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Banana flowers photo by Martin Sharman

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The title of the article should be written in compact, clear, and informative sentence, preferably not more than 20 words. Name of author(s) should be completely written, especially for the first and the last name. **Name and institution** address should also be completely written with street name and number (location), postal code, telephone number, facsimile number, and email address. We choose local names in Bahasa Indonesia for universities in Indonesia. The mention of "strata" program, should be avoided. Manuscript written by a group, author for correspondence along with address is required (marked with "♥"). **The title page** (first page) should include title of the article, full name(s), institution(s) and address(es) of the author(s); the corresponding authors detailed postage and e-mail addresses (P), and phone (O) and fax numbers (O).

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Figures and Tables of a maximum of three pages should be clearly presented. The title of a picture is written down below the picture, while the title of a table is written above the table. Colored figures can only be accepted if the information in the manuscript can lose without those images; the chart is preferred to use black and white images. The author could consign any picture or photo for the front cover, although it does not print in the manuscript. All images property of others should be mentioned the source. Author is suggested referring to Wikipedia for international boundaries and Google Earth for satellite imagery. If not specifically mentioned, it is assumed to refer to these sources. **There is no appendix**, all data or data analysis is incorporated into Results and Discussions. For broad data, it can be displayed on the website as a supplement.

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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. DOI: 10.13057/biodiv/d070213.

The usage of "et al." in long author lists will also be accepted:

Smith J. Jones M Jr. Houghton L et al. 1999. Future of health insurance. N Engl J Med 965: 325-329. DOI: 10.10007/s002149800025.

Book:

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

Chapter in the 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 the 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. DOI: 10.1038/msb.2008.24. www.molecularsystembiology.com.

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Physiological characteristics of *Sisyrinchium palmifolium* with fertilization treatment and IAA hormone

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Abstract. *Alifah EN, Mudyantini W, Solichatun. 2022. Physiological characteristics of* Sisyrinchium palmifolium *with fertilization treatment and IAA hormone. Cell Biol Dev 6: 51-60. Dayak* onion (*Sisyrinchium palmifolium* L., Syn.: *Eleutherine palmifolia* L. Merr.) is a typical Central Kalimantan, Indonesia medicinal plant. Optimizing the function of the *S. palmifolium* is done to increase cultivation and community interest; more fertilization and growth regulator treatments are needed to accelerate plant growth. In this study, the physiological properties of S. palmifolium will be examined regarding fertilization and the IAA (Indole Acetic Acid) hormone. CRD factorial pattern with four levels of fertilization variation (soil; soil + chicken manure (1:1); soil + vermicompost fertilizer (1:1); soil + chicken manure + vermicompost (1:1:1)) and three levels of hormone concentration (0 ppm; 100 ppm; 200 ppm) resulting in 12 treatment combinations which are used in this experiment. It was repeated three times for each therapy. It was shown that the number and length of leaves, the number of flowers and their flowering time, bulb weights, chlorophyll content, and flavonoid levels were all correlated with the number of leaves. The data were examined for significant changes between the treatments using the Analysis of Variant (ANOVA) and Duncan's Multiple Test (DMRT) at a 5% significance level. The physiological and biochemical features were altered by fertilization and the IAA hormone. The IAA hormone and fertilization on *S. palmifolium* enhanced the number of leaves, their width, the ratio of shoot roots to roots, and flavonoids. The maximum yields of wet weight, dry weight, and leaf carotenoids were obtained with vermicompost (1: 1) and a hormone at 200 ppm (P2H2) adding. Combining vermicompost treatment with the IAA hormone produced the highest level of flavonoids (P2H0).

Keywords: Biochemical, fertilization, hormones, physiological, *Sisyrinchium palmifolium*

INTRODUCTION

Indonesia is a country rich in medicinal plant germplasm. *Sisyrinchium palmifolium* L (Syn.: *Eleutherine palmifolia* L. Merr.), also known as a *Dayak* onion or *bawang Dayak*, is a native plant of Central Kalimantan, Indonesia. *S. palmifolium* is not very popular in contemporary Indonesian society. The majority are unaware of the functions and benefits of *S. palmifolium* as a medicine for various diseases, despite their potential as medicinal raw materials. The *Dayak* community has used *S. palmifolium* as a medicinal plant for generations. According to Utami and Desty (2013), *S. palmifolium* contains alkaloids, saponins, triterpenoids, steroids, glycosides, tannins, phenolics, and flavonoids used as raw materials in the manufacture of pharmaceuticals. *S. palmifolium* is used in traditional medicine to treat various conditions ranging from constipation to intestinal inflammation, dysentery, jaundice, hypertension, hypercholesterolemia, and diabetes mellitus. The bulbs and leaves of plants are frequently used medicinally.

Given the enormous potential of *S. palmifolium* as a multifunctional medicinal plant, efforts must be made to increase public awareness of the *S. palmifolium* plant. Among the efforts that can be made is applying appropriate cultivation techniques via fertilization to increase soil fertility or enhances a plant's quality and quantity. Therefore, fertilization is critical to the growth and production of *S. palmifolium*. At the moment, farmers prefer chemical fertilizers to organic fertilizers. While chemical fertilizers are considered more effective than organic fertilizers, excessive chemical fertilizer application can negatively affect soil fertility. Therefore, organic fertilizers are one way to reduce reliance on chemical fertilizers.

Organic fertilizers have several advantages, including the ability to improve the chemical and physical properties of the soil, soil water absorption, the effectiveness of soil microorganisms as food sources for plants, and the fact that they are environmentally friendly, less expensive, and improve production quality. In addition, organic matter could increase the number of nutrients available to plants (Pranata 2010). Moreover, to boost the growth of *S. palmifolium*, organic fertilizers such as manure and vermicompost can be employed. According to Jasmine et al. (2019), chicken manure enhances the quantity of *S. palmifolium* tillers. According to Aryani et al. (2019), vermicompost fertilizer can enhance shallot growth and production by improving soil structure and nutrient absorption.

Another strategy is to supply growth regulators to assist with substandard *S. palmifolium* growing. Fertilization, helped by hormones, is critical for plant growth and metabolic efficiency. External growth regulators can stimulate plants' development; hence, IAA (Indole Acetic Acid) is a growth regulator promoting growth (Wijayati et

al. 2005). According to Pranata (2010), the IAA hormone is one of the most significant types of auxin. These hormones perform various tasks, including accelerating plant growth, assisting in developing early roots, and promoting stem and leaf elongation. Exogenous administration of the IAA hormone is crucial for eliciting stimulation and effect, notably on plant growth and physiological parameters. Mandang (1993) believes that the combination of exogenous and endogenous auxin can accelerate root development.

Hormones must be administered at the appropriate dose; the hormone supports optimal plant development at ideal concentrations. For example, Mondal and Alam (2003) observed that employing IAA at a 200 ppm concentration yielded the greatest results for all *Allium cepa* L. growth parameters, including leaf number, bulb diameter, average bulb weight, and bulb yield. Therefore, *S. palmifolium* fertilized with IAA should demonstrate a physiological characteristic of accelerated growth.

The objectives of this study were (i) to ascertain the effect of fertilization on the physiological characteristics of the *S. palmifolium*, (ii) to ascertain the effect of the IAA hormone on the physiological characteristics of the *S. palmifolium*, (iii) to ascertain the effect of fertilization and the IAA hormone on the physiological characteristics of *S. palmifolium*, (iv) to ascertain the most effective combination of treatments for increasing *S. palmifolium* growth.

MATERIALS AND METHODS

Ingredient

The research was carried out at the Universitas Sebelas Maret Integrated Laboratory, FMIPA Biology Integrated Laboratory, Surakarta, Central Java, Indonesia, from October 2020 to March 2021. The material needed is *S. palmifolium*.

Procedure

The experiment design was a Completely Randomized Design (CRD) with a factorial pattern consisting of two factors, namely fertilization variation consisting of four levels and hormone concentration consisting of three levels, to obtain 12 treatment combinations. Fertilization consisted of soil (control), soil + chicken manure fertilizer (1:1), soil + chicken manure fertilizer + vermicompost fertilizer (1:1), and soil + chicken manure fertilizer + vermicompost fertilizer (1:1:1). The hormone concentrations were 0, 100, and 200 ppm.

Preparation of *S. palmifolium* bulb

The *S. palmifolium* is taken directly from the Kelurahan Besar, Pontianak District, West Kalimantan, Indonesia. The *S. palmifolium* bulbs were selected uniformly, i.e., with the same harvest age (± five months) and initial weight (5-12 grams).

Preparation of treatment media and hormone concentration

Planting media was soil with fertilizer in four types of treatments. As it was readied, it was put into polybags. The IAA hormone was made with a concentration of 100 ppm (0.1 gram) and 200 ppm (0.2 gram), which was added with a small amount of 70% alcohol solvent and 1 liter of distilled water.

Soaking *S. palmifolium* bulbs and planting

The *S. palmifolium* bulbs were soaked in IAA hormone according to the concentration of each treatment (0 ppm; 100 ppm; 200 ppm). Immersion is carried out at the same interval for 40 minutes (Alpriyan and Karyawati 2018). The soaking of the *S. palmifolium* bulbs was calculated using a stopwatch. The bulbs that had been soaked in the IAA hormone were then planted in polybags containing the media with the treatment. Finally, *S. palmifolium* bulbs were soaked on their basal plates.

Parameter measurement

The observed growth parameters included the number of leaves and the leaf width. Both measurements were carried out once a week until harvest time. The number of tiller bulbs was calculated at harvest, while the wet weight and shoot-root ratio were measured post-harvest.

Chlorophyll and carotenoid levels

The levels of chlorophyll and carotenoids in the leaves were carried out by taking *dayak* leeks. First, 0.1 grams was crushed and added with 70% alcohol for as much as 20 ml of the leaves. Next, the finely chopped leaves were filtered using a funnel in a test tube with Whatman filter paper no. 42 (Prastyo and Laily 2015). Next, the levels of chlorophyll and carotenoids in tubers were assessed by crushing 1 gram of tubers, adding 20 mL of 70% alcohol, and filtering with Whatman No. 42 filter paper. Next, the obtained filtrate was put into a cuvette of as much as 3 mL. Finally, the cuvette was inserted into a UV-Vis spectrophotometer with wavelengths of 480 nm, 645 nm, and 663 nm. According to Hendry and Grime (1993), the measurement of chlorophyll and carotenoid levels is as follows:

Chlorophyll a = 12.7(A.663) - 2.69(A.645)
Chlorophyll b = 22.9(A.645) - 4.68(A.663)
Total Chlorophyll Level = 8.02(A.663) + 20.2(A.645)

$$\text{Carotenoid } \mu\text{mol/g} = \frac{(A480 + 0.114 \times A663 - 0.638 \times A645) \times V \times 10^3}{112.5 \times 0.1 \times 10}$$

Flavonoid level

The quercetin standard was weighed at 0.06 mg, 0.08 mg, 0.010 mg, 0.012 mg, and 0.014 mg and dissolved in 10 mL of aquabides to produce a quercetin solution with a concentration of 6 ppm, 8 ppm, 10 ppm, 12 ppm, and 14 ppm. In addition, 1 mL of 2% $AlCl_3$ and 120 mM potassium acetate was added. At room temperature, samples were incubated for one hour. Then, at a maximum

wavelength of 435 nm, the absorbance was measured using the UV-Vis spectrophotometric method (Stankovic 2011).

Total flavonoid content of S. palmifolium *bulb*

The resulting extract was diluted in 1 mL of 96 % PA ethanol to dissolve any remaining extract residue on the porcelain cup. Next, the solution was transferred to a test tube, adding 1 mL of 2% $AlCl_3$ solution and 1 mL of 120 mM potassium acetate. At room temperature, samples were incubated for one hour. Finally, the absorbance was determined using the UV-Vis spectrophotometric method at a maximum wavelength of 435 nm (Stankovic 2011).

Data analysis

The data were analyzed using Analysis of Variance (ANOVA) and continued with Duncan's Multiple Range Test (DMRT) at the 5% test level to determine the significant difference between treatments.

RESULTS AND DISCUSSION

Number of leaves

Leaves are one of the plant organs that have an important role in photosynthesis. Observations on leaf organs included the number of leaves, leaf length, and leaf width. All three are important factors in plant growth. Therefore, the number of leaves is closely related to photosynthesis in plants. The number of leaves is calculated when the leaves are fully developed until harvest time. Data on the number of leaves are presented in Table 1.

The ANOVA findings indicated that fertilizer affected the number of leaves (Appendix 1). There was no effect of the IAA hormone on the number of leaves. This condition is most likely due to an insufficient concentration of hormones, which prevents the *S. palmifolium* plant from growing more leaves. The mixture of fertilizer and hormones substantially increased the number of leaves (Table 1). The largest number of leaves was seen when vermicompost (1:1) was combined with the IAA hormone 0 ppm (P2H0). The standard deviation for some treatments, such as P2H0, was excessive, owing to the death of some repetitions. Because vermicompost is high in nutrients, one of which is nitrogen, it influences the rising number of leaf parameters. Nitrogen absorbed by plants is necessary for the production and growth of vegetative parts such as leaves, which allows for a rise in the number of leaves in this study.

Additionally, vermicompost contains phosphorus and other nutrients necessary for developing plants' vegetative components. According to Rekha et al. (2018), vermicompost balances macro and micronutrients, and nutrient uptake benefits plant nutrition, growth, photosynthesis, and leaf chlorophyll content. As Mulat (2003) argues, applying vermicompost fertilizer can also improve the soil's physical properties, such as structure, permeability, water holding capacity, and chemical properties, such as boosting the soil's ability to absorb cations. Vermicompost can stimulate vegetative plant growth, particularly leaf development, increasing the number of leaves. The increased leaf count results in photosynthesis, which increases photosynthesis results. Figure 1 depicts the weekly average statistics for the number of leaves on the *S. palmifolium.*

Based on Figure 1, it can be seen that the number of leaves of *S. palmifolium* plants always increases every week. The available macronutrients supported the increase in the number of leaves. Still, in the final week of observation, several treatments experienced a decrease in the number of leaves caused by internal and external factors that affect plant growth and development. The main external influencing factor was climate change. Plant physiology is disturbed by climate change factors, such as extreme temperatures (too high or too low) and too high rainfall. The erratic weather could disrupt the nutrient transport process, which will affect the growth and development of the *S. palmifolium* plant.

Leaf length

Observation of leaf length is needed as an indicator of plant growth. Therefore, leaf length calculation was carried out on the leaves that appeared first. Leaf length data are presented in Table 1.

According to the ANOVA results, neither fertilizer nor IAA hormone had a statistically significant effect on leaf length. Additionally, the interaction of fertilizers and hormones had no significant influence on leaf length (Table 1). It could be because the plant requires macronutrients and micronutrients, which were not supplied by chicken manure or vermicompost. Cell division and differentiation could normally occur if sufficient macronutrients such as nitrogen were available during vegetative growth. The given IAA hormone concentration was less than optimal for improving leaf length characteristics. IAA is a hormone required for cell elongation and division. Mondal and Alam (2003) indicated the treatment of 200 ppm IAA hormone to *A. cepa* affected all growth metrics except plant height. Figure 2 depicts data on the weekly mean leaf length of the *S. palmifolium.*

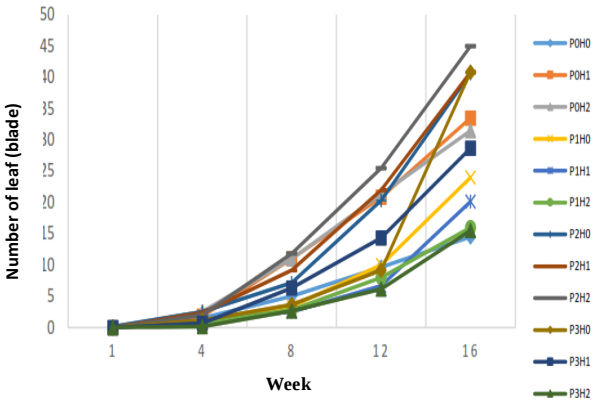

Figure 1. The weekly average number of leaves of *Sisyrinchium palmifolium* after fertilization and IAA hormone treatment

Table 1. Number of leaves of *Sisyrinchium palmifolium* at harvest after fertilization and IAA hormone treatment

Type of fertilizer	Hormone concentration (ppm) ± SD 0	100	200
Number of leaves			
P0	6.67 ± 6.42^{a}	25.00 ± 9.54^{abc}	9.33 ± 5.03^{a}
P1	19.00 ± 2.64^{ab}	23.67 ± 9.60^{abc}	42.33 ± 14.50^{cd}
P2	48.00 ± 18.52^{d}	39.33 ± 13.87^{bcd}	34.00 ± 15.72^{bcd}
P3	20.00 ± 12.12^{abc}	33.33 ± 14.50^{bcd}	20.33 ± 5.13^{abc}
Leaf length			
P0	31.00 ± 16.64	48.03 ± 0.06	40.67 ± 2.52
P1	42.67 ± 0.57	40.50 ± 4.36	43.50 ± 1.32
P2	43.10 ± 1.56	40.20 ± 4.70	43.17 ± 2.02
P3	33.73 ± 3.40	39.23 ± 1.25	42.33 ± 2.88
Leaf width			
P0	2.00 ± 0.86^{abcd}	2.43 ± 0.12^{cde}	1.50 ± 0.10^{a}
P1	2.20 ± 0.17^{bcde}	3.07 ± 0.11^{f}	2.73 ± 0.20^{ef}
P2	2.36 ± 0.32^{bcde}	1.83 ± 0.15^{abc}	1.80 ± 0.20^{ab}
P3	2.03 ± 0.25^{abcd}	2.76 ± 0.20^{ef}	2.50± 0.36^{def}
Flower number			
P0	0.00 ± 0	3.00 ± 6.16	5.80 ± 6.34
P1	0.00 ± 0	2.80 ± 3.42	2.20 ± 4.92
P2	4.00 ± 4.64	4.00 ± 5.48	3.20 ± 4.32
P3	2.80 ± 3.27	0.00 ± 0	0.60 ± 1.34
Number of tillers			
P0	3.60 ± 3.51^{a}	8.00 ± 1.73^{cd}	7.20 ± 2.39^{bcd}
P1	7.00 ± 1.60^{abc}	8.00 ± 2.12^{cd}	3.80 ± 3.11^{ab}
P2	8.80 ± 2.77^{cd}	10.80 ± 2.77^{d}	8.40 ± 1.52^{cd}
P3	6.20 ± 2.49^{abc}	9.20 ± 2.86^{cd}	6.60 ± 2.30^{abc}
Wet weight (grams)			
P0	19.05 ± 14.76 ^{ab}	42.23 ± 15.44^{cd}	42.54 ± 6.74^{cd}
P1	20.82 ± 10.16^{ab}	26.69 ± 2.07^{b}	15.24 ± 4.87^{a}
P2	38.06 ± 1.59^{c}	37.96 ± 1.65^{c}	48.20 ± 1.22^{d}
P3	18.32 ± 2.28^{ab}	27.21 ± 0.61^{b}	13.72 ± 0.54^{a}
Dry weight (grams)			
P0	8.39 ± 6.50^{ab}	16.20 ± 3.37^{f}	17.28 ± 2.06^{f}
P1	8.46 ± 0.88^{ab}	11.20 ± 0.86^{bcd}	6.65 ± 3.35^{a}
P2	14.95 ± 1.99^{ef}	12.23 ± 0.83^{cde}	23.14 ± 0.48^{g}
P3	9.06 ± 4.56^{abc}	13.83 ± 1.65^{def}	6.44 ± 1.07^{a}
Shoot-root ratio			
P0	0.27 ± 0^{a}	0.54 ± 0.05^{a}	0.30 ± 0.05^{a}
P1	1.97 ± 0.35^{bc}	2.86 ± 0.27^{de}	1.84 ± 0.09^{bc}
P2	1.55 ± 0.13^{b}	1.49 ± 0.01^{a}	2.43 ± 0.13^{cd}
P3	2.77 ± 1.5^{de}	3.46 ± 0.32^{f}	2.53 ± 0.25^{cd}
Total chlorophyll (mg/g) leaves			
P0	10.9869 ± 3.90^{d}	3.2172 ± 1.69^{abc}	4.0710 ± 1.14^{abc}
P1	2.0761 ± 1.52^{a}	2.5562 ± 0.05^{ab}	3.3856 ± 1.24^{abc}
P2	3.7365 ± 1.15^{abc}	3.9356 ± 1.26^{abc}	6.2437 ± 0.73^{c}
P3	4.2247 ± 2.61^{abc}	3.0112 ± 0.80^{abc}	5.8188 ± 2.97^{bc}
Total chlorophyll (mg/g) bulbs			
P0	2.5249 ± 0.85^{a}	3.5163 ± 1.03^{abc}	6.1868 ± 0.41^{d}
P1	5.6906 ± 2.46^{d}	4.9855 ± 2.04^{bcd}	5.3133 ± 1.25^{cd}
P2	3.4882 ± 0.21^{abc}	1.5498 ± 0.31^{a}	2.3588 ± 1.10^{a}
P3	1.8735 ± 0.15^{a}	3.0276 ± 0.63^{ab}	2.5661 ± 0.36^{a}
Carotenoids (mg/g) leaves			
P0	18.5559 ± 4.53^{cd}	12.2481 ± 1.83^{abc}	10.6084 ± 3.29^{ab}
P1	7.3706 ± 1.22^{a}	7.5383 ± 1.17^{a}	11.1962 ± 3.22^{ab}
P2	14.4574 ± 2.70^{bcd}	15.9487 ± 3.57^{bcd}	19.1647 ± 6.96^{d}
P3	12.1711 ± 0.52^{abc}	14.3905 ± 2.90^{bcd}	13.3263 ± 4.38^{abcd}
Carotenoids (mg/g) bulbs			
P0	57.3879 ± 4.97^{cd}	54.5748 ± 9.22^{bcd}	61.5276 ± 1.95^{d}
P1	44.1941 ± 14.38^{abcd}	37.1034 ± 2.66^{ab}	39.0421 ± 17.26^{ab}
P2	39.5984 ± 1.63^{abc}	35.9126 ± 8.97^{a}	41.4039 ± 15.47^{abc}
P3	30.9099 ± 7.27^{a}	42.6914 ± 0.41^{abc}	44.3153 ± 8.35^{abcd}
Levels of flavonoid (mgQE/g)			
P0	8.3176 ± 0.30^{a}	8.3994 ± 0.21^{ab}	8.4847 ± 0.22^{abc}
P1	8.5458 ± 0.19^{abc}	8.4462 ± 0.17^{ab}	8.6766 ± 0.15^{abc}
P2	8.8454 ± 0.44^{c}	8.7593 ± 0.05^{bc}	8.2912 ± 0.14^{a}
P3	8.4113 ± 0.16^{ab}	8.5419 ± 0.11^{abc}	8.3588 ± 0.06^{ab}

Note: Numbers accompanied by the same letter in the row/column show no significant difference (P>0.05) in the 5% DMRT test. P0 : Ground (Control). P1 : Soil + Chicken manure Fertilizer (1:1) P2 : Soil + Vermicompost (1:1). P3 : Soil + Chicken Manure + Vermicompost (1:1:1) SD: Standard deviation

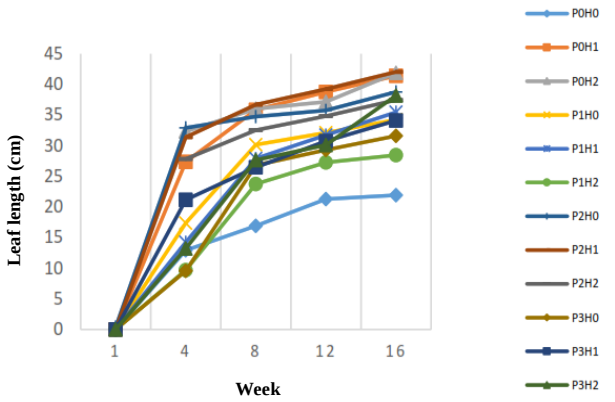

Figure 2. Weekly average of leaf length of *Sisyrinchium palmifolium* after fertilization and IAA hormone treatment

As illustrated in Figure 2, the length of the leaves of the *S. palmifolium* plant rises week after week. In contrast, the leaves that were initially generated appear to fall after the 16th week in some treatments. Compared to other plant organs, leaves have a limited growth rate (they grow to a particular size and then stop abruptly) (Loveless 1991). The nutrients collected in the early to midweeks of the plant period were sufficient to carry out cell elongation. However, the nutrients obtained began to deplete dramatically in the last week. The decreased nutrients taken by the *S. palmifolium* plant and other environmental conditions resulted in a fall in leaf length parameters over the last week.

Leaf width

Besides being a place for photosynthesis, leaves also serve as respiration organs. Additionally, the leaf width parameter must be considered. Leaf width and leaf length metrics are inextricably linked. Table 1 contains statistics on leaf width.

ANOVA revealed that fertilizer or IAA hormone affected leaf width, and combining fertilizers and hormones increases leaf width (Table 1). The interaction between manure treatment and 100 ppm IAA hormone (P1H1) resulted in the greatest leaf width value. The control combination with 200 ppm IAA hormone (P0H2) resulted in the smallest leaf width value of 1.5 cm. Sutedjo (2008) asserts that chicken manure contains the highest concentration of nitrogen required by plants to establish vegetative parts such as leaves. Hanaa and Safaa (2019) found that 100 ppm IAA increased plant growth and physiological parameters such as leaf area and chlorophyll content. Figure 3 shows data on the weekly mean leaf width of the *S. palmifolium.*

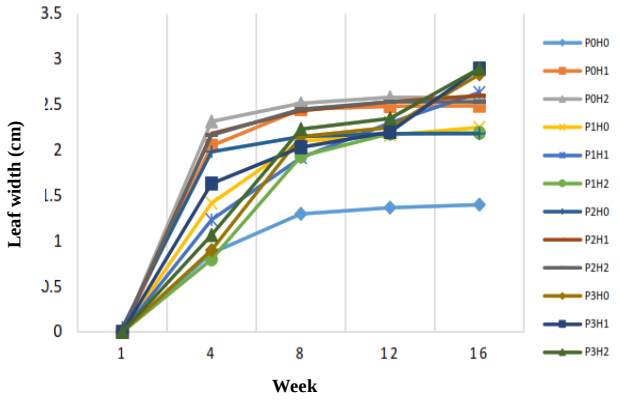

Figure 3. The weekly mean of leaf width of *Sisyrinchium palmifolium* after fertilization and IAA hormone treatment

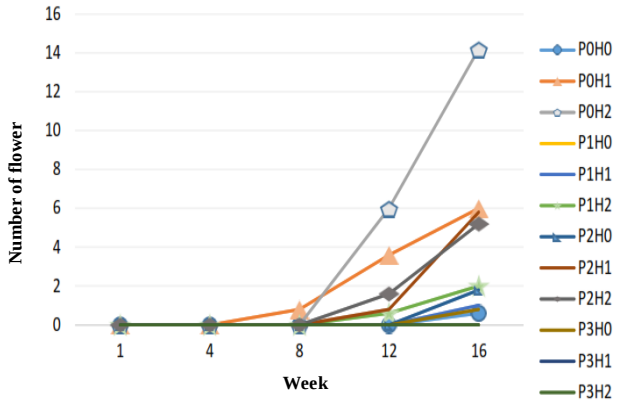

Figure 4. Average flower number of *Sisyrinchium palmifolium* at the end of fertilization and IAA hormone treatment

Based on Figure 3, the width of the leaves of the *S. palmifolium* plant always increases, but after the 16th week, some of the leaves were found to fall in the last week of observation due to limited growth. The nutrients absorbed from the fertilizer content began to decline, resulting in a drop in leaf width at the end of the observation. External growth and development factors such as temperature and sunlight also contributed to a reduction in leaf width.

In general, fertilization and the IAA hormone affected the number of leaves and leaf width of *S. palmifolium* plants. However, chicken manure or vermicompost could stimulate plant growth compared to controls because both types of fertilizers had enough nutrients to stimulate *S. palmifolium* growth. Additionally, the IAA hormone could boost plant growth parameters. Srivastava (2002) asserts that this hormone regulates numerous aspects of plant growth and development.

Number of flowers

Flowers are a form of self-reproduction. Therefore, metrics relating to the number of flowers were observed from blossoming through harvest. In general, fertilization and Indole Acetic Acid hormone did not affect flowering characteristics. Table 1 contains information on the amount of interest.

According to the ANOVA results, neither fertilizer nor the IAA hormone affected the flowering (Appendix 4). Additionally, the interplay of fertilizers and hormones did not influence the number of flowers (Table 1). It could be because fertilization and the IAA hormone have a reduced effect on flowering. Fertilizer increases the availability of phosphorus (P) nutrients, which stimulates flowering, but if applied excessively, it can also impede the *S. palmifolium* plant's generative phase. The *S. palmifolium* plant's flower development is not boosted by nutrition and hormone absorption. Internal and external factors have a greater influence on the appearance of flowers than the treatment used. Figure 4 illustrates the average number of flowers on the *S. palmifolium* plant.

As illustrated in Figure 4, the flowering amount always increases each week. Flowers begin to appear on *S. palmifolium* plants in week 8 or within two months. The control treatment with 200 ppm IAA hormone produced optimum results compared to other treatments. The presence of flowers shows that the *S. palmifolium* plant has entered a reproductive phase. Therefore, the hormone IAA at a concentration of 200 ppm is considered ideal for flower development. The IAA auxin hormone could influence the growth of roots, stems, and flowers (Agboola et al. 2014). It exhibited the highest graph on the number of flowers in the soil treatment alone (the control), likely due to the inhibited nutrients in fertilizers, such as phosphorus (P). Phosphorus stimulates the production of various proteins and accelerates the development of flowers and fruit.

Number o tiller bulbs

The number of tiller bulbs on a plant is a measure to indicate the health of the plant. The quantity of tiller bulbs harvested reflects the plant's excellent output. Fauzi et al. (2018) state that onion plants have a disc-shaped basal plate. The basal plate contains buds that can develop into lateral shoots or tillers; these tillers can generate new basal plates and bulbs. Table 1 contains information on the number of tiller bulbs.

According to the ANOVA results, the fertilizer and the IAA hormones affected the number of tiller bulbs produced by *S. palmifolium* plants (Appendix 5), contributing to crop production growth. Fertilizer-hormone interactions also affected all treatments (Table 1). The interaction between vermicompost fertilizer treatment and the IAA hormone 100 ppm (P2H1) resulted in the maximum number of tillers, whereas the control (P0H0) resulted in the lowest number of tillers (3.60). It was because vermicompost contained more NPK nutrients than chicken manure. The fertilizer application and the IAA hormone at 100 ppm were supposed to be best for raising the number of *S. palmifolium* tillers. Bulb formation would increase in a favorable climate, allowing bulbs to create new shoots and grow and develop into tillers. The average number of tiller bulbs produced by the *S. palmifolium* plant is depicted in Figure 5.

As shown in Figure 5, the highest yield was achieved with vermicompost fertilizer combined with 100 ppm of the IAA hormone. Vermicompost contains a variety of vital macronutrients, one of which is important for meristematic growth and the synthesis and transfer of photosynthetic products for production and storage in plants (seeds, fruits, and tubers), particularly potassium (K) (Havlin et al. 2005). Because the roots could take the 100 ppm of IAA hormone, optimal root conditions influenced the effective absorption of nutrients necessary for the *S. palmifolium* plant's development and productivity. In addition, optimal light intensity and environmental conditions were critical for creating blooms and bulbs in *S. palmifolium* seedlings.

Wet mass

Wet weight is a growth parameter that is often used and important to study. Therefore, the wet weight of the plant was measured after harvesting before the plant lost water. Table 1 contains information about the wet weight of *S. palmifolium* plants.

ANOVA revealed that fertilizer influenced the wet weight of the *S. palmifolium* plant. In addition, the IAA hormone increases wet weight. Therefore, the interplay of fertilizer and hormones affects the *S. palmifolium* plant's wet weight (Table 1). For example, adding vermicompost fertilizer with the IAA hormone at 200 ppm (P2H2) resulted in the highest wet weight value. In contrast, the lowest treatment consisted of a mixture of soil amended with chicken manure and vermicompost containing IAA hormone at 200 ppm (P3H2).

The amount of water absorbed by plants, particularly bulbs, affects the rise in wet weight. For example, the lowest yield of 13.72 grams was obtained when the three treatments were combined with the IAA hormone at 200 ppm (P3H2). On the other hand, the wet weight of the plant indicated high yields due to the fast rate of water absorption, which promoted cell elongation and expansion. The wet weight is determined by the water content of the harvested *S. palmifolium* plant. Figure 6 shows data on the average wet weight of *S. palmifolium* plants.

Based on Figure 6, adding vermicompost fertilizer with the IAA hormone at 200 ppm showed the highest yield. Vermicompost fertilizer is rich in N, P, and K nutrients, which *S. palmifolium* plants need for growth and development. According to Fauzi et al. (2018), sufficient nutrient content causes biosynthesis to run smoothly so that the produced carbohydrates are greater, increasing the wet weight of the plant. Therefore, 200 ppm of IAA hormone could support plant growth and trigger cell enlargement, providing the highest graphic results.

Dry weight

Growth can also be measured by measuring plant dry biomass. Dry weight indicates the accumulation of organic compounds that plants have successfully synthesized from inorganic compounds. The accumulation in question is especially CO_2 carried out during plant growth and development. Dry weight data are presented in Table 1.

Based on the ANOVA results, it was shown that fertilizer affected the dry weight of the *S. palmifolium* plant (Appendix 7). In addition, the IAA hormone affects increasing dry weight parameters. Therefore, the interaction of fertilizers and hormones affects the dry weight of *S. palmifolium* plants (Table 1). The addition of vermicompost fertilizer with the IAA hormone at 200 ppm (P2H2) showed the highest dry weight value. The lowest treatment was a combination of soil added with both types of fertilizers with the IAA hormone at 200 ppm (P3H2) of 6.44 grams. Data on the average dry weight of *S. palmifolium* plants are presented in Figure 7.

Figure 7 shows that adding vermicompost fertilizer with the IAA hormone at 200 ppm showed high yields. Vermicompost fertilizer provides nutrients in balanced amounts and increases the organic matter content to benefit plants. The availability of nutrients increases the rate of photosynthesis so that photosynthate can increase, and photosynthate can be translocated to all parts of the plant; as a result, it can increase dry weight (Dwijoseputro 1981). According to Nurhidayati et al. (2019), the dry weight of plants is a representation of wet weight, which is a condition that states the amount of accumulation of organic matter contained in plants without the water content.

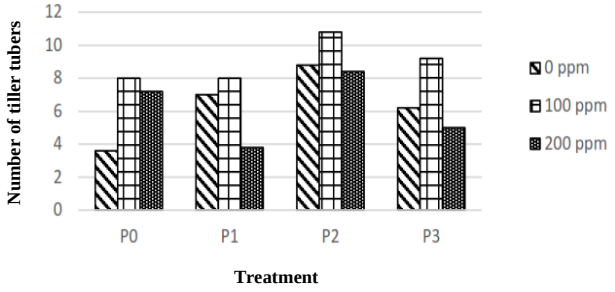

Figure 5. The average number of tiller bulbs of *Sisyrinchium palmifolium* at the end of fertilization and IAA hormone treatment

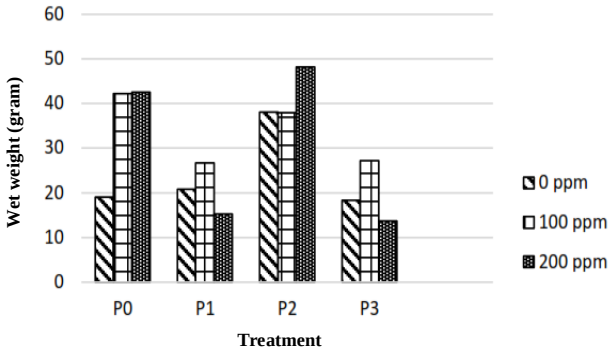

Figure 6. Average wet weight of *Sisyrinchium palmifolium* after fertilization and IAA hormone treatment

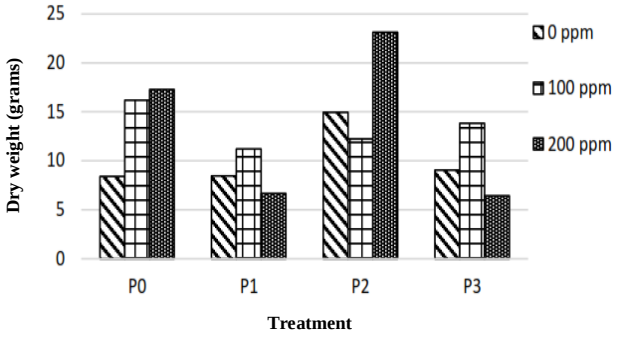

Figure 7. The average dry weight of *Sisyrinchium palmifolium* after fertilization and IAA hormone treatment

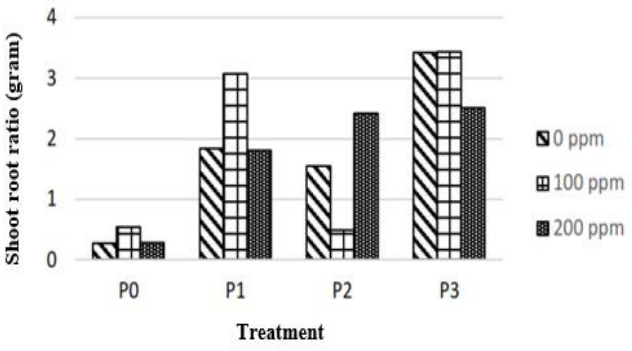

Figure 8. The average shoot-root ratio of *Sisyrinchium palmifolium* after fertilization and IAA hormone treatment

Organic fertilizers such as vermicompost are rich in nutrients to support growth and development to maximize the process of water transport to all parts of the plant and can affect the wet and dry weight. The sunlight intensity is also one factor that can increase the wet and dry weight of *S. palmifolium* plants. Heavy rainfall occurs almost every day in the last two months of the planting season. It is thought to trigger the sub-optimal size of the bulb weight due to the inhibition of plant metabolic processes. Rosmawaty et al. (2019) determined that the optimal dry weight of the *S. palmifolium* plant (5 months harvesting age) is between 19.00 and 23.00 grams.

Plant root canopy ratio

Wet weight, dry weight, and shoot-root ratio on *S. palmifolium* generally showed varying results. The shoot-root ratio shows the growth balance between the crown and roots. The shoot-root ratio value in this study was not too large because the growth of the *S. palmifolium* plant was more dominant towards the roots. The shoot-root ratio data can be presented in Table 1.

Based on the ANOVA results, fertilizer increased the shoot-root ratio of *S. palmifolium* plants. On the other hand, the Indole Acetic Acid hormone did not affect the shoot-root ratio. Therefore, the interaction of fertilizer and hormones increases the shoot-root ratio of *S. palmifolium* plants (Table 1). The combination treatment of soil added with chicken manure and vermicompost (1:1:1) with the IAA hormone at 100 ppm (P3H1) showed the highest shoot-root ratio value. In contrast, the lowest treatment was the control (P0H0) of 0.27. The data on the average shoot-root ratio of the *S. palmifolium* is presented in Figure 8.

Figure 8 shows the result the ratio that was undergone the treatment was higher than the control. Soil treatment plus both types of fertilizers with the IAA hormone at 100 ppm showed the graph of the highest shoot-root ratio. Both manure and compost contain high N, P, and K elements, so their interaction can give high yields on shoot and root weight and shoot-root ratio. Therefore, the concentration of the IAA hormone at 100 ppm was thought to be the optimum concentration for increasing the shoot-root ratio of *S. palmifolium* compared to other concentrations.

Total chlorophyll level

Chlorophyll plays an important role in photosynthesis, and the pigment is needed in a plant's photosynthesis. Therefore, the parameters of total chlorophyll and carotenoids measured were obtained from the leaves and bulbs of the *S. palmifolium*. Data on the total chlorophyll content of leaves and bulbs are presented in Table 1.

The ANOVA results showed that fertilizer or IAA hormone affected the total chlorophyll count of *S. palmifolium* leaves (Appendix 9). Therefore, the interaction of fertilizers and hormones affects the total chlorophyll of *S. palmifolium* leaves (Table 1). The control treatment (P0H0) showed the highest total leaf chlorophyll value, while the lowest treatment was the addition of chicken manure with the IAA hormone at 0 ppm (P1H0), which was 2.0761 mg/g. Data on the average total chlorophyll of the leaves of *S. palmifolium* are presented in Figure 9.

The results of ANOVA showed that fertilizer affected the total chlorophyll count of the *S. palmifolium* bulb (Appendix 10). The IAA hormone did not affect the total chlorophyll of bulbs. The interaction of fertilizers and hormones affects the total chlorophyll of the *S. palmifolium* bulb (Table 1). The control treatment with IAA hormone at 200 ppm (P0H2) showed the bulbs' highest total chlorophyll value. The lowest treatment was the addition of vermicompost fertilizer with 100 ppm IAA hormone (P2H1) of 1.5498 mg/g. The IAA hormone at 200 ppm is thought to favor chlorophyll formation. Data on the average total chlorophyll of the *S. palmifolium* bulb are presented in Figure 9.

Figure 9 shows that the control treatment had a high yield of leaf chlorophyll, while the control combination with 200 ppm IAA hormone showed the highest yield of bulb chlorophyll. Leaves play a very important role in the photosynthesis process because they contain chloroplasts, so the chlorophyll content in the leaves is greater than the bulbs. The control treatment (soil only) showed high yields compared to the treatment with the addition of fertilizer, even though chicken manure or vermicompost was rich in nutrients. These results were caused by the condition of the *S. palmifolium* plants harvested for further testing. The control treatment showed a greener color than the other

treatments. It is because treatment leaves in some parts are deficient in nutrients (yellowing). Muhuria et al. (2006) reported that the greener a leaf, the higher the chlorophyll content. Nutrients that play a role in photosynthesis, such as phosphorus absorption, may be low, so the formation of chlorophyll is disrupted. IAA hormone synthesis may also indirectly affect the chlorophyll content.

Carotenoid level

Carotenoids are companion pigments for chlorophyll, which play a role in absorbing light energy for photosynthesis. Carotenoids are a group of yellow, orange, or red pigments commonly found in plants. The content of carotenoids and chlorophyll vary in plant species. Leaf and bulb carotenoid data are presented in Table 1.

Based on the results of ANOVA, it was shown that fertilizer affected the carotenoid content of the leaves of the *S. palmifolium* plant. On the other hand, the IAA hormone did not affect leaf carotenoids. It is presumably because the exogenously applied IAA hormone was less able to trigger an increase in leaf carotenoid levels. The interaction of fertilizers and hormones affects leaf carotenoids (Table 1). The addition of vermicompost fertilizer with the IAA hormone at 200 ppm (P2H2) showed the highest leaf carotenoid values, while the combination treatment with chicken manure with the IAA hormone at 0 ppm (P1H0) was the lowest at 7.3706 mg/g. The average data for carotenoids in the leaves of *S. palmifolium* is presented in Figure 10.

ANOVA showed that fertilizer affected the carotenoid content of *S. palmifolium* bulbs. IAA hormone did not affect tuber carotenoids. IAA hormone transport to affect the increase in tuber carotenoid levels may not run optimally, and the concentration of hormones received by plants is less than optimal. According to Asra et al. (2020), after being synthesized, auxin will be translocated to all parts of the plant, but not all parts get the same concentration. The part close to the synthesis site gets higher auxin, while the high concentration of IAA hormone can inhibit the formation of carotenoids. The interaction of fertilizers and hormones affects the carotenoids of tubers (Table 1). The control treatment with the hormone IAA at 200 ppm (P0H2) showed the highest tuber carotenoid values, while the interaction of the combination of chicken manure and vermicompost (1:1:1) with the hormone IAA at 0 ppm (P3H0) was the lowest at 30.9099 mg/g. The average carotenoid data of *S. palmifolium* bulbs shows in a histogram in Figure 10.

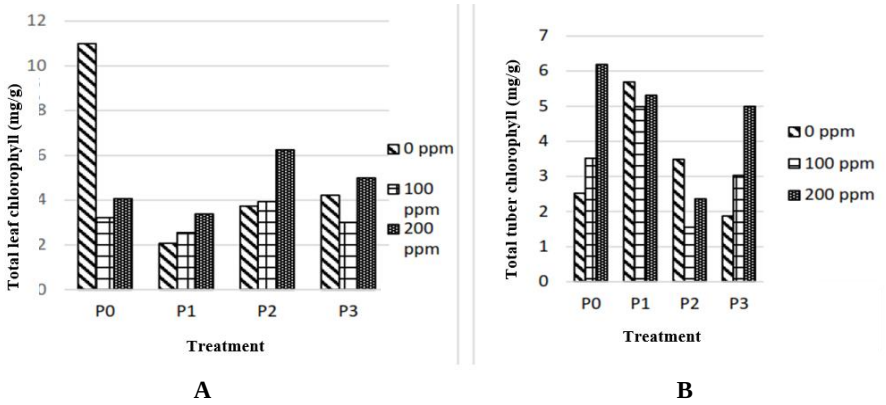

Figure 9. Average total leaf chlorophyll (A), total chlorophyll of *Sisyrinchium palmifolium* bulb (B) after fertilization, and IAA hormone treatment

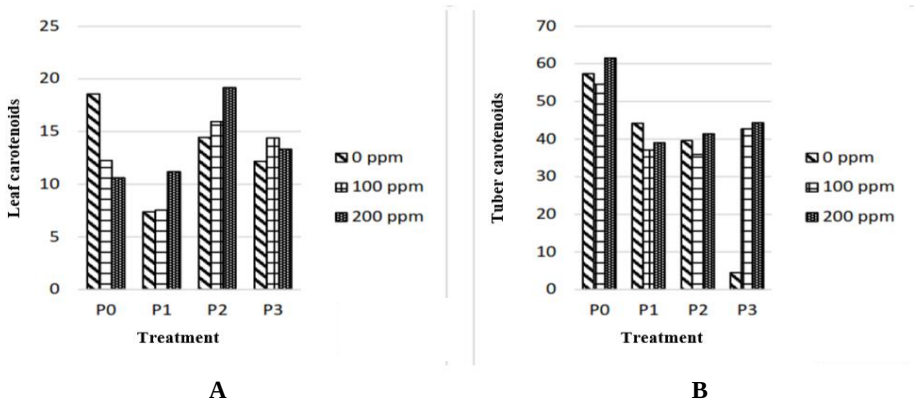

Figure 10. Average leaf carotenoids (A), bulb carotenoids of *Sisyrinchium palmifolium,* (B) after fertilization and IAA hormone treatment

Figure 10 shows that the highest graph of leaf carotenoids was shown in the addition of vermicompost fertilizer with 200 ppm IAA hormone. The control combination treatment with 200 ppm IAA hormone was the highest graph for bulb carotenoids. The carotenoid content was greater in the *S. palmifolium* bulbs because the red pigment of the bulbs was very dominant. Vermicompost fertilizer application can improve soil structure so that the root system and nutrient absorption process run more optimally. Optimal absorption of nutrients can cause physiological processes to run well, so the carotenoid levels are quite large. In another study, the application of exogenous IAA also increased the concentration of photosynthetic pigments, including total chlorophyll and carotenoids (Singh and Prasad 2015).

Carotenoids as photosynthetic light energy absorbers generally increase when the chlorophyll content decreases (Yang et al. 2014). Chlorophyll and carotenoids are labile and easily degraded. Internal and external factors in plants affect the levels of chlorophyll and carotenoids. For example, environmental temperatures that are too extreme due to climate change can inhibit the formation of pigments because various environmental factors easily degrade, such as chlorophyll.

Bulb flavonoid levels

Flavonoids are one of the secondary metabolites of plants that have an important role. The flavonoid content of the *S. palmifolium* extract was measured at a wavelength of 435 nm. The flavonoid levels were calculated using the quercetin curve's previously measured linear regression equation. Based on the measurement results of the quercetin standard curve, the equation y = 0.1544x + 1.7821 with a correlation value (R^2) = 0.9684, a correlation value close to 1 indicates that there is a relationship between the concentration of quercetin solution and the absorbance value. According to Kelly (2011), quercetin is a standard solution because it belongs to the flavonoid group, which can react with $AlCl_3$ to form complexes. The measurement of flavonoid levels shows in Table 1.

ANOVA results showed fertilizer or IAA hormone did not affect flavonoid levels in *S. palmifolium* plants (Appendix 12). It could be due to the plant's inability to carry out IAA hormone transport optimally and the long stage of flavonoid formation. The interaction of fertilizers and hormones affects the levels of flavonoids (Table 1). The interaction treatment of vermicompost fertilizer with IAA hormone 0 ppm (P2H0) showed the highest flavonoid value, while the addition of vermicompost fertilizer (1:1) with the IAA hormone at 200 ppm (P2H2) the lowest was 8,291 mgQE/g. Data on the average flavonoid content of *S. palmifolium* is presented in Figure 11.

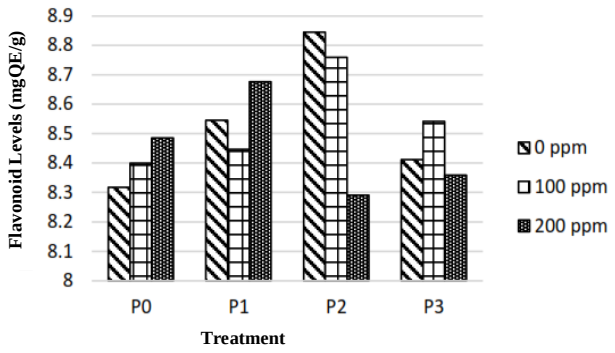

Figure 11. Average flavonoid content of *Sisyrinchium palmifolium* after fertilization and Indole Acetic Acid hormone treatment

Figure 11 shows that adding vermicompost fertilizer with 0 ppm IAA hormone showed the highest flavonoid content. Vermicompost fertilizer showed the highest yield on the flavonoid content of *S. palmifolium* bulbs. It could be because this fertilizer was rich in nutrients, so it could trigger the formation of secondary metabolites. In a different study by Putri et al. (2018), vermicompost fertilizer gave the best results and could increase the content of flavonoid compounds. High N uptake also encourages the enzymes that form flavone compounds to run more optimally to increase the phenolic and flavonoid content. The high flavonoid content proves that the *S. palmifolium* is a medicinal plant that can be used to treat various diseases. The lowest flavonoid levels were found at 200 ppm IAA hormone concentration. The administration of the IAA hormone is thought to be more directed at increasing the growth and development of the *S. palmifolium* plant so that it does not affect the secondary metabolite content of *S. palmifolium* bulbs (flavonoids). Precursors in IAA formation (tryptophan) may also begin to decrease. Their synthesis does not run optimally, causing the hormone to have less effect on the biosynthesis of secondary metabolites (flavonoids).

This study concluded that (i) fertilization affected leaf width, wet weight, dry weight, total chlorophyll content, leaf and bulb carotenoids, and flavonoid content. In addition, fertilizer affected the increase of the number of leaves, the number of tiller bulbs, and shoot-root ratio; (ii) IAA (Indole Acetic Acid) hormone affected increasing leaf width, the number of tiller bulbs, wet weight, and dry weight; (iii) the interaction of fertilization and the IAA (Indole Acetic Acid) hormone increase the number of leaves, leaf width, shoot-root ratio, and levels of flavonoids in *S. palmifolium* bulbs; (iv) the most optimal combination of treatments to increase the growth of *S. palmifolium* was a combination of soil and vermicompost fertilizer (1:1) with the IAA hormone of 200 ppm concentration (P2H2). The highest flavonoid level was in the combination of vermicompost fertilizer treatment with 0 ppm hormone (P2H0).

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Diversity of plant-parasitic nematodes in potato fields at different altitudes in Probolinggo District, East Java, Indonesia

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Abstract. *Prada AP, Pratiwi GNC, Wagiyana. 2022. Diversity of plant-parasitic nematodes in potato fields at different altitudes in Probolinggo District, East Java, Indonesia. Cell Biol Dev 6: 61-67.* Plant parasitic nematodes (PPNs) are microorganisms sensitive to environmental conditions. Therefore, plant parasitic nematode diversity in a field may affect the decision of effective management tactics. This study aimed to assess the diversity of plant parasitic nematodes in potato fields at three different altitudes. The study was carried out in Probolinggo District, East Java, Indonesia. The sampling area was divided into three groups based on altitude variations, namely 1,008 m asl., 1,413 m asl., and 1,875 m asl. Soil samples were collected randomly in a zigzag pattern. Samples were collected from 20 points across each field at a depth of 20-30 cm below the soil surface. Each sub-sample included up to 500 g of soil. The white head tray method extracted nematodes from 500 g of soil from each subsample. After characterizing the extracted nematodes, the absolute density and dominance values were determined. The results revealed variations in the three areas with different altitudes. The total number of plant-parasitic nematodes (PPN) discovered on the field at an altitude of 1,008 m asl. was 57.00 ± 16.43. Furthermore, at an altitude of 1,413 m asl., the total number of PPNs discovered is 41.67 ± 10.98. The total number of nematodes on the field at an altitude of 1,875 m asl. was 50.50 ± 12.60. The findings of this study also indicated that *Meloidogyne* sp. was the genus with the highest population on the field, at an altitude of 1,008 m asl. On the other hand, *Pratylenchus* sp. is the most abundant nematode in the field between 1,413 and 1,875 m asl. An identical situation also occurred with the nematode dominance values in the three fields studied.

Keywords: Abiotic, dominance, elevation, environment, population

INTRODUCTION

Potato is one of the most important commodities produced in Indonesia, and it now holds the fourth spot in terms of the top agricultural value in the horticulture industry. Probolinggo District, located in East Java Province, is one of the major production hubs for potatoes in Indonesia. However, the production of potatoes in numerous highland areas is suffering due to several issues. According to research data, fungal, bacterial, and nematode diseases are significant challenges in potato cultivation. Fungi such as *Phytophthora infestans* have caused massive losses in potato production worldwide. This fungal infection can swiftly induce blight symptoms and plant mortality (Nowicki et al. 2012). Pathogenic bacteria, such as *Ralstonia solanacearum*, have also been linked to yield losses in the field and post-harvest potatoes (Kurabachew and Ayana 2017). According to the data, infection with plant-parasitic nematodes (PPNs) is also a severe threat to potato production. Infections with nematodes in potato plants have been recorded globally. Even countries with modern agricultural technologies, such as the United States, the Netherlands, Germany, Japan, and Australia, are exposed to nematode infection in their potato fields (Jones et al. 2013). That demonstrates that PPNs are a significant threat to potato production globally (İmren 2018; Maleita et al. 2018; Orlando et al. 2020).

Infection by plant parasitic nematodes in various horticulture products results in common symptoms like wilting, reduced plant development, and lower yield (Coyne et al. 2018). Certain nematodes, such as those belonging to the genus *Meloidogyne*, can produce particular symptoms, such as the development of gall on the roots. It has been found that potato cyst nematodes develop syncytium in root tissue (Collange et al. 2011). In addition, nematodes of the genera *Pratylenchus* and *Radopholus* are endoparasitic migratory nematodes (Sarah 1989). The feeding activity of *Pratylenchus* and *Radopholus* on root tissue may directly destroy root tissue (Riley and Wouts 2001). Infection with ectoparasitic nematodes such as *Xiphinema* can reduce the length of root hairs (Van Zyl et al. 2012). These symptoms have a direct impact on the capacity of plants to absorb soil nutrients and water. As a result, the plant will eventually lack nutrients and undergo stunted development. In addition, a reduction in a plant's ability to absorb nutrients and water makes it more vulnerable to infection by other pathogens (Chitwood 2003). *Globodera* sp. and *Meloidogyne* sp. have been identified as the nematodes that pose the most issue in potato production worldwide. Depending on the plant variety, the environmental conditions, and population density, both nematodes can reduce yields by 30% to 75% (Collange et al. 2011; Trudgill et al. 2014).

Nematode infestation in potato fields has reached a serious point and must be dealt with immediately.

Nematode infections may even be responsible for a potential loss of up to 80 billion dollars worldwide (Kumar et al. 2020). The *Meloidogyne* species is responsible for root gall symptoms in most horticultural plants and most commonly infects potato plants (Onkendi et al. 2014; Rusinque et al. 2021). Potato tubers infected with these nematodes reportedly indicate symptoms similar to scabies (Majeed and Muhammad, 2018). Aside from *Meloidogyne* sp., *Globodera* sp. is another common nematode affecting potato crops (Trudgill et al. 2014; Mwangi et al. 2015).

A variety of biotic and abiotic variables regulates the PPNs diversity. The kind of plant, the age of the plant, and the presence of other microorganisms in the rhizosphere are all biotic variables that influence the diversity and abundance of nematodes in a field (Coyne et al. 2018; Silva et al. 2018). Furthermore, the diversity of nematodes in the soil is influenced by soil type, humidity, temperature, altitude, and other physical and chemical conditions surrounding plant roots (Mateille et al. 2014). For example, potato fields in the low-temperature highlands have a greater *Globodera* sp. infection rate than those in the high-temperature lowlands (Phillips et al. 2015). In addition, *Meloidogyne graminicola* is typically more prevalent in rice fields with sandy soils. Nematodes are typically found in lower population density in loamy soils, another phenomenon supporting the claim that environmental factors influence the diversity of nematodes (Bouwman and Arts 2000; Kandji et al. 2001).

The presence of PPNs on potato plants in Indonesia has been documented in some prior studies; these studies indicated that major potato-growing regions across the country, including Wonosobo, Batu City, and several locations in West Java, were all likely to be infected by nematodes (Aprilyani et al. 2015; Syafii et al. 2018). However, in Indonesia, the diversity of PPNs that infect potato plants and their diversity at various altitudes still needs further study.

Nematodes are highly sensitive to environmental changes. Therefore, the nematodes that predominate at various altitudes may be of distinct species. Considering the unique lifespan of nematodes, it is important to observe information on the diversity at various field altitudes. Different species of nematodes that infest a field may necessitate distinct management strategies (Ralmi et al. 2016). For example, ectoparasitic and endoparasitic nematodes would require specific management measures. In addition, the prevalent nematode genus or species will influence the selection of nematicide. For instance, certain active nematicide components are effective against *Meloidogyne* and *Globodera* species but less against *Pratylenchus* species (Ebone et al. 2019). In contrast, trap crops and crop rotation significantly impact the nematode genus to be managed. Some nematode species resist control with trap plants (Vestergård 2019). Also, crop rotation does not affect some nematodes if the plant used in crop rotation is still the nematode's host plant (Nusbaum and Ferris 1973).

In light of the information presented above, it is necessary to research the inventory of plant-parasitic nematodes in potato fields located at different elevations. This study is important since there has yet to be a study done on the identification and inventory of nematodes in the Probolinggo District at the various field elevations before this investigation.

MATERIALS AND METHODS

Study area

The research was conducted between December 2020 and June 2021. Three potato fields in Probolinggo District, East Java Province, Indonesia, were sampled for this study. The first land was located at 7° 55' 37.0"S 113° 07' 27.0"E and was 1,008 meters above sea level. The second land was located at 7°57'22.0"S 113°03'36.0" E, at an altitude of 1,413 m asl. Furthermore, the third land has coordinates of 7°58'02.0"S 113°00'52.0" E and an 1,875 m asl. altitude. Nematodes were extracted and observed at the Plant Pest Organism Control Technology Laboratory, Plant Protection Study Program, Faculty of Agriculture, Universitas Jember, Jember, East Java, Indonesia.

Soil sampling

The sampling was done on potato crops 60 days after planting. The sampling area was divided into three groups based on altitude variations, namely 1,008 m asl., 1,413 m asl., and 1,875 m asl. Following soil sampling protocols for regular nematode examination, samples were taken in a zigzag pattern. Samples were collected with a shovel from 20 points across each field at a depth of 20–30 cm below the soil surface. Each sub-sample included up to 500 g of soil. The samples were labeled and placed in a cool box before being transported to the laboratory for observation. In addition to taking samples, we also made a record of the general situation of the land at the time. The most widely used active ingredients in pesticides, the average temperature and humidity, the area of land, crop varieties, crop rotation systems, crop intercropping systems, fertilization, and temperature and humidity averages were recorded. The information was gathered by discussing with the farmers in charge of the land surveyed.

Nematodes extraction

The white head tray method extracted nematodes from 500 g of soil from each subsample for 48 hours (Bell and Watson 2001). The nematodes were then filtered using a 400-mesh sieve. Then, the filtered nematodes were kept in DESS solution, with a pH of 8, 0.25 M EDTA, 20% DMSO, and saturated with NaCl (Yoder et al. 2006). After that, the nematode samples were kept at room temperature until observed.

Nematodes analysis

The plant-parasitic nematodes were observed under a light microscope and classified by genus. A Nikon Eclipse E200 stereo microscope and a calibrated camera were used to identify nematodes. The absolute population density of nematodes and their dominance values on land were calculated using the formula below (Mirsam et al. 2020):

$$PD = \frac{n}{500\ g\ soil}$$

$$D = \frac{PD \times \sqrt{absolute\ frequency}}{100} \times 100\%$$

Where: PD: population density; n: nematodes number; D: dominance value

RESULTS AND DISCUSSION

Field condition

In this study, we collected data from farmers on the common agricultural methods employed in the area. Based on the findings of interviews, it is known that the cultivation techniques of the three fields employed in this study have both similarities and variations. In this study, all fields were fertilized using organic and inorganic fertilizers. Organic fertilizer from fermented animal dung, such as cow and horse manure, is the average organic fertilizer utilized in the three assessed fields. Furthermore, it is known that the potato plants planted in the three assessed fields are Granola varieties, frequently cultivated in the Probolinggo District because of their suitability to the district's microclimate and general weather conditions. Moreover, according to farmers, the yields of the Granola varieties are easier to sell than those of other kinds of potato varieties.

The three fields investigated had distinctively diverse patterns of crop rotation and intercropping. For example, leeks are grown in certain areas as part of a rotational cropping system. On the other hand, some farms did not engage in crop rotation and instead planted potatoes throughout the year. In addition, the species of pests that attacked each of the three fields were not substantially different. Nevertheless, the farmers reported that all three fields were infected with nematodes. Data related to cultivation techniques in the field surveyed in this study are presented in Table 1.

Differences in farming practices have an impact on soil nematode populations. Nematodes are sensitive to environmental changes such as temperature fluctuations, pH, and soil structure changes (Bongers and Ferris 1999; Sun et al. 2013). Fertilizer use will also influence plant development and, in turn, the root exudate secreted by plants. Differences in the quantity and variety of root exudate secreted by plants will impact the nematode population in the soil, either directly or indirectly. Root exudates have a direct part in nematode-plant communication signals. Because the nematodes have a chemotaxis response to root exudates emitted by plants, variations in root exudates will influence the type of nematode attracted to the plant root area (Huang et al. 2014; Williams and De Vries 2020). Changes in root exudates will indirectly affect bacterial and fungal communities in the soil, and changes in bacterial and fungal communities will indirectly affect nematode communities in the soil. It was noted in several earlier studies that variations in cultivation methods would influence nematode diversity in the soil (Narula et al. 2012; Lareen et al. 2016). Even in some studies, it is mentioned that cultivating methods are one approach to controlling plant-parasitic nematodes (Atandi et al. 2017).

Table 1. General field condition of the research area

Altitude	Variable		Description
1,008 m asl.	Land area	:	0.25 Ha
	Plant variety	:	Granola
	Crop rotation system	:	Crop rotation with leeks
	Fertilization system	:	Organic fertilizers (bran and animal manure), inorganic fertilizers (Phonska)
	Commonly used active pesticide ingredients	:	Mancozeb
	Average air temperature	:	26°C
	Average air humidity	:	84%
1,413 m asl.	Land area	:	0.25 Ha
	Plant variety	:	Granola
	Crop rotation system	:	No crop rotation
	Fertilization system	:	Organic fertilizer (animal manure), inorganic fertilizer (ZA, SP, Phonska)
	Commonly used active pesticide ingredients	:	Mancozeb
	Average air temperature	:	21°C
	Average air humidity	:	83%
1,875 m asl.	Land area	:	0.25 Ha
	Plant variety	:	Granola
	Crop rotation system	:	There is no crop rotation, but intercropping with leeks
	Fertilization system	:	Organic fertilizers (animal manure) and inorganic fertilizers (Phonska, NPK, ZA)
	Commonly used active pesticide ingredients	:	Mancozeb
	Average air temperature	:	15°C
	Average air humidity	:	76%

The kinds of weeds present will be greatly influenced by both the average temperature and the different altitudes (Nowak et al. 2015). In addition, the species of plants that grow above ground influence the diversity and quantity of root exudates found directly below the ground's surface. Therefore, it is possible that nematodes also be discovered in some weeds, which leads one to speculate that these plants could act as alternative hosts (Munif et al. 2022). For example, *Cyperus rotundus* is a kind of weed with an impressive level of resistance to infection by *Meloidogyne* sp. On the other hand, the root systems of some weeds, such as *Centrosema pubescens*, *Cyperus kyllingia*, and *Cyperus irria*, may provide an ideal environment for the growth of *Meloidogyne* sp. (Widiyanto 2016). This finding also explains how and why nematode populations are impacted by the environment in which they are located.

PPNs population density at various altitudes

Plant-parasitic nematodes have variable population densities at different altitudes. For example, the PPN population per 500 g of soil was 57.00 ± 16.43 at 1,008 m asl., which is also 36.7% greater than the number of parasitic nematodes observed at 1,413 m asl. (41.67 ± 10.98). However, there are 21% higher parasitic nematodes at an altitude of 1,875 m asl. than at 1,413 m asl. (50.50 ± 12.60). Details of the PPNs population density in this study are presented in Table 2.

A broad variety of variables, such as temperature and the soil's physical qualities, might stimulate differences in nematode densities in a particular region. The amount of fertilizer typically applied by farmers and the type of fertilizer directly impact the soil's physical characteristics. According to several research findings, the number of Globodera species has been observed to grow with altitude (Jones et al. 2017). The difference in altitude has a direct bearing on the temperature differential that exists between the three locations. In this research, the temperature at an altitude of 1,008 m asl. was much higher than at an altitude of 1,875 m asl. It is known that the difference between 26°C and 15°C is considerably important. In addition, the air pressure in regions that are higher in height tends to be lower than the air pressure in areas that are lower in altitude. Because nematodes are sensitive microorganisms, it is considered that these factors explain why there is a disparity in the total number of nematodes found at various elevations (Nyang'au et al. 2021; Zhang et al. 2021).

There have been several studies done in the past that have indicated that the density of nematodes varies in response to different environmental conditions (Barker and Olthof, 1976). In general, the differences in altitude will influence the farming practices that farmers adopt at each different altitude. For instance, farmers usually utilize mulch to reduce the amount of water present at higher altitudes (Liu and Siddique 2015). In addition, the likelihood of a plant pathogen infection being present at higher elevations is often increased. Because of this condition, farmers are pressured to use more pesticides to reduce the number of plant diseases that arise (Shunthirasingham et al. 2011). However, the use of agrochemicals such as fungicides and bactericides will, in an indirect way, change the biological and chemical composition of the soil. These changes will cause a shift in the basic features of the soil's microbial ecology, including a change in the number of nematodes (Ney et al. 2019).

PPNs dominance index at various altitudes

There were six different parasitic nematode genera at an altitude of 1,008 m asl., with *Meloidogyne* sp. having the most members. The other genera were *Helicotylenchus* sp., *Longidorus* sp., *Pratylenchus* sp., *Rotylenchus* sp., and *Trichodorus* sp. Furthermore, there were six different parasitic nematode genera at an altitude of 1,413 m asl.: *Criconema* sp., *Globodera* sp., *Longidorus* sp., *Meloidogyne* sp., *Pratylenchus* sp., and *Trichodorus* sp. The genus with the greatest population was *Pratylenchus* sp. While there were four different genera at an altitude of 1,875 m asl., including *Longidorus* sp., *Pratylenchus* sp., *Trichodorus* sp., and *Tylenchulus* sp., the genus with the largest number was from the genus *Pratylenchus* sp. Detail on the nematode population found in this study is presented in Table 3.

This study shows that changes in altitude considerably impact PPN population density and dominance; however, this is mainly acceptable because of the varying environmental conditions present in each area. For example, table 3 shows that the total number of parasitic nematodes at an altitude of 1,008 m asl. is 36.7% greater than the total number of parasitic nematodes at 1,413 m asl. and that at 1,875 m asl., with the total number of parasitic nematodes is 21.1% higher than the entire population.

In this study, it was shown that *Meloidogyne* sp. had the maximum dominance value at 1,008 m asl. *Meloidogyne* sp. was the most dominant nematode, followed by *Pratylenchus* sp. Even more remarkably, *Pratylenchus* sp. was discovered to be the most predominant nematode in both fields at 1,413 m asl. and 1,875 m asl. This study's findings are consistent with previous research indicating that *Meloidogyne* nematodes are more common at intermediate and low altitudes and that *Meloidogyne* does well in warmer areas (Whitehead 1969). Moreover, *Pratylenchus* was more prevalent in two areas with greater altitudes. Several investigations corroborate this finding, showing that *Pratylenchus* sp. is abundant in higher elevations (Gaidashova et al. 2004).

Table 2. PPNs population density found in different potato field altitudes

Altitudes	Population density 500 g^{-1} soil
1,008 m asl.	57.00 ± 16.43
1,413 m asl.	41.67 ± 10.98
1,875 m asl.	50.50 ± 12.60

Table 3. PPNs genera and their absolute population found from different potato field altitudes

Altitude	Genera	Ordo	Sub ordo	Family	PPNs absolute population
1,008	*Helicotylenchus* sp.	Tylenchida	Tylenchina	Hoplolaimidae	1.68
	Longidorus sp.	Dorylaimida	Dorylaimina	Longidoridae	8.38
	Meloidogyne sp.	Tylenchida	Tylenchina	Meloidogynidae	20.12
	Pratylenchus sp.	Tylenchida	Tylenchina	Pratylenchidae	16.76
	Rotylenchus sp.	Tylenchida	Tylenchina	Hoplolaimidae	3.35
	Trichodorus sp.	Dorylaimida	Diphtheroporina	Trichodoridae	6.71
1,413	*Criconema* sp.	Tylenchida	Tylenchina	Criconematidae	1.49
	Globodera sp.	Tylenchida	Tylenchina	Heteroderidae	1.49
	Longidorus sp.	Dorylaimida	Dorylaimina	Longidoridae	8.93
	Meloidogyne sp.	Tylenchida	Tylenchina	Meloidogynidae	5.95
	Pratylenchus sp.	Tylenchida	Tylenchina	Pratylenchidae	14.88
	Trichodorus sp.	Dorylaimida	Diphtheroporina	Trichodoridae	8.93
1,875	*Longidorus* sp.	Dorylaimida	Dorylaimina	Longidoridae	17.57
	Pratylenchus sp.	Tylenchida	Tylenchina	Pratylenchidae	21.96
	Trichodorus sp.	Dorylaimida	Diphtheroporina	Trichodoridae	4.39
	Tylenchulus sp.	Tylenchida	Tylenchina	Tylenchulidae	6.59

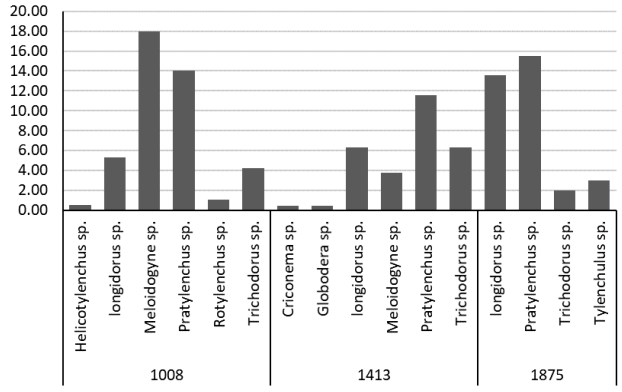

Figure 1. The dominance index of PPNs based on altitude area

PPNs dominance index found in this study is presented in Figure 1. The nematode species, however, have a major impact on the diversity of nematodes in a field. Different species of nematodes within the same genus may have different environmental preferences. Since we did not investigate at the species level in this study, future research is needed to uncover the unresolved issues with its limitations.

Meloidogyne sp. was present and had a dominating value that tends to be high at various elevations, particularly on land at the height of 1,008 m asl., where the PV value is 17.99. According to Elling (2013), *Meloidogyne* sp. is a nematode widely found in tropical countries with warm, humid climate zones, including the lowlands and the highlands. Additionally, this nematode has a broad host range and the potential to disseminate globally. According to Tapia-Vázquez et al. (2022), this nematode has more than 2,000 hosts and is classified as cosmopolitan. Therefore, this nematode species may live and thrive in various soil conditions, cropping techniques, crop rotations, and multiple commodities (Singh et al. 2013).

Pratylenchus sp. is a parasitic nematode with various hosts, particularly in agricultural crops. The nematodes of the species *Pratylenchus* sp. induce root lesions, which can result in yield loss and crop failure if the infection is severe (Jones and Fosu-Nyarko 2014). The habitat of the nematode *Pratylenchus* sp. tends to be dense in plant rhizosphere regions where a sufficient number of hosts are present (Bucki et al. 2020). According to calculations and analyses, this nematode has the largest population and dominance value at altitudes of 1,413 m asl. and 1,875 m asl. It is in line with previous research that the habitat of this nematode is plant root areas with a range of hosts. Therefore, it is suspectible that these nematodes are abundant in potato plants and other types of host plants in the crop rotation system (Jones and Fosu-Nyarko 2014).

The value of the dominance difference between the two nematodes is often impacted by changes in the characteristics of the environment in which they live. This research reveals that the average temperatures of the air in each of the three areas that were investigated had significant differences from one another. Due to the land being located at lower altitudes, the average air temperature is more critical.

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Effective decontamination and multiplication of *Croton membranaceus* in vitro

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Abstract. *Adukonu IA, Ayeh KO, Acheampong E. 2022. Effective decontamination and multiplication of* Croton membranaceus in vitro. *Cell Biol Dev 6: 68-81. Croton membranaceus* Müll. Arg. is a useful herb with medicinal properties, and its leaves, roots, and bark are used to treat diverse ailments. However, the harvesting method by traditional medical practitioners without replacement exposes it to extinction. Therefore, the only means of propagating is by relatively slow use of seeds, and an alternative propagation method is needed for field establishment and nursery. Therefore, this study aims at determining an effective propagation by sterilization regime and subsequent in vitro regeneration using different explants; intact seeds, isolated embryos, coatless seeds, and nodal-cutting. The explants were decontaminated using double sterilization; the best was achieved by pre-treatment with 70% ethanol for 3 min before immersion in sodium hypochlorite (NaOCl). Intact seeds were effectively decontaminated by immersion in 15% NaOCl solution for 20 min, then by 10% NaOCl for 15 min. Conversely, coatless seeds were effectively decontaminated when isolated from intact seeds immersed in 20% NaOCl solution for 20 min and 15% NaOCl for 15 min. Further, embryos isolated from intact seeds were effectively decontaminated in 20% NaOCl for 15 min, then by 15% NaOCl for 10 min sequentially. These sterilization regimes successfully decontaminated 86% intact seeds, 100% isolated embryos, and 80% coatless seeds. Nodal-cutting explants were best decontaminated by immersion in 20% NaOCl solution for 15 min, followed by 15% NaOCl for 10 min sequentially without ethanol pre-treatment. This sterilization regime successfully decontaminated 100% of the nodal-cutting explants. However, the development of shoot explants varied in response to sterilization. Intact seeds did not develop into shoots, while isolated embryos, coatless seeds, and nodal-cutting explants developed into shoots independent of the sterilization regime. Shoot development was highest with the medium's shoot-tip explants added with BAP, NAA, and GA3. Shoot multiplication was best achieved on an MS basal medium with 5.0 μM BAP, 0.5 μM NAA, and 5.0 μM GA3 amendment.

Keywords: *Croton membranaceus*, decontamination, in vitro, multiplication

INTRODUCTION

Croton membranaceus Müll. Arg. is a singly used plant by almost herbal hospitals to manage benign prostatic hyperplasia (BPH) and other prostate-related diseases (Obu 2015). The extracts of the roots exhibit general cytotoxicity and growth inhibition against three cancer cell lines; MCF-7(breast), DLD-1(colon), and M14(melanoma) (Bayor et al. 2007; Salatino et al. 2007; Bayor 2008; Nath et al. 2013). Root and stem extracts are used to relieve symptoms of BPH (Afriyie et al. 2014b; Sarkodie et al. 2014). Also, the aqueous root extract is used to treat measles (Schmelzer and Gurib-Fakim 2008), while it is non-toxic yet exhibits cardio-protective, anti-atherogenic, and anti-ischemic potentials (Afriyie et al. 2013). Furthermore, the ethanolic and aqueous root extracts have improve glycemic index in diabetic patients and reduce glucose levels (Afriyie et al. 2013; Sarkodie et al. 2014; Asare et al. 2015a); it demonstrates their potential to treat type I diabetes (diabetes mellitus), which justifies the use of leaf extract by Ghana traditional healers (Afriyie et al. 2013; Sarkodie et al. 2014; Asare et al. 2015a). The leaf and root extracts are used to aromatize tobacco in the Bahamas and improve digestion in Nigeria. While, the bark oil is essential in treating fever, flatulence, cough, diarrhea, and nausea (Asare et al. 2011; 2015b).

The *C. membranaceus* is an economically important medicinal plant derived from the wide range of the plant's leaves, stems, and roots of phytochemicals. The root bark contains julocrotine (a glutarimide alkaloid), scopoletin, and calcium oxalate crystals (Schmelzer and Gurib-Fakim 2008) which are potent in treating cancer, tumors, BPH, and diabetes (Aboagye 1997; Bayor et al. 2007; Sarkodie et al. 2014). The methanolic root extracts contain cajucarinolide to induce apoptosis (programmed cell death) in cancer cells (Block et al. 2005), and recent findings by Bayor et al. (2009) revealed antimicrobial activity, antihyperglycaemic properties, and cytotoxic effects against human prostate cells. The ethanolic and aqueous root extracts contain a compound, N[N-(2-methylbutanoyl) gluta minoyl]-2-phenylethya mine, to improve glycemic index in diabetic (type I diabetes, i.e, diabetes mellitus) and reduce glucose levels (Afriyie et al. 2013; Sarkodie et al. 2014; Asare et al. 2015a). Despite its rich phytochemicals, the plant still grows without propagating commercially in the wild.

The propagation of croton species is by asexual and sexual means; Asexually, techniques such as cutting, division, budding, layering, and grafting (Ingram and Yeager 2003; Relf and Ball 2009; Lott and Lindgren 2012; Devi and Shanthi 2013). Besides, a few species of the genus (*Croton antisyphyliticus, Croton sublyratus,*

Codiaeum variegatum, *Croton urucurana*, and *Croton bonpladinum*) have been cultured and multiplied successfully in vitro (Nasib et al. 2008; Kondamudi et al. 2009; Ashish and Sharma 2011; Oliveira et al. 2011). The *C. membranaceus* is propagated only sexually on seeds by dehiscence and dispersed (Aboagye 1997; Lott and Lindgren 2012). Several Center for Plant Medicine Research attempts by asexual means yielded low results, specifically stem cutting and air layering.

Ghana's report to the Food and Agriculture Organization (FAO) (1996) indicated that *C. membranaceus* is a rare species that would become extinct due to the high demand required in treating and managing diseases such as BPH, diabetes, cough, fever, flatulence, diarrhea, measles, and nausea (Mshana et al. 2000; Bayor et al. 2009; Asare et al. 2011; Devi and Shanthi 2013;). Therefore, its barks and roots are prone to unsustainable harvesting (Abbiw et al. 2002) and bad environmental such as drought and bush fire. As a result, several large-scale propagation attempts using seeds or stem cuttings have been unsuccessful (Aboagye 1997).

The *C. membranaceus* is in rising demand in Ghana and the other tropics (Asare et al. 2011; Afriyie et al. 2013), even though endangered in the wild because of exposure to adverse environmental conditions, mainly drought and bushfires and over-exploitation. In contrast with the increased demand (Abbiw et al. 2002; Afriyie et al. 2014a; Sarkodie et al. 2014), no alternative propagation methods have been established yet. The increasing demand may wipe out its existence if measures are not taken, hence the need to study propagation using in vitro techniques; Apart from using seeds, no report has been published yet. However, it is possible to work on the multiplication using tissue culture techniques on some croton species (Shibata et al. 1996; Ashish and Sharma 2011; Salamma and Rao 2013; Silva et al. 2013).

MATERIALS AND METHODS

Research site

The current research was undertaken in the Tissue culture section of the Department of Botany, University of Ghana, Legon, Ghana, with funding from the United Nations University Institute for Natural Resources in Africa (UNU/INRA).

Plant materials

Seeds and seedlings of *C. membranaceus* were obtained from the Center for Plant Medicine Research (CPMR), Mampong-Akuapem, Ghana. Mature fruits of *C. membranaceus* were obtained randomLy from field-grown plants. Four-week-old seedlings received from CPMR were kept outside the screen house under ambient conditions in the Department for four months to allow the development of shoots to be used as explants. Explants used for the research were dry-mature seeds with seed coats (intact seeds), seeds without seed coats (coatless seeds), embryos isolated from seeds (isolated embryos), and cuttings (nodal cuttings) from young shoots of *C. membranaceus.*

Culture medium

Murashige and Skoog (MS) (1962), produced by Sigma-Aldrich Company, Spain, used the basal medium for the experiment. Stock solutions of macronutrients, micronutrients, vitamins, and growth hormones were prepared based on the required composition and quantity of nutrients. All stock solutions were kept in a refrigerator and stored at 4^{o}C. Media were prepared from stock solutions and used for culturing explants. The pH of the medium was adjusted to 5.7. Agar was added as a gelling agent to harden the medium and dispensed into test tubes, after which they were sterilized in an autoclave at a temperature of 121^{o}C and a pressure 102.97KPa for 15 min. The medium was allowed to cool after autoclaving before being used.

Sterilization of labware

All dissecting kits (scalpel, forceps), and glassware (petri dishes, covered bottles, etc.), were sterilized in an autoclave at a temperature of 121^{o}C and a pressure of 102.97KPa for 15 min. Distilled water was also sterilized under the same condition.

Experiment 1: Decontamination of explants

A decontamination experiment was done using seeds and nodal cuttings to determine the optimal sterilization regime for Croton explants. In addition, the effects of ethanol and various concentrations of sodium hypochlorite were determined. Seeds of an average length of 4mm were selected, and young shoots of the potted plants were used for the various experiments. Intact seeds were double sterilized by immersing in 15% NaOCl for 20 min, followed by 10% NaOCl for 15 min, without ethanol pre-treatment (A0) or with ethanol pre-treatment (A1), 20% NaOCl for 15 min, followed by 15% for 10 min, without ethanol pre-treatment (B0) or with ethanol pre-treatment (B1) and 20% NaOCl for 20 min followed by 15% NaOCl for 15 min, without ethanol pre-treatment (C0) or with ethanol pre-treatment (C1). Nodal cuttings were first washed under running tap water containing a few drops of household liquid detergent (Klene lene) containing sodium Laureth sulfate for 5 min and rinsed with sterile distilled water, trimmed to a length of 1.5 cm with two nodes and sterilized as described for intact seeds.

Seeds and nodal cuttings were placed in separate bottles, completely immersed in NaOCl, and carefully agitated by hand. Four drops of household liquid detergent (Klene lene) were added to every 100 mL NaOCl, then thoroughly washed with sterile distilled water to remove traces of the sterilant. Coatless seed and embryo explants were aseptically isolated from sterilized intact seeds previously soaked for 48 hours. The nodal cuttings were trimmed to 1 cm. Sterilized explants were inoculated on MS basal medium supplemented with 5.0 µM BAP and 5.0 µM NAA. One explant was inoculated in a test tube containing 10 mL of the medium. The cultures were incubated in 16 hours of light and 8 hours of darkness at a temperature of 26±1^{o}C. Each explant was replicated five times, and the experiment was repeated three times. The

number of decontaminated explants and the number of explants that developed shoots or plantlets were recorded.

Experiment 2: Initiation of cultures

Initiation of cultures from seeds

Intact seeds were sterilized using the best sterilization regime from the decontamination experiment and cultured on MS basal medium supplemented with 100 mg/L *myo*-inositol, 0.1 mg/L thiamine HCl and varying concentrations of BAP, NAA, and GA3 (Table 1). Coatless seeds and embryos were obtained from sterilized intact seeds and cultured on MS medium as described for intact seeds.

One explant was inoculated per test tube containing 10mL of the medium. One explant in each test tube formed an experimental unit and was replicated ten times. Data on the number of explants that developed shoots, the number of shoots per explant, and the height of shoots were recorded four weeks after culture.

Initiation of cultures from nodal-cutting and shoot-tip explants

Sterilized nodal-cutting and shoot-tip explants bearing two buds were cultured on MS basal medium amended with 100 mg/L *myo*-inositol, 0.1 mg/L thiamine HCl, 5.0 µM BAP only, 5.0 µM BAP and 0.5 µM NAA, 5.0 µM BAP and 5.0 µM NAA, 5.0 µM BAP and 5.0 µM GA3, 5.0 µM

BAP, 0.5 µM NAA and 5.0 µM GA3 or 5.0 µM BAP, 5.0 µM NAA and 5.0 µM GA3. One explant was cultured per test tube containing 10mL of the medium. The medium was sterilized as described in section 3.3. One explant in each test tube formed an experimental unit and was replicated ten times. The number of explants that developed shoots, the number of shoots per explant, and the number of roots per explant were counted. In addition, the height of shoots was measured with a meter rule. All data were recorded four weeks after culture.

Experiment 3: Comparison of nodal-cutting and shoot-tip explants for shoot initiation in *Croton membranaceus*

This experiment was carried out to investigate the most responsive part of nodal explants for culture initiation, using nodal cuttings and shoot-tips. First, the explants were washed in running tap water containing four drops of household liquid detergent by Klene Lene, Ghana, which contains the active ingredient, sodium Laureth sulfate, per 100mL water for five min and rinsed thoroughly with sterile distilled water. That was followed by double sterilizing with 15% sodium hypochlorite (NaOCl) for 20 min and 10% NaOCl solution for 15 min sequentially. Finally, the nodal explants were rinsed thoroughly with sterile distilled water to remove any remaining sterilant, then aseptically trimmed to obtain cuttings of 1cm long with two buds each. The sterilized explants were cultured on solid MS basal medium amended with 100 mg/L *myo*-inositol, 0.1 mg/L thiamine HCl, 30 g/L sucrose without growth hormone, 5.0 µM BAP and 5.0 µMNAA. One nodal explant was cultured in one test tube containing 10mL of MS medium and replicated ten times. The cultures were incubated in the growth room in light for 16 hours and darkness for 8 hours at a temperature of 26 ± 1^0C for four weeks. The number of shoots and leaves formed by each explant was counted, and the height of the shoot was measured with a meter rule.

Multiplication of *Croton membranaceus*

Two experiments were conducted to determine an optimum medium for the multiplication; the first experiment was in vitro shoots, which were subcultured on MS basal medium containing 100 mg/L *myo*-inositol, 0.1 mg/L thiamine HCl, 30 g/L sucrose without growth hormone, with 5.0 µM BAP only or in combination with 0.5 µM or 5.0 µM NAA, 5.0 µM GA3, 0.5 µM NAA, and 5.0 µM GA3 or 5.0 µM NAA and 5.0 µM GA3; Other was cultured in a test tube, and this was replicated ten times. All cultures were incubated under growth room conditions of 25 ± 1^0C and light intensity of 3000 Lux for four weeks; the number of cultures that developed shoots were recorded. Also, the number of shoots and leaves per culture was counted. Finally, the shoot height was measured with a meter rule.

The second experiment investigated the response of in vitro nodal cuttings of *C. membranaceus* on an MS basal medium amended with BAP, NAA, and varying concentrations of GA3. In vitro shoots were subcultured on MS basal medium amended with 5.0 µM BAP and 0.5 µM NAA (control) and in combination with 5.0 µM GA3 or 50 µM GA3. One nodal-cutting was cultured in a test tube and replicated ten times. All cultures were incubated as described above. The number of shoots per explant, the number of leaves per shoot, and the height of shoots were recorded after four weeks.

Design and data analysis

The experiments were laid out in a Completely Randomized Block Design. Data collected were quantified and analyzed statistically by ANOVA or Two-Sample T-Test, using the minitab software version 17 where necessary. Means were separated using the Fisher Least Significant Difference (LSD) method at α level of 0.05.

Table 1. Concentrations of BAP, NAA, and GA3 in MS basal medium used for the initiation experiments

Growth hormones (µM)		
BAP	**NAA**	**GA3**
0.0	0.0	0.0
5.0	0.0	0.0
5.0	0.5	0.0
5.0	5.0	0.0
5.0	0.0	5.0
5.0	0.5	5.0
5.0	5.0	5.0

RESULTS AND DISCUSSION

Effect of double sterilization with sodium hypochlorite (NaOCl) on decontamination of explants

The decontamination of explants using the double sterilization method with or without immersion in ethanol is shown in Table 2.

When intact seeds were double sterilized with 15% NaOCl for 20 min and 10% NaOCl solution for 15 min sequentially, the least explant decontamination was 33% and was obtained without pre-treatment with ethanol (A0), while the highest explant decontamination was 86.7% and was obtained when intact seeds were immersed in 70% ethanol for 3 min before immersion in NaOCl (A1). When intact seeds were sterilized with treatment B, the least explant decontamination was 60% and obtained when intact seeds were double sterilized with 20% NaOCl for 15 min and 15% NaOCl for 10 min sequentially without pre-treatment with ethanol (B0) while the highest explant decontamination was 80% and was obtained when intact seeds were immersed in 70% ethanol for 3 min followed by double sterilization with 20% NaOCl solution for 15 min and 15% NaOCl solution for 10 min sequentially (B1). With treatment C, the least explant decontamination was 46.7% and obtained when intact seeds were double sterilized with 20% NaOCl for 15 min and 15% NaOCl for 10 min sequentially without pre-treatment with ethanol (C0), while the highest explant decontamination was 80% and was obtained when intact seeds were immersed in 70% ethanol for 3 min followed by double sterilization with 20% NaOCl for 20 min and 15% NaOCl for 15 min sequentially (C1). None of the intact seeds developed shoots or roots when sterilized using all the sterilization regimes (Table 2).

Coatless seeds were least decontaminated (13.7%) when sterilized with treatment A0 (15% NaOCl for 20 min, 10% NaOCl for 15 min). Still, the decontamination rate increased to 66.67% when coatless seeds were treated with A1 (70% ethanol for 3 min, 15% NaOCl for 20 min, and 10% NaOCl for 15 min) (Table 2). With treatment B, 66.67% of coatless seeds were decontaminated when sterilized with B0 (20% NaOCl for 15 min, 15% NaOCl for 10 min), while 80% of the coatless seeds were decontaminated with B1 (70% ethanol for 3 min, 20% NaOCl for 15 min, 15% NaOCl for 10 min). With treatment C0 (20% NaOCl for 20 min, 15% NaOCl for 15 min), 73.33% coatless seeds were decontaminated, whereas C1 (70% ethanol for 3 min, 20% NaOCl for 20 min, 15% NaOCl for 15 min), 80% decontamination was recorded. There was no development of shoot or root when coatless seeds were treated with A1; however, when coatless seeds were treated with A0, 6.7% developed shoots and roots. Shoot increased (13.33%) with treatment B0 though this was lower than 26.67% shoot development when coatless seeds were decontaminated with treatment C1. The optimum number of 46.7% coatless seeds developed shoots and roots when treated with B1 and C0.

Embryos isolated from intact seeds sterilized with treatment A0 (15% NaOCl for 20 min, 10% NaOCl for 15 min) were least decontaminated 73.3%, while with Treatment A1 (70% ethanol for 3 min, 15% NaOCl for 20 min, 10% NaOCl for 15 min), 93.33% isolated embryos were decontaminated. When the percentage of NaOCl was increased as in B0 (20% NaOCl for 15 min, 15% NaOCl for 10 min), the number of decontaminated embryos observed was 93.33%, but 100% decontamination was observed when explants were pretreated with ethanol before immersion in the same concentration of NaOCl for the same time, B1 (70% ethanol for 3 min, 20% NaOCl for 15 min, 15% NaOCl for 10 min). With treatment C, 80% of isolated embryos were decontaminated when ethanol was not used C0 (20% NaOCl for 20 min, 15% NaOCl for 15 min). In comparison, an optimum of 100% isolated embryos was decontaminated when sterilized treatment C1 (70% ethanol for 3 min, 20% NaOCl for 20 min, 15% NaOCl for 15 min). The Embryos decontaminated with the various sterilization regimes developed shoots and roots (Table 2). The least number of embryos that developed shoots with roots was 33.3%, which was observed in embryos decontaminated with treatment A0. The number increased to 40% for embryos treated with A1 and B0. A further increase of 60% shoot development was observed for embryos treated with C0 and C1, while an optimum of 80% of embryos developed into shoots with roots when decontaminated with Treatment B1, as described earlier.

Table 2. Effect of ethanol and various concentrations of sodium hypochlorite (NaOCl) on decontamination of explants. Explants were cultured on MS basal medium amended with 5 μM BAP and 5 μM NAA

Treatment	No. of cultures	Intact seeds		Coatless seeds		Isolated embryos		Nodal explants	
		Decontaminated (%)	Plantlets (%)	Decontaminated (%)	Plantlets (%)	Decontaminated (%)	Plantlets (%)	Decontaminated (%)	Plantlets (%)
A0	15	33.33	0.00	13.33	6.67	73.33	33.33	80.00	93.33
A1	15	86.67	0.00	66.67	0.00	93.33	40.00	93.33	80.00
B0	15	60.00	0.00	66.67	13.33	93.33	40.00	100.00	66.67
B1	15	80.00	0.00	80.00	46.70	100.00	80.00	93.33	66.67
C0	15	46.67	0.00	73.33	46.70	80.00	60.00	93.33	73.33
C1	15	80.00	0.00	80.00	26.67	100.00	60.00	100.00	66.67

Note: A0-15% NaOCl for 20 min, 10%NaOCl for 15 min; A1-70% ethanol for 3 min, 15% NaOCl for 20 min, 10% NaOCl for 15 min; B0-20% NaOCl for 15 min, 15% NaOCl for 10 min; B1-70% ethanol for 3 min, 20% NaOCl for 15 min, 15% NaOCl for 10 min; C0-20% NaOCl for 20 min, 15% NaOCl for 15 min; C1-70% ethanol for 3 min, 20% NaOCl for 20 min, 15% NaOCl for 15 min

Nodal-cutting explants were least decontaminated (80%) when sterilized with treatment A0 (15% NaOCl for 20 min, 10% NaOCl for 15 min). The decontamination rate increased (93.33%) when nodal-cutting explants were sterilized with treatment A1 (70% ethanol for 3 min, 15% NaOCl for 20 min, 10% NaOCl for 15 min), B1 (70% ethanol for 3 min, 20% NaOCl for 15 min, 15% NaOCl for 10 min) and C0 (20% NaOCl for 20 min, 15% NaOCl for 15 min). An optimum rate of 100% decontamination was observed when nodal-cutting explants were sterilized with treatments B0 (20% NaOCl for 15 min, 15% NaOCl for 10 min) and C1 (70% ethanol for 3 min, 20% NaOCl for 20 min, 15% NaOCl for 15 min). Nodal-cutting explants developed shoots with roots when decontaminated using all the sterilization regimes (Table 2). However, nodal-cutting explants decontaminated with treatments B0, B1, and C1 had the least rate of plantlet regeneration. With these treatments, 66.7% of nodal-cutting explants developed shoots with roots. The rate of shoot development increased to 73.33% and 80% when nodal-cutting explants were sterilized with treatments C0 and A1, respectively. An optimum number of 93.3% of nodal explants developed shoots and roots when nodal explants were sterilized with treatment A0.

Response of explants to BAP, NAA, and GA3 in the culture medium

The effect of BAP, NAA, and GA3 in MS basal medium on shoot development is shown in Tables 3 to 5.

Intact seeds failed to develop into shoots when cultured on MS basal medium amended with BAP alone, BAP with NAA or BAP, NAA, and GA3. However, only one intact seed developed radicle on MS basal medium amended with 5.0 µM BAP and 5.0 µM NAA after twelve weeks of culture (Figure 1). Coatless seed explants developed into shoots nine days after culture on MS basal medium with or without hormone (the control). However, the number of shoots developed depended on the combination of growth hormones (Table 3).

All explants cultured on MS basal medium with 5.0 µM BAP only, did not develop shoots. Similarly, all explants cultured on MS basal medium amended with either 0.5 or 5.0 µM NAA and GA3 also did not develop shoots. These explants were swollen by the second week of culture but turned brown at four weeks of culture. Shoots were only developed on MS basal medium without hormone (the control) and MS basal medium amended with BAP and NAA only or BAP with GA3 only (Table 3). The mean number of shoots per explant was 0.10, independent of the hormone manipulation and combination. However, the number of leaves and roots per explant and the height of the shoots varied. The mean number of leaves was 0.2 on MS medium without hormone and MS basal medium fortified with 5.0 µM BAP and 5.0 µM NAA or GA3. The mean number of leaves increased (0.5) when an MS basal medium was amended with 5.0 µM BAP and 0.5 µM NAA. Statistically, the differences observed were not significant ($P \leq 0.712$).

The mean number of roots per explant on MS basal medium without hormone treatment was 0.4. That was higher than the mean number (0.1) observed on MS basal medium amended with 5.0 µM BAP and 5.0 µM NAA. The mean number of roots increased to 0.5 per explant when an MS basal medium was amended with 5.0 µM BAP and 5.0 µM GA3 with an optimum of 0.7 roots per shoot on an MS basal medium amended with 5.0 µM BAP and 0.5 µM NAA. Statistical analysis showed that the observed differences were insignificant ($P \leq 0.700$). The mean height of the shoot was 0.12cm on MS basal medium without hormone, which was higher than 0.10cm and 0.11cm as observed on MS basal medium amended with 5.0 µM BAP and 5.0 µM NAA or 5.0 µM GA3, respectively. The highest shoot (0.38cm) was obtained on an MS basal medium amended with 5.0 µM BAP and 0.5 µM NAA (Figure 2). However, the differences in shoot height observed were not statistically significant ($P \leq 0.649$).

Isolated embryos developed into shoots five days after culture. However, the shoots' survival rate and development depended on a combination of growth hormones in the MS medium (Table 4).

Figure 1. Intact seed explant developing root on MS basal medium amended with 5.0 µM BAP and 5.0 µM NAA after twelve weeks of culture

Table 3. Response of coatless seeds to MS basal medium amended with BAP and NAA. Data were collected four weeks after culture

Growth hormone (µM)	Number of explants	Surviving explants	Shoot per explant	Leaf per explant	Root per explant	Shoot height (cm)
0	10	0.10±0.32[a]	0.10±0.32[a]	0.20±0.63[a]	0.40±1.27[a]	0.12±0.38[a]
5.0 BAP	10	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]
5.0 BAP + 0.5 NAA	10	0.10±0.32[a]	0.10±0.32[a]	0.50±1.58[a]	0.70±2.20[a]	0.38±1.20[a]
5.0 BAP + 5.0 NAA	10	0.10±0.32[a]	0.10±0.32[a]	0.20±0.63[a]	0.10±0.32[a]	0.10±0.32[a]
5.0 BAP + 5.0 GA3	10	0.10±0.32[a]	0.10±0.32[a]	0.20±0.63[a]	0.50±1.58[a]	0.11±0.35[a]
5.0 BAP + 0.5 NAA + 5.0 GA3	10	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]
5.0 BAP + 5.0 NAA + 5.0 GA3	10	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]

Note: Each value in the table is the mean and standard deviation of ten replicates. Values that do not share a letter in a column differ significantly by LSD ($\alpha = 0.05$)

Table 4. The response of isolated embryos to MS basal medium was amended with BAP, NAA, and GA3. Data were collected four weeks after culture

Growth hormone (µM)	Number of explants	Surviving explants	Shoot per explant	Leaf per explant	Root per explant	Shoot height (cm)
0	10	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]
5.0 BAP	10	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]
5.0 BAP + 0.5 NAA	10	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]
5.0 BAP + 5.0 NAA	10	0.20±0.42[a]	0.20±0.42[a]	0.70±1.89[a]	0.80±2.53[a]	0.28±0.89[a]
5.0 BAP + 5.0 GA3	10	0.10±0.32[a]	0.10±0.32[a]	0.70±2.21[a]	0.20±0.63[a]	0.25±0.79[a]
5.0 BAP + 0.5 NAA + 5.0 GA3	10	0.10±0.32[a]	0.10±0.32[a]	0.20±0.63[a]	0.00±0.00[a]	0.08±0.25[a]
5.0 BAP + 5.0 NAA + 5.0 GA3	10	0.10±0.32[a]	0.10±0.32[a]	0.10±0.32[a]	0.40±1.27[a]	0.10±0.32[a]

Note: Each value in the table is the mean and standard deviation of ten replicates. Values that do not share a letter in a column differ significantly by LSD (α = 0.05)

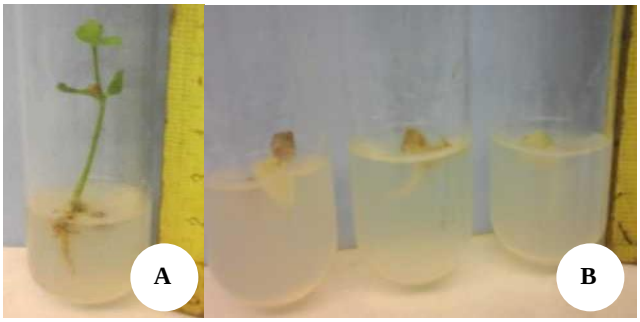


Figure 2. Coatless seed explants developing shoots on MS basal medium amended with A. 5.0 µM BAP and 0.5 µM NAA and B. 5.0 µM BAP and 5.0 µM NAA four weeks after culture

All isolated embryos cultured on MS basal medium without any growth hormone failed to develop shoots. Also, all explants cultured on MS basal medium with 5.0 µM BAP only or 0.5 µM NAA did not develop shoots. Shoot development occurred when the concentration of NAA in the culture medium was increased or when GA3 was added to the medium. Isolated embryos cultured on MS basal medium amended with 5.0 µM BAP and 5.0 µM GA3, 5.0 µM BAP, 0.5 µM NAA, and 5.0 µM GA3 or 5.0 µM BAP, 5.0 µM NAA and 5.0 µM GA3 had a mean of 0.1 surviving over four weeks period. However, this was not statistically different from the highest survival rate of 0.2 when an MS basal medium was amended with 5.0 µM BAP and 5.0 µM NAA (P≤0.546). Similarly, shoot development was lower with a mean of 0.1 shoots per explant on an MS basal medium amended with 5 µM BAP and 5 µM GA3 or with 0.5 or 5.0 µM NAA. In contrast, an optimum of 0.2 shoots per explant was observed on an MS basal medium amended with 5.0 µM BAP and 0.5 µM NAA but not statistically different (P≤0.546).

Leaf development was least (0.1) when an MS basal medium was amended with 5.0 µM BAP, 5.0 µM GA3, and 5.0 µM NAA, but the number increased to 0.2 when NAA was reduced to 0.5 µM. An optimum mean (0.7) was observed when an MS basal medium was amended with 5.0 µM BAP and 5.0 µM NAA or 5.0 µM GA3. Statistically, the differences observed for leaf development were not significant (P≤0.587).

Root development was not evident for shoots on MS basal medium amended with 5.0 µM BAP and 0.5 µM NAA and 5 µM GA3. When an MS basal medium was amended with 5.0 µM BAP and 5.0 µM GA3, a mean of 0.2 roots per explant was observed. That increased to 0.4 roots per explant when an MS basal medium was amended with 5.0 µM BAP and 5.0 µM NAA and 5.0 µM GA3, while the highest mean of 0.8 roots per shoot was observed on an MS basal medium amended with 5.0 µM BAP and 5.0 µM NAA (Table 4). However, the differences observed were not statistically significant (P≤0.590).

The height of the shoot was the least (0.08cm) on MS basal medium amended with 5.0 µM BAP, 5.0 µM GA3, and 0.5 µM NAA but increased to 0.1cm when NAA was increased to 5.0 µM. The height of the shoot increased from 0.25cm when an MS medium was amended with 5.0 µM BAP and 5.0 µM GA3 to 0.28cm when an MS medium was amended with 5.0 µM BAP and 5.0 µM NAA. However, the differences observed in the height of the shoot were not statistically significant (P≤ 0.757).

Thus, only a medium amended with 5.0 µM BAP in combination with 5.0 µM NAA, 5.0 µM GA3, and 5.0 µM BAP in combination with both NAA and GA3 irrespective of NAA concentration, developed into shoots (Figure 3).

Nodal-cutting and shoot-tip explants

Of all the explants used, nodal-cutting explants had the best shoot development as all the media combinations developed shoots (Table 5).

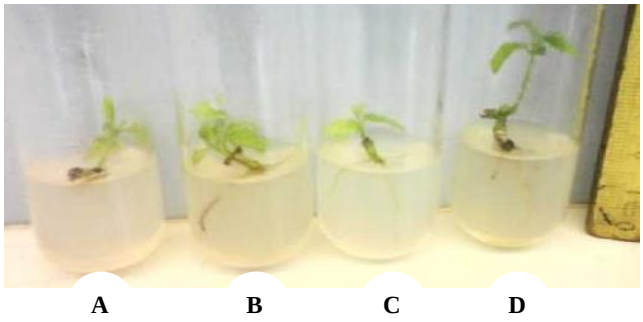


Figure 3. Response of isolated embryos to BAP, NAA, and GA3 treatments; A. 5.0 µM BAP, 0.5 µM NAA and 5.0 µM GA3, B. 5.0 µM BAP and 5.0 µM GA3, C. 5.0 µM BAP, 5.0 µM NAA and 5.0 µM GA3 and D. 5.0 µM BAP and 5.0 µM NAA. Photos were taken four weeks after culture

Explants cultured on MS basal medium without hormone (the control) had a survival rate of 0.3 (Table 5). That was higher than 0.10 as observed for explants cultured on an MS basal medium amended with 5.0 µM BAP and 5.0 µM NAA or 5.0 µM BAP, 0.5 µM NAA and 5.0 µM GA3. When an MS basal medium was amended with 5.0 µM BAP and 0.5 µM NAA, the survival rate increased to 0.2 explants. A further increase (0.4) was observed when an MS basal medium was amended with 5.0 µM BAP only or combined with 5.0 µM GA3. The survival rate was optimum (0.6) when an MS basal medium was amended with 5.0 µM BAP, 5.0 µM NAA, and 5.0 µM GA3. Statistically, the differences observed in the explants' survival were insignificant ($P \leq 0.147$).

The number of shoots per explant was 0.5 when nodal-cutting and shoot-tip explants were grown on MS basal medium without hormone (control). When an MS basal medium was amended with 5.0 µM BAP and 5.0 µM NAA or 5.0 µM BAP, 0.5 µM NAA, and 5.0 µM GA3, the number of shoots per explant reduced to 0.1. The number of shoots per explant was observed (0.2) when an MS basal medium was amended with 5.0 µM BAP, and 0.5 µM NAA was still lower than the control medium. An increase of 0.6 shoots per explant was observed when explants were cultured on an MS basal medium amended with 5.0 µM BAP and 5.0 µM GA3. When an MS basal medium was amended with 5.0 µM BAP, 5.0 µM NAA, and 5.0 µM GA3, 0.7 shoots per explants were observed. The shoot number was optimum (0.8) when nodal-cutting and shoot-tip explants were cultured on MS basal medium amended only with 5.0 µM BAP. Statistically, the differences in shoot development observed from nodal-cutting and shoot-tip explants were insignificant ($P \leq 0.165$).

Leaf development was 2.2 leaves per shoot, as observed when an MS medium was not amended with hormones (control) (Table 5). The number of leaves per shoot was reduced to 0.2 when an MS basal medium was amended with 5.0 µM BAP, 0.5 µM NAA, and 5.0 µM GA3. An increase of 0.4 leaves per explant was observed when an MS basal medium was amended with 5.0 µM BAP and 5.0 µM NAA. That was followed by 0.8 leaves per explant when an MS basal medium was amended with 5.0 µM BAP and 0.5 µM NAA. Leaf development increased to 2.3 per shoot when an MS basal medium was amended with 5.0 µM BAP and 5.0 µM GA3. The optimum number of leaves per explant (3.2) was observed when nodal-cutting explants were cultured on an MS basal medium amended with either 5.0 µM BAP only or 5.0 µM BAP, 5.0 µM NAA and 5.0 µM GA3. However, the differences observed in leaf development were not statistically significant ($P \leq 0.183$).

The shoot height observed on the MS basal medium without hormone was 0.61cm (Table 5). That was higher than the height of the shoot observed on four treatments with growth hormones. When an MS basal medium was amended with 5.0 µM BAP, 0.5 µM NAA and 5.0 µM GA3, 5.0 µM BAP and 0.5 µM NAA, 5.0 µM BAP and 5.0 µM NAA and 5.0 µM BAP only, the height of shoot observed were 0.10cm, 0.18cm, 0.23cm, and 0.59cm respectively. Shoot height was enhanced when an MS basal medium was amended with 5.0 µM BAP and 5.0 µM GA3 (Figure 4C). On this medium, nodal-cutting and shoot-tip explants grew to a height of 0.64cm. The optimum shoot height (0.80cm) was observed when an MS basal medium was amended with 5.0 µM BAP, 5.0 µM NAA, and 5.0 µM GA3 (Figure 4E). Statistically, the differences observed were not significant ($P \leq 0.270$). However, the overall response of nodal-cutting explants to the development of shoot leaves, roots, and the height of shoots was optimum on MS basal medium amended with 5.0 µM BAP, 5.0 µM NAA, and 5.0 µM GA3 (Figure 4).

Comparison of nodal-cutting and shoot-tip explants

The finding that an MS basal medium amended with a combination of the growth hormones 5.0 µM BAP, 5.0 µM NAA, and 5.0 µM GA3, was effective in the response of nodal-cutting and shoot-tip explants (Table 5) led to the investigation of the comparison between the use of nodal-cutting and shoot-tips explants in micropropagation of *C. membranaceus*. Thus, in this experiment, nodal-cutting and shoot-tip explants were cultured on MS basal medium amended with 5.0 µM BAP, 5.0 µM NAA, and 5.0 µM GA3, to compare their regeneration potential. Shoot-tip explants developed into shoots earlier and faster than nodal-cutting explants. It was observed that shoot tips started sprouting 5 days after culture, whereas it took ten days for nodal cuttings to start sprouting. There was a significant effect of shoot-tip and nodal-cutting explants on shoot regeneration from *C. membranaceus.* The effect was observed in the number of surviving explants, shoot per explant, leaves per explant, and shoot height (Table 6).

Table 5. Effect of MS basal medium amended with BAP, NAA, and GA3 on nodal-cutting and shoot-tip explants. Data were collected four weeks after culture

Growth hormone (µM)	Number of explants	Surviving explants	No. of shoot per explant	No. of leaves per explant	Shoot height (cm)
0	10	0.30 ± 0.48^{ab}	0.50 ± 0.85^{ab}	2.20 ± 3.85^{ab}	0.61 ± 1.03^{ab}
5.0 BAP	10	0.40 ± 0.52^{ab}	0.80 ± 1.14^{a}	3.20 ± 4.83^{a}	0.59 ± 0.93^{ab}
5.0 BAP + 0.5 NAA	10	0.20 ± 0.42^{ab}	0.20 ± 0.42^{ab}	0.80 ± 2.20^{ab}	0.18 ± 0.42^{ab}
5.0 BAP + 5.0 NAA	10	0.10 ± 0.32^{b}	0.10 ± 0.32^{b}	0.40 ± 1.27^{ab}	0.23 ± 0.73^{ab}
5.0 BAP + 5.0 GA3	10	0.40 ± 0.52^{ab}	0.60 ± 0.97^{ab}	2.30 ± 3.83^{ab}	0.64 ± 0.94^{ab}
5.0 BAP + 0.5 NAA + 5.0 GA3	10	0.10 ± 0.32^{b}	0.10 ± 0.32^{b}	0.20 ± 0.63^{b}	0.05 ± 0.16^{b}
5.0 BAP + 5.0 NAA + 5.0 GA3	10	0.60 ± 0.52^{a}	0.70 ± 0.68^{ab}	3.20 ± 3.08^{a}	0.80 ± 0.91^{a}

Note: Each value in the table is the mean and standard deviation of ten replicates. Values that do not share a letter in a column differ significantly by LSD ($\alpha = 0.05$)

Table 6. Comparison of nodal-cutting and shoot-tip explants on shoot regeneration of *Croton membranaceus*. Explants were cultured on MS basal medium amended with 5.0 µM BAP, 5.0 µM NAA, and 5.0 µM GA3. Data were collected four weeks after culture

Explant	Number of explants	Surviving explants	No. of shoot per explant	No. of leaves per explant	Shoot height (cm)
Nodal cutting	10	0.50±0.53[a]	0.50±0.53[a]	1.30±1.57[a]	0.30±0.41[b]
Shoot-tip	10	0.80±0.42[a]	1.20±0.79[b]	3.00±2.58[a]	1.29±0.83[a]

Note: Each value in the table is the mean and standard deviation of ten replicates. Values that do not share a letter in a column differ significantly by LSD ($\alpha = 0.05$)

A B C D E F G

Figure 4. Response of nodal explants to BAP, NAA, and GA3 treatments; A. Control, B. 5.0 µM BAP only, C. 5.0 µM BAP and 5.0 µM GA3, D. 5.0 µM BAP and 5.0 µM NAA, E. 5.0 µM BAP, 5.0 µM NAA and 5.0 µM GA3, F. 5.0 µM BAP and 0.5 µM NAA and G. 5.0 µM BAP, 0.5 µM NAA and 5.0 µM GA3. Photos were taken four weeks after culture

The number of surviving explants from nodal-cutting explants was not significantly lower (0.50) ($P \leq 0.177$) than shoot-tip explants (0.80). Similarly, shoot development in nodal-cutting explants was lower (0.50) shoots per explant. On the other hand, the number of shoot-tip explants that developed into shoots was more than twice (1.20) that of nodal-cutting explants. Statistically, there is evidence of a significant difference ($P \leq 0.031$) in the development of shoots from nodal-cutting and shoot-tip explants.

Leaf development was also less in nodal-cutting explants than in shoot-tip explants. A mean of 1.30 leaves per explant was observed when nodal-cutting explants were used, whereas a mean of 3.00 leaves per explant was observed for shoot-tip explants. Although the difference observed was more than twice, there was no statistical evidence of a significant difference in leaf development for nodal-cutting and shoot-tip explants ($P \leq 0.092$).

The mean height of shoots observed in nodal-cutting explants was 0.30, while that of shoot-tip explants was 1.29. Statistically, there was evidence of a significant difference ($P \leq 0.003$) in the shoot height for nodal-cutting and shoot-tip explants.

Thus, the comparison between the response of nodal-cutting and shoot-tip explants showed that shoot-tip explants enhanced the regeneration of shoots better (Figure 5).

Effect of BAP, NAA, and GA3 on in vitro nodal-cutting explants

This experiment was conducted to investigate the effect of BAP, NAA, and GA3 on shoot development using in vitro nodal cuttings as explants. Shoot development was observed on MS basal medium without growth hormone and MS basal medium amended with BAP, NAA, and GA3 irrespective of the concentration or combination (Table 7).

The mean number of explants that survived on the MS basal medium without hormonal modification (the control) (0.5) was less than the number of surviving explants on the MS basal medium amended with growth hormones. When an MS basal medium was amended with 5.0 µM BAP only or combined with 5.0 µM GA3, the mean number of surviving explants increased to 0.8. The mean number of surviving explants further increased to 0.9 when the MS medium was amended with 5.0 µM BAP and 5.0 µM GA3 combined with either 0.5 µM or 5.0 µM NAA. The highest mean of surviving explant was 1.0, and this was observed on an MS basal medium amended with either 5.0 µM BAP and 0.5 µM NAA or 5.0 µM BAP and 5.0 µM NAA. However, there was no statistical evidence of a significant difference ($P \leq 0.187$) in the number of surviving explants.

The mean number of shoots per explant observed on an MS basal medium without hormone (0.6) was the least, which increased to 0.9 when the MS medium was amended with 5.0 µM BAP, 5.0 µM NAA, and 5.0 µM GA3. A further increase (1.0) was observed when an MS medium was amended with 5.0 µM BAP and 5.0 µM GA3 or (1.2) when an MS medium was amended with 5.0 µM BAP, 0.5 µM NAA, and 5.0 µM GA3. Finally, the highest mean number of shoots per explant (1.4) was observed on an MS medium amended with 5.0 µM BAP only and 5.0 µM BAP combined with either 0.5 µM or 5 µM NAA. Statistically, the differences observed were not significant ($P \leq 0.121$).

Leaf development was the least (1.4) on the MS medium without hormones. When 5.0 µM BAP and 5.0 µM GA3 were added to an MS medium, leaf development increased to 2.3 leaves per shoot. When an MS basal medium was amended with 5.0 µM BAP, 5.0 µM NAA, and 5.0 µM GA3, or 5.0 µM BAP and 5.0 µM NAA, the mean number of leaves per shoot observed were 3.0 and 3.1 respectively. The number of leaves per shoot increased to 3.7 when 5.0 µM BAP only was added to an MS medium, 3.8 when 5.0 µM BAP and 0.5 µM NAA were added to an MS basal medium. The highest mean number

of leaves per explant (3.9) was observed when an MS basal medium was amended with 5.0 µM BAP, 0.5 µM NAA, and 5.0 µM GA3. However, there was no statistical evidence of a significant difference (P≤ 0.193) in the number of leaves per explant on each MS medium.

The height of the shoot observed when an MS basal medium was not manipulated with growth hormone (0.51cm) was less than when an MS basal medium was amended with growth hormones. A mean height of 0.63cm was observed when an MS basal medium was amended with 5.0 µM BAP and 5.0 µM GA3. The shoot height increased to 0.70cm on an MS basal medium amended with 5.0 µM BAP, 5.0 µM NAA, and 5.0 µM GA3. A further increase in shoot height (0.80cm) was observed when an MS basal medium was amended with 5.0 µM BAP and 0.5 µM NAA, while a mean height of 0.84cm was observed when an MS basal medium was amended with 5.0 µM BAP only. A mean shoot height of 0.92cm was observed on an MS basal medium amended with 5.0 µM BAP, 0.5 µM NAA, and 5.0 µMGA3 with an optimum mean height of 1.03cm observed when an MS basal medium was amended with 5.0 µM BAP and 5.0 µM NAA. Statistically, the differences observed were not significant (P≤0.156).

Effect of different GA3 concentrations on shoot multiplication of in vitro nodal cuttings

Modification of an MS basal medium amended with 5.0 µM BAP and 0.5 µM NAA with either 5.0 µM GA3 or 50 µM GA3 had different effects on shoot development of in vitro nodal-cutting explants (Table 8).

The mean number of surviving explants on an MS basal medium amended with 5.0 µM BAP and 0.5 µM NAA without GA3 (control) was 0.8, and the same was observed when an MS basal medium was amended with 5.0 µM BAP, 0.5 µM NAA and 50.0 µM GA3 (Table 8). Explant survival was enhanced when an MS basal medium was amended with 5.0 µM BAP, 0.5 µM NAA, and 5.0 µM GA3. The number of surviving explants increased when the concentration of GA3 increased from 0 to 5.0 µM and then decreased as the hormone concentration increased to 50.0 µM, suggesting that GA3 was phytotoxic at this concentration. However, there was no statistical evidence of a significant difference (P≤0.354) in the survival rate.

The number of shoots per explant observed when an MS basal medium was amended with 5.0 µM BAP and 0.5 µM NAA without GA3 (the control) was 1.8. That increased to 2.0 shoots per explant when GA3 was increased to 50.0 µM. An optimum number of shoots per explant (2.7) was observed when GA3 was reduced to 5.0 µM. However, there was no evidence of statistical significance (P≤0.169) in the differences observed.

Leaf development on MS basal medium amended with 5.0 µM BAP and 0.5 µM NAA without GA3 was 7.0 leaves per explant, which was higher than 6.8 leaves per explant observed when an MS basal medium was amended with 5.0 µM BAP, 0.5 µM NAA and 50.0 µM GA3. An increase to 9.3 leaves per explant was observed when an MS basal medium was amended with 5.0 µM BAP, 0.5 µM NAA, and 5.0 µM GA3. Statistically, the differences observed for leaf development were insignificant (P≤0.281).

The height of the shoot followed a similar trend as leaf development. Shoot height was 1.3cm on an MS basal medium that was amended with 5.0 µM BAP and 0.5 µM NAA without GA3. Shoot height increased to 1.4cm on an MS basal medium amended with 5.0 µM BAP, 0.5 µM NAA, and 5.0 µM GA3, while it reduced to 1.2cm when an MS basal medium amended with 5.0 µM BAP, 0.5 µM NAA and 50.0 µM GA3. Although the differences observed in the height of the shoot were not statistically significant (P≤ 0.643), shoot development was vigorous on MS basal medium amended with 5.0 µM BAP, 0.5 µM NAA, and 5.0 µM GA3 (Figure 6).

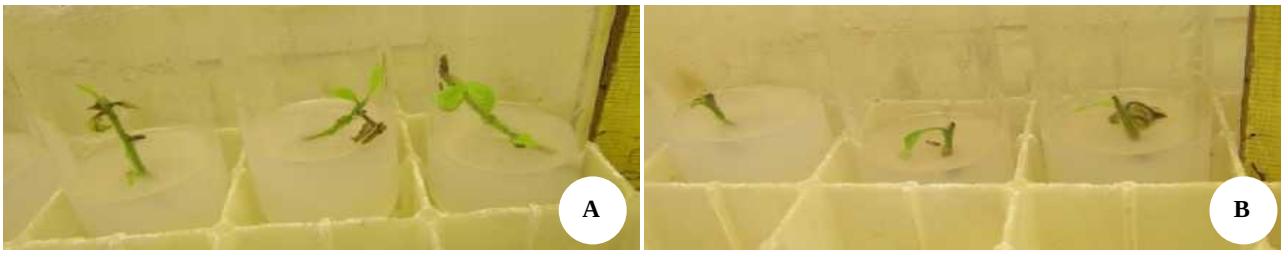
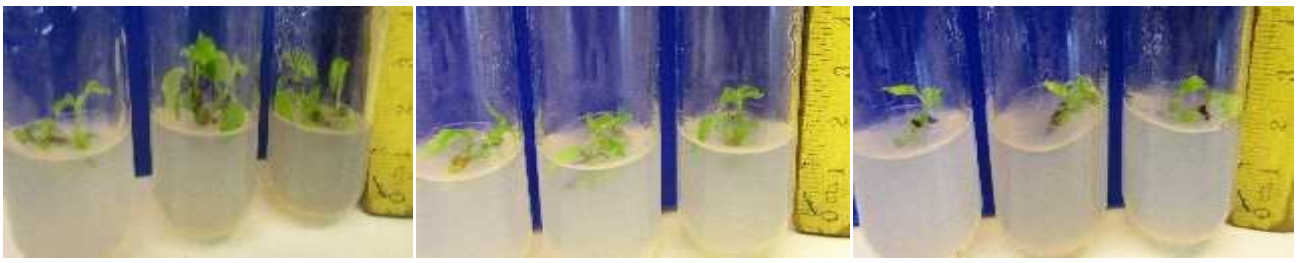


Figure 5. Shoot development from A) shoot-tip and B) nodal-cutting explants at four weeks of culture on MS basal medium amended with 5.0 µM BAP, 5.0 µM NAA, and 5.0 µM GA3



A **B** **C**

Figure 6. Effect of different GA3 concentrations on shoot multiplication of in vitro nodal cuttings; A. 5.0 µM BAP, 0.5 µM NAA and 5.0 µM GA3, B. 5.0 µM BAP and 0.5 µM NAA and C. 5.0 µM BAP, 0.5 µM NAA and 50 µM GA3

Table 7. Response of in vitro nodal-cutting explants to BAP, NAA, and GA3. Data were collected four weeks after the culture

Growth hormone (µM)	Number of explants	Surviving explants	No. of shoot per explant	No. of leaves per explant	Shoot height (cm)
0	10	0.50±0.53^{b}	0.60±0.70^{b}	1.40±1.71^{b}	0.51±0.62^{c}
5.0 BAP	10	0.80±0.42^{ab}	1.40±0.97^{a}	3.70±2.45^{a}	0.84±0.46^{abc}
5.0 BAP +0.5 NAA	10	1.00±0.00^{a}	1.40±0.70^{a}	3.80±3.16^{a}	0.80±0.29^{abc}
5.0 BAP +5.0 NAA	10	1.00±0.00^{a}	1.40±0.52^{a}	3.10±1.10^{ab}	1.03±0.30^{a}
5.0 BAP +5.0 GA3	10	0.80±0.42^{ab}	1.00±0.67^{ab}	2.30±1.42^{ab}	0.63±0.37^{bc}
5.0 BAP +0.5 NAA +5.0 GA3	10	0.90±0.32^{ab}	1.20±0.79^{ab}	3.90±3.67^{a}	0.92±0.49^{ab}
5.0 BAP +5.0 NAA +5.0 GA3	10	0.90±0.32^{ab}	0.90±0.57^{ab}	3.00±1.83^{ab}	0.70±0.62^{abc}

Note: Each value in the table is the mean and standard deviation of ten replicates. Values that do not share a letter in a column differ significantly by LSD ($\alpha = 0.05$)

Table 8. Response of in vitro nodal-cutting explants to different GA3 concentrations on an MS basal medium supplemented with BAP and NAA. Data were collected four weeks after the culture

GA3 concentration (µM)	Number of explants	Surviving explants	No. of shoots per explant	No. of leaves per explant	Shoot height (cm)
0	10	0.80±0.42^{a}	1.80±1.14^{a}	7.00±3.92^{a}	1.33±0.77^{a}
5.0	10	1.00±0.00^{a}	2.70±0.82^{a}	9.30±3.40^{a}	1.44±0.40^{a}
50.0	10	0.80±0.42^{a}	2.00±1.25^{a}	6.80±4.08^{a}	1.19±0.67^{a}

Note: Each value in the table is the mean and standard deviation of ten replicates. Values that do not share a letter in a column are different from each other significantly by LSD ($\alpha = 0.05$)

Discussion

Decontamination of explants

Reports on in vitro propagation of *C. membranaceus* are scanty, with few propagation methods using seeds as explants with low germination rates due to the sterility (Aboagye 1997). However, there are reports on in vitro multiplication of many species belonging to the same genus which have been successfully cultured in vitro (Shibata et al. 1996; Orlikowska et al. 2000; Nasib et al. 2008; Ashish and Sharma 2011; Salamma and Rao 2013). Therefore, the present study was conducted to establish an effective sterilization regime and determine an appropriate totipotent tissue for the rapid multiplication of *C. membranaceus*.

An appropriate sterilization scheme may greatly affect the efficiency of subsequent explants regeneration (Sen et al. 2013; Evtushenko et al. 2016). In addition, the exposure duration to the sterilants may be vital in producing viable explants in vitro (Badoni and Chauhan 2010). These treatments tend to remove pathogens in seeds, nodal cuttings, and shoot tips (Srivastava et al. 2010; Donnaruma et al. 2011; Garg et al. 2011). The explants' contamination depends on plant species, donor age, source, and weather (Srivastava et al. 2010); single or double sterilization regimes may be used depending on the source. Before culture, this study used double sterilization with NaOCl to effectively sterilize explants, including seeds obtained from the field under ambient conditions, which may be highly susceptible to contaminations. Even though NaOCl was used, double sterilization of field-grown *Aloe vera* was effectively decontaminated before culture using 0.1% mercuric chloride and 0.04% streptomycin in 5-6 min (Singh and Sood 2009). Coatless seeds and embryos were not immersed in the sterilant after isolation since they are well protected in intact double sterilized seeds. With double sterilization of explants, 86.7%, 80%, and 100% of intact, coatless seeds and isolated embryos were decontaminated, respectively. The frequency of decontamination was high when seed explants were immersed in ethanol before sterilizing in NaOCl solution. While a lower percentage of NaOCl and more time was optimum for decontaminating intact seeds, a higher percentage of NaOCl, irrespective of time, was optimum for decontaminating seeds before the seed coat removal and isolation of embryos. Thus, the increase in the concentration of sterilant and timing did not improve the decontamination of intact seeds but was necessary when the seed coat ought to be removed. Using 15% NaOCl solution for 20 min, followed by 10% NaOCl, given the optimal decontamination frequency and did not improve when the concentration and time were increased. Pre-treatment with ethanol and double sterilization may have accounted for the high decontamination rates at a lower sterilant concentration. For each treatment A, B, and C, using ethanol in the sterilization regime (A1, B1, and C1) resulted in higher numbers of decontaminated intact seeds than when ethanol was not used before sterilization (A0, B0, and C0). Although the frequency of decontaminated coatless seeds and embryos was higher when isolated from seeds immersed in ethanol before sterilizing in NaOCl solution, the frequency improved with increased NaOCl solution (20%) while the time was either maintained (20 min) or reduced (15 min). A detailed review of work on other croton species revealed that only single sterilization was employed. Salamma and Rao (2013) reported that seeds of *Croton scabiosus* were best sterilized when immersed in 70% ethanol for 60 seconds after sterilizing with 30% Sodium hypochlorite solution for 15 min. Again, effective double sterilization of *Croton variegatum* (Croton) leaf explants, using ethanol pre-treatment and followed by 25% NaOCl and $HgCl_2$, has been reported by Radice (2010). That may support the generally high numbers of decontaminated seeds, coatless seeds, and

isolated embryos obtained when ethanol precedes double sterilization in this study, even though $HgCl_2$ was used as the second sterilization agent by Radice (2010).

The current study also tested the effect of sterilization on explants regeneration. None of the decontaminated intact seeds developed shoots; this failure could be due to factors other than the sterilant. One factor may be the hard seed coat limiting the water uptake to initiate germination and the embryo's size. According to Koornnef et al. (2002), seed germination is influenced by the embryo's growth potential and the limitation caused by the surrounding tissues. The underdeveloped but differentiated embryo size and the presence of a seed coat are reported as the most limiting germination (Finch-Savage and Leubner-Metzger 2006).

Coatless seeds and isolated embryos formed plantlets irrespective of the use or non-use of ethanol and the concentration of NaOCl in the sterilization regime used. Since plantlet formation was not limited by ethanol pre-treatment and decontamination was highest with ethanol pre-treatment for both coatless seeds and isolated embryos, an established sterilization regime for effective decontamination of both explants should include ethanol pre-treatment.

Nodal-cutting explants were decontaminated with double sterilization, irrespective of ethanol as pre-treatment. There was a 100% rate of decontamination with or without ethanol pre-treatment, which established the effectiveness of double sterilization. With double sterilization, 20% NaOCl for 15 or 20 min, followed by 15% NaOCl for 10 or 15 min, was optimum for decontaminating nodal explants. The ineffectiveness of single sterilization with NaOCl was reported by Salamma and Rao (2013). They found that a single application of 5-20% NaOCl was ineffective for surface sterilization of nodal explant of *C. scabiosus* from the wild. A comparison of the three sterilization regimes showed that the higher percentage of 20% NaOCl (treatments B and C) resulted in an optimum number of decontaminated nodal explants (Table 2). However, shoot development from nodal-cutting explants was higher (73.3- 93.3%) when explants were not pretreated with ethanol than (67-80%) pretreated. The low rate of shoot development explants pretreated with ethanol and sterilized with high concentrations of NaOCl could be delicate meristematic tissues that may have been scorched by ethanol and higher concentrations of NaOCl (Sen et al. 2013).

Initiation of cultures from seeds

The successful multiplication of plants in vitro is determined by the type of explant used (Gitonga et al. 2010; Alagumanian et al. 2004; Ali and Mirza 2006; Kumar et al. 2011), the growth regulators (Kumar et al. 2011) in the culture medium and the different levels of endogenous phytohormones present in the plant tissues (Kumar and Reddy 2011). Gitonga et al. (2010) observed varying responses of different explants in culture media with only nodal segments and cotyledons of *Macadamia* spp. form shoots while leaf explants dried up within the first week of culture. This thesis used four explants to initiate in vitro multiplication for Croton. These were intact seeds, coatless seeds, isolated embryos, and nodal cuttings. The explants were grown on media amended with various concentrations and combinations of BAP, NAA, and GA3 and compared with MS basal medium without growth hormone (the control).

Intact seeds did not show any shoot development at four weeks independent of the media composition, except that only one seed developed radicle on an MS medium amended with 5.0 µM BAP and 5.0 µM NAA after twelve weeks. Rather than growth hormones, the hard seed coat may be the limiting factor to shoot development from intact seeds as inadequate water is absorbed, and the comparatively small embryos cannot break through the seed coat. Further, the intact seeds did not develop shoots in this study, which suggests that the media formulated for *C. membranaceus* may require further additives such as peptone to aid shoot development. Even though peptone on seed germination was not investigated, peptone has been reported to support in vitro germination of *Dendrobium lasianthera* (Utami et al. 2017) and *Phalaenopsis* hybrid (Shekarriz et al. 2014).

Development of coatless seeds into plantlets was observed on MS basal medium (the control) and MS basal medium amended with BAP combined with NAA or GA3; those several plantlets depended on hormonal treatment. A high BAP to low NAA concentration ratio gave the highest plantlet formation (Table 4). A similar result on high BAP to low NAA combination has been reported by Vijendra et al. (2017) for *Mentha piperitau*, though shoot-tip and nodal explants were used. Further, a combination of BAP and NAA in MS basal medium has been reported in inducing shoots from different sources of explants, such as embryos in *C. scabiosus* (Salamma and Rao 2013) and callus induction and shoot regeneration of *Lantara camara*, a medicinal plant (Veraplakron 2016). On the other hand, a combination of BAP, NAA, and GA3 did not support plantlet formation from coatless seeds. Similar results were obtained by Masekesa et al. (2016). They reported a near similar observation that 0.5 mg/L (2.7 µM) NAA + 1 mg/L (4.4 µM) BAP and 10 mg/L (28.9 µM) GA3 did not support the induction of shoot from cultured meristems of sweet potato. Therefore, it seems that the presence of GA3 may have an inhibitory effect on NAA for producing shoots from coatless seeds.

Unlike coatless seeds, plantlet development from isolated embryos was highest (Table 6) when cultured on an MS basal medium amended with 5.0 µM BAP and 5.0 µM NAA. Contrary to the present findings, Karami (2016) has reported that the addition of 3mg/L (17.1 µM) IAA+ 0.5 mg/L (2.2µM) BAP, a high auxin to cytokinin ratio enhanced the development of embryos in *C. scabiosus*.

The high regeneration rate of isolated embryos in comparison with coatless seed and intact seed explants may be explained by the fact that embryos have a fully differentiated morphogenetic structure with potential shoots and roots that may undergo full regeneration into matured plants on a medium containing BAP and GA3 (Freitas et al. 2016) as compared to coatless seeds and intact seeds with no well-defined morphogenetic structure.

Further, the use of embryo culture for the regeneration of plantlets from recalcitrant plant species has been reported by Moura et al. (2009) while working with macaw palm (*Arocomia aculeata*).

Initiation of cultures from nodal cuttings and shoot-tips

Considering the type of explants for shoot induction in the current study, explants from nodal segments showed the best regeneration of shoots regarding the number of shoots, leaves per explant, and height. These observations were similar to findings in *Croton bonplandinum*, as reported by Ashish and Sharma (2011). Nodal-cutting explants showed the best response and developed into shoots independent of the concentration of growth hormones in the culture medium. However, the development of multiple shoots was highest, with a mean of 0.8 shoots per explant on an MS basal medium amended with 5.0 µM BAP alone, followed by 0.7 shoots per explant when an MS basal medium was amended with 5.0 µM BAP, 5.0 µM NAA, and 5.0 µM GA3. Multiple shoot induction from nodal cuttings of *C. bonplandinum* was obtained on MS basal medium amended with 0.5mg/L (2.22 µM) BAP alone (Ashish and Sharma 2011). 5.0 µM BAP alone or in combination with 5.0 µM NAA and 5.0 µM GA3 equally promoted leaf development but shoot height was optimum with all three hormones at the same concentration. The addition of GA3 was more effective in the formation of shoot from nodal explants of *C. membranaceus* than BAP alone or BAP combined with NAA as GA3 enhanced the effect of BAP and NAA, resulting in shoot elongation, thereby making the nodes distinct for subculturing. It was therefore established that the combined effects of BAP, NAA, and GA3 on shoot development were the best for nodal-cutting explants of *C. membranaceus*.

However, this is not in line with shoot regeneration from other croton species, as Nasib et al. (2008) reported. They observed that the formation of shoot from nodal cuttings of *Co. variegatum* produced axillary buds on MS basal medium amended with 0.5mg/L (2.22 µM) BAP alone. That may have resulted from the differences in concentration of endogenous hormones in the plant (Schween and Schwenkel 2003) and the type of plant growth hormones being cultured in the medium plant (Thompson et al. 2008). Apart from growth hormones, shoot regeneration depended on the part of the stem segment used. Shoot-tip explants showed faster bud break and rapid shoot growth over nodal cuttings, more leaves, and longer internodes from shoot-tip explants. The response of shoot-tip explants over nodal segments in shoot induction has been observed in *Spilanthes mauritiana* (Sharma et al. 2009) and *Coleus blumei* (Rani et al. 2006) while in *Simarouba glauca*, nodal cuttings showed higher frequencies of axillary bud initiation than shoot-tip explants (Shukla and Padmaja 2014), which may suggest that the response of nodal cuttings and shoot-tips would be species-specific.

Shoot multiplication from in vitro nodal cuttings

The investigation of the effect of various combinations and concentrations of BAP, NAA, and GA3 on the multiplication of in vitro nodal explants showed that multiple shoots were established on MS basal medium with and without growth hormones. An MS basal medium amended with 5.0 µM BAP alone or in combination with 0.5 µM NAA or 5.0 µM NAA resulted in the highest number of shoots per explant. That is comparable to optimum multiple shoot formation on MS medium amended with 5.0 µM BAP and 0.5 µM IAA for shoot multiplication of *Celastrus paniculatus* (De Silva and Senarath 2009). On the contrary, Thangavel et al. (2016), working on the medicinal plant *Janakia arayalpathra*, reported that adding NAA and BAP in shoot multiplication medium induced basal callus formation but suppressed the growth of adventitious shoots. The different responses reported may be related to the genotype, and the level of hormones already existing in the plant part used (Li et al. 2002; Schween and Schwenkel 2003, and Thompson et al. 2008).

In the current study, adding 5.0 µM GA3 to 5.0 µM BAP and 0.5 µM NAA resulted in the highest number of leaves per explant. Further experimentation with two different concentrations (5.0 µM and 50 µM) of GA3 combined with 5.0 µM BAP and 0.5 µM NAA to investigate their effect on the multiplication of *C. membranaceus* in vitro showed the highest shoot regeneration when 5.0 µM GA3 was added and the least when 50 µM GA3 was added. Generally, in culture, GA3 is added in the medium to prevent rosette growth as it enhances shoot elongation and the growth of meristems and buds (Kumar and Reddy 2011). However, the current study observed that the concentration of GA3, in combination with BAP and NAA, had a profound effect on shoot development. This situation conforms to the fact that interaction and ratio between growth hormones in the medium can affect the growth and development of plants in vitro (Kumar and Reddy 2011).

Adding 5.0 µM GA3 to 5.0 µM BAP and 0.5 µM NAA greatly enhanced the number of shoots and leaves per explant and the height of shoots. This study showed that a ten-fold increase in the concentration of GA3 from 5.0 µM to 50 µM was inhibitory to shoot development in *C. membranaceus*. Even though in all reports on the multiplication of other croton species, GA3 was not used, similar findings were observed and reported by Fotopoulos and Sotiropoulos (2004) that the addition of 0.028 to 0.28 µM GA3 enhanced vigorous shoot formation while a ten-fold increase from 0.28 to 2.8 µM GA3 in combination with various concentrations of BAP and NAA resulted in a reduction of the rate of shoot proliferation in *Prunus persica*, indicating that a higher concentration of GA3 may be inhibitory to shoot development.

In conclusion, the current research has established a protocol for effective decontamination of explants for initiation and multiplication of culture of *C. membranaceus* in vitro. Decontamination was achieved using 70% ethanol for 3 min and double sterilization with NaOCl solution.

Different explants responded differently to the double sterilization regimes used. Sterilized intact seeds did not develop into shoots which may be due to inhibitory effects associated with the seed. Explants from nodal sources showed better shoot formation over seed explants, with shoot-tip explants as the best for shoot induction and rapid shoot development. The culture medium for rapid multiplication is an MS medium amended with 5.0 µM BAP, 0.5 µM NAA, and 5.0 µM GA3. The various hormone combinations and concentrations used did not promote the root development of *C. membranaceus*.

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Interactions between soil additives and a variety of naturally occurring nematode-demolishing fungi in banana fields of Meru and Embu Counties, Kenya

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Abstract. *Wairimu WJ, Kimenjul JW, Muiru WM, Wachira PM. 2022. Interactions between soil additives and a variety of naturally occurring nematode-demolishing fungi in banana fields of Meru and Embu Counties, Kenya. Cell Biol Dev 6:* 82-93. Plant-parasitic nematodes pose a significant danger to banana production, as they reduce the productivity, quality, and lifetime of banana orchards. This study aimed to evaluate the diversity, quantity, and occurrence of nematode-demolishing fungi in banana production farms to use them to manage plant-parasitic nematodes. Also examined was the impact of organic and inorganic soil additives on nematode-demolishing fungi. The study region was divided into three agroecological zones: UM3 (Low), UM2 (Middle), and UM 1 (Upper). Ten farms were chosen randomly for a soil sample in each zone to determine the diversity and abundance of nematode-demolishing fungi. One farm was chosen randomly for soil additive treatments in each zone. The gathered soil samples were used to isolate and identify nematode-demolishing fungi at the species level. The diversity of nematode-demolishing fungi varied significantly between zones, with the highest variety and number of fungi found in the highest zone. *Arthrobotrys, Monacrosporium, Nematoctonus, Harposporium,* and *Paecilomyces* were the identified genera. *Arthrobotrys* was the most often isolated genus, with a frequency of 45%, followed by *Harposporium,* with a frequency of 18%. The remaining three genera each had a frequency of 9%. The *A. dactyloides, A. oligospora, A. robusta, A. longispora, A. superba, H. anguillulae, H. crassum, Meria coniospora, Monacrosporium cionopagum, N. leiosporus,* and *P. lilacinus* were among the species identified. The *A. oligospora* had captured and destroyed 98 plant parasite nematodes, whereas *M. cionopagum* and *Dactyllela phymatopaga* had eliminated 88 and 76 plant parasitic nematodes, respectively, within the same time frame. The amount of nematode-demolishing fungi was significantly different between the various soil additives, with chicken dung having the greatest number of 74 isolates, followed by cow manure, goat manure, the control treatment, and inorganic additive with 71, 69, 54, and 39 isolates, respectively. The amount of isolated nematode-demolishing fungi fluctuated throughout time, from 89 pre-treatment isolates to 122 after three months and 96 after six months. The variation of nematode-demolishing fungi over time was significantly different, demonstrating the impact of diverse soil additives on their existence. *Arthrobotrys* spp. is a suitable option for field efficacy studies since it was the most diversified, had the highest demolishing rate, and the organic additives facilitated its dispersion in banana plantations.

Keywords: Banana farm, Embu, fungi, Meru, nematode, soil additives

INTRODUCTION

Bananas (*Musa* spp.) are the most widely consumed fruit in the world, with consumers spending over £10 billion on bananas every year (Trade Fair 2013; Hapsari et al. 2017). About two percent of all international trade is in fresh bananas. Evidence of widespread dependence on fruit shows that only 15% are traded on the global market (FAO 2005), with the remaining 85% consumed inside individual countries. The banana is the fourth most valuable crop in the world (Muchui et al. 2013), after rice, wheat, and maize, to ensure food security in developing countries.

Bananas, both raw and cooked, are a nutritious choice because they include a variety of useful nutrients. For subsistence farmers, their attractive features include adaptability to intercropping, rapid growth, and a prolonged harvesting time (Macharia et al. 2010). Bananas are consumed not only by humans but are also utilized as animal fodder and material for roofing, flooring, and even matting. Furthermore, they provide a beneficial ground cover that shields the soil from wind and decreases the likelihood of erosion.

Bananas are widely traded and consumed in both underdeveloped and developed nations. Bananas play an important role in East African agriculture, both a staple crop and a source of revenue for subsistence farmers (Seshu et al. 1999). Almost 15 million tons of bananas are produced annually in the East African highlands (Ng'ang'a et al. 2011), making this food staple for over 20 million people. According to the data compiled by Ng'ang'a et al. (2011), small-scale farmers in Kenya are responsible for cultivating the vast bulk of Kenya's banana crop. Lacatan, Uganda Green, Apple, Gros Michel, Dwarf Cavendish, Giant Cavendish, Grand Nain, Williams, Valery, Muraru, Sukari, and Kiganda are some of the most popular types planted in Kenya.

Giant Cavendish, Apple (Sweet Banana), Valery, Uganda Green (for use in cooking), and Red Banana (used primarily in baking) are the most desirable export kinds (Mburugu 2013). Bananas are mostly grown in Kenya's Central, Eastern, Western, Nyanza, and Coast provinces (Ministry of Agriculture 2008; Kabunga et al. 2012; Mwombe et al. 2013; Karienye and Kamiri 2020; Kirimi et al. 2021; Wahome et al. 2021). Recently, coffee fields in the East and Central have given way to banana plantations (Muchui et al. 2013). As a result, growing bananas has been singled out as an industry that helps those in need. However, the banana product is in decline because of

knowledge gaps and constraints experienced by small-scale farmers and other players in the banana value chain, as indicated in a situational analysis of Imenti south districts conducted by Muchui et al. (2013).

Production of bananas in Kenya has dropped drastically during the past few decades. An increase in pests and illnesses, made worse by a lack of efficient control techniques, is mostly to blame for the decline, say Kahangi et al. (2002). Deteriorating soil fertility, poor crop management, inadequate sanitary planting material, poor marketing arrangement, post-harvest damage, genetic corrosion, and high costs are only some of the factors contributing to this decrease (Macharia et al. 2010).

Banana yields can drop by a factor of one hundred due to pests and illnesses, and the quality of the crop will suffer as a result (Viljoen 2010). Given their minuscule size, high rate of destruction, and the section of the plant they attack, root-lesion nematodes are a serious and economically significant pest of many cultivated crops worldwide (Trifonova and Karadjova 2009). Above-ground symptoms of nematode damage to roots include nutrient deficiency, incipient wilt, stunting, and poor yield; however, these symptoms are often misinterpreted as being caused by a lack of soil nutrition (Viljoen 2010), delaying the diagnosis and, as a result, management measures and increasing crop losses. In addition, crop damage caused by root-invading nematodes sometimes goes unnoticed by cultivators because of a lack of awareness of the symptoms created by parasitic nematodes, such as root galls, root lesions, and cysts. As a result, the banana crop is still threatened by nematodes in every place it is farmed (Mitreva et al. 2005).

This research aimed to examine the impact of organic and inorganic additives on the population and diversity of nematode-demolishing fungi in various agroecological zones and estimate the abundance and diversity of nematode-demolishing fungi in banana fields.

MATERIALS AND METHODS

Study site

Embu and Meru Counties, both in Kenya's Eastern province, were the focus of the research (Figure 1). Embu is found 120 kilometers northeast of Nairobi, at the height of 1,350 meters above sea level on the southern slopes of Mount Kenya (coordinates: 0.5333° S, 37.4500° E). At 1,462 meters above sea level and 275 kilometers from Nairobi, the town of Meru is a popular spot. The soils are rich, deep, and volcanic in origin, making them ideal for growing tea, coffee, bananas, and other crops. Study areas included the UM1 (upper midland high zone), UM2 (upper midland intermediate zone), and UM3 (lower upper midland zone). Since most bananas are grown in these areas, they made for good study locations. Land in the higher zone is used mostly for growing tea, intercropped with maize and banana; in the middle zone, tea and coffee are grown alongside banana plantations and horticultural fields; and in the lower zone, banana plantations predominate. Livestock was also kept in the upper and middle zones.

Assessing the diversity and abundance of nematode-demolishing fungi in banana fields

Experimental design

To contrast the effects of cultural practices and climate on the distribution and prevalence of nematode-demolishing fungi, the region was separated into three zones ranging from high altitude to low altitude at each study site. In Embu and Meru counties, 60 farms were chosen using a completely random design. Banana plantations were prioritized in the selection process of farms. Thirty samples of soil were gathered from each of the two sites. In June 2013, ten (10) samples were obtained randomly from each zone.

Soil sampling procedure

A random sampling technique was employed to collect five soil samples from each banana farm in Meru and Embu counties and three samples from each banana stool from the Embu trial locations. Soil cores were collected from a depth of 10-20 cm around the banana roots using core collection equipment (soil auger). The 500-gram composite sample was sealed in plastic and placed in a cool box for transit to the lab, where it was kept in a cold room at 10°C until the nematode-demolishing fungi could be isolated. To prevent contamination across sites, the auger was sterilized by dipping it in 70% ethanol between each sampling. Subsequently, the soil samples were utilized to extract nematode-demolishing fungi and plant parasitic nematodes.

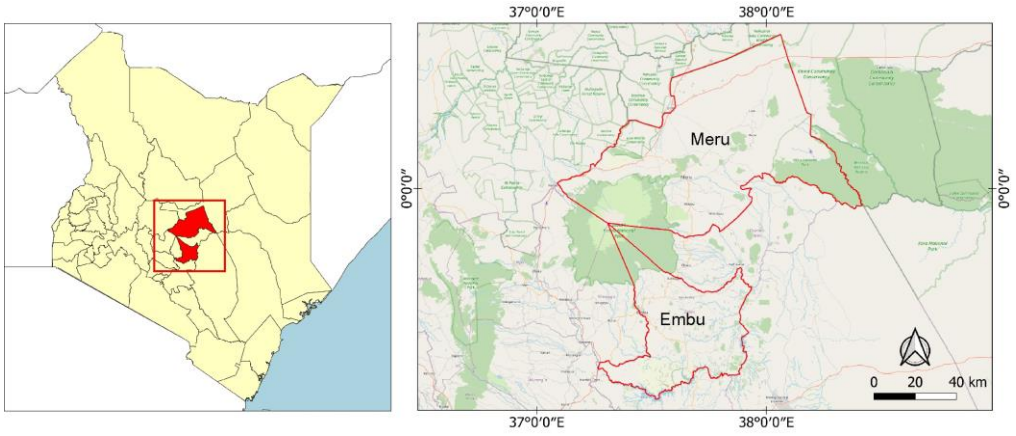

Figure 1. Maps of Embu and Meru, Kenya (Google Maps 2016)

Isolation of nematode-demolishing fungi

The 60 samples collected from Embu and Meru counties and the 45 samples collected from farms in Embu County that had been treated with organic and inorganic manure were used in an isolation process to look for nematode-demolishing fungi. Tap water agar was made by dissolving 20 grams of agar in 1 liter of regular tap water. The medium was autoclaved at 121 ° C for 15 minutes, cooled to 45 ° C, and then modified with 0.1 grams of streptomycin sulfate to prevent further bacterial growth. To isolate nematode-demolishing fungi, the soil was dusted on plates following the method described by Jaffe (1996). On the surface of the tap water agar plates, one gram of soil from each sample was evenly distributed, and this process was repeated three times for each soil sample. *Melodogyine* spp. J2 plant parasitic nematodes were used as bait, and plates were incubated at room temperature before being checked daily beginning in the third week and continuing through the sixth week using a dissecting microscope to see how much growth had occurred. Experiment nematodes were collected from soil and root samples following Kleynhans's method (1999). Prepared slides of the deceased nematodes were studied with a compound microscope. Fungal nematode demolishers in the study region were classified by genus and species based on their capturing organ and conidia form. Each farm's status regarding the presence or lack of fungi capable of demolishing nematodes was noted. The detected fungi were subcultured on potato dextrose agar to obtain pure cultures for the in-vitro nematode-demolishing fungi trapping ability trial. Prepared by dissolving 39.5 grams of potato dextrose agar into one liter of purified water. When the medium was modified with 0.1 g of streptomycin sulfate, it was autoclaved at 121°C for 15 minutes, cooled to 45°C, and then used to inhibit bacterial growth.

Identification of the nematode-demolishing fungi

Identification of the fungi to the species level was achieved by analyzing their morphology and trapping characteristics in relation to nematodes. Conidial shape, septation, conidiophore morphology, adhesive hyphae, adhesive traps, non-constricting rings, constricting rings, adhesive knobs, and inward invasions were the key features used. The fungi were observed on glass slides using a 1000x compound microscope. Nematodes, their trapping organs, and the conidia of the nematode-demolishing fungi were all photographed.

Determining the potential of the nematode-demolishing fungi under laboratory conditions

The 60 samples collected from Embu and Meru counties and the 45 samples collected from farms in Embu County that had been treated with organic and inorganic manure were used in an isolation process to look for nematode-demolishing fungi. Tap water agar was made by dissolving 20 grams of agar in 1 liter of regular tap water. The medium was autoclaved at 121°C for 15 minutes, cooled to 45°C, and then modified with 0.1 grams of streptomycin sulfate to prevent further bacterial growth. To isolate nematode-demolishing fungi, the soil was dusted on plates following the method described by Jaffe (1996). On the surface of the tap water agar plates, one gram of soil from each sample was evenly distributed, and this process was repeated three times for each soil sample. *Melodogyine* spp. J2 plant parasitic nematodes were used as bait, and plates were incubated at room temperature before being checked daily beginning in the third week and continuing through the sixth week using a dissecting microscope to see how much growth had occurred. Experiment nematodes were collected from soil and root samples following Kleynhans's method (1999). Prepared slides of the deceased nematodes were studied with a compound microscope. Fungal nematode attackers in the study region were classified by genus and species based on their capturing organ and conidia form. Each farm's status regarding the presence or lack of fungi capable of demolishing nematodes was noted. To test the fungi's ability to capture nematodes in vitro, each species was subcultured on potato dextrose agar to produce pure cultures. Prepared by dissolving 39.5 grams of potato dextrose agar into one liter of purified water. When the medium was modified with 0.1 g of streptomycin sulfate, it was autoclaved at 121°C for 15 minutes, cooled to 45°C, and then used to inhibit bacterial growth.

Field trials, design, and application of treatments

To study the impact of organic and inorganic soil additives on the presence and diversity of nematode-demolishing fungi, researchers applied several treatments on soil in the Embu county region. In the experiment, Randomized Complete Block Design was utilized. The soil additive studies were conducted on a single farm in each zone with a monoculture banana crop and employed good agronomic techniques before the trials. Each treatment was applied three times on the farm, yielding a total of 45 samples for each sampling period and 135 samples for the three sampling periods. Species cumulative curves were utilized to validate the required sample size. Banana stools comprised four/three to six-month-old Israel cultivar banana plants per treatment application. The treatments employed were (i) cow manure, (ii) goat manure, and (iii) chicken manure that had been composted, cured, and dried at the recommended rate of 5% wet weight for six months (Wachira et al. 2009a), (iv) a compound fertilizer, and (v) a control banana stool where no treatment was administered. Cow manure, goat manure, and chicken manure were each applied at a rate of 40 kg per banana stool, along with compound fertilizer (17:17:17 100g/stool) and a control stool where no treatment was administered. The carbon-to-nitrogen ratio of organic additives for cow, goat, and chicken was 16:0.8, 26:2.3, and 20:0.6, respectively. The additives were mixed into the soil to a depth of 10 cm in each banana stool.

To allow for full decomposition and interaction with the rhizosphere, soil samples were taken (i) immediately before the application (pre-treatment) of the additives in the third week of July 2013, (ii) three months after the application date in the second week of September 2013, and (iii) six months after the application date in the second week of December 2013 to determine the presence and diversity of nematode-demolishing fungi.

Data analysis

Microsoft Excel 2013 was used to input data on the presence/absence of various nematode-demolishing fungi, and this information was then analyzed using tables and charts. Frequency of occurrence, Renyi profiles, the Shannon diversity index, and evenness were examined with R commander software to determine the diversity of nematode-demolishing fungi (Kindt and Coe 2005). The predatory fungal activity was calculated using Microsoft Excel 2013 by comparing the number of plant parasitic nematodes caught to the total number of plant parasitic nematodes. Soil additives' effects on the fungal population were compared by analyzing variance in the number of fungal isolates collected from each additive using the Genstat 15th version. The Least Significant Difference (LSD) test was used to compare the means at the 5% confidence interval.

RESULTS AND DISCUSSION

Diversity and abundance of nematode-demolishing fungi in banana fields

Characterization of nematode-demolishing fungi in Meru and Embu counties

This research determined 138 different fungal isolates to be nematode-demolishing agents. There were a total of 11 different species, divided into 6 different genera. *Arthrobotrys, Meria, Monacrosporium, Nematoctonus, Harposporium,* and *Paecilomyces* were the recognized genera. In terms of frequency, *Arthrobotrys* was the most often isolated species (45%), followed by *Harposporium* (18%) and the other three genera (9% each). There were many different types of *Arthrobotrys* found in the samples taken, including *A. oligospora, A. longispora, A. superba, A. dactyloides, A. robusta, Harposporium anguillulae, Harposporium crassum, Meria coniospora, Monacrosporium cionopagum, Nematoctonus leiosporus,* and *Paecilomyces lilacinus.* The species with the highest prevalence was *M. cionopagum* (26.6%), followed by *A. oligospora* (17.4%) and *A. robusta* (0.7%) (Table 1). The prevalence of *M. cionopagum* was highest in Embu (40.5%) and Meru (21%). The second most common species in both locations was *Arthrobotrys* spp., whereas the least common was *P. lilacinus*. The agroecological zone had a significant impact on only two species: *A. longispora* and *H. crassum*, with P values of 0.014 and 0.059, respectively ($P<0.05$) (Table 1).

There was no noticeable difference in the distribution of nematode-demolishing fungi, which was discovered in all three agroecosystems. The highest concentration of nematode-demolishing fungi (53 isolates) was found in the uppermost zone (UM1). The highest incidence (49 occurrences) was found in the intermediate zone, while the lowest incidence (36 occurrences) was found in the lowest altitude (Figure 2).

Diverse nematode-demolishing fungi were found in only one of the agroecological regions, while the other two had many more. The mean Shannon diversity of nematode-demolishing fungi was 0.511 in the low zone, 0.758 in the middle zone, and 0.799 in the upper zone (see Figure 3). The mean Shannon value across all samples was 0.689.

Figure 4 shows that the upper zone had the most diversity richness, followed by the middle and low zones. The *M. coniospora, A. robusta, N. leiosporus, H. anguillulae,* and *H. crassum* were detected in Meru but not in Embu (Table 2).

The total species cumulative curve for this study showed that the 60 soil samples used were sufficient for estimating nematode-demolishing fungi (Figure 5).

Table 1. Frequency of occurrence of nematode-demolishing fungi in Embu and Meru Counties, Kenya

Isolate	Rank	No. of isolates	%	Cumulative frequency	P- value (P=0.05)
M. cionopagum	1	37	26.8	1.6	0.6233
A. oligospora	2	24	17.4	44.2	0.8199
A. longispora	3	21	15.2	59.4	0.01396
A. superba	4	19	13.8	73.2	0.5964
A. dactyloides	5	15	10.9	84.1	0.3564
H. anguillulae	6	7	5.1	89.1	0.8572
H. crissum	7	5	3.6	92.8	0.0590
N. leiosporus	8	4	2.9	95.7	0.3547
P. lilacinus	9	3	2.2	97.8	0.3613
M. coniospora	10	2	1.4	99.3	0.1305
A. robusta	11	1	0.7	100	0.3742

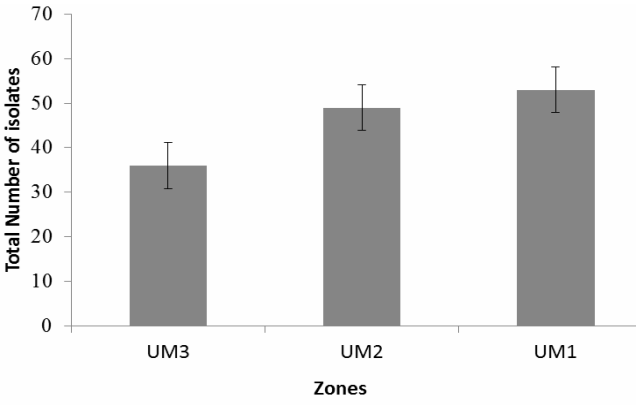

Figure 2. Frequency of nematode-demolishing fungi in Embu and Meru Regions, Kenya

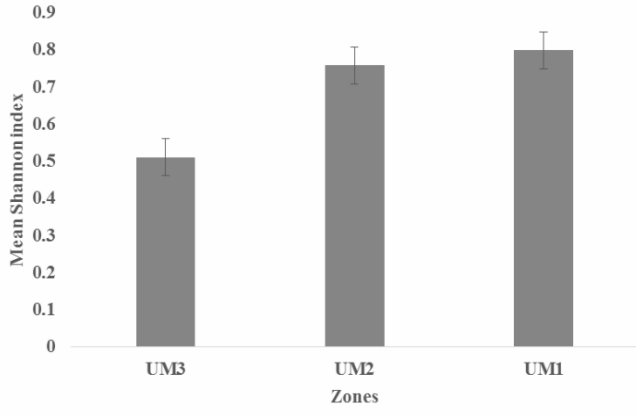

Figure 3. Mean Shannon indexes of the nematode-demolishing fungi in the study area

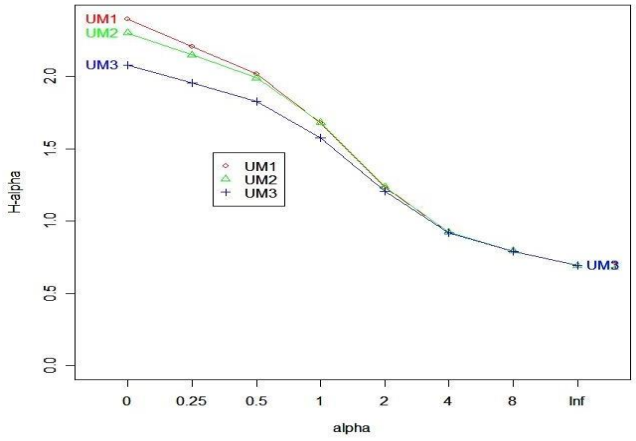

Figure 4. Renyi diversity profile for nematode-demolishing fungi in the study area

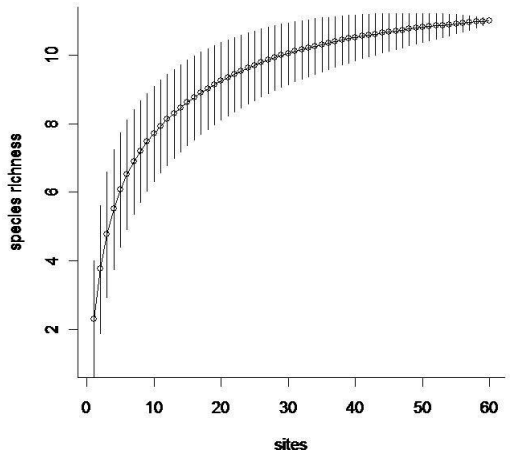

Figure 5. Frequency cumulative curve for the species of nematode-demolishing fungi

Table 2. Comparison of the nematode-demolishing fungi species present in banana production sites in Embu and Meru Counties, Kenya

Isolate	Embu in %	Meru in %
M. cionopagum	12	14.5
A. oligospora	5.8	11.6
A. longispora	3.6	10.7
A. superba	2.9	10.7
A. dactyloides	4.3	7.2
H. anguillulae	-	5.1
H. crassum	-	3.6
N. leiosporus	-	2.9
P. lilacinus	1.4	1.4
M. coniospora	-	0.7
A. robusta	-	0.7
Total	30	69.1

Occurrence of nematode-demolishing fungi in Embu

The Embu research site yielded 42 nematode-demolishing fungal isolates. The fungi in the Embu research site belonged to *Arthrobotrys*, *Monacrosporium*, and *Paecilomyces*. Seven *Arthrobotrys* were discovered: *oligospora, longispora, superba, dactyloides, robusta, cionopagum,* and *lilacinus*. The three agroecological zones in the Embu study site did not differ significantly in terms of the prevalence of the fungi that demolish nematodes. The number of isolates was largest in the upper zone (15), then in the middle zone (14), and lowest in the lower zone (13). It was found that nematode-demolishing fungi were less likely to be isolated at lower altitudes (Figure 6). The presence or absence of agroecological zones has no effect on their dispersal.

Occurrence of nematode-demolishing fungi in Meru

Ninety-six fungal isolates were found to be nematode-demolishing agents in the Meru investigation. Nematode-demolishing fungi were found in six different genera and eleven different species. There were 40 isolates in the high zone, 35 in the middle zone, and only 21 in the low zone. The results showed that ecological zones significantly (P=0.04) influenced the occurrence of nematode-demolishing fungi, with the frequency of occurrence decreasing with altitude from upper, middle, to low zones (Figure 7).

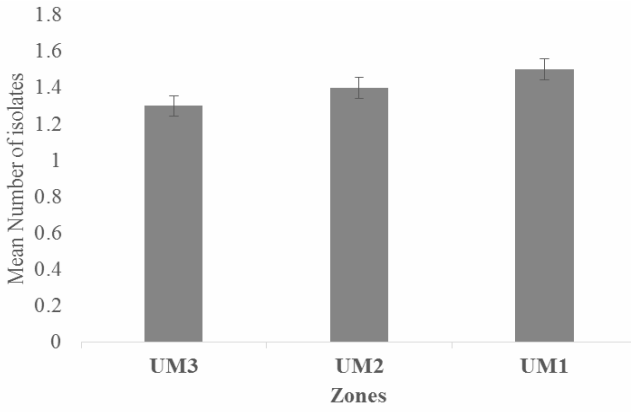

Figure 6. Occurrence of nematode-demolishing fungi in banana orchards in Embu County, Kenya

Determining the potential of the nematode-demolishing fungi under laboratory conditions

The growth habits, conidial morphology, and nematode-trapping structures of the isolated fungi were characterized (Figure 8). Characteristics of *Arthrobotrys* include a conidium made up of two cells, one at each end, and a mycelium that grows primarily outside of the nematode hosts. Conidia of *A. oligospora* had a small distal cell and a huge proximal cell, and the conidiophore was erect and tree-like in appearance (Figures 8A and 8B). The conidium of *A. dactyloides* was long and slender, and the distal cell was nearly the same size as the proximal cell. The conidia were born in a terminal cluster on the conidiophore and formed constricting rings. Although the conidia of *A. superba, A. robusta,* and *A. longispora* all possessed a single septum and clustered together in three dimensions (Figure 8C), the three species' conidium were significantly different in size and shape. The conidia of the genus Harposporium were carried on phialides and typically were lodged outside of the dead nematode's body, contrasting with the mycelium's internal location. Arcuate conidia can be seen in *H. anguillulae* (Figure 8E) and *H. crassum*, with the latter having smaller conidia. In contrast to *N. leiosporus*, whose conidium does not create chlamydospores, Nematoctonus was identified based on its

hyphae, which lacked adhesive cells but developed adhesive knobs on the conidium. Mycelium that became tangled around nematode eggs allowed us to identify the fungi as belonging to the species Paecilomyces, which is distinguished by its cylindrical conidia that hang in chains from phialides. The worm became tangled in the sticky mycelium of *M. cionopagum*, which formed a trap, and a single apical conidium was carried on an erect conidiophore (Figure 8D). The endoparasitic fungi *M. coniospora* was distinguished by its nearly conical conidium, which formed a knoblike structure at the apical end. The *Dactyllela phymatopaga* may be identified by the nematodes it caught on its distinctive sticky knobs (Figure 8F).

Trapping ability of the isolated nematode-demolishing fungi

The average plant parasitic nematodes trapped and destroyed by the three selected nematode-demolishing fungi were 262 (72%) out of approximately 360 juveniles of *Meloidygyne spp*. After 96 hours, *A. oligospora* trapped and destroyed 98 plant parasitic nematodes, while *M. cionopagum* destroyed 88 plant parasitic nematodes. The least was *D. phymatopaga*, with 76 plant parasitic nematodes destroyed in the same period (Table 3). No trapping was recorded from all isolates between 0-24 hours. *A. oligospora* had the highest trapping ability at 82%, followed by *M. cionopagum* at 73%, and the least was *D. phymatopaga* at 63%. A paired test showed that the time taken by *A. oligospora* to trap nematodes was significantly different from that of *D. phymatopaga* (P value= 0.047) but did not differ significantly from the time taken by *M. cionopagum (P value 0.378).* The time taken by M. cionopagum to trap nematodes was not significantly different from that of *D. phymatopaga* (P value= 0.075).

The effect of organic and inorganic additives on the population and diversity of nematode-demolishing fungi

The highest number of nematode-demolishing fungi were isolated from the soils amended with chicken manure, with 74 isolates, followed by soils amended with cow manure 71, goat manure 69, and control plot 54, while the least was from the soils amended with fertilizer, with 39 isolates (Table 4). A significant difference was observed in the nematode-demolishing fungi population on different soil additives. Application of cow, goat, and chicken manure did not differ significantly in their interaction with the nematode-demolishing fungi in the banana plots, as indicated by the means. The mean for control banana plots where no additive was applied was significantly compared with the plots where a synthetic additive (fertilizer) was applied (Table 4).

In the soils amended with chicken manure, the population of nematode-demolishing fungi increased from 24 isolates to 31 isolates in the first 3 months. It decreased to 19 isolates below the initial numbers isolated in the sixth month. In soils amended with cow manure and goat manure, the populations increased after three months from 19 to 30 and were maintained above the initial isolation after 6 months at 22 and 20 isolates, respectively. Soils with no additive increased the population of nematode-demolishing fungi from 14 isolates to 20 isolates after 3 months, and the numbers did not change after six months. Soils amended with fertilizer decreased the nematode-demolishing fungi population in the first three months from 13 to 11 isolates; the population, however, increased after six months to reach 15 isolates (Table 4).

Seventy-four nematode-demolishing fungi were isolated from soils added with chicken manure, 71 from soils amended with cow dung, 69 from soils modified with goat manure, and 54 from the control plot. Only 39 nematode-demolishing fungi were identified from soils improved with fertilizer (Table 4). The number of fungi that feed on nematodes varied significantly across treatments. The results showed no discernible difference between the effects of applying cow, goat, or chicken manure to the banana plots and the fungi that kill nematodes. However, there was a considerable difference between the mean of amended and unamended banana plots (Table 4).

Soil nematode-demolishing fungi increased from 24 to 31 isolates in the first three months after being introduced to the soil treated with chicken manure before declining to 19 isolates by the sixth month. After three months, populations in soils altered with cow manure and goat manure rose from 19 to 30, and after six months, they remained at 22 and 20 isolates, respectively. After 3 months, the number of nematode-demolishing fungi in soils that had not been amended had increased from 14 to 20. However, this rise had stalled by 6 months. Table 4 shows that after six months of fertilizer replenishment, the number of nematode-demolishing fungi in soils rose from 11 to 15.

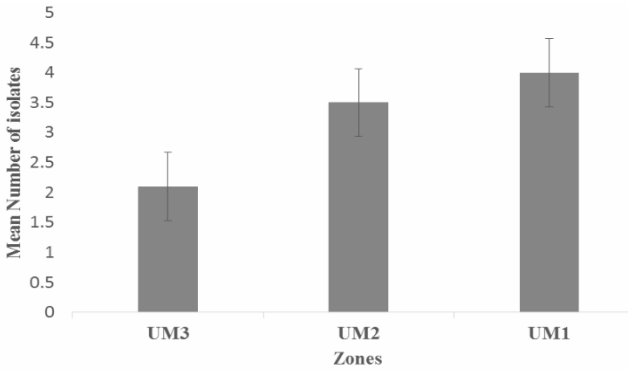

Figure 7. Occurrence of nematode-demolishing fungi in banana orchards in Meru County, Kenya

Table 3. The trapping ability of the nematode-demolishing fungi

Isolate/hours	0	6	12	24	30	36	42	48	54	60	66	72	78	84	90	96	Total
A. o	0	0	0	0	9	11	8	11	11	11	11	13	7	3	2	1	98
M. c	0	0	0	0	4	4	3	10	11	12	15	14	8	4	2	1	88
D. p	0	0	0	0	2	3	7	8	10	11	12	12	6	3	1	1	76

Note: *A.o= Arthrobotrys oligospora, M.c= Monacrosporium cionopagum, D. p= Dactyllela phymatopaga.* Figures in the table represent the mean numbers of plant parasitic nematodes trapped in three experiments

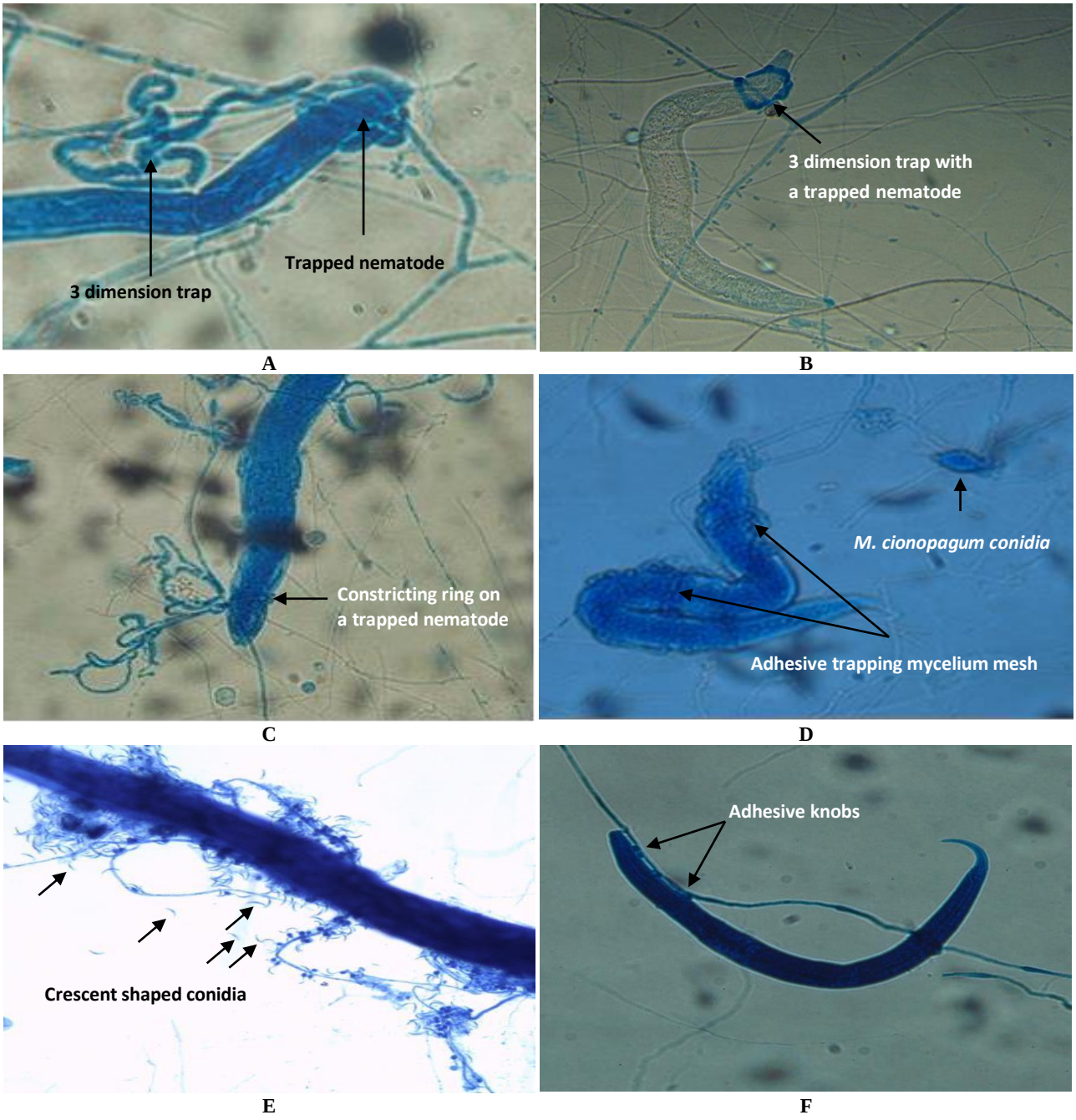

Figure 8.A. *A.oligospora* three dimension trap with the fungal (Magnification X1000). B. *A. oligospora* three dimension trap (Magnification X1000). C. Constricting rings of *A. longispora* (Magnification 1000). D. Adhesive mycelium and conidia of *M. cionopagum* (Magnification X1000). E. Arcuate conidia of *H. anguillulae* emanating from a digested nematode (Magnification X1000). F. Sticky knobs of *Dactyllela phymatopaga* (Magnification X1000)

Individual isolates were affected by the soil additives. *M. cionopagum* had the highest population in all treatments, followed by *A. longispora*, while *H. anguillulae* and *D. phymatopaga* had the lowest populations in all the treatments. Only *A. dactyloides* distribution was significantly affected by the different additives (P= 0.003) (Table 5).

Soil additives had an impact on individual isolates. The most abundant species across treatments were *M. cionopagum* and *A. longispora*, while *H. anguillulae* and *D. phymatopaga* were consistently the least numerous. The distribution of *A. dactyloides* was the only factor that changed noticeably due to the various adjustments (P= 0.003) (Table 5). The total number of nematode-demolishing fungi varied from month to month. When nematode-demolishing fungi were isolated before and after treatments, there was a notable difference between the two populations; however, by the sixth month, there was no longer a discernible difference between the two (Table 5).

Following the implementation of changes, the number of retrieved isolates grew from 89 to 122, then decreased to 96 by the sixth month (Table 6, Figure 9).

Table 4. The population of nematode-demolishing fungi (NDF) in banana plots treated with different additives

Additives	Months after application			Total isolates	NDF means
	Pre-treatment	3	6		
Chicken	24	31	19	74	2.8 a
Cow	19	30	22	71	2.6 ab
Goat	19	30	20	69	2.6 ab
Control	14	20	20	54	2.0 bc
Fertilizer	13	11	15	39	1.4 c

Note: Means with the same letter along the columns are not significantly different. LSD = 0.7

Table 5. The abundance of nematode-demolishing fungi in different banana plots treated with organic soil additives

Fungal isolates	Additives						
	Chicken	Cow	Goat	Control	Fert.	Total	P value
M. cionopagum	22	18	22	19	15	96	0.182
A. longispora	16	17	17	13	10	73	0.24
A. oligospora	16	14	19	13	9	71	0.08
A. dactyloides	12	10	5	5	1	33	0.003
A. superba	7	9	4	4	3	27	0.215
H. anguillulae	2	0	2	0	0	4	0.188
D.phymatopaga	1	1	0	0	1	3	0.735
Total	74	71	69	54	39	307	

Note: Systematic presentation of the species names in full (*Monacrosporium cionopagum, Arthrobotrys longispora, Arthrobotrys oligospora, Arthrobotrys dactyloides, Arthrobotrys superba, Harposporium anguillulae, Dactyllela phymatopaga.*

Table 6. Monthly variation of the nematode-demolishing fungi in soils amended with organic soil additives

Months	No. of isolates	Means
Pre-treatment	89	1.977b
3	122	2.7111a
6	96	2.1333b

Note: Means with the same letter along the columns are not significantly different. LSD = 0.518

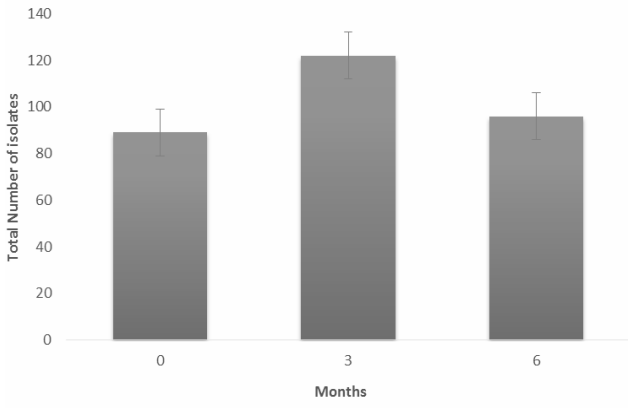

Figure 9. Nematode-demolishing fungi in amended soil over time

Discussion

This study has confirmed that nematode-demolishing fungi are widespread, and their diversity differs from one agroecological zone. Furthermore, they were present in all banana production farms in the two study sites; this concurs with previous studies as reviewed by Swe et al. (2011) that reported the presence of nematode-demolishing fungi in a wide range of environments.

The fungi isolated used various mechanisms to capture and destroy plant parasitic nematodes: these included; constricting rings, non-constricting rings, adhesive nets, adhesive knobs, and ingested spores. Different genera and species isolated were; *M. cionopagum* and *Arthrobotrys* spp. from all three regions were the highest in occurrence. It reflects past research work, as recorded by Birgit et al. (2002), on the occurrence of nematode-demolishing fungi. Wachira et al. (2014) also recorded that *Arthrobotrys* and *Monacrosporium* species were the most diverse in the three zones in the Embu region in banana farms.

Various agricultural practices and systems significantly impact the soil biota, their occurrence, and diversity (Scott et al. 2010). For example, cleared forest land for cultivation negatively disturbs the soil environment and decreases the number and species of beneficial soil organisms. High zones had the highest occurrence and diversity of nematode-demolishing fungi, which decreased with altitude. The variations in nematode-demolishing fungi populations can be attributed to the farming and cultural practices practiced in the study sites. High altitudes were characterized by mixed cropping and animal farming. Animal farming may have resulted in the use of animal manure in the farms hence the higher soil organic matter content in the high zone (Kaskavalci 2007), which positively influences the establishment and diversity of nematode-demolishing fungi (Wachira et al. 2011). In addition, Intercropping in the high zone offered alternative hosts to the nematodes, hence higher numbers of plant parasitic nematodes and a subsequent high number of the antagonists.

This research confirms the ubiquitous prevalence of nematode-demolishing fungi and shows that these fungi vary between agroecological zones. Furthermore, it is consistent with previous research evaluated by Swe et al. (2011), which indicated the prevalence of nematode-demolishing fungi in various environments. It was found in all banana production farms in the two study sites.

The separated fungi used strategies to trap and eliminate plant parasitic nematodes, including constricting rings, non-constricting rings, adhesive nets, adhesive knobs, and ingested spores. Common to all three areas, *M. cionopagum* and *Arthrobotrys* spp. were the most frequently found isolated genera and species. It is consistent with the findings of previous studies, as Birgit et al. (2002) documented on the prevalence of nematode-demolishing fungi. Furthermore, Wachira et al. (2014) found that across all three zones in the Embu region's banana fields, Arthrobotrys and Monacrosporium species exhibited the highest levels of diversity.

The diversity and abundance of soil biota are strongly influenced by farming methods (Scott et al. 2010). When

forests are cut down to make way for farms, the soil is thrown out of balance, and the number and variety of beneficial organisms in the soil decline. The abundance and variety of nematode-demolishing fungi peaked at higher altitudes and gradually declined. It has been hypothesized that differences in farming and cultural methods at the research sites are responsible for the observed variability in nematode-demolishing fungi populations. A variety of crops and livestock were raised at high elevations. Soil organic matter content is higher in the high zone, possibly due to the application of animal manure in farming (Kaskavalci 2007), which has a beneficial effect on the establishment and diversity of nematode-demolishing fungi (Wachira et al. 2011). Plant parasitic nematodes proliferated because of the increased availability of alternate hosts provided by intercropping in the high zone.

The low zone had fewer nematode-demolishing fungi, characterized by banana monoculture and high irrigation practices. Moisture in soil is known to affect fungal spore dispersal (Dieterich and Sommer 2009). The running water and soaked soils may have harbored the growth of fungal spores, resulting in the few nematode-demolishing fungi in this zone. There was also minimal land disturbance in low altitudes as tillage was reduced in the banana plantations (Wachira et al. 2009a). Cultivation increases the chance of dispersing fungal spores to other parts of the farm.

The three nematode-demolishing fungi used for the trapping ability test were positive. They managed to trap plant parasitic nematodes within the first thirty hours and exhausted the available nematodes in 96 hours. The high trapping ability of the nematode-demolishing fungi reveals their potential for field trial tests to confirm their viability for commercial production in managing vermiform plant parasitic nematodes. Commercial products of nematode-demolishing fungi of the species *Paecilomyces* that parasitizes nematode eggs have been released in the market.

Some nematode-demolishing fungi, such as *A. oligospora*, have been shown to boost plant growth and improve the nutritional value of fruits, all while being kind to the environment and lowering farmers' agricultural production costs (Singh et al. 2013). The nematode-demolishing fungus *A. oligospora*, as documented by Bíró-Stingli and István (2011), significantly decreased the population of female *Meloidogyne* larvae in a pepper field by 35%. The use of microorganisms in the control of agricultural pests and disease pathogens is becoming increasingly common. One example is *Beauveria bassiana* formulations, which are becoming popular for controlling insect pests.

The study confirmed a significant difference between the various soil additives tested; the highest population of nematode-demolishing fungi was isolated from the soils treated with organic additives. Conversely, soils added with inorganic fertilizers had the lowest nematode-demolishing fungal population. Mugwe et al. (2009) noted that fertilizer strongly influenced the soil microbial community structure and function.

Various farm practices, such as monoculture, tillage, soil pollution, and pesticide use, have undesirable impacts on many soil organisms (Scott et al. 2010; Xue and Zhang 2011). Levels of soil organic matter are decreasing due to intensive land use and excessive use of high external chemical inputs for crop production. Disturbance of soil physicochemical and biological processes leads to soil and water pollution, which results in the destruction and reduced effectiveness of useful soil microorganisms leading to increased numbers of pathogens and parasitic organisms.

Organic additives are known to increase organic carbon and nitrogen in the soil, as recorded by Bouajila and Sanaa (2011). Swe et al. (2011) also noted that animal manure is high in nitrogen, phosphorous, and potassium which the nematode-demolishing fungi utilize saprophytically as they establish themselves. An example is *Arthrobtrys* spp., known to thrive in the soil as a saprophyte and as a predator; this reflects earlier findings as documented by Jaffe (2004), Farell et al. (2006), Wachira et al. (2009b) and Xue and Zhang (2011) who noted that the *A. oligospora* obtains its carbon and energy from organic matter as a saprophyte and from trapping nematodes as a parasite which makes it adaptable to the big range of habitations. It explains the abundance and diversity of *Arthrobotrys* spp. in all agroecological zones and across all additives (Connell et al. 2006).

Since there were fewer nematode-demolishing fungi in the low zone, typified by its high rates of irrigation and banana monoculture, nematodes thrived there. Soil moisture is known to play a role in spreading fungi spores (Dieterich and Sommer 2009). The low concentration of nematode-demolishing fungi in this area may be because rushing water and saturated soils did not provide ideal conditions for germinating fungal spores. Tillage was reduced in banana plantations, causing less land disturbance at low elevations (Wachira et al. 2009a). The spread of fungi spores to unaffected areas of the farm is made more likely by cultivation.

Three nematode-demolishing fungi were tested for their ability to trap nematodes; all three passed, successfully entrapping plant parasitic nematodes within the first 30 hours and demolishing them all in 96 hours. For the control of vermiform plant parasitic nematodes, field trial testing is needed to confirm the viability of the nematode-demolishing fungi for commercial production due to their high trapping ability. Fungi of the genus *Paecilomyces*, which feed on nematode eggs, are now available commercially.

When correctly introduced into a farm, nematode-demolishing fungi eliminate the need for repeated sprays, which helps the environment and the farmers' bottom-line needs. According to the research of Singh et al. (2013), *A. oligospora* both stimulate plant development and improves the dietary value of harvested fruits. The nematode-capturing fungi were documented by Bíró-Stingli and István (2011). In a pepper field, oligospora cut the population of female Meloidogyne larvae by 35 %. Pests and disease-causing organisms in crops can be controlled with the help of certain microorganisms nowadays. Beauveria bassiana formulations, increasingly used to control insect pests, are gaining favor.

An abundance of nematode-demolishing fungi was isolated from soils treated with organic additions, confirming a significant difference between the soils treated with the different additives examined. The fungal population that feeds on nematodes was lowest in soils supplemented with inorganic fertilizers. The fertilizer regime's composition and activity of soil microbes are profoundly affected by Mugwe et al. (2009).

Many soil organisms are negatively impacted by common farming techniques such as monoculture, tillage, soil contamination, and pesticide use (Scott et al. 2010; Xue and Zhang 2011). Intense land usage and the overuse of high external chemical inputs have resulted in declining soil organic matter levels. When the soil's physicochemical and biological processes are disrupted, soil and water pollution leads to the death or impairment of beneficial soil microorganisms and the proliferation of diseases and parasites.

According to the research of Bouajila and Sanaa (2011), organic additions raise the levels of organic carbon and nitrogen in the soil. Animal dung is rich in nitrogen, phosphorus, and potassium, which the nematode-demolishing fungi use saprophytically throughout their establishment (Swe et al. 2011). Soil-dwelling Arthrobtrys spp., for instance, are both saprophytes and predators, before corroborating observations published by Jaffe (2004), Farell et al. (2006), Wachira et al. (2009b) and Xue and Zhang (2011), who all mentioned that the *A. oligospora* can thrive in various environments thanks to its ability to derive its carbon and energy needs from a combination of saprophytic (carbon from organic matter) and parasitic (energy from nematodes) sources. It explains why Arthrobotrys spp. can be found in every agroecological zone and every type of additive (Connell et al. 2006).

Farms amended with inorganic fertilizer decreased the nematode-demolishing fungal population after three months, and the population increased after six months. It can be attributed to the deterioration of soil microbe diversity and health by the chemicals in the inorganic fertilizers (Kar et al. 2007). The population, however, increased steadily but remained below the initial population after six months; this shows the recovery of the soils from the effects of the inorganic fertilizer. Using inorganic sources of phosphorus and nitrogen may enhance soil organic carbon. Still, long-term use of synthetic fertilizers is not advised as it negatively impacts soil structure and decreases soil macro aggregates. Using fertilizers pollutes the environment and degrades the soil (Lazcano et al. 2012).

Soils amended with chicken manure had the highest number of nematode-demolishing fungal isolates, which concurs with the findings of similar research work as Wachira et al. (2009b) and Iqbal et al. (2012). It can be attributed to the chicken manure composition. Chicken manure is slightly basic with a pH of 8.4, has a high electrical conductivity compared to other organic manures, and contains higher levels of nitrogen, phosphorus, and potassium than cow and goat manure (Karanja et al. 2007) which the fungi thrive on as it establishes itself for predatory activities. Analysis of different animal manure reveals that goat and chicken manure have more potential to provide nutrients, mainly phosphorous and nitrogen, than other manure sources. The highest amount of potassium was also delivered by the chicken manure (Noling 2012).

Previous work on *Bacillus subtilis* by Miriam et al. (2011) shows that a combination of *B. subtilis* and cow manure led to a reduction of 54% in the number of plant parasitic nematodes compared to the untreated control. In addition, it has been indicated that poultry litter reduced *Rotylenchulus reinformis* by 55 % (Ravichandra 2014) in a field experiment. It was attributed to the reduction of the number of eggs in the roots.

It is evident that soil additive management practices impact nematode-demolishing fungi (Romy and Robert 2014); the availability of various nutrients which the fungi feeds on as they establish their colonies in the soil to attack the nematodes. Therefore, using locally available animal manures could restore the regulatory processes of nematode-demolishing fungi and maintain effective populations of plant parasitic nematodes in the soil. In addition, the release of substances with nematicidal effect and organic acid during additives decomposition has been attributed to the reduction of the nematode population (Kimenju 2004; Georgis et al. 2006).

Soils contain huge amounts of various living organisms. These life forms play essential ecosystem services such as filtering water, parasitizing pests and pathogens, removing pollutants, and providing plant nutrients. Therefore, applying animal manure to enhance the establishment and conservation of beneficial soil organisms is one of the most convenient ways farmers can conserve nematode-demolishing fungi to reduce the number of plant parasitic nematodes in their farms to maintain general soil health (Renčo (2013).

After three months, the number of nematode-demolishing fungi decreased on farms that had been altered with inorganic fertilizer, and after six months, the population grew. This decline can be related to the inorganic fertilizers' detrimental effect on soil microbial variety and health (Kar et al. 2007). Despite being lower than the initial population, the steady increase in numbers suggests that the soils have recovered from the effects of the inorganic fertilizer. It's possible that using inorganic sources of phosphorus and nitrogen could increase soil organic carbon, but this wouldn't be a good idea as a prolonged application of synthetic fertilizers tends to reduce soil macro aggregates, which has a detrimental effect on soil structure. In addition, fertilizers impair soil quality and contribute to environmental pollution (Lazcano et al. 2012).

This finding is in agreement with those of related studies by Iqbal et al. (2009a) and Wachira et al. (2009b), which found that the greatest number of nematode-destructive fungal isolates were found in soils modified with chicken manure. Chicken manure's chemical makeup explains why this is the case. Due to its slightly basic pH of 8.4 and high electrical conductivity compared to other organic manures, as well as its higher levels of nitrogen,

phosphorus, and potassium than cow and goat manure, the fungi flourish in chicken manure as it sets up shop for predatory activities (Karanja et al. 2007). Compared to other types of manure, goat and chicken are the most promising in providing essential nutrients like phosphorus and nitrogen. Chicken manure also provided the highest levels of potassium (Noling 2012). Miriam et al. (2011) found that when *B. subtilis* was combined with cow manure, the quantity of plant parasitic nematodes dropped by 54 percent compared to the untreated control. Furthermore, according to data from a field study, the amount of *R. reinformis* was cut in half by using poultry litter (Ravichandra 2014). A decrease in the total number of eggs in the roots was blamed for this phenomenon.

Soil additive management strategies affect nematode-demolishing fungi (Romy and Robert 2014) because they affect the nutrients used by the fungi as their colonies spread across the soil in search of nematodes to devour. Keeping functional populations of plant parasitic nematodes in the soil requires balancing beneficial and harmful organisms. Using locally available animal manures could reestablish regulatory processes of nematode-demolishing fungi. In addition, the decomposition of additives causes the release of chemicals having a nematicidal impact, such as organic acid, which helps keep nematode populations in check (Kimenju 2004; Georgis et al. 2006).

Numerous creatures call soil home, and these organisms perform crucial ecosystem functions like filtering water, controlling pests and pathogens, detoxifying soil, and nourishing plants. To preserve nematode-demolishing fungi to decrease the number of plant parasitic nematodes on farms and maintain general soil health, one of the most practical methods farmers can employ is the application of animal manure to enhance the establishment and conservation of beneficial soil organisms (Renčo 2013).

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Gamma irradiation effect on the microbial load and physicochemical properties of honey from Ghana

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Abstract. *Larbi DA, Klutse CK, Adotey DK. 2022. Gamma irradiation effect on the microbial load and physicochemical properties of honey from Ghana. Cell Biol Dev 6: 94-107.* The honey's high sugar concentration and low pH give it antimicrobial properties and make it difficult for microorganisms to grow. This study is to ascertain microorganisms' presence in Ghanaian honey, the sources of microbial contamination, the physico-chemical properties of honey, and the effect of gamma radiation on the microbial load. Furthermore, 90 honey samples were collected from Brong Ahafo, Ashanti, and Greater Accra, Ghana, with 30 from each region. Honey was sampled directly from the beehive with the comb before the farmer harvested, and honey was sampled from retailers who buy directly from the farmer. The effect of gamma radiation on the microbial load was studied using a 60Co source gamma irradiation facility at doses of 20 kGy, 30 kGy, and 40 kGy on the presence of microbes and the physicochemical properties (pH, reducing sugar, apparent sucrose, and ash content) of honey. The pH values obtained for the Honey Comb samples were in the range of 3.6-3.9, and the pH for the Retail samples was in the range of 4.9-5.6. Microorganisms were not detected in about 70% of the honey sampled directly from the honeycomb. The mean microbial count in the remaining 30% was within the range of 30-35%, whereas all the honey sampled from the retailers was contaminated with microbes. The mean microbial counts in the retailer samples were 148 CFU/g, 183 CFU/g, and 271 CFU/g for Ashanti, Brong Ahafo, and Greater Accra Regions, respectively. These values were significantly higher than the required maximum relative to the MERCOSUR (Mercado Comun del SUR) standard (≤ 100 CFU/g). The low level of microbial detection in the Honey Comb samples (30-35 CFU/g) could be due to their relatively low pH levels (3.6-3.9) compared to the retailer samples with pH within 4.9-5.6. The ash content of all the honey sampled and analyzed was within the required standard, with an average of 0.16% in the honeycomb samples and an average of 0.62% for the retail samples. The apparent sucrose concentration (in percentage) in honey sampled from the retailers was within the range of 22-33%, which is beyond the required maximum as stipulated by the CODEX Alimentarius Commission (≤ 10%). The study showed that a 20 kGy gamma radiation dose was enough to denature the microbes and preserve the honey's essential qualities. Finally, to ensure good quality honey on the Ghanaian market, it is recommended that honey meant for human consumption should undergo gamma irradiation (cold pasteurization).

Keywords: Antimicrobial, honey, primary and secondary sources, radiation

INTRODUCTION

Honey is a food product consumed by many people throughout life due to its high nutritional value. It is essentially composed of reducing sugars hence a major source of energy. It is used as a substitute for sugar by people and helps digestion and removal of free radicals from the body, among other benefits. Besides sugars, honey also contains proteins, organic acids, amino acids, vitamins, and lipids, making it a rich source of other nutrients (White 1975; Da Silva et al. 2016; Agussalim et al. 2019).

Research has shown that honey physicochemical has the potential to prevent cancer (Beretta et al. 2007), can also be used to cure some eye defects (Kwapong et al. 2013) and other physicochemical (Suntiparapop et al. 2012; Truzzi et al. 2014; Biluca et al. 2016; Chuttong et al. 2016; Nordin et al. 2018; Ranneh et al. 2018; Agus et al. 2019; Villacrés-Granda et al. 2021). Economically, honey is a product of international value serving as a source of foreign exchange for many countries, including Ghana, contributing significantly to the gross domestic product. It is also a major source of livelihood for many people who are into apiculture. But honey is only as good as its quality, and honey quality cannot be judged just by its physical appearance. For these reasons, there is a need to ensure that honey is free from microorganisms and that it is wholesome for human consumption.

Honey is a flavourful product consumed globally as a high-nutritional food. It is composed of a complex mixture of carbohydrates (glucose and fructose account for nearly 85-95%, the rest being sucrose) and other minor substances such as organic acids, amino acids, proteins, minerals, vitamins, and lipids (White 1975). Due to its high sugar concentration, high osmotic pressure, and low pH, it is difficult for microorganisms to grow. However, research has shown that microorganisms have been detected in honey, including pollen, molds, yeasts, and the spores of *Clostridium* sp. and *Bacillus* sp. (Snowdon and Cliver 1996). There are two main sources of microorganism contamination: primary sources include pollen, digestive tracts of honeybees, dust, air, soil, and nectar; The next sources are those arising from animals, such as some rodents, insects, etc., that may visit the beehive while honey is maturing. Secondary sources of contamination are manipulation by people, including food handlers, cross-

contamination, equipment, and materials where harvested honey is stored (Snowdon and Cliver 1996).

The microorganisms are inactive in honey, but they could present different results when transferred into a living host through ingestion. Sulfite-reducing *Clostridium* is an indicator organism whose presence in honey provides evidence of contamination or pollution (Snowdon and Cliver 1996). Clostridium spores are especially dangerous for infants and small children (Centorbi et al. 1999). Botulism is a neuroparalytic disease caused by *Clostridium botulinum* which can survive in honey and be transferred into an infant.

Some North American and European countries have recorded reports of anaphylactic shock in people with allergens (pollen) who eat raw honey. At its worst, anaphylactic shock could cause breathing difficulties, low blood pressure, dizziness, fainting, heart failure, weakness, sweating, nausea, vomiting, and prickling sensations in the brain (Bartkowski 2014). Symptoms of less severe allergic reactions resulting from raw honey include itching, puffy skin, and rash.

Honeybees obtain their nectar from flowers of different plants, including Rhododendrons, with the nectar containing a substance called grayanotoxin, according to the America Food and Chemical Toxicology journal (Koka and Koka 2007). It is explained in this article that grayanotoxins are chemicals that are toxic to the nervous system; they prevent nerve cells from functioning effectively. In addition, pollen grains stick to the bees' bodies during foraging, are transferred into the honeycomb, and mature as part of the honey. This pollen causes allergic reactions in people when exposed to pollen-contaminated honey.

There is a scarcity of published information on the microbiological properties of Ghanaian honey; and very limited information on the physicochemical characteristics of Ghanaian honey. Therefore, the main objective of this research was to investigate the presence of microorganisms in Ghanaian honey and to assess how to improve the quality of Ghanaian honey through cold pasteurization (Gamma irradiation).

MATERIALS AND METHODS

Sampling locations

This research was conducted in Ghana, West Africa, in three (3) regions; Brong Ahafo, Ashanti, and Greater Accra Table 1 and Figure 1 show the regions and sample locations and the map of the locations, respectively. Two (2) of the regions-Brong Ahafo and Ashanti-are believed to be among the largest honey production centers in the country (Alaazi et al. 2010). Brong Ahafo has an estimated 2188 apiaries, the Ashanti has 2243 apiaries, and the Greater Accra region has 1536 apiaries (Alaazi et al. 2010).

The sampling locations were small towns with relatively low vehicular and construction activities compared to the major cities in the regions. The beehives are mostly located away from residences or places where human activities are predominant to protect the residences and ensure minimal human influence.

The sampling started from August through December 2014, samples were taken from the sample points in the Ashanti and Brong Ahafo Regions. From January through March 2015, samples were taken from the Greater Accra Region. Sampling was conducted with the available resources (period within which to complete the project, beekeepers, and finance), and most of the places visited were rural communities. Considering the complex nature of the sampling process and the locations, sampling within the same month proved quite difficult with the available resources. These informed the decision to spread the sampling across this period.

Questionnaire administration

Based on the objectives of this study, a questionnaire was designed to find out the activities and apiculture practices that contribute to honey contamination. In addition, oral interviews were conducted based on the questionnaire with those who could neither read nor write.

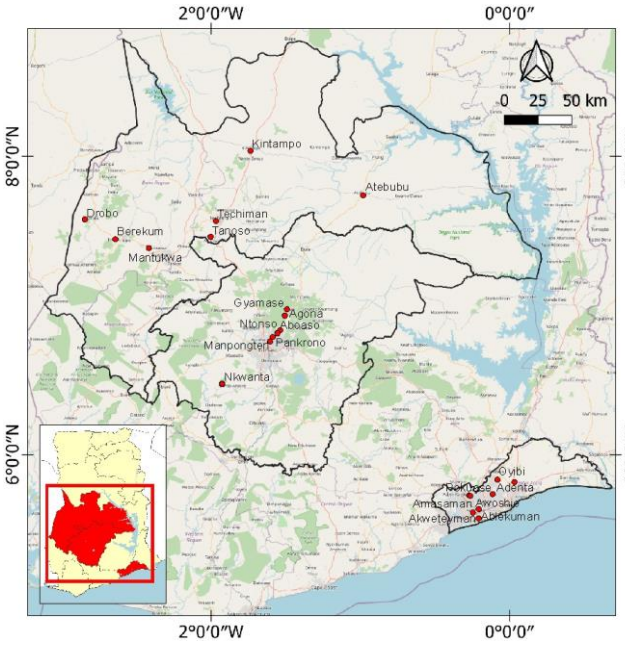

Figure 1. Map of Ghana showing sampling locations

Table 1. Sampling locations in Ghana

Region	Towns
Ashanti	Aboaso, Agona, Ahwiaa, Asenua, Fawode, Jamasi, Mampongten, Nkwanta, Ntonso, Pankrono
Brong Ahafo	Atebubu, Berekum, Drobo, Dumasua, Fiapre, Kintampo, Mantukwa, Nsoatre, Tanoso and Techiman
Greater Accra	Ablekuma, Adenta, Afienya, Akweteyman, Amasaman, Awoshie, Ayi Mensah, Oyibi, Pokuase and Weija

Sampling

The samples were taken along the farmer/hunter-to-trader route. The first set of samples was essentially honey taken directly from the honeycomb before the farmer harvested it for extraction and packaging. The next set of samples was honey harvested, processed, and ready for selling to the retailers.

The last set of samples was taken from the retailers; only the retailers who bought from the sampled farmers were considered; it is efficient because the retailers personally assisted in locating the farmers from whom they obtained their stock. Some of the samples were obtained from honey hunters. At each sampling location, 3 samples were taken; in all, 30 samples were taken from each region. A total of 90 samples were collected. Before the analysis, the honey samples were stored in clean plastic bottles and tightly covered to prevent external contaminants.

The samples were systematically coded based on the regions, sampling site, and collection date. They were then sealed in transparent polyethylene bags (to prevent, as many dust particles as possible, from the bottled samples) and packed into paper boxes for transport to the laboratory. The samples were removed from the bags at the laboratory and kept on clean shelves at room temperature, still in their original packages, before analysis. The temperature conducive for microbial growth varies concerning their type. For example, mesophyllic bacteria grow best within 30^{o}C to 37^{o}C; keeping the honey samples at room temperature and below (when air conditioning is available) could inhibit microbial growth. As much as possible, care was taken to prevent contamination in the storage shelves. For each sampling point, a control was added. The control consisted of an empty bottle treated the same way as the sampling bottles, except that honey was not added. All the plastic containers for the sampling were pre-washed and steam sterilized.

Analysis of honey samples

The flow diagram (Figure 2) illustrates a general overview of the analyses performed on the honey samples;

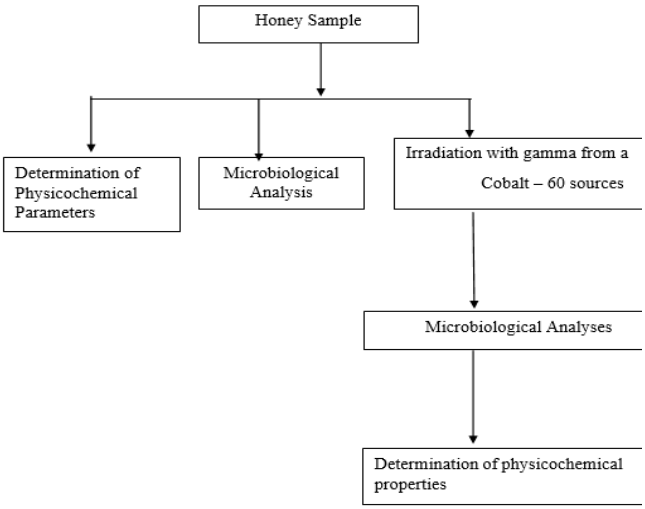

Figure 2. Flow diagram of analyses

Microbial analysis

The microorganisms in the honey samples (farmer-to-trader route) were determined using the Colony Count Technique (ISO 4832: 2006-Microbiology of food and Animal Feeding Stuffs-Horizontal Method for Enumeration of Coliform). That was followed by decontamination of the honey samples by irradiation with gamma rays using a cobalt 60. The samples were analyzed for Total Viable Counts, *Faecal* sp., *Clostridium* sp., *Staphylococcus* sp., and *Salmonella* sp. These are common causative microorganisms of foodborne illness and are also indicator organisms showing the unhygienic processing, packaging, and other treatment of food. The total viable count estimates the samples' possible aerobic and anaerobic microbes.

Media

Media used to culture microbes can be seen in Table 2.

Preparation of agar

The method of preparation was the same for each agar. First, about 22 g of the agar powder was weighed into a 0.5 L Erlenmeyer flask based on the manufacturer"s instruction. Next, about 50 mL aliquot of distilled water was added to the flask's content and then shaken gently to allow for mixing. When it blended, more distilled water was added to the content to reach the 0.5 L mark (Marshall 1993). The prepared media were then sterilized at 100^{o}C for about 30 minutes allowing the mixture to melt further and enhance blending. Next, the media was tempered, which was done whiles ensuring that the water level in the bath was about 1 cm above the level of the medium in the bottle.

Inoculation and incubation

The working surface of the laminar chamber was cleaned with 70% isopropyl alcohol. Duplicate inoculations were done for each sample; hence each duplicate petri dish was marked per sample per microbe analyzed. About 10 g of each honey sample was weighed and homogenized in 90 mL of peptone water solvent by agitating in a dilution bottle for about 10 seconds. About 1ml aliquot of the prepared sample was pipetted into five other 10 mL bottles with peptone water.

A cotton-coated piece of the plastic rod was used to wipe the inner surface of the control plastic bottle and the content dissolved in 5 mL of peptone water which was then transferred into a labeled Petri dish for inoculation with Plate Count Agar and incubation. That was done for each control taken at the sample points visited. The controls were taken mostly at the honey processing areas.

Table 2. Media

Total viable count	**Plate count agar**
Faecal sp.	Eosin Methylene Blue agar
Clostridium sp.	Perfringens agar
Staphylococcus sp.	Baird Parker agar
Salmonella sp.	Xylose Lysine Deoxycholate agar

About 1 mL aliquot of the prepared sample was pipetted into the labeled Petri dishes, and 15 mL of the prepared agar was added. That was repeated for each agar for the various plate counts; Perfringens, Baird-Parker, eosin methylene blue, and Xylose Lysine Deoxycholate. These transfers and sample dilutions were done in a laminar chamber where the temperature was controlled at 45ºC. The agar was transferred 15 minutes after the sample mixtures were pipetted into the petri dishes. In addition, for each sample inoculated for incubation, a blank Petri dish (without sample) was inoculated with plate count agar to verify if the pre-sterilized Petri dishes were free of any possible microorganisms which could influence the results.

The sample-agar mixtures in the Petri dishes were gently swirled to mix thoroughly and left for 15 minutes to settle, after which 4 mL of the respective agar was added to the mixtures as a second layer and left to solidify, which was to ensure that the entire sample was well covered with the agar. The inoculated samples were then incubated at 37ºC for 48 hours, monitoring the changes every 24 hours until the incubation time had elapsed.

Counting of colonies

Colonies were counted immediately after the incubation period. A colony represents cells well separated on the plate and can be distinguished after growth; it could be one cell or several thousand (Sutton 2006). Petri dishes (plates) that have microbial growths up to at least 30 colonies or at most 300 colony units formed per plate are the most favored in colony counting, sometimes the range could be 25 to 250 CFU/plate, and these were selected for counting (Sutton 2006). For plates that contained colonies that spread out, a representative portion of the plate was selected for counting; if a quarter of the plate was counted for colonies, it was estimated that the colonies formed on the plate were four times the count values for the quarter portion.

Counts were calculated using the formula (ISO 4832 formula for the microbial count):

$$N = \frac{(C1 + C2 + C3)}{v} \times d$$

Where C1, C2, C3 = count values for the triplicate plates.

V = volume of sample on each plate

d = dilution factor for the plates counted

N = the Colony Forming Unit (CFU) per gram or mL of product. Count results were rounded off to two (2) significant Figures

Determination of physicochemical parameters

The physicochemical parameters (pH, Apparent Reducing sugar, Apparent Sucrose, and Ash content) of 60 honey samples were determined to assess the honey quality. In addition, the results were compared to International Food Standards for honey as stated in the CODEX Alimentarius Standards. The samples selected were the honeycomb samples (S1) and the consumer samples (S3) from all 3 regions. The samples taken after harvesting and treating were not subjected to any physical and chemical test because it was assumed there would be no significant alteration to the physicochemical properties during percolation and sieving. Instead, the physicochemical tests were conducted before and after "decontamination" by gamma radiation to determine how they are affected by the high-energy photons.

The quality of honey depends on several parameters. These parameters include reducing sugar and sucrose concentration, pH, and ash percentage. By CODEX Alimentarius Standards, good quality honey must have less than 60% reducing sugars and not more than 10% sucrose.

Ash content

The Ash dish was heated in an electrical furnace at 600ºC, cooled in a desiccator to room temperature, and weighed to the nearest 0.001 g (M_2). Exactly 5 g of the honey sample was weighed to the nearest 0.001 g into the ash dish that had been prepared (M_0). Two drops of olive oil were added to the sample. Ashing was commenced at a temperature of 350ºC and progressively increased to a maximum of 600ºC. After heating for an hour, the ash dish was cooled in the desiccator and weighed (M_1) (Bogdanov 2009).

The ash proportion (W_A) in $[\frac{g}{100}\ g]$ honey was calculated using the formula below as stipulated by the International Honey Commission;

$$W_A = \frac{M_1 + M_2}{M_0} \times 100$$

M_0 = Weight of sample

M_1 = Weight of ashed sample on the dish

M_2 = Weight of pre-ashed dish without sample

Reducing sugar

"Apparent reducing sugars" are the sugars that reduce Fehling" s solutions from blue to brick red under specified conditions (Bogdanov 2009). Honey primarily comprises reducing sugars (85% to 95%) and apparent sucrose (5% to 10%). The high sugar concentrations give honey its osmotic pressure, which helps inhibit microorganisms' growth.

The Lane and Eynon procedure modified the method employed in this research. This procedure involved reducing Soxhlet"s modification of Fehling"s solution by titration at the boiling point against a solution of reducing sugars in honey. About 50 mL of 1% sample (prepared by diluting 2 g of honey in 200 mL distilled water) were placed into a burette as the titrant (Table 3). In the preparation of the analyte, 10 mL of each Fehling solution A and B with 8 mL of distilled water were transferred into a 250 mL conical flask. That was followed by adding 2 drops of 0.2% Methylene Blue Indicator. The mixture was heated until it started boiling. Titration started at this point until the initial blue color changed to brick red. Titration was stopped, and the volume of titrant was noted and recorded.

The % Total Reducing Sugar was calculated using the equation:

$$\% \text{ total reducing sugar} = \frac{2 \times 10.2}{titre\ value \times mass\ of\ sample} \times 100$$

10.2 = Glucose factor

Apparent sucrose

The apparent sucrose was determined by the concentration of inverted reducing sugar, which was done by measuring about 10 mL of 6.34 M HCL and 50 mL of 1% honey and transferred into a conical flask and kept in a water bath at 60°C for 20-30 minutes. First, the sample was cooled and neutralized with a 5 M NaOH $_{(aq)}$ to a pH of 7 (the pH was monitored with the pH meter). Next, the samples were transferred into a burette. Next, about 10 mL each of Fehling solutions A and B and 8 mL water were transferred into a 250 mL flask and heated till the solution started boiling. Titration started at this stage after adding 2 drops of 0.2% Methylene Blue Indicator and titration was completed while the solution was boiling. At the endpoint, the blue color of the solution changed to brick red.

Calculations

The % Reducing sugar was calculated using the formula

$$= \frac{2 \times 10.2}{titre\ value \times mass\ of\ sample} \times 100$$

From the results obtained, the percentage sucrose was calculated by:

Percent sucrose = % sugar (after inversion) × 0.95

The "Harmonised Methods of the International Honey Commission for honey quality analyses.

pH

Honey is a product of high acidity with pH values ranging from 3.6 to about 6.5 for different types of honey (Bogdanov 2009). The low pH also inhibits the growth of microorganisms in honey, contributing to the honey product's long shelf life. The pH meter was calibrated using buffer solutions of pH 3.7 and 9.0. About 10 g aliquots of the sample were weighed into 75 mL of carbon dioxide-free water in a 250 mL beaker. The solution was stirred to homogenize, the pH electrode was immersed in the solution, and the pH was recorded.

Sterilization of honey by irradiation with gamma rays

Instrumentation. The Gamma Irradiation facility is a ^{60}Co (category IV wet storage gamma radiator) with an initial source strength of 50 kCi. It was manufactured and installed at the Radiation Technology Centre, Gamma Irradiation facility at Ghana Atomic Energy Commission in Accra, by the "Isotope Company Ltd," a company based in Hungary, in 2010 (RTC-GAEC). The cobalt 60 (^{60}Co) source with the current strength of 26 Kci with a dose rate of 1.4 kGy/hr around the shroud.

Packaging of the honey samples for irradiation. About 50 mL of the honey samples were packaged in 100 mL polyethylene bottles for irradiation. Next, 20 bottles, each per dose rate, were secured with masking tape, and the samples were transferred into the gamma chamber by the pneumatic transfer system attached to the facility. The samples were irradiated with gamma radiation from a Co-60 source at varying doses of 20 kGy, 30 kGy, and 40 kGy. The doses were varied in the control room.

The Ethanol Chlorobenzene (ECB) dosimeters were stuck to the samples and subjected to gamma radiation for half the predetermined period for each dose; this is the time taken to irradiate the samples at each dose completely. For example, at a dose rate of 1.4 kGy/hr, irradiating at 20 kGy will take approximately 14 hours to complete. The honey samples were turned through 180 degrees and then subjected to gamma radiation for the other half of the predetermined period to ensure homogenous distribution of the dose delivered under the same conditions.

The ECB dosimeters were removed from the honey samples after the irradiation period. Then the absorbed dose was determined using a calibrated readout instrument (High-Frequency Dosimeter System, Model 2131, version 2.5, produced by SENSOLAB LTD).

The expected and absorbed/delivered doses for the Honey samples are as follows Table 4.

RESULTS AND DISCUSSION

All foods, including honey, should be free of microorganisms. Therefore, the honey samples were analyzed for microorganisms to ascertain whether the treatment given to the product in the percolation, sieving, and packaging processes affected its quality before it finally reached the consumer. The microorganisms considered in this research are very common microbes frequently encountered where food products are treated in conditions suspected to be unhygienic.

Staphylococcus sp. is common in man" s respiratory passages, skin, and superficial wounds. Although the heat of cooking can denature them, the toxins produced by these microorganisms can resist heat and may not be destroyed in the human alimentary, causing food poison (Wagner 2008). In addition, since honey is used raw, it will be dangerous to have these microbes.

Table 3. Apparatus and reagents for reducing sugar and sucrose determination

Apparatus	**A 50 mL burette, Erlenmeyer flask, electric heater**
Chemicals and Reagents:	Fehling solution A (7% CuSO4) and Fehling solution B (25% KOH); 0.2% Methylene Blue Indicator

Table 4. Absorbed and expected doses

Expected dose(kGy)	**Delivered dose (kGy)**
20	20.83
30	33.08
40	41.64

Note: Uniformity ratio: 1.09

Responses from interviews (Through questionnaire administration)

Figures 3 to 6 are graphical representations of some of the responses of the beekeepers regarding honeybee farming and its practices. Most beekeepers (20%) were between the ages of 51 and 60 (Figure 3). For a greater percentage of the age group of 60 years and above, beekeeping was a full-time business, whereas those below 60 years are mostly crop and livestock farmers who keep bees as part of their business.

Most of the apiaries (about 53.3%) were located away from places of residence, as shown in Figure 4. However, some apiaries were not far from fuel stations, and about 20% were located close to quarries, wood processing factories, and places where (human activities) were quite common. In addition, 47% of the beekeepers used metallic and wooden ladles to scoop the honey from their comb (Figure 5). For others, they keep the combs in containers over a period to get the honey out of the combs by gravity.

After extraction from the combs, the product was sieved and finally stored in containers made of various materials. About 63% of the retailers used plastic containers, of which 43% of these containers were pre-used water bottles. 17% also used glass bottles of different products ranging from beer to soft drinks to store and sell honey (Figure 6).

Retailers carry honey around for sale, from house to house, workplaces, etc. From Figure 7, about 27% of the retailers sell their honey by hawking, and only a few retailers (about 14%) sell theirs in their homes because they have customers who trust their honey and come to the house to purchase. Moreover a combined 46% of the product are sold on markets and roadside, most travelers stop their cars to purchase honey before continuing their journey.

Results of microbiological analyses

Total viable count values for honey ranged from 0 to several thousand per gram. This variation in bacterial counts may be due to the type of sample (Honeycomb samples, finished or retailed), the freshness of the honey, the time of harvest, and the analytical techniques used (Snowdon and Cliver 1996). The microbial quality of honey in this research will be compared with the publication by Sereia et al. (2011). They used the MERCOSUR GMC, number 15/94 technical rules for identity fixation in honey quality, approved by ordinance number 367 on 4 September 1997, which states that honey may contain a maximum of 100 CFU/g.

Figure 8 represents the results obtained for Microbial Counts in honey sampled in the Brong Ahafo Region. The microbial count values in about 80% of the honey samples were below the MERCOSUR stipulated maximum (100CFU/g) for wholesome honey. However, there were samples from a few locations where the microbial counts were significantly above this level. The samples from Fiapre and Berekum were purchased from the market. The honey from these locations was purchased from honey hunters who have specific locations where bees make their honey. On their way from their farms, they visit these locations to extract the product to the market directly for sale.

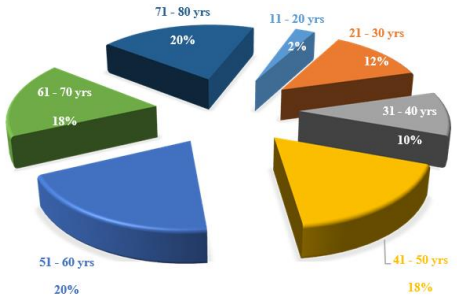

Figure 3. Age distribution of beekeepers for all three (3) Regions

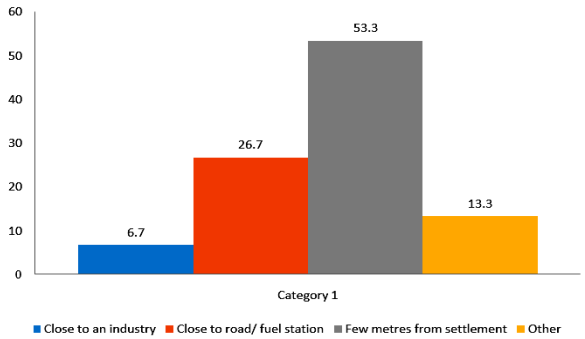

Figure 4. Location of Apiaries

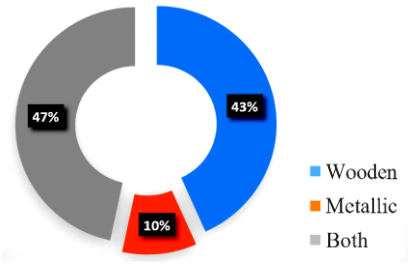

Figure 5. Extraction equipment

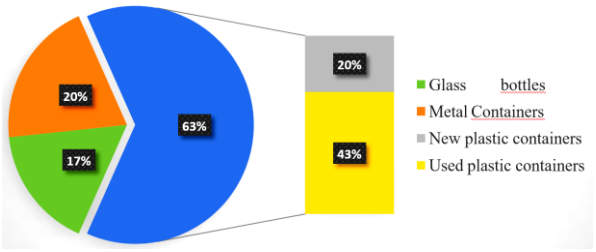

Figure 6. Storage containers for honey

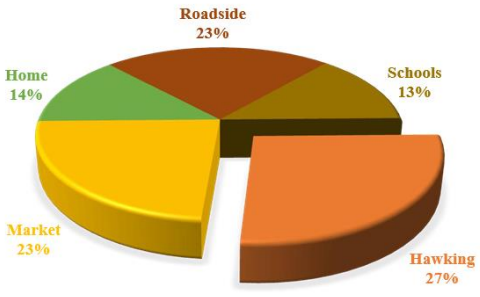

Figure 7. Places where honey is sold

In the Ashanti Region, the apiary in Agona was located just a few meters away from a fuel station, and honey extraction happens on-site when harvested. The beekeepers admitted that it was a good location for marketing since people come around for re-fueling and shopping in the mini-mart. That could have resulted in the contamination of the samples from these locations (Figure 9). The honey sampled from Jamase was purchased from a farmer who had collected some honeycomb into a plastic bucket to be taken home for extraction. Honey was heat-drained from the comb by leaving it in the sun.

In the Greater Accra Region, the retailers from whom honey was sampled were selling in the markets (23%), by the roadside (23%), and hawking (27%) at places where vehicular activities were quite common and where most roads were untarred. In most cases, the retailers transfer the honey into smaller containers after buying it in bulk. These factors could have played a pivotal role in the contamination of the honey hence the relatively higher Microbial Count readings recorded in this region (Figure 10). All the honey sampled from the 3 regions tested negative for *Salmonella* sp.

Relative microbial counts per region

Brong Ahafo Region

The absolute values in Table 5 indicate the total microbial counts in samples from apiaries in this region, whereas the mean values represent the microbial count per sample. After incubation at 37ºC for 48 hours, the Total Viable Count in the honeycomb samples was an average of 3 CFU/g per sample analyzed, the mean values of *Faecal* sp., *Clostridium* sp., and *Staphylococcus* sp. were 1 CFU/g. The mean Total Viable Counts, *Faecal* sp., *Clostridium* sp., and *Staphylococcus* sp., in the "Harvested and Treated samples" (S2), were (5, 2, and 3) CFU/g, respectively. These count values were relatively higher than the microbial counts for the Honeycomb Samples. That indicates that the local treatment methods introduce microbes into the product. Generally, the honey sampled from the honeycombs and that sampled from the treated honey from the beekeeper were low in microbial count and within the MERCOSUR maximum, except for the retail samples, which recorded a mean value of 183±20 CFU/g for Total Viable Count. The most prominent among the microbes detected was *Faecal* sp. with a mean count value of 112±10 CFU/g.

Ashanti Region

Figure 11 represents the mean values of the various microbes analyzed in each sample from the apiaries in the Ashanti Region. It can be observed that the Microbial Counts in the Honey Comb Samples (S1) were relatively very low per sample compared to the Extracted and Sieved Samples (S2), which could be a result of the treatment procedure, the equipment used in collecting honey from the beehives and in sieving, and the method of harvesting from the hive. A higher number of Coliform Units were detected in the Retail Samples (S3). Generally, the microbial count values were within the MERCOSUR maximum level, except for the Retail Samples where mean values of 148 CFU/g and 110 CFU/g were recorded for Total Viable Count and *Faecal* sp. respectively.

Greater Accra Region

The trend in the Greater Accra Region was similar to those observed in the Ashanti and Brong Ahafo regions. *Salmonella* sp. was not detected in any samples, from the apiary to the samples from the retailers. However, *Faecal* sp., spore-forming *Clostridium* sp., and *Staphylococcus* sp. were detected in all the samples (Figure 12). *Faecal* sp. was relatively higher than *Clostridium* sp. and *Staphylococcus* sp. in all the samples analyzed. The significant difference between the Total Microbial Count and the specific microbes analyzed represents the possibility of other microbes, which were not ascertained in this research.

Microbial counts per sample per region

Honeycomb samples (S1)

Table 6 represents the average Microbial Counts in Coliform Units per gram of sample (CFU/g) counted in honeycomb samples after incubation at 37 ºC for 48 hours. The mean value for Total Viable Count was less than 10 CFU/g for all the sample locations, whereas the mean values for *Faecal* sp., *Clostridium* sp., and *Staphylococcus* sp. in these samples were less than 5 CFU/g. Comparing these values with the MERCOSUR standards, they are reasonably below the allowed maximum of 100 CFU/g. The honey sampled directly from the beehive is relatively wholesome for human consumption.

Figure 13 represents the distribution of microbial contaminants in Honeycomb samples per region. It is observed that the highest mean recorded per honeycomb sample was the samples from the Greater Accra Region. It is worth mentioning that the limit of microbes detected in these samples was significantly below harmful limits for honey relative to the MERCOSUR maximum.

Table 5. Microbes Detected in Samples from the Brong Ahafo Region, Ghana

Microorganisms	Counts (CFU/g)					
	Sample One (S1)		Sample Two (S2)		Sample Three (S3)	
	Absolute	Mean	Absolute	Mean	Absolute	Mean
TVC	30	3.0 ± 1	144	16±4	1462	183±20
Faecal sp.	12	1.0 ± 0.4	37	5±2	895	112±10
Clostridium sp.	7	1.0 ± 0.3	19	2±1	186	24±3
Staphylococcus sp.	7	1.0 ± 0.4	20	3±1	102	13±2
Salmonella sp.	Negative	Negative	Negative	Negative	Negative	Negative

Note: TVC-Total viable count; S1-Honeycomb sample, S2-Treated, S3-Retail sample; Mean values (Average ± SD)

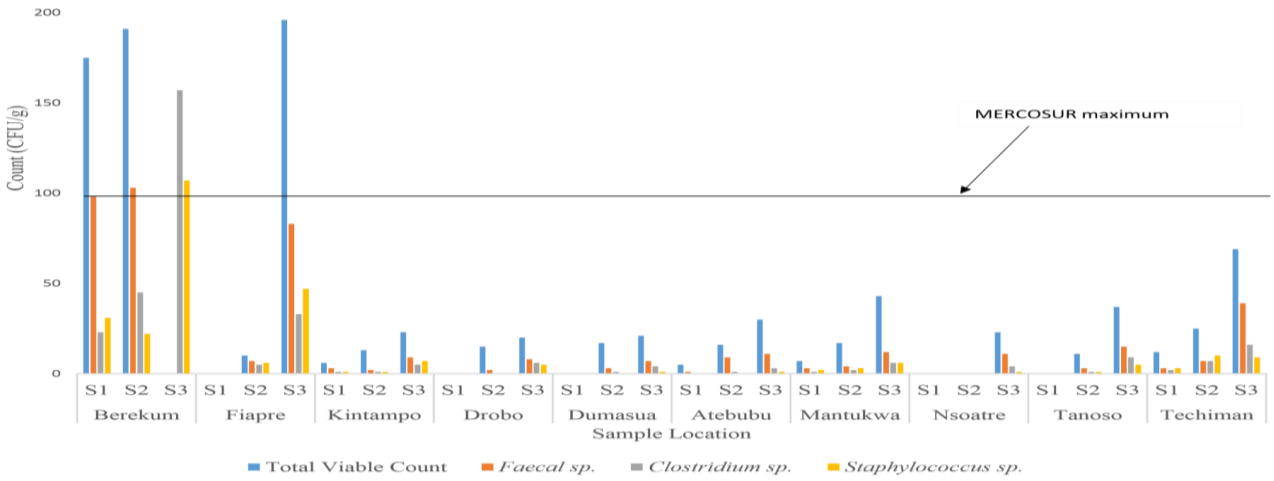

Figure 8. Microbial count in honey sampled from the Brong Ahafo Region, Ghana

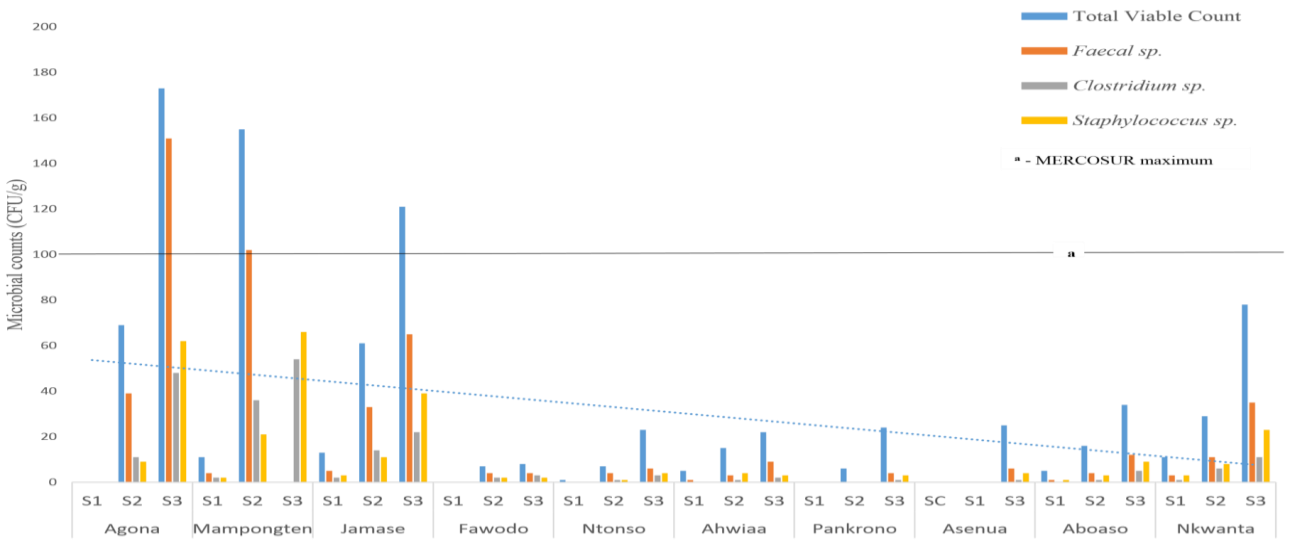

Figure 9. Microbial count per sample in honey sampled from the Ashanti Region, Ghana

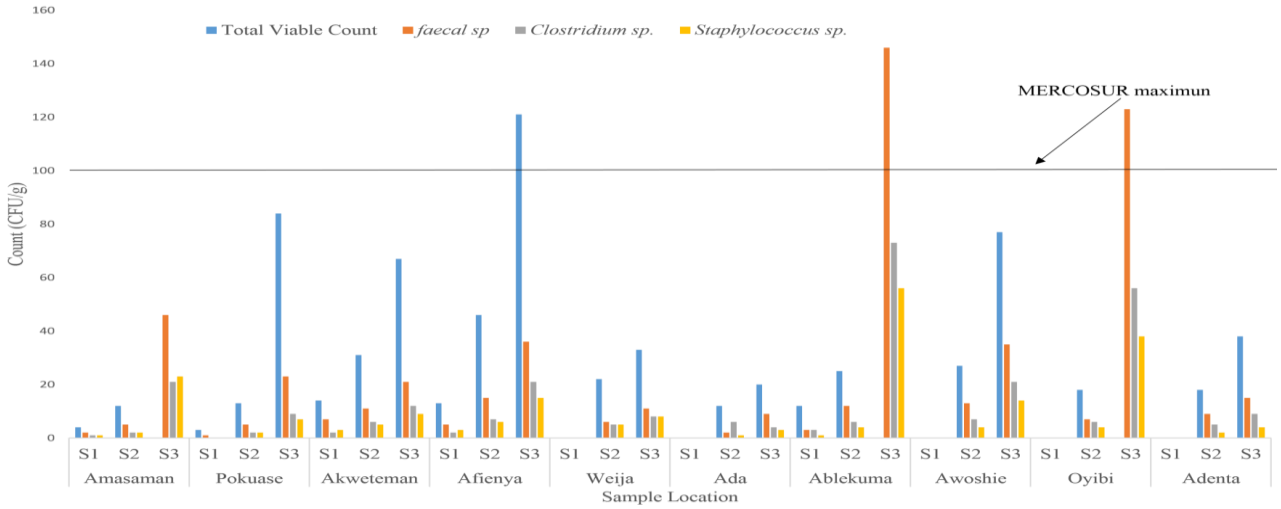

Figure 10. Microbial Count per in Honey sampled in the Greater Accra Region, Ghana

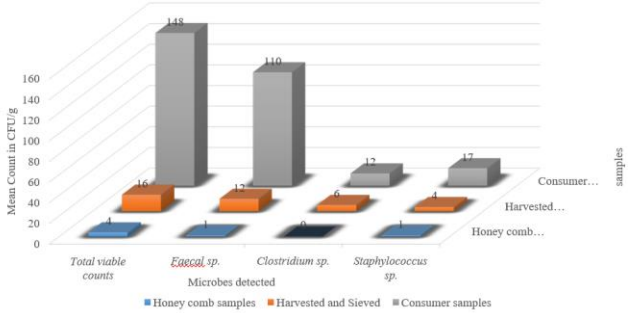

Figure 11. Microbial count per honey sample in Ashanti Region, Ghana

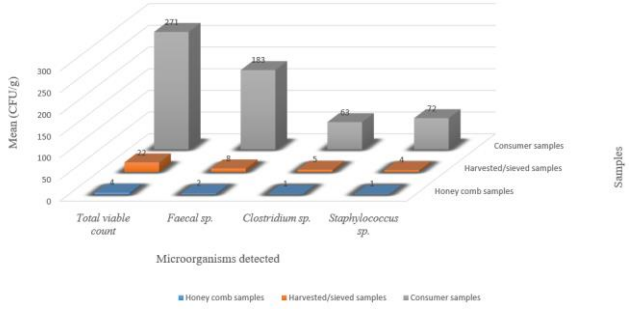

Figure 12. Microbial count per sample in Greater Accra Region, Ghana

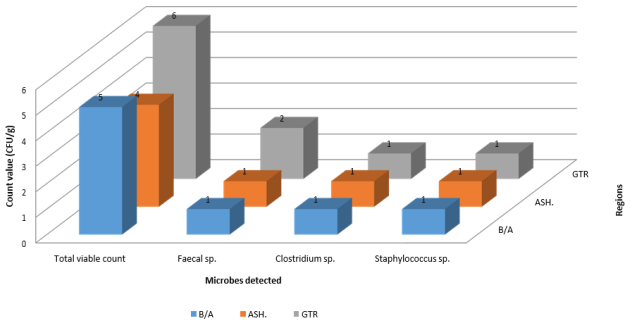

Figure 13. Mean microbial count in honeycomb samples per region

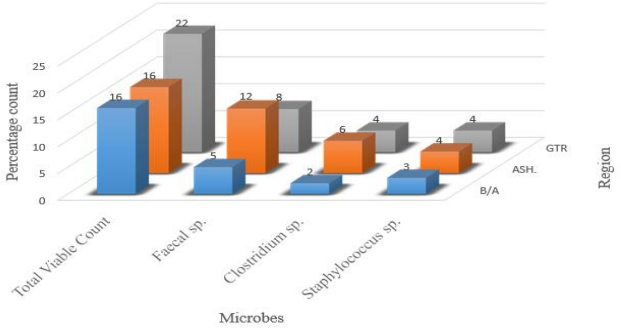

Figure 14. The average count of microbes detected in the treated samples per region

Sieved samples (S2)

Figure 14 represents data for the mean microbial counts in the Extracted and Sieved (S2) samples. The Mean Value (i.e., the Microbial Count per sample) for the Total Viable Count for the Brong Ahafo, Ashanti, and Greater Accra Regions were 16, 16, and 22 CFU/g, respectively. These values fall within 10 to 10^2 CFU/g, as reported by Snowdon and Cliver (1996), hence honey treated by the beekeeper can be deemed wholesome in terms of microbial quality. The Mean Values for *Faecal* sp., *Clostridium* sp., and *Staphylococcus* sp. for each sample in all the sample areas were below 10 CFU/g.

TVC-Total Viable Count

Consumer (Retail) Samples (S3)

In Table 7, it is observed that the mean microbial count for the Retailer Samples (183, 148, and 271) CFU/g for B/A, ASH, and GAR, respectively, were relatively higher than the mean microbial counts of the honey sampled directly from the honeycomb (S1) as well as honey sampled from the farmer after harvesting, extracting and sieving (S2). In addition, the count values were significantly higher than the MERCOSUR maximum standard of 100 CFU/g for all the sample locations, which indicates that the honey sampled from the retailers was contaminated. The honey sampled from the Greater Accra Region had the highest mean microbial count for Total Viable Count.

Table 6. Microbial counts in honeycomb samples per sample region

Microorganisms	Counts (CFU/g)					
	B/A		ASH		GAR	
	Abs.	Mean	Abs.	Mean	Abs.	Mean
Total aerobic count	30	5.0±2	35	4.0±1	35	6.0±2
Faecal sp.	12	1.0±0.3	10	1.0±0.3	14	2.0±0.4
Clostridium sp.	7	<1	3	<1	9	1.0±0.02
Staphylococcus sp.	7	<1	7	<1	9	1.0±0.03

Table 7. Microbial count in retailer samples per sample location

Microorganisms	Counts (CFU/g)					
	B/A		ASH		GAR	
	Absolute	Mean	Absolute	Mean	Absolute	Mean
TVC	1462	183±16	1332	148±12	2440	271±18
Faecal sp.	895	112±10	992	110±9	1465	183±15
Clostridium sp.	186	24±4	196	25±4	425	53±5
Staphylococcus sp.	102	13±3	153	17±5	577	72±6
Salmonella sp.	Negative	Negative	Negative	Negative	Negative	Negative

Results of physicochemical analyses of the honey samples

Figures 15, 16, and 17 represent results from the physical and chemical parameters analyzed for 60 samples comprising the Honey Comb Samples (S1) and the Retail Samples (S3) from all 90 sample locations. It is part of the hypothesis of this research that the harvested and processed samples (S2) would have no significant difference in reducing sugar and apparent sucrose concentrations, ash content, and pH. However, should there be any significant change to honey in terms of these qualities, it would happen during the products" transition to the consumer.

Pre-irradiation

The physicochemical analyses were conducted to identify any significant changes resulting from the dose of gamma radiation used in decontamination. It is the high concentration of reducing sugars and the low pH (acidic nature) of honey that makes it difficult to support microbial growth; in case these parameters alter, the shelf life of honey could be affected because it may improve the conditions of microbial survival and perhaps their multiplication and other food spoilage factors.

As much as decontamination is necessary, it is equally significant to maintain the physicochemical parameters to maintain or improve the shelf life and natural quality of honey. Therefore, the parameters analyzed were total reducing sugars, apparent sucrose, pH, and ash content.

The graphs (Figures 15, 16, and 17) show the concentration (in percentage) of reducing sugar and apparent sucrose in the honey sampled from the 30 sample points. The sucrose concentrations of the Honey Comb samples (S1) from all the sample points in the Brong Ahafo region were found to be within the Codex standards for honey (≥ 60% for Reducing Sugar concentration and ≤ 10% for Apparent Sucrose concentration), except for the Berekum sample (12.24% for Apparent Sucrose and 54.16% for Reducing sugar).

Apart from the sample from Aboaso in the Ashanti Region, all the Honey Comb samples (S1) from this region had apparent sucrose concentrations within the acceptable range. About 20% of the Honey Comb samples from the Greater Accra Region had sucrose concentrations slightly over the required maximum by the Codex standards (≤10%). The Codex standard gives precedence to a type of honey (honeydew) concerning the reducing sugar and apparent sucrose concentrations with reducing sugar concentration (≥53%) and apparent sucrose (≤15%); hence these samples could still be deemed wholesome.

All the Retail Samples (S3) from the thirty (30) sample locations had sucrose concentrations significantly above the average maximum of 10%. The reducing sugar concentrations in the Retail Samples compared to the Honey Comb samples were reduced for all the samples analyzed. The samples' pH was within the Codex standards of 3.8±1 to 6.0±1.

The Reducing Sugar (fructose and glucose) for honey, by Codex standards and the directive of the European Commission (EC Directive 2001/110), should be ≥60 g/100g of sample (i.e., 60%). In contrast, the Apparent Sucrose concentration should be ≤10 g/100g (10%) of the sample. Furthermore, the pH of honey should fall within 3.6 ±0.1 and 6.1±0.1, and the values for ash content by international standards (EC directive 2001; CODEX STAN 12-1981) should not exceed the range of (0.6-1.2)%.

Table 8 shows the physicochemical parameters for honey sampled from the Brong Ahafo Region. The Reducing Sugar values recorded for the Honey Comb Samples were mostly within the required standard, with a mean value of 70%. In comparison, the mean value recorded for the Apparent Sucrose for these samples was 8 %, falling within the required values for wholesome honey. For the Retail Samples (S3), the mean value for "Total Reducing Sugar" content of 54% falls below the expected standard according to Codex (≥60%). Concerning the Apparent Sucrose, the mean value of 23 % is significantly higher than the expected range of ≤10%. The ash content for the consumer samples was all within the required standard values.

Tables 9 and 10 are representations of the physicochemical qualities of the honey sampled from the Ashanti and Greater Accra Regions, respectively. The results trend was similar to those obtained for honey sampled in the Brong Ahafo Region. The mean values of the Total Reducing sugars for the Honey Comb samples were 68% and 66% for Ashanti and Greater Accra samples, respectively. The Apparent sucrose concentrations were 9.0% and 9%, respectively. These results satisfy the Codex standards for honey quality. For the Retail Samples, there was a reduction in the Reducing sugar concentration and a significant increase in the Apparent Sucrose concentrations for both regions, as seen in Tables 9 and 10. The ash content for the honeycomb samples was within the range of 0.1-0.2 (%), which falls below the codex range of 2-4%.

Results from microbiological analysis after irradiation

The effect of gamma-irradiation on the microbiological decontamination of the honey sampled from the sample points in the Brong Ahafo, Ashanti, and Greater Accra regions are shown in Tables 11, 12, and 13.

All the honey samples irradiated tested negative for microorganisms. The gamma radiation at 20 kGy, 30 kGy, and 40 kGy was enough to decontaminate the honey samples.

Physicochemical results after gamma-irradiation

The Table 14 represents the physicochemical results of the irradiated samples. There were no significant changes to the Reducing Sugar, Apparent Sucrose, Ash, and pH of the honey samples after irradiation with gamma energy from a Cobalt-60 source.

Results compared to standards

The microbiological analyses' results were compared with those of similar research undertaken by Finola et al. (2007) in Argentina. These researchers reported that microbial contaminants in honey were within "MERCOSUR stipulated values" of 10^1-10^2 CFU/g of honey.

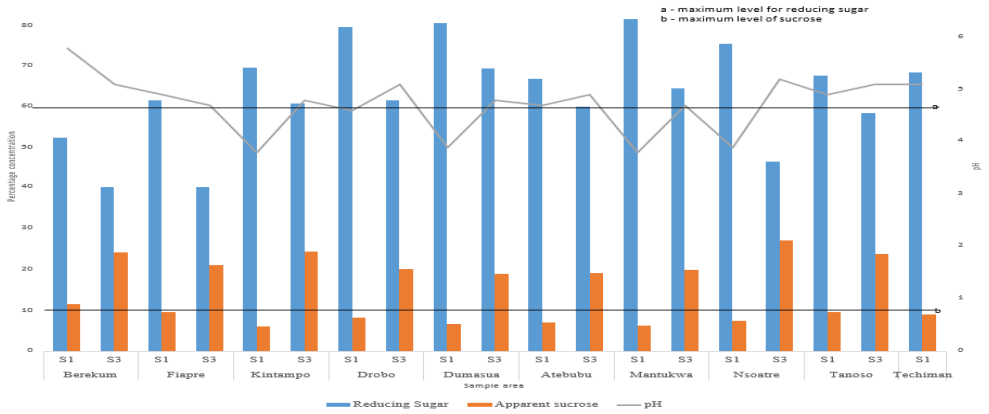

Figure 15. Pre-irradiation physicochemical parameters of samples from the Brong Ahafo Region, Ghana

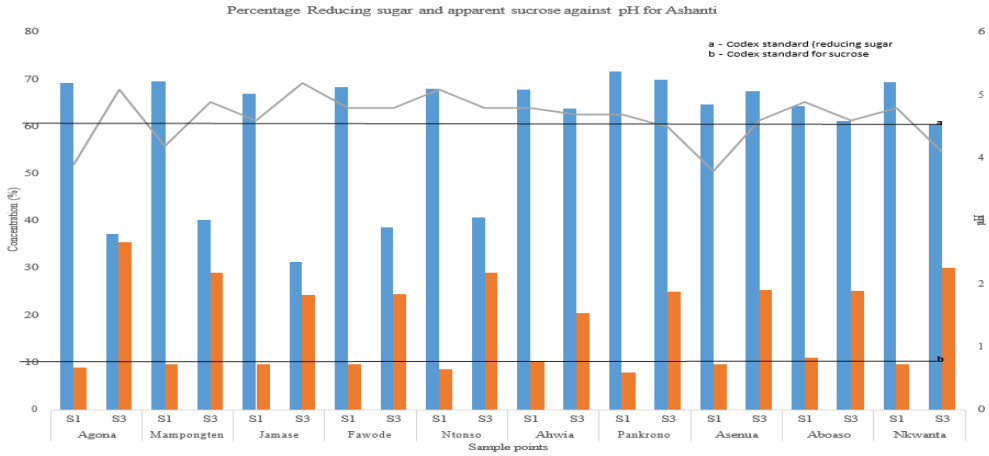

Figure 16. Pre-irradiation physicochemical parameters of samples from the Ashanti Region, Ghana

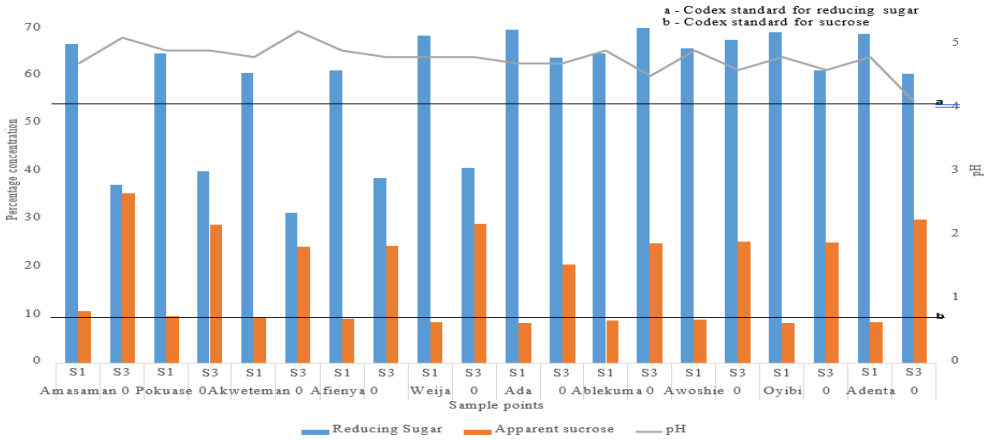

Figure 17. Pre-irradiation physicochemical parameters of samples from the Greater Accra Region, Ghana

Following this standard, the mean viable counts in microbes detected in the honeycomb samples (S1) from all the sample points were within 30-35 CFU/g, which falls within the MERCOSUR stipulated value. The mean count for *Faecal* sp. in all the honeycomb samples was below 10 CFU/g. The count for sulfite-reducing *Clostridium* sp. and *Staphylococcus* sp. were below 10 CFU/g. About 40% (12 out of 30) of the honeycomb samples from all 3 regions sampled were contaminated with sulfite-reducing *Clostridia* sp. In contrast, approximately 43% (13 out of 30) of these honeycomb samples were contaminated with *Staphylococcus* sp. Most of these contaminations were due to the locations of the beehives and a lack of proper maintenance of the hives. However, from the microbiological point of view, the relatively low microbial count in the honeycomb samples indicates the proper management of the beehive by most honeybee keepers.

Table 8. Physicochemical composition of samples from the B/A before gamma irradiation

Parameter	Type of Honey Sample			
	Honey Comb Sample		Retail Sample	
	Range	Mean ±SD	Range	Mean ±SD
TRS (%)	52.6-81.6	70±9	40.34-69.45	54±10
AS (%)	6.2-11.6	8±2	19.09-27.46	23±4
AC (%)	0.04-0.27	0.1±0.05	0.06-0.27	0.16±0.06
pH	3.8-5.6	5.0±0.6	3.8-5.3	5.0±0.2

Note: TRS-Total Reducing Sugar; AS-Apparent Sucrose; AC-Ash Content

Table 9. Pre-irradiation physicochemical parameters of the honey sampled from Ashanti Region, Ghana

Parameter	Type of honey sample			
	Honey Comb Sample (S1)		Retail Sample (S3)	
	Range	Mean ±SD	Range	Mean ±SD
TRS (%)	61.28-71.85	68±3	30.87-70.50	51.0±6
AS (%)	7.45-10.23	9.0±0.8	21.66-35.46	26.0±4
AC (%)	0.04-0.15	0.1±0.07	0.11-0.75	0.2±0.04
pH	3.9-5.1	5.0±0.3	4.4-5.6	4.9±0.3

Note: TRS-Total Reducing Sugar; AP-Apparent Sucrose; AC-Ash Content

Table 10. Pre-irradiation physicochemical parameters of the honey sampled from Greater Accra Region, Ghana

Parameter	Type of honey sample			
	Honey Comb Sample (S1)		Consumer Samples (S3)	
	Range	Mean ±SD	Range	Mean ±SD
TRS (%)	60.71-69.68	66±3	31.57-66.96	43±12
AC (%)	8.42-10.88	9.0±0.7	21.02-41.49	32±6
AC (%)	0.12-0.24	0.2±0.09	0.45-0.78	0.7±0.02
Ph	4.7-4.9	5.0±0.07	4.9-5.6	5.0±0.2

Note: TRS-Total Reducing Sugar; AP-Apparent Sucrose; AC-Ash Content

Table 11. Post-irradiation microbial analyses of honey from the Brong Ahafo Region, Ghana

Irradiation dose (kGy)	Microbes (CFU/g)							
	Total viable count		Coliform		*Clostridium* sp.		*Staphylococcus* sp.	
	S2	S3	S2	S3	S2	S3	S2	S3
0	16	183	5	112	2	23	3	13
20	ND	ND	ND	ND	ND	ND	ND	ND
30	ND	ND	ND	ND	ND	ND	ND	ND
40	ND	ND	ND	ND	ND	ND	ND	ND

Note: ND-No detection (negative); S2-Extracted/Sieved Sample; S3-Retail Samples

Table 12. Post-irradiation microbial analyses of native honey from the Ashanti Region, Ghana

Irradiation dose (kGy)	Microbes (CFU/g)							
	Total viable count		Coliform		*Clostridium* sp.		*Staphylococcus* sp.	
	S1	S3	S1	S3	S1	S3	S1	S3
0	16	148	12	110	6	12	4	17
20	ND	ND	ND	ND	ND	ND	ND	ND
30	ND	ND	ND	ND	ND	ND	ND	ND
40	ND	ND	ND	ND	ND	ND	ND	ND

Note: ND-No detection (negative)

Table 13. Post-irradiation microbial analyses of native honey from the Greater Accra Region, Ghana

Irradiation dose (kGy)	Microbes (CFU/g)							
	Total viable count		Coliform		*Clostridium* sp.		*Staphylococcus* sp.	
	S1	S3	S1	S3	S1	S3	S1	S3
0	22	271	8	183	5	63	4	72
20	ND	ND	ND	ND	ND	ND	ND	ND
30	ND	ND	ND	ND	ND	ND	ND	ND
40	ND	ND	ND	ND	ND	ND	ND	ND

Note: ND-No detection (negative)

Table 14. Post-irradiation physicochemical analyses

Dose (kGy)	Reducing sugar			Apparent sucrose			Ash content			pH		
	BA	ASH	GAR	BA	ASH	GAR	BA	ASH	BAR	BA	ASH	GAR
Pre-irradiation	54.3	51.13	42.70	22.16	26.23	32.25	0.16	0.22	0.67	4.8	4.9	4.8
20	55.2	51.46	43.10	22.62	26.25	32.31	0.15	0.23	0.67	4.7	4.8	4.8
30	54.7	51.25	42.74	22.40	27.10	32.11	0.14	0.25	0.66	4.8	4.9	4.9
40	52.1	50.3	40.41	20.20	27.9	34.65	0.16	0.23	0.66	4.4	4.3	4.4

The microbial count values in samples harvested and treated by the farmers (S2) were relatively higher for all the 30 samples analyzed than the honeycomb samples. About 90% (27 out of 30) of these samples were contaminated with *Faecal* sp. Approximately 18% of the samples were from the Ashanti Region (the highest among the three regions) for *Faecal* sp. Sulfite-reducing clostridium was detected in all the contaminated samples, and *Staphylococcus* sp. The soil is the main source of *Clostridium* sp., although dust, equipment, buildings, and the environment could also contain this genus of microbe. The presence of these microorganisms indicates contamination or pollution. The chain of manufacturing and maturity at harvest should be monitored to decrease the chances of making honey impure.

Most of the beekeepers used their bare hands to remove the comb from the beehive, and very little attention was given to the possibility of contamination during harvesting. The combs are kept in plastic containers, sometimes metallic containers, and left overnight to drain. During the day, the containers with the honey are brought out into the

sun to heat-drain, exposing the honey to dust and other particles.

Microorganisms were detected in all 30 honey samples from the retailers. The mean count of *Faecal* sp. in the samples was 111 CFU/g, 124 CFU/g, and 183 CFU/g for Brong Ahafo, Ashanti, and Greater Accra Regions, respectively. These count values were higher than the maximum values stipulated by the MERCOSUR regulations, 10^2 CFU/g. Samples from the Greater Accra region had the highest recorded values for microbial counts. The mean values for *Clostridium* sp. and *Staphylococcus* sp. were 53 CFU/g and 72 CFU/g. Comparing the areas sampled in the Greater Accra Region to those sampled in the other two regions, the Greater Accra Region has the highest population density; hence, human activities are higher in this region than in other regions. That is a major factor in the contribution of contaminants to any food sample; honey is not an exception. All the samples were negative for *Salmonella* sp.

Mean values for Clostridium sp. and Staphylococcus sp. for the Ashanti Region were 25 and 19 CFU/g, respectively. In contrast, the mean values of *Clostridium* sp. and *Staphylococcus* sp. detected in samples from the Brong Ahafo Region were 24 and 13 CFU/g, respectively. Therefore, honey sampled from the Brong Ahafo Region can be said to be the most wholesome for human consumption.

Honey from the honeycombs sampled from all the regions had reducing sugar values within the required concentrations as in the standards of the International Honey Commission (≥60%) and the CODEX Alimentarius Commission. About 70% of the sugars in the honeycombs samples from the Brong Ahafo Region were reducing sugars and contained the required sucrose concentrations (average; 8%) according to the Codex standards.

The results showed relatively high concentrations of sucrose in the honey sampled from the retailers. The mean reducing sugar concentration for the honeycomb samples from the Ashanti Region was 68%, within the accepted concentration range, and an average of 9% for sucrose. Honey sampled from the retailers in the Greater Accra Region had the highest mean concentration of sucrose (34%) compared to 26 % and 22% in the samples from the Ashanti and Brong Ahafo regions, respectively. Relative to the Codex, EC directive, and the standards from the International Honey Commission, these results were above the required concentration of sucrose in natural honey. The great disparity in apparent sucrose concentration in the honeycomb samples (S1) relative to the retail samples (S3) indicates adulteration of honey with white sugar. This inference concerns the percentage increase for the samples from the Brong Ahafo Region,

The ash content helps to interpret the honey's origin and indicates the foraging area of the honeybees. Ash content standards are set at a minimum of 0.6 g/100 g of sample and a maximum of 1.2 g/100 g (i.e., 0.6%-1.2%) of honey by the Codex Alimentarius Commission, the IHC, and the EC directive among other standards all of which follow a similar quality assurance criteria. The ash content of all the honey sampled and analyzed was within the required standard, with an average of 0.16% in the honeycomb samples and an average of 0.62% for the retail samples.

This research showed that the honey sampled from all the sample areas was found to have an acidic character. The pH values ranged from 3.8 to 5.6 in the Brong Ahafo Region (Table 11), 3.9 to 5.6 in samples from the Ashanti Region (Table 12), and 4.7 to 5.6 in the samples from the Greater Accra Region. These results conform to the standard values. The results also showed that the pH in the retail samples was higher than in the honeycomb samples, which could have contributed to the fewer detection values of microorganisms in the honeycomb samples. The pH meets the values reported by Bera et al. (2009), with pH values in the 3.8 to 4.2 and the Codex standard range of 3.8±1 to 6.0±1.

Gamma irradiation at a temperature of 25°C at doses ranging from 20 kGy to 30 kGy had no significant effect on the physical and chemical qualities of the honey. The mean reducing sugar concentration reduced insignificantly after irradiating at 40 kGy relatives to the average concentration before irradiation. On average, the change in apparent sucrose concentration was insignificant, whereas there was a slight reduction in pH after irradiation at 40 kGy.

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