($P < 0.05$). C_{AAM}: alpha-amylase concentration; DE: dextrose equivalent; PP: partial precipitation; FD: freeze-drying.

[1]RSH (rice starch hydrolysates) obtained by PP of AAM-untreated rice starch.

[2]RSH obtained by FD of AAM-untreated rice starch.

[3]RSH obtained by PP of 0.1% AAM-treated rice starch.

[4]RSH obtained by FD of 0.1% AAM-treated rice starch.

[5]RSH obtained by PP of 0.2% AAM-treated rice starch.

[6]RSH obtained by FD of 0.2% AAM-treated rice starch.

[7]RSH obtained by PP of 0.3% AAM-treated rice starch.

[8]RSH obtained by FD of 0.3% AAM-treated rice starch.

3.2. Morphological characteristics

The NRS, PP-2003 (DE 10.2), FD-2001 (DE 13.5), and commercial maltodextrin GLUCIDEX-12 (DE 12) powders were chosen for SEM observations in Figure 1 to see the influence of alpha-amylase treatments, purification and drying method on the morphologies of RSH when compare to those of NRS and commercial maltodextrin. SEM micrographs showed that granules of the NRS had irregular cubic shapes with size less than 10 mm (Figure 1A), the PP-2003 has non-granular condense body shape with particle size larger than 50 mm (Figure 1B), while the FD-2001 (Figure 1C) and the commercial maltodextrin GLUCIDEX-12 (Figure 1D) have fragment structures with various sizes. The morphological differences found could be attributed to the PP and FD method of RSH which led to their different physicochemical properties mentioned below.

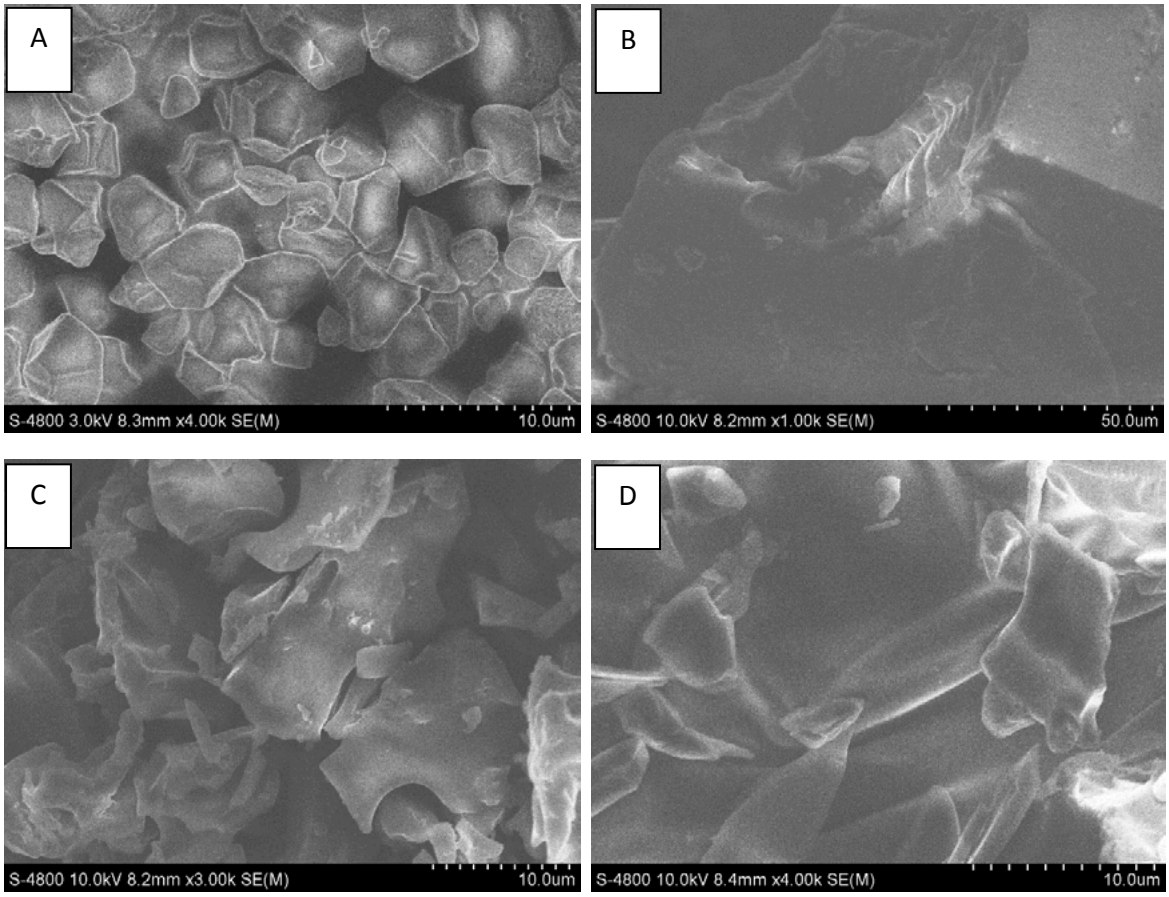

Figure 1. SEM micrographs of NRS (A), PP-2003 (B), FD-2001 (C), and GLUCIDEX-12 (D). NRS: normal rice starch; PP: partial precipitation; FD: freeze-drying.

3.3. Physicochemical properties

Table 2 showed the physicochemical properties of native normal rice starch (NRS), commercial maltodextrin GLUCIDEX-12, and RSH obtained by PP and FD method. The RSH produced by PP method (PP-RSH) have higher bulk densities (0.416 - 0.647 g/mL) than those of RSH produced by FD method (FD-RSH) (0.377 - 0.479 g/mL) at same enzyme treatment conditions, however their lower bulk densities were lower than that of commercial maltodextrin (0.746 g/mL). Higher bulk densities of PP-RSH and commercial maltodextrin could be explained from their morphological characteristics since they have larger particle sizes and more condense microstructures which have been observed in Figure 1B and 1D. In terms of moisture content and hygroscopicity, the PP method produces RSH with higher moisture content (9.3 - 11.9%) but lower hygroscopicity (20.4 - 48.9%) at same enzyme treatment conditions when compared to those obtained by FD method (4.4 - 6.0% and 22.0 - 66.7%), respectively. In terms of solubility, the PP method produced RSH with lower solubility (5.8 - 34.8%) compared to that obtained by FD method (9.8 - 76.4%). The higher moisture content, lower hygroscopicity and solubility of PP-RSH can result from the large size and condense microstructure of product particles, narrow higher DP and lower DE values; whereas the lower moisture content, higher hygroscopicity and solubility of FD-RSH can result from the smaller particle sizes, less condense microstructures, and higher DE values. From these obtained results, the PP and FD method produced RSH with different morphological and physicochemical properties that might be beneficial or unbeneficial for technological applications. For instance, the FD-RSH have dramatic high hygroscopicity would not be suitable for keeping the powder state for a long time, while the PP-RSH have dramatic low solubility would not be suitable for product formulation without heating.

Table 2. Physicochemical properties of normal rice starch, commercial dextrin, and rice starch hydrolysis products

Sample	Bulk density (g/mL)	Moisture content (%)	Hygroscopicity (%)	Solubility (%)
NRS (DE 0)	0.573 ± 0.035^{e}	11.5 ± 0.2^{h}	8.2 ± 1.9^{a}	0.6 ± 0.0^{a}
PP-control[1] (DE 1.3)	0.416 ± 0.003^{b}	9.3 ± 0.1^{f}	20.4 ± 0.5^{b}	5.8 ± 0.3^{b}
FD-control[2] (DE 1.1)	0.446 ± 0.011^{c}	5.7 ± 0.1^{d}	22.0 ± 0.8^{c}	9.8 ± 0.3^{c}
PP-2001[3] (DE 3.9)	0.563 ± 0.018^{e}	10.9 ± 0.0^{g}	32.1 ± 0.2^{d}	34.8 ± 0.6^{f}
FD-2001[4] (DE 13.5)	0.472 ± 0.006^{d}	6.0 ± 0.0^{e}	46.0 ± 2.5^{f}	76.4 ± 0.0^{i}
PP-2002[5] (DE 7.2)	0.647 ± 0.013^{f}	11.9 ± 0.0^{i}	39.2 ± 1.7^{e}	20.2 ± 0.3^{d}
FD-2002[6] (DE 20.7)	0.479 ± 0.009^{d}	5.1 ± 0.2^{b}	64.9 ± 1.3^{h}	74.4 ± 0.6^{h}
PP-2003[7] (DE 10.2)	0.642 ± 0.003^{f}	11.9 ± 0.1^{i}	48.9 ± 0.1^{g}	26.2 ± 0.3^{e}
FD-2003[8] (DE 37.6)	0.377 ± 0.019^{a}	4.4 ± 0.0^{a}	66.7 ± 0.7^{h}	73.0 ± 0.8^{g}
GLUCIDEX-12 (DE 12)	0.746 ± 0.021^{g}	5.4 ± 0.3^{c}	34.5 ± 0.7^{d}	100.0 ± 0.0^{j}

The data within a column followed by the same superscript letter are not statistically significant difference (P > 0.05). NRS: normal rice starch; DE: dextrose equivalent; PP: partial precipitation; FD: freeze-drying.

[1]RSH (rice starch hydrolysates) obtained by PP of AAM-untreated rice starch.

[2]RSH obtained by FD of AAM-untreated rice starch.

[3]RSH obtained by PP of 0.1% AAM-treated rice starch.

[4]RSH obtained by FD of 0.1% AAM-treated rice starch.

[5]RSH obtained by PP of 0.2% AAM-treated rice starch.

[6]RSH obtained by FD of 0.2% AAM-treated rice starch.

[7]RSH obtained by PP of 0.3% AAM-treated rice starch.

[8]RSH obtained by FD of 0.3% AAM-treated rice starch.

4. Conclusions

The differences in physicochemical properties of starch hydrolysis products from the same botanical source and enzyme treatment conditions can be explained from the differences in morphological characteristics, DE values and the purification and drying method. The partial precipitation method produces starch hydrolysis products with large condense particles, lower DE values and yields, higher bulk densities and moisture contents, lower hygroscopicity and solubility; while the freeze-drying method produces starch hydrolysis products with less condense fragments, higher DE values and yields, lower bulk densities and moisture contents, higher hygroscopicity and solubility. Thus, based on these findings, suitable purification and drying method can be applied for production of starch hydrolysates with desired morphological and physicochemical properties.

Conflict of interest

The authors declare no conflict of interest.

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