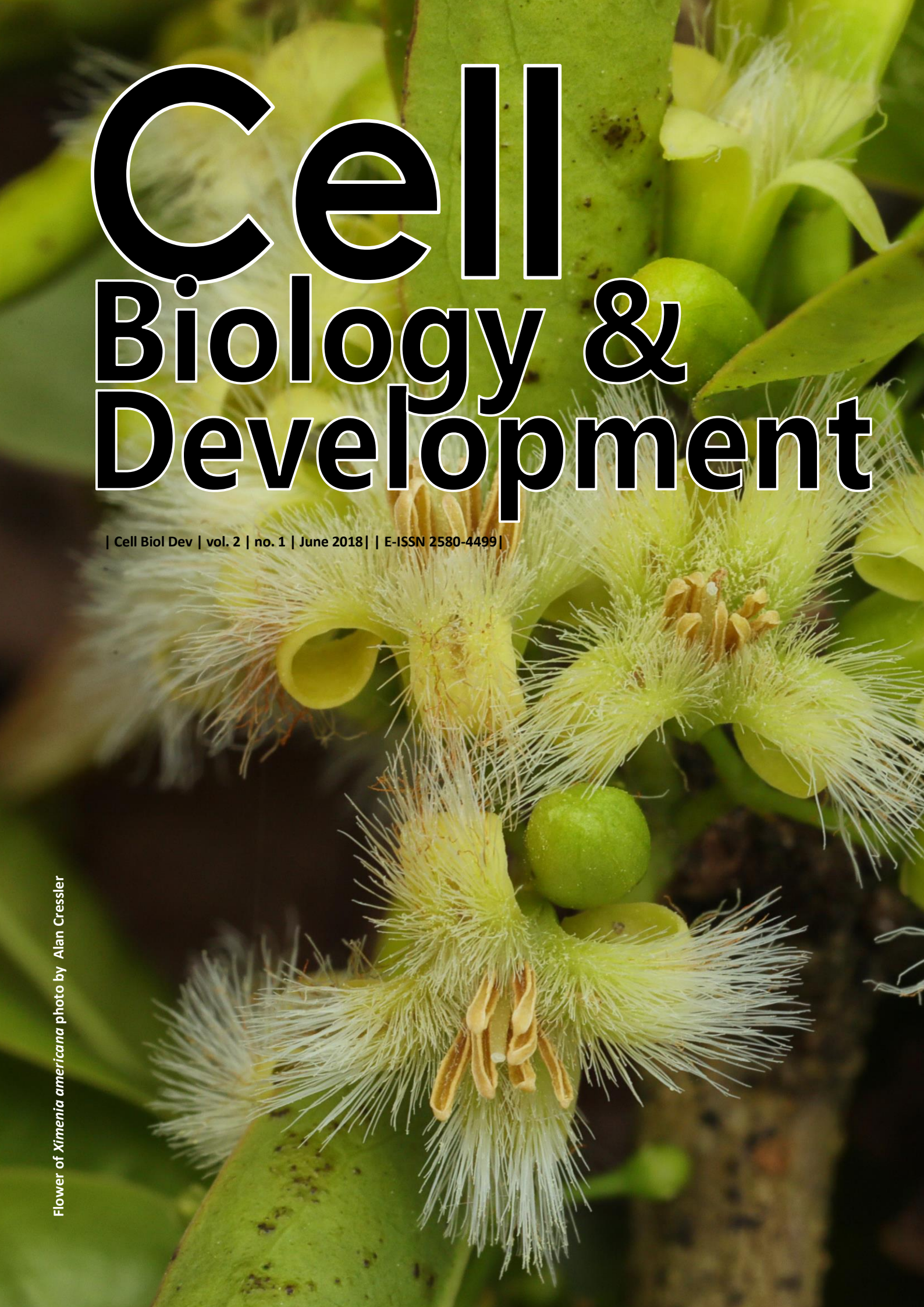

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Flower of *Ximenia americana* photo by Alan Cressler

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Journal:

Saharjo BH, Nurhayati AD. 2006. Domination and composition structure change at hemic peat natural regeneration following burning; a case study in Pelalawan, Riau Province. Biodiversitas 7: 154-158.

Book:

Rai MK, Carpinella C. 2006. Naturally Occurring Bioactive Compounds. Elsevier, Amsterdam.

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:

Alikodra HS. 2000. Biodiversity for development of local autonomous government. In: Setyawan AD, Sutarno (eds.) Toward Mount Lawu National Park; Proceeding of National Seminary and Workshop on Biodiversity Conservation to Protect and Save Germplasm in Java Island. Universitas Sebelas Maret, Surakarta, 17-20 July 2000. [Indonesian]

Thesis, Dissertation:

Sugiyarto. 2004. Soil Macro-invertebrates Diversity and Inter-Cropping Plants Productivity in Agroforestry System based on Sengon. [Dissertation]. Universitas Brawijaya, Malang. [Indonesian]

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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Genetic analysis of *Ximenia americana* based on the cross-species DNA-DNA hybridization technique

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Abstract. *Ekandjo A, Naomand E, Kahaka G. 2018. Genetic analysis of* Ximenia americana *based on DNA-DNA based on the cross-species hybridization technique. Cell Biol Dev 2: 1-7.* Genetic analysis of species without the availability of complete genome arrays can be studied using the cross-species hybridization technique. The preliminary microarray studies have created DNA-DNA hybridization of *X. americana* to the GeneChip of *Arabidopsis thaliana* (ATH1). Based on the perfect-match (PM) probe signal, the probe pairs of *A. thaliana* that hybridized with the genomic DNA of *X. americana* were selected and analyzed using a cel file parser script. This selection was to generate new high-density probe mask files. The *X. americana* DNA-DNA hybridization data were effectively represented by this file. In addition, this study carried out gene ontology analysis of hybridization data of *X. americana/A. thaliana*. This gene ontology analysis showed that genes for abiotic stress response were over-expressed in respective comparison to model species of *A. thaliana* under natural conditions. This analysis was confirmed independently with PCR amplification of the orthologous genes using genomic DNA of *X. americana*, such as AT4G15910.1, SAD2, HXK1, ACC, and ERF/AP2. Due to the lack of genomic sequence data in X. americana, the primers for genomic amplification were designed using a genomic sequence of *A. thaliana*. The primers were designed to produce a genomic PCR product of 100 bp. Each selected gene was successfully amplified. Therefore, it gave evidence of homology within primer binding sites. However, the genomic amplification of these crucial abiotic factors in *X. americana* confirms the response type, which supports the adaptation of *X. americana* under natural conditions with stress associated (with heat and drought stress). Although transcript levels of these unique abiotic response factors could not be observed in absolute or relative terms, the study showed the inherent existence of such genes at the genomic level of *X. americana*. Further studies are needed to confirm that some modification of the factors of these abiotic responses or other abiotic responses within the genome of *X. americana* gives the key basis for its adaptation to the comparatively dry and hot climatic conditions.

Keywords: ATH1, cross-species hybridization, gene ontology, abiotic response factors, microarray

INTRODUCTION

Ximenia is also found in other parts of the world, such as South America, although it is primarily indigenous to Africa. *Ximenia* includes in the family of Olacaceae and is widely spread in Africa. *X. americana* and *X. caffra* are the most common species. These two species are distributed in the northern part of Namibia. In this study, we focused on *X. americana*. The *X. americana* has rough dark-grey bark and grows up to 6 meters tall. It thorny branches and has hairless green leaves. Regardless of climate change, *X. americana* bears flowers and fruits annually (Orwa et al. 2009). *X. americana* is a drought-resistant plant where it mostly grows at low altitudes in a wide range of environments, such as savannahs, dry woodlands, dry forests, and along with coastal areas or on river banks (Orwa et al. 2009)

Ximenia americana is of economic importance because of its wood yield. Its fruit is often used for jam and jellies, and its kernel contains a high quantity of oil. *X. americana* fruits are also used for medicinal purposes, treating skin problems, sexually transmitted diseases, headaches, relieving cough, and healing wounds as a topical ointment. X. americana for medicinal purposes due to its free radical scavenging and antioxidant activity (Maikai et al. 2009).

This study analyzed and compared X. americana and Arabidopsis thaliana DNA-DNA hybridization data using gene ontology and independently confirmed ontology results with PCR amplification of orthologous genes within a given biological process. This study requires designing primers in *A.thaliana* background and examining the presence of genomic fragments in *X. americana* genomic DNA.

MATERIALS AND METHODS

Sample collection

The samples (*X. americana* leaves) were collected from Waterberg, Otjiwarongo, in the Otjozondjupa region. The sample collection was conducted during the early summer (September) when the average daytime temperature reached 30˚C, and no rainfall was recorded during that month. The leaves samples were kept on ice from the field to the laboratory. The samples were subsequently stored in the freezer at -20 ˚C until the analysis time.

DNA extraction

DNA was extracted from the leaves of *X. americana* using a DNeasy Plant mini kit (Qiagen). First, 20 mg of *X.*

americana leaves was ground buffer using a mortar and pestle in 800 µL API to get a homogeneous solution. Next, the buffer mixture of *X. americana* - was transferred to two 1.5 mL microcentrifuge tubes (400 µL). Then, 4 µL RNase was added to each tube. The mixture of the samples was incubated at 65˚C for 10 minutes. During the incubation, it was mixed 3 times by inverting the tubes. First, a 130 µL was added to the lysate and then mixed and incubated for 5 minutes on ice. The lysate was centrifuged at 14,000 rpm for 5 minutes. Then the lysate was transferred to a QIAshredder Mini spin column (lilac), put in the collection tube of 2 mL, and centrifuged at 14,000 rpm for 2 minutes. The flow-through fraction from the previous step was moved into a new tube without disturbing the cell-debris pellet. The flow-through volume was measured, and sodium acetate (3M, pH5.2) with 0.1 volume was added. A t room temperature, a 0.7 volume of 50% isopropanol was added to the mixture. This mixture (650µL), including precipitate, was moved to the DNeasy Mini spin column within a 2 mL collection tube, and then it was centrifuged at 13,400 rpm for 1 minute. For the remaining sample, the previous step was repeated. The DNeasy Mini spin column was put into a new 2 mL collection tube, and Buffer AW of 500 µL was added, and it was centrifuged at 13,400 rpm for 1 minute. The flow-through was discarded and centrifuged for 2 minutes at 14,000 rpm. Next, the DNeasy Mini spin column was moved to a 1.5 mL microcentrifuge tube. As much as 50 µL Buffer AE (warmed at 65˚C for 2 minutes) was added directly onto the DNeasy membrane. It was incubated for 5 minutes at room temperature and then centrifuged at 14,000 rpm for 1 minute to elute. The extracted DNA was stored at -20 ˚C.

Quantification of DNA

The quantity of DNA of *X. americana* leave samples (1.5 µL) was analyzed using an ND-NanoDrop 2000c spectrophotometer. The results of the quantification were recorded and tabulated.

Hybridization

DNA-DNA hybridization was proceeded by a commercial service of The Nottingham NASC arrays in the United Kingdom. NASC arrays processed about 50 µL DNA samples. The pre-defined experimental conditions were needed before the DNA was sent to The Nottingham NASC arrays. First, the DNA was labeled with the Invitrogen BIoPrime kit. Then, about 100 ng DNA was dissolved in a microcentrifuge tube with 20 µL of the dilute buffer, and 20 µL of 2.5X random primers solution were added. Denaturation was done by heating in a boiling water bath for 5 minutes; then, it was cooled immediately on ice. Five µL of 10X dNTP mixture and distilled water to a volume of 49 µL were added to the solution on ice. The solution was mixed quickly, and 1 µL of Klenow fragment was added. The solution was mixed softly but thoroughly and centrifuged for 30 seconds. After that, the solution was incubated for 60 minutes at 37˚C, and 5 µL of a stop buffer was added. When incubating, the array was loaded with 200 µL 1X hybe buffer, and later prehybe the array in the oven at 45˚C, at 60,000 rpm for 10 minutes. Next, the probe was incubated at 45˚C for 5 minutes. The prehybe buffer was discarded from the array, 200 µL probes were added, and the debris at the bottom of the tube was eluded. Next, to prevent leakage, the septa were covered with tight spots, and then the arrays were put in the oven for 20 hours at 45˚C, 60,000 rpm. Finally, using the GeneChip operating software, the arrays were washed. After washing, CEL files data were transferred and scanned.

The homologous A. thaliana GeneChip array PM probes were selected by selecting the probe pairs from each probe-set on the *A. thaliana* GeneChip array derived from the hybridization efficiency of genomic DNA from *X. americana* (Hammond et al. 2005). First, biotin-labeled was applied to genomic DNA from *X. americana*, and it was hybridized to the *A. thaliana* ATH1 GeneChip array. Then, selecting a probe-set using Perfect Match probes with the intensities of gDNA hybridization above a set threshold was done to analyze if the probe-set was represented. The selection was carried out using a .cel file parser script written in the programming language of Perl (Xspecies Version 1.1, http: //affymetrix.*Arabidopsis*.info/xspecies/).

The overlapped regions (defined by probe sets) during cross-hybridization were chosen. Then, the primers were designed from those regions' probe sets (Hammond 2005).

Primer design

Using the ProbeFinder Assay Design software, designing primers were performed (www.lifescience.roche.com). Sequences of genomic DNA of *A. thaliana* genes (putative *X. americana* orthologous) as templates for primer design were retrieved from a database of Gene Bank (from The *Arabidopsis* Information Resources-TAIR website). The designed primers were 15-30 bases in length, and the region of interest was designed without any other nonspecific binding possibility. When primers were designed, the Promega kit was followed as per manufacturer instruction to contain 40-60% (G+C). To avoid the production of primer-dimers, the 3'-ends of the primers were not complimentary.

Furthermore, to avoid nonspecific primer annealing, none of the primers contained three G or C nucleotides in a row near the 3'-end. Primers were designed to produce similar melting temperatures and a PCR product of between 70 and 100 bp. The advantage of the fragments of this size is that they are suitable for analysis using multiple platforms such as standard PCR or Real-Time PCR.

Gene ontology classification

Analysis of gene ontology was done to evaluate if particular biological functions were over or under-represented during cross-hybridization. The Gene Ontology terms (Gene Ontology Consortium 2001) were used to perform a classification analysis and to classify the genes based on their putative function. The GO analysis was performed using the TAIR Web site (www.arabidposis.org).

Gene ontology analysis of A. thaliana *genome*

Using the bulk GO annotation, genomic annotations of *A. thaliana* were sub-classified. The retrieval tool is available on the TAIR website. Using the same bulk GO annotation, uploading Data Locus identifiers of more than 11,000 probes (between *A. thaliana* and *X. americana*, the homology was positive) were made. The lists of all GO annotations for a given set of genes using the locus identifiers were obtained. The number of genes per category was noted and tabulated.

Gene ontology analysis of X. americana *PCR confirmed loci*

Uploading locus identifiers of the 5 PCR positive *X. americana* loci (putative orthologous) were performed using the same bulk GO annotation. This analysis retrieved a list of all GO annotations and the number of genes per category.

Independent confirmation using standard PCR

Standard PCR was used to obtain genes and, during cross-hybridization, were analyzed to confirm their presence in *X. americana* (Table 1).

Standard PCR amplification reactions

The isolated DNA of *X. americana* was performed to amplify the targeted amplicon using standard PCR. The reagents (Table 1) were added to the PCR tubes placed on ice according to Fermentas life science protocol, kit no: #K0171. For each tube, there was a 25 µL reagent. For PCR amplification, 10 primers were used (Table 2).

PCR reaction mixtures were thoroughly mixed, and PCR was done using thermal cycling conditions (Table 3). The PCR was running overnight. The PCR conditions were set according to Fermentas life science protocol, kit no: #K0171 (Table 3).

PCR products gel electrophoresis

Approximately 2,200 lg of agarose powder was placed in a 500 mL screw cap reagent bottle. After that, 200 mL of 1×TBE buffer was added. The mixture was dissolved in the microwave until no bubbles appeared in the solution. Under running tap water, the solution was cooled until the bottle could be held for at least 6 seconds. Under the fume hood, 5 µL of ethidium bromide was added to the solution and poured into the gel tray. For 30 minutes, the 1% agarose gel was allowed to stand. Then, the comb was gently removed from the gel. The gel was moved into the electrophoretic tank filled with 1×TBE buffer until the gel was covered with buffer. The mix of PCR products (10 µL) and loading dye (3 µL) was loaded on the gel. In the first well, DNA lader100 bp (8µL) was loaded. At 120V, the gel was run for 60 minutes. On the UV transilluminator, the DNA bands were visualized.

RESULTS AND DISCUSSION

DNA quantification and quality analysis

Finally, to measure the concentration and quality of DNA samples, a NanoDrop 2000c was used at the ratio of 260/280 nm to assess the purity of DNA. The DNA is accepted as "pure" if the ratio is ~1.8-2.2. This ratio is a secondary measure of nucleic acid purity. If the ratio is considerably lower, it may indicate that protein, phenol, or other contaminants are present that strongly absorb at or near 280 nm (www.info@nanodrop.com). For example, the ratio derived from the graph of DNA samples was 2.05, indicating that the DNA was pure with a concentration of 139 ng/µL at 260/280 (Table 4; Figure 1).

Table 1. Reaction master mix for 25 µL (Fermentas life science protocol, kit no: #K0171)

Reagents/components	Final concentration	1× reaction
PCR Master Mix 2×	1×	12.5 µL
Primer forward	0.3µM	1.2 µL
Primer Reverse	0.3µM	1.2 µL
Template DNA	500ng	2.5 µL
Nuclease-free water	Makeup 25 µL	7.6 µL
Total	25 µL	25 µL

Table 3. Standard PCR reaction parameters

Segment	Number of cycles	Target temperature	Hold time
Pre-denaturation	1	94 °C	2 minutes
Denaturation		94 °C	1 minute
Annealing	45	60 °C	1 minute
Extension		72 °C	2 minutes
Final extension	1	72 °C	5 minutes
Hold	0	4 °C	0 minutes

Table 2. The primers IDs and the gene IDs for each primer

Primers ID	Forward primer	Reverse primer	Gene ID	Expected size (bp)
252921	CTTCCATGGGAACTCACGTT	CCCCATACATTGCACATCCT	AT4G39030.1	100
256075	CAAATTCAGAGCGTGAACCA	TTTCTCTCAAAGTCAAATTCAAGC	AT1G18150.2	100
260133	AACCGCTCTTGTCACCAAGT	CCAGTTGGCACGACAAAAA	AT1G66340.1	100
245523	AGGTTTAGATCAAGAGGCAGAGTC	GCACGATTGGAAGGTCTGTAG	AT4G15910.1	100
263448	AACTGCAGAAGTTAGCTGCACA	TCAGAATCGTCGTCATCATCA	AT2G31660.1	100
253705	GGTATTCTGAAGAAACTGGGAAGA	CCATGGCTATAACCGATTTCTG	AT4G29130.1	100
259439	TTCAAGCTCCGACAATTTCA	TGATGGGTTGGTCAAAATCA	AT1G01480.1	100
264415	ACATGGACGATGGGTCATAAG	GCTGAACCGGTAATCAATGG	AT1G43160.1	100
249036	AGCTGCGCCAACGAATTAT	CGATTTTGTCCTCCTTGACC	AT5G44200.1	100
255220	GAAGTTCAATGTTTCGTTTCATGT	GGATTATACAAGGCCCCAAAA	AT4G05320.2	100

A. thaliana/X. americana DNA-DNA hybridization data

The putative orthologous genes of *X. americana* were studied using *A. thaliana* ATH1 GeneChip arrays. A similar technique was applied to investigate ovine and *Brassica oleracea* (Graham et al., 2011; Hammond et al., 2005, 2006). The gDNA of *X. americana* was labeled and then hybridized to the ATH1 GeneChip arrays. The hybridization signals were detected for all probes on the GeneChip for *X. americana* gDNA samples.

In this study for *X. americana*, the average hybridization signal intensities of gDNA for 10 selected putative orthologous genes of the ATH1 GeneChip array was 6.6589 (Table 5). Since gDNA from *X. americana* produced hybridization signals on the ATH1 GeneChip array, this information can be applied in genetic analysis to identify probes and probe sets with good homology between *A. thaliana* and *X. americana*. Only one replication was done in this study since fewer replications are required for experiments incorporating confirmatory testing such as PCR.

Gene ontology analysis of *A. thaliana* genome annotations

The number of genes associated with a particular GO biological process in the genome of *A. thaliana* is shown in Table 6. There were 47 and 46 genes that carried annotations relating to response to abiotic or biotic stimuli and response to stress, respectively. Furthermore, about 66 genes in the *A. thaliana* genome were not ascribed to a biological function. About 5% of genes were assigned to biological function genes that control protein metabolism and transport in the *A. thaliana* genome. Moreover, the least number of genes (3%) was assigned to signal transduction and transcription.

Gene ontology analysis of *A. thaliana/X. americana* hybridization data

Table 7 shows the number of genes related to biological processes that were retrieved from the locus identifiers of the set of the hybridized probe (putative orthologous).

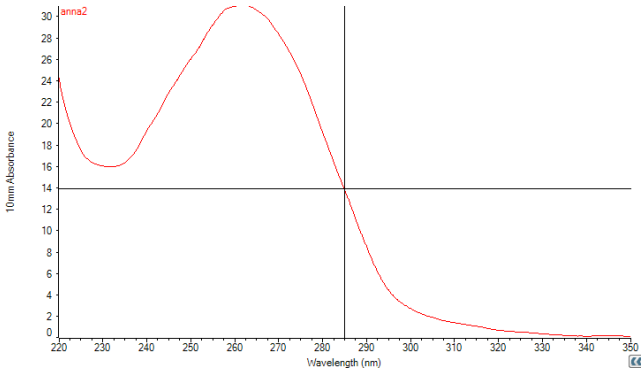

Figure 1. The graph of *X. americana* DNA concentration (139ng/µL) was measured using nanodrop.

Table 6 shows that the number of genes related to response to abiotic or biotic stimuli and stress response were 1,333 and 1,036, respectively. Further, the data indicate that 10,226 probe sets that represented putative orthologous genes of *X. americana* were not assigned to a biological function. The number of this probe set was higher than the A. thaliana because the genetic makeup of X. americana is not yet known. The putative orthologous genes associated with signal transduction had a least 906 probe set.

Table 4. The concentration of *X. americana* DNA samples

Sample ID	Nucleic acid [ng/µL]	A_{260}	A_{280}	$A_{260/280}$	$A_{260/230}$	Sample type
XimeniaDNA	139	2.779	1.356	2.05	1.75	DNA
XimeniaDNA	62.5	1.251	0.611	2.05	1.44	DNA
XimeniaDNA	703	14.061	8.718	1.61	1.87	DNA

Table 5. The genomic DNA hybridization intensity values

Probe-set ID	Corresponding *A. thaliana*	*X. americana* DNA Leaves intensities
252921_at	AT4G39030	8.72618
256075_at	AT1G18150	7.74376
260133_at	AT1G66340	5.6179
245523_at	AT4G15910	6.54669
263448_at	AT2G31660	9.27173
253705_at	AT4G29130	5.6248
259439_at	AT1G01480	5.19156
264415_at	AT1G43160	7.21048
249036_at	AT5G44200	5.54982
255220_at	AT4G05320	5.10657

Table 6. GO biological process in *A. thaliana* genome

GO biological process	Gene count	%
Response to abiotic or biotic stimulus	47	6
Response to stress	46	6
Unknown biological processes	66	6
Protein metabolism	50	5
Transport	34	5
Signal transduction	11	3
Developmental processes	13	6
Transcription	9	3

Table 7. DNA-DNA hybridization GO annotations

GO biological process	Gene count	%
Response to abiotic or biotic stimulus	1,333	9
Response to stress	1,036	12
Unknown biological processes	10,226	1
Protein metabolism	3,961	2
Transport	1,699	3
Signal transduction	906	6
Developmental processes	1,488	2
Transcription	1,701	3

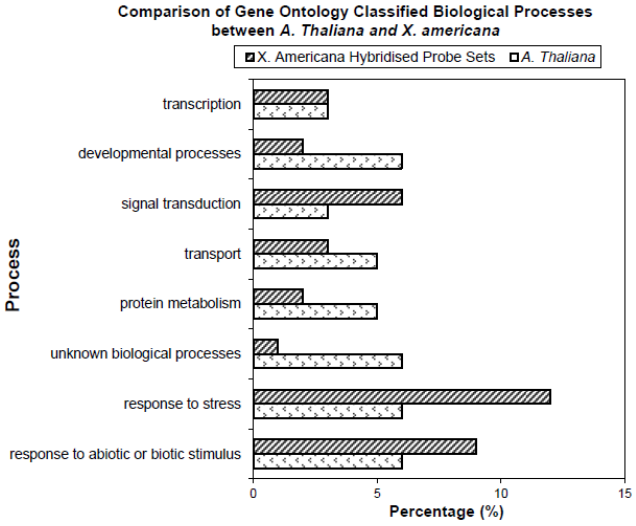

Figure 5. Comparison of gene ontology classified biological processes between *A. thaliana* and *X. americana*

Comparative gene ontology analyses

Between *A. thaliana* and *X. americana*, the gene ontology classifications of biological processes were compared. Figure 5 compares gene-ontology of the biological process presented in *Arabidopsis thaliana* with biological processes represented by hybridized regions (probe set) between *A. thaliana* and *X. americana* (http: //www.geneontology.org/; http: //www.arabidopsis.org).

The results illustrated that only 6% of biological processes in *A. thaliana are* associated with the stress response. The further GO analysis revealed that 6% of the *A. thaliana* genome was not assigned to any specific biological process. The data suggest that 12% of the biological processes were assigned to stress response in the cross-hybridized region. The representation of stress responses increased by 6% in *X. americana*. The over-representation of stress response was shown in *X. americana* under natural conditions. This observation was confirmed by an increased response to abiotic or biotic stimuli from 6% to 9%. Given the significance of the representation, an independent confirmation was required.

Independent confirmation

The DNA-DNA hybridization identified more than 20,000 probes (genes) in this study. In the study of *B. oleracea* with the same techniques, 40,099 genes were identified (Hammond et al. 2005). In this study, putative orthologous genes of X. americana demonstrating abiotic and stress responses were selected for independent confirmation.

DNA quality analysis and quantification

For PCR amplification, the quality of DNA extracted was at a reasonable standard, although it was not pure (ratio of 260/280 was 1.61). On the two extracts, the concentration of DNA samples of *X. americana* were 1546.2 ng/µL and 700 ng/µL, respectively (Table 8).

After the DNA of *X. americana* was quantified using ND-NanoDrop 2000c (Table 4), DNA amplification was performed using primers designed to independently confirm if the ten genes were found on the microarray results presented in *X. americana*. Figure 6 demonstrated that five out of ten genes (AT4G15910.1; AT2G31660.1; AT4G29130.1; AT1G01480.1; AT1G43160.1) of targeted orthologous genes were positive compared to a housekeeping gene (AT4G05320.2) as the positive control. Furthermore, these five genes from the targeted ten genes that are present in *X. americana* were confirmed from PCR results. These genes had sizes around 100 bp.

Independent confirmation of X. americana *biological process*

Gene ontology analysis of *X. americana* GeneChip showed that abiotic stress response genes were over-represented (12%) than *A. thaliana* (6%). The observation was independently confirmed with standard PCR amplification on the orthologous genes of AT4G15910.1; SAD2; HXK1; ACC, and ERF/AP2 (Figure 7). Furthermore, it was confirmed that biological processes responding to abiotic and biotic stimuli, other metabolic processes, other cellular processes, and transport were over-represented. The other biological processes that presented and were active were signal transduction, protein metabolism, transcription, and developmental processes.

The data may reflect the environmental interaction of *X. americana*. All five genes confirmed independently by PCR and GO found in *X. americana* were associated with environmental changes. Moreover, these genes were over-represented in *X. americana* than in *A. thaliana.* It is because *X. americana* grows in dry, rocky places and harsh conditions, while *A. thaliana* grows in a sandy and cold environment (Orwa et al. 2009). For instance, a gene whose transcript level in roots and leaves increases progressive drought stress is encoded by AT4G15910.1. This particular transcript level is also shown to be affected by the changes in three levels of endogenous or exogenous abscisic acid (Kawaguchi et al. 2004).

An important beta-domain family protein is encoded by SAD2 (super sensitive to ABA and drought 2); it is likely involved in the nuclear transport of ABA signaling. The mutation in SAD2 shows higher tolerance for UV stress, higher production of UV protective secondary metabolites, and reduced nuclear localization of MYB4 (a repressor of UV stress response genes) (Verslues et al. 2006; Zhao et al. 2007). ABA is an important mediator in activating the plant's responses to adverse environmental stimuli. Water stress condition during somatic growth increases ABA levels, which can be limited by reducing stomata opening when ABA levels increase (Leung and Giraudat 1998). In other aspects of stress adaptation, ABA is also involved. For example, in cold acclimation, the ABA-deficient mutant in *A. thaliana* is impaired (Mantyla et al. 1995) compared to *X. americana* which grows in dry and hot environments and has a root morphogenetic response to drought conditions. In signaling stress conditions, the role of ABA has also been considerably reported by molecular studies showing that ABA-deficient mutants are influenced in the regulation of several genes by drought, salt, or cold (Leung and Giraudat 1998).

Table 8. The DNA concentration using ND- NanoDrop 2000c

Sample ID	Nucleic acid [ng/µL]	A260	A280	A260/280	A260/230	Sample type
XimeniaDNA	1546.2	30.925	19.226	1.61	1.97	DNA
XimeniaDNA	700	14.061	8.718	1.61	1.87	DNA

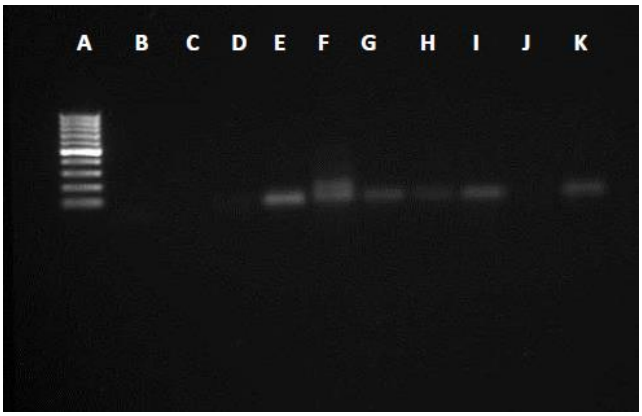

Figure 6. DNA amplifications of *X. americana* with different primers. A) DNA ladder (100bp); B) AT4G39031.1 (not present); C) AT1G18150.2 (not present); D) AT1G66340.1 (not present); E) AT4G15910.1 (present); F) AT2G31660.1 (present); G) AT4G29130.1 (present); H) AT1G01480.1 (present); I) AT1G43160.1 (present); J) AT5G44200.1 (not present) and K) AT4G05320.2 (present, positive control)

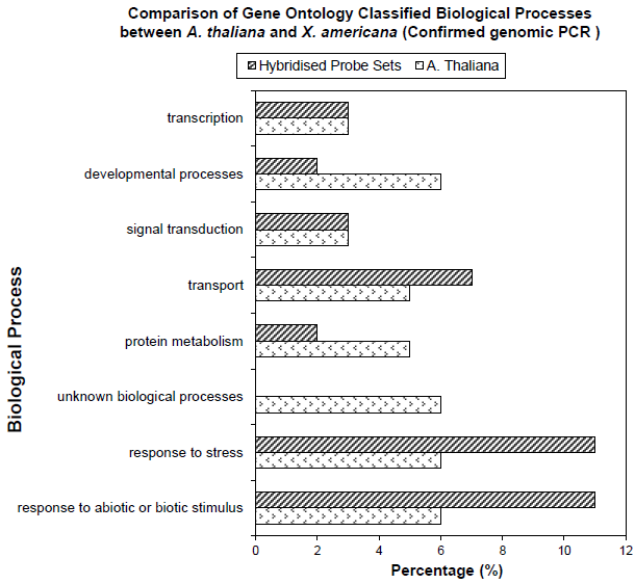

Figure 7. Genomic PCR-based independent confirmation of *X. americana* biological process.

The data of ontology results (Figure 5) suggests that stress response representation in *X. americana* increased by 6%, and over-representation of stress response occurred in *X. americana* under natural conditions, drought, and heat dependently examined using PCR (Figure 6). An increase in response to abiotic or biotic stimuli from 6% to 9% confirmed the observation. According to Jiang and Zhang (2004), the phytohormone abscisic acid (ABA) is one of the most important regulators of plant responses to abiotic stresses. Plants store higher amounts of ABA under drought, cold, or salt stress conditions, where drought stress has the most prominent effect. It elucidates the more abiotic stress genes in *X. americana* compared to *A. thaliana* due to *X. americana* growing in a dry environment.

The gene of AT4G29130.1 encoding a hexokinase (HXK1) in the plant glucose-signaling network roles as a glucose sensor for signaling networks of nutrients, light, and hormones in controlling growth and development responding to the changing environment (Moore et al. 2003; Cho et al. 2006; Karve et al. 2012). Therefore, all metabolism, growth, development, and abiotic and biotic stress responses are controlled, at least partially, by sugars (Harrington and Bush 2003). The changes in hexokinase levels by HXK1/ HXK2 affect plant tolerance to methyl viologen (Sarowar et al. 2008). Methyl viologen is a compound that damages the tissues of green plants by disturbing their photosynthesis. Hence, methyl viologen can be served as an herbicide. The alteration of bipyridyl residues to mono-cation radicals affects its herbicidal property. The radical reacts with oxygen, lead the formation of a superoxide anion, which can initiate the formation of hydroxyl and H_2O_2 radicals. These products cause oxidative destruction to cellular components, including DNA damage, lipid peroxidation, and protein inactivation (Timbrell 2000).

AT1G01480.1 is one of the 1-aminocyclopropane-1-carboxylate (ACC) synthases. It catalyzes the conversion of 5-adenosylmethionine (SAM) to ACC. The oxidation of ACC to ethylene is catalyzed by ACC oxidase. This plant hormone ethylene is produced to respond to various environmental stress types, such as low temperature and chemicals and water stress, wounding, physical load, and disease (Karve et al. 2012; Kato et al. 2000; Li et al. 2012; Liang et al. 1992).

A member of the ERF (ethylene response factor)/AP2 (The APETALA2) is encoded by AT1G43160.1. The essential roles of these AP2/ERF proteins are presented in the transcriptional regulation of a kind of biological processes associated with growth and development and various environmental stimuli responses (Jung et al. 2010; Licausi et al. 2013; Nakano et al. 2006). Ethylene is noticed as a stress hormone because its production is induced by stress signals, such as excess chemicals and metals, mechanical wounding, drought, extreme temperatures, and pathogen infection. In higher plants, ACC is an immediate precursor of ethylene. Endogenous levels of ACC highly influence ethylene production in plants. Hence, in the early phase of plant response to stress, ACC accumulates along with a rapid burst in ethylene synthesis (Bibi et al. 2006).

In this study, transcript levels of these essential abiotic response factors could not be determined in absolute or relative terms. Nevertheless, the study showed the inherent

presence of such genes at the *X. americana* genomic level. Further studies will be needed to show that some kind of modification of the factors of these abiotic responses or other abiotic responses within the genome of *X. americana* gives the key basis for its adaptation to the comparatively dry and hot climatic conditions.

In conclusion, During DNA-DNA cross-hybridization between *X. americana* with *A. thaliana*, gene ontology analysis of perfectly matched probe-pairs showed an increase in ontologies related to the biological process and abiotic stress compared to the ontology database of *A. thaliana*. Such evidence indicates that molecular mechanisms govern its adaptation to the prevailing natural conditions. *X. americana* that grows under harsh conditions leads the evolutionary adaptation to survive under these conditions. This study investigated the presence of 10 probe pairs of more than 20,000 perfectly matched probe pairs. The selected probe pairs confirmed the hybridization data and the presence of stress that related orthologous at the genomic level between *A. thaliana* and *X. americana*. Furthermore, successful amplification of *X. americana* genomic fragments provided evidence of homology within primer binding sites and some biological processes that integrate external stress signals, such as heat stress and drought stress in *X. americana*.

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Morphological and cytological analysis of yellow skin dragon fruit (*Selenicereus megalanthus*)

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Abstract. *Setyowati A, Sukaya, Yuniastuti E. 2018. Morphological and cytological analysis of yellow skin dragon fruit (*Selenicereus megalanthus*). Cell Biol Dev 2: 8-14.* Dragon fruit is a tropical fruit that has the potential to be developed. Information on morphological and cytological characters of dragon fruit plants is still small and simple, so morphological and cytological analysis is necessary. The morphological and cytological analysis yielded useful information to support the dragon fruit plant breeding program. This study aims to study the morphological and cytological characteristics (karyotype) of the yellow skin dragon fruit (*Selenicereus megalanthus* (Schum. ex Vaupel) Moran). This study took samples from "Kusumo Wanadri Agrotourism" Glagah Indah Beach, Yogyakarta, Indonesia. Chromosomal identification was carried out at the Plant Breeding Laboratory, Faculty of Agriculture, Universitas Sebelas Maret, Indonesia. The research was carried out from December 2006 to June 2008. The methods used were field observations and treatment methods in water for 24 hours at a temperature of 5-8ºC, fixation in 45% acetic acid solution for 2 hours at a temperature of 5-8ºC, and hydrolysis in an aqueous solution. One (1) N HCl for 3-4 minutes at 60ºC, staining in 2% aceto-orcein solution for 24 hours at 5-8ºC and squashing. Morphological and cytological data were analyzed and presented descriptively. The morphology of the plant *S. megalanthus* is a white thread-shaped root with a fibrous plant root system. The stem is a smooth, faceted wet stem with concave edges. The fruit is oval, surrounded by short spines, the skin is yellow, and the flesh is white. The *S. megalanthus* has a tetraploid chromosome 2n = 4X = 44 with a chromosome length ranging from 2 + 0.098 m to 4.75 + 0.98 m. The karyotype formula for the *S. megalanthus* chromosome 2n = 4X = 44 = 40 m + 4 sm.

Keywords: Cytology, morphology, *Selenicereus megalanthus*

INTRODUCTION

Dragon fruit plants originating from Central and South America have not been widely cultivated. This plant has only been cultivated intensively in several countries such as Israel, Colombia, Nicaragua, Vietnam, Thailand, China, and Australia (Lichtenzveig et al. 2000).

Dragon fruit plants have tufts that resemble dragon scales. This plant is a vine-shaped tree with thorns that grow along the tendrils and look unique, especially if the fruit has appeared on the tendrils. At first, this plant was only used as an ornamental plant. However, after it was discovered that the fruit was delicious to eat, it started to be cultivated as a fruit crop.

Dragon fruit can be consumed in fresh or processed form. Dragon fruit has a fairly high nutritional and water content of about 90.20% of the fruit weight (Kristanto 2003; Aji et al. 2013). In addition, it tastes quite sweet because the sugar in the fruit is quite high. Besides being cultivated as a fruit plant, dragon fruit is also cultivated as a medicinal plant because it has properties for human health. These benefits include: balancing blood sugar, preventing colon cancer, protecting oral health, reducing cholesterol, preventing bleeding, and treating vaginal discharge complaints.

This plant was recognized and cultivated in Indonesia in 2000. However, the development of this plant cultivation is very slow, even though Indonesia's climatic conditions are very supportive of the development of this plant. In 2006, there were only a few areas that cultivated this plant, namely Malang, Kediri, Tawangmangu, Semarang, and Kulon Progo, with various planting areas. In recent years, after it was discovered that dragon fruit has medicinal properties, dragon fruit cultivation continues to be carried out because it is very profitable. However, the cultivation of yellow skin dragon fruit (*Selenicereus megalanthus* (Schum. ex Vaupel) Moran). is still rarely done. It is because *S. megalanthus* requires an environment to grow in the highlands, in contrast to other types of dragon fruit cultivated in the lowlands.

Introducing dragon fruit plants based on morphological and cytological characters will greatly support the success of the dragon fruit plant breeding program. However, until now, knowledge of dragon fruit plants' morphological and cytological characteristics is still small and simple. By knowing with certainty the morphological and cytological characteristics of a plant, genetic information of a plant can be known so that appropriate methods can be sought for its development and cultivation.

There are 2 genera of dragon fruit cultivated in Indonesia, namely *Hylocereus* and *Selenicereus*. The dragon fruit cultivated is dragon fruit from the genus *Hylocereus*, namely *H. undatus* (Haw.) Britton & Rose (white flesh), *H. polyrhizus* (F.A.C.Weber) Britton & Rose (dark red flesh), and *H. costaricensis* (F.A.C.Weber) Britton & Rose (pink flesh), which are all diploid plants

with 2N = 2X = 22. Meanwhile, the *Selenicereus* genus cultivated is *S. megalanthus*, a tetraploid plant 2n = 4x = 44. However, although the ploidy is known, the karyotype is not known.

This study aims to study the morphological and cytological characteristics (karyotype) of the yellow skin dragon fruit (*S. megalanthus*).

MATERIALS AND METHODS

Materials

Plant material of yellow skin dragon fruit (*S. megalanthus*) obtained from "Agrowisata Kusumo Wanadri" Glagah Indah Beach, Kulon Progo District, Yogyakarta, Indonesia. Chromosomal identification was carried out at the Plant Breeding Laboratory, Faculty of Agriculture, Universitas Sebelas Maret, Surakarta, Central Java, Indonesia.

The main chemicals are 2% aceto-orcein, 45% acetic acid, and 1 N HCl solutions.

Research design

Morphology

Morphological research was carried out using survey observation methods in the field. The survey is aimed at knowing the conditions at the research site, including observing environmental factors and identifying dragon fruit plants. A sampling of 5 plants was done randomly (random sampling).

Cytology

Cytological research was carried out using the squashing method, a method for obtaining preparations by squeezing a piece of tissue or an organism. Thus, a dispersed preparation was obtained and thus can be observed under a microscope.

Morphological observation

Observations of morphological characteristics were carried out by randomly taking plant samples of 5 plants. The observed variables were based on plant morphology stated by Tjitrosoepomo (2003). These variables include the nature of roots, stems, fruits, and seeds.

Root morphology

The number of roots; (i) The observation was on the number of roots growing (very little, little, medium, a lot, or very much). (ii) Root form; The observation is on the shape of plant roots, whether they are spear-shaped (fusiform), top-shaped (napiform), or thread-shaped (filiform). (iii) Presence of root hairs; The observation was on the number of root hairs that grew (none, a little, moderate, a lot, or a lot). (iv) Root color; observations are on root color (white, yellowish white, yellow, or other colors). (v) Root system; The observation is on the root system, whether the taproot system or the fibrous root system.

Stem morphology

(i) Types of stems, observations were made by observing whether the stems were wet (herbaceus), woody stems (lignosus), grass stems (calmus), or *mendong* stems (calamus). (ii) The shape of the stem is observed by observing the shape of the stem in its cross-section, whether it is round (teres), square (angular), or flat. (iii) The surface of the stem, observations were made by observing the surface, whether it was smooth (laevis), ribbed (costatus), grooved (sulcatus), winged (alatus), hairy (pilosus), thorny (spinosus), showing leaf marks, showing traces of leaves. (iv) Leaf marks show many lenticels or conditions, such as crusting. In the branching of stems, observations were made by looking at the presence or absence of branching on the stem and then determining whether the branching method was monopodial, sympodial, or forking. (v) The number of branches was observed by counting the number of branches from the main trunk. (vi) Stem color (green, dark green, yellowish green, or other colors). (vii) The shape of the stem circumference observations was made by observing the shape of the stem circumference, whether it was curved inward (convex) or curved outward (concave).

Fruit morphology

(i) Fruit weight observations were made by weighing the observed fruit. (ii) Fruit shape is observed by observing its shape and determining whether it is round, elliptical, oval, or star. (iii) Observing fruit skin color is made by carefully observing the fruit skin color. (iv) The color of the fruit's flesh is done by carefully observing the color of the fruit's flesh. (v) The aroma of taste and observations are made by tasting the fruit's flesh, whether it tastes sour, sweet, or very sweet. The presence of thorns or tassels. (vi) The observations are made by observing whether the fruit's skin has thorns or tassels.

Seeds

(i) The number of seeds per fruit; observations were made by counting the number of seeds per fruit. (ii) Weight of 100 seeds, observations were made by weighing every 100 seeds. (iii) Seed shape, observations are made by observing the shape of the seed and determining whether it is round, elliptical, or oval. (iv) The color of the seeds is done by carefully observing the color of the seeds.

Nursery

Dragon fruit seeds are obtained from vine cuttings. Cuttings are grown in pots using a planting medium like Malang sand. After the roots appear, these cuttings are cut and used as material for making preparations.

Preparation making

Preparation of preparations using the squash method (squeezing) and sealing it semi-permanently, which is adapted from the method used by Anggarwulan et al. (1999) and Parjanto et al. (2003). Pre-treatment started with root cutting. First, the roots are washed with clean water. Then, the meristematic part of the root was cut about 5 mm

from the root tip and soaked in distilled water for 24 hours in the refrigerator at a temperature of 50º-80ºC.

Root pieces were fixed using a 45% acetic acid solution and stored in the refrigerator for 2 hours. Next, the root pieces were taken and washed with distilled water three times. Root pieces that have been fixed are then hydrolyzed with 1 N HCl solution for 3-4 minutes and stored in an oven at 60ºC. Then, 1 N HCl is removed and washed with distilled water three times (Anggarwulan et al. 1999).

Chromosomal staining was carried out by soaking the root pieces in 2% aceto-orcein solution (Anggarwulan et al. 1999; Parjanto et al. 2003) for 24 hours in the refrigerator. After this process, the root cap at the root tip is removed. Next, the meristematic part (approximately 0.5 mm from the root tip) was taken and placed on a glass slide. Next, the root pieces were covered with a cover glass placed on top of the root pieces and squeezed with the thumb or using the tip of a pencil slowly (Anggarwulan et al. 1999; Damayanti and Mariska 2003). Then the pressed preparations were sealed using clear nail polish (Anggarwulan et al. 1999) and observed using a light microscope at 1,000 times magnification.

Visible cells do not accumulate and exhibit prometaphase or metaphase are selected. At this stage, the chromosomes appear to be spread out well, making it easier to observe. Selected cells were photographed with a Nikon photomicroscope and micrographed. This method is a modification of the method used by Parjanto et al. (2003).

Chromosomal observation

Chromosomal morphology observations include:

Number of chromosomes

Observation of the number of chromosomes is done by taking pictures of them after they are visible on a light microscope and printing them with an enlarged print so that the number of chromosomes can be counted (Anggarwulan et al. 1999).

Chromosome size

On the chromosome image, the length of the two arms (Anggarwulan et al. 1999) and the length of the chromosome (the sum of the length of the long arm and the length of the short arm) were measured (Parjanto et al. 2003).

Chromosome shape

The location of the centromere determines the shape of the chromosome. The location of the centromere is determined by the ratio of the long arm to the short arm. Determination of the shape of this chromosome refers to the method of Ciupercescu et al. (1990) cit. Parjanto et al. (2003).

Karyotype

Chromosomes in prometaphase or metaphase showing the good distribution of chromosomes are photographed with a photomicroscope. The chromosomal images were then observed for morphology and arranged sequentially from the longest to the shortest size as a karyotype. The arrangement of the karyotype is made by pairing homologous chromosomes, which are determined based on the similarity of the size and shape of the chromosomes (Parjanto et al., 2003).

Data analysis method

Morphology

Morphological data were analyzed and presented descriptively based on the results of morphological observations in the field.

Cytology

Cytological data were analyzed and presented descriptively based on observations from the photographed chromosome images and observations of the length and shape of the chromosomes.

RESULTS AND DISCUSSION

Location

The land in "Kusumo Wanadri Agrotourism" Glagah Indah Beach, Kulon Progo District, Yogyakarta, Indonesia is a sandy land with an altitude of 5 m asl. The environmental conditions for growing dragon fruit plants in the study area have an average daily intensity of sunlight between 2,860-3,950 lux. The average daily temperature is between 27-3°C. The average daily humidity is between 88-93%. Rainfall is between 1,580-2,300 mm/yr. The growing environmental conditions are by the requirements for growing dragon fruit plants as stated by Kristanto (2003), that the ideal temperature for dragon fruit plants is between 26-36°C, and the humidity needed by plants is between 70-90%. However, the growth of *S. megalanthus* plants will be optimal if planted in cold areas with an altitude of more than 800 m asl.

Description of root

S. megalanthus roots are similar to the roots of dragon fruit plants of the genus *Hylocereus*. These similarities include filiform roots, a moderate number of yellowish-white root hairs, and a fibrous root system. The root morphology of *S. megalanthus* also differs from the roots of dragon fruit plants from the *Hylocereus* genus in terms of the number of roots. The number of roots of *S. megalanthus* is not as much as the number of roots of dragon fruit plants of the genus *Hylocereus.*

The difference in the number of roots between *Selenicereus* and *Hylocereus* may affect plant growth. *Selenicereus* growth tends to be slower compared to *Hylocereus* growth. It is possible because of the influence of the number of minerals that plants can absorb that, it also affects the production of food substances that are distributed to all parts of the plant.

S. megalanthus roots are also the same as the roots of other types of dragon fruit, which are epiphytic, where the roots propagate and attach to other plants. In its cultivation, this plant requires a propagation medium. The required propagation medium can be concrete or bamboo. The roots

of dragon fruit plants are very drought tolerant and can not tolerate puddles.

Stem description

The stem is a very important part of the plant body. The stem can be equated with the body's axis and the plant's support. The stem of *S. megalanthus* is a type of herbaceous that contains a lot of water with a laevis surface because it is coated with wax. The shape of the stem is an angular rod, to be precise, a triangle. The stem of the *S. megalanthus* plant has a fork branching system in which 2 branches will appear on each stem. The stem of the *S. megalanthus* plant is green with a concave ridge shape. From the stem will come out short thorns from which flowers and fruit will grow.

The description of the stem of the *S. megalanthus* plant has similarities with the description of the dragon fruit plant of the genus *Hylocereus*, namely in terms of stem type, stem shape, stem surface, branching, number of branches on the stem, and the presence of thorns coming out of the stem. However, in addition to these similarities, there are differences in the stem's color and the rim's shape. The stem of the dragon fruit plant of the *Hylocereus* genus is dark green and has a convex edge shape.

The waxy coating that covers the stems of dragon fruit plants reduces plant evaporation. The stems have a high water content, so without the protection of the wax coating, the plant will lose a lot of water and become dry. Dragon fruit plants do not have leaves, so the stems do not grow leaves. Instead, the leaves have modified their shape into thorns that grow along the stems (vines). Similar to the wax coating, the spines function in reducing plant evaporation.

Fruit description

Dragon fruit plant fruit results from pollination, which causes the ovary to grow into fruit. Immature fruit skin is still light green. The color of the fruit gradually changes. Each type of dragon fruit has a different fruit color. For example, in *S. megalanthus* plants, the color will change to yellow. It differs from *H. undatus* whose skin color changes to pink, *H. polyrhizus*, whose skin color turns red, and *H. costarinensis*, whose fruit skin color turns dark red. The color of the fruit flesh of each type of dragon fruit plant is also different. *S. megalanthus* and *H. undatus* have the same flesh color, i.e., white. However, it differs from *H. polyrhizus*, which has pink flesh, and *H. costarinensis*, which has red flesh.

The shape of the fruit of each type of dragon fruit plant is not the same. For example, the fruit of *S. megalanthus* is oval. It is different from *H. undatus* and *H. polyrhizus*, which have elliptical fruit shapes, while the shape of *H. costarinensis* fruit is round.

Apart from being different in terms of fruit shape, skin color, and fruit flesh color, there is still one difference. There is no tuft on the fruit of *S. megalanthus* as in the three types of *Hylocereus*. The tufts on the fruit of *S. megalanthus* are replaced by the growth of short spines. The fruit of the dragon fruit plant appears along the stem, precisely near the thorns that grow on the stem. The fruit appears singly in groups of two or three or even more.

The tufts on the *Hylocereus* dragon fruit are the same as the spines on the *S. megalanthus* fruit. Both are modifications of flower petals. The flower crown will wither and fall when the ovary begins to form. However, it is not the case with flower petals. The petals do not wither and fall in fruit growth but still grow and develop. It's just that in this growth, the flower petals have modified their shape. In the Hylcereus dragon fruit, the petals will be modified into tassels. At the same time, the fruit of *S. megalanthus* was modified into thorns. Both grow around the fruit.

In fruit morphology, fruit weight and aroma taste could not be observed. The observed fruit is still small and growing on the tree. In addition, the sample for observation of fruit is only one fruit. Therefore, it does not meet the sample requirements.

Description of seeds

Seeds are the primary means of reproduction for seed plants. Seed observation can only be done on *Hylocereus* dragon fruit. Meanwhile, observation of seeds on *S. megalanthus* could not be done because there were no samples. Dragon fruit seeds are small, so many seeds are in one fruit.

Based on observations, the average number of seeds of *H. undatus* was 4,399, *H. polyrhizus* was 4,790, and *H. costaricensis* was 5,232. Meanwhile, the average weight of 100 seeds of *H. undatus* was 0.2005 g, *H. polyrhizus* was 0.1783 g, and *H. costaricensis* was 0.1973 g. The shape of the seeds of the three *Hylocereus* species is the same: oval. Similarly, the color of the seeds. All three types of *Hylocereus* have black seeds.

Number of chromosomes

The number of chromosomes is a characteristic of chromosomes that is easy to observe and stable. The results showed that the *S. megalanthus* had a chromosome number of 2n= 4X = 44. The chromosome number of *S. megalanthus* in Figure 1 shows that the number of chromosomes is quite large. Therefore, there are still chromosomes attached.

The number of chromosomes *S. megalanthus* is tetraploid, where 1 set of chromosomes consists of 4 homologous chromosomes. This number differs from the number of chromosomes of the dragon fruit genus *Hylocereus*, which is 2n= 2X = 22 (diploid). The difference in the number of chromosomes will be expressed in several plant morphological characters.

In general, tetraploid plants produce larger fruit than diploid plants. However, this is not the case with *S. megalanthus.* The size of the fruit of this plant is even smaller than the size of the fruit of the *Hylocereus* type. Furthermore, *S. megalanthus* fruit is not surrounded by tufts as in the *Hylocereus* type fruit but is surrounded by thorns. In addition, the appearance of the stem of *S. megalanthus* is also different from the dragon fruit of the genus *Hylocereus*. The stem of *S. megalanthus* is relatively thinner and has a concave rim, while the stem of the dragon fruit genus *Hylocereus* is thicker with a convex rim.

Another difference is that *S. megalanthus* has slower growth than the dragon fruit of the *Hylocereus* genus.

Chromosome size

One of the criteria for identifying chromosome morphology is chromosome size. Observations of chromosome size include the total length of the chromosome (q+p), the length of the long arm of the chromosome (q), and the length of the short arm of the chromosome (p).

The length of the *S. megalanthus* chromosome ranged from 2 + 0.098 µm to 4.75 + 0.98 µm. The length of the short arm of the chromosome (p) ranged from 0.76 + 0.053 µm to 2.09 + 0.053 µm, while the length of the long arm of the chromosome (q) ranged from 1.05 + 0.06 µm to 2.85 + 0.06 µm.

Based on the average length of chromosomes, *S. megalanthus* has a fairly large chromosome size. In addition, it has many chromosomes compared to the dragon fruit of the *Hylocereus* genus. The length of the DNA sequence will determine the size of the chromosome, and it varies from species to species. Thus, the longer the chromosome size, the longer the DNA sequence. According to Damayanti et al. (2005), the amount of DNA content in the cell nucleus positively correlates with the length and total volume of chromosomes.

Chromosomal shape

Determination of the shape of the chromosome can be based on the location of the centromere. The location of the centromere is one of the important morphological characteristics of chromosomes in identifying chromosomes. The classification of the shape of the chromosomes is based on the ratio of the chromosome arms (r = q / p).

The shape of the *S. megalanthus* chromosome is metacentric and submetacentric. The chromosome form of *S. megalanthus* consists of 40 metacentric chromosomes and 4 submetacentric chromosomes. The *S. megalanthus* chromosomes which have a metacentric shape are on chromosomes number 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, while the *S. megalanthus* chromosome which has the shape submetacentric, namely on chromosome numbers 21, 22, 23, and 24. In general, plants have metacentric chromosomes.

Karyotype

A karyotype is an arrangement of chromosomes in order from longest to shortest. Karyotyping is done by pairing one chromosome with a homologous chromosome. The result of this pairing of chromosomes is called a karyogram. The determination of homologous chromosomes is based on the similarity of the shape and size of the chromosomes. Karyogram is useful for knowing chromosomal aberrations in the number and structure of chromosomes that occur during cell division. In addition, it can be searched for its relationship with abnormalities found in a living creature's anatomy, morphology, and physiology.

Based on the number of chromosomes of *S. megalanthus* in the research results, 2n = 4X = 44, and the chromosomes of *S. megalanthus* are tetraploid so that each set of chromosomes consists of 4 homologous chromosomes. The karyogram of *S. megalanthus* can be seen in Figure 2.

The karyotype formula can be made from the karyotype arrangement of *S. megalanthus*. The formula for the karyotype of *S. megalanthus* is 2n = 4X = 40 m + 4 sm with m = metacentric chromosome and sm = submetacentric chromosome. The arrangement of chromosomes in the form of an idiogram can be seen in Figure 3.

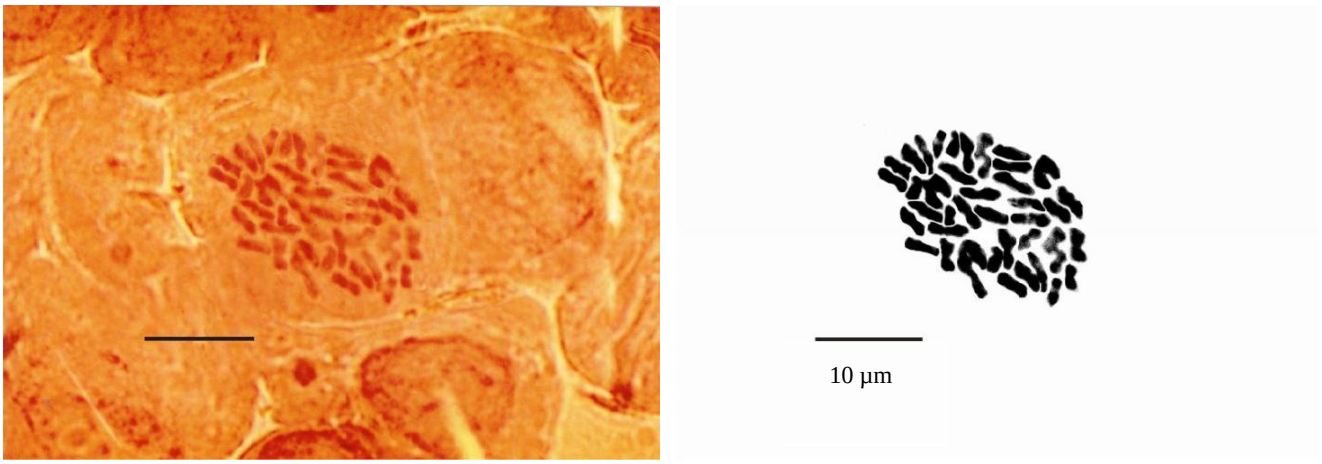

Figure 1. Chromosomes of yellow skin dragon fruit (*S. megalanthus*) are tetraploid 2n = 4X = 44. Bar 10 um

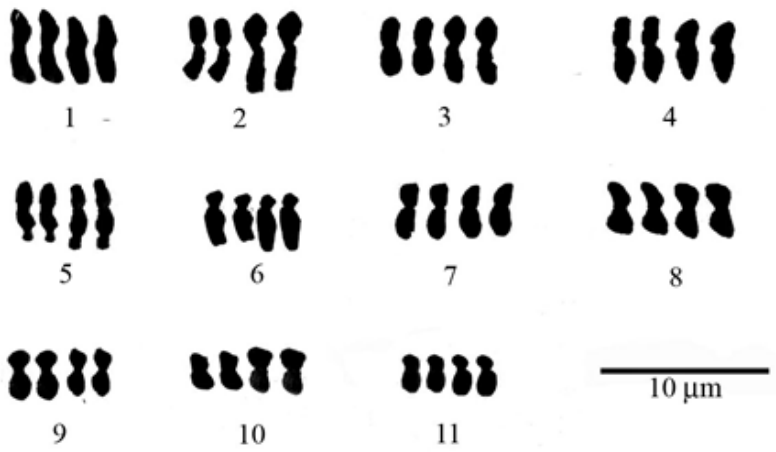

Figure 2. Karyogram of yellow skin dragon fruit (*S. megalanthus*)

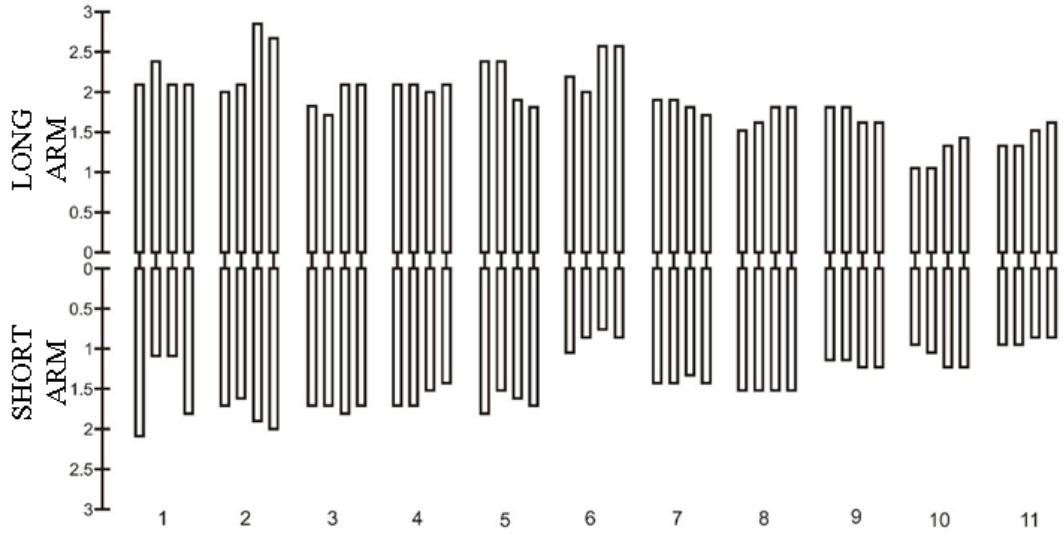

Figure 3. Idiogram of yellow skin dragon fruit (*S. megalanthus*)

Chromosomes paired with homologs often have similar shapes and sizes, making it difficult to determine homologous pairs. Therefore, it is necessary to identify chromosomes using a chromosome banding technique to overcome this. Thus, the identification of individual homologous chromosomes can be carried out so that the determination of homologous chromosome pairs can be carried out accurately (Parjanto et al., 2003).

Based on the research that has been done, it can be seen: (i) The roots of *S. megalanthus* are filiform in shape, have a moderate amount of root hairs, the root color is yellowish white, has a fibrous root system. The number of roots is not as much as the number of roots of dragon fruit plants genus *Hylocereus*. (ii) The stem of *S. megalanthus* is a type of herbaceus with a laevis surface because it is coated with wax. The trunk shape is an angular fork branching system where each stem will appear in 2 branches. The stem of the *S. megalanthus* plant is green with a concave ridge shape. (iii) The color of the fruit skin of *S. megalanthus* is yellow, and the color of the flesh is white. The *S. megalanthus* has oval fruit. On the fruit of *S. megalanthus*, there are no tufts but short spines. (iv) *S. megalanthus* has a tetraploid chromosome number of 2n = 4X = 44 with a chromosome length ranging from 2 ± 0.098 m to 4.75 ± 0.098 m. The *S. megalanthus* has 40 metacentric chromosomes and 4 submetacentric chromosomes with the karyotype formula 2n = 4X = 44 = 40 m + 4 sm.

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Effect of organic and inorganic fertilizers on growth, yield and nutrient use efficiency of clonal tea (*Camellia sinensis*)

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Abstract. *Mokaya BN, Chemining'wa GN, Ambuko JL, Nyankanga RO. 2018. Effect of organic and inorganic fertilizers on growth yield and nutrient use efficiency of clonal tea (*Camellia sinensis*). Cell Biol Dev 2: 15-26.* This research observed how to boost tea yields in small-scale tea producers using inorganic NPK fertilizers yearly while the cost of fertilizers has been rising, resulting in a decrease in net returns. The influence of different rates of organic and inorganic fertilizers on the soil chemical characteristics, growth, yield, and nutrient usage efficiency of clonal tea was investigated in an experiment. In the 2014/2015 production year, experiments were put up in Kianjokoma, Embu County. Organic Rutuba® and inorganic NPK fertilizers were employed (26.5.5). No-fertilizer control, 625 kg NPK/ha, 937.5 kg NPK/ha, 1875 kg NPK/ha, 625 kg Rutuba/ha + 625 kg NPK/ha, 625 kg Rutuba/ha, 937.5 kg Rutuba/ha, and 1875 kg Rutuba/ha were the treatments. These treatments were repeated three times in a randomized full-block design. Green leaf yield, black made tea yield, leaf length, and leaf breadth were among the crop statistics obtained. The soil data collected at the trial included the pH, exchangeable acidity, organic carbon, micronutrients, and macronutrients. Plant utilization efficiency for nitrogen, phosphate, and potassium were also measured. The cost and net revenue of each fertilizer treatment were calculated as well. The data were subjected to analysis of variance, and the least significant difference test was used to separate the groups at p 0.05. Adding organic Rutuba to the soil raised the pH and the amounts of Mn, Cu, Fe, Zn, Ca, and Mg. Compared to the no-fertilizer control and farmers' practice, the application of 625 kg Rutuba/ha + 625 kg NPK/ha resulted in enhanced leaf length, breadth, fresh green leaf yield, and made tea yield. According to the findings, the application of Rutuba fertilizer considerably improved agronomic efficiency, apparent nutrient recovery, and partial factor productivity of N, P, and K compared to inorganic NPK fertilizer. The study also found that applying NPK 26.5.5 at rates more than 625 kg/ha did not affect growth, yield, nutrient usage efficiency, or net returns. However, because organic Rutuba has a low macronutrient level, the study indicated that it should not be used alone in tea manufacturing. More research is needed to discover the best NPK and Rutuba fertilizer mix, as well as the influence of organic Rutuba fertilizer on tea quality.

Keywords: *Camellia sinensis,* fertilizers, growth, yield, nutrient use, tea

INTRODUCTION

The economy of Kenya is heavily reliant on agriculture, which accounts for 25% of the country's GDP and 75% of its industrial raw materials (MOA 2013). Furthermore, the sector accounts for 65 % of the total exports of Kenya, 18 % of formal and informal job opportunities, and 60 % of total employment chances (MOA 2013). Forestry, fisheries, livestock, food crops, horticulture, and industrial crops are part of the agricultural industry. Tea is one of the most important industrial crops (*Camellia sinensis* L.). Tea contributes 4% of the national GDP and 26% of overall export revenues in Kenya, according to the Tea Board of Kenya (TBK) (Tabu et al. 2015). According to TBK, Tea brought in Ksh.114 billion in export earnings and Ksh.22 billion in local sales in 2013 (TBK 2013), while it brought in Ksh.94 billion in export earnings and Ksh.123 billion in local sales in 2014 and 2015 (KNBS 2016). Tea is also significant for providing direct employment to over 639,521 farmers, as well as various job opportunities along the value chain (Onduru et al. 2012) and the development of rural infrastructure.

Next to water, tea is the most popular and cheapest beverage in the world (Onduru et al. 2012), and it is a major commercial crop in both subtropical and tropical climates. Tea is grown on plantations throughout the world, but in Kenya and Sri Lanka, small-scale farmers play a crucial role (Onduru et al., 2012). China, India, Sri Lanka, and Kenya are the world's top tea producers (Gunathilaka et al., 2016). India is the world's largest producer of black tea, accounting for 25% of global production, with China and Kenya following closely behind (Gunathilaka et al., 2016). India consumes 21% of the world's tea production, with approximately 70% of the tea consumed in the country, while Kenya exports most of its tea (Gunathilaka et al. 2016).

Tea was initially introduced to Kenya in the early twentieth century by the Caine brothers in Limuru in 1904, but commercial manufacturing did not commence until 1924. (Owuor 2011; Kagira ,2012). Tea cultivation is supported by a wide range of ecological variables, all of which significantly impact the rate of growth and quality of the tea plant, resulting in different yields and quality of tea in different places. Tea is grown in Kenya on the foothills of the Aberdare ranges in the west and Mount Kenya in the east of the Great Rift Valley (Owuor 2011). Drought, temperature, frost, high radiation, and soil pH are all factors that affect tea yield and quality. Tea is grown in

warm, humid tropical regions with soils ranging from loamy to volcanic red clays with reasonably evenly distributed rainfall throughout the year (Owuor 2011). It grows best in deep, well-drained soils with a pH of 4.0-5.0. (Mwaura et al. 2007). The particular goals of the study were (i) to assess the influence of various rates of inorganic NPK and organic Rutuba fertilizers on soil nutrients in a clonal tea field; (ii) to determine the effect of different rates of inorganic NPK and organic Rutuba fertilizers on clonal tea growth and yield; and (iii) to determine the effect of varying rates of inorganic NPK and organic Rutuba fertilizer on clonal tea growth and yield.

MATERIALS AND METHODS

Description of the experimental sites

Kianjokoma, Embu county agroecological zone (AEZ) UM1 (Sombroek et al. 1984), is 20 kilometers from Embu town and 152 kilometers from Nairobi, in which the experiment was set up. The trial lasted from June 2014 to June 2015, throughout the production of 2014/2015. The site is located at latitude $0^0 23^0$S and longitude $37^0 17.3^0$E, on the tea-growing slopes of Mt. Kenya, at an elevation of 1,831m above sea level. The average temperature at the site is 17.4 to 24.5^0 Celsius, with an annual rainfall of 700 to 900 millimeters. The site experiences a bimodal rain pattern, with long rains in April and May and brief rainfall in October and December. Red clays, classed as ando-humic nitsols, comprise this area's soils (FAO-UNESCO, 1988). pH, exchangeable acidity, total nitrogen, total organic carbon, phosphorus, potassium, calcium, magnesium, manganese, copper, iron, zinc, and sodium were all measured in the soil (Table 1).

During the study, the rainfall was evenly dispersed. The brief rains occurred between July and December 2014, with November 2014 receiving the most rainfall. In January and February 2015, there was little or no rain, severely influencing the plucking rounds and, as a result, the bush productivity. From March through May 2015, there was a lot of rain, with May 2015 being the wettest month of the year. Table 2 shows the study's rainfall, minimum, and maximum temperatures.

Experimental design, treatments, and crop husbandry

The experiment was carried out three times in a randomized complete block design. The treatments were: 1. No-fertilizer control; 2. 625 kg NPK/ha (26.5.5) (farmer practice); 3. 937.5 kg NPK/ha (26.5.5); 4. 1875 kg NPK/ha (26.5.5); 5. 625 kg Rutuba/ha + 625 kg NPK/ha (26.5.5); 6. Application of 625 kg Rutuba/ha; 7. Application of 937.5 kg Rutuba/ha (Recommendation to farmers); and 8. Application of 1875 kg Rutuba/ha. Each plot included 20 tea bushes spaced 1.5 m apart by 0.762 m apart. The tea bushes of clone TRFK 6/8 were 20 years old and already established. A row of mature-grown shrubs was established between the plots to avoid interaction with adjoining crops. Weeding of the plots was low because the bushes had grown a full cover; therefore, no or few weeds were growing during that time. Weeds around the edges were eradicated by hand before fertilizer was applied. Rutuba® organic fertilizer from Rutuba Bio Agric & Organic Fertilizers CO.LTD was used in the experiment, as well as inorganic NPK 26.5.5. The inorganic fertilizer NPK, 26.5.5 is the sort of fertilizer used by KTDA tea producers to top-dress their tea yearly. Rutuba® fertilizer was subjected to a thorough examination (Table 3).

Table 1. Soil analysis before fertilizer application

Chemical component	Value
Ph	4.39
Exchangeable acidity	0.50
Total nitrogen (%)	0.34
Total organic carbon (%)	3.51
Phosphorus (ppm)	35.00
Potassium (Me%)	0.80
Calcium (Me%)	4.90
Magnesium (Me%)	0.19
Manganese (Me%)	0.24
Copper (ppm)	2.91
Iron (ppm)	62.8
Zinc (ppm)	3.12
Sodium (Me%)	0.22

Table 2. Monthly rainfalls and maximum and minimum temperature at Kianjokoma from June 2014 to June 2015

Month	Monthly rainfall (mm)	Max. monthly temp. (°C)	Min. monthly temp. (°C)
June	59.7	21.4	12.9
July	68.9	25.4	11.7
August	76.5	28.5	12.9
September	150.1	25.4	12.0
October	100.7	23.2	13.3
November	200.7	16.8	14.9
December	154.5	19.2	14.4
January	0.0	16.9	14.1
February	25.3	21.3	15.7
March	150.9	18.7	15.1
April	191.0	17.3	15.7
May	234.4	18.6	14.3
June	50.7	17.1	14.5
Monthly mean	112.6	20.8	14.0

Table 3. Analytical results of the organic Rutuba fertilizer that was used during the experiment

Chemical component	Value
Nitrogen (%)	2.1
Phosphorus (%)	1.0
Potassium (%)	1.2
Calcium (%)	3.1
Magnesium (%)	0.4
Iron (mg/kg)	5233.0
Copper (mg/kg)	26.7
Manganese (mg/kg)	512.0
Zinc (mg/kg)	427.0
pH	8.2
Total organic carbon (%)	2.1

Data collection

The information gathered included soil chemical characteristics, second leaf length, and width, green leaf yield, made tea yield, N P and K use efficiency, and fertilizer regime profitability.

Soil chemical data

Soil samples were collected at random from 30 different locations in the experimental field for laboratory analysis. Before scooping the earth to a depth of 30 cm with a soil auger, roots and other plant wastes were removed. The samples were properly mixed to create a composite sample weighing around 500 g for examination. Next, soil samples were scooped from each plot at a depth of 30 cm using a soil auger at 3 separate sites at the end of the experiment (June 2015) and carefully mixed to generate a composite sample from each plot. The samples were then placed in a plastic bag, labeled, and transferred to the National Agricultural Research Laboratories (NARL) in Nairobi, Kenya, for analysis on the same day. pH, total organic carbon, exchangeable acidity, accessible micronutrients, and macronutrients were all measured in the soil samples.

The elements were extracted in a 1:5 ratio (w/v) with a combination of 0.1 N HCl and 0.025 N H2SO4 after the soil samples had been sieved through a 2 mm sieve. A flame photometer was used to measure sodium, calcium, and potassium. In addition, AAS was used to assess phosphorus, magnesium, and manganese (Mukai et al. 1992).

The calorimetric method was used to estimate total organic carbon. For full oxidation, acidified dichromate was used to oxidize organic carbon in an oven-dried soil sample at 150°C for 30 minutes. Next, the cold digests were given a shot of barium chloride. After carefully mixing the cool digests, they were allowed to sit overnight. Finally, at 600 nm, the carbon concentration was measured using a spectrophotometer (Walkley et al. 1934).

The Kjeldahl method was used to determine the total nitrogen. The soil samples (0.5 mm) were oven dried at 40^0 Celsius and digested with concentrated sulphuric acid comprising potassium sulphate, selenium, and copper sulphate that had been hydrated to approximately 35^0 degrees Celsius. Distillation followed by titration with diluted standards 0.007144N H_2SO_4 was used to estimate total N. (2002) (Okalebo et al.).

Exchangeable acidity was evaluated by heating 5 g of oven-dried soil sample (2 mm) to 40^0C in a 50 ml container, adding 12.5 ml of 1 M KCl, and stirring with a clean glass rod for half an hour. The solutions were filtered using a funnel. Next, five successive 12.5 ml aliquots of 1 M KCl were utilized to leach the solutions. The indicator solution phenolphthalein was added and titrated with 0.1 M NaOH until the first permanent pink hue was achieved. The amount of NaOH used was noted. The available trace elements (Cu, Fe, and Zn) were determined using 0.1 M HCl extraction. The elements were extracted with 0.1 M HCl in a 1:10 (w/v) ratio. AAS was used to determine the elements (Mukai et al. 1992). The pH of the soil was tested using a pH meter in a 1:1 (w/v) soil–water suspension.

Length and width determination

At the time of harvest, a random sample of 100 shoots with two leaves and a bud was collected from each plot's gathered shoots. The length and width of the second leaf were measured with a ruler, and each plot's average length and width were recorded 20 times over 7-10 days, depending on crop availability.

Assessment of yield

Leaf plucking began two weeks following fertilizer application in each area. First, the tea was harvested by hand using the traditional method of harvesting two leaves and a bud every 7-10 days, depending on crop availability, for 20 harvests. Then, weigh and record the plucked leaves. Finally, the calculation below was used to convert the green leaves harvested per plot to made tea (kg/ha/year) (Sitienei et al. 2013). Made tea is the tea that has been manufactured from harvested green shoots, i.e., after withering, fermenting, and drying (De Costa et al. 2007).

$$\text{Made tea yield/ha/year} = \frac{N*a*0.225}{b}$$

Where: **a** is the plant population per hectare, **N** is the green leaf yield per plot, **b** is the number of tea bushes per plot, and **0.225** is the factor converting green leaf to made tea.

Determination of leaf nutrient and nutrient use efficiencies

Each plot received 100 mature leaves for nutritional analysis, which calculated nutrient utilization efficiencies. Hydrogen peroxide was used to oxidize the leaf samples at 100^0C. After the breakdown of the excess hydrogen peroxide and evaporation of water, the digestion was completed with concentrated H2SO4 at 330^0C using SE as a catalyst. Nitrogen was then determined using distillation and titration with standardized 0.3 N HCl. Potassium, phosphorus, and nitrogen concentrations were determined using a flame photometer, spectrophotometry, and the Kjeldahl method.

The apparent nutrient recovery, agronomic efficiency, and partial factor productivity of nitrogen, phosphorus, and potassium were calculated. The following equations were used to determine the efficiencies (Sitienei et al., 2013; Jagadeeswaran et al., 2005).

$$\text{Agronomic efficiency (AE)} = \frac{\text{Yield in fertilized plot (kg/ha) -yield in control plot (kg/ha)}}{\text{Quantity of fertilizer nutrient applied (kg/ha)}}$$

Apparent nutrient recovery efficiency (**ANR)** was used to determine the ability of the plant to acquire nutrients from the soil.

ANR= Nutrient uptake in the fertilized plot (kg/ha)- Nutrient uptake in control plot (kg/ha) Quantity of fertilizer nutrient applied (kg/ha)

$$\text{Nutrient uptake} = \frac{\text{Nutrient concentration}*\text{dry matter Dry matter-Kg of made tea/ha}}{\text{Partial factor productivity } (P_fP)}$$

P_fP= Yield of made tea (kg/ha)

Amount of fertilizer nutrients applied (kg/ha)

Determination of the economic benefits of the treatments

Each fertilizer regime's total expected cost and income were computed to determine each application's profit. The overall cost included the price of fertilizer, the cost of fertilizer application, and the cost of plucking/harvesting. The entire revenue includes the farm gate fee, which is paid monthly for each delivered green leaf, and the annual payment, which varies per factory. One kg was assessed at Ksh. 14/= as the farm gate price per kg of total green sent to the factory in a month, the price paid to small-scale farmers affiliated with KTDA for monthly green leaf deliveries. The yearly payment was 35.05/= of the cumulative green leaf in the financial year, which corresponded to the payment made to farmers who brought the green leaf to the Mungania tea factory in the 2014/2015 financial year the experiment was conducted. As a result, the green leaf was evaluated at Ksh.49.05 per kg. The overall cost comprised labor for fertilizer application (Ksh/ha), fertilizer purchase (Ksh/ha), and plucking (Ksh/ha). Labor costs for harvesting were projected to be Ksh.10/= each kg of harvested green leaf.

In contrast, labor costs for fertilizer application were estimated to be Ksh.50 for every 50 kg bag of fertilizer. Therefore, a 50 kg bag of NPK 26.5.5 and Rutuba fertilizer was sold on the market at Ksh.2,250 and Ksh.3,000, respectively. The entire revenue was estimated by multiplying the cumulative number of green leaves by the total amount paid to farmers per kg.

The total cost = the purchasing cost of fertilizer applied + the cost of harvesting + the cost of applying fertilizer.

Total revenue =cumulative green leaf (kg/ha)* Ksh.49.05 Net revenue=Total Revenue-total cost

Data analysis

All data collected were subjected to analysis of variance (ANOVA) using GEN STAT discovery edition 14 software, and means were separated using the least significant difference (LSD) at p=0.05.

RESULTS AND DISCUSSION

Effects on soil nutrients, pH, total carbon, and exchangeable acidity in a clonal tea field at the end of the experiment

Fertilizer treatment regimes significantly affected the soil's pH, exchangeable acidity, total carbon, manganese, copper, iron, and zinc levels ($p \leq 0.05$). (Table 4). The pH value of 1,875 kg Rutuba/ha was considerably higher than the pH value of all other fertilizer rates except 625 kg Rutuba/ha + 625 kg NPK/ha, which was not statistically different from 937.5 kg Rutuba/ha, 625 kg Rutuba/ha, and the no-fertilizer control. There were no significant differences in the rates of solo NPK. The pH of the soil varied between 3.94 and 5.36 (1875 kg NPK/ha). The 1,875 kg NPK/ha application rate resulted in significantly higher exchangeable acidity than the other rates. The exchangeable soil acidity of 625 kg Rutuba/ha + 625 kg NPK/ha, 625 kg NPK/ha, 937.5 kg NPK/ha, 625 kg Rutuba/ha, and the no-fertilizer control were not substantially different. There was no discernible difference in exchangeable soil acidity between the 1,875 kg Rutuba/ha and the 937.5 kg Rutuba/ha treatments. The soil acidity was changeable between 0.3 Me% (1,875 kg Rutuba/ha) and 0.57 Me% (1875 kg NPK/ha). The 1,875 kg Rutuba/ha application exhibited a significantly greater total carbon content than all other fertilizer rates except the 937.5 kg Rutuba/ha application.

There was no significant change in total carbon content between 937.5 kg Rutuba/ha, 625 kg Rutuba/ha, 625 kg NPK/ha, and 625 kg Rutuba/ha + 625 NPK/ha applications. The total carbon content of 1,875 kg NPK/ha, 937.5 kg NPK/ha, and the no-fertilizer control was not substantially different. The total carbon content varied between 2.96 % (control with no fertilizer) to 4.32 % (1875 kg Rutuba/ha). 1875 kg Rutuba/ha application had a considerably greater Mn content than all other fertilizer regimes except the 937.5 kg Rutuba/ha application, which was not statistically different from the 625 kg Rutuba/ha application. The Mn content was not significantly different between the NPK rates and the no-fertilizer control. Mn concentrations ranged from 0.18 Me% (all NPK rates) to 0.33 Me% (1,875 kg Rutuba/ha).

The three sole Rutuba application rates and 625 kg Rutuba/ha + 625 kg NPK/ha exhibited significantly higher Cu content than the sole NPK application rates and the no-fertilizer control. Cu concentration was substantially greater in the no-fertilizer control than in the pure NPK application regimes. Copper levels ranged from 2.28 ppm (1,875 kg NPK/ha) to 4.37 ppm (1,875 kg Rutuba/ha). The application of 1,875 kg Rutuba/ha resulted in the highest Fe content of all treatments. The application of 937.5 kg Rutuba/ha and 625 kg Rutuba/ha resulted in the highest Fe content of all NPK treatments and the no-fertilizer control.

The only application of NPK resulted in a lower Fe concentration than the no-fertilizer control. Fe concentrations ranged from 45.83 ppm (1,875 kg NPK/ha) to 94.5 ppm (1,875 kg Rutuba/ha), and the 1,875 kg Rutuba/ha application showed a considerably greater Zn content than all other rates except 937.5 kg Rutuba/ha. There was no significant difference in Zn concentration between applications of 937.5 kg Rutuba/ha and 625 kg Rutuba/ha. However, these two treatments contained more Zn than all NPK treatments, the no-fertilizer control and 625 kg Rutuba/ha + 625 kg NPK/ha. The application of 625 kg Rutuba/ha in combination with 625 kg NPK/ha resulted in a significantly higher Zn soil concentration than any other NPK application rate. The zinc soil content was not significantly different for any NPK rates or the no-fertilizer control. Zinc concentrations ranged from 2.45 ppm (625 kg NPK/ha) to 7.99 ppm (1,875 kg Rutuba/ha).

The application regimens unaffected TN and Na content; however, P, K, Ca, and Mg were altered considerably (p 0.05). (Table 4.2). The application of 1875 kg NPK/ha resulted in the greatest P content of all the fertilizer regimes. The P content of applications of 937.5 kg NPK/ha and 625 kg NPK/ha was not significantly different. The P content of 625 kg Rutuba + NPK, 625 kg NPK/ha,

1875 kg Rutuba/ha, and 937.5 kg Rutuba/ha was not substantially different. The P content of 937.5 kg Rutuba/ha and 625 kg Rutuba/ha applications was not significantly different. The no-fertilizer control had the lowest P content compared to all treatments except 625 kg Rutuba/ha. The P content ranged from 23.33 ppm (control) to 65 parts per million (1,875 kg Rutuba/ha).

The three NPK-only applications and the 625 kg Rutuba/ha + 625 kg NPK/ha application rates all exhibited significantly higher K content than the other. There was no discernible difference in the K content of different applications. 625 kg Rutuba + 625 kg NPK/ha, 937.5 kg NPK/ha, 625 kg NPK/ha, and 625 kg Rutuba/ha. The K content was not substantially different between 625 kg Rutuba + 625 kg NPK/ha and all other Rutuba application rates, which were also not significantly different from the no-fertilizer control. The K concentration varied between 0.75 and 1.5 Me percent (non-fertilized control) and 1,875 kg NPK/ha (1,875 kg NPK/ha). All applications of 625 kg Rutuba/ha, 625 kg Rutuba/ha + 625 kg NPK/ha, and 625 kg NPK/ha exhibited significantly higher Ca contents than the other treatments. The Ca content of all NPK rates tested, 625 kg Rutuba/ha+ 625 kg NPK/ha, and the no-fertilizer control were not significantly different. Ca concentration varied between 4.47 Me% (control) to 8.13 Me% (1,875 kg Rutuba/ha). The application of 1,875 kg Rutuba/ha resulted in a significantly higher Mg content than the application of 937.5 kg Rutuba/ha, which resulted in a significantly higher Mg content than all other treatments. The no-fertilizer control had an Mg level that was not substantially different from 625 kg NPK/ha, but was significantly higher than 937.5 kg NPK/ha and 1,875 kg NPK/ha. The Mg content of the three sole NPK fertilizer application rates was not significantly different. The magnesium level varied between 0.21 and 0.55 ppm (1,875 kg NPK/ha and 937.5 kg NPK/ha).

Table 4. Effect of different fertilizer regimes on soil pH, Exchangeable acidity, total carbon, and micronutrients in a clonal tea field at Kianjokoma at the end of the experimental period

Treatments	pH	Ea (Me%)	TC (%)	Mn (Me%)	Cu (ppm)	Fe (ppm)	Zn (ppm)
1,875 kg NPK/ha	3.94^{c}	0.57^{a}	3.03^{d}	0.18^{c}	2.28^{d}	45.83e	2.70^{d}
625 kg Rutuba/ha +625 kg NPK/ha	4.83^{ab}	0.47^{b}	3.60^{bc}	0.23b^{c}	4.06^{a}	61.27^{c}	3.74^{c}
937.5 kg NPK/ha	3.98^{c}	0.47^{b}	3.05^{cd}	0.18^{c}	2.66^{cd}	47.47^{e}	2.71^{d}
625 kg NPK/ha	4.06^{c}	0.43^{b}	3.47^{bc}	0.18^{c}	2.89^{c}	49.63^{de}	2.45^{d}
1,875 kg Rutuba/ha	5.36^{a}	0.30^{d}	4.32^{a}	0.33^{a}	4.37^{a}	94.50^{a}	7.99^{a}
937.5 kg Rutuba/ha	4.41^{bc}	0.33^{cd}	3.92^{ab}	0.28^{ab}	4.34^{a}	76.40^{b}	7.43^{ab}
625 kg Rutuba/ha	4.27^{bc}	0.43^{b}	3.63^{bc}	0.26^{b}	4.01^{a}	69.43^{b}	6.75^{b}
Control	4.71^{b}	0.43^{b}	2.96^{d}	0.19^{c}	3.44^{b}	54.67^{cd}	3.05^{cd}
P-value	0.003	0.002	<.001	<.001	<.001	<.001	<.001
LSD p≤ 0.05	0.63	0.10	0.52	0.06	0.46	7.04	0.99
CV%	9	17	8.6	15.5	7.4	6.4	12.3

Note: Treatments with different letters in the same column are significantly different according to LSD at p≤ 0.05; CV: coefficient of variation; Ea: Exchangeable acidity; TC: Total carbon

Table 5. Effects of different fertilizer regimes on total nitrogen, phosphorus, potassium, calcium, magnesium, and sodium in a clonal tea field at Kianjokoma from June 2014 to June 2015

Treatments	TN (%)	P (PPM)	K (Me%)	Ca (Me%)	Mg (Me%)	Na (Me%)
1,875 kg NPK/ha	0.35^{a}	65.00^{a}	1.50^{a}	4.57^{b}	0.21^{f}	0.25^{a}
625 kg Rutuba/ha + 625 kg NPK/ha	0.33^{a}	43.33^{c}	1.26^{abc}	6.73^{ab}	0.34^{c}	0.35^{a}
937.5 kg NPK/ha	0.31^{a}	53.33^{b}	1.40^{ab}	4.80^{b}	0.21^{f}	0.23^{a}
625 kg NPK/ha	0.33^{a}	45.00^{bc}	1.40^{ab}	5.90^{ab}	0.23^{ef}	0.28^{a}
1,875 kg Rutuba/ha	0.38^{a}	43.33^{c}	0.94^{cd}	8.13^{a}	0.55^{a}	0.40^{a}
937.5 kg Rutuba/ha	0.35^{a}	36.67^{cd}	0.96^{cd}	7.90^{a}	0.45^{b}	0.40^{a}
625 kg Rutuba/ha	0.33^{a}	30.00^{de}	1.02^{bcd}	7.57^{a}	0.28^{d}	0.32^{a}
Control	0.32^{a}	23.33^{e}	0.75^{d}	4.47^{b}	0.25^{de}	0.25^{a}
p-value	0.20	<.001	0.01	0.04	<.001	0.31
LSD p≤ 0.05	NS	9.16	0.38	2.67	0.04	NS
CV%	18	12.3	12.8	24.4	7	23

Note: Treatments with a different letter(s) in the same column are significantly different according to LSD at p≤ 0.05; CV: coefficient of variation; TN: total nitrogen; LSD: least significant difference

Effects on the average width and length of the second leaf of the harvestable shoot

In both seasons, fertilizer treatment regimes had a significant (p0.05) effect on the average breadth of the second leaf (Table 6). In the first season, the three solo NPK application rates and the application of 625 kg Rutuba/ha + 625 kg NPK/ha resulted in much wider average widths than the other treatments. The rates of application of the three sole Rutuba were not significantly different. The average width of the second leaf was not substantially different, whether 625 kg Rutuba/ha, 937.5 kg Rutuba/ha, or no fertilizer was applied. In season one, the average width of the second leaf was 1.8 cm (no fertilizer control) to 2.8 cm (625 kg Rutuba/ha + 625 kg NPK/ha). In season two, applications of 625 kg Rutuba+625 kg NPK/ha, 1875 kg Rutuba/ha, and three sole NPK fertilizer rates resulted in significantly wider average widths than all other treatments. The average width of the second leaf did not differ significantly between applications of 1875 kg Rutuba/ha and 937.5 kg Rutuba/ha.

There was no significant difference in the average width of the second leaf between applications of 937.5 kg Rutuba/ha and 625 kg Rutuba/ha. The average width of the second leaf varied between 1.8 cm (no fertilizer control) and 2.9 cm (625 kg NPK/ha and 625 kg Rutuba/ha + 625 kg NPK/ha) in the second season. In both seasons, the fertilizer treatment regimes had a significant (p0.05) effect on the second leaf length (Table 6). The combination of 625 kg Rutuba/ha and 625 kg NPK/ha, as well as the three NPK application rates, resulted in significantly longer average second leaf lengths than the three solo Rutuba treatments and the no-fertilizer control. The 1875 kg Rutuba/ha application rate resulted in a significantly longer average length than all other Rutuba treatment rates and the no-fertilizer control. The average length of the second leaf did not differ significantly between applications of 937.5 kg Rutuba/ha, 625 kg Rutuba/ha, and the no-fertilizer control. The average length of the second leaf varied between 4.1 cm (control) to 7.4 cm (1,875 kg NPK/ha and 625 kg Rutuba/ha+ 625 kg NPK/ha) in season one. In both seasons, the administration of 625 kg rutuba/ha had no discernible effect on the average length of the second leaf compared to the no-fertilizer control. The length of the second leaf did not differ significantly between the Rutuba treatment rates. Except with 625 kg Rutuba/ha, all solitary Rutuba treatments exhibited significantly longer average leaf length than the no-fertilizer control. The average length of the second leaf ranged from 4.4 cm in the absence of fertilizer to 7.6 cm in the presence of 1,875 kg NPK/ha.

Effects on the green leaf and made tea yields

In both seasons, green leaf and made tea yields differed considerably ($p \leq 0.05$) according to the fertilizer treatment regime (Table 7). In the first season, fertilizer application boosted green leaves and improved tea yields significantly regardless of regime or kind. 625 kg Rutuba/ha + 625 kg NPK/ha produced much more green leaves and made tea than all other fertilizer treatment regimes. The three solo NPK fertilizer rates did not differ considerably in green leaf and made tea yields, but they were significantly greater than the three Rutuba treatment rates. 1875 kg Rutuba/ha and 937.5 kg Rutuba/ha considerably increased green leaf and made tea yields compared to 625 kg Rutuba/ha, which was not significantly different from the no-fertilizer control. Season one green leaf yields ranged from 1521 kg/ha (no fertilizer) to 6587 kg/ha (625 kg Rutuba + 625 kg NPK/ha), while made tea yields ranged from 342 kg/ha (no fertilizer) to 1482 kg/ha (625 kg Rutuba + 625 kg NPK/ha).

In season two, as compared to all other fertilizer regimens, 625 kg Rutuba/ha + 625 kg NPK produced considerably more green leaves and made tea yields (Table 7). The three single NPK rates did not affect green leaf or made tea yields. There were no significant variations in green leaf and brewed tea yields when applied 1,875 kg NPK/ha, 625 kg NPK/ha, or 1,875 kg Rutuba/ha. The three Rutuba application rates did not differ considerably in green leaf and made tea yields, but they were significantly greater than the no-fertilizer control. Season two leaf yields ranged from 2621 kg/ha (no-fertilizer control) to 6444 kg/ha (625 kg Rutuba+ 625 kg NPK/ha), whereas made tea yields ranged from 588 kg/ha (no-fertilizer control) to 1450 kg/ha (625 kg Rutuba+ 625 kg NPK/ha).

Table 6. Length and width (cm) of the second leaf of the harvestable shoot for different fertilizer regimes in the first and second seasons at Kianjokoma from June 2014 to June 2015

Treatments	Season 1		Season 2	
	Average width (cm)	Average length (cm)	Average width (cm)	Average length (cm)
1,875 kg NPK /ha	2.7^{a}	7.4^{a}	2.7^{a}	7.0^{a}
625 kg Rutuba/ha +625kg NPK/ha	2.8^{a}	7.4^{a}	2.9^{a}	7.1^{a}
937.5 kg NPK/ha	2.7^{a}	6.9^{a}	2.7^{a}	7.1^{a}
625 kg NPK/ha	2.7^{a}	6.9^{a}	2.9^{a}	7.2^{a}
1,875 kg Rutuba/ha	2.3^{b}	5.7^{b}	2.6^{ab}	5.6^{b}
937.5 kg Rutuba/ha	2.0^{bc}	4.6^{c}	2.2^{bc}	5.2^{b}
625 kg Rutuba/ha	2.1b^{c}	4.5^{c}	2.2^{c}	4.9^{bc}
Control	1.8^{c}	4.1^{c}	1.8^{d}	4.4^{c}
p-value	<.001	<.001	<.001	<.001
LSD($p \leq 0.05$)	0.3	0.5	0.4	0.7
CV%	8	4.9	8.5	6.2

Note: Treatments with different letters in the same column are significantly different according to the least significant difference test ($p \leq 0.05$)

Table 7. Green leaf (kg/ha) and made tea yields (kg/ha) under different fertilizer regimes at Kianjokoma from June 2014 to June 2015

Treatments	Season 1		Season 2		Cumulative yield	
	GL	MT	GL	MT	GL	MT
1,875 kg NPK/ha	4162^{b}	936 ^{b}	5339^{bc}	1201^{bc}	9501^{b}	2137^{b}
625 kg Rutuba/ha +625 kg NPK/ha	6587^{a}	1482^{a}	6444^{a}	1450^{a}	13031^{a}	2932^{a}
937.5 kg NPK/ha	4105^{b}	924^{b}	5382^{b}	1211^{b}	9486^{b}	2135^{b}
625 kg NPK/ha	4004^{b}	900^{b}	5296^{bc}	1192^{bc}	9300^{b}	2093^{b}
1,875 kg Rutuba/ha	2856^{c}	642^{c}	4435^{cd}	998^{cd}	7291^{c}	1641^{c}
937.5 kg Rutuba/ha	2669^{c}	600^{c}	4004^{d}	901^{d}	6674^{c}	1501^{c}
625 kg Rutuba/ha	1708^{d}	384^{d}	3660^{d}	823^{d}	5368^{d}	1207^{d}
Control	1521^{d}	342^{d}	2612^{e}	588^{e}	4133^{e}	930^{e}
p-value	<.001	<.001	<.001	<.001	<.001	<.001
LSD ($p \leq 0.05$)	362.8	73.53	920.5	207.1	1036	233.3
CV%	5.4	5.4	11.3	11.3	7.3	7.3

Note: Treatments with different letters in the same column are significantly different according to the least significant difference test ($p \leq 0.05$). GL-Green leaf; MT-Made tea; CV: coefficient of variation; LSD: least significant difference

Table 8. Nitrogen, potassium, and phosphorus leaf uptake under different fertilizer regimes at Kianjokoma from June 2014 to June 2015

Treatments	Nitrogen (%)	Potassium (%)	Phosphorus (%)
1,875 kg/ha NPK	3.5^{c}	2.4^{d}	0.3^{de}
625 kg Rutuba/ha + 625 kg NPK/ha	4.5^{a}	4.4^{b}	0.5^{a}
937.5 kg NPK/ha	3.9^{b}	2. 6^{d}	0.4^{cd}
625 kg NPK/ha	4.1^{b}	2.7^{cd}	0.4^{bcd}
1,875 kg Rutuba /ha	3.2^{cd}	5.4^{a}	0.5^{a}
937.5 kg rutuba/ha	3.1^{de}	4.8^{ab}	0.5^{a}
625 kg Rutuba/ha	2.9^{e}	3.5^{c}	0.5^{a}
Control	2.1^{f}	2.4^{d}	0.3^{e}
p-value	<.001	<.001	<.001
LSD $p \leq 0.05$	0.3	0.9	0.1
CV%	4.6	14.4	10.7

Note: Treatments with different letters in the same column are significantly different according to LSD at $p \leq 0.05$; CV: coefficient of variation

Effects on leaf nitrogen, potassium, and phosphorus uptake

Fertilizer application demonstrated a substantial ($p \leq 0.05$) effect on the nitrogen, potassium, and phosphorus content of the leaves (Table 8). When 625 kg Rutuba/ha was combined with 625 kg NPK/ha, the nitrogen content was much higher than when no fertilizer was applied, or other fertilizer regimens were used. There were no significant variations in leaf nitrogen content between 937.5 kg NPK/ha and 625 kg NPK/ha applications, nor between 1,875 kg NPK/ha and 1,875 kg Rutuba/ha applications. The latter treatment was comparable to applying 937.5 kg rutuba/ha. The leaf nitrogen concentration of 937.5 kg Rutuba/ha and 625 kg Rutuba/ha was not substantially different. The N leaf content varied between 2.1 and 4.5 percent (no fertilizer control) and 625 kg Rutuba+ 625 kg NPK/ha. The application of 1875 kg Rutuba/ha resulted in the highest potassium content when compared to the no-fertilizer control and all other treatments, except for the application of 937.5 kg Rutuba/ha, which did not differ substantially from the application of 625 kg Rutuba/ha + 625 kg, NPK/ha. The leaf K content was not significantly different between the three sole NPK application rates and the no-fertilizer control. The leaf K content varied between 2.4 and 5.4 % (no fertilizer control and 1,875 kg NPK/ha). All rates of solitary Rutuba fertilizer and 625 kg NPK/ha + 625 kg Rutuba/ha treatment exhibited significantly higher leaf P content than other fertilizer regimes. There was no statistically significant difference in leaf P content between the solo NPK application rates and between the 1875 kg NPK/ha application and the no-fertilizer control. The P content ranged from 0.3 % (control with no fertilizer and 1,875 kg NPK/ha) to 0.5 % (all sole rutuba applications and 625 kg NPK/ha+ 625 kg Rutuba/ha).

Effects on apparent nutrient recovery, partial factor productivity, and agronomic efficiencies of nitrogen, potassium, and phosphorus in clonal tea

The fertilizer treatment regimes had a significant effect (p0.05) on nitrogen, potassium, and phosphorus apparent nutrient recovery (ANR) (Table 9). The application of 625 kg Rutuba/ha and 937.5 kg Rutuba/ha resulted in all treatments' highest apparent nutritional recovery of nitrogen (ANRN). 1875 kg Rutuba/ha application had a considerably greater ANR_N than all other NPK application rates and 625 kg Rutuba/ha + 625 kg NPK/ha application. 625 kg NPK/ha and 625 kg Rutuba/ha + 625 kg NPK/ha had an ANR_N of 625 kg NPK/ha that was not substantially different from 1875 kg NPK/ha, and 937.5 kg NPK/ha, which were not significantly different. The ANR_N varied from 0.11 (1875 kg NPK/ha) and 1.69 (937.5% Rutuba/ha). All Rutuba-only treatments had significantly better apparent nutrient recovery of potassium (ANR_K) than all other fertilizer rates. While in ANR_K, there was no significant difference between 625 kg NPK/ha and 625 kg Rutuba/ha + 625 kg NPK/ha. In ANR_K, the rates of sole NPK application were not significantly different, and in ANR_K, values varied between 0.32 (1875 kg NPK/ha) and 4.01 (1875 kg Rutuba/ha). Using 937.5 kg Rutuba/ha and 625 kg Rutuba/ha resulted in a significantly greater apparent nutrient recovery of phosphorus (ANR_P) than any other fertilizer regime. Furthermore, 1,875 kg Rutuba/ha had a considerably higher ANR_P than all other NPK

fertilizer rates but was not significantly different from 625 kg Rutuba/ha + 625 kg NPK/ha, which was not significantly different from 625 kg NPK/ha. The application of 937.5 kg/ha NPK was not significantly different from that of 1875 kg/ha NPK and had the lowest ANR_P. The ANR_P was between 0.05 (1875 kg NPK/ha) to 0.52 (937.50 kg Rutuba/ha). The fertilizer regimens considerably affected the nitrogen, potassium, and phosphorus partial factor productivity (PfP) (Table 9). The PfP_N levels were considerably greater when 625 kg Rutuba/ha and 937.5 kg Rutuba/ha were applied, compared to all other treatments. The application of 1875 kg Rutuba/ha resulted in significantly better nitrogen partial factor productivity (PfP_N) than the other NPK treatments, including the application of 625 kg Rutuba/ha + 625 kg NPK/ha, whose PfP_N was not statistically different. The PfP_N concentrations were between 4.38 (1875 kg NPK/ha) and 92.04 (625 kg Rutuba/ha). 625 kg Rutuba/ha significantly increased the partial factor productivity of potassium (PfP_K) compared to all other treatments.

There were no significant variations in PfPk between applications of 625 kg Rutuba/ha + 625 kg NPK/ha, 625 kg NPK/ha, 1875 kg Rutuba/ha, and 937.5 kg Rutuba/ha. The application of 625 kg Rutuba/ha with 625 kg NPK/ha and 937.5 kg NPK/ha did not differ substantially from the application of 625 kg NPK/ha, which did not change significantly from the application of 1875 (NPK/ha. The PfPK concentrations were 22.8 (1875 kg NPK/ha) and 158.39 (625 kg Rutuba/ha). The application of 625 kg Rutuba/ha and 937.5 kg Rutuba/ha resulted in considerably greater phosphorus partial factor productivity (PfP_P) than all other treatments. In PfP_P, the application of 1875 kg Rutuba/ha, 625 kg NPK/ha, 937.5 kg NPK/ha, and 625 kg Rutuba/ha + NPK 625 kg NPK/ha did not differ significantly. In partial PfP_P, the applications of 937.5 kg Rutuba/ha NPK and 1875 kg NPK/ha NPK were not substantially different. The PfPP concentrations were between 22.8 (1875 kg NPK/ha) to 195.18 (625 kg Rutuba/ha).

The nitrogen, potassium, and phosphorus agronomic efficiency were significantly ($p \leq 0.05$) affected by the fertilizer application regimes (Table 10). Compared to all other fertilizer regimes, applications of 937.5 kg Rutuba/ha and 625 kg Rutuba/ha exhibited the highest agronomic efficiency of nitrogen (AEN). The AEN of 625 kg Rutuba/ha was not substantially different from the AEN of 1875 kg Rutuba/ha or 625 kg Rutuba/ha + 625 kg NPK/ha. In AEN, there were no significant differences in the rates of sole NPK fertilizer and 625 kg Rutuba/ha Plus 625 kg NPK/ha. Nitrogen's agronomic efficiency (AEN) varied between 2.48 (1875 kg NPK/ha) and 29.03 (937.5 kg Rutuba/ha). The treatment of 625 kg Rutuba/ha in combination with 625 kg NPK/ha, 625 kg NPK/ha, and just Rutuba had the maximum agronomic efficiency of potassium (AEK). There were no significant variations in AEK between the application rates of single Rutuba, 625 kg NPK/ha and 937.5 kg NPK/ha, which was not significantly different from 1875 kg NPK/ha. The AEK ranged from 12.88 kg (1,875 kg NPK/ha) to 51.51 kg (625 kg Rutuba/ha + 625 kg NPK/ha). 937.5 kg Rutuba/ha and 625 kg Rutuba/ha+ 625 kg NPK/ha exhibited significantly greater agronomic phosphorus efficiency (AEP) than all other fertilizer regimes. In AEP, the application of 1875 kg Rutuba/ha, 625 kg Rutuba/ha, 625 kg NPK/ha, and 625 kg Rutuba/ha+ 625 kg NPK/ha did not differ significantly. The AEP for 1875 kg NPK/ha was not statistically different from the AEP for 937.5 kg NPK/ha, which was not substantially different from the AEP for 625 kg NPK/ha, 1875 kg Rutuba/ha, and 625 kg Rutuba/ha. The AEP varied between 12.88 (1,875 kg NPK/ha) and 61.58 (937.5% Rutuba/ha).

Table 9. Effects of varying inorganic and organic fertilizer regimes on the apparent nutrient recovery and the partial factor productivity of nitrogen, potassium, and phosphorus in clonal tea at Kianjokoma from June 2014 to June 2015.

Treatment	ANR_N	ANR_K	ANR_p	PfP_N	PfP_K	PfP_P
1,875 kg NPK/ha	0.11^{d}	0.32^{d}	0.05^{e}	4.38^{c}	22.80^{d}	22.80^{c}
625 kg Rutuba/ha+ 625 kg NPK/ha	0.64^{bc}	2.77^{abc}	0.31^{bc}	16.69^{c}	75.43^{bc}	78.31^{b}
937.5 kg NPK/ha	0.26^{d}	0.74^{cd}	0.12^{de}	8.76^{c}	45.53^{c}	45.53^{bc}
625 kg NPK/ha	0.41^{c}	$1.08b^{cd}$	0.19^{cd}	12.88^{c}	66.96^{bcd}	66.96^{b}
1,875 kg Rutuba/ha	0.84^{b}	4.01^{a}	0.32^{b}	41.66^{b}	101.07^{b}	88.37^{b}
937.5 kg Rutuba/ha	1.69^{a}	3.41^{a}	0.52^{a}	76.26^{a}	102.34^{b}	161.78^{a}
625 kg Rutuba/ha	1.35^{a}	2.96^{ab}	0.46^{a}	92.04^{a}	158.39^{a}	195.18^{a}
P-Value	<.001	0.01	<.001	<.001	0.003	<.001
LSD $p \leq 0.05$	0.33	2.09	0.13	21.52	52.3	43.87
CV%	27.2	31.1	26.2	33.5	21.2	26.2

Note:

Treatments with different letters in the same column are significantly different according to the least significant difference at $p \leq 0.05$; ANR_N: Apparent nutrient recovery of nitrogen; P_fP_N: Partial factor productivity of nitrogen ANR_K: Apparent nutrient recovery of potassium; PfP_K: Partial factor productivity of potassium; ANR_P: Apparent nutrient recovery of phosphorus; P_fP_P: Partial factor productivity of phosphorus. CV: coefficient of variation; LSD: least significant difference

Table 10. Effects of varying inorganic and organic fertilizer regimes on the agronomic efficiencies of nitrogen, potassium, and phosphorus in clonal tea at Kianjokoma from June 2014 to June 2015

Treatment	AE_N	AE_K	AE_P
1,875 kg NPK/ha	2.48^{d}	12.88^{c}	12.88^{d}
625 kg Rutuba/ha+ 625 kg NPK/ha	11.04^{cd}	51.51^{a}	53.46^{ab}
937.5 kg NPK/ha	4.94^{d}	25.70^{bc}	25.7^{cd}
625 kg NPK/ha	7.15^{d}	37.20^{ab}	37.2^{bc}
1,875 kg Rutuba/ha	18.04^{bc}	43.90^{ab}	38.27^{bc}
937.5 kg Rutuba/ha	29.03^{a}	37.55^{ab}	61.58^{a}
625 kg Rutuba/ha	21.17^{ab}	36.42^{ab}	44.89^{abc}
P-Value	<.001	0.04	0.005
LSD ≤0.05	9.51	21.72	21.03
CV%	39.7	15.5	30.2

Note: Treatments with different letters in the same column are significantly different according to LSD at p≤ 0.05; CV: coefficient of variation; AE_N: Agronomic efficiency of nitrogen; AE_K: Agronomic efficiency of potassium; AE_P: Agronomic efficiency of phosphorus

Effects on the cost of production and profitability in tea production

There was a significant (p≤0.05) impact on the estimated total production costs and estimated total income from the fertilizer application regimes (Table 11). The cost of applying 625 kg Rutuba + 625 kg NPK/ha was much higher than that of applying 1,875 kg Rutuba/ha. The projected total cost of application of 1,875 kg NPK/ha was significantly higher than the estimated total cost of application of other sole NPK rates, 937.5 kg Rutuba/ha and 625 kg Rutuba/ha, but was not significantly different from the anticipated total cost of application of 1875 kg Rutuba/ha. The projected total cost of application of 625 kg NPK/ha was significantly higher than that of 625 kg Rutuba/ha, but was not significantly different from that of 937.5 kg Rutuba/ha. The projected total cost per hectare varied between Ksh. 41,333 (control) to Ksh. 196, 557 (625 kg Rutuba + 625 kg NPK). The farmers' approach (625 kg NPK/ha) resulted in a total cost savings of 61.45 % compared to the application of 625 kg Rutuba/ha + 625 kg NPK/ha. Applying 625 kg Rutuba/ha combined with 625 kg NPK/ha generated significantly more predicted total revenue than any other fertilizer regime. It generated 40% and 215% more revenue than farmers' practices and the no-fertilizer control, respectively. Although the total expected revenue from the sole NPK fertilizer application regimes was not significantly different, it was much greater than the total estimated revenue from all the sole Rutuba application rates. Although the expected total revenue from the application of 1875 kg rutuba/ha was not significantly different from the estimated total revenue from the application of 937.5 kg rutuba/ha, both regimes generated significantly more revenue than the application of 625 kg rutuba/ha. The no–fertilizer control group generated much less revenue than the other treatments. Total income estimates ranged from Ksh. 144, 871 (no-fertilizer control) to Ksh. 456, 748 (625 kg NPK/ha+ 625 kg Rutuba/ha).

The application regimes for fertilizer had a substantial effect on the net returns. 625 kg Rutuba + 625 kg NPK/ha generated significantly more net revenue than all other treatments (Table 11), but 1,875 kg Rutuba/ha generated significantly less net revenue. However, there was no statistically significant difference in predicted net revenue between 937.5 kg NPK/ha and 625 kg NPK/ha. Those applications generated significantly more revenue than 1,875 kg NPK/ha, than all the sole Rutuba and the no-fertilizer control. The estimated net return on 1,875 kg NPK/ha was substantially greater than the anticipated net return on all other Rutuba rates and the no-fertilizer control. The net return was not substantially different between application regimes of 937.5 kg Rutuba/ha, 625 kg Rutuba/ha, and no-fertilizer control. Net revenue was anticipated to range between Ksh. 68, 256 (1,875 kg Rutuba/ha) to Ksh. 260, 191 (625 kg Rutuba/ha + 625 kg NPK/ha). 625 kg Rutuba + 625 kg NPK application yielded 27.4 % and 151.3 % more than farmers' practice and no-fertilizer control, respectively. Fertilizer rates increased from 625 kg NPK/ha (farmers' practice) to 937.5 kg NPK/ha and 1875 kg NPK/ha, respectively, reducing net returns by 4.7 % and 25.7 %.

Table 11. Cost of each fertilizer regime and the net revenue at Kianjokoma from June 2014 to June 2015

Fertilizer regime	CoF (Ksh/ha)	CoP (Ksh/ha)	CoAF (Ksh/ha)	TC (Ksh/ha)	TR (Ksh/ha)	NR (Ksh/ha)
1875 kg NPK/ha	84356	95008^{b}	1875	181239b	333003^{b}	151764^{c}
625 kg Rutuba/ha+ 625 kg NPK/ha						
625 kg NPK/ha	65619	130313^{a}	625	196557^{a}	456748^{a}	260191^{a}
937.5 kg NPK/ha	42178	94865^{b}	937.5	137980^{c}	332500^{b}	194520^{b}
625 kg NPK/ha	28119	92999^{b}	625	121743^{d}	325961^{b}	204218^{b}
1875 kg Rutuba/ha	112500	72906^{c}	1875	187281^{ab}	255537^{c}	68256^{e}
937.5 kg Rutuba/ha	56250	66735^{c}	937.5	123923^{d}	233907^{c}	109984^{d}
625 kg Rutuba/ha	37500	53675^{d}	625	91800^{e}	188132^{d}	96331^{d}
Control	_	41333^{e}	_	41333^{f}	144871^{e}	103539^{d}
p-value		<.001		<.001	<.001	<.001
LSD p≤ 0.05		10358		10358	36305	25947
CV%		7.3		4.4	7.3	10

Note: CoF-Cost of fertilizer; Cop-Cost of plucking; CoAF-Cost of applying fertilizer; NR-Net revenue; TC-Total cost; TR-Total revenue

Discussion

Using organic Rutuba resulted in a higher soil pH than all NPK fertilizer rates. Chong et al. reported similar findings (2008). The recent findings may be explained by the high pH of organic Rutuba (pH= 8.2). Thus, the addition of organic Rutuba to a generally acidic tea crop has the ability to raise the pH. When compared to NPK fertilizer rates and the no-fertilizer control, the application of organic Rutuba increased total carbon. That means continued use of organic Rutuba fertilizer increases total organic carbon, increasing soil fertility. In addition, using Rutuba fertilizer raised Mn, Cu, Fe, and zinc soil concentrations. That could be because the Rutuba fertilizer contains a higher concentration of Mn, Cu, Fe, and Zn than the NPK (26.5.5) fertilizer. The elevated pH caused by Rutuba fertilizer may also have increased nutrient availability. Nath (2013b) showed that the pH of the soil was positively connected with the micronutrients Mn, Fe, Cu, and Zn in a study on the status of micronutrients in tea plantations. That means continued use of Rutuba fertilizer will improve the soil's micronutrient condition, resulting in increased tea yield and quality (Sedaghathoor et al. 2009).

Because of leaching, denitrification, and plant uptake of nitrogen (Chong et al. 2008), as well as the low levels of nitrogen in the organic fertilizer, there was no significant difference in total nitrogen content between the Rutuba and NPK fertilizer regimes. Chong et al. reported similar findings (2008). The NPK fertilizer regimes produced soils with a greater P content than all Rutuba rates and no-fertilizer control. Generally, the P content of the soil increased as the NPK and Rutuba fertilizers rate increased. That could be explained by the poor mobility of phosphorus in the soil and the increase in P concentration as Rutuba and NPK fertilizer rates are increased. Nath (2013b) and Kekana et al. (2012) reported similar results, in which soil P increased as fertilizer rates increased. Compared to NPK fertilizer regimes and no-fertilizer controls, the organic Rutuba treatments enhanced soil Ca and Mg content. That could be attributable to the increased Ca and Mg concentrations in organic Rutuba, which were 3.1 percent and 0.4 percent, respectively, compared to NPK fertilizer. That means farmers' continued use of Rutuba fertilizer has the ability to raise the calcium and magnesium content of soils, whereas repeated use of NPK fertilizer alone results in soil depletion of calcium and magnesium. The fertilizer application boosted the second leaf's breadth and length. Chong et al. reported similar findings (2008). Increased NPK fertilizer rates from 625 to 1875 kg NPK/ha had no discernible effect on the average breadth and length of the second leaf. That indicates that 625 kg NPK/ha contained an acceptable amount of nitrogen, phosphorus, and potassium for leaf development. Sole Rutuba rates did not significantly increase the average length of the second leaf compared to the no-fertilizer control in the first season. Still, they increased it significantly in the second season. That could be explained by the fact that organic fertilizers take longer to release nutrients; nutrients were likely more readily available to the plant in the second season (Hazra 2016). Fertilizer use increased green leaf yields and made tea yields considerably. Applying 625 kg Rutuba/ha + 625 kg NPK/ha yielded the highest green leaf and black made tea yields. Because the organic fertilizer increased the availability of nutrients, the integrated system had the highest yield. Tabu et al. (2015) discovered that supplementing cattle manure with inorganic fertilizer increased tea yield.

When the Rutuba fertilizer rate was increased from 625 kg Rutuba/ha to 1,875 kg Rutuba/ha, the yields of made tea and green leaf rose, and to improve tea yields, larger Rutuba rates may be required. When NPK fertilizer rates increased from 625 kg NPK/ha to 1875 kg NPK/ha, there were no significant ($p \leq 0.05$) increases in green leaf and made tea yields. That could be because the tea plants received enough N, P, and K by applying 625 kg NPK/ha. When it comes to nitrogen, Hamid et al. (2002) found that applying more than 225 kg N/ha did not improve tea yield appreciably. 937.5 kg NPK/ha and 1875 kg NPK/ha provided 243.75 kg N/ha and 487.5 kg N/ha, respectively, in the current study, both of which were more than 225 kg N/ha. Compared to plots with NPK fertilizers, green leaf and made tea yields were lower in sole Rutuba plots. The low levels of mineralizable N in Rutuba could be attributable to this.

The leaf nitrogen content did not considerably increase as NPK fertilizer rates rose from 625 kg NPK/ha to 1875 kg NPK/ha. That may be due to inorganic nitrogen leaching and denitrification, which means that using higher NPK rates than 625 kg NPK/ha would result in N loss because the plant will not consume it. The leaf nitrogen content rose as the Rutuba fertilizer was raised from 625 kg Rutuba/ha to 1875 kg Rutuba/ha, showing that organic Rutuba may not be lost as quickly as inorganic NPK fertilizer by leaching. Compared to the other fertilizer rates, the application of 625 kg Rutuba/ha with 625 kg NPK/ha resulted in the greatest leaf nitrogen content, showing that the NPK and Rutuba improved N uptake. According to Tabu et al. (2015), increasing NPKS with organic manure resulted in higher leaf N content. The N leaf content of sole Rutuba was lower than that of sole NPK fertilizer, probably because of the low N content of Rutuba fertilizer. Increases in NPK fertilizer rates from 625 to 1,875 kg NPK/ha showed no influence on potassium leaf content, although increases in Rutuba fertilizer rates did. Micronutrient availability may have increased as a result. Applying 625 kg Rutuba/ha + 625 kg NPK/ha rates resulted in higher leaf phosphorus content than NPK fertilizer rates or the no-fertilizer control. That could be because the Rutuba fertilizer's organic P was less fixed in the soil, making it more available to the plant because of the higher pH. (Sultana et al., 2014), organic Rutuba fertilizer can be used as a phosphorus source for long-term tea cultivation. Rutuba rates were increased from 625 kg Rutuba/ha to 937.5 kg Rutuba/ha, which increased the apparent nutrient recovery (ANR) of N, K, and P. However, increasing Rutuba rates to 1875 kg Rutuba/ha decreased the ANR_N and ANR_P while increasing the ANR_K, which suggests that Rutuba, when used in moderation, improves the apparent nutritional recovery of N, K, and P. The ANR_N, ANR_K, and

ANR_P all reduced when NPK fertilizer application rates were increased from 625 kg NPK/ha to 937.5 kg NPK/ha. That shows low levels of NPK contribute to the recovery of N, P, and K.

The partial factor productivity (PfP) of N, K, and P decreased when rates were increased from 625 kg/ha to 1,875 kg/ha for both NPK and organic Rutuba fertilizers. With an increase in potassium from 40 K20/ha to 80 K20/ha, Calvin et al. (2013) found that increasing nitrogen rates from 100 kg N/ha to 200 kg N/ha lowered the partial factor productivity of nitrogen (PfP_N) and partial factor productivity of potassium (PfP_K). NPK fertilizer nitrogen rates increased from 162.5 kg N/ha (625 kg NPK/ha) to 487.5 kg N/ha (1,875 kg NPK/ha) in the current study, while Rutuba fertilizer nitrogen rates grew from 13.13 kg N/ha (625 kg Rutuba/ha) to 39.38 kg N/ha (1,875 kg Rutuba/ha).

According to Jagadeeswaran et al. (2005), the partial factor productivity of NPK decreased as NPK levels increased. Increased Rutuba levels from 625 kg to 937.5 kg Rutuba/ha raised the agronomic efficiency of nitrogen (AEN). Still, additional increases to 1,875 kg Rutuba/ha decreased AEN, and increased NPK fertilizer from 625 kg to 1,875 kg NPK/ha decreased AEN. Increased the agronomic efficiency of potassium (AEK) on increased Rutuba fertilizer rates from 625 kg Rutuba/ha to 1,875 kg Rutuba/ha, meanwhile the AEK was reduced by increasing NPK fertilizer rates from 625 kg NPK/ha to 1875 kg NPK/ha. According to Calvin et al. (2013), raising potassium rates from 40 kg/ha to 80 kg/ha raised the AEK from -0.59 to 0.04. Increased Rutuba fertilizer rates from 625 kg Rutuba/ha to 937.5 kg Rutuba/ha improved AEP, while increases to 1,875 kg Rutuba/ha resulted in AEp reduction; meanwhile increase in NPK fertilizer rates from 625 kg NPK/ha to 1,875 kg NPK/ha decreased AEP. That means using moderate amounts of Rutuba fertilizer improves N, P, and K efficiency, lowering pollution consequences. On the other hand, higher NPK fertilizer rates lower N, P, and K efficiency, potentially resulting in pollution in tea crops in the long term.

Compared to farmers' practices and other fertilizer regimes, the application of 625 kg Rutuba/ha + 625 kg NPK/ha yielded the highest net yields. That means farmers in Kianjokoma (the experimental location) should employ this fertilizer regime instead of the current one. Furthermore, as compared to the farmers' practice, this fertilizer regime has the potential to improve soil fertility. However, the net yields from the application of 625 kg Rutuba/ha and 937.5 kg Rutuba/ha were not significantly greater than the no-fertilizer control.

In summary, compared to the no-fertilizer control and NPK fertilizer, the study found that organic Rutuba increases soil pH, total organic carbon, Mn, Cu, Fe, Zn, Ca, and Mg. Soil pH was lowered after a sole application of NPK (26.5.5). Compared to sole NPK treatment rates, co-application of 625 kg Rutuba/ha and 625 kg NPK/ha increased soil pH, Cu, Fe, Zn, and Mg concentrations. Increases in NPK fertilizer rates from 625 to 1,875 kg NPK/ha did not affect leaf growth or yield. While compared to sole Rutuba and NPK, the application of 625 kg Rutuba/ha + 625 kg NPK/ha resulted in significantly higher cumulative green leaf and made tea yields. The leaf N concentration of Rutuba fertilizer was lower than that of NPK fertilizer, but the leaf P content was higher. Compared to Rutuba fertilizer rates, NPK fertilizer rates resulted in low K leaf content.

The ANRN increased when Rutuba fertilizer rates climbed from 625 kg Rutuba/ha to 937.5 kg Rutuba/ha, but decreased as Rutuba rates increased further. While compared to sole NPK fertilizer rates, the application of 625 kg Rutuba/ha + 625 kg NPK/ha improved N, P, and K utilization efficiency. On the other hand, the sole rutuba application rates had greater nutrient usage efficiencies of N, P, and K than the sole NPK fertilizer application rates. Therefore, the farmers' approach of applying 625 kg Rutuba/ha and 625 kg NPK/ha resulted in greater net returns.

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Effect of cytokinin and gibberellic acid applications on seed germination and growth of *Rauvolfia verticillata* plant

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Abstract. *Lestari S, Solichatun, Anggarwulan E. 2018. Effect of cytokinin and gibberellic acid applications on seed germination and growth of* Rauvolfia verticillata *plant. Cell Biol Dev 2: 27-32.* The purposes of this research are to study the effect of cytokinin and gibberellic acid (GA_3) on seed germination and growth of *Rauvolfia verticillata* Lour. or *pule pandak* and to determine the best treatment combination that has an optimal effect on the seed germination and growth of *R. verticillata*. This study was done in a complete randomized design by combination treatment of gibberellic acid and cytokinin that consist of 6 treatment combinations including GA_3 0 ppm/ cytokinin 0 ppm, GA_3 50 ppm/ cytokinin 0 ppm, GA_3 0 ppm/ cytokinin 50 ppm, GA_3 50 ppm/ cytokinin 50 ppm, GA_3 50 ppm/ cytokinin 75 ppm, GA_3 75 ppm/ cytokinin 50 ppm and 8 replicates. The treatments were given by soaking the mature seed in the hormone solution. Some parameters such as germination and growth parameter were measured. The result shows that presoaking treatment with GA_3 50 ppm had a greater influence on germinating time, germination percentage, plant height, leaf number, leaf wide, and dry weight than presoaking use cytokinin 50 ppm. Presoaking treatment using a combination of GA_3 and cytokinin ($G_{50}S_{50}$) has a significant influence on increasing fresh weight and dry weight. The combination of GA_3 and cytokinin in the concentration of $G_{50}S_{75}$ influences fresh weight and dry weight plants. The combination of GA_3 and cytokinin in concentration $G_{75}S_{50}$ influences increased germination percentage, plant height, leaf number, and leaf wide.

Keywords: Cytokinin, GA_3, germination, gibberellic acid, growth, *Rauvolfia verticillata*

INTRODUCTION

Utilizing medicinal plants without considering the sustainability aspect can be viewed as affecting the sustainability and decline of the medicinal plant population, increasing the scarcity of medicinal plant species. Given the Indonesian people's attachment to their cultural traditions of herbal medicine, the use of Indonesian medicinal plants is expected to continue. Some herbal raw materials have proven reliable export commodities, helping the country's foreign exchange reserves. The increased use of medicinal plants as export commodities has not been accompanied by rational cultivation and germplasm preservation (Sulandjari 2008).

One currently widely used plant is the *pule pandak*, a collective name for Genus *Rauvolfia,* such as *Rauvolfia verticillata* Lour and *Rauvolfia serpentina* (L.) Benth. ex Kurz. *R. verticillata*, also known as rat root, is a member of the Apocynaceae family. This plant is found throughout Indonesia, including Sumatra, Java, and Nusa Tenggara islands. It typically grows at elevations ranging from 1000 m to 2100 m above sea level (asl) (Iptekda-LIPI 2001).

Rauvolfia verticillata is effective as a preventative increase in body temperature, sedatives, high blood pressure drugs, and normalized heart rate, among other things. The most important alkaloid found in the root of *R. verticillata* is reserpine, which is commonly used as a hypertension medication (Lilly 1990; Duke 1992; Nigg and Seigler 1992).

Until now, there has been no extensive cultivation of *R. verticillata* in Indonesia (Rosita et al. 1992). *R. verticillata* plants can be propagated by seed, cuttings, or tissue culture. Seeds of *Rauvolfia serpentina* (L.) Benth. ex Kurz species germinated 10-12 days after planting due to the hardness of the seed shell (Iptekda-LIPI 2001). The same occurred to *R. verticillata*. When compared to the germination of peanuts (*Arachis hypogea* L.) in 5 days, corn seeds (*Zea mays* L.) in 2-3 days, and cotton seeds (*Gossypium* sp.) in 5-7 days, this germination time is considered long (Goldsworthy and Fisher 1992).

Pule pandak reproduces naturally by seed, but the germination percentage is low, ranging from 7 to 15%, due to the hard seed coat on pule pandak seeds. The percentage of successful propagation of *R. serpentina* by seed can be increased by removing half of the seed shell before planting or soaking the seeds in a concentrated or semi-concentrated sulfuric acid (H_2SO_4) solution for five minutes before planting (Sulandjari 2008).

Many researchers believe that the growth hormones gibberellins (GA_3) and cytokinins influence enzyme activity in plant metabolic processes (Wringler et al. 1998; Leitei et al. 2003). Previous research on *Brucea javanica* (L.) Merr. seeds by Setyowati and Utami (2008) revealed that soaking seeds in 1000 mg/L GA_3 solution effectively accelerated and increased *B. javanica* germination.

The Forestry Research and Development Agency (2005) researched balsa seeds (*Ochroma* sp.) to improve seedling growth. The findings revealed that soaking hot water for 12 hours effectively increased the seed

germination percentage. Soaking the seeds in hot water for 12 hours, then applying 500 ppm atonic to seedlings, increased height, root length, and seedling dry weight. Combining two types of hormones is more effective than combining three, four, or even more types of hormones (Cavusoglu and Kabar 2007). This is due to the synergistic effect of each hormone, which supports each other because each hormone used has a different effect on growth. Based on this, two types of hormones were used in this study, which were treated separately and then combined to determine their effect on *R. verticillata* seed germination and growth.

The purpose of this study is to determine the effect of the hormones cytokinin and gibberellic acid (GA_3) on the germination and growth of *R. verticillata* and the optimal concentration of cytokinin and gibberellic acid (GA_3) hormones on increasing plant germination and growth of *R. verticillata*.

MATERIALS AND METHODS

Materials

The main materials used in this study were old *R. verticillata* seeds taken from the *R. verticillata* plant that grows in the Boyolali area; cytokinin hormone (kinetin); gibberellic acid (GA_3); and Dhitane M45 2%.

Research design

This study used a completely randomized design (CRD), with each treatment with 5 replications. The types of treatment carried out include:

I = Control (G_0S_0)
II = GA_3 50 ppm: Cytokinin 0 ppm ($G_{50}S_0$)
III = GA_3 0 ppm: Cytokinin 50 ppm (G_0S_{50})
IV = GA_3 50 ppm: Cytokinin 50 ppm ($G_{50}S_{50}$)
V = GA_3 50 ppm: Cytokinin 75 ppm ($G_{50}S_{75}$)
VI = GA_3 75 ppm: Cytokinin 50 ppm ($G_{75}S_{50}$

Research methods

This study begins with collecting seeds from old plants distinguished by their gray color (black). For 2-3 days, the seeds are dried. The seeds were extracted from the seed coat and treated by immersing them in hormones (cytokinins and GA_3). The hormone solution was created by dissolving the powdered hormone in ethanol and aquadest. Before adding aquadest, this ethanol was used to dissolve the hormone. Proceed with the soaking of the seeds once the seeds are ready. The seeds were soaked overnight in the hormone solution at each concentration. These steps were based on Setyowati and Utami's (2008) study on *B. javanica* seeds, which have a seed structure similar to *R. verticillata*. After that, the seeds were planted in polybags. The media was soil collected from the Boyolali area in ¾ polybags (±1 kg/polybag). To avoid mold during germination, the seeds were soaked in a 2 percent Dhitane M45 solution for 5 minutes before planting (Setyowati and Utami 2008). Each polybag contains three seeds spaced about 3-4 cm apart. After the seeds had been planted, the hole was filled with soil and doused with 100 mL of water (Lestari 2008). The polybags containing the planted seeds were placed in a greenhouse with limited sunlight.

Watering was used to treat plants from planting to the end of treatment. Watering was performed regularly to meet the needs of the plant. It was not done if the soil in the polybag was not too dry because it is feared that the seeds will rot if it is too wet.

Data were collected during or at the end of treatment depending on the parameters to be observed. Observations of germination parameters include: (i) the time of sprout emergence. (ii) Germination percentage. The percentage of germination was calculated for each polybag using the formula:

$$\text{Germination percentage} = \frac{\text{Number of sprouted seeds}}{\text{Total of all seeds}}$$

Plant growth observation begins when the plant's first leaves appear. The development of the *R. verticillata* plant was studied. The following parameters were observed: (i) Plant height was measured from the soil surface to the plant's highest tip. (ii) Leaf area and the number of leaves: The number of leaves was calculated after the first leaf appeared, whereas the leaf area was calculated at the end of treatment or at harvest, which was 1 week after the specified time had passed. The gravimetric method was used to calculate leaf area. Leaves were drawn on a piece of paper whose weight and area were known as leaf replicas, which were then cut out, and the leaf area was measured using the following equation:

$$LD = \frac{Wr}{Wt} xLK$$

Where:
LD : leaf area
Wr : leaf replica paperweight
Wt : total paperweight
LK : total paper area (Sitompul dan Guritno 1995)

(iii) Fresh weight: Fresh weight of plants was measured by weighing the plants at the end of the treatment. (iv) Dry Weight: Plants were dried in an oven at a temperature of 60°C until dry, and then the dry weight was weighed. Observations were made daily to determine the germination process and once a week for other parameters. Observations were stopped when the plants were 8 weeks old (± 2 months).

Data analysis

The collected data were then analyzed with analysis of variance (ANOVA) to determine the effect of treatment on the measured parameters. If there is a significant difference between treatments, a second test using the Duncan's Multiple Range Test (DMRT) at a test level of 5% is performed.

RESULTS AND DISCUSSION

Sprouts emergence time

The results of the average germination time of pule seeds pandak with GA_3 and cytokinin treatments are presented in Table 1.

The germination time observations in Table 1 revealed that the seeds began to germinate on the sixth day. Each treatment (control, G_0S_0, $G_{50}S_0$, $G_{50}S_{50}$, $G_{75}S_{50}$, $G_{50}S_{75}$) produced germinated seeds, but the G_0S_{50} concentration treatment showed no signs of seeds beginning to germinate. This suggests that pule seed germination is influenced by internal factors, such as seed age, and external factors, such as hormones, particularly GA_3.

Embryo growth during germination depends on preparing food materials in the endosperm. The embryo's survival depends on the occurrence of enzymatic decomposition, specifically the conversion of starch into sugar, which is then translocated to the embryo as an energy source for its growth, which requires amylase activity (Abidin 1994).

GA_3 is exogenous (from outside) and endogenous (from within the plant). Endogenous GA_3 increases the hydrolysis of starch, fructan, and sucrose into glucose and fructose molecules. The hexose sugar provides energy through respiration, aids cell formation, and causes the cell's water potential to become more negative at times. Water will enter more quickly due to decreased water potential, causing cell enlargement (Salisbury and Ross 1955).

The embryo's production of GA_3 stimulates cells in the aleurone layer to synthesize and produce α-amylase and protease enzymes, which convert endosperm starch into sugar for the growth of young seeds. After the seed absorbs water, the embryo releases GA_3 as a signal to the aleurone, a thin endosperm membrane. Aleurone responds to the response by synthesizing and secreting enzymes that hydrolyze the endosperm's food reserves. α-amylase, for example, hydrolyzes carbohydrates (much like the salivary enzymes in saliva that help break down bread and other carbohydrates). The scutellum (cotyledon) absorbs sugars and nutrients from the endosperm and stores them for use from embryonic development to adulthood (Sponsel 1987).

Cytokinins did not have a significant effect on germination in this study. This could happen if the cytokinin concentration is not optimal. Cytokinins can influence plant germination, growth, and development. Generally, this hormone interacts with the hormone auxin (Abidin 1994). Cytokinins and auxins (IAA) collaborate to promote embryonic growth; cytokinins promote cell division, while auxins (IAA) promote cell elongation (Hess 1970).

Germination percentage

Germination percentage was measured by comparing the number of seeds that germinated with all seeds planted and multiplying by 100%. The results of the average percentage germination of *R. verticillata* seeds with GA_3 and cytokinin treatments are presented in Table 2.

Table 2 shows the germination percentages for 30 days after planting. Table 2 shows that the control had the highest rate of germination. The treatment with the $G_{50}S_{50}$ combination produced the poorest results. Hormone treatment at concentrations greater than cytokinins produced positive results. This suggests that the GA_3 hormone affects seed germination. Gibberellins can remove germination inhibitors, break dormancy, and activate enzymes, resulting in increased metabolic activity (Salisbury and Ross 1995).

Non-germinating seeds mostly rot. This is because too much water ingestion by the seeds creates anaerobic conditions, allowing many rotting seeds to be supported by the state of the seeds, which causes the rot. These findings were reported in a study on the germination of jelutung (*Dyera costulata* (Miq.) Hook.fil.), which found that prolonged soaking of seeds sensitive to anaerobic conditions caused the seeds to rot and become incapable of germinating (Utami et al. 2007).

Germination is an embryonic growth process that includes morphological activity, which is characterized by the appearance of plant organs such as roots, stems, and leaves, as well as chemical activity, which includes several stages such as imbibition, secretion of hormones and enzymes, and hydrolysis of food reserves, particularly carbohydrates and proteins, from simple into complex forms, photosynthesis, translocation of dissolved food and hormones to the growing point area and other parts (Hidayat 1995). Several early germination hormones are activated by water in the cells. Abscisic acid, a phytohormone, decreased while gibberellins increased. Furthermore, the presence of water in the seeds activates active enzymes. The amylase enzyme breaks down flour into maltose, which is then hydrolyzed into glucose by maltase. Proteins are also degraded into amino acids. Glucose compounds enter the metabolic process and are broken down into energy and carbohydrate compounds, which form the body's structure. These amino acids are combined to form proteins, which build cell structures and new enzymes. Fatty acids are primarily used to construct cell membranes (Salisbury dan Ross 1995).

Table 1. Average germination time of *Rauvolfia verticillata* seeds with GA_3 and cytokinin treatment

Cytokinin and GA_3 concentrations	Average germination time (days)
G_0S_0	6
G50S0	6
G0S50	12
G50S50	6
G50S75	6
G75S50	6

Note: G = GA_3 concentration (ppm), G_0= 0, G_{50}= 50, G_{75}= 75; S = Cytokinin concentration (ppm), S_0= 0, S_{50}= 50, S_{75}= 75

Table 2. The average percentage of germination of *Rauvolfia verticillata* seeds with GA_3 and cytokinin treatment 30 days after planting

Cytokinin and GA_3 concentrations	Average (%) of germination
G_0S_0	62.50
G50S0	41.62
G0S50	33.25
G50S50	24.88
G50S75	33.25
G75S50	45.88

Note: G = GA_3 concentration (ppm), G_0= 0, G_{50}= 50, G_{75}= 75; S = Cytokinin concentration (ppm), S_0= 0, S_{50}= 50, S_{75}= 75

Plant height

Plant height is a plant size commonly observed as an indicator of growth and a parameter used to assess the effect of the environment or the treatment used. This is done because plant height is the most visible measure of growth. Plant height is sensitive to environmental factors as a parameter of environmental influence (Sitompul dan Guritno 1995).

The results of the average plant height with GA_3 and cytokinin treatment at the end of the study are presented in Table 3. The table of average plant height shows that for the treatment of hormone administration, $G_{50}S_0$ and $G_{75}S_{50}$ increased plant height compared to other hormone treatments and the control.

The application of GA_3 from the outside on the development of stolons of wild potato plants by Wareing and Philips in Abidin (1994) revealed internodal elongation in shoots growing in leaf axils. GA_3 applied to the plant's tip and kinetin applied to the leaf axils demonstrated that plant height and lateral branch growth were balanced (Abidin 1994).

Internode elongation causes stem elongation, caused by the growth of rib meristems, which form long rows of cells in the cortex and pith meristems. Cell elongation and an increase in the number of cells in the rib meristem occur during stem elongation (Hidayat 1995). Rib meristem is a meristematic tissue composed of a vertical series of cells that divide transversely (Fahn 1995).

Gibberellins stimulate the formation of enzymes that soften cell walls, particularly proteolytic enzymes that release the amino tryptophan (a precursor/former of auxin), increasing auxin levels. Gibberellins also promote the formation of polyhydroxy cinnamic acid, which inhibits the action of the IAA oxidase enzyme (Green Tect 2009).

When used alone, cytokinins (kinetin) stimulate DNA synthesis and are required in the process of mitosis, though IAA is usually more dominant in this phase. Cytokinins work with nucleic acids in cells to increase nuclear RNA synthesis and regulate the amount of RNA in the cytoplasm (Abidin1994).

Number of leaves

Because leaves are the primary photosynthetic organs, they must be observed as a growth parameter to explain the growth process. Once a week, observations on the number of leaves were made. Table 4 shows observation data on the number of leaves.

The best hormone treatment results are shown in the table above for hormone treatment with a concentration of $G_{50}S_0$ and the lowest concentration of G_0S_{50}. This is because gibberellins and cytokinins have opposite effects on leaf formation and meristem development when present in the same concentration or greater concentrations than cytokinins. Some of the effects of cytokinins on epidermal differentiation can also be reversed by GA_3. However, GA_3 and cytokinins can stimulate the formation of epidermal structures known as trichomes (Gan et al. 2007), so the concentration of cytokinins used has no effect.

The increased number of leaves is thought to be due to the increased division of leaf primordia cells and the differentiation of stem tip cells (Hidayat 1995). Leaves, as a means of photosynthesis, will be able to perform optimally if sufficient water, light, and nutrients are available (Loveless 1991; Salisbury and Ross 1995). According to Windarsih (2007), the effect of GA_3 on the process of leaf formation is that administration of the GA_3 hormone does not affect the number of leaves of flax plants (*Boehmeria nivea* (L.) Gaudich.).

Leaf area

Leaf area is a parameter that can be used to determine the rate of photosynthesis per unit plant. The method used to determine leaf area is the gravimetric method, which uses simple tools (Sitompul and Guritno 1995). Observations were made at harvest. The results of the average leaf area can be seen in Table 5.

Table 3. Average plant height of *Rauvolfia verticillata* with GA_3 and cytokinin treatment 60 days after planting

Cytokinin and GA_3 concentrations	Average Plant Height (cm)
G_0S_0	4.14
G50S0	4.64
G0S50	4.06
G50S50	2.2
G50S75	3.38
G75S50	5.06

Note: G = GA_3 concentration (ppm), G_0= 0, G_{50}= 50, G_{75}= 75; S = Cytokinin concentration (ppm), S_0= 0, S_{50}= 50, S_{75}= 75

Table 4. The average number of leaves of *Rauvolfia verticillata* with GA_3 and cytokinin treatment 60 days after planting

Cytokinin and GA_3 concentrations	The average number of leaves
G_0S_0	5.8
G50S0	6.2
G0S50	3.6
G50S50	4.4
G50S75	4.4
G75S50	5.0

Note: G = GA_3 concentration (ppm), G_0= 0, G_{50}= 50, G_{75}= 75; S = Cytokinin concentration (ppm), S_0= 0, S_{50}= 50, S_{75}= 75

Table 5. Average leaf area of *Rauvolfia verticillata* with GA_3 and cytokinin treatment 60 days after planting

Cytokinin and GA_3 concentrations	Average leaf area (cm^2)
G_0S_0	0.519
G50S0	0.663
G0S50	0.362
G50S50	0.362
G50S75	0.504
G75S50	0.612

Note: G = GA_3 concentration (ppm), G_0= 0, G_{50}= 50, G_{75}= 75; S = Cytokinin concentration (ppm), S_0= 0, S_{50}= 50, S_{75}= 75

Table 5 shows that the *R. verticillata* plant with a 50 ppm concentration of GA_3 hormone treatment had the highest leaf area of 0.663 cm2, while the G_0S_{50} and $G_{50}S_{50}$ treatments had the lowest leaf area.

Increased leaf area is one type of plant growth caused by cell division and elongation activity. The hormone GA_3 acting on cells and cytokinins, which are responsible for regulating the degree of leaf development according to soil conditions such as the availability of water and mineralized nitrogen, is one of the effects of cell division and elongation (Goldsworthy dan Fisher 1992).

Plant fresh weight

Plant fresh weight is one of the parameters to describe plant biomass. Fresh weight gain of plants is carried out by harvesting all or part of the plant and weighing it quickly before too much water evaporates from the material (Salisbury and Ross 1995). The results of the average fresh weight of the *R. verticillata* plant are presented in Table 6 below.

The average fresh weight in the control treatment was 0.3308 grams. The results for the $G_{50}S_0$ and $G_{75}S_{50}$ treatments were 0.2662 grams and 0.1244 grams, respectively, which were the lowest results compared to other treatments. The fresh weight of the $G_{50}S_{75}$ treatment was 0.2706 grams. The G_0S_{50} treatment yielded 0.2842 grams, while the $G_{50}S_{50}$ treatment yielded 0.3992 grams, the highest yield of all treatments.

The treatment with the same concentration of GA_3 as cytokinin produced a better fresh weight of the plant based on the average fresh weight obtained from all treatments. These findings suggest that a well-balanced concentration of gibberellins and cytokinins promotes optimal growth in the *R. verticillata* plant. These two hormones mutually benefit plant development, particularly in encouraging cell development (Cavusoglu and Kabar 2007).

Increased endogenous GA_3 content can cause the cell potential to become more negative and water to enter more quickly, resulting in cell enlargement (Lakitan 1996). Increased water uptake by these cells may increase fresh plant weight (Wattimena 1988).

Dry weight

When expressed in terms of dry weight, both the whole plant and its parts, growth as an increase in the material is more accurate. Dry and fresh weight will significantly change water status from time to time, which can vary throughout the day. Water loss causes a significant loss of fresh weight as older tissue dries. Because photosynthesis accounts for 90 percent of plant dry matter, growth analysis is expressed in dry weight, particularly to assess plants' ability to produce photosynthate (Goldsworthy and Fisher 1992). Table 7 shows the results of the average dry weight of the *R. verticillata* plant.

According to Table 7, the highest average dry weight was obtained in the plant treated with $G_{50}S_{50}$, while the lowest was obtained in plants treated with $G_{75}S_{50}$. This indicates that combining the same concentration of GA_3 and cytokinins at 50 ppm produced the best results for plant dry weight. The optimal results for fresh weight are also shown at the concentration of $G_{50}S_{50}$.

A balanced concentration of cytokinins and gibberellic acid has a synergistic effect that supports each other, resulting in optimal cell growth and development, which also affects plant dry weight (Cavusoglu and Kabar 2007).

The addition of cytokinins and exogenous gibberellins will increase the content of cytokinins and gibberellins in the plant (header) as well as the number of cells (via cytokinin hormones) and cell size (via gibberellin hormones), which will accelerate the process along with the increased photosynthate yield at the start of planting. Plant vegetative growth (including the formation of new shoots) and plant stunting are both addressed (Green Tect 2009).

Table 6. Average fresh weight of *Rauvolfia verticillata* in combination treatment with GA_3 and cytokinins 60 days after planting

Cytokinin and GA_3 concentrations	Average of fresh weight (gram)
G_0S_0	0.3308^b
G50S0	0.2662^b
G0S50	0.2842^b
G50S50	0.3992^b
G50S75	0.2706^b
G75S50	0.1244^a

Note: (i) G = GA_3 concentration (ppm), G_0= 0, G_{50}= 50, G_{75}= 75; S = Cytokinin concentration (ppm), S_0= 0, S_{50}= 50, S_{75}= 75. (ii) The numbers followed by the same letter in the column show no significant difference at the 5% DMRT test level

Table 7. The average dry weight of *Rauvolfia verticillata* in combination treatment with GA_3 and cytokinins 60 days after planting

Cytokinin and GA_3 concentrations	Average of dry weight (gram)
G_0S_0	0.0188^{ab}
G50S0	0.0246^b
G0S50	0.0148^{ab}
G50S50	0.0252^b
G50S75	0.0180^{ab}
G75S50	0.0088^a

Note: (i) G = GA_3 concentration (ppm), G_0= 0, G_{50}= 50, G_{75}= 75; S = Cytokinin concentration (ppm), S_0= 0, S_{50}= 50, S_{75}= 75. (ii) The numbers followed by the same letter in the column show no significant difference at the 5% DMRT test level

The increase in dry weight is caused by an increase in the rate of photosynthesis, which produces photosynthate as a byproduct of the metabolic process. Carbohydrates are the end product of the photosynthesis process. Carbohydrates are the basic organic matter building blocks in plant cells, such as structural, metabolic, and important food reserves. These organic materials comprise plant cell components such as cytoplasm, cell nucleus, and cell wall. The accumulation of dry weight resulted from this process (Salisbury and Ross 1992).

According to the research findings, immersion with GA_3 at a concentration of 50 ppm has a greater influence on accelerating germination time, increasing germination percentage, plant height, number of leaves, leaf area, and dry weight than immersion with 50 ppm cytokinins. Immersion treatment with a combination of GA_3 and cytokinin ($G_{50}S_{50}$) increased fresh and dry weight significantly. The combination of GA_3 and cytokinins at a concentration of $G_{50}S_{75}$ increased plants' wet and dry weight. The combination of GA_3 and cytokinins in $G_{75}S_{50}$ increases germination percentage, plant height, number of leaves, and leaf area.

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The effect of adding coconut pulp resulting from *Aspergillus oryzae* fermentation in commercial feed on the growth of Nile tilapia (*Oreochromis niloticus*)

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Abstract. *Elyana P, Pangastuti A, Nugraheni ER. 2018. The effect of adding coconut pulp resulting from* Aspergillus oryzae *fermentation in commercial feed on the growth of Nile tilapia (*Oreochromis niloticus*). Cell Biol Dev 2: 33-42.* Nile tilapia (*Oreochromis niloticus* Linnaeus, 1758) is one type of fish with the potential as an animal protein source. Quality feed, particularly feed containing basic protein nutrients, is required to increase fishery product production. Coconut pulp is one type of household waste with a relatively high nutritional content, particularly protein, and the potential to be processed into fish feed ingredients. Coconut pulp is initially processed through a fermentation process with *Aspergillus oryzae* (Ahlb.) Cohn, which is expected to increase protein digestibility. This study aims to determine the effect of adding fermented coconut pulp as a mixture in feed on the protein content and growth of Nile tilapia. This study used an experimental method called Completely Randomized Design (CRD). This study lasted 60 days and included four treatments containing fermented coconut pulp and commercial pellets. The composition of each treatment I, II, III, and IV was 75%:25%; 50%:50%; 25%:75%; and 0%:100%. Each treatment was replicated three times. Data analysis revealed that adding 75% fermented coconut pulp to the feed increased the water, fat, and crude fiber content by 25.72%, 20.36%, and 10.56%, respectively. In addition, the growth of Nile tilapia increased after they were fed fermented coconut pulp. The concentration of adding coconut pulp to the optimal feed for Nile tilapia growth and protein content was 25%. However, it is necessary to improve the nutritional composition of the feed to increase growth and meat protein.

Keywords: Coconut pulp, feed, fermentation, *Oreochromis niloticus*

INTRODUCTION

Indonesia is a country rich in biodiversity. One of these riches is the diversity of germplasm species and fishery resources in freshwater, coastal, and marine waters. That is an excellent natural potential for developing the Indonesian fisheries industry. Fish is an outstanding source of animal protein for meeting the Indonesian people's nutritional needs. Nile tilapia (*Oreochromis niloticus* Linnaeus, 1758) is a freshwater fish that has the potential to be a source of animal protein (Azwan et al. 2011). Nile tilapia meat contains 17.5% protein, 4.1% fat, and 74.8% water (Suyanto 2002). Nile tilapia has several advantages, including quick growth, high environmental tolerance, a large body size, good taste, a high survival rate, and ease of maintenance. However, to meet the nutritional needs of humans as consumers, the nutritional quality of the fish must also be considered.

Feed is essential for aquaculture species to grow properly and effectively. Quality feed ingredients can help increase fishery product production. Unfortunately, cost constraints are still a frequent impediment. Feed is the most expensive component of aquaculture production (can reach 60-70%). High feed prices in Indonesia are caused by imported raw materials such as soybean meal, fish meal, and even corn, even though the country has been self-sufficient (Amri 2007). Indonesia is vulnerable to market fluctuations due to its reliance on imported raw materials for feed. As a result, it is critical to investigate more sustainable approaches and strategies, such as developing the domestic feed raw material industry, while also ensuring that the origin of these ingredients can be easily traced. Plant protein sources can be used as animal protein sources.

Coconut pulp as a potential vegetable source for animal feed should be investigated as a fish feed additive. Aside from being readily available, using coconut pulp as a vegetable component in fish feed is expected to boost the nutritional value of the feed. Coconut pulp contains 13.35% water, 17.09% protein, 9.44% fat, 23.77% carbohydrates, 5.92% ash, and 30.4% crude fiber (Mujiman 1985). According to Derrick (2005), the crude protein content of coconut pulp reaches 23%, and its easily digestible fiber content makes coconut pulp suitable for use as feed ingredients. Using a biotechnology approach through fermentation is one way to increase the usability of protein and the value of the benefits of coconut pulp. According to Miskiyah et al. (2006), fermentation coconut pulp with *Aspergillus niger* Tiegh. mold increased protein content by 130%, from 11.35% to 26.09%.

Aside from *A. niger*, the mold of *Aspergillus oryzae* (Ahlb.) Cohn can be used to boost the nutritional value of feed ingredients, particularly protein content. The *A. oryzae* is the mold that produces the most enzymes, including a-amylase, a-galactosidase, glutaminase, protease, and β-

glucosidase. The most important of these enzymes are protease and amylase enzymes, which work to break down protein and starch from substrates. For example, the a-amylase enzyme produces glucose by breaking the a-1,4 bond, whereas the β-glucosidase enzyme breaks the β-1,6 bond in the branched-chain and converts dextrins to glucose (Purwoko 2007). The *A. oryzae's* protease enzyme converts long polymer chains from proteins into amino acids, increasing amino acid nitrogen and total acid levels (Gandjar 1977).

The objectives of this study were as follows: (i) to determine the nutritional content of the feed, which included protein, fat, carbohydrate, ash, and water content after the addition of coconut pulp fermented by *A. oryzae*; and (ii) in determining the nutritional content of the feed after the addition of coconut pulp fermented by A. oryzae. (ii) To assess the growth of Nile tilapia after feeding with coconut pulp fermented *A. oryzae* at various concentrations. (iii) To identify the optimal concentration of coconut pulp fermented *A. oryzae* to feed to boost Nile tilapia growth.

MATERIALS AND METHODS

Research site and time

This study was carried out at the Biology Sub-Laboratory of the Faculty of Mathematics and Natural Sciences Central Laboratory, the Soil Science Laboratory, Faculty of Agriculture, Universitas Sebelas Maret, Surakarta, and the PBIAT Janti Satker, Klaten, Central Java, Indonesia.

Materials

The main materials used were a pure culture of *A. oryzae* from the Inter-University Center, Universitas Gajah Mada, Yogyakarta, Indonesia. Other materials were fresh coconut pulp from household waste and male Nile tilapia.

Experimental design

This experiment used a completely randomized design consisting of four treatments, each with three replications. The treatment is shown in Table 1.

Creating work culture

PDA (3 g) and distilled water (77 mL) were put in a beaker, then heated to boiling on a hot plate and stirred with a magnetic stirrer until homogeneous. Next, the PDA solution was placed in 4 ml test tubes, tightly closed with cotton and aluminum foil, tied with a rubber band, and autoclaved at 121°C for 15 minutes. The tube was then tilted so that the PDA inside was tilted and no moisture was present before inverting its position and waiting for it to cool. Next, *A. oryzae* spore suspension was inoculated into slanted agar media and incubated for 3-5 days at room temperature. Finally, the culture was ready for use as a working culture, while the remainder was stored as stock culture in an incubator at 4ºC.

Coconut pulp fermentation

Ten (10) kg of coconut pulp were sun-dried. After the coconut pulp had dried, it was mashed, and 800 ml of water was added. The mixture of water and coconut pulp was steamed for 30 minutes, then cooled on a Formica plastic. After cooling, minerals containing 360 g $(NH_4)2SO_4$, 200 g Urea, 75 g NaH_2PO_4, 25 g $MgSO_4$, and 7.5 g KCl were added, followed by 10 oses of *A. oryzae* spores. It was then mixed and stirred until homogeneous. The mixture was placed on a plastic tray with a thickness of 1 cm and then fermented aerobically at room temperature for 2 days. Afterward, the mixture was wrapped in plastic, compacted without air (enzymatic process occurred), and incubated at room temperature for 2 days. Then, the mixture was dried, ground into pellets, and stored (Purwadaria et al. 1995a,b).

The pond with a size of 15m x 3m x 0.5m was divided into 12 plots with a size of 1m x 1m each. Each plot was filled with water up to 30 cm from the bottom of the pool. Then 180 male Nile tilapia fish that were 2 months old were put in plots, each plot consisting of 15 fish. Each plot was also given an aerator to increase water aeration. Before treatment, the Nile tilapia were acclimatized for 10 days. After acclimation, 5 fish were taken in each plot to collect data on the initial protein content of the study.

Supporting data to determine pond water quality is the water's physical and chemical conditions, which is the fish's environment. The data measured include DO and temperature measured by DO meter, pH measured by pH meter, and ammonia content measured by distillation.

The weight of the fish as measured by the O'Hauss scale and the standard length of the fish as measured with a ruler were the variables used to determine the growth of the fish. Fish were fed various rations that included varying concentrations of fermented coconut pulp. Feeding occurs thrice daily, at 08.00, 12.00, and 16.00. According to Afrianto and Liviawaty (2005), the feeding interval is 4 hours because the fish require food every 3-4 hours after eating. When the temperature rises, Nile tilapia activity rises, and the fish become hungry quickly. Feed concentration was up to 5% of the Nile tilapia's body weight, with 3 replications in each treatment. Every day, before feeding in the morning, the rest of the feed and feces were cleaned, and as much water as was issued was added (Rukmana 1997).

Water quality data was collected once every 10 days, followed by measurements of total weight and standard length of fish, and data collection was done in the morning before feeding. The value of protein retention was determined by observing the fish protein content test at the beginning and end of the study. In addition, the muscle tissue (meat) of the dorsal part of Nile tilapia from each treatment was collected.

Table 1. Comparison of fermented coconut pulp concentration and commercial pellets

Commercial treatment (%)	Fermented coconut pulp (%)	Pellets (%)
P_I	75	25
P_{II}	50	50
P_{III}	25	75
P_{IV}	0	100

Analysis of feed nutrition before and after fermentation

Measurement of protein content using the Kjeldahl Method

Samples of 1-2 grams were taken and placed in a Kjeldahl flask with 3 grams of the digestion mixture (1 part $CuSO_4$ and 9 parts K_2SO_4), and 20 ml concentrated H_2SO_4. The Kjeldahl flask was heated on a heating stove until the solution's original black color changed to a clear color during heating. After the digestion, the Kjeldahl flask was cooled, the inner surface of the flask was rinsed with distilled water, and the solution was mixed until homogeneous. The destruction sample solution was placed in a steam distillation device, and three drops of phenolphthalein indicator were added. The collecting solution was placed in a beaker (containing 50 mL of a 2% boric acid solution and 5 drops of Tashiro's indicator) under the cooler tip, which was immersed in the collecting solution. The concentrated NaOH solution was then gradually poured until the sample solution became alkaline. The distillation was complete when the distillate dripping reacted neutrally to red litmus, and the reservoir solution turned green. Next, the reservoir solution was titrated with 0.1 N HCl solution until the color returned to pink.

Protein content was calculated by the following formula (Sudarmadji 1997):

$$\text{Protein content} = \frac{\text{mL HCl x N HCl titrate x 14 x 6.25}}{\text{g sample x 1000}} \text{ X 100\%}$$

Measurement of fat content

The filter flask (extraction) containing boiling stone grains was dried in a dryer at 105°-110°C for 1 hour, then cooled in a desiccator and weighed (a). The sample was weighed to 1 g (X), placed in a filter sleeve, and wrapped in cotton. The filter sleeve was inserted into the Soxhlet, and then chloroform filtered until clear. The filter flask was dried in a dryer at 105°-110°C for 1 hour, then cooled in a desiccator until a constant weight was obtained. (b).

The following formula calculated fat content:

Fat content = b-a/x.100%

Where:
b : Constant (final) weight of the pumpkin
a : Pumpkin initial weight
X : Sample weight (Anggorodi 1979)

Measurement of the crude fiber content

The filter flask (extraction) containing boiling stone grains was dried in a dryer at 105°-110°C for 1 hour, then cooled in a desiccator and weighed (a). The sample was weighed to 1 g (X), placed in a filter sleeve, and wrapped in cotton. The filter sleeve was inserted into the Soxhlet, and then chloroform filtered until clear. The filter flask was dried in a dryer at 105°-110°C for 1 hour, then cooled in a desiccator until a constant weight was obtained:

Crude fiber content = (Y-Z-A)/X.100%

Where:
Y : Weight of filter paper after final drying
Z : weight of filter paper after initial drying
A : Weight of filter paper after curing
X : Sample weight (Anggorodi 1979)

Water level measurement

The bottles and caps were weighed and dried at a temperature of 105°-110°C for 10-12 hours before being cooled in a desiccator for 30 minutes and weighed. Next, a 1 g sample was placed in a dried bottle. Finally, the bottle and its contents were weighed, and the contents were dried at 105°-110°C until a constant weight was obtained (B). The following formula calculated the water level:

Water content = (A-B)/A.100%

Where:
A: Initial bottle weight
B: Constant bottle weight (Tillman et al. 1989)

Measurement of ash content

Porcelain was dried in a dryer at a temperature of 105-110°C, then cooled in a desiccator and weighed (X). Next, a sample of 1 gram was put into the porcelain (Y), then burned on a bunsen until no smoke came out. Next, the porcelain dish and the burned sample were put in an oven at 400°C until the sample turned white, then cooled and weighed (Z). The following formula calculated ash content:

Ash content = Z-Y/X.100%

Where:
Z : Final weight of porcelain cup and sample
X : Porcelain cup weight
Y : Sample weight (Anggorodi 1979)

Measurement of carbohydrate levels

The measurement of carbohydrate content is carried out using the "Carbohydrate by Difference" method (Nugroho 1999):

% carbohydrate = 100% - (protein+lipid+ash+water)%

Nile tilapia growth analysis

Measurement of the growth of Nile tilapia: (i) The Weight of the Nile tilapia was weighed using an O'Hauss scale. (ii) The standard length of the Nile tilapia was measured from the tip of the front of the head to the crease of the base of the caudal fin using a ruler and millimeter paper.

The following formula calculated the degree of Survival (Effendi in Fuad 1996):

S = Nt/No x 100%

Where:
S : Survival rate
Nt : Number of fish at the end of the study
No : Number of fish in the initial study

The following formula calculated the daily Growth Rate (Effendi in Fuad 1996):

$$SGR = \frac{\text{In Wt} - \text{In Wo}}{t2 - t1} \times 100\%$$

Where:
Wt : Final Weight of fish
Wo : Initial Weight of fish
t_1 : Start time (days)
t_2 : Finish time (days)
SGR : Daily growth rate (%)

Protein retention (PR), according to Buwono (2004), was calculated by the following formula:

$$PR \quad \frac{\text{JPS end (g)} - \text{JPS start (g)}}{\text{JPB (g)}} \quad X\ 100\%$$

Where:
JPS *end*: The amount of protein stored in the fish body at the end of the study (g)
JPS *start*: The amount of protein stored in the fish body at the start of the study (g)
JPB: The amount of protein given (g)

Feed Efficiency (FE), according to Huisman in Ing Mokoginta et al. (1995) calculated by the following formula:

$$FE = \frac{(Wt + D - Wo)}{F} \times 100\%$$

Where:
Wt : Final weight of Nile tilapia
Wo : Initial Weight of Nile tilapia
D : The Weight of dead Nile tilapia
F : weight of feed given

Sampling technique

This study used random sampling as its sampling technique. Three replications were performed to test the nutritional content of the feed, fish protein content, and growth of each treatment group.

Data collecting technique

For 60 days, data on Nile tilapia growth was collected every 10 days. The growth of Nile tilapia was observed three times by weighing and measuring the standard length of Nile tilapia as a growth parameter. The research data includes observational data. In addition, the protein content of the fish was measured at the beginning and end of the study.

Data analysis technique

The results of the observations were analyzed using analysis of variance to determine whether or not the effect on the parameters measured in this study is real (ANOVA). If the treatment has a significant effect or is significantly different, the DMRT (Duncan's Multiple Ranges Test) was used with a test level of 5% to pinpoint the location of the difference in influence between treatments.

RESULTS AND DISCUSSION

Coconut pulp fermentation

One of the methods used to convert coconut pulp into feed using *A. oryzae* is fermentation. The fermentation process consists of two stages: aerobic fermentation and anaerobic fermentation. Similar studies on coconut cake have been conducted in the past (Purwadaria et al. 1995a,b). Mycelium was present during the growth of *A. oryzae* during the fermentation process. The appearance of fine thread-like fibers and the pulp compaction indicates the mycelium's growth. As a result, after fermentation, unfermented coconut pulp has a different structure, color, odor, and chemical composition (Table 2).

Table 2 shows that the water content increased by 12.84% after fermentation. The high increase in water content was caused by *A.* oryzae's respiration process, which increased the water content in the pulp. Meanwhile, after fermentation, the ash content of coconut pulp decreased from 5.92% to 3.15%. This decrease was caused by the fact that *A. oryzae* required mineral salts as cofactors for enzymes involved in metabolism during its growth (Advisory Committee on Technology Innovation 1979).

After fermentation, the fat content of coconut pulp increased from 9.44% to 20.35%. This increase in fat content is possible due to the *A. oryzae* mold's low lipase activity, which prevents it from optimally degrading fat into fatty acids, and its high amylase activity, which allows it to remodel carbohydrates optimally.

Furthermore, fat is not used as an energy source during fermentation; carbohydrates are the primary energy source. This metabolism allows for the conversion of various carbohydrates into fat (Kasmidjo 1990). Acetyl Co-A is the key compound that connects carbohydrate metabolism to fatty acid synthesis. If the cells in the body have more glucose than they require for energy, they will convert some of the acetyl Co-A produced by glucose catabolism into fatty acids (Wilbraham and Matta 1992).

Table 2. Proximate analysis of coconut pulp before and after fermentation using *Aspergillus oryzae*

Analysis	Before fermentation (%)	After fermentation (%)
Water	13.35	26.19
Ash	5.92	3.15
Fat	9.44	20.35
Protein	13.09	13.63
Crude fiber	30.40	10.15
Carbohydrate	23.77	26.53

Coconut pulp's protein content increased from 13.09% to 13.63%. Compared to previous studies, the increase in protein levels was very small. According to Miskiyah et al. (2006), the protein content of coconut pulp increased from 11.35% to 26.07% or 130% after fermentation with *A. niger*. This minor increase in protein content was most likely caused by *A. oryzae*'s activity of low protein consumption compared to amino acid synthesis. Fungi can synthesize amino acids, including phosphoenolpyruvate and a-ketoglutarate. Molds produce proteins and amino acids by utilizing the carbon and nitrogen skeletons found in the substrate (Cochrane 1958 in Gusmanizar and Rahman 2000).

The amount of energy in the feed can be estimated using crude fiber. The three components of crude fiber are cellulose, hemicellulose, and lignin. After fermentation, the crude fiber content of coconut pulp decreased from 30.40% to 10.15%. The high activity of the cellulase enzyme in *A. oryzae* mold allowed it to degrade cellulose and hemicellulose in coconut pulp, decreasing fiber content. A fish feed with a low crude fiber content will be easily digested because fish do not have cellulase enzymes in their digestive tract that can degrade cellulose and hemicellulose (Gusmanizar and Rahman 2002).

Fish feed

According to Sahwan (2002), one of the most important factors in maximizing fishery productivity is the availability of feed in sufficient quantities, on time, and with good nutritional value. That is because the natural feed content available at the location is insufficient to meet the needs of the fish, necessitating the use of additional feed. However, feed quality must also be considered because it significantly impacts the fish growth rate.

There were four types of feed treatments in this study, each with a different composition: treatment I was 75% fermented coconut pulp and 25% commercial pellets, treatment II was 50% fermented coconut pulp and 50% commercial pellets, treatment III was 25% fermented coconut pulp and 75% commercial pellets, and treatment IV (control) was 100% commercial pellets. A feed quality test was performed to determine the nutritional value of feed in each treatment, which included protein, fat, carbohydrate, ash, crude fiber, and water content levels. According to statistical analysis (appendices 1-6), each test in each treatment produces a significant difference (Table 3).

The water content test determines the amount of water in the feed for each treatment. According to Sahwan (2002), the water content in the feed should not exceed 10% so that fungus does not overgrow the feed. Statistical analysis showed a significant difference between treatments III and IV, indicating that treatments III and IV had different water content. Treatments I and II were also noticeably different from treatments III and IV. The higher the percentage of fermented coconut pulp, the higher the water content in each treatment, as shown in Table 3. This high-water content is caused by *A. oryzae* respiration in the pulp during the fermentation process, which increases the water content in the pulp, causing an increase in the water content of the feed.

The ash content of the feed indicates the amount of mineral content in the feed (Jangkaru 1974). Fish require minerals during the growth process, but only in trace amounts. Calcium (Ca) and phosphorus (P) is required for bone formation and to keep body tissues functioning normally. Iron (Fe) is required for the formation of red blood cells, and manganese (Mn) is required for reproduction (Sahwan 2002). Statistical analysis shows a significant difference between treatments, indicating that each has a different ash content. According to Table 3, the higher the percentage of fermented coconut pulp added to the feed, the lower the ash content of the feed. The highest ash content was 9.35% in treatment IV (control), and the lowest ash content was 3.40% in treatment I. The decrease in ash content was caused by the fact that *A. oryzae* required mineral salts as cofactors for enzymes that were important in metabolism during its growth, resulting in a decrease in ash content in the feed in each treatment with varying levels of fermented coconut pulp.

The presence of fat in the feed affects its taste and texture. Mudjiman (1985) states that the ideal fat content for fish feed ranges from 4 to 18%. According to statistical analysis, there was a significant difference between treatments, indicating that the fat content of each treatment differed. According to Table 3, the percentage of high-fat content in the feed was followed by a high percentage of fermented coconut pulp addition. Treatment IV (control) had the lowest fat content of 5.73%, and treatment I had the highest fat content of 20.36%. The increase in fat content was possible because the lipase enzyme activity in *A. oryzae* was low, making degrading the fat in coconut pulp into fatty acids less efficient. Furthermore, fat is not used as an energy source during fermentation; carbohydrates are the primary energy source. This metabolism enables the conversion of various carbohydrates to increase the fat content of coconut pulp, thereby influencing feed treatment.

Table 3. Nutritional data for feed after the addition of fermented coconut pulp

Treatment	The nutritional content of the feed					
	Water level (%)	Ash (%)	Fat (%)	Protein (%)	Crude fiber (%)	Carbohydrate (%)
P_I	25.72^{c}	3.40^{a}	20.36^{d}	13.40^{a}	10.56^{b}	37.13^{a}
P_{II}	24.11^{c}	5.03^{b}	14.79^{c}	18.17^{b}	8.22^{ab}	37.91^{a}
P_{III}	19.35^{b}	6.68^{c}	12.23^{b}	23.46^{c}	6.03^{a}	38.29^{a}
P_{IV}	10.61^{a}	9.35^{d}	5.73^{a}	29.34^{d}	5.66^{a}	44.98^{b}

Notes: Numbers followed by different letters indicate a significant difference in the DMRT test at the 5% test level in the vertical direction. PI: 75% fermented coconut pulp and 25% commercial pellets, PII: 50% fermented coconut pulp and 50% commercial pellets, PIII: 25% fermented coconut pulp and 75% commercial pellets, PIV: 100% commercial pellets (Control)

Carbohydrates are the last element in the fish growth process because the fish's ability to digest carbohydrates is very low. Therefore, fish carbohydrate needs are relatively small and tend to be used as a source of carbon skeletons for protein synthesis (Tacon 1987). The highest carbohydrate content in treatment IV (control) was 44.98%, and the lowest in treatment I was 37.13%. The analysis showed treatments I, II, and III significantly differed from treatment IV. From Table 3, it can be seen that the low carbohydrates in treatments I, II, and III were probably because carbohydrates were used as the main energy source during the coconut pulp fermentation process by *A. oryzae*, causing low carbohydrates in the feed.

Protein is a chemical compound needed by the fish body as a source of energy and for growth. The research results show that the relationship between growth and protein content is directly proportional; the more protein content in the feed, the higher the growth. Table 3 above shows that the highest protein content in treatment IV was 29.34%, and the lowest protein content in treatment I was 13.40%. The higher the addition of fermented coconut pulp in the feed, the lower the protein content of the feed. That was due to the low activity of protein consumption compared to the synthesis of amino acids by *A. oryzae* during the fermentation process. Molds carry out the biosynthesis of proteins and amino acids by utilizing the carbon and nitrogen skeletons available in the substrate.

Table 3 shows that the crude fiber content in the feed is increasing along with the large percentage of fermented coconut pulp, even though the crude fiber is difficult to digest by fish. From Table 3, it can be seen that in treatment I, with the addition of 75% of fermented coconut pulp, the highest crude fiber content was 10.57%. The high crude fiber content in fish feed will affect the digestibility and absorption of food substances in the fish's digestive tract. The crude fiber content of less than 8% will improve the structure of the feed, but if the crude fiber exceeds 8% will reduce feed quality (Djajasewaka 1995).

Feed efficiency (FE)

The growth of fish with indicators of fish weight and the amount of feed given during the study can show feed efficiency (Figure 1). Feed efficiency calculates the amount of feed that enters the fish's digestive system for the body's ongoing metabolic processes, one of which is growth. Protein is fish's primary energy source, followed by fat and carbohydrates. Therefore, protein is a nutrient that is required for growth. Protein utilization for growth is influenced by several factors, including fish size/age, protein content, feed energy content, water temperature, and feeding level (Mokoginta et al. 1995).

Food is digested in the intestine and then transported to the liver via blood vessels via passive diffusion, active transport, and pinocytosis. Protein is absorbed in the form of amino acids, which are then transported to the liver to be converted back into protein, which has been tailored to the needs of the fish body (Wedemeyer 1996). The qualitative and quantitative composition of the amino acid mixture determines the pattern of amino acid absorption in the intestine. Amino acids are taken up by blood capillaries from the mucosa after active absorption by intestinal mucosal cells and transported in plasma and body tissues for metabolism (Noor 1990).

The higher the feed efficiency value, the faster the growth rate. According to the above study's findings, treatment IV had the highest feed efficiency of 0.28 grams, while treatment I had the lowest. The feed efficiency value (FE) was 0.28, meaning that for every gram of feed, 0.28 grams was digested into the fish's digestive system. This high FE value is followed by a good nutritional content of the feed (Table 3), one of which is the low crude fiber content of 5.66%, making the feed easy to digest for fish. Furthermore, protein and carbohydrate levels are high, allowing for rapid growth. Meanwhile, the low FE value was followed by the feed's low nutritional content, with a high crude fiber content of 10.56%. Whereas crude fiber in feed should not exceed 8% because it interferes with digestion and absorption of food substances, lowering the quality of fish feed (Mudjiman 2004).

Nile tilapia growth

Growth is an increase in length and weight over a specific period. Growth is influenced by nutrients (food), which include protein, fat, carbohydrates, vitamins, and minerals, as well as water and oxygen, in addition to genetic factors and hormones. Protein is the most important feed substance for fish weight gain, according to Djajasewaka and Suhenda (1992). Feeds with a high protein content will be more effective and efficient for growth because protein, like fat and carbohydrates, is an important substance. Growth hormone has the greatest influence on protein utilization for growth. Growth hormone increases the transport of amino acids across the membrane or speeds up the chemical process of protein synthesis, increasing tissue protein.

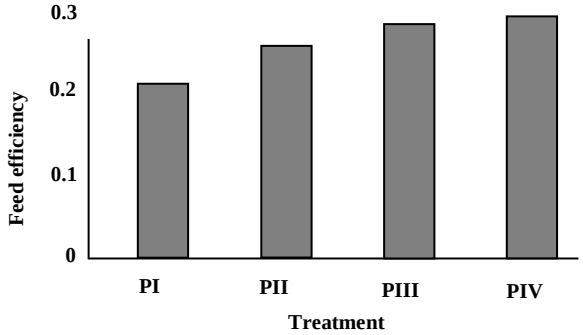

Figure 1. Feed efficiency during research. Notes: PI: 75% fermented coconut pulp and 25% commercial pellets, PII: 50% fermented coconut pulp and 50% commercial pellets, PIII: 25% fermented coconut pulp and 75% commercial pellets, PIV: 100% commercial pellets (control)

Nile tilapia fish weight

The results of the research and statistical analysis of Nile tilapia growth with weight indicators revealed a significant difference ($p<0.05$) from each treatment. Treatment IV (control), which contained 100% commercial pellets without the addition of fermented coconut pulp, produced the greatest weight gain. While treatment I, which contained 75% fermented coconut pulp and 25% commercial pellets, produced the lowest weight gain (Table 4).

This Nile tilapia weight difference demonstrates a proportional relationship between protein quantity and fish weight growth. The more feed protein there is, the more effective it is at increasing fish weight. According to Utojo (1995), the amount and type of essential amino acids in the feed determines the amount and type of protein in the body, among other things.

Compared to the results obtained with commercial pellet feed alone, the value of weight gain in Nile tilapia with a combination of fermented coconut pulp and commercial pellet feed was still relatively small. That does not, however, preclude the use of feed with this fermented coconut pulp mixture. According to the DMRT test, there was no significant difference between treatment III, which still contained 25% fermented coconut pulp, and treatment IV, which contained 100% commercial pellets, so feed that only consisted of commercial pellets could be replaced with feed that also contained fermented coconut pulp, a 25% content in addition to commercial pellets as one of the raw materials. That is due to the nutritional content of the feed being sufficient to support fish growth.

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Nile tilapia fish protein content

The fish's body converts the protein in the feed into protein based on its requirements (Table 5). There are two basic chemical processes for protein synthesis: amino acid synthesis and the conjugation of suitable amino acids to form each type of protein in each cell. That is the most fundamental growth process because growth is impossible without large-scale protein production (Fujaya 2004).

Protease and peptidase enzymes secreted by the intestines catalyze the breakdown of proteins into amino acids. Amino acids are required for protein synthesis, which plays a role in replacing damaged cells and forming body tissues, increasing tissue protein. This increase in tissue protein is reflected in the fish's increased body weight and length (Murray et al. 1996).

Protein analysis and analysis of variance ($p<0.05$) revealed no significant difference ($p>0.05$) between treatments. According to Djuanda (1981), some food consumed is converted into energy used for living activities, and some food is expelled from the body. As a result, not all of the protein in the fish's diet is converted into meat. Furthermore, the physiological ability of fish influences the formation of meat protein.

Table 4. Nile tilapia fish weight and length

Treatment	Fermented coconut pulp level (%)	Fish weight (gram)	Fish length (cm)
PI	75	26.66^{a}	11.38^{a}
PII	50	27.78^{a}	11.84^{ab}
PIII	25	32.09^{ab}	12.83^{bc}
PIV	0	35.97^{b}	13.76^{c}

Note: Numbers followed by letters indicate a significant difference in the DMRT test at the 5% test level. PI: 75% fermented coconut pulp and 25% commercial pellets, PII: 50% fermented coconut pulp and 50% commercial pellets, PIII: 25% fermented coconut pulp and 75% commercial pellets, PIV: 100% commercial pellets (Control)

Table 5. Nile tilapia fish protein content

Treat-ment	Fermented coconut pulp level (%)	Feed protein content (%)	Fish protein content (%)
PI	75	13.40	13.40
PII	50	18.17	18.17
PIII	25	23.50	23.50
PIV	0	29.34	29.34

Notes: Numbers followed by letters indicate a significant difference in the DMRT test at the 5% test level. PI: 75% fermented coconut pulp and 25% commercial pellets, PII: 50% fermented coconut pulp and 50% commercial pellets, PIII: 25% fermented coconut pulp and 75% commercial pellets, PIV: 100% commercial pellets (Control)

Protein retention

Protein retention depicts the amount of protein provided that can be absorbed and used to build or repair damaged cells and by the fish body for daily metabolism (Figure 2). The amount of protein that can be absorbed and used by the body as a building block determines whether or not fish grow quickly. For fish to grow normally, the ration or feed must contain enough energy to meet daily metabolic energy needs and enough protein to meet the development needs of new body cells.

The protein retention value represents the protein deposition index in body tissues (used for growth). Protein retention refers to the amount of protein stored in the body of a fish. The use of feed protein is expected to increase body protein or cause growth (Suhenda et al. 2003).

According to Figure 2, treatment I had the lowest protein retention of 0.056%, while treatment IV (control) had the highest protein retention of 0.089%. The low protein retention is most likely caused by the feed's low protein content, which cannot meet the energy requirements to build or repair damaged body cells and fish metabolism daily. Protein is a very important nutrient that is required for body maintenance, tissue formation, replacement of damaged body tissues, and increasing body protein in growth, according to Cowey and Sargent (1979).

The energy content of the feed also influences protein utilization to form tissue. The higher the energy content of the feed, the better the utilization of protein by the fish body, resulting in increased body tissue formation.

Daily growth rate

The growth rate of fish will accelerate as the protein content of the feed increases. Fish use protein feed for body maintenance, tissue growth, protein addition to the body, and tissue replacement (Cowey and Sargent 1979). Figure 3 depicts the daily growth rate for each treatment.

Figure 3 shows that treatment IV had the highest daily growth rate (control). The treatment with the lowest daily growth rate was treatment I, which contained 75% fermented coconut pulp with a protein content of 13.40%. In addition to the low protein content, the crude fiber content of the treatment I fed was still high, causing Nile tilapia growth to be lower than in other treatments. Fiber has several physiological effects, including reducing nutrient availability. Additionally, fish digestive organs are less able to digest fiber perfectly, resulting in decreased nutritional intake and low growth.

The low daily growth rate in each feed treatment with the addition of fermented coconut pulp indicates that Nile tilapia is less responsive to the feed given and cannot consume feed optimally, resulting in a low growth rate because the body's energy supply is reduced.

The amount and balance of feed nutrients affect the growth rate, which means that the nutritional composition of feed raw materials can complement each other's nutrient needs, increasing the growth rate and nutritional content of fish. For example, Table 3 shows that the feed containing fermented coconut pulp generally has a less balanced nutritional composition than the control feed, with high water, fat, and crude fiber content.

Survival rate

The survival rate in this study was relatively high. Suyanto (2002) believes that a 30-50% mortality rate is still considered normal. Nile tilapia generally died after sampling, specifically during length measurement and weighing. The most common causes of fish mortality are bacterial, fungal, vitamin C deficiency, and nutritional imbalances in feed (Sutarmat et al. 2003).

There was no significant difference based on the analysis of variance, implying that the survival percentage in each treatment is nearly the same (Table 6). That demonstrates that the inclusion of fermented coconut pulp in the feed does not affect Nile tilapia survival.

The observations during the study's 60-day period revealed that dead Nile tilapia did not indicate disease attacks on Nile tilapia. That is demonstrated by the lack of morphological organ damage in the fish body due to bacterial or fungal attacks. After 24 hours, dead fish usually float to the water's surface.

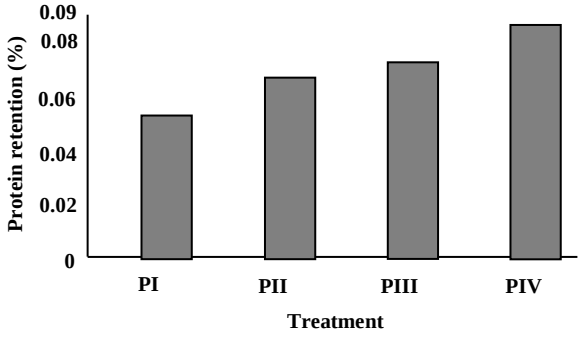

Figure 2. Protein retention. Notes: PI: 75% fermented coconut pulp and 25% commercial pellets, PII: 50% fermented coconut pulp and 50% commercial pellets, PIII: 25% fermented coconut pulp and 75% commercial pellets, PIV: 100% commercial pellets (control)

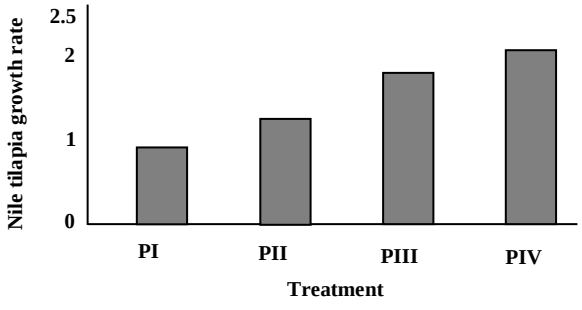

Figure 3. Nile tilapia growth rate during the research. Notes: PI: 75% fermented coconut pulp and 25% commercial pellets, PII: 50% fermented coconut pulp and 50% commercial pellets, PIII: 25% fermented coconut pulp and 75% commercial pellets, PIV: 100% commercial pellets (control)

Table 6. The survival rate of Nile tilapia

Treatment	Survival rate (%)
PI	98.89[a]
PII	96.67[a]
PIII	96.67[a]
PIV	97.78[a]

Notes: PI: 75% fermented coconut pulp and 25% commercial pellets, PII: 50% fermented coconut pulp and 50% commercial pellets, PIII: 25% fermented coconut pulp and 75% commercial pellets, PIV: 100% commercial pellets (Control)

Table 7. Water quality data during the study

Day -	Water quality parameters		
	Temperature (ºC)	pH	DO (ppm)
0	26.9	7.47	2.10
10	27.1	7.07	2.82
20	28.7	7.47	2.55
30	27.3	7.90	2.21
40	30.0	8.01	3.75
50	29.5	7.93	3.48
60	29.6	7.74	3.80

Water quality

Water is the most important medium or habitat for fish life. Therefore, an adequate water supply will solve various problems in intensive fish farming. In addition, good water quality is one of the keys to success in fish farming. The water parameters observed in this study include temperature, pH, and DO (oxygen content). Water quality parameter data can be seen in Table 7.

Table 7 shows that the water temperature ranges from 26.9 to 30.0ºC. The temperature range is still ideal for Nile tilapia growth. Suyanto (2002) states that the ideal temperature for Nile tilapia growth is between 25 and 30ºC. Water temperature influences fish appetite and metabolic processes. Food digestion in fish occurs slowly at low temperatures, whereas digestion occurs more quickly at high temperatures.

In this study, the degree of acidity (pH) ranged from 7.07-8.01. This pH range is favorable for Nile tilapia habitat and growth. According to Sherif and Feky (2009), the pH range for optimal growth is 7-8, while the pH range for Nile tilapia habitat is 6-8.5. One of the most important environmental factors for fish life is oxygen content. If the dissolved oxygen concentration is low, the organism being reared appetite decreases, affecting growth.

In this study, the dissolved oxygen (DO) content ranged from 2.10 to 3.80 ppm. This range falls below fish life's 4 ppm minimum oxygen concentration limit. Therefore, the low DO levels in the waters in this study were caused by the water coming from the earth's bowels, which had a low oxygen content. Nile tilapia, on the other hand, can tolerate DO levels as high as 1 ppm, but its growth is not optimal (Kordi 2000).

According to the study, the following conclusion is drawn: (i) adding 75% fermented coconut pulp to commercial pellets increases the water, fat, and crude fiber content by 25.72%, 20.36%, and 10.56%, respectively. On the other hand, the control feed had the highest ash content, protein content, and carbohydrates, which were 9.35%, 29.34%, and 44.98%, respectively. (ii) The growth of Nile tilapia was increased after feeding with fermented coconut pulp. (iii) The concentration of addition of fermented coconut pulp in the optimal feed for the growth of tilapia is 25%.

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