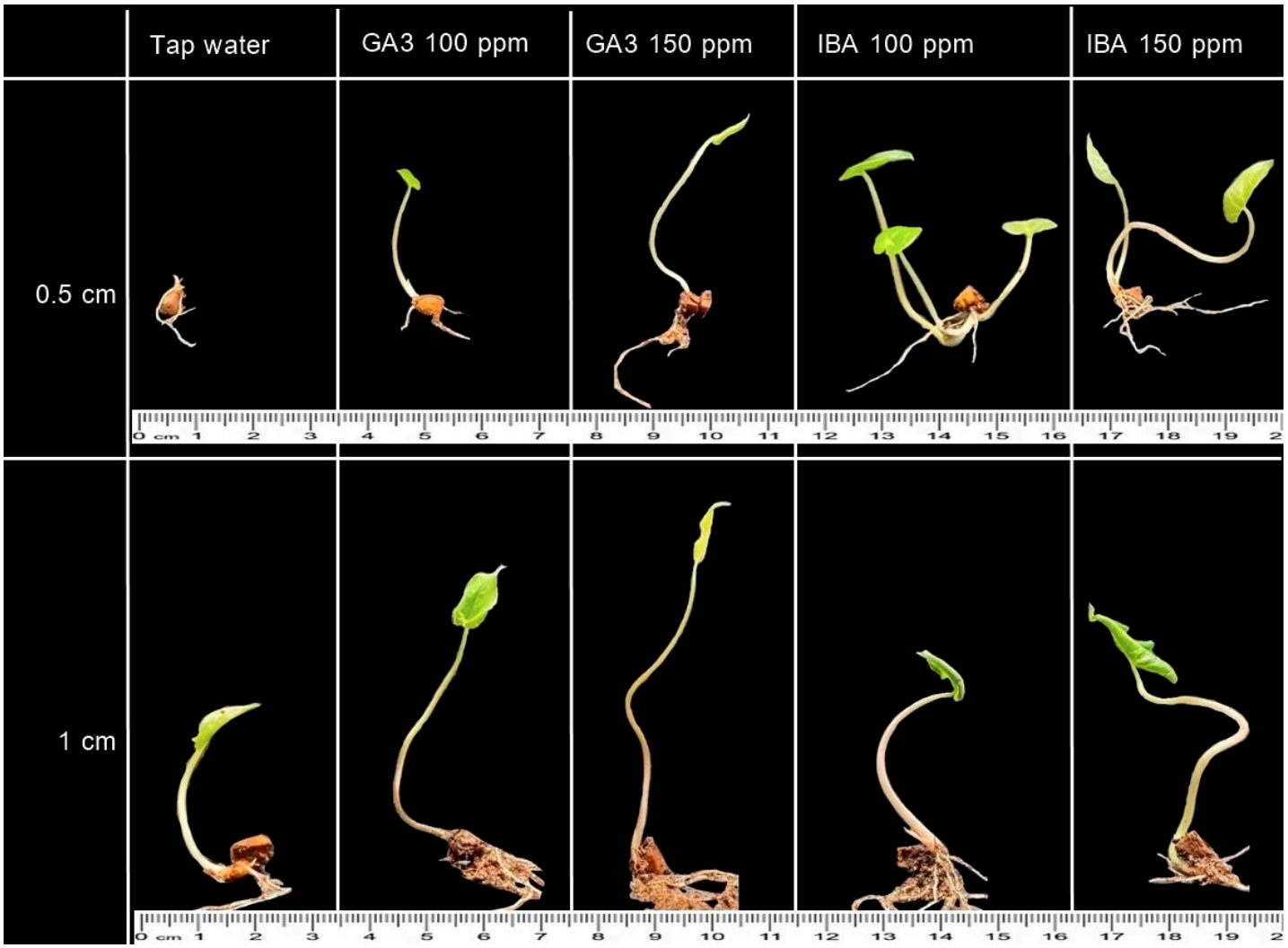
JSTR
ASEAN Journal of Scientific and Technological Reports
Vol. 26 No. 2, April - June 2023



ISSN 2773-8752 (online)
https://ph02.tci-thaijo.org/index.php/tsujournal/issue/view/16950

ASEAN Journal of Scientific and Technological Reports (AJSTR)

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Editorial

The ASEAN Journal of Scientific and Technological Reports (AJSTR) Vol. 26 No. 2 (April-June 2023) ISSN 2773-8752 Published eight worth-reading research articles. Experts from various universities and institutions reviewed and answered these exciting research articles. We sincerely hope some research papers will help guide and motivate our active researchers in ASEAN to produce and create more valuable research shortly. The AJSTR has served our energetic readers and customers on an international level.

This issue details the post-harvesting with different photoperiods under artificial light sources on nitrate and vitamin C contents in hydroponic green oak lettuce, disruptive technology Impacts industrial engineering professionals under the context of Thailand 4.0, the effect of eliminating the application of phosphorus-containing fertilizer for the bulking period of sweet potato (*Ipomoea batatas)* production, increased growth of *Caladium* by tuber section and plant growth regulators, applying the flyweight design pattern to android application development, the effect of *Durio zibethinus* murr. cv. monthong rind as a dietary ingredient in feed on the growth performance and disease resistance against *Aeromonas hydrophila* in red tilapia (*Oreochromis niloticus x Oreochromis mossambicus*), and antimicrobial activity of extremely halophilic archaea isolated from southern thai salt-fermented products and solar saltern of Pattani, Thailand, biocompatibility study of gelatin-blended fibroin scaffold

The AJSTR and an editorial team are ready to organize, manage, publish, and deliver all good quality articles written in well-organized English to the world of academic society.

Assoc. Prof. Dr. Sompong O-Thong
Journal Editor-in-Chief

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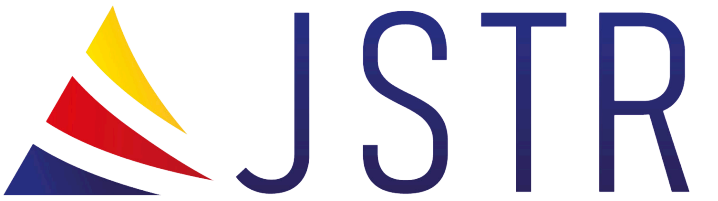

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Research article

A Study of Disruptive Technology Impacts on Industrial Engineering Professionals under the Context of Thailand 4.0

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Citation:
Sangkhiew, N.; Karot, T.; Kamphukhieo, K.; Thirawongprawes, T.; Pornsing, C.; Sanonok, A.; Inthawongse, C. A Study of disruptive technology impacts on industrial engineering professional under the context of Thailand 4.0. *ASEAN J. Sci. Tech. Report.* **2023**, *26*(2), 1-9. https://doi.org/10.55164/ajstr.v26i2.247893.

Article history:
Received: December 20, 2022
Revised: March 12, 2023
Accepted: March 20, 2023
Available online: March 26, 2023

Abstract: The purpose of this study is to examine the impact of disruptive technologies on the industrial engineering profession and learn how to deal with such disruptions. The study uses questionnaires to collect data from 8,149 individuals practicing industrial engineering at the Associate Engineering level and Senior Professional Engineering levels. After that, the sample is calculated using the Taro Yamane method with a confidence level of 95 percent. Then, the main reasons for the impact of disruptive technology on industrial engineers were analyzed using the Affinity Diagram, and the problem structure was clarified using the Relations Diagram. The main cause was analyzed to find a solution with the help of a Tree Diagram. Finally, the matrix diagram was used to find the correlation of solutions by comparing 11 technologies that will affect global change, according to research by McKinsey Global Institute. The result of this research shows that disruptive technology directly impacts the work of the sample, affecting Decision Making and investment in industry and technology rather than replacing people. Therefore, industrial engineers should further explore technology and engineering such as Big Data, the Internet of Things (IoT), and maintenance engineering. This also includes improving one's language skills and ability to collaborate with others.

Keywords: Disruptive technology; industrial engineering; Thailand 4.0

1. Introduction

Industrial engineering emerged simultaneously with the Industrial Revolution when production systems required mass production at a lower cost [1]. Frederick W. Taylor, the father of Industrial Engineering, invented an approach to study guidelines and applied it successfully at Bethlehem Steel Co. to increase productivity. He also created scientific management principles that have been continuously applied and developed [2].

However, the challenges of a changing world mean a constant struggle for the profession of industrial engineering. In the 21st century, these challenges may be more pressing than ever. This is especially true considering the impact of disruptive technologies and innovations [3-4] that will fundamentally change

manufacturing and the service sector. In addition, the Thai government has embraced change by encouraging everyone to escape the middle-income trap by producing and providing high-value, high-technology products to prevent the country from becoming a low-performing country and to support the country's target industries [5]. Thailand's Industry 4.0 policy proposes a new technological development and manufacturing paradigm with profound economic, environmental, and safety implications. It is believed that the exploration of Industry 4.0 is a viable way to create sustainable manufacturing [6]. The division between the S-Curve and New S-Curve groups has raised concerns about the adaptability and competence of the industrial engineering profession.

This study aims to answer the question of what the industrial engineering profession will look like in the future. Adapting to the situation and the impact of disruptive technology under the accelerator of Industry 4.0. This is a survey to test hypotheses and draw conclusions based on the interest of interested groups regarding the profession of industrial engineering.

2. Methodology

This study is exploratory and based on data collected through a stakeholder questionnaire. The specific characteristics of the population, sample and analytical instruments are listed below:

2.1 Population and sampling

This study considered a population of the Council of Engineers working in industrial engineering, divided into two levels: 8,002 people with Associate Engineering levels and 147 people with Senior Professional Engineering levels (2019 data). Then, as indicated in equation (1), the Taro Yamane method is used to determine the number of samples with a 95% confidence level [7].

$$n = \frac{N}{1+N(e)^2} \tag{1}$$

Where: N = number of people in the population

n = sample size requires

e = allowable error

After calculating the sample size by substituting the numbers into the Yamane formula, $N_1 = 8{,}002$ persons, $N_2 = 147$ persons, and $e = 0.05$, the number of samples is $n_1 = 381$ persons (Associate Engineering levels) and $n_2 = 108$ persons (Senior Professional Engineering levels).

2.2 Tools for research

This study used surveys and interviews to collect data, feedback, and recommendations. The data is analyzed using four new high-quality tools to find answers to the question: Affinity Diagram, Relationship Diagram, Tree Diagram, and Matrix Diagram, which are as follows:

2.1.1 Questionnaires and interviews

The questionnaire consists of four parts: personal information and comments on specific industrial engineering topics suitable for the era of Industry 4.0. Comments on the impact of disruptive technologies on the industrial engineering profession and other recommendations. The interviews assessed industrial engineering stakeholders to obtain the most accurate and relevant preliminary information possible.

In this study, the validity of the questionnaires and interview forms was assessed by three qualified individuals to determine the quality of the questionnaires. Then, the IOC approach was used to check the content and language concordance index values.

2.1.2 The New QC tools in research

The review revealed secondary data, which are qualitative data that cannot be quantitatively calculated mathematically. Therefore, these data were analyzed using contemporary qualitative tools to ensure the data's viability and reliability [8]. The analysis tools are described below:

1) Affinity diagrams organize secondary data combining citations and opinions on technical subjects for industrial engineering disciplines.
2) Relationship diagrams identify cause-effect relationships for primary and secondary data with various correlations. It shows the appropriateness of the quotes from the interview and the characteristics of the others.
3) Tree diagrams evaluate solutions from multiple key sources at different levels to understand the potential cause-and-effect relationships.
4) Matrix diagrams are used to compare 11 solutions and technologies impacting global change [8]. This has sudden implications for the industrial engineering profession.

3. Results

3.1 Findings from the analysis of the research tool

The three experts will read all questionnaires to assess validity. The evaluation criteria for the questions are as follows:

1 means that they are convinced that the questions meet the objectives.

0 means they are unsure whether the question meets the purpose.

-1 means that it is certain that the question does not meet the purpose.

Then, the experts evaluated the questionnaire for its content and index of consistency (IOC). The results were used to evaluate the average value of 12 questions using the IOC. All questions had a concordance index greater than 0.60, indicating that the questionnaire was appropriate for the subject. The results of this survey were valid because respondents consistently understood the questions.

3.2 Results of questionnaire analysis

According to a survey of industrial engineering stakeholders, 36.4% of respondents had worked in industry and education for over 20 years. This level of experience helps to understand better how the industrial engineering profession has changed over time and helps to shed light on the true impact of disruptive technology adoption on labor market demands. Therefore, the data collected in this study are acceptable and reliable. In addition, the respondents have a working duration of 16-20 years (24.2%), 11-15 years (22.2%), and others (17.2%), respectively. According to the above findings, the respondents have high industry knowledge and expertise. Therefore, it is very likely that the questionnaires used in this research will provide actual data for an in-depth study when evaluating the hypothetical results of the research.

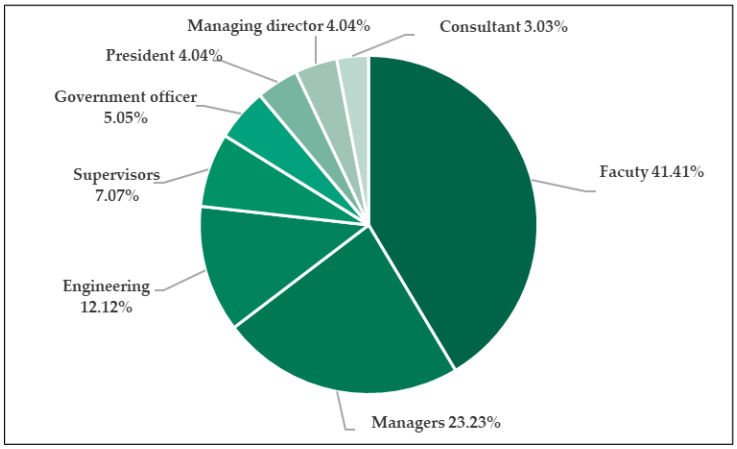

Figure 1. The proportion of respondents' positions

Regarding the categorization of the operating group, the respondents included individuals working in industrial engineering: Associate Engineering (78%) and Senior Professional Engineering (22%), both of which are stakeholder groups directly related to the industrial engineering career field. Explain in detail how technology has evolved and how much the field of industrial engineering has changed. Faculty respondents made up most respondents at 41.41%. They were followed by managers (23.23%), engineers (12.12%), supervisors (7.07%), government officials (5.05%), presidents (4.04%), general managers (MD.) (4.04%), and consultants (3.03%), as shown in Figure 1.

Respondents' responsibilities are divided into two categories: 1) 229 industry professionals divided among Electronics (24%), Automotive (19%), Food (19%), Consumer Products (8%), and Other (30%); and 2) 152 faculty members in Industrial Engineering who currently have teaching loads, divided among Work Study (15.6%), Plant Design (15.6%), Maintenance Engineering (13.3%), Production Planning and Control (11%), Safety Engineering (6.7%), Engineering Economic (6.7%), and Other (31.1%), as shown in Figure 2.

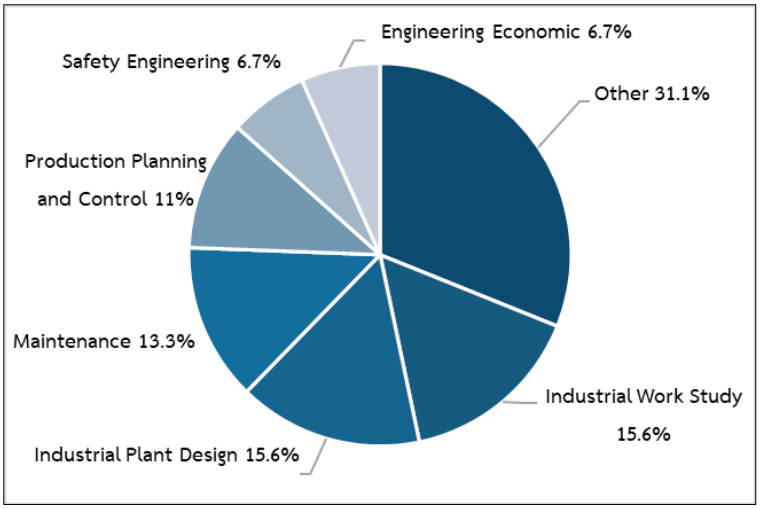

Figure 2. Faculty proportions

The importance of engineering-specific subjects for industrial engineering meets Industry 4.0 with a matrix diagram of eight fundamental subjects in the Council of Engineers industrial engineering certification exam. Respondents prioritize the application of knowledge to their work areas and tasks. The subjects in the rankings are as follows: 1) Safety Engineering, 2) Industrial work study, 3) Production Planning and Control, 4) Quality Control, 5) Industrial Plant Design, 6) Operations Research, 7) Engineering Economics, and 8) Maintenance Engineering, as shown in Table 1.

Table 1. Prioritization of subjects

	Subjects	Prioritization of subjects							
		1	2	3	4	5	6	7	8
1	Safety Engineering	**76**	53	50	38	34	30	50	50
2	Industrial Plant Design	53	38	72	42	**57**	46	34	38
3	Production Planning and Control	57	61	**88**	50	38	50	19	19
4	Quality Control	69	72	57	**50**	46	30	27	30
5	Industrial Work Study	69	**88**	34	27	57	50	23	34
6	Operation Research	53	46	46	38	53	**72**	30	42
7	Engineering Economics	53	65	53	30	38	46	**61**	34
8	Maintenance Engineering	57	46	30	34	46	27	53	**88**

Table 1 shows respondents with first-hand knowledge of industrial engineering ranked safety engineering as the most important. Therefore, it should be taught in this degree program. The subjects of secondary importance are Industrial Work Study and Production Planning and Control. To prioritize 8 basic subjects, all of which are basic subjects of the profession and taught in the current curriculum. It is based on the actual application in the respondents' industry. The survey shows that the subjects taught are still important and applicable in the industry. However, to train industrial engineers capable enough to meet the market's demands, education must still adapt to the changes brought about by disruptive technology.

3.3 Analysis of disruptive technology affecting industrial engineering

Based on interviews with stakeholders in the industrial engineering profession: the Associate Engineering level and the Senior Professional Engineering level, to examine the impact of disruptive technology on the industrial engineering profession. It was found that these issues can be divided into three groups: 1) technology, 2) investment and decision-making, and 3) labor. We analyzed all the collected data with four new quality tools to find the exact correlation and cause, which led to the following results:

3.3.1 Affinity diagram

After collecting the stakeholder interviews, the data is organized into groups to work together on any issues that arise. This makes it easy to see and understand. Figure 3 shows how the comments are grouped.

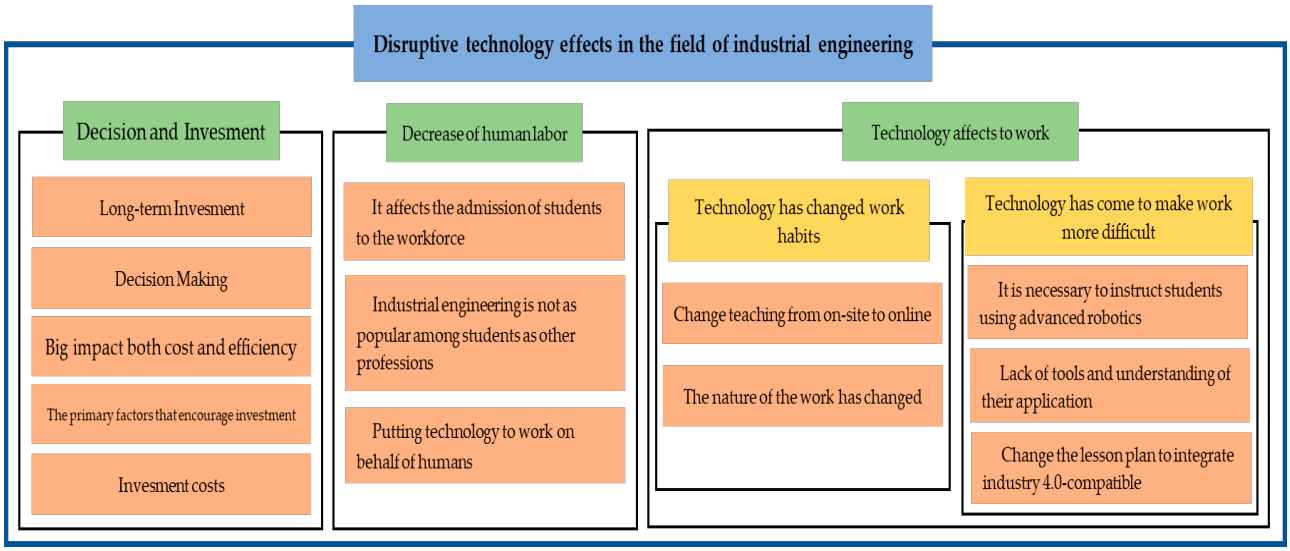

Figure 3. Affinity diagram

3.3.2 Relations diagram

The problem was classified using the affinity diagram. We defined the relationships between the three groups of problems to understand the results better. As shown in Figure 4, the field of industrial engineering is highly affected by disruptive technology, which can be divided into problem groups as follows:

1) Technological problems are changing our work, making it more difficult and complex. On the other hand, the technology we use at work is very useful, especially in supporting humans. Humans must try to adapt to life with technology. Therefore, the semester should familiarize students with technological learning opportunities to cope with rapid technological changes and constantly prepare them to pursue knowledge.

2) Due to disruptive technology and expensive investment, investment and decision-making problems affect executives and entrepreneurs. For this reason, the company is exposed to relatively high risks when deciding to invest in technology. Therefore, executives and entrepreneurs must consider usability and cost-effectiveness when making investment decisions.

3) The workforce problem is becoming more serious as technology replaces personnel in areas that do not require critical thinking or problem-solving skills. Therefore, using technology to increase production and efficiency while reducing costs is wise for businesses.

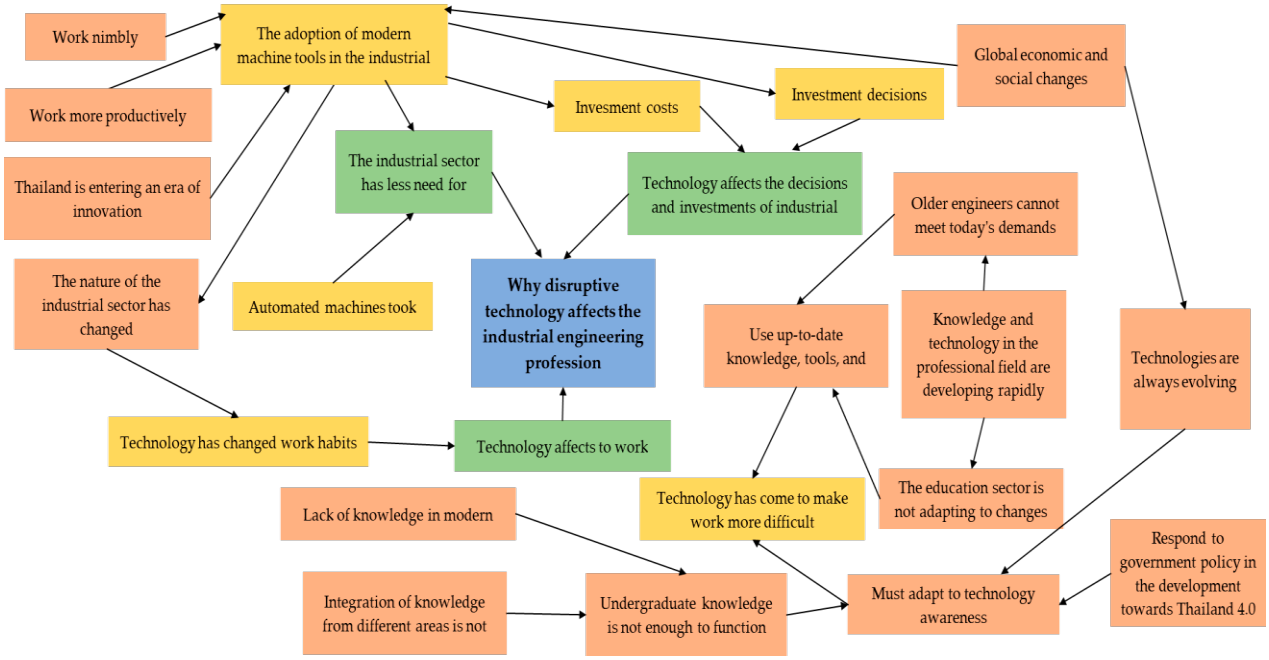

Figure 4. Relations diagram

3.3.3 Tree diagram

We analyze the solutions and improvements using the tree diagram. As shown in Figure 5, it is necessary to understand the causes of disruptive technology and adaptation approaches. Data were obtained from questionnaires and interviews with industrial engineering stakeholders.

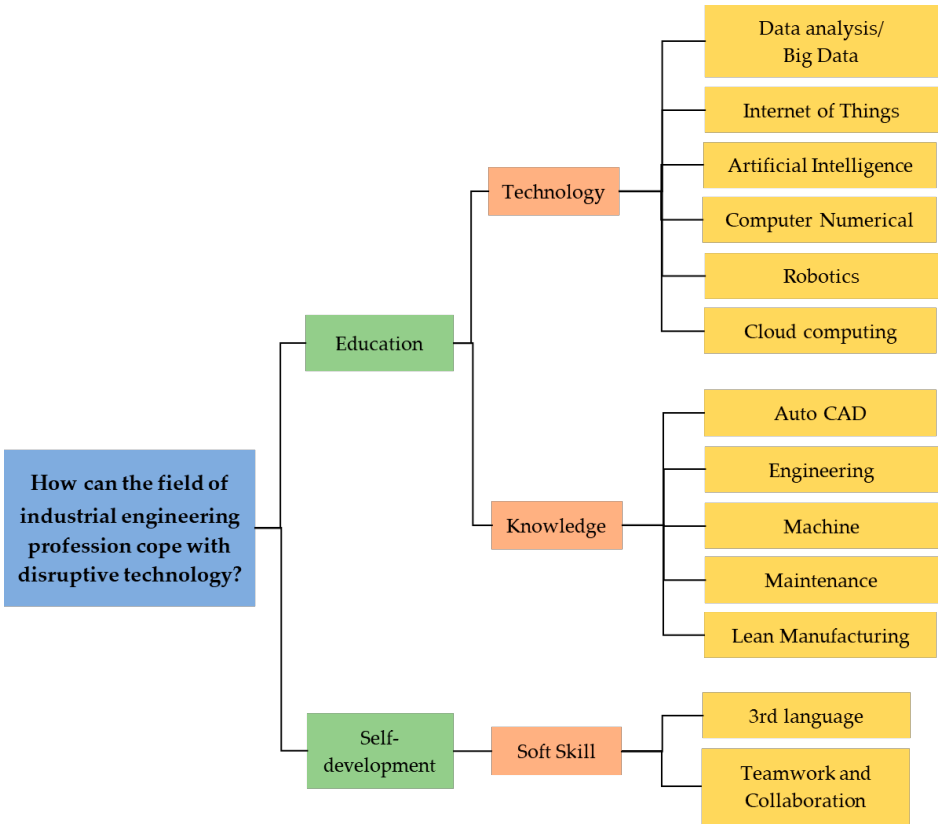

Figure 5. Tree diagram

From the analysis, solutions and improvements to address the impact of disruptive technologies consist of two approaches:

1) Industrial engineering stakeholders should educate themselves in the areas of technology and engineering. Technology knowledge focuses on learning more about Big Data, Computer Numerical Control (CNC), the Internet of Things (IoT), Artificial Intelligence (AI), Robotics, and Cloud technology. The details are as follows:
    - 1.1 Big Data: There is a large amount of structured and unstructured data. Understanding how to analyze and manage data to use it effectively is important. Knowledge of Big Data is an essential tool for direct data management.
    - 1.2 CNC: It significantly reduces errors in the production process.
    - 1.3 IoT: The IoT is indispensable today as it enables all forms of communication, including the operation of devices that collect data and search for data quickly. Undoubtedly, this technology is already part of our daily lives.
    - 1.4 AI: One of the factors that are critical to the performance of industrial engineering is AI. For example, AI can quickly determine the best solutions for engineers and is often used in cost-conscious planning of production processes.
    - 1.5 Robotics: robotics is a popular technology used in various industries where humans cannot perform all tasks themselves due to their limited cognitive abilities in certain tasks.
    - 1.6 Cloud technology: Cloud technology is also important for the knowledge that enables the industrial engineering profession to deal with disruptive technologies.

Engineering fundamentals assume that engineers should already have these skills. Still, learning more about software such as computer-aided design (CAD), engineering statistics, machine learning, maintenance engineering, and lean manufacturing is a good idea. This is important knowledge that is used in most tasks and is specific to the field of industrial engineering [10]. According to the survey, 36.4% of the industrial engineers surveyed have more than 20 years of professional experience. Twenty years ago, Computer Numerical Control (CNC) was not widely used in the Thai industry, so such content did not cover teaching in industrial engineering.

It was found that there is an imbalance between the number of students who have graduated in this field and the number of students applying for engineering licenses. As a result, there are few recent engineering graduates in this research population. This suggests that few industrial engineers know disruptive technology's real impact. Stakeholder in the field of industrial engineering must strengthen their soft skills. According to interviews, the abilities required for today's industrial engineering career include communication and interpersonal skills. Engineers need to continue to grow to develop hard skills and their hard specialty to keep up with the changes occurring. Therefore, knowing many aspects of coping with change in every way is very helpful.

3.3.4 Matrix diagram

We will use the solutions and improvements the tree diagram offers to guide our response to 11 technologies that will affect global change. According to research by the McKinsey Global Institute, USA [9]. Figure 6 shows that Big Data/Data Analytics studies received the highest with 11 points, followed by IoT (7 points), AI (6 points), machine learning (6 points), robotics (5 points), maintenance engineering (5 points), lean manufacturing (5 points), CNC (5 points), AutoCAD (4 points), and cloud technology (3 points).

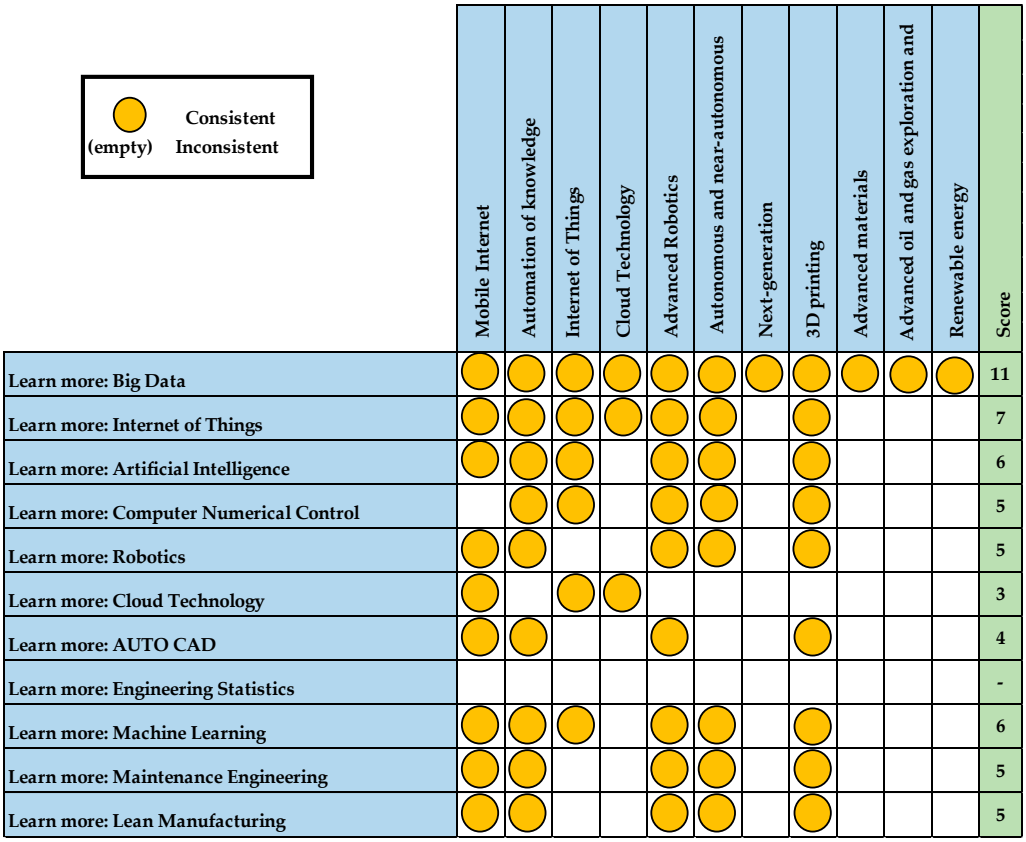

Figure 6. Matrix diagram

4. Conclusion

This research is an application of new quality tools to study the impact of disruptive technologies on the engineering profession. This includes examining how sudden technological changes prepare people in educational systems to become industrial engineering practitioners. The findings of the research can be summarized as follows:

1) Three effects of disruptive technology in industrial engineering:

1.1 Increased labor productivity due to technology reduces the need for human labor in the industrial sector.

1.2 Technology affects business and investment decisions. The cost-effectiveness of long-term technology investment decisions will have a direct impact.

1.3 Technologies that change the nature of the job and the aptitude of the engineer by making it more demanding.

In addition, it was noted that the curriculum used in classrooms to train graduates is not in line with the twenty-first century, forcing stakeholders to adapt to new modern technologies.

2) The approach to addressing disruptive technology in the industrial engineering profession is as follows:

All continents worldwide have increasingly accepted the Internet in the industrial sector in the twenty-first century. As a result, technological advancements have occurred rapidly to keep pace with the demands of the entire supply chain. The industrial sector is changing rapidly technologically, but the education sector has not adapted. Therefore, it is time for collaboration between industry and the education sector. The industrial sector is seen as an important variable, indicating that the industrial engineering curriculum and instruction in the education sector need to change. There is a need to ensure graduates can meet the sector's demands and find employment. Curriculum adjustments must consider the future for 3-10

years. In the past, technology and innovation did not play a major role in industrial engineering, so the curriculum did not deal much with the application of technology. Today, technology and innovation are becoming increasingly important in the industrial sector. Those in the industrial engineering profession need to learn more about their areas of expertise, such as engineering statistics, maintenance engineering, and lean manufacturing, and learn more about technology to apply that knowledge to the emerging technology and innovation groups that are coming into play in the industry. This will help to improve the skills of the entire Thai industrial engineering profession in the future.

5. Acknowledgements

This study was supported by Silpakorn University Research, Innovation and Creative Fund.

Author Contributions: Conceptualization, C.P. and White paper report, N.S. and amp; T.K., Data Collection, K.K, and amp; T.T, Translation first draft, A.S., Editing, and Corresponding author, C.I. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Silpakorn University Research, Innovation and Creative Fund.

Conflicts of Interest: The authors declare no conflict of interest.

References

[1] Pornsing, C. Industrial work study. Silpakorn University, 2019. (in Thai).
[2] Blair, R. N; Whiston, C. W. Elements of industrial systems engineering. 1st ed. New Jersey: Prentice-Hall, 1971.
[3] Malisuwan, S. The End of Nokia, Lessons leaders need to learn. (in Thai), 2016. Available online https://www.it24HRS.COM/2016/Nokia-case-study/ (accessed on 27 March 2020)
[4] Martinez-Vergara, S. J.; Valls-Pasola, J. Clarifying the disruptive innovation puzzle: a critical review. *European Journal of Education and Management.* 2021, *24*(3), 893-918.
[5] Ministry of Industry. Industrial Development Strategy of Thailand 4.0 for 20 years (2017 – 2036). (in Thai), Available online https://waa.inter.nstda.or.th/stks/pub/2017/20171114-oie.pdf (accessed on 6 April 2020)
[6] Nascimento, D. L.M.; Alencastro, V.; Quelhas, O. L. G.; Caiado, R. G. G.; Garza-Reyes, J. A.; Rocha-Lona, L.; Tortorella, G. Exploring Industry 4.0 technologies to enable circular economy practices in a manufacturing context A business model proposal, *Journal of Manufacturing Technology Management*. 2018, *30*(3), 607-627.
[7] Yothongyot, M.; Sawasdisan. Determination of sample size for research. (in Thai). Institute for the Promotion of Research and Development Innovation, Available online http://www.fsh.mi.th/km/wp-content/ uploads/2014/04/resch.pdf (accessed on 18 March 2020)
[8] Pongpornsub, W. Let's get to know 7 New QC Tools. (in Thai). Technology Promotion Association (Thailand-Japan). Available online http://www.tpa.or.th/publisher/pdfFile DownloadS/QM206_P24-25.pdf (accessed on 19 March 2020)
[9] McKinsey & Company. Disruptive technologies: Advances that will transform life. business, and the global economy. Available online https://www.mckinsey.com/~/media/ McKinsey (accessed on 16 March 2020)
[10] Manarangsan, S.; Apiwanworarat, T. 'PIM' continues to build on "Ready to Work". (in Thai). Available online https://www.techtalkthai.com/pim-through-disruptive-technology-trend/ (accessed on 29 February 2020)

JSTR
ASEAN Journal of Scientific and Technological Reports

Research article

Effect of Post-harvesting with Different Photoperiods under Artificial Light Sources on Nitrate and Vitamin C Contents in Hydroponic Green Oak Lettuce

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Citation:
Htwe, NMPS.; Rawangpai, M.; Ruangrak, E. Effect of post-harvesting with different photoperiods under artificial light sources on nitrate and vitamin C contents in hydroponic green oak lettuce. *ASEAN J. Sci. Tech. Report.* **2023**, *26*(2), 10-19. https://doi.org/10.55164/ajstr.v26i2.247845

Article history:
Received: December 15, 2022
Revised: March 15, 2023
Accepted: March 20, 2023
Available online: March 26, 2023

Abstract: Green oak lettuce (*Lactuca sativa* L.) is a popular vegetable for consumers, but it is concerned about nitrate contamination that may harm human health. However, light can affect nitrate reduction and contribute to the accumulation of vitamin C in vegetables. Therefore, this study focused on the effects of post-harvesting with different photoperiods under artificial light sources on vitamin C and nitrate content in hydroponic green oak lettuce. Green oak lettuces were grown in the NFT system, harvested, and post-harvested under Bulb-LED (Experiment I), Bar-LED (Experiment II), and fluorescent lamp (FL) (Experiment III) for 6, 12, and 24 h photoperiods and replaced the nutrient solution with tap water. The nitrate content was significantly reduced after post-harvesting for 12 h photoperiods under FL (9,012 μg NO_3^- -N/g dry weight) followed by Bulb-LED (13,985 μg NO_3^- -N/g dry weight) and 24 h photoperiods for Bar-LED (10,727 μg NO_3^- -N/g dry weight). Vitamin C content was highest after post-harvesting for 24 h photoperiods under Bar-LED (45.47 μg/ml), followed by Bulb-LED (44.73 μg/ml) and FL (35.40 μg/ml). Post-harvesting with artificial light sources for 12 to 24 h photoperiods can improve hydroponic green oak lettuce quality.

Keywords: Green oak lettuce; nitrate; vitamin C; hydroponic system; photoperiods; post-harvest; artificial light source

1. Introduction

Recently, vegetables have been playing an important role in promoting human health and helping people avoid COVID-19, which affects human health worldwide [1]. Because vegetables contain phytochemicals such as flavonoids, carotenoids, soluble sugars, proteins, vitamin E, and vitamin C, suitable for human health [2,3]. In particular, vitamin C is key in promoting human immunity by supporting several innate immune system cellular functions [4]. In plants, vitamin C is an abundant component in all cell compartments. A biosynthetic of the vitamin C begins from D-glucose-6-P (product of photosynthesis), D-fructose-6-P, GDP-mannose, GDP-L-galactose, L-galactose, and L-galactono-1,4-lactone. Photosynthesis plays a major role in the biosynthetic of vitamin C.

Alternatively, light is the key factor in promoting vitamin C content in the plant [4]. Dowdle et al. [5] reported that vitamin C could be increased 20-fold by exposure to high light intensity for 24 h. Laing et al. [6] found that photon flux density levels (PFD) affect vitamin C content after 2 days.

Lettuce (*Lactuca sativa* L.) has a high nutritional value for humans; it is rich in minerals and vitamins. Aćamović-Djoković et al. [7] researched vitamin C content in several lettuce varieties, and it was in the range of 3.50–9.60 mg/100 g of fresh weight. However, lettuce is not always good for consumer health as people have concerned about excessive nitrate content, especially in hydroponic lettuce.

Nitrate is a nitrogen source that is the essential plant nutrient, a main constituent of chlorophyll, protein, and genetic material [8]. However, high intake of nitrate content by consumers may cause several types of cancer, such as bladder cancer [9], gastric cancer [10], and prostate cancer [11]. After consuming excessive nitrate, saliva, and gastric juice in the human body include nitrate reductase that can reduce nitrate to nitrite [12-13]. Nitrite has formed N-nitroso compounds, which are carcinogenic and can cause a variety of cancers [12–14]. For the plant, nitrate is needed, and it is taken up through the root hair, xylem, mesophyll cells, and cell walls. Then nitrate is reduced into nitrite, ammonium, glutamine acid, and other amino acids [15]. The process of nitrate reduction in plants needs energy from the photosynthesis system. The energy from photosynthesis has activated the suitable light wavelength [8].

Nitrate can be reduced by pre-harvest or post-harvest, especially post-harvest. Post-harvesting reduces health risks while preserving and improving the quality of fruits and vegetables. Recently, light has been used as one of the options for post-harvest treatment tools because artificial light technology has been successfully developed in the horticulture industry. For example, post-harvest artificial light has affected the chemical composition and bioactive compounds of tomatoes, garlic, and lettuce [16,17]. Perera et al. [18] found that continuous lighting and photoperiod with UV-B and UV-C irradiation affect senescence and deterioration delays. Liu et al. [19] reported that irradiation of white light, red light, and UVC increases citrus quality after post-harvest. Nassarawa et al. [20] and Poonia et al. [21] found that LEDs can accumulate phytochemicals and antioxidants, reduce microbial spoilage, reduce senescence delay, extend shelf life, and increase disease resistance and nutritional quality. However, little is known about artificial light sources' vitamin C and nitrate content. Thus, this study mainly focused on the effects of post-harvesting with different photoperiods under artificial light sources on vitamin C and nitrate content in hydroponic green oak lettuce.

2. Materials and Methods

2.1 Plant materials and growth conditions

The green oak lettuce (*Lactuca sativa* L.) was used in this experiment. The lettuce plants were grown in a Nutrient Film Technique (NFT) hydroponic system with open air under 50% aluminum net shade for 28 days. The hydroponic nutrient solution was applied at an EC of 1.5–2.0 mS/cm and pH of 5.5–6.5. Formular A of nutrient solution contained Magnesium Sulphate ($MgSO_4 \cdot 7H_2O$, 500 g/10 L), Potassium Nitrate (KNO_3, 780 g/10 L), Mono Ammonium Phosphate ($NH_4H_2PO_4$, 130 g/10 L), Mono Potassium Phosphate (KH_2PO_4, 100 g/10 L), Manganese EDTA (MnEDTA, 8 g/10 L), Micro element (Boron EDTA, MnEDTA, MgO, CuEDTA, MoEDTA and FeEDTA, 10 g/10 L). Calcium Nitrate ($Ca(NO_3)_2\,4H_2O$, 1000 g/10 L) and Iron Chelate (FeEDTA, 10 g/10 L) were included in Nutrient Solution Formular B [22]. After 28 days of growth in the NFT system, lettuce plants were transferred into post-harvest treatments by lighting.

2.2 Light conditions and treatments

Lettuce plants were moved and placed into a post-harvest chamber with three shelves. The shelf has three cells [80 length x 40 width x 30 height (cm)] (More information is available in the previous experiment [23]). This post-harvest chamber was set up with three different artificial light sources 1); Bulb-LED (1:1:1 ratio of blue 460 nm: red 630 nm: red 660 nm, PPFD 2005.05 ± 53.62 μmol/m^2/s), 2); Bar-LED (2:1:1 ratio of blue 460 nm: red 630 nm: red 660 nm, PPFD 256.32 ± 11.56 μmol/m^2/s) and 3); fluorescence light (FL) (spectral wavelength 400 – 700 nm PPFD; 51.64 ± 11.20 μmol/m^2/s). The nutrient solution in the post-harvest chamber was replaced by tap water using DFT (Deep Floating Technique) system and supplied oxygen with an air stone bubbler.

Thus, the study was designed into three experiments, including 1) the effect of post-harvesting with different photoperiods under Bulb-LED source on vitamin C and nitrate contents in hydroponic green oak lettuce;

2) the effect of post-harvesting with different photoperiods under Bar-LED source on vitamin C and nitrate contents in hydroponic green oak lettuce; and 3) the effect of post-harvesting with different photoperiods under FL source on vitamin C and nitrate contents in hydroponic green oak lettuce. All three experiments were conducted using the same method, except for the light condition. These experiments were replicated three times and performed at the Laboratory of Urban Agriculture Technology, Division of Agricultural Technology, Department of Agricultural and Fishery Science, Faculty of Science and Technology, Prince of Songkla University, Pattani Campus.

2.3 Determination of nitrate content

Lettuce leaves were harvested and washed with a running tap and distilled water three times. Then, the lettuce leaves were dried at 65 °C for 48 h. The samples were weighed to 1 g after being ground and blended, suspended in 10 mL of distilled water, allowed to stand at 45 °C for 1 h, and filtered through No. 40 Whatman filter paper. The extraction solution was added to 0.1 mL in a 50 mL tube with 0.4 mL of 5% (w/v) salicylic acid (Ajax Finechem, Australia). After that, the samples were left at room temperature for 20 min. Then, 9.5 mL of 2N sodium hydroxide (NaOH) solution was added to the samples. The absorbance of 412 nm (Biochrom, Libra S12, England) was used immediately for determination [24].

2.4 Determination of vitamin C content

The method of vitamin C determination was modified by Jagota and Dani [25]. Fresh samples were used, cut into small pieces, and weighed for 7 g. The samples were then homogenized and 30 ml of oxalic acid (0.5% w/v) was added. Moreover, homogenous extract solutions were filtered through No. 40 Whatman filter paper. 1 mL of homogeneous extract solution was mixed with 4 mL of 10% trichloroacetic acid, shacked, and placed on ice for 5 min. Then, the extract solution was centrifuged at 8,000 rpm for 5 min. After that, 3 mL of the extract solution was used, 0.2 mL of 0.2 M Folin-Ciocalteu reagent was added, and the extract solution was left at room temperature for 60 min. The absorbance at 760 nm was measured with a spectrophotometer (Biochrom, Libra S12, England).

2.5 Statistical analysis

The statistics of these studies were performed with a completely randomized design (CRD). A one-way analysis of variance (ANOVA) was used for data analysis with MS Excel software (version 7.0). Treatment means were analyzed by Least Significant Difference (LSD) at a confidence level of 95%.

3. Results

3.1 Experiment I: Effect of post-harvesting with different photoperiods under Bulb-LED on nitrate and vitamin C contents in hydroponic green oak lettuce

This experiment studied the effects of post-harvesting with different photoperiods under a Bulb-LED source on nitrate and vitamin C contents in hydroponic green oak lettuce. The nitrate content ranged from 13,984-27,016.46 μg NO_3^--N/g dry weight, as shown in Figure 1A. The nitrate content decreased significantly after post-harvesting with Bulb-LED for 6, 12, and 24 h photoperiods, but 12 h treatment did not show a significant difference with 24 h treatment. The nitrate content was the lowest under post-harvested for 12 h (13,984 μg NO_3^--N/g dry weight, 48% reduction), followed by treatments of 24 h (14,293.55 μg NO_3^--N/g dry weight, 47% reduction), 6 h (16,248.29 μg NO_3^--N/g dry weight, 40 % reduction) and control (27,016.46 μg NO_3^--N/g dry weight) (Figure 1A).

In contrast, the vitamin C content was increased significantly under different photoperiods in hydroponic green oak lettuce after post-harvest with Bulb-LED for 12 (37.67 μg/ml, 22% increased) and 24 h (44.78 μg/ml, 45% increased) of photoperiod when compared with control treatment. However, the vitamin C content in the earlier photoperiod of 6 h (28.12 μg/ml) was decreased to 9%, which suggests that 12 h of photoperiod is necessary to enhance vitamin C content (Figure 1B).

3.2 Experiment II: Effect of post-harvesting with different photoperiods under Bar-LED on nitrate and vitamin C contents in hydroponic green oak lettuce

This experiment studied the effect of post-harvesting with different photoperiods under Bar-LED on nitrate and vitamin C content in hydroponic green oak lettuce. The nitrate content ranged from 10,727.02 to

27,016.46 μg NO_3^- -N/g dry weight in different photoperiods and it was significantly reduced by 53% to 60% when compared with the control treatment (27,016.46 μg NO_3^- -N/g dry weight) (Figure 2A). The lowest nitrate content was detected in 24 h of photoperiod (10,727.02 μg NO_3^- -N/g dry weight, 60% reduction) and followed by 12 h (12,544.58 μg NO_3^- -N/g dry weight, 54% reduction) and 6 h of photoperiod (12,647.46 μg NO_3^- -N/g dry weight, 53 % reduction), in which no significant difference was observed between them (Figure 2A).

Under post-harvest with Bar-LED of different photoperiods, the vitamin C content varied from 30.13 to 45.47 μg/ml. Although vitamin C content was significantly increased during the 12 h (35.82 μg/ml, 16% increase) and 24 h (45.47 μg/ml, 47% increase) photoperiods, it was decreased during the 6 h (30.13 μg/ml, 2% decreased) photoperiod (Figure 2B). The pattern of vitamin C content in Bar-LED treatment is similar to that of Bulb-LED treatment (Figure 1B, 2B).

3.3 Experiment III: Effect of post-harvesting with different photoperiods under FL on nitrate and vitamin C contents in hydroponic green oak lettuce

This experiment studied post-harvesting effects with different photoperiods under FL on nitrate and vitamin C content in hydroponic green oak lettuce. The nitrate content in different photoperiods ranged from 9,012.35 to 27,016.46 μg NO_3^--N/g dry weight and was significantly reduced from 22% to 67% compared to the control treatment (27,016.46 μg NO_3^--N/g dry weight), as shown in Figure 3A. The lowest nitrate content was detected in 12 h of photoperiod (9,012.35 μg NO_3^- -N/g dry weight, 67% reduction) which was almost three times the reduction and followed by 6 h (14,327.85 μg NO_3^- -N/g dry weight, 47% reduction) and 24 h (21,117.97 μg NO_3^- -N/g dry weight, 22 % reduction) than the control treatment (Figure 3A).

Hydroponic lettuces were post-harvested with FL and measured for vitamin C content in different photoperiods. The results showed that the vitamin C content increased by 6% to 15% in different photoperiods compared with the control treatment (30.87 μg/ml) and ranged from 30.87 to 35.40 μg/ml, as shown in Figure 3B. Unlike the post-harvest with Bar-and Bulb-LED lighting, the vitamin C content was significantly increased in hydroponic lettuce after being post-harvested with FL for 6, 12, and 24 h. The vitamin C content showed the highest under post-harvested for 24 h (35.40 μg/ml, 15% increase) followed by 12 h (34.56 μg/ml, 12% increase), 6 h (32.71 μg/ml, 6% increase) and control (30.87 μg/ml, 0% increased) in which 12 h photoperiod treatment was not a significant difference with 24 h treatment. It could be concluded that post-harvest treatment by Bar-LED, Bulb-LED, and FL decreased nitrate content in all different photoperiods, indicating that post-harvest by light can improve the quality of vegetables for human consumption. In addition, except for 6 h of photoperiod in the Bar-LED and Bulb-LED treatments, vitamin C content was significantly increased in other photoperiods and all of the FL, compared to the control treatment. This result also showed that post-harvest with different lights could improve the quality of lettuce by enhancing vitamin C content.

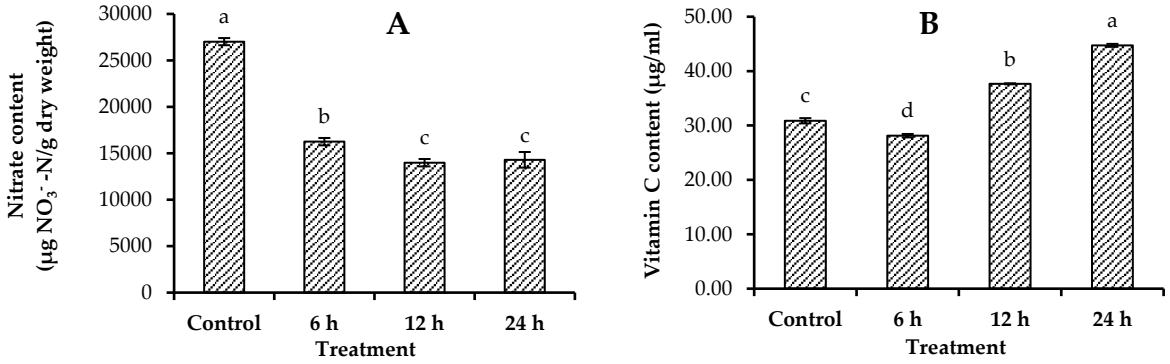

Figure 1. Effect of post-harvesting with different photoperiods under Bulb-LED on nitrate content (A) and vitamin C content (B) in hydroponic green oak lettuce. The error bars represent the standard deviation over three replications. The means denoted by the various letters differ significantly at $p < 0.05$, according to the least significant difference.

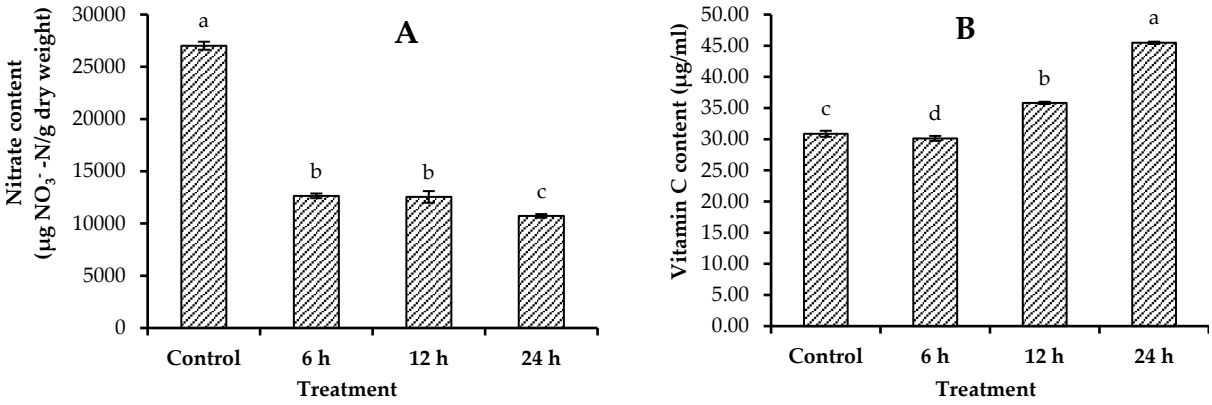

Figure 2. The effect of post-harvesting with different photoperiods under Bar-LED on nitrate content (A) and vitamin C content (B) in hydroponic green oak lettuce. The error bars represent the standard deviation over three replications. The means denoted by the various letters differ significantly at $p < 0.05$, according to the least significant difference.

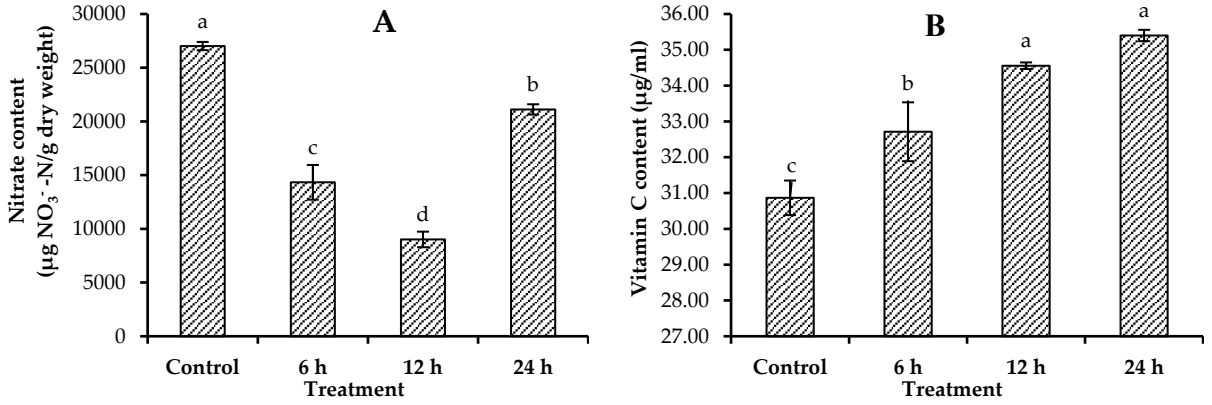

Figure 3. The effect of post-harvesting with different photoperiods under FL on nitrate content (A) and vitamin C content (B) in hydroponic green oak lettuce. The error bars represent the standard deviation over three replications. The means denoted by the various letters differ significantly at $p < 0.05$, according to the least significant difference.

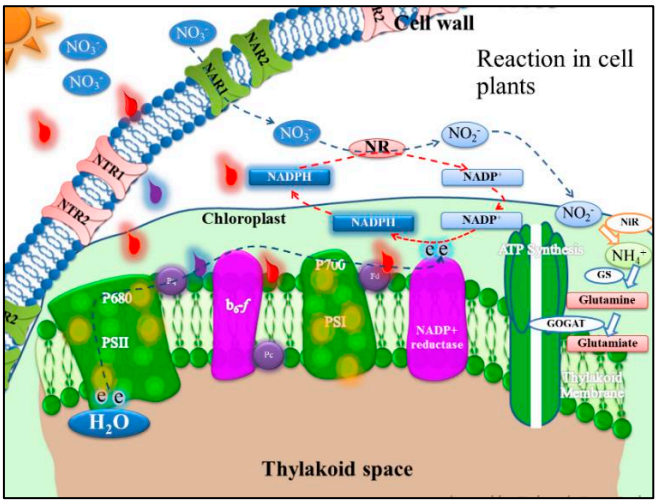

Figure 4. The mechanism of light affects nitrate reduction in plant cells (Adapted from Chow [26] and Sanz-Luque et al. [27]). Water molecules (H_2O) break down molecules and release two electrons in photosynthesis II (PSII P680). Two electrons move to photosynthesis I (PSI P700), and NADP+ reductase uses these two electrons to change NADP+ to $NADPH_2$. $NADPH_2$ carries two electrons from the chloroplast to the cytosol. Two electrons from $NADPH_2$ activate Nitrate Reductase (NR) in the cytosol for the conversion of nitrate molecules (NO_3^-) to nitrite molecules (NO_2^-). The nitrite molecule moves into the chloroplast and has been changed to ammonium by Nitrite Reductase (NiR). Then, ammonium is transformed into glutamin and glutamate acids by the glutamine synthetase (GS) and glutamine oxoglutarate aminotransferase (GOGAT), respectively.

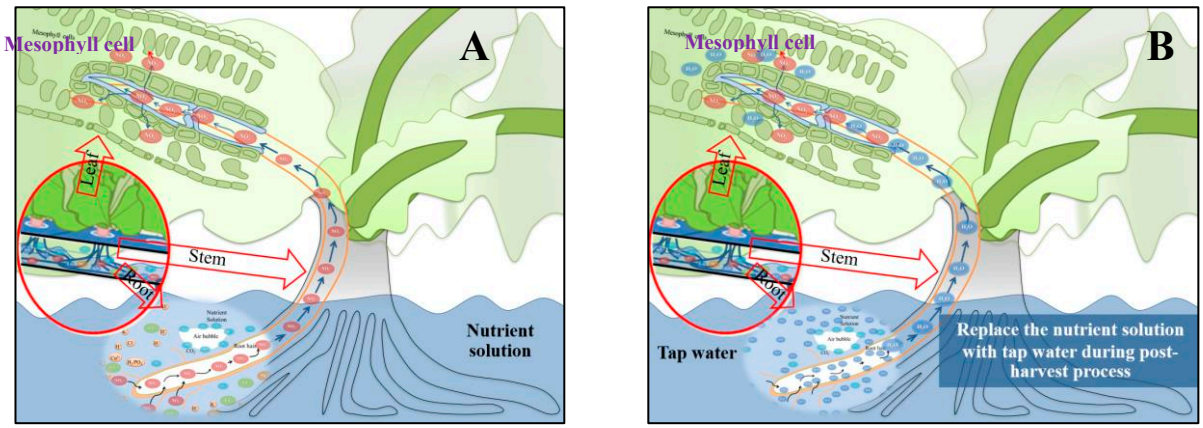

Figure 5. The root zone before post-harvest shows nitrate transportation via xylem vessel under conditions of nutrient solution (A). The root zone is shown during post-harvest with photoperiods and replacing the nutrient solution with tap water (B).

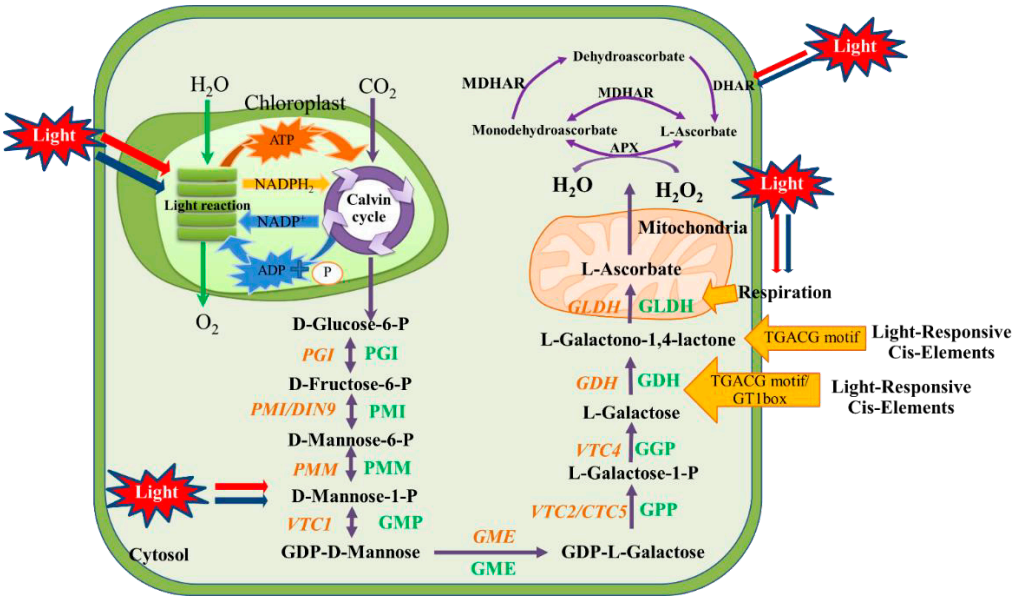

Figure 6. Summary of the effects of light on vitamin C (ascorbate) biosynthesis (adapted from Rosado-Souza et al. [32] and Paciolla et al. [31]). The genes of the pathway are highlighted in orange and written in italics. The enzymes are highlighted in green. Abbreviations: PGI, Phosphoglucose isomerase; PMI, phosphomannose isomerase; PMM, phosphomannomutase; VTC1, Vitamin C1; GMP, GDP-D-mannose pyrophosphorylase, GME, GDP-mannose-30-50-epimerase; GGP, GDP-L-galactose transferase; GPP, L-galactose-1-phosphate phosphatase; GDH, GDP-L-galactose transferase; GLDH, L-galactono-1,4-lactone dehydrogenase; APX, ascorbate peroxidase; MDHA, monodehydroascorbate; MDHAR, monodehydroascorbate reductase; DHAR, dehydroascorbate reductase, the details show in the text.

4. Discussion

Nitrate content has been reduced after post-harvest because light acts to produce electrons in the photosynthesis process. The electrons have been received by NADP+ and ADP+, which have been converted to $NADPH_2$ and ATP, respectively. $NADPH_2$ and ATP provide energy to activate Nitrate Reductase and Nitrite Reductase, which catalyze nitrate to nitrite and nitrite to ammonium. Ammonium then assimilates to glutamine and glutamate acids by GS and GOGAT (Figure 4) [27,28]. However, the nitrate assimilation process helps to assimilate nitrate to glutamate acid, but nitrate residue in the lettuce remains in the xylem vessel of the root, stem, petiole, and leaf veins [29]. As mentioned in materials and methods, these experiments were designed to replace the nutrient solution with tap water to clean up the xylem vessel of roots, stems, and leaves. After the nitrate assimilation process, tap water occurs in a xylem vessel, as shown in Figure 5. Thus, the results of these experiments indicated that nitrate was reduced after post-harvest light treatment. These results are similar to those of Guffanti et al. [30], Wanlai et al. [31], and Cometti et al. [32].

These experiments studied effect of post-harvesting effects with different photoperiods under artificial light sources on nitrate and vitamin C content in hydroponic green oak lettuce. We found that vitamin C content was increased by -2% to 47% when compared with the control treatment, and the results of vitamin C content ranged from 30.13 to 45.47 µg/ml, as shown in Figure 1B-3B. The vitamin C content significantly differed in hydroponic green oak lettuce after post-harvest with all light sources for 6, 12, and 24 h, except for Bar-LED and Bulb-LED for the 6 h treatment. The vitamin C content showed the highest under post-harvested Bar-LED for 24 h following Bulb-LED for 24 h, Bulb-LED for 12 h, Bar-LED for 12 h, FL for 24 h, FL for 12 h, FL for 6 h, control, Bar-LED for 6 h, and Bulb-LED for 6 h, respectively. According to the findings of these studies, light is an important factor in vitamin C biosynthesis [4]. Similarly, Dowdle et al. [5] and Laing et al. [6] found that vitamin C content can increase after exposure to high light intensity.

Light is essential for photosynthesis, which generates energy to stimulate the D-Glicose-6-P to D-Mannose/L-Galactose pathway of ascorbate biosynthesis in plants, as shown in Figure 6 [33,34]. Light is

responsible for stimulating CO_2 to D-Glucose-6-P in the Calvin cycle by using ATP and $NADPH_2$, which are generated from the photosynthesis process in the chloroplast. Following that, D-Glucose-6-P was converted into D-Fructose-6-P, D-Mannose-6-P, D-Mannose-1-P, GDP-D-Mannose, and D-Fructose-6-P. GDP-L-Galactose, L-Galactose-1-P, L-Galactose, L-Galactono-1,4-lactone (cytosol), and L-Ascorbate (mitochondria) stimulate phosphoglucose isomerase (PGI), phosphomannose isomerase (PMI), phosphomannomutase (PMM), GDP-mannose-30-50-epimerase (GME), GDP-L-galactose transferase (GGP), L-galactose-1-phosphate phosphatase (GPP), L-galactose dehydrogenase (GDH), and L-galactono-1,4-lactone dehydrogenase (GLDH), respectively. Plant genes play the main role in the D-Mannose/L-Galactose pathway of ascorbate biosynthesis in plants, including PGI, PMI/DIN9, PMM, VTC1, GME, VTC2/CTC5, VTC4, GDH, and GLDH, respectively [34]. L-Ascorbate has been stimulated in mitochondria and transported into the cytosol. L-ascorbate has been used to reduce H_2O_2, a relative oxygen species (ROS), and it has been converted to monodehydroascorbate by the APX enzyme. However, the MDHAR enzyme can convert monodehydroascorbate to L-Ascorbate, or it can change to dehydroascorbate and then to L-Ascorbate by DHAR and MDHAR enzymes, respectively [33].

5. Conclusions

This study focused on the effect of post-harvesting with different photoperiods under artificial light sources on vitamin C and nitrate content in hydroponic green oak lettuce. The nitrate content was reduced lowest after post-harvested for 12 h photoperiods under Bulb-LED and FL and 24 h photoperiods under Bar-LED. Vitamin C content was the highest after post-harvested for 24 h photoperiods under Bar-LED, Bulb-LED, and FL. Thus, post-harvesting with artificial light sources for 12 to 24 h photoperiods can help to improve the quality of hydroponic green oak lettuce by increasing vitamin C and reducing nitrate content.

6. Acknowledgements

The authors thank the Urban Agriculture Technology Research Group team at the Department of Agricultural and Fisheries Science, Faculty of Science and Technology, Prince of Songkla University, Pattani Campus, for helping in this work.

Author Contributions: E.R.: Conceptualization, methodology, software, formal analysis, investigation, writing—original draft preparation, M.R.: data curation, N.M.P.S.H.: writing—review and editing, visualization

Funding: This research was funded by the Faculty of Science and Technology, Prince of Songkla University (grant number SAT6104067S, reference No. 21915)

Conflicts of Interest: The authors declare no conflict of interest.

References

[1] Alagawany, M.; Attia, Y.A.; Farag, M.R.; Elnesr, S.S.; Nagadi, S.A.; Shafi, M.E.; Khafaga, A.F.; Ohran, H.; Alaqil, A.A.; Abd El-Hack, M.E. The strategy of boosting the immune system under the COVID-19 pandemic. *Front. Vet. Sci*. 2020, *7*, 570748, https://doi.org/10.3389/fvets.2020.570748.

[2] Bian, Z.H.; Yang, Q.C.; Liu, W.K. Effects of light quality on the accumulation of phytochemicals in vegetables produced in controlled environments: a review. *J. Sci. Food Agric.* 2015, *95*, 869–877, https://doi.org/10.1002/jsfa.6789.

[3] Chang, A.C.; Yang, T.Y.; Riskowski, G.L. Ascorbic acid, nitrate, and nitrite concentration relationship to the 24 hour light/dark cycle for spinach grown in different conditions. *Food Chem.* 2013, *138*, 382–388, https://doi.org/10.1016/j.foodchem.2012.10.036.

[4] Carr, A.C.; Maggini, S. Vitamin C and immune function. *Nutrients* 2017, *9*, 1211, https://doi.org/10.3390/nu9111211.

[5] Dowdle, J.; Ishikawa, T.; Gatzek, S.; Rolinski, S.; Smirnoff, N. Two genes in Arabidopsis Thaliana encoding GDP-L-Galactose phosphorylase are required for ascorbate biosynthesis and seedling viability. *Plant J. Cell Mol. Biol.* 2007, *52*, 673–689, https://doi.org/10.1111/j.1365-313X.2007.03266.x.

[6] Laing, W.; Norling, C.; Brewster, D.; Wright, M.; Bulley, S. Ascorbate concentration in Arabidopsis Thaliana and expression of ascorbate related genes using RNAseq in response to light and the diurnal cycle 2017, 138008.
[7] Aćamović-Djoković, G.; Pavlović, R.; Mladenović, J.; Djurić, M. Vitamin C content of different types of lettuce varieties. *Acta Agric. Serbica* 2011, *17*, 83–89.
[8] Htwe, N.M.P.S.; Ruangrak, E. A review of sensing, uptake, and environmental factors influencing nitrate accumulation in crops. *J. Plant Nutr.* 2021, *44*, 1054–1065, https://doi.org/10.1080/01904167.2021.1871757.
[9] Abdel Mohsen, M.A.; Hassan, A.A.; El-Sewedy, S.M.; Aboul-Azm, T.; Magagnotti, C.; Fanelli, R.; Airoldi, L. Biomonitoring of N-nitroso compounds, nitrite and nitrate in the urine of egyptian bladder cancer patients with or without schistosoma haematobium infection. *Int. J. Cancer* 1999, *82*, 789–794, https://doi.org/10.1002/(sici)1097-0215(19990909)82:6<789::aid-ijc3>3.0.co;2-c.
[10] Eroğlu, A.; Demirci, S.; Ayyildiz, A.; Kocaoğlu, H.; Akbulut, H.; Akgül, H.; Elhan, H.A. Serum concentrations of vascular endothelial growth factor and nitrite as an estimate of in vivo nitric oxide in patients with gastric cancer. *Br. J. Cancer* 1999, *80*, 1630–1634, https://doi.org/10.1038/sj.bjc.6690573.
[11] Wu, T.; Wang, Y.; Ho, S.-M.; Giovannucci, E. Plasma levels of nitrate and risk of prostate cancer: a prospective study. *Cancer Epidemiol. Biomark. Prev. Publ. Am. Assoc. Cancer Res. Cosponsored Am. Soc. Prev. Oncol.* 2013, *22*, 1210–1218, https://doi.org/10.1158/1055-9965.EPI-13-0134.
[12] Du, S.; Zhang, Y.; Lin, X. Accumulation of nitrate in vegetables and its possible implications to human health. *Agric. Sci. China* 2007, *6*, 1246–1255, doi:10.1016/S1671-2927(07)60169-2.
[13] Santamaria, P. Nitrate in vegetables: toxicity, content, intake and EC regulation. *J. Sci. Food Agric.* 2006, *86*, 10–17, https://doi.org/10.1002/jsfa.2351.
[14] Petpiamsiri, C.; Siritientong, T.; Kangsadalampai, K.; Tongyonk, L. The nitrate content in some green leafy vegetables with different cultivation methods in Thailand. *Thai J. Public Health* 2018, *48*, 385–397.
[15] Stitt, M. Nitrate regulation of metabolism and growth. *Curr. Opin. Plant Biol.* 1999, *2*, 178–186, https://doi.org/10.1016/S1369-5266(99)80033-8.
[16] Arah, I.K.; Amaglo, H.; Kumah, E.K.; Ofori, H. Preharvest and postharvest factors affecting the quality and shelf life of harvested tomatoes: a mini review. *Int. J. Agron.* 2015, *2015*, e478041, https://doi.org/10.1155/2015/478041.
[17] Martins, N.; Petropoulos, S.; Ferreira, I.C.F.R. Chemical composition and bioactive compounds of garlic (Allium Sativum L.) as affected by pre- and post-harvest conditions: a review. *Food Chem.* 2016, *211*, 41–50, https://doi.org/10.1016/j.foodchem.2016.05.029.
[18] Perera, W.P.T.D.; Navaratne, S.B.; Wickramasinghe, I. Review on effect of postharvest illumination by fluorescent and ultraviolet light waves on the quality of vegetables. *J. Food Process Eng.* 2022, *45*, e13960, https://doi.org/10.1111/jfpe.13960.
[19] Liu, S.; Hu, L.; Jiang, D.; Xi, W. Effect of post-harvest LED and UV light irradiation on the accumulation of flavonoids and limonoids in the segments of newhall navel oranges (Citrus Sinensis Osbeck). *Mol. Basel Switz.* 2019, *24*, E1755, https://doi.org/10.3390/molecules24091755.
[20] Nassarawa, S.S.; Abdelshafy, A.M.; Xu, Y.; Li, L.; Luo, Z. Effect of light-emitting diodes (LEDs) on the quality of fruits and vegetables during postharvest period: a review. *Food Bioprocess Technol.* 2021, *14*, 388–414, https://doi.org/10.1007/s11947-020-02534-6.
[21] Poonia, A.; Pandey, S.; Vasundhara. Application of light emitting diodes (LEDs) for food preservation, post-harvest losses and production of bioactive compounds: a review. *Food Prod. Process. Nutr.* 2022, *4*, 8, https://doi.org/10.1186/s43014-022-00086-0.
[22] Sirinupong, M. *Practical for Soilless Culture in Thailand*; 4th ed.; Fram-up design, Bangkok, 2017.
[23] Etae, N.; Wamae, Y.; Khummueng, W.; Utaipan, T.; Ruangrak, E. Effects of artificial light sources on growth and phytochemicals content in green oak lettuce. *Hortic. Bras.* 2020, *38*, 204–210, https://doi.org/10.1590/s0102-053620200213.
[24] Lastra, O.C. Derivative spectrophotometric determination of nitrate in plant tissue. *J. AOAC Int.* 2003, *86*, 1101–1105.

[25] Jagota, S.K.; Dani, H.M. A new colorimetric technique for the estimation of vitamin C using folin phenol reagent. *Anal. Biochem.* 1982, *127*, 178–182, https://doi.org/10.1016/0003-2697(82)90162-2.

[26] Chow, F. Nitrate assimilation: the role of in vitro nitrate reductase assay as nutritional predictor. In; 2012 ISBN 978-953-51-0061-4.

[27] Sanz-Luque, E.; Chamizo-Ampudia, A.; Llamas, A.; Galvan, A.; Fernandez, E. Understanding nitrate assimilation and its regulation in microalgae. *Front. Plant Sci.* 2015, *6*, 899, https://doi.org/10.3389/fpls.2015.00899.

[28] Bucher, M.; Kossmann, J. Chapter 15 - molecular physiology of the mineral nutrition of the potato. In *Potato Biology and Biotechnology*; Vreugdenhil, D., Bradshaw, J., Gebhardt, C., Govers, F., Mackerron, D.K.L., Taylor, M.A., Ross, H.A., Eds.; Elsevier Science B.V.: Amsterdam, 2007; 311–329 ISBN 978-0-444-51018-1.

[29] Qiu, W.; Wang, Z.; Huang, C.; Chen, B.; Yang, R. Nitrate accumulation in leafy vegetables and its relationship with water. *J. Soil Sci. Plant Nutr.* 2014, *14*, 761–768, https://doi.org/10.4067/S0718-95162014005000061.

[30] Guffanti, D.; Cocetta, G.; Franchetti, B.M.; Ferrante, A. The effect of flushing on the nitrate content and postharvest quality of lettuce (Lactuca Sativa L. Var. Acephala) and rocket (Eruca Sativa Mill.) grown in a vertical farm. *Horticulturae* 2022, *8*, 604, https://doi.org/10.3390/horticulturae8070604.

[31] Wanlai, Z.; Wenke, L.; Qichang, Y. Reducing nitrate content in lettuce by pre-harvest continuous light delivered by red and blue light-emitting diodes. *J. Plant Nutr.* 2013, *36*, 481–490, https://doi.org/10.1080/01904167.2012.748069.

[32] Cometti, N.N.; Martins, M.Q.; Bremenkamp, C.A.; Nunes, J.A. Nitrate concentration in lettuce leaves depending on photosynthetic photon flux and nitrate concentration in the nutrient solution. *Hortic. Bras.* 2011, *29*, 548–553, https://doi.org/10.1590/S0102-05362011000400018.

[33] Paciolla, C.; Fortunato, S.; Dipierro, N.; Paradiso, A.; De Leonardis, S.; Mastropasqua, L.; de Pinto, M.C. Vitamin C in plants: from functions to biofortification. *Antioxidants* 2019, *8*, 519, https://doi.org/10.3390/antiox8110519.

[34] Rosado-Souza, L.; Fernie, A.R.; Aarabi, F. Ascorbate and thiamin: metabolic modulators in plant acclimation responses. *Plants* 2020, *9*, 101, https://doi.org/10.3390/plants9010101.

JSTR
ASEAN Journal of Scientific and Technological Reports

Research article

Increased Growth of *Caladium* by Tuber Section and Plant Growth Regulators

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Citation:
Kosinwattana, S.; Anuwong, C. Increased growth of *Caladium* by tuber section and plant growth regulators. *ASEAN J. Sci. Tech. Report.* **2023**, *26*(2), 20-29. https://doi.org/10.55164/ajstr.v26i2.247558.

Article history:
Received: October 12, 2022
Revised: March 16, 2023
Accepted: March 20, 2023
Available online: March 26, 2023

Abstract: *Caladium* is a tuberous plant propagated by the tuber section technique to increase the plant quantity. Gibberellin (GA_3) and indole-3-butyric acid (IBA) have great capability in various agricultural practices, such as growing rapidly and enlargement tuber size. The effects of different tuber section sizes and PGRs levels having the following constituents (GA_3 and IBA) in different levels by 2x5 factorial in a completely randomized design (CRD) with ten replications. The treatments involved 2 factors: The first factor was separating two sizes of tuber section into a squall length of each size 0.5 and 1 cm, and the second factor was drenching the tuber in two different levels of each IBA (100 and 150 ppm) and GA_3 (100 and 150 ppm). The results obtained from this experiment showed that the 1 cm of tuber section size was a higher significant difference in bud germination, tuber size, plant height, canopy width, and the number of leaves than the 0.5 cm of tuber section size. However, IBA at 150 ppm was a higher significant difference in the average survival rate, bud germination, plant height, canopy width, and the number of leaves than others. The interaction between the tuber size at 1 cm and IBA at 150 ppm gave the highest survival rate, bud germination, plant height, canopy width, and the number of leaves. Therefore, the *Caladium* was separated at 1 cm size and immersed in 150 ppm of IBA appropriated to induce growth after the section within two months.

Keywords: IBA; GA_3; *Caladium*; tuber; section size

1. Introduction

Caladium is a member of the Araceae family. It is popular for its colorful foliage and is widely used as a pot plant or for outdoor bedding. It has few complications and is easier to grow. Relatively insensitive to diseases and few pests. Therefore, commercial crops have a lower cost advantage. In Thailand, *Caladium* production was located in the regions around Bangkok, including the provinces of Pathum Thani and Nonthaburi. *Caladium* production is worth around 3 million Thai baht annually [1-4]. Caladium 'Candidum' is generally a potted plant produced as a tuber. Growing *Caladium* was a popular technique for propagation in the landscape or home garden or as a bedding plant for late spring or early summer [1, 5].

The possible techniques for *Caladium* propagation were micropropagation, seeding, or tuber cutting. The tissue culture technique is rarely used due to the

complicated protocol and costs. Seeds are predominantly employed in breeding programs because it is a time-consuming process with laborious that introduces significant variability to the crop. Consequently, tubers cutting are the most widely used technique for commercial purposes for *Caladium* [6]. Reproduction through the tuber section is the most used method among tuber plant production methods. Reproduction through the tuber section is propagation by using a piece taken from the tuber of a plant. The tuber section is put in a suitable environmental light and is forced to form roots and suckers [7]. The new plants obtained in this way retained the entire rootstock gene structure [8]. It was stated that large rhizomes yielded significantly higher yields than smaller ones. Commercial growers typically use plant growth regulators (PGRs) to improve the marketable characteristics of their crops for economic gains. Sarkar et al. [9] explained that using various PGRs could increase the number of tubers per plant, the weight of the bulbs, and the overall bulb yield when applying a dip treatment method in northern India. Singh [10] reported that IBA is a widely used rooting hormone that promotes rooting in stem cuttings or air layering, enhances early establishment, bud formation, vegetative growth, and increases the survival of seedlings. According to Amin et al. [11], the most effective PGRs in tuberose growth is GA_3 at 300 ppm. The effects of PGRs tend to be rather inconsistent because environmental factors also influence them. Under increased humidity or when longer drying times are used, the uptake of PGRs can be increased according to laboratory test results [12].

However, the results of respective scientific research on this *Caladium* plant are inadequate, and the tuber section size and PGRs concentrations in its growth. This study examines the capability of various tuber section sizes and PGRs concentrations with the following constituents (GA_3 and IBA) in different concentrations for *Caladium* quantities and faster sales growth within two months.

2. Materials and Methods

2.1. Area of reserach

The experiment was managed under greenhouse conditions at the Department of Horticulture, King Mongkut's Institute of Technology Ladkrabang (KMITL) in Bangkok, Thailand, between March 2022 and July 2022.

2.2. Treatments

The experimental design was 2x5 factorial in a completely randomized design (CRD) with ten replications. The treatments compose of 2 factors: factor 1 used two sizes of tuber section: 0.5 and 1 cm., and the other factor used four concentrations of each plant growth regulator: IBA (100 and 150 ppm) and GA_3 (100 and 150 ppm), saturated to the tuber of *Caladium* for 30 minutes. The tap water was assigned to be the controlled treatment.

2.3 Plant materials

The tuber of Caladium 'Candidum' with an average diameter of a tuber of about 6-8 cm. The tuber was prepared by removing the roots. Then the section was divided into a squall length of each size 0.5 and 1 cm (There will be one side that is the growth point of the buds, and the rest will be accumulated food). Then, the tubers were saturated with the four different plant growth regulator solutions or tap water for 30 minutes, followed by the above treatments. After that, the tubers were dried and planted in a 12 x 17.5 (width x length) cm plastic box using a 1:1 (by volume) sand and coconut coir ratio. After bud germination, planted in a 4.25 x 3.5 (width x length) cm pot using a 2:1 (by volume) ratio of decomposed rain tree leave and soil. The plants were transplanted into the planting material and placed in a greenhouse with 60% shading, average temperature 30-35 °C, relative humidity 60-70%, light intensity 150-220 μmol $s^{-1}m^{-2}$, watering rate 500 ml/pot every 2 days.

2.4 Plant growth analysis

All growth parameters of the Caladium 'Candidum' growth were collected 1 month after planting. The survival rate was evaluated by counting a normal tuber bud germination 30 days after section [4]. The

survival percentage [% survival= (number of survival tubers/number of total tubers) x 100], the days to sprouting was collected after planted at 30 days or the sprouted shoot about 1-2 cm length, and were determined growth at 4 months are as follows: The plant height in a unit of cm was set to be extended from the base of the ground to the tip of the plant, the number of leaf per plant, the canopy width at length and width of the leaf, and color of leaf samples were measured by a Colorimeter Color Reader CR-10 Plus, in the CIE L*, a*, b* (Lightness (L*), redness (a*), and yellowness (b*) values were recorded.

2.5 Statistical analyses

All plant growth parameters were determined using the statistical analysis system IBM SPSS Statistics version 25 and statistix version 10 program. A comparison of treatment methods can be made using Duncan's Multiple Range Test (DMRT) at the 0.05 probability level.

3. Results and Discussion

3.1 The percentage of survival rate and bud germination of Caladium 'Candidum'

The survival rates were non-significant differences ($P<0.05$) after the tuber section in a different size (Table 1), while bud germination showed significant differences after the tuber section in a different size. A 1 cm tuber was the earliest days to bud germination (27.58 days) than a 0.5 cm tuber size, after planting at one month than a 1 cm size (Figure 1). The tuber cutting size at 1 cm has more eyes that are larger with the volume of higher reserve food and may help in cracking shoots in the early stages, as in line with the reported [13]. These results are encouraged by the research literature of Shakh et al. [14], who investigated potatoes that had early days to bud germination with the large-sized tuber, and Satyavir and Singh [15], who investigated gladiolus that had early days to bud germination with the large-sized corms.

Similarly, the growth and development of the *Caladium* when saturated with different concentrations of GA_3 and IBA indicated that survival rate and days to bud germination were significant differences (Table 1). The percentage of survival rate was shown to be the highest at the 150 ppm IBA (91.30 %), the earliest days to bud germination (20.65 days) (Figure 1). These findings concern the research results of Dhiman and Gupt [16]. The highest bud germination levels were reported after treating the seeds with 100 ppm IBA, which can be considered like GA_3 when the concentrations are the same. The improvements in seed germination following treatment with GA_3 could be attributed to the greater levels of hydrolase synthesis diffusion (alpha amylase) of endogenous auxin and gibberellin-like substances. On the other hand, the cause might be the increased metabolic rate during the germination process. The synthesis of specific proteins can lead to more rapid cell division, allowing quicker and more vigorous germination [17-18]. The process of seed metabolism can be assisted during germination by enzyme and coenzyme production, which can then mediate the protein synthesis necessary to promote cell division and the following growth [19].

The interaction between tuber section size and plant growth regulator concentrations affects the survival rate. A 1 cm tuber saturated with 150 ppm IBA gave the highest percentage of survival rate (91.30%), while the 0.5 cm tuber with saturated-in tap water was the lowest rate (67.90%). However, the days to bud germination were found the earliest in the 1 cm tuber with saturated in at 150 ppm IBA (19.00 days), while the 0.5 cm tuber saturated in tap water was the latest days (43.00 days) after planting for one month. This is consistent with the research results of Chaudhary *et al*. [20] that the application of auxin IBA 100 ppm significantly the earliest bud germination of Gladiolus x hybridus Hort. tubers. IBA breaks down and decays rapidly to low concentrations, suitable for converting root tissue into the root [21].

Table 1. Effect of tuber section size and the different concentrations of GA_3 and IBA on the percentage of survival rate and days to the bud germination of Caladium 'Candidum' after planting at 1 month.

Tuber size	Survival rate (%)	Bud germination (day)
Tuber size		
0.5 cm	81.84	31.24 a
1 cm	80.58	27.58 b
PGRs		
Tap water	69.35 e	39.00 a
GA_3 100 ppm	77.00 d	35.85 b
GA_3 150 ppm	80.80 c	28.85 c
IBA 100 ppm	87.75 b	22.70 d
IBA 150 ppm	91.15 a	20.65 e
Tuber size x PGRs (ppm)		
0.5 cm + Tap water	67.90 d	43.00 a
1 cm + Tap water	70.80 d	35.00 c
0.5 cm + GA_3 100	76.10 c	38.00 b
1 cm + GA_3 100	77.90 c	33.70 d
0.5 cm + GA_3 150	79.80 bc	30.50 e
1 cm + GA_3 150	81.80 b	27.20 f
0.5 cm + IBA 100	88.10 a	22.40 gh
1 cm + IBA 100	87.40 a	23.00 g
0.5 cm + IBA 150	91.00 a	22.30 h
1 cm + IBA 150	91.30 a	19.00 i
F-test		
Tuber size	ns	*
PGRs	*	*
Tuber size x PGRs	*	*
CV (%)	9.10	2.50

1/ Means with the same letter within a column are not significantly different at $P<0.05$ by the least significant difference by using the DMRT test.
ns= non-significant.

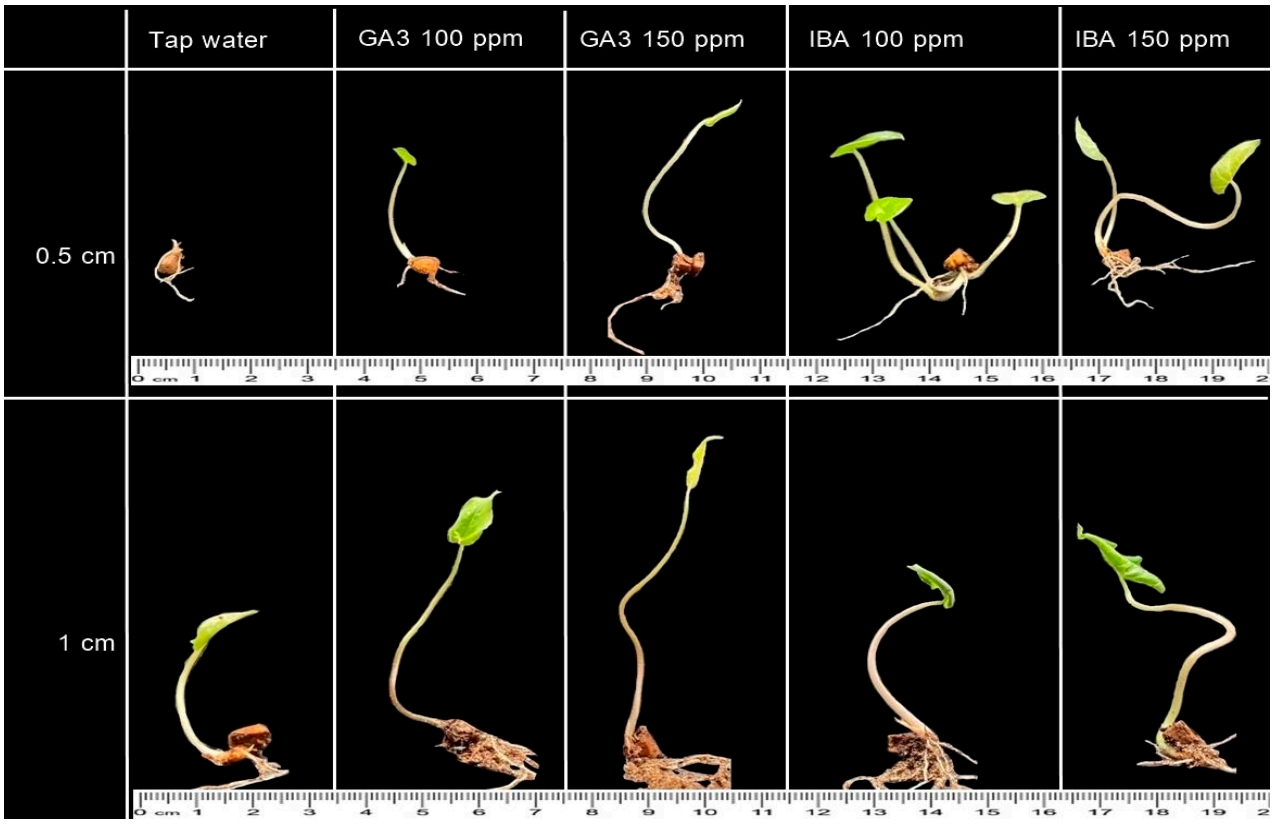

Figure 1. The bud germination of Caladium 'Candidum' after section and saturated within the different concentrations of GA_3 and IBA at 2 months**.**

3.2 Plant height, the canopy width, and the number of leaves of Caladium

The growth and development of the tuber section size differences were indicated by the plant height and the canopy width significant differences ($P<0.05$). The 1 cm tuber was the highest of the plant height (17.88 cm) and the canopy width (15.65 cm), while the number of leaves showed nonsignificant differences after planting at 2 months (Table 2).

The various concentrations of plant growth regulators were significantly different ($P<0.05$). The plant height, the canopy width, and the number of leaves were highest at 100 and 150 ppm IBA. There were 15.09 and 16.07 cm in height, respectively, 15.01 and 14.28 cm in width, respectively, and the highest number of leaves was shown in the tap water, and both IBA levels (100 and 150 ppm) were 2.75, 2.85, and 3.05 leaves/plant, respectively, after planted at 2 months (Table2).

The interaction between tuber section size and plant growth regulator concentrations on the plant height, canopy width, and the number of leaves after planting at 4 months resulted that a 1 cm tuber saturated within IBA at 150 ppm giving the highest plant height (20.81 cm) while the 0.5 cm tuber with saturated with tap water was the lowest height (5.99 cm).

While, the canopy width found that the highest canopy width 14.86, 15.16, 15.34, 15.42, 15.78, and 16.58 cm in a 1 cm tuber and saturated with IBA 100 ppm, IBA 150 ppm, tap water, GA_3 100 ppm, IBA 150 ppm, and GA_3 150 ppm, respectively, while the 0.5 cm tuber and saturated with tap water was the lowest (8.83 cm) in a 1 cm tuber and saturated with at tap water, 100 and 150 ppm IBA, respectively. Besides, the number of leaves showed the highest number of leaves, 2.90, 3.20, and 3.30 leaves/plant in a 0.5 cm tuber and saturated with at an IBA 150 ppm, 1 cm tuber and saturated with at an IBA 150 ppm and 0.5 cm tuber and saturated with at IBA 100 ppm, respectively. In comparison, the 0.5 cm tuber saturated with GA_3 at 100 ppm was the lowest leaf number (2.00 leaves /plant) (Table 2). Generally, Auxin plays a critical role in the growth characteristics because it can rapidly stimulate cells inside tubers, while higher levels of auxin in tissues

support the conversion of tryptophan to IAA, leading to the promotion of cell division and elongation. This can be attributed to IBA, leading to the formation of root initiation and, thus, root formation and, eventually, the uptake of a greater amount of nutrients from the soil, resulting in higher plant height and canopy width [11, 22]. While GA could not stimulate plant height because of the later germination time than IBA, the growth was lower than in the IBA treatment. These findings concurred with the results reported by Bhattacharjee [23] in the context of gladiolus, Jana, and Biswas [24] and Mukhopadhyay and Bankar [25] in the case of tuberose. The use of plant growth regulators to enhance both cell division and cell elongation can potentially lead to plant height in tuberose, according to Shanker et al. [26] and Tiwari and Singh [27] in tuberose. This is consistent with the research results of Chaudhary et al. [20] that the application of auxin IBA 100 ppm the highest plant height, leaf length, leaf width, and number of leaves of tubers of Gladiolus x hybridus Hort. (Figure 2)

Table 2. Effect of tuber section size and the different concentrations of GA_3 and IBA on the plant height, the canopy width, and the number of Caladium 'Candidum' leaves after planting at 2 months.

Tuber size	Plant height (cm)	Canopy width (cm)	Number of leaves
Tuber size			
0.5 cm	8.33 b	11.44 b	2.66
1 cm	17.88 a	15.65 a	2.54
PGRs			
Tap water	11.24 b	12.68 bc	2.75 a
GA_3 100 ppm	11.12 b	12.12 c	2.10 b
GA_3 150 ppm	12.00 b	13.64 ab	2.25 b
IBA 100 ppm	15.09 a	15.01 a	2.85 a
IBA 150 ppm	16.07 a	14.28 a	3.05 a
Tuber size x PGRs (ppm)			
0.5 cm + Tap water	5.99 e	8.83 c	2.80 bcd
1 cm + Tap water	16.50 c	15.34 a	2.70 cde
0.5 cm + GA_3 100	6.71 e	10.02 c	2.00 f
1 cm + GA_3 100	15.54 c	15.42 a	2.20 f
0.5 cm + GA_3 150	6.70 e	10.70 c	2.30 ef
1 cm + GA_3 150	17.31 bc	16.58 a	2.20 f
0.5 cm + IBA 100	10.94 d	14.86 a	3.30 a
1 cm + IBA 100	19.25 ab	15.16 a	2.40 def
0.5 cm + IBA 150	11.34 d	12.78 b	2.90 abc
1 cm + IBA 150	20.81 a	15.78 a	3.20 ab
F-test			
Tuber size	*	*	ns
PGRs	*	*	*
Tuber size x PGRs	*	*	*
CV (%)	17.65	16.50	19.02

[1/] Means with the same letter within a column are not significantly different at $P<0.05$ by the least significant difference by using the DMRT test.
ns=non-significant.

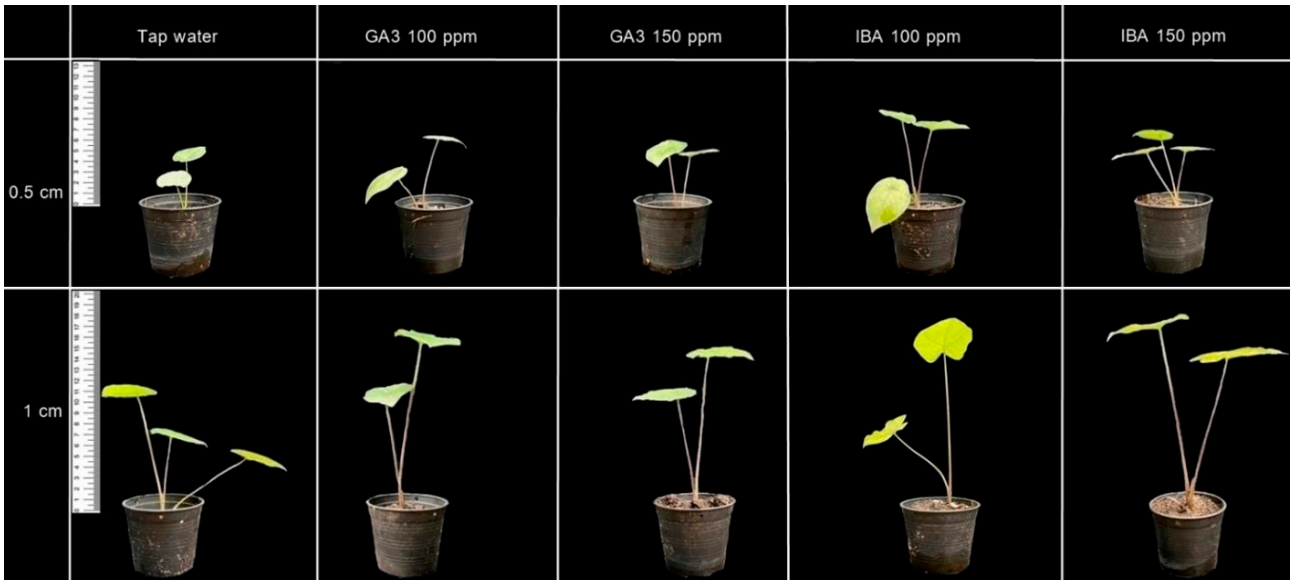

Figure 2. Effect of tuber section size and the different concentrations of GA_3 and IBA on the plant height, the canopy width, and the number of Caladium 'Candidum' leaves after planting at 2 months.

3.3 Leaf shapes and Leaf color of Caladium

The interaction between tuber section size and plant growth regulator concentration affects leaf shape 2 months after planting (Figure 3). Tubers size of 1 cm and saturated with IBA or GA_3 gave the larger leaf than saturated in tap water. Moreover, IBA increased leaf width and length more effectively than GA_3. This could possibly be due to IBA leading to root initiation and thus root formation and eventually uptake of a greater amount of nutrients from the soil, resulting in greater leaf area. Jawanda *et al.* [22] and Diwakar and Katiyar [28] also reported that IBA promotes leaf area.

The interaction between tuber section size and plant growth regulator concentrations on the leaf color changes after planting at 2 months resulted in a 1 cm tuber and drench in GA_3 at 100 ppm had lower L* values (brightness) and lower yellow components (b* values) of the control plants. As a result, the plants are darker and less bright. This results in darker and less bright colors. These results adapted with results of Mynett et al. [29] in *Freesia perennis* about the effect of GA_3 on the increase of the greenness index. GA_3 has a structural role in the membrane of chloroplasts and stimulates photosynthesis [30].

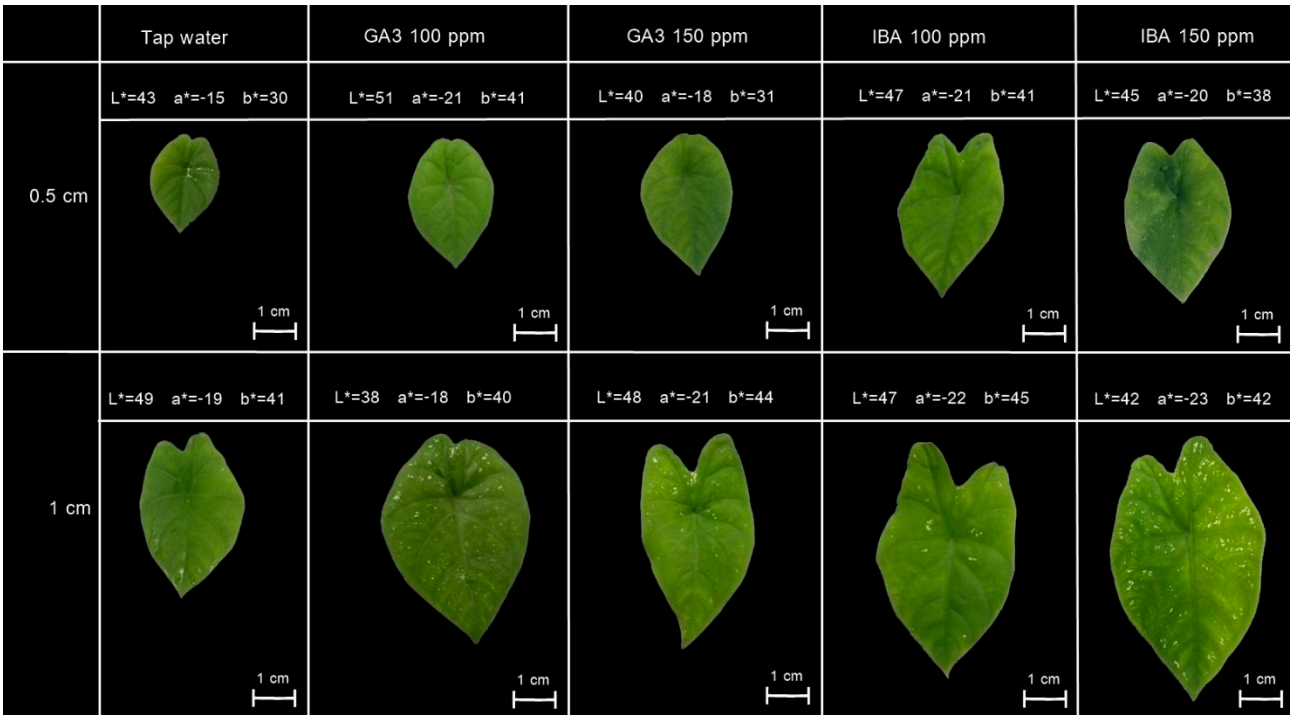

Figure 3. Leaf shapes and color (L*, a*,b*) of Caladium 'Candidum' after planting at 2 months.

4. Conclusions

The result showed that a 1 cm of tuber section size with drenched in IBA at 150 ppm was shown the highest effect on days taken for bud germination, tuber size, plant height, canopy width, and the number of leaves than other treatments and control. Therefore, the Caladium 'Candidum' was cut at 1 cm size and drenched in 150 ppm of IBA appropriated to induce growth after section within four months.

5. Acknowledgements

Thank you for the research fund from the Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang provides research funding and places to conduct research.

Author Contributions: Conceptualization, C.A.; methodology, S.K.; software, S.K.; validation, C.A.; formal analysis, S.K.; investigation, C.A.; resources, S.K. and C.A.; data curation, S.K., and C.A.; writing—original draft preparation, S.K., and C.A.; writing—review and editing, S.K. and C.A.; visualization, S.K.; supervision, C.A.; project administration, C.A.

Funding: This research was funded by the Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang, grant number 2565-02-04-007.

Conflicts of Interest: The authors declare no conflict of interest.

References

[1] Caladium Association of Thailand. *Caladium bicolor edition of the Caladium bicolor*. Amarin Printing and Publishing Co., Ltd.: Thailand, Bangkok, 1997; 108.

[2] Deng, Z. *Caladium* genetics and breeding: recent advances. *Floriculture and Ornamental Biotechnology*. 2012, *6*(1), 53-61.

[3] Essien, E.E.; Jacob, I.E.; Thomas, P.S. Phytochemical composition, antimicrobial and antioxidant activities of leaves and tubers of three *Caladium* species. *International Journal of Medicinal Plants and Natural Products*. 2015, *1*(2), 24-30.

[4] Hussain, R.; Younis, A.; Riaz, A.; Tariq, U.; Ali, S.; Ali, A.; Raza, S. Evaluating sustainable and environment friendly substrates for quality production of potted *Caladium*. *International Journal of Recycling of Organic Wastein Agriculture*. 2017, *6*(1), 13-21.

[5] Miranda, B.D.; Wilfret, G.H.; Harbaugh b.k. 'Florida white ruffles'—A white strap leaved *Caladium* for small pots, hanging baskets, or sunny landscapes. *Journal Hort Science*. 2002, *37*(5), 838-840.

[6] Shah, M.; Khattak, A.M.; Amin, N. Effect of various amended organic media on the tuberization of *Caladium* cultivar. *Journal of Agricultural and Food Chemistry*. 2007, *23*(4), 899-904.

[7] Turhan, H. Türk Zambağı *(Lilium martagon* L.*)* Soğanlarında IBA *(*Indole butirik asit*)* nın Yavru Gövde Oluşumu ve Köklenmeye Etkisi MsC. Thesis, Department of Forest Engineering, Graduate School of Natural and Applied Sciences, Kastamonu University, Turkey. 2015.

[8] Mahanta, P.; Paswan, L.; Siddique, A.B. Effect of bulb size on growth and flowering of tuberose (*Polianthes tuberosa* L). cv. single. *An international peer reviewed semi-annual journal*. 1998, 3, 35-38.

[9] Sarkar, J.; Mishra, R. L.; Singh, S. K.; Prasad, K. V.; Arora, A. Effect of growth regulators on growth and flowering in tuberose under north India conditions. *Indian Journal of Horticulture*. 2009, *66*(4), 502-507.

[10] Singh, M. Response of plant growth regulators and wrappers on air-layering of guava (*Psidium guajava* L.). *Advances in Plant Sciences (Muzaffarnagar)*. 2002, 15, 1, 153-157.

[11] Amin, M. R.; Pervin, N.; Nusrat, A., Mehraj, H.; Jamal Uddin, A. F. M. Effect of plant growth regulators on growth and flowering of tuberose (*Polianthes tuberosa* L.) cv. single. *Journal of Bioscience and Agriculture Research*. 2017, 12, 1016-1020.

[12] Stover, E.W.; Watkins, C.B.; Fargione, M. J.; Iungerman, K. A. Harvest management of marshall 'McIntosh' apples: Effects of AVG, NAA, ethephon and summer pruning on preharvest drop and fruit quality. *HortScience*. 2003, 38, 1093-1099.

[13] Kapoor, S. L. The role of type of seed in the culture of potato. Influence of number of eyes in a seed piece and size of seed tuber (whole) on growth, yield and grade of potato. *Indian Potato Association*. 1951, 8, 11-18.

[14] Shanker, K.; Singh, A. K.; Singh, H. K. Effect of plant growth regulators on spike yield and bulb production of tuberose double. *Plant Archives*. 2010, *11*(1), 169-171.

[15] Satyavir, S.; S. Singh. Effect of corm size on flowering and corm production. *Journal of Horticultural Science and Ornamental Plants*. 1998, 1, 79-80.

[16] Dhiman, R.; Gupta, N. K. Effect of soaking conditions with growth regulators and nutrient solutions on germination behavior of Ratela: A fuelwood species of Shiwalik Hills. *Environment & Ecology*. 2015, *33*(1), 41-45.

[17] Bewley, J. D.; Black, M. 1994. *Seeds Physiology of Development and Germination. Second Edition*. Plenum Press: New York, US, 1994; 445.

[18] Chen, S. Y.; Kuo, S. R.; Chien, C. T. Roles of gibberellins and abscisic acid in dormancy and germination of red bayberry (*Myrica rubra*) seeds. *Tree Physiology*. 2008, *28*(9), 1431-1439.

[19] Sajid, G. M.; Kaukab, M.; Ahmed, Z. Foliar application of plant growth regulators (PGRs) and nutrients for improvement of lily flowers. *Pakistan Journal of Botany*. 2009, *41*(1), 233-237.

[20] Chaudhary, P.; Moond, S. K.; Bola, P. Effect of bioregulators on vegetative growth and flower production of gladiolus (Gladiolus x hybridus Hort.). *International Journal of Current Microbiology and Applied Sciences*. 2018, 7, 463-470.

[21] Shakh, M. N. U.; Awal, M. A.; Ahmed, S. U.; Baten, M. A. Effect of eyes number in cut seed tuber and different levels of nitrogen on growth, yield and grading of tuber size in potato (*Solanum tuberosum* L.). *Pakistan Journal of Biological Sciences*. 2001, *4*(10), 1184-1187.

[22] Jawanda, J. S.; Singh, A.; Singh, S.; Bal, J. S. Effect of indolebutryic acid and shoot portion on the rooting of cuttings in Japanese plum. *Acta Horticulturae.* 1991, 283, 189-197.

[23] Bhattacharjee, S. K. Influence of growth regulating chemicals on *Hippeastrum hybridum*. *Journal Gardens' Bulletin. Singapore*. 1983, 36, 237-242.

[24] Jana, B.K.; Biswas, S. Effect of growth substances on growth and flowering of tuberose (*Polianthus tuberosa* L.). *Haryana Journal of Horticultural Sciences*. 1979, *8*(3-4), 216-219.

[25] Mukhopadhyay, A.; Banker, G. L. Regulation of growth and flowering in tuberose. *Journal of Ornamental Horticulture*. 1983, 6, 80-81.

[26] Shanker, K.; Singh, A. K.; Singh, H. K. Effect of plant growth regulators on spike yield and bulb production of tuberose double. *Plant Archives*. 2010, *11*(1), 169-171.

[27] Tiwari, J. K.; Singh, R. P. Effect of preplanting GA_3 treatment on tuberose. *Journal of Ornamental Horticulture*. 2002, *5*(2), 44-55.

[28] Diwakar, P. S.; Katiyar P.N. Regeneration of Kagzi lime (*Citrus aurantifolia*) through stem cuttings with the aid of IBA and PHB. *HortFlora Research Spectrum.* 2013, *2*(3), 271-273.

[29] Mynett, K.; Startek, L.; Zurawik, P.; Ploszaj, B. Effect of gibrescol and flordimex on the emergence and growth of freesia Rocz. *international journal Acta Agrobotanica*. 2001, 33, 103-110.

[30] Janowsk, B.; Jerzy, M. Effect of gibberrelic acid on postharvest leaf longevity of *Zantedeschia elliottiana*. *Journal of Fruit and Ornamental pland Research.* 2003, 11, 69-76.

JSTR
ASEAN Journal of Scientific and Technological Reports

Research article

Antimicrobial Activity of Extremely Halophilic Archaea Isolated from Southern Thai Salt-Fermented Products and Solar Saltern of Pattani, Thailand

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Citation:
Niyasom, C.; Mamimin, C. Antimicrobial activity of extremely halophilic archaea isolated from southern Thai salt-fermented products and solar saltern of Pattani, Thailand. *ASEAN J. Sci. Tech. Report.* **2023**, *26*(2), 30-38. https://doi.org/10. 55164/ajstr. v26i2.248121.

Article history:
Received: March 1, 2023
Revised: March 19, 2023
Accepted: March 20, 2023
Available online: March 26, 2023

Abstract: This research aimed to study the diversity and antimicrobial activity of culturable haloarchaea in soil samples of solar saltern in Pattani Province, Thailand and Southern Thai salt-fermented food. Seventy-seven extremely haloarchaea were isolated on Halophilic medium agar containing 25% NaCl at 37°C for 7-21 days. They were grouped by Amplified Ribosomal DNA Restriction Analysis (ARDRA) with two restriction enzymes, *RsaI* and *HindIII*. The ARDRA patterns illustrated 6 different Operation Taxonomic Units (OTUs). Partial 16S rRNA gene sequences (938 bp) of the representative of each OTUs were aligned with the GenBank database. The results showed that the representatives of OTU1, OTU2, OTU3, OTU4, OTU5, and OTU6 had 99-100% similarity *to Halobacterium salinarum*, 99-100% similarity to *Halostagnicola larsenii*, 99% similarity to *Natronococcus* sp., 99-100% similarity to *Haloferax alexandrinus*, 99-100% similarity to *Natrialba* sp. and 97% similarity to *Halococcus* sp., respectively. The antimicrobial activity testing of the 17 isolated haloarchaeal strains from solar saltern was performed against 5 tested strains of *Hbt. salinarum* and 1 strain of *Natrialba* sp. were isolated from fermented food. Seven (41.2%) were candidate halocin-producing strains. In addition, no phage activity was observed. The 14-day fermentation broth of *Natronococcus* sp. SS13 showed the highest antimicrobial activity against *Hbt. salinarum* PK08, BD07 and BD09 (>5,120 AU/ml).

Keywords: Halophilic archaea; antagonistic activity; salt-fermented product; solar saltern

1. Introduction

Halophilic archaea (haloarchaea) are the members of the family *Halobacteriaceae*, order *Halobacteriales*, class *Halobacteria*, phylum *Euryarchaeota* in the domain *Archaea*. Halobacteriaceae, including at least 51 genera, have been validly characterized, reflecting their considerable ecophysiological diversity [1,2]. They require at least 1.5 M NaCl (9% w/v) for growth but grow optimally in 2.5 (15% w/v) to 5 M NaCl (30% w/v), and lower concentrations of salinity generally cause cell lysis [3]. Haloarchaea accumulates salts, mainly KCl, to adapt the entire intracellular machinery to function in the presence of high salts. Moreover, they can produce compatible organic osmotic solutes to maintain the concentration of ions inside and outside the cell to keep themselves intact and

remain alive in the hypersaline environment and salt-saturated ecosystem [4]. Several studies focusing on the microbial diversity in these ecosystems using culture-dependent or culture-independent approaches have shown that haloarchaea are the dominant colonizers [5,6]. When salinity reaches values close to saturation of NaCl, some archaeal populations become predominant to the exclusion of others. An important factor that may provide haloarchaea with a competitive advantage in competing for nutrients and other resources is the production of gene-encoded antimicrobial peptides or halocin proteins [7].

Halocins are either peptide (<10 kDa, microhalocins) or protein (>10 kDa) antibiotics. They have been reported to generally kill the indicator organisms by cell swelling followed by cell lysis [8]. Several previous studies have screened halocin-producing microorganisms from hypersaline environments [5,9,10]. Halocins have been reported to be produced among the Halobacteriaceae by species of *Haloferax*, *Haloterrigena*, *Natrinema,* and *Halobacterium* [11]. Moreover, the unique character of haloarchaea and their enzymes enabling them to sustain catalytic activity in hypersaline environments make them attractive resources for use in various industrial conditions. Several applications of haloarchaea and their value-added molecules have been characterized, i.e., halophilic and thermostable enzymes [12], bacteriorhodopsin [13], biodegradable polymers [14], bioremediation of polluted hypersaline environment [15], salt-fermented food [16] and carotenoid production [17].

Solar salterns, consisting of a series of shallow interconnected ponds filled with natural water from the sea, have been used for sea salt production. In Thailand, sea salts were produced by evaporating seawater and used in fermented food, especially fish sauce, fermented fish, salted fish, and pickled mussels. These products are rich in nutrients, particularly amino acids, and contain a high NaCl concentration, allowing several halophilic microorganisms to thrive. Some investigations of extremely halophilic archaea in Thai fermented food have been reported [18-21], but the haloarchaeal communities inhabiting the solar saltern of Southern Thailand have never been examined. Several studies have isolated and identified the strains of halophilic archaea from various fermented fish products. New species of halophilic archaea have been proposed, such as *Halobacterium piscisalsi* [18], *Natrinema gari* [19], *Haloarcula salaria*, *Haloarcular tradensis* [20], and *Halococcus thailandensis* [21]. However, the potential of such strains to produce halocins has also never been reported. Protein antibiotics can potentially preserve agents in the food and leather industries and control infectious bacteria. Hence, there is great interest in isolating potential proteinaceous bioactive substances. Therefore, in this study, members of the family *Halobacteriaceae* isolated from several Southern Thai salt-fermented products and the solar saltern soil of Pattani Province, Thailand, were screened to produce antimicrobial substances. For the first time, we also report members of the genera *Halostagnicola* and *Natronococcus* as producers of growth-inhibitory substances.

2. Materials and Methods

2.1 Isolation of extremely halophilic archaea

Extremely halophilic archaea were isolated from various Southern Thai traditional salt-fermented food products such as *budu* (Southern Thai fermented fish sauce), *pla-kem* (dried salted fish), *jing-jung* (fermented anchovy) and *hoi-dong* (pickled mussel), and solar saltern soils located in Pattani Province (Latitude 6° 53' 22.4592" N Longitude 101°16' 41.2795" E), Thailand. Samples were diluted at 1:10 in 25% NaCl. One-hundred microliters of diluted samples were plated on halophilic medium (HM) agar containing (l^{-1}) 250 g NaCl, 5 g casamino acids, 5 g yeast extract, 1 g sodium glutamate, 2 g KCl, 3 g trisodium citrate, 20 g $MgSO_4.7H_2O$, 0.036 g $FeCl_4.4H_2O$, 0.00036 $MnCl_2.4H_2O$, and 20 g agar (pH 7.5). The plates were incubated at 37 °C for 14-21 days. Representative colonies were transferred to fresh HM agar to obtain a pure culture.

2.2 Amplified ribosomal DNA restriction analysis

Genomic DNA was extracted from log-phase cells by lysed in purified water. Amplification of 16S rRNA gene fragment using archaeal primers set, 21F (5′-TTCCGGTTGATCCYGCCGGA-3′) and 958R (5′-YCCGGCGTTGAMTCCAATT- 3′) as previously described [22]. Each 50 µl reaction PCR mixture contained 5 µl of 10x PCR buffer, 1 µl of dNTP (10 µM each), 1 µl of each primer (10 µM), 0.25 µl (1.25 U) *Taq* polymerase,

and 1 µl of template DNA. After a denaturation step of 5 min at 94 °C, amplification reactions were performed with 30 cycles of denaturation (1 min, 94 °C), primer annealing (1 min, 55 °C), and primer extension (1 min, 72 °C) with a final extension step at 72 °C for 7 min. The amplified DNA fragment (938 bp) was single-digested with *RsaI* and *HindIII* restriction endonucleases (Fermentas, Promega). Digestion reactions were performed in 25 µl containing 2 µl amplified 16S rDNA of the strains following protocol. The final concentration of restriction digestion mixture containing 1X appropriate buffer, 20-unit restriction enzyme, and make up the final volume with PCR grade water, restriction digestion was carried out at 37 °C for 2 hours, followed by enzyme inactivation at 65 °C for 30 minutes. The fragments from digested PCR products were analyzed in 1.5% agarose gel electrophoresis. PCR products were purified using an E.Z.N.A cycle pure kit and sequenced using primer 21F and 958R by the Macrogen sequencing facility (Macrogen Inc., Korea).

2.3 Antagonistic activity assay

To screen the antimicrobial activity of haloarchaeal strains, the antagonistic activity was carried out by using the agar double-layer diffusion assay. Briefly, 7 ml of melted soft HM agar (7.5 g/L agar maintained at 50 °C) was mixed with 10 µl of exponential phase culture of the target strain (OD_{600} = 0.4-0.6) and poured over HM agar (15 g/l). The target strains were BD06, BD07, BD09, PK08, JJ02, and HD07. Upon solidification, 5 µl of exponential phase culture of the potentially producing strain was spotted on the top agar. Incubation was carried out at 37 °C for 7 days until a homogenous microbial lawn and inhibition halos were observed.

2.4 Antimicrobial activity (Halocin activity) assay

The halocin activity was performed using the agar well diffusion assay as described previously [23] with some modifications. The selected bacterial strain with halocin activity was grown in 10 ml halophilic medium at 37 °C for 5 days. After growth, the culture obtained was centrifuged at 10,000 rpm at 4 °C for 15 min. Cells were harvested, washed with brine solution (25% NaCl), and resuspended in the brine solution. The cell suspension concentration was adjusted to 0.2 OD at 600 nm and used as inoculum at a 2% (v/v) level. 100 ml of halophilic medium in a 250 ml conical flask was inoculated with the prepared inoculums and incubated for 14 days. The bacterial-free supernatant was used for halocin assay after centrifugation (10,000 rpm for 10 min) of the culture broth. The halocin activity was checked by the agar well diffusion method. The tested strains (according to the result from 2.3) were grown until the OD reached 0.3 at 600 nm, and 200 µl of the tested strain was mixed with 20 ml of halophilic medium containing half-strength (0.75%) agar and overlaid on halophilic agar plates containing 1.5% agar. The halocin activity was checked by adding 50 µl of cell-free supernatant of selected producing strains into the well (0.5 cm diameter) made on the plate containing the top agar and performing the halocin assay after incubation for 7 days. The halocin activity was determined using serial twofold critical endpoint dilutions to extinction and expressed as arbitrary units (AU), defined as the reciprocal of the first dilution at all traces of inhibitory activity disappear. The two-fold dilution ratio of halocin follows a geometric progression where the halocin activity can be calculated by Arbitrary unit (AU/ml) = (1,000/A)/B. Where "A" is the volume of supernatant and "B" is the highest dilution of the supernatant at which inhibitory activity still appears.

2.5 Detection of phage activity

To determine the phage activity, a fragment of agar was cut from the halo zone of inhibition of the sensitive strain and added to 100 ml of HM inoculated with 100 µl of the culture of the sensitive strain. The culture (with and without agar fragments) was then incubated at 37°C for 7-14 days. The growth was determined by measuring OD_{600} (compared to the growth without the agar fragment). Plague assay was performed by inoculating crude culture supernatant with sensitive strain in soft agar medium as described above.

3. Results and Discussion

This study isolated 77 halophilic archaeal strains from Southern Thai traditional salt-fermented food products and solar saltern soils. Amongst 77 isolates, 20, 15, 15, 10, and 17 isolates were from *budu* (designated as BD01-BD20), *pla-kem* (PK01-PK15), *jing-jung* (JJ01-JJ15), *hoi-dong* (HD01-HD10) and solar saltern soil

(SS01-SS17), respectively. Colonies of all selected isolates were circular with entire edges, pink and orange to red, except all 15 HD isolates, which were colorless. All isolates were catalase-positive, oxidase-positive, gram-negative, aerobic rods with optimum growth at 37-42 °C, pH 7-8 in the medium containing 20-25% salt concentration. When the ARDRA method was used to group all haloarchaeal isolates, the restriction profiles revealed significant genetic differences among the strains, confirming that the isolates represented at least 6 different operational taxonomic units (OTUs) groups from 77 isolated haloarchaea. *RsaI* digestion classified all isolates into 4 restriction patterns, while *HindIII* digestion generated 3 patterns (Figure 1).

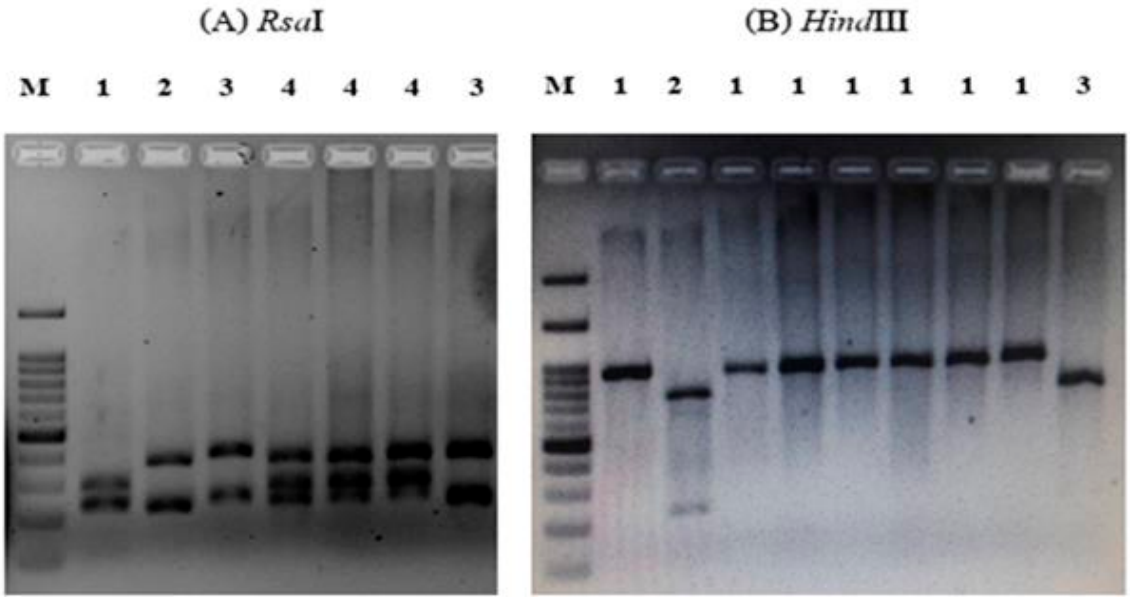


Figure 1. Four and three restriction fragment patterns of 938 amplified DNA of haloarchaeal strains digested with *RsaI* (A) and *HindIII* (B), respectively. M; 100 bp DNA ladder, 1-4; restriction patterns.

When combined *RsaI* with *HindIII* digested patterns, 6 OTUs were classified. The isolate belonging to OTU1 was found in all samples, while the isolate belonging to OTU2, 3, and 4 were found in solar salterns. Partial 16S rRNA gene sequencing was performed for at least 2 representatives of each OTUs and was analyzed using the BLAST algorithm. The representative strains of each OTUs were identified to the different genera, which were *Halobacterium* (OTU1), *Halostagnicola* (OTU2), *Natronococcus* (OTU3), *Haloferax* (OTU4), *Natrialba* (OTU5) and *Halococcus* (OTU6) by the similarity of 97-100% compared with the previously reported strains (Table 1).

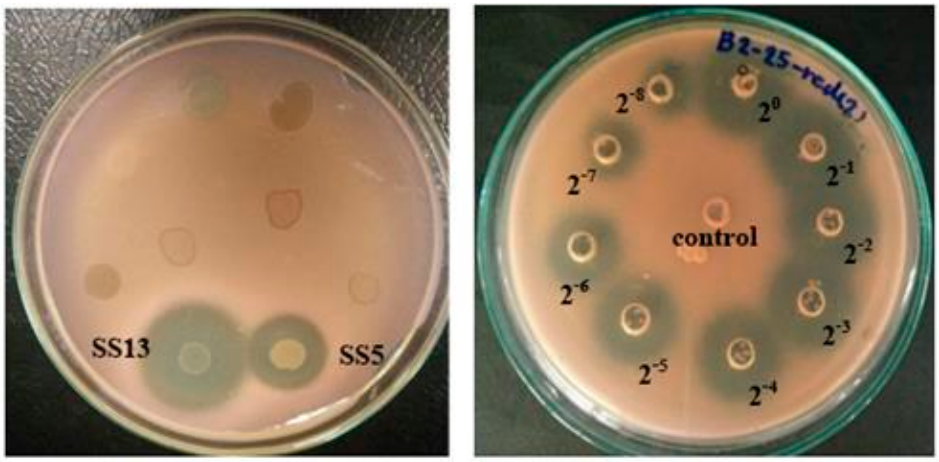
Together with the colony pigmentation and the requirement for high salt concentrations, these properties suggested that all strains might be members of the family *Halobacteriaceae*. The isolates revealed high similarity with the closest described species regarding the colony, cell morphologies, and physiological characteristics. Only 3 haloarchaeal genera (*Halobacterium*, *Halococcus,* and *Natrialba*) were isolated from salt-fermented food products, but all 6 genera were found in soil samples from the solar saltern of Pattani, Thailand. *Hbt. salinarum* seems to be the most abundant haloarchaea in all salt-fermented food products except *hoi-dong* (pickle mussel), in which Natrialba predominates. ARDRA technique was applied for several archaeal diversity studies, such as in superficial hypersaline sediments of solar salterns in Tunisia [24] and saltpan sediment in India [25]. Our results showed that *RsaI* and *HindIII* could be helpful for the primary grouping of haloarchaea isolated from a solar saltern in Thailand, as they can classify at least 6 restriction patterns.

All 17 (SS01-17) halophilic archaeal strains from solar saltern were tested for their antagonistic ability against 6 tested halophilic microorganisms (BD06, BD07, BD09, JJ02, PK08, and HD07), which were isolated from fermented food samples. Among those, 7 strains (41.2%) showed antagonistic activity against at least 2 of 6 testing haloarchaeal strains (Table 2). SS01, SS05, SS10, and SS16 exhibited antagonistic activity against

only two target strains, while three other strains (SS03, SS13, and SS14) could inhibit the growth of several testing isolates. Different degrees of activity were observed. The genus *Natronococcus* (SS03 and SS13) were the most active and might be the best producer of antimicrobial compounds (Figure 2, Table 2-3). *Hbt. salinarum* PK08 was the most sensitive. It showed sensitivity to all 7 producing strains.

Table 1. The results and maximum identification percentage were obtained from the NCBI nucleotide sequencing program and 6 OTU groups of the isolated haloarchaeal strains.

OTU group (*Rsa*I-*Hind*III)	Strains	Representing strain	Identification/accession no.	%Similarity
OTU 1	JJ01-15	JJ02	*Halobacterium salinarum* KP751341.1	99
(1-1)	PK01-15	PK08	*Halobacterium salinarum* KR611163.1	99
	BD01-19	BD06	*Halobacterium salinarum* KR611163.1	100
	HD10	BD07	*Halobacterium salinarum* NR113428.1	99
	SS07-09, 11-12	BD09	*Halobacterium salinarum* NR113428.1	99
	SS14-16	SS14	*Halobacterium salinarum* NR113428.1	99
		SS16	*Halobacterium salinarum* NR113428.1	99
OTU 2	SS01-02, 10	SS01	*Halostagnicola larsenii* KP117067.1	99
(2-1)		SS10	*Halostagnicola larsenii* NR113506.1	100
OTU 3	SS03, 13	SS03	*Natronococcus* sp. JX481739.1	99
(3-2)		SS13	*Natronococcus* sp. DQ373054.1	99
OTU 4	SS04-05	SS04	*Haloferax alexandrinus* NR113438.1	99
(4-1)		SS05	*Haloferax alexandrinus* NR113438.1	100
OTU 5	HD01-09	HD02	*Natrialba taiwanensis* AB663459.1	99
(3-1)	SS17	HD03	*Natrialba taiwanensis* AB663459.1	99
		HD07	*Natrialba aegyptia* JX481742.1	99
		SS17	*Natrialba aegyptia* JX481742.1	99
OTU 6	BD20	BD20	*Halococcus thailandensis* EU984192.1	97
(1-3)	SS06	SS06	*Halococcus* sp. AB904834.1	97



(A) (B)

Figure 2. The antagonistic activity of *Haloferax* SS05 and *Natronococcus* SS13 against sensitive strain *Hbt. salinarum* BD07 (A) and antimicrobial activity of the supernatant of strain *Natronococcus* SS13 against *Hbt. salinarum* BD07 (B)

Table 2. Antagonistic activity of haloarchaea from solar saltern (SS) against at least 1 strain of tested haloarchaea from fermented food products (BD, PK, JJ, and HD)

Producer strains	Inhibition zone of target strains (mm)					
	BD06	**BD07**	**BD09**	**PK08**	**JJ02**	**HD07**
SS01	-	-	13	15	-	-
SS03	18	-	28	28	15	-
SS05	-	20	-	14	-	-
SS10	-	-	-	12	12	-
SS13	-	28	27	26	32	-
SS14	12	-	12	11	-	-
SS16	-	-	12	12	-	-

Table 3. Antimicrobial activity of 7 candidate halocin-producing strains measured in arbitrary unit (AU)/ml

Producer strain	Target strain	Arbitrary Units (AU)/ml
Halostagnicola SS01	*Hbt. salinarum* PK08	640
Natronococcus SS03	*Hbt. salinarum* PK08	1,280
	Hbt. salinarum BD06	1,280
	Hbt. salinarum BD09	≥ 5,120
Haloferax SS05	*Hbt. salinarum* BD07	≥ 5,120
Halostagnicola SS10	*Hbt. salinarum* JJ02	80
	Hbt. salinarum PK08	160
Natronococcus SS13	*Hbt. salinarum* PK08	≥ 5,120
	Hbt. salinarum BD07	≥ 5,120
	Hbt. salinarum BD09	≥ 5,120
Halobacterium SS14	*Hbt. salinarum* PK08	20
	Hbt. salinarum BD09	40
Halobacterium SS16	*Hbt. salinarum* PK08	40
	Hbt. salinarum BD09	80

The halophilic archaeal strains with antagonistic activity against other microorganisms were selected for further halocin activity assay observation. The culture supernatants of those producing strains were determined for their activities in arbitrary units (AU)/ml against their sensitive strains. SS01, SS03, SS05, and SS13 showed inhibitory activity against *Hbt. salinarum* PK08 of 640, 1,280, >5,120 and >5,120 AU/ml, respectively. SS05 and SS13 showed the equal highest inhibitory activity against *Hbt. salinarum* BD07 of >5120 AU/ml. From the inhibitory assay in AU/ml, the culture supernatant of the strain of the genus *Natronococcus* (SS03 and SS13) and *Haloferax* (SS05) was the most active. It might be the strongest producer of antimicrobial compounds.

The diversity of haloarchaeal strains from the solar saltern of Pattani Province, Thailand, and the potential antimicrobial production of those strains have not yet been reported. In this study, 17 haloarchaeal strains were isolated and were examined for their ability to exert antimicrobial activity against 6 testing haloarchaeal strains from several salt-fermented foods. This work isolated 19 strains of *Halobacterium* and 1 strain of *Halococcus* from *budu*, the Southern Thai fermented fish sauce. Previous studies consistently reported that several haloarchaeal genera were isolated from Thai fish sauce products, including *Halobacterium*, *Natrinema, Haloarcula,* and *Halococcus* [18-21]. Furthermore, non-pigmented *Natrialba* spp. were the major haloarchaea in hoi-dong (salt-fermented mussel), consistent with the previous report that *Natrialba aegyptia* was isolated using culture-dependent methods of *jeotgal*, Korean fermented fish and shellfish [26]. For the diversity of haloarchaea in the solar saltern of Pattani, 6 genera were identified, including *Halobacterium, Halococcus*, *Halostagnicola, Haloferax*, *Natronococcus,* and *Natrialba*. This result correlates with several studies that reported that haloarchaea had been isolated from different hypersaline habitats, especially solar saltern.

Culture-based assessment of archaeal diversity in solar saltern from other regions of the world has been identified, which *Haloarcula, Halobacterium, Halorubrum,* and *Haloferax* as the main genera [27]. Atanasova and coworkers have isolated 11 haloarchaeal genera from the solar saltern of Samutsakorn Province, another solar saltern of Central Thailand, including *Halorubrum, Halolamina, Halobacterium, Halobellus, Haloarcula, Halogeometricum, Haloterrigena, Halogranum, Haloferax, Halosarcina* and *Natrinema* [28-29].

Seven strains exhibiting antimicrobial activity belong to 4 genera (2 *Halobacterium,* 2 *Halostagnicola,* 1 *Haloferax*, and 2 *Natronococcus*). It has been reported in previous studies that a significant fraction of haloarchaea inhabiting hypersaline areas produce antimicrobial agents [30]. A previous study reported the antagonistic interactions among halophilic archaeal isolates of distant geographical sites [31]. Several genera of halophilic archaea, including *Halorubrum, Haloferax, Haloplanus, Haloarcula, Halogranum, Halobacterium, Halosarcina, Halogeometricum* [32], *Natrinema, Halopiger* [9] and *Haloterigena* [5] are halocin-producers. For the first time, in the present work, we reported that genera *Natronococcus* and *Halostagnicola* had also been shown to be candidate halocin producers.

Antimicrobial activities produced by haloarchaeal strains may be due to agents of various nature-lytic viruses or antimicrobial peptides/proteins that either may be secreted or remain bound to the wall of the producing cells [5]. Multiple dilutions of producer strain culture supernatants were applied on indicator lawns to confirm inhibition was not a result of phage infection. No plaques were observed in a plaque assay using crude or diluted culture supernatant inoculated together with sensitive strain in a soft agar medium, demonstrating the absence of the virus. Halophilic archaea were the first archaea domain members found to produce archaeocin, bacteriocin-like proteins, also known as halocin. Halocins were initially discovered during a survey of antagonistic interactions among different members of the class *Halobacteria* [31]. At least 11 halocins have been reported, including HA1, HA3, A4, H1, H4, H6/H7, R1, Sech7A, SH10, S8, and C8 [7,8,23,30,32-35]. Among these, only 3 genes, *halH4*, *halS8*, and *halC8* coding halocin H4, S8, and C8, have been identified and described [36-38]. However, all our producing strains gave negative results for PCR using *halH4*, *halS8*, and *halC8* specific primers (data not shown). The halocin will be purified and characterized in a future study to confirm that the antagonistic activity of 7 candidate halocin-producing strains was due to halocin production.

4. Conclusions

ARDRA analysis of 77 haloarchaeal strains was classified into 6 OTUs. According to the comparison of the 16S rDNA sequences, 17 representative strains were affiliated with six different genera, including *Halobacterium, Halococcus, Natrialba, Haloferax, Natronococcus,* and *Halostagnicola*. The first 3 genera were found in Southern Thai salt-fermented food products, while all 6 were found in solar saltern. Some isolates of the 4 genera; 2 isolates of *Halostagnicola* (SS01 and SS10), 1 isolate of *Haloferax* (SS05), 2 isolates of *Halobacterium* (SS14 and SS16), and 2 isolates of *Natronococcus* (SS03 and SS13) showed antagonistic activity against testing haloarchaea. Candidate halocin-producing strain *Natronococcus* sp. SS13 showed the highest antimicrobial activity against *Hbt. salinarum* PK08, BD07 and BD09.

5. Acknowledgements

We would like to thank the owners of the solar saltern in Pattani Province, Thailand, for their kind accommodation and sample collection.

Author Contributions: Conceptualization, C.N.; methodology, formal analysis, investigation, data curation, writing—original draft preparation, visualization, funding acquisition; C.M. resources, writing—review and editing,

Funding: This work was supported by The Thailand Research Fund (Grant No.: MRG5580073)

Conflicts of Interest: The authors declare no conflict of interest.

References

[1] Williams, T.J.; Allen, M.A.; DeMaere, M.Z.; Kyrpides, N.C.; Tringe, S.G.; Woyke, T.; Cavicchioli, R. Microbial ecology of an Antarctic hypersaline lake: Genomic assessment of ecophysiology among dominant haloarchaea. *ISME J* 2014, *8*, 1645-1658.

[2] Parte, A.C.; SardàCarbasse, J.; Meier-Kolthoff, J.P.; Reimer, L.C.; Goker, M. List of prokaryotic names with standing in nomenclature (LPSN) moves to the DSMZ. *Int J Syst Evol Microbiol* 2020, *70*, 5607-5612.

[3] Oren, A. Life at high salt concentrations, intracellular KCl concentrations, and acidic proteomes. *Front Microbiol* 2013, *4*, 315. https://doi.org/10.3389/fmicb.2013.00315

[4] Gunde-Cimerman, N.; Plemenitas, A.; Oren, A. Strategies of adaptation of microorganisms of the three domains of life to high salt concentrations. *FEMS Microbiol Rev* 2018, *42*, 353-375.

[5] Ghanmi, F.; Carré-Mlouka, A.; Vandervennet, M.; Boujelben, I.; Frikha, D.; Ayadi, H.; Peduzzi, J.; Rebuffat, S.; Maalej, S. Antagonistic interactions and production of halocin antimicrobial peptides among extremely halophilic prokaryotes isolated from the solar saltern of Sfax, Tunisia. *Extremophiles* 2016, *20*, 363-374.

[6] Najjari, A.; Stathopoulou, P.; Elmnasri, K.; Hasnaoui, F.; Zidi, I.; Sghaier, H.; Ouzari, H.I.; Cherif, A.; Tsiamis, G. Assessment of 16S rRNA gene-based phylogenetic diversity of archaeal communities in halite-crystal salts processed from natural Saharan saline systems of Southern Tunisia. *Biology* 2021, *10(5).* https://doi.org/10.3390/biology10050397

[7] Besse, A.; Peduzzi, J.; Rebuffat, S.; Carre-Mlouka, A. Antmicrobial peptides and proteins in the face of extremes: lessons from archaeocins. *Biochimie* 2015, *118*, 344-355.

[8] O'Connor, E.M.; Shand, R.F. Halocins and sulfolobicins: the emerging story of archaeal protein and peptide antibiotics. *J Ind Microbiol Biotechnol* 2002, *28(1),* 23-31.

[9] Quadri, I.; Hassani, I.I.; Haridon, S.; Chalopin, M.; Hacene, H.; Jebbar, M. Characterization and antimicrobial potential of extremely halophilic archaea isolated from hypersaline environments of the Algerian Sahara. *Microbiol Res* 2016, *186-187*, 119-131.

[10] Mazguene, S.; Rossi, M.; Gogliettino, M.; Palmieri, G.; Cocca, E.; Mirino, S.; Imadalou-Idres, N.; Benallaoua, S. Isolation and characterization from solar salterns of North Algeria of a haloarchaeon producing a new halocin. *Extremophiles* 2018, *22*, 259-270.

[11] Besse, A.; Vandervennet, M.; Goulard, C.; Peduzzi, J.; Isaac, S.; Rebuffat, S.; Carré-Mlouka, A. Halocin C8: an antimicrobial peptide distributed among four halophilic archaeal genera: *Natrinema, Haloterrigena, Haloferax* and *Halobacterium*. *Extremophiles* 2017, *21*, 623-638.

[12] Oren, A. Industrial and environmental applications of halophilic microorganisms. *Environ Technol* 2010, *31*, 825-834.

[13] Ispirli, N.H.; Gulluce, M.; Karadayi, M.; Demir, A.Y. Culturable bacteriorhodopsin-producing haloarchaea of Tuz Lake (Turkey). *Geomicrobiol J* 2019, *36*, 831-836.

[14] Don, T.M.; Chen, C.W.; Chan, T.H. Preparation and characterization of poly (hydroxyalkanoate) from the fermentation of *Haloferax mediterranei*. *J Biomater Sci Polym Edn* 2006, *17*, 1425-1438.

[15] Martínez-Espinosa, R.M.; Zafrilla, B.; Camacho, M.; Bonete, M.J. Nitrate and nitrite removal from salted water by *Haloferax mediterranei*. *Biocatal Biotransform* 2007, *25*, 295-300.

[16] Akolkar, A.V.; Durai, D.; Desai, A.J. *Halobacterium* sp. SP1 as a starter culture for accelerating fish sauce fermentation. *J Appl Microbiol* 2010, *109*, 44-53.

[17] Rodrigo-Baños, M.; Garbayo, I.; Vílchez, C.; Bonete, M.J.; Martínez-Espinosa, R.M. Carotenoids from haloarchaea and their potential in biotechnology. *Mar Drugs* 2015, *13(9),* 5508-5532.

[18] Yachai, M.; Tanasupawat, S.; Itoh, T.; Benjakul, S.; Visessanguan, W.; Valyasevi, R. *Halobacterium piscisalsi* sp. nov., from fermented fish (pla-ra) in Thailand. *Int J Syst Evol Microbiol* 2008, *58,* 2136-2140.

[19] Tapingkae, W.; Tanasupawat, S.; Itoh, T.; Parkin, K.L., Benjakul, S.; Visessanguan, W.; Valyasevi, R. *Natrinema gari* sp. nov., a halophilic archaeon isolated from fish sauce in Thailand. *Int J Syst Evol Microbiol* 2008, *58*, 2378-2383.

[20] Namwong, S.; Tanasupawat, S.; Kudo, T.; Itoh, T. *Haloarcula salaria* sp. nov. and *Haloarcula tradensis* sp. nov., isolated from salt in Thai fish sauce. *Int J Syst Evol Microbiol* 2011, *61*, 231-236.

[21] Namwong, S.; Tanasupawat, S.; Visessanguan, W.; Kudo, T.; Itoh, T. *Halococcus thailandensis* sp. nov., from fish sauce in Thailand. *Int J Syst Evol Microbiol* 2007, *57*, 2199-2203.

[22] Delong, E.F. Archaea in coastal marine environments. *PNAS* 1992; *89*, 5685-5689.

[23] Karthikeyan, P.; Bhat, S.G.; Chandrasekaran, M. Halocin SH10 production by an extreme haloarchaeon *Natrinema* sp. BTSH10 isolated from salt pans of South India. *Saudi J Biol Sci* 2013, *20(2),* 205-212.

[24] Boujelben, I.; Martínez-García, M.; van Pelt, J.; Maalej, S. Diversity of cultivable halophilic archaea and bacteria from superficial hypersaline sediments of Tunisian solar salterns. *Antonie van Leeuwenhoek* 2014, *106*, 675-692.

[25] Ahmad, N.; Johri, S.; Sultan, P.; Abdin, M.Z.; Qazi, G.N. Phylogenetic characterization of archaea in saltpan sediments. *Indian J Microbiol* 2011, *51(2),* 132-137.

[26] Roh, S.; Kim, K.H.; Nam, Y.D.; Chang, H.W.; Park, E.J.; Bae, J.W. Investigation of archaeal and bacterial diversity in fermented seafood using barcoded pyrosequencing. *ISME J* 2010, *4*, 1-16.

[27] Birbir, M.; Calli, B.; Mertoglu, B.; Bardavid, R.E.; Oren, A.; Ogmen, M.N.; Ogan, A. Extremely halophilic Archaea from Tuz Lake, Turkey, and the adjacent Kaldirim and Kayacik salterns. *World J Microbiol Biotechnol* 2007, *23*, 309-316.

[28] Atanasova, N.S.; Roine, E.; Oren, A.; Bamford, D.H.; Oksanen, H.M. Global network of specific virus-host interactions in hypersaline environments. *Environ Microbiol* 2012, *14(2),* 426-440.

[29] Atanasova, N.S.; Pietila, M.K.; Oksanen, H.M. Diverse antimicrobial interactions of halophilic archaea and bacteria extend over geographical distances and cross the domain barrier. *MicrobiologyOpen* 2013, *2(5),* 811-825.

[30] Rodríguez-Valera, F.; Juez, G.; Kushner, D.J. Halocins: salt-dependent bacteriocins produced by extremely halophilic rods. *Can J Microbiol* 1982, *28*:151-154.

[31] Li, Y.; Xiang, H.; Liu, J.; Zhou, M.; Tan, H. Purification and biological characterization of halocin C8, a novel peptide antibiotic from *Halobacterium* strain AS7092. *Extremophiles* 2003, *7(5),* 401-407.

[32] Torreblanca, M.; Meseguer, I.; Rodríguez-Valera, F. Halocin H6, a Bacteriocin from *Haloferax gibbonsii*. *J Gen Microbiol* 1989, *135*, 2655-2661.

[33] Pasic, L.; Velikonja, B.H.; Ulrih, N.P. Optimization of the culture conditions for the production of a bacteriocin from halophilic archaeon Sech7a. *Prep Biochem Biotechnol* 2008, *38(3),* 229-45.

[34] Kumar, V.; Tiwari, S.K. Halocin HA1: An archaeocin produced by the haloarchaeon *Haloferax larsenii* HA1. *Process Biochem* 2017, *61*, 202-208.

[35] Mazguene, S.; Rossi, M.; Gogliettino, M.; Palmieri, G.; Cocca, E.; Mirino, S.; Imadalou-Idres, N.; Benallaoua, S. Isolation and characterization from solar salterns of North Algeria of a haloarchaeon producing a new halocin. *Extremophiles* 2018, *22(2),* 259-270.

[36] Meseguer, I.; Rodriguez-Valera, F. Production and purification of halocin H4. *FEMS Microbio Lett* 1985; *28(2)*, 177-182.

[37] Cheung, J.; Danna, K.J.; O'Connor, E.M.; Price, L.B.; Shand, R.F. Isolation, sequence, and expression of the gene encoding halocin H4, a bacteriocin from the halophilic archaeon *Haloferax mediterranei* R4. *J Bacteriol* 1997, *179(2)*, 548-551.

[38] Sun, C.; Li, Y.; Mei, S.; Lu, Q.; Zhou, L.; Xiang, H. A single gene directs both production and immunity of halocin C8 in a haloarchaeal strain AS7092. *Mol Microbiol* 2005, *57(2)*, 537-549.

JSTR
ASEAN Journal of Scientific and Technological Reports

Research article

Effect of *Durio zibethinus* Murr. cv. Monthong Rind as a Dietary Ingredient in Feed on the Growth Performance and Disease Resistance Against *Aeromonas hydrophila* in Red Tilapia (*Oreochromis niloticus x Oreochromis mossambicus*)

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Citation:
Sangkhonkhet, N.; Phanchindawan, N.; Lerdsuwan, S.; Nalinanon, W.; Pisuttharachai, D. Effect of *Durio zibethinus* Murr. cv. Monthong rind as a dietary ingredient in feed on the growth performance and disease resistance against *Aeromonas hydrophila* in Red Tilapia (*Oreochromis niloticus* x *Oreochromis mossambicus*). *ASEAN J. Sci. Tech. Report.* **2023**, *26*(2), 39-48. https://doi.org/10.55164/ajstr.v26i2.248572.

Article history:
Received: February 20, 2023
Revised: March 17, 2023
Accepted: March 22, 2023
Available online: March 26, 2023

Abstract: Monthong durian is a durian species mainly cultivated in Thailand. The rind makes up about 60-75% of whole fruit and is often discarded. Here, we evaluated the effect of durian rind, specifically the inner white peel, as a supplement for a fish diet on the growth performance and disease resistance against *Aeromonas hydrophila* in Red Tilapia. Fish with an initial average weight of 42.68 ± 0.11 g were fed diets supplemented with durian rind at 0, 10, 15, and 20% for 140 days. Subsequent results showed that growth performance in terms of weight gain, average daily gain, length gain, and specific growth rates were not significant among treatments ($P > 0.05$). In addition, the feed conversion ratio and survival rate of fish fed with diets supplemented with durian rind were also not significantly different from the control ($P > 0.05$). After the 140-day feeding trial, fish were injected intraperitoneally with *A. hydrophila*, and the cumulative mortality was recorded for 14 days. The survival rate of fish-fed durian rind-supplemented diets at all levels was higher than that of the control. The highest survival rate and relative survival percentage were fish fed with 10% and 15% durian rind in the diet. Taken together, the white peel of the durian rind can be a potential fish feedstuff and can act as a natural antibiotic to improve fish resistance against *A. hydrophila*. Recommended level of durian rind supplementation is 10%-15% in the diet which will not affect growth but can enhance disease resistance to *A. hydrophila*.

Keywords: *Durio zibethinus* Murr. cv. Monthong; *Oreochromis niloticus x Oreochromis mossambicus*; growth performance; disease resistance

1. Introduction

Tilapia is often called the "Aquatic chicken" due to its high growth performance, low cost, and palatability [1]. It has become one of the most popular cultured aquatic species worldwide and is farmed under semi-intensive and intensive

systems. Unfortunately, intensive culture practices for tilapia resulted in low water quality and a high incidence of diseases, especially bacterial infections, which subsequently led to reduced production. "Motile Aeromonas Septicemia" caused by *Aeromonas hydrophila*, a Gram-negative bacilliform, is one of the most concerning diseases in farmed fish. This disease affects various freshwater fish species, including Channel catfish (*Ictalurus punctatus*) [2], Nile tilapia (*Oreochromis niloticus*) [3], and Red tilapia (*Oreochromis niloticus* × *Oreochromis mossambicus*) [4]. In tilapia farms, Aeromonas infections have been reported to occur frequently [5]. The symptoms of this disease include skin ulcerations and fin erosion [6]. To curb the devastating effects of this disease, stimulating fish immunity has been postulated as a solution. In the past decade, there has been an increased interest in developing fish feed additives that can serve as immunostimulants [7].

Durian (*Durio zibethinus* Murr.) is a popular fruit in many countries such as Malaysia, Philippines, Indonesia, China, and Thailand [8-10]. It has a thick rind that constitutes about 60-75% of the whole fruit [11], which are discarded and form part of agricultural waste. Previous studies have shown that polysaccharides (PG) can be isolated from durian-rind waste [12-13]. Bioactivities of PG extracted from durian rind have been reported to have antibacterial [8, 14] and immunomodulating properties [15-17]. These studies, however, used the extracted form, which may be laborious and costly for fish farmers. Moreover, there is very little information on durian rind-supplemented diets for fish. Hence, the present study aimed to determine the effect of dried and ground durian rind supplemented in fish diets on the growth performance and disease resistance against *A. hydrophila* in Red Tilapia (*O. niloticus* x *O. mossambicus*).

2. Materials and Methods

2.1 Experimental design

All experiments were approved by the Ethics Committee of King Mongkut's Institute of Technology Ladkrabang (Approval no. ACUC-KMITL-RES/2021/034). The Fingerlings of Red tilapia were sourced from the Aquaculture Genetics Research and Development Center, Department of Fisheries, Chumphon Province, Thailand. These fish were cultured in the pond of King Mongkut's Institute of Technology Ladkrabang, Prince of Chumphon Campus, Chumphon Province, for two months. Only fish with an average weight of 40-45 g were used in this study. After initial acclimatization, 400 Red tilapia were randomly distributed to 16, 500-L plastic tanks, each holding 25 fish. The tanks were again randomly assigned to feed diets supplemented with durian rind at 0, 10, 15, and 20%. Each treatment has four replicates. The Red tilapia were trained to feed on sinker pellet feed using a control diet for 14 days before the start of the experiment.

2.2 Preparation of durian rind and experimental diet

Ripe durian fruits from the organic garden in Chumphon Province were selected, and only the white inner rind was used. The rind was cleaned with tap water. After that, the rind was cut into 1-2 cm thick pieces, dried at 60°C for 48 h, and ground and sifted through a 0.5 mm sieve. The chemical composition of the processed rind was analyzed following earlier methods [18], and the minerals present were evaluated using ICP-OES (Perkin Elmer version AVIO500); samples were prepared following Allan [19] before it was mixed with the other components of the diet. The composition of each diet is shown in Table 1. The ingredients of each diet were homogeneously mixed using a Hobart mincer (model HL200) for 10 min and pelleted to make a diet at 3.0 mm in size. All diets were dried in a hot air oven at 60°C for 12-24 h. The experimental diets were kept in plastic bags and refrigerated at 4°C until use. The proximate analysis of all experimental diets was also analyzed following AOAC [18].

2.3 Feeding management

Red tilapia were fed with experimental diets twice daily, at 8:30 a.m. and 4:00 p.m., at 3% of their body weight for 140 days. Water quality, including temperature, pH, dissolved oxygen, ammonia, and nitrites, was checked every 2 weeks. The tanks were cleaned daily, and water exchange at about 50% was done every 2 days.

Table 1. Composition of experimental diets

Diet ingredients (g)	Durian rind (%)			
	0	**10**	**15**	**20**
Fish meal	35	35	35	35
Soybean meal	17	17	17	17
Rice bran	20	13.76	8.87	3
Durian rind	0	10	15	20
Broken rice	12.04	11	11.75	15.15
Yellow corn	6	6	5.8	3
Rice hulls	4.7	1.4	0.3	0
Soybean oil	2.04	2.62	3.06	3.63
Tuna oil	1	1	1	1
Vitamins and minerals mix	2	2	2	2
Butylated hydroxyl toluene (BHT)	0.02	0.02	0.02	0.02
Calcium propionate	0.2	0.2	0.2	0.2
Total (g)	**100**	**100**	**100**	**100**

Vitamins and minerals mix/kg: vitamin A 10.00 MIU; vitamin D3 2.50 MIU; vitamin E 5,000 IU; vitamin K 1.60 g; vitamin B1 1.20 g; vitamin B2 3.20 g; vitamin B6 1.20 g; niacin 5.00 g; pantothenic acid 4.00 g; folic acid 5.00 g; biotin 0.12 g; vitamin C 30.00 g

2.4 Growth performance

The weight, length, feed intake, and survival rate were noted at the end of the experiment. All the data collected were used to calculate growth efficiency, including weight gain, average daily gain, length gain, specific growth rate, feed conversion ratio, and survival rate.

2.5 Challenge test

The *Aeromonas hydrophila* strains were kindly provided by the Aquatic Animal Health Research and Development Division, Department of Fisheries, Bangkok Province, Thailand. The protocol was modified by Julie et al. [20]. In brief, one loop of *A. hydrophila* was cultured in 100 mL of tryptic soy broth (DifcoTM, France) at 37^{o}C for 24 h. The bacterial cultures were centrifuged at 5000 rpm for 10 min (Eppendorf 5920 R, Germany), and then the supernatant was discarded. The pellets were washed once and adjusted to the optical density of 1.0 at 540 nm (Thermo Scientific-Evolution 201, America) using sterile 0.85% NaCl corresponding to a concentration of 4.1 x 10^7 CFU/ml. After the 140 days feeding trial, 10 fish from each tank were randomly collected and placed in 200-L plastic tanks. The fish were injected intraperitoneally with 0.3 ml of the *A. hydrophila* suspension (4.1 x 10^7 CFU/ml). The mortality pattern was examined for 14 days. The formulas calculated the cumulative mortality rate and relative percentage survival of fish [21-22].

Cumulative mortality rate (%) = (total of dead fish/number of injected fish) x 100

Cumulative survival rate (%) = (total of live fish/number of injected fish) x 100

Relative percentage survival (%) = [1- (% mortality of experimental group / % mortality of control group)] x 100

2.6 Data analysis

The data were analyzed using Analysis of Variance (ANOVA). Duncan's multiple range test compared the mean difference in each experiment with a statistical program at a 95 percent confidence level. Statistical differences in mineral contents in rind were analyzed using a t-test at a significance level of 0.05.

3. Results and Discussion

3.1 Composition of durian rind and experimental diets

Our results show that the proximate composition of Monthong durian processed white inner rind (Table 2) appear to be similar to the white inner rind of Chanee durian (*Durio zibethinus* L. cultivar Chanee), showing high levels of carbohydrate except for the fiber. In Monthong, dietary fiber (22.10 ± 0.58%) is lower than in Chanee durian rind, which was reported to be as high as 50.18 ± 0.43% [23]. Wanlapa et al. [24] reported that the dietary fiber of whole Monthong durian shells, the white inner plus green thorny outer, was 79.18 ± 3.46% higher than only the white inner rind. This information suggests that using durian rind, either the whole shell or the inner part only, as a dietary ingredient in feeds for other fish species is possible. Feed efficiency for diets high in fiber in herbivorous fish is better than in omnivorous fish and predatory fish because of the differences in the structure of the digestive tracts, especially the length of the intestines [25-27].

The mineral contents of the durian's white inner and green outer rind were also analyzed, as shown in Table 3. Comparing the minerals in the two types of rind, potassium, magnesium, phosphorus, iron, manganese, zinc, copper, and boron significantly differed between the white inner and green outer rind of durian. Focusing on a mineral related to immune response, it was found that the white inner rind contained radically higher magnesium, manganese, and copper than the green outer rind. Magnesium is a mineral that is very important in regulating vital biological functions such as cofactor for more than 300 enzymes, regulating mechanisms of the heart and neurons, muscle contraction [28], and the immune system [29-30]. Manganese had been reported to be involved in the defense against infectious agents and mentioned as well in nutritional immunity [31]. Wu et al. [32] described that manganese could increase the immune response in many species of vertebrates, for instance, humans, mice, birds, and fish. Copper is also reportedly associated with immune functions [33]. Several research reviewed the effects of copper deficiency, which include higher susceptibility to bacterial infections [34], reduced antibody production by spleen cells [35], impaired neutrophil function [36], and decreased bactericidal activity [37].

As mentioned earlier, we chose only the white inner rind of the Monthong durian to produce the experimental diets used in this study. The proximate composition of the experimental diets shows that all treatments are isonitrogenous and isocaloric. It should be noted, however, that crude fiber increases along with the amount of durian rind included in the feed ration (Table 2).

Table 2. Proximate composition of the white inner rind of Monthong durian and experimental diets

Proximate composition	White inner rind	Durian rind (%)			
		0	10	15	20
Crude protein (%)	5.25 ± 0.20	23.15 ± 0.04	24.10 ± 0.11	23.80 ± 0.17	23.38 ± 0.07
Crude lipid (%)	0.66 ± 0.03	6.45 ± 0.07	6.79 ± 0.04	7.53 ± 0.07	7.19 ± 0.01
Crude fiber (%)	22.10 ± 0.58	5.12 ± 0.08	5.73 ± 0.06	5.80 ± 0.02	6.17 ± 0.06
Moisture (%)	6.69 ± 0.03	4.55 ± 0.10	4.68 ± 0.04	4.77 ± 0.04	4.59 ± 0.21
Ash (%)	6.88 ± 0.05	9.54 ± 0.07	10.09 ± 0.23	10.52 ± 0.21	11.54 ± 0.14
Gross energy (Kcal/100g)	3473.53 ± 7.36	3994.30 ± 2.49	4030.70 ± 6.65	3985.10 ± 1.94	3977.80 ± 7.91

Table 3. Mineral contents of the white inner rind and green outer rind of Monthong durian rind.

Parameters	White inner rind	Green outer rind	p-values	Parameters	White inner rind	Green outer rind	p-values
Macro mineral (%)				**Micromineral (ppm)**			
K	2.33 ± 0.05	1.98 ± 0.05	0.0011*	Fe	13.77 ± 1.08	18.59 ± 1.98	0.0208*
Ca	0.16 ± 0.00	0.15 ± 0.01	0.1583	Mn	22.14 ± 0.20	2.42 ± 0.20	0.0000*
Mg	0.41 ± 0.00	0.22 ± 0.00	0.0000*	Zn	6.93 ± 0.19	5.34 ± 0.14	0.0003*
Na	0.02 ± 0.00	0.02 ± 0.00	0.3739	Cu	7.57 ± 0.22	4.40 ± 0.11	0.0000*
P	0.18 ± 0.00	0.21 ± 0.00	0.0022*	B	18.64 ± 0.06	22.73 ± 0.05	0.0000*
S	0.08 ± 0.00	0.08 ± 0.00	0.3739	Al	5.81 ± 1.79	7.99 ± 0.87	0.1309

Potassium (K); Calcium (Ca); Magnesium (Mg); Sodium (Na); Phosphorus (P); Sulphur (S); Iron (Fe); Manganese (Mn); Zinc (Zn); Copper (Cu); Boron(B); Aluminium (Al); *significant ($p < 0.05$) difference between the white inner rind and green outer rind in a row

3.2 Growth performance

Growth-related parameters of red tilapia reared with a diet supplemented with durian rind at 0, 10, 15, and 20% for 140 days are shown in Table 4. The results revealed that growth-related parameters in terms of final weight, weight gain, average daily gain, final length, length gain, and specific growth rate of fish fed with durian rind supplemented diet at 10, 15 and 20% were not significantly different ($P > 0.05$) from that of the control. Moreover, we analyzed the whole-body composition of Red tilapia fed with the experimental diet for 140 days, shown in Table 5. The crude protein, moisture, and ash of fish were not significantly different ($P > 0.05$) among treatments. Crude lipids of fish fed with durian rind supplemented diets at 10, 15, and 20% seem lower than the control. This may be due to the increased levels of fibers in the feed, which may affect the ability of tilapia to utilize feed [38]. Furthermore, feeds with high fiber consumption enhance the movement passing rate in the digestive tract, decreasing digestion and absorption [38-39] and increasing fat in the fecal matter [40]. However, there was no significant difference in growth performance among the fish-fed diets supplemented with durian rind, and the survival rate of these treatments was still more than 90%. Additionally, the feed conversion ratio of fish-fed diets with different concentrations of durian rind was also not significantly different from the control ($P>0.05$) (Table 4). This is similar to the results conducted earlier in common carp (*Cyprinus carpio* Linn.), an omnivorous fish, [41] in which they were fed a diet substitution of broken rice with ground dried durian peel and durian seed at 0, 25, 50, 75 and 100% for 16 weeks. The growth of common carp fed with 25% ground-dried durian peel substitution was not different from the control. In another study by Lommetta et al. [42], which used the same treatment diets but conducted on another fish, silver barb (*Barbonymus gonionotus*), an herbivorous fish, found that the growth of silver barb fed with 50% ground dried durian peel substitution was not different from control. It can be concluded from these findings that omnivorous and herbivorous fish can be fed diets supplemented with durian rind at 20% and 50%, respectively, with no effect on growth performance.

Water quality throughout the culture period measured in the morning, including temperature, pH, dissolved oxygen, ammonia, and nitrites, were 26.55-29.83°C, 6.36-7.35, 3.55-5.70 mg/L, 0.01-0.56 mg/L and 0.04-0.25 mg/L, respectively. Boyd and Lichkoppler [43] recommended that the range of temperature and pH were suitable for aquaculture, while the range of dissolved oxygen was enough for fish to survive. Still, the effect on growth is slow if under prolonged culture. The values of ammonia and nitrite, especially in the last 6 weeks of rearing, tend to increase due to the fish growth. They produce more feces and waste. Ali et al. [44], who worked on the effects of stocking density and ammonia excretion on Nile tilapia (*O. niloticus*), suggested raising the stocking density from 2.81 g fish/L to 14.07 g fish/L increased the ammonia level from 1.48 to 26.44 mg/L and yielded lower growth rates. Considering the increasing mass of fish per 1 liter of water, it can be

concluded that as the fish grows, the ammonia content will also increase. From our result, the slow growth may be because of water quality. However, the Department of Fisheries [45] previously reported that Nile tilapia cultured in cages for 6 months should be 200 g of body weight. In comparison, our fish cultured in 500-L plastic tanks for 140 days (about 5 months) was 154.36 ± 9.51 g, which is relatively comparable with the above-growth rate.

Table 4. Growth performance of Red tilapia fed with diets supplemented with durian rind at different concentrations for 140 days.

Growth performance	Durian rind (%)				
	0	10	15	20	p-values
Initial weight (g/fish) ^{ns}	42.62 ± 0.73	42.82 ± 0.47	42.70 ± 0.35	42.58 ± 0.38	0.5340
Final weight (g/fish) ^{ns}	157.81 ± 12.96	163.55 ± 17.67	141.15 ± 16.84	154.92 ± 10.23	0.0690
Weight gain (g/fish) ^{ns}	111.19 ± 12.43	120.73 ± 17.42	98.45 ± 16.99	112.34 ± 15.54	0.0670
ADG (g/fish/day) ^{ns}	0.82 ± 0.09	0.86 ± 0.12	0.70 ± 0.12	0.80 ± 0.07	0.0670
Initial length (cm) ^{ns}	13.65 ± 0.11	13.98 ± 0.25	13.65 ± 0.42	13.88 ± 0.28	0.1500
Final length (cm) ^{ns}	21.80 ± 0.55	22.09 ± 0.63	21.44 ± 0.65	21.64 ± 0.44	0.1660
Length gain (cm) ^{ns}	8.14 ± 0.63	8.10 ± 0.68	7.79 ± 0.31	7.76 ± 0.54	0.3850
SGR (%/day) ^{ns}	0.86 ± 0.18	1.00 ± 0.06	0.91 ± 0.18	0.92 ± 0.05	0.2180
FCR ^{ns}	1.16 ± 0.11	1.16 ± 0.08	1.19 ± 0.13	1.18 ± 0.12	0.7240
Survival rate (%) ^{ns}	94.00 ± 9.52	93.00 ± 6.00	95.00 ± 10.00	94.00 ± 8.00	0.7730

Non statistically significant difference at $P > 0.05$ (ns); Average daily gain (ADG); Specific growth rate (SGR); Feed conversion ratio (FCR)

Table 5. Composition of whole-body fish fed the experimental diet for 140 days**.**

Proximate composition	Durian rind (%)				
	0	10	15	20	p-values
Crude protein (%)^{ns}	58.61 ± 0.25	62.03 ± 2.32	60.25 ± 2.49	61.39 ± 2.92	0.0680
Crude lipid (%)	12.33 ± 0.09^{a}	8.08 ± 0.36^{b}	11.76 ± 2.26^{a}	9.32 ± 0.15^{b}	0.0006
Moisture (%)^{ns}	4.54 ± 3.02	5.67 ± 0.50	4.68 ± 0.41	5.00 ± 0.36	0.3600
Ash (%)^{ns}	22.60 ± 1.64	23.89 ± 2.51	22.71 ± 1.40	23.14 ± 0.35	0.3270

Non statistically significant difference at $P > 0.05$ (ns); Different letters in the row indicate a statistically significant difference at $P < 0.05$.

3.3 Antibacterial property

Girddit et al. [46] reported that polysaccharide gel isolated from durian peel contained antimicrobial compounds. Hence, at the end of the growth performance study, fish were intraperitoneally injected with *A. hydrophila*. The mortality pattern was recorded for 14 days (Fig. 1). The cumulative mortality increased after one-day post *A. hydrophila* injection until the 4th day. After that, the cumulative mortality was stable until the 14th day. The lowest mortality rate and the highest relative percentage survival were observed in fish fed with supplemented durian rind diets at 10% and 15%, followed by 20%. In addition, the survival rate of a fish-fed diet supplemented with durian rind at 10% and 15% was statistically significantly different from the control group ($P<0.05$) (Table 6). The results agreed with the study of Mohammadi et al. [47], which reported resistance against *A. hydrophila* of Nile tilapia-fed diets added with polysaccharide derived from *Pistacia vera* hulls at 2.5, 5, and 10 g/kg were higher than fish fed a diet without polysaccharide. Priya et al. [16] also evaluated the

efficacy of a polysaccharide extracted from durian rind on disease resistance against *Vibrio anguillarum* in Zebrafish (*Danio rerio*). The fish fed with the polysaccharide encapsulated with *Bacillus subtilis* had a higher survival rate than the non-polysaccharide encapsulated group after 5 days of the *Vibrio* immersion challenge. Their result also revealed a significant upregulation of lysozyme in fish fed with polysaccharide-encapsulated probiotics. Lysozyme is an enzyme produced from leucocytes involved in the phagocytosis of Gram-negative bacteria [48]. Previous studies have also reported that polysaccharides extracted from durian peel can inhibit the growth of Gram-positive bacteria such as *Staphylococcus aureus* [8, 14]. This study and these previous reports collectively indicate that polysaccharides from durian rind have broad-spectrum antibacterial properties.

Moreover, polysaccharide also has immunomodulating properties. For instance, polysaccharide gel (PG) extracted from *D. zibethinus* could improve prophenoloxidase activity in black tiger shrimp (*Penaeus monodon*) [49], increasing immune-related genes such as interleukin 1 beta (Il1b), lysozyme (lyz), tumor necrosis factor-alpha (TNF-α), superoxide dismutase (SOD) genes in Zebrafish (*D. rerio*) [16]. In addition, the minerals, including magnesium, manganese, and copper, were elevated in fish-fed diets supplemented with durian rind and may improve immune response, resulting in higher survival rates than the control. On the other hand, too many of these minerals may cause detrimental effects. For instance, excess manganese can cause neurotoxicity [32], and excess manganese and copper can be toxic to vertebrates by inducing cell death [50-51]. This could be why fish fed a diet supplemented with durian rind at 20% have a survival rate lower than those fed with 10 and 15%.

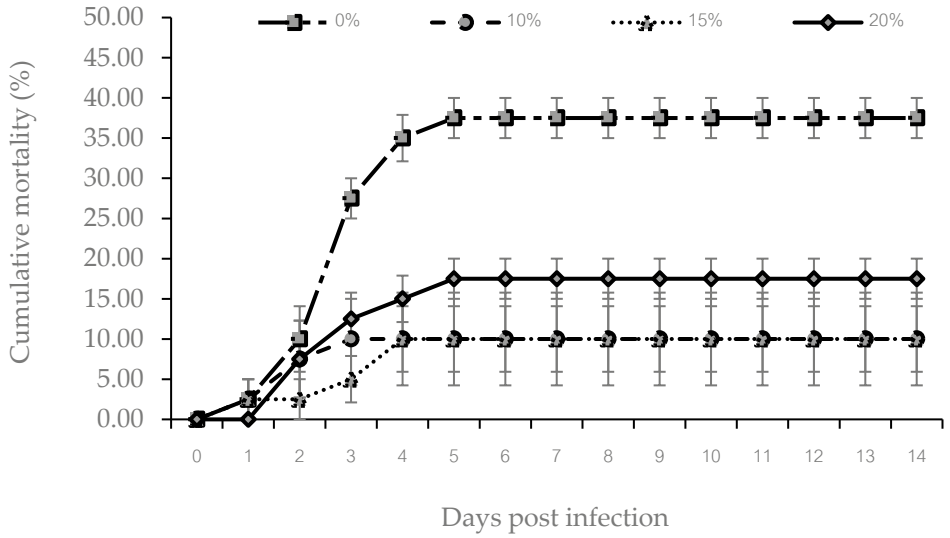

Figure 1. Cumulative mortality rate (%) of Red tilapia after being challenged with *A. hydrophilla.* Values are shown as means ± S.E. (n=4)

Table 6. Relative percentage survival of Red tilapia fed different levels of durian rind supplementation for 140 days and infected with *A. hydrophila*

Durian rind (%)	Mortality rate (%)	Survival rate (%)	Relative percentage survival
0	37.50 ± 5.00	62.50 ± 5.00^{b}	-
10	10.00 ± 11.55	90.00 ± 11.55^{a}	73.33
15	10.00 ± 8.16	90.00 ± 8.16^{a}	73.33
20	17.50 ± 5.00	82.50 ± 5.00^{ab}	53.33

Different letter in the same column indicates a statistically significant difference at $P < 0.05$.

4. Conclusions

Our results revealed that fish-fed diets supplemented with white durian rind at 20% maximum concentration for 140 days still displayed growth performance (final weight, weight gain, average daily gain, final length, length gain, and specific growth rate), feed conversion ratio and survival rate that were not significantly different ($P > 0.05$) from that of the control. However, the challenge test with *A. hydrophila* showed that fish fed a diet supplemented with durian rind at 10% and 15% have the highest survival rate, significantly different from the control ($P<0.05$). Taken together, our results suggest that the white durian rind can be used as feedstuff and a natural antibacterial ingredient in diets of Red tilapia. The recommended level of supplementation is at 10%-15%, which can elicit improved disease resistance against *A. hydrophila* but has no effect on the growth performance of Red tilapia. Furthermore, we show here that durian rind with minimal processing methods, dried and ground, which is not as laborious and costly as extraction methods and, therefore, ideal for small-scale fish farming, can be used as a diet supplement.

5. Acknowledgements

The authors would like to thank Dr. Fernand F. Fagutao for his help in proofreading the manuscript.

Author Contributions: Conceptualization, D. P.; performed the experiments, N. S., D. P., S. L., and W. N.; formal analysis, N. S. and N. P.; data collection, N. S. and D. P.; writing—original draft preparation, N. S and D. P.; writing—review and editing, D. P.; supervision, D. P. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Conflicts of Interest: The authors declare no conflict of interest.

References

[1] Perschbacher, P.W. Tilapia: The "Aquatic Chicken" - At Last. *Journal of Fisheries and Livestock Production*. 2014, 2, 120. doi: 10.4172/2332-2608.1000120.

[2] Zhang, D.; Xu, D.H.; Shoemaker, C. Experimental induction of motile *Aeromonas* septicemia in channel catfish (*Ictalurus punctatus*) by waterborne challenge with virulent *Aeromonas hydrophila*. *Aquaculture Report*. 2016, 3, 18-23.

[3] Aboyadak, I.M.; Ali, N.G.M.; Goda, A.M.A.; Aboelgalagel, W.H.; Salam, A. Molecular detection of *Aeromonas hydrophila* as the main cause of outbreak in tilapia farms in Egypt. *Journal of Aquaculture and Marine Biology*. 2015, 2, 237-240.

[4] Pauzi, N.A.; Mohamad, N.; Azzam-Sayuti, M.; Yasin, I.S.M.; Saad, M.Z.; Nasruddin, N.S.; Azmai, M.N.A. Antibiotic susceptibility and pathogenicity of *Aeromonas hydrophila* isolated from red hybrid tilapia (*Oreochromis niloticus*×*Oreochromis mossambicus*) in Malaysia. *Veterinary World*. 2020, 13, 2166-2171.

[5] EI-Asely, A.; Amin, A.; El-Naby A.S. Abd.; Samir, F.; El-Ashram, A.; Dawood, M.A.O. *Ziziphus mauritiana* supplementation of Nile tilapia (*Oreochromis niloticus*) diet for improvement of immune response to *Aeromonas hydrophila* infection. *Fish Physiology and Biochemistry*. 2020, 46, 1561-1575.

[6] Swann, L.; White, M.R. Diagnosis and treatment of "*Aeromonas hydrophila*" infection of fish. *Aquaculture Extension Illinois- Indiana Sea Gran Program*. 1991, 461- 462.

[7] Kumar, M.; Kumar, V.; Roy, D.; Kushwaha, R.; Vaswani, S. Application of herbal feed additives in animal nutrition -a review. *International journal of livestock research*. 2014, 4, 1-8.

[8] Arlofa, N.; Ismiyati, I; Kosasih, M.; Fitriyah, N.H. Effectiveness of durian peel extract as a natural anti-bacterial agent. *Journal of Chemical Engineering and Environment*. 2019, 14, 163-170.

[9] Noodaeng, S. Export competitiveness of Thai durian in China market. *European Journal of Business and Management*. 2017, 9, 48-55.

[10] Foo, K.Y.; Hameed, B.H. Transformation of durian biomass into a highly valuable end commodity : Trends and opportunities. *Biomass and Bioenergy*. 2011, 35, 2470-2478.

[11] Jana, S. L.; Nurcahyani, O. H.; Dewi, W. The using of durian peels trashes as a potential source of fiber to prevent colorectal cancer. Surakarta: Sebelas Maret University. 2010.
[12] Pongsamart, S.; Panmuang, T. Isolation of polysaccharide from fruit-hulls of durian (*Durio zibethinus* L.). *Songklanakarin Journal of Science and Technology*. 1998, 20, 323-332.
[13] Hokputsa, S.; Gerddit, W.; Pongsamart, S.; Inngjerdingen, K.; Heinze, T.; Koschella, A.; Harding, S.E.; Paulsen, B.S. Water-soluble polysaccharides with pharmaceutical importance from Durian rinds (*Durio zibethinus* Murr.): isolation, fractionation, characterization and bioactivity. *Carbohydrate Polymers.* 2004, 56, 471-481.
[14] Lipipun, V.; Nantawanit, N.; Pongsamart, S. Antimicrobial activity (in vitro) of polysaccharide gel from durian fruithulls. *Songklanakarin Journal of Science and Technology*. 2002, 24, 31–38.
[15] Pholdaeng, K.; Pongsamart, S. Studies on the immunomodulatory effect of polysaccharide gel extracted from *Durio zibethinus* in *Penaeus monodon* shrimp against *Vibrio harveyi* and WSSV. *Fish and Shellfish Immunology*. 2010, 28, 555–561.
[16] Priya, P.S.; Ashwitha, A.; Thamizharasan, K.; Harishkumar, M.; Dinesh, S.; Nithya, T.G.; Kamaraj, M. Synergistic effect of durian fruit rind polysaccharide gel encapsulated prebiotic and probiotic dietary supplements on growth performance, immune-related gene expression, and disease resistance in Zebrafish (*Danio rerio*). *Heliyon*. 2021, 7, https://doi.org/10.1016/j.heliyon.2021.e06669.
[17] Kitprathaung, N.; Ngamrojanavanich, N.; Chansiripornchai, P.; Pongsamart, S.; Chansiripornchai, N. Effect of polysaccharide gel extracted from *Durio zibethinus* rind on immune responses, bacteria counts and cholesterol quantities in chickens. *Thai Journal of Veterinary Medicine*. 2013, 43, 251-258.
[18] AOAC. Official methods of analysis (supplement). Association of Official Analytical Chemists (AOAC), Washington DC. 1995.
[19] Allan, S.E. Chemical analysis of ecological materials. Blackwell Scientific Publications, London. 1989, 368.
[20] Julie, B.; Julio, C.G.; Ahmed, D. Effect of copper sulfate on *Aeromonas hydrophila* infection in channel catfish fingerlings. *North American Journal of Aquaculture*. 2012, 74, 494-498.
[21] Lee, S.W.; Sim, K.Y.; Wendy, W.; Zulhisyam, A.K. *Peperomia pellucida* leaf extract as immunostimulator in controlling motile aeromonad septicemia due to *Aeromonas hydrophila* in red hybrid tilapia, *Oreochromis* spp. farming. *Veterinary World*. 2016, 9, 231-234.
[22] Achmad, M.J.; Isnansetyo, A.; Andriani, R.; Samman, A.; Marus, I. The analysis of challenges test of catfish (*Clarias* sp.) with fatty acid compounds from starfish (*Acantaster planci*). *IOP Conference Series: Earth and Environmental Science*. 2020, 584. 012014. 10.1088/1755-1315/584/1/012014.
[23] Bunyasawat, J.; Bhoosem, C. Effect of using durian rind powder substitution with wheat flour on brownies cake quality. *RMUTP Research Journal*. 2018, 12, 113-124.
[24] Wanlapa, S.; Wachirasiri, K.; Sithisam-ang, D.; Suwannatup, T. Potential of selected tropical fruit peels as dietary fiber in functional foods. *International Journal of Food Properties*. 2015, 18, 1306-1316.
[25] Kapoor, B.; Smit, H.; Verighina, A. The alimentary canal and digestion in teleosts. *Advances in Marine Biology*. 1976, 13, 109–239.
[26] Horn, M.H. Biology of marine herbivorous fishes. *Oceanography and Marine Biology, An Annual Review*. 1989, 27, 167–272.
[27] Kramer, D.L.; Bryant, M.J. Intestine length in the fishes of a tropical stream: 2. Relationships to diet-the long and the short of a convoluted issue. *Environmental Biology of Fishes*. 1995, 42, 129–141.
[28] Mathew, A.A.; Panonnummal, R. 'Magnesium'-the master cation-as a drug-possibilities and evidences. *Biometals*. 2021, 34, 955-986.
[29] Galland, L. Magnesium and immune function: an overview. *Magnesium*. 1988, 7, 290-299.
[30] Weyh, C.; Krüger, K.; Peeling, P.; Castell, L. The Role of Minerals in the Optimal Functioning of the Immune System. *Nutrients*. 2022, 14, 644.
[31] Haase, H. Innate immune cells speak manganese. *Immunity. 2018, 48,* 616–618.
[32] Wu, Q.; Mu, Q.; Xia, Z.; Min, J.; Wang, F. Manganese homeostasis at the host-pathogen interface and in the host immune system. *Seminars in Cell and Developmental Biology*. 2021, 115, 45-53.
[33] Percival, S.S. Copper and immunity. *American Journal of Clinical Nutrition*. 1998, 67, 1064-1068.

[34] Jones, D.G.; Suttle, N.F. The effect of copper deficiency on the resistance of mice to infection with *Pasteurella haemolytica*. *Journal of Comparative Pathology*. 1983, 93, 143-149.
[35] Lukasewycz, O.A.; Prohaska, J.R. The immune response in copper deficiency. *Annals of the New York Academy of Sciences*. 1990, 587, 147–59.
[36] Jones, D.G.; Suttle, N.F. Some effects of copper deficiency on leucocyte function in sheep and cattle. *Research in Veterinary Science*. 1981, 31, 151-156.
[37] Boyne, R.; Arthur, J.R. Effects of selenium and copper deficiency on neutrophil function in cattle. *Journal of Comparative Pathology*. 1981, 91, 271-276.
[38] Dioundick, O.B.; Stom, D.I. Effects of dietary α-cellulose levels on the juvenile tilapia, *Oreochromis mossambicus* (Peters). *Aquaculture*. 1990, 91, 311-315.
[39] Maina, J.G.; Beames, R.M.; Higgs, D.; Mbugua P.n.; Iwama, G.; Kisia, S.M. Digestibility and feeding value of some feed ingredients fed to tilapia *Oreochromis niloticus* (L.). *Aquaculture Research*. 2002, 33, 853-862.
[40] Kritchevsky, D.; Tepper, S.A. Influence of a fiber mixture on serum and liver lipids and on fecal fat excretion in rats. *Nutrition Research*. 2005, 25, 485-489.
[41] Lommetta, K.; Phaewcham, S.; Koolkalya, S.; Matchakuea, U. The use of ground dried durian peel and durian seeds instead of broken rice in fish feed formula for common carp (*Cyprinus carpio* Linn.). *Rajabhat Rambhai Barni Research Journal*. 2016, 10, 109-117.
[42] Lommetta, K.; Phaewcham, S.; Koolkalya, S.; Matchakuea, U. Use of ground dried durian peel and durian seeds replacement of broken rice in diet for Silver barb. *Integrating Multidisciplinary Research and Sustainable Development towards ASEAN Community*. 2013, 6, 474-486.
[43] Boyd, C.E.; Lichkoppler, F. Water quality management in pond fish culture. Auburn University, Alabama, International for aquaculture. Agricultural Experiment Station. Research and Development Series, 1979, 22, 30.
[44] Ali, M.S.; Stead, S.M.; Houlihan, D.F. Effects of socking density on ammonia excretion and the growth of Nile tilapia (*Oreochromis niloticus* L.). *Bangladesh Journal of Fisheries Research*. 2006, 10, 13–24.
[45] Department of fisheries. Aquaculture of Tilapia. Inland Fisheries Research and Development Division, Ministry of Agriculture and Cooperatives. Bangkok, Thailand. 2010, 32.
[46] Girddit, W.; Tipayakul, C.; Lertchaiporn, J.; Sutanthavibul, N.; Pongsamart, S. Characterization and properties of polysaccharide gel from durian fruit-hulls. *Thai Journal of Pharmaceutical Sciences*. 2001, 25 Suppl:6.
[47] Mohammadi, G.; Rafiee, G.; El Basuini, M.F.; Abdel-Latif, H.M.R.; Dawood, M.A.O. The growth performance, antioxidant capacity, immunological responses, and the resistance against *Aeromonas hydrophila* in Nile tilapia (*Oreochromis niloticus*) fed *Pistacia vera* hulls derived polysaccharide. *Fish and Shellfish Immunology*. 2020, 106, 36-43.
[48] Saurabh, S.; Sahoo, P.K. Lysozyme: an important defense molecule of fish innate immune system. *Aquaculture Research*. 2008, 39, 223-239.
[49] Pholdaeng, K.; Pongsamart, S. Studies on the immunomodulatory effect of polysaccharide gel extracted from *Durio zibethinus* in *Penaeus monodon* shrimp against *Vibrio harveyi* and WSSV. *Fish and Shellfish Immunology*. 2010, 28, 555–561.
[50] Liu, X.F.; Li, Z.P.; Tie, F.; Liu, N.; Zhang, Z.W.; Xu, S.W. Effects of manganese-toxicity on immune-related organs of cocks. *Chemosphere*. 2013, 90, 2085-2100.
[51] Liang, Q.; Zhou, B. Copper and manganese induce yeast apoptosis via different pathways. *Molecular Biology of the Cell*. 2007, 18, 4741-4749.

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Research article

Applying the Flyweight Design Pattern to Android Application Development

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Citation:
Ngaogate, W. Applying the flyweight design pattern to Android application development. *ASEAN J. Sci. Tech. Report.* **2023**, *26*(2), 49-57. https://doi.org/10.55164/ajstr.v26i2.247607.

Article history:
Received: October 24, 2022
Revised: March 17, 2023
Accepted: March 22, 2023
Available online: March 26, 2023

Abstract: This research aims to demonstrate how the Flyweight design pattern enhances programmers' ability to develop mobile applications while being aware of memory consumption flexibly. By following the systematic approach to software development, the two Android applications have been designed using a class diagram based on two paradigms: general object-oriented programming and the Flyweight design pattern. Both applications are developed in Java and installed on a physical Android phone. Memory usage is monitored by using the Android Profiler and using programming. The results show that both Android applications consume almost the same amount of memory, given all classes or package classes. Therefore, applying the Flyweight design pattern to mobile software development does not affect memory usage and follows a professional software design.

Keywords: Design patterns; flyweight design pattern; memory usage; Android application

1. Introduction

Some programmers develop software based on their familiar coding styles without concern for resource consumption, especially memory usage in mobile applications. In some situations, various information is shown in one view of the software, for example, a view of several items on one web page or mobile view. Each type of item contains information retrieved from different sources. Some programmers then create a new object for each item whenever needed. However, the information may be retrieved from the same source. It causes the applications to contain too many unnecessary objects. Moreover, a new source of information may be available after the software has been deployed. The programmers are asked to update the software to provide new items from the new source in the software. Based on the basics of object-oriented programming, they might recode the software by creating a new object for each new source without awareness of the memory usage. Also, the programmers might be confused with many objects and their messy codes.

It is important to disclose how coding styles or paradigms affect software performance so that programmers can easily decide which paradigms suit their work. The design patterns, frequently recognized as the best practices in programming, can be applied in various application development scenarios. Rimawi and Zein [1] encourage developers to use design patterns properly. They developed the PatRoid framework to detect design patterns in 1,400 Android applications. They found that only 4 design patterns were used, with a percentage ranging from 20% to 55%, and 38% of applications did not apply any

design pattern. Design patterns enhance programmers' ability to develop more flexible and scalable software as reusable solutions for general problems, shorten software development time, and improve software quality. [2] New software engineers can also apply design patterns, as Lartigue and Chapman [3] confirmed.

This research aims to demonstrate how the design pattern paradigm can support programmers to flexibly develop mobile applications under the realization of memory consumption based on a systematic software design and development. Software performance is measured by comparing two coding paradigms; general object-oriented programming and the Flyweight design pattern. Both applications are installed and tested on a physical Android mobile. The memory usage of both applications developed by both paradigms is monitored using the Android Profiler and programming.

2. Materials and Methods

2.1 Software Performance: Memory Usage

Coding styles affect the software's performance. Therefore, software testing must be done carefully. The Android Studio Profiler is an efficient tool for monitoring a mobile application's performance. Hidayat and Sungkowo [4] used it for analyzing the CPU and the RAM usage when animations in JSON, PNG, and GIF types are run. Fatima et al. [5] compared the CPU and memory usage of a simple quiz Android application when it is developed using ListView compared to RecyclerView. They found that RecyclerView produced better application performance than ListView. Muhammad Ehsan Rana and Wan Nurhayati Wan Ab. Rahman [6] concluded that the Observer and the State design patterns are more effective for real-time application.

To measure the software performance of Android applications, Ghari et al. [7] are interested in problems around reliability, maintainability, and security. They studied the source codes of 10 applications and monitored their performances using MonkeyRunner and adb. Their findings confirm that quality is a multi-dimensional software complex and needs more tools to support software quality assurance. Abebaw Degu [8] reviewed 31 empirical papers on Android application memory and energy performance, resource leaks, and performance testing techniques and challenges. They identified several research gaps, such as memory and energy memory utilization optimization, including resource leaks, programming techniques, performance enhancement, and source code analysis tools. Cross-platform tools are also an option in mobile development. Dorfer et al. [9] are concerned about mobile applications built with cross-platform development approaches as they might consume system resources. So, they compared an application built using React Native to one built using native Android code and found that the React Native application consumed between 6% and 8% more energy than the Android native code.

This paper focuses on the memory usage of two Android applications: the first is developed using general object-oriented programming, and the second is developed by applying the Flyweight design pattern.

2.2 The Flyweight Design Patterns

Object-oriented design and programming are important paradigms in software development. It has been developed as a best practice called design patterns. Some open-source software with good architecture is built based on best practices, so the code is cleaner and has fewer infractions. The programs written using most of the design patterns were simpler compared to the ones written without them. Although the values of CK metrics, the number of classes, and the SLOC (Source Lines of Code) increased. [10-11] The GoF design patterns introduced by Erich et al. [12] are the most famous and applied in various business domains. For example, Bruno L. Sousa et al. [13] investigated large classes in five Java projects developed with design patterns. They concluded that the Composite and Factory Method patterns have a low co-occurrence with long methods. In contrast, the Template and Observer methods have a high co-occurrence with large classes and long methods.

The GoF Flyweight pattern intends to efficiently support large amounts of small-grained objects. A flyweight behaves as an independent object in each context and can be used in various contexts simultaneously. Therefore, if particular objects are stored on a server that many clients frequently request simultaneously, Flyweight can support those requests. The Flyweight pattern was compared with the Proxy [14] on software efficiency. They were applied to an online shooter game. The result showed that both design patterns spent less execution time and consumed less memory than expected. But the Proxy pattern consumes more memory

than the Flyweight pattern. Peng Zhang et al. demonstrated how to apply Abstract Factory, Flyweight, Proxy, and Publisher-Subscriber patterns in a healthcare system called Smart Health (DASH), a distributed application that uses blockchain technology in the healthcare sector. They concluded that data sharing with the flyweight registry decreased the cost of changes to the common intrinsic state in blockchain-based apps. [15-16]

Dimitrichka Nikolaeva et al. [17] developed a Unity video game. They explained that the Singleton and Flyweight design patterns are applied to the MonoBehavior, such as the Update, LateUpdate, and other methods. The Flyweight is an object that minimizes memory usage by sharing as much data as possible with similar objects. Kirill Pupynin and Oleg Golovnin [18] developed the toolkit for modeling traffic flows based on a microscopic simulation model and the multimodal modeling system SUMO. They applied the Flyweight design pattern and found that the loading on memory was significantly reduced. Sepideh Maleki et al. [19] studied the impact of five design patterns on four object-oriented programmings (OOP) features inheritance, polymorphism, dynamic binding, and overloading. They found that the flyweight design pattern remarkably improved performance and energy efficiency.

However, Boyan Bontchev and Emanuela Milanova [20] received 82 usable responses from intermediate or higher-experienced software professionals in Bulgaria, with 65 experiencing one of the Gang of Four patterns. Most of them demonstrate good knowledge and a reasonable and responsible attitude regarding the advantages and drawbacks of design patterns. Their responses reveal that Singleton, Factory Method, and Iterator are the most recognized and valuable, while Memento and Flyweight are the most unrecognized and useless.

According to the benefits of the Flyweight design pattern discussed above, this research introduces how this pattern can be applied to developing an Android application embedded with information items retrieved from various sources. To reduce the number of unnecessary item objects created by programmers, which causes too much memory consumption, the design and development of an Android application using the Flyweight design pattern are demonstrated.

2.3 Method

The following subsections are the case study, two class diagrams for object-oriented programming and the Flyweight design pattern, the Java codes, and the software performance testing.

2.3.1. The case study

Systematic software development is important. Dong Kwan Kim [21] proposed guidelines for the software development activities and procedures for building mobile applications on the cloud service by applying UML diagrams and artifacts such as the UML profile extensions, class diagrams, and deployment diagrams. The experimental results suggest that the proposed guidelines can improve the productivity, scalability, and maintainability of software design models. For the case studies, they used the Android mobile platform, Amazon Web Service for cloud computing, and MySQL for data management.

To demonstrate the situation where many objects can be created in one particular view of the software, this research proposes a case study of a food list shown in one view of an Android application. The food has several types: meal, cocktail, dessert, or starter. Many types of food are randomly shown in one view of the application. Each type of food is retrieved from different sources, such as themealdb.com and thecocktaildb.com. Each source is managed using a different application object, created every time it retrieves the food information. Because the application needs to use different controller objects for different food types. Moreover, new food types might be added to the application later. For example, instead of the four food types described above, more types, such as seafood, noodles, or pizza, could be added to the application after deployment. Therefore, programmers must know the new controller objects for such new food types. Programmers create them whenever they retrieve the data, either by the new or existing controller objects. Consequently, the code is getting messy, and the controller objects are getting too many.

Instead of creating a new controller object every time the application retrieves the food information, the Flyweight design pattern, which was claimed to support the flexible use of the objects, has been applied. This research follows a systematic approach to software development by designing two class diagrams; one for the application developed by general object-oriented programming and another for the application developed by the Flyweight design pattern. The application is developed in Java using Android Studio. The

food data are stored on a public server and provided freely via the REST API (REpresentational State Transfer Application Programming Interface). The speed of information retrieval depends on the data communication and does not impact memory usage.

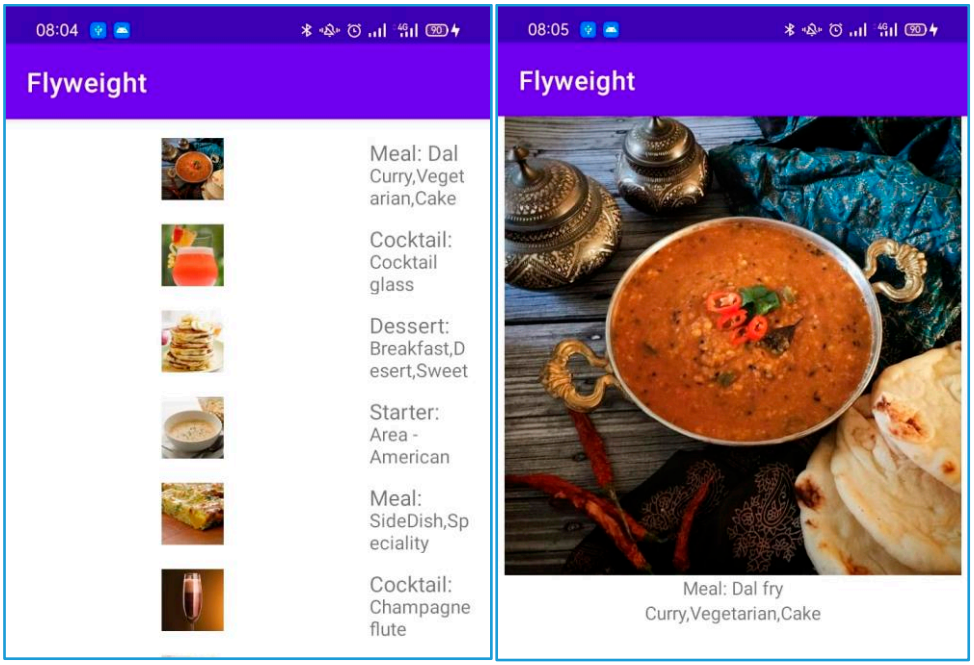

Figure 1. Main page and ShowDetail page

The list of various types of food is the main view of both applications. Types are selected in the same amount to be shown in the main view. The data of each type is randomly retrieved from a different REST API. Figure 1 is the main view, which shows a list of various kinds of food. Once the user clicks on a particular image, the details of that image are shown in the view on the right.

2.3.2. The class diagram for General Object-Oriented Programming

The common attributes of the food are id, name, description, and image. Therefore, Data is defined as an abstract class with two children at the beginning: Meal and Cocktail. Each data object's child has its controller class for data retrieval. Those controller classes implement the interface class RESTData, as shown in the class diagram below.

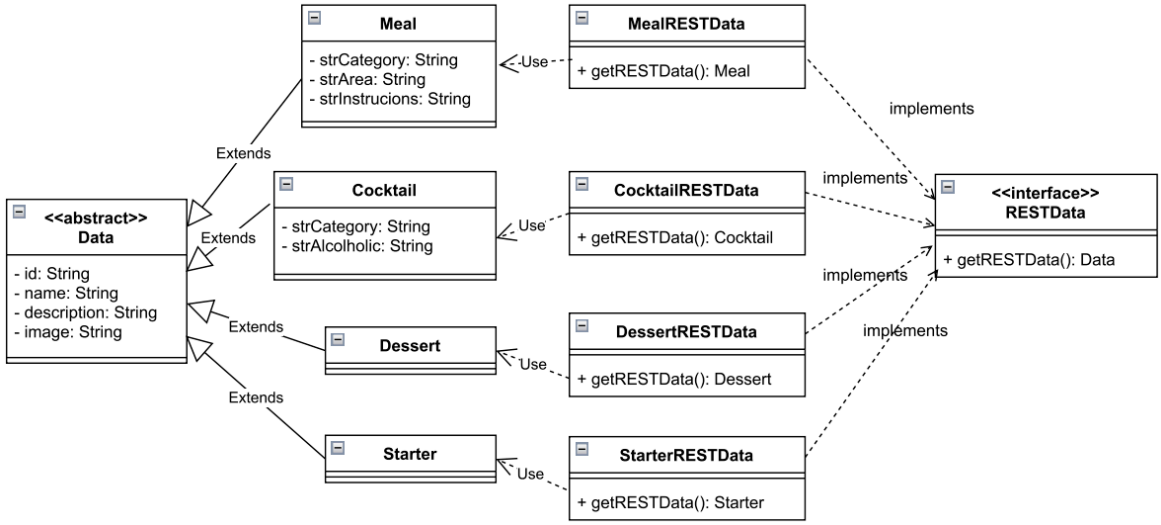

Figure 2. The class diagram for general object-oriented programming

2.3.3 The class diagram for the Flyweight design pattern

The class diagram for the Flyweight design pattern is almost the same as the one in the above subsection, except for the class FoodRESTFactory. According to the Flyweight design pattern, the controller classes are kept in one collection, such as an ArrayList or HashMap. Once the main program needs to retrieve a particular piece of data provided by the controller, it is checked to see if it has already been created and stored in the collection. If it is stored, it will be retrieved and used. Otherwise, it is created and stored in the collection.

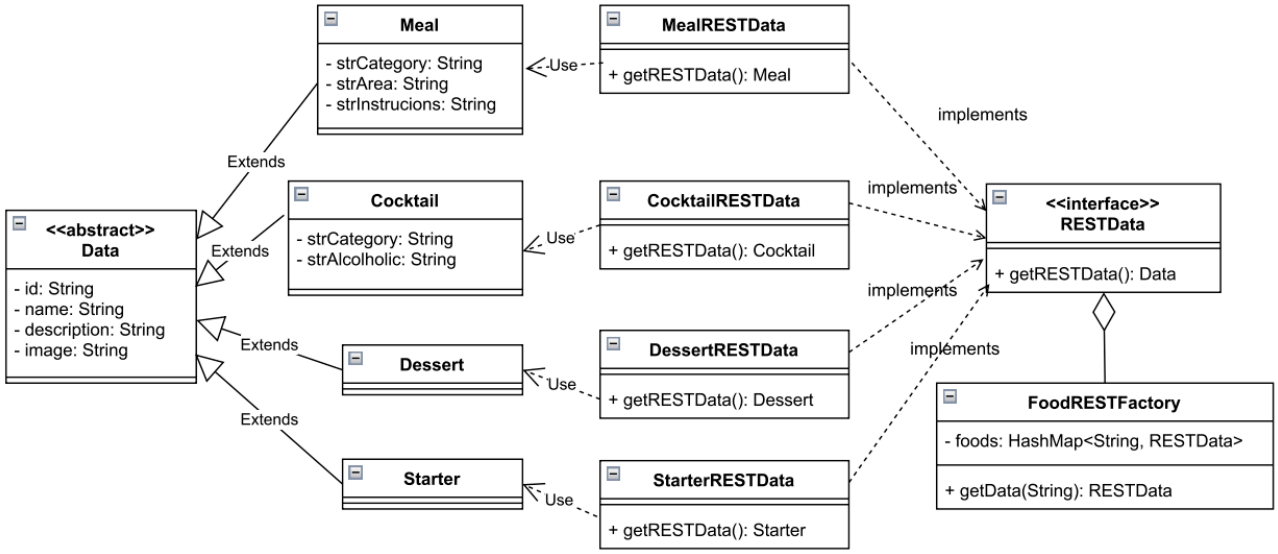

Figure 3. The class diagram for the Flyweight design pattern

2.4 Java Codes

The following codes show how both applications launch four services, 10 times and 50 times, respectively.

2.4.1. General Object-Oriented Programming

The application developed using general object-oriented programming creates each object of MealRESTData, CocktailRESTData, DessertRESTData, and StarterRESTData one by one every time it needs more REST data.

Table 1. General Object-Oriented Programming: MainActivity.java

General Object-Oriented Programming: MainActivity.java

List<Data> dataList = new ArrayList<Data>();
for(int i=0; i < 50; i++){
  if(i%4 == 0){
  dataList.add(new MealRESTData().getRESTData());
  }else if(i%4 == 1){
  dataList.add(new CocktailRESTData().getRESTData());
  }else if(i%4 == 2){
  dataList.add(new DessertRESTData().getRESTData());
  }else{
  dataList.add(new StarterRESTData().getRESTData());
  }
}

[1] This table demonstrates only two instances of a Data object creation to minimize the page space.

2.4.2. Flyweight Design Pattern Programming

The application creates only one instance of FoodRESTData, which creates an instance of MealRESTData, CocktailRESTData, DessertRESTData, or StarterRESTData when it is necessary. Once one of them is created, it is kept in a HashMap and retrieved when it is needed again without creating another instance.

Table 2. Flyweight Design Pattern Programming: MainActivity.java

Flyweight Design Pattern Programming: MainActivity.java

```
List<Data> dataList = new ArrayList<Data>();
for(int i=0; i < 50; i++){
  if(i%4 == 0){
    dataList.add(FoodRESTFactory.getData("meal").getRESTData());
  }else if(i%4 == 1){
    dataList.add(FoodRESTFactory.getData("cocktail").getRESTData());
  }else if(i%4 == 2){
    dataList.add(FoodRESTFactory.getData("dessert").getRESTData());
  }else{
    dataList.add(FoodRESTFactory.getData("starter").getRESTData());
  }
}
DataAdapter dataAdapter = new DataAdapter(dataList, this);
recyclerView.setAdapter(dataAdapter);
```

[1] This table demonstrates only two instances of a Data object creation to minimize the page space.

Table 3. Flyweight Design Pattern Programming: FoodRESTFactory.java

Flyweight Design Pattern Programming: FoodRESTFactory.java

```
public class FoodRESTFactory {
  // HashMap of RESTData
  private static final HashMap<String, RESTData>
        foods = new HashMap<String, RESTData>();

  // method: getData()
  public static RESTData getData(String foodType){
    RESTData restData = foods.get(foodType);
    if (restData == null){
      restData = new MealRESTData();
    }else if (foodType.equals("cocktail")){
      restData = new CocktailRESTData();
    }else if (foodType.equals("dessert")){
      restData = new DessertRESTData();
    }else if (foodType.equals("starter")){
      restData = new StarterRESTData();
    }
    foods.put(foodType, restData);
   }
   return restData;
  }
}
```

As the code in Table 3, programmers do not need to know the controller class names, such as MealRESTData or CocktailRESTData, as in Table 2. They pass a string of their names to the FoodRESTFactory, which is simpler, for example, "meal" or "cocktail." Once the new controller classes are introduced, for example, "seafood," "noodle," or "pizza,." Programmers pass those strings to the FoodRESTFactory. The code is clean and easy to manage.

2.5 Software Performance Testing: Memory Usage

This research deployed both applications on the same physical Android device: Oppo A5 2020 RAM 3GB Qualcomm Snapdragon 665, Device storage: available 29.1GB, total 64.0 GB, Android version 10, and ColorOS version v7.1. Memory usage is detected by using the Android Profiler embedded in the Android Studio and by programming.

3. Results and Discussion

The two Android applications are deployed on a physical mobile device, Oppo A5 2020 RAM 3GB Qualcomm Snapdragon 665. The memory usage is then monitored by the Android Profiler embedded in Android Studio. Table 4 shows the amount of memory used by each application; the application developed based on a general object-oriented paradigm and the application developed based on the Flyweight design pattern. Each application is tested twice: once with 10 items of food and again with 50 items.

There are 3 views shown in the Android Profiler; View all classes, Show project classes, and the class MainActivity.

Table 4. Memory usage monitored by the Android Profiler (in bytes)

	General Object-Oriented		**Flyweight design pattern**	
View app heap: Arrange by class: **View all classes**				
	Show 10 items	Show 50 items	Show 10 items	Show 50 items
Classes	1,289	1,435	1,263	1,400
Count	24,443	29,975	20,564	29,723
Native size	382,989	3,001,440	312,273 bytes	2,899,867
Shallow size	2,666,908	2,309,909	2,166,562 bytes	2,121,671
Retained size	8,585,130	25,547,875	6,667,429	24,058,038
View app heap: Arrange by package: **Show project classes**				
	Show 10 items	Show 50 items	Show 10 items	Show 50 items
Classes	8	9	12	13
Count	32	83	36	87
Native size	0	0	0	0
Shallow size	1,892	3,984	1,904	4,016
Retained size	6,167	87,448	6,296	121,348
View app heap: **Class MainActivity**				
	Show 10 items	Show 50 items	Show 10 items	Show 50 items
Allocations	1	1	1	1
Native Size	0	0	0	0
Shallow Size	324	324	324	324
Retained Size	4,040	4,076	4,220	4,040

The shallow size means the total amount of Java memory used by this object, and the retained size means the total amount of memory being retained due to all instances of this class and ready to be cleaned by the garbage collector. As with Table 4, the memory usage of both applications is nearly the same, whether showing only 10 items or 50 items. The Flyweight application consumes a little bit less memory than all other classes. But it consumes a little bit more memory from the perspective of project classes. The memory consumption in the MainActivity is also nearly similar.

Table 5. Memory usage monitored programmatically: ActivityManager.MemoryInfo (in bytes)

	General Object-Oriented		Flyweight design pattern	
	Show 10 items	Show 50 items	Show 10 items	Show 50 items
Available memory	1,060,016,128	976,662,528	1,098,248,192	1,024,471,040
Threshold	467,364,864	467,364,864	467,364,864	467,364,864
Total Memory	2,771,116,032	2,771,116,032	2,771,116,032	2,771,116,032

Table 5 shows memory usage in a physical mobile device: the Oppo A5 2020. It is detected programmatically using a class called ActivityManager, showing its memory information at the Android Studio's run shell. According to the available memory of MainActivity, the application developed using the Flyweight design pattern consumes almost the same amount of memory as the general object-oriented application. They both have the same threshold, a memory level at which the system begins to kill processes.

4. Conclusions

Programmers need a delicate software design before coding. They might be familiar with the general object-oriented paradigm, which is insufficient for building better software. This research demonstrates applying the Flyweight design patterns to Android application development. Following a systematic approach to software development, two class diagrams were designed; one for the application, which is developed based on the general object-oriented paradigm, and another for the application, which is developed based on the Flyweight design pattern. The example data is retrieved from a REST API. The applications are deployed on a physical mobile device. Memory usage of both Android applications was monitored by using the Android Profiler and programming. The results show that both applications consume almost the same amount of memory, either a view of all classes or project classes. The MainActivity class, which is the application's main view, also consumes the same amount of memory.

Applications of design patterns could be used in various problem domains, either GoF design patterns or other patterns experienced software engineers introduce. Especially software designers are responsible for making decisions about the software components. Design patterns should be applied along with software testing to prove that the patterns do not affect the software's performance.

5. Acknowledgements

Author Contributions: Literature review, software design, development and testing, discusssion and conclusion. Wasana Ngaogate.

Funding: -

Conflicts of Interest: The author declares no conflict of interest..

References

[1] Rimawi, D.; Zein, S. A Model Based Approach for Android Design Patterns Detection. Proceedings of the 3rd International Symposium on Multidisciplinary Studies and Innovative Technologies (ISMSIT), Ankara, Turkey, 11-13 October 2019; IEEE: Turkey.

[2] Karavokyris, A.; Alpis, E. Software Measures for Common Design Pattern Using Visual Studio Code Metrics. Proceedings of the International Conference on Information, Intelligence, Systems and Applications, Zakynthos, Greece, July 23-25, 2018, ACM.

[3] Lartigue, W. J.; Chapman, R. Comprehension and Application of Design Patterns by Novice Software Engineers. Proceeding of the Annual ACM Southeast Conference, Kentucky, United States, March 29 – 31, 2018, ACM.

[4] Hidayat, T.; Sungkowo, B. D. Comparison of Memory Consumptive Against the Use of Various Image Formats for App Onboarding Animation Assets on Android with Lottie. Proceedings of the 3rd International Conference on Computer and Informatics Engineering (IC2IE), Yogyakarta, Indonesia, 15-16 September, 2020; IEEE.

[5] Fatima, S.; Steffy, N.; Stella, D.; Nandhini D.; Devi, S. Enhanced Performance of Android Application Using RecyclerView. *Advanced Computing and Intelligent Engineering. Advances in Intelligent Systems and Computing*, Springer, Singapore, 2020, *1089*, 189–199.

[6] Ehsan, M. R.; Wan Nurhayati, W. W. The Effect of Applying Software Design Patterns on Real Time Software Efficiency. Proceedings of the Future Technologies Conference (FTC), Pan Pacific Hotel Vancouver, BC, Canada, 29-30 November 2017.

[7] Ghari, S.; Hadian, M.; Rasolroveicy, M.; Fokaefs, M. A multi-dimensional quality analysis of Android applications. Proceedings of the 29th Annual International Conference on Computer Science and Software Engineering, Markham, Ontario, Canada, November 4-6, 2019. ACM.

[8] Abebaw, D. Android Application Memory and Energy Performance: Systematic Literature Review. *Journal of Computer Engineering (IOSR-JCE)*, 2019, *21*(3), 20-32.

[9] Dorfer, T.; Demetz, L.; Huber, S. Impact of mobile cross-platform development on CPU, memory, and battery of mobile devices when using common mobile app features. Proceedings of the 17th International Conference on Mobile Systems and Pervasive Computing (MobiSPC), Leuven, Belgium, August 9-12, 2020.

[10] Feitosa, D.; Ampatzoglou, A.; Avgeriou, P.; Chatzigeorgiou, A.; Nakagawa, Y. E. What can violations of good practices tell about the relationship between GoF patterns and run-time quality attributes?. *Information and Software Technology*, 2019, 105, 1-16.

[11] Qamar, N.; Malik, A. A. Impact of Design Patterns on Software Complexity and Size. *Mehran University Research Journal of Engineering and Technology*, (S.l.), *39*(2), 342-352.

[12] Gamma, E.; Helm, R.; Johnson, R.; Vlissides, J. *Design Patterns: Elements of Reusable Object-Oriented Software*. Addison-Wesley, United States, 1994.

[13] Sousa, L. B.; Bigonha, A. S. M.; Ferreira, A. M. K. Evaluating Co-Occurrence of GOF Design Patterns with God Class and Long Method Bad Smells. Proceedings of the XIII Brazilian Symposium on Information Systems, Lavras, MG, Brazil, June 5-8, 2017.

[14] Rana, E. M.; Rahman, W. N.; Murad, A. A. M.; Atan, B. R. The Impact of Flyweight and Proxy Design Patterns on Software Efficiency: An Empirical Evaluation. *International Journal of Advanced Computer Science and Applications*, 2019, *10*(7), 161-170.

[15] Zhang, P.; White, J.; Schmidt, C. D.; Lenz, G. Design of Blockchain-Based Apps Using Familiar Software Patterns with a Healthcare Focus. Proceedings of the 24th Conference on Pattern Languages of Programs, Vancouver British Columbia, Canada, October 23 - 25, 2017; ACM.

[16] Zhang, P.; Schmidt, C. D.; White, J.; Lenz, G. Blockchain Technology Use Cases in Healthcare. *Advances in Computers*, Elsevier, 2018, 111, 1-41.

[17] Nikolaeva, D.; Safi, M.; Mihailov, M.; Georgiev, A.; Bozhikova, V.; Stoeva, M. Algorithm A* and Design Patterns used in Unity Video Game development. Proceedings of International Conference Automatics and Informatics (ICAI), Varna, Bulgaria, 2020, 1-3.

[18] Pupynin, K.; Golovnin, O. A Microscopic Traffic Simulation Web Toolkit. Proceedings of the 8th Scientific Conference on Information Technologies for Intelligent Decision Making Support, Ufa, Russia , 6-9 October 2020.

[19] Maleki, S.; Fu. C.; Banotra, A.; Zong, Z. Understanding the impact of object oriented programming and design patterns on energy efficiency. Proceedings of the 8th Int Green and Sustainable Computing Conference, Orlando, FL, USA, 23-25 October 2017, 1-6.

[20] Bontchev, B.; Milanova, E. On the Usability of Object-Oriented Design Patterns for a Better Software Quality. *Cybernetics and Information Technologies*, 2020, *20*(4), 36–54.

[21] Kim, K. D. Development of Mobile Cloud Applications using UML. *International Journal of Electrical and Computer Engineering* 2018, *8*(1), 596-604.

JSTR
ASEAN Journal of Scientific and Technological Reports

Research article

The Effect of Eliminating the Application of Phosphorus-Containing Fertilizer for the Bulking Period of Sweet Potato (*Ipomoea batatas)* Production

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Citation:
Mustacisa-Lacaba M.; Tan, N.; Villanueva, R. The Effect of eliminating the application of phosphorus-containing fertilizer for the bulking period of sweet potato (*Ipomoea batatas*) production. *ASEAN J. Sci. Tech. Report.* **2023**, *26*(2), 58-66. https://doi.org/10.55164/ajstr.v26i2.248752.

Article history:
Received: March 8, 2023
Revised: March 23, 2023
Accepted: March 24, 2023
Available online: March 26, 2023

Abstract: The Philippine Root Crop Research and Training Center (PhilRootcrops) divided fertilizer applications for sweet potato farming into various phases. Full fertilizer is applied two weeks after planting for the vegetative growth period. Apply another set of fertilizers containing a combination of nitrogen and potassium during the bulking period one and a half months later; this is one way to give the tuber a sweetened flavor. Because fresh sweet potato tubers are more marketable if their sizes are more or less consistent, the study hypothesized that ceasing to apply fertilizer containing phosphorus will adversely affect the uniformity of the size of sweet potato tubers. The application of mixtures of muriate of potash and urea-containing fertilizer increased sweet potato tubers with the highest average circumference and the average weight in Treatment 2 [Circumference (C)=12.4 cm; weight (W)=0.35 kg], which demonstrates the elimination of phosphorus-containing fertilizer for the bulking period of sweet potato production. According to the one-way analysis of variance (ANOVA) and F test ($F = 1.09$, $p = 0.41$), there was no statistically significant difference in the dependent variable between the various groups. The study finds that using muriate of potash and urea-containing fertilizer combinations generated the maximum yield in terms of circumference and weight per tuber for sweet potato farming with semi-loam varieties (Treatment 2). The usual application of the farmers under study to Treatment 2 is to reduce the use of synthetic fertilizers. Based on the study results, phosphorus-containing fertilizer does not affect sweet potato tuber yield.

Keywords: Circumference; urea; muriate of potash; sweet potato; eliminated phosphorus fertilizer

1. Introduction

Sweet potato (*Ipomoea batatas)* is one of the most important crops in the Philippines. Its marketability has high potential outside and within the country. It is called "food for calamity" since its survival rate is flexible in any type and weather [1]. It is used for relief operations as an alternative for rice, especially amidst catastrophes and pandemics. More than anything, it can utilize in various food products such as flour, fries, muffins, cookies, chips, and the like [2].

Sweet potatoes are important in many world regions, and their economic importance cannot be overstated. In many developing countries, sweet potatoes are the main food source because they are cheap, full of nutrients, and easy to grow and care for. They are also used as animal feed and raw materials for industrial processing. In addition to their role as a food source, sweet potatoes are also an important cash crop, generating significant income for farmers and other stakeholders in the value chain [3–4].

Given the high demand for sweet potatoes for food and the economy, finding and using the right cultural management practices to get the best crop yields and quality is important. One important part of cultural management is applying fertilizer, which can greatly affect crops' growth, development, and production. The current quality of soil nutrients differs from the previous time due to anthropogenic activities; thus, there is a need to shift to proper cultural management, which requires the necessary nutrients in the soil. By identifying the best fertilizer application strategies for sweet potatoes, farmers can increase yields and improve the quality of their crops, which can ultimately lead to more significant economic benefits. [5-6].

It is noted that most of the fertilizer applied for SP production is a complete fertilizer (14-14-14) with chemical compositions of Nitrogen (N), Phosphorus (P), and Potassium (K). Recently, the Philippine Root Crop Research and Training Center-PhilRootcrops [7] divided fertilizer applications into different periods for SP farming. A complete fertilizer is applied two weeks after planting for vegetative growth/period to help in the photosynthetic activity and initiate the development of storage roots where the tubers are formed. Then one and half months after planting, another set of fertilizers using a combination of nitrogen and potassium for the bulking period. This is also one way to add sweetened flavor to the tuber.

Based on the above-cited facts, especially on the PhilRootcrops system of SP farming, there is a phosphorus elimination in the bulking period. The study intends to find out the effect of the said nutrient elimination, a fact that some literature claims that the primary role of phosphorus in the plant is for growth and reproduction processes [8-9].

The phosphorus application for sweet potato cropping is important for the consistency of the length and shape of the tuber storage roots [10]. It was proven that phosphorus significantly contributes to better quality sweet potato tubers and appreciable vegetative growth [11]. However, recent literature indicates that phosphorus has negative effects on sweet potato crop production from the vegetative period until the bulking period because it prolongs the days required to attain the crop phenology, and the higher the amount applied, the longer it delays the reported days to flowering in sweet potato plants [12-13].

Sweet potatoes are an important crop in many parts of the world, and proper fertilization practices can significantly impact their yield. Several studies have investigated the effects of different fertilizer applications on sweet potato growth and yield. For example, research by Xu et al. (2019) found that high phosphorus fertilization improved the yield of sweet potatoes by increasing the number and weight of tubers produced [14]. On the other hand, other studies have shown that low phosphorus fertilization can also positively impact sweet potato yield. According to Zhang et al. (2020), applying low-phosphorus fertilizer combined with organic fertilizer can increase sweet potato yield by promoting tuber growth and improving soil fertility [15].

Several assumptions were made to help the researcher determine what would happen to sweet potato crop production if phosphorus was removed. First, it is assumed that there is a significant difference in sweet potato crop production between those that received phosphorus and those that did not. Second, it is thought that phosphorus is a very important part of the growth and quality of sweet potato tubers and that if it isn't there during the bulking period, the tubers might not all be the same size. Third, it is assumed that the way sweet potatoes are fertilized in the Philippines is insufficient and may need to be changed to increase crop yield and quality. These assumptions helped shape how the study was made and how the results were interpreted. They also helped find the best ways to fertilize sweet potato fields.

So, the end goal of this study is to determine the effect of eliminating phosphorus-containing fertilizer during the bulking period of sweet potato production. It specifically aimed to (1) apply 14% by volume of Phosphorus using a complete fertilizer in the vegetative period and 100% volume of combined urea and muriate of potash for a bulking period or 1 and half months after planting; (2) apply a combination of 14% by volume of phosphorus using a complete fertilizer in the vegetative period or 2 weeks after planting and 100% volume of phosphate for the bulking period or 1 and half months after planting; (3) compare in kilograms the sweet potato tubers with phosphorus application to the absence of all nutrient requirements (natural with no N, P, and K additives) and with the presence of all nutrient additives both in the vegetative and bulking periods and (4) determine if the elimination of phosphorus-containing fertilizer in the bulking period of sweet potato production will be effective**.**

2. Materials and Methods

2.1 Materials

2.1.1 Use of Clean Planting Materials

The source of the clean planting materials was acquired from Philippine root crops, Baybay Leyte, and adapted by the farmers of Brgy. Balante Basey, Samar, Philippines. The demonstration farms are adjacent to Samar State University's Basey Campus in Basey, Samar, and are a half-hectare agricultural land where the experimental activities occur. The needed clean planting materials are approximately 60 cuttings of NSIC SP 36; this variety was used because it is resilient to any weather, thus minimizing extraneous factors due to unexpected weather interruption. The stem cutting system (SCS) refers to the stage of planted seedlings with six leaves, in which stems are cut from the plantlets using a sterile surgical blade. The *stem cutting* consisted of at least one axial bud and two leaves.

2.1.2 Fertilizers

Twenty-five kilograms of muriate of potash (potassium) and 25 kilograms of urea (nitrogen)— were applied during the bulking period of Treatment 1 with 20 replications in a ridge.

Twenty-five kilograms sack of complete fertilizer (N, P, and K)—these were applied during the vegetative period for Treatments 1 and 2 with 20 replications in a ridge.

Fifty kilograms of Doufos (phosphate)—these were applied for the bulking period of Treatment 2, with 20 replications in a ridge.

2..1.3 Labels

These are used to ensure that the treatments were not interchanged before, during, and after the intended fertilizer was applied for the specific treatment.

2.2 Methods

2.2.1 Methodology Design

The study design for this was a randomized complete block design (RCBD) with six treatments and 20 replications. Each replication consisted of a plot with 20 sweet potato cuttings planted in a ridge. The purpose of using an RCBD was to reduce variability and ensure that differences in outcomes were due to the treatments and not to other factors. The study was a randomized controlled trial (RCT) where different treatments (fertilizer applications) were compared to determine their effects on the size and weight of sweet potato tubers. According to the experimental design, the average circumference and weight of the tubers were collected and analyzed using statistical methods to determine the differences between the treatments. Table 1 shows the tabular presentation of experimental setups, which shows the kind of treatment, target completion,

As for the fertilizer application, each treatment received a specific combination of fertilizers applied at different times. The amount of fertilizer applied per tree was 0.50 grams for all treatments. Treatment 1 (T1) received a complete fertilizer of 0.50 grams two weeks after planting and a combination of urea and muriate of potash of 0.50 grams each 1.5 months after planting. Similarly, treatment 2 (T2) received a combination of muriate of potash and urea of 0.50 grams each 1.5 months after planting. Treatment 3 (T3) received a complete fertilizer of 0.50 grams two weeks after planting and muriate of potash of 0.50 grams 1.5 months after planting, while treatment 4 (T4) received a complete fertilizer of 0.50 grams two weeks after planting and urea of 0.50 grams 1.5 months after planting. Treatment 5 (T5) received only a complete fertilizer of 0.50 grams (Table 1).

In the given context, the sweet potato plants were applied 0.50 grams of fertilizer each to provide them with the required nutrients for growth and development [16]. Fertilizers are often used to add to the natural nutrients in the soil, which may not always be enough for plants to grow at their best [17]. Regarding the different treatments, each got a different set of fertilizers at different times. 0.50 grams of fertilizer were put on each tree for all treatments to ensure the fertilizers were placed similarly [18].

The NSIC Sp-36 variety of sweet potatoes is a low-maintenance crop that does not require a water management system. The plant spacing for this variety is 25 centimeters between each plant, and the ridge height should be at least 40 centimeters. This variety doesn't have any pests, so there is less need for pest control. The harvesting of NSIC Sp-36 was done through the piecemeal method 105 days after planting to

protect the roots from damage during storage. This method involved removing individual sweet potatoes from the ridge as they matured rather than harvesting the entire crop at once [19].

Table 1. Experimental Set-ups of the Study

Treatment	Description	Target Date of Completion	Actual Date of Application
T_o	Control	No fertilizer applied	No fertilizer applied
T_1	Application of: -complete fertilizer 2 weeks after planting -combination of urea and muriate of potash 1 month and half after planting	Aug. 4, 2022 Aug. 27, 2022	Aug. 4, 2922 Aug. 26, 2022
T_5	Complete fertilizer only	Aug. 27, 2022	Aug. 27, 2022
T_2	Application of: Combination of muriate of potash and urea 1 month and half after planting	Aug. 4, 2022 Aug. 27, 2022	Aug. 4, 2922 Aug. 26, 2022
T_3	Application of: -complete fertilizer 2 weeks after planting -muriate of potash 1 month and half after planting	Aug. 27, 2022	Aug. 27, 2022
T_4	Application of: --complete fertilizer 2 weeks after planting -urea 1 month and half after planting	Aug. 27, 2022	Aug. 27, 2022
T_5	Complete fertilizer only	Aug. 27, 2022	Aug. 27, 2022

2.2.2 Collection Method

The yield data was collected 105 days after planting, when the tubers had plenty of time to grow and mature, allowing for accurate tuber size and weight measurements. The way this study collected data was suitable for its purpose, which was to look at how different fertilizer treatments affected the size and weight of sweet potato tubers. In agricultural research, it is common to use a caliper to measure the diameter of the tubers in centimeters and a scale to measure their weight in kilograms. This gives an estimate of the crop yield per tuber. Also, figuring out the average diameter and weight of the sweet potato tubers for each treatment gave a clear picture of how the different fertilizers affected the tubers.

The two parameters, tuber size and weight were chosen in this study because they are widely accepted indicators of sweet potato yield. They are essential factors in evaluating the effectiveness of different fertilizer treatments on sweet potato production. Moreover, the study aimed to determine the impact of different fertilizers on sweet potato tuber size and weight, critical factors in assessing crop productivity and profitability.

Measuring the circumference of the tubers using a caliper and their weight using a scale are standard methods in agricultural research for estimating crop yield because they are objective, reliable, and precise. These methods allow for accurate measurements and minimize errors that may arise from subjective judgments or variations in data collection. Additionally, calculating the average circumference and weight for each treatment provides a clear picture of the effects of different fertilizers on the size and weight of sweet potato tubers, enabling researchers to draw meaningful conclusions from the data.

Using statistical methods to compare treatment differences was likewise suitable and required for drawing meaningful inferences from the data. This reduced bias and ensured that any differences found were related to the treatments and no other factors. Overall, the data collection strategy employed in this study was appropriate, dependable, and valid, enhancing the quality and trustworthiness of the research findings.

2.2.3 Data Analyses

For the data analyses, descriptive statistics were used using means and weighted means for the circumference in centimeters and weights in kilograms. Inferential statistics based on a one-way analysis of variance were also used to test the hypothesis with a 0.01 margin of error for differences in the study parameters. If the data showed a significant difference in the study parameters based on the one-way analysis of variance (ANOVA), the mean comparison method would be the post hoc test. The post hoc test using Tukey's HSD (Honestly Significant Difference) test is employed to determine which specific groups or treatments differ significantly after finding a significant result from the ANOVA.

3. Results and Discussion.

3.1. Experimental Set-ups Results

The study design for this was a randomized controlled trial (RCT) where different treatments (fertilizer applications) were compared to determine their effects on the size and weight of sweet potato tubers. The treatments were randomly assigned to different plots or groups to reduce bias and ensure that differences in outcomes were due to the treatments and no other factors. The outcome measures (average circumference and average weight) were collected and analyzed using statistical methods to determine the differences between the treatments.

Specifically, Table 2 presented the results of the study, indicating that Treatment 2 (C = 12.4 cm; w = 0.35 kg) had average tuber circumference and weight, where mixtures of muriate of potash and urea-containing fertilizer were applied. This treatment eliminated phosphorus-containing fertilizer during the bulking period of sweet potato production. On the other hand, the lowest quality sweet potato tubers were found in Treatment 4 (C = 11.4 cm; w = 0.18 kg), which applied complete fertilizer two weeks after planting and applied urea-containing fertilizer one month and a half after planting. This treatment contained phosphorus, as complete fertilizer is a blend of nitrogen (N), phosphorus (P), and potassium (K) in the forms of potash, phosphoric acid, and nitrogen.

Table 2. Experimental Results of the Applications

Treatment and Replication	Average Circumference (cm)	Average Weight in (kg)
To	13.9	0.28
T_1	14.7	0.32
T_2	14.9	0.35
T_3	12.4	0.26
T_4	11.4	0.18
T_5	14.4	0.24

These findings provide empirical evidence that phosphorus has a detrimental effect on sweet potato crop production during the vegetative period, consistent with previous research [12-13]. In addition, these results agree with other studies that have shown a negative relationship between phosphorus and sweet potato yield during the vegetative stage [20-21]. Therefore, the current practice of applying complete fertilizer during both the vegetative and bulking periods should be avoided by farmers, as recommended by the researchers. The data collection and statistical analysis methods employed in this study underscore the importance of using appropriate fertilizer mixtures for sweet potato crop production, which can lead to increased yield and improved quality.

The importance of understanding the mechanism of a fertilizer application strategy for sweet potato crop production has been emphasized in previous studies by Alabi et al. [26] and Omoigui et al. [27]. The current study's findings support the previous research, showing that eliminating phosphorus-containing fertilizer during the bulking period may improve sweet potato yield [28-29]. However, the need for further research to optimize fertilizer application strategies for sweet potato production under different environmental conditions has also been noted [30-31].

Figure 1. Shows the yield of sweet potato tubers for each of the different study treatments. The data is presented subjectively in terms of circumference, with the scale in centimeters (cm), and weight, with the scale ranging from 0 to 1 kilogram (kg). The figure provides a visual representation of the differences in yield among the treatments, allowing readers to compare and interpret the data easily.

3.2 Computation of Yield Difference

3.2.1. Differences in Sweetpotato Tuber Yield Based on Circumference

The findings in Table 3 suggest that phosphorus-containing fertilizers do not significantly affect sweet potato tuber yield. The one-way ANOVA analysis of the data revealed a non-significant difference in sweet potato tuber yield between the different groups ($F = 1.09$; $p = 0.41$). These results indicate that regardless of the content of the fertilizers in the six treatments, the size of the sweet potato tubers may be more or less the same. This finding is in line with the study conducted by Bonneau et al. (2012), where the researchers found that using phosphorus-containing fertilizers did not significantly increase sweet potato yield [22].

Table 3. Differences in Sweetpotato Tuber Yield Based on Circumference

Source	Sum of Squares	Df	MS	F test tab	F test result	p-value
Between SS	29.76	5	5.95	2.76	1.09	0.41 (Not
Within SS	65.13	12	5.43			Significant)
Total	94.89	17				

Legend: Level of Significance at 0.01; Significant if the p-value is less than 0.01; Not Significant if the p-value is greater than 0.01

Although the study did not find a significant effect of phosphorus on sweet potato yield, it is important to note that it was conducted in a specific location and under specific conditions. Thus, further research is needed to confirm these findings in other locations and under different conditions [23]. Additionally, appropriate fertilizers, including those that do not contain phosphorus, remain important for crop production.

The data presented in Table 3 suggest that using phosphorus-containing fertilizers does not significantly affect sweet potato tuber yield. The findings of this study contribute to the existing body of knowledge on the effects of fertilizers on sweet potato yield and highlight the need for further research in this area.

3.2.2. Differences in Sweetpotato Yield Based on Weight per Tuber

Table 4 shows how the sweet potato varies depending on the yield per tuber. The one-way analysis of variance (ANOVA), F test revealed no statistically significant difference between the groups in the dependent variable ($F = 1.37$; $p = 0.30$).

Table 4. Differences in Sweetpotato Tuber Yield Based on Weights

Source	Sum of Squares	Df	MS	F test tab	F test	p-value
Between SS	0.05	5	0.01	2.76	1.37	0.30 (Not
Within SS	0.09	12	0.00			Signficant)
Total	0.15	17				

Legend: Level of Significance at 0.01; Significant if the p-value is less than 0.01; Not Significant if the p-value is greater than 0.01

The results presented in Table 4 indicate no significant difference in sweet potato tuber yield based on weights, regardless of the type of fertilizer used. This is consistent with previous studies [24-25] that have also found no significant difference in yield based on tuber weight. However, it is important to note that soil fertility and environmental conditions can significantly impact sweet potato yield. A comprehensive approach to soil management is necessary to achieve optimal yield [23].

While the current study did not find a significant difference in yield based on tuber weight, it is still important for farmers to pay attention to factors that may affect yields, such as soil quality, climate, and pest management. Additionally, future research should continue exploring the impact of different fertilizers and soil management strategies on sweet potato yield in different environments.

4. Conclusions

After a randomized controlled trial, it was found that removing phosphorus-containing fertilizers during the bulking stage of sweet potato production did not affect the yield of sweet potato tubers. The study results show that using a complete fertilizer with phosphorus during the bulking period may produce sweet potato tubers that aren't as good as those in Treatment 4. However, it is important to note that there were no significant differences in the yield of sweet potato tubers among the six treatments. It's important to remember that this study was only done in one place and under certain conditions. More research is needed to see if these results hold up in other places and under different circumstances. The study also found that the amount of fertilizer may not significantly affect how big sweet potato tubers are. This study's careful statistical analysis shows the importance of using suitable experimental methods in agricultural research to get accurate and trustworthy results. Overall, this study shows how important it is to grow sweet potatoes with the right

fertilizer mixtures, which can lead to a higher yield and better quality. Using fertilizers with phosphorus during the bulking period may not greatly affect sweet potato yield. It is important to consider other things like soil type, climate, and crop variety when determining the best fertilizer mix for a specific location and set of conditions.

5. Acknowledgments

The authors of the research article acknowledged the funding support of the Philippine Council for Agriculture, Aquatic, and Natural Resources Research and Development (*PCAARRD*) with a research grant of six million pesos and Samar State University *(SSU)* with seventy-six thousand pesos.

Author Contributions: Conceptualization, methodology, formal analysis, writing—original draft preparation, funding acquisition is contributed by M. M.L.; Investigation, resources, and supervision is contributed by N. T.; Writing-review and editing, project administration is contributed by R. B.V.
Funding: The Philippine Council for Agriculture, Aquatic, and Natural Resources Research and Development (*PCAARRD*)
Conflicts of Interest: The authors declare no conflict of interest.

References

[1] CIP International Potato Center. Available online: https://cipotato.org/research/sweet-potato/sweetpotato-gives-hope-ensuring-climate-smart-food-systems-livelihoods-resilience/ (accessed: January 15, 2023).
[2] Arianti, Yoesti Silvana, and Yos Wahyu Harinta. "Sweet potatoes: development and potential as alternative food ingredients in Karanganyar Regency, Indonesia." In *E3S Web of Conferences*, 226, 00050. EDP Sciences, 2021.
[3] Mukhongo, Ruth W.; John B. Tumuhairwe, Peter Ebanyat, AbdelAziz H. AbdelGadir, Moses Thuita, and Cargele Masso. "Combined application of biofertilizers and inorganic nutrients improves sweet potato yields." *Frontiers in plant science*, 2017, 8, 219.
[4] Guodong, L.; Simonne, H. E.; Morgan, T. K.; George J. Hochmuth, Shinsuke Agehara, Rao Mylavarapu, and Phillip B. Williams. "Fertilizer Management for Vegetable Production in Florida: CV296, rev. 4/2021." *EDIS* (2021).
[5] Steffan, J. J.; Brevik, E. C.; Burgess, L. C.; A. Cerdà. The effect of soil on human health: an overview. *European journal of soil science*, 2018, *69*(1), 159-171.
[6] Tony, Y.; Siddique, H. M. K.; Liu, K. Cropping systems in agriculture and their impact on soil health-A review, *Global Ecology and Conservation*, 2020, 23, e01118.
[7] PhilRootcrops. Available online: https://philrootcrops.vsu.edu.ph/?option=com_content&view=article&id=8. (Accessed: January 15, 2023).
[8] Kambewa, E. Casava commercialization in Malawi. *Gates Open Res*, 2019, *3*(379), 379.
[9] Harman Parks, Mary, Maria Elisa Christie, and Isidra Bagares. Gender and conservation agriculture: constraints and opportunities in the Philippines, *GeoJournal*, 2015, *80*(1), 61-77.
[10] Villordon, A.; Gregorie, J. C.; LaBonte, D.; Khan, A.; Selvaraj, M. Variation in 'Bayou Belle'and 'Beauregard'sweetpotato root length in response to experimental phosphorus deficiency and compacted layer treatments. *HortScience*, 2018, *53*(10), 1534-1540.
[11] Kareem, I.; Akinrinde, E. A.; Oladosu, Y.; Eifediyi, E. K.; Abdulmaliq, S. Y.; Alasinrin, S. Y.; Kareem, S. A.; Adekola, O. F. Enhancement of phosphorus uptake, growth and yield of sweet potato (*Ipomoea batatas*) with phosphorus fertilizers. *Journal of Applied Sciences and Environmental Management*, 2020, *24*(1), 79-83.
[12] Moreda, G. M.; Gedebo, A.; Beshir, M. H.; Haile, A. Ensuring food security of smallholder farmers through improving productivity and nutrition of potato. *Journal of Agriculture and Food Research*, 2022, 10, 100400.
[13] Agezew, A. M.; Vad, A.; Bytyqi, B.; Babett Ábrahám, B.E. Harnessing diversity in durum wheat (*Triticum turgidum* L.) to enhance climate resilience and micronutrient concentration through genetic and agronomic biofortification. *Acta Agraria Debreceniensis* 2022, 2, 9-20.

[14] Xu, Z.; Ma, Z.; Ding, Y. Effects of phosphorus fertilizer on yield and quality of sweet potato (*Ipomoea batatas* (L.) Lam.). *Journal of Plant Nutrition*, 2019, *42*(11-12), 1426-1435.
[15] Zhang, X.; Ma, Q.; Li, Y.; Hu, Y. Effects of low phosphorus fertilization combined with organic fertilizer on yield and quality of sweet potato. Plant Nutrition and Fertilizer Science, 2020, *26*(1), 89-95.
[16] Jones, B. The benefits and risks of fertilizer use in agriculture. *Journal of Agricultural and Environmental Ethics,* 2018, *31*(3), 279-293. doi: 10.1007/s10806-018-9729-9
[17] Johnson, C. Fertilizer application rates and methods for sweet potato cultivation. *Agricultural Research Journal*, 2015, *2*(1), 17-24.
[18] Smith, A.; Brown, E.; Lee, J. The effects of fertilizer application on sweet potato yield and quality. *Journal of Plant Nutrition*, 2020, *43*(6), 868-880. doi: 10.1080/01904167.2019.1687335
[19] Serrano, E. P.; Barroga, A. F.; Lales, J. S. Growth and yield performance of sweet potato (*Ipomoea batatas* L.) cultivars under rainfed and irrigated conditions. *Philippine Journal of Crop Science*, 2019, *44*(2), 46-54.
[20] Umana, P. B.; Udo-Inyang, M. A.; Ekaette, E. R. Effect of phosphorus on the growth and yield of sweet potato (*Ipomoea batatas* L.) in Uyo, Nigeria. *African Journal of Agricultural Research*, 2011, *6*(6), 1387-1391. doi: 10.5897/AJAR10.522
[21] Devarajan, H.; Vijayakumar, P. M.; Govindasamy, S. Effect of different levels of nitrogen, phosphorus and potassium on growth and yield of sweet potato. *International Journal of Agricultural Sciences*, 2015, *5*(4), 852-855. doi: 10.9735/0975-3710.5.4.852
[22] Bonneau, J.; Graffin, V.; Vanderschuren, H. The use of mineral fertilizers in sweetpotato production in sub-Saharan Africa: any opportunity for an agronomic nutrient use efficiency increase? *Frontiers in Plant Scienc*e, 2012, 3, 116. doi: 10.3389/fpls.2012.00116
[23] Baral, R.; Shrestha, J.; Gautam, S.; Chaulagain, T.; Dahal, K. R. The effect of organic and inorganic fertilizers on the yield and yield components of sweet potato (*Ipomoea batatas* L.) in eastern Nepal. *Journal of Agricultural Science and Technology*, 2020, *22*(4), 1001-1016. doi: 10.24200/JAST.2020.1006
[24] Asare, R.; Ntiamoah, A.; Amoatey, H.; Ofori, K.; Safo-Kantanka, O. Effect of planting dates and sweetpotato cultivars on yield and quality in the forest-savannah transitional agro-ecological zone of Ghana. *Journal of Agricultural Science*, 2010, *2*(2), 41-49.
[25] Njoku, D. N.; Akobundu, I. O. Response of sweet potato (*Ipomoea batatas* L.) to varying rates of poultry manure and NPK fertilizer. Journal of Sustainable Agriculture, 2005, *25*(1), 123-137.
[26] Alabi, T.O.; Ojeniyi, S.O.; Adetunji, M.T. Effect of NPK Fertilizer Rates on the Growth and Yield of Two Sweet Potato (*Ipomoea batatas* L. Lam) Varieties in Ibadan, Nigeria. *American Journal of Experimental Agriculture,* 2015, *5*(4), 308-317. doi: 10.9734/AJEA/2015/15552
[27] Omoigui, L.O.; Abaidoo, R.C.; Kamara, A.Y. (2017). Fertilizer Microdosing for Improved Livelihoods and Food Security in Africa: An Introduction. In Fertilizer Microdosing in Africa: A Field Practical Guide. 1-9. Springer International Publishing.
[28] Abdulmalik, R.O.; Balogun, O.O.; Fashogbon, O.A. Effect of Organic Manure and NPK Fertilizer on Growth and Yield of Sweet Potato (*Ipomoea batatas* L.) in a Tropical Environment. *Journal of Agricultural Science*, 2021, *13*(2), 74-85. doi: 10.5539/jas.v13n2p74
[29] Oyedeji, S.; Olowu, R.; Oluwafemi, T. The Effect of Inorganic and Organic Fertilizers on the Growth, Yield and Quality of Sweet Potato (*Ipomoea batatas* L.). *Journal of Agricultural Science and Technology*, 2016, *6*(1), 31-36.
[30] Akanbi, W.B.; Togun, A.O.; Oyelami, O.O. Influence of Organic and Inorganic Fertilizers on the Yield and Quality of Sweet Potato. *Journal of Agriculture and Sustainability*, 2018, *11*(2), 54-68.
[31] Zerihun, A.; Assefa, F.; Ferede, Y. Effects of Different Organic and Inorganic Fertilizers on Growth and Yield of Sweet Potato (*Ipomoea batatas* L.) in the Humid Highlands of Ethiopia. *Journal of Soil Science and Environmental Management*, 2020, *11*(6), 83-93. doi: 10.5897/JSSEM2019.0809

JSTR
ASEAN Journal of Scientific and Technological Reports

Research article

Fabrication, Characterization, and Biocompatibility Study of Gelatin-Blended Fibroin Scaffold

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Citation:
Naksuwan, K.; Chaipayang, S.; Charoeythornkhaijhornchai, P. ; Phosri, S. Fabrication, characterization, and biocompatibility study of gelatin-blended fibroin. *ASEAN J. Sci. Tech. Report.* **2023**, *26*(2), 67-76. https://doi.org/10.55164/ajstr.v26i2.248655.

Article history:
Received: February 27, 2023
Revised: March 28, 2023
Accepted: March 29, 2023
Available online: March 31, 2023

Abstract: This research investigates the biocompatibility of gelatin-blended fibroin scaffold fabricated by freeze-dry process. The morphology showed interconnected spongy spheres and polygons shapes with a pore size of 14.144 ± 11.31 and 19.4822 ± 18.71 micrometers, respectively. The formula ratio of fibroin:gelatin of 8:2 contained more random coil, while the formula ratio of fibroin:gelatin of 7:3 exhibited more β-sheet structure. The result revealed that gelatin influences fibroin conformational transition from random coil to beta, which might result from better hydrophobic characteristics. The surface of the formula ratio of fibroin:gelatin at 8:2 and 7:3 was 125.88 ± 9.85 and 130.07 ± 3.72 degrees, indicating the formula ratio of fibroin:gelatin at 7:3 had more hydrophobicity than the formula ratio of fibroin:gelatin at 8:2. The biocompatibility of scaffolds was determined for both formulas of human keratinocyte (HaCaT) and African green monkey kidney (Vero) cell lines by MTT assay. The formula ratio of fibroin: gelatin at 8:2 with various concentrations of 62.50, 125, 250, 500, and 1,000 μg/mL had cell viability percentages of 96.31, 101.74, 97.81, 104.15, and 103.22%, respectively. The formula ratio of fibroin:gelatin at 7:3 with various concentrations of 62.50, 125, 250, 500, and 1,000 μg/mL had cell viability percentages of 106.38, 104.28, 108.54, 97.42, and 97.65%, respectively. The growth of cell lines showed better performance with the hydrophilic characteristics of the scaffold, and it did not make it toxic to the HaCaT and Vero cell lines. These results demonstrated the possibility of gelatin-blended scaffold as a supporting material for skin tissue engineering, especially for wound healing.

Keywords: Biocompatibility; scaffold; fibroin; gelatin

1. Introduction

Diseases and accidents lead to injuries and damage to tissues and organs, especially skin tissue. The treatment of skin tissue generally focuses on tissue and organ transplantation [1]. Transplantation is the standard method of treating extensive injuries or wounds to the injured part of the skin tissue. However, tissue grafts and organ transplantation methods may not achieve this entirely because they do not have enough space for skin tissue. Therefore, tissue engineering has been developed to fabricate cell scaffolds for treating or closing acute and chronic wounds.

A scaffold is three-dimensional for cell culture that creates an artificial environment as the condition of cell growth in the body. It mimics the state of tissue physiology resulting in creation of new usable tissues. The scaffold could be made from natural or bio-based materials, an extensive study in tissue engineering applications. The scaffold production would be made the extracellular matrix of tissues that could maintain structural integrity in the body and eventually degradation. The scaffold product might be natural or synthetic materials with biodegradable and good biocompatibility, which does not cause any adverse effects on cell culture and does not stimulate the immune system or react against any organs of the human body [2]. Many materials are required for this application depending on biological, chemical, and mechanical properties.

The silk fibroin from the cocoon is a protein from natural fibers similar to the human body protein. It is a promising biomaterial for tissue engineering because it is biocompatible, degrades slowly, is chemically modified, and can be processed into various structures [3]. Likewise, it has excellent physical properties, being lightweight, strong, highly elastic, and thermally stable [3]. It can reduce inflammation and heal skin tissue wounds owing to good biocompatibility with human tissues [3-4]. Nevertheless, silk fibroin has poor cell adhesion, and some biomaterials that encourage cell adhesion, especially gelatin, to improve its properties. Gelatin is a protein product extracted from collagen by partial hydrolysis of collagen from animal skin, bone, and tissue. Arginine-glycine-aspartate (RGD) tripeptide in gelatin's bioactivities indicates cell-matrix interactions recognition binding site [5]. It could be the adhesive material between fibroin and skin tissue, promote cell growth [6], and have anti-inflammatory properties.

The silk-based scaffolds are a wide range. For example, freeze-dried sponges, nanofibers, and hydrogels have been investigated for tissue engineering and repair [7]. Therefore, this study aims to fabricate a gelatin-blended fibroin scaffold and confirm the physical properties and cell compatibility of blended scaffolds.

2. Materials and Methods

2.1 Silk fibroin extraction

Bombyx mori silk cocoons were purchased from Yasothon province. Silk fibroin (SF) was prepared by chopping 10 g of *Bombyx mori* silk cocoons into small pieces and degumming in 0.2 M of Na_2CO_3 twice the time at 100°C for 30 min. Next, the degummed cocoons were rinsed throughout to remove the glue-like sericin proteins with water. After degumming, silk fibers were dried in a hot air oven at 60°C overnight. The degummed silk fibers were then dissolved in a $CaCl_2/C_2H_5OH/H_2O$ solution (molar ratio 8:2:1) and continuously stirred at 70°C for 5 h. The obtained solution was centrifuged at 6,000 rpm to remove any impurities. The solution was then dialyzed with deionized water for three days to remove the salt that could remain in the solution. Finally, the SF solution was stored at 4°C until usage.

2.2 Fabrication of the composite scaffolds

Five percent of the gelatin solution was mixed with 5% of the silk fibroin solution at different ratios between fibroin and gelatin. Two formula scaffolds of fibroin:gelatin at 8:2 (v/v) and 7:3 (v/v), were investigated. Then, 2.5% of glutaraldehyde was added to the scaffold solution as a crosslinking agent. The answer was transferred to the 15 mL centrifuge tube and frozen at -40°C overnight, followed by freeze-dry process at -80°C for 48 h. The blended scaffolds were stored in aluminum foil at 4°C for characterization and testing.

2.3 Characterization

The microstructure of the blended scaffold was observed by scanning electron microscopy (SEM, ZEISS, LEO 1450 VP, USA). The standard procedures prepared samples: fixation in glutaraldehyde 2.5%vol at 4°C for 6 h and coating with gold by sputtering. The accelerating voltage of SEM was 10 kV. Fourier Transform Infrared Spectroscopy (FTIR) was used to characterize the intermolecular interactions between the components and conformational changes of each scaffold formula. The spectra of the individual and SF/Gelatin scaffolds were performed by a Bruker tensor 27 DTGS detector. Spectral data were measured using a platinum diamond ATR with the reflection mode of 64 scans at a resolution of 4 cm^{-1} over a measurement

range of 4,000-600 cm^{-1}. Data acquisition and analysis were performed by OPUS 7.5 software (Bruker Optics Ltd., Ettlingen, Germany).

The Image J software quantified porosity measurement with the analyze particles function. Axial and perpendicular sections of morphological change after preparation were analyzed. Furthermore, the hydrophilicity of the scaffolds was measured by the contact angle method. Five microliters of distilled water were dropped on the surface of each formula, and the contact angle was recorded after 2 s. The drop shape analysis software was used to calculate the contact angle. The results are shown as an average mean value with standard deviation.

The crystal structure of blended scaffolds was analyzed using wide-angle synchrotron X-ray scattering using 1.3 W of the beamline: SAXS/WAXS of the Synchrotron Light Research Institute (SLRI), Nakhon Ratchasima, Thailand that has energy 1.2 GeV. The distance between the sample and detector was 153 mm with LX170 as a detector. The Wide Angle X-ray Scattering (WAXS) intensity was obtained at a photon flux of 2×10^9 photons/s at 9 keV. The WAXS patterns were corrected using air as a background. The 2D WAXS patterns were reduced and radially averaged by SLRI (SAXSIT) staff-developed software to obtain 1D WAXS curves. The percentage of crystallinity (Pc) was calculated based on the data obtained from the WAXS profiles using the following equation (1)

$$\text{Percentage of crystallinity (Pc)} = (A_c/A_c+A_a) \times 100 \quad (1)$$

Where A_c is, and A_a are the areas under the crystalline peak of interest and the amorphous halo, respectively [8].

For the biocompatibility assay, the scaffold was placed into 1.5 mL micro centrifugal tubes and sterilized under UV light for 15 min. After that, FBS-free DMEM was added from a stock solution at 20 mg/mL for 24 h to extract the scaffolds. After incubation, the extracted medium was sterilized by a 0.22 μM PES filter. The filtrated medium was kept at -20°C until usage. The human keratinocyte (HaCaT) and African green monkey kidney (Vero) cell lines were seeded into 96 well plates with a density of 1×10^4 cells per well and incubated at 37°C in an atmosphere containing 5% of CO_2 for 24 h. After culturing for each period, the cells were treated with the extracted medium at 31.25, 62.5, 125, 250, 500, and 1,000 μg/mL for 24 h. Then, the surviving cells were quantified by 3-(4,5-dimethyl thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay. The medium was discarded from each well. The MTT solution (0.5 mg/mL in PBS) was added into each well at around 150 μL and incubated for 1 h to establish cell viability. After that, the formazan crystals were dissolved in 150 μL of pure DMSO, and the absorbance of the solution was measured at 570 nm using a microplate reader (Molecular Devices SpectraMax ABS, USA).

3. Results and Discussion

The microstructure of scaffolds formed by freeze-dry process under SEM image shows in Figure 1. The porous and spongy cells could be observed. The formula of fibroin:gelatin scaffolds at 8:2 reveal some spherical spongy connections, as shown in Figures 1a and 1b, while the formula of fibroin:gelatin scaffolds at 7:3 scaffolds exhibit spongy, spherical, and polygon interconnected evenly shape as shown in Figures 1c and 1d. This result is similar to a previous study by Chamchongkaset et al. [2]. They also found a spongy and uniformly connected fibroin fiber. In addition, Wang et al. [9] studied the structure of scaffolds as a porous spherical lattice that could provide an ideal environment for cell proliferation and better cell growth.

Pore size analysis was performed with the Image J software for the cellular scaffold. The blended scaffold formula ratios of fibroin:gelatin at 8:2 is shown in Figure 2a. The pore size displays approximately 14.14 ± 11.31 micrometers, whereas the pore size for formula ratios of fibroin:gelatin at 7:3 Figure 2b exhibits approximately 19.48 ± 18.71 micrometers. The pore size of these two cell scaffolds was smaller than the scaffolds with a cellulose nanofiber (CNF) to poly (vinyl) alcohol (PVA) ratio at 0.4:0.1 wt% had the average pore size of around 90.71 ± 2.4 micrometers, which was the same as the dermis. The average pore size is approximately 10-15 micrometers, especially the pore size of the scaffold allows the fibroblast cells to penetrate and produce the extracellular matrix (ECM). Moreover, the scaffold with CNF to PVA ratio at 1.4:0.35 wt% had an average pore size of 19.72 ± 3.6 micrometers, similar to the skin cells in the epidermis [10]. Furthermore,

the study of pore size of the agarose-chitosan-collagen type I cell scaffold at a ratio of 3:1:0.5 and 3:1:3 exhibited approximately 170.37 and 241.99 micrometers [10]. Besides, the optimal pore size for bone formation should be 200-400 micrometers [11]. Another previous study of scaffolds fabricated with 75:25 poly (lactic-co-glycolic) acid (PLGA) by the solvent casting particulate leaching technique with NaCl examined the suitable optimum pore size between 150-750 micrometers, exhibiting a pore size comparable to the size of the salt particles used in the fabrication process [12]. From those research, our blended scaffolds between fibroin and gelatin have appropriate pore sizes, allowing the cells to penetrate and produce the extracellular matrix. They might be better for skin tissue engineering.

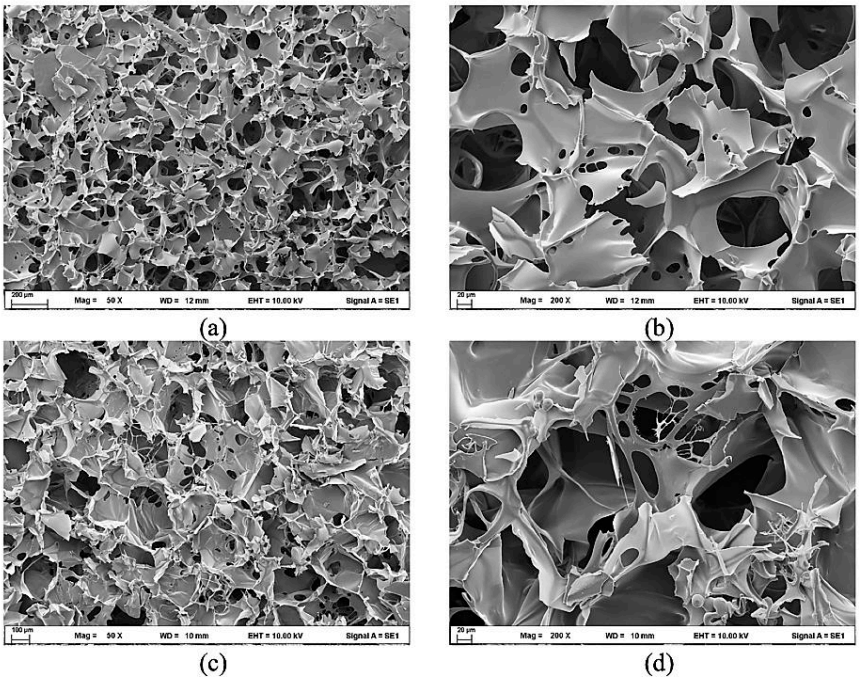

Figure 1. SEM images: (a) scaffold formula ratios of fibroin:gelatin at 8:2 with a scale bar of 50 μm, (b) scaffold formula ratios of fibroin:gelatin at 8:2 with a scale bar of 200 μm, (c) scaffold formula ratios of fibroin: gelatin at 7:3 with a scale bar of 50 μm, (d) scaffold formula ratios of fibroin:gelatin at 7:3 with a scale bar of 200 μm.

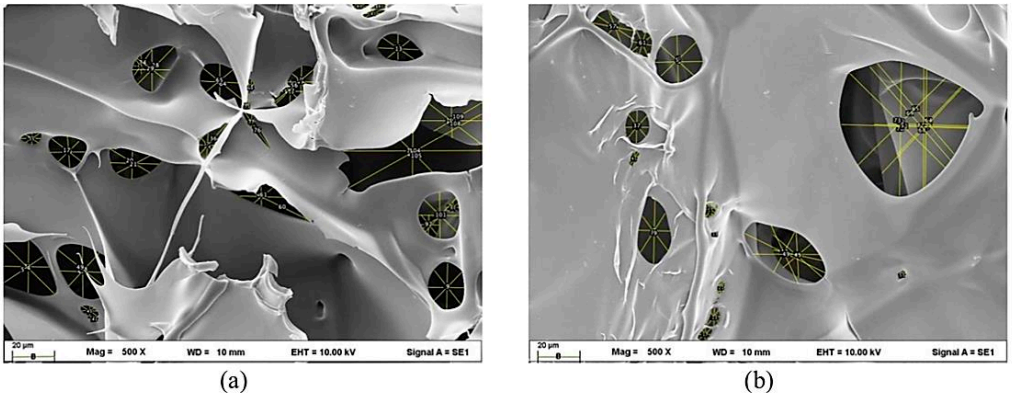

Figure 2. Pore size: (a) scaffold formula ratios of fibroin:gelatin at 8:2 with a scale bar of 20 μm, (b) scaffold formula ratios of fibroin:gelatin at 7:3 with a scale bar of 20 μm.

FTIR analysis was measured to obtain the conformational changes of each scaffold formula (Figure 3a). The spectrum clearly showed the similar chemical structures between fibroin and gelatin that are characteristic of protein absorption. FTIR spectrum of fibroin comprised of amide I (1640 cm^{-1}), indicating the carbonyl (C=O) stretching; amide II (1515 cm^{-1}), showing the vibration on the plane of the N-H bond and C-N stretching; amide III (1236 cm^{-1}) referring to C-N stretching and N-H deformation, and O-H stretching appearing at 3276 cm^{-1} [13-15]. Likewise, the gelatin spectrum presented the main components of amide I, II, and III with a slight shift of wavelength number. In addition, the wavelength number 1399 cm^{-1} indicated the C=O symmetric stretching of the COO-group in the amino acid of gelatin was different from fibroin which has a double peak at 1411 and 1384 cm^{-1} as well as scaffold formula ratios of fibroin:gelatin at 8:2 and 7:3. Therefore, their amino acid and secondary structure were different. The secondary structure of the proteins was estimated thoroughly by the deconvolution and curve fitting of the amide I band [13, 16]. The formula ratios of fibroin: gelatin at 8:2 exhibited higher percentages of random coil structure compared to those of fibroin:gelatin at 7:3 and contained less β-sheet. The results indicated that the increase influenced a slight increase in β-sheet gelatin content (Table 1). This conformation may affect the scaffold's ability to support cells [17].

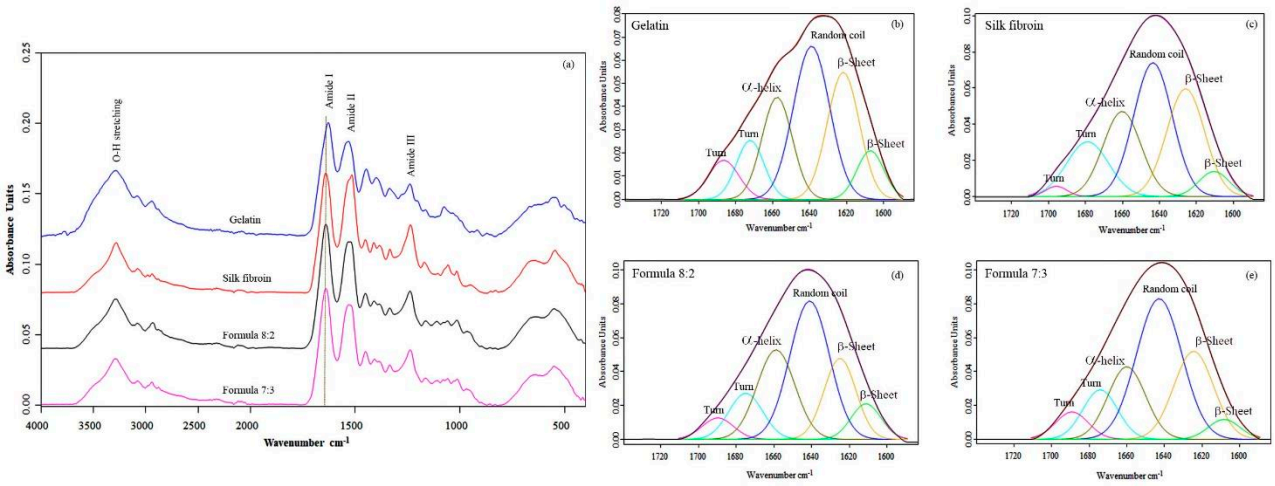

Figure 3. FTIR functional group detection results of gelatin-infused fibroin cell scaffolds (a), The secondary structure of samples determined by Fourier deconvolution of the amide I band of each sample, Gelatin (b), Silk fibroin (c), formula ratios of fibroin:gelatin at 8:2 (d) and formula ratios of fibroin: gelatin at 7:3 (e).

Table 1. The structural conformation ratios in gelatin, silk fibroin, and silk fibroin-gelatin scaffolds derived from deconvoluted amide I FTIR spectra.

Samples	Conformation content of samples [a)]			
	β-sheet	Random coil	α-helix	β-turns
Gelatin	32.96 ± 1.0	34.12 ± 0.5	17.72 ± 0.7	17.20 ± 0.3
Silk fibroin	31.00 ± 1.2	32.69 ± 1.2	19.89 ± 1.5	16.41 ± 1.4
Scaffold 8:2	24.88 ± 0.5	36.58 ± 0.6	21.61 ± 1.2	23.54 ± 0.6
Scaffold 7:3	27.75 ± 1.6	29.47 ± 2.1	21.39 ± 0.5	23.38 ± 1.0

[a)] Values are average ± standard derivation (N=3)

Contact angle measurements were determined to identify the hydrophilic properties of the scaffold. The formula ratios of fibroin:gelatin at 8:2 had a slightly lower contact angle than those of fibroin:gelatin at 7:3. The formula ratios of fibroin:gelatin at 8:2 shows 125.88 ± 9.85 degrees, while the formula ratios of fibroin: gelatin at 7:3 display 130.07 ± 3.72 degrees Figures 4a, 4b, 4c. This might indicate the binding of the N-H group of collagens caused by the polarity of the carboxyl groups. The reduction of polarity with various water contact angles versus time showed that the formula ratios of fibroin:gelatin at 8:2 scaffolds decreased faster than those

of fibroin:gelatin at 7:3 scaffolds, as shown in Table 2. Likewise, a previous study has shown that the water contact angle of silk/collagen (0.25) was 103.27 ± 5.91, silk/collagen (0.50) was 104.27 ± 4.20, silk/collagen (1.00) was 106.55 ± 6.18, and silk/collagen (2.00) was 103.95 ± 2.03 degrees [18]. The angle of contact with water was reduced depending on the measurement time. The high measurement variability is caused by the droplet's location and the scaffold absorption [19].

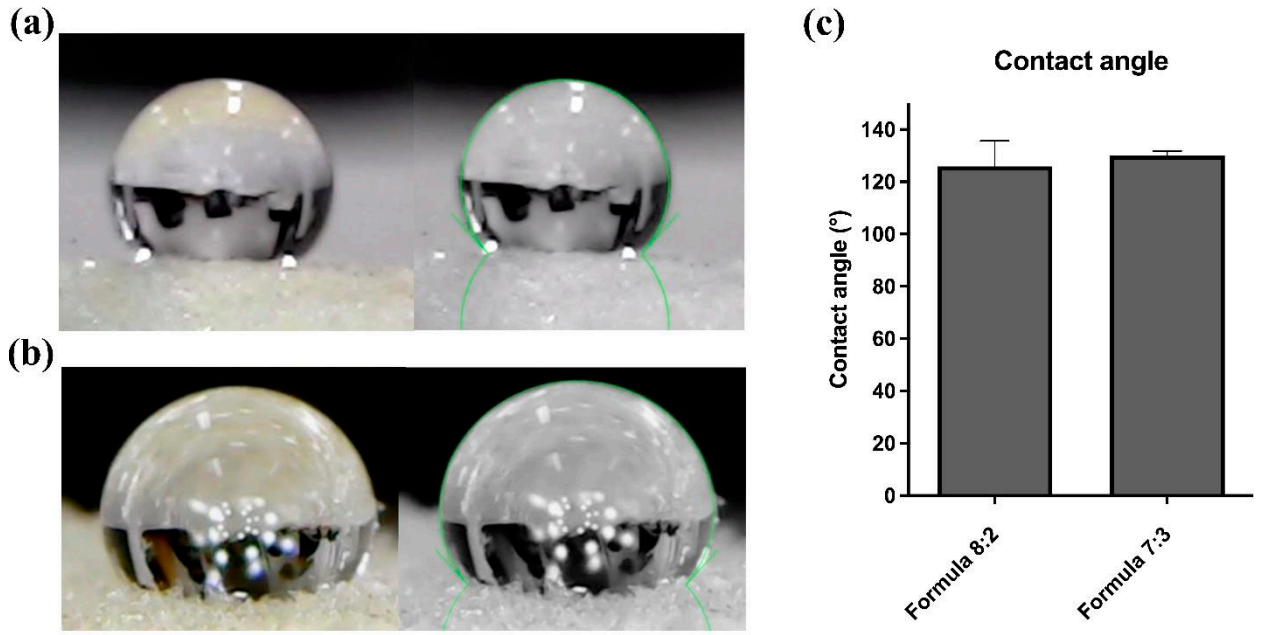

Figure 4. Contact angle: (a) scaffold formula ratios of fibroin:gelatin at 8:2, (b) scaffold formula ratios of fibroin: gelatin at 7:3, (c) a comparison between scaffold formula at 8:2 and 7:3.

Table 2. Water contact angle.

Scaffolds	Water contact angle					
	0 sec	10 sec	15 sec	20 sec	25 sec	30 sec
formula 8:2	125.88 ± 9.85	110.72 ± 7.80	95.29 ± 0.85	82.75 ± 5.84	67.22 ± 7.94	51.34 ± 5.11
formula 7:3	130.07 ± 3.72	109.22 ± 6.14	84.73 ± 5.35	72.74 ± 10.57	64.08 ± 7.84	49.79 ± 6.62

The crystal structure of scaffolds was studied using Wide Angle X-ray scattering. The Gaussian function was used to fit the diffraction peaks, and the Lorentz function was used to hold the amorphous background to estimate crystallinity, as shown in Figure 5. The WAXS pattern of the two scaffolds demonstrated diffraction peaks at 7.29°, 19.50°, and 39.38°. The crystallinity percentage of formula ratios of fibroin: gelatin at 8:2 was 39.039%, whereas the formula ratios of fibroin: gelatin at 7:3 was 41.936%. For the WAXS result, the crystallinity of formula ratios of fibroin:gelatin at 7:3 was slightly higher than that of fibroin: gelatin at 8:2. The gelatin induced fibroin conformational conversion from random coil to beta crystal [20]. Therefore, the presence of gelatin influences gelatin-blended gelatin-blended fibroin scaffold crystallization. The characteristic of these two scaffolds was almost the same as those of the β-sheet crystalline structure (silk II) of silk fibroin [21-22] due to the lower ratio of gelatin.

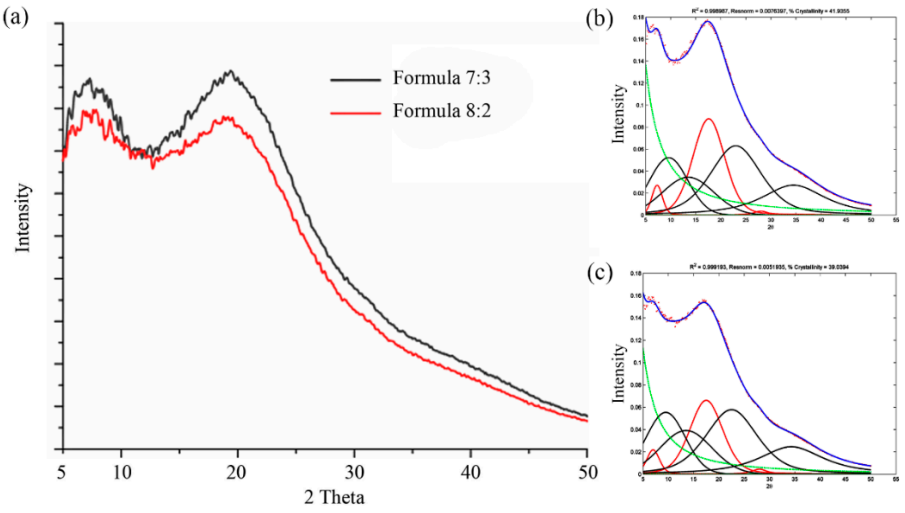

Figure 5. The WAXS pattern of scaffolds (a), fitting curves of formula ratios of fibroin:gelatin at 7:3 (b) and fitting curves of formula ratios of fibroin:gelatin at 8:2 (c).

The biocompatibility test was measured to evaluate the cytotoxicity on regular cell lines. Vero cells were treated with scaffold formula ratios of fibroin:gelatin at 8:2 with various concentrations of 62.50-1000 μg/mL. Cell viability percentages were in the range of 96.31-103.22%. Similarly, for scaffold formula ratios of fibroin:gelatin at 7:3 (62.50-1000 μg/mL), the cell viability percentages were 106.38, 104.28, 108.54, 97.42, and 97.65%, respectively. The results show that both scaffolds at different concentrations do not produce toxicity to Vero cells and unchanged cell viability compared to the control, as shown in Figures 6a and 6b. HaCaT cells viability after treatment with the scaffold formula ratios of fibroin:gelatin at 8:2 (62.50-1000 μg/mL) had cell viability percentages around 104.86, 111.71, 109.58, 113.23, and 119.90%, respectively, while the formula ratios of fibroin:gelatin at 7:3 displayed cell viability percentages around 99.62, 101.02, 113.16, 114.38 and 101.36%, respectively. The result revealed that the scaffolds at different concentrations were also not toxic to HaCaT cells or did not decrease cell viability due to cytotoxicity on the Vero cell, as shown in Figures 6c and 6d. These results confirmed that the scaffold could be applied to the human body for medical application and tissue engineering. Similarly, the previous study cultured the fibroblast cells on agarose-chitosan-collagen type I cell scaffolds at a ratio of 3:2:0.5, showing that fibroblast cells could grow and multiply well in the scaffold. Thus, this scaffold was not toxic to fibroblast cells [10]. An additional study was tested on gelatin-fibroin-chitosan-produced cell scaffolds. It was found that all ratios of scaffolds had a percentage of viability of more than 80%, indicating that scaffolds had no cytotoxicity [23]. Therefore, our results confirmed the scaffolds are biocompatible with skin (HaCaT) and kidney (Vero) cell lines, indicating that they can be used in the human body and might restore damaged skin or internal organs for tissue engineering applications.

4. Conclusions

This study investigated the fabrication of blended scaffolds between fibroin and gelatin by the freeze-drying process for tissue engineering. The porous scaffold by SEM images is suitable for penetrating fibroblast cells and producing the ECM. The chemical functional structure of scaffolds revealed an amide group inside the scaffold, exhibiting a lattice-like crystalline form. The microstructure of the blended scaffolds had porous and connected evenly, which was a suitable environment for cell proliferation. Furthermore, these scaffolds were non-cytotoxic and biocompatible with Vero and HaCaT cells. Our results demonstrated that fibroin/gelatin scaffolds exhibited good biological properties and could be supported because they might apply to the human body. Hence, our scaffolds are a strong candidate biomaterial appropriate for manufacturing as innovative skin tissue engineering products for further applications.

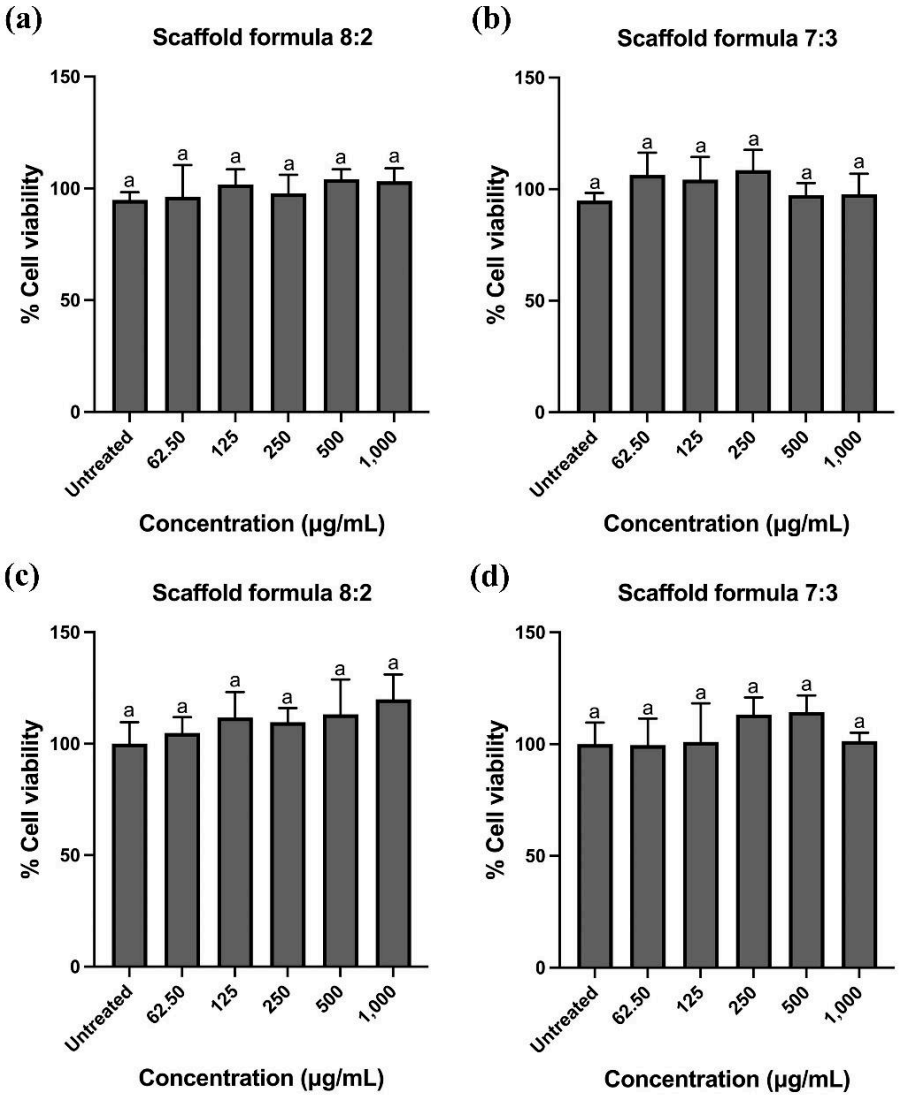

Figure 6. Cell viability with MTT assay: (a) cell viability analysis of Vero cells on scaffold formula ratios of fibroin: gelatin at 8:2, (b) Vero cells on scaffold formula ratios of fibroin:gelatin at 7:3, (c) cell viability analysis of HaCaT cell on scaffold formula ratios of fibroin:gelatin at 8:2, (d) HaCaT cell viability on scaffold formula ratios of fibroin:gelatin at 7:3. The data were displayed as means ± S.D. of triplicate determinations. Different letters on the top of each bar indicate statistically significant differences ($p < 0.05$).

5. Acknowledgements

This study was supported by the Faculty of Engineering, Burapha University. The authors gratefully acknowledge the support of the Synchrotron Light Research Institute (Public Organization), Nakhon Ratchasima, Thailand, for a wide-angle synchrotron X-ray scattering instrument.

Author Contributions: Conceptualization, S.P.; methodology, K.N., and S.C.; formal analysis, K.N., S.C., P.C., and S.P.; data curation, K.N.; writing-original draft preparation, K.N.; writing-review and editing, K.N., S.C., P.C., and S.P.; supervision, S.P. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Conflicts of Interest: The authors declare no conflict of interest.

References

[1] O'Brien, F.J. Biomaterials & Scaffolds for Tissue Engineering. *Materials Today*, 2011, 14(3), 88-95.

[2] Chamchongkaset, J.; Kanokpanont, S.; Kaplan, D.L.; Damrongsakkul, S. Modification of Thai Silk Fibroin Scaffolds by Gelatin Conjugation for Tissue Engineering. *Advanced Materials Research*, 2008, 55, 685-688.

[3] Rockwood, D.N.; Preda, R.C.; Yucel, T.; Wang, X.; Lovett, M.L.; Kaplan, D.L. Materials Fabrication from *Bombyx mori* Silk Fibroin. *Nature Protocols*, 2011, 6, 1612-1631. https://doi.org/10.1038/nprot.2011.379.

[4] Luangbudnark, W.; Viyoch, J.; Laupattarakasem, W.; Surakunprapha, P.; Laupattarakasem, P. Properties and Biocompatibility of Chitosan and Silk Fibroin Blend Films for Application in Skin Tissue Engineering. *The Scientific World Journal*, 2012, 697201. https://doi.org/10.1100/2012/697201.

[5] Duangpakdee, A.; Laomeephol, C.; Jindatip, D.; Thongnuek, P.; Ratanavaraporn, J.; Damrongsakkul, S. Crosslinked Silk Fibroin/Gelatin/Hyaluronan Blends as Scaffolds for Cell-Based Tissue Engineering. *Molecules*, 2021, 26(11), 3191. https://doi.org/10.3390/ molecules26113191.

[6] Cole, C.G.B. Gelatin. In *Encyclopedia of Food Science and Technology*, 2nd ed; Frederick, F.J., Ed.; John Wiley & Sons, New York, 2000; 1183-1188.

[7] Luetchford, K.A.; Chaudhuri, J.B.; De Bank, P.A. Silk Fibroin/Gelatin Microcarriers as Scaffolds for Bone Tissue Engineering. *Materials Science and Engineering: C*, 2020, 106, 110116. https://doi.org/10.1016/ j.msec.2019.110116.

[8] Surya, I.; Waesateh, K.; Masa, A.; Hayeemasae, N. Potency of Urea-Treated Halloysite Nanotubes for the Simultaneous Boosting of Mechanical Properties and Crystallization of Epoxidized Natural Rubber Composites. *Polymers*, 2021, 13, 3068. https://doi.org/10.3390/polym13183068.

[9] Wang, J.; Yang, Q.; Cheng, N.; Tao, X.; Zhanga, Z.; Sun, X.; Zhang, Q. Collagen/Silk Fibroin Composite Scaffold Incorporated with PLGA Microsphere for Cartilage Repair. *Materials Science and Engineering: C, Materials for Biological Applications*, 2016, 61, 705-711.

[10] Ghafari, R.; Jonoobi, M.; Amirabad, L.M.; Oksman, K.; Taheri, A.R. Fabrication and Characterization of Novel Bilayer Scaffold from Nanocellulose Based Aerogel for Skin Tissue Engineering Applications. *International Journal of Biological Macromolecules*, 2019, 136, 796-803.

[11] Chonanant, C. Fabrication the Biomaterial Scaffold for Tissue Engineering by Freeze-Drying Technique. *Journal of Medical Technology and physical therapy*, 2019, 31(3), 370-381.

[12] Boyan, B.D.; Hummert, T.W.; Dean, D.D.; Schwartz, Z. Role of Material Surfaces in Regulating Bone and Cartilage Cell Response. *Biomaterials*, 1996, 17(2), 137-146.

[13] Martin, C.; Subathra, R.; Jayanthi, V.; Reddy, S.; Varghese, J.; Rela, M.; Kalkura, N. Collagen Rich Scaffolds for Liver Engineering: a Step Forward to an Articicial Liver. *Journal of Clinical and Experimental Hepatology*, 2016, 6, S86-S87.

[14] Yang, H.; Yang, S.; Kong, J.; Dong, A.; Yu, S. Obtaining Information about Protein Secondary Structures in Aqueous Solution using Fourier Transform IR Spectroscopy. *Nature Protocols*, 2015, 10(3), 382-396.

[15] Nagai, T.; Suzuki, N.; Tanoue, Y.; Kai, N. Functional Property of Honey from *Echium vulgare*. *Food and Nutrition Sciences*, 2012, 3(8), 614-620.

[16] Silva Júnior, Z.S.; Botta, S.B.; Ana, P.A.; Franca, C.M.; Fernandes, K.P.S.; Mesquita-Ferrari, R.A.; Deana, A.; Bussadori, S.K. Effect of Papain-Based Gel on Type I Collagen-Spectroscopy Applied for Microstructural Analysis. *Scientific Reports*, 2015, 5, 11448. https://doi.org/10.1038/srep11448.

[17] Chadefaux, C.; Le Hô, A.-S.; Bellot-Gurlet, L.; Reiche, I. Curve-Fitting Microe-ATR-FRIR Studies of the Amide I and II Bands of Type I Collagen in Archaeological Bone Materials. *e-PRESERVATION Science*, 2009, 6, 129-137.

[18] Efraim, Y.; Schoen, B.; Zahran, S.; Davidov, T.; Vasilyev, G.; Baruch, L.; Zussman, E.; Machluf, M. 3D Structure and Processing Methods Direct the Biological Attributes of ECM-Based Cardiac Scaffolds. *Scientific Reports*, 2019, 9, 5578. https://doi.org/10.1038/s41598-019-41831-9.

[19] Yeelack, W. Hybrid Biomimetic Scaffold of Silk Figroin/Collaget Type I Films for Tissue Engineering: Preparation and Characterization. M.Sc. thesis, Prince of Songkla University, Songkla, 2014.

[20] Paxton, N.; Woodruff, M. Measuring Contact Angles on Hydrophilic Porous Scaffolds by Implementing a Novel Raised Platform Approach: A technical note. *Polymer Advanced Technologies*, 2022, 33, 3759-3765.

[21] Gil, E.S.; Frankowski, D.J.; Bowman, M.K.; Gozen, A.O.; Hudson, S.M.; Spontakm, R.J. Mixed Protein Blends Composed of Gelatin and *Bombyx mori* Silk Fibroin: Effects of Solvent-Induced Crystallization and Composition. *Biomacromolecules*, 2006, 7, 728-735.

[22] Asakura, T.; Kuzuhara, A.; Tabeta, R.; Saito, H. Conformational Characterization of *Bombyx mori* Silk Fibroin in the Solid State by High-Frequency Carbon-13 Cross Polarization-Magic Angle Spinning NMR, X-ray Diffraction, and Infrared Spectroscopy. *Macromolecules*, 1985, 18, 1841-1845.

[23] Kim, U.J.; Parka, J.; Kima, H.J.; Wada, M.; Kaplan, D.L. Three-Dimensional Aqueous-Derived Biomaterial Scaffolds from Silk Fibroin. *Biomaterials*, 2005, 26, 2775-2785.

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3.1.1. Subsubsection

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