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Article

The optimization of high-protein duckweed cultivation in eutrophicated water with mutualistic bacteria

Sunyapat Akkarajeerawat[1], Thanatkorn Chauvanasmith[1], Chanan Keatsirisart[1]
[1] Bangkok Christian College, Bangkok, Thailand

SUMMARY

The exponential increase in the human population is the leading cause of food crises in Thailand and Southeast Asia, and it is affecting global food security while increasing humanity's carbon footprint. One approach to resolving these issues is the cultivation of the aquatic plant, duckweed (*Wolffia globosa*) for consumption in the Northern and Northeastern provinces of Thailand. Duckweed has drawn increasing attention due to its rapid growth rate, adaptability to extreme conditions, and potential as an alternative protein; however, due to the difficulty in achieving these properties, they could potentially be greatly enhanced. Our research investigates two optimizations for the cultivation of duckweed: (1) the use of phosphorus- and nitrogen-rich growing media and (2) the use of plant growth–promoting bacteria (PGPB). Based on evidence from past research on the biochemical and metabolic functions of nitrogen and phosphorus, the absorption rates for duckweed, and the nitrogen-fixing properties of PGPB, we hypothesized that using phosphorus-rich water and PGPB would significantly increase duckweed biomass production, while nitrogen-rich water would increase protein content. We found that using a phosphorus-rich medium boosted duckweed biomass greatly, while using a nitrogen-rich medium significantly enhanced the protein value of the duckweed. Furthermore, PGPB yielded the highest increase in duckweed biomass but had an insignificant impact on protein content. Based on our results, we recommend the use of nitrogen- and phosphorus-rich water and PGPB to optimize duckweed cultivation and help address the global food crisis and reduce the environmental impact from meat production.

INTRODUCTION

As the world population is projected to exceed 9.1 billion by 2050, Southeast Asian countries like Thailand is currently facing an increase in food shortages. The prevalence of food insecurity in Thailand in 2023 has increased by 1.5 times compared to 2017 (1, 2). In order to meet this food demand, finding a promising alternative protein should be the top priority. Aside from proteins derived from insects, plant-based proteins offer an alternative to meat and are gaining mainstream attention, particularly as the number of vegans and vegetarians increase, largely due to concerns about animal welfare and the environmental impact of animal products (3, 4). This has led to increase in consumption of superfoods, which are foods with high nutritional value due to large amounts of nutrients and bioactive ingredients (5).

Duckweeds, categorized as aquatic macrophytes in the Lemnaceae subfamily, are the smallest angiosperms, measuring only 0.4–0.9 mm in length (6). In Thailand, two species of duckweed, *Wolffia globosa* and *Wolffia arrhiza,* can be found naturally in still ponds and marshes (7). Known for their high protein content (45.54% dry weight) and considerable amount of carbohydrates and lipids, duckweeds have become popular superfoods in Thailand (8). Furthermore, duckweeds contain all nine essential amino acids and have more bioactive ingredients than most grains, such as wheat, corn, or rice (20). In addition, recent research has highlighted *Wolffia globosa's* functional properties, such as its high protein content and antioxidant activity, as well as its antimicrobial capabilities, suggesting its potential development into functional food ingredients (21). Not only does *W. globosa* have a high nutritional value, but they are also one of the fastest-growing plants under ideal environmental conditions (9).

Various species of duckweed are being cultivated for their low water consumption compared to other plant-based protein sources, their ability to reduce water evaporation, and their water recovery rate exceeding 95% (10). Duckweed cultivation can also reduce the production of carbon dioxide, offering a sustainable option that can be cultivated in both polluted and non-polluted waters (10).

Additionally, cultivation of duckweed offers a promising approach for addressing long-standing environmental concerns associated with traditional farming practices, such as the use of pesticides, herbicides, and chemical-based fertilizers. Pollution from these sources continues to pose a significant challenge for the agricultural community, leading to issues like algae blooms and ocean acidification (11). While chemical-based fertilizers can enhance the rate of production to supply the growing demand for food, they can also cause major health issues and disrupt the ecological balance of the aquatic environment (12). The accumulation of fertilizers, mainly nitrogen and phosphate-based in water contributes to eutrophication, a destructive process, leading to algae blooms. These blooms can reduce the oxygen and sunlight levels from underwater organisms, causing disruption to the environment and eventually leading to agricultural damage (13, 14). However, previous research highlights duckweeds in the genus *Lemna*'s ability to efficiently utilize excessive nutrients and adapt to a diverse range of environments, while also remediating pollutants in wastewater, with absorption and usage rates of nitrogen and phosphorus at 1.3g/m^2/day and 0.18 1.3g/m^2/day, respectively (15). Coupled with the use of mutualistic plant growth-promoting bacteria, which enhance

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nutrient uptake through nitrogen fixation and protect plants against pathogens, duckweed could offer a dual benefit. It may not only effectively remediate pollutants from wastewater but also be harvested as a protein-rich food source. This approach could reduce environmental impact while providing a sustainable food option during long-term cultivation (16).

Recent studies have showcased the potential of bacteria such as *Acinetobacter calcoaceticus* P23 and *Acinetobacter magnusonii* H3 to enhance nutrient absorption and growth rates of duckweed (*Lemna minor* Linnaeus) (17, 18). Additionally, commercialized bacteria in the genus *Azospirillum*, *Burkholderia*, and *Gluconacetobacter* also have positive effects on various agricultural crops, further revealing the potential for mutualistic relationships between bacteria and plants in agriculture (19).

Little research has been done on duckweed in the *Wolffia* genus and specifically the effects of the environment on their properties. The purpose of this study was to investigate the effects of nitrogen- and phosphorus-rich media and plant growth–promoting bacteria (PGPB)-enhanced media on the growth rate and nutritional value of *Wolffia globosa*. We chose to study *Wolffia globosa* due to its abundance and popularity to be used as a food source in Thailand, and the lack of sufficient research focus on *Wolffia* species. Since nitrogen and phosphorus are the main components of fertilizers that lead to eutrophication, and duckweeds can effectively absorb these nutrients, along with PGPB's ability to enhance nutrient uptake, we hypothesized that the addition of phosphorus and PGPB to the growing media would significantly increase duckweed biomass production due to phosphorus being components of significant intermediates in both cellular respiration and photosynthesis, and growth-promoting properties of PGPB. We also hypothesized that the addition of nitrogen would increase protein content due to nitrogen being a direct component in chlorophyll, promoting photosynthetic activity and increasing macromolecular composition (15, 19). We conducted our research by simulating eutrophication in the growth medium and using commercialized PGPB to create a mutualistic environment. The growth rate was then collected from analyzing the change in duckweed density. We also measured the final duckweed biomass and protein content.

The hypothesis was supported by our results, which showed that the protein content and biomass production of duckweed significantly increased in the nitrogen-rich group and the phosphorus-rich group, respectively, and that biomass production was significantly boosted in PGPB-enhanced groups. Our research may provide an environmentally friendly approach to the optimization of high-protein duckweed cultivation, which could reduce the global food shortage sustainably and cost-effectively.

RESULTS

Due to the scarcity of research on duckweed in the *Wolffia* genus and specifically on the effects of the environment on their properties, we hypothesized that by studying environmental factors like nitrogen, phosphorus, and PGPB, we could enhance the biomass production and protein content of duckweed. Therefore, our research investigated the effects of nitrogen-rich, phosphorus-rich, and PGPB-enhanced media on duckweed biomass production and protein content. Over a span of 15 days, duckweed was cultured in plastic tubs with water at pH 7 and 28°C, under a light intensity of 12,500 lux and a 12-hour light/12-hour dark photoperiod (**Figure 1**). We monitored the growth rate of duckweed and quantified the net biomass (the increase from the initial mass at day 0) and protein content as a percentage of dry weight at the end of this period. We averaged the data and then performed statistical analyses to determine differences.

Effects of nitrogen- and phosphorus-rich media on duckweed

We prepared four different experimental groups: a control group, a nitrogen-rich group, a phosphorus-rich group, and a combined nitrogen- and phosphorus-rich group. The nutrients were dissolved into the culturing media with nitrogen and phosphorus at concentrations of 43.7 mg/L and 7.8 mg/L, respectively. These nutrient concentrations were specifically chosen to replicate conditions characteristic of eutrophication based on 53 global wastewater quality datasets (21). To effectively measure the growth rate over a large area, we chose to analyze the changes to chroma values in duckweed images based on previous research (23) (**Figure 2A**). The growth rate of duckweed was assessed by photographing the plants every three days under consistent lighting conditions and analyzing the average color values using the color space, defined by the International Commission on Illumination (CIELAB) **(Figure 3)**. CIELAB is a three-dimensional color space based on human perception, comprising three values: L* for lightness, and a* and b* for the red-green and blue-yellow color axes, respectively (23). Given that CIELAB is grounded in human perception, it was used to calculate the chroma values of the duckweed samples. Since the duckweed tubs are black, an increase in chroma value (the color has a higher intensity and saturation) would indicate increase in density, which indicates duckweed growth (23). After 15 days, the control group experienced an average change of chroma value at 0.279/day. The duckweed grown in nitrogen-rich media and those in nitrogen- and phosphorus-rich media displayed statistically insignificant differences from the control group, with the chroma value increasing 0.442/day (p = 0.565, one-way ANOVA) and 0.511/day (p = 0.329, one-way ANOVA), respectively. As expected, the phosphorus-rich

Figure 1: The cultivation of duckweed (*Wolffia globosa*). Wolffia globosa were grown in plastic tubs (93 x 128 x 28 cm) filled with water maintained at a level of approximately 15 cm inside a controlled room for 15 days.

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media compared to the control group significantly elevated the change in chroma value to 0.854/day (p = 0.009, one-way ANOVA) after the 15-day period.

We then evaluated the net biomass of duckweed after 15 days by subtracting the initial weight from the final weight (**Figure 2B**). The control group had an average net biomass of 68.96 grams. Both nitrogen-rich and the combined nitrogen- and phosphorus-rich groups presented an insignificant increase in net biomass compared to the control group, with values of 111.80 grams (p = 0.217, one-way ANOVA) and 125.25 grams (p = 0.085, one-way ANOVA), respectively. Additionally, the phosphorus-rich group compared to the control group had a significantly increased net biomass of 203.36 grams (p = 0.001, one-way ANOVA), supporting our hypothesis.

Next, we analyzed the protein content of the duckweed after 15 days. The control group had a protein content of 27.41% dry weight (**Figure 4**). The nitrogen-rich group exhibited the most substantial increase in protein content compared to the control group, reaching 52.76% dry weight (p = 0.001, one-way ANOVA). It was closely followed by the combined nitrogen- and phosphorus-rich group with a protein content of 52.50% dry weight (p = 0.001, one-way ANOVA), while the phosphorus-rich group exhibited a protein content of 41.58% dry weight (p = 0.029, one-way ANOVA) when compared to the control group.

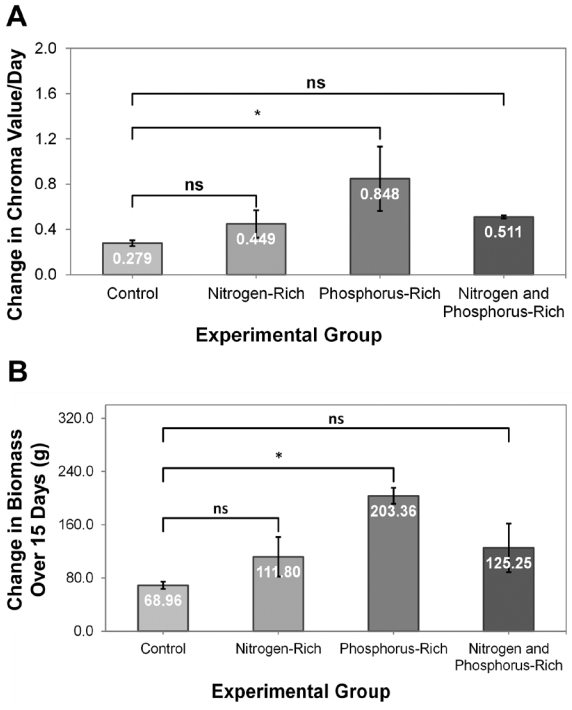

Figure 2: Changes in chroma value and biomass of duckweed (*Wolffia globosa*) under nutrient-rich conditions. The height of the bar represents the mean, and the error bars depict the standard deviation (SD) (A) The change in chroma value of duckweed (the relative growth rate) of the control group, nitrogen-rich group, phosphorus-rich group, and the combined nitrogen- and phosphorus-rich group (n = 3). (B) The net biomass of duckweed cultured in the control group, nitrogen-rich group, phosphorus-rich group, and the combined nitrogen- and phosphorus-rich group (n = 3). One-way ANOVA was used to analyze the statistical significance; p > 0.05 (ns) and p < 0.05 (*).

Effects of PGPB-enhanced conditions on duckweed

Recent research shows that some bacteria have plant-growth-promoting properties. There are many species of PGPB available for experiment. However, we selected three species sold commercially (19). We created three experimental groups: a control group, a PGPB-I group consisting of *Azospirillum brasilense* and *Burkholderia vietnamiensis*, and a PGPB-II group comprised of *Azospirillum brasilense* and *Gluconacetobacter diazotrophicus*. Upon examining the growth rate of duckweed, we found that the control group demonstrated a chroma value change of 0.465/day (**Figure 5A**). In contrast to this, both PGPB-I and PGPB-II groups showed significant increases in their chroma values when compared to the control group. Specifically, PGPB-I exhibited a change of chroma value at 1.253/day (p < 0.001, one-way ANOVA), while PGBB-II showed an increase of chroma value at 1.148/day, aligning with our hypothesis (p < 0.001, one-way ANOVA).

We then analyzed net biomass in each of the duckweed conditions, the control group yielded an average net biomass of 82.70 grams (**Figure 5B**). As expected, when compared to the control group, the PGPB-I group exhibited the highest net biomass output at 310.11 grams (p = 0.005, one-way ANOVA), which was comparable to that of the PGPB-II group, which had a net biomass of 284.66 grams (p = 0.009, one-way ANOVA). Finally, we examined the protein content of duckweed (**Figure 6**). The control group displayed a protein percentage of 30.00% dry weight. The PGPB-I group exhibited an insignificant increase in protein content of 34.67% dry weight (p = 0.548, one-way ANOVA), while the PGPB-II group achieved a slightly significant increase to 35.44% dry weight (p = 0.454, one-way ANOVA) when compared to the control group.

DISCUSSION

Despite advances in agriculture and technology, research on *Wolffia* duckweed, particularly concerning the effects of environmental factors on enhancing its properties as a high-protein functional food, remains limited. We hypothesized that studying environmental factors such as nitrogen, phosphorus, and plant growth–promoting bacteria (PGPB) could enhance the biomass production and protein content of duckweed. This hypothesis is supported by literature indicating that these nutrients are critical components in essential processes like cellular respiration and photosynthesis in duckweed, and that PGPB possess growth-promoting properties (15, 19). Therefore, our research investigated the effects of nitrogen-rich, phosphorus-rich, and PGPB-enhanced media on duckweed biomass production and protein content over a period of 15 days.

The results from this study suggest that duckweed growth is significantly enhanced in a phosphorus-rich medium, as seen through the highest change observed in chroma value. Furthermore, there was also an increase in net biomass in the phosphorus-rich group. These results align with our initial hypothesis that the addition of phosphorus to the growing medium would significantly enhance duckweed biomass production. However, the data collected from the combined nitrogen and phosphorus group did not exhibit a significant difference in biomass production when compared to the control group. This unexpected outcome could be because of the complex dynamics of duckweed growth, particularly the decrease of biomass production when duckweed is introduced

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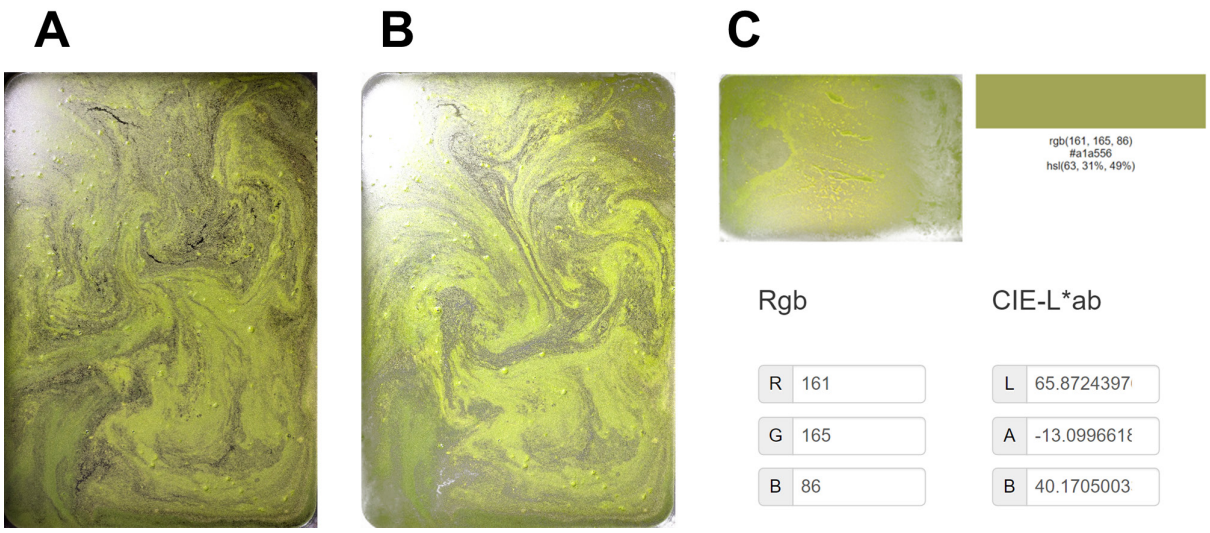

Figure 3: The process of chroma value evaluation in duckweed images. (A) Duckweed tubs were photographed every three days. (B) The duckweed in the images were isolated in Adobe Photoshop 2020 to remove background color and find the average RGB value of the image. (C) The RGB value were translated to the CIELAB value using an external website (https://colormine.org/convert/rgb-to-lab).

to a nitrogen-rich medium.

Additionally, due to limitations regarding equipment and time constraints, we were only able to examine one concentration level for each nutrient. Further studies are required to confirm the data, as the number of samples tested may have not been sufficient to fully reveal trends in the larger population.

The protein content of duckweed grown in nitrogen-rich media was significantly increased. Thus, our initial hypothesis was partially supported, as nitrogen-rich media did lead to the most increase in protein content as expected. However, phosphorus-rich media, both alone and in combination with nitrogen, also demonstrated significant increases in protein content. This can be attributed to the biochemical and metabolic functions of nitrogen and phosphorus. Studies showed that

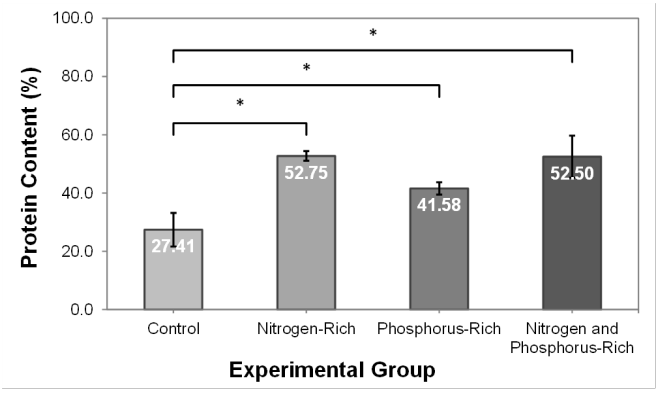

Figure 4: Changes in protein content of duckweed (*Wolffia globosa*) under nutrient-rich conditions. The height of the bar represents the mean, and the error bars depict the standard deviation (SD). The protein content of duckweed cultured in the control group, nitrogen-rich group, phosphorus-rich group, and the combined nitrogen- and phosphorus-rich group (n = 3). One-way ANOVA was used to analyze the statistical significance; $p > 0.05$ (ns) and $p < 0.05$ (*).

nitrogen is directly a component in chlorophyll, promoting photosynthetic activity and increasing macromolecular composition such as protein, while phosphorus is a component of significant intermediates in processes such as cellular respiration and photosynthesis, which may also have effects on increasing protein composition (15, 19).

We also examined the effects of PGPB on duckweed growth. Both PGPB-I (consisting of *Azospirillum brasilense* and *Burkholderia vietnamiensis*) and PGPB-II (consisting of *Azospirillum brasilense* and *Gluconacetobacter diazotrophicus*) significantly enhanced the growth rate of duckweed, as seen from the high change in chroma value. Additionally, the net biomass of the duckweed was also significantly boosted after 15 days, for both the PGPB-I-and PGPB-II-enhanced groups. These results aligned with our hypothesis that the addition of PGPB would significantly enhance duckweed biomass production and support previous findings that PGPB can promote growth using mechanisms such as enhancing plant nutrient uptake via nitrogen fixation (19).

While PGPB significantly improved in duckweed growth, it did not have a significant impact on its protein content. This may be because, although PGPB enhanced nutrient uptake and contributed to biomass increase, the limited nitrogen availability in the control medium restricted the bacteria's nitrogen fixation, preventing a corresponding increase in protein content. More research using different concentrations of nitrogen is needed to investigate the specific influence of PGPBs on enhancing protein content within duckweed.

We predicted that a potential source of error could stem from the chroma value analysis program. Despite its development based on a previous study (23) and our efforts to control variables such as angle and lighting, the program still processes images we captured, which could be influenced by subtle variations in lighting and angle. Future research should focus on refining image analysis techniques to reduce potential bias and on exploring the long-term impact of nutrients and PGPB on duckweed growth and protein production.

Despite some unexpected results during this study, we

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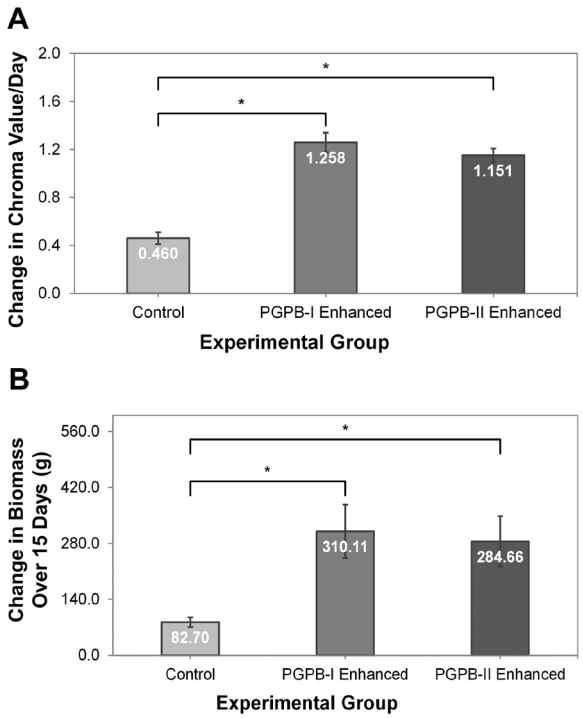

Figure 5: Changes in chroma value and biomass of duckweed (*Wolffia globosa*) under PGPB-enhanced conditions. The height of the bar represents the mean, and the error bars depict the standard deviation (SD). (A) The change in chroma value of duckweed (the relative growth rate) cultured in the control group, PGPB-I-enhanced group, and the PGPB-II-enhanced group (n = 3). (B) The net biomass of duckweed cultured in the control group, PGPB-I-enhanced group, and the PGPB-II-enhanced group (n = 3). One-way ANOVA was used to analyze the statistical significance; $p > 0.05$ (ns) and $p < 0.05$ (*).

explored how eutrophicated water and the application of PGPBs can optimize both *Wolffia globosa* growth and protein content for the production of superfoods. Moreover, the present study is the first to investigate the effect of PGPB on *Wolffia globosa* growth and protein content. It is evident that duckweed has the potential to serve as a sustainable and cost-effective solution to address global food shortages. In the future, the agricultural industry must continue to advance research and innovations to ensure that hunger crises, along with the current challenges of global warming, may one day be eradicated.

MATERIALS AND METHODS

Duckweed Acquisition and Preliminary Preparation

Duckweed (*Wolffia globosa*) were sourced from a local private farmer in Chonburi, Thailand. Before incubation, the duckweed were repeatedly washed with filtered water to remove bacteria, algae, and other compounds. The plants were then cultured inside plastic tubs (93 x 128 x 28 cm) filled with water maintained at a level of approximately 15 cm for 15 days. The parameters were set to 28 °C for temperature, light intensity of 12,500 lux, a 12-hour light/12-hour dark photoperiod, and a pH level of 7. The control cultivation medium consisted of a mixture of 16-16-16 nitrogen-phosphorus-potassium fertilizer (Chemrich, Thailand) and filtered water at a ratio of 1 g of fertilizer in 10 L of water. All plant cultivations used as controls in this study were conducted under the same conditions as described above, unless stated otherwise.

Experimental Groups

After the incubation period of 48 hours in separate tub filled with the control solution, fresh duckweed was evenly distributed among four groups, with three replicates per group, each containing 180 grams of duckweed, to investigate the effect of nitrogen- and phosphorus-rich media. The groups included a control group (nitrogen and phosphorus concentration of 3 mg/L and 2.5 mg/L, respectively), a nitrogen-rich group (nitrogen and phosphorus concentration of 43.7 mg/L and 2.5 mg/L, respectively), a phosphorus-rich group (nitrogen and phosphorus concentration of 3 mg/L and 7.8 mg/L, respectively), and the combined nitrogen- and phosphorus-rich group (nitrogen and phosphorus concentration of 43.7 mg/L and 7.8 mg/L, respectively). The nitrogen and phosphorus, in the form of soluble urea pills and monocalcium phosphate, respectively (Chemrich, Thailand), were added to the control water solution and were used as the experimental groups. The final concentrations described above accounted for the nitrogen and phosphorus already present in the water.

For the investigation into PGPB-enhanced conditions, fresh duckweed was evenly distributed among three groups, with three replicates per group, each containing 180 grams of duckweed. This included a control group, a PGPB-I group containing *Azospirillum brasilense* and *Burkholderia vietnamiensis*, and a PGPB-II group consisting of *Azospirillum brasilense* and *Gluconacetobacter diazotrophicus*. Each bacteria species was present in the media at an approximate concentration of 1.0 × 106 CFU/g. The commercially available PGPBs were purchased from the Department of Agriculture, Thailand. The PGPB was diluted in the cultivation medium at a ratio of 1:20 (PGPB:water) 24 hours before cultivation. The water added had a nitrogen and phosphorus concentration of 3 mg/L and 2.5 mg/L, respectively.

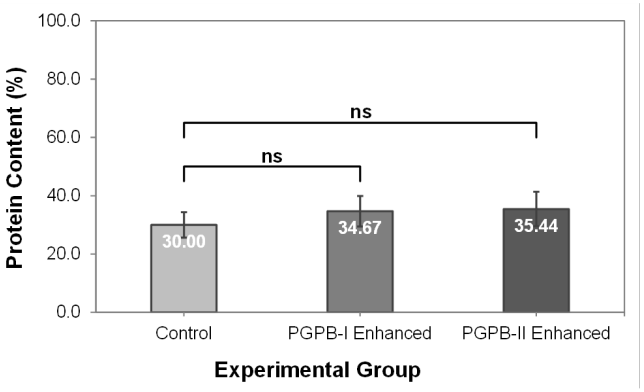

Figure 6: Changes in protein content of duckweed (*Wolffia globosa*) under PGPB-enhanced conditions. The height of the bar represents the mean, and the error bars depict the standard deviation (SD). The protein content of duckweed cultured in the control group, PGPB-I-enhanced group, and the PGPB-II enhanced group (n = 3). One-way ANOVA was used to analyze the statistical significance; $p > 0.05$ (ns) and $p < 0.05$ (*).

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Growth Rate Evaluation

To evaluate the growth rate of duckweed, the plastic tubs were photographed every three days and analyzed in Adobe Photoshop 2020 to find the average CIELAB color value of the duckweed (**Figure 6**). The CIELAB values were then used to find the chroma value of the duckweed tubs (the distance of the color from the achromatic axis). Since the duckweed tubs are black, an increase in chroma value would indicate duckweed growth. The experiments were conducted indoors under controlled conditions to eliminate variations in sunlight, and the cameras were fixed on set stands to ensure consistency in image capture. The chroma value was calculated using the following formula.

$$C^* = \sqrt{(a^*)^2 + (b^*)^2}$$

In this equation: C^* = chroma value, a^* = red/green coordinate, and b^* = yellow/blue coordinate. The change in chroma value (ΔC^*) was used to evaluate the growth rate of duckweed, which was determined by calculating the slope of the linear regression line fitted to the ΔC^* values over time. This method was adapted from the research of Azetsu & Suetake (23).

Net Biomass Evaluation

To evaluate the net biomass of duckweed after 15 days, the plants were harvested and placed on a cloth to allow excess water to drain off from the fronds. The final biomass was weighed using a precision scale (FR-H-1000 digital scale, E-scale). The net biomass was calculated using the following formula.

$$Nb = tb - ib$$

In this equation: *Nb* = net biomass gained after 15 days, *tb* = total biomass of the duckweed obtained after 15 days, and *ib* = initial biomass of the duckweed, which equaled 180 grams.

Protein Content Evaluation

To evaluate the total protein content of duckweed after 15 days, the biomasses were analyzed by Central Laboratory Co., Ltd. in Bangkok, Thailand using an in-house method based on AOAC Official Method 994.12 (2000). The nitrogen content of the samples was determined using the Micro-Kjeldahl method with a conversion factor of 6.25 to calculate crude protein (24).

Statistical Analysis

The experiments followed a CRD with three replications. IBM SPSS Statistics 29.0 was used to conduct One-Way ANOVA for statistical analysis and Tukey's Honest Significant Difference to identify significant differences ($p < 0.05$) across samples.

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