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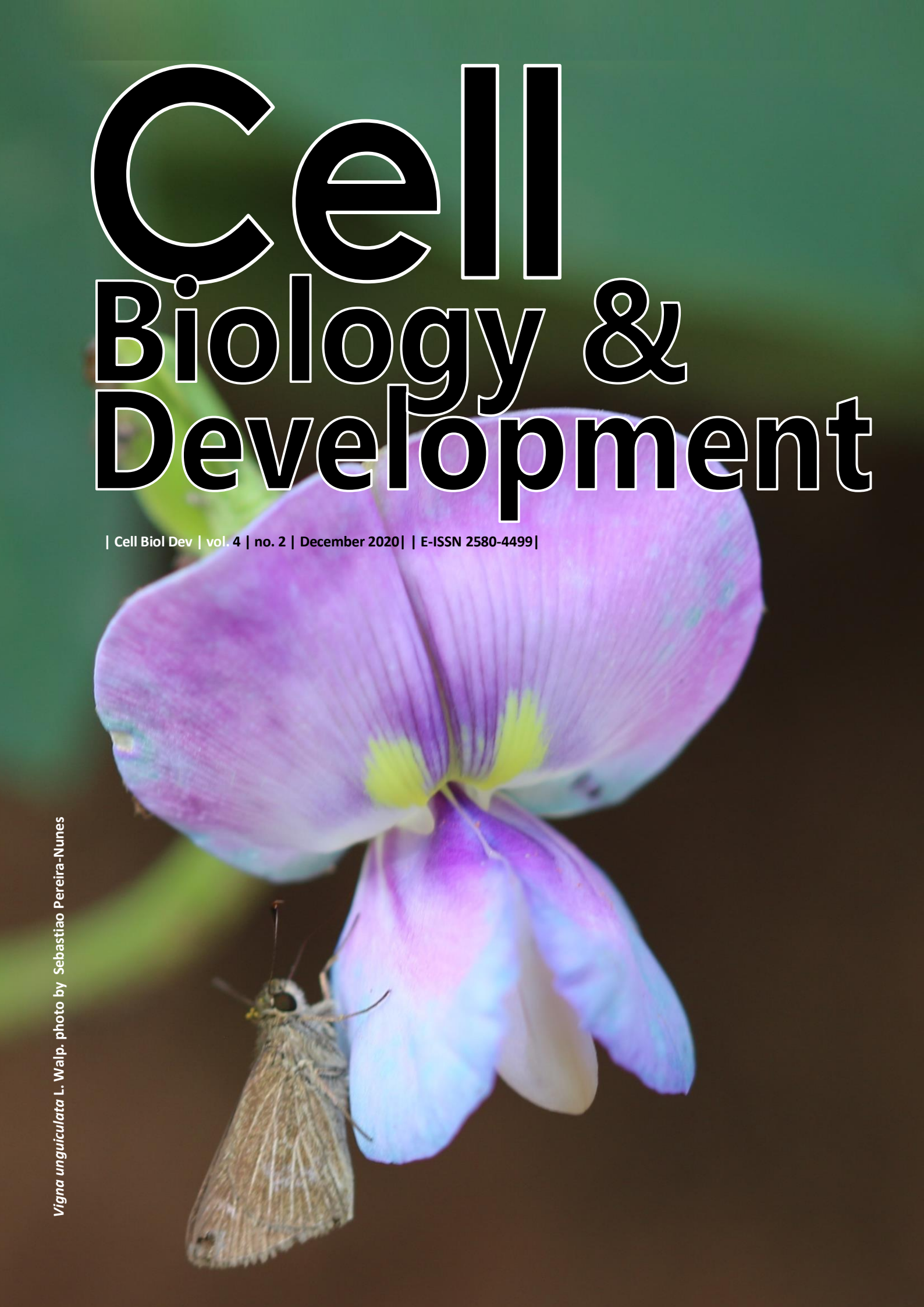

Vigna unguiculata L. Walp. photo by Sebastiao Pereira-Nunes

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Association of cowpea (*Vigna unguiculata* L. Walp.) (var. Ife Brown) with *Colletotrichum destructivum* O'Gara: A special reference to nutrients lost by the host **41-46**
DAVID NWAZUO ENYIUKWU, GRACE AMARACHI NWAOGU, INEMESIT N. BASSEY

Changes in color of betta fish (*Betta splendens*) by feeding of *Artemia salina* enriched with *Tagetes erecta* flower flour **47-51**
CHIKA ANNISA KISWARA, AGUNG BUDIHARJO, SITI LUSI ARUM SARI

Plant growth and total flavonoid content of *Sisyrinchium palmifolium* after light intensity and gibberellin treatment **52-63**
PUJI ASTUTI KISWANTARI ZUAINI, WIDYA MUDYANTINI, SOLICHATUN

Study of seed maturity level and duration of immersion in auxin solution on growth of *Anthurium hookeri* seedlings **64-70**
SONY PRIHANDONO, EDDY TRI HARYANTO, BAMBANG PUJIASMANTO

Factors influencing micropropagation and somatic embryogenesis of two cassava varieties, Kello and Qulle **71-81**
ROZA BERHANU, TILEYE FEYISSA

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Chapter in book:

Webb CO, Cannon CH, Davies SJ. 2008. Ecological organization, biogeography, and the phylogenetic structure of rainforest tree communities. In: Carson W, Schnitzer S (eds) Tropical Forest Community Ecology. Wiley-Blackwell, New York.

Abstract:

Assaeed AM. 2007. Seed production and dispersal of *Rhazya stricta***.** 50th Annual Symposium of the International Association for Vegetation Science, Swansea, UK, 23-27 July 2007.

Proceeding:

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Thesis, Dissertation:

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Information from internet:

Balagadde FK, Song H, Ozaki J, Collins CH, Barnet M, Arnold FH, Quake SR, You L. 2008. A synthetic *Escherichia coli* predator-prey ecosystem. Mol Syst Biol 4: 187. www.molecularsystemsbiology.com. DOI:10.1038/msb.2008.24

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Association of cowpea (*Vigna unguiculata* L. Walp.) (var. Ife Brown) with *Colletotrichum destructivum* O'Gara: A special reference to nutrients lost by the host

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Abstract. *Enyiukwu DN, Nwaogu GA, Bassey IN. 2020. Association of cowpea (*Vigna unguiculata *L. Walp.) (var. Ife Brown) with Colletotrichum* destructivum *O'Gara: A special reference to nutrients lost by the host. Cell Biol Dev 4: 41-46.* Cowpea or southern pea (*Vigna unguiculata* L. Walp.) is an important African grain and leafy vegetable. Anthracnose caused by *C. destructivum* represents a major biotic drawback to the profitable production of cowpeas in the incontinent. The disease culminates in the loss of grain yield, produce quality, and nutritional values of the crop. This work assessed and quantified the nutrients lost from the aerial organs of cowpea (var. Ife Brown) challenged by anthracnose disease in the field and storage 4 weeks after inoculation (WAI) by classical and spectrometric analyses. The results showed that the disease affected the nutrient contents of the inoculated crop's aerial organs, decreasing the mean quantities of all proximate constituents and major mineral nutrients such as calcium and phosphorus in the test plant materials. Amongst the aerial organs, fat was the most depleted nutrient (36.30%), followed by protein (28.52%), carbohydrate (26.67%), and crude fiber, with 20.04%, was the least. Generally, the highest mean loss of proximate nutrients due to anthracnose disease per organ of the test crop of 27.97% was recorded in the leaf sample, followed by 22.13% in the seeds and 18.03% in the husks was the least. Similarly, the highest mean mineral loss of 42.63 mg 100 g^{-1} occurred in the husks, followed by the stem (26.14 mg 100 g^{-1}), while the seed recording (15.41 mg 100 g^{-1}) was the least. Controlling this important fungal disease of the crop will undoubtedly result in better leaf, grain, and haulm quality, which will rub off as improved farm economy and public health in the continent.

Keywords: Anthracnose, nutrient loss, proximate composition, protein, elemental nutrients, cowpea

INTRODUCTION

Cowpea, or southern pea (*Vigna unguiculata* L. Walp.), is an important leafy vegetable or grain legume eaten in several varieties of food in tropical third worlds (Chikwendu et al. 2014; Igbatim et al. 2014; Enyiukwu et al. 2018). In bean-growing areas of West Africa, anthracnose (*Colletotrichum* spp.) is endemic, destructive, and affects all the aerial organs of the crop. Thus, the disease remains one of the major biotic challenges to economic cowpea production in the sub-region, especially in Nigeria (Adegbite and Amusa 2008; Sabo et al. 2014; Falade et al. 2018). *Colletotrichum destructivum* O'Gara has been identified as the causal agent of the disease in Nigeria (Ogu and Owoeye 2013; Awurum and Enyiukwu 2013). The pathogen is a hemibiotrophic fungus; which survives no-crop seasons as dormant mycelia or spores in bean debris or seed coats, cotyledon, and embryo of infected cowpea seeds (Begum et al. 2007). About 80%, 50%, and 88% of infection of *Colletotrichum* spp. has been reported from surveys on guava fruits, soybean, and cowpea seed lots in Nigeria, Brazil, and India, respectively (França Neto et al. 1989; Emechebe and Florini 1997; Amusa et al. 2006). Similarly, as high as 80-83% seed to the disease's seedling transmission has been reported from studies on common and lima beans in Brazil (Da Mota et al. 2019).

Under warm moist conditions or free moisture on cowpea surfaces; spores of the fungus germinate on susceptible varieties of the crop to initiate anthracnose (Latunde-Dada et al. 1999; Enyiukwu and Awurum 2013b). Affected cowpea develops small, round or irregular tan to brown, depressed lesions with characteristic black acervuli, bearing single-celled setae. Incidence of 83% and mean severity of 6 on a 10-point scale have been recorded on some accessions of cowpea in studies in eastern Nigeria (Enyiukwu and Awurum 2013b; Awurum 2013). Grain yield losses averaging 50% due to the disease have also been reported in Thailand and some parts of the humid rainforest agro-ecological zone of Nigeria; and this may be exacerbated to total crop failure in severe instances of attacks by the disease occasioned by very wet weather (França Neto et al. 1989; Begum et al. 2008, Enyiukwu and Awurum 2013b; Awurum 2014).

Crop pathogenic fungi, including *Colletotrichum* species sap, are susceptible hosts of vital nutrients, energy, and electrolytes to grow, reproduce, build their protoplasm and survive in the ecosystem (Amusa et al. 2006; Amadioha and Enyiukwu 2019a). *Colletotrichum truncatum* has been implicated in sapping electrolytes and a

variety of nutrients in infected seeds of legumes (Begum et al., 2013). Recently Amadioha and Enyiukwu (2019a, b) reported that the interaction of cowpea (var. IAR-48) with *C. destructivum* within 4 weeks resulted in mean protein losses, fat, carbohydrates, calcium, and phosphorus ranging up to 20-30 percent. The Ife Brown is a very popular and commonly grown variety of cowpea in humid southern Nigeria. The variety is known to be seriously affected and constrained by anthracnose. So far, however, there is little or no documented information on the effects of the association of *C. destructivum* on the proximate nutrient composition and basic electrolytes such as K, Na, Mg, and Ca of cowpea (var. Ife Brown),

Therefore, this paper assessed the interaction between anthracnose and cowpea (var. Ife Brown) in field and storage situations; with the aim of quantifying the level of depletion of proximate nutrients and electrolytes due to activities of *C. destructivum* in affected organs of the crop.

MATERIALS AND METHODS

Source of seeds and environmental parameters

The seeds of cowpea (var. Ife Brown) obtained from the Research and Training (R&T) Unit of the University were used for the study. The environmental parameters of the study location were 1,072.10 mm of rainfall over 60 days, temperature range of 29-33°C, and relative humidity of 80.0-87.0% within the study months of August-November, 2018. The soil type was sandy loam with organic carbon recorded at 68000 ppm, pH (water) of 4.17, and altitude of 121.08 meters above sea level (GPS Coordinates 2017).

Isolation and identification of the causal agent

Pods of infected cowpea (*Vigna unguiculata* (L.) Walp.) with typical anthracnose symptoms were collected from the University Research field. The pods were cut in bits using a surgical blade, sterilized in 70% ethanol, and washed in several changes of 200 mL of sterile distilled water. The tissues were plated in Petri dishes containing moistened Whatman No 1. filter paper and incubated for 7 days at 27°C. Then 39.50 g of dehydrated potato dextrose agar (PDA) (Oxoid® ThermoScientific Product, England, UK) was dissolved in 1,000 mL of sterile distilled water in a 2L flask, stirred thoroughly with a glass stirrer, then stoppered, and autoclaved at 15 Psi for 30 minutes. The mycelial growth from the plated tissues was repeatedly sub-cultured to obtain a pure culture of the organism maintained on PDA as prepared above. Finally, the isolate was subjected to pathogenicity tests by re-inoculating it into 2-week-old cowpea seedlings. About 4 days after inoculating the seedlings, typical anthracnose symptoms resembling those observed on the diseased pods were observed on the leaf blades and petioles of the seedlings (Ogu and Owoeye, 2013; Markson et al., 2014; Enyiukwu et al. 2020). Thus, confirming that the organism is pathogenic.

Slides of the organism were then prepared, mounted, and examined under a microscope. The organism's identity was confirmed to be *C. destructivum* with the aid of fungi identification manual by Barnett and Hunter (1995), annotated species of *Colletotrichum* by Damn et al. (2009; 2014), and monographs of the International Mycological Institute IMI (1995).

Preparation of spore suspension

The spores of the pathogen *C. destructivum* were collected from 8 days old culture-agar stock in a Petri dish by lifting a 60 cm^2 piece into a beaker containing 200 mL of sterile distilled water. Next, this spore was sieved through 4-folds of sterile cheesecloth to remove agar and mycelial mesh, and the filtrate was then centrifuged for 10 minutes. After that, the spore suspension was standardized using a hemocytometer counting slide to 10^5 spores mL^{-1} (Awurum and Enyiukwu 2013b; Alberto 2019).

Preparation of test cowpea materials

Four-week-old cowpea seedlings (var. Ife Brown) were grown in pots containing sterilized topsoil (4 kg). At this period, they were spray-inoculated with spore suspension (1 x 10^5 spores/mL of sterile distilled water) of *C. destructivum*. At eight weeks after planting (8 WAP), the percentage (%) incidence of anthracnose on the matured inoculated cowpea plants was calculated using the protocol adopted by Amadioha (2003). The control experiment of the cowpea plants was kept anthracnose disease-free with bi-weekly sprays with benomyl sprays (Awurum 2014). The percentage incidence of anthracnose on the test cowpea cultivar was calculated using the formula adopted by Amadioha (2003):

$$\% \text{ incidence} = \frac{\text{Number of plants affected by anthracnose}}{\text{Total number of plants assessed}} \times \frac{100}{1}$$

Healthy husks and seeds of cowpea (var. Ife Brown) (50 g) were soaked in spore suspension of *C. destructivum* (1 x 10^5 spores mL^{-1} of sterile water) for 1 day and then air-dried on sterile filter papers (Whatman No 1) placed on the laboratory bench for 24 h and incubated in the inoculation chamber for 3 weeks. The control (un-inoculated seeds) were similarly treated but soaked in sterile distilled water alone for the same period; after that, the specimens were separately enveloped and oven-dried at 60°C for 3 days.

One hundred grams of each specimen (leaves, stems, husks, and seeds) from both the infected and healthy specimens were weighed out with a digital balance and ground separately into powder using a hand milling machine (Corona Lavesh 250) (Amadioha and Enyiukwu 2019a, b). Each powder was stored in a dark, air-tight bottle and kept in the laboratory cupboards until required for biochemical analysis.

Biochemical analyses

Samples of healthy and infected cowpea organs (stem, leaf, husks, and seeds) were analyzed separately for protein, carbohydrate, fats, crude fiber, and ash contents after 4 weeks of inoculation. The proximate composition of both the healthy and infected test specimens was determined by the procedure adopted from the AOAC (2000) and Chikwendu et al. (2014); while the elemental nutrients were done by the absorbances of the individual

specimens from the atomic absorption spectrometer (AAS) (Model: AA 7000, Shimadzu, Japan) as described by Enyiukwu et al. (2018).

Determination of the effects of the association of the test fungus on cowpea specimens

The quantitative effects of the pathogenesis of the test fungus on the respective individual and mean nutrient contents of the cowpea materials were assessed and calculated using the formulae adopted by Amadioha (1994) and Amadioha and Enyiukwu (2019a, b) as:

$$\% \text{ loss of each nutrient per organ} = \frac{Ha - Hb}{Ha} \times \frac{100}{1}$$

Whereas the mean% loss of individual nutrients per organ or across organs was calculated as:

$$\text{Mean\% loss of each nutrient per organ or amongst organs} = \frac{\sum [Ha - Hb]}{Ha} \times \frac{100}{1}$$

Where:

Ha = value of a parameter in a healthy cowpea tissue(s)

Hb = value of a parameter in anthracnose-infected cowpea tissue(s)

$\sum$ = Summation of the values of parameters per organ or across organs divided by the number of variables (n) considered in the sum.

Data analysis

Data collected from this study were obtained in three determinations. First, they were analyzed by ANOVA (analysis of variance). The general linear model program (Genstat Release, Windows/PC Vista Version 12.10) at a significant level of 0.05 was used for the analysis. Means were separated and compared using Fisher's LSD at the probability of 5%.

RESULTS AND DISCUSSION

The results of this study showed that a mean percentage incidence of 78.21% of anthracnose disease was recorded on the *C. destructivum* inoculated cowpea (var. Ife Brown). The results of the major fingerprinting of nutrients of cowpea presented in Table 1 also showed that the disease affected the quantities of protein, carbohydrate, fat, crude fiber, ash, calcium, and phosphorus within and amongst the aerial organs of the assayed plant materials. Furthermore, it showed that fats (54.62% and 45.23%) and protein (38.92% and 26.30%) were the highest lost nutrients in the leaf and stem specimens, respectively. Also, carbohydrates recorded at (32.01%) and protein (31.04%) were the most depleted nutrients in the leaf and husk specimens, respectively, due to the activities of the fungus, in terms of loss of macro-elementals, Ca (26.05 mg/ 100 g and 34.69 mg/ 100 g) for the leaf and stem tissues and P recorded at (54.16 mg 100 g^{-1} and 21.04 mg 100 g^{-1}) for the husk and seed represent the most depleted elemental nutrient per organ of the test cultivar.

The results of the nutrient fingerprinting in Table 1 also show the comparative mean depletion of different individual proximate nutrients amongst the aerial organs of cowpea. Overall, it showed that the mean nutrient loss profile amongst the aerial organs of the crop was fat (36.30%) ≥ protein (28.52%) ≥ carbohydrate (26.67%) ≥ crude fiber (20.04%). Similarly, the nutrient profiling on the comparative mean depletion of different individual minerals amongst the aerial organs of the test cultivar revealed that P (27.75 mg 100 g^{-1}) was the most depleted element. However, it was statistically ($P \leq 0.05$) at par with 25.39 mg 100 g^{-1} recorded for Ca in the evaluation.

The mean comparative proximate and elemental nutrient composition per organ of the test crop is presented in Figure 1. The pathogen attack on the test cowpea caused the highest mean loss of proximate nutrients (27.97%) in the leaf specimen, followed by 22.13% and 21.61%, respectively, for the stem and seed samples, while the husk recording 18.05% was the least. Mean losses of 42.63 mg 100 g^{-} followed by 26.14 mg 100 g^{-} recorded in the husks and stem specimens of the inoculated cowpea represented the highest mean losses of minerals in the test organs, while the seed recording a mean loss of 15.41 mg 100 g^{-} was the least (Figure 1).

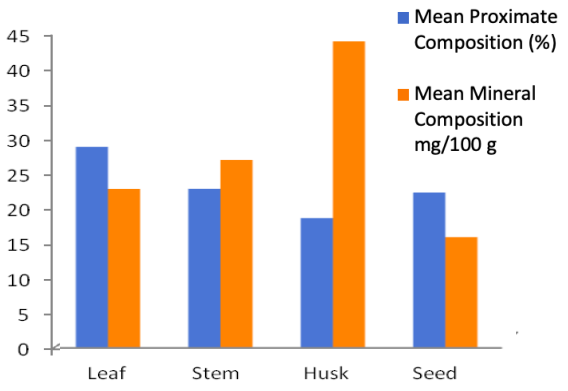

Figure 1. Mean percentage depletion of proximate nutrients (%) and minerals (mg 100 g^{-1}) per organ of cowpea Legend: Y-axis =% composition while X-axis = organs of cowpea

Table 1. Percentage nutrient loss in cowpea aerial organs due to activities of *Colletotrichum destructivum*

Nutrients	Nutrient loss (%) from					
	Leaf	Stem	Husks	Seeds		
		Proximate	Composition	(%)	Means	LSD (0.05)
Moisture content	10.28*	8.61*	10.00*	9.12*	--	0.11
Protein	38.92	26.30	17.83	31.04	28.52	4.41
Fat (lipid)	54.62	45.23	20.16	25.68	36.30	4.80
Carbohydrate	26.08	16.48	32.01	28.49	26.67	3.22
Ash	1.57	1.22	0.99	2.85	1.66	0.21
Crude fiber	18.66	21.43	19.08	20.97.	20.04	3.94
LSD (0.05)	6.28	5.13	4.20	3.81		
		Elemental	Minerals	(mg/100 g)		
Calcium	26.05	34.69	31.09	9.77	25.39	3.06
Phosphorus	18.19	17.58	54.16	21.04	27.75	2.89
LSD (0.05)	0.21	0.17	0.44	0.18	3.45	-

Note: Data are means from 2 separate experiments. *represents% increase

Discussion

The association and infection of cowpea in the field and storage with *Colletotrichum destructivum* have been reported (Enyiukwu and Awurum 2013a, b; Awurum et al. 2014). The results of this study indicated up to 78.21%% incidence of anthracnose in the inoculated cowpea plants. Moreover, this agrees with submissions of other workers who reported a high incidence (80%) of anthracnose (*C. gloeosporioides*) on guava fruit grown in humid Southwest Nigeria (Amusa et al. 2006). It is also consistent with the reports of a high incidence of 83% due to *C. destructivum* induced anthracnose on cowpea in humid southeast Nigeria (Enyiukwu and Awurum 2013b) as well as over 50% incidence due to brown blotch − a closely related disease to anthracnose − caused by *C. truncatum* recorded on soybean in Brazil and Thailand (França Neto 1989). Furthermore, Amusa et al. (2006) observed high humidity and rainfall to have encouraged a high incidence of anthracnose (*C. gloeosporioides*) on guava fruits grown in Ibadan, Southwest Nigeria. Hence, the high ambient temperature, humidity, and rainfall in Umudike, Southeast Nigeria, during the study period, amongst other environmental factors, may partly explain the high incidence of the disease recorded on the crop in this study.

High reductions of all proximate compounds were observed on *Colletotrichum destructivum*-infected aerial organs of the test crop in this study. Furthermore, this is in agreement with reports by Naikoo et al. 2013 who found marked depletions of energy, sugars, and lipids due to storage mold on three varieties of groundnut (Arachis hypogeal L.). The high loss of proximate nutrients (fat, protein, carbohydrate, and crude fiber) from the individual aerial organs and high percentage mean loss of same across the different organs of cowpea uphold that the pathogen is very destructive in deriving energy, amino acids, and other growth factors for its metabolic activities from all parts of the host crop. This finding thus sustained the views of Amusa et al. (2006), Begum et al. (2008; 2013), and Abd-Allah et al. (2018) that *Colletotrichum truncatum*, *C. gloeosporioides*, *Alternaria alternata*, *Fusarium spp. Aspergillus* spp. and some other storage fungi deprived lupine, pea, soybean, common bean seeds, and guava fruits of the same factors during infection in the field and storage. It also agrees with submissions of Amadioha and Enyiukwu (2019a, b), who reported up to 20-30% mean proximate nutrient losses from the interaction of aerial organs of another cultivar of cowpea with *C. destructivum*.

Calcium and phosphorus foster proper cell division and growth, maintain cell wall rigidity and tissue integrity, and resist pathogenic invasion (Better Crops 1999; Easterwood 2002; Imran et al. 2016). The high loss profile of calcium and phosphorus, especially in the husks and stems of the infected host, is in tandem with findings from Begum et al. (2008) and Abbasi et al. (2013). They observed marked reductions of these factors in their studies on the association of *C. truncatum* with soybean and groundnuts, respectively. Therefore, this suggests that the pathogen deprived the host of electrolytes (Amusa et al. 2006) needed for the formation and accumulation of xylans, lignans, and interlocking cross-walls in the host tissues; and thus impaired structural integrity and strength in the matrices of the infected host tissues (Easterwood, 2002; Amadioha and Enyiukwu, 2019a). This depletion of vital mineral factors may explain the girdling, tipping over and breakage of the stems, branches, and pods due to the disease observed in this study.

In all four organs of cowpea tested, the mean percentage losses of both proximate and mineral compositions (Ca and P) were higher in the leaves than in the seeds (Figure 1). This finding agrees with the assertions by Amadi and Oso (1996) and Amadioha and Enyiukwu (2019a, b). They reported higher mean losses of biochemicals in the leaves than in seed samples due to the interaction of *C. cruenta* and *C. destructivum* with cowpea, respectively. The higher loss of nutrients in the leaf, stem, and husk may be due to higher numbers of lenticels and stomata, which provided more and easier entry for the pathogen than the other crop organ (Latunde-Dada et al. 1999). It may also be due to the production of certain exudates such as mannitol, fructose, sucrose, alanine, and xylose-amino acid, which is known to encourage growth, conidiation, and infectivity of the pathogen from these organs than the seeds (Sangeetha and Rawal 2008; Boyette and Hoagland 2012). Or it may suggest the presence of

certain long-chain fatty acids on the cuticle of the severely affected specimens, which has been reported to stimulate the formation of certain adhesive substances, appressoria development, and effective penetration of fungal infection pegs into host tissues than in the seeds (Podilla et al. 1993). Or on the other hand, it may connote the presence of lower concentrations of certain phytochemical inhibitors to the pathogenic fungi such as tannins, polyphenols, flavonoids, and saponins in the husks, leaves, and stem than in the seeds of the test crop (Enyiukwu and Awurum 2013c); since the seed coats of legumes (including cowpea) contain high levels of these fungi-fighting phytochemicals especially flavonoids (Okwu and Orji 2007; Tajoddin et al. 2010).

In conclusion, the challenge of *C. destructivum* causing anthracnose disease of the crop remains a fundamental biotic constraint to profitable cowpea production in sub-Saharan Africa. Besides causing yield losses and crop failure in extreme cases, the disease can lead to serious loss in the quality of cowpea produce. Substantial losses in the proximate and mineral constituents such as protein, carbohydrate, fat, prebiotics, ash, calcium, and phosphorus of the crop were depicted in this study. The mean losses of these 7 nutrients were higher in the leaf, stem, and husk but least in seeds. Therefore adequate control of anthracnose disease will not only improve the farm economy but lead to the production and storage of high-quality, nutritious produce that could contribute to food security in the sub-continent.

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Changes in color of betta fish (*Betta splendens*) by feeding of *Artemia salina* enriched with *Tagetes erecta* flower flour

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Abstract. *Kiswara CA, Budiharjo A, Sari SLA. 2020. Changes in color of betta fish (*Betta splendens*) by feeding of* Artemia salina *enriched with* Tagetes erecta *flower flour. Cell Biol Dev 4: 46-50.* Betta fish are attractive due to their varied and vibrant colors and aggressive temperament. Carotenoids are the pigments found in betta fish. They are kept in chromatophores. While fish cannot synthesize carotenoids in their bodies, they can absorb them from their diet and use them as pigments to enhance the color intensity of their bodies. The purpose of this study was to determine the degree of color change and survival of betta fish fed with *Artemia salina* (Linnaeus, 1758), which has *Tagetes erecta* L. flower flour as their food. This study used a completely randomized design (CRD) and three treatments with varying compositions of rice flour: *T. erecta* flower flour, namely, 5:1, 2:1, and 1:1, respectively. *Artemia salina* fed simply with rice flour was a control. Betta fish require 40 days of care. Color intensity was determined once every ten days using a color scale with a score of 1 (white), 2 (weak red), 3 (medium red), and 4 (dark red) (red). The results indicated that *A. salina* fed in a 1:1 ratio had the best effect on improving the color brightness of betta fish. All parts of the betta fish's body received a score of 4 in this treatment, including the head, fins, and tail.

Keywords: *Artemia salina,* Betta, *Betta splendens,* degree of discoloration, *Tagetes erecta*

INTRODUCTION

Betta fish (*Betta splendens* Regan, 1910) are freshwater fish native to various tropical Asian nations, including Indonesia, Thailand, Malaysia, and Vietnam (Witte and Schmidt 1992). Male betta fish have long tails, gorgeous colors, and a tendency to defend their territory aggressively, frequently used as ornamental fish or fighting fish. This fish's primary attraction is its long dangling fins with vibrant colors. Round (wild type) fins, half-moon (shovel-shaped), spade-tailed (lanced form), long fin (flowing and extended), delta (triangular with vertical end), and ribbon (elongated flowing) fins are found in betta fish (Goldstein 2004). Betta fish are classified according to their color into three categories: solid color, combination, and mascot.

Moreover, it is called solid color if all body parts and fins are the same color, such as red, blue, gray, black, yellow, or white. However, if the body or fins are a combination of two or more different hues, such as blue-red, black-red, red-steel, or green-red, it is classified as a color combination. At the same time, it is referred to as a mascot if the betta fish's body is predominantly red and white or other hues such as gray, blue, or green (Goldstein 2011).

Carotenoids are the pigments found in betta fish. They are kept in chromatophores. Carotenoids are organic pigments found naturally in the chromoplasts of plants. Although animals, including fish, lack chromoplasts, they can retain carotenoids in their bodies (Britton et al. 2001). By and large, fish will absorb the carotenoids in their meal and use them as a pigment-forming agent, thereby intensifying the color of their bodies (Torrlssen et al. 1989). Carotenoids are absorbed into tissue cells and affect the chromatophores in the epidermal layer of fish. Fish can change color due to chromophores found in their skin. Chromatophore cells are pigment cells with a spherical form that are distributed throughout the fish skin's epidermal cell layer. Chromatophores produce distinct hues; each chromatophore has only one color (Sally 1997; Anderson 2000). Fish modify the pigment their food receives physiologically, resulting in color differences. Color changes generated by the movement of pigment grains or chromatophores are physiological color alterations (Evan 1993). Chromatophores are pigment-containing cells that store, manufacture, absorb, and reflect light in a specific color form. There are six types of chromatophores, which are generally classified according to their color range when observed under light: melanophores (black-brown), erythrophores (red-orange), xanthophores (orange-yellow), iridophores (metallic), leucophores (beige-white), and cyanophores (bright green-blue) (Sköld et al. 2013).

Carotenoid-rich feeds can accelerate pigment production in fish and modify the brightness of color. For example, Çapar et al. (2007) studied rainbow trout feeding (Oncorhynchus mykiss) with *Tagetes erecta* L. flower flour, red chili, and pure astaxanthin. The results indicated that using *T. erecta* flower flour at a concentration of up to 1.6% (carotene about 65 mg/kg) in the feed was the most effective way to raise the carotene content in the fish body while reducing the fish performance (fish weight) just little

when compared to control feed. According to Kusuma's (2012) research, adding 1.5% marigold flower flour to total artificial feed increased the color intensity by up to 127.53%, with a final chroma content of 54.54.

Carotenoids are abundant in *T. erecta* flowers; moreover, the carotenoid concentration of *T. erecta* flower petals is around 7,000 mg/kg dry weight. This number is more than the carotenoids found in algae (2,000-4,000 mg/kg) or yeast (30-800 mg/kg), which are frequently added to the fish diet as supplements (Hertrampf and Puscual 2008). Therefore, the purpose of this study was to examine the effect of adding *T. erecta* flowers to Betta's fish native diet, *Artemia salina* (Linnaeus, 1758), on the color brightness of Betta fish.

MATERIALS AND METHODS

Research materials

The main ingredients used were 56 betta fish seeds with an age of ± 60 days and size of ± 1 cm, 2 kg of *T. erecta* flowers, 2 kg of rice flour, and *A. salina* eggs.

Research design

This study used a completely randomized design (CRD) and three enrichment treatments with *T. erecta* flower flour on the *A. salina* diet. The composition of *A. salina* feed is shown in Table 1.

The making of *Artemia salina* feed

Tagetes erecta flowers are carefully washed under running water and separated into petals and other parts. After washing, the petals were weighed wet with a scale. The petals were then dried in an oven set to 70-80°C for 15 minutes. Next, they were blended until smooth and in the form of flour after drying. Then the feed was made by diluting a mixture of rice flour and *T. erecta* flower flour into 10 mL of salt suspension water with a concentration of 55% according to the variation of treatment, namely 3 mg of rice flour and a mixture of rice flour and *T. erecta* flower flour with the composition according to the research design.

Maintenance of *Artemia salina*

Artemia salina eggs are incubated in bottles until they hatch. Bottles for egg incubators are constructed with two sections of insulated space, one for darkness (on top) and the other for illumination (bottom). An aerator is used to aerate saltwater that has a concentration of 55%. The water is placed in a container and aerated. *Artemia salina* eggs are laid in a single chamber, after which the chamber is sealed. As a result of the phototactic properties of the hatched *A. salina*, the other side of the bulkhead was left exposed, and a lamp was used to pull it through the opening. The eggs will hatch into larvae after approximately 24 hours. Larvae were fed as much as 1 mL of feed suspension twice a day when they were 48 hours old, in the morning at 08.30 and in the afternoon at 15.00. After being kept for two days, *A. salina* was fed betta fish as food.

Betta fish care

Eight pieces were used to construct the aquarium, which measured 20 cm in length, 15 cm in height, and 10 cm in width. First, the aquarium is cleaned with alcohol and thoroughly rinsed, filled with water to a height of up to 12 cm.

Betta fish seeds are acclimated in advance for approximately one night, after which they are placed in the aquarium that has been previously prepared. Betta fish seeds were maintained over 40 days by feeding them one teaspoon of sugar daily (5% of fish biomass). Therefore, it is necessary to feed the brine shrimp three times a day, in the morning at 09.00, in the afternoon at 13.00, and in the evening at 17.00. Daily, 25% of the water was siphoned and replaced to keep the system running correctly during maintenance.

Color change observation

Every ten days, the body color parameters of betta fish were observed. The head (from the mouth to the front of the operculum), the middle body (from the tip of the operculum to the base of the anal fin), and the fin were studied. Color parameters were observed using the scoring method, explicitly calculating the color scale on the body of betta fish, as Satyani and Sugito (1997) and Brake et al. (2013) did. The color scheme is depicted in Figure 1.

Identification of carotenoid compounds in *Tagetes erecta*

Tagetes erecta flower flour was soaked in a solvent mixture of acetone and methanol (7:3) with a sample-solvent ratio of 1:10 (w/v). Next, $CaCO_3$ was added as a neutralizing agent and ascorbic acid as an antioxidant to prevent oxidation during extraction. Next, the extracted pigments were filtered, and the residue was re-extracted with the same solvent using a stirrer at a speed of 750 rpm for 10 minutes or until all pigments were lifted (became colorless). Next, the extraction findings were partitioned using diethyl ether at a sample ratio of 1:1 (v/v), which is compatible with distilled water and concentrated sodium chloride (if the pigment solution is difficult to separate). Furthermore, to bind H_2O, the ether layer containing the pigment (colored) was removed, and anhydrous Na_2SO_4 was added. Following that, a filter was applied to this layer. Finally, the filtrate was concentrated using a rotary evaporator (Britton et al. 1995).

The carotenoid pigment extract was briefly affixed to the silica gel TLC plate 60 F254 and then eluted with a 3:2:1 (v/v/v) mixture of hexane, diethyl ether, and acetone. After observing the TLC pattern of each pigment, the Rf was calculated and compared to the standard marker's TLC pattern.

Table 1. Combination of treatments

Feed concentration *Artemia salina*	Weight (mg)			
	Control	Treatment 1	Treatment 2	Treatment 3
T. erecta flour	0	0,5	1	1,5
Rice flour	3	2,5	2	1,5

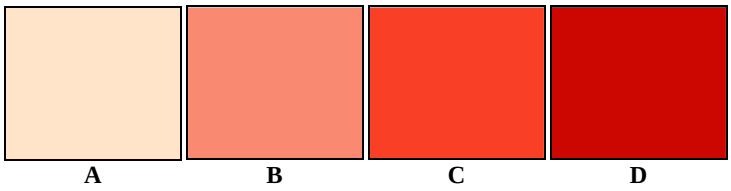

Figure 1. Color Intensity Scale on Betta fish body: A. scale 1 (white), B. scale 2 (weak red), C. scale 3 (medium red), and D. scale 4 (red)

RESULTS AND DISCUSSION

Discoloration

All treatments were classified according to their color appearances in categories: body, fins, tail, and head. The results indicated that adding *T. erecta* flower flour imparted a reddish hue to betta fish. This condition was demonstrated by the rise in color intensity observed in each treatment throughout the investigation (Table 2).

At the commencement of the study, the fish's tail and fins were a medium red, while the head and body were weak red. On day 10th, after treatment with *A. salina* fed with *T. erecta* flower meal, betta fish experienced a higher color intensity than the control treatment. Colors occurred exclusively on the control treatment's body, tail, and fins. In contrast, other treatments that included *T. erecta* flower flour underwent an increase in color intensity on the tail, fins, body, and head. The initial treatment resulted in a color alteration of weak red into a medium red on the head and body, while the fins and tail changed from a medium red to a red rise.

On the tenth day of treatment, the color intensity of betta fish increased. The highest increase in color was noted in the head of betta fish fed with *A. salina*, which was given by rice flour and *T. erecta* flower flour at a 1:1 ratio. Meanwhile, the greatest average increase in color intensity ratings was recorded in the body and tail at 5:1 and 2:1 feed compositions.

On the twentieth day, the greatest rise in color intensity occurred. Color changes, particularly on the head and body, were found in betta fish fed with *A. salina*, which had *T. erecta* flower flour as the meal. In addition, the tail and fins developed pigment more subtly than on the tenth day. The greatest rise was recorded in betta fish fed with *A. salina*, which had a diet of rice flour and *T. erecta* flower flour at a ratio of 2:1. This color change happened on the head, tail, fins, and body which started on day 10th and culminated on day 20th (Figure 2). In contrast, the control treatment had a lower average score in the head, body, and tail than the other treatments.

Observations from the 30th to 40th days revealed a reasonably consistent color intensity, practically identical to that on the 20th day. There was a small drop in color intensity in certain treatments. After 20 days of treatment, betta fish development was at its peak. On the 30th day, the Kruskal Wallis test using SPSS revealed that the findings were substantially different only for the body part for which the calculated F (0.005) was less than the F table (0.05).

Table 2. Changes in color intensity of betta fish fed *A. salina* feed enriched with *T. erecta* flower flour

Fish body parts	Feed composition rice flour: *T. erecta* flower flour	The average color intensity on the day-				
		0th	10th	20th	30th	40th
Head	1:0	2.0	2.0	3.0	3.4	3.3
	5:1	2.0	2.4	3.9	3.9	3.1
	2:1	2.0	2.6	4.0	4.0	3.7
	1:1	2.0	2.9	3.7	3.7	4.0
Body	1:0	2.0	2.1	3.0	3.0	3.1
	5:1	2.0	2.7	3.9	3.7	3.6
	2:1	2.0	2.7	3.9	4.0	3.7
	1:1	2.0	2.6	3.9	3.9	4.0
Tail	1:0	3.0	3.4	3.6	3.9	3.7
	5:1	3.0	3.6	3.9	4.0	3.9
	2:1	3.0	3.6	4.0	4.0	4.0
	1:1	3.0	3.3	4.0	4.0	4.0
Fins	1:0	3.0	3.4	3.9	3.7	3.7
	5:1	3.0	2.9	3.6	4.0	3.7
	2:1	3.0	2.9	4.0	4.0	4.0
	1:1	3.0	3.3	4.0	4.0	4.0

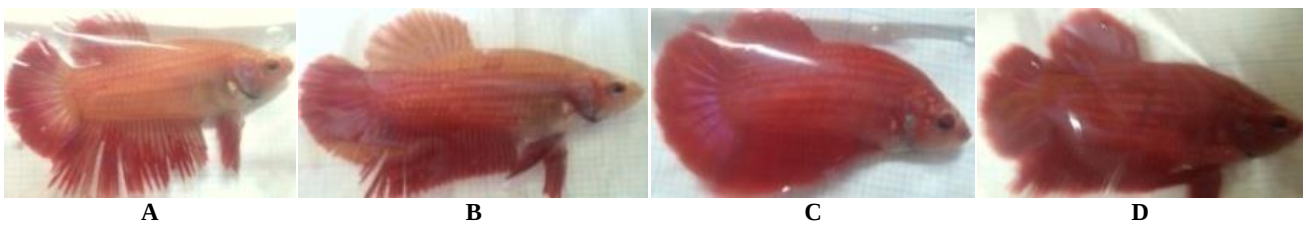

Figure 2. Color of betta fish before treatment (A), after treatment with *A. salina* feed fed with rice flour and *T. erecta* flower flour with a composition of 2:1 on day 10th (B), 20th (C), and 30th (D)

According to Evan (2008), as cited in Indarti et al. (2012), carotene in the form of lutein found in *T. erecta* flower flour is altered by fish into astaxanthin and is used as a source of red pigment. Fish can brighten their color physiologically by the food they ingest, and the pigments are dispersed throughout the body's chromatophore cells via the blood. On the tenth day, it is conceivable that the red pigment has not been spread evenly throughout the body, resulting in the coloration of the fins of certain Betta fish remaining unchanged.

Hormones and the central nervous system also affect fish pigmentation. Sally (1997), cited by Puspita (2012), states that the pituitary gland generates three types of hormones: Melanocyte Stimulating Hormone (MSH), Melanin Concentrating Hormone (MCH), and Melatonin (MT). MSH allows the pigment to permeate throughout the cells, giving the scales a brilliant and clear appearance. MCH causes the pigment to collect in cells, giving fish scales a lighter appearance. MT, produced in the epiphyseal gland, is the third hormone that affects fish color. These hormones act on chromatophores of pigment cells, causing pigment granules to aggregate within cells, resulting in a decrease in color. The food consumed by fish highly controls these hormones' activity. According to Fujaya (2004), dietary sources contribute significantly to the secretion of hormones that directly produce and store a variety of colors in the fish body.

Betta fish convert astaxanthin to zeaxanthin in their bodies, and zeaxanthin also exists in three optical isomers in the fish body (Maoka 2009). Tunaxanthin is most abundant in fish of the order of Perciformes. The fish's tail and skin are brilliant red due to the presence of tunaxanthin, whereas tunaxanthin is the product of astaxanthin metabolism via zeaxanthin, according to research.

Color changing pattern

The direction of the color change pattern in betta fish is determined by comparing the average color change score on each body part of the fish after 40 days of observation, beginning with the fins, tail, body, and head. According to the observations, the most noticeable color changes occurred on the fins and tail, while the head and body remained relatively unchanged (Figure 3).

Betta fish that consumed *A. salina* fed on rice flour (control), and a mixture of rice flour and *T. erecta* flower flour in a ratio of 5:1 and 2:1 would almost result in the same color change pattern; that is, the head is better than the body. However, Betta fish fed *A. salina* fed rice flour, and *T. erecta* with a ratio of 1:1 showed a better pattern of color change throughout the body.

Identification of carotenoid compounds in *Tagetes erecta* flowers

Thin Layer Chromatography (TLC) technology was used to identify carotenoid pigments. Figure 4 illustrates the color separation pattern. TLC analysis revealed three yellow and yellow-orange spots with Rf values of 0.8, 0.68, and 0.25 in the carotenoid extract *T.erecta.*

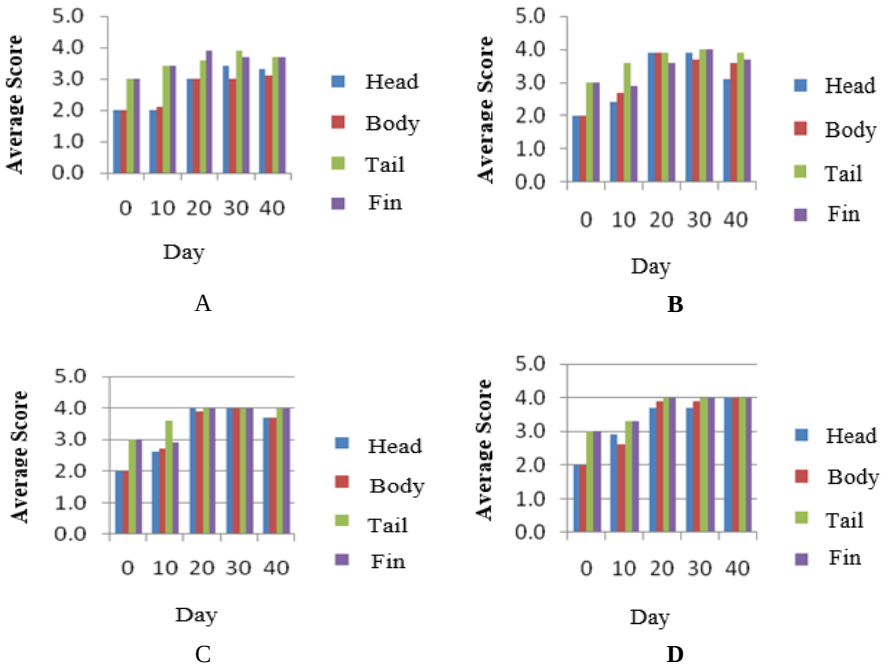

Figure 3. The average distribution of the total color of betta fish on each limb after 40 days of treatment by feeding *A. salina* fed rice flour (control) (A), rice flour, and *T. erecta* in a ratio of 5:1 (B), 2: 1 (C) and 1:1 (D)

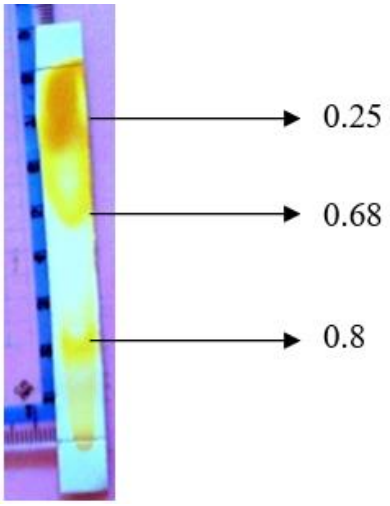

Figure 4. TLC Results of *T. erecta* carotenoid pigment extract

The diversity of pigment Rf values is closely related to the pigment content of the *T. erecta* flower. Spot 1 produces an orange color on the TLC plate, which is thought to be beta-carotene, as the mobile phase used is predominantly nonpolar, which is also a property of beta-carotene, as beta-carotene has a higher Rf value and a faster rate of movement than other pigments. Spot 2 with yellow-orange color is astaxanthin, while spot 3 with yellow-orange color represents lutein. The research results of Monika (2009) show that lutein, astaxanthin, and beta-carotene have an RF values of 0.3, 0.6, and 0.8. This RF value is almost the same compared to this study's results.

In conclusion, feeding *T. erecta* flower flour to betta fish natural food can cause the color change of the betta fish to become more noticeable. The *A. salina* feed, composed of rice flour and *T. erecta* flower flour in a 1:1 ratio, was shown to be the most effective in boosting the color of betta fish. The initial direction of color change in betta fish is on the limbs, but the fastest color changes are on the fins and tail. The body and head tend to be slower to change color.

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Plant growth and total flavonoid content of *Sisyrinchium palmifolium* after light intensity and gibberellin treatment

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Abstract. *Zuaini PAK, Mudyantini W, Solochatun. 2020. Plant growth and total flavonoid content of* Sisyrinchium palmifolium *after light intensity and gibberellin treatment. Cell Biol Dev 4: 52-63.* Dayak onion (*Sisyrinchium palmifolium* L Syn.: *Eleutherine palmifolia* L. Merr.) was a plant that has the potential to be developed as a traditional medicine because it contains flavonoid compounds. This research aimed to determine the effect of the application of differences in light intensity and gibberellins on the plant growth and flavonoid content of *S. palmifolium*. This research used uniform *S. palmifolium* with a harvest age of 3-4 months and an 8-9 g weight range from Pasir Besar Village, South Pontianak District, Pontianak, Indonesia. The experiment used the Factorial Completely Randomized Design method with two factors treatment and six replications: light intensity (100%, 75%, 50%) and gibberellin concentration (0, 10, 20 ppm). The control was 100% light intensity treatment and 0 ppm gibberellin concentration. The parameters measured were the number of leaves, length, width, time of flowering, number of flowers, and number of bulbs. In the post-harvest, dry and wet weights of bulbs were measured. Then, chlorophyll, carotenoid, and flavonoid contents were assessed using a UV-Vis spectrophotometer, and stomata density was also analyzed. ANOVA analyzed the data, and if the difference was significant, it was continued with the DMRT test at a significance level of 5%. The results showed that the growth of *S. palmifolium* was significantly affected by light intensity and gibberellins on the parameters of leaves length; leaves, midrib, and bulbs wet weight; leaves and midrib wet weight; leaves and midrib dry weight; bulbs dry weight; shoot root ratio; leaves carotenoid content, and bulbs carotenoid content. However, parameters had no significant effect on the number of leaves, leaves width, time of flowering, number of flowers, number of bulbs, chlorophyll contents, flavonoid, and stomata density. The 50% light intensity and 10 ppm gibberellins were the best treatments to increase leaves length, leaves, midrib, bulbs wet weight, leaves, and bulbs chlorophyll content of *S. palmifolium*.

Keywords: Flavonoid, gibberellin, light intensity, plant growth, *Sisyrinchium palmifolium*

INTRODUCTION

Indonesia was a country with abundant biodiversity. One medicinal plant with very little utilization was the *Dayak* onion (*Sisyrinchium palmifolium* L. Syn.: *Eleutherine palmifolia* L. Merr.). Based on several previous studies, *S. palmifolium* bulbs have the potential to be developed as traditional medicine. The *S. palmifolium* bulbs contain secondary metabolites such as phenolics, polyphenols, flavonoids, alkaloids, anthraquinones, tannins, glycosides, steroids, polysaccharides, saponins, and naphthoquinones (Naspiah et al. 2014). Based on empirical data, it was known that *S. palmifolium* could help cure diabetes and hypertension, lower cholesterol, prevent stroke, and treat stomach aches (Galingging 2009). The *S. palmifolium* plants can also act as antibacterial (Harlita et al. 2018), skin antimicrobial (Puspadewi et al. 2013), and agents for lowering blood glucose levels (Galingging 2009). The *S. palmifolium* has a distinctive character, including a smooth bulb surface and a bright red color. *S. palmifolium* leaves have a double pinnate shape that is located in pairs. The midribs of *S. palmifolium* were parallel (rectinervis) with smooth leaf margins (entire). Ribbon leaf shape in the form of a line (linearis). This plant can adapt easily to various climates and soil types. The *S. palmifolium* can be propagated and harvested relatively quickly (Galingging 2009). Harvesting can be done after the plant is 3-4 months after planting (Yusuf 2009).

There were both exogenous and endogenous influences on plant growth. Light intensity was one of the exogenous factors that impact a plant's growth. Indirect or direct, light has a significant impact on the growth and development of plants. Light directly affected plant photosynthesis, while plant growth and development were indirectly affected by light (Fitter and Hay 1998). High light intensity impacts bulb or fruit formation in some plants, while low light intensity reduces bulb and fruit formation, resulting in vegetative overgrowth (Bahruddin 2004). Vegetative organs such as leaves, stems, and roots can expand to a greater volume and impede the growth of generative organs such as flowers and fruit.

Growth regulators can be given to the bulbs to speed up their growth. One class of growth regulators to consider was gibberellins. There are various physiological functions that gibberellins can induce in plants. Numerous vegetable and fruit crops benefit from gibberellins. Leaf growth, root extension, and fruit ripening were examples of these features in action (Miceli et al., 2019).

The amount of chlorophyll in plants can be influenced by changes in light intensity. High light levels can reduce chlorophyll content in leaves. According to other studies, carotenoids and nitrogen levels rose when the light

intensity was high (Salisbury and Ross 1995). According to Bruce et al. (2001), the shadow affects plants' physiological and biochemical conditions. Increasing chlorophyll b levels was a strategy for plant survival that reduced the chlorophyll a/b ratio. In addition, increased chlorophyll b concentration benefits the optimal absorption of radiation energy under shady conditions.

According to Handriawan et al. (2016), 50% shade can significantly inhibit soybean plant development compared to 25% paranet shade. The findings of this study indicate that the intensity of light absorption was diminishing because increased shade levels can result in etiolation symptoms. The symptoms of etiolation were produced by light inhibition, which resulted in a rise in the hormone auxin.

According to Ekawati's (2018) research, darkened environments can increase the overall flavonoid content of *Talinum fruticosum* (L.) Juss. Because *S. palmifolium* grows more rapidly in full light than in a shady setting, shallots were classified as C4 plant species that require full light. Therefore, without shade, 50% shade and no shade on the growth of *S. palmifolium* will produce optimal results. The growth of *S. palmifolium* was determined by the number of bulbs, tillers, leaves, flower percentage, and fresh weight of bulbs per sample (Yusuf 2009).

According to Atif et al. (2020), photoperiod and temperature affected the development of garlic bulbs, and phytohormonal signals induced alterations. In addition, the length of the photoperiod has been shown to boost endogenous gibberellin levels. Therefore, light intensity treatment and growth regulators likely affect *S. palmifolium*'s growth characteristics and flavonoid content. With the potential for *S. palmifolium* to be developed as a medicinal plant, it was vital to research the effect of light intensity and growth regulators on the growth and flavonoid levels of *S. palmifolium.*

The aims of this study were: (i) to determine the effect of light intensity on the growth and levels of *S. palmifolium* flavonoids; (ii) to determine the effect of gibberellins on the growth and levels of *S. palmifolium* flavonoids; (iii) to know the effect of the interaction of the combination of light intensity treatment and gibberellin hormone on the growth and levels of *S. palmifolium* flavonoids; (iv) to know the most optimum combination of treatments from the effect of light intensity and gibberellins on the growth and levels of flavonoids of *S. palmifolium.*

MATERIALS AND METHODS

This research was carried out from October 2020 to April 2021 at the (i) Biology Laboratory, Faculty of Mathematics and Natural Sciences, (ii) Integrated Biology Sub-Laboratory, Faculty of Mathematics and Natural Sciences, and (iii) greenhouse of UPT Integrated Laboratory, Faculty of Mathematics and Natural Sciences, Universitas Sebelas Maret, Surakarta, Central Java, Indonesia.

Ingredient

The materials used in this study were *S. palmifolium* bulbs from Pasir Besar village, South Pontianak District, Pontianak, Indonesia, with a harvest age of 3-4 months and a weight range of 8-9 g.

Research design

This study used a completely randomized design (CRD) 2 factorial and 6 replications, namely:

Light intensity:
C50 : 50% light intensity
C75 : 75%light intensity
C100: 100% light intensity
Gibberellin hormone concentration:
G0 : 0 ppm gibberellin hormone (control)
G10 : 10 ppm gibberellin hormone
G20 : 20 ppm gibberellin hormone

The treatment variation was carried out nine times and the repetition six times.

Procedure

Paranet shade making

The treatment of paranet shading was to install 50% black paranet and 75% intensity separately to pass the light on to the plants. The paranet was installed 2 m above the polybag, and the light intensity was measured with a lux meter during installation. Light shade treatment was given from planting time to harvest time.

Preparation of planting media

The composition of the planting media was a mixture of soil and compost in a ratio (1:1) which was put in a polybag. Polybags were arranged in rows under a paranet sheath and left for a week to stabilize the planting medium.

Field capacity test

Polybags containing soil were dried to a consistent weight. Weighing and recording the weight of the sample polybag was done then. Following drying, the soil was thoroughly watered until no water drips. The amount of water required was kept on track. After watering, the soil was weighed again, and the volume was recorded.

Gibberellin solution preparation and planting

In distilled water, the gibberellin hormone was dissolved. Concentrations of 0, 10, and 20 ppm were used. Next, the gibberellin hormone was diluted with one drop of 70% alcohol and dissolved in distilled water until a solution between 10 and 20 ppm was obtained. After the solution had formed, it was stored in an Erlenmeyer flask covered with aluminum foil, and then it was put in a labeled dark bottle in the refrigerator.

The gibberellin stock solution was prepared by calculating the formula:

1 ppm = 1 mg/L
10 ppm = 10 mg/1 L of distilled water
20 ppm = 20 mg/1 L of distilled water

Information:

10 mg and 20 ppm of gibberellins were dissolved in 1 L of distilled water.

The readied bulbs were soaked in a solution of the gibberellin hormone for 40 minutes. Next, soaking 1/3 of the bulbs near the basal plate in water was done. It would promote rapid growth. Polybags filled with planting media were then planted with one bulb.

Preparation of S. palmifolium *to be planted*

The *S. palmifolium* bulbs to be planted were opened and cleaned of dirt. Onion bulbs to be planted were sorted based on uniform clove size. Basal plates of *S. palmifolium* were kept from being damaged. The onion cloves were weighed, and the cloves with a bulb weight of 8-9 grams were selected.

Plant maintenance

In the morning, the polybags containing the bulbs were hydrated. If there were weeds in the soil, they were weeded out. Fungi are removed from the mushroom soil media and discarded before the polybag soil media is progressively scraped to the bottom so air can flow through it. Biosoil was applied if the media was still infested with fungus.

Observation

Every week, the daily growth of *S. palmifolium*, including the number of leaves, length and width of the leaves, the time of flower appearance and the number of flowers, and the number of tillers, was documented. First, dry and wet bulb weights were detected as post-harvest characteristics. Next, wet and dry bulbs' chlorophyll and carotenoid content were analyzed. Finally, the wet and dry weights of bulbs were measured after harvest. The wet weight was the weight of produce at harvest, whereas the dry weight was derived after baking at a temperature of 60ºC to maintain a constant weight.

Compound analysis

Chlorophyll and carotenoid pigments

The amounts of chlorophyll a, chlorophyll b, and carotenoids were determined using spectrophotometric techniques. This study tested chlorophyll and carotenoids in *S. palmifolium* leaves and bulbs. Onion bulbs and leaves were cut into little pieces that weighed no more than 0.1 g each and were utilized in the experiment. The leaves and bulbs were mashed separately into a pulp in a mortar and pestle, and then 20 mL of 70% alcohol was added. After some time, the filter paper collected the fluid in a test tube. Aluminum foil was placed over the test tube to prevent the fluid from evaporating. Three milliliters of the filtrate were added to the cuvette. The cuvette was analyzed using a spectrophotometer. The absorbance of the solution was measured using three wavelengths (A). The formula for calculating chlorophyll content can be found here:

Chlorophyll a = [(12.7 x (A663) – (2.69 x (A645))) µmol
Chlorophyll b = [(22.9 x (A645) – (4.68 x (A663))) µmol
Total Chlorophyll = [(8.02 x (A663)) + (20.2 x (A645))] µmol

The following formula can calculate carotenoid content:

$$\frac{[(A480)+0.114(A663)-0.638(A645)]\ x\ 3\ ml\ x\ 1000\ \mu mol}{112.5\ x\ 10}$$

Information:
A480 = absorbance value at a wavelength of 480 nm
A645 = absorbance value at a wavelength of 645 nm
A663 = absorbance value at a wavelength of 663 nm

Flavonoid content test

The onion bulbs were washed and then sliced into thin pieces to begin the extraction process. The bulbs were kept in an oven at 60ºC for three days. Next, the bulbs were taken out of the oven and pulverized into a fine powder using a blender. Next, five grams of simplicia powder was macerated with 10 mL PA 96% ethanol until completely submerged with slow stirring. Next, 10 mL of PA ethanol was added to the macerated bulb powder and allowed to sit for a while before filtering. It was then re-macerated in PA ethanol (96 %) and filtered.

The flavonoid content of simplicia powder can be measured by dissolving 5 g in 10 ML of 96% PA ethanol. The solution was macerated and filtered. The sample solution was mixed with 1 mL of potassium acetate solution containing 120 mM potassium and 1 mL of $AlCl_3$ solution containing $AlCl_3$ 2 % for 30 minutes in a test tube.

The amount of $AlCl_3$ 2% content was made by the formula:

$$\text{Total volume x } \frac{2}{100} = 18 \text{ x } \frac{2}{100} = 0.36 \text{ gram}$$

The formula made the total content of Potassium Acetate:

$$M = \text{mol x } V = \frac{m}{Mr}\ x\ \frac{1000}{ml}$$

Information:
M = molarity (mmol/L)
V = volume of solvent required (mL)
m = weight of potassium acetate (mg)
Mr = molecular mass of potassium acetate (gr/mol)
mL = required volume (mL)

The absorbance of the solution was measured at a wavelength of 435 nm. The mean absorbance was considered the y value in the standard curve equation of quercetin. Therefore, the x value was the equivalent of quercetin milligrams in 100 mg of sample (QE / Quercetin Equivalent).

$$\text{Flavonoid content} = \frac{c\ x\ v\ x\ fp\ x\ 10^{-3}}{m}\ x\ 100\%$$

Information:
C = concentration of flavonoid levels (mg/L)
V = total volume of ethanol extract (mL)
fp = dilution factor
m = sample weight (mg)

Quercetin standard curve creation

Quercetin was made into solution concentrations of 6 ppm, 8 ppm, 10 ppm, 12 ppm, and 14 ppm. Quercetin was weighed as much as 0.06 mg, 0.08 mg, 0.10 mg, 0.12 mg, and 0.14 mg and dissolved in 10 ml of aquabidest. Then 1 mL of 120 mM potassium acetate was added, 1 mL of 2% $AlCl_3$ was added, and incubated for 30 minutes in a test tube. The absorbance of the sample was measured by UV-Vis spectrophotometry at a wavelength of 435 nm.

Stomata observation

Observation of stomata on the leaf surface was the imprinting method. *S. palmifolium* leaves that have been harvested are lightly smeared on the lower surface with clear nail polish. Wait until the nail polish dries. The tape was attached to the nail polish that had dried until it was glued. After feeling sticky enough, the tape was peeled off, placed on a glass object, and labeled. Glass objects were arranged in the preparation box. Furthermore, the preparations can be observed per field of view with a light microscope with a magnification of 400 times. The Fiji application calculated the number of stomata and their density values.

Stomata density was the field of view used at a magnification of 10x40 with a diameter of 0.5 mm. The following was the formula for stomatal density (Lestari 2006):

$$\text{Stomata density} = \frac{\text{number of stomata}}{\text{width of field of view}}$$

Where:

Width of field of view $= \frac{1}{4} \times 3.14 \times d^2$

$= \frac{1}{4} \times 3.14 \times 0.52$

$= 0.19625\ mm^2$

Data analysis

The research data were analyzed by two-way ANOVA (Analysis of Variance) to determine the effect of light intensity and gibberellins on the growth and levels of *S. palmifolium* flavonoids from all treatments given. Significantly different characters were tested using DMRT (Duncan's Multiple Range Test) at a significance level of 5%.

RESULTS AND DISCUSSION

This study used two combinations of treatments, namely light intensity and gibberellins. The light intensity treatments include paranet with 50% light intensity, which produces 15,000-18,000 lux of light, paranet with 75% light intensity produces 6,000-7,000 lux of light, and 100% light intensity produces 18,000-20,000 lux of light. The gibberellins were treated with 0 ppm concentration (control), 10 ppm concentration, and 20 ppm concentration. The basal plate of this study includes the effect of light intensity and gibberellins on leaf length and width, flowering time, number of flowers, number of tillers, wet and dry weight of plants, shoot root ratio, chlorophyll and carotenoid levels in leaves, and bulbs, bulb flavonoid content, and leaf density of *S. palmifolium* plant (Figure 1).

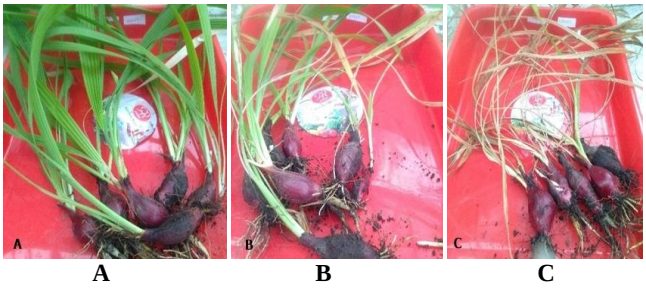

Figure 1. The condition of the Dayak onion plants at the time of harvest treatment: A. 50% paranet light intensity, B. 75% paranet light intensity, and C. 100% light intensity

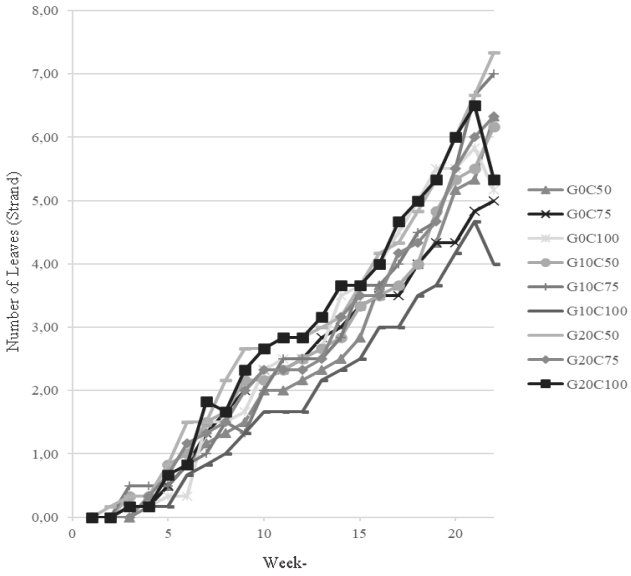

Figure 2. The average number of leaves of *Sisyrinchium palmifolium* during 22 weeks of planting

Number of leaves

The number of leaves of *S. palmifolium* in the light intensity treatment tended to increase weekly. The average number of leaves in Figure 2 shows that the highest number of leaves was treated with 50% paranet light intensity and 20 ppm gibberellin hormone (G20C50) on as many as 7 leaves. The least number of leaves was 100% light intensity treatment and 10 ppm gibberellin hormone (G10C100). The average number of leaves in the shade was higher than the light control treatment (100% light intensity). The gibberellin concentration control treatment showed a higher mean number of leaves than the 10 ppm and 20 ppm gibberellin concentrations. The growth of the number of leaves in the last week before harvest decreased due to the control of light intensity plants so that the leaves dry.

Based on the analysis of variance, light intensity and gibberellins did not affect the number of leaves of *S. palmifolium* plants. Therefore, complete data on the number of leaves of *S. palmifolium* was presented in Table 1.

Table 1. *Sisyrinchium palmifolium* parameters treated by light intensity and gibberellin concentration at yields time

Light intensity (%)	Giberelin concentration ± SD (ppm)		
	0	**10**	**20**
Number of leaves average			
50	6.33 ± 2.50	6.17 ± 1.47	7.33 ± 2.07
75	5.00 ± 0.89	7.00 ± 2.37	6.33 ± 2.50
100	5.17 ± 1.17	4.00 ± 2.83	5.33 ± 1.86
Leaf length average (cm)			
50	43.13 ± 1.25^{a}	61.43 ± 0.59^{b}	48.03 ± 0.61^{a}
75	46.93 ± 3.00^{a}	56.60 ± 0.36^{b}	45.03 ± 5.28^{a}
100	44.67 ± 0.91^{a}	45.73 ± 6.28^{a}	41.93 ± 5.35^{a}
Leaf width average (cm)			
50	1.47 ± 0.57	1.57 ± 0.06	1.57 ± 0.12
75	1.60 ± 0.17	1.50 ± 0.10	1.57 ± 0.12
100	1.57 ± 0.06	1.60 ± 0.40	1.00 ± 0.87
Flowering time (days)			
50	127 ± 73.32	0	0
0	0	0	0
0	0	0	112 ± 77.98
Amount of flower			
50	3.00	0	0
75	0	0	0
100	0	0	6.00
Number of tillers			
50	0	0	4.00 ± 1.16
75	2.00 ± 1.16	3.00 ± 1.73	4.00 ± 1.16
100	2.00 ± 1.16	2.00 ± 1.16	6.00 ± 0.00
Wet weight of leaves, midribs, and bulbs (gram)			
50	12.60 ± 0.0^{bcd}	13.25 ± 0.12^{d}	12.47 ± 0.53^{bcd}
75	12.03 ± 1.69^{bcd}	12.97 ± 1.58^{cd}	10.28 ± 1.52^{a}
100	11.75 ± 0.91^{bc}	11.48 ± 0.59^{ab}	12.85 ± 1.32^{bcd}
Average wet weight (grams) of leaves and midribs			
50	3.94 ± 0.00^{e}	2.51 ± 0.00^{c}	2.78 ± 0.00^{cd}
75	3.03 ± 0.99^{d}	4.33 ± 0.00^{e}	1.93 ± 0.00^{b}
100	0.50 ± 0.00^{a}	0.53 ± 0.00^{a}	1.55 ± 0.00^{b}
Average wet weight (grams) of bulbs			
50	8.83 ± 0.00^{a}	10.21 ± 0.00^{bc}	9.02 ± 1.27^{ab}
75	9.25 ± 1.54^{ab}	10.65 ± 0.61^{cd}	8.81 ± 1.15^{a}
100	11.20 ± 0.98^{cd}	10.03 ± 1.06^{abc}	11.50 ± 1.08^{d}
Average dry weight (grams) of leaves and midribs			
50	3.94 ± 0.00^{h}	2.51 ± 0.00^{f}	2.80 ± 0.02^{g}
75	2.12 ± 0.00^{e}	4.32 ± 0.00^{i}	1.93 ± 0.00^{d}
100	0.50 ± 0.00^{a}	0.53 ± 0.00^{b}	1.55 ± 0.00^{c}
Average dry weight (grams) of bulbs			
50	8.83 ± 0.00^{a}	10.21 ± 0.00^{b}	9.02 ± 0.00^{a}
75	8.77 ± 0.36^{a}	10.65 ± 0.61^{bc}	8.64 ± 0.00^{a}
100	10.93 ± 0.82^{cd}	10.03 ± 1.06^{b}	11.45 ± 0.00^{d}
Average shoot root ratio			
50	0.44 ± 0.00^{g}	0.24 ± 0.00^{d}	0.30 ± 0.00^{e}
75	0.24 ± 0.01^{d}	0.40 ± 0.02^{f}	0.22 ± 0.00^{c}
100	0.04 ± 0.00^{a}	0.05 ± 0.00^{a}	0.13 ± 0.00^{b}
Average leaf chlorophyll content (µmol)			
50	4.19 ± 0.57	6.66 ± 4.57	3.66 ± 0.72
75	5.30 ± 1.00	3.40 ± 0.26	4.77 ± 0.57
100	4.14 ± 2.23	5.39 ± 5.63	1.88 ± 0.17
Average chlorophyll content (µmol) of bulbs			
50	1.44 ± 0.05	1.63 ± 0.59	1.17 ± 0.21
75	1.18 ± 0.31	1.46 ± 0.53	1.07 ± 0.03
100	1.28 ± 0.36	0.75 ± 0.41	1.11 ± 0.19
Average carotenoid content (µmol) of leaves			
50	11.84 ± 1.52^{bc}	12.01 ± 3.13^{bc}	10.74 ± 1.60^{bc}
75	14.97 ± 1.99^{c}	8.28 ± 0.83^{ab}	10.86 ± 1.00^{bc}
100	11.84 ± 6.23^{bc}	3.17 ± 5.47^{a}	8.28 ± 1.09^{ab}
Average bulbs' carotenoid content (µmol)			
50	43.28 ± 2.99^{c}	38.78 ± 4.58^{bc}	37.25 ± 3.76^{bc}
75	36.41 ± 7.92^{bc}	39.99 ± 7.99^{bc}	30.04 ± 3.85^{ab}
100	32.06 ± 7.77^{bc}	19.97 ± 4.66^{a}	31.92 ± 8.87^{bc}
Average percentage of bulbs' flavonoid content (%)			
50	0.65 ± 0.01	0.66 ± 0.01	0.65 ± 0.02
75	0.66 ± 0.02	0.67 ± 0.01	0.65 ± 0.02
100	0.65 ± 0.01	0.64 ± 0.03	0.66 ± 0.01
Average stomatal density (/mm^{2})			
50	122.29 ± 35.67	105.30 ± 7.78	118.89 ± 22.98
75	139.27 ± 23.53	122.29 ± 40.45	135.84 ± 29.48
100	183.43 ± 30.57	149.46 ± 31.13	149.46 ± 10.61

Note: Numbers accompanied by the same letter in the same column show no significant difference in the DMRT = 5% test. SD: Standard deviation

Light intensity was important in leaf growth and development (Fan et al., 2013). The 50% paranet light intensity showed the highest value of the other treatments (Table 1). It was presumably due to etiolation, which causes plants to grow faster so that the number of leaves was more. The highest concentration of gibberellin hormone that affects the number of plant leaves was the 20 ppm gibberellin hormone presented in Table 1. Gibberellin hormones generally accelerate stem growth and cell propagation in plants but have no effect because the concentration is too small (Putrasamedja and Permadi, 2004). Other influencing factors are genetic and environmental (Gardner et al. 1991). Genetic factors were also thought to affect the growth of the number of plant leaves so that the leaves begin to appear after the third week of planting *S. palmifolium* bulbs. In addition, media that was too wet because it was exposed to raindrops affects the plants treated with 100% light intensity.

Leaf length

The mean leaf length of *S. palmifolium* (Table 1) showed that the highest leaf length was treated with 50% paranet light intensity and 10 ppm gibberellin hormone concentration along 56.60 cm. The lowest light intensity treatment was 100% light intensity treatment and 20 ppm gibberellin hormone due to plant leaves drying in the final week. As a result, the average leaf length growth was lower before harvest at the control light intensity (100%). The average leaf length growth data is presented in Figure 3.

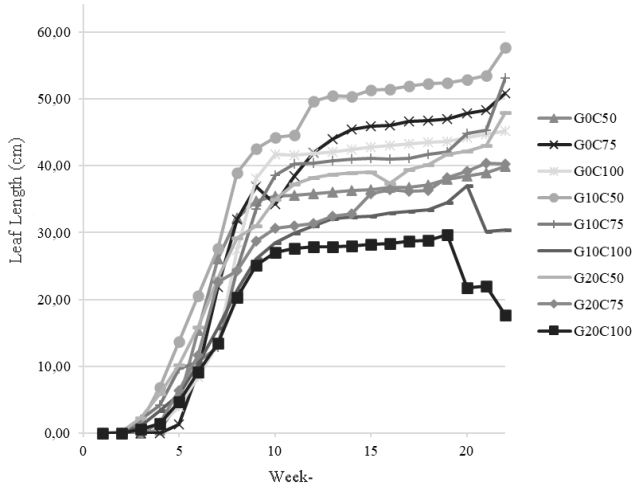

Figure 3. Average leaf length of *Sisyrinchium palmifolium* during 22 weeks of planting

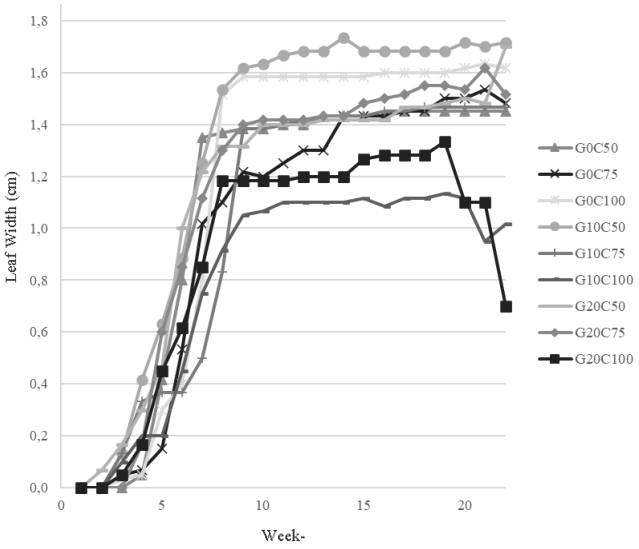

Figure 4. Average leaf width of *Sisyrinchium palmifolium* plants during 22 weeks of planting

The results of the ANOVA showed that the light intensity treatment affected the leaf length of *S. palmifolium* plants. In contrast, the gibberellin hormone treatment did not affect the leaf length growth of *S. palmifolium* plants. The interaction of the two treatments affected the leaf length growth of *S. palmifolium*. The light intensity of 50% paranet and 10 ppm gibberellin hormone was the most influential combinations on *S. palmifolium* leaf length.

Light intensity affects leaf growth. *S. palmifolium* leaf length that produces the most optimal length was 50% paranet light intensity. It was following the research of Rezai et al. (2018) that the light level under 50% shade has the largest leaf size. Plant leaf length growth decreased at 100% light intensity because the leaves were dry in the final weeks before harvest.

Leaf width

The growth of the leaf width of *S. palmifolium* tends to increase during the planting period. The most significant growth occurred in the eighth week of planting for *S. palmifolium* (Figure 4). Based on Table 1, the highest leaf width of *S. palmifolium* was treated with 100% light intensity and 10 ppm gibberellin hormone. Treatment of 50% light intensity and 0 ppm gibberellin hormone was the lowest mean leaf width of *S. palmifolium* plants. The mean leaf width of *S. palmifolium* was almost evenly distributed with 100% light intensity control treatment and 0 ppm gibberellin concentration.

The analysis of variance showed that the light intensity affected the leaf width of *S. palmifolium.* At the same time, the gibberellin hormone did not affect the leaf width of the *S. palmifolium* plant. Therefore, the interaction between light intensity treatment and gibberellin hormone did not affect the growth of leaf width of *S. palmifolium* plants.

The application of gibberellins did not affect the leaf width of *S. palmifolium* plants. The concentration of gibberellins would have an effect if the appropriate dose were given. In general, gibberellins function to increase leaf length and width (Salisbury and Ross 1995). Growth regulators must be given at the right dose so as not to inhibit plant production and work properly (Farida and Rohaeni 2019).

The intensity of light affects the leaf width of *S. palmifolium* plants. The quantity and quality of light can affect leaf morphology (Xu et al., 2011). The leaf width of plants at 100% light intensity decreased because the leaves dried up in the final weeks before harvest. The results of the average leaf width and length of *S. palmifolium* plants were thought to be genetically influenced. Endogenous hormones act as precursors (Wiraatmaja 2017). Plants will grow and develop as influenced by the environment. The environment will stimulate hormones to express genes so that they can change the development and metabolism of these plants.

Flowering time

The flowering stage of *S. palmifolium* plants begins with the formation of flower buds on the newly grown leeks. Based on Table 1, flowering plants of *S. palmifolium* were treated with 100% light intensity and 20 ppm gibberellin hormone and 50% paranet light intensity treatment and 0 ppm gibberellin hormone. The fastest flowering time of *S. palmifolium* was treated with 100% light intensity and 20 ppm gibberellins. The 50% light intensity treatment was a light treatment that can stimulate flowers compared to the 100% light intensity control treatment. In addition, a gibberellin concentration of 20 ppm can stimulate flower formation more than the control treatment.

The results of ANOVA showed that light intensity treatment and gibberellins did not affect the flowering time of *S. palmifolium* plants. Furthermore, the interaction between the treatment combinations did not affect the flowering time of *S. palmifolium* plants.

Flowers on plants were used as a parameter to determine the speed of the growth cycle from vegetative to

generative (Sari et al., 2020). The *S. palmifolium* plants that do not flower were thought to be caused by internal and external factors. External factors include the length of irradiation of plants. The length of irradiation can accelerate the plant to flower in large quantities. In addition, the length of irradiation depends on the type of plant (Lakitan 1996). Plant genetics also affects the time of flower emergence on the growth of *S. palmifolium* (Sari et al. 2020). Another factor was that the concentration of gibberellins was too low. The gibberellins concentration used for flowering in onions was between 500-1,000 ppm (Corgan and Motano 1975). The flowering process of the plant begins with the appearance of flower buds on the stem. The fastest flowering time was 112 days after planting. Flowering time on *S. palmifolium* was about 3-4 months after planting (Yusuf 2009). These factors cause the generative growth cycle of plants to be delayed.

Amount of flower

The *S. palmifolium* plants that were able to flower were plants that received treatment with 50% paranet light intensity and 0 ppm gibberellin hormone (G0C50) and 100% light intensity and 20 ppm gibberellin hormone (G20C100). Most flowers were treated with 100% light intensity and 20 ppm gibberellin hormone (Table 1). The treatment which stimulated flower formation was better than the control treatment (100% light intensity and 0 ppm gibberellin hormone).

The analysis of variance showed that the light intensity treatment did not affect the number of flowers of *S. palmifolium* plants. Likewise, the gibberellin hormone did not affect the number of flowers of *S. palmifolium* plants. Therefore, the interaction between light intensity and gibberellins did not affect the number of flowers of *S. palmifolium* plants.

The number of *S. palmifolium* flowers resulted from a combination of 2 treatments (Table 1). Flowers that appear as markers of plant growth end from the vegetative period to become generative, requiring sufficient nutrients to carry on their lives (Sari et al. 2020). Factors that could affect the hormone gibberellins in *S. palmifolium* plants were the concentration of substances, the response of plant parts to the given regulatory substances, and external environmental factors (Salisbury and Ross 1995). However, the unstable endogenous plant hormone caused the gibberellin hormone not to affect the number of *S. palmifolium* flowers (Hidayati et al. 2019).

Number of tillers

Based on Table 1, *S. palmifolium* tillers on 20 ppm hormone treatment tended to be more numerous than other hormone treatments. The treatments that produced the highest number of tillers were 100% light intensity and 20 ppm gibberellin hormone (G20C100). The combination of light intensity treatment and gibberellin hormone resulted in a higher average number of tillers than the control treatment.

The results of ANOVA showed that light intensity affected the number of tillers, while gibberellin hormone treatment did not affect the number of tillers of Sisyrinchium palmifolium. Furthermore, the interaction between light intensity and gibberellins did not affect the number of tillers of *S. palmifolium* bulbs.

With full light intensity treatment, *S. palmifolium* plants produced the most dominant number of bulbs. A large number of bulb tillers was caused by the plants' photosynthesis rate, which was proportional to the high light intensity. If the light intensity received were low, it caused slow plant growth because the photosynthesis process causes much water loss during respiration (Lakitan 1996). The small number of tillers was suspected because photosynthate was distributed for plant vegetative growth and development, such as leaf length, so the production of tiller bulbs was inhibited (Ekawati 2020). The number of tillers will affect the wet and dry weight of the plant.

The treatment of gibberellins did not affect the number of tillers, but a concentration of 20 ppm resulted in a higher number of tillers than other concentrations. One of the functions of gibberellins was the formation of bulb tillers. Sufficient hormone concentrations will stimulate assimilation so that the number of tillers increases within a certain limit (Wicaksono et al., 2016).

Wet weight of leaves, midribs, and bulbs

Wet weights of leaves, midribs, and bulbs were determined following harvest. According to Table 1, the 50% light intensity treatment resulted in the maximum wet weight, leaf, and midrib. The maximum value of wet leaf, midrib and bulb of *S. palmifolium* was obtained after treatment with 50% paranet light intensity and 13.25 grams of gibberellin hormone at a concentration of 10 ppm. At 10.28 grams, the lowest wet weight was treated with 75% paranet light intensity and 20 ppm gibberellin hormone. The combination of shade treatment and gibberellin administration resulted in a larger wet weight than neither shade treatment nor exogenous hormone administration.

The analysis of variance results indicated that light intensity affected the moist weight of *S. palmifolium* leaves, midribs, and bulbs. On the other hand, gibberellin treatment did not affect the moist weight of *S. palmifolium* leaves, midribs, or bulbs. However, the interaction between the two treatments affected the moist weight of S. palmifolium leaves, midribs, and bulbs. The most effective treatments were 50% paranet light intensity and 10 ppm gibberellin hormone.

Wet weight parameters of plants were used to show the ability of nutrient and water uptake in plants and their distribution to all plant parts (Ekawati 2020). Nutrients and water in the tissue would be used for plant metabolic activities so that plants would be able to maintain their lives. Treatment under paranet shade yielded higher weight yields than under 100% light intensity. In addition, not much water was lost in the respiration process, so the photosynthesis process of plants was not hampered, and the accumulation of photosynthate became more (Lakitan 1996).

The treatment of gibberellins did not affect the wet weight, midrib, and bulb of *S. palmifolium* plants. This condition showed that plants were not affected by gibberellins, so photosynthetic translocation was not

inhibited (Putrasamedja and Permadi 2004). Furthermore, applying external hormones to plants has a good physiological process, so it will not affect them significantly because the hormone functions well as a stimulant for plant physiological processes (Sitanggang et al. 2015).

Wet weight of leaves and midribs

Wet weight of leaf and midrib was obtained after harvest and separated from *S. palmifolium* bulbs. The heaviest wet weight of leaves and midribs resulted from treatment with 75% shade light intensity and 10 ppm gibberellin hormone. On the other hand, 100% light intensity treatment and 0 ppm gibberellin hormone were the lowest average. The data on the average wet weight of leaves and midribs of *S. palmifolium* are presented in Table 1.

The analysis of variance showed that the light intensity treatment affected the wet weight of the leaves and midrib of *S. palmifolium*, as well as the gibberellin hormone treatment, which affected the wet weight of the leaves and midrib of *S. palmifolium*. In addition, the interaction between light intensity and gibberellins also affected the wet weight of leaves and midribs of *S. palmifolium*.

The treatment of 75% paranet light intensity and 10 ppm gibberellin hormone was the combination treatment that had the highest effect on the wet weight of leaves and midrib of *S. palmifolium* plants. The wet weight of leaves and plant midrib at 75% paranet light intensity (under shade) was heavier than the wet weight at 100% light and 0 ppm gibberellin hormone. Apart from the plants being at 100% dry light intensity, the wet weight yield in the shade was due to the soil's high moisture and moisture content.

Bulbs wet weight

After harvesting and separating the bulbs from the leaves and midrib, the wet weight of the bulbs was determined. The 100% light intensity treatment resulted in the bulbs' highest average wet weight. The lowest light intensity and gibberellins were treated with 75% paranet light intensity and 20 ppm gibberellin hormone. The *S. palmifolium* plant bulb had the highest wet weight in the 100 % ppm light intensity and 20 ppm gibberellin hormone treatments. Table 1 contains statistics on the average wet weight of *S. palmifolium* bulbs.

The ANOVA analysis revealed that light intensity affected the wet weight of *S. palmifolium* bulbs. On the other hand, Gibberellins did not influence the wet weight of *S. palmifolium* bulbs. The interaction between treatment combinations affected *S. palmifolium* bulb weight.

The combination of 100% light intensity and 20 ppm gibberellin hormone had the greatest effect on the wet weight of *S. palmifolium* bulbs. Bulb wet weight at 100% light intensity resulted in more weight than treatment with paranet shade. Meanwhile, gibberellin concentrations were significantly higher than in the control condition. Wet weight under the shade of paranet reduces plant weight which was thought to be because the results of the photosynthesis process were distributed to the process of plant growth and vegetative development (Ekawati 2020). The concentration of gibberellins must follow the dose to be effective for plant growth and development. Therefore, the concentration of growth regulators must be given according to the dose so that the plant production process is not hampered and works properly (Farida and Rohaeni 2019).

The dry weight of leaves and midribs

The leaves and midribs that have been harvested and the wet weight were known, then dried using an oven to get the dry weight. The highest average dry weight of leaves and midribs of *S. palmifolium* was treated with 75% paranet light intensity and 10 ppm gibberellin hormone (G10C75). In comparison, the lowest average was 100% light intensity and 0 ppm gibberellin hormone (G10C100) of 0, 5 grams (Table 1).

Based on the ANOVA results, it was known that light intensity affected the dry weight of leaves and midrib of *S. palmifolium*, the same effect as the hormone gibberellin on the dry weight of leaves and midrib of *S. palmifolium*. Therefore, the interaction of light intensity treatment and gibberellin hormone affected the dry weight of leaves and midribs of *S. palmifolium*.

The most effective treatments on leaf and midrib dry weight were 75% paranet light intensity treatment and 10 ppm gibberellin hormone. These results showed that the dry weight of leaves and midribs was more in paranet shaded conditions than in full light intensity conditions. In addition, the gibberellin concentration treatment resulted in a heavier leaf and midrib dry weight than the control treatment (0 ppm gibberellin hormone). Dry and wet weight will show high yields proportional to the number of leaves (Salfia et al., 2020). The wet weight and the weight of leaves and plant midribs would increase due to many leaves. The leaves and midribs will absorb water and nutrients and be used in photosynthesis. As a result of photosynthesis, the wet and dry weights of leaves and midribs of plants would grow.

Bulb dry weight

After drying the wet weight of *S. palmifolium* bulbs in an oven, the dry weight was determined. According to Table 1, the treatment with 100 % light intensity resulted in the highest mean dry weight of *S. palmifolium* bulbs at 11.45 g. In contrast, the treatment with 20 ppm gibberellin hormone resulted in the lowest mean dry weight at 11.45 g. On the other hand, the treatment with 75% light intensity and 20 ppm gibberellin hormone resulted in the lowest mean dry weight of *S. palmifolium* bulbs at 8.64 g.

Light intensity and gibberellin hormone therapy affected the dry weight of *S. palmifolium* bulbs. The effect of light intensity treatment and gibberellins on the dry weight of *S. palmifolium* bulbs was considerable. The dry weight of the bulbs that had the most influence on the treatment combination's results was 100% light intensity, and a gibberellin concentration of 20 ppm was known. The dry weight of *S. palmifolium* bulbs was greater under control light intensity (100% light intensity) than under paranet shade stress conditions. Giving gibberellins led to a

greater dry weight of *S. palmifolium* bulbs than the control behavior (0 ppm).

Table 1 shows the wet and dry weights of *S. palmifolium* bulbs grown in shadow yielded a lighter weight than those grown in full sun. It was because shade-grown plants develop faster and have fewer leaves, resulting in a lighter wet and dry weight for the bulbs (Ekawati 2020). However, it differed with the wet and dry weights of leaves and midribs, which were lower since some plants without shade have dried up over the last week.

The terms "dry weight" and "wet weight" were used to describe plants' nutritional content and metabolic activity. If the weight gained were significant, the nutritional content and metabolic activity were enough. The dry weight parameter compares the photosynthesis-respiration balance in plants (Sari et al., 2020).

The plant's dry weight was gained through the photosynthetic light response mechanism. Photosynthesis occurs in the mesophyll of the leaf. Photosynthesis is a process that requires sunshine energy. Plant photosystems absorb vast amounts of sunlight, converting it to oxygen and carbon dioxide for the dark reaction. Starch and energy from the light reaction are accumulated in the dark process. The effects of photosynthetic assimilation will be sent to all plant organs, including leaves, bulbs, and roots. Larger photosynthetic assimilation will produce larger plant organs (Salisbury and Ross 1995).

Ratio shoot root

Based on Table 1, the highest mean shoot root ratio of *S. palmifolium* was obtained when the plants were under 50% light intensity. The highest average shoot root ratio was treated with 50% light intensity and 0 ppm gibberellin hormone. A 100% light intensity treatment and 0 ppm gibberellin hormone were the lowest average root shoot ratio for *S. palmifolium* plants. The shoot root ratio of plants under paranet shade was higher than under conditions of 100% light intensity. The control treatment with 0 ppm gibberellin concentration resulted in a higher shoot root ratio than the 10 ppm and 20 ppm gibberellin concentrations.

The ANOVA results show that light intensity affects the shoot root ratio of *S. palmifolium* plants. The gibberellin hormone had the same effect on the shoot root ratio of *S. palmifolium* plants. The interaction of treatment combinations also affected the shoot root ratio of *S. palmifolium* plants. The most influential shoot root ratio was the ratio after treatment with 50% paranet light intensity and 0 ppm gibberellin hormone.

The shoot root ratio increased in proportion to the increase in roots and number of leaves. The shoot root ratio was defined in plant growth as an important factor in determining plants' ability to absorb nutrients and metabolic processes. The dry weight ratio of shoot roots was used to determine the absorption of nutrients by the roots circulated to the plant canopy (Rudiansyah et al., 2017).

Plants need a sufficient shoot root ratio to circulate nutrients to all plant parts. Plant growth factors, namely genetics, influenced the shoot root ratio. Endogenous hormones act as precursors (Wiraatmaja 2017). The shoot root ratio of plants was influenced by the dry weight of the leaves, midribs, and bulbs of *S. palmifolium* plants. Another factor that affected plant growth and development was the environment. The environment will stimulate hormones to express genes so that they can change the development and metabolism of these plants. For example, gibberellins' concentration affected the growth rate of shoots or roots. If the concentration of gibberellins was increasing, the growth increased, but the diameter of the wee was getting narrower. This situation caused the plant not to experience additional shoot root weight (Rudiansyah et al., 2017).

Chlorophyll content

Based on Table 1 presented, it can be seen that the mean leaf chlorophyll content of the *S. palmifolium* plant ranges from 1.88 to 6.66 mol. The highest mean leaf chlorophyll content of *S. palmifolium* was treated with 50% paranet light intensity and 10 ppm gibberellin hormone. In contrast, the lowest leaf chlorophyll level was treated with 100% light intensity and 20 ppm gibberellin hormone. Treatment under paranet shade resulted in a higher mean leaf chlorophyll content than treatment with *S. palmifolium* without shade (100% control light intensity). Meanwhile, the control gibberellin concentration treatment (0 ppm) resulted in a lower mean leaf chlorophyll content than the 10 ppm gibberellin concentration.

The analysis of variance showed that the light intensity treatment did not affect the leaf chlorophyll content of *S. palmifolium*. Furthermore, the gibberellin hormone also did not affect the chlorophyll content of the leaves of *S. palmifolium* plants. Therefore, the interaction between light intensity treatment and gibberellins did not affect leaf chlorophyll levels.

Table 1 presents data on the average chlorophyll content of *S. palmifolium* bulbs. The highest chlorophyll content of *S. palmifolium* bulbs was the treatment of 50% paranet light intensity and 10 ppm gibberellin hormone; meanwhile, the 100% light intensity treatment and 10 ppm gibberellin hormone was the lowest chlorophyll content of *S. palmifolium* plant bulbs. The average yield of bulb chlorophyll content under shade conditions was higher than bulb chlorophyll content under 100% light intensity (control). The results of ANOVA analysis showed that light intensity did not affect the chlorophyll content of *S. palmifolium* bulbs. The gibberellin hormone also did not affect the chlorophyll content of *S. palmifolium* bulbs. The interaction between the two treatments did not affect the chlorophyll content of *S. palmifolium* bulbs.

Table 1 shows that the chlorophyll content of the leaves of *S. palmifolium* plants was higher than that of the bulbs of *S. palmifolium*. The highest chlorophyll content in the leaves and bulbs of *S. palmifolium* was found in the same combination of treatments, namely 50% paranet light intensity and 10 ppm gibberellin hormone. The result of chlorophyll content under 100% light intensity was lower than the light intensity under the paranet shade. Plants that absorb full light intensity produce lower total chlorophyll

content than plants that absorb limited light. Full light intensity functions to carry out photosynthesis and increase metabolic processes (Wulandari et al. 2016). Plant pigments' content was influenced by light intensity, temperature, and soil pH (Hasidah et al., 2017). Absorption of small amounts of light intensity for the photosynthesis process produced a greater amount of chlorophyll to be optimal for absorbing light (Salisbury and Ross 1995). Enzymes that play a role in chlorophyll synthesis can increase their role with light stimulation. Light accelerates the catalytic process of the chlorophyllase enzyme in converting protochlorophyllide into protochlorophyll a (Hasidah et al. 2017).

Carotenoid level

The carotenoids level in the leaves of *S. palmifolium* were measured for absorbance using a UV-Vis spectrophotometer. The results showed that the average carotenoid content showed that the highest leaf carotenoid content was the combination of 75% paranet light intensity treatment and 0 ppm gibberellin hormone. In comparison, 100% light intensity treatment and 10 ppm gibberellin hormone were the lowest average leaf carotenoid content (Table 1). The mean levels of leaf carotenoids in the control condition (100% light intensity) resulted in lower levels than the light intensity treatment under the paranet shade. Moreover, the mean leaf content in the control condition of the gibberellin hormone concentration was higher than in the treatment with the gibberellin hormone concentration.

The analysis of variance showed that the light intensity treatment affected the carotenoid levels in the leaves of *S. palmifolium* plants. The same thing happened to treat gibberellins hormones, affecting the carotenoid levels in the leaves of *S. palmifolium* plants. The interaction between the light intensity treatment and the gibberellin hormone affected the carotenoid levels in the leaves of *S. palmifolium* plants.

The average carotenoid content of *S. palmifolium* bulbs presented in Table 1 showed that the carotenoid content was in the range of 19.97-43.28 mol. The highest levels of carotenoids in *S. palmifolium* bulbs were treated with 50% paranet light intensity and 0 ppm gibberellin hormone at 43.28 mol. The mean carotenoid content of *S. palmifolium* bulbs was higher when treated under paranet shade than under full light intensity (100% light intensity). The average carotenoid levels' average results given the gibberellin hormone were lower than the control treatment (gibberellin hormone 0 ppm).

Based on the analysis of variance, it was known that the light intensity affects the carotenoid content of the bulb of *S. palmifolium.* On the other hand, the gibberellin hormone does not affect the carotenoid levels of *S. palmifolium* plant bulbs. Therefore, the interaction between the light intensity treatment and the gibberellin hormone affected the carotenoid levels of *S. palmifolium* plant bulbs.

Carotenoid levels of plants under paranet were higher than those with less than 100% light intensity. This result was allegedly due to plants that received full light, which increased the metabolic process for the photosynthesis process. Factors that affect the process of photosynthesis are the amount of light intensity absorbed, permanent pigments that absorb light, and complementary pigments such as carotenoids (Wulandari et al., 2016). Higher carotenoid content under paranet shade. The genetics of the plant also influenced carotenoids in plants. Each plant has genetic differences, affecting gene expression ability in the carotenoid synthesis process (Hasidah et al. 2017).

Light also plays a role in the process of carotenoid biosynthesis. In carotenoid biosynthesis, the enzyme mRNA level will increase if the enzyme were stimulated for its catalysis by light. For example, carotenoid hydroxylase (CH) and phytoene synthase (PSY) enzymes that function in carotenoid biosynthesis. Therefore, the light will increase the mRNA carotenoid hydroxylase and phytoene synthase levels, then phytoene (components of carotenoids) will increase so that carotenoid levels will increase too (Hasidah et al. 2017).

Flavonoid level

The flavonoid content of the *S. palmifolium* plant was obtained from the crushed part of the *S. palmifolium* bulb, then dissolved, and the absorbance was calculated using a UV-Vis spectrophotometer. The flavonoid content of the bulb of *S. palmifolium* was in the range of 0.64-0.67%. The highest flavonoid content of *S. palmifolium* bulb was obtained on the treatment combination of 75% paranet light intensity and 10 ppm gibberellin hormone 0.67%. The lowest flavonoid content of *S. palmifolium* was treated with 100% light intensity and 10 ppm gibberellins. The average levels of flavonoids did not affect the control treatment. The data on the average flavonoid content of *S. palmifolium* bulbs are presented in Table 1.

The ANOVA results indicated no effect of light intensity on the flavonoid content of *S. palmifolium* bulbs. Similarly, gibberellin hormone administration did not influence the flavonoid content of *S. palmifolium* plant bulbs. As a result, there was no effect of the interaction between the two treatments on the flavonoid content of S. palmifolium bulbs.

The light intensity affects the flavonoid content of *S. palmifolium* plants. Through flavonoid production, light can raise overall flavonoid levels. Flavonoids are synthesized via two distinct pathways: the polyketide pathway (malonic pathway/three acetate units) and the phenylpropanoid pathway (shikimate pathway). The polyketide pathway begins with the interaction of acetyl CoA with CO to form malonate CoA. Additionally, acetyl CoA combines with malonic CoA to form acetoacetyl CoA. Acetoacetyl CoA is formed and reacts with malonate CoA to generate poly acetyl. Next, this poly acetyl product will react with and condense the phenylpropanoid pathway product. The reaction results of the two pathways will produce flavonoid compounds (Mariana et al., 2013). The phenylpropanoid pathway reaction will use shikimic acid using phosphoenolpyruvate and erythrose. Shikimic acid was converted to phenylalanine and tyrosine. The resultant phenylalanine will release NH_3 and form cinnamic acid. Tyrosine will produce derivative cinnamic acid compounds

(Julianto 2019). Plants' most significant phenolic compounds were flavonoids (Taiz and Zeiger 2012).

Flavonoids have an important role as growth regulators. Flavonoids are critical for the long-term viability of plant physiological processes. Gibberellins have a crucial role in the production of flavonoids (Kim et al., 2009). Secondary metabolite production and chlorophyll synthesis occur in plants (Sukartini and Syah 2009). High light intensity is suggested to promote the accumulation of the wet and dry weight of *S. palmifolium.* Plant weight accumulation due to respiration and photosynthesis decreased flavonoid levels (Sari et al. 2020). This condition can occur due to chlorophyll production inhibiting the activity of flavonoid synthesis (Hasidah et al., 2017).

The calibration curve for quercetin in Figure 5 indicates that the higher the concentration, the greater the absorbance. The standard quercetin curve yielded the regression equation y = 0.0574x + 1.5737 with an R2 value of 0.9198. The quercetin calibration curve equation is a reference point for determining the total flavonoid concentration in a sample.

Stomata density

Stomata density was obtained from observing stomata on the lower epidermis of the leaves of *S. palmifolium* plants. The average leaf stomata density of *S. palmifolium* (Table 1) was found in the observation area with 100% light intensity treatment and 0 ppm gibberellin hormone of 183.43/mm^2. The average stomatal density was at least 50% paranet light intensity treatment, and 10 ppm gibberellin hormone was 105.30/mm^2. The mean leaf stomata density of *S. palmifolium* resulted in higher data in the control treatment. It shows that the treatment of light intensity under the shade and the concentration of gibberellins do not affect the amount of stomatal density.

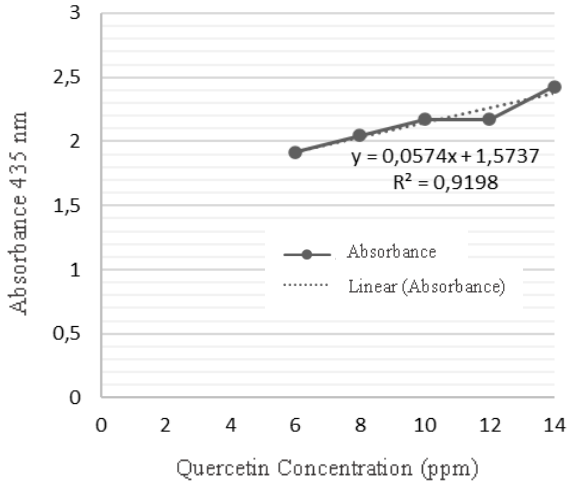

Figure 5. Quercetin calibration curve at a maximum wavelength of 435 nm

Based on the analysis of variance, light intensity affects the stomata density of *S. palmifolium* leaves. However, the gibberellin hormone also did not affect the stomata density of the leaves of *S. palmifolium* plants. Therefore, the interaction between the combination of light intensity treatment and gibberellins did not affect the stomata density of *S. palmifolium* leaves.

Full light intensity resulted in higher leaf stomata density than low light intensity. The reason was that sunlight received at a high frequency would increase the rate of photosynthesis, respiration, and metabolism. Based on research Bowen (1991) states that the process of photosynthesis will increase if many stomata are open. If many stomata were open, much carbon dioxide would enter the light reaction process of photosynthesis. Photolysis of water produces hydrogen, which will be used for the dark reaction, and oxygen, which is released for respiration. In addition, the photosynthesis stage also produces glucose which could be distributed and accumulated in plant organs. A lot of carbon dioxide will increase the production of flavonoids through respiration. As a result of the Krebs cycle, acetyl CoA will react with CO in the polyketide cycle to produce poly acetyl compounds and react-condensate with the results of the phenylpropanoid pathway to form flavonoid compounds (Mariana et al. 2013). This condition correlates with the fact that the more stomata were open, the more photosynthate accumulation in plants increased.

In conclusion, 50% paranet light intensity affects the growth of *S. palmifolium* plants. The increased parameters were leaf number, length, wet leaf weight, midrib, bulb, shoot root ratio, chlorophyll, and carotenoid content of *S. palmifolium* bulbs. The concentration of gibberellins at 10 ppm affected the growth and flavonoid content of *S. palmifolium* plants. Growth parameters included leaf length, width, midrib and bulb wet weight, leaf and midrib wet weight, and leaf and bulb chlorophyll content. The interaction of light intensity and gibberellin hormone affected the growth of *S. palmifolium* plants. It was shown on leaf length parameters, wet weight of leaves, midrib, and bulbs, wet weight of leaves and midribs, wet weight of bulbs, dry weight of leaves and midribs, dry bulb weight, shoot root ratio, as well as leaf and bulb carotenoid content. The interaction of light intensity treatment did not affect the growth and flavonoid content of *S. palmifolium* plants, including the number of leaves, leaf width, flowering time, number of flowers, number of tillers, chlorophyll content, leaf stomata density, and flavonoid content of *S. palmifolium* plants. The combination of 50% paranet light intensity and 10 ppm gibberellin hormone affected the growth of *S. palmifolium* plants. This interaction can increase the parameters of leaf length, wet weight of leaves, midrib, bulbs, and chlorophyll content of leaves and bulbs of *S. palmifolium* plants.

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Study of seed maturity level and duration of immersion in auxin solution on growth of *Anthurium hookeri* seedlings

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Abstract. *Prihandono S, Haryanto ET, Pujiasmanto B. 2020. Study of seed maturity level and duration of immersion in auxin solution on growth of Anthurium hookeri seedlings. Cell Biol Dev 4: 64-70.* Anthurium is one of the favorite indoor potted plants to decorate the room. One type of anthurium is *Anthurium hookeri* Kunth. The problem in anthurium cultivation is very slow plant growth. This study aimed to study the effect of seed maturity level and duration of immersion in auxin solution on the growth of *A. hookeri* seedlings. This study used a completely randomized design arranged in a factorial manner and consisted of two factors. The first factor is the level of maturity of the seeds (seeds aged 1-3 days after harvest, seeds aged 4-6 days after harvest, and seeds aged 7-9 days after harvest). The second factor was the duration of immersion (without soaking in the IAA solution, soaking for 15 minutes in the IAA solution, soaking for 30 minutes in the IAA solution, and soaking for 45 minutes in the IAA solution). The variables observed were germination variables (moisture content of seeds at harvest, germination fastness, germination potency, germination value, and germination unison) and growth variables (root length, seedling height, number of leaves, and leaf length). The data obtained on the germination variable were analyzed descriptively. In contrast, the data obtained on the growth variable was analyzed by the F test at the 5% level. If there was a significant difference, it was continued using Duncan's Multiple Distance Test (DMRT) at the 5% level. The results showed that on the germination variable, treatment of 1-3 days old seeds and soaking in IAA solution for 30 minutes gave better results than other treatments. Seed age treatment did not affect all growth variables except the number of leaves. The immersion treatment in the IAA solution only affected the number of leaves and did not affect other growth variables. There was an interaction between the treatment of seed age and immersion in the IAA solution on the variable of the number of leaves.

Keywords: Immersion duration, seed maturity level, seedling growth

INTRODUCTION

Ornamental plants in Indonesia still have very potential market prospects to be developed in type and variety. Therefore, opportunities to develop ornamental plant cultivation are useful to meet domestic and foreign needs. In addition, ornamental plant agribusiness will be able to encourage the economy of the people who develop it (Sastrapradja 1975).

Anthurium is a tropical ornamental plant with high appeal as a room decorator because of its beautiful leaf and flower shape. Types of anthurium in terms of aesthetics are classified into two: flower-type of anthurium and leaf-type of anthurium. The leaf type of anthurium is one of the favorite indoor potted plants to decorate the room (Chen et al. 2004; Henny and Chen 2004). One type of leaf type of anthurium is *Anthurium hookeri* Kunth.

Anthurium hookeri is an ornamental plant with high appeal as a room decoration because of its beautiful leaf and flower shape. This plant is in great demand by consumers and has high economic value, so it is very potential to be developed. Demand for various species of anthurium is currently very high and demands good plant quality. Some of the criteria consumers want are broad-leaved plants with a large number of leaves and tough and fresh plants. The quality of this plant is very influential on the selling price of the plant (Sakya et al. 2008).

Anthurium hookeri is a flowering plant species belonging to the genus *Anthurium.* Almost typically, the specimens sold are hybrids and not the species. *A. hookeri* is a unique plant because it has short internodes, dense roots, and cataphylls shaped like lances. The stems of the leaves are either triangular or D-shaped and are 2-9 cm long. The leaves are rosulate, 10-26 cm wide and 35-89 cm long. The veins on the leaves are scalariform and supervolute. There are tiny black glands all over the leaves. The white berries that the plant produces are easy to spot. *A. hookeri* has a plain inflorescence that spreads out and hangs down along with the blue spadix, which stands straight up. The spathe is cylinder-shaped and green with a hint of purple. Most people think the seed berries on the infructescence are red, but they are oval or oblong and not red. *Anthurium* species are known to have a lot of differences, and not every leaf on every plant will look the same. However, the sexual traits stay the same (https://www.exoticrainforest.com/Anthurium%20hookeri%20pc.html).

Anthurium hookeri loves humid environments. Since epiphytes normally do not have soil-based roots, it also requires very well-draining soil to thrive. The roots are linked to the host tree and hang in the air. Therefore, this plant is suitable for planting in soil that dries quickly and does not retain moisture (https://www.exoticrainforest.com/Anthurium%20hookeri%20pc.html)

The problem in cultivating *A. hookeri* or other types of Anthurium is that plant growth is very slow. Some obstacles faced in anthurium nurseries are the slow growth of sprouts and seedlings, many dead seeds, abnormal growth of sprouts, and non-uniform growth of sprouts and seedlings. Several alternative ways to overcome these problems include ripe seeds in the nursery and immersing the seeds in a phytohormone solution. One solution to be used is auxin. Chemically ripe seeds have sufficient food reserves and a perfect embryo so that they are ready to germinate. In addition, immersing seeds in auxin allows stimulation of somatic embryos in the formation of shoots and roots more quickly and uniformly (George et al. 2008; Liu et al. 2013).

This research aimed to study the effect of seed maturity level and soaking duration in auxin solution on the growth of *A. hookeri* seedlings.

MATERIALS AND METHODS

This research was conducted in Margorejo, Gilingan, Banjarsari, Surakarta, Central Java, Indonesia, at an altitude of 97 masl. This research started from March 2009 to May 2009.

This study used a completely randomized design (CRD) which was arranged in a factorial manner and consisted of two factors. The first factor was the level of maturity of the seeds (seeds aged 1-3 days after harvest, seeds aged 4-6 days after harvest, and seeds aged 7-9 days after harvest). The second factor was the duration of immersion (no immersion, immersion for 15 minutes in IAA solution with a concentration of 100 ppm, immersion for 30 minutes in IAA solution with a concentration of 100 ppm, and immersion for 45 minutes in IAA solution with a concentration of 100 ppm). Thus, 12 treatment combinations were obtained. Each treatment combination was repeated 3 times so that 36 experimental pots were obtained.

The variables observed were germination variables (moisture content of seeds after harvest, germination fastness, germination potency, germination value, and germination unison) and growth variables (root length, seedling height, number of leaves, and leaf length). The data obtained on the germination variable were analyzed descriptively. In contrast, the data obtained on the growth variable was analyzed by the F test at the 5% level. If there was a significant difference, it was continued using Duncan's Multiple Distance Test (DMRT) at the 5% level.

RESULTS AND DISCUSSION

Seed water content after harvest

Water content is weight loss when the seeds are dried according to a certain technique or method. Determining a group of seeds' water content is very important. It is related to the success of germination because water content influences a seed's rate of decline. Within a certain limit, the lower the water content of the seed, the longer the viability of the seed.

The maturity level of anthurium seeds affects the water content of the seeds to be sown. For example, Table 1 shows that the seeds aged 1-3 days after harvest have a water content of 33%, the seeds aged 4-6 days after harvest have a moisture content of 34%, and the seeds aged 7-9 days after harvest have a water content of 35%. It also shows that the longer the harvest, the higher the water content. It is thought to be in line with the harvesting process, which gradually increases rainfall. So, the water content of the fruit increases and affects the water content of the seeds as well.

The germination process and growth are strongly influenced by the availability of water in the seed and water in the growth medium for absorption and stimulating the activity of enzymes for germination metabolism in the seed. Therefore, water content that is too high can cause seeds to germinate before planting. Besides, the too-high water content can stimulate the development of pathogens in the storage area, but keep in mind that too-low water content will cause damage to the embryo (Sutopo 1985).

Germination fastness

Technically, germination is the beginning of active growth that produces seedlings. During germination, biochemical, physiological, and morphological changes occur. Through these reactions, the food reserves in the seeds are broken down, which are then used for the formation and growth of parts of the sprouts, such as the plumule and radicle. Unfavorable sub-optimum conditions in the field can increase the seeds' weakness, decrease the germination percentage, and slow seed growth. Therefore, it is necessary to test the germination fastness. According to Sadjad (1974), in Sutopo (1985), the speed of growth indicates a fast-growing seed will be better to face sub-optimum field conditions.

Table 2 shows that *A. hookeri* seeds have a low mean germination fastness. Plant genetic factors caused it. It was per the opinion of Heydecker (1972) in Sutopo (1985), which states that certain cultivars are more sensitive to unfavorable environmental conditions. Unsuitable environmental conditions can result in a decrease in seed vigor. Low seed vigor can result in a decrease in the fastness of seed germination. Although the average seed germination fastness of *A. hookeri* was relatively low, the treatment of a 1-3 days old seed and immersion in IAA solution for 30 minutes gave a better percentage of germination fastness than other treatments.

Table 1. The water content of seeds at harvest

Seed age	Water content (%)
1-3 days after harvest	33
4-6 days after harvest	34
7-9 days after harvest	35

Table 2. Average germination fastness of *Anthurium hookeri*

No immersion (days old)		Immersion for 15 minutes	Immersion for 30 minutes	Immersion for 45 minutes	Average
1-3	30.00	23.30	43.30	36.70	33.32
4-6	23.30	36.70	23.30	26.70	27.50
7-9	10.00	13.30	30.00	23.30	19.15
Average	21.10	24.40	32.20	28.90	

Seed aged 1-3 days gave a better germination fastness than the treatment of 4-5 days or 7-9 days of seed age. Seeds aged 1-3 days are suspected of having the lowest water content among other treatments. Water content is very influential on germination; with excessive seed water content, the level of water absorption at the time of germination cannot be optimal because, during the ripening period of the seed, water is reduced or lost from the seed. Still, for germination, it is necessary to add water again. Although according to Kamil (1979), the water absorbed by the seeds is useful for softening the seed coat and causing rupture or tearing, water is also useful in diluting the protoplasm to activate various functions of the protoplasm. Moreover, water can also stimulate the activity of enzymes for germination metabolism in seeds.

The treatment of immersion in IAA solution affects the fastness of seed germination so that the seeds can produce more sprouts. The immersion in the IAA solution for 30 minutes resulted in a better percentage of germination fastness than the immersion treatment for 15 minutes and 45 minutes. It is suspected that at the time of immersion for 30 minutes, the amount of absorbed IAA had reached the optimum concentration for the plant's growth, so adding more auxin at 45 minutes decreased germination's fastness. According to Wilkins (1989), the hormone auxin increases growth until it reaches an optimal concentration. Still, if the concentration exceeds optimal, it will interfere with plant metabolism and development.

Germination potency

Seed germination potency provides information to seed users on the ability of seeds to grow normally into plants that produce reasonable yields under optimum field biophysics conditions.

The germination test is to germinate the seeds under conditions suitable for the germination needs of the seeds, then calculate the percentage of seed germination. The germination percentage is the number of proportions of seeds that have produced germination under certain conditions and periods. The purpose of the germination test was to obtain information on the value of planting seeds in the field that had been treated with immersion in IAA solution and to compare seed quality between seed groups.

Table 3 shows that *A. hookeri* seeds have a low average germination potency (<70%). As with the variable of germination fastness, the low germination potency was caused by plant genetic factors that decreased seed vigor and viability. Although the average normal germination potency of *A. hookeri* seeds was low, treatment of 1-3 days old seed and immersion in IAA solution for 30 minutes gave a better germination fastness than the other treatments.

Treatment of 1-3 days old seeds gave better germination results than treatments of 4-5 days old or 7-9 days old. It is related to the water content of seeds. 1-3 days old seeds have the lowest water content compared to other treatments. According to Kuswanto (2003), at high water content, seed respiration runs faster. It produces heat which will increase the temperature of the seed so that the seed will rapidly decrease vigor and viability. With lower moisture content, the seeds could undergo normal germination because of the higher viability of the seeds.

The treatment of immersion in IAA solution for 30 minutes and 45 minutes affected the germination of the seeds so that the seeds could produce more normal sprouts. The treatment of immersion in IAA solution for 30 minutes gave a better percentage of normal germination than the treatment of immersion for 45 minutes. It is assumed that at the time of immersion for 30 minutes, the amount of absorbed IAA had reached the optimum concentration to encourage germination. PGR from the auxin group at the right concentration can stimulate cell division. It is suspected that cell division occurred at this concentration; cell permeability and water absorption by seeds increased so that seed food reserves could be broken down for the seed germination process.

Germination value

The germination value is the multiplication of the peak value with the average value of daily germination. The peak value is the percentage of germination at the time the germination fastness begins to decrease, divided by the number of days required to reach it. The average daily germination is the percentage of germination at the stop point of the percentage of germination divided by the total test days (Sutopo 1985).

Table 4 shows that the treatment of 1-3 days old seeds and immersion in IAA solution for 30 minutes gave a better average germination value than the other treatments. In addition, the treatment of immersion in IAA solution for 30 minutes gave a better average germination value than the treatment of immersion for 15 minutes or 45 minutes. However, it is suspected that at the time of immersion for 30 minutes, the amount of absorbed IAA had reached the optimum concentration for the plant's growth, so adding more auxin at 45 minutes decreased the germination value.

Table 3. Average germination potency of *Anthurium hookeri*

No immersion (days old)		Immersion for 15 minutes	Immersion for 30 minutes	Immersion for 45 minutes	Average
1-3	70.00	53.30	83.30	66.70	68.33
4-6	40.00	66.30	56.70	66.70	57.43
7-9	60.00	50.00	66.70	50.00	56.68
Average	56.67	56.53	68.90	61.13	

Table 4. Average germination value of *Anthurium hookeri*

No immersion (days old)		Immersion for 15 minutes	Immersion for 30 minutes	Immersion for 45 minutes	Average
1-3	24.40	27.50	30.50	28.50	27.70
4-6	27.50	19.80	27.50	29.50	26.08
7-9	25.40	28.50	29.00	27.50	27.60
Average	25.77	25.27	29.17	28.50	

Table 5. Average germination unison of *Anthurium hookeri*

No immersion (days old)		Immersion for 15 minutes	Immersion for 30 minutes	Immersion for 45 minutes	Average
1-3	66.70	50.00	80.00	63.30	65.00
4-6	40.00	60.00	56.70	63.30	55.00
7-9	56.70	46.70	60.00	46.70	52.53
Average	54.47	52.23	65.57	57.77	

Treatment of seeds aged 1-3 days gave a better germination value than the treatment of seeds aged 4-5 days or 7-9 days. It is suspected that seeds at the age of 1-3 days have the lowest water content among other treatments. Moisture content is very influential on germination. Excessive seed moisture content will cause the level of water absorption at the time of germination to not take place optimally because, during the ripening period of the seeds, water is reduced or lost from the seeds. On the contrary, for germination, additional water is needed. It is clarified by Kamil (1979) that the water absorbed by the seeds is useful for softening the seed coat and causing the seed coat to break or tear. It is also useful in diluting the protoplasm so that it can activate various functions of the protoplasm. Besides, water can also stimulate the activity of enzymes for germination metabolism in seeds.

Germination unison

Germination unison is influenced by the seed's size, weight, and physiological maturity. In addition, it is related to the amount and content of organic matter in the seeds needed in the germination process (Mugnisjah 1994).

Table 5 shows that the treatment of seeds aged 1-3 days and immersion in IAA solution for 30 minutes gave a better average germination unison than other treatments. In addition, the treatment of immersion in IAA solution for 30 minutes gave a better average of germination unison than the treatment of immersion for 15 minutes or 45 minutes. However, it is suspected that at the time of immersion for 30 minutes, the amount of absorbed IAA had reached the optimum concentration for the plant's growth, so adding more auxin in 45 minutes' immersion treatment decreased the germination unison.

In the treatment of seed age, the older age of seeds led to a lower average of germination unison. Seeds aged 1-3 days had better germination unison compared to treatments of seeds aged 4-5 days or 7-9 days. It is suspected that seeds at the age of 1-3 days have the lowest water content among other treatments. According to Kuswanto (2003), at high water content, seed respiration runs faster. During the respiration process, heat is generated, which will increase the temperature of the seed, so the seed will rapidly decrease both vigor and viability. With lower moisture content, the seeds could undergo normal germination because of the higher viability of the seeds.

Germination unison is also influenced by the level of maturity of the seeds. Still, this study was not too influential because all the seeds used had reached physiological maturity. The maturity level affects the amount and type of food reserves. Three kinds of food substances affect germination and growth: carbohydrates, fats, and proteins. Carbohydrates are the largest part of seeds. They function as a source of energy needed for growth and germination. Fat is found in the vegetative and reproductive organs but mostly in seeds as a food reserve that serves as a source of energy for growth. Protein is a food substance in seeds and functions as a protoplasm former at the beginning of growth (Kamil 1979). According to Sutopo (1985), seed food reserves influence seed viability. If the content of food reserves in the food network is too small in number and variety, then the viability of the seeds is low at harvest time.

Root length

Roots are one of the most important plant organs supporting plant growth. Roots function as an absorber of nutrients contained in the planting medium. Long roots will expand the area of nutrient absorption so that the distribution of nutrients from the planting medium to the plants can run smoothly. In addition, the roots serve as a reinforcement for the establishment of the plant.

From the analysis of variance, it was found that the treatment of seed age and immersion in IAA solution had no effect on root length, and there was no interaction between seed age and immersion time on root length. It is presumably because the concentration of endogenous IAA in seedlings is already optimum to stimulate the division and elongation of cells in the roots, so adding IAA will inhibit root elongation. Per the opinion of Salisbury and Ross (1995), the application of relatively high concentrations of IAA to the roots will cause inhibition of root elongation but increase the number of roots. On the other hand, IAA can promote root elongation at very low concentrations.

Inhibition of root growth is strongly influenced by endogenous control in plants. This inhibition is not only caused by the concentration of auxin, which is too high, but also by root inhibitor compounds in the form of phenol and manganese compounds (Jarvis 1986). Phenol compounds, namely monophenols and manganese (Mn^{2+}), are important cofactors in the activity of the IAA oxidase enzyme (Krisnamoorthy 1981). Monophenol is a growth inhibitory substance because it affects increasing IAA oxidase activity so that it will reduce the auxin content in the plant body.

Although statistically, the results of the analysis showed that the results were not significantly different, there was a tendency for the best mean root length to be obtained in the treatment without immersion in IAA solution and seed age of 4-6 days (Table 6).

Table 6. Average root length of *Anthurium hookeri* at 12 WAP

No immersion (days old)		Immersion for 15 minutes	Immersion for 30 minutes	Immersion for 45 minutes	Average
1-3	9.10	8.90	9.50	7.00	8.63
4-6	9.50	8.50	8.50	8.20	8.68
7-9	8.00	7.80	7.50	7.60	7.73
Average	8.87	8.40	8.50	7.60	

Table 7. Average seedling height of *Anthurium hookeri* at 12 WAP

No immersion (days old)		Immersion for 15 minutes	Immersion for 30 minutes	Immersion for 45 minutes	Average
1-3	3.60	3.50	3.10	3.30	3.38
4-6	4.20	3.70	3.30	3.70	3.78
7-9	3.20	3.40	3.50	3.90	3.50
Average	3.67	3.53	3.30	3.63	

Seedling height

Seedling height is a plant size often observed both as a growth indicator and as a parameter to measure the effect of the treatment. So, it is because plant height is the most easily observed growth measure (Sitompul and Guritno 1995). From the analysis of variance, it was found that the treatment of seed age and immersion in IAA solution had no effect on seedling height, and there was no interaction between seed age and soaking time on seedling height. It is thought to be due to the relatively high concentration of IAA used. The main site of auxin synthesis in plants is in the apical meristem of the tip of the shoot. IAA produced in the shoot tip is transported to the bottom and promotes the elongation of stem cells. IAA promotes stem cell elongation only at a certain concentration of 0.9 g/l. Above this concentration, IAA will inhibit stem cell elongation. This inhibitory effect probably occurred because the high concentration of IAA resulted in plants synthesizing other PGRs, namely ethylene, which gave the opposite effect to IAA (Purwanto 2006).

It is clarified by Salisbury and Roos (1995) that auxin is effective at a certain amount. Concentrations that are too high can damage. Otherwise, concentrations below the optimum are ineffective. According to Wilkins (1989), the hormone auxin increases growth until it reaches an optimal concentration, but if it is higher than the optimal concentration, metabolism and plant development will be disrupted.

Although the analysis showed that the results were not significantly different, there was a tendency for the best seed height to be obtained in the treatment of no immersion and seeds aged 4-6 days (Table 7).

Number of leaves

The beauty of the leaves is one of the attractions of the *A. hookeri* plant. However, *A. hookeri* growth is very slow, characterized by a slow increase in the number of leaves. According to Gardner et al. (1991), leaves are carbohydrate factories for cultivated plants, where leaves are needed for absorption and converting sunlight through photosynthesis, which is used for plant growth and development. Therefore, the number of leaves indicator can be used as supporting data to explain the growth process. From the analysis of variance, it was found that the treatment of seed age and immersion in IAA solution had a significant effect on the number of leaves, and the interaction between the two treatments had a very significant effect on the variable number of leaves of *A. hookeri*.

The immersion treatment was related to the IAA absorption process that occurred on the entire surface of the seeds. According to Lakitan (1996), the absorption process in plant cells is influenced by the permeability of the cell membrane and the difference in water potential between the inside and outside the cell. Therefore, absorption by plant cells will increase the turgor pressure in the cells, which in turn will cause cell enlargement.

IAA can enter plant cells because the cell membrane contains auxin receptors in the form of proteins (Salisbury and Roos 1995). IAA enters through the cell membrane by osmosis, where water can diffuse from a solution with a high potential to a low potential until it reaches the same water potential (Campbell et al. 2002).

Table 8 shows the highest average number of leaves obtained in the treatment of no immersion, namely 4 leaves. The lowest average number of leaves was found in the immersion treatment for 15 minutes, as many as 3.33 (3 blades). The treatment of no immersion was significantly different from the treatment of immersion for 15 minutes. The immersion treatments for 30 minutes or 45 minutes resulted in an average number of leaves that were not significantly different from the other two treatments. The condition showed that the immersion treatment for 30 minutes or 45 minutes produced the same number of leaves when the seeds were soaked for a shorter time, although the treatment of no immersion produced the highest number of leaves.

Auxin is a growth hormone that is inseparable from the process of plant growth and development. This compound can support cell elongation in shoots. By providing growth regulators, auxin can stimulate roots, affecting shoot growth and leaf formation (Soedjono 1997). However, in this study, the IAA immersion treatment did not provide a better plant response than the control. It was because the endogenous hormone content was optimal to stimulate the process of cell division and cell differentiation. The supply of hormones from outside can stimulate plant physiological processes, but the response depends on the level of endogenous hormones (Hidayanto et al. 2003).

Table 9 shows the highest average number of leaves was obtained in the treatment of seeds aged 1-3 days and ages 4-6 days, while the lowest average number of leaves was found in the treatment of seeds aged 7-9 days. Treatment of seeds aged 1-3 days was not significantly different from those aged 4-6 days, but the two treatments differed significantly from those aged 7-9 days. The seeds aged 7-9 days had higher moisture content than the other two treatments. According to Kuswanto (2003), at high water content, seed respiration runs faster, and heat is generated during the respiration process, which will

increase the seed's temperature, so the seed will rapidly decrease both vigor and viability.

Sutopo (1985) states that high water content can cause respiratory activity to increase, which can cause food reserves in the seeds to be depleted so that the vigor and viability of the seeds decrease. Therefore, seeds that have low vigor can cause a decrease in plant production. It can be seen in the average number of leaves of seeds aged 7-9 days which is lower than the average number of leaves in the treatment of seeds aged 1-3 days and seeds aged 4-6 days.

Leaf length

Leaves are very important organs for plants. Leaves are needed to absorb and convert light energy to grow and produce crops. Leaf length is one component of the leaf. From the analysis of variance, it was found that the treatment of seed age and immersion in IAA solution had no effect on leaf length, and there was no interaction between seed age and soaking time on leaf length. It is presumably due to the nature of the IAA itself, which is easy to spread to other parts, so the IAA's effectiveness is lost. Another possibility is that IAA is basipetal (moving towards the base), accumulating in the roots and affecting root formation (Gardner et al. 1991).

Table 8. The average effect of immersion time on the number of leaves of *Anthurium hookeri* at 12 WAP

Treatment	Number of the leaf (blade)
IAA immersion for 0 minutes	4.00 a
IAA immersion for 15 minutes	3.33 b
IAA immersion for 30 minutes	3.56 ab
IAA immersion for 45 minutes	3.56 ab

Note: Numbers followed by the same letter are not significantly different at the 5% level of DMRT

Table 9. The average effect of seed age on the number of leaves of *Anthurium hookeri* at 12 WAP

Treatment	Number of the leaf (blade)
Seeds aged 1-3 days	3.75 a
Seeds aged 4-6 days	3.75 a
Seeds aged 7-9 days	3.33 b

Note: Numbers followed by the same letter are not significantly different at the 5% level of DMRT

Table 10. Average leaf length of *Anthurium hookeri* at 12 WAP

No immersion (days old)		Immersion for 15 minutes	Immersion for 30 minutes	Immersion for 45 minutes	Average
1-3	4.50	3.20	4.10	3.10	3.73
4-6	5.00	3.80	3.00	3.30	3.78
7-9	3.50	2.80	3.20	3.40	3.23
Average	4.33	3.27	3.43	3.27	

Although the analysis showed that the results were not significantly different, the best trend in the mean leaf length was found in the treatment of no immersion and seeds aged 4-6 days (Table 10). Compared with the control, the plants showed a decrease in leaf length. Endogenous auxin content in seedlings was sufficient for seedling growth, so immersion in IAA with a concentration of 100 ppm could not provide a better growth effect. However, giving IAA can stimulate leaf cell enlargement. By giving the right concentration, the growth activity of cell enlargement becomes higher (Purwanto 2006).

Based on the research, it can be concluded: (i) treatment of seeds aged 1-3 days and immersion in IAA solution for 30 minutes gave better results than other treatments on germination variables; (ii) treatment of seeds age only affects the number of leaves variable. Therefore, the long age of seeds leads to a decrease in the number of leaves; (iii) the immersion treatment in IAA solution only had an effect on the variable number of leaves but had no effect on other growth variables. Therefore, the longer immersion time will produce a lower number of leaves; (iv) there is an interaction between the treatment of seed age and immersion in IAA solution on the variable number of leaves.

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Factors influencing micropropagation and somatic embryogenesis of two cassava varieties, Kello and Qulle

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Abstract. *Berhanu R, Feyissa T. 2020. Factors influencing micropropagation and somatic embryogenesis of two cassava varieties, Kello and Qulle. Cell Biol Dev 4: 71-81.* Cassava (*Manihot esculenta* Crantz) is a perennial Euphorbiaceae shrub cultivated in the tropics, Africa, and Asia for its tuberous starchy roots. Lack of good-quality seed, low productivity and profit, virus and insect pests, high heterozygosity, low natural fertility, poor seed set, and seed germination limit its cultivation. The conventional breeding system is ill-equipped to address these issues. Therefore, additional biotechnological solutions are required to address the abovementioned issues. This research aimed to devise a method for obtaining a maximum number of shoots and morphologically sound in vitro materials by micropropagation, as well as to investigate the various parameters related to the micropropagation of the two cassava varieties, 'Kello' and 'Qulle.' The research also contains a somatic embryogenesis technique that is an optimal source of in vitro materials for large-scale multiplication. In this experiment, a Solid MS medium with different salt concentrations, sucrose concentrations, TDZ, a two-step procedure involving pre-soak of nodal explants in a liquid MS medium prior to culture on a solid MS medium, with different pH values, repeated subcultures, and somatic embryo induction on MS medium supplemented with 2,4-D alone and in combination with 2 µM $CuSO_4$ were utilized. At a concentration of 0.2 mg/L TDZ, both 'Kello' and 'Qulle' exhibited the highest average number of shoots per explant and improved morphological properties of in vitro material. At a TDZ concentration of 0.2 mg/L, the two-step cultivation strategy produced the greatest mean number of shoots per plant for both kinds. The maximum mean number of shoots per explant was produced by 'Kello' at a medium salt strength of a quarter, but 'Qulle' required a medium salt strength of full. 'Kello' and 'Qulle' produced the greatest average number of shoots per explant at pH values of 5.6 and 6.6, respectively. At 0.15% sucrose, 'Kello' and 'Qulle' attained their highest mean values for all parameters. During somatic embryo induction, treatments with varying doses of 2,4-D generated FEC and somatic embryos, but the somatic embryos failed to progress through the globular stage. The micropropagation property of the two types through sequential subculturing suggested that repeated subculturing causes the in vitro materials to lose their multiplication property.

Keywords: 2,4-D, FEC, *Manihot esculenta*, salt tolerance, shoot multiplication, sucrose, TDZ

INTRODUCTION

Manihot esculenta Crantz is a perennial shrub of the Euphorbiaceae family. It is mostly cultivated in the tropics for its starchy tuberous roots, consumed by humans, fed to animals, and used as raw material in the starch industry. Among the most important tropical crops in calorie supply, which include rice, sugarcane, maize, and cassava, it is more inexpensive to plant cassava (Mathews et al. 1993; Raemakers et al. 1993; Nweke 2004). Cassava ranks five as a world food crop but two for tuber crops after potatoes (Lestari and Apriyadi 2017). In addition, due to its vast adaptation to a variety of soil, climate, drought tolerance, and capacity to grow on marginal soil, it is vital to the agricultural economies of several tropical nations (Mathews et al. 1993; Raemakers et al. 1993; Le et al. 2007).

The centers of diversity include central and north-eastern Brazil, south-western Mexico, and eastern Bolivia. Cassava is also cultivated on the continents of Africa and Asia. However, the global average yield is merely a small fraction of the maximum yield. One of the reasons for this low yield is the use of diseased and insect-infested cuttings as the starting material (Raemakers et al. 1993).

Cassava, sometimes referred to as manioc, mandioc, tapioca, and yucca is Africa's second most important crop regarding calories consumed. Its starchy tuberous roots generate 25-35% starch, which provides food for about 500 million people in developing nations through small-scale and subsistence agriculture (Smith et al. 1986; Li et al. 1998).

Cassava is the third largest source of calories in the tropics and has the potential to become a major feed and industrial crop in the coming decades. With more than one billion people suffering from hunger and malnutrition, it is evident that staple crops such as cassava must play a significant role in addressing these issues. Since 1970, the output of cassava has increased by 75%, with the majority of this increase attributable to greater acreage devoted to the crop rather than yield enhancements. In contrast to the major cereals, scientific and technical advancements over the previous decades have not significantly impacted cassava yield (Fauquet 2001).

In the tropics, cassava is an inexpensive source of carbohydrates. However, low protein and high cyanogen concentrations drastically reduce the nutritional value of cassava (Konan et al. 1994). With the traditional breeding technique, addressing these issues, such as the sharply

declining output caused by viruses, insects, and pests, is difficult. In addition, allopolyploid cassava plants demonstrate significant heterozygosity, low natural fertility, poor seed set, and germination. Therefore, additional strategies are required to address the issues mentioned above. Cassava biotechnology provides potent tools to supplement conventional breeding techniques and can expand the useful gene source pool outside the species (Raemakers et al. 1993; Hankoua et al. 2006; Saelim et al. 2006; Danso and Ford-Llyod 2008).

Cassava is propagated mostly through stem cuttings, a slower method than cereal crops. It is mostly cultivated on small farms, and cuttings are typically planted at the beginning of the rainy season. Depending on whether early or late cultivars are planted, the crop cycle is either eight or eighteen months after planting (Santana et al. 2009).

According to Santana et al. (2009), despite its significance, the average global yield over the past 30 years has been only 12 - 13 tonne/hectare, which is considerably below its potential yield of 80 tonne/hectare. Furthermore, it is difficult to cultivate on a wide scale due to the scarcity of high-quality seeds and the low yield and profit. However, these obstacles can be solved by tissue culture techniques that provide high-quality vegetative planting material, resulting in a productivity improvement of more than 30 tonnes per hectare (Santana et al. 2009). Furthermore, utilizing genetic variants resistant to pests, diseases, and environmental restrictions may also increase crop yield.

As one of the key components of biotechnology, plant tissue culture plays a significant role in developing crop varieties and complementing traditional breeding techniques. It is always involved in improving the quality of the crops we consume. Plant tissue culture with the notion of cellular totipotency, media formulation, and cell, tissue, organ, and protoplast culture led to the formation of haploids, somatic hybrids, and pathogen-free plants in numerous resistant plant species. Tissue culture techniques have also been used to investigate the fundamental features of plant development, metabolism, differentiation, and morphogenesis, and they provide the ideal opportunity to control these processes (Gupta and Ibaraki 2006).

This study aimed to determine the optimal method for obtaining a maximum number of shoots and suitable in vitro materials through micropropagation, as well as the various factors associated with the micropropagation of the two cassava varieties "Qulle and "Kello to overcome the obstacles as mentioned above. Furthermore, to develop a method with fewer limitations for producing micropropagated cassava plantlets, and because in vitro propagation may be influenced by several environmental factors, it is necessary to understand the effects of various factors associated with cassava micropropagation.

MATERIALS AND METHODS

Stock plant preparation

Hawassa Agricultural Research Center's Root Crops Research Division provided the fresh stem cuttings of two types of cassava, "Kello" and "Qulle," which were planted in containers with a 1:2:1 mixture of sand, soil, and compost. The glasshouse at Addis Ababa University's College of Natural Sciences, Ethiopia, was kept at a steady 25°C ± 2°C with natural light shining on the plants all day.

Stock Solutions and medium preparations

MS medium and growth regulators stock solution preparation

Plant growth regulators of the right type and concentration were applied alongside the Murashige and Skoog (1962) MS nutrition. The mixture of macronutrients, micronutrients, a Fe-Na-EDTA/FeSO4 mixture, and vitamins was used at maximum dosage. Stock solutions of the MS components (Appendix 1) were prepared by dissolving the correct amount of each component into double-distilled water and then chilling the mixture to -20°C. For example, to prepare a 1 mg/mL concentration, the plant growth regulators were weighed, dissolved in 1 N NaOH using drops, and dissolved in double-distilled water while swirling on a magnetic stirrer. The stock solutions were kept at +4°C for quick access.

The making of a culture medium

MS Basal Medium Preparation

With 100 mL/L of MS macronutrients, 10 mL/L of MS micronutrients, 10 mL/L of MS vitamin, 10 mL/L of Fe-Na-EDTA and $FeSO_4$ mixture, and 2% sucrose, a full-strength MS basal medium was created. First, a pH of 5.8 was reached by adding 1 N HCl and 1 N NaOH, and then 7.0 g of agar was added and melted on a swirling hot plate or in the microwave. As soon as the agar solution was clear, 25 mL was dispensed into baby food jars for starting the shoot to culture, and 50 mL was put onto Magenta GA7 culture vessels for multiplying the shoots before being autoclaved at 121°C with 0.15 Kpa pressure for 15 minutes.

Culture medium for the effect of TDZ on solid medium

TDZ was given to the MS basal medium at 0.1 mg/L, 0.15 mg/L, 0.2 mg/L, and 0.25 mg/L. There were four TDZ treatment groups, and each group was replicated five times.

Liquid media for growing cultures at various salt concentrations

Full strength culture medium was made with 100 mL/L of MS macronutrient, 10 mL/L of MS micronutrient, 10 mL/L of MS vitamin, 10 mL/L of Fe-Na-EDTA and $FeSO_4$ mixture, and 2% sucrose. In the case of half and quarter salt strengths, the amount of macronutrient, micronutrient, Fe-EDTA, and $FeSO_4$ mixture, and vitamin stock solutions utilized was reduced by half and quarter, respectively, and supplemented with 0.5 mg/L BAP + 1 mg/L GA_3 + 0.01 mg/L NAA. A pH of 5.8 was reached by adding 1 N HCl and 1 N NaOH, and then 7.0 g of agar was added and melted on a swirling hot plate or in the microwave. When the agar solution was clear, 25 mL was dispensed into baby food jars to start the shoot to culture, and 50 mL was put onto Magenta GA7 culture containers for multiplying the shoots before being autoclaved at 121°C with 0.15 Kpa

pressure for 15 minutes. Three salt concentrations were utilized in the experiments (full, half, and quarter), and each was replicated five times.

Media preparation for the different types of pH

The MS medium (preparation instructions may be found in section 4.3.1 of this publication) was used, and the pH was changed to 5.0, 5.6, 5.8, 6.0, and 6.6 with 1 N HCl and 1 N NaOH, respectively. After the pH was corrected, different amounts of agar were added: 1.3%, 1.1%, 0.8%, 0.7%, and 0.6%. Five replicates were performed using pH 5.0, 5.6, 5.8, 6.0, and 6.6 as the treatments.

Culture medium for different sucrose concentrations

2%, 1.5%, and 1% sucrose were added to MS baseline medium that had been supplemented with 0.5 mg/L BAP, 0.01 mg/L NAA, 1 mg/L GA_3. Five replicates were performed on three treatments (2%, 1.5%, and 1% sugar concentrations).

Media preparation for somatic embryo induction

A final volume of 1000 mL of CIM (Cassava induction medium) was made by combining MS basal medium with various concentrations of 2,4-D (4.0 mg/L, 6.0 mg/L, 8.0 mg/L, 10.0 mg/L, and 12.0 mg/L) or 7.0 mg/L, 10.0 mg/L, 12.0 mg/L, 16.0 mg/ L 2,4-D with 2 µM $CuSO_4$. Then, 1 N HCl and 1 N NaOH were used to bring the pH to 5.8. To the pH-balanced mixture, 8.0 g of agar was added and melted using either a stirring hot plate or a microwave. Autoclaving at 121°C with 0.15 Kpa pressure for 15 minutes, sterilized the agar medium before being distributed into baby food jars (holding 25 mL) and Magenta GA7 culture pots (holding 50 mL).

Somatic embryo induction was performed using ten different treatments (4.0, 6.0, 8.0, 10.0, and 12.0) mg/L 2,4-D and (7.0, 10.0, 12.0, 16.0) mg/L 2,4-D with 2 µM $CuSO_4$ and six replicates.

Cassava maturation medium (CMM) was made in half MS medium supplemented with (0.1, 0.2, and 0.3) mg/L BAP alone or with 0.01 mg/L 2,4-D. When the medium's pH was at 5.8, it was placed into a 100 mL Erlenmeyer flask and a Magenta GA7 culture vessel fitted with a membrane raft and autoclaved for 15 minutes at 121ºC, 0.15 Kpa pressure. In this case, six replicates were employed across six treatments (0.1 mg/L, 0.2 mg/L, and 0.3 mg/L BAP alone or in combination with 0.01 mg/L 2,4-D).

Explant collection and surface sterilization

Every four to seven days, shoot tips measuring 5-10 cm in length were picked from "Qulle" and "Kello" varieties in the greenhouse to trim them down to 2-3 cm of explants. The cuttings were given the first wash in tap water to remove any loose debris and mud. The second wash was in OMO powder detergent and a thorough rinsing until all traces of detergent were gone, followed by two rinses in double-distilled water. After a minute of rubbing with 70% ethanol, it was disinfected and rinsed three times with double-distilled sterile water. After soaking in sterile double-distilled water for 10 minutes for "Qulle" and 11 minutes for "Kello," the explants were cleaned with 1% Clorox bleach containing 5.25% active chlorine and 1 drop of tween 20. The explants were gently shaken by hand while being treated with Clorox, and the entire disinfection process, beginning with 70% ethanol, was carried out inside a laminar airflow cabinet.

Culture initiation

After being sterilized, shoot explants were cut to 2-3 cm before being utilized for shoot culture, whereas explants with at least two nodes were used for node cultures. Next, all leaves except the two youngest leaf lobes at the tip and the scales were removed using sterile forceps and a scalpel. Following parafilming of the vessels, the explants were grown in the various shoot initiation media employed to investigate the factors. A total of four explants were utilized in each culture vessel, and the experiment was repeated five times for each factor except for the liquid medium treatment, which was conducted with a total of twenty test tubes.

Initially, nodal explants were immersed in liquid MS media supplemented with various doses of TDZ. Then, the cultures were kept on an orbital shaker at 110 rpm for a week. For the next four weeks, the cells were maintained in a solid MS medium containing 0.5 mg/L BAP, 1 mg/L GA_3, and 0.01 mg/L NAA.

Shoot multiplication

Explants for shoot multiplication were grown from cultures obtained from both shoots and nodes. Each Magenta GA7 culture vessel contained six replicates of five explants (five shoots or five nodes).

Culture conditions

The cultures were maintained in the culture chamber at 29±2°C with 16 h/day light and 8 h/day dark cycles (light intensity of 2000 lux) and subcultured every four weeks.

Somatic embryogenesis

Induction of somatic embryos

Young leaf lobes were employed to induce somatic embryogenesis from in vitro propagated cassava shoots of the "Kello" and "Qulle" types. There were a wide range of hormone concentrations utilized, including 4 mg/L, 6 mg/L, 8 mg/L, 10 mg/L, and 12 mg/L 2,4-D, as well as 7 mg/L, 10 mg/L, 12 mg/L, and 16 mg/ L 2,4-D with 2 µM $CuSO_4$. There were six copies of each therapy with five explants, and the results were averaged. After sterile scalpels were used to create small incisions in the leaf lobe explants, the adaxial side of the leaves was placed in contact with the culture medium for a total of 60 days (4, 6, and 8 milligrams per liter of 2,4-D) or 40 days (10 and 12 milligrams per liter of 2,4-D). The cells were moved to a maturation medium consisting of half MS supplemented with 0.1 mg/L BAP alone and in combination with 0.01 mg/L 2,4-D to create cyclic somatic embryos (Saelim et al. 2006).

Culture conditions

Baby food jars were used for inducing somatic embryos. The explants were kept in dim light for a week before being moved to a condition with 16 h/day light and

8 h/day dark cycles at a light intensity of 2000 lux and a temperature of 29±2°C.

Micropropagation scheme of the two cassava varieties

Cassava "Qulle" and "Kello" were subcultured, and their micropropagation schemes were analyzed. Subcultured and multiplied shoots were recorded at the start of each subculture to examine the pattern of shoot multiplication. Full-strength MS media containing 0.5 mg/L BAP, 0.01 mg/L NAA, and 1 mg/L GA_3 was employed in this experiment.

Rooting and acclimatization

They were put in a hormone-free MS medium to burgeon roots in the propagated shoots. After the cuttings had established roots, the agar was washed away, and they were moved to a greenhouse. Putting the containers in plastic bags allowed them to sit for a week or two.

Study design

The research was performed in the Plant Propagation and Tissue Culture Laboratory at Addis Abeba University, Ethiopia. CRD (Completely Randomized Design) was employed as the statistical approach to this problem, and the data were analyzed with SPSS 17.0 and then loaded into Sigma plot 10.0 for graphic illustration. Mean separation at the 5% probability level was performed using one-way ANOVA.

RESULTS AND DISCUSSION

Shoot induction using solid MS medium with different concentrations of TDZ

Five to seven days after being cultured, shoot explants began reacting across all treatments, and by the end of the second week, shoot elongation and leaf development were obvious. Both types showed mature in vitro development after four weeks (Figure 1). In the case of "Kello," statistical analysis of the number of shoots produced by explants showed that all treatments were substantially different except for those containing 0.1 mg/L and 0.2 mg/L TDZ. In the instance of "Qulle," there is a statistically significant difference between the three treatments (0.5 mg/L BAP, 0.01 mg/L NAA, and 1 mg/L GA_3), but not between the treatments and the control (1 mg/L GA_3).

In the instance of "Kello," the optimal concentration of TDZ was 0.25 mg/L, where the average number of shoots and leaves per explant were 2.05 and 5.7, respectively, and the optimal length of shoot per explant was 4.55 cm (Table 1, Figure 1). In contrast, in the instance of "Qulle," the maximum mean the number of shoots, leaf, and shoot length per explant were 6.1, 9.45, and 1.90 cm at a TDZ dosage of 0.2 mg/L, 0.1, and that of the control, respectively (Table 2, Figure 2).

Table 1. The average number of shoots, leaves, nodes, and shoot length of the "Kello" variety after 4 weeks on medium supplemented with different concentrations of TDZ

Mg/L of TDZ	Mean		
	No. of shoots	No. of leaves	Length of shoots
Control	1.95 ± 1.05^{d}	3.0000 ± 0.85^{a}	4.2250 ± 0.78^{c}
0.10	1.45 ± 0.68^{abd}	2.3500 ± 2.03^{bc}	3.2750 ± 0.95^{abc}
0.15	1.60 ± 1.04^{c}	3.9000 ± 3.83^{d}	4.0750 ± 1.55^{d}
0.20	1.55 ± 0.68^{a}	4.6500 ± 3.13^{b}	4.5500 ± 2.15^{a}
0.25	2.05 ± 0.75^{b}	5.7000 ± 4.36^{ac}	4.4250 ± 1.41^{b}

Numbers connected by the same superscript letters in the same column are not significantly different at a 5% probability level

Table 2. The average number of shoots, leaves, nodes, and shoot length after 4 weeks per explant of the variety "Qulle" on medium supplemented with different concentrations of TDZ

Mg/L of TDZ	Mean		
	No. of shoots	No. of leaves	Length of shoots
Control	2.9500 ± 1.16^{c}	2.3000 ± 1.45^{c}	1.9000 ± 1.16^{d}
0.10	4.1500 ± 0.51^{b}	9.4500 ± 5.13^{a}	0.9500 ± 0.51^{c}
0.15	4.8250 ± 0.63^{b}	8.6000 ± 2.60^{a}	1.2500 ± 0.63^{bc}
0.20	6.1000 ± 0.96^{a}	8.6000 ± 2.32^{a}	1.1000 ± 0.96^{a}
0.25	4.8500 ± 0.60^{b}	5.1500 ± 1.63^{b}	0.9500 ± 0.60^{b}

Numbers connected by the same superscript letters in the same column are not significantly different at a 5% probability level

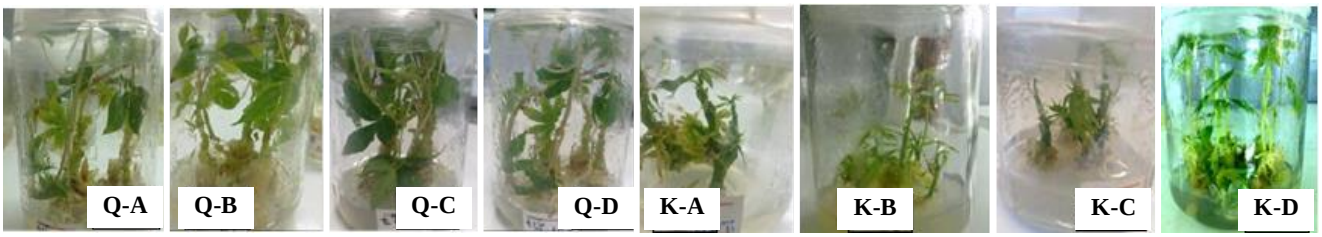

Figure 1. Culture initiation of the two cassava varieties "Qulle" (Q) and "Kello" (K) on MS medium supplemented with different concentrations of TDZ 0.1, 0.15, 0.2, 0.25 mg/L TDZ for A, B, C, and D, respectively

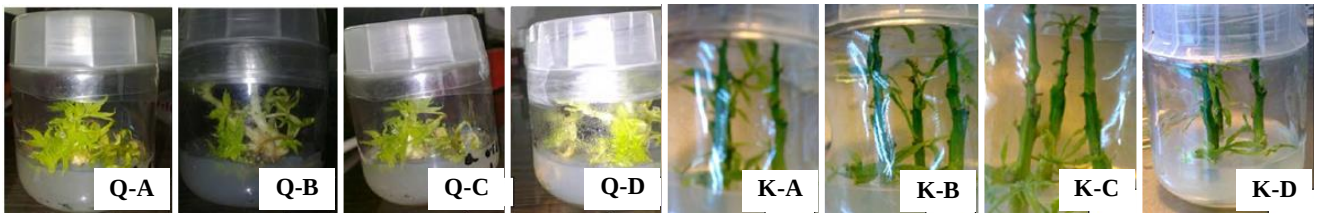

Figure 3. Prior to shoot induction, nodal cells grown in liquid MS media containing varying doses of TDZ A = 0.1 mg/L, B = 0.15 mg/L, C = 0.2 mg/L D = 0.25 mg/L of TDZ

Table 3. The average number of shoots per explant of "Kello" and "Qulle" varieties pre-immersed in liquid medium supplemented with various doses of TZ, followed by initiation of culture on medium supplemented with 0.5 mg/L BAP, 1 mg/L GA_3, and 0.01 mg/L NAA after four weeks

Mg/L of TDZ	Mean no. of shoots/explant	
	"Kello"	"Qulle"
Control	1.9500 ± 1.05^{cbd}	1.9000 ± 1.16^{bc}
0.1	2.2000 ± 1.00^{b}	1.9000 ± 0.71^{bc}
0.2	3.6500 ± 0.87^{a}	3.2000 ± 0.89^{a}
0.3	2.2000 ± 0.83^{c}	2.3500 ± 0.67^{b}
0.4	1.5000 ± 0.60^{d}	1.8000 ± 0.69^{c}

Numbers connected by the same superscript letters in the same column are not significantly different at a 5% probability level

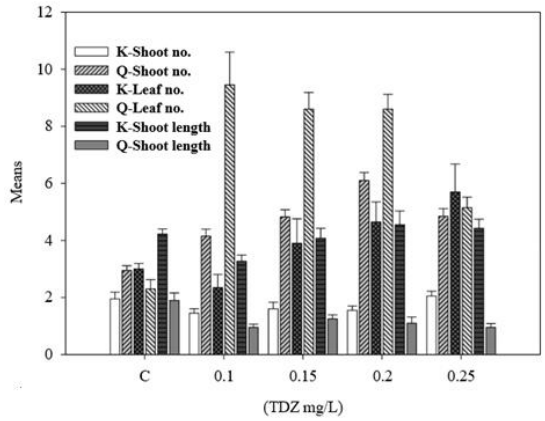

Figure 2. Mean shoot length and number of shoots, leaves, and nodes of "Kello" (K) and "Qulle" (Q) varieties based on various TDZ doses

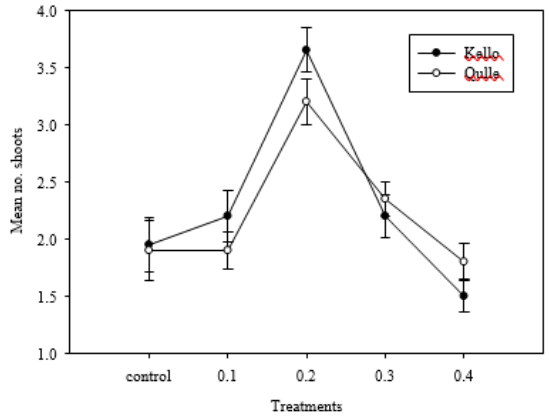

Figure 4. The average amount of shoots induced per explant on a liquid MS media supplemented with various dosages of TDZ before transfer to a solid medium containing 0.5 mg/L BAP, 1 mg/L GA_3, and 0.01 mg/L NAA

Shoot induction using a two-step procedure

By the end of the week, nodal explants that had been soaked in various TDZ pre-culture treatment concentrations had expanded at the nodes and, in some cases, had begun to initiate new shoot growth (Figure 3). In addition, after being transplanted to solid media, shoots were shown to proliferate. The maximum mean number of shoots per explant was 3.6 for "Kello" and 3.2 for "Qulle" when explants were treated with 0.2 mg/L TDZ (Table 3, Figure 4). Compared to a control group grown in MS media containing 0.5 mg/L BAP, 0.01 mg/L NAA, and 1 mg/L GA_3, the in vitro materials showed enhanced leaf proliferation. As for leaf proliferation, the in vitro materials surpassed the control, which had been grown in an MS medium containing 0.5 mg/L BAP, 0.01 mg/L NAA, and 1 mg/L GA_3.

Salt strength

By the end of the first week in culture, shoot explants grown on MS media with varying salt concentrations showed signs of life. By the end of the month, mature cultures of both the "Kello" and "Qulle" types had been obtained at all three salt concentrations (Table 4).

For "Kello," the optimal growth was achieved at one-fourth, one-half, and full salt strengths, with mean values of 1.60 shoots, 3.50 leaves, 3.30 nodes, and 4.10 cm shoot length per explant. While in the case of "Qulle," the maximum mean number of shoots, leaves, nodes, and shoot length was attained at full (control) and half salt strength (1.80, 5.20, 3.40, and 3.40 cm, respectively) (Table 5, Figure 5).

Response to medium with different pH

Approximately five to seven days after the commencement of culture, the cultures exposed to varying pH levels began to show signs of life. By the end of the month, explants from throughout the pH spectrum had grown into fully formed in vitro structures (Figure 6). In the case of "Kello," the optimal pH was 5.6, where the average number of shoots, leaves, nodes, and shoot length were 4.10, 12.10, 4.30, and 5.00, respectively (Table 6, Figure 7). The optimal pH for "Qulle" is 6.6, where the mean values for shoots, leaves, nodes, and shoot length are 2.40, 9.80, 4.12, and 4.90, respectively (Table 7, Figure 7).

Response to different sucrose concentrations

Explants began responding during the first week of culture initiation with leaf emergence, shoot elongation, and shoot multiplication, all per the outcomes of earlier treatments. The average number of shoots, leaves, nodes, and shoot length per explant was greatest at a sucrose concentration of 0.15% for both the "Kello" and "Qulle" kinds (Tables 8 & 9, Figure 8). The explants looked healthier and showed almost no signs of necrosis, which is common on a medium supplemented with greater sucrose content like the control (0.2% sucrose concentration).

Somatic embryo induction

After 60 days of culture, those with 4.0 mg/L, 6.0 mg/L, and 8.0 mg/L of 2,4-D had a yellowish friable callus. Those with 4.0 mg/L, 6.0 mg/L, and 8.0 mg/L of 2,4-D had a yellowish friable callus after 40 days of culture (10.0 and 12.0). After a month, none of the other treatments, including 2 µM $CuSO_4$, produced an FEC and went brown (Figure 9).

Table 4. Mean number of shoots, leaves, nodes, and length of shoot per explant of variety "Kello" on medium supplemented with 0.5 mg/L BAP, 0.01mg/L NAA, 1 mg/L GA_3 with different salt strengths after 4 weeks

Medium salt strength	Mean			
	No. of shoots	No. of leaves	No. of nodes	Length of shoots
Control	1.5667 ± 1.10^{a}	3.1667 ± 1.01^{a}	2.9000 ± 0.71^{a}	4.1667 ± 4.16^{a}
Half	1.5333 ± 0.89^{a}	3.5333 ± 1.92^{a}	3.3333 ± 0.92^{a}	3.5000 ± 3.5^{ac}
Quarter	1.6667 ± 0.88^{a}	2.9667 ± 2.25^{a}	2.1333 ± 1.22^{b}	3.3500 ± 3.35^{bc}

Note: Numbers connected by the same superscript letters in the same column are not significantly different at a 5% probability level

Table 5. Mean number of shoots, leaves, nodes, and length of shoot per explant of variety "Qulle" on medium supplemented with 0.5 mg/L BAP, 0.01 mg/L NAA, 1 mg/L GA_3 with different salt strengths after 4 weeks

Medium salt strength	Mean			
	No. of shoots	No. of leaves	No. of nodes	Length of shoots
Control	1.8667 ± 1.07^{a}	2.8333 ± 1.68^{b}	2.8000 ± 0.76^{b}	3.1333 ± 0.88^{ab}
Half	1.0667 ± 0.63^{b}	5.2000 ± 1.60^{a}	3.4000 ± 1.06^{a}	3.4667 ± 0.93^{a}
Quarter	0.6333 ± 0.49^{c}	3.6000 ± 1.49^{b}	2.8333 ± 1.28^{b}	2.9333 ± 1.17^{b}

Note: Numbers connected by the same superscript letters in the same column are not significantly different at a 5% probability level

Table 6. Mean number of shoots, leaves, nodes, and length of shoot per explant of variety "Kello" on medium supplemented with 0.5 mg/L BAP + 0.01 mg/L NAA + 1 mg/L GA_3 with different pH after 4 weeks

pH	Mean			
	No. of shoots	No. of leaves	No. of nodes	Length of shoots
Control	1.95 ± 1.05^{b}	3.00 ± 0.85^{c}	3.050 ± 0.75^{c}	4.225 ± 0.78^{b}
5.0	3.80 ± 1.47^{a}	10.20 ± 2.09^{b}	3.850 ± 0.81^{ab}	4.300 ± 0.89^{b}
5.6	4.10 ± 1.33^{a}	12.15 ± 1.95^{a}	4.300 ± 0.86^{a}	5.000 ± 0.94^{a}
6.0	3.45 ± 0.68^{a}	11.85 ± 2.41^{a}	4.050 ± 0.82^{a}	4.950 ± 0.80^{a}
6.6	2.40 ± 1.09^{b}	9.00 ± 1.94^{b}	3.450 ± 0.75^{bc}	4.750 ± 1.14^{ab}

Note: Numbers connected by the same superscript letters in the same column are not significantly different at a 5% probability level

Table 7. Mean number of shoots, leaves, nodes, and length of shoot per explant of variety "Qulle" on medium supplemented with 0.5 mg/L BAP + 0.01 mg/L NAA + 1 mg/L GA_3 with different pH after 4 weeks

pH	Mean			
	No. of shoots	No. of leaves	No. of nodes	Length of shoots
Control	1.90 ± 1.16^{ab}	2.30 ± 1.45^{c}	2.80 ± 0.76^{d}	2.95 ± 0.74^{d}
5.0	1.70 ± 0.80^{ab}	8.05 ± 1.35^{b}	3.90 ± 0.64^{ab}	3.55 ± 0.53^{c}
5.6	2.30 ± 1.12^{ab}	9.05 ± 2.98^{ab}	3.50 ± 1.00^{bc}	5.075 ± 0.81^{a}
6.0	1.70 ± 0.80^{b}	9.50 ± 2.87^{ab}	2.95 ± 1.09^{cd}	4.300 ± 0.78^{b}
6.6	2.40 ± 0.82^{a}	9.80 ± 2.85^{a}	4.10 ± 0.91^{a}	4.9250 ± 0.83^{a}

Note: Numbers connected by the same superscript letters in the same column are not significantly different at a 5% probability level

Table 8. Mean number of shoots, leaves, nodes, and length of shoot per explant of variety "Kello" on medium with different sucrose concentrations supplemented with 0.5 mg/L BAP, 0.01 mg/L NAA, 1 mg/L GA_3 after 4 weeks

Sucrose concentration	Mean			
	No. of shoots	No. of leaves	No. of nodes	Length of shoots
Control	1.9500 ± 1.05^{b}	3.0000 ± 0.85^{c}	3.0500 ± 0.75^{b}	4.2250 ± 0.78^{a}
0.15%	3.7000 ± 1.55^{a}	12.1500 ± 6.57^{a}	5.8000 ± 1.57^{a}	5.1000 ± 1.22^{a}
0.10%	2.0000 ± 1.55^{b}	7.2000 ± 3.86^{b}	3.5000 ± 1.76^{a}	2.8500 ± 1.33^{b}

Note: Numbers connected by the same superscript letters in the same column are not significantly different at a 5% probability level

Table 9. Mean number of shoots, leaves, nodes, and length of shoot per explant of variety "Qulle" on medium with different sucrose concentrations supplemented with 0.5 mg/L BAP, 0.01 mg/L NAA, 1 mg/L GA_3 after 4 weeks

Sucrose concentration	Mean			
	No. of shoots	No. of leaves	No. of nodes	Length of shoots
Control	1.9000 ± 1.16^{a}	2.3000 ± 1.45^{b}	2.8000 ± 0.76^{ab}	2.9500 ± 0.74^{b}
0.15%	2.1500 ± 1.03^{a}	4.7500 ± 1.48^{a}	3.5000 ± 1.76^{a}	3.9500 ± 1.29^{a}
0.10%	1.4500 ± 1.27^{a}	3.6500 ± 2.58^{a}	2.3500 ± 1.38^{b}	2.9600 ± 1.69^{b}

Note: Numbers connected by the same superscript letters in the same column are not significantly different at a 5% probability level

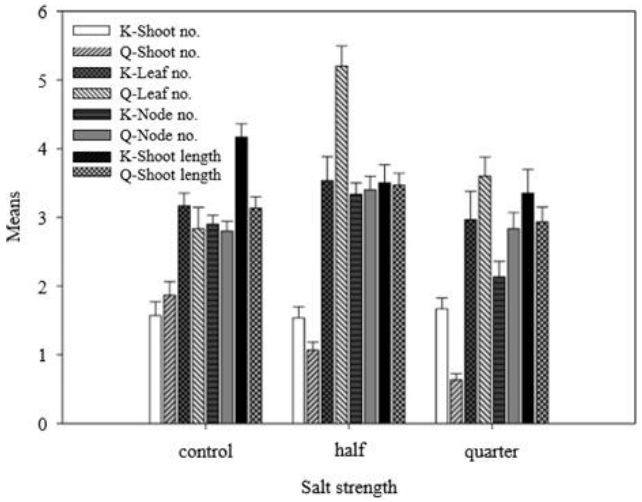

Figure 5. Mean number of shoots, leaves, and nodes and mean shoot length of "Kello" (K) and "Qulle" (Q) varieties based on different salt strengths

Although all leaf lobes were successful in inducing somatic embryos, no somatic embryos obtained from the induced callus on a 2,4 D medium showed embryo maturation and shoot induction on a medium containing different concentrations of BAP alone and along with a drastically lowered concentration of 2,4- D compared to those used while inducing the somatic embryos.

Micropropagation scheme

Subculturing the induced shoot tips every four weeks on a complete MS medium with 2% sucrose and 0.5 mg/L BAP + 0.01 mg/L NAA + 1 mg/L GA_3 has proven successful. Initially, "Qulle" cultured shoot tips responded very well to subculturing, but as the number of subcultures continued to increase, the average number of shoots began to decrease. In contrast, "Kello" showed a very opposite pattern; the average number of shoots grew linearly up to the third month, then suddenly reached an exponential high in the fourth month, and again showed a drastic decline in the fifth subculture (Table 10, Figure 10).

Rooting and acclimatization

By the end of the third week, the plantlets had grown roots and were ready to be moved to the field. Half of the "Qulle" plantlets and 66.6% of the "Qello" plantlets that had been acclimated survived (Figure 11).

Table 10. Mean number of shoots per explants on different successive subculturing stages

Monthly subculture	Mean no. of shoots/explant	
	"Kello"	"Qulle"
Subculture 1	1.5667 ± 1.10^{b}	1.8667 ± 1.07^{b}
Subculture 2	1.7000 ± 1.26^{b}	2.9333 ± 1.33^{a}
Subculture 3	1.8667 ± 1.10^{b}	1.6333 ± 1.47^{b}
Subculture 4	3.5333 ± 1.83^{a}	1.3000 ± 0.98^{b}
Subculture 5	2.0333 ± 1.35^{b}	1.2667 ± 2.22^{b}

Numbers connected by the same superscript letters in the same column are not significantly different at a 5% probability level

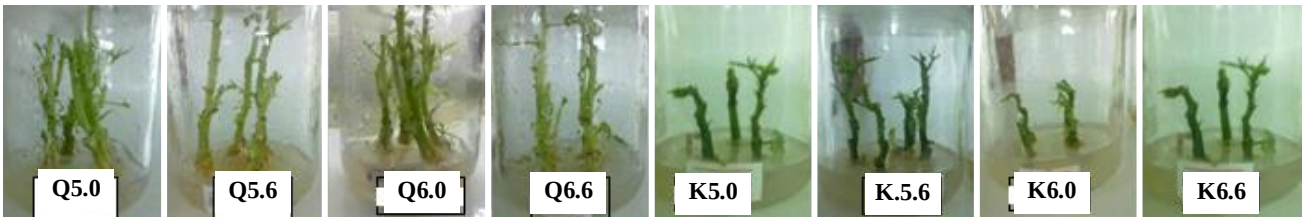

Figure 6. Shoot induction in a medium containing 0.5 mg/L BAP + 0.01 mg/L NAA + 1 mg/L GA_3 at different pH levels (5.0, 5.6, 6.0 and 6.6 pH). Q = Qulle, K = Kello

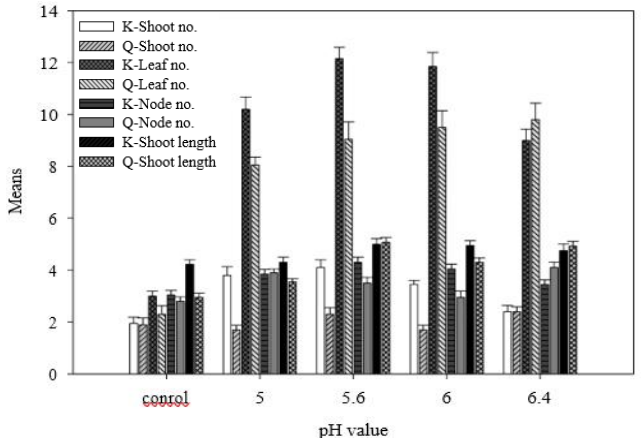

Figure 7. Mean number of shoots, leaves, and nodes and mean shoot length of "Kello" (K) and "Qulle" (Q) varieties based on different pH values

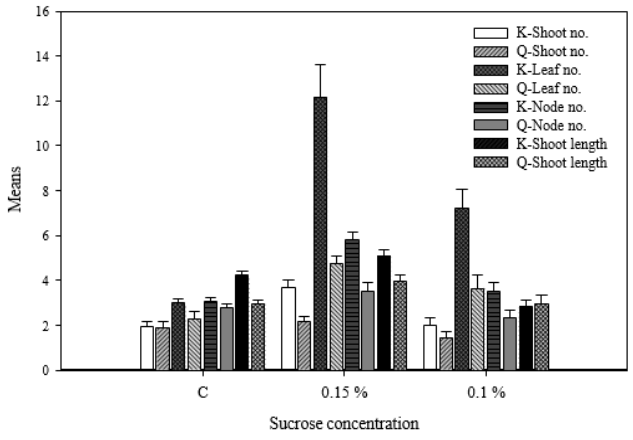

Figure 8. Mean number of shoots, leaves, and nodes and mean shoot length of "Kello" (K) and "Qulle" (Q) varieties based on different sucrose concentrations

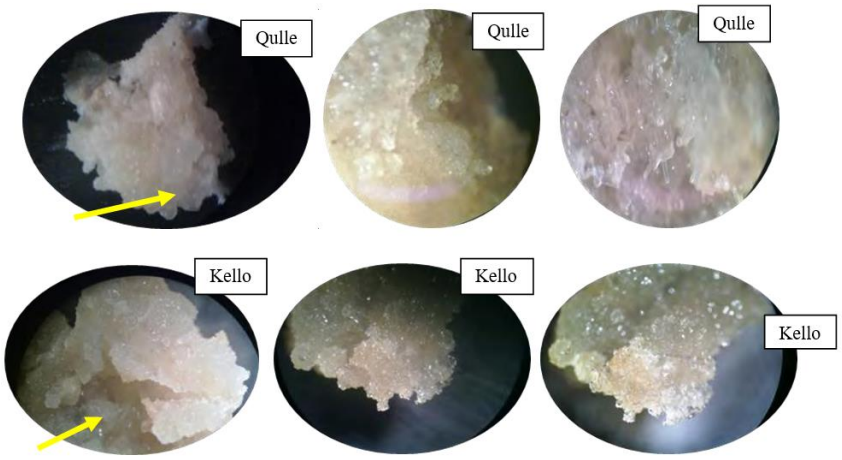

Figure 9. Structure of globular somatic embryos as seen under a dissecting microscope

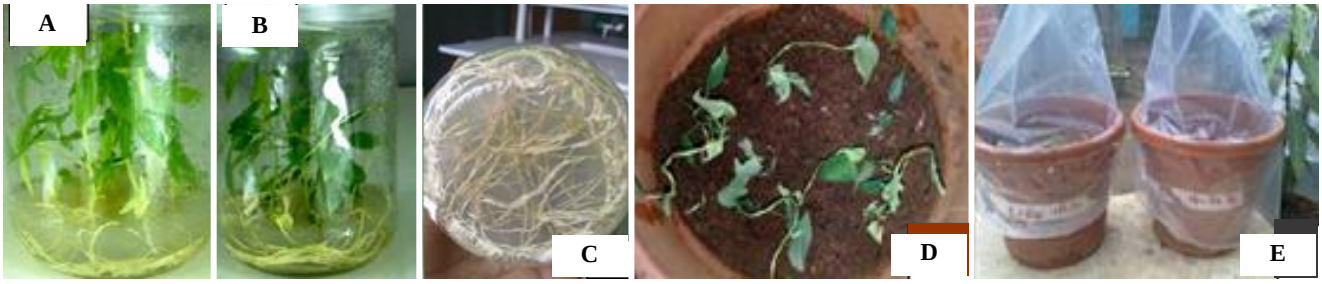

Figure 11. Rooting and acclimatization. Rooting in "Qulle" (A), rooting in "Kello" (B), roots at the bottom of the culture vessels (C), rooted plants after being planted (D), Plants covered in a polyethylene bag (E)

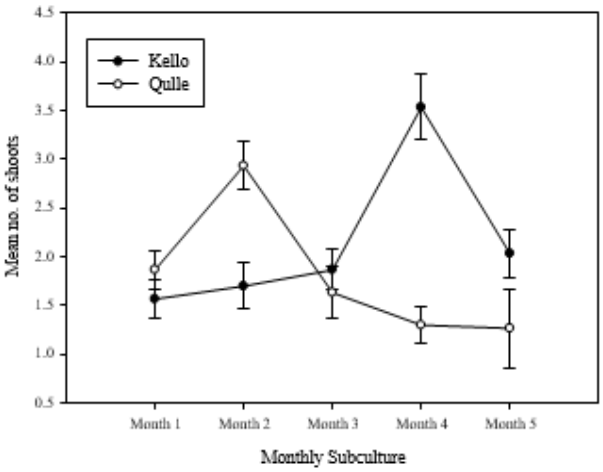

Figure 10. Subculturing schemes of the two cassava varieties in successive subcultures

Discussion

Shoot induction using solid MS medium with different concentrations of TDZ

The results of shoot induction on MS medium with different concentrations of TDZ were the same as those of Escobar et al. (2001) and Siddique and Anis (2006). Their results also showed that using different concentrations of TDZ alone and with IAA from cotyledonary node explants caused multiple shoots to grow. It was shown that multiple shoots grew from the explants as early as 15 days after they were taken. It is very similar to the results of this study, which showed that the shoots were growing well by the end of the first week and that the growth and number of shoots continued as early as the second week. They also said that when there was more than 5.0 µM of TDZ, there was a big drop in the number of shoot buds and the number of times they grew back. They thought this might be because of too much callus growth, while its specific concentration supports the most shoot bud formation. In the same way, this study on "Qulle" showed that at a concentration of 0.25 mg/L, the average number of shoots produced per explant dropped, and the explants showed a mass of callus at the base.

Sajid and Aftab (2009) reported in 2009 that TDZ stimulates growth when added to a tissue culture medium at a low concentration (10-1000 times lower than the concentration of other PGRs). The results of this study agree with those of Sajid and Aftab (2009). The same goes for the results of Huetteman and Preece (1993). In addition, Aasim et al. (2009) said that TDZ is the most active

cytokinin substance that causes more shoot growth in vitro than many other cytokinins in many plant species.

The results of Konan et al. (1997), which involved growing cassava shoots on MS medium with BAP, kinetin, thidiazuron, and zeatin, don't match up with this study. However, their results found that BAP was the most effective at causing shoot growth and development because of the abovementioned cytokinins.

Shoot induction using a two-step procedure

Bhagwat et al. (1996) found that an open-ended shoot proliferation process produced the most shoots per nodal explant after 10 weeks. It was based on a two-step procedure that involved putting the nodal explants in TDZ-containing liquid MS-medium for 6-8 days and then growing them on agar solidified medium with 22.2 µM BAP and 1.6 µM GA_3. Consistent findings were found between the current investigation and a previous one that used a liquid MS-medium pre-treatment for a week followed by culture on agar solidified MS-medium supplemented with 0.5 mg/L BAP, 0.01 mg/L NAA, and 1 mg/L GA_3.

According to Bhagwat et al. (1996), TDZ stimulates the growth of nodal explants, and this growth (expansion) persists into the second stage of culture on agar solidified medium. The enlarged explant gives rise to new shoots by continuously producing new bud clusters and fasciated stems.

For example, in hybrid tea rose cultivars, Singh and Syamal (2001) report that axillary shoot proliferation can be greatly increased by exposing explants to TDZ for a brief time prior to subculturing onto a conventional shoot proliferation medium.

Salt strength

Variety "Kello" generated the highest mean number of shoots, leaves, nodes, and shoot length per explant (1.60, 3.50, 3.30, 4.10, respectively) at quarter-MS, half-MS, half-MS, and full-MS medium salt concentrations. However, when grown in complete (control), half (half), and a half (half) salt strength of the medium, the "Qulle" variety produced the greatest mean number of shoots, leaves, nodes, and shoot length per explant (1.80, 5.20, 3.40, and 3.40, respectively).

However, the highest number of nodes was found at full-strength salt, which contradicts the mean number of nodes for "Kello" found by Mantell and Hugo (1989). However, the results of this study are consistent with the work of Mantell and Hugo (1989) regarding the mean number of shoots for "Qulle," which is reached at a full-strength mineral salt concentration of the medium.

Response to medium with different pH

The results of this investigation demonstrated that specific pH ranges are tolerable in the micropropagation of cassava, supporting the advice of Kozai et al. (1997) that pH is controlled in the mass production of plants by in vitro procedures. By the end of the month, all of the explants across all pH ranges had successfully given rise to in vitro material. Optimal growth for "Kello" occurred at a pH of 5.6, as measured by the mean number of shoots, leaves, nodes, and shoot length. Maximum averages for shoot number, leaf count, node count, and shoot length were all reached at a pH of 6.6 in "Qulle." In "Kello," there was no discernible difference in the average number of shoots produced by explants when the pH was either 5.0, 5.6, 6.0, or 5.8 or 6.6. A comparison of the pH levels 6.0 with the control, 5.0 and 5.6, and 6.0 with 5.0 and 5.6, showed no significant differences. The performance and pH variation tolerance of "Qulle" is particularly impressive, especially given that the medium is often created within the pH range of 5.0 to 6.0.

Response to different sucrose concentrations

The maximum number of shoots and nodes per explant was found to be attained at a lower concentration of sucrose, which is consistent with the findings of Mantell and Hugo (1989), who investigated the effects of various factors on the root, shoot, and microtuber development in shoot cultures of *Dioscorea alata* L. and *D. bulbifera* L. yams.

Nhut et al. (2001) studied plant and shoot regeneration in *Lilium longiflorum* Thunb. and found that using 3% or 4% sucrose resulted in a higher frequency of shoot development. In contrast, using 2% sucrose resulted in a slightly lower frequency of shoot creation. While at 0.15% sucrose concentration, the "Kello" and "Qulle" varieties showed the highest mean values for shoots, leaves, nodes, and shoot length per explant. This finding holds great potential for the practice of cassava tissue culture in developing nations. The significant benefit is the savings in purchasing costly plant tissue culture grade sucrose from a reduced need for that carbon source.

Sugar-free (photoautotrophic) micropropagation was recommended for use on a large scale by Kozai et al. (1997) due to its many benefits over heterotrophic photomixotrophic micropropagation, including the in vitro plantlets' faster growth and development, fewer physiological and morphological disorders, and lower risk of biological contamination.

Somatic embryo induction

None of the somatic embryos obtained from the induced callus on a 2,4 D medium showed embryo maturation and shoot induction on a medium containing different concentrations of BAP alone as well as together with a drastically lowered concentration of 2,4-D, in contrast to the result of Stamp (1987), who used a consistent procedure in inducing and maturing somatic embryos. The outcome was also at odds with the work of (Groll et al. 2002), which induced somatic embryos and shot germination using full and reduced-strength MS medium.

Browning of the callus and the absence of somatic embryo formation were the results of the present experiment on both "Kello" and "Qulle," in contrast to the result of Saelim et al. (2006). The latter used 2 µM $CuSO_4$ as a supplement along with different concentrations of 2,4-D to have the best embryo induction frequency. Consistent

with the present observation, Konan et al. (1994) found that 2,4-D generated globular somatic embryos.

Similar results were found by Atehnkeng et al. (2006). They demonstrated that pro-embryos created by two cassava genotypes did not survive past the globular developmental stage and that the pro-embryo formation level was insufficient to indicate embryogenic competence.

According to Sudarmonowati and Henshaw's unpublished research, only six of sixteen cassava cultivars tested successfully inducing somatic embryogenesis when grown in a medium enriched with 2,4-D. The authors hypothesized that this was because various genotypes react differently to 2,4-D and that genotypes substantially influence somatic embryogenesis. However, when given a media with picloram and dicamba, Sudarmonowati and Henshaw (1996) reported success in inducing somatic embryogenesis in cultivars that had failed to do so when given a medium with 2,4-D. In addition, cassava's immature leaf lobes and apical shoot meristems have been found to differ in their genotype's propensity to generate primary somatic embryos, as reported by Hankoua et al. (2005).

Although both 2,4-D and picloram induced embryogenic calli and matured primary embryos, Danso et al. (2010) reported that picloram enhanced early calli development more than 2,4-D. It is because picloram can acidify and loosen the cell wall earlier than 2,4-D, which is critical in weakening the cell-cell interaction gradient that coordinates the normal bipolar development of the embryo.

Micropropagation scheme

According to Beyene (2009), the study's multiplication data was gathered from the first subculture of plantlets after four weeks in the multiplication medium. For this reason, the second and third subcultures may significantly increase their numbers. Thus, it was suggested that other subculturing take place.

In the instance of "Qulle," the apex of cultured shoots responded well during the initial subcultures, but the mean number of shoots began to decline as the frequency of subculturing increased. While "Kello" revealed a completely different pattern, with a linear increase in the mean number of shoots through the third month, an exponential peak in the fourth month, and a subsequent sharp fall in the fifth subculture, the two trends were in stark contrast to one another. These may have arisen because frequent subcultures caused the plantlets to lose their ability to reproduce.

Mbanaso (2008) found that after four successive subcultures, shoots obtained from starch-gelled medium were significantly less strong than those grown in regular soil. The higher content of starch in the medium exacerbated this tendency toward diminished resilience over time.

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