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Development of *in-vitro* propagation technique for onion (*Allium cepa* L.) regeneration

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Abstract Tissue culture media with pH 5.0 resulted in the tallest plants for onion at 25.09 cm on Day 10 and maximum leaf count on Day 14, yet shoot proliferation was best observed at pH 6.5 with 2–3 shoots per plantlet. Thiamine supplementation always improved plant height, which was up to 17.33 cm, while indole-3-acetic acid (IAA) triggered more shoot formation of 2–3 shoots on Day 14. The other hormones, naphthaleneacetic acid (NAA), gibberellic acid (GA), and kinetin, had moderate growth responses. Controlled environmental conditions also impacted plantlet showed the performance, with refrigerator conditions yielding plants of up to 20.76 cm on Day 21 and dark conditions eliciting the greatest elongation at 37.36 cm. Light exposure, by contrast, favored more developed shoot and leaf growth. Temperature control, especially cooler conditions under initial growth, favored leaf emergence and plant height, while room temperature maintained overall growth performance. Collectively, these results confirmed the important role of pH of the culture medium, certain plant hormones, and controlled environments in optimizing onion tissue culture. The outcomes are offered empirical evidence that is useful for enhancing micropropagation protocols, which can be extended to onion production systems, as well as future genetic breeding programs focused on maintaining and increasing onion yields under controlled conditions

Keywords: In-vitro, Nueva Ecija, Onion, Production, Regeneration

Introduction

Onion is a highly valued horticultural crop worldwide, consumed not only on special occasions but also for everyday use. Onion (*Allium cepa* L.) is a high-value crop of significant economic importance in Central Luzon. In 2019, the Philippines' onion production volume totalled approximately 222.1 thousand metric tons and a total production value of 6.7 billion pesos in 2018. The

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production cost per hectare of onions reaches around 148,212 for red onions and 102,590 for multipliers. Region 3-Central Luzon remains one of the top-producing regions of the country, contributing to the total volume of onion harvest, which is highly concentrated in Luzon. Nueva Ecija, commonly known as "Rice Granary of the Philippines", has also been the number one onion producer for decades among the 22 onion-producing provinces of the country, accounting for around 115,474.37 MT (62.65%) of the total onion harvest in the whole region (PSA, 2017).

The province holds 98% of regional onion production, with the remainder from Tarlac (PSA, 2020). Five municipalities, namely Bongabon, Rizal, Laur, Gabaldon, and Talavera, account for most of the onion output from this province (Gavino *et al*., 2020). Despite its significant impact on the local economy, onion production faces several challenges such as susceptibility to unfavorable weather conditions, susceptibility to pest and disease attacks, high costs of production due to relatively high inputs, labor-intensive postharvest handling practices, as well as smuggling and a high risk for huge losses.

Common onion varieties cultivated in the province are red creole, yellow garnet, and multipliers or shallots. However, its value chain became an interesting area when market prices were hiked to as high as 300 to 450 pesos per kilo in 2022. For years, a limited amount of research focusing on improving onion production in the country has been noticeable despite its evident importance as a highly valued crop. On the part of the local and national governments, programs for onion producers to upgrade production quality and increase income have been implemented. According to the Philippine News Agency in 2020, to further enhance the facilities and programs for High-Value Crops Development, the Department of Agriculture allocated an additional 94.6 million pesos focused on crucial onion farming in Nueva Ecija.

Onion (*Allium cepa* L.) can be propagated either from seeds or bulbs, though onion seeds used in production here in the country are obtained through importation only. There is no known onion seed producer in the Philippines. The reason why onion propagation is still a challenge may also be because the entire crop cycle takes almost two years to complete a single seed cycle. This longer duration simply means a tremendous investment of time, labor, and cost, and explains why only a few researchers are trying to perform studies focusing on its production. Relying on this long cycle involves significant risks, such as crop failure and market fluctuations, which can negatively impact overall productivity and profitability (Sidhu *et al*., 1992). Among the limitations in onion open-field propagation include low seed viability, high outcrossing, bulblet formation, plantlet dormancy, vitrification of tissues, and reduced renewability for natural vegetative multiplication (Passi and Awan, 2020).

Given these challenges, onion-focused research, such as in vitro propagation of onion seedlings for production, is not only timely but imperative, especially for the weakening onion industry of the province. In-vitro onion propagation allows mass production of healthy plantlets free from disease, rapid multiplication that leads to faster production cycles, and preservation of unique varieties. In addition, it aids research efforts to develop improved and new onion varieties that are less susceptible to pests, diseases, and environmental stresses (Basu and Reddy, 2018).

Other species within the *Allium* genus are now being reproduced through in vitro regeneration, including garlic (Robledo-Paz *et al*., 2000, Haque *et al*., 2003, Luciani *et al*., 2006), *Allium wallichii* (Wawrosch *et al*., 2001), and *Allium chinense* (Xu *et al*., 2008). Although there are studies concerning in vitro callus induction, shoot regeneration, and micropropagation (Martinez *et al*., 200; Khalid *et al*., 2001; and Kahane *et al*., 1992), this technique is not yet established for onion research and production in the country. Specifically, the study focuses on determining the optimal pH and combination of plant hormones for media preparation and identifying optimal physical factors and laboratory conditions, such as temperature and light exposure, to establish a baseline protocol for onion *in vitro* propagation and regeneration. Hence, this study aimed to develop an in vitro propagation technique for onion regeneration that can be used for both the production of healthy seedlings utilized to improve onion production in controlled environments and future applications in onion genetic breeding.

Materials and methods

This experiment was conducted at the Tissue Culture Laboratory, College of Agriculture, Nueva Ecija University of Science and Technology-Gabaldon Campus, Brgy. Poblacion, Gabaldon, Nueva Ecija, from September 2022 to August 2024.

Collection and preparation of explant

Healthy and disease-free mature onion bulbs constituted the source of meristems. Onion bulbs were washed with running tap water and the outer dry scales to ensure that there would be no dirt and soil residue, decreasing the possibility of contaminants. A modified method of plant tissue preparation (Hasnain *et al*., 2022) was used for disinfection and sterilization before inoculation. Washed bulbs were transferred to a sterilized container soaked in bleach and a liquid soap ratio of 10:1. After 10 minutes, the bulbs were washed with distilled water. For another five minutes, onion bulbs were soaked with

bleach and liquid soap at the same ratio. After soaking, the onion bulbs were cut to excise shoots. Excised explants were then sterilized by soaking them in a liquid soap for five minutes and then rinsed thrice with sterile distilled water. Then, explants were again soaked in 15% bleach solution for 10 minutes and then rinsed again three times with sterile distilled water. Final disinfection was done by soaking the explants in 70% isopropanol for 2 minutes and then rinsing with sterile distilled water, and they were ready for inoculation in sterile media.

Media preparation and inoculation of explants

Sterile media were prepared based on the method of direct formation of adventitious shoots obtained from explants of the basal plate (Zeng, S. 2010; George *et al*., 2008; and Hussey and Falavigna, 1980) using the Murashige and Skoog (1962) basic nutrient medium with modifications applied optimized for onion tissue culture plantlet production. Combinations of 0.04 sucrose, 0.004 Agar, 0.002 Activated charcoal, and 0.004 MS Media were used for the media base of 1 Liter. Experimental treatments, such as the determination of pH and a set of plant hormones, are incorporated into the process of media preparation.

The prepared media solution was boiled and stirred to dissolve the different components. After boiling, 5 mL of the prepared modified MS media was transferred to each 50 mL test tube using a 50 mL glass syringe. Cotton plugs were then put in each test tube to serve as a seal. All test tubes were then placed in polypropylene (PP) bags sealed with rubber bands before sterilization. Prepared media were subjected to sterilization using an autoclave at 121˚C at 15 psi for 30 minutes. After sterilization, the media prepared in the test tubes were cooled, and then 0.5 mL of streptomycin was added to each test tube to minimize the occurrence of contamination during experiments. Prepared sterile explants were then inoculated in prepared nutrient media in the test tube on the laminar flow following the basic aseptic technique procedure.

Determination of pH and plant hormone combination

Using the above media preparation, pH (Wang and Liu, 2015) was adjusted for each treatment from pH 5.0 to pH 7.0 with 0.5 intervals. Five treatments were implemented as follows: Treatment 1 (pH 5.0), Treatment 2 (pH 5.5), Treatment 3 (pH 6.0), Treatment 4 (pH 6.5), and Treatment 5 (pH 7.0). Moreover, different plant hormones (Zhao and Zhang, 2017) were also assessed, such as Treatment 1 using Naphthalene Acetic Acid (NAA), Treatment 2 with Gibberellic Acid (GA_3), Treatment 3 using Pyridoxine, Treatment 4 with

Kinetin, Treatment 5 using Indole Acetic Acid (IAA), and Treatment 6 (Thiamine). All treatments were replicated three times.

Exposure to different temperatures and light conditions

Once the pH and combination of plant hormones were determined, the media used for the subsequent experiment were adjusted based on the identified pH and combination of plant hormones. Then, explants were inoculated and incubated in tissue culture cabinets, exposing them to light conditions and being exposed to dark conditions (Zhang and Zhang, 2011). Furthermore, exposure to different temperatures was done by exposing the inoculated explants at room temperature (24°C to 31°C), air-conditioned (18°C to 23°C), and refrigerated temperatures (7°C to 12°C) based on the study of Kumar and Sharma (2018). As with previous experiments, all treatments were in triplicate.

Data gathering and analysis

Different plant growth parameters were observed, such as days to leaves, days to roots, days to shoot, plant height (cm), leaf number, shoot number, and root number at 3, 7, 10, 14, and 21 days for each experimental set up including determination of pH, combination of plant hormone, exposure to light and varying temperature. Data were gathered using a Vernier caliper for measurement of height, and other parameters were recorded in the data sheet. Data were analyzed for each factor using a Completely Randomized Design (CRD) with a comparison of means using Analysis of Variance (ANOVA) and post-hoc tests like LSD (Least Significant Difference) and Duncan's Multiple Range Test (DMRT).

Results

Effect of varying pH level in onion in-vitro propagation

Onion plantlets were assessed on their growth performance towards pH stress, alkalinity, and acidity tolerance, by growing them in a plant growth medium with varying pH levels, 5.0 to 7.0, as presented in Figure 1. Results indicated that plant growth, such as days to produce shoots, plant height, shoot number, and leaf number, is significantly influenced by changes in the pH of the plant growth medium. Maximum shoot production was observed on Day 1,0 while shoot production started as early as three days at a pH ranging from 5.0 to 5.5 (Figure 1A). The tallest plant height was recorded at pH 5.0 with 25.09 cm

on Day 10 of observation, while the shortest plant height was obtained from pH 6.0 (Figure 1B). Maximum leaf number was produced by Day 14, peaking at pH 5.0, and maximum shoot number at pH 6.5, both with the highest leaf and shoot number of 2-3 leaves, as shown in Figure 1C and 1D, respectively.

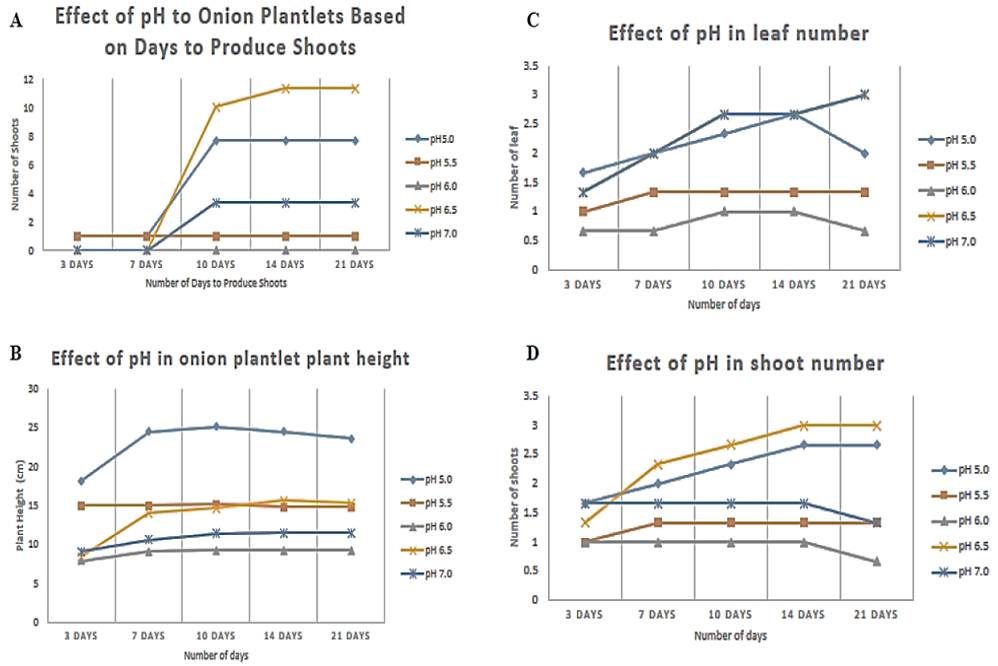

Figure 1. Effect of varying pH on the growth performance of onion plantlets

Effect of different plant hormones on onion in vitro propagation

The role of different plant hormones highly affected plant growth performance in both field and laboratory settings, such as onion in vitro propagation. Determination of the combination of plant hormones can result in optimal growth and development of onion tissues in a controlled condition. In Figure 2, the influences of six plant hormones, namely naphthalene acetic acid (NAA), Gibberellic Acid (GA), pyridoxine, kinetin, Indole acetic acid (IAA), and thiamine were assessed based on parameters for growth performance of onion plantlet (Figure 2). Among the growth performance parameters observed, plant height among onion plantlets showed a significant difference in response to the application of plant hormones.

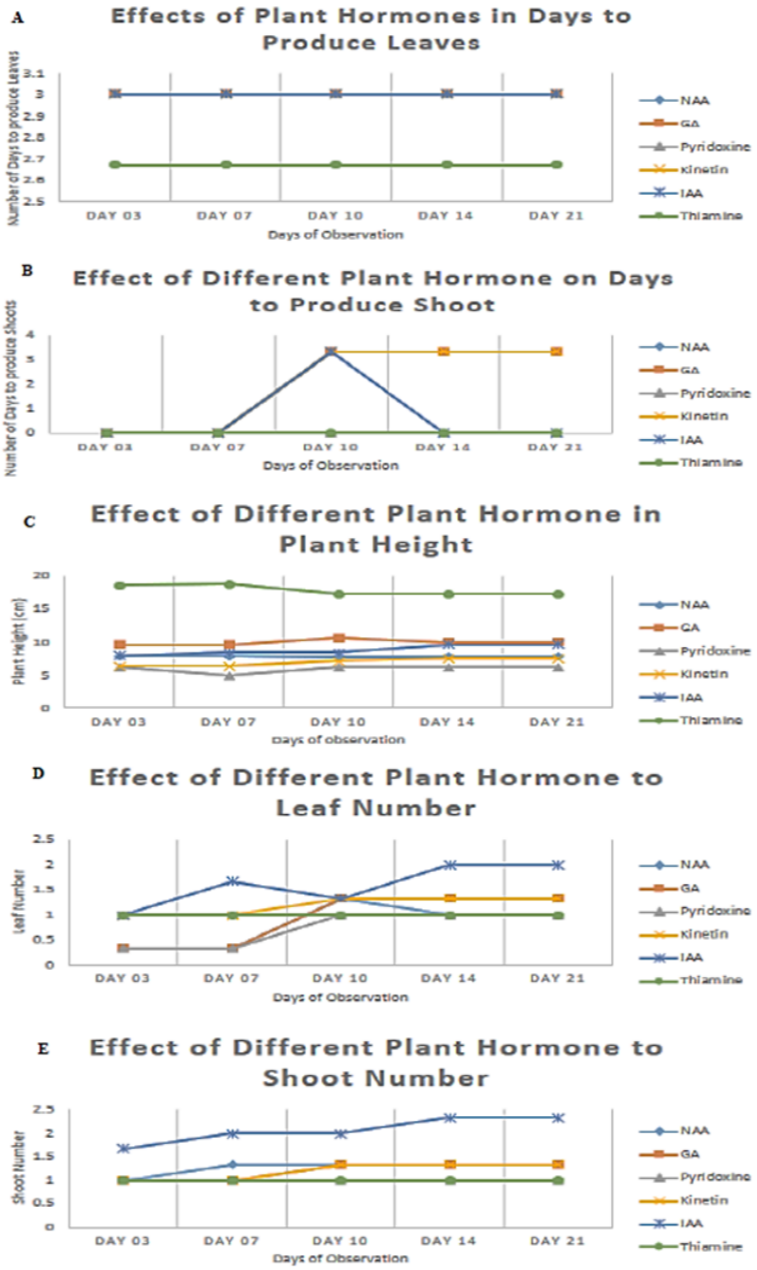

Figure 2. Effect of different plant hormones on the growth performance of onion plantlets

Thiamine revealed the earliest production of leaves in between 2-3 days (Figure 2A). On Day 10, the emergence of 3-4 (3.33) shoots was observed in treatments incorporated with NAA, GA, kinetin, and IAA (Figure 2B). The same pattern with days to produce leaves and shoots, applications of plant hormones such as NAA, GA, and thiamine had a variety of impacts on onion growth. Notably, thiamine induced the tallest plants at 17.33 cm by day 10 (Figure 2C), which was also observed consistently from Day 03 to 21. Moreover, the maximum number of leaves produced was 2 leaves recorded on Day 14 in the plantlets treated with IAA, as shown in Figure 2D. In terms of shoot number, the same pattern was revealed wherein IAA promoted the largest number of shoots at about 2-3 shoots (2.33) by day 14, followed by NAA and GA, which showed moderate shoot induction of 1-2 shoots (Figure 2E).

Influence of physical factors in onion in-vitro propagation

Temperature

In this study, onion plantlets were observed under three ranges of temperature such as room temperature, air-conditioned, and refrigerated temperature (Figure 3) revealed that exposing onion plantlet to air-conditioned temperature (18°C to 23°C) induced early emergence of leaves at 3 days after inoculation of explant (Figure 3A) while shoot emergence was noted between 3-4 days (3.33) after inoculation under treatment exposed to room temperature (24°C to 31°C) shown in Figure 3B. Meanwhile, the tallest plantlet was recorded under refrigerated temperature with 20.77 cm at 21 days. However, consistent tall plant height was noted under room temperature from Day 03 to Day 21 (Figure 3D). With the same pattern, the optimal number of leaves and shoots was observed at treatment under room temperature, with 2 leaves and shoots developed (Figures 3C and 3E). Moreover, shoot development under air-conditioned rooms also attains a maximum number of 2 shoots on Day 21.

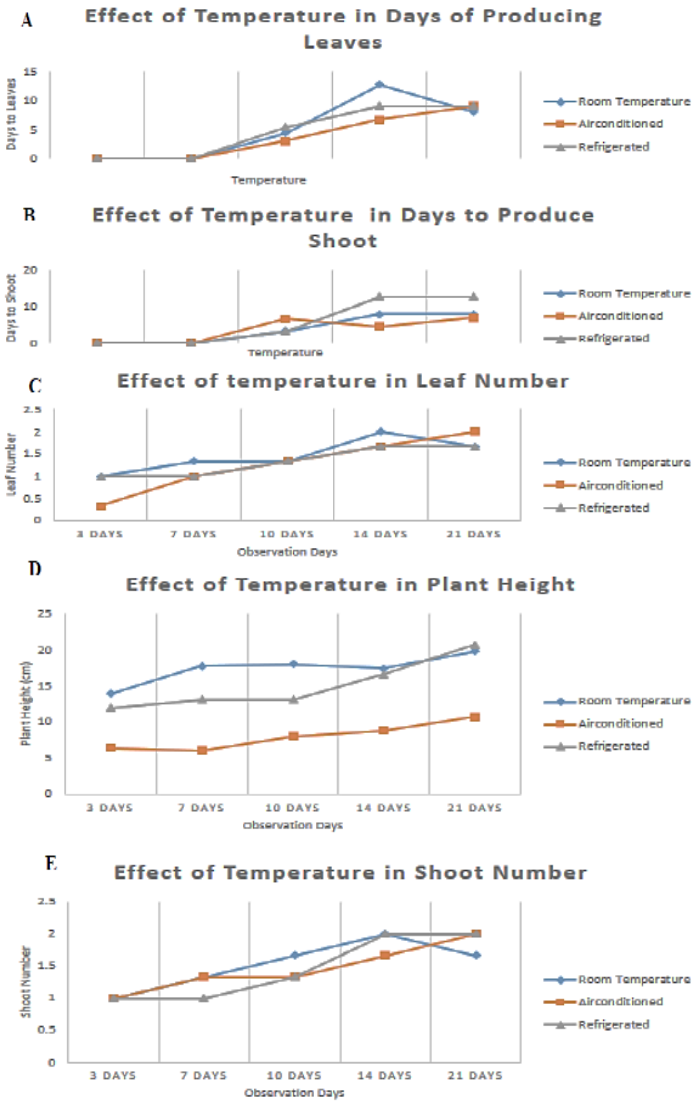

Figure 3. Influence of varying temperature on the growth performance of onion plantlets

Exposure to light

The parameters, such as leaf and shoot emergence, plant height, and leaf and shoot number of onion plantlets, were obtained under two treatments, light and dark conditions (Figure 4). In this study, treatment under lighted conditions showed earlier days of both leaves and shoot emergence, and a greater number of leaf and shoot developed (Figure 4A,4B,4C, and 4E) with 1 to leaves and

shoots produced as early as 2-6 days. In contrast, plant height was the tallest in dark conditions compared to the lighted condition, reaching 37.27 cm tall onion plantlet on Day 21 (Figure 4D), surpassing the lightroom condition, indicating that onions might grow better in darker environments during tissue culture.

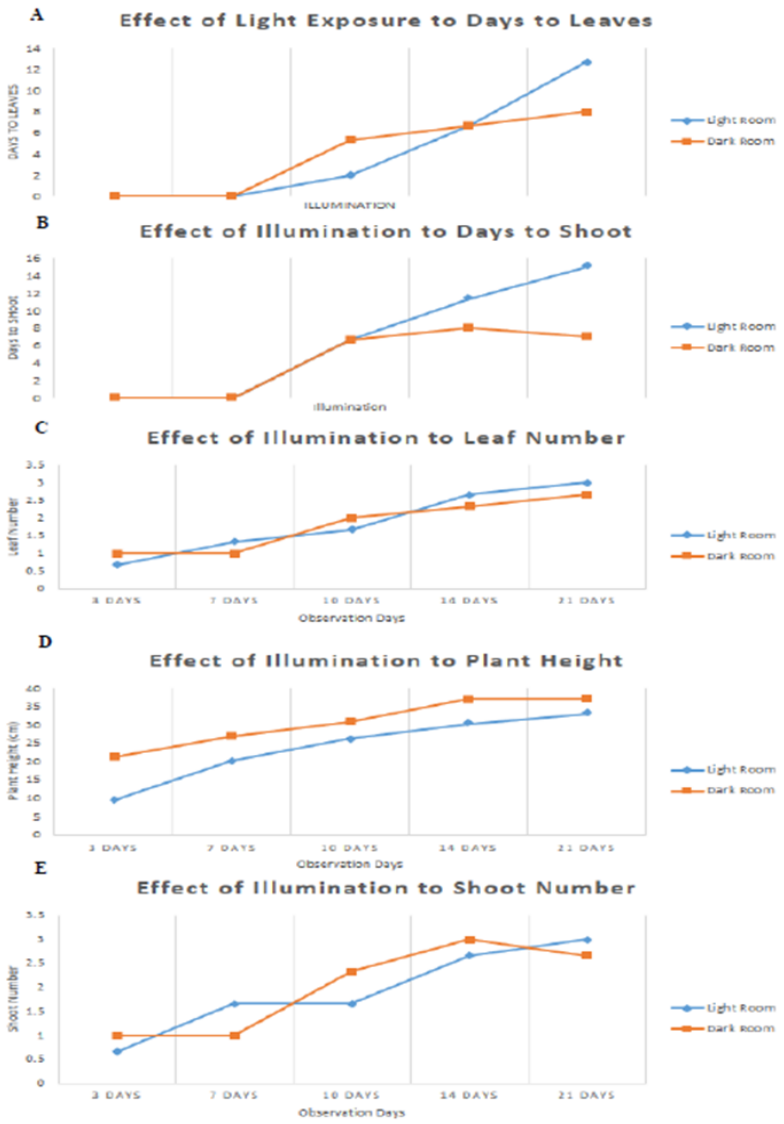

Figure 4. Comparison of the growth performance of onion plantlets in light and dark conditions

Discussion

This study showed that various components of growth medium, such as pH and a combination of plant hormones, can affect the growth performance of micropropagated plantlets as well as environmental factors such as exposure to light and different temperature ranges. Assessment of the effect of these factors can provide comprehensive data and a basis to develop an optimized in vitro propagation protocol for the production of onion plantlets. The result of this research will serve as a guide to promote practices in tissue culture improvement for an increased number of quality plantlets that can be used for sustainable agricultural management in onion production and utilization for research in plant biotechnology.

In plant tissue culture, pH is an important criterion for nutrient uptake and the action of enzymes. A major number of investigations have shown that the optimal pH value brings about a tremendous increase in in vitro growth. It has been reported that the optimum pH level affects root induction in many varieties. The highest root production in hydroponics is observed in *Allium cepa* at pH 5.5-6.5 (Savvas and Passam, 2002). Similarly, pH influences nutrient solubility and further affects cellular metabolism and plant development (George *et al*., 2008). The results of this experiment confirm the notion that the best way to grow plants in tissue culture is by keeping the pH environment balanced.

The effects of plant growth regulators in tissue culture are quite documented. For example, NAA and IAA fall under the category of auxins, which have been used to induce cell elongation, root initiation, and shoot proliferation. Cytokinin, such as kinetin, promotes cell division and shoot formation. Another hormone that plays a role in plant metabolism, particularly in the synthesis of carbohydrates, is thiamine, vitamin B1. However, the direct involvement of thiamine in growth parameters is far less significant than that of auxins and cytokinins (Thorpe, 2007). Research on other *Allium* species indicates that auxins promote root development, whereas cytokinins promote shoot formation (Rout and Samantaray, 2000). This literature agrees with the observed trends in growth responses. Variability in the in vitro effects of plant hormones requires that such an experiment be thoroughly and carefully conducted, focusing on optimizing hormone type as well as concentration, and hence, success in propagation with some increased growth is possible, considering the environment.

Various physical factors play a basic role in the successful in vitro propagation. Optimization and control of the variables can improve efficiency as well as increase yields in tissue culture practices. In this study, onion plantlets were subjected to light and dark conditions as well as varying ranges of temperature. The result suggests that exposure to temperatures in the range of

some lower temperatures can significantly promote leaf emergence and increase the plant height of the cultured onion tissue. Where desirable shoots and leaf development conditions are to be obtained, the growth medium should be kept at room temperature. The relationship of temperature to plant tissue culture is closely allied to metabolic activities (Zhao and Cheng, 2016). Lower temperatures often inhibit increased rates of respiration while accumulating carbohydrates, which is beneficial since they provide energy for growth, thus ensuring greater development of the plant as a whole (Bourget, 2008).

On the other hand, regarding onion tissue culture, research has been able to establish that low temperatures may favor shoot regeneration in in vitro conditions. It has been proven that one means of attaining these positive results is the reduction of oxidative stress in plant tissues. Low temperatures reduce damaging reactive oxygen species; thus, this maintains cellular integrity and encourages healthier cellular functioning (Oggema *et al*., 2007). To sum it up, this study finds that lower temperatures may support the early phase of growth by promoting leaf development and increasing plant height. However, the temperature itself has to be optimized during the culture period to sustain steady growth and good tissue development.

Plant development and growth are highly affected by the quality of light, on which the photosynthetic process depends (Vitale *et al*., 2022). In in vitro propagation, light is one important factor that dictates the growth, morphology, and regeneration of plant tissues that is needed for the success of any in vitro cultured plants (Fan *et al*., 2022). In Figure 4, parameters such as leaf and shoot emergence, plant height, and leaf and shoot number of onion plantlets were obtained under two treatments, light and dark conditions.

In support of these findings, light and dark condition separately affects specific growth metrics, such as changes in light conditions directly influence shoot and leaf development of plants, while exposure to a dark environment can boost other growth parameters like plant height (Kumar and Sharma, 2013). Exposure to light also impacts the efficiency of photosynthesis and the morphology of plants. In lower light conditions, there will be a high possibility for increased stem elongation since there is decreased activity in photosynthesis, according to studies of other plant species (Tsegaye *et al*., 2002). This is a crucial essential in understanding how such interactions between dark and light conditions would affect growth parameters that would help in the optimization of the onion tissue culture technique.

The result of this study is highlighted the optimal conditions for media preparation (pH and combination of plant hormones) and physical conditions (varying temperature and exposure to light) intended for onion in vitro propagation. The results suggested that optimizing tissue-cultured onions

involves maintaining pH levels around 5.0-6.5 to attain optimal plant height and promote shoot and leaf development. Combinations of plant hormones such as NAA, GA, kinetin, and IAA induce growth and development for the onion plantlets. For physical conditions such as temperature, regulating temperature from the cooler temperature at an early stage of plant development promotes leaf emergence and an increase in plant height, while the growth medium should be kept at room temperature to maintain growth performance. Meanwhile, the interaction of light and dark conditions has affected on different parameters, wherein the dark condition promoted taller plantlets and the light condition favored shoot and leaf development. Such results follow the literature established on tissue culture practices for other *Allium* species and offer new information to enhance onion tissue culture protocols for higher yield and better plant health.

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Conflicts of interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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