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Echinacea purpurea (L.) Moench) 'Harvest Moon' photo by Achim

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Introduction is about 600 words, covering the aims of the research and provide an adequate background, avoiding a detailed literature survey or a summary of the results. **Materials and Methods** should emphasize on the procedures and data analysis. **Results and Discussion** should be written as a series of connecting sentences, however, for a manuscript with long discussion should be divided into subtitles. Thorough discussion represents the causal effect mainly explains why and how the results of the research were taken place, and do not only re-express the mentioned results in the form of sentences. **Concluding** sentence should be given at the end of the discussion. **Acknowledgements** are expressed in a brief; all sources of institutional, private and corporate financial support for the work must be fully acknowledged, and any potential conflicts of interest are noted.

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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]

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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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Association of thyroid disorders with diabetes: A cross-sectional study

MUBASHIR ZAFAR[1], SYED MONWAR ALAM SHAHID[2,♥], REEM FALAH ALSHAMMARI[3], MOHD ADNAN KAUSAR[2], TARIG A. N. GINAWI[2], ABDUL WAHAB HATIM[2], ABDUL MAJEED WADI[2], HAMAD ALI[2], ABDUL AZIZ HAMED[2], MOATH SALEH AL-ZAHRANI[2], ALI HUSSAIN[2], ABDULAZIZ SAUD ALDUHAIM[2], ABDULMAJEED MOHAMMED[2]

[1] College of Public Health, Imam Abdul Rehman bin Faisal University. Dammam 34212, Kingdom of Saudi Arabia.
[2]Department of Biochemistry, College of Medicine, University of Hail. Hail 55476, Kingdom of Saudi Arabia. Tel./fax.: +96-6551803367, ♥email: shahahid@uoh.edu.sa
[3]Department of Family and Community Medicine, University of Hail. Hail 55476, Kingdom of Saudi Arabia

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Abstract. *Zafar M, Shahid SMA, Alshammari RF, Kausar MA, Ginawi TAN, Hatim AW, Wadi AM, Ali H, Hamed AA, Al-Zahrani MS, Hussain A, Alduhaim AS, Mohammed A. 2022. Association of thyroid disorders with diabetes: A cross-sectional study. Nusantara Bioscience 14: 135-140.* Two common endocrine disorders that correlate with each other are diabetes mellitus (DM) and thyroid dysfunction (TD). Undiagnosed thyroid disorders (TD) have a high risk for diabetes mellitus (DM) patients. A common complication among these patients is cardiovascular disease. This study aims to assess the association of TD among diabetes patients. It is a cross-sectional study, and 338 patients with type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM) were observed through a simple random sampling method from a public sector hospital. The diabetes status of patients was confirmed through clinical and laboratory investigation. Those patients who were under treatment of thyroid were excluded from the study. The chi-square test was used for analysis, and a p-value <0.05 was considered significant. The frequency of TD among diabetic patients was 47.6%. The main type of TD was subclinical hypothyroidism, and its prevalence is 43.8% and 23.5% among patients with T1DM and T2DM, respectively. Subclinical hyperthyroidism prevalences are 12.3% and 24.4% among TIDM and T2DM patients, respectively. The study found a high-frequency rate of TD among DM patients. Therefore, there is a need for regular screening of DM patients for TD and increased awareness regarding TD among DM patients.

Keywords: Diabetes mellitus, hyperthyroidism, hypothyroidism, prevalence, thyroid dysfunction

INTRODUCTION

Diabetes is one of the most common disorders in the human population. There is a link between diabetes and other metabolic disorders (Kalra et al. 2019). The association between these two disorders is recognized. The prevalence of thyroid disorders in the diabetic population varies widely between studies (Gu et al. 2017). The pancreas releases insulin, and the thyroid gland releases T3 and T4, which affect the body's metabolism (Al-Omrani et al. 2018). Thyroid-stimulating hormone (TSH) levels in the blood regulate the T3 and T4 levels in the serum because TSH stimulates the thyroid gland to release the T3 and T4 (Ogbonna et al. 2019). In diabetes, patients have thyroid disorders (TD) symptoms, including hypothyroidism and hyperthyroidism among type 2 diabetes patients. A study conducted in a hospital outpatient medicine department found a high prevalence of TD among type 2 diabetes patients (T2DM) (Rubaye 2019). Diabetes mellitus (DM) has been affected on multiple levels of the thyroid gland through thyroid-stimulating hormone (TSH). Also known as non-communicable, DM is a systemic disease with a high prevalence and mortality rate (Haryta et al. 2021). It increased the serum's T4, T3, and Insulin levels (Gronich et al. 2015; Chuang et al. 2016; Shahid et al. 2020). The first study, which was conducted in 1979 and found that a correlation between TD and diabetes mellitus had been established (Bellastella et al. 2018). The burden of TD among diabetes (DM) patients ranges from 3.2% to 57.6% (Bano et al. 2019). There are different types of TD in diabetes patients, and the most common type is sub-clinical hypothyroidism, specifically in type 2 diabetes patients. This prevalence in diabetes patients is comparatively high in the general population (Luna et al. 2014; Mehran et al. 2014). Various studies found that subclinical hypothyroidism is the common type in diabetes patients (Palma et al. 2013; Hoermann et al. 2021).

Poor glucose control in the serum leads to TD. The reason for this disorder is a high level of TSH in the serum at night which inhibits the thyroid from releasing hormones in the hypothalamus, which leads to a decreased level of T3 and T4 in the serum, which causes hypothyroidism (Hussain et al. 2019). Decreased serum T3 level is observed in diabetes patients. A low T3 level in the serum leads to a low level of T4 due to low conversion from T3 to T4. This mechanism is reversed if glycaemic control is good. The main enzyme present in the liver is thyroxine 5 deiodinase, which is converted from T3 to T4, and when the concentration of this enzyme is low, ultimately, the outcome is hypothyroidism. Insulin also contributes to TD, increased levels of insulin in the serum lead to insulin resistance, thyroid tissue proliferative mechanism has

started, leading to nodule formation in the thyroid, and goiter disease has developed (Hasan et al. 2016).

In diabetes, a common cause is insulin resistance due to multiple factors such as sendatory lifestyle, obesity, smoking, etc. This disease develops for years after a disturbance of hormone regulation (Chen et al. 2019). At the initial stage, the pancreas β cells produce a high insulin level to decrease the glucose level because it increases resistance in the cell and muscular tissues. At the later stage, *β* cells burn out, increase serum glucose levels, and start diabetes. Metabolic syndrome is a cluster of diseases, including diabetes, due to insulin resistance (Evron et al. 2020). It is also comprised of cardiovascular diseases, polycystic ovaries, hypertension, and other endocrine disorders, with additional risk factors, are increased age, smoking, and genetic disorders (Biondi et al. 2019). The TD is part of a metabolic syndrome caused by autoimmunity against thyroid cells. Common TD are hypothyroidism, thyrotoxicosis, and goiter (Hoermann et al. 2021). Previous studies found that the prevalence of TD among diabetes patients is 6-25% in North America and Europe (Giugliano et al. 2020; Groothof et al. 2021).

A previous study was conducted on a sample size of 386 patients with diabetes at the hospital's outpatient department. The results found that hypertension and dyslipidemia are risk factors for TD among diabetes patients. These patients had undiagnosed TD, and the common type of TD was sub-clinical hypothyroidism (Jun et al. 2017a,b). In addition, a previous study was conducted on diabetes patients (T2DM), and the results showed that T3 and T4 levels in the serum were very high in diabetic patients (Vries et al. 2019).

In Middle East countries, the prevalence of diabetes is very high, and limited data is available for TD among diabetes patients. This study determines the actual burden of TD among diabetes patients. Diabetes has been associated with myocardial infarction and other diseases of endocrine disorders, specifically the thyroid gland. This study's results will benefit the community by increasing the awareness level among the general population, and it will also benefit the physician in managing diabetic patients with TD and preventing the complication of diabetic patients. It is, therefore, important to make an early diagnosis of thyroid dysfunction in diabetic patients to prevent further complications, and this practice is carried out in the clinic.

MATERIALS AND METHODS

Study area

This cross-sectional study was conducted in a primary health Centre (Figure 1).

Procedures

The sample size was calculated using world health organization (WHO) software for sample size determination in health studies. To measure sample size by using a prevalence of TD was 17% among DM patients from the previous study (Hall et al. 2020), at a confidence level of 95% and a bound of error of 4%, the estimated sample size came out to be 338. Therefore, taking the largest sample, a minimum of 338 participants were included in the study. The sampling technique employed was a multi-stage cluster sampling, first selecting the cluster, then selecting the strata within the cluster, and then selecting the household through systematic random sampling.

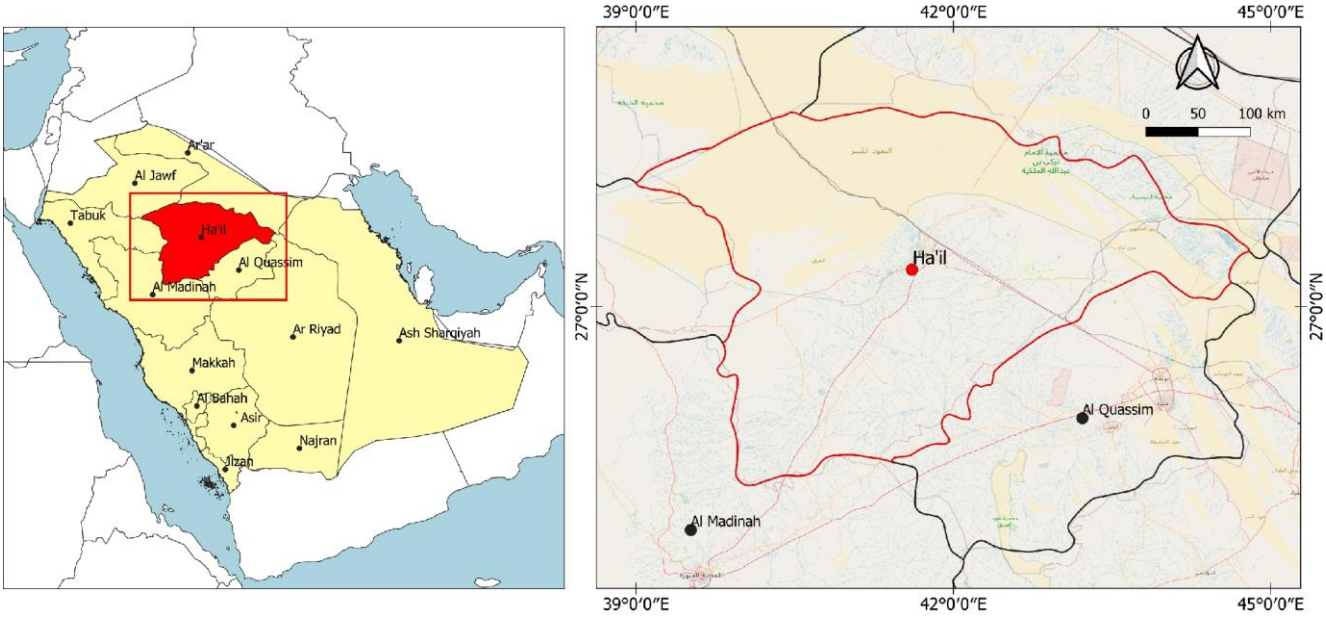

Figure 1. Location of Hail, Kingdom of Saudi Arabia

Instrument and data collection

All persons aged 18 to 60 years were included in the study. The study instrument was a validated questionnaire (Chang et al. 2017) comprising three sections. Part 1 relates to the socio-demographic characteristics, and part 2 relates to the study participants' clinical characteristics.

First, check the reliability and validity of the questionnaire and translate it into Arabic. To check reliability and validity, we collected data from other cities in the northern region and improved the quality of the questionnaire. There is sampling bias in the study because it is a cross-section study and this bias minimize through the blinding of study participants. Participants with diabetes and those patients above 60 years and below 18 years were excluded from the study. Study variables are age, gender, smoking status, type of diabetes, and status of TD.

Data collection procedure

The inclusion criteria of study participants were 6 months duration of DM among T2DM and one year for T1DM. T2DM patients are diagnosed at an age ≥30 years, without using insulin in the first year after diagnosis, and without a history of ketosis or ketonuria. The T1DM patients were diagnosed with clinical presentation, weight loss, polydipsia, polyphagia, polyuria, and the need to use insulin continuously since the diagnosis without discontinuation and at least one year medical follow-up. Written informed consent for the study was obtained from all patients aged 18 years or older or from the parents or guardians of patients younger than 18. Exclusion criteria for study participants were those unable to understand and sign the informed consent, pregnant women, and past medical history of hospitalization for less than 6 months. There are standard classification of TD, which is classified as Subclinical hyperthyroidism (SC-Hyper) if TSH < 0.27 μUI/mL, Sub-clinical hypothyroidism (SC-Hypo) if TSH > 4.20, Clinical hyperthyroidism(C-Hyper) if TSH < 0.27, clinical hypothyroidism (C-Hypo) if TSH > 4.20 μUI/mL, FT4 in the normal range (0.93 and 1.7 ng/dL), FT4 < 0.93 ng/dL; μUI/mL and FT4 ranged from 0.93 to 1.7 ng/dL.

Data analysis

Epi Data Entry software version 1.3 was used for data entry; data were entered twice and cleaned for any missing variables. Data were analyzed using software SPSS version 23. Descriptive statistics analysis was done for categorical variables and is present as frequency (percentage) and mean ±standard deviation. Student T was used for differences between the two groups. The Chi-square test was used for categorical variables to compare two or more groups. The p-value was statistically significant when it was less than or equal to 0.05.

RESULTS AND DISCUSSION

The mean age of patients was 33.51±1.51SD. Mostly (52.1%) are male patients and 219(64.8%) with T1DM, and 119(35.2%) with T2DM. The frequency of TD in diabetes mellitus patients is 47.6% (Table 1).

The prevalence of SC-Hypothyroidism was 43.8% in T1DM and 23.5% in T2DM. The prevalence of C-Hypothyroidism was 3.56% and 1.7% among TIDM and T2DM, respectively. The prevalence of SC-hyperthyroidism was 12.3% and 24.4% in T1DM and T2DM, respectively. C-hyperthyroidism prevalence was 3.8% and 0.7% among TIDM and T2DM, respectively. (Table 2).

The prevalence of SC-Hypothyroidism in females is 59.9% and 15.3% in male patients (Figure 2).

Discussion

The study found that 47.6% prevalence of TD among diabetic patients. Among thyroid diseases, Subclinical hypothyroidism was the top type of TD, found at 43.8%, which is consistent with the other studies (Journy et al. 2017; Mehran et al. 2017). Another study found that 16% prevalence of thyroid among T2DM (Journy et al. 2017). The basic cause of TD among people with diabetes is that Thyroid hormones and insulin antagonize each other, and both affect the biochemistry of human body cells (Ogbonna et al. 2019). The prevalence of TD among the general population is 7.6% (Bano et al. 2019).

Table 1. Clinical and demographic characteristics of study participants (n= 338)

Characteristics	Frequency (%)
Age (Years) (Mean ±SD)	33.51±1.51
18-40	187(55.3)
41-60	151(44.7)
Gender	
Male	176(52.1)
Female	162(49.9)
Smoking	
Ever	122(36.1)
Never	216(63.9)
Type of Diabetes Mellitus	
Type 1(T1DM)	219(64.8)
Type 2(T2DM)	119(35.2)
Thyroid Dysfunction(TD)	
Yes	166(47.6)
No	177(52.4)

Table 2. The frequencies of thyroid dysfunction among T1DM and T2DM patients

Thyroid function	TIDM (219) (%)	T2DM (119) (%)
Euthyroidism	37.16	49.7
SC-Hypothyroidism	43.8	23.5
C-Hypothyroidism	3.56	1.7
SC-Hyperthyroidism	12.3	24.4
C-Hyperthyroidism	3.8	0.7

Note: SC: subclinical, C: clinical

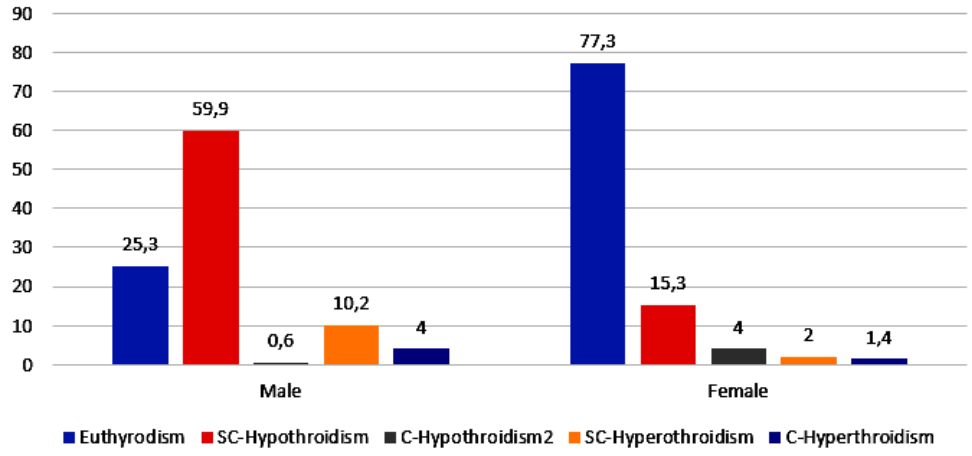

Figure 2. Gender distribution of thyroid function among diabetic patients

This study found that the prevalence of hyperthyroidism among T2DM was higher by 24.4% compared to T1DM. However, another study showed that TD among T2DM (Ittermann et al. 2018) found 31.4%, and SC-hyperthyroidism was the highest-burden among TD in these patients, followed by C-hyperthyroidism (24.2%), similar to our study results. The reason for this difference is that TSH is linked with insulin resistance and endothelial function, which leads to the increased serum level of TSH and dyslipidemia and disturbs the endothelial function of cells (Zhao et al. 2016).

Different literature also found that the correlation between diabetes and TD might be bidirectional. T2DM patients may have increased thyroid tissue hyperplasia, which leads to goiter and multinodular goiter (Tang et al. 2017; Chen et al. 2019). It also affects the serum level of glucose due to insulin resistance. It also found that SC-Hypothyroidism will increase with age. Another factor is obesity, which is also correlated with hypothyroidism (Song et al. 2017; Song et al. 2019).

Among the type of thyroiditis, postpartum thyroiditis is common in diabetes patients due to autoimmune among women in the postpartum stage. The symptoms include a hyperthyroid state to a hypothyroid state, which leads to mental disorders in women (Moleti et al. 2020). Postpartum thyroiditis prevalence is from 20% to 30% (Jun et al. 2017a,b; Tang et al. 2017; Zhang et al. 2019).

Diabetic patients with TD have an increased risk of retinopathy and cardiovascular disease, and limited studies are available regarding TD impairment in diabetic patients. A previous study showed that TD prevalence among female and male diabetic patients was 23.7% and 79%, respectively (Giugliano et al. 2020). Insulin resistance is the main cause of disturbing the TD, such as T3 and T4 levels in the serum blood. A previous study found 15% TD in female diabetic patients compared to male diabetic patients (Ryödi et al. 2018).

Diabetic patients with TD affect the metabolic disorders on glucose utilization in hypothyroidism. In the liver, glucose metabolism is also affected, decreasing gluconeogenesis and glucose utilization in tissue. In addition, it affects the glucose level in the serum (Ralli et al. 2020). Hypoglycemia was common among children and adults with an increased TSH level and decreased T3 and T4 (Song et al. 2019).

A previous study found that TD among poorly controlled diabetes patients this dysfunction was subclinical and was reversed after a normal level of glucose. Therefore, the treatment of TD is also affected due to diabetic conditions and may increase the dose of T3 to control thyroid function (Sert et al. 2020).

A recent study found that TD patients among elderly diabetic patients correlated with thyroid hormone levels, and this association are not associated with other non-communicable diseases (Zhou et al. 2019).

There are different types of hypothyroidism; subclinical hypothyroidism is the most common type among diabetic patients; in these patients, a high level of TSH is in the serum with a normal level of T3 and T4. SC-hypothyroidism prevalence among diabetic patients was 6%. Specifically, its prevalence among female diabetic patients is around 7%. A previous study was conducted on adult diabetic patients and found that the prevalence rate is 6.5%. A highly significant finding in healthy papulation regarding TSH level in the serum. In diabetic patients, TSH levels increased as the disease progressed toward chronic disease (Tang et al. 2017).

There is no consistent finding on the screening of TD in diabetic patients, and the basic difference was a type of TD test to find the thyroid function test. Also, there are different opinions about the timing of the test, either routine or specific time to conduct the test. There are different guidelines regarding screening tests for TD, endorsing one guideline that routine screening for TSH levels among diabetic patients is contradictory to other guidelines. Some guidelines say only T1DM patients need to screen their TSH level, but others recommend that T2DM patients screen their TSH level. Without the standard guideline, there is a dependence on the physician's experience and knowledge to follow the TSH level process (Sawin et al. 1994).

In T2DM patients, it recommends periodic screening of TSH levels at the period of every three months. In T1DM patients, randomly screened T3 and T4 levels were every

six months. Testing the level of thyroid antibodies and serum TSH is a strong predictor of TD (Shahid et al. 2020).

A previous study found that serum concentration of TSH above 3mU/L was correlated with hypothyroidism and another study found that serum concentration of TSH above 4mU/L was associated with hypothyroidism. A cross-sectional study found that serum concentration of TSH above 1.90mU/L was associated with hypothyroidism (Ralli et al. 2020).

Among diabetic pregnant women, a routine specific screening test for TSH and T3 levels every 2 years. In addition, thyroid antibody levels are also screened yearly to check the TD among diabetic pregnant women. This test should be done in the first trimester of pregnancy, along with other basic tests in antenatal care. If weight changes during the first semester, then screen the test every 6 months. Start thyroid management immediately if an abnormality is found (Palma et al. 2013). Both types of TD, hypothyroidism, and hyperthyroidism, affect insulin and glucose metabolism. This relationship has an indirectly related L and U-shaped association which leads to metabolic syndrome in a different population of diabetes.

There are several limitations of this study. First, it is a cross-sectional study that did not determine the causality of the association between risk factors and outcome. Second is the study's selection bias because these patients are already under medical treatment. The major strength of this study is the sample size (n= 338), which is higher than other studies conducted in the Kingdom of Saudi Arabia. The study found a high prevalence of TD among diabetic patients, specifically SC-hypothyroidism, which recommends regular screening of TD among these patients and early diagnosis and prompt treatment started. There is a need to make the physician aware of screening diabetic patients routinely and identifying the risk factors which may lead to cardiovascular diseases.

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Effect of storage temperature and packing materials on seed germination and seed storage behavior of *Schefflera abyssinica*

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Abstract. *Bareke T, Addi A, Roba K, Kumsa T. 2022. Effect of storage temperature and packing materials on seed germination and seed storage behavior of* Schefflera abyssinica. *Nusantara Bioscience 14: 141-147.* Knowledge of seed storage behavior is crucial for developing appropriate ex-situ conservation strategies. The main objective of this study was to determine the seed storage behavior of *Schefflera abyssinica* (Hochst. ex A. Rich.). A factorial combination of three temperatures (-10, 0, and 22°C) levels, two types of packing containers (polythene bag and aluminum bag), and eight periods of storage (0, 30, 60, 90, 120, 150, 180 days, and for a year) level was used to determine the germination capacity and storage behavior of the seeds. Accordingly, seed storage temperatures and storage period (up to 1 year) have a significant effect on the germination ($p<0.01$) of *S. abyssinica*. The highest average germination percentage of *S. abyssinica* seeds was obtained after 2 months of storage under all conditions. The seed storage period influences the germination of *S. abyssinica* by 47.8%. The germination percentages of *S. abyssinica* seeds have shown significant differences in storage temperatures. Seeds stored at -10°C showed the highest germination percentage in all storage periods compared to the two storage temperatures. Generally, the highest germination capacity of *S. abyssinica* seeds was between 7% and 9% moisture content after 60 and 90 days of storage. Packing materials have no significant difference in the survival of seeds. No stored seeds germinated after one year of storage at room temperature, 0°C, and -10°C. Based on the definition of seed storage behavior, we conclude that seeds of *S. abyssinica* have intermediate storage.

Keywords: Germination, moisture content, seed, storage behavior

INTRODUCTION

Knowledge of the seed storage behavior is a key feature for determining the success of seed storage protocols used to develop appropriate ex-situ conservation strategies (Hong and Ellis 1996; Jaganathan et al. 2019). Successful storage enables the maintenance of seed viability over time to improve the plant breeding program (Ibraheem et al. 2021). Seed storage behavior varies from one species to another and with the storage environment. Hence, proper seed storage minimizes the rate of deterioration and the loss of viability (Singh et al. 2017). Environmental storage conditions such as temperature and seed water content are the most relevant factors for conserving seed viability (Silva et al. 2019). The impact of moisture content and temperature on seed quality is, therefore, of particular significance in tropical countries where ambient conditions tend to lead to a rapid loss in seed quality (Singh et al. 2017; Ibraheem et al. 2021).

Seed storage behavior is classified as orthodox, intermediate, or recalcitrant based on the responses to moisture content and storage temperature (Hong et al. 1998; Chmielarz 2009). In addition, seed storage behavior is affected by seed origin (provenance) and seed storage containers (Bareke et al. 2018). Orthodox seeds tolerate severe desiccation from 2 to 5% without damage (Roberts 1973), while recalcitrant seeds survive high moisture content (>31%) (ISTA 1999). Recalcitrant seeds do not survive drying to low moisture content and are sensitive to desiccation (Pammenter and Berjak 2014). Due to this, recalcitrant seeds are viable only for a very limited period and exhibit an inability for medium- and long-term storage (Hong and Ellis 1996). Intermediate seed storage behavior is found between orthodox and recalcitrant (Bareke 2018). The difference between the three types of seed storage behavior is presented based on evolution, environmental influences, and seed maturation (José 2018).

Schefflera abyssinica (Hochst. ex A. Rich.) is an indigenous tree belonging to Araliaceae that is branched and small/medium to 30 m tall in height. It produces creamy-yellowish or creamy-white flowers from March to April. It grows in Afromontane forests, secondary forests, and woodlands within the altitudinal range of 1,450-2,800 masl, often in association with *Hagenia abyssinica* (Bruce) J.F.Gmel. (Addi et al. 2014). It is also usually left as scattered trees when forests are cleared for farmlands.

The *S. abyssinica* is one of Ethiopia's most important honey plants (Bareke and Addi 2019). It is a high producer of nectar and significantly contributes to honey production. One hectare of *S. abyssinica* plants has the potential to produce 895.5 kg of harvestable honey (Bareke et al. 2020). Due to its high potential, monofloral honey can be produced from this species which has high demand in the market and could generate high income (Addi et al. 2014; Bareke and Addi 2018).

The *S. abyssinica* is considered an epiphyte, which grows on another tree species, finally overwhelms it, and becomes an independent tree in highland areas.

Currently, *S. abyssinica* can be propagated by seed using an aqueous smoke solution (Bareke et al. 2014), and smoke treatment improve seedlings' capacity to survive the effect of aphids. However, the appropriate seed storage temperature, storage material, storage periods, and seed storage behavior of *S. abyssinica* are not known, which is detrimental to developing conservation strategies for the species. Therefore, the study was designed to identify the appropriate storage temperature, materials, and period and determine the seed storage behavior of *S. abyssinica*.

MATERIALS AND METHODS

Study site

Seeds were collected from the Munessa forest in Ethiopia, which was recommended as a good provenance for the multiplication of *S. abyssinica* by seedlings (Bareke et al. 2014). The experiment was conducted at Holeta Bee Research Center, Oromia region, Ethiopia.

Collection of fruits/seeds and processing

After 5 to 10 preferred (elite) mother trees were randomly selected, mature fruits (Figure 1) were collected from each tree's crown's top, middle and lower parts (ISTA 2007). Moreover, to ensure maximum genetic variation within the population, the selected trees were at least 100 m apart (FAO 1975). The mixture of fruits was packed in perforated sacks, transported to Holeta Bee Research Center, and placed on the laboratory bench at room temperature for about a week. Seeds from dehiscing fruits were manually extracted and allowed to dry for 1 day on the same bench.

Seed storage behavior testing

For practical seed storage purposes, the difference among the orthodox, intermediate, and recalcitrant categories of seed storage behavior enables one to determine whether the species can be maintained successfully over the long term, the medium term, or only the short term, respectively. The two-stage procedure of Hong and Ellis (1996) was used to classify seed storage behavior: desiccation tolerance and cold tolerance. Furthermore, seeds were dried to about 12-18% moisture content (MC) to determine desiccation tolerance using ambient relative humidity and room temperature (Hong and Ellis 1996). Tolerance of desiccation to these levels of seed MC is usually sufficient to differentiate recalcitrant seed storage behavior from orthodox and intermediate seed storage behavior (Hong and Ellis 1996).

Determination of seed moisture content

Samples of fresh seeds were used to determine the initial MC. Then, for three replicates of 5 g of seeds each, seeds were weighed, dried in an oven for 2 hrs at 120°C, and then placed in a desiccator for cooling before weighing again. Finally, dry weight was measured, and calculation was done for MC determination based on a fresh weight basis (ISTA 2005; Schmidt 2007), as shown in the following equation.

$$MC\ (\%) = \left(\frac{W_2 - W_3}{W_2 - W_1}\right) x100$$

Where MC is moisture content, w_1 is the weight of the container, w_2 is the weight of the container with seed sample before oven drying, and w_3 is the weight of the container with seed sample after oven drying.

Identifying orthodox and intermediate storage behavior

Seeds were stored over a range of temperatures to determine cold tolerance, i.e., the second step distinguishes orthodox and intermediate storage behavior (Hong and Ellis 1996; ISTA 2007). A factorial combination of three temperatures (-10, 0, and 22°C) levels, two types of packing containers (Polythene bag and aluminum bag), and eight periods of storage (0, 30, 60, 90, 120, 150, 180 days, and for a year) was used to determine the germination capacity and storage behavior of the seeds. If all or most seeds die during 12 months of storage, then the seeds have intermediate storage behavior. On the other hand, if no loss in viability is evident during this period, then the species shows orthodox seed storage behavior (Hong and Ellis 1996).

Figure 1. Seeds of *Schefflera abyssinica* during collection

Germination experiment

The germination test was conducted using Whatman filter paper in 12-cm-diameter Petri dishes. Four replicates of 25 seeds each for each storage condition were incubated in a lightroom at room temperature. Distilled water was added to the Petri dishes to moisten the filter paper. The germination percentage was computed using the following equation (ISTA 1999; Davies et al. 2015).

$$G\ (\%) = \frac{n}{N} x100$$

Where G is germination, n is the number of seeds germinated, and N is the sum of the number of germinated seeds, fresh seeds, and those destroyed by fungi.

Data collection methods

For the entire experiment, seed germination counts were made every three days after the commencement of the seed germination. Furthermore, to facilitate future counts, germinated seeds were removed after recording. The experiment was continued until at least 90% of the replication from each treatment showed no new germination for 3 consecutive counts. A seed is considered germinated when the radicle protrusion occurs on the Petri dish's surface (Tigabu et al. 2007; Dayamba et al. 2016).

Data analysis

Data were analyzed using factorial analysis, Oneway ANOVA, and regression analysis to see their effect on the seeds' germination capacity. Therefore, the effect of each factor was analyzed independently using One way ANOVA.

RESULTS AND DISCUSSION

Effect of storage temperature, containers, and storage period on seed germination

Seed storage temperatures and storage period significantly affected the germination capacity of *S. abyssinica*, whereas storage containers and the interaction between temperature and containers did not (Table 1).

The germination percentages of seeds stored at -10°C were significantly higher than those stored at the other two temperature treatments (Table 2). These results indicated that -10°C is an appropriate temperature for storing *S. abyssinica* seeds.

Table 1. The combined effect of storage temperature, storage container, and time of storage on the germination capacity of *S. abyssinica* seeds

Treatment	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Storage temperature	1	849	849	9.406	0.00238 **
Storage container	1	4	4	0.041	0.83999
Storage period	6	12913	3228	35.749	< 2e-16 ***
Storage temperature* storage container	1	43	43	0.477	0.49045
Residuals	276	24924	90		

Note: Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ‘’ 1

Table 2. Effect of storage temperature on mean germination percentage of *S. abyssinica* seeds

Storage temperature	Mean germination %	Minimum germination %	Maximum germination %
-10°C	56 0[a]	41.20	91.00
0°C	46[c]	5.00	88.00
22°C	50[b]	12.50	90.00

Note: Since all seeds were not germinated at a year of storage, the data of this result is only up to six months of storage

Table 3. The mean moisture content (MC) and germination capacity of *S. abyssinica* seeds after different periods of storage

Seed longevity	Mean MC	Mean germination%	Minimum germination%	Maximum germination%
Before storage	9.5[a]	56[bc]	45	65
First month (30 days)	8.58[ab]	56[bc]	45	91
Second month (60 days)	8.37[b]	70[a]	46	91
Fourth month (120 days)	7.65[bc]	48[cd]	43	73
Third month (90 days)	7.58[bc]	65[ab]	44	85
Fifth month (150 days)	6.75[c]	40[d]	20	71
Sixth month (180 days)	6.5[c]	20[e]	5	30
Year	6.00[d]	0[f]	0	0

Note: Treatments with the same letter are not significantly different along a column of mean moisture content and germination percentage.

Seed moisture content and germination

The highest average germination capacity of *S. abyssinica* seeds was seen after 60 days of storage. On the other hand, the lowest germination capacity of *S. abyssinica* was seen at the end of the fifth month (after 150 days) of seed storage (Table 3). After 180 days of storage, germination capacity was less than 20%. The maximum germination capacities of the seeds were seen from the first to third months of storage with 7.58 to 8.58% of average seed MC. After 1 year of storage, no seeds germinated, which indicates that the storage behavior of *S. abyssinica* seeds is intermediate since the seed MC is within the intermediate seed category.

Effect of storage period on germination

The effect of the seed storage period on the germination capacity of *S. abyssinica* was about 47.8% (Figure 2). The peak germination time was in the first and second months of the storage period. Therefore, the seed germination capacity of *S. abyssinica* has an indirect relationship with the seed storage period, and as the storage period increases, the germination capacity of the seeds decreases.

Most of the seeds (50%) stored at -10°C were germinated up to 120 days of storage (Figure 4). This result is statistically similar to that for seeds stored at room temperature. On the other hand, seeds stored at 0°C had the highest germination capacity only after the second and third months of storage. At the end of 6 months of storage, the lowest germination percentage was seen for the seeds stored at 22°C. For all months of storage, the germination capacity of seeds stored at -10°C was the best compared to the other two storage temperatures. After 1 year of storage, the seeds of *S. abyssinica* did not germinate.

Most seeds stored at -10°C germinated 50% after 120 days of storage (Figure 3).

Seed storage behavior

The highest germination capacity of *S. abyssinica* seeds was stored at an MC between 7% and 9% (Figure 5). Thus, most seeds tolerate desiccation to about 7-9% MC. Further, desiccation lowered the MC and reduced the germination capacity of *S. abyssinica* seeds. Therefore, this shows intermediate seed storage behavior.

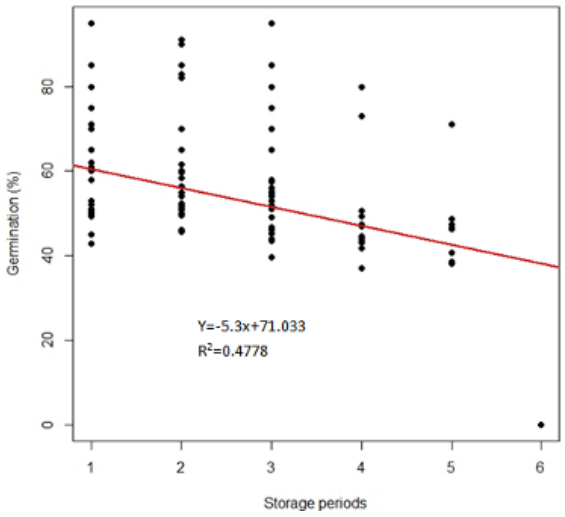

Figure 2. The effect of storage duration on the germination of *S. abyssinica* seeds

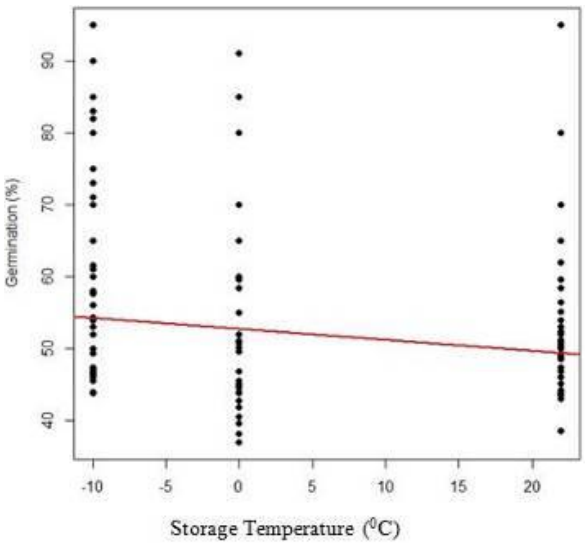

Figure 3. The overall germination percentages of *Schefflera abyssinica* seeds stored at -10°C, 0°C and 22°C for 6 months

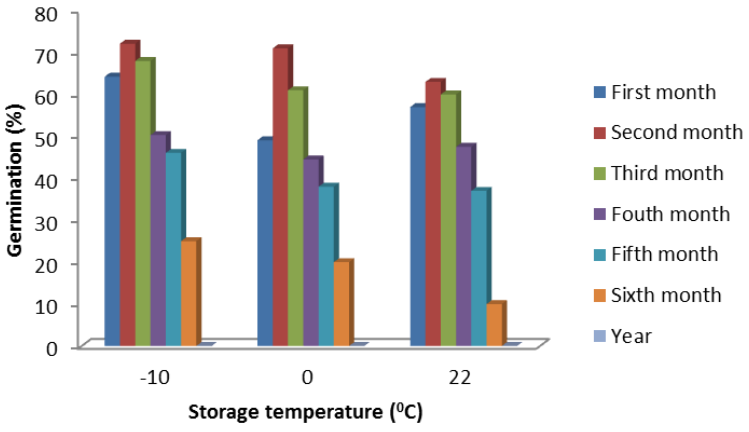

Figure 4. The effect of storage temperature on germination percentages of *S. abyssinica* seeds after different periods of storage

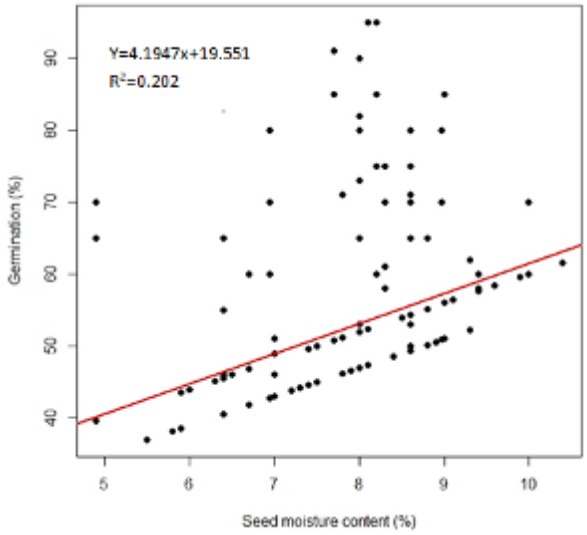

Figure 5. The relationship between seed moisture content and germination capacity of *S. abyssinica*

Discussion

Seed moisture content and germination capacity of S. abyssinica seeds

The growth of a new plant from a seed is called germination, the protrusion of the radicle from its enclosing tissue (Rai and Kim. 2020). The germination process starts with imbibition (initial absorption of water to hydrate seed) and activation of metabolism (Bareke 2018). On the other hand, seed germination is a multifaceted physiological process controlled by genetic and environmental factors (Marcos 2015; Guo et al. 2020). Seed germination is affected by temperature, water potential, oxygen, light, pH, seed storage period, and storage container (Guo et al. 2020; Rai and Kim 2020).

Seed MC had no significant variation between storage containers but varied among storage periods. Parimala et al. (2013) reported that the MC of seeds is the most important factor influencing the germination capacity of seeds during storage.

Seed storage containers

Polythene and aluminum bags were the two packing materials used for this study. Statistically, the two packing materials had no significant variation. Both packing materials are waterproof. For seed preservation, types of containers that can provide suitable conditions to maintain seed quality for a longer period are preferred. Packing material or container normalizes relative humidity, seed moisture content, and temperature (Akter et al. 2014). The appropriate storage container is varied from plant species to species. For example, Akter et al. (2014), tin containers for Soybean (Akter et al. 2014) and mungbean (Mohammad et al. 2017), and sealed containers for *Lens culinaris* Medik. (Kamrul et al. 2017) and polylined bags for *Trifolium alexandrinum* L. (Bahukhandi et al. 2017).

Seed storage behavior

Seed storage behavior is the way to identify whether the seeds of a plant species can be maintained successfully over a long, medium, or short period (Hong and Ellis 1996). This information is essential for developing appropriate ex-situ conservation strategies (Hong et al. 1996). The MC of *S. abyssinica* seeds was an average of 9.5% after they were allowed to air dry at room temperature for 1 week. At the end of the fifth month of storage, seed MC had decreased to an average of 6.75%. A similar study conducted by Zheng et al. (2016) on Kapok (*Ceiba pentandra* (L.) Gaertn.) also indicated that the moisture content was 11.1% at the initial measurement and decreased to 4.98% after 5 days of desiccation. As the storage period increased, the MC of *S. abyssinica* seed decreased, while germination capacity increased somewhat and decreased after reaching a germination peak. After the fifth month of seed storage, the MC and germination capacity of *S. abyssinica* seeds decreased. In a study conducted by Joshi et al. (2019) on seed germination and seed storage behavior of *Pittosporum eriocarpum* Royle, the MC and germination capacity of stored seeds gradually decreased with an increase in storage period. The highest germination capacity of *S. abyssinica* seeds was 7 and 9% MC. That shows the intermediate seed storage behavior. However, the determination of desiccation tolerance does not alone enable the determination of seed storage behavior.

Seed storage period and temperature

The germination capacity of *S. abyssinica* increased from the first month (30 days) of storage to the end of the third month (90 days) of storage and then decreased. After the fifth month of storage, most seeds did not germinate. Mohammad et al. (2017) and Boadu and Siaw (2019) also reported that the duration of seed storage of *Triplochiton scleroxylon* K.Schum. and mungbean, respectively, had a significant influence on their germination capacity. In addition, Olosunde et al. (2017) also mentioned that the seed storage period influences the final germination percentage and the health of the stands of the *Abelmoschus esculentus* (L.) Moench plant.

Duration of seed storage period and temperature are very important in determining seed storage behavior associated with desiccation tolerance. For instance, seeds of *Cattleya aurantiaca* (Bateman ex Lindl.) P.N.Don tolerated desiccation to 3.7 and 2.2% MC with 94% germination; however, only 10% germinated after storage for 90 days at -18°C with 3.7% MC (Pritchard and Seaton 1993).

Many authors have mentioned that seed storage longevity is affected by many factors. Some of them are seed maturation and ways of pre-harvesting handling. Also, harvesting time and weather conditions affect seed storage longevity (Hong and Ellis 1996; Hay and Probert 2011; Hay et al. 2013; Bareke 2018; Ellis et al. 2018). If matured seeds are not collected from mother trees, they have drastic consequences on the quality of seeds. As a result, seeds rapidly deteriorate when exposed to less favorable

environmental conditions (Bareke 2018). Visual identification of the physiological maturity of seeds is used to identify the maturity of seeds.

The germination capacities of *S. abyssinica* seeds have shown significant differences in storage temperatures. Accordingly, seeds stored at -10°C had the highest germination capacity in all storage months compared to the two storage temperatures. Many authors also reported that low temperature and low relative humidity are required to maintain the quality of the seeds (Schwallier et al. 2011; Silva et al. 2019). Hong and Ellis (1996) also reported that the storage environment influences the response of seed storage behavior. According to Harrington's rule, seeds with an MC of 5-14% will have a double germination potential when the MC of the seeds decreases by 1% (Parimala et al. 2013). In addition, moisture content above 14% increases the chance that seeds will be attacked by insects and mold, whereas an MC below 5% causes physiochemical changes in the seeds.

Orthodox seeds can be dried to an MC of about 5% without damage; if most or all seeds tolerate desiccation to about 10-12.5% MC, they are said to have intermediate seed storage behavior. On the other hand, if most or all seeds are killed by desiccation to 15-20% moisture content, they have recalcitrant seed storage behavior (Hong and Ellis 1996).

In conclusion, on average, the MC of S. abyssinica seeds was 9.5% after they were dried at room temperature for 1 week. At the end of the sixth month of storage, seed MC decreased to an average of 6.75%. The germination capacity of *S. abyssinica* seeds indirectly correlates with the seed storage period and MC. The seed storage period influences the germination capacity of *S. abyssinica* by 47.8%. The germination capacities of *S. abyssinica* seeds have shown significant differences in the storage temperature. Seeds stored at -10°C had the highest germination capacity in all storage months compared to the other storage temperatures. Generally, the highest germination capacity of *S. abyssinica* seeds was found to be between 7 and 9% moisture content at the end of the second and third months of storage. All stored seeds were not germinated after 1 year of storage at room temperature, 0°C, and -10°C. Therefore, based on the definition of seed storage behavior given by Hong and Ellis (1996), we conclude that seeds of *S. abyssinica* have intermediate storage behavior.

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The addition of vermicompost and biostarter affects the growth, total phenolic and antioxidant activity of *Echinacea purpurea*

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Abstract. *Choirunnisa LF, Widiayastuti Y, Solichatun, Yunus A. 2022. The addition of vermicompost and biostarter affects the growth, total phenolic and antioxidant activity of* Echinacea purpurea. *Nusantara Bioscience 14: 148-154. Echinacea purpurea* (L.) Moench or purple coneflower is a medical plant that originated in North America and contained various bioactive compounds, one of which is phenolic. Applying organic fertilizer like vermicompost has been reported to increase plants' bioactive compounds' components and antioxidant activity. The purpose of this study was to determine the addition of vermicompost and biostarter on the growth, total phenolic and antioxidant activity of *E. purpurea*. Split-Plot Randomized Complete Block Design was used with dosages of vermicompost 0, 40, 60, and 80 g/plant and different types of biostarter from Banana peel waste and effective microorganisms (EM). The results showed that treatment of 80 g/plant vermicomposts and EM highest resulted in the growth rate parameters (plant high, leaf numbers, leaf area, roots volume, plant fresh and dry weight) and total phenolic content with 1.802%. On the other hand, the herb extracts had the highest result with the treatment of 40 g/plant vermicomposts and EM (4.96%). The antioxidant activity was tested using the DPPH method with TLC and showed that all treatments indicated positive antioxidant activity.

Keywords: Antioxidant, biostarter, *Echinacea purpurea*, phenolic, vermicompost

INTRODUCTION

Echinacea purpurea (L.) Moench or purple coneflower is a medical plant that originated in North America and contained various bioactive compounds, one of which is phenolic. This plant is widely cultivated as medicinal because it increases the human body's immunity. The morphology characteristics of *E. purpurea* turned out to have changed after being developed and cultivated in Indonesia. The clear morphological difference is the flower.

Bioactive compounds were most commonly found in *E. purpurea*, such as alkamides, polysaccharides, lipoproteins, betaine, sesquiterpenes, polyacetylenes, saponins, and phenolic compounds (echinacoside and caffeic acid). Various components of bioactive compounds found in medicinal plants can be obtained with different results due to various internal and external factors. The *E. purpurea* were reported to have the most efficient free radical scavenging activity than another genus *Echinacea*. The antioxidant activity comes from bioactive compounds such as flavonoids, phenolic acids, or phenolic diterpenes (Sharifi-Rad et al. 2018; Oniszczuk et al. 2019; Coelho et al. 2020).

Adding organic fertilizer is very important in supporting growth and development because it provides essential nutrients needed by plants. Humic acid or humic substances contained in vermicompost can increase plants' phenolic compounds and antioxidant activity. In addition, humic acid increases the biosynthesis of phenolics such as flavonoids and anthocyanins. Other than that, humic acid can increase the number of microorganisms in the soil and the availability of plants' nitrogen uptake (Gholami et al. 2018; Hosseinzadeh et al. 2018).

Biostarter can be made by mixing organic waste (fruits or vegetables) with melted sugar and water and fermenting for about 2-3 weeks. Sugar is used to accelerate the growth of microorganisms so that microorganisms are obtained (Wiryanti 2014). The biostarter used in this study is EM or an effective microorganism. According to Mayer et al. (2010), EM is a combination of various types of beneficial microorganisms selected and isolated from various environments. Microorganisms contain in EM are the populations of lactic acid bacteria, yeasts, smaller populations of phototropic bacteria, filamentous fungi, and actinomycetes.

Organic materials such as vermicompost and biostarter can be a way to help increase the growth rate, the accumulation of total phenolic content, and the antioxidant

activity of *E. purpurea*. However, since *E. purpurea* is an introduced species from a subtropical country, a proper method is needed to improve this species to adjust and cultivate in a tropical area like Indonesia. Therefore, the purpose of this study was to determine the addition of vermicompost and biostarter on the growth, total phenolic, and antioxidant activity of *E. purpurea* cultivated in a lowland area (± 300 meters above mean sea level) in Experimental Garden Faculty of Agriculture, Universitas Sebelas Maret, Sukosari, Jumantono, Karanganyar, Central Java, Indonesia.

MATERIALS AND METHODS

Experimental design

The *E. purpurea* is cultivated in lowland areas (± 300 meters above mean sea level), in Experimental Garden Faculty of Agriculture, Universitas Sebelas Maret, Sukosari, Jumantono, Karanganyar, Central Java, Indonesia (7º37'829" S, 110º56'901" W). Split-Plot Randomized Complete Block Design was used with dosages of vermicompost 0, 40, 60, and 80 g/plant and different types of biostarter from Banana peel waste and EM with the trademark "EM4". The treatments were done twice when the plants were 6 and 10 weeks after transplanting in the experimental garden.

Procedures

Collection of seeds

The seeds were from the collection of the Research Center of Medical Plants and Traditional Medicines (B2P2TOOT), Tawangmangu, Karanganyar, Central Java, Indonesia. We used and cultivated accession 4 in the field.

Planting and harvesting

There were 12 combinations of treatments with a total of 270 plants of *E. purpurea*. The plants were transplanted into beds consisting of 10 per bed, with a total of 27 beds. The temperature was captured between 27°C to 33°C, and the humidity was around 50-60%. The plants were cultivated during the 2021 rainy season. The plant's watering was done every day (morning or afternoon). Harvesting was done after the plants were 80% flowering or about 12 weeks after transplanting. The qualitative observation was the antioxidant activity using the DPPH method with TLC. In contrast, quantitative observations measured growth rate parameters (plant high, leaf numbers, leaf area, roots volume, fresh and dry weight), total phenolic content, and herb extract. The leaf area measurement was done with non-destructive models by combining width length and maximum width. Based on Aminifard et al. (2016), leaf area estimation on *E. purpurea* can be done by measuring leaf dimension and resulting in the most accurate formulation [LA = 0,575 (Length x width) – 0,934]. The length and width of leaves were measured manually by a ruler.

Analysis of herb extract

The plant parts that are extracted are the aerial parts (stems, leaves, and flowers). The drying method was directly exposed to the sun and carried out for about 14 days. After completely drying, it was ground into a powder and weighed 5 grams for herb extract residue. Next, the powder was put into a bottle and macerated with 50 mL of 70% ethanol for three days. Then, it was filtered, and the filtrate was put into a cup that had been weighed previously as the empty weight of the cup. After that, it dried in the oven at 50ºC. The herb extract calculation was done by subtracting the weight of the herb extract and the initial sample weight (Choirunnisa et al. 2021; Ferdyana et al. 2022). Then, the percentage of the herb extract was done using the formula:

$$r\,(\%) = \frac{x}{y} \times 100\%$$

Where:
r: Herb extract content (%)
x: Weight of herb extract (g)
y: Initial sample weight (g)

Analysis of total phenolic content

The accumulation of phenolic content was using the Folin-Ciocalteau (FC) method with modifications from the Research Center of Medical Plants and Traditional Medicines (B2P2TOOT) based on the research of Sidhiq et al. (2020). The analysis of phenolic content using this method is based on the reaction of phenol with the Folin-Ciocalteau reagent. The indication of this reaction was a changed color turned into complex blue.

For the preparations, the sample extract of *E. purpurea* weighed 15 mg in 10 mL of 50% ethanol solution per treatment, then sonicated at 40 Hz for 15 minutes at 60ºC and left for 1 night. After that, prepare the gallic acid stock solution and Na_2CO_3 solution. First, the stock solution was prepared by dissolving 1 g of gallic acid with 1 L of aquadest in a liter measuring cup. Next, the solution was shaken and poured into a bottle covered with aluminum foil to prepare the Na_2CO_3 solution by dissolving 10.5 g of Na_2CO_3 in 100 mL of aquadest in a measuring cup.

Various concentrations of 40, 60, 80, 100, and 120 ppm were made from gallic acid solutions. In the sample solution, 500 mL of each concentration of the gallic acid solution was taken, 500 mL of FC reagent and 8 mL of aquadest were added, then waited for 5 minutes. After that, 1 mL of 20% Na_2CO_3 solution was added. The solution was transferred into a test tube and incubated for 30 minutes. Each solution concentration was read at a wavelength of 760 nm with a UV-Vis Spectrophotometer, and the results were calculated by the equation $y = bx – a$.

The total phenol content contained in the extract of *E. purpurea* was calculated based on the absorbance value using the standard curve obtained previously, $y = 0.0041x + 0.0296$ and $R^2 = 0.9971$. Then, the percentage of the total phenolic was calculated with the following formulation:

$$Total\ phenolic\ content\ (\%) = c\ \frac{V}{m} \times 100\%$$

Where:
c: concentration of gallic acid (mg/mL)
V: volume of extract (mL)
m: mass of extract (mg)

Analysis of antioxidant activity

The antioxidant activity was tested using DPPH (*1,1-diphenyl-2-picrylhydrazyl*) with TLC or thin-layer chromatography modifications from B2P2TOOT. The spot that turned yellow with a purple background indicates the extract positively has antioxidant activity.

Extract powder weighed 1 g and dissolved with 10 mL of methanol in a bottle. After that, it was sonicated for 30 minutes, filtered, and evaporated until a thick extract was obtained. As the stationary phase, G plate F254 nm was used, which had previously been activated on the oven at 100ºC for 1 hour. Then, mark the G plate with a distance of 1 cm from the bottom and top sides as the elution limit. Afterward, prepare a solution used in the mobile phase by mixing 25 mL of ethyl acetate; 15 mL of n-butanol; 5 mL of formic acid; 5 mL of aquadest and pouring it into a bottle.

Took 5 µL of the prepared extract for each treatment using a micropipette and dropped it slowly on the plate. Each treatment was spaced 1.5 cm on the plate. Next, prepare a TLC vessel filled with a mixed solution that has been made to saturate the plate. The TLC vessel was then tightly closed using silicon grease and waited until the elution limit reached 1 cm from the top side of the plate. After the mobile phase was complete, the plate dried and was observed on a TLC visualizer with UV light at 245 nm and 366 nm. Then, the dried plate was sprayed with DPPH solution using a sprayer in LAF.

Data analysis

The quantitative data from growth rate parameters were analyzed using ANOVA (Analysis of Variance) and continued with the Duncan Multiple Range Test (DMRT) at the 5% level to know the significant effect of the treatments. In addition, the herbs extract and total phenolic content were analyzed from the average of each observation based on the available graphs. Meanwhile, the qualitative data as antioxidant activity were analyzed descriptively.

RESULTS AND DISCUSSION

Growth rate

The growth rate is important to observe and indicates the plant's physiological function. This measurement is frequently used to know the effect of each treatment. In this study, we measured the growth rate parameters such as plant high, leaf numbers, leaf area, root volume, and fresh and dry weight. The quantitative analysis of growth rate parameters using ANOVA and continued with the DMRT at the 5% level among all treatments significantly showed different results (Table 1). The whole plant of *E. purpurea* used in this study is shown in Figure 1.

The results showed that the combination treatment of 80 g/plant vermicomposts and EM highest resulted in the growth rate parameters such as plant high, leaf numbers, leaf area, roots volume, and fresh and dry weight compared to the other treatments. Using organic fertilizer like vermicompost can increase plant growth properties. Based on Choirunnisa et al. (2022), plant regulators such as auxin can also increase cell walls and longitudinal growth in the plant. On the other hand, the macro and microelements on vermicompost increase the plant's nutrition status and nourish the leaf and leaf morphology. Amiri et al. (2017) stated that plant growth regulators such as auxin and cytokinin play an important role in increasing the microorganism activity in the soil. The humic acid found in vermicompost has many elements for improving its growth development properties.

On the other hand, effective microorganisms result in many beneficial microorganisms introduced, which enhance the decomposition of organic materials, release nutrients for plant uptake, and improve soil's physical and chemical characteristics. Therefore, EM was applied in combination with organic fertilizer, which can be attributed largely to the activity of the introduced beneficial microorganisms, which enhanced the decomposition of organic materials and the release of nutrients for plant uptake (Joshi et al. 2019; El-Mageed et al. 2020).

Figure 1. The whole plant of *E. purpurea was* used in this study (A-L). A: T_{11} (vermicompost 80 g/plant with EM). B: T_8 (vermicompost 60 g/plant with EM). C: T_5 (vermicompost 40 g/plant with EM). D: T_2 (vermicompost 0 g/plant with EM). E: T_{10} (vermicompost 80 g/plant with banana peel waste). F: T_7 (vermicompost 0 g/plant with banana peel waste). G: T_4 (vermicompost 40 g/plant with banana peel waste). H: T_1 (vermicompost 0 g/plant with banana peel waste. I: T_9 (vermicompost 80 g/plant without biostarter). J: T_6 (vermicompost 60 g/plant without biostarter). K: T_3 (vermicompost 40 g/plant without biostarter). L: T_0 (vermicompost 0 g/plant without biostarter or control)

Table 1. The addition of vermicompost and biostarter on the growth rate of *E. purpurea*

Characters	Treatments											
	T_0	T_1	T_2	T_3	T_4	T_5	T_6	T_7	T_8	T_9	T_{10}	T_{11}
Plant high (cm)	72.60c	73.60b	77.00a	73.20c	77.20b	82.60a	76.60c	80.40b	87.60a	78.60c	82.40b	88.80a
Leaf numbers	45.40b	55.20a	63.20a	46.33b	70.80a	72.60ab	47.75b	74.00a	75.20a	54.50b	68.40a	82.60a
Leaf area (cm^2)	59.34a	65.21a	67.02a	69.80ab	64.42ab	69.84ab	70.39b	72.82b	78.93b	79.67c	87.21c	83.83c
Roots volume (ml)	18.80c	20.20b	22.10d	21.20c	22.20b	24.40a	25.60c	28.40b	29.80b	27.80c	31.20a	34.50a
Plant fresh weight (g)	201.48c	243.49b	280.89a	215.49c	247.38c	303.13a	262.73b	288.43b	340.27b	319.98a	340.27a	392.21a
Plant dry weight (g)	53.35c	70.20b	73.66a	63.73b	75.85b	85.20b	68.67a	86.33a	88.20a	71.20c	89.43a	91.40a

Note: Numbers followed by different letters in the same row show a significant difference in DMRT at the 5% level. T_0 (vermicompost 0 g/plant without biostarter or control). T_1 (vermicompost 0 g/plant with banana peel waste). T_2 (vermicompost 0 g/plant with EM). T_3 (vermicompost 40 g/plant without biostarter). T_4 (vermicompost 40 g/plant with banana peel waste). T_5 (vermicompost 40 g/plant with EM). T_6 (vermicompost 60 g/plant without biostarter). T_7 (vermicompost 0 g/plant with banana peel waste). T_8 (vermicompost 60 g/plant with EM). T_9 (vermicompost 80 g/plant without biostarter). T_{10} (vermicompost 80 g/plant with banana peel waste). T_{11} (vermicompost 80 g/plant with EM)

Herb extract

The maceration method carried out the herb extraction process of E. purpurea. Before the extraction, this plant needed to be dried and powdered because the texture of the aerial parts (stems, leaves, and flowers) that we used was quite hard, so it must be grounded until the texture became smooth. This method prevented the growth of microbial activity and fungi so that they could be stored for quite longer. The maceration process is a simple extraction method that uses more solvents and is easy to obtain.

Figure 2 presents the graph of the percentage of herb extract among all treatments. The results showed the best result on the combination treatment of 40 g/plant vermicomposts and EM with 4.96%. The treatment of vermicompost 80 g/plant without biostarter showed the lowest result of herb extract percentages with 4.16%. The higher value of herb extract is correlated to the more active compounds attached to the solvent or indicates many bioactive compounds in the plant's extract. But according to Choirunnisa et al. (2021), the high value of herb extract does not correlate to the specific amount of bioactive compounds found in the plant because this is an accumulation of all the bioactive compounds contained in plants.

Total phenolic content

The accumulation of phenolic content was done using the Folin-Ciocalteau (FC) method. The analysis of phenolic content using this method is based on the reaction of phenol with FC reagent using gallic acid as the standard. The indication of this reaction was changed color and turned into complex blue. The absorbance values obtained at different concentrations (40, 60, 80, 100, and 120 ppm) were used to construct the calibration curve. The data were analyzed from the average of each observation based on the available graphs.

Figure 3 presents the percentage of total phenolic content among all treatments. The results showed the best result on the combination treatment of 80 g/plant vermicomposts and EM with 1.802%. The lowest result of total phenolic content was on the combination treatment of vermicompost 0 g/plant and EM with 0.808%. Extracts of *E. purpurea* may contribute significantly to the antioxidant properties. Because of these properties, this plant has been used in several herbal medications and is known for increasing the human body's immunity. In addition, some interference may arise from other chemical components in the extract.

The accumulation of total phenolic content in the plant is related to antioxidant activity. Phenolic compounds act as reducing agents and hydrogen donors and can scavenge free radicals. Phenolic compounds are important plant constituents because of their scavenging ability on free radicals due to their hydroxyl groups. The antioxidant response of phenolic compounds varies remarkably, depending on their chemical structure. Several investigations of the antioxidant activity of plant extracts have confirmed a correlation between total phenolic content and antioxidant activity (Ahmed et al. 2019; Phuyal et al. 2020).

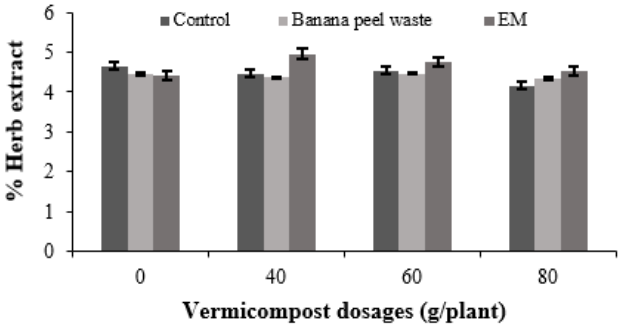

Figure 2. Percentage of herb extract *E. purpurea* on each treatment

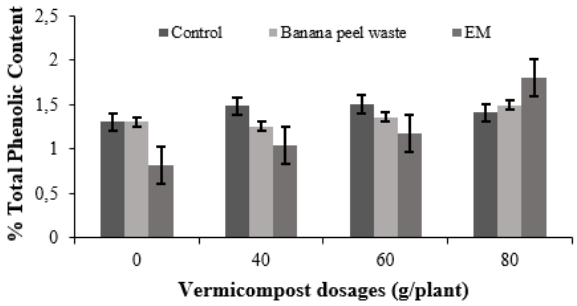

Figure 3. Percentage of the total phenolic content of *E. purpurea* in each treatment

Antioxidant activity

The DPPH method is based on the presence of an electron donor or hydrogen radical (H), which produces antioxidant compounds. This method is selected because it is simple, easy, fast, and sensitive and requires only a small number of samples. Qualitative antioxidant activity analysis is done using TLC (thin-layer chromatography).

The antioxidant activity test of *E. purpurea* showed that all treatments indicated positive antioxidant activity (Figures 4, 5, and 6). After the plate was sprayed with DPPH solution, the spot turned yellow with a purple background, as shown on the picture's left side. TLC visualizer detects UV light at 245 nm and 366 nm. The detection with a UV light at 245 nm showed on the middle side of the picture, while UV light at 366 nm showed on the right side.

Based on Pratiwi et al. (2013), the detection using a UV light was to find the spot that can fluoresce so that it can be seen visually due to the interaction between UV rays with a chromophore group attached to an auxochrome at that spot. Visible light fluorescence results from the emission of light emitted by the component when electrons are transferred to a higher energy level and then come back again while releasing energy.

The TLC plate was eluted using eluents in the form of n-butanol, ethyl acetate, and formic acid. The eluents were used, so the color's appearance and the distance of the stain were quite clear on the sample spotted on the TLC plate. According to Handayani et al. (2014), based on its polarity, the eluent is more nonpolar, so separated compounds are also nonpolar, like on the principle of TLC, "like dissolves like." After the TLC plate was eluted and then sprayed with DPPH, spots that changed color to yellow indicated the presence of antioxidant activity. Antioxidant compounds will react with DPPH radicals through the mechanism of hydrogen atom donation and cause color decay from purple to yellow.

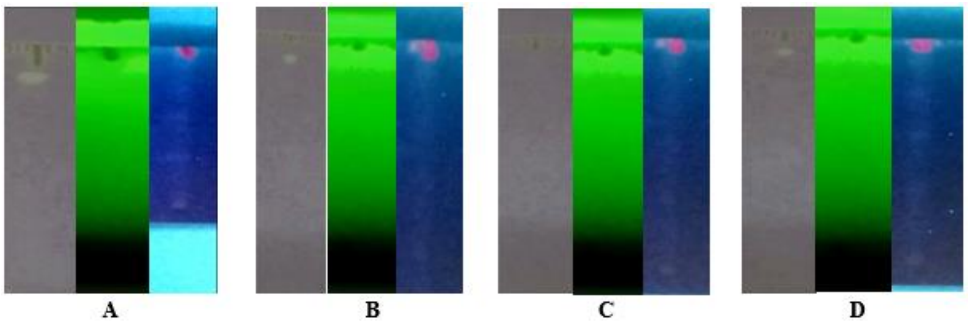

Figure 4. Antioxidant activity using the DPPH method with TLC visualizer at 245 and 265 nm. A: T_0 (vermicompost 0 g/plant without biostarter or control). B: T_1 (vermicompost 0 g/plant with banana peel waste). C: T_2 (vermicompost 0 g/plant with EM). D: T_3 (vermicompost 40 g/plant without biostarter)

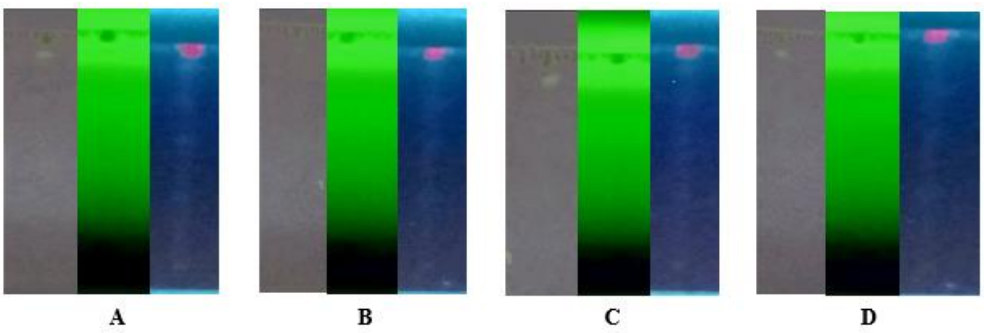

Figure 5. Antioxidant activity using the DPPH method with TLC visualizer at 245 and 265 nm. A: T_4 (vermicompost 40 g/plant with banana peel waste). B: T_5 (vermicompost 40 g/plant with EM). C: T_6 (vermicompost 60 g/plant without biostarter). D: T_7 (vermicompost 0 g/plant with banana peel waste)

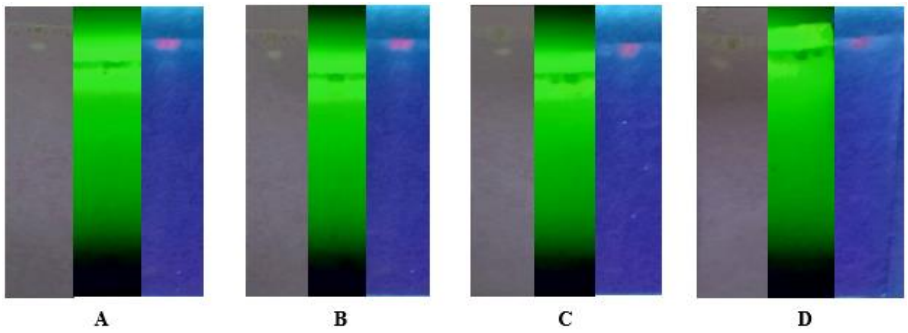

Figure 6. Antioxidant activity using the DPPH method with TLC visualizer at 245 and 265 nm. A: T_8 (vermicompost 60 g/plant with EM), B: T_9 (vermicompost 80 g/plant without biostarter). C: T_{10} (vermicompost 80 g/plant with banana peel waste). D: T_{11} (vermicompost 80 g/plant with EM)

The antioxidant activity of *E. purpurea* extract has the highest antioxidant capacity (relative to ascorbic acid and gallic acid). The antioxidant activity of *E. purpurea* extract was measured using the DPPH method test. The high ability to scavenge free radicals from the extract was found to be related to the increase in antioxidant concentration. The *E. purpurea* were reported to have the most efficient free radical scavenging activity compared to the other genus of *Echinacea* (Oniszczuk et al. 2019; Banica et al. 2020).

Using fertilizer added to *E. purpurea* increases the antioxidant activity in counteracting hydroxyl radicals. This antioxidant mechanism is described as eliminating free radicals and metal ions. The antioxidant activity is considered from bioactive compounds such as polyphenol components (flavonoids, phenolic acids, or phenolic diterpenes). Applying organic fertilizer is crucial in supporting growth and development because it provides essential nutrients plants need (Ahmadi et al. 2020).

This study concluded that there is an effect on the growth, total phenolic, and antioxidant activity of *E. purpurea* by adding vermicompost and biostarter. The combination treatment of 80 g/plant vermicomposts and EM highest resulted in the growth rate parameters (plant high, leaf numbers, leaf area, roots volume, plant fresh and dry weight) and total phenolic content of 1.802%. The herb extracts highest resulted in the combination treatment of 40 g/plant vermicomposts and EM (4.96%). The antioxidant activity was tested using the DPPH method with TLC and showed that all treatments indicated positive antioxidant activity. Further studies are needed to promote the integration between vermicompost and biostarter, especially given *E. purpurea* in Indonesia. A good adaptation and better improvement to increase the bioactive compounds are important because this plant is promising for medicinal uses and products.

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Short Communication: Antioxidant activity of ethanol extract of *Chlorella sorokiniana* cultured in tofu wastewater

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Abstract. *Mursandi H, Susanty D, Nurhayati L, Okasari AA. 2022. Short Communication: Antioxidant activity of ethanol extract of* Chlorella sorokiniana *cultured in tofu wastewater. Nusantara Bioscience 14: 155-159.* Microalgae are microorganisms that grow quickly and produce secondary metabolites with antioxidant activity. Antioxidants of microalgae can be utilized in various aspects such as cosmetics, pharmaceuticals, supplements, and feed. Microalgae utilization will be more profitable if the microalgae can be cultured on waste media. This study aims to determine the concentration of a suitable medium for the growth of *Chlorella sorokiniana* Shihira & R.W.Krauss, total flavonoids, total phenolics, and the potential of ethanolic extract of *C. sorokiniana* as an antioxidant. This study cultured the microalgae *C. sorokiniana* on tofu liquid waste media at various concentrations (15, 20, 25, and 30%). The growth of *C. sorokiniana* on the media was observed using a spectrophotometer at 680 nm wavelength. *C. sorokiniana* biomass was collected on the 7th day. The biomass was extracted using ethanol as a solvent. Phytochemical analysis was performed using the standard method, Total phenolic content, total flavonoid content, and antioxidant activity using 2,2-diphenyl-1-picrylhydrazyl (DPPH) were conducted to determine the IC_{50} value. The results showed that the best growth of *C. sorokiniana* was on TLW media at a concentration of 30%. The ethanolic extract of *C. sorokiniana* showed the presence of alkaloids, flavonoids, steroids, tannins, and saponins. The total phenolic content in the ethanolic extract of *C. sorokiniana* was 18.39 ± 0.29 mgGAE/g, and the total flavonoid content was 31.93 ± 5,60 mgQE/g. The IC_{50} of the ethanolic extract of *C. sorokiniana* was 288.95 mg/L, which shows this extract has a potent antioxidant.

Keywords: Antioxidant, *C. sorokiniana,* flavonoid, phenolic

INTRODUCTION

Microalgae are photosynthetic microorganisms with varied cell morphology, i.e., unicellular and multicellular. Microalgae have advantages, namely high photosynthetic efficiency, high mass production, fast-growing, and can use certain wastes as a source of nutrients. The diversity of microalgae globally is estimated to reach millions of species, most of which are not yet identified and cannot be cultivated. As many as 200,000-800,000 microalgae live in nature, 35,000 species have been identified, and 15,000 chemical components from microalgae have been identified (Hadiyanto and Azim 2012). Microalgae have been applied in various fields, including energy (Li et al. 2011; Makareviciene et al. 2011) and health as antioxidants (Li et al. 2007). Microalgae that have been known to have antioxidant activity, including *Botryococcus* (Rao et al. 2006), *Dunaliella* (Herrero et al. 2006), and *Haematococcus* (Cerón et al. 2007), *Chlorella* (Lai, 2017; Napitupulu 2019), and 32 selected microalgae (Goiris et al. 2012). The antioxidant activity of these microalgae is related to the produced metabolites. One of the microalgae capable of antioxidants is *Chlorella sorokiniana* Shihira & R.W.Krauss.

The *C. sorokiniana* can grow in mixotrophic conditions with various carbon and nitrogen sources, making it ideal for the cultivation of raw waste materials (Ramanna et al. 2014). The *C. sorokiniana* has a high growth rate and can absorb the nutrients contained in the media (Lizzul et al. 2018). The optimum growth temperature of *C. sorokiniana* is 35°C-45°C (de-Bashan et al. 2008) with less than 4-6 hours of light (Janssen et al. 1999). The *C. sorokiniana* produced carotenoids at 0.69% dry weight under extremophilic conditions (Matsukawa et al. 2000). A previous study by Lai (2017) showed that the ethanolic extract of *C. sorokiniana* had an IC_{50} of 7.36 mg/mL.

The *C. sorokiniana* can utilize waste for culture medium. Waste as microalgae culture reduces production costs and the solutions for handling waste problems. Previous studies showed that *C. sorokiniana* grew well in wastewater, such as domestic waste (Ramanna et al. 2014) and livestock waste (Chen et al. 2020; Susanty and Oksari 2020). Tofu wastewater can also be used as a medium for cultivating *Chlorella*. In Indonesia, tofu industries contribute about 20 million cubic meters of liquid waste annually (Widayat and Hadiyanto 2016). Utilization of tofu wastewater for microalgae culture media can be an alternative to waste treatment. Tofu wastewater contains large amounts of carbon, nitrogen, and phosphorus that can be used as a nutrient source for microalgae (Syaichurrozi and Jayanudin 2016). In this study, *C. sorokiniana* was cultured on tofu wastewater media at various concentrations (15, 20, 25, and 30%), and the antioxidant

activity was conducted on the ethanolic extract of *C. sorokiniana*.

MATERIALS AND METHODS

Materials

The microalgae used in this study was *C. sorokiniana* (InaCCM 38). Tofu wastewater was obtained from the Ciriung area, Bogor, West Java, Indonesia. Chemicals and equipment: universal pH indicator, distilled water, filter paper, 70% alcohol, NaOH 2N, gallic acid, hydrochloric acid, sulfuric acid, acetic anhydride, 2,2-diphenyl-1-picrylhydrazyl (DPPH) (SIGMA-ALDRICH), 96% ethanol (SMART LAB), $FeCl_3$, Folin Ciocalteu (MERCK), chloroform, quercetin (HIMEDIA), Na_2CO_3, Dragendorff's reagent, Mayer's reagent, Wagner's reagent, Mg band, $AlCl_3$ 10%, potassium acetate 1 M. Equipment: glass jars, analytical balance, TL lamp, hemocytometer, laminar airflow, autoclave, oven, centrifuge (Hettich Zentifugen EBA 20), water bath, rotary evaporator, Ultra Violet spectrophotometer. -Visible (UV-Vis) (OPTIZEN™ POP-SPECTROPHOTOMETER UV-VIS SMART) and laboratory glassware.

Preparation of tofu wastewater media

The tofu wastewater was taken from the tofu pressing process unit. The N, P, and K concentration in tofu wastewater was then measured. The nitrogen content was determined using the Kjeldahl method (IKLab-102-188), while phosphorus and potassium levels were determined using the spectrophotometric method (IKLab-105-191). The microalgae culture media used four concentrations of tofu wastewater (15, 20, 25, and 30%). Culture media were sterilized before being used as growth media of *C. sorokiniana*.

The measurement of *Chlorella sorokiniana* growth by optical density (Haneda 2015)

The growth of *C. sorokiniana* in various concentrations of tofu wastewater was observed using a UV-VIS spectrophotometer at 684 nm wavelength at 10 days of cultivation.

Biomass production of *Chlorella sorokiniana*

The biomass of *C. sorokiniana* was collected at optimum growth by separating the media with microalgae using a centrifuge, then air-dried at room temperature to obtain dry biomass.

Extraction of secondary metabolic compounds of *Chlorella sorokiniana*

The *C. sorokiniana* biomass with the best growth was extracted with ethanol 96% (1:10 (w/v)), using a shaker for 3 days. The ethanol extract was evaporated using a rotary evaporator at a temperature of 60°C to obtain a concentrated extract.

Phytochemical testing of *Chlorella sorokiniana* extract

Alkaloids, flavonoids, terpenoids/steroids, tannins, and saponins content were analyzed based on Harbone's (1996) methods.

Determination of total phenolic content of *Chlorella sorokiniana* extract

Total phenolic content was analyzed using the Folin Ciocalteu reagent, referring to the method of Azaman et al. (2017). Fifty (50) mg of microalgae ethanolic extract was dissolved into 50 mL of distilled water. One ml of the solution was taken and added with 2.5 mL of Folin Ciocalteu reagent, allowed to stand for 5 minutes at room temperature. After that, 2.5 mL of 7.5% Na_2CO_3 was added and allowed to stand again for 45 minutes. The absorbance of the solution was measured using a spectrophotometer at a wavelength of 760 nm. Standard gallic acid was used at 5, 10, 15, 20, and 25 mg/L concentrations. Each concentration of standard gallic acid has the same treatment as the extract solution. Therefore, the total phenolic extract of the sample is expressed in mg gallic acid equivalent /g sample (mg GAE/g sample).

Determination of total flavonoid content of *Chlorella sorokiniana* extract (Ukieyanna 2012)

Fifty mg of extract of *C. sorokiniana* was dissolved into 50 mL of solution with ethanol. One (1) mL of extract solution was added with 0.2 mL of $AlCl_3$ 10%, 0.2 mL of sodium acetate, and 3 mL of ethanol and calibrated with distilled water. The absorption was measured at the maximum wavelength. The resulting absorbance was entered into the regression equation of the quercetin standard curve.

Analysis of antioxidant activity using 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Jerez-Martel et al. 2017; Kusumah 2019)

The *C. sorokiniana* extract and quercetin (as positive control) were put into a test tube with 2 mL of 0.1 mg/L DPPH solution, shaken with a vortex until homogeneous, and incubated in a dark room for 30 minutes. The absorption was measured at a wavelength of 515 nm. The sample concentration and the percent inhibition are plotted on the x and y axes of the linear regression equation, respectively, and that equation determines the IC_{50} (Nurjana et al. 2011).

RESULTS AND DISCUSSIONS

The cell density of *Chlorella sorokiniana*

The density of microalgae cells was determined by the optical density (OD) method, which describes the growth of microalgae cells per unit of time and can be used as a benchmark to determine the carrying capacity of media or nutrients for cell growth and division (Istirokhatun et al. 2017). The best growth of *C. sorokiniana* was obtained at the treatment of 30% tofu wastewater (Figure 1).

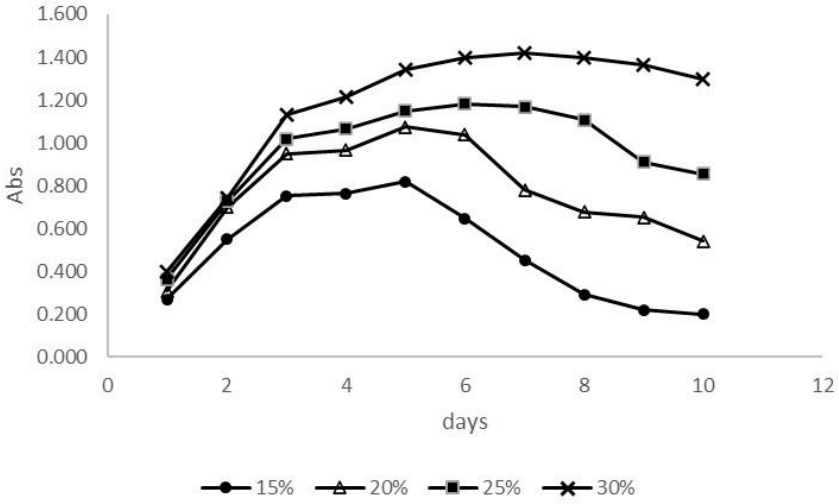

Figure 1. Optical density (OD) of *C. sorokiniana* in various concentrations of tofu wastewater media

The *C. sorokiniana* quickly adapted to tofu waste media and utilized the nutrients in the media. It was shown by the rapid adaptation/lag phase. The length of the lag phase depends on the amount and age of the inoculum and the substrate used as the medium. The availability of nutrients is the limiting growth factor and affects the adaptability of microalgae. Nutrient imbalance causes inhibition of the reduction process of organic compounds and the growth of microalgae (Rini 2012).

The results showed that *C. sorokiniana* could be cultivated in tofu liquid waste media because it contains many organic substances, such as proteins, carbohydrates, and fats. The growth of microalgae *C. sorokiniana* is influenced by various factors, i.e., the availability of macro and microelements in the medium. The limited amount of nutrients can eliminate the ability of cells to build functional structures (Chrismadha et al. 2006). Nitrogen, phosphorus, and potassium are the macro elements that play a role in C. sorokiniana growth. The tofu wastewater used in this study contains 0.02% nitrogen, 0.01% phosphor, and 0.07% potassium.

Nitrogen is essential in the growth and formation of essential compounds such as proteins, chlorophyll, lipids (Yusandi 2010), amino acids, nucleic acid amides, coenzymes, and many essential compounds. Phosphorus in the growth media could be used as an energy reserve and a constituent of the energy-rich compound; it is also used for genetic information systems, cell membranes, and phosphoproteins (Intaglietta 1977). The limited availability of phosphorus influences the production of nucleic acids. It can reduce protein formation, cell formation, or cell division (Komarawidjaja 2011) and microalgae growth rate (Ji and Sherrell 2008). Meanwhile, potassium plays a role in carbohydrate metabolism and is a cofactor for several coenzymes (Munir et al. 2017).

Secondary metabolites in ethanol extract of *C. sorokoniana*

The results of phytochemical screening showed that the ethanolic extract of *C. sorokiniana* contained alkaloids, flavonoids, steroids, saponins, and phenolics (Table 1).

The alkaloid test of the ethanolic extract of microalgae *C. sorokiniana* showed positive results in all three reagents. There are three alkaloid biosynthesis, i.e., the mevalonic acid, phenylpropanoid, and poly acetic acid pathways. Furthermore, based on GC-MS analysis, chlorella extract contained several secondary metabolites, especially alkaloid compounds (Olasehinde et al. 2019) with antioxidant activity (Bariyyah et al. 2013).

The total phenolic content of the ethanolic extract of *C. sorokiniana* was 18.39 ± 0.29 mgGAE/g. Phenolic compounds can scavenge DPPH free radicals, and the OH group in phenolic compounds strongly influenced the ability to scavenge DPPH (Nakiboglu et al. 2007). The chemical structure, number, and position of hydroxy and methyl groups on the ring determine the difference in the antioxidant activity of phenolic. The more substituted hydroxyl groups in the molecule, the stronger the free radical scavenging ability because more hydrogen atoms can be donated (Lin et al. 2009). Phenolic compounds have several biological activities, including antioxidant, anti-inflammatory, and antimicrobial effects (Cotta et al. 2012).

Table 1. Phytochemical test results of ethanol extract of *Chlorella sorokiniana*

Test	Results
Alkaloid	+++
Flavonoids	+++
Steroids	+++
Saponins	+
Tannins	+++

Note: (-) Negative; (+) Not Concentrated; (++) Concentrated; (+++) Very Concentrated

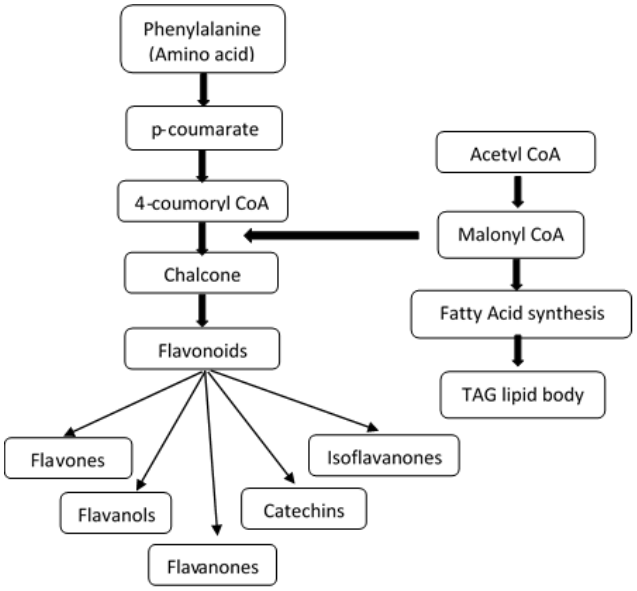

Figure 2. Flavonoid biosynthetic pathway (Yadavalli et al. 2020)

The total flavonoid content of the ethanol extract of *C. sorokiniana* was 31.93 ± 5.60 mgQE/g. Flavonoids produced by the phenylpropanoid metabolism pathway lead to nine main sub-groups: colorless chalcone, aurone, isoflavonoids, flavones, flavonols, flavandiols, anthocyanins, condensed tannins and phlobaphene pigments (Winkel-Shirley 2001; Yadavalli et al. 2020). Algae produce polyketides by condensing acetyl-CoA, leading to flavones and flavonols catalyzed by chalcone synthase (CHS). Phenylalanine produced 4-coumaroyl CoA (Figure 2) (Yadavalli et al. 2020).

Antioxidant activity of ethanol extract of *C. sorokoniana*

Based on the results, the ethanolic extract of *C. sorokiniana* had an IC_{50} value of 288.95 mg/L, which was classified as weak antioxidant activity (Jun et al. 2003), while quercetin, as a positive control, had an IC_{50} value of 7.74 mg/L which was classified as very strong antioxidant activity. The antioxidant activity of *C. sorokiniana* ethanol extract cultured in tofu waste (7,360 µg/mL) was higher than that of *C. sorokiniana* cultured in Seuoka Culture Medium, which was 2,062 µg/mL (de Carvalho et al. 2020). Therefore, tofu wastewater could be used as a culture medium for *C. sorokiniana*, and further research should be done to isolate bioactive compounds to get strong antioxidant activity.

Secondary metabolites in the ethanolic extract of *C. sorokiniana* contributed to the antioxidant activity. Flavonoids are compounds that act as antioxidants. The antioxidant mechanism of flavonoids is to scavenge reactive oxygen species (ROS) directly, prevent regeneration and indirectly increase the antioxidant activity of cellular antioxidant enzymes (Akhlaghi and Bandy 2009). There are several ways to prevent ROS formation by flavonoids, namely by inhibiting xanthine oxidase and Nicotinamide Adenine Dinucleotide Phosphate (NADPH) oxidase enzymes and chelating metals (Fe^{2+} and Cu^{2+}) to inhibit redox reactions that produce free radicals (Atmani et al. 2009).

In conclusion, microalgae *C. sorokiniana* cultured in tofu wastewater had the best growth at a concentration of 30% at 7 days of cultivation. The ethanol extract of *C. sorokiniana* contained phenolic compounds (18.39 ± 0.29 mgGAE/g) and flavonoids (31.93 ± 5.60 mgQE/g). The IC_{50} of the ethanolic extract of *C. sorokiniana* was 288.95 mg/L, categorized as a weak antioxidant.

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Somatic embryogenesis of the selected intergeneric hybrid between *Phalaenopsis* 2166 and *Vanda'* Saint Valentine': Application of NAA and TDZ

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Abstract. *Dwiati M, Susanto AH, Budisantoso I. 2022. Somatic embryogenesis of the selected intergeneric hybrid between* Phalaenopsis *2166 and* Vanda*' Saint Valentine': Application of NAA and TDZ. Nusantara Bioscience 14: 160-165.* Intergeneric hybridization between *Phalaenopsis* 2166 and *Vanda*' Saint Valentine' has produced a hybrid seedling with several characters potentially developing into plant individuals with flowers of better performance. Therefore, identical clones of the selected hybrid should be developed into PLBs using an *in-vitro* culture technique employing somatic embryogenesis supported by the application of plant growth regulators. This study aims to unveil the effect of NAA and TDZ in stimulating the formation of identical clones of the selected intergeneric hybrid between *Phalaenopsis* 2166 and *Vanda*' Saint Valentine'. The experiment was arranged in a factorial Randomized Complete Block Design (RCBD) involving two factors, i.e., types of plant growth regulators and the levels of concentrations of each substance. It was found that the combination of NAA and TDZ significantly affected the growth of the identical clones. Furthermore, the combination of NAA 0.5 mgL^{-1} and TDZ 1.5 mgL^{-1} resulted in clones that potentially differentiate into PLBs. This finding indicates that NAA and TDZ should be applied appropriately to stimulate somatic embryogenesis in the intergeneric hybrid.

Keywords: Intergeneric hybrid, NAA, *Phalaenopsis* 2166, TDZ, *Vanda* 'Saint Valentine'

INTRODUCTION

The members of the Family Orchidaceae are mostly known as ornamental plant species due to their distinctive characteristics of flowers. However, overexploitation and alteration in land use have caused some orchid species to be vulnerable to extinction. For instance, all of the 115 identified orchid species from Mount Ungaran, Central Java, Indonesia, are listed in Appendix II of the CITES, and four are even listed in the IUCN Red List (Kurniawan et al. 2021). On the other hand, some wild orchid species can be potentially used as parental lineages to produce hybrid varieties of desirable better performances, including flower color, shape, and resistance (Li et al. 2021).

Intergeneric hybridization between *Phalaenopsis* 2166 and *Vanda*' Saint Valentine' has resulted in several hybrid seedlings characterized phenotypically and molecularly. Based on the leaf shape, edge, and color, the intergeneric hybrids likely resemble *Phalaenopsis* 2166 as the female parent. However, some variations of leaf shape and color were also observed. In general, it could be said that maternal inheritance of the phenotypic characters in the intergeneric hybridization occurred. Hence, it is reasonable that the hybrid seedlings showed the best growth when grown in New Phalaenopsis (NP) medium (Dwiati et al. 2020a). Then, molecular characterization using the ndhE partial gene revealed that 11 of the 14 hybrids obtained had the same sequences of the ndhE partial gene as that of *Phalaenopsis* 2166. The sequences are now registered in the NCBI database with accession number MH646649. The other three hybrids, i.e., F1.9, F1.11, and F1.14, showed slightly different ndhE sequences from that of *Phalaenopsis* 2166, and they have also been registered in the NCBI database as MH646651. One of the three hybrids, i.e., F1.14, has a partially spotted reddish-purple leaf that is predictable to produce conspicuously attractive flowers, thus potentially to be developed into a large number of plant individuals (Dwiati et al. 2020b; Dwiati and Susanto 2021).

Furthermore, to develop the promising hybrid, an in vitro culture technique should be employed, by which the hybrid clones are grown using ½ MS media enriched with NAA and TDZ. In this case, NAA is used for stimulating clone formation, while TDZ is intended to promote the propagation of the somatic embryos (Mayer et al. 2010; Gantait and Sinniah 2012). Some previous studies on the stimulation of somatic embryogenesis using TDZ alone or in combination with NAA have been reported, such as those in *Phalaenopsis amabilis* (L.) Blume (Mose et al. 2017), *Dendrobium aqueum* Lindl. (Parthibhan et al. 2018), commercial *Phalaenopsis* hybrids (Zanello and Cardoso 2019), and *Paphiopedilum niveum* (Rchb.f.) Stein (Soonthornkalump et al. 2019). Therefore, the objective of this study is to demonstrate the effect of NAA and TDZ application on the stimulation of somatic embryogenesis of the selected intergeneric hybrid between *Phalaenopsis* 2166 and *Vanda*' Saint Valentine'. Once the somatic

embryos of the hybrid are produced, they could be developed further into PLBs.

MATERIALS AND METHODS

Experimental design

The study was conducted as an experiment arranged in a factorial Randomized Complete Block Design (RCBD) using two factors, i.e., types of plant growth regulators (NAA and TDZ) and their respective concentrations levels. The NAA concentrations consisted of 0 mgL^{-1}, 0.5 mgL^{-1}, 1.0 mgL^{-1}, while those of TDZ comprised 0 mgL^{-1}, 1.0 mgL^{-1}, 1.5 mgL^{-1}, 2.0 mgL^{-1}. Each of the 12 treatment combinations thus made was given three replications resulting in a total of 36 experimental units.

Procedures

Preparation of media

A half-strength modified MS was prepared as the basal medium, which was then supplemented with 2 gL^{-1} peptones, 150 mLL^{-1} coconut water, 150 mLL^{-1} alkaline water, 75 mgL^{-1} vitamin C, 0.50 mgL^{-1} PVP, 0.25 mgL^{-1} Na pantothenate, 0.25 mgL^{-1} pyridoxine HCl, 2 gL^{-1} active charcoal, and 20 gL^{-1} sucrose. NAA solution was applied to the medium corresponding to the respective treatment. The pH of individual treatment was adjusted to 5.2 by dripping NaOH or HCl as necessary. Each medium was added with 1.2 g agar and sterilized in an autoclave at 121°C; 0.15 MPa for 20 minutes. All the media were cooled at approximately 45ºC and shaken gently for homogeneity. Each medium was added with TDZ according to the respective treatment and poured onto a Petri dish.

Planting of leaf explants

The leaves of the selected hybrid were washed under running water, air-dried, and put into sterile bottles. These were then added with sterile-distilled water and Tween-20 of three drops, after which the leaves were rinsed using sterile aquadest until the foams were totally removed. Then, the leaves were sterilized using 70% (v/v) ethanol for 5 minutes, followed by $HgCl_2$ for 5 minutes, and rinsed three times with sterile distilled water. Finally, the leaves were put into a sterile Petri dish lined with filter paper, where they were cut into 0.5 x 0.5 cm pieces which served as explants. These were then planted onto aseptic media in the previously prepared Petri dishes corresponding to the respective treatment. Each medium was filled with two explants and put on the culture rack in the dark at a temperature of 22°C and an air humidity of 90%. The clone growth was observed daily. Since the 30th day after incubation, the explants were subjected to light exposure for 12 hours and dark exposure for 12 hours alternately until they were 108 days old.

Parameters

The parameters that were examined comprised the date of clone formation, the number of embryogenic clones formed (%), the thickness of clones formed (mm), clone diameter (mm), and clone color and consistency. All parameters were examined weekly from the date of explant incubation until the clones were 21 days old. Meanwhile, the development of somatic embryos was still examined 108 days after explant incubation.

Data analysis

The quantitative data obtained were analyzed using ANOVA. When a significant effect of treatments was observed, further analysis was performed using Duncan Multiple Range Test (DMRT). Meanwhile, descriptive analysis was applied to the qualitative data.

RESULTS AND DISCUSSION

Clone formation

Clone formation had been observed since the third day of explant incubation, showing sufficiently friable, green, and compact clone characteristics. Depending on the treatment applied, these would grow into maximum at approximately three to four weeks. It can be seen in Table 1 that some treatment combinations of NAA and TDZ resulted in 100% of somatic embryo formation.

Table 1. Identical clones formed in the dark condition on the 14th day after explant incubation

Treatment		Clone color	Clone consistency	Clone formation (%)	Somatic embryos (%)	Clone thickness (mm)	Clone diameter (mm)
NAA (mgL^{-1})	TDZ (mgL^{-1})						
0.0	0.0	light green	sticky	50 ^{f}	50 ^{c}	0.565 ^{i}	0.305 ^{b}
0.0	1.0	light green	sticky	60 ^{ef}	70 ^{b}	0.698 ^{e}	0.279 ^{b}
0.0	1.5	light green	sticky	65 ^{de}	70 ^{b}	0.669 ^{f}	0.306 b
0.0	2.0	light green	sticky	65 ^{de}	80 ^{ab}	0.639 ^{g}	0.317 ^{ab}
0.5	0.0	light green	less friable	70 ^{cde}	90 ^{a}	0.591 ^{h}	0.320 ^{ab}
0.5	1.0	light green	friable	100 ^{a}	90 ^{a}	0.763 ^{b}	0.312 ^{ab}
0.5	1.5	light green	friable	100 ^{a}	90 ^{a}	0.759 ^{bc}	0.302 ^{ab}
0.5	2.0	fresh green	friable	100 ^{a}	90 ^{a}	0.730 ^{d}	0.303 ^{b}
1.0	0.0	fresh green	friable	90 ^{ab}	80 ^{ab}	0.740 ^{cd}	0.291 ^{b}
1.0	1.0	dark green	friable	95 ^{a}	90 ^{a}	0.706 ^{e}	0.323 ^{ab}
1.0	1.5	dark green	friable	80 ^{bc}	80 ^{ab}	0.842 ^{a}	0.289 ^{b}
1.0	2.0	dark green	friable	75 ^{cd}	85 ^{a}	0.663 ^{f}	0.355 ^{a}

Note: Values followed by the same letter in the same column show the non-significant difference after DMRT at α 0.05

It was found in this study that clone formation of 100 % was obtained in the combination of NAA 0.5 mgL^{-1} and TDZ 1.0 mgL^{-1}; NAA 0.5 mgL^{-1} and TDZ 1.5 mgL^{-1}; NAA 0.5 mgL^{-1} and TDZ 2.0 mgL^{-1}. Therefore, it seemed likely that NAA of 0.5 mgL^{-1} was the optimum concentration for promoting clone formation. In addition, the somatic embryo thus produced was 90 % (Table 1). It was also shown from the table that a combination of NAA 0.5 mgL^{-1} and TDZ 1.0 mgL^{-1} on the 14th day was the most optimum treatment in producing clone thickness and clone cell diameter, i.e., 0.763 mm and 0.312 mm, respectively. As a result, light green and friable clones were obtained.

The selected intergeneric hybrid clone's development stage began when the cells had reached their maximum size. On the 16th day, the sufficiently friable clones started to enlarge, gradually reaching their maximum size (Figure 1A). Then, the clone cells would enlarge, followed by the formation of initial globular structures, which the division of clone cells indicated. The next stage was characterized by forming several new cells inside the previous big clone cell, as shown in Figure 1B, which was found on the 21st day.

Clone development

Table 2 and Figure 2A show that the combination of NAA 0.5 mgL^{-1} and TDZ 1.0 mgL^{-1} resulted in light green and friable clones forming dome-like protuberances of 50 % on the 21st day. Here the explants started to be subjected to light for an hour, and on the 25th day, the light condition was prolonged for six hours. Then, on the 28th day, the somatic embryo cells under treatment began to differentiate (Figure 2B).

Further development of globular structures was observed in all treatments, except in control (NAA 0 mgL^{-1} and TDZ 0 mgL^{-1}). On the other hand, some treatments, i.e., the combination of NAA 0.5 mgL^{-1} and TDZ 0 mgL^{-1}; NAA 0.5 mgL^{-1} and TDZ 1.0 mgL^{-1}; 0.5 mgL^{-1} and TDZ 1.5 mgL^{-1}; NAA 1.0 mgL^{-1} and TDZ 1.0 mg L^{-1} had even begun to form dome-like protuberance (Figure 2A).

On the 49th day, the somatic embryos developed, forming structures characterized by protuberances in some parts of the cell side (Figure 2B). These would then develop into structures resembling scutella (Figure 3B).

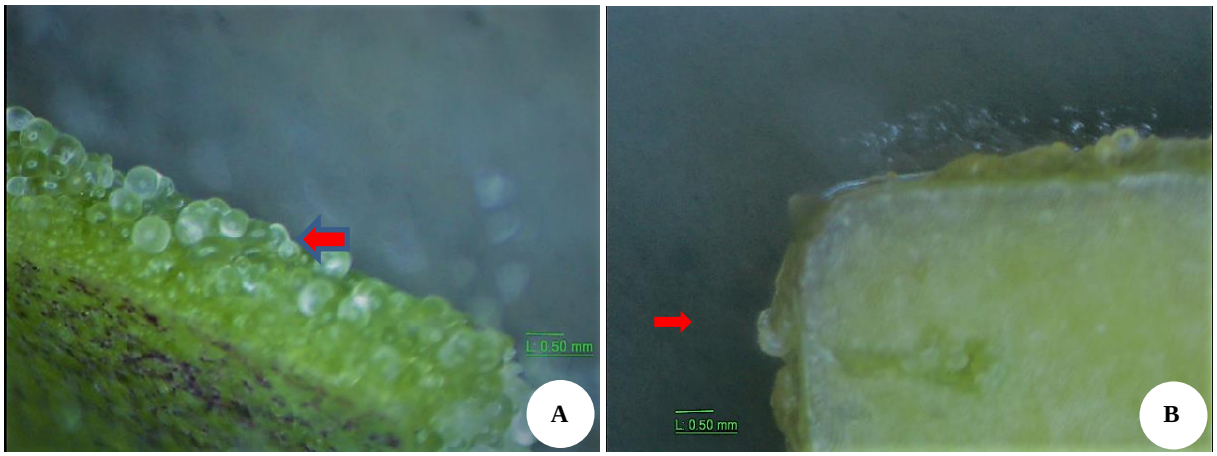

Figure 1. Development of identical clones of the selected intergeneric hybrid between *Phalaenopsis* 2166 and *Vanda*' Saint Valentine' (A) diameter of clone cells with NAA 0.5 mgL^{-1} and TDZ 1.0 mgL^{-1} at 16th day; (B) clone obtained at 21st day

Table 2. Clones formed in the dark condition on the 21st day after explant incubation

Treatment		Clone color	Clone consistency	Further development	Clones undergoing further development (%)
NAA (mgL^{-1})	TDZ (mgL^{-1})				
0.0	0.0	whitish green	sticky	clone not develop	0 ^{d}
0.0	1.0	light green	compact	initial globular	30 ^{bc}
0.0	1.5	light green	compact	tree-like	20 ^{c}
0.0	2.0	light green	compact	initial globular	40 ^{ab}
0.5	0.0	light green	friable	dome-like	40 ^{ab}
0.5	1.0	light green	friable	dome-like	50 ^{a}
0.5	1.5	light green	friable	dome-like	30 ^{bc}
0,5	2.0	fresh green	friable	tree-like	30 ^{bc}
1.0	0.0	fresh green	friable	globular	35^{b}
1.0	1.0	dark green	friable	dome-like	40 ^{ab}
1.0	1.5	dark green	friable	last globular	50 ^{a}
1.0	2.0	dark green	friable	globular	35 ^{b}

Note: Values followed by the same letter in the same column show the non-significant difference after DMRT at α 0.05

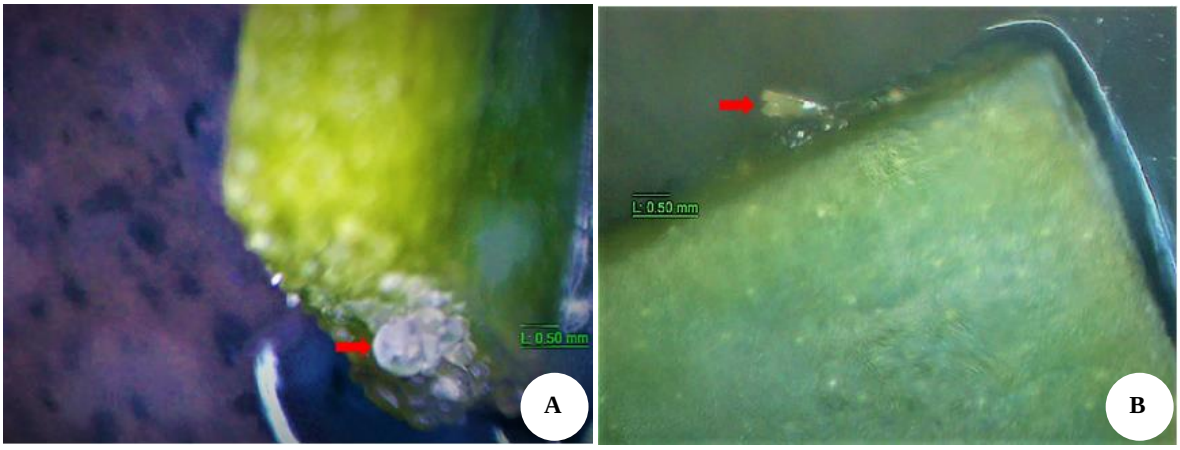

Figure 2. Further development of globular structures of the selected intergeneric hybrid between *Phalaenopsis* 2166 and *Vanda*'Saint Valentine' (A) dome-like protuberance with NAA 0.5 mgL^{-1} and TDZ 1.0 mgL^{-1} at 28th day; (B) somatic embryo with NAA 0.5 mgL^{-1} and TDZ 1.5 mgL^{-1} at 49th day

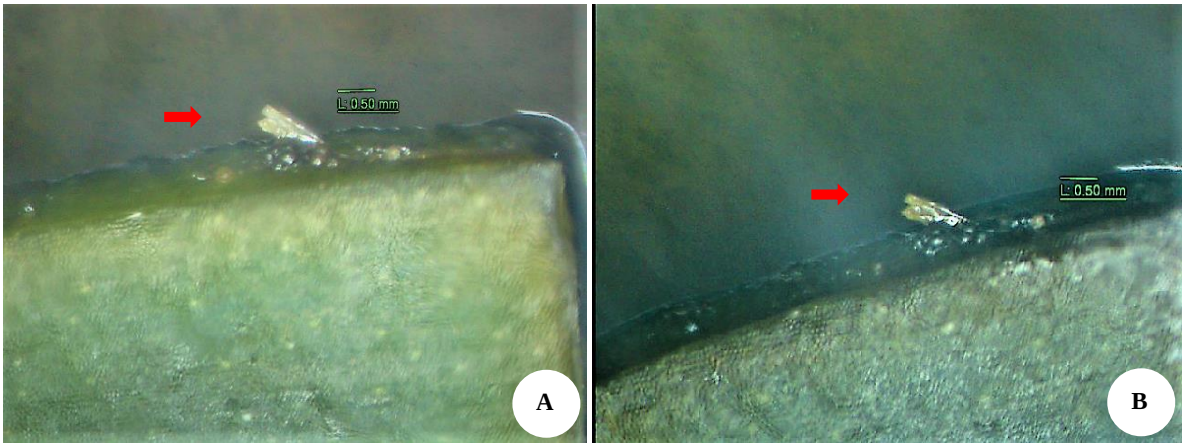

Figure 3. The next development of somatic embryos of the selected intergeneric hybrid between *Phalaenopsis* 2166 and *Vanda*' Saint Valentine' (A) somatic embryo with NAA 0.5 mgL^{-1} and TDZ 1.5 mgL^{-1} under light condition for an hour on the 70th day; (B) somatic embryo with NAA 0.5 mgL^{-1} and TDZ 1.5 mgL^{-1} under 12 hour-light and 12 hour-dark conditions at 108th day

Discussion

Slightly different from our results in that 50% of clones were still formed in the medium without the supply of NAA and TDZ (Table 1), recalcitrant leaf explants in *Cymbidium eburneum* Lindl. were observed in the basal medium of MS. No regeneration occurred where the explants turned brown and died within 10 weeks (Sembi et al. 2020). A similar finding (no somatic embryo formation) was reported in leaf explant in *Spathoglottis plicata* Blume when cultured in MS medium without a plant growth regulator (Manokari et al. 2021). Another report on indirect somatic embryogenesis where calli of the intergeneric hybrids between *Aranda* Wan Chark Kuan' Blue' and *Vanda coerulea* Griff. ex Lindl. appeared from the incision scar relatively fast since the third day of explant incubation (Gantait and Sinniah 2012). Wounding is considered important in assisting both direct and indirect somatic embryogenesis because it stimulates cell division (Rojas-Herrera and Loyola-Vargas 2002)

Regeneration responses in the leaf explant culture of *C. eburneum* were observed in the applications of NAA and BA. The highest response (83.3%) was obtained using 2 mgL^{-1} NAA and 0.5 mgL^{-1} BA (Sembi et al. 2020). In our study, we use TDZ instead of BA to stimulate the further development of the clones into somatic embryos since TDZ has been generally used in orchid tissue culture to enhance somatic embryogenesis (Wu et al. 2012; Mahendran and Bai 2016). It was also reported that TDZ showed better efficacy over other purine types of cytokinins, such as BA, in inducing somatic embryogenesis in orchids (Bhattacharyya et al. 2018). The fastest PLB induction in the intergeneric hybrids between *Aranda* Wan Chark Kuan' Blue' and *V. coerulea* using leaf explants was observed in the treatment of TDZ 1.5 mgL^{-1} (Gantait and Sinniah 2012). Some other studies showed that TDZ was proved very effective in inducing somatic embryogenesis in several orchid species, such as *Renanthera* Tom Thumb' Qilin' (Wu et al. 2012), *P. amabilis* (Mose et al. 2017), and

P. niveum (Soonthornkalump et al. 2019). TDZ can replace cytokinin as a plant growth regulator and auxin in an in-vitro culture media (Kou et al. 2016). As a plant growth regulator, TDZ should not exceed 3 mgL^{-1}. The high level of TDZ (3 to 5 mgL^{-1}) will inhibit cytokinin oxidase (Soonthornkalump et al. 2019). Variation in the application of growth regulators, especially auxin and cytokinin, in an in vitro culture media could affect somatic embryogenesis (Guo et al. 2011; Moradi et al. 2017).

Since the 30th day after incubation, the explants of the selected intergeneric hybrid of *Phalaenopsis* 2166 x *Vanda*' Saint Valentine' were subjected to 12 hour-exposure to light and another 12 hours in the dark. As a comparison, the explants of *Phalaenopsis* Classic spotted pink started to form the last globular structure on the 23rd day, and the embryos began to form coleoptelar (Pereira et al. 2019). Meanwhile, direct somatic embryogenesis from leaf explants in *Phalaenopsis*' Little Steve' revealed that somatic embryos were formed on the 30th day after explant incubation in the dark (Kuo et al. 2005).

Somatic embryogenesis in *P. amabilis* was reported to begin in the 8th week when grown in NP media supplemented with TDZ. However, the most rapid somatic embryogenesis was obtained with TDZ 3.0 mgL^{-1} on the 11th day using leaf explants, while the slowest one was found with TDZ 3.0 mgL^{-1} using stem explants (Mose et al. 2017). In this study, we found that somatic embryogenesis of the intergeneric hybrid between *Phalaenopsis* 2166 and *Vanda*' Saint Valentine' began on the 21st day in the modified ½ MS medium. Meanwhile, *Vanda tricolor* Lindl. shoots produced from somatic embryogenesis showed the best development when subcultured in the New Dogashima (ND) medium without applying any plant growth regulator (Ashihah et al. 2022).

Regeneration of somatic embryogenesis in orchids began on the 15th to 30th day in the concentration of TDZ ranging between 0.001 and 5 mgL^{-1} (Shen et al. 2018). In the last development stage of somatic embryogenesis, reduced auxin was needed, especially for stimulating PLB proliferation and differentiation (Yang and Zhang 2010). TDZ concentration and its interaction with light spectra were found highly determining direct somatic embryogenesis in *Phalaenopsis* orchids. The concentration of 3mgL^{-1} in interaction with red and far red light spectra was the efficient treatment to induce direct somatic embryogenesis in the orchids without somaclonal variation (Boldaji et al. 2021).

While no callus was formed in our study, pre-embryo is a further callus development in the indirect somatic embryogenesis, which two bipolar centers of meristems characterize. These structures will develop into the root and stem meristem, respectively (Seth et al. 2017; Shen et al. 2018). In addition, histological examination shows that callus resulting from somatic embryogenesis will develop sequentially into PLBs, which consist of some meristematic tissues undergoing further development to form roots, stems, and leaves (Sherif et al. 2018).

Some factors have direct effects on the somatic embryogenesis of orchids. They are genotypes, growth regulators, and media (Campos et al. 2017; Zanello and Cardoso 2019). Half-strength MS is the most common media used, in which N is in the form of nitrate (NH_4NO_3) in a sufficiently high concentration, i.e., 1.7 mgL^{-1}. In addition, KNO_3 of 1.9 mgL^{-1} is also contained in ½MS media. Most plants absorb N in the form of nitrate. Both NH_4NO_3 and KNO_3 can be used to stimulate somatic embryogenesis (Zanello and Cardoso 2019). NP was reported as the media with N in the form of nitrate suitable for *P. amabilis*. This media contained NH_4NO_3 of 82 mgL^{-1}, KNO_3 of 424 mgL^{-1}, $Ca(NO_3)_2.4H_2O$ of 443.04 mgL^{-1}, and $Mg(NO_3)_2.6H_2O$ of 256.4 mgL^{-1} (Mose et al. 2017). Nitrogen, in the form of either potassium nitrate or calcium nitrate, is very good at stimulating somatic embryogenesis, while that in the form of ammonium nitrate less stimulates somatic embryogenesis. Nevertheless, explants in the absence of ammonium nitrate in the growth media will fail to undergo somatic embryogenesis (Méndez-Hernández et al. 2019).

The presence of TDZ in the culture media of *Oncidium flexuosum* (Kunth) Lindl. without light would stimulate the regeneration of PLBs. Pre-embryos with no chlorophyll were formed in the dark condition, so somatic embryogenesis occurred in the absence of chlorophyll. After treatment with no growth regulator and incubation in the light condition, embryos would be greenly initiated to form PLBs (Zanello and Cardoso 2019). It was proved that in the early stages of PLB formation, characteristics of somatic embryonic callus similar to zygotic embryo development were observed, indicating that PLBs were truly somatic embryos of orchids (Lee et al. 2013).

Somatic embryogenesis could result from the proliferation of young PLBs cultured in the MVW media containing NAA 0.1 mgL^{-1}. Therefore, the increasing accumulation of endogenous auxin through exogenous auxin application in the early stages of somatic embryogenesis was needed. In the next stages of development, reduced levels of auxin enabled rapid proliferation and differentiation of meristems, which in turn would stimulate the emergence of shoots. Then, the plantlets thus produced were moved into MVW media without a growth regulator (Soonthornkalump et al. 2019).

In conclusion, our present study found that ½ MS medium supplemented with the combination of NAA 0.5 mgL^{-1} and TDZ 1.5 mgL^{-1} produced identical clones of the intergeneric hybrid between *Phalaenopsis* 2166 and *Vanda* 'Saint Valentine' that showed high potential of differentiating into PLBs. That indicates that applying NAA and TDZ could stimulate somatic embryogenesis in the selected hybrid.

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Fruit development and capsaicin content of hot pepper (*Capsicum annuum*) plant cultivated in different soil salinity stress

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Abstract. *Purnama PC, Sumardi I, Nugroho LH. 2022. Fruit development and capsaicin content of hot pepper (*Capsicum annuum*) plant cultivated in different soil salinity stress. Nusantara Bioscience 14: 166-171.* Land scarcity for cropping at Java Island is a challenge for scientists to look for alternative cropping land. The use of saline land for cropping needs to have further discussed. Red pepper (*Capsicum annuum* L.) can be used as a plant model because, aside from being used as a vegetable, it is also used as natural medicine because of its secondary metabolite, capsaicin. A harsh environment could induce changes in the primary metabolism, which leads to secondary metabolite decomposition. For example, plants respond to stress, such as salt stress, by synthesizing flavonoids and phenolic acid as defense systems to reduce damage. However, the total sugar level and organic acids are decreased. This research aimed to study the fruit development and capsaicin content of hot pepper grown on various coastal soil sand to know whether or not different growth medium affects the size of each part of the fruit. The design of this research was a Completely Randomized Block Design (CRBD). In this research, five different salinity mediums were used, they were A. 15.20 dS/m, B. 5.70 dS/m, C.1.10 dS/m, and D. 2.85 dS/m obtained from Pandansimo and E. 3.25 dS/m obtained from Sleman, Yogyakarta, Indonesia, as comparation. Seedlings were transferred to the polybag after having four truly expanded leaves. Fruit development was observed every week, starting from the first day after flowering (DAF) to 35 DAF. Pericarpium and placenta thickness, fruit diameter, number, length, and width of the giant cell were recorded appropriately from the slides prepared using the paraffin method. Capsaicin content was determined at 14 and 35 DAF, performed with Gas Chromatography-Mass Spectrometry (GC-MS). The results show structural changes in the exocarpium; on the first day after flowering, there was only one layer of epidermis cells, but at 7 DAF, there was one layer of epidermis cells and one layer of collenchyma cells. Next, at 14 DAF, one layer of epidermis cells and two layers of collenchyma cells are observed. The structure of the mesocarpium, endocarpium, and placenta were not changed. The capsaicin content of the green fruit (14 DAF) was lower than the mature one (35 DAF) in all survival mediums. The highest capsaicin content at 14 and 35 DAF was obtained from a plant grown at medium C. Different growing mediums affected pericarpium and placenta thickness, number, length, and width of the giant cell fruit diameter.

Keywords: Capsaicin, *Capsicum annuum*, development, GC-MS, saline

INTRODUCTION

Agricultural land scarcity in Java, Indonesia, is a challenge for researchers to find alternative agricultural land; one way is to utilize available marginal lands, for example, peatland, saline land, and swamps. In 1974, Massoud (1974) estimated that the saline land in Indonesia had reached 13,213 thousand ha, which had expanded due to the increase of invasive fertilization, which caused higher salt levels in the soil. Moreover, seawater intrusion that pollutes groundwater sources used for irrigation is also the cause of excess salt in the soil, especially in coastal areas (Rhoades and Miyamoto 1990). Too much salt in the soil inhibits plant growth by preventing nutrients and water from being absorbed by the roots. In the case of *Sapium sebiferum* (L.) Dum. Cours. grown on the soil closer to the coast, with its growth further slowing down (Barrileaux and Grace 2000).

Aside from influencing growth, environmental factors also affect the secondary metabolites content of plants, as in the case of *Capsicum annuum* L. (jalapeno) grown on sand medium with the addition of nitrogen showing an increase in growth, yield, and capsaicin content (Johnson and Decoteau 1996). In other cases, NaCl solution added in the planting medium of the *C. annuum* (jalapeno) cultivar is known to affect capsaicin content. Proving that environmental conditions and genetic factors can affect capsaicin content (Arrowsmith et al. 2012).

Some plants are also known to have a tolerance to high salt levels, for example, corn, chili, cassava, long beans, and soybeans which are widely cultivated in the coastal areas of Pandansimo, Yogyakarta, Indonesia. The *C. annuum* is one of the chili varieties grown in Indonesia. (Solichatun et al. 2022). Moreover, national demand increased yearly; in 2000, the total consumed reached 427,018 tons (Rukmana and Oesman 2002). The food industry is the principal user of chili fruits. It is often used as a coloring and flavoring agent in sauce, soup, processed meat, snacks, candies, soft drinks, and alcoholic beverages. In addition to their sensory features, oleoresin is extracted from pepper fruits and used as an ingredient in numerous commercial products such as insect repellent or self-

defense sprays. *Capsicum* fruits can also be employed in medicinal applications since they are an important source of bioactive compounds that provide health benefits to consumers (Baenas et al. 2018). It is known that the content of secondary metabolites of chili peppers is widely used to overcome obesity, cardiovascular diseases, and gastrointestinal diseases (Sharma et al. 2013). Chili fruit is also reported to contain antioxidants (Loizzo et al. 2017), hypoglycemic activity (Tundis et al. 2012), suppress intracellular accumulation of triglycerides (Feng et al. 2014), and have anticancer properties (Corson and Crews 2007).

Capsicum fruit varies in size, shape, color, flavor, and pungency (from nonspicy varieties to the hottest species). The pungency in chili peppers is known to come from its secondary metabolite compound known as capsaicin, which is the component of capsaicinoids, along with four other compounds, namely nordihydrocapsaicin, dihydrocapsaicin, homocapsaicin, and homodihydrocapsaicin. About 90% of the spiciness in chili peppers is determined by capsaicin and dihydrocapsaicin (Barbero et al. 2014). In addition to capsaicin, chili peppers contain proteins, fats, carbohydrates, minerals (calcium, phosphorus, iron), vitamins (A, B1, B2, B3, C), essential flavon oils, and carotenoids. The known fatty acid content is palmitic, stearic, oleic, and linoleic by 76% (Jarret et al. 2013).

Capsaicinoids are the compounds responsible for chili pungency, and it is also valued as an excellent source of natural pigments and antioxidant compounds (de Sa Mendes and Gonzales 2020). Capsaicinoids are found in different amounts in *Capsicum* fruits, depending mainly on the variety. The color of the chili can vary as green, yellow, or white (for the immature fruit), orange as semi-ripe, and it becomes red, dark red, brown, and sometimes almost black in the ripe stage. These colors originate from the carotenoids produced in the fruit during its ripening stages. Once the fruits are harvested, they change mainly in color, but changes also happen in the aroma, flavor, and antioxidant activity (Manikharda et al. 2018). *Capsicum* fruit, especially in its dehydrated form, is an excellent source of tocopherols which are recognized for their high resistance to lipid oxidation; in such a way, these compounds could serve as protective agents for the antioxidant capacity in the pericarp (Wildman et al. 2017).

The capsaicinoids increase during maturation until they reach the maximum content and immediately decrease, presenting a degradation greater than 60%. On the other hand, it has been reported that the activity of peroxidases that degrade these compounds increases at the same time that the content of capsaicinoids is reduced (Cisneros-Pineda et al. 2017). Therefore, both external factors and the maturity state of chili may have generally affected the secondary metabolites production.

It is remained unclear until now the compartmentalization of each metabolite in the hot pepper fruit because the placenta, pericarp, and seed produce the enzyme important for capsaicinoid production. However, according to Zamljen et al. (2021) placenta is the main synthesis point of capsaicinoid, and the other two produces in smaller amounts. But some compounds known to be found in a certain part of the fruits, such as pericarpium, placenta, and seed, for example, capsaicin, are mainly synthesized in the placenta (Gamboa-Becerra et al. 2015), while anthocyanins are described as being accumulated in pericarpium during fruit development (Aza-Gonzales et al. 2013).

This study aims to determine the development and capsaicin content of hot pepper fruit in different coastal soil planting mediums and whether different planting mediums affect the size of the constituent components of red chili fruit.

MATERIALS AND METHODS

All experiments are conducted in the greenhouse. The seeds used are *C. annuum* North Red Star variety produced by PT Sang Hyang Seri (Persero), which is transferred into polybags that have been filled with soil medium obtained from Pandansimo beach, Bantul, namely A. 15.20 dS/m, B. 5.70 dS/m, C.1.10 dS/m, and D. 2.85 dS/m and as a comparison medium E with salt content 3.25 dS/m was obtained from Sleman, Yogyakarta, Indonesia. Transfer of seedlings to polybags is carried out after the seedlings have 4 leaves. Each treatment has five replications separated into different polybags. Observations were carried out on 1, 7, 14, 21, 28, and 35 DAF to determine the development of fruits preserved with preparations using the single staining paraffin method. The parameters observed were: pericarpium thickness, placenta thickness, number, length, and width of giant cells, as well, as fruit diameter. Gas Chromatography-Mass Spectrometry technique (Agilent GC 6890N 5975B MSD) was used to analyze capsaicin content in fruits aged 14 and 35 DAF. The capillary column is an Agilent 19091S-433 model, HP-5MS 5% Phenyl Methyl Siloxane. Capsaicin standard was obtained from Sigma Chemical Co. Single-stain paraffin embedding method (Ruzin 1999) was applied in the preparation of microscopic slides for examination of fruit development. The quantity of capsaicin compound was analyzed with Gas Chromatography-Mass Spectrometry using capsaicin as standard injected simultaneously with the samples. The experimental design used was CRBD (Completely Randomized Block Design) followed by Variance Analysis (ANOVA), which continued with the LSD-Least Significance Difference with a 5% significance level.

RESULTS AND DISCUSSION

Response of flowering to the growing medium

The results of planting *C. annuum* on growing mediums with various salt levels, namely A (15.20 ds/m), B (5.70 ds/m), C (1.10 ds/m), D (2.85 ds/m), and E (3.25 ds/m), was plants cannot survive more than two weeks in planting mediums A and D. All of them died suffered from high salinity and drought since its texture is 100% sand so that the growing medium cannot hold water. Conversely, in planting mediums B, C, and E, plants can survive, produce flowers, and eventually bear fruit (Table 1).

Table 1. The speed of chili fruits flowering on various growing medium

Planting medium	A	B	C	D	E
Flowering speed (day)	X^7	59.6^b	55.8^b	X^9	45.8^a

Notes: The value followed by the same character showed no significant differences among each other based on LSD analyses at 5 % significance. X^7: Die on day 7th, X^9: Die on day 9th

Anatomy of fruit development

Pericarpium thickness

Pericarpium is the further development of ovary walls that are not rapidly differentiated before. At the time of anthesis, most of it is composed of parenchyma, transport tissue, and the epidermis layer, which has a cuticle at the outermost part.

The pericarpium thickness of the red pepper fruit grown on planting mediums B, C, and E began to differ markedly in fruits aged 7 to 35 DAF. Whereas at the age of 21 and 28 DAF in medium C and E, there is no noticeable difference, indicating the increase in pericarpium thickness is the same. At 35 DAF of the three mediums, the lowest pericarpium thickness is at medium B (Table 2, Figure 1).

Placenta thickness

Chili seeds are attached to the placenta, composed of parenchymal cells similar to the ones found in mesocarpium (Figure 2). The stele type observed in the placenta is bicolateral, the xylem is situated between the outer and inner phloem, and the cambium is found.

Capsaicin content

In all survival growing mediums, the capsaicin content of green fruits (14 DAF) is lower than that of brownish-green fruits (35 DAF) (Table 4). The capsaicin content of green and brownish-green fruits in medium B (5.7 dS/m) was lower than in medium C (1.1 dS/m), both mediums coming from a coastal region. The capsaicin content of green and brownish-green fruits in medium E (3.25 dS/m) as the control medium is lowest compared to the other two mediums, probably due to the texture of the medium, which in the form of *geluhan* sand and also the source of the salt which are not coming from the coastal area but unknown salt from the ground completely far away from the shoreside, approximately 25 km.

Table 2. Pericarpium thickness of chili on various growing media

Day	Pericarpium thickness (µm)		
	B	C	E
1	249.6^{aq}	249.6^{ap}	348.0^{aq}
7	428.4^{bq}	393.0^{bp}	526.5^{br}
14	531.0^{cp}	747.0^{cq}	778.5^{cr}
21	688.5^{dp}	976.5^{dq}	987.0^{dq}
28	773.5^{ep}	1155.0^{eq}	1125.0^{eq}
35	802.5^{ep}	943.5^{dq}	1018.5^{dr}

Notes: The value followed by the same character showed there were no significant differences among each other based on LSD analyzes at 5 % significance

Table 3. The placenta thickness of chili fruit grown on a different medium

Day	Placenta thickness (µm)		
	B	C	E
1	438.0^{aq}	216.6^{ap}	210.0^{ap}
7	$713.4b^{cp}$	468.0^{br}	526.5^{bq}
14	819.0^{dp}	1065^{cq}	1158^{cr}
21	913.5^{cp}	1209^{dq}	2058^{dq}
28	765.0^{cdp}	1552.5^{eq}	1156.5^{cr}
35	670.5^{bp}	1063.5^{cq}	1306.5^{dr}

Notes: The value followed by the same character within one column (a, b, c) and one row (p, q, r) showed there were no significant differences among each other based on LSD analyses at 5 % significance

Table 4. The relative content of capsaicin (%) of chili grown on different growing mediums

Planting medium	14DAF	35DAF
B	23.97	58.78
C	77.99	100
E	17.17	32.27

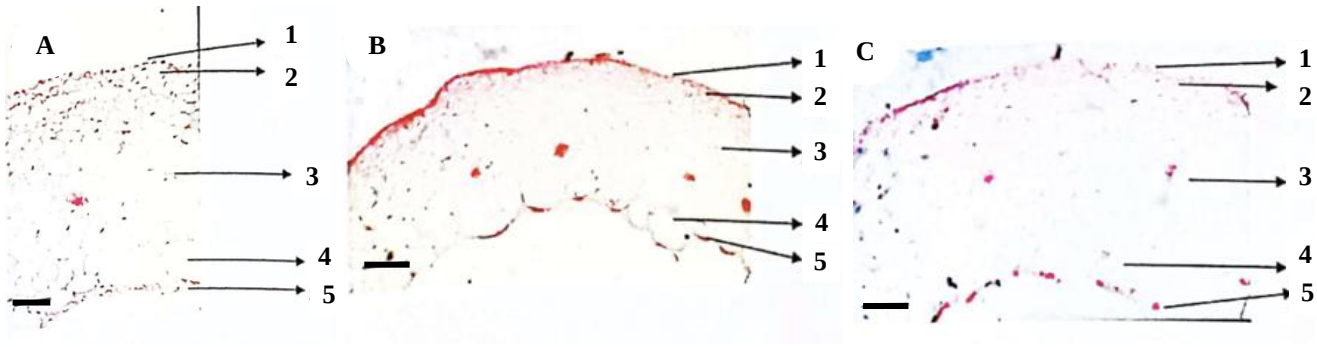

Figure 1. Cross section of chili pericarpium fruit aged 35 DAF on the growing medium: A. 5.7 dS/m, B.1.1 dS/m, C. 3.25 dS/m observed under Nikon light microscope: 1. Epidermis with cuticle, 2. Collenchyma, 3. Parenchyma, 4. Giant cell, 5. Sclerenchyma. 1 Bar A= 25 µm, 1 Bar B, C= 50 µm

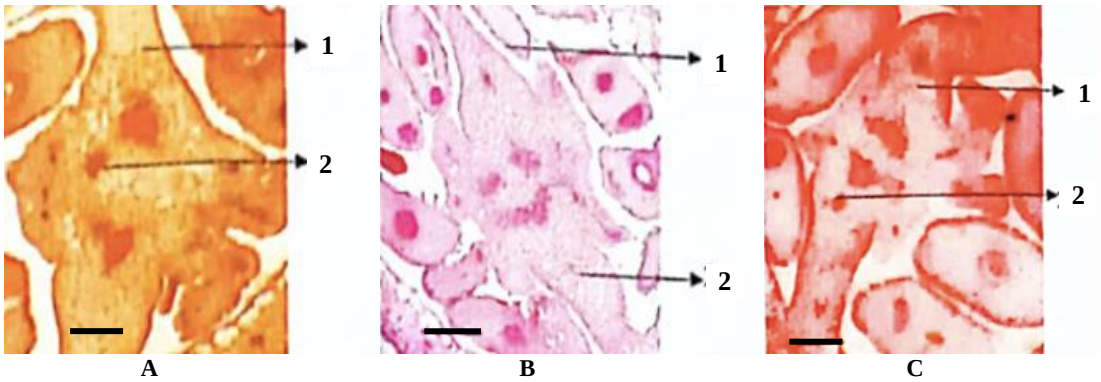

Figure 2. Cross-section of chili fruit through placenta aged 7 DAF grown on planting medium: A. 5.7 dS/m, B. 1.1 dS/m, C. 3.25 dS/m observed under Nikon light microscope: 1. Parenchyma, 2. Stele in the placenta. 1 Bar = 50 µm

Discussion

From planting hot pepper in mediums A, B, C, D, and E, none of the plants could survive in mediums A and D, and the plants died on days 7th and 9th, respectively (Table 1). It could be that in medium A the P content is 10.43 ppm (low), the total N is 0.0056% (very low), the K is 0.63 ppm (medium), and the salt level is far above the normal limit of 4 dS / m which is 15.20 dS/m (Wilkinson 1994). Therefore, the texture is 100% sand which cannot hold water; as a result of which, plants cannot get water. Likewise, in medium D, although the salt content is 2.85 dS/m, the texture is 100% sand, and the nutrient content is low. On the other hand, in medium B (5.70 dS/m), the texture is in the form of *geluhan* sand, allowing the roots to absorb water and nutrients, the P content is 20.79 ppm, the total N is 0.015%, and the K available is 1.45 ppm relatively high. In medium C (1.1 dS/m), the texture is *geluh*, the P content is 51.76 ppm (very high), the N total is 0.11%, and the K available is 0.12 ppm (low). While E (3.25 dS/m), soil texture is in the form of *geluhan* sand, the P is 41.26 ppm, the N total is 0.025%, and the K is 1.56 ppm (high). Thus, it is clear that plants can not survive at medium A and D due to their salt level, which is beyond the normal level, and their texture which is 100% sand.

As one of the site productions of capsaicinoid, pericarpium is important to measure growth. The results from pericarpium measurement showed that the pericarpium at medium B is less developed compared to the other two planting medium, C and E, mainly due to the salt level of growing medium B (5.70 dS/m) being high. Salt inhibits the growth of plants by affecting their osmotic potential. As a result, cell turgor decreases and inhibits the elongation of plant cells. The high level of Na+ causes K+, which plays a role in activating enzymes involved in pyruvate synthesis, and protein translation cannot be absorbed (Manchanda and Garg 2008). K also acts as a cofactor in protein synthesis, functioning to maintain water balance and the movement of the stomata. The low K causes the closed stomata so that the rate of photosynthesis is reduced, as a result of which the energy for growth decreases (Salisbury and Ross 1995).

The placenta is the site of capsaicinoids, of which 90% consist of capsaicin and dihydrocapsaicin (Topuz and Ozdemir 2017). Though the production site is at the placenta, epidermal cells accumulate them in the vacuoles and eventually excrete them in the seeds and on the inner surface of the pericarp (Cisneros-Pineda et al. 2017). In the placenta thickness measurement, at day 35 DAF overall showed a reduction in the decreasing nutrient supply in the growing medium. Therefore, the placenta of hot pepper at medium B is less developed than in the other two growing mediums, C and E (Table 3, Figure 2).

Several researchers have confirmed that in the epidermal cells of the placenta, where capsaicinoids (mainly capsaicin and dihydrocapsaicin) are synthesized, a higher concentration of capsaicin is found. They are secreted into the cell wall and finally accumulate within the structures named vesicles located at the surface of the placenta. It is worth mentioning that the placenta was the one that presented the highest content of capsaicin, but in turn, it was the one that conserved less bioactive compounds, similar to the pericarp (Palma-Orozco et al. 2021).

The results of capsaicin measurement showed that the content at 35 DAF is higher than 14 DAF (Table 4). That indicates that the older the fruit, the higher the capsaicin content. Changes during fruit ripening include modification of cell walls that become softer, conversion of carbohydrates to sugars, increased susceptibility to pathogens, raised production of aromas and volatile compounds, changes in biosynthesis, and accumulation of pigments. The softening of the cell wall is in line with the decrease in lignin content which reduces the formation pathway so that precursors (phenylalanine) will be more widely used to form alkaloids, namely capsaicin (Diaz et al. 2004). Among all, the capsaicin concentration at medium C is the highest. That is likely because the C growing medium is collected from Pandansimo, which has salt naturally from the coastal region and has a texture in the form of a *geluh* so that it has enough fine particles to provide a larger water absorption surface and more nutrients attached to these particles. In addition, the medium also contains P 51.76 ppm, N total 0.11%, and K

0.12 ppm. Potassium can increase pigment biosynthesis and affect pungency, and N can increase chili production (Johnson and Decoteau 1996). It is the finding that the content of bioactive compounds is influenced by climatic conditions, maturation time, genotype, and cultivation techniques (Aza-Gonzales et al. 2013).

As we noticed, the pericarpium and the placenta thickness at medium C are higher than in B, which were 943.5 µm and 1,063.5 µm, respectively. Moreover, the capsaicin concentration of red pepper fruits grown at medium C is higher than B. Thus, in line with what Materska (2014) reported, the pericarpium and placenta is the most prominent site of metabolites production found in chili. He reported the comparison of the pericarpium and placenta in chili and found that the placenta was richest in flavonoids while the pericarpium presented larger diversity in glycosylated compounds.

The degradation of capsaicin in the fruit of plants treated with high salt concentration may be due to peroxidase enzymes. Studies in capsicum show the involvement of peroxidases in the degradation of capsaicinoids in the capsicum cultivar (Fujiwake et al. 1980). In addition to that, Contreras-Padilla and Yahia (1998) also revealed that peroxidases are most likely candidates for capsaicinoid degradation. That may also be the possible reason for the degradation of capsaicin in the high salt-exposed plant. These results suggest that the degradation of capsaicin in these fruits could be a response to salt stress conditions and needs further investigation to understand this process properly. It is known that the component of capsaicinoids is not only capsaicin, thus could be the deposition is not only in the form of capsaicin. According to Castro-Concheat et al. (2014), capsaicinoids were considered part of the phenol content of various fruits. Therefore, the content in all parts of chili was much higher than capsaicin, as found in their work, 3 to 6 times higher. Moreover, the right method to extract also highly influence the yield; for example, the Folin Ciocalteu method, despite being easy, sensitive, and precise, can be affected by the presence of aromatic amines, carbon dioxide, ascorbic acid, and other reducing compounds and thus interfering results (Prior et al. 2015). It has been reported that capsaicinoids are synthesized in the superficial cells of the placenta, and these are specialized as parts that secrete these compounds (González-Estrada et al. 2018)

The increase in salt affects primary metabolism, plant growth, and development due to ion toxicity, which induces nutrient and water deficiencies and oxidative stress. Plant cells survive in dealing with osmotic pressure by adjusting the osmotic, accumulating compounds to maintain osmotic pressure, regulating oxidative stress, inducing the formation of certain proteins, and altering some physiological adaptations such as modification of stem and root growth and transpiration. In addition, it also affects the production of secondary metabolites, which physiologically play a role in dealing with or tolerating stress. Most secondary metabolites are produced through intermediates from primary carbon metabolism via phenylpropanoids, shikimate pathways, mevalonates, or MEPs (non-mevalonate pathways). The most studied secondary metabolites include chlorophyll, carotenoids, and phenols. Increased synthesis of secondary metabolites is used to protect cellular structures from oxidative damage (Machanda and Garg 2008). Thus, the possibility that occurs why the capsaicin content of green and brownish-green fruits in medium B is lower than C is that even though in terms of salt levels of growing medium B are higher than C, there is a possibility of an increase in secondary metabolites collected not only as capsaicin but also in the form of flavonoids or other phenol compounds. These phenol compounds are produced to increase antioxidant activity that can prevent the formation of free radical fats. In general, increasing secondary metabolites is expected to increase the internal concentration of cells to prevent the release of fluid from inside plants to the external environment, whose concentration is more concentrated.

In principle, water loss can cause losses in chili's sensory and nutritional quality; such losses are related to the change in color and textures and a decrease in the content of vitamins and other bioactive compounds (Arslan and Ozcan 2019). Therefore, it can be concluded that the accumulation among the different varieties differs, and the difference could be due to environmental and cultivation conditions (Ruiz-Lau et al. 2017).

In conclusion, the anatomical features observed (pericarpium, placenta, parenchyma, giant cell, and stele) are not changed by the saline soil medium; indeed, it affects its size. Overall the thickness of the pericarpium and placenta decreased at 35 DAF, likely due to the depletion of nutrients in the medium. The capsaicin content of green fruits (14 DAF) is lower than that of brownish-green fruits (35 DAF) in all growing mediums. The saline soil influenced the capsaicin content, proving that both hot pepper fruits grown in the coastal saline soil had higher capsaicin contents than the control soil. For improvement, more experiments should be conducted to separate each part of the anatomical features and analyze the capsaicin content to understand its production site.

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Diversity, floral phenology, and socio-economic importance of melliferous plants in Eastern Ethiopia

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Abstract. *Fassil A, Habtamu T, Tahir M, Terefe T. 2022. Diversity, floral phenology, and socio-economic importance of melliferous plants in Eastern Ethiopia. Nusantara Bioscience 14: 172-181.* Beekeeping is a supply of extra money and financial gain for many thousands of farmer beekeepers in Ethiopia and plays a big role in preserving natural resources. Honeybees and flowering plants have co-evolved in their special symbiotic relationship. Bee plant types and their flowering duration differ from one place to another due to variations in topography, climate, and other cultural and farming practices. This study investigated and documented the diversity and floral phenology of honeybee plants in Doba, Gemechis, and Mi'eso Districts, Oromia National Regional State, Eastern Ethiopia, from January 2019 to July 2021. Ethnobotanical data were collected to reveal the diversity of melliferous plants, practices, and communities' attitudes about honey production and melliferous plant conservation. A total of 422 respondents participated through semi-structured interviews, focus group discussions, and field walks for socio-economic data collection. Descriptive statistics such as frequencies, ranking, and scores were used and presented with tables and figures to analyze ethnobotanical data. A total of 120 melliferous plant species were distributed under 108 genera and 55 families, of which 70 plants were found in the Gemechis District, followed by Doba and Mi'eso Districts with 47 and 42 plants each, respectively. Sorenson's Similarity Index values showed the wide-ranging melliferous plant species distribution patterns in the three districts with 50.4 (between Doba and Mi'eso), 37.5 (between Doba and Gemechis), and 15.3 (between Gemechis and Mi'eso) species overlaps. Fabaceae and Asteraceae contribute a significant number of species, with 12 (10 %) and 9 (7.5 %) melliferous plants, respectively. Local communities have a good awareness of the seasonal availability of melliferous plants, indicating adequate supply (June to early December) and critical shortage (November to early May) of melliferous plant resources favoring strong and weak colony strength, respectively. Lack of nutrition, improper management practices, honey bee predators, and lack of beekeeping knowledge and equipment were the most important constraints deleteriously influencing the honey quality and amount in the study area. The shortage of pollen and nectar flow during the dearth periods (January to March) needs interventions like hive migration and bee floral plantations. Hence, there is an urgent need for intervention through awareness creation, campaign-based melliferous plant plantations, and technology transfers.

Keywords: Ethiopia, floral phenology, honey production constraints, melliferous plant

INTRODUCTION

Beekeeping is a floral-based industry where honey bees entirely depend on flowering plants (Olana and Demrew 2019) and vice versa for their mutual benefit of pollination and provision of food in the form of pollen and nectar, respectively (Urbanowicz et al. 2020; Khalifa et al. 2021). Reproduction, productivity, and diversification successes of flowering plants are attributed to honey bees' behavior and practices of varying vegetarian diets, flower-visiting habits, hairy bodies that readily pick up pollen grains, and visit many flowers of the same species during a single trip (Bhalchandra et al. 2014). The implication is that honey bees greatly subsidize ecosystem conservation and agricultural production while they produce important products such as honey and wax (FAO 2009; Minja and Nkumilwa 2016).

Beekeeping in Ethiopia is a longstanding, quick, off-farm, environmentally friendly, and major income-generating agricultural activity, providing diverse income and employment opportunities (Sahle et al. 2018; Olana and Demrew 2019). Annual honey production in the country was estimated at 43,373 metric tons, sharing about 23.5% and 2.35% of Africa's and the world's honey production, respectively (Sahle et al. 2018). These blessings ranked Ethiopia the leader in Africa and the ninth in the world in honey production and stand first in Africa and third in the world in beeswax production (Legesse 2014).

Agro-ecology-based management of honey bees and their beehives during short or long dearth periods (Teferi 2018) and clear awareness of the effects of floral composition on honey bee food stores (Donkersley 2017) are vital, among others, for efficient honey productivity. By the same token, local beekeepers' perception and knowledge of identifying the important melliferous plant and their flowering patterns during the dry and rainy seasons greatly impact the stable production of quality honey (Novac 2017; Coh-Martínez et al. 2019). Some ready reckoner documentation of melliferous plant type, density, flowering period, and quality of melliferous plants

to their nectar and pollen resource potentials help beekeepers of that particular area to start beekeeping (Harugade and Chaphalkar 2013). Development of a bee calendar based on the melliferous plant blooming period is a basic decision-making tool for beekeepers (Novac 2017), if or whether the supply of pollen substitute (Rachna et al. 2011; Pande and Karnatak 2013; Pande and Karnatak 2014), nectar supplement (Pande and Karnatak 2013; Pande et al. 2015) and/or migration of beekeeping is required.

A considerable number of previous studies conducted in Ethiopia on the melliferous plant were identification, floral establishment and honey harvesting seasons (Kebede and Samuel 2016; Tadele et al. 2016; Degaga 2017), status and production systems (Abebe et al. 2015; Tadele et al. 2016; Teferi 2018; Lango and Lomba 2020), working knowledge and perception of the community on beekeeping practices (Berhe et al. 2016; Tulu et al. 2020), trends, opportunities and challenges of honey production (Mohammed et al. 2018; Arega et al. 2020) among others. That successively needs the correct identification of honey bee plants and association with the floral calendar.

Generally, beekeeping activity in West Hararghe Zone, and Gemechis District, particularly, is relatively marginalized compared to other agricultural sub-sectors (Dawud et al. 2020). This study investigates common melliferous plants, their seasonal availability, farmers' perception and practices on honey production, and management activities in Doba, Gemechis, and Mi'eso Districts, Eastern Ethiopia. This study is expected to answer the following three questions. (i) Do Doba, Gemechis, and Mi'eso Districts have adequate melliferous plants to support sustainable honey production? (ii) What are the threats encountered in honeybee production systems in the study area? (iii) Is the floral calendar informative for the seasonal availability of honeybee sources?

MATERIALS AND METHODS

Study area

Doba, Gemechis, and Meiso Districts are found in West Hararghe Zone, Eastern Ethiopia. Doba District is located at 9˚10′0″-9˚30′0″N and 40˚55′0″E-41˚16′0″E, with elevations ranging from 1,149-2,773 m.a.s.l., Gemechis District is located at 8˚40′0″-9˚04′0″N and 4˚50′0″-41˚12′0″E, with elevations ranging from 1,300-3,017 m.a.s.l., whereas Mi'eso is geographically found at 8˚48′12″-9˚19′52″N and 40˚9′30.1″-40˚56′44″E, with elevations ranging from 1,107-1,523 m.a.s.l., Eastern Ethiopia (Figure 1).

Study design

A community-based cross-sectional study was conducted from January 2019 to July 2021in Doba, Gemechis, and Mi'eso Districts following (Berhe et al. 2016). Socio-economic data, including informant characteristics, honey production management constraints, and perception of the local community on seasonal availability of melliferous plants, were collected using semi-structured questionnaires and group discussions following Coh-Martínez et al. (2019). Questions on the seasonal availability of honey plants and honey production management were addressed to hive owners in the study area. A stratified sampling technique was employed to select the three districts based on resource availability, agroecology, and environmental variations (Binford et al. 2004). In addition, field surveys were conducted on neighboring natural forest areas and community home gardens to portray the pollen and nectar potentials of plants. Quni Forest from Gemechis District, Ades Forest from Doba District, and Asebot Forest from Mi'eso District were purposively selected for the same purpose.

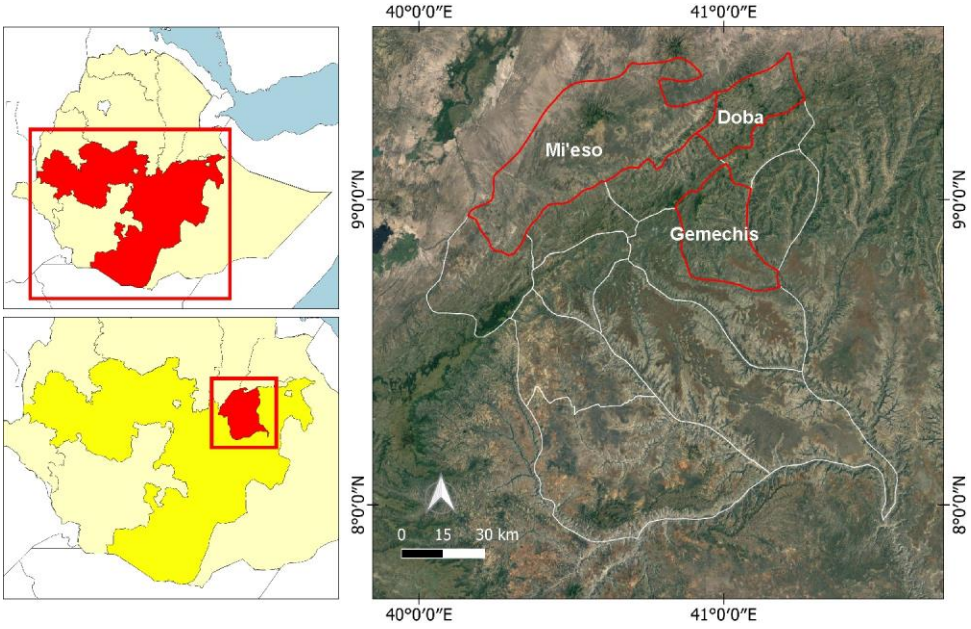

Figure 1. Map of Doba, Gemechis, and Mi'eso District in Ethiopia

Sample size and informant selection

The total sample size was determined following (Berhe et al. 2016) and using an assumption of a 95% Confidence Level (CL), 0.05 margin of error, 50% proportion, and 10% non-respondent rate. The sample size was determined using the following formula:

$$n = (Z\alpha/2)2 * P(1 - P)/W2$$

Where n is the sample size, W is the margin of error, and P is the population proportion.

Thus, 422 household sample sizes were proportionally allocated to each district (Doba, n= 126; Gemechis, n= 173; Mi'eso, n= 123) using the single population proportion formula following (Berhe et al. 2016).

Vegetation and field surveys

To portray the real experiences of the local communities on beekeeping practices, assess the challenges and constraints they face, and substantiate the reliability of data gathered through interviews and group discussions, guided field surveys were conducted in the three districts (Cheng et al. 2020). Plant specimen collection, processing, and identification were done in the field by allowing the involvement of elderly community members for vernacular name identification and specimen collection following (Martin 1995; Kent 2012). Before processing identification, specimens were stored in the Oda Bultum University Biology Department, Ethiopia, for referencing purposes following (Kent 2012). Our field study, including plant material collection, complied with the IUCN policy statement on research involving species at risk of extinction, the convention on the trade in endangered species of wild fauna and flora, and other relevant institutional and national guidelines and legislation.

Data collection instruments

Both qualitative and quantitative data collection approaches were employed to collect relevant data. Field surveys, focus group discussions (FGD), and semi-structured questionnaires were used to collect data from study participants. The semi-structured questionnaire included the following main questions, among others, i.e., (i) What are the major melliferous plants found in your locality? In which month/s do they flower? (ii) Which proper bee management practices do you prefer to do? (iii) What type of beehive do you use for honey production? (iv) In which seasons do you think the scarcity of bee forages happened? (v) In which season do colony performance become high? (vi) What are the main constraints in honey production systems?

Data analysis

Vegetation data analysis

Sorenson's similarity index was used to analyze the relative occurrence of melliferous plants in the three districts and its implication for honey productivity. Plant species were grouped into four plant habits: trees, shrubs, lianas, and herbs.

Socio-economic data analysis

Data were analyzed using descriptive statistics such as mean frequency and percentage, and graphics were presented using tables and figures.

RESULTS AND DISCUSSION

Socio-economic characteristics of respondents

A total of 422 respondents were randomly selected for the socio-economic survey (Table 1). The respondents were selected from Doba (n= 126), Gemeches (n= 173), and Mi'eso (n= 123) to include the relatively higher and lower altitudinal ranges. Most of the respondents (n=92; 85.18%) were males indicating males' great interest in devoting their time to honey production and management than their female relatives. Concerning the different age groups, respondents ranging from 30-49 showed the highest (44.79%), followed by people 50 years and above (28.43%). Concerning their educational status, the majority of the respondents (46.92%) were illiterates, followed by respondents who completed their basic education (22.52%), Primary school (19.19%), and Secondary school and above educational levels (11.37%), respectively.

Floral diversity

A total of 120 melliferous plants distributed under 108 genera and 55 families were identified from the three districts (Figure 2). Fabaceae contains the highest number of species (n= 12, 10%), followed by Asteraceae (n= 9, 7.5%). Euphorbiaceae, Poaceae, and Rosaceae were represented by 5 species (4.17%). Finally, Flacourtiaceae and Myrsinaceae were represented by 4 species each (3.33%). Hence, the above seven families were exclusively represented by 44 melliferous plants (36.66%). Gemechis District comprises 70 melliferous plants (58.33%), followed by Doba and Mi'eso Districts with 47 (39.17%) and 42 (35%) plant species, respectively.

Melliferous species similarity in the study districts

Our study evidenced wide-ranging melliferous plant species distribution patterns in the three districts (Table 2). Some 37.5% of species overlap was observed between Gemechis and Doba Districts. Moreover, 50.40% and 15.3% melliferous species similarities were observed between Doba and Mi'eso and Gemechis and Mi'eso Districts, respectively.

The melliferous species were distributed under 44 (36.66%) trees, 42 (35%) shrubs, 29 (24.16%) herbs, and 5 (4.16%) climber plant habits (Figure 3).

Seasonal availability of melliferous plants

Melliferous plant resource availability across the different months was recorded from group discussions and field surveys. First, results were converted into numerical values as 1 (plants blooming in that specific month) and 0 (plants that cannot bloom in that specific month). Then, the values were summed up and computed into percentages to ascribe the relative proportion of blooming melliferous plants each month for seasonal availability analysis (Figure

4). Our result evidenced nonconformity of floral resource availability across different months. For example, there was a critical shortage of bee forage in the dry season (January to March). In addition, in June, December, and April, the majority of the respondents viewed that there was also a moderate shortage of bee forage. On the other hand, there was less/mild shortage of bee forage in July and November.

Apart from a presence-absence inquiry for melliferous plant blooming, respondents were also asked to classify the 12 months of the year against the extent of floral resource availability (Figure 5). The criteria used to evaluate the different months against floral resource availability were adequate to supply of melliferous plant resources (where there is sufficient access to melliferous plant resources like pollen, nectar, and others to support the honey bee colony), less/moderate supply of melliferous plant resources (where the honey bee could support its colony but surplus production of honey is restricted), and a critical shortage of melliferous plant resources (where it become hard for the honey bee colony to collect floral resources to support the colony resulting in colony size decrement and migration). Most of the local community agreed that adequate melliferous plant resources supply was mown from late June to early December. At the same time, a critical shortage of melliferous plant resources occurred from the end of November to early May.

Table 2. Sorenson's similarity index in the study districts

No.	Study site	Sorenson's similarity index (%)		
		Gemechis	Doba	Mi'eso
1	Gemechis			15.3
2	Doba	37.5		
3	Mi'eso		50.40	

Table 1. Socio-economic status of respondents

Description	Range	Study districts		
		Doba (N = 133,939)	Gemechis (N = 184,238)	Mi'eso (N = 130,709)
Sex	Male	116	160	116
	Female	10	13	7
Age group	18-29	40	43	30
	30-49	48	82	59
	>50	38	48	34
Educational status	Illiterate	73	77	48
	Basic Education	18	43	34
	Primary School	25	29	27
	Secondary Schools and above	10	24	14
Altitudinal range		1149-2773 m.a.s.l.	1300-3017 m.a.s.l.	1107-1523 m.a.s.l.

Note: N: Inhabitants

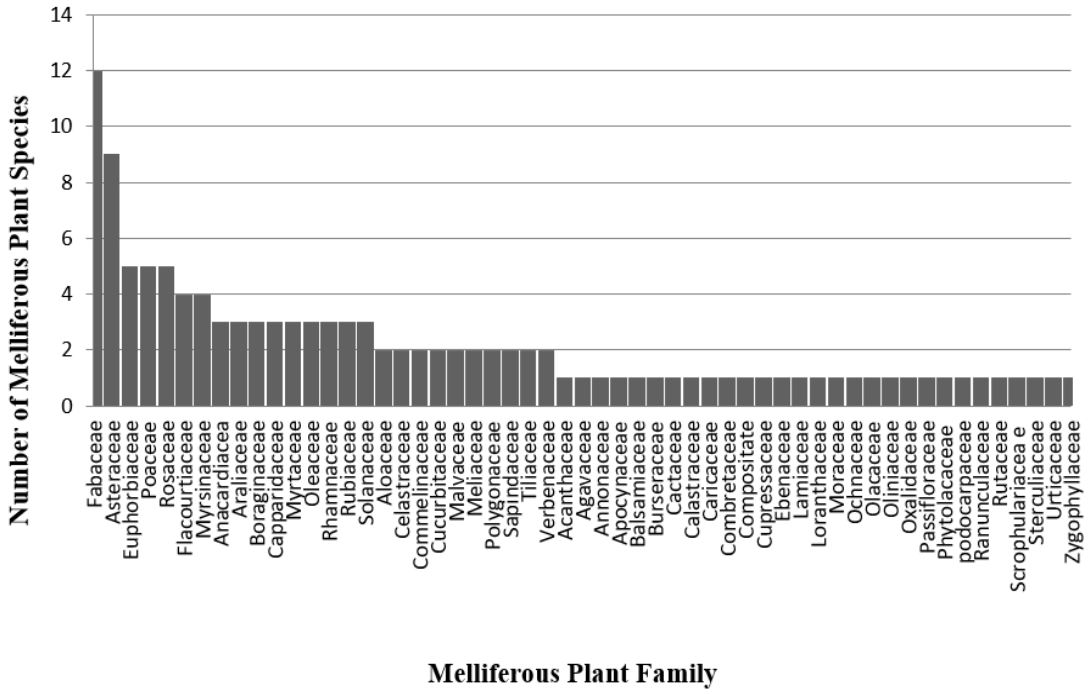

Figure 2. Distribution of melliferous plants across plant families

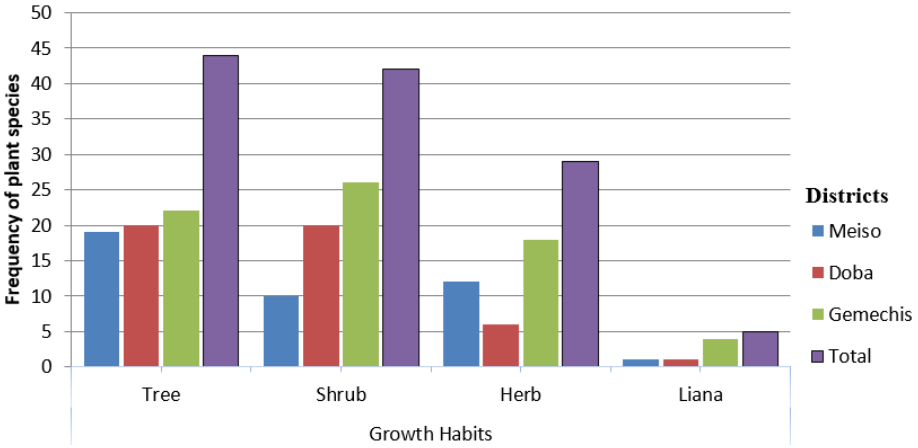

Figure 3. Plant habits of Gemechis, Doba, and Mi'eso Districts in Ethiopia

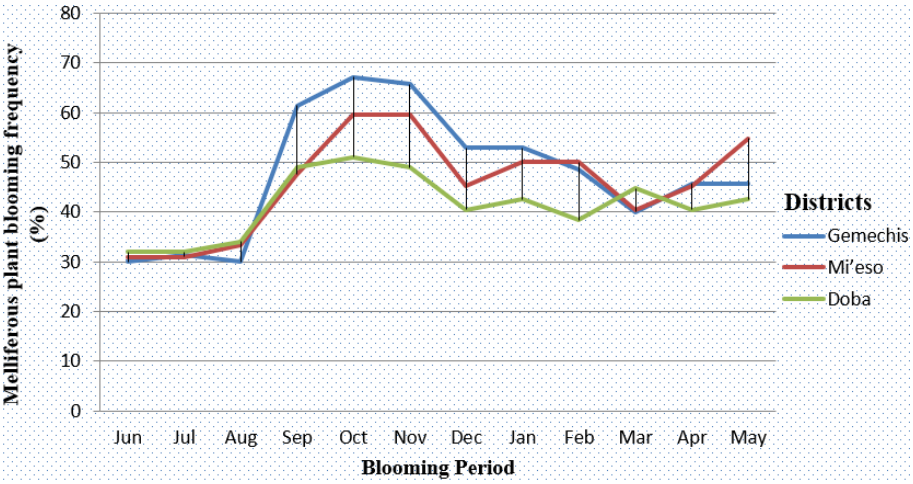

Figure 4. Floral phenology of Gemechis, Doba, and Mi'eso Districts in Ethiopia

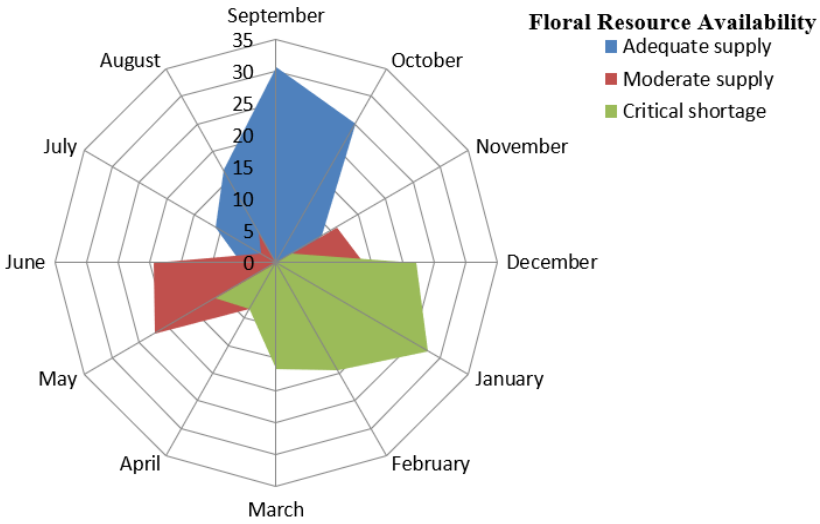

Figure 5. Annual floral resource availability in the study area

Table 3. Honey production management and constraints

No.	Constraints	Frequency	Percentage
1	Lack of nutrition, including melliferous honey plant and adequate water,	131	31.04%
2	Improper practices such as smoking, crowdedness, pesticide usage, poisonous weeds, and poor swarming management	59	13.98%
3	Honey bee predators, such as pests and birds	67	15.88%
4	Lack of Beekeeping knowledge and management skills	93	22.04%
5	Lack of equipment, such as a modern beehive and others	72	17.06%
Total		422	100%

Honey production management and constraints

A total of 422 respondents participated in this socio-economic survey to identify the honey production constraints (Table 3). The study revealed that lack of nutrition, improper management practices, honey bee predators, and lack of beekeeping knowledge and equipment were the five most important constraints deleteriously influencing the honey quality and amount in the study area. Among the above-listed constraints, lack of nutrition expressed in honey melliferous plant availability and adequate water accessibility were ranked first (31.04%) for reducing the quality and quantity of honey production by consuming honeybee products and/or killing bees themselves. Of all the 422 respondents, 93 (22.04%) cited the second honey productivity constraint: lack of beekeeping knowledge and management skills.

Farmers' perception and knowledge of the contribution of melliferous plants to honey productivity

The local community was asked whether they have experience planting honey melliferous plants to increase their honey productivity. The results showed that most respondents (61.37%) poorly understand the shortage of bee forage plants in the locality and have little experience planting melliferous honey plants for productivity. However, the rest of the respondents (39%) have a good awareness of the shortage of bee forage plants resulting in experiences of planting honey melliferous plants for honey production, which also positively impacts gross floral diversity.

Honey production practices and prospects

A total of 106 beekeepers were purposively selected for the honey productivity survey. Of these beekeepers, 70.75% preferred an exclusively traditional type of beehive; while, 19.81% had a modern type of beehive. The remaining 9.44% of beekeepers have both traditional and modern types of beehives. The honey productivity of traditional hives ranged from 2 to 10 kg/hive/year with a 4.8 kg/hive/year average yield. The honey productivity of modern hives ranged from 8-18 kg/hive/year with a mean yield of 12 kg.

Honey bee colony strength

The number of bees/colony, total comb area, and the proportion of comb that contained honey, brood, and pollen storage were the parameters used to assess the colony strength across the following season (Mushonga et al. 2019). From this study, it has been revealed that from strong to very strong, colony strength has been shown starting from the end of August to November (Figure 6).

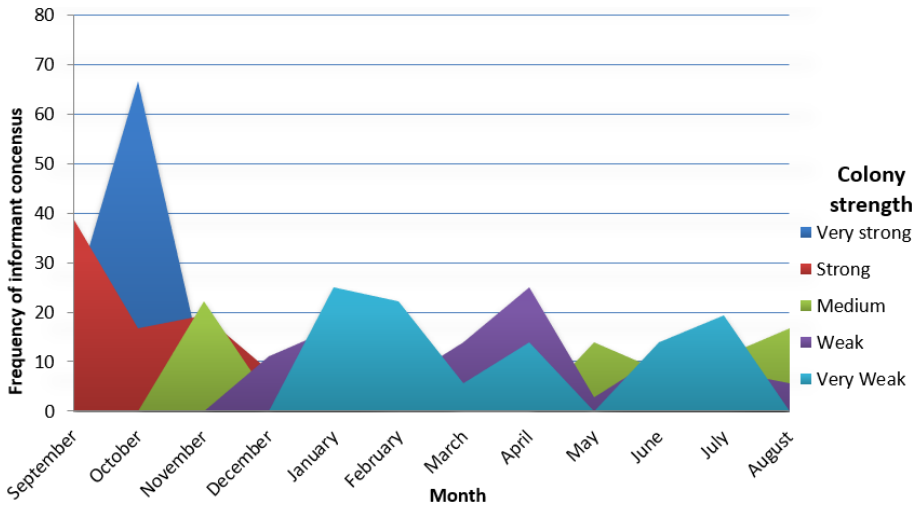

Figure 6. Months of the year in which bee colonies become strong

This intensive colony strength was observed following the winter season when floral diversity was also outsized. On the contrary, weak and very weak colony strength has been observed from mid-November to August, where either a small amount of precipitation is collected or the huge rain during July and August distracts the floral resources. Similarly, medium colony strength is also observed from May to June, apart from November. As a result, this finding revealed that the colony strength period is directly related to the floral availability time.

Discussions

Domination of male's traditional perception and practice of honey production activities in the study area showed that the perception of original male works should be done only by males (Kiptot and Franzel 2012; Tulu et al. 2020; Bihonegn and Begna 2021). The result implicated that all beekeeping interventions should include gender issues into consideration to utilize the generous women's power to enhance the productivity of the sector (Cohen and Lemma 2011; Mburu 2015; Mushonga et al. 2019). Furthermore, our study evidenced a relatively higher number of illiterate respondents than educated ones, as reported by Coh-Martínez et al. (2019) and Olana and Demrew (2019). The high illiterate proportion may challenge the honey productivity sector by hindering technology adoption and exploiting advanced training for innovative working knowledge acquisition (Coh-Martínez et al. 2019; Mulatu et al. 2021).

The floral diversity of the study area (with 120 species, 108 genera, and 55 families) is attributed to agroecological variabilities, including topography, climate, and farming practices (Teferi 2018). Our study evidenced that Fabaceae and Asteraceae have high melliferous species richness in agreement with other studies elsewhere (Dukku 2013; Olana and Demrew 2019; Khalid and Hamed 2021). According to (Venjakob et al. 2022), the families of Fabaceae and Asteraceae (both very attractive for pollinators) are particularly pronounced for their carbohydrate contents. Therefore, honey bees could benefit from many floral resources from these richest botanical families with the highest number of individuals (Filho et al. 2015). We evidenced a relatively higher procurement of plants in the Gemechis District than in the Doba and Mi'eso Districts, with 70, 47, and 42 plants, respectively. As with studies by (Fikadu et al. 2014; Sewale and Mammo 2022), the high species diversity of the Doba District was possibly ascribed to the existence of a preference for environmental/ecological gradients with which the biotic community interacts for the same token. In contrast, the low plant diversity procurements in Doba and Mi'eso Districts might be attributable to anthropogenic, topographic, and/or biological factors. Our study evidenced that the study area as a whole and/or individual study districts have higher or comparable melliferous plant diversities than the Gergera watershed in the Eastern Zone of Tigray with 52 plant species (Tekeba 2011), Zone two of the Afar region with 31 plant species (Reda et al. 2018), and Kilte Awlaelo District in Eastern Zone of Tigri with 20 plant species (Yetimwork et al. 2015).

Similar to our findings, the dominancy of melliferous plants with tree and shrub habits over herbs and climbers was documented elsewhere in the country (Dukku 2013; Kebede and Samuel 2016; Olana and Demrew 2019). The higher availability of melliferous plants with tree and shrub habits may contribute to sustainable honey production, especially at times of longer drought, as perennials often have a specific but lengthy blooming time. According to Coh-Martínez et al. (2019), the largest number of tree species in the study area is related to the knowledge of the local beekeepers, who focus primarily on the trees that bloom most of the year. However, some studies controvert lower tree and shrub melliferous plant habits than herbs and climbers (Wubie et al. 2014; Gebru et al. 2015; Addi and Bareke 2019).

Adequate knowledge of the melliferous plants and flowering times are prerequisites for establishing an apiary site (Novac 2017; Olana and Demrew 2019; Shegaw and Giorgis 2021). In addition, the temporal dynamics of plant phenology resulting in colony strength variations need proper interventions employed during those dearth periods to harvest honey sustainably and other honey bee resources (Tefera 2005; Teklu 2017; Dereje et al. 2020).

Lack of nutrition, improper management practices, honey bee predators, and lack of beekeeping knowledge and equipment are constraints hindering potential honey productivity in the study area, which is in agreement with previous studies (Sahle et al. 2018, Sebho and Baraki 2018; Dereje et al. 2020; Bihonegn and Begna 2021). Dependency on traditional honey production systems in the study area, which is also in line with the study of (Olana and Demrew 2019), may be attributed to the high financial demands of modern bee hives (Sahle et al. 2018). This study revealed that honey productivity varies with the type of beehive that the beekeepers use, which is congruent with similar studies by (Tarekegn and Ayele 2020). Similar results were also recorded in the Jimma and Illubabur Zones of the country (Welay and Tekleberhan 2017).

The colony strength and honeybee products mostly depend on the availability and type of melliferous plant next to the level of colony management practices (Bista and Shivakoti 2001; Gebru et al. 2016). The study area's major and minor honey harvesting periods are from September to December and May to June, respectively. This presence of a significant relationship between floral availability and the strength of bee colonies is also supported by the study by (Olana and Demrew 2019).

In conclusion, the present finding from the three districts (i.e., Doba, Gemechis, and Mi'eso) revealed that the study area is enriched with diversified melliferous plants for a sustainable honey production system. About 101 honey bee plant species in 88 genera and 48 families were identified from the study area. The plant species have shown variation in their distribution against the 48 families. The agroecology variation among the three study districts defines the type of flora and their floral phenology. The comprehensive floral phenology shows that there are major and minor honey harvesting seasons in the study area besides the dearth period, where there is poor floral flowering time resulting in weak colony strength and poor

honey productivity. The local community has appreciably good knowledge of the types of honey melliferous plant, their floral phenology, and the management constraints on honey productivity. However, the community has poor experiences of purposeful planting of honey melliferous plants and little participation of females in the beekeeping business. Therefore, technology transfer, such as using modern beehives, awareness creation on progressive females and youths participation, and improved beekeeping activities for enhanced honey production, should improve honey productivity in the study area.

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Effect of foliar zinc application on growth and yield of rice (*Oryza sativa*) in the Indo-Gangetic Plains of India

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Abstract. *Saikh R, Murmu K, Sarkar A, Mondal R, Jana K. 2022. Effect of foliar zinc application on growth and yield of rice (*Oryza sativa*) in the Indo-Gangetic Plains of India. Nusantara Bioscience 14: 182-187.* A field experiment was conducted on rice cv. Satabdi (IET-4768) to investigate the effect of foliar zinc application at different stages during the post-Kharif season of 2019. The field experiment was carried out at 'C' block farm of (B.C.K.V), Kalyani, India, with eight different foliar 0.5% 0.5% $ZnSO_4$ ($ZnSO_4$) are T_1: Control (without foliar application), T_2: Foliar application of 0.5% $ZnSO_4$ at Panicle Initiation, T_3: Foliar application of 0.5% $ZnSO_4$ at Booting, T_4: Foliar application of 0.5% $ZnSO_4$ at Panicle Initiation and 1 week after flowering, T_5: Foliar application of 0.5% $ZnSO_4$ at Panicle at 1 week after Flowering, T_6: Foliar application of 0.5% $ZnSO_4$ at Panicle at 2 weeks after flowering, T_7: Foliar application of 0.5% $ZnSO_4$ at Panicle at 1 week and 2 weeks after flowering and T_8: Foliar application of 0.5% $ZnSO_4$ at Panicle Initiation, Booting, 1 week and 2 weeks after flowering respectively in randomized complete block design with three replication. The result of the experiment revealed that rice plants treated with the combination of T_4i.e. Foliar application of 0.5% $ZnSO_4$ at Panicle Initiation and 1 week after flowering have resulted in the highest grain yield of 5.09 t/ha, which was 50.59% higher (3.38 t/ha) than the T_1i.e. the control. Furthermore, residual nutrient status was also highest in the plot treated with T_4i.e. Foliar application of 0.5% $ZnSO_4$ at Panicle Initiation and 1 week after flowering.

Keywords: Foliar application, rice, seed zinc, seedling growth, $ZnSO_4$

INTRODUCTION

Cereals are the prime contributor of Zn to the world's population, particularly for rural communities, which is an essential source for most of the cereal-based food products that are quite deficient in meeting human demands (Juliano 1993; Siahpoush and Darvishnia 2019). Among the cereals, rice (*Oryza sativa* L.) is the principal food source, contributing to a major dietary energy requirement of more than 90% of the global population consumed (Jana et al. 2020). A foliar spray of Zn is the most effective way, rather than soil application, to improve the quality production of crops (Yuan et al. 2013). On average, about 30% of arable lands in West Bengal are under deficiency of available Zn (Singh 2009). Zinc deficiency is most commonly adjusted through zinc sulfate ($ZnSO_4.7H_2O$) because of its high solubility and low cost (Mollah et al. 2009; Fageria et al. 2011). Based on the discussion above, providing Zn to plants (for example, by applying Zn-fertilizers to soil and/or to foliar) appears vital to ensure breeding efforts' success in boosting zinc concentration in grains. Zn's Foliar applications improve grain quality significantly (Mondal et al. 2019). Foliar application is linked with the advantage of fast and effective utilization of nutrients, reduction of losses through leaching, fixation, and regulating the nutrient uptakes of plants. Foliar nutrition is regarded as an essential approach to fertilization at appropriate growth stages as the applied nutrients can easily penetrate through leaf cuticles that can improve the better utilization of the crop, causing rapid utilization of nutrients to reduce the cost of cultivation and minimize crop production. Therefore, applying nutrients such as foliar spray has great potential in enhancing the higher content of this nutritionally important element, and assessing their Zn use efficiencies upon different modes of Zn fertilization has become high-priority research for overcoming Zn-related nutritional disorders in humans and plants. Developing rice variety with high Zn content through the process of "biofortification" aims to combine high mineral content with grain quality (Prasad et al. 2012), improving yield as well as resistance to pests and disease (Graham et al. 2001). Finally, it is the most economical way to achieve quality production and yield, especially when sink competition for carbohydrates occurs while nutrient uptake is restricted.

MATERIALS AND METHODS

Experimental site and weather data

The field experiment was conducted in the post-Kharif season of 2019 at 'C' Block Farm of Bidhan Chandra Krishi Viswavidyalaya (B.C.K.V.), Kalyani, Nadia, West Bengal, India, to study on "Effect of foliar zinc application at different growth stages on seed zinc concentration and yield of rice." The farm where the experiment was conducted is situated in the New Alluvial Zone (NAZ) of West Bengal. The farm is situated at 22°57′ N latitude and 88°20′E longitude with an altitude of 9.75 m above mean

sea level, and the ecosystem is on medium land. The farm is situated in the New Alluvial Zone of West Bengal under the sub-tropical climate with high summer temperature, erratic rainfall, high humidity, and short-mild winter. The Monthly weather phenomenon of rice (*O. sativa*) during the growing period is presented in Figure 1. The long-term average annual rainfall is about 1,396 mm; 70-80% comes from the southwest monsoon, with its onset in the region during the second week of June.

Treatments details

Foliar application of zinc was applied with 0.5% zinc sulfate ($ZnSO_4.7H_2O$) solution in rice at different growth stages. The solution was prepared by dissolving $ZnSO_4$ powder with triple distilled deionized (TDI) water. The solution was poured into the sprayer and applied to the whole plant during the morning hours. The rate of applications was 900-1,000 L ha^{-1}.

The treatments are T_1: Control, T_2: Foliar application of 0.5% $ZnSO_4$ at Panicle Initiation, T_3: Foliar application of 0.5% $ZnSO_4$ at Booting, T_4: Foliar application of 0.5% $ZnSO_4$ at Panicle Initiation and 1 week after Flowering, T_5: Foliar application of 0.5% $ZnSO_4$ at Panicle at 1 week after Flowering, T_6: Foliar application of 0.5% $ZnSO_4$ at Panicle at 2 weeks after flowering, T_7: Foliar application of 0.5% $ZnSO_4$ at Panicle at 1 week and 2 weeks after Flowering and T_8: Foliar application of 0.5% $ZnSO_4$ at Panicle Initiation, Booting, 1 week and 2 weeks after flowering.

Crop and soil management

The Rice variety Satabdi (IET-4786) was taken for the entire field experiment. A 100% RDF (120 kg N, 60 kg P_2O_5, and 60 kg K_2O per ha) or 75% of it was given to the respective plots as per the treatments. The entire dose of phosphate, potash, and ½ N was applied at basal, and the remaining ½ of N was top-dressed in two equal splits at 30 and 60 days after transplanting (DAT). Urea, single super phosphate, and muriate of potash were used to supply nitrogen, phosphorus, and potassium, respectively. Soil pH was determined from soil-water suspension in 1:2.5 ratios with the help of a systronics processor-based pH meter (Model-361) described by Jackson (1973). Organic carbon of soil was estimated by oxidizing the soil with a mixture of 1(N) $K_2Cr_2O_7$ and concentrated H_2SO_4 and back-titrating the excess $K_2Cr_2O_7$ with standard ferrous ammonium sulfate solution using diphenylamine indicator following the wet digestion method of Walkley and Black (1934) as outlined by Jackson (1973). The available nitrogen content of the soil was determined using hot alkaline potassium permanganate for oxidative hydrolysis of the soil organic matter, and liberated ammonia was then absorbed and condensed in boric acid and titrated against standard 0.02 (N) H_2SO_4 following the method as proposed by Subbiah and Asija (1956). The available phosphorus content of the soil was extracted with 0.5 (M) $NaHCO_3$ solution at pH 8.5 following Olsen's method (Olsen et al. 1954). The available potassium in the soil was determined by shaking 5 g of the soil sample with 25 mL neutral 1 (N) ammonium acetate solution for 5 minutes. The soil-extractant suspension was leached through Whatman No.1 filter paper.

Statistical analysis

All the data were statistically analyzed following the standard procedures Gomez and Gomez (1984) described. In addition, the data were treated for analysis of variance and least significant difference (P=0.05) to compare the effect of foliar zinc application on the growth and yield of rice.

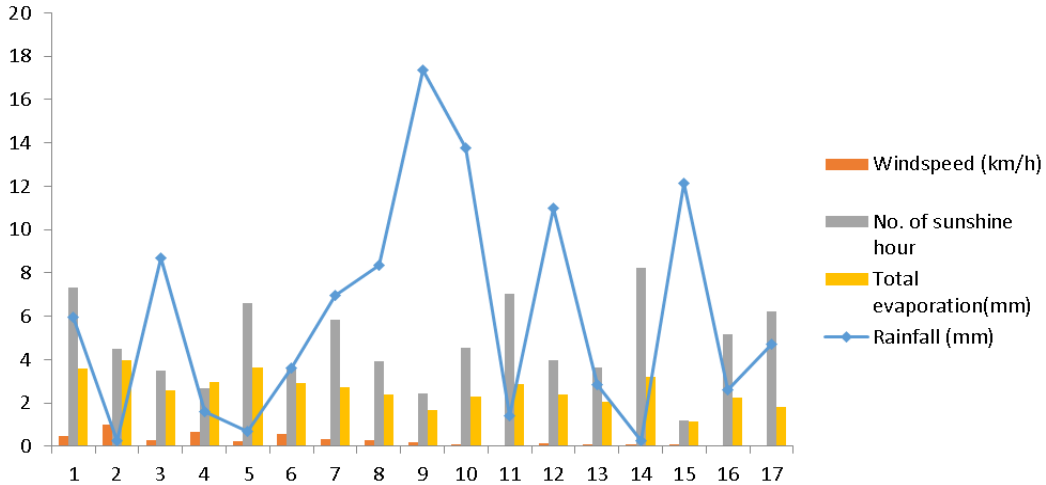

Figure 1. Monthly weather phenomenon of rice (*Oryza sativa*) during the growing period **(*** Source: AlCRlP on Agro-Meteorology. Directorate of Research, BCKV. Kalyani, Nadia)

RESULT AND DISCUSSION

Growth attributes

The height is greatly influenced by the foliar application of 0.5% $ZnSO_4$ at different growth stages of rice plants (Table 1). A maximum plant height of 93.00 cm was observed in the T_4 treatment (Panicle initiation+1week after flowering). The increase in growth parameters might be ascribed to an adequate supply of zinc that might have increased the availability and uptake of other essential nutrients and thereby resulted in improved crop growth. The minimum plant height (82.27 cm) was recorded in the control. At harvesting (100 DAT), the maximum dry matter accumulated in the case of treatment T_4 (983.66 g/m^2) followed by T_8 (952.93 g/m^2) treatment. Dry matter production was very poor (825.06 g/m^2) in the control situation, i.e., where no fertilization was done. Muthukumararaja et al. (2012) reported that maximum dry matter production of (2.98 gm per pot) at tillering and (40.93 gm per pot) at panicle initiation was obtained with the application of 5 mg Zn per kg, which was about 44 to 60% greater as compared with the treatment that did not receive zinc. Notable changes were also reflected in the Crop Growth Rate (CGR) of Kharif rice from 60 DAT to harvest with the different times of foliar management. The crop growth rate of rice at the stage of 60 to 100 DAT varied from 11.77 to 13.34 g/m^2/day, with a variation of 13.33%. The maximum crop growth rate (13.34 g/m^2/day) was observed in T_4, and the lowest CGR (11.77 g/m^2/day) was recorded in the control treatment (T_1), but the treatments were non-significant. At harvesting (100 DAT), there was a significant difference between the LAI of rice under different treatments. The maximum LAI of rice (2.44) was recorded at treatment T_5, followed by T_8 treatment. The lowest value of LAI was recorded from treatment T1, i.e., the control obtained a value of 1.95. At harvesting (100 DAT), there was a significant difference between the root length of rice under different treatments.

Foliar zinc application resulted in a significant impact on root length over control plots. The maximum root length of rice (25.73 cm) was recorded at treatment T4 followed by T_8 treatment (Panicle initiation + Booting + 1 week and 2 weeks after flowering). The root volume of the rice crop was found to vary from 20.34 to 25.82 cc/hill, with a variation of 26.94%. Amongst all treatments, the T_4 treatment (Panicle initiation + 1 week after flowering) recorded a maximum root volume of 25.82 cc/hill, whereas the control treatment recorded the least value of root volume. At harvesting (100 DAT), there was a significant difference between root volumes of rice under different treatments. Foliar zinc application resulted in a significant impact on root volume over control plots. The maximum root volume of rice (31.12 cc per hill) was recorded at treatment T_4 followed by T_8 treatment (Panicle initiation + Booting + 1 week and 2 weeks after flowering. At harvesting (100 DAT), there was a significant difference between the root dry weight of rice under different treatments. Foliar zinc application resulted in a significant impact on root dry weight over the control plots. The maximum root dry weight of rice (10.64 gm) was recorded at treatment T_8 followed by T_4 treatment (Panicle initiation + 1 week after flowering). The lowest root value by dry weight of rice (4.23gm) was recorded from T_1i.e the control.

The increase in growth parameters might be ascribed to an adequate supply of zinc that might have increased the availability and uptake of other essential nutrients, thereby resulting in improved crop growth in rice. Foliar Zn application significantly increased Zn concentration in rice seeds of paddy and other crops. This result is in good agreement with the previous studies in wheat, during which seed Zn concentration was increased by foliar Zn application up to 3 times compared with no Zn application (Yilmaz et al. 1997; Karim et al. 2012). Foliar Zn applied is well absorbed and transported through the phloem, as shown in wheat using radiolabeled Zn (65Zn), especially in plants grown under low Zn supply (Erenoglu et al. 2002). Although xylem transport of Zn has been indicated to be more important for Zn accumulation in rice grain than re-translocation of Zn from the leaves (Palmgren et al. 2008), the results of this study, however, suggested that phloem transport of Zn from leaf and stem tissue may additionally play a greater role in the enrichment of grains with Zn.

Yield attributes and yield

All yield attributes and yield are presented in (Table 2). The most important yield component of rice in terms of panicle per square meter area was found to be statistically significant as influenced by different times of foliar zinc application during the Kharif season. It has been observed that the panicles/m^2 was to tune 182.33 to 270 with a variation of 48.08% among the treatments. The highest number of panicles/ m^2 was recorded at T_4 (270 /m^2), followed by treatment T_8 (267/m^2) which were statistically at par. The lowest number of effective tiller /m^2 was observed in the T_1i.e., the control treatment (182.33/m^2). It has been observed that the panicle length of rice varied from 23.52 to 26.06 cm with a variation of 13.09% over the control. The maximum panicle length (26.06cm) was achieved in the T_5 treatment, i.e., 1 week after flowering, followed by the T_2 treatment, i.e., Panicle Initiation (23.52 cm). The control treatment observed a very short panicle length (23.52 cm). The inflorescence length of rice, called panicle, was significantly different from the treatment. It has been observed that the panicle length of rice varied from 23.52 to 26.06 cm with a variation of 13.09% over the control. The maximum panicle length (26.06cm) was achieved in the T_5 treatment, i.e., 1 week after flowering, followed by the T_2 treatment, i.e., Panicle Initiation (23.52 cm). The control treatment observed a very short panicle length (23.52 cm).

However, the number of filled grains/panicles varied from 91.08 to 116, and the variation was recorded at 27.06%. The number of filled grains/panicle was maximum in T_8 treatment, i.e., panicle initiation+ Booting + 1 week and 2 weeks after flowering (116). The lowest number of filled grains/panicles was obtained in the T_1 treatment (91.08), which was the control plot. The other treatments significantly differ from each other. Foliar zinc application

at different growth stages significantly improved rice grain yield due to the improvement in yield attributing characters. The grain yield of rice cv. IET 4786 (Satabdi) varied to the range of 3.38 to 5.09 t/ha, and the variation was recorded by 50.59%. The highest grain yield (5.09 t/ha) was recorded in the T_4 treatment Panicle initiation + 1 week after flowering, which was significantly higher than other treatments. The control plot recorded the lowest yield (3.38 t/ha). The straw yield of rice significantly increased from 5.56 to 6.88 t/ ha, and the variation was recorded by 23.74%. Application at Panicle initiation + 1 week after flowering (T_4) recorded the highest straw yield of 6.88 t/ha, followed by T_8 (6.86 t/ha) and T_7 (6.82 t/ha). The lowest straw yield was recorded in the control plot (5.56 t/ ha). The harvest index of rice increased from 37.80% to 42.52%. The harvest index differed significantly among different treatments. However, treatment T_4 (Panicle initiation + 1 week after flowering) gave the highest value of harvest index (42.52%) compared to the rest of the treatment combinations. The lowest value of 37.80 was recorded in T_1. The higher harvest index values indicated the greater translocation of photosynthates from source to sink and better portioning towards reproductive growth.

It seems obvious that only a small amount of foliar-applied Zn is translocated into paddy rice after early foliar application, while a greater amount of Zn is translocated into paddy rice grain and enters into brown rice after the late foliar application of Zn. This result agrees with Phattarakul et al. (2012), who showed that a foliar Zn spray applied at late growth to rice grown under field conditions caused a greater increase in grain Zn than a foliar Zn spray before the flowering stage. Similar results were also found in field-grown-wheat (Cakmak et al. 2010). Furthermore, Abid et al. (2002) observed that the growth and rice yield were significantly enhanced by applying Zn, Fe, and Mn alone or in various combinations.

Table 1. Effect of foliar zinc application on growth parameters at different growth stages of rice (*Oryza sativa*)

Treatment	Plant height (cm)	Dry matter accumulation (g/m^2)	Crop growth rate (g/m^2/day)	Leaf area index	Root length (cm)	Root volume (cc/hill)	Root dry wt.(gm)
T_1	82.27	825.06	11.77	1.95	22.02	26.58	4.23
T_2	89.36	948.38	12.95	2.24	22.45	27.46	5.03
T_3	83.02	844.53	12.22	2.02	23.38	28.56	6.38
T_4	93.00	983.66	13.34	2.15	25.73	31.12	10.30
T_5	86.96	882.60	12.74	2.44	21.25	29.29	8.50
T_6	91.44	925.60	12.92	2.32	22.86	28.45	6.82
T_7	84.36	933.47	13.31	2.37	23.24	29.76	8.28
T_8	84.34	952.93	12.30	2.45	23.74	29.90	10.64
SD	4.02	55.75	0.55	0.19	1.34	1.44	2.32
CD at 5%	NS	3.45	NS	0.03	0.05	0.32	2.41

Note: T_1: the Control, T_2: Foliar application of 0.5% $ZnSO_4$ at Panicle Initiation, T_3: Foliar application of 0.5% $ZnSO_4$ at Booting, T_4: Foliar application of 0.5% $ZnSO_4$ at Panicle Initiation and 1 week after flowering, T_5: Foliar application of 0.5% $ZnSO_4$ at Panicle at 1 week after flowering, T_6: Foliar application of 0.5% $ZnSO_4$ at Panicle at 2 weeks after flowering, T_7: Foliar application of 0.5% $ZnSO_4$ at Panicle at 1 week and 2 weeks after flowering and T_8: Foliar application of 0.5% $ZnSO_4$ at Panicle Initiation, Booting, 1 week and 2 weeks after flowering

Table 2. Effect of foliar zinc application on yield parameters at different growth stages of rice (*Oryza sativa*)

Treatment	No. of panicle/m^2	No. of filled grain/panicle	Panicle length (cm)	Grain yield (t/ha)	Straw yield (t/ha)	Harvest Index (%)
T_1	182.33	91.08	23.53	3.38	5.56	37.80
T_2	263.00	96.73	25.51	4.60	6.65	40.88
T_3	212.00	93.00	25.27	4.26	5.96	41.68
T_4	270.00	110.33	25.00	5.09	6.88	42.53
T_5	240.00	104.00	26.06	4.58	6.22	42.40
T_6	247.00	101.00	24.29	4.55	6.77	40.19
T_7	252.00	105.00	25.16	4.68	6.82	40.69
T_8	267.00	116.00	24.77	4.72	6.86	40.75
SD	30.33	8.51	0.77	0.50	0.50	1.50
CD at 5%	3.36	1.81	1.06	0.02	0.01	0.09

Note: T_1: the Control, T_2: Foliar application of 0.5% $ZnSO_4$ at Panicle Initiation, T_3: Foliar application of 0.5% $ZnSO_4$ at Booting, T_4: Foliar application of 0.5% $ZnSO_4$ at Panicle Initiation and 1 week after flowering, T_5: Foliar application of 0.5% $ZnSO_4$ at Panicle at 1 week after flowering, T_6: Foliar application of 0.5% ZnSO4 at Panicle at 2 weeks after flowering, T_7: Foliar application of 0.5% $ZnSO_4$ at Panicle at 1 week and 2 weeks after flowering and T_8: Foliar application of 0.5% $ZnSO_4$ at Panicle Initiation, Booting, 1 week and 2 weeks after flowering

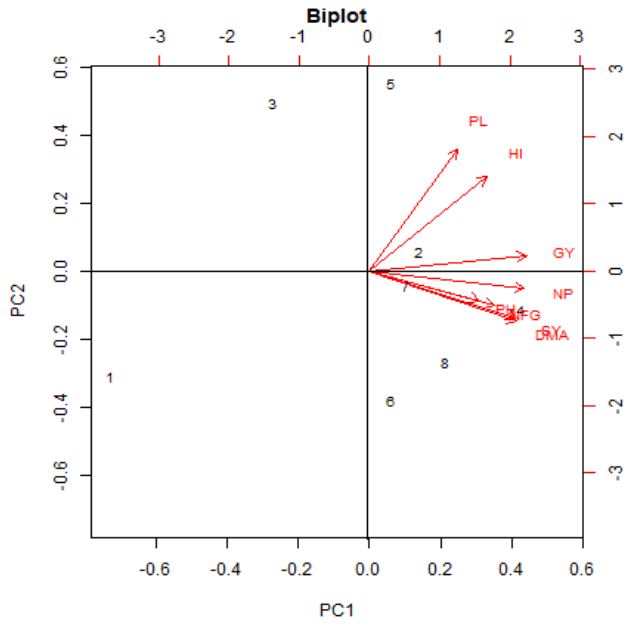

Figure 2. PCA graphs of 8 treatments for yield, yield components, and agronomic traits of the experiment. PH: plant height; DMA: dry matter accumulation; NP: numbers of panicle m-2; NFG: numbers of filled grain per panicle; PL: panicle length; GY: grain yield; SY: seed yield; HI: harvest index; (1-8) denotes (T1-T8)

Table 3. Effect of foliar zinc application on soil nutrient status at post-harvest soil

Treatment	Available N (kg/ha)	Available P_2O_5 (kg/ha)	Available soil K_2O (kg/ha)
T_1	162.41	25.81	167.41
T_2	188.01	37.19	207.77
T_3	204.62	40.45	208.61
T_4	215.53	44.93	219.56
T_5	208.27	42.34	213.19
T_6	189.25	38.54	185.02
T_7	192.40	37.65	191.00
T_8	186.64	37.51	183.36
SD	16.43	5.64	17.98
CD at 5%	2.32	2.10	2.51

Note: T_1: the Control, T_2: Foliar application of 0.5% $ZnSO_4$ at Panicle Initiation, T_3: Foliar application of 0.5% $ZnSO_4$ at Booting, T_4: Foliar application of 0.5% $ZnSO_4$ at Panicle Initiation and 1 week after Flowering, T_5: Foliar application of 0.5% $ZnSO_4$ at Panicle at 1 week after Flowering, T_6: Foliar application of 0.5% $ZnSO_4$ at Panicle at 2 weeks after Flowering, T_7: Foliar application of 0.5% $ZnSO_4$ at Panicle at 1 week and 2 weeks after Flowering and T_8: Foliar application of 0.5% $ZnSO_4$ at Panicle Initiation, Booting, 1 week and 2 weeks after flowering

Principal Component Analysis (PCA)

The PCA comprising two principal components (PC1 and PC2) explained 86.49% of the total variation in the experiment (Figure 2). In the experiment, PC1 explained 69.81%, and PC2 explained 16.68% of the total variation. A strong correlation was observed between various components, yield, plant height, dry matter accumulation, panicle number, filled grain per panicle, panicle length, stover yield, and harvest index. Superimposition of 8 treatments on rice yield and yield components revealed that foliar application of 0.5% $ZnSO_4$ at panicle initiation and 1 week after flowering with RDF on rice cv Satabdi (IET 4786) produced the highest values for the given attributes and showed significant correlation with these parameters (Figure 2).

Soil nutrient status

After harvesting Kharif rice, available soil nitrogen, phosphorus, and potassium varied significantly with different treatments (Table 3). The available nitrogen in the soil varied from 162.41 to 215.53 kg/ha with a variation of 32.70%. The available nitrogen was more (215.53 kg/ha) in the plot fertilized with foliar management at Panicle initiation + 1 week after flowering (T4), followed by the plot fertilized with foliar management at (T5). The lowest available nitrogen (162.41 kg/ha) was recorded in the control plot. Conversely, the soil's phosphorus availability varied from 25.81 to 44.93, with a variation of 74.07%. The highest available phosphorus was recorded in treatment T4,i.e., Panicle initiation + 1 week after flowering, followed by treatment T5 and T3, and the lowest available phosphorus was recorded in the control plot (T1). The treatments significantly differ from each other. The available potassium in the soil varied from 167.41 to 219.56 kg/ha with a variation of 31.15%. The highest available potassium was obtained from the T4 treatment, i.e., Panicle initiation + 1 week after flowering (219.56 kg /ha) followed by T5 (1 week after flowering) treatment (213.86 kg /ha), and the lowest data was obtained in the control plot where no foliar were applied (167.41 kg/ ha). The highest value of available N, P_2O_5, and K_2O was found in T4 treatment, maybe because they can improve soil properties and structure, leading to an increase in soil fertility. The results are close finding with Murmu et al. (2013).

In conclusion, the effect of foliar zinc application at different growth stages significantly influenced the growth as well as yield and yield attributes of rice cv. Satabdi (IET-4786) grew in the post-Kharif situation. Therefore, Zn in rice grain can be effectively raised by foliar Zn application, particularly when Zn is sprayed after flowering. Therefore, considering values and based on the results obtained in the present study, it may be concluded that foliar application of 0.5% $ZnSO_4$ at Panicle Initiation, Booting, 1 week and 2 weeks after flowering and foliar application of 0.5% $ZnSO_4$ at Panicle Initiation and 1 week after flowering, could be recommended due to better grain and straw yield obtaining values of 4.72t/ha and 6.88t/ha respectively.

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Development of semi-artificial feed in the larva stage of the black soldier fly *Hermetia illucens* (Diptera: Stratiomyidae)

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Abstract. *Anasya AD, Sugiarto, Mahajoeno E. 2022. Development of semi-artificial feed in the larva stage of the black soldier fly* Hermetia illucens *(Diptera: Stratiomyidae). Nusantara Bioscience 14: 188-194.* Alternative food sources in fish farming besides pelleted feed are generally used, but many have also used biotic materials, larvae of the black soldier fly (*Hermetia illucens* L.). Maggot stadia larvae have a high protein content of more than 19%, could be mass-produced, are low-priced, and has a fast growth time. Therefore, high levels of protein and nutrients in maggots can be increased through suitable semi-artificial formulations. The purpose of this study was to determine the nutritional value of the semi-artificial feed recipe given and to determine the effectiveness of the semi-artificial feed recipe in increasing the nutritional value and survival of larvae. Data analysis used qualitative analysis with descriptive analysis method and quantitative analysis by determining the amount of increased nutritional value of *H. illucens*. The research method was carried out in several stages, including (i) insect rearing obtained 2nd generation of tillers (F2); (ii) manufacture of semi-artificial feed recipes; (iii) calculation of insect survival; (iv) measurement of larval mass weight after treatment; (v) testing of nutritional content value includes water content, fat content and protein content of *H. illucens* after treatment. Results of the evaluation larval survival after treatment were effective enough 100%, larval instar life phase was between 28-32 days, while the highest increase in the nutritional value of larvae water content was 45.90%, fat content 7.25%, and protein content 45.95%, the average increase in mass weight of larvae was 12.50%.

Keywords: Artificial feed, black soldier fly, *Hermetia illucens*, larval survival, the nutritional value of maggot

INTRODUCTION

Indonesia has the potential for livelihood in the field of fisheries after agriculture. Indonesia occupies the number three position in world aquaculture fish production and is a fish supply country that meets the international fish market (FAO 2020). Many fish ponds are currently being developed for export and import purposes. Fish farmers require special fish feed to increase the weight and quality of fish. The feeds usually used are pellets, fish meals, essential amino acids, fatty acids, and other micronutrients. The high price of fish feed causes farmers to need other solutions to reduce feed costs, one of which is replacing pellets with artificial feeds that are high in protein and at low costs.

The feed that can be used as an alternative source of animal feed, especially fish is the black maggot soldier fly *Hermetia illucens* (Linnaeus, 1758) (Diptera: Stratiomyidae). The selection of *H. illucens* as an insect substitute for animal feed because it has a high protein content and can be mass-produced, is rich in protein at every stage of its metamorphosis with good protein quality. In addition, it is more environmentally friendly and can be bred sustainably for diet protein. Therefore, *H. illucens* provides adequate nutritional value for animal feed and ensures optimal digestive problems and intestinal health in fish (English et al. 2021). According to (Riddick 2014), another aspect considered for using insect species for feed purposes is that they can be reared in bulk to provide large quantities at an affordable price.

Improving the quality of live insects, especially in the *H. illucens*, can be used accurately as a natural animal feed resource for aquaculture. The insect has a short life cycle, is included as a source of protein, and is rich in nutrients at each stage of the larval phase. The high protein content and the ability of *H. illucens* to be used as animal feed encourage the production of *H. illucens* of high quality. Using *H. illucens* as a feed ingredient must have guaranteed chemical safety (Lieven et al. 2021). The composition of the larval-rearing substrate is an important factor that must be considered because it has a bioaccumulative risk of various organic compounds in the larvae being bred, so semi-artificial feed for *H. illucens* is an alternative that can be developed.

Mass rearing of insects requires the development of artificial feeds that can meet nutritional needs and ensure good insect rearing. In addition, feed formulation greatly influences larvae's survival, developmental rate, and yield (Danieli et al. 2019). Therefore, although the semi-artificial feed is used to reproduce insects, it is necessary if insects are needed in large quantities regularly and continuously.

Semi-artificial feed is expected to produce rapid growth and development of larvae. It has a high nutritional value, and it is necessary to multiply insects so that good-quality larvae will be produced, such as high protein, low-fat

content, and no bioaccumulative risk of organic compounds using feed semi-artificial. Maggot growth is largely determined by the medium in which the larvae grow. The type of *H. illucens* likes the distinctive aroma of the media, but not all media can be used to lay eggs for *H. illucens* (Tomberlin et al. 2018).

The objective of the study was to determine the nutritional value of the semi-artificial feed recipe to increase the nutritional value and survival of larvae. The survival, growth, and bioconversion ability of black soldier fly larvae are determined by the type of food consumed by the larvae (Lalander et al. 2019). Food has a very large role in insect nutrition, so semi-artificial feeding must be adjusted to the needs and nutrition of insects. Research on artificial feeds for insects, especially *H. illucens*, has not yet been widely developed. Given the excellent benefits of *H. illucens* for animal feed, especially in the fisheries sector, it encourages efforts to increase the nutritional value so that they are good for use as an alternative fish feed.

MATERIALS AND METHODS

Materials

The materials for making artificial feed include ground sweet corn, rice polish, soybean flour, chicken feather flour, DL-methionine, vitamins and premixes, limestone, salt, monocalcium phosphate, sodium bicarbonate, and tetracycline formaldehyde 37%. The materials used to analyze fat content include filter paper and hexane solvent. Analyzing protein content includes carrageenan, nitrogen, hydrochloric acid (HCl) solution, 15% phenol, HgO, aquadest, 50% NaOH, K_2SO_4 concentrated, gauze, and red metal indicator.

Procedures

This research was carried out from March to June 2022 at the Integrated Laboratory, Universitas Sebelas Maret, Surakarta, Indonesia. The research method was an experimental method using a Completely Randomized Design (CRD) with four treatments, each repeated twice. The treatment used is as follows: (i) diet 1 treatment was formulated according to standard specifications of feed sources that are often used for rearing larvae for the control feed formulations with a feed intake of 120 mg per day. The nutritional composition of this feed was used as the control because it was formulated according to the nutritional specifications needed by insects per day; (ii) treatment using rice polish by mixing 10% chicken feather flour; (iii) treatment resembles the ideal amino acids profile of *H. illucens*; and (iv) treatment with rice polish added 10% chicken feather flour, each component of which has been adjusted.

Population and sample

The population in this study was the black soldier fly (BSF) insect (*H. illucens*), obtained from around community farms in the Surakarta area. First, wild parent BSF insects (F0) were caught using insect nets (Hoffman et al. 2021). Then, BSF breeding would be carried out. The sample in this study was the larvae resulting from the mating of three pairs of brooders which would then be transferred to a 100 mL pill pot until the age of five days and then transferred to a 15x15x15 box until the age of 18 days before being transferred to the cage and became generation 1 (F1). Breeding was continued to obtain offspring until the second generation, and further testing using larvae in second-generation 2 (F2).

Insect propagation

Insect propagation initially, three pairs of F1 adult insects were taken randomly (Woods et al. 2019), later becoming brood stock, then transferred to cages of 30x30x30 cm^3 to produce eggs. After the eggs hatch, they are transferred to 100 mL pill pots. Then after reaching the age of five days, they are transferred to a 15x15x15 cm^3 box where the environment is very concerned with both temperature and humidity, then 100 larvae will be occupied in on nursery box (Figure 1). The propagation of the test insects was carried out in 8 boxes, with each being reared and repeated twice.

Preparation of semi-artificial feed

The manufacture of semi-artificial feed recipes that will be used adapts Woods et al. (2019) research by replacing additional feeds made compatible with *H. illucens* larvae, such as spray-dried blood meal replaced with rice polish because they have the same high protein and can be used properly as a safe food. In addition, pig brains are replaced with chicken feather flour, adding more DL-methionine tailored to the needs of *H. illucens* and changing the composition dose ingredient. Artificial feed comprises the needs for the growth and development of *H. illucens* (Table 1). The feed that will be used is wet feed during the larvae phase until it reaches the 4th larva stage, then it will be converted into dry feed until the pupa phase. According to Bekker et al. (2021), *H. illucens* grows and develops at 30-70% substrate humidity. Optimal moisture content for larval development, ultimate weight, feed conversion efficiency, and yield is found in substrates with moisture content in the range of 50-80% (Cheng et al. 2017).

The parameters observed in this study were the survival rate of larvae, larval weight, totals of the larval instar phase, and analysis of the nutrients contained in the larvae after being treated.

Calculating the survival rate of larvae

The survival rate of larvae can be seen from the ability of *H. illucens* to adapt to artificial feeds and can be calculated based on the percentage (%) of its effectiveness by reducing the standard feed. The survival rate, including the percentage of the number of live larvae and the number of larvae used during rearing, was calculated using Wirawan et al. (2021) as follows:

$$SR = \left(\frac{Nt}{No}\right) \times 100\%$$

Where:
NT: Number of live larvae
No: Number of initial larvae that the research carried out

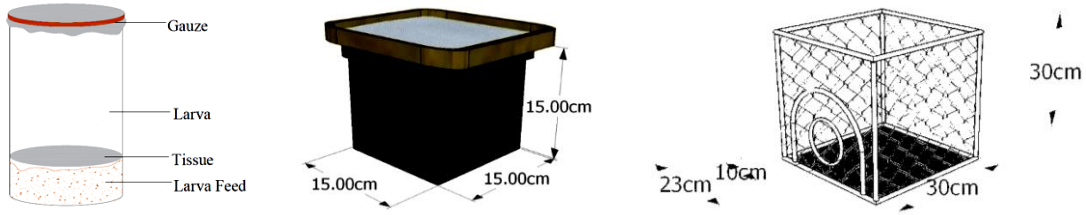

Figure 1. Insect propagation. A. 100 mL pill pot for five days old larvae, B. cage box for larvae, C. net cages for adult insect

Table 1. Preparation of semi-artificial feed recipes for *H. illucens*

Component	Diet 1 (control) %	Diet 2 %	Diet 3 %	Diet 4 %
Sweet corn	77.18	-	75.47	-
Rice polish	-	85.63	-	79.62
Soybean flour (46% crude protein)	18.62	0.22	20.12	4.43
Chicken feather flour	-	10.00	-	10.00
DL-methionine	0.07	0.07	0.29	0.27
Vitamin + premix	0.15	0.15	0.15	0.15
Limestone	1.82	1.87	1.81	3.13
Salt	0.25	0.21	0.25	0.21
Monocalcium phosphate	1.67	1.64	1.67	1.64
Sodium bicarbonate	0.24	0.21	0.24	0.21
Tetracycline formaldehyde 37%	0.01	0.01	0.01	0.01

Testing the weight of the larvae after being given treatment

The weight of the larvae was observed by measuring the weight of the larvae produced in the media for each treatment. Data collection on larval weight was obtained after the larvae were harvested at prepupa age; each treatment medium would be weight using a digital scale with a specification of 0.001 g. The result of the weight of larvae in each treatment medium was then recorded, and the total weight was calculated.

Larvae instar phase

The initial phase can indicate the feed media's success in meeting the nutritional needs of insects. The total instar phase of *H. illucens* shows the larval growth rate on the artificial feed given. Semi-artificial feed is expected to produce rapid growth and nutritional development and has high value. Therefore, feed nutrition is very influential on the total insect instar period.

Testing the nutritional content of larvae after treatment

After treatment, testing the nutritional status of *H. illucens* was done by analyzing the water, fat, and protein content contained in the larvae. The test method used in analyzing the moisture content by the oven (thermogravimetry), fat content using the micro-Soxhlet method (Zozo et al. 2022), and protein content using the Kjeldahl method (Maehre et al. 2018).

Analysis of water content

The principle of water content analysis is the distillation method, which is based on the direct measurement of the amount of water removed from the sample by evaporation (Deman et al. 2018). Water content is based on the difference by weight sample that determines the actual moisture before and after drying (Muchdar et al. 2021). Therefore, water in materials can be used as an index of stability during storage and a determinant of organoleptic. The water content analysis, according to Deman et al. (2018) procedure is as follows: (i) petri dish to be used is dried in the oven for 15 minutes, then cooled in a desiccator; after it has cooled, the weight will be calculated; (ii) the sample is weighed as 5 g put in a petri dish after that is dried in an oven for 6 hours at a temperature adjusted of 105°C; (iii) petri dish is cooled in a desiccator for 30 minutes and weighed again; (iv) petri dish dried in the oven again so that a constant weight can be obtained; (v) the following formula can calculate the percentage of water content.

$$\%= \frac{W1-W2}{W} \times 100\%$$

Where:
W1: Weight of sample and petri dish before drying (g)
W2: Weight of sample and petri dish after drying (g)
W : Sample weight (g)

Analysis of fat content

Fat content analysis is defined as separating fat from the sample using a specific fat solvent. Analysis of the fat content profile was aimed at the essential fatty acid profile, especially the content of linoleic acid, which is important for the physiological process and the creation of linolenic acid (Adamkova et al. 2017). The procedure of analyzing fat content, according to Zozo et al. (2022), is as follows: i) 5 g homogenized sample was weighed and wrapped using filter paper, placed in a Soxhlet extraction device mounted above the condenser, and a fat flask below; ii) hexane solvent is used, and reflux is carried out until the solvent drops into the fat flask. Next, the solvent in the fat flask is distilled and collected; iii) the fat flask containing the extracted fat was then dried in an oven at 105°C for 5 hours; iv) the fat flask is then cooled in a desiccator for 20 minutes and weighed; v) the percentage of fat content can be calculated using the following formula.

$$\% = \frac{W2-W1}{W} \times 100\%$$

Where:
W2: Final weight
W1: Initial weight
W: Sample weight

Analysis of protein content

Analysis of the protein content is the process of releasing nitrogen from protein in the material using sulfuric acid, which is carried out by heating. The Kjeldahl method is widely used to quantify insects' crude protein content ranging from 8 to 70% dry mass. The procedure evaluates the total Nitrogen (N) concentration, converted to a protein conversion factor (Levi and Jean 2017). The procedures for analyzing protein levels, according to Zozo et al. (2022), i) a 5 g sample is weighed, mashed, and put in a 30 mL Kjeldahl flask, added HgO concentrated; ii) destruction was carried out until a clear green color; iii) adding 60 mL of water and 50% NaOH solution before being transferred to a distillation flask; iv) distillate is accommodated in an Erlenmeyer flask which has previously been filled with 0.1 N and red metal indicator and then distilled accommodated; v) contents of the Erlenmeyer flask are titrated with 0.1 N NaOH until the yellow color is obtained; vi) the protein content is calculated based on the N content in the material by multiplying the conversion factor. The formula for calculating protein content is as follows.

$$\%N = \frac{(V1 - V2) \times N \times 14{,}007}{W\ (mg)} \times 6.25 \times P \times 100\%$$

Where:
%protein= % conversion factor (6.25)

Data analysis

Qualitative data analysis using descriptive and quantitative analysis was applied to determine the increase in the nutritional value of *H. illucens*. Quantitative analysis was used to determine the impact of changes in nutritional value due to the diet carried out with the amount of food given and the time required for larval growth after being given an artificial Diet, all compared in all treatments. Tests using the Tukey HSD test included calculating the survival rate of *H. illucens* and testing the weight of the larvae after being treated (F2). Meanwhile, the water content analysis, fat content analysis, protein content analysis, number of eggs, and total instar mass used analysis of difference test (significance was set $p<0.05$) were tested using ANOVA paired t-test. In addition, Duncan's distance test and statistical data analysis were calculated using the IBM SPSS version 22 variance to test the comparative hypothesis between samples and compare the highest increase in nutritional value in each treatment.

RESULTS AND DISCUSSION

The *H. illucens* is an insect with very good benefits, and it has a high nutritional and protein content, so that it can be used as an alternative to animal feed at a low price. Previous studies reported the nutritional content of *H. illucens* by providing feed from household waste for larval development and insect pupa stages. However, this study provides insight into changes in nutritional value and variations in the larval phase of *H. illucens*. The evidence of the information obtained supports insect breeders, fish farmers, researchers, and the entire animal feed industry, especially fish. In addition, research is valuable to improve mass breeding and development of livestock products using the *H. illucens* to meet future challenges in providing safe and viable protein as a priority for the global community. In this study, artificial feed contributed to a significant increase in larval weight of 12.50% and a short maggot life span compared to natural feeding. In addition, artificial feed increases the nutritional value of larvae with high protein and low-fat content, making it suitable for fish feed.

Larvae survival rate

The survival of insects is closely related to abiotic factors such as humidity. Temperature and relative humidity affected the survival of eggs. The relationship between development rate and temperature fits well with the linear relative humidity models (Mourao et al. 2021). The humidity in the study was 71%, *and H. illucens* had a high survival rate of 100% (Table 2). Environmental humidity is another important requirement for egg development and the survival rate of insects (Mourao et al. 2021). Humidity influences insect life. The optimum humidity of each insect varies according to each development's type and stage of life.

The higher humidity, the insect's body temperature increases; if the humidity decreases, the insect's body temperature will decrease. Humidity that's too high or too low can inhibit the activity and life of an insect, except for insects that can live in wet environments. The optimum humidity of each insect varies according to the development type and stage of life. In addition, humidity affects the evaporation of the insect's body preference for places to live and hide (Hasan et al. 2017). Calculation of the relationship between humidity and survival of larvae using the ANOVA was $1{,}6 \times (10)^{(-4)}$). At the same time, the Tukey HSD test, which was determined to be $p<0.05$, got a p-value of $2{,}54 \times (10)^{(-8)}$). It was concluded that humidity and feed nutrition significantly affected the survival rate of *H. illucens* in all treatments.

Humidity in this study tends to be stable. Efforts were made to stabilize the humidity by spraying with water using a spray in the study area during the day. The environmental humidity should not be too low or too high. Humidity that is too high encourages the growth of fungi and microorganisms. Air humidity that is too high or too low can inhibit the activity and life of an insect, except for insects that can live in wet environments.

Table 2. Percentage of survival *H. illucens*

Feed medium	RH (%)	Nt		$\overline{X}$ SR (%)
		1	2	
Diet 1	71	100	100	100
Diet 2	71	100	100	100
Diet 3	71	100	100	100
Diet 4	71	100	100	100
Σ		400	400	

Testing the weight of larvae after being treated

Testing the weight of the larvae after harvesting shows the success or failure of the given feed medium. The success index of larval weight is based on the high or low results of weighing the larvae after being harvested. The results showed that the weight of larvae (Table 3), the highest occurred in the Diet 3 of 20.85 g total weight, with an average weight of larvae per individual at 0.21 g. In comparison, the lowest weight occurred in Diet 2 at 18.45 g of total weight, with the weight of the larvae per individual at 0.18 g. The average weight of larvae in all Diet treatments was 19.34 g, while the average weight per individual was 0.19 g.

Meanwhile, according to calculations using ANOVA analysis where the significance is set at $p<0.05$, the p-value ANOVA is 0.00, while according to calculations using the Tukey HSD analysis with a significance set $p<0.05$, the results are 0.00, which means that the feed medium used is by the nutrients needed by *H. illucens*, besides that the nutrition of the feed has a significant effect on increasing the weight of the larvae after treatment. Based on level, Diet 1 and 4 feeds did not significantly affect larval weight after treatment. Diet 2 and 4 feeds did not significantly affect larval weight after treatment. Diet 3 significantly differs from Diet 1, 2, and 4. Diet treatments had a highly significant effect on the weight of larvae after treatment (Table 3).

The weight of the larvae after harvest indicates the ability of the larvae to accept the given feed medium and an indication of the success of the feed media used. The quality and availability of food affect the growth and development of insects. The stunted development and growth will cause individuals to have small sizes when the larvae to adult insects. Slower development leads to high mortality (Holmes et al. 2020). Differences in nutrition in the feed will cause various nutritional content of larvae. Weighed of *H. illucens* larvae is strongly influenced by the media they breed (Tschirner and Simon 2015). Media that has good quality and quantity has a good impact on the nutritional value of *H. illucens* produced, as well as accelerates its growth and development. Feed formulation affects larvae's survival, developmental level, and yield (Pacheco et al. 2022).

Larval instar stage

The fastest instar phase results occurred in Diet 3 with a total larval instar period of 28 days, while the longest larval instar period occurred in Diet 2 with a total larval instar period of 32 days (Figure 2). The relationship between the nutrition of the feed given and the total larval instar mass was calculated using the ANOVA paired t-test, which was set at $p<0.05$. The results were $1{,}5x\ (10)^{(-8)}$, and it can be concluded that the nutrition of the feed given has a very significant effect on the total insect instar period. Nutrition has a very large influence on the length of the insect instar. The instar period is faster, allowing the nutritional needs to grow and develop to be met properly (Holmes et al. 2020).

The nutrients an organism absorbs from the diet are essential for development and determine how organisms can maximize their fitness. Alteration in diet quality during development has a wide-ranging effect on many life history characteristics (Chapman et al. 2013). A diet's two major nutritional components that contribute to development are proteins and carbohydrates. Protein provides essential amino acids necessary for viability. Imbalances in dietary amino acids can significantly affect development and the total instar stage. Carbohydrates provide energy for development and represent the mechanism by energy stored for the future (Nash and Tracey 2014).

Water content

The results of testing the water content in this study showed that the highest water content was found in Diet 3, with a total water content of 45.90%, while the lowest water content occurred in Diet 1, with a water content of 41.90%. Based on analysis using the ANOVA paired t-test obtained a significant result of 0.02. Furthermore, based on Duncan's Multiple Distance analysis at a significance level of 5%, the treatment of Diet 1 (control) was significantly different compared to diets 2, 3, and 4. While feeds 2, 3, and 4 had no significant difference (Table 4).

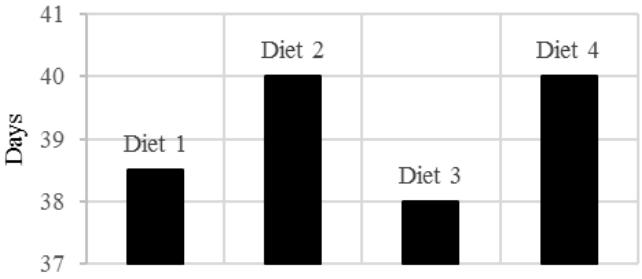

Figure 2. Chart total larval instar phase after treatment

Table 3. The average weight of larvae after being treated

Diet	Treatment (gr)		Average Weight (g)	Standard Deviation
	1	2		
Diet 1	19.40	19.20	19.30	0.14 b
Diet 2	18.70	18.20	18.45	0.35 a
Diet 3	21.30	20.40	20.85	0.64 c
Diet 4	19.00	18.50	18.75	0.35 ab

Note: Average value followed by the same letter in the same column shows that it is not significantly different according to Duncan's Multiple Distance Test at a 5% significance level

Table 4. Analysis of nutrient levels in *H. illucens* larvae

Feed medium	Water content	Fat content	Protein content
Diet 1	41.90±1.41 a	7.25±0.90 b	43.65±1.73 bc
Diet 2	44.20±0.57 b	7.00±1.80 b	41.74±0.54 ab
Diet 3	45.90±0.99 b	5.2±0.10 a	45.95±0.62 d
Diet 4	43.90±0.42 b	6.64±0.87 b	40.25±0.30 a

Note: Average value followed by the same letter in the same column shows that it is not significantly different according to Duncan's Multiple Distance Test at a 5% significance level

Water is the largest part of the body composition of living insects. Almost all reactions in the body of insects require fluids to carry out body metabolism. Nearly 30-60% of the insect body consists of water. The water content in the insect's body helps improve blood circulation in the insect's body. Insects are often dehydrated and freeze to reduce their body air conductivity (Takikawa et al. 2020). For the body's metabolism to run well, it takes a good intake of fluids in the form of feed containing minerals to replace the lost fluids properly. Food has a physiological impact on insects. Most insects are water content, stored in bonds to minimize water evaporation from the body. Therefore, the water content in the insect's body will be greater when compared to the water content in the food. According to Bekker et al. (2021), *H illucens* grow and develop in 30-70% substrate humidity. Substrate 50-80% water content, including optimum moisture content for larval development, final weight, feed conversion efficiency, and yield.

Fat content

Determination of the success of feed nutrition by testing proximate larvae using fat content analysis. Fats and oils are one of the groups belonging to the lipid group. These organic compounds have characteristics that are not soluble in water but in organic solvents such as ether, benzene, and chloroform. Fat is an energy reserve for periods of high energy intensity, such as flying or moving. The role of fat content in the formation of cell membrane structure. The phospholipid content of insects is usually less than 20%, varying according to the life stage and species of insects. Although the fatty acids profile in insects is influenced by the food eaten, cholesterol is the most abundant sterol in insects (Lenka and Anna 2016).

The results of the fatty acid analysis showed that the highest fat content of *H. illucens* maggot occurred in Diet 1 at 7,25%, while the lowest fat content was in Diet 3 with total fat of 5,20%. The calculation data using the ANOVA paired t-test was set at $p<0.05$ the p-value was 0.04. Duncan's multiple distance test was conducted to determine the difference in the increased fat content value in each treatment and to place a distance difference test on each data based on the notation that stated the difference occurred. It was shown that Diet 3 had a significantly different effect on fat content after treatment. Diets 1, 2, and 4 had no significant effect on fat content after treatment (Table 4). This study's low-fat content of *H. illucens* was due to the high water content of the larvae. According to Adamkova et al. (2017), insects that contain relatively large amounts of fat in fish feed are high, and it will cause liver damage in fish, causing death. The fat content allowed in fish feed is between 4-18%. Fat content in each treatment can be used as fish or other livestock feed because the fat content is not more than 18%. The low-fat content in *H. illucens* is due to the high water content in *H. illucens* maggots. Insects have high water, protein, and fat content. Fat content has the opposite relationship with water content; the higher the water content, the lower the fat content (Kroncke and Rainer 2022).

Protein content

Protein is needed in the insect body in large quantities because protein has a role as a neurotransmitter in the insect nervous system (Roriz and Joachim 2013). In addition, ultrastructural studies suggest that proteins are involved in sperm maturation in insects. Protein has an essential function in ovarian maturation in egg formation, so insects require large amounts of protein. *H. illucens* have significant protein and fat content and good amino acids (Xavier et al. 2018).

Analysis of protein content levels in this study was conducted to determine the increase in the nutritional value of *H. illucens* after being given treatment. Data from the analysis of protein levels in this study showed that the highest protein content occurred in Diet 3, with an average protein content of 45.95%. In contrast, the lowest protein content occurred in Diet 4, with an average of 40.25%. The analysis of protein content using ANOVA was determined to have a significant value of $p<0.05$; the results were 0.02. Continued the test with Duncan's multiple distances, and the significance was set at 5%. The treatment feed had a significant effect on all treatments. Diets 1, 2, and 4 showed significantly different protein content effects. At the same time, the Diet 3 feed gave a significantly different effect than other Diet treatments. Therefore, it can be concluded that all feeds in each treatment affected the larvae's protein content (Table 4).

The factor that supported the amount of protein in *H. illucens* is due to the addition of the composition of ingredients added in the feed media, such as DL-methionine and limestone, which is a source of protein and essential amino acids needed by insects. In addition, corn and rice polish have a high protein content. As a result, the *H. illucens* larvae have significant protein and good amino acids. The nutritional composition of the fatty acid and protein profile depends on the development stage, making it a good source of nutrients for animal feed.

The artificial feed given had a significant effect on water content and protein content in all treatments. The feed with the highest nutritional effect was found with basic ingredients of ground corn in the Diet 1 and 3 treatments with high protein content. Still, feed using rice polish is also good for *H. illucens* because the larvae have high water and high-fat content. Relatively low and has a protein content of more than 40%. All treatment feeds can be used and have a good effect on *H. illucens*. The low protein content in larva feed rice polish mixed with chicken feather flour has high protein but is not followed by a digestibility rate of only 5.58% (Nursinatrio and Rudy 2019).

In conclusion, semi-artificial feed in the study on all treatments gave good results on the survival rate of instar larvae to pupae reaching 100%. Feed that gave the greatest larval weight on Diet 3 with 20.85 gr when compared to larvae feed with other media. Diet 3 feed media gave the best number of instars, with a total of 38 days compared to other diets. A feed with increased nutritional value to larva water content occurred in Diet 3 with an average of 45,90% compared to Diet 1 as a control. Fat content analysis in all treatments was lowest on Diet 3 with 5,20% compared to

control. The increase in protein levels was highest in Diet 3, with 45.95% compared to the control and all treatment media. Diet 3 feed medium gave a higher percentage increase in water and protein content compared to all treatments. Decreased fat content is a marker of increasing water content in larvae. The percentage of insect protein content is higher at each instar phase due to the presence of chitin. Diet 3 feed is considered the best for *H. illucens* larvae because it gives higher yields when compared to other diets.

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Suitability of selected legume (*Vachellia* spp.) tree species for forest restoration in the Central Ethiopian highlands

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Abstract. *Asmelash F, Getachew E. 2022. Suitability of selected legume (*Vachellia *spp.) tree species for forest restoration in the Central Ethiopian highlands. Nusantara Bioscience 14: 195-202.* This study aimed to evaluate the comparative suitability of the legume tree species viz., *Vachellia abyssinica* (Hochst. ex. Benth.) Kyal. & Boatwr., *Vachellia etbaica* (Schweinf.) Kyal. & Boatwr., *Vachellia lahai* (Steud. & Hochst. ex Benth.) Kyal. & Boatwr., and *Vachellia seyal* (Delile) P.J.H.Hurter for restoring forests in Central Ethiopian highlands. The suitability of three accessions of *V. seyal* was also compared. The correlation between root nodule number and root Arbuscular Mycorrhizal Fungi (AMF) colonization (RC) and seedlings' growth variables were computed, and the effect of seedling age on nodule number and RC was determined. Seedlings were grown for nine months on degraded local soil in a mesh house in central Ethiopia. We measured shoot height, shoot fresh weight, rooting depth, root nodule number, and RC in the third and ninth months. The one-way ANOVA results indicated that tree species and/or accession (for *V. seyal*) had a significant (p<0.05) effect on all the measured variables except shoot height and rooting depth at the ninth month. Generally, nodule number and RC increased with seedling age. However, according to the independent t-test results, significant (p<0.05) differences were recorded for *V. abyssinica,* with a 57.16% reduction in nodule number, and *V. seyal* accession-1, with a 418.52% increase in RC. The Spearman's rank correlation results indicated that the correlation between nodule number and RC was weak and non-significant (p>0.05) both in the third and ninth months. Based on the measured growth variables, nodule number (N-fixation potential), and RC, *V. etbaica* was the least suitable species for forest restoration in central Ethiopian highlands. The remaining species/accessions are comparably suitable. The *V. abyssinica* lost its comparative fitness with seedling age, maybe because it is a provenance far away from central Ethiopia. However, *V. seyal* accession-3, the furthest provenance, has performed much better. The legume trees of Ethiopia are less studied. Their role as environmental engineers could be better understood by knowing more about their root traits. Therefore, this study could motivate future research in this regard. Long-term experiments are required to consider more legume tree species and provenances in the future.

Keywords: Arbuscular mycorrhizal fungi, nitrogen fixation, nodulation, rhizobium, *Vachellia etbaica*

INTRODUCTION

Forest restoration and conservation are at the core of Ethiopia's climate-resilient green economy strategy; hence it has committed to restoring more than 200 million hectares of forests by 2030 (MEFCC 2018). Most of these forests could be restored on the dry highlands (Pedercini et al. 2021). If this commitment succeeds, it could be crucial to improve the livelihoods of millions of Ethiopians and contribute significantly to climate change mitigation (Strassburg et al. 2020). However, the soils of the Ethiopian dry highlands are too deficient in the essential nutrients (particularly N&P) required for tree/shrub seedlings' field survival, growth, and forest development (Asmelash et al. 2021a). Accordingly, past experiences indicate that despite several forest restoration efforts in these areas, there has been a limited success (Asmelash et al. 2019). One of the reasons for little restoration success could be related to tree species selection (Bekele et al. 2021). Therefore, future forest restoration programs should prioritize tree species selection. In addition, planting legume trees/ shrubs with better N-fixation potential and AMF association could be crucial.

Arbuscular Mycorrhiza (AM) and Rhizobium-Legume (RL) are two of the most important root endosymbiosis that plays key roles in plant nutrition (Barea et al. 2013). Rhizobium can fix N_2 and allow legumes to grow independently of a mineral nitrogen source (Barea et al. 2013; Mahmud et al. 2020). Moreover, legumes could potentially supply N to the associated or succeeding plants (Mahmud et al. 2020). When the soil is deficient in phosphate and nitrates, plants initiate root infection by soil-available AM fungi (Gutjahr 2014). Then, the fungi colonize the root cortex and develop Extraradical Mycelia (ERM), which are very extensive and can significantly increase plants' soil resource acquisition (Asmelash et al. 2016). The role of AM fungi in phosphorus nutrition and forest restoration (Suharno et al. 2017; Asmelash et al. 2016) is well documented. Therefore, legume trees with better N-fixation potential and Arbuscular Mycorrhizal Fungi (AMF) association could preferably be used in forest restoration in the dry Ethiopian highlands.

Currently, about 115 accessions of 20 Vachellia tree and shrub species are conserved in the Ethiopian Biodiversity Institute (EBI) forest gene bank. In the past, there has not been any attempt to evaluate the N-fixation potential and AMF association on these collections.

Therefore, one of the main objectives of this study was to evaluate the N-fixation potential and AMF association of the four *Vachellia* tree species, viz., *Vachellia abyssinica* (Hochst. ex. Benth.) Kyal. & Boatwr., *Vachellia etbaica* (Schweinf.) Kyal. & Boatwr., *Vachellia lahai* (Steud. & Hochst. ex Benth.) Kyal. & Boatwr., and *Vachellia seyal* (Delile) P.J.H.Hurter, conserved in the EBI forest gene bank. Evaluating the comparative N-fixation potential and AMF association of three *V. seyal* accessions was the second main objective of this study. These tree species and accessions were selected since they were the only ones currently conserved in the EBI gene bank suitable for the Ethiopian dry highlands (Hedberg and Edwards 1989).

Previous studies have indicated that the AM and the RL symbioses require a common set of plant genes, constituting a common symbiotic pathway (Barea et al. 2013). Therefore, there could potentially be synergistic AM and RL interactions (Primieri et al. 2021). Hence, we hypothesize that there could be a strong positive correlation between root nodule number and root AMF colonization (RC). Therefore, the third objective of this study was to determine the correlation between nodule number and RC and seedlings' growth variables, i.e., shoot height and shoot fresh weight. Moreover, seedling age could significantly influence root nodulation and/or mycorrhization (Azad et al. 2013; Azad et al. 2016). Hence, determining the effect of seedling age on root nodule number and RC was the fourth objective of the study.

MATERIALS AND METHODS

Seedlings preparation and experiment setup

Seeds of the study species were obtained from the Ethiopian Biodiversity Institute (EBI) forest gene bank (Table 1). The seeds were germinated on filter paper, and then 5 individuals per accession, i.e., 60 seedlings in total, were transplanted on 1-lit plastic pots filled with soil collected from a central Ethiopian highland. The soil is highly degraded (pH = 6.245±0.015, EC = 32.35±0.35ds/m, TN = 0.07%, P (Bray-II) = 4.74 ppm, OM = 5.36%, and CEC = 19.04 m equi/100 g) and its AMF spore abundance was quantified to be 13.95±1.6 g^{-1} (Asmelash et al. 2021a). Treatments were arranged in a split-plot design with third and nine months treatments arranged in separate blocks, and within each block, treatments were arranged in a completely randomized design. Seedlings were watered to field capacity every other day and were grown in the mesh house in EBI, central Ethiopia. The experiment lasted about nine months, counted after seedlings transplantation from 07 October 2021 to 10 June 2022.

Data collection

Data was collected after three and nine months of seedling growth to determine the effect of age on root nodulation and root Arbuscular Mycorrhizal Fungi (AMF) colonization (RC). Seedling shoot height and shoot fresh weight, Relative Growth Rate (RGR) in shoot height and shoot fresh weight, rooting depth, root nodule number, and RC were the measured variables. Shoot height and rooting depth were measured by a ruler, while fresh shoot weight was measured using an analytical balance. The relative growth rate was determined according to Hunt (1990) by the formula RGR= 1/Xt ($\Delta X/\Delta T$), where ΔX is the change in seedling growth (Xn-Xt), and between ranked values, Xn is the measurement at the ninth month, Xt is the measurement at the third month, ΔT is the time for the change in days.

The N-fixation potential was estimated by counting the root nodule number (Brockwell et al. 2005). When possible, the different types of nodules were counted separately (Figure 1). Moreover, the pink/red pigmented nodules were counted separately to represent the effective nodules (Azad et al. 2016). Arbuscular mycorrhizal fungi association was estimated by determining root AMF colonization (Asmelash et al. 2021c). AMF colonization was estimated from 100 intersection points observed under a NOVEX light stereomicroscope at (45x) magnification.

Data analysis

Parametric and, when appropriate, non-parametric one-way ANOVA was computed to determine the effect of tree species and accession on the various seedlings' traits measured at the third and ninth months of growth. When a significant ($p<0.05$) effect was found, pair-wise mean comparisons were carried out between tree species and accessions using Tukey honestly significant difference (HSD) test ($p<0.05$) or Dunn-Bonferroni test ($p<0.05$) respectively for parametric and non-parametric ANOVA. An independent t-test (for equal and non-equal variances) was also computed to determine seedling age's effect on nodule number and root AMF colonization (RC). Finally, Spearman's rank correlation was computed to know the correlation between nodule number and RC and the various seedlings' growth variables. R software version 4.1.1 was used to do all the statistical analyses.

Table 1. *Vachellia* species and accessions selected for the study

Species name	Treatm. code	Acc. no.	Altitude (m a.s.l)	Location	Coordinate	Areal distance to the planting site (km)
Vachellia abyssinica (Hochst. ex. Benth.) Kyal. & Boatwr.	VA	244601	1880	Ngele Borena	N5.02° E40.10°	468
Vachellia etbaica (Schweinf.) Kyal. & Boatwr.	VE	20645	1793	North Wollo	N11.73° E39.65°	312
Vachellia lahai (Steud. & Hochst. ex Benth.) Kyal. & Boatwr.	VL	20647	1971	South Wollo	N11.32° E39.68°	270
Vachellia seyal (Delile) P.J.H.Hurter accession-1	VS1	20652	1889	South Wollo	N11.34° E39.68°	272
Vachellia seyal (Delile) P.J.H.Hurter accession-2	VS2	20651	1599	North Shewa	N9.92° E39.85°	149
Vachellia seyal (Delile) P.J.H.Hurter accession-3	VS3	20673	2038	Tigray/Wukro	N13.93° E39.38°	547

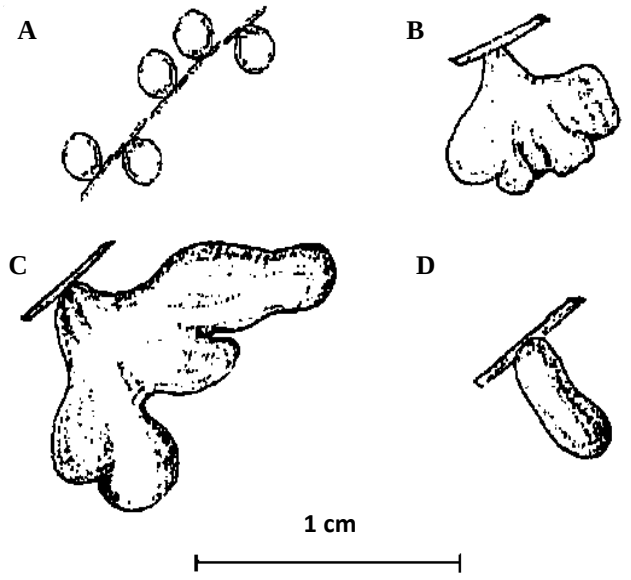

Figure 1. Classification of the shapes of nodules of *Vachellias* (Acacias). A. Globose, B. Coralloid, C. Elongate with branching, and D. Elongate/delicate (Brockwell et al. 2005)

RESULTS AND DISCUSSION

Seedlings growth

None of the seedlings died, and the seedlings' survival was 100% for this experiment. The one-way ANOVA results indicated that, in the third month, tree species and accession had significant effects (p<0.05) on both shoot height and shoot fresh weight. The biggest mean shoot height was recorded for *V. abyssinica* (13.5 cm), which was significantly (p<0.05) and 91.76% greater than the mean shoot height of *V. etbaica* only. The biggest mean shoot fresh weight was also recorded for *V. abyssinica* (0.51 g), which was significantly (p<0.05) and 96.15%, 121.74%, and 131.82%, and 200% greater than the mean shoot fresh weights of *V. seyal* accession-1, *V. lahai, V. seyal* accession-2, and *V. etbaica* respectively. In the ninth month, a significant (p<0.05) tree species and accession effect were found for fresh shoot weight, relative growth rate (RGR) in shoot height, and RGR in fresh shoot weight. No significant effect was found for shoot height and rooting depth (Table 2). Although a significant (p<0.05) one-way ANOVA result was found for fresh shoot weight, the mean difference between species and accessions was not significant (p<0.05) for the Tukey HSD test. However, the biggest mean fresh weight was recorded *V. seyal* accession-3 (0.66 g), and the smallest similar to the third month, was recorded for *V. etbaica* (0.29 g). The biggest mean RGR in shoot height was recorded for *V. seyal* accession-2 (0.0057 cm/cm/day) and was significantly (p<0.05) greater than the mean RGR in shoot height of the remaining species and accessions except for *V. lahai*. The RGR in the height of *V. seyal* accession-3 had a decay rate of -0.0001 cm/cm/day. The biggest mean RGR in fresh weight was also recorded for *V. seyal* accession-2 (0.012 g/g/day) and was also significantly (p<0.05) greater than the mean RGR in fresh weight of the remaining species and accessions except for *V. lahai*. The RGR in shoot fresh weight of *V. abyssinica* was a decay rate of -0.0003 g/g/day. Regarding the difference in mean growth between *V. seyal* accessions, a significant (p<0.05) difference was recorded for mean shoot fresh weight at the third month, RGR in shoot height, and RGR in fresh shoot weight. In comparison, the mean shoot fresh weight of *V. seyal* accession-3 was significantly (p<0.05) and 109.10% greater than the mean fresh shoot weight of *V. seyal* accession-2 (third month), it was the mean RGR in shoot height and fresh shoot weight of *V. seyal* accession-2 that was significantly (p<0.05) greater than the mean RGR in height and weight of both *V. seyal* accession-1 and 3 (Figure 2).

Nitrogen fixation potential and arbuscular mycorrhizal fungi association

Almost all of the nodules we recorded were globose. No branched elongate nodule was recorded, while few elongate/delicate (10%) and coralloid (1.6%) nodules were recorded. Moreover, all the nodules recorded were not pigmented as viewed externally (Figure 3). According to the one-way ANOVA results, tree species and/or accession was found to have a significant (p<0.05) effect on nodule number both at the third and ninth months of seedlings' growth (Table 2). In the third month, no nodule was recorded for *V. etbaica*. However, the biggest nodule number was recorded for *V. seyal* accession-3 (19.2), which was significantly (p<0.05) and 200% and 540% more than the nodule number recorded, respectively, for *V. seyal* and *V. lahai* accession-2. In the ninth month also, the highest nodule number was recorded for *V. seyal* accession-3 (33.8), and this was significantly (p<0.05) and 5,533.33% greater than the nodule number recorded for *V. etbaica* only. Regarding the mean nodule number difference between the *V. seyal* accessions, a significant (p<0.05) difference was recorded in the third month of seedlings' growth but not in the ninth month. Hence, in the third month, the mean nodule number of *V. seyal* accession-3 was significantly (p<0.05) and 200% and 242.86% greater than the mean nodule number of *V. seyal* accession-2 and 1 (Figure 2).

Regarding root AMF colonization (RC), the one-way ANOVA results indicated tree species and/or accession effect at the third and nine months of seedlings' growth (Table 2). *Vachellia lahai* had the highest mean RC at the third (39%) and ninth (68%) months. These values were significantly (p<0.05) and 1,672.73% greater than the mean RC of *V. etbaica* in the third month and 183.33% and 1,600% greater than the mean RC of *V. seyal* accession-2 and *V. etbaica* at the ninth month. In the third month, the mean RC of *V. abyssinica* was also significantly (p<0.05) and 1563.64% greater than the mean RC of *V. etbaica* (Figure 2). Moreover, the mean RC of *V. seyal* accessions was not significantly (p>0.05) different both in the third and ninth months (Figure 2). Arbuscules were the predominant form of root AMF colonization, although extra and intracellular spores and hyphae were also observed (Figure 3).

Generally, both nodule number and RC increased by the age of seedlings. However, according to the independent t-test results, except the nodule number of *V. abyssinica* and the RC of *V. seyal* accession-1, none of the remaining tree species and/or accessions had their nodule number or RC significantly (p<0.05) affected by seedling age, i.e., three vs. nine months (Table 3). Accordingly, the roots *V. abyssinica* produced significantly (p<0.05) and 57.16% fewer nodules in the ninth month compared to the third. On the contrary, the roots of *V. seyal* accession-1 seedlings were significantly (p<0.05) and 418.52% more colonized by AMF in the ninth month compared to the third.

Correlation

The correlation between nodule number and root AMF colonization (RC) was a weak positive non-significant (p>0.05) both in the third and ninth month (Figure 4). The nodule number, shoot height, and fresh shoot weight correlations in the third month were significant (p<0.05), respectively strong and positive. However, nodule number, shoot height, and fresh shoot weight correlations in the ninth month were weakly positive and non-significant (p>0.05). Contrary to this pattern observed regarding nodule number correlation, the RC correlation with shoot height and fresh shoot weight were insignificant (p>0.05) and were respectively very weak and weakly positive in the third month. In contrast, in the ninth month, they were significant (p<0.05) and were moderate and weakly positive (Figure 4).

Discussion

Using legume trees/shrubs in tropical forest restoration is important to significantly improve restoration success (Gei and Powers 2013; Van Haren et al. 2013; Werden et al. 2018; Mira et al. 2022). In Ethiopia, there have been massive forest restoration programs. However, there has been little attention to tree/shrub species selection, and the deliberate incorporation of the different tree/shrub functional groups, particularly legumes, in forest restoration projects is overlooked. Moreover, very few studies have been conducted to compare the suitability of native legume trees (*Vachellia* spp.) for forest restoration projects. Previously, Tuffer (2017) evaluated the effectiveness of species-specific Rhizobium inoculant on *V. abyssinica*, *V. negrii*, and *V. seyal*. The mean shoot height of *V. seyal* we recorded during the third month of seedlings' growth ranged from 8.48 to 9.86 to 12.4 cm, respectively, for accession-2, accession-1, and accession-3 and was comparable to the mean shoot height (10.6 cm) reported for *V. seyal* seedlings of the same age (Tuffer 2017). However, the mean shoot height of *V. abyssinica* that Tuffer (2017) reported (10 cm) was smaller than the mean shoot height we recorded (13.5 cm).

Table 2. One-way ANOVA result for the effect of tree species and accession on desirable seedling trait

Seedling traits	Third month			Ninth month			
	df	F value	p-value	df	F value	Chi-Sq.	p-value
Shoot height	24	3.9304	0.009585**	24	1.1498	-	0.3621
Fresh shoot weight	24	8.437	0.0001018***	24	2.7242	-	0.04363*
Rooting Depth	-	-	-	24	1.7401	-	0.1638
RGR in shoot Height	-	-	-	24	14.014	-	0.000001844***
RGR in fresh shoot weight	-	-	-	24	20.796	-	0.00000005158***
Nodule number	24	7.1402	0.0003224***	24	-	20.483	0.001014**
Root AMF colonization	24	3.9822	0.009009**	24	4.288324	-	0.00628**

Note: Significant species and/or accession effect at *0.05, **0.01, and ***0.001. RGR: relative growth rate

Table 3. Mean nodule number, AMF root colonization, and the effect of seedling age was tested by independent t-test

Tree species/accession	Nodule number (Mean±std.Err.)		Independent t-test p-value	Root AMF colonization (Mean±std.Err.)		Independent t-test p-value
	3rd month	9th month		3rd month	9th month	
Vachellia seyal (Delile) P.J.H.Hurter -accession 1	5.6±2.04	12.6±5.22	0.247	5.4±3.71	28±8.89	0.04703*
Vachellia etbaica (Schweinf.) Kyal. & Boatwr.	0±0.00	0.6±0.24	0.9648	2.2±1.56	4±1.70	0.4584
Vachellia lahai (Steud. & Hochst. ex Benth.) Kyal. & Boatwr.	3±1.14	3.4±0.60	0.7641	39±11	68±11.47	0.1055
Vachellia seyal (Delile) P.J.H.Hurter - accession 2	6.4±2.73	13.4±1.94	0.07004	18.4±6.27	24±11.73	0.6849
Vachellia abyssinica (Hochst. ex. Benth.) Kyal. & Boatwr.	16.8±3.48	7.2±1.24	0.03184*	36.6±11.87	29.6±10.57	0.6714
Vachellia seyal (Delile) P.J.H.Hurter - accession 3	19.2±5.01	33.8±10.54	0.2462	16.8±6.14	34±11.97	0.237

Note: *significant diffrence

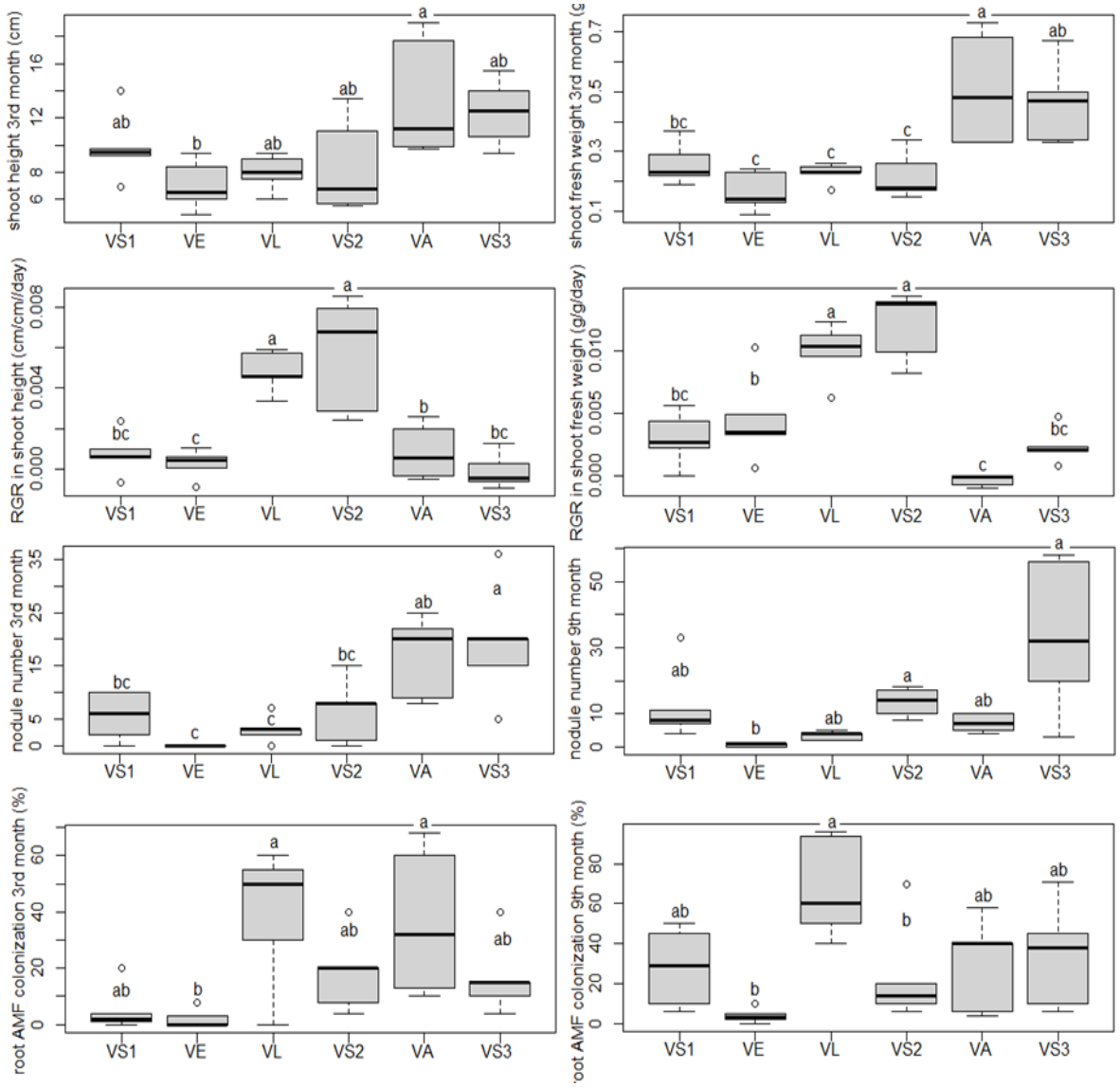

Figure 2. Mean difference in seedling traits between tree species and accessions. Data containing means with a significant difference for Tukey HSD (p<0.05) or the Dunn-Bonferroni test (p<0.05) do not contain similar letter labels. AMF= Arbuscular Mycorrhizal Fungi, VS1= *Vachellia seyal* accession-1, VE= *Vachellia etbaica*, VL= *Vachellia lahai*, VS2= *Vachellia seyal* accession-2, VA= *Vachellia abyssinica*, VS3= *Vachellia seyal* accession-3, RGR=Relative Growth Rate

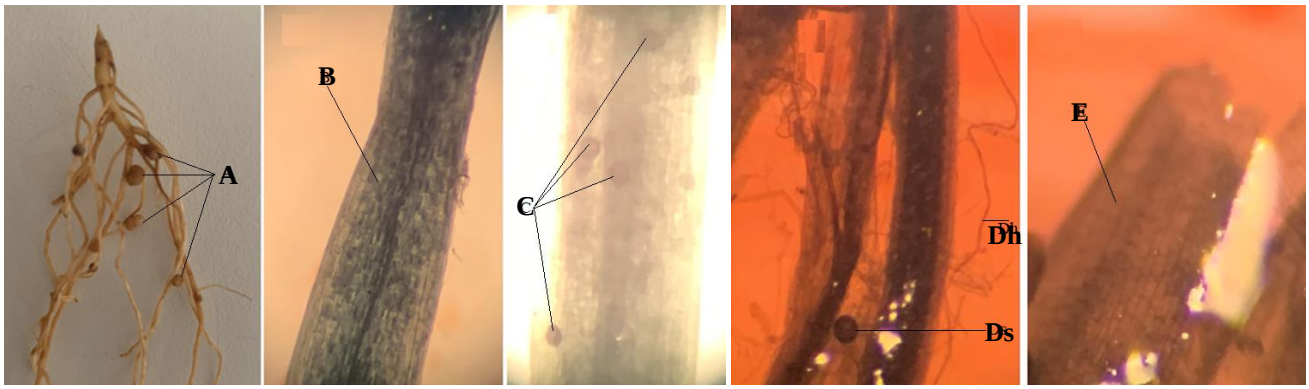

Figure 3. Picture to show nodulation and arbuscular mycorrhizal fungi colonization. A: Nodulation of the root (globose, big sized), B: AMF colonization with arbuscules, C: AMF colonization with intraradical spore, Ds: AMF colonization with extraradical spore, Dh: AMF colonization with extraradical hyphae, E: No AMF colonization. Pictures are not on the scale

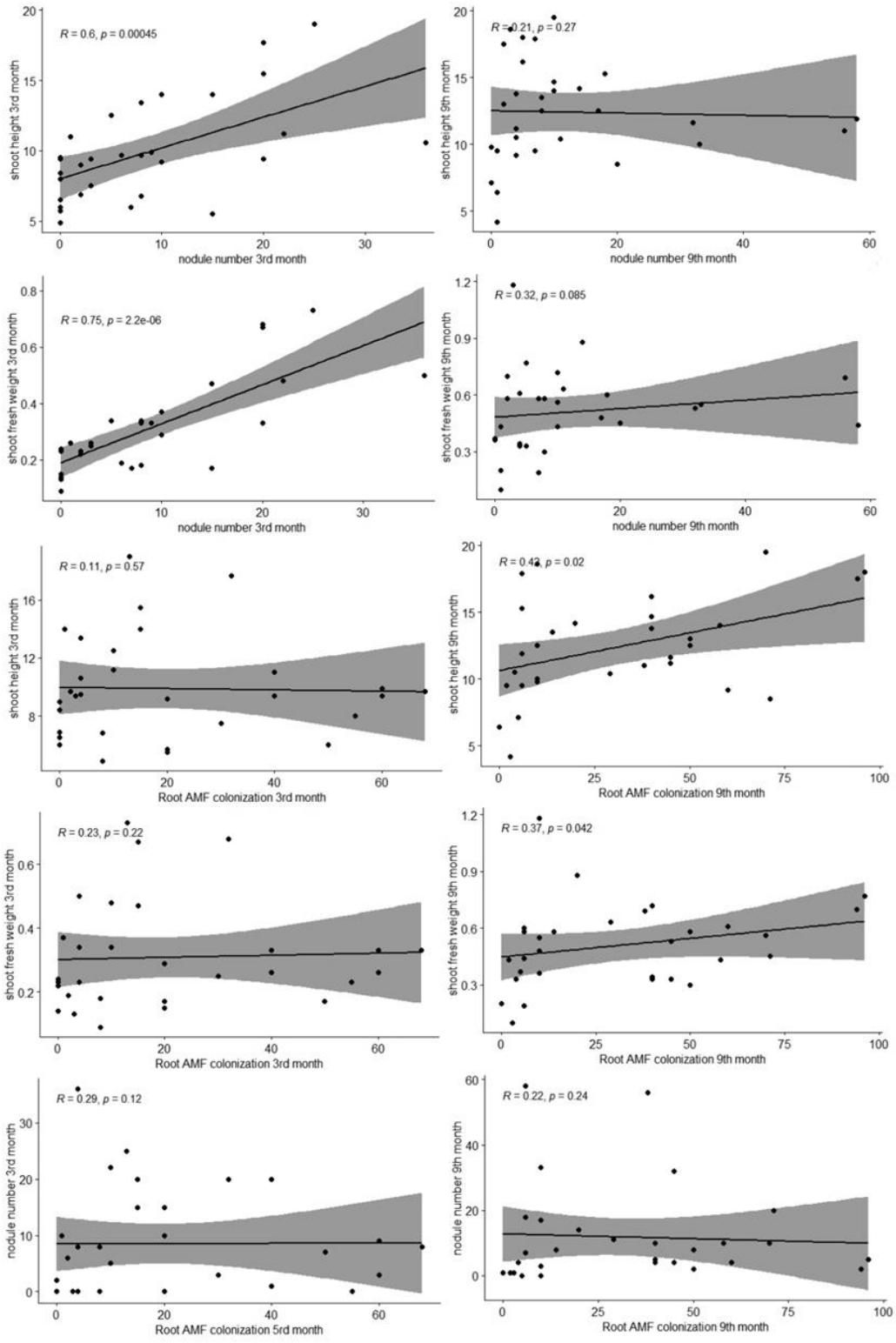

Figure 4. Spearman's rank correlation between root nodule number, root arbuscular mycorrhizal fungi colonization, seedling shoot height, and shoot fresh weight

We used nodule number and pigmentation to determine N-fixation potential. Our pigmentation assessment was ineffective as we only observed nodule color from the outside. Effective nodules are those pigmented red/pink on the inside and not outside (Brockwell et al. 2005) and could be observed by cutting the nodules (Hultman 2018) or using light microscopy (Ardley et al. 2013). However, nodule number is still an important variable in determining N-fixation potential. Accordingly, an increase in mean nodule number has been reported to increase the mean N-fixation potential (computed as the shoot dry mass ratio between inoculated and N^+ fertilized plants) of three

months old *V. abyssinica* and *V. seyal* seedlings (Tuffer 2017). Nodule number and nodule position in the root are tightly controlled by the plant (Ferguson et al. 2010), and thus, nodule number could be an important variable in comparing legume trees. The mean nodule number we recorded for *V. seyal* (5.6 to 6.4 to 19.2 for the three accessions) and *V. abyssinica* (16.8) in the third month are very much smaller than the mean values, i.e., 42 and 37, reported by Tuffer (2017) respectively for the seedlings of the same species of similar age. These differences could be attributed to seedlings being inoculated with species-specific rhizobium bacteria in the previous experiment. Elongate/delicate nodules, i.e., cylinder-shaped nodules, are considered to be more involved in N-fixation in Vachellias (Acacias), while globose (spherical) nodules, particularly the small sized, are ineffective (Brockwell et al. 2005). Therefore, the fact that we recorded a few elongated nodules could indicate that our study species had low N-fixation potential in our experiment condition, resembling the central Ethiopian highlands.

We recorded a general trend in an increase of nodule number in the ninth month compared to the third. Similarly, a general increase in nodule number has been reported for seedlings of other legume tree species (Azad et al. 2013; Azad et al. 2016). On the contrary, in the case of *V. abyssinica*, we recorded a significant (p<0.05) reduction in nodule number with an increase in seedling age. That also coincided with a decay rate in fresh weight (-0.0003 g/g/day) recorded for *V. abyssinica*. Nodulation/N-fixation could be affected by several factors that also affect seedlings' growth. For example, leaf removal has significantly reduced nodule growth and N-fixation (Marschner 2012). Similarly, other factors such as light, soil nutrients, soil moisture, and temperature could also significantly reduce nodule number and N-fixation (Brockwell et al. 2005). Hence, in our case, weather change-induced reduction in light intensity and soil temperature and hence, leaf shedding (observed during watering) could have resulted in the nodule number reduction, particularly by the *V. abyssinca* seedlings. That could also be related to the particular provenance (accession no: 244601) being located at the hottest range of the species, which is also far from central Ethiopia.

In this experiment, we also evaluated root AMF colonization (RC) to determine the legume tree species or accession with a better potential for N-fixation and phosphorus acquisition. The mean RC levels we recorded in most cases vary from the mean RC levels reported previously. Accordingly, the mean RC we recorded for *V. etbaica* (2.2% and 4%, respectably for the third and ninth months) is very small as compared to the mean RC reported (73%) for adult individuals of the same species in north Ethiopia (Birhane et al. 2017). Whereas the mean RC we recorded for *V. abyssinica* at the third (36.6%) and ninth (29.6%) months were much higher than the mean value previously reported (18.3%) by (Zerihun et al. 2013) for adult *V. abyssinica* trees in central Ethiopia, these mean RC values were very much lower than the mean RC reported (96%) for *V. abyssinica* seedlings (Asmelash et al. 2021c). Variable mean RC levels have also been reported previously for adult *V. seyal* trees, 78.2% (Birhane et al. 2017) and 42.1%, 48.8%, and 96.4% (Zerihun et al. 2013). Likewise, we recorded variable mean RC at the third and ninth months of seedling growth. Regarding *V. lahai*, (Birhane et al. 2017) previously reported a mean RC of 83% for adult trees which is much higher than the mean RC we recorded in the third month (39%) but relatively comparable to the mean RC we recorded at month nine (68%). Root AMF colonization is determined more by the host species (John St. 1980; Schüßler et al. 2016; Silva-Flores et al. 2019). Therefore, the variable and wide range of RC we recorded and previously reported may indicate that the study species have a wide range of RC. Moreover, soil property could also be responsible for these variable RC values. Root AMF colonization is generally lower for degraded soils than fertile or virgin soils (Asmelash et al. 2021b). Hence, the generally low mean RC we recorded for the study species could be expected since the potting soil used in this experiment was highly degraded. Except for the non-significant (p>0.05) reduction of RC by *V. abyssinica*, the remaining RC values recorded increased with age and particularly significantly (p<0.05) for *V. seyal* accession-1. Root AMF colonization and age relationships could vary widely between host species (Asmelash et al. 2021c). However, in our case, there seem to be more or less similar RC and age relationships for the study species and accessions. That could be because the species are taxonomically related, being from a similar genus (John St. 1980).

In the third month, nodule number correlated significantly (p<0.05) with shoot height and fresh shoot weight, while RC did not. On the contrary, in the ninth month, RC correlated significantly (p<0.05) with shoot height and shoot fresh weight, not nodule number. That may indicate that with an increase in seedlings' age, rhizobia's role in the legumes' nutrition declines while the role of AMF increases. Moreover, nodule number and root AMF colonization were not significantly (p>0.05) correlated in the third and ninth months. That could be because the nodule numbers we recorded were not all active/pigmented inside or because nodule number/RC correlations are species-dependent.

In this study, we evaluated the variation in N-fixation potential (nodule number) and arbuscular mycorrhizal fungi (AMF) association determined by root AMF colonization (RC) between *V. abyssinica*, *V. etbaica*, *V. lahai*, and *V. seyal* (represented by three accessions). We also evaluated the comparative growth of these species and accessions on the degraded soil of central Ethiopia in a mesh house in central Ethiopia. We also determined the correlation between nodule number and RC and between nodule number and RC with seedlings' shoot height and fresh weight. We also determined the effect of seedling age on nodule number and RC. Based on growth, nodule number, and RC, *V. etbaica* (accession number: 20645), could be considered the less suitable species for forest restoration in central Ethiopian highlands. The remaining species and accessions have distinct qualities considering growth, N-fixation potential, or AMF association. *V. lahai* is the most suitable if considering mainly AMF association.

V. seyal accession-3 is the furthest provenance to central Ethiopia; it performed better in N-fixation potential, growth, and AMF association than provenances collected from near central Ethiopia. Hence, the general assumption of using local provenances in forest restoration should be evaluated per tree species. The legume trees of Ethiopia are less studied; particularly, their role as environmental engineers could be better understood by knowing more about their root traits. Therefore, this study could motivate future research in this regard. Long-term experiments are important as experiments on seedlings alone, although crucial, may not be sufficient. In this experiment, a few legume tree species and provenances were evaluated. The comparative suitability of more tree species and provenances/ accessions is required in the future.

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Diversity studies on insect pests of high altitudinal transitional zones of North-western Himalayas

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Abstract. *Kumar P, Thakur TS, Deepika, Sharma N. 2022. Diversity studies on insect pests of high altitudinal transitional zones of North-western Himalayas. Nusantara Bioscience 14: 203-210.* Class Insecta constitute a major fauna and comprise many species of economic importance. Due to climate change and increase in temperature, many insect species are changing their habitat considerably and shifting their hosts, which leads to changes in the diversity of insect pests at different altitudinal gradients. High altitude forest cover is not large and massive as plains forest cover, but it supports some of the very important economical tree species like- *Quercus* sp., Himalayan Poplar, *Betula* sp., *Abies pindrow* (Royle ex D.Don) Royle, *Juniper* spp., Birdcherry, Maple, etc. The present study analyzed any change in insect pest incidences and diversity of pest species due to the change in host preferences or climatic patterns. The study was conducted at four selected sites viz. Rohtang area (Kullu Forest Division), Chanshal area (Rohru Forest Division), Sach area (Churah Forest Division), and Indrahar area (Dharamshala Forest Division) of high altitudinal transitional zones of Himachal Pradesh, India, to study insect pest diversity. A total of 32 insect species were recorded during the study period comprised of the insect orders viz., Coleoptera, Hymenoptera, Hemiptera, Orthoptera, Dermaptera, and Lepidoptera. The present investigation led to the finding that species of Coleoptera (beetles) were the most dominant insects attacking trees of the high altitudinal transitional zone, followed by Lepidoptera (Butterflies and moths) and Hemiptera (aphids).

Keywords: Altitude, dominant, habitat, insect pests, transitional zones

INTRODUCTION

Like many other plants, forests and trees are attacked by insect pests and diseases that inflict extensive damage, resulting in poor tree growth, poor timber quality, and, in rare cases, full forest destruction and decline (Sharma 2016). In addition, various forest pests threaten forests and their services worldwide, causing significant ecological and economic losses (Wingfield et al. 2015; Diagne et al. 2021). The most common insect pests can be grouped into three categories: regeneration, defoliators, and bark beetles (Björkman et al. 2015). The fundamental concepts underlying forest insect population dynamics involve top-down (natural enemies) and bottom-up (host plant) forces operating on insect reproductive success and survival. Furthermore, at large population densities, lateral forces (competition) substantially impact population dynamics (Martin et al. 2013).

Insect pests are major limitations on agricultural and forestry output (Pureswaran et al. 2018; Trisnawati et al. 2022). Despite the lack of comparable global estimates for forestry systems (Niquidet et al. 2016), forest pests such as the gypsy moth (*Lymantria dispar* Linnaeus, 1758) in Appalachian Plateau and mountain pine beetle (*Dendroctonus ponderosae* Hopkins, 1902) in western North America are known to have major ecological consequences. These include eradicating indigenous tree species and widespread defoliation and mortality, which disturb ecosystem processes and decrease biodiversity (Fajvan and Wood 1996; Janes et al. 2014).

The current and anticipated challenges posed by phytophagous insect pests are likely to be exacerbated by projected global warming, which may promote pest population growth, increase outbreak frequencies, and facilitate the geographic expansion of many pest species, resulting in greater economic losses and food security threats (Andrew et al. 2013; Thackeray et al. 2016).

Several criteria govern the level of impact of a novel insect or illness, including the pathogen's virulence and the sort of harm caused by the bug (e.g., phloem- or wood-boring, sap-feeding, or defoliation. In addition, other pest characteristics, such as host specificity and reproductive and dispersal potential, as well as characteristics of the host tree, such as dominance in the forest, role in productivity and nutrient cycle, and provisioning of wildlife food and habitat impacts, can all influence the severity and extent of the damage (Lovett et al. 2006).

Except in the tropics, where season length does not change with elevation, decreasing temperature, shortening of the growing season, increasing climatic variability, increasing exposure to sunlight and wind, and, occasionally, decreasing water availability and soil fertility are all common effects of increasing elevation (Pellissier et al. 2014; Rasmann et al. 2014; De Long et al. 2016). Generally, it has been observed that as elevation rises, the diversity and number of plants, insect herbivores, and predators decrease (Pellissier et al. 2012). A mountain

range's geological age influences communities along elevational gradients since older mountains allow for greater species colonization and diversification (Schemske and Mittelbach 2017). Additionally, the regional species pool and the species inhabiting a particular mountain range influence local diversity at various elevations (Ricklefs and He 2016). Numerous species of insect herbivores regularly attack plant species across the entirety of their distribution range. These herbivores may differ in their vulnerability to varying environmental conditions, which could lead to variations in their abundance and the amount of harm they cause to focal host plants along environmental gradients (Pratt et al. 2017). A recent study suggested that in addition to critically reevaluating the evidence supporting spatial gradients in plant-insect herbivore interactions, a new mechanistic framework for forecasting the existing patterns should also be developed (Moreira et al. 2018).

The present study has been conducted at four selected sites in the forests of High Altitudinal Zones of the NW Himalayas to investigate insect pest diversity.

MATERIALS AND METHODS

Study area

The study was conducted from April 2016 to March 2021. The survey was carried out in areas of different high altitudinal transitional zones of Himachal Pradesh, India, to study the pest status of insects and collect the insect pests specimens.

The study was conducted at four selected sites viz. Rohtang area (Kullu Forest Division), Chanshal area (Rohru Forest Division), Sach area (Churah Forest Division), and Indrahar area (Dharamshala Forest Division) (Table 1). Insect fauna and pest status were recorded from selected sites covering different altitudes and forest types viz., Himalayan moist temperate forests: 1,500-3,000 m (Both coniferous and broad-leaved species), Sub-alpine forests: 3,000-3,400 m (Birch and Fir), Moist alpine scrubs: 3,000-3,500 m (Rhododendron), Dry alpine scrubs: 3,400m-3,600m (*Junipers* species) and Alpine meadows:>3,600 m (alpine pasture).

Methodology for insect collection

All four sites were regularly visited, and five transects were established at each study site. Insect samples were collected with the help of different insect collection methods such as using a sweep net (diameter 30 cm and 1.5 mm mesh) light trap only for four hours (if required) of all specimens encountered, hand-picking, pitfall trap, beating tray samples, search method (Figure 1, Figure 2) and by line transect method. Five transects have been established in each forest division, and the collection has been made from selected sites. The start points for each transect were chosen using a random number, and there are 10 collection sites on each transect separated by 20-30 m. These collection sites are placed at right angles to the transect. Each forest category has 25 collection sites (15 pitfalls and 10 leaf litter).

Data collection: Four techniques were used to collect data in the collection sites

Leaf litter: In each of the 10 sites, 1 m^2 quadrate was established, and leaf litter was collected into plastic bags and put in Berlese funnels to dry. The beetles and other insects moved to collected tubes and sorted them out for identification and preservation.

Pitfall traps: In each of the 15 collection sites, 5 pitfall traps (8 cm diameter and 10 cm deep) were dug into the soil surface, a total of 75 traps per habitat. The traps were left for 2 days, then another two days were emptied into sample bottles every 48 hours (Samways et al. 1996) and then taken to the laboratory for sorting and identification.

Beating tray samples: Three transects were randomly selected along with 15 collection sites placed at an interval of 10 meters; beetles were obtained by beating plants' leaves and branches, up to 2.0 meters in height, onto the tray. Insects will be collected for sorting and identification.

Light traps: As insects attract toward the light sources, the collection was done near the lamp posts/street lights in the various sites in the eco-development area. Apart from this, several attempts were made to use the light traps.

Hand-picking: Small Coleopteran beetle like Coccinellidae, Chrysomelidae, Curculionidae, Scolytidae, etc., were collected by hand with the help of fine forceps. (Kumar and Thakur 2014; Kumar et al. 2016).

Pitfall traps primarily capture ground-dwelling insects (Lundgren and McCravy 2011). Sweep netting only traps insects residing on plants at vertical levels that the collector can access (McCravy 2018). Therefore, many alternative sampling techniques should be used in a comprehensive investigation (Spafford and Lortie 2013; González et al. 2020).

Table 1. Geographical positions of different localities

Area	Site	Latitude	Longitude
Dharamsala	Triund	$32^0$15’34.50’’	$76^0$21’23.37’’
Dharamsala	Indrahar Pass	$32^0$17’42.21’’	$76^0$23’01.67’’
Dharamsala	Galu Forest	$32^0$14’55.07’’	$76^0$19’18.72’’
Rohru	Larot	$31^0$14’10.97’’	$77^0$56’49.69’’
Rohru	Chanshal Pass	$31^0$11’48.93’’	$77^0$59’20.11’’
Rohru	Dodra	$31^0$11’45.67’’	$78^0$03’13.18’’
Manali	Jagatsukh	$32^0$12’06.50’’	$77^0$12’16.51’’
Manali	Gulaba	$32^0$19’30.03’’	$77^0$11’50.48’’
Manali	Kothi	$32^0$18’52.77’’	$77^0$11’24.76’’
Manali	Marhi	$32^0$20’55.98’’	$77^0$13’05.67’’
Manali	Rohtang Pass	$32^0$22’17.91’’	$77^0$14’47.84’’
Chamba	Sach Pass	$33^0$00’20.89’’	$76^0$14’23.26’’
Chamba	Satrundi	$32^0$59’31.34’’	$76^0$12’36.95’’
Chamba	Tissa	$32^0$49’49.21’’	$76^0$09’03.02’’
Chamba	Devi Kothi	$32^0$54’31.92’’	$76^0$14’05.55’’
Chamba	Bairagarh	$32^0$54’05.48’’	$76^0$09’47.37’’

Figure 1. Assessment of insect-infested trees at high altitude transition zone in Sach Pass area, Himachal Pradesh, India

Figure 2. Monitoring heavy infestation of tent caterpillar-affected Moru oak trees in Sach Pass, Himachal Pradesh, India

Preservation

During field surveys, the freshly collected specimens of butterflies were kept in a triangular paper envelope, whereas specimens like beetles, bugs, and moths were kept in small specimen containers. Insects were pinned by entomological pins of 38 mm in length. In the laboratory, insect specimens were put in a relaxing chamber, followed by pining perpendicularly through the middle of the thorax at a point equidistant between the bases of forewings. Next, the wings were spread using paper stripes (for butterflies and moths. After that, the insect specimens were allowed to dry in desiccators for 2-3 weeks, depending on climatic conditions. Finally, the dried specimens were transferred to airtight insect boxes containing powdered naphthalene balls. A label written with black Indian ink was fixed on each specimen (Arora 1990). This methodology was also followed by Kumar et al. (2015), Thakur and Kumar (2015), and Kumar (2016).

Identification of insects

Insects collected during the survey using various methods were identified and studied taxonomically by comparing the specimens with the authentic identified collections available at Forest Research Institute, Dehradun, India.

Data analysis

Interpretive data analysis was done using Simpson's index and Shannon-Wiener Diversity Index (H) (Shannon-Wiener 1963) to determine the abundance and diversity of insect species. Next, the concentration of dominance (D) was measured by Simpson's Index (Simpson 1949). Finally, the Evenness (E) Index was calculated by Hill (1973).

RESULTS AND DISCUSSION

During the present study, a total of 32 species of insects belonging to 28 genera, 18 families, and 6 orders were collected and identified (Table 2). Of the six orders, Coleoptera was the most represented, with 15 species corresponding to seven families and seven genera, followed by Lepidoptera with 11 species, nine genera, and six families. On the other hand, Orthoptera and Dermaptera were represented by one species. In terms of population abundance, Lepidoptera was the dominating order with 65.15 relative abundance, followed by Coleoptera (30.17), Hemiptera (2.22), Orthoptera (1.15), Hymenoptera (0.84) and Dermaptera (0.45) (Figure 3). A similar pattern was reported by Singh (2013).

High altitudinal plant species like *Betula utilis* D.Don, *Abies pindrow* (Royle ex D.Don) Royle, *Rhododendron* spp., *Prunus cornuta* (Wall. ex Royle) Steud., *Acer* spp., *Quercus* spp., and *Poplar* spp. are mostly affected by the insect pests.

Distribution of insect pests

The amount and distribution of vegetation in forests depend on the nature of the plant community, the regional climate, past management practices, and the invasion of non-native species. These variations are believed to have significant effects on animal diversity, distribution, and migration patterns, and different canopy components are anticipated to have various impacts on functional and trophic guilds (Heidrich et al. 2020; Tinya et al. 2021). That is especially true for insects, which not only have a significant variety of species but also in terms of life strategies and resource consumption. For instance, variations in tree composition may favor some insect groups' diversity while diminishing or having no effect on others (Leidinger et al. 2021). The complexity of the canopy, as measured by the number of plant layers, the distribution of the vegetation, or other comparable classifications, appears to generally favor increased insect abundance (Knuff et al. 2020) and higher species richness as well (Müller et al. 2018).

High-mountain regions are undergoing tremendous change globally (Pörtner et al. 2019). The rapid increase in air temperature in these places, which are often above tree line between 2,000 and 4,000 meters, is the primary indicator of change and has significant effects on glacier cover (McKernan et al. 2018), streamflow (Hotaling et al. 2017), and dissolved oxygen levels (Jacobsen 2020), among other things. Insects (Moret et al. 2016; Wu et al. 2019) and the creatures they interact with are moving upslope due to warming in mountain environments (Guo et al. 2018; Anderson and Wadgymar 2020). High-mountain environments are experiencing a fast change in their insect communities (Shah et al. 2020). A study on the spatial distribution of insect pests was carried out in four sites, i.e., Chanshal (Shimla), Triund (Dharamshala), Rohtang (Kullu), and Sach (Chamba) areas of Himachal Pradesh, during different seasons of the year. In Figure 5, it was observed that there is clear segregation in species as well as population diversity along with altitude gradient, and climatic factors like temperature and relative humidity also play a pivotal role in the spatial distribution of insects in different habitats. Figure 6 shows that the insects specimens collected from different sites of high altitudinal transitional zones, North-western Himalayas

Table 2. List of identified insect

Order	Family	Species	Triund	Chanshal pass	Rohtang pass	Sach pass
Lepidoptera	Nymphalidae	*Junonia iphita* (Cramer, 1779)		√		√
Lepidoptera	Pieridae	*Pieris canidia* (Sparrman, 1768)	√	√	√	√
Lepidoptera	Pieridae	*Pieris brassicae* (Linnaeus, 1758)	√		√	
Lepidoptera	Pieridae	*Pieris napi* (Linnaeus, 1758)	√	√	√	√
Lepidoptera	Noctuidae	*Asota caricae* (Fabricius, 1775)	√	√	√	√
Lepidoptera	Noctuidae	*Thysanoplusia* spp.	√	√	√	√
Lepidoptera	Noctuidae	*Agrotis ipsilon* (Hufnagel, 1766)	√	√	√	√
Lepidoptera	Lasiocampidae	*Malacosoma* sp.	√	√	√	√
Lepidoptera	Erebidae	*Lymantria Concolor* (Walker,1855)	√	√	√	√
Lepidoptera	Notodontidae	*Thaumetopoea processionea* (Linnaeus, 1758)	√	√	√	√
Lepidoptera	Lasiocampidae	*Lasiocampa trifolii* (Denis & Schiffermüller, 1775)			√	
Coleoptera	Scarabaeidae	*Melolontha furcicauda* (Ancey, 1881)	√	√	√	√
Coleoptera	Scarabaeidae	*Clinteria* sp.	√	√	√	√
Coleoptera	Scarabaeidae	*Brahmina comata* (Blanchard, 1851)	√	√	√	√
Coleoptera	Scarabaeidae	*Mimela amphichroma* (Prokofiev & Zorn, 2016)	√		√	√
Coleoptera	Chrysomelidae	*Phratora vulgatissima* (Linnaeus, 1758)	√		√	√
Coleoptera	Scarabaeidae	*Xylotrupes* sp.	√		√	
Coleoptera	Chrysomelidae	*Plagiodera versicolora* (Laicharting, 1781)	√	√	√	√
Coleoptera	Cerambycidae	*Arhopalus rusticus* (Linnaeus, 1758)	√	√	√	√
Coleoptera	Curculionidae	*Curculio glandium* (Marsham, 1802)		√		√
Coleoptera	Coccinellidae	*Coccinella magnifica* (Redtenbacher, 1843)	√	√	√	√
Coleoptera	Dytiscidae	*Cybister tripunctatus* (Olivier, 1795)		√		√
Coleoptera	Carabidae	*Carabus coriaceus* (Linnaeus, 1758)		√		√
Coleoptera	Scarabaeidae	*Xylotrupes beckeri* (Schauffus, 1885)		√		√
Coleoptera	Scarabaeidae	*Hylocereus* sp.			√	
Coleoptera	Curculionidae	Scolytinae				√
Hemiptera	Pentatomidae	*Halyomorpha* sp.	√	√	√	√
Hemiptera	Pentatomidae	*Podisus* sp.		√		
Dermaptera	Labiduridae	*Labidura* sp.				√
Hymenoptera	Tenthredininae	*Tenthredo cretata* (Konow, 1898)			√	
Hymenoptera	Tenthredininae	*Tenthredo* sp.			√	
Orthoptera	Acrididae	*Gesonula punctifrons* (Stål, 1861)	√	√		√

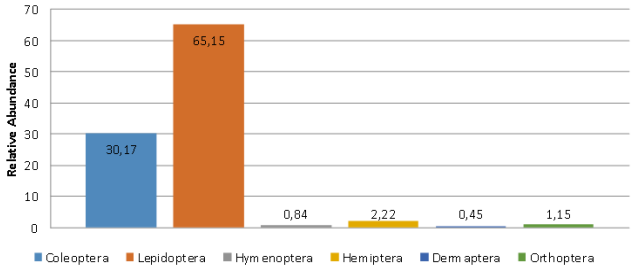

Figure 3. Relative abundance of insect pests in all sites

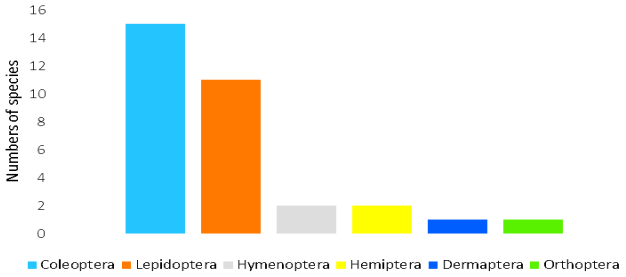

Figure 4. Species richness of different orders of insect pests at different sites

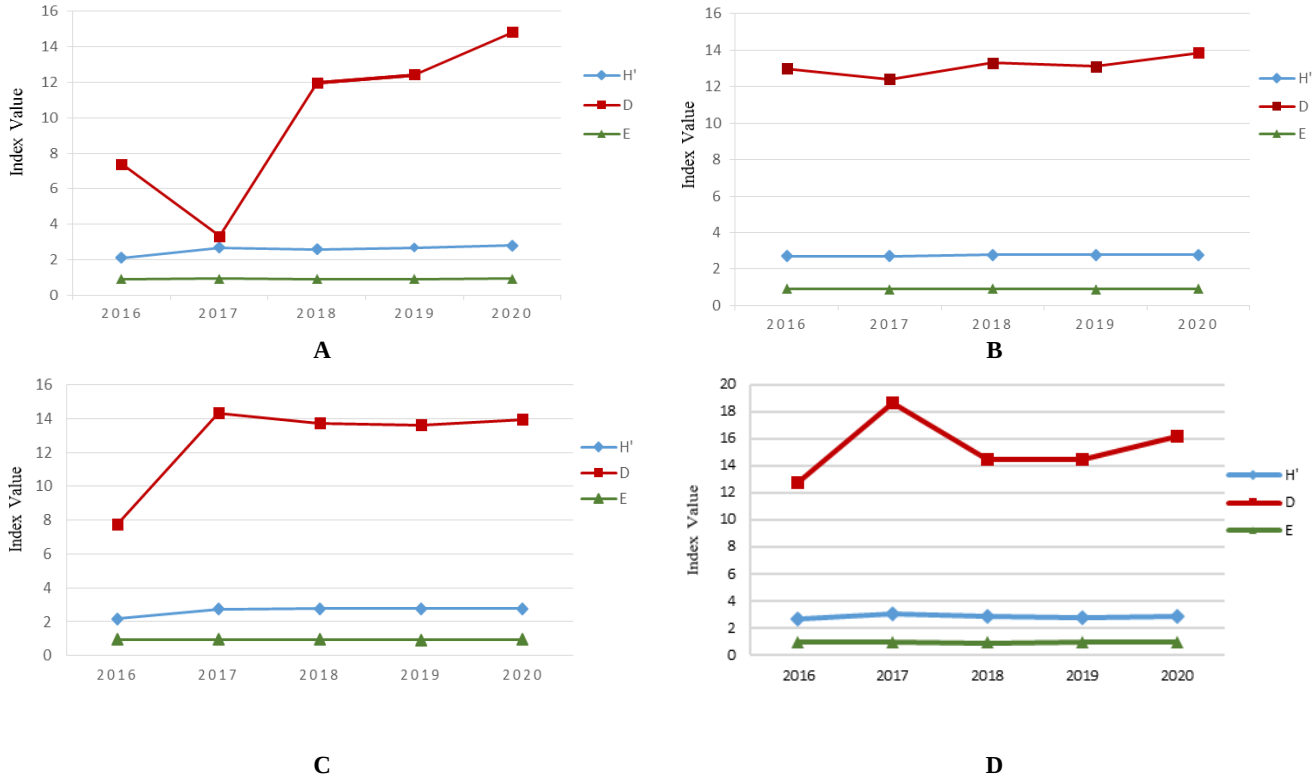

Figure 5. Diversity values of insect pests in the North-western Himalayas: A. Triund area, B. Chanshal area, C. Rohtang area, D. Sach area

Depending on the spatial and temporal variation, insects vary in diversity and abundance (Bashir et al. 2019; Khan et al. 2021). In the present study, the most dominating order Coleoptera (Figure 4) which represented by 15 species belonging to 7 families, the maximum beetles were represented by the family Scarabaeidae followed by Chrysomelidae (2 spp.), Curculionidae (2 spp.), Cerambycidae (1 spp.), Coccinellidae (1 spp.), Dytiscidae (1 spp.) and Carabidae (1 spp.). Coleoptera is one of the most diverse orders of class Insecta as only from India about 15,500 species belonging to 104 families under three sub-orders have been reported (Sengupta and Pal 1998). A study on the species diversity of insects in the southern forest-steppe zone of the Chelyabinsk region also revealed that the largest number of species observed were the representatives of the order Coleoptera (Makarova et al. 2022).

Concerning population abundance Lepidoptera was the dominating order with a relative abundance of 65.15, represented by 11 species belonging to 6 families, the maximum butterflies were represented by the family Pieridae (3 spp.) followed by Nymphalidae (1 spp.), and the maximum moth represented by Noctuidae (3 spp.) followed by Lasiocampidae (2 spp.), Notodontidae (1 spp.), Erebidae (1 spp.). Lepidopterans were also found to be most abundant in the diversity study of Sg. Tiagau Forest Reserve, Malaysia (Razy et al. 2022). More dense vegetation has been observed to boost the number of moths (Müller et al. 2012).

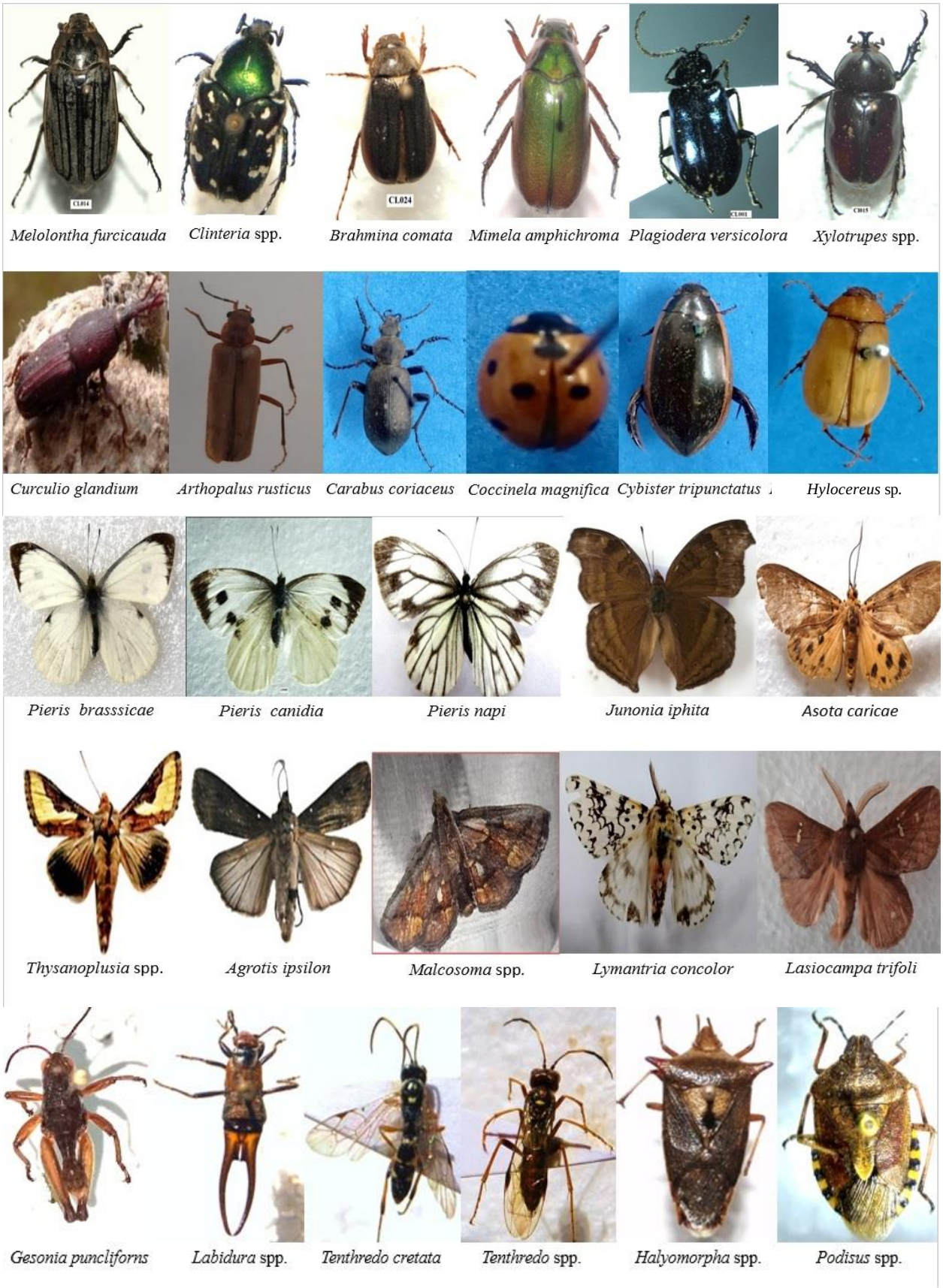

Figure 6. Insects specimens collected from different sites of high altitudinal transitional zones, North-western Himalayas

Order Hymenoptera (Bees and Wasps) is probably the most economically important and beneficial class of Insecta. It contains many insects that are of value as parasites or predators of various insect pests, pollinators, and many commercially important insects, like honey bees. 2 species of Hymenoptera were recorded in the present study, which is represented by 1 family Tenthredininae (2 spp.). Hemipteran fauna of Himachal Pradesh is poorly known except for the family Aphididae, which is rather well explored in comparison to the other families. Some 368 species belonging to 186 genera under 25 families of Hemiptera are so far known from Himachal Pradesh (Varshney 1992). The majority of Hemipteran insects are phytophagous and widely distributed all over the world. Many of them, including aphids, scale insects, leafhoppers, and planthoppers, are significant pests in agriculture and forestry and have a variety of host plants (Guo and Yuan 2016). In the present study, 2 genera of Hemiptera were recorded, which belong to the family Pentatomidae. Just like the Hemiptera order, the order Dermaptera is also very poorly investigated in Himachal, and only one genus of this order was recorded as belonging to the family Labiduridae.

Shishodia and Gupta (2009) have reported Acrididae as the largest family of Orthoptera in Himachal Pradesh. One genus of order Orthoptera was reported to belong to the family Acrididae. The study will help develop the conservation plan to restore the diversity in the region. The biodiversity data revealed that the insects are shifting or expanding their habitat from lower altitude to higher altitude areas as the temperature, relative humidity, and seasonal pattern change. Some insects were also collected from the navel zones of sites, which is an indicator that the area under study and other such areas should be continuously surveyed and monitored to assess the changing spatial distribution of insects to add new taxa to the existing biodiversity. Due to habitat destruction, many species are already extinct, but with the help of this study, we can identify and conserve the threatened taxa. The variation caused by environmental changes can also cause drastic changes in the spatial distribution of high-altitude insect fauna, and only periodic surveys of these areas can help assess the pest incidences and check them by applying effective control measures. Insect pests must be monitored regularly, as well as studies on their ecology, biology, host and distribution patterns, effects on forest ecosystems, and interactions with natural enemies. However, precise knowledge of pests, diseases, and forest ecosystems is required to develop successful pest management strategies (Bista and Thapa 2012; Mohammed et al. 2012).

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The use of Indole Butyric Acid on the growth of dragon fruit plant stem cuttings

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Abstract. *Hernosa SP, Tampubolon SDR, Siregar LAM. 2022. The use of Indole Butyric Acid on the growth of dragon fruit plant stem cuttings. Nusantara Bioscience 14: 211-216.* The difficulty of root growth of dragon fruit cuttings is affected by stem rot; one way to increase the growth of cuttings is to use a growth regulator, namely Indole Butyric Acid (IBA), which is used to stimulate the growth of dragon fruit rootstock cuttings. This research was conducted in Sidorukun Village, Pangkatan Sub-district, Labuhan Batu District, North Sumatra, Indonesia, at an altitude of ± 40 m above sea level, from June to October 2020. The research design used was a randomized block design with five treatments. The concentration of the IBA hormone consists of B0 : 0 ppm, B1: 4,500 ppm, B2: 5,500 ppm, B3: 6,500 ppm, B4: 7,500 ppm, and five replicates, so the entire experimental plot was 25. Parameters observed were percentage of shoot emergence, age of shoot emergence, shoot length (cm), shoot fresh weight (g), shoot dry weight (g), root length (cm), fresh root weight (g), root volume (g), and root dry weight (g). Data analysis used a linear model followed by Duncan's test. It is known that the administration of Indole butyric acid (IBA) significantly affected the observed parameters on shoot emergence, age of shoot emergence, number of shoots, root length, fresh root weight, root volume, and root dry weight. However, it did not affect the observed parameters, namely, shoot length, shoot fresh weight and shoot dry weight. On the other hand, B4 concentration (7,500 ppm) gave better shoot growth, root weight, and root dry weight.

Keywords: Cutting, dragon fruit, growth, Indole Butyric Acid

INTRODUCTION

Dragon fruit which belongs to the Cactaceae family was once widely grown as an ornamental plant and is now considered a fruit crop (Kasim et al. 2019; Siregar et al. 2021). Red dragon fruit (*Hylocereus polyrhizus* Britton and Rose) is one cactus plant with the potential as an ornamental plant and edible fruit (Utaminingsih et al. 2019). Red dragon fruit is a popular tropical fruit highly regarded for its health benefits, including its high antioxidant content (Jiang et al. 2020). Enthusiasts have been practicing plant propagation through cuttings for a long time. It is simple, rapid, and cheaper than other sexual or asexual methods (seeds) of plant propagation (Stokes et al. 2020). Breeding by cuttings is expected to guarantee the same traits as the mother and a relatively shorter fruiting time.

The auxin plant hormone is a central regulator of plant growth and development; due to auxin playing a critical role in cell division and expansion, plants use several cellular mechanisms to regulate auxin levels and response (Frick and Strader 2018). Plant hormone auxin is critical in plant growth, dependent on its heterogeneous distribution in plant tissues (Allen and Ptashnyk 2020). The plant hormone auxin drives plant growth and morphogenesis. The active auxin Indole-3-Acetic Acid (IAA) levels and distribution are tightly controlled through synthesis, inactivation, and transport (Korasick et al. 2013). Among these mechanisms is regulating the input from the auxin precursor Indole-3-Butyric Acid (IBA) into the pool of active auxin (IAA) (Frick and Strader 2018).

In the propagation of dragon fruit cuttings, some points need attention are; First, the disease that often occurs is stem rot. This disease attacks new dragon fruit plants; Dragon fruit plants often rot on the rootstock, are brown, and have white threads. The decay is caused by excessive soil moisture so that a fungus appears that causes rottenness, namely *Sclerotium rolfsii* Sacc. All stems become rotten, which can occur if the stems are planted directly. The sap that is still wet gives a rotten effect on red dragon fruit cuttings.

Growth regulators are chemicals that regulate all growth and development factors in plants. The application of plant growth regulators modifies hormone balance and growth, leading to increased yield, enhanced crop tolerance against abiotic stress, and improved physiological traits of crops (Desta and Amare, 2021). The concept of growth regulators begins with the plant hormones concept. Plant growth regulators are mainly exploited to achieve this because of their essential role in plant growth and development (Amoanimaa-Dede et al. 2022). Growth regulators are widely used in modern horticulture,

agriculture, and horticultural crop maintenance. The "correct" plant growth regulators directly interfere with the hormonal status of the plant. They are represented by hormone biosynthesis or translocation inhibitors, plant hormones or synthetic analogs, and hormone receptor blockers (Rademacher 2015). For example, one way to increase the growth of cuttings is to use a type of hormone Indole Butyric Acid (IBA), which is used to stimulate root formation. Using exogenous plant growth regulators is essential for Nepalese cucumber producers as growth regulators have a hasty effect on vegetative and crop yield quality (Gosai et al. 2020).

Based on the description above, the author is very interested in studying the effect of Indole Butyric Acid (IBA) on the growth of rootstock cuttings of red dragon fruit (*Hylocereus costaricensis* (F.A.C. Weber) Britton & Rose) IBA on the growth of rootstock cuttings. Therefore, this study aims to determine the effect of IBA on the red dragon fruit (*H. costaricensis*).

MATERIALS AND METHODS

This research was carried out in Sidorukun Village, Pangkatan Sub-district, Labuhan Batu District, North Sumatra, Indonesia, with a flat topography of sandy loam soil at an altitude of ± 39 m from sea level. This study was conducted from June to October 2020.

The materials used include Indole Butyric Acid (IBA) with various concentrations and rootstock cuttings of red dragon fruit plants with a length of 20 cm (Ross et al. 2021). The additional material includes; topsoil, chicken manure, sand label paper, and 25 x 35 cm polybags. The number of each component in the experimental field: number of repetitions; 5 replicates, number of plots; 25 plots, number of plants per plot: 5 plants, number of sample plants per plot; 3 plants, total number of plants, 125 plants, number of sample plants; 75 plants.

Dragon fruit stems are planted for 60 days by watering in the morning and evening with sufficient clean water. The temperature is 25-30 degrees Celsius, and the relative humidity needed by dragon fruit plants is between 70%-95%. The tools used are hoes, shovels, drills, meters, stationery, calculators, analytical scales, ovens, and other tools that support this research. A randomized block design was used with 1 factor, namely the concentration of IBA consisting of B0:0 ppm, B1:4,500 ppm, B2:5,500 ppm, B3:6,500 ppm, and B4:7,500 ppm. The data from the study were analyzed using a linear model to show the result in fingerprint, using the equation as follows:

$$Y_{ij} = \mu + t_i + \beta j + \varepsilon_{ij}$$

ε_{ij} = experimental error

The data were then analyzed using IBM Statistical Program for Social Science (SPSS) version 20 (Hernosa et al. 2022). If the fingerprint results show a noticeable difference, it will be continued with the Duncan Multiple Range Test (DMRT) test with a confidence level of 5% (Nikmah et al. 2019).

RESULTS AND DISCUSSION

The percentage of the emergence of shoots (%)

Based on the analysis of fingerprints, it is known that the treatment of administering IBA has observed a significant effect on the percentage of shoot emergence (%), as shown in Table 1. Furthermore, the application of various concentrations of IBA has a significant effect on the parameters of the percentage of shoot emergence (%), namely B0 (control) by 80%, then B1 (4,500) ppm, B2 (5,500) ppm, B3 (6,500) ppm, and B4 (7,500) ppm, all by 100% (Figure 1).

Age appears shoots (days)

The results in Table 2 showed that the cuttings of the dragon fruit plant that sprouted the fastest were obtained at the B2 (5,500 ppm) treatment, with an average of 26 days of seedlings. Meanwhile, the cuttings that sprouted the longest were obtained at the B4 treatment (7,500) ppm, with an average of 33 days (Figure 2).

Shoot length (cm)

Based on the analysis of fingerprints, it is known that the treatment of giving various concentrations of Indol Butyric Acid (IBA) has no noticeable effect on the parameters of the longevity of shoots on 30, 45, and 60 days after planting (cm). The average length of the shoots on 30, 45, and 60 can be seen in Table 3.

Number of shoots

Table 4 shows that the highest number of shoots of stem cuttings at 30 days after planting the dragon fruit plant was found in the B2 treatment with 0.87 shoots, and the lowest number of shoots was found in the B0 treatment with 0.27 shoots. Furthermore, the highest number of dragon fruit stem cutting shoots at 45 days after planting was found in the B2 treatment with 1.60 shoots, which were significantly different from the B0, B3, and B4 treatments, and the lowest was found in the B0 control treatment with 0.33 shoots. The highest number of shoots at 60 days after planting was found in the B4 treatment with 2.33, which was significantly different from the B0, B1, and B3 treatments, and then the lowest number at 60 days after planting was found in the B0 treatment with 0.53 shoots.

Table 1. The percentage of the emergence of shoots (%) on the treatment of Indole Butyric Acid (IBA)

Treatment	Percentage of emergence of shoots (%) Average (%)
B0 (control)	80b
B1 (4500 ppm)	100a
B2 (5500 ppm)	100a
B3 (6500 ppm)	100a
B4 (7500 ppm)	100a

Note: Numbers followed by the same letter in columns and rows are insignificant according to DMRT 0.05

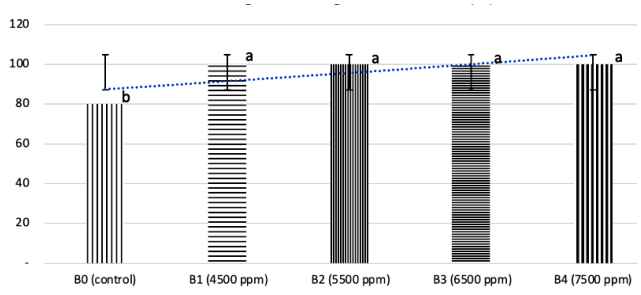

Figure 1. The percentage of the emergence of shoots (%) on the treatment of Indole Butyric Acid (IBA) with standard deviation display

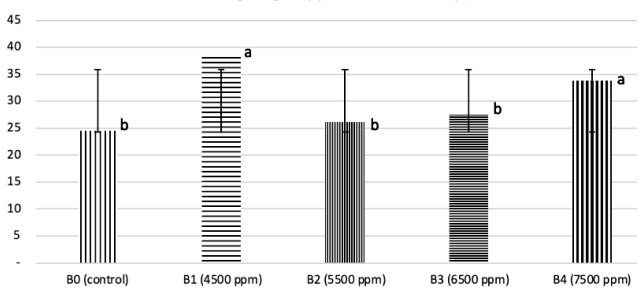

Figure 2. The average age of emergence of shoots with a standard deviation display

Table 2. Age of emergence of shoots (days) seedlings of dragon fruit plant cuttings on various treatments and their concentration

Treatment	Repeat					Total	Average
	1	2	3	4	5		
B0	-	-	-	24.00	25.00	49.00	24.50b
B1	32.00	35.00	25.00	20.33	29.00	141.33	28.27a
B2	25.50	20.00	30.50	31.33	23.33	130.66	26.13b
B3	32.00	20.00	33.66	26.00	26.00	137.66	27.53b
B4	27.00	49.75	34.00	28.66	29.50	168.91	33.78a
Total	116.50	124.75	123.16	130.32	132.83	627.56	-
Average	23.30	24.95	24.63	26.06	26.57	-	28.04
SD	0.13	0.36	0.19	0.19	0.11	2.72	-

Note: -: No shoots appear

Table 3. Shoot length (cm) aged 30, 45, and 60 days after planting on the treatment of various centration of Indole Butyric Acid (IBA)

Treatment	Shoot length (cm)		
	30 DAP	45 DAP	60 DAP
B0 (control)	2.53	4.50c	7.80d
B1 (4500 ppm)	4.28	6.09a	11.19a
B2 (5500 ppm)	4.28	6.52a	14.35a
B3 (6500 ppm)	1.47	5.40b	8.77c
B4 (7500 ppm)	0.28	2.23d	8.19c
Average	2.51	4.94c	10.06b
Standard Deviation	0.0722	0.0720	0.1243

Note. DAP: days after planting. Numbers followed by the same letter in columns and rows are insignificant according to DMRT 0.05

Table 4. Number of shoots aged 30, 45, and 60 days after planting in the treatment of various concentrations of Indole Butyric Acid (IBA)

Treatment	Number of shoots (shoots)		
	30 DAP	45 DAP	60 DAP
B0 (control)	0.28	0.34c	0.54c
B1 (4500 ppm)	0.74	1.41a	1.80b
B2 (5500 ppm)	0.88	1.61a	1.74b
B3 (6500 ppm)	0.48	0.88b	1.80b
B4 (7500 ppm)	0.34	1.01b	2.34a
Average	0,54	1.05	1.64
Standard Deviation	0.0179	0.0236	0.0278

Note. DAP: days after planting. Numbers followed by the same letter in columns and rows are insignificant according to DMRT 0.05

Figure 3. Comparison of control treatment B0 (control), B1 (4,500 ppm), B2 (5,500 ppm), B3 (6,500 ppm), and B4 (7,500 ppm)

Fresh weight of shoots (g)

Table 5 shows that the highest fresh weight of the stem cuttings of the dragon fruit plant was found in the B1 treatment at 25.65 g, and the lowest shoot fresh weight was found in the B0 dose at 10.68 g. The observations of fingerprints on the parameters of fresh weight of shoots Table 5 found that the treatment of giving various concentrations of IBA was insignificant.

The dry weight of shoots (g)

Based on the analysis of fingerprints, it is known that the treatment of various concentrations of Indole Butyric Acid (IBA) has no noticeable effect on the parameters of the dry weight of shoots (g). Therefore, the average dry weight of the shoots can be seen in Table 5.

Table 5. Several parameters in the treatment of various concentrations of Indole Butyric Acid (IBA)

Treatment	Fresh weight of shoots (g)	The dry weight of shoots (g)	Root length	Fresh weight of the roots (g)	Root volume (mL)	The dry weight of roots (g)
			60 days after planting			
B0 (control)	10.69	1.64	9.31c	0.43d	2.01c	0.24d
B1 (4500 ppm)	25.66	3.85	11.91b	0.69c	2.31b	0.35c
B2 (5500 ppm)	23.84	3.34	11.65b	0.91b	2.61a	0.32c
B3 (6500 ppm)	18.90	2.17	13.45a	0.79b	2.41b	0.40b
B4 (7500 ppm)	18.37	2.65	14.18a	1.16a	2.71a	0.52a
Average	19.49	2.73	12.1	0.79	2.41	0.36
Standard deviation	0.227	0.0399	0.077	0.0123	0.982	0.0072

Note: Numbers followed by the same letter in columns and rows are insignificant according to DMRT 0.05

The observation of the dry weight of shoots in Table 5 showed the highest value in the B1 (4,500 ppm) treatment at 3.84 g and the lowest in the B0 (the control) treatment at 1.63 g. That shows that the dry weight of the shoots depends on the amount of nutrient uptake.

Root length (cm)

The average length of the roots can be seen in Table 5. The results of the observation of root length parameters are known that the treatment of giving various concentrations of Indole Butyric Acid (IBA) has a significant effect on the root length parameters (cm), with the highest average in the B4 (7,500 ppm) treatment at 14.17 cm.

Fresh weight of roots (g) and volume of roots

The results of the observation of the parameters of fresh weight of roots and root volume in Table 5 are known that the treatment of giving various concentrations of Indole Butyric Acid (IBA) has a significant effect on the parameters of fresh weight of roots (g) and root volume (mL), with each of them the highest for the fresh weight of roots B4 (7,500 ppm) at 1.15 g and root volume B4 (7,500 ppm) at 2.71 mL.

The dry weight of roots (g)

Based on the analysis of fingerprints, it is known that the treatment of giving various concentrations of Indole Butyric Acid (IBA) has an areal impact on the dry weight parameters of the roots (g). The average dry weight of the roots can be seen in Table 5. The root's dry weight is the root's weight after being dried in the oven, so its moisture content has been lost, and only chemical compounds are left in the root. Table 5 shows that the dry weight of the roots of the lower stem cuttings of the dragon fruit plant was highest in the B4 treatment at 0.51 g, which was significantly different from the B0, B1, and B3 treatments, and the lowest was found in the B0 the control treatment of 0.23 mL.

Discussion

The application of various concentrations of IBA was significant to the parameter of the percentage of shoot emergence (%), namely B0 (the control) of 80%, then B1 (4,500 ppm), B2 (5,500ppm), B3 (6,500ppm) and B4 (7,500 ppm) of 100%. The most effective method for promoting adventitious rooting was IBA addition, especially in top-performing individuals (Barron et al. 2020). The higher concentration of IBA increased the thickness root production (Barroso et al. 2018). That is because IBA (Indole Butyric Acid) can stimulate root growth because IBA has a chemical content that is more stable and works longer so that it can stimulate root growth

The results in Table 2 show that the cuttings of the dragon fruit plant that sprouted the fastest were harvested at B2 (5,500 ppm) treatment, with an average of 26 days. Meanwhile, the cuttings that sprout the longest were obtained at the B4 treatment (7,500 ppm), with an average of 33 days. Because the B4 (7,500 ppm) application has exceeded the optimum value, cell lengthening and division activity have decreased. Auxin is a major phytohormone that coordinates plant responses to the environment and controls numerous aspects of plant development (Brumos et al. 2018). Auxin is a crucial phytohormone involved in multiplying plant developmental processes. Spatiotemporal regulation of auxin levels is necessary to develop organs in the proper place and at the proper time (Damodaran and Strader 2019).

Indole Butyric Acid's (IBA) behavior was insignificant by observing lifelong length parameters of 30, 45, and 60 days after planting in Table 3. It is suspected that environmental factors affect the growth of dragon fruit cuttings, among others; temperature, the intensity of sunlight, and the influence of care in dragon fruit cuttings. Plant growth factors control or influence plant characteristics as well as adaptation. Light is one of the most factors that affect plant growth. Although photosynthesis has been extensively investigated, knowledge is still limited about how light quality affects the production of horticultural crops, especially fruits and vegetables (Kuniga 2020).

The number of shoots describes the plant's nutritional status and determines growth and yield quality. In this study, Table 4 shows the observation results on the number of shoots that significantly influenced the application of Indole Butirat Acid (IBA). Figure 3 shows that for 45 days after planting and 60 days after planting, Indole Butyric Acid (IBA) given to the cuttings of dragon fruit plants affects by the lengthening of plant cells, namely by spurring specific proteins in the plasma membrane of plant cells to pump H + ions into the cell wall. The plasma membrane (PM) H+-ATPase is an important ion pump in the plant cell membrane. This pump energizes the PM by

extruding protons from the cell and generating a membrane potential, a prerequisite for growth (Falhof et al. 2016).

Table 5 shows that the treatment of administration of various concentrations of Indole Butyric Acid (IBA) was insignificant. However, it is clear that the highest value treatment is B1 (4,500 ppm) at 25.65 g, and the lowest is the B0 (the control) treatment at 10.68 g, which means there remains a difference between the B0 treatment (the control) and the B1, B2, B3, and B4 treatments. Indole Butyric Acid (IBA) influences cell lengthening, cell division, and cell differentiation, stimulating cambium activity and forming phloem and xylem vessels. Plant hormones regulate the interactions between plants and their complex biotic and abiotic environments. Each hormone initiates a specific molecular pathway, and these different hormone pathways are integrated into a complex network of synergistic, antagonistic, and additive interactions (Aerts et al. 2021).

Table 5 shows the highest values at treatment B1 (4,500 ppm) at 3.84 g and the lowest at treatment B0 (the control) at 1.63 g, which shows that the dry weight of the shoots depends on the amount of nutrient uptake. High nutrient uptake causes photosynthesis to increase, contributing to the plant's increasing dry weight. Therefore, the plant will grow well if photosynthesis proceeds well, followed by increased dry weight.

In the dragon fruit cultivation case, techniques for the subsequent development of these shoots should be removed, and only one should be maintained. Cuttings with two or more shoots must be removed, and only one sprout must be maintained, considering dragon fruit is nutrient-greedy cacti. Selected shoots are large shoots with no attacks of pests or diseases, long shoots, and usually growing ones. The removal of the shoots is carried out after the seedlings are planted in the field; this is because when transferring the seedlings to the field, there is often damage to the seedlings, such as broken shoots, so these broken shoots must be removed and what is maintained is a good sprout. If the removal of shoots is carried out in a nursery, it is feared that there is no shoot reserve to be maintained, waiting for the next shoot, which is very detrimental.

The results showed that indole butyric acid (IBA) treatment had no noticeable effect on observing the parameters of longevity shoots of 30, 4,5, and 60 days after planting. Therefore, it is suspected that environmental factors affect the growth of dragon fruit cuttings, among others; temperature, the intensity of sunlight, and the influence of care in dragon fruit cuttings. Factors affecting growth are internal factors and external factors; internal factors consist of the rate of photosynthesis, respiration, differentiation, and influence of genes, while external factors include light, temperature, water, organic matter, and nutrient availability.

Indole Butyric Acid (IBA) has better and more effective properties in increasing the percentage of rooted plants, accelerating root growth, increasing the number of roots, and improving root quality. Phytohormones are chemical substances that regulate plant growth, reproductive processes, longevity, development, and even death. One of the most typical representatives of this group is indole-3-butyric acid (IBA), which is widely applied in various branches of agriculture (Kaczmarek et al. 2020)

Suppose the fresh weight of the roots formed is high. In that case, the ability of the roots to absorb nutrients is also higher, and the photosynthesis process goes well so that the photosynthetic allocated to all parts of the plant is included for root growth, increasing root growth and root volume. Different IBA applications are likely to continue to be developed in the future with a concern for environmental protection and the sustainability of human life (Bandarra et al. 2021).

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Acute toxicity, biochemical and histological of fenitrothion and thiobencarb on fish Nile tilapia (*Oreochromis niloticus*)

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Abstract. *Fouad MR, El-Aswad AF, Aly MI. 2022. Acute toxicity, biochemical and histological of fenitrothion and thiobencarb on fish Nile tilapia (*Oreochromis niloticus*). Nusantara Bioscience 14: 217-226.* The results show that the tested fenitrothion and thiobencarb are highly toxic to fish. However, fenitrothion is more toxic (1.6 times) on *Oreochromis niloticus* (Linnaeus, 1758) than thiobencarb. The determined 96-h LC_{50} values using a static bioassay system to Nile tilapia fingerlings (8-10 g) were 0.20 and 0.32 mg L^{-1} for fenitrothion and thiobencarb, respectively. The mortality rate of fish exposed to ½ 96-h LC_{50} of fenitrothion (0.10 mg/L) and thiobencarb (0.16 mg/L) for four days demonstrated was 20% mortality rate. Fish showed tremors, lethargy, decreased movement, and increased respiratory rhythm. The total activity of AChE in control was 5.61 ±0.03; it was significantly reduced to 4.92 ±0.03 in fenitrothion treatment and 1.13 ±0.02 in thiobencarb treatment. Fenitrothion decreased the specific activity from 0.83 ±0.01 for the control to 0.68 ±0.01, whereas thiobencarb reduced the specific activity to 0.22 ±0.01. Generally, thiobencarb inhibited AChE activity much more than fenitrothion; it produced 80% inhibition, while fenitrothion produced 12.5% inhibition. It showed a significant increase in liver GST and SOD activity of Nile tilapia exposed to the tested pesticides compared to the control. There were no histological alterations in the tissues of the control individuals. It was found that the herbicide thiobencarb affected the gills, kidneys, and liver of Nile tilapia more than the insecticide fenitrothion.

Keywords: Biochemical, fish, histological, Nile tilapia, pesticides, toxicity

INTRODUCTION

Over the world, fish is an important human food due to its protein content. About 25,000 different known species of fish, including 15,000 marine species and 10,000 freshwater species (Nelson 1994). The annual capture (direct and from fish farms) was about 149 million tons (FAO 2012). Egypt is considered the second largest producer of tilapia in the world, where Nile tilapia accounts for about 80% of fish production (Tahoun et al. 2008). Fish is a source of important nutrients essential for human health (Stanley et al. 2016; Aboagye et al. 2020). Fish farming is practiced on farmlands as a component of sustainable agriculture in many tropical countries. Thus, to supplement the risk assessment, it is crucial to gather information on the impact of pollutants on fish (Peebua et al. 2007). Fish serve as a bioindicator of water quality due to two key characteristics: their availability in a larger range of habitats and their stronger reactivity to contaminants (Stanley et al. 2016).

Once a pesticide is applied to a field, it travels through the watershed and may have negative consequences far away from the application site (Akan et al. 2014). Even though pesticides are diluted in rivers and streams, various mechanisms cause the pesticide to become concentrated and harmful (Stanley et al. 2016). Through soil erosion, the pesticides may reach the rivers (de Melo Plese et al. 2005). However, surface runoff due to rainfall is the major mechanism for pesticide transport into water bodies. It was reported that pesticide exposure to aquatic biota was achieved in three ways; direct absorption of polluted food, water absorption through gills, and integument. Each of the three routes varies with pesticide type, organism, and environmental conditions. The absorption through the gut and gills are the two important pesticide routes. The relative importance of absorption via water or food depends on the exposure, dose level, duration, and the individual organism (Stanley et al. 2016). The absorption of pesticides by an aquatic organism occurs through partitioning. The organisms living in the water (fish) must combat the water pollutants, especially the break-down products of pesticides, since they live in that water medium. Fish is exposed to contaminants by direct absorption through the skin and dermal contact (Akan et al. 2014). Fish is affected by pesticides, due to acute mortality or by sublethal effects. Commonly, acute mortality is expressed in LC_{50}, typically done for 96 hours. Behavioral changes such as excitation, avoidance, respiration, and feeding can be due to pesticide toxicity. Chemicals can also cause affect reproduction, blood cells, enzymes, and hormones (Akan et al. 2014).

Pesticide contamination is common in the agricultural sector. It can cause damage to human and beneficial organisms and eventually lead to aquatic environment pollution; thus, it becomes hazardous to aquatic life (El-Murr et al. 2015). Sub-lethal effects of pesticides could be studied in different beneficial organisms such as birds, fish, earthworms, and bees with a highlight to identify the biochemical responses that may be useful to monitor sub-

lethal levels of exposure in the field. The biochemical responses may be detected by measuring enzyme activities in different tissues. In general, the enzyme activities measurement may be useful for exposure assessment and sub-lethal effects of chemicals on the non-target organisms, causing structural and functional changes in liver tissues (Dahamna et al. 2004). Pesticides at low levels may produce various biochemical changes, some of which could not necessarily lead to observable symptoms (Araoud et al. 2012). Environmental pollution due to extensive usage of pesticides without proper management has affected the survival potential of fish as some of these toxic chemicals in the environment may persist for long periods (Gill et al. 1988), producing many physiological, biochemical, and histological changes in freshwater organisms, particularly of the fish. Therefore, histology is a useful tool to determine the pollution degree, particularly for sublethal and chronic effects. Histological examination of fish tissues, especially their liver, proved to be an extraordinarily sensitive tool to detect detrimental effects in fish induced by organic contaminants since the fish liver is a major storage site and pesticide biotransformation and excretion (Cengiz and Unlu 2006).

MATERIALS AND METHODS

Experimental materials

Tested pesticides

Fenitrothion

UPAC name: O, O-dimethyl O-4-nitro-m-tolyl phosphorothioate, Chemical formula: $C_9H_{12}NO_5PS$, Solubility: Water 0.038 g/L, Pesticide type: Insecticide, miticide, Group: Organophosphate, Production Company: Shandong Chuangying Chemical Co., Available formulations: EC 50%; ULV 100%; WP 25, 40, 50%; G 5%; D 2, 2.5, 3, 5%, Usage: For controlling chewing and sucking insects on rice, orchard fruits, vegetables, cereals, cotton, and forest.

Thiobencarb

UPAC name: S-4-chlorobenzyl diethyl thiocarbamate, Chemical formula: $C_{12}H_{16}ClNOS$, Solubility: Water 0.030 g/L, Pesticide type: Herbicide, Group: Thiocarbamate, Production Company: Shandong SanYoung Industry Co., Ltd, Available formulations: EC 50%; G 10%, Usage: It is herbicide for weed control for pre and early post-emergence in rice paddy fields and other situations.

Fish

Nile tilapia was used (*Oreochromis niloticus* Linnaeus, 1758), a common type in Egypt, particularly in rice fields. Tilapia of both genders weighing 8-10 g and measuring 6-8 cm total length were obtained from a Department of Animal Production, Faculty of Agriculture, Alexandria University, Egypt, without pesticide exposure. Fish were acclimated to laboratory conditions for 10 days in the tank (50 L) containing dechlorinated water with aeration and a natural photoperiod (12 h light/12 h dark) before the experiments. During acclimation and pesticide exposure, commercial fish pellets were fed once a day at a proportion of 1% of the total weight of fish in each tank (Peebua et al. 2007).

Experiments

Toxicity of tested pesticides on fish by static bioassay technique

A series of concentrations (0.1, 1, 2.5, 5, 10, 25, 50, and 100 mg L^{-1}) of fenitrothion and thiobencarb were added to the dechlorinated water. The pesticide solutions (2 L for each) were added into 4 L plastic containers, 3 replicates per concentration, and the control was tested. Five Nile tilapia fishes (*O. niloticus*) were exposed per replicate. Under aeration conditions and a natural photoperiod (12 h light/12 h dark), commercial fish pellets were fed daily for 4 days. The mortality percentages were recorded daily, and the LC_{50} values were calculated by LdP line software (Saka 2010).

Side-effects of tested pesticides on biochemical indicators

The side effects of fenitrothion and thiobencarb were studied on the AChE as a target of action of many pesticides, GST, which plays a key role in cellular detoxification, and SOD which prevent oxidative stress. First, the enzyme activities were tested in vivo; Nile tilapia fish (*O. niloticus*) was exposed to concentrations that achieved LC_{50} of fenitrothion and thiobencarb for 4 days, and the fish was taken to extract the enzymes.

Acetylcholinesterase (AChE) assay

AChE activity was determined by colorimetric method according to the procedure of Ellman et al. (1961) using acetylthiocholine iodide (ATChI) as a substrate. The definite weight of fish brain tissue was homogenized in 0.1M potassium phosphate buffer (pH 7.0) using a glass/Teflon homogenizer with ice. Determined protein concentrations by the Lowry et al. (1951) method.

Superoxide Dismutase (SOD) assay

The SOD activity was determined in the liver tissue of fish. The enzyme activity was determined according to Nishikimi et al. (1972). The assay depends on the ability of an enzyme to inhibit the phenazine methosulphate-mediated reduction of nitroblue tetrazolium dye.

Glutathione S-transferees (GST) assay

Total GST activity (cytosolic and microsomal) was determined according to Habig et al. (1974) by measuring the conjugation of 1-Chloro-2,4-dinitrobenzene (CDNB) as substrate with reduced glutathione. An increasing absorbance accompanies the conjugation at 340 nm.

Side-effects of tested pesticides on histological characteristics

The live exposed fish to concentrations of fenitrothion and thiobencarb equivalent to their LC_{50} for 4 days was taken the histological examination. Moreover, to study the changes in the histology of these organs, different organs of fish, such as the liver, kidney, and gills, were isolated from the control and the exposed fish. First, the physiological

saline solution (0.75% NaCl) was used to rinse and clean the tissues. Next, they were fixed in aqueous bouin's fluid. Then, they were dehydrated through gradual ethanol (70-100%). Finally, they were xylene-cleared and embedded in paraffin wax. Five microns thick paraffin sections were prepared, stained with Ehrlich hematoxylin/eosin (H&E) dyes, dissolved in 70% alcohol, and mounted in Canada balsam (Humason 1972). The slides were examined under a microscope. The possible changes in the tissues of fish exposed to tested pesticides were observed, and photo monographs were taken from the Olympus Microscope (El-Murr et al. 2015).

Statistical analysis

The Ldp line software performed experimental fish data presented as LC_{50} statistical analysis. In addition, a probit analysis developed by Chi (1997) was employed to assess the acute toxicity of pesticides to fish Nile tilapia.

RESULTS AND DISCUSSION

Toxicity of tested pesticides on fish by static bioassay technique

The acute toxicity as mortality percentages of fenitrothion and thiobencarb (50% EC commercial grade formulation for each) using a static bioassay system to Nile tilapia (*O. niloticus*) was tested. While using only the active ingredient in the tests is insufficient (Benli and Özkul 2010). The operational formulations of the herbicide are more toxic than the technical grade chemical (Jiraungkoorskul et al. 2002). Therefore, in this investigation, the results were given regarding the herbicide's commercial formulation, not the active ingredient used, because farmers only use the commercial formulation for agricultural uses.

Mortality percentages of fish exposed to concentrations of 0.1, 1, 5, 10, 25, and 50 mg L^{-1} were recorded at 24, 48, 72, and 96 h. The control mortality was zero during the experiment. Regarding fenitrothion, the lowest tested concentration (0.1 mg L^{-1}) caused 13.33, 20.00, 33.33, and 53.33 % mortality. Consequently, the concentration of 5 mg L^{-1} caused 33.33, 40.00, 53.33, and 80.00 % mortality at 24, 48, 72, and 96 h, respectively. The highest tested concentration (50 mg L^{-1}) of fenitrothion caused 100% mortality at 24 h; the concentration of 25 mg L^{-1} gave 100% mortality after 72 hours of exposure. Concerning thiobencarb, the mortality percentages were (20.00, 26.67, 33.33, and 40.00 %) for 0.1 mg L^{-1}, (40.00, 53.33, 60.00, and 73.33 %) for 5 mg L^{-1} at an exposure time of (24, 48, 72, and 96 h), respectively (Table 1). The concentrations of 25 and 50 mg L^{-1} gave 53.33 and 86.67 %, then 100% at 24 and 72 h, respectively. The results indicated that all tested concentrations' effects on fish increased mortality, with the increasing concentration of the pesticides and exposure time. It was observed that the mortality rate of tested pesticide exposure ranged from 15-55% in acute doses, which began high and decreased gradually. This observation also supported the mortality rate of thiobencarb exposure ranged from 25-30%, which began high and decreased gradually (Eissa et al. 2015).

The calculated 96-h LC_{50} value (95% confidence limits) of fenitrothion, using a static bioassay system to Nile tilapia (*O. niloticus*) fingerlings, was 0.20 mg L^{-1} (0.04-0.22). LC_{50} at 24 h was found to be 4.07 mg L^{-1} (3.28. 5.01) (Table 2). Similarly, the calculated 96-h LC_{50} value of thiobencarb was 0.32 µg /mL (0.19. 0.49). LC_{50} at 24 h was found to be 5.63 mg L^{-1} (4.23. 7.48). The selected species are as recommended by the standard methods (OECD 1993). The slope value was ≤ 1 at all probability levels. The Chi-Square values ranged from 13.36 to 85.99 for fenitrothion and from 22.63 to 61.92 for thiobencarb. The p-values were ≤ 0.01 for fenitrothion and thiobencarb at different time intervals. Moreover, based on the LC_{50} values, the toxicity of fenitrothion at 96 h was 20.7, 14.1, and 6.5 times more than its toxicity at 24, 48, and 72 h. Also, thiobencarb toxicity at 96 h was 17.7, 7.4, and 3.5 times more than its toxicity at 24, 48, and 72 h, respectively. Therefore, from comparing the effect of two pesticides according to 96-h LC_{50} values, fenitrothion was 1.6 times more toxic than thiobencarb. Additionally, from comparing according to the relative toxicity, at 48 and 72 h, thiobencarb has relative toxicity = 100, with fenitrothion having relative toxicity of (85.82 and 85.57) compared to thiobencarb. In contrast, at 24 and 96 h, fenitrothion has relative toxicity = 100, while thiobencarb has relative toxicity of 72.17 and 61.44 compared to fenitrothion. The results show that the tested pesticides are highly toxic to fish. However, fenitrothion is additional deadly on *O. niloticus* than thiobencarb. This result agreed with those obtained by Benli and Özkul (2010), who stated that fenitrothion is highly toxic to Nile tilapia.

The results demonstrated the calculated 96-h LC_{50} value of fenitrothion, using a static bioassay system to Nile tilapia (*O. niloticus*) of body weight (8-10 g) was 0.20 mg L^{-1}. Despite being the same species, the difference in LC_{50} was referred to as the difference in body weight (Eissa et al. 2015). The results showed that fenitrothion is highly toxic to Nile tilapia. However, it was reported that fenitrothion is considered moderately toxic to fish (Benli and Özkul 2010). Still, it is less toxic to most other species, such as 48-h LC_{50} values for carp (*Cyprinus carpio*) 8.2 mg L^{-1}, eel (*Anguilla anguilla*) 3.2 mg L^{-1}, and 96-h LC_{50} values compared with guppy (*P. reticulata*), peppered corydoras (*Corydoras paleatus*), medaka (*Oryzias latipes*) and mullet (*M. cephalus*) 3.21, 3.51, 2.1 and 2.6 mg L^{-1}, respectively (Sarikaya et al. 2007). Also, the experiments showed that the LC_{50} at 96 h of fenitrothion was 0.2 mg L^{-1} in *A. anguilla* (Ferrando et al. 1991) and 1.64 mg L^{-1} for top mouth gudgeon (Solomo et al. 2000). Experimentally, the LC_{50} of thiobencarb in this study on *O. niloticus* (8-10 g of body weight) was found to be 0.32 mg L^{-1} for 96 h, which conformed by Eissa et al. (2015), which was 0.40 mg L^{-1} while; was less than that recorded by Abbas et al. (2007), and Abumourad et al. (2010) which was 0.72 mg L^{-1} on Nile tilapia of body weight (15-20 g). Thiobencarb, at very low doses, is widely used to eradicate the larvae of mosquitoes and control argulus disease and milkfish during pond preparation, which can be toxic to aquatic organisms

(Abbas et al. 2007). It should be emphasized that sublethal concentrations are as significant as acute concentrations to understand the earlier toxicity in non-target species (Benli and Özkul 2010). There is a global interest concerning the problems of a polluted ecosystem, which comprises hazards to fish health and human health (Ulrich et al. 2004).

Side-effects of tested pesticides on biochemical indicators

Effects of tested pesticides on AChE activity

Measurement of acetylcholinesterase activity is routinely used as a biomarker of exposure to different groups of contaminants, such as organophosphate and carbamate insecticides. Previous experiments carried out in this study showed that 0.20 and 0.32 mg/L for fenitrothion and thiobencarb were the LC_{50}-96 h in Nile tilapia (*O. niloticus*). Based on these results, constant sublethal exposure of Nile tilapia fish to ½ LC_{50}-96 h (0.10 and 0.16 mg/L) of fenitrothion and thiobencarb for 96 h resulted in the inhibition of the AChE activity in the brain tissue. The mortality rate of fish exposed to ½ 96-h LC_{50} of two pesticides for 4-days demonstrated about (20%) mortality rate, the dead fish were avoided, and live fish were examined. No mortality occurred in control during the experiment, but fish in treatments showed signs of tremors, lethargy, decreased movement, and increased respiratory rhythm. As reported in Table 3, total and specific brain AChE activity were significantly reduced. The total activity of AChE in un-exposed control was 5.61 ±0.03; it was significantly reduced to 4.92 ±0.03 in fenitrothion treatment and significantly reduced to 1.13 ±0.02 in thiobencarb treatment. On the other hand, fenitrothion decreased the specific activity from 0.83 ±0.01 for the control to 0.68 ±0.01, while thiobencarb reduced the specific activity to 0.22 ±0.01. In general, thiobencarb inhibited AChE activity much more than fenitrothion; it produced 80% inhibition, whereas fenitrothion produced 12.5% inhibition. Similar findings have been described by Sancho et al. (1998). They reported 57% inhibition of AChE activity at 96 h exposure to 0.04 mg L^{-1} and 0.02 mg L^{-1} of fenitrothion produced only a 51% reduction in AChE at 96 h, too. A 20% decline in AChE activity in fish was used as evidence of exposure to OPs (Zinkl et al. 1991), those obtained in fish that have encountered fenitrothion concentration of O. 4 mg/L (Solomo et al. 2000). Fenitrothion insecticides induced significant inhibitory effects on the AChE activity of *A. anguilla*, ranging from > 40% inhibition at a sublethal concentration of 0.02 mg L^{-1} to > 60% inhibition at a sublethal concentration of 0.04 mg L^{-1} (Sancho et al. 1997). A few fish studies indicate a greater decrease in AChE activity with exposure to higher concentrations of OP insecticides such as fenitrothion (Morgan et al. 1990).

Table 1. Toxicity of fenitrothion and thiobencarb on fish (Mortality% ±SE) by static bioassay technique

Conc. (mg L^{-1})	24h	48h	72h	96h
		Fenitrothion		
0.1	13.33 ±6.67	20.00 ±11.55	33.33 ±6.67	53.33 ±6.67
1	20.00 ±11.55	26.67 ±13.33	40.00 ±11.55	73.33 ±6.67
2.5	26.67 ±6.67	33.33 ±6.67	46.67 ±6.67	73.33 ±6.67
5	33.33 ±6.67	40.00 ±0.00	53.33 ±6.67	80.00 ±0.00
10	66.67 ±6.67	73.33 ±6.67	73.33 ±6.67	86.67 ±6.67
25	93.33 ±6.67	93.33 ±6.67	100.00 ±0.00	100.00 ±0.00
50	100.00 ±0.00	100.00 ±0.00	100.00 ±0.00	100.00 ±0.00
		Thiobencarb		
0.1	20.00 ±0.00	26.67 ±6.67	33.33 ±6.67	40.00 ±0.00
1	26.67 ±6.67	33.33 ±6.67	40.00 ±11.55	66.67 ±6.67
2.5	33.33 ±6.67	46.67 ±6.67	46.67 ±6.67	66.67 ±6.67
5	40.00 ±0.00	53.33 ±6.67	60.00 ±0.00	73.33 ±6.67
10	46.67 ±13.33	53.33 ±17.64	73.33 ±6.67	93.33 ±6.67
25	53.33 ±6.67	73.33 ±6.67	100.00 ±0.00	100.00 ±0.00
50	86.67 ±13.33	93.33 ±6.67	100.00 ±0.00	100.00 ±0.00

Table 2. Toxicity indices and their parameters for fenitrothion and thiobencarb on fish by static bioassay technique

Pesticide	Time (day)	LC_{50} (mg L^{-1})[a]	Confidence limits at 95%	Slope[b]	χ^{2} [c]	P	Relative toxicity[d]
Fenitrothion	24	4.07	3.28-5.01	1.19 ±0.07	85.99	0.001	100
	48	2.76	2.15-3.52	1.03 ±0.01	72.20	0.001	85.82
	72	1.28	0.86-1.82	0.72 ±0.06	45.27	0.001	88.57
	96	0.20	0.04-0.22	0.57 ±0.01	13.36	0.010	100
Thiobencarb	24	5.63	4.23-7.48	0.77 ±0.04	61.92	0.001	72.17
	48	2.37	1.71-3.22	0.77 ±0.01	45.07	0.001	100
	72	1.13	0.80-1.54	0.86 ±0.01	58.31	0.016	100
	96	0.32	0.19-0.49	0.80 ±0.01	22.63	0.001	61.44

Note: a: Concentration causing 50% mortality of the fish. Results of LC_{50} are expressed by the mean of three replicates ± standard error (SE). b: Slope of the concentration-mortality regression line ± SE. c: Chi-square value. d: Relative toxicity = (LC_{50} level for the most effective/LC_{50} level for the other pesticide) × 100

Table 3. In-vivo AChE activity in the brain of the Nile tilapia exposed to ½ LC50 for 96 h of fenitrothion and thiobencarb individually

Parameters	Control	Fentrothion	Thiobencarb
Total AChE activity	5.61^{a} ±0.03	4.92^{b} ±0.03	1.13^{c} ±0.02
Specific activity	0.83 ±0.01	0.68 ±0.01	0.22 ±0.01
Inhibition %	-	12.35 ±0.96	79.93 ±0.51

Note: Total activity (OD 412/min g tissue), Specific activity (OD 412/min mg protein). I%, Inhibition % ((activity of control - activity of treatment)/activity of control) * 100. The means that do not share a letter differ greatly. Data significantly different from the control at $P < 0.05$

Effects of tested pesticides on GST and SOD activities

Results showed a significant increase in liver GST activity of treated fish compared to the control (Table 4). On 96 h, it showed a significant increase of GST activity as Unit/g tissue (OD_{340}/min g tissue) from control (155.63 ± at 2.82 to 2107.45 ±20.86 for fenitrothion and 3475.67 ±25.24 for thiobencarb. GST activity in the liver, gills, and muscle of fish *Labeo rohita* was significantly increased by endosulfan and chlorpyrifos compared to the control (Naz et al. 2019). Our results also show induction in GST-specific activity at 96 h exposure; the specific activity of GST for fish exposed to thiobencarb and fenitrothion individually was more than 30 times and 10 times that of the control (Table 4). Therefore, it seems likely that GST-dependent detoxification of fenitrothion might have also contributed to low acute toxicity. The acute toxicity level and liver esterases' susceptibility to fenitrothion were inversely related (Solomon et al. 2000).

On the other hand, Superoxide Dismutase (SOD) plays a very important role in the process of scavenging reactive oxygen species (ROS) (Livingstone 2001). The results showed that the activity of SOD in Nile tilapia liver exposed to fenitrothion and thiobencarb (Table 4) significantly increased in the two individual exposure pesticides. Fenitrothion promoted SOD activity after a 96 h exposure period from 43.10 ±0.71 in the control to 135.00 ±2.18. Also, thiobencarb significantly increased the SOD activity to 106.80 ±2.89. In addition, the specific activity of SOD was 3.81 ±0.12 in the control.

In contrast, it was 4.50 ±0.16 and 7.51 ±0.39 in the treatment of fenitrothion and thiobencarb, respectively, was revealed a significant increase in SOD in the treatment of fenitrothion-exposed fish compared with the control; this induction may be attributed to the high production of superoxide anion radical after fenitrothion exposure (Zeid and Khalil 2014). SOD is one of the most important defense mechanisms against the toxic effects of oxygen metabolism. SOD catalyzes the conversion of superoxide radicals to hydrogen peroxide, maintaining low steady-state concentrations of ROS and alleviating their toxic effects (Oruc et al. 2004). Furthermore, the dismutation of the superoxide anion radical is catalyzed by SOD to H_2O and H_2O_2, which is detoxified (Monteiro et al. 2006). Considering this, pro-oxidant conditions elicited by pesticides could trigger increases in the activity of this antioxidant enzyme as an adaptive response (Oruc and Usta 2007). In addition, it has been found that SOD as an antioxidant maintains the enzyme balance in cells and protects them from oxidative damage to the tissues (hepatopancreas, muscle) to fish after chronic exposure to the herbicide prometryne (Stará et al. 2014).

Side-effects of tested pesticides on histological characteristics

Water pollution induces pathological changes in fish. Therefore, histology is a valuable method for assessing pollution degree, particularly for sublethal and chronic impacts, as an indicator of pollutant exposure (Cengiz and Unlu 2006). Tissue damage brought about by water-borne pollutants can be easily observed because the fish gills come into immediate contact with the environment (Cengiz and Unlu 2006). No morphological changes were observed in fish exposed to 1.0 and 1.6 mg L^{-1} concentrations of fenitrothion and thiobencarb; the fish were unchanged, similar to the control group. However, respiratory distress was observed, one of the early symptoms of pesticide poisoning. Control individuals did not show any histological changes in the tissues examined by the light microscope.

Table 4. In-vivo GST and SOD activities in the liver of the Nile tilapia exposed to ½ LC_{50} for 96 h of fenitrothion and thiobencarb individually

Parameters	Control	Fentrothion	Thiobencarb
GST-Activity	155.63^{c} ±2.82	2107.45^{b} ±20.86	3475.67^{a} ±25.24
GST-Specific activity	0.36 ±0.02	3.51 ±0.27	11.88 ±0.65
SOD-Activity	43.10^{c} ±0.71	135.00^{a} ±2.18	106.80^{b} ±2.86
SOD-Specific activity	3.81 ±0.12	4.50 ±0.16	7.51 ±0.39

Note: GST-Activity U/g tissue (OD_{340}/min g tissue), GST-Specific activity (OD_{340}/min mg protein). SOD-Activity U/g tissue (OD_{560}/min g tissue), SOD-Specific activity (OD_{560}/min mg protein). Data significantly different from the control at $P < 0.05$

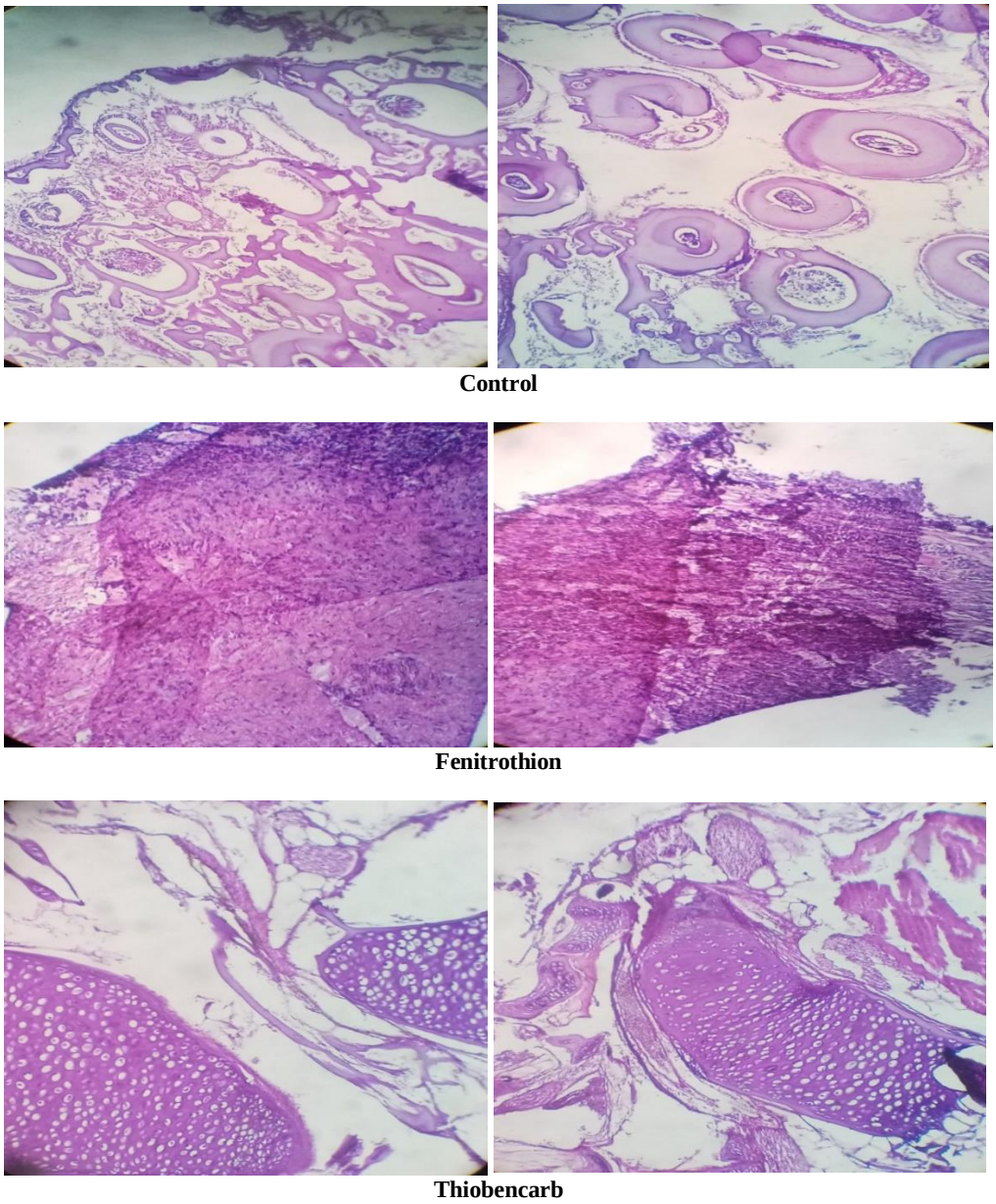


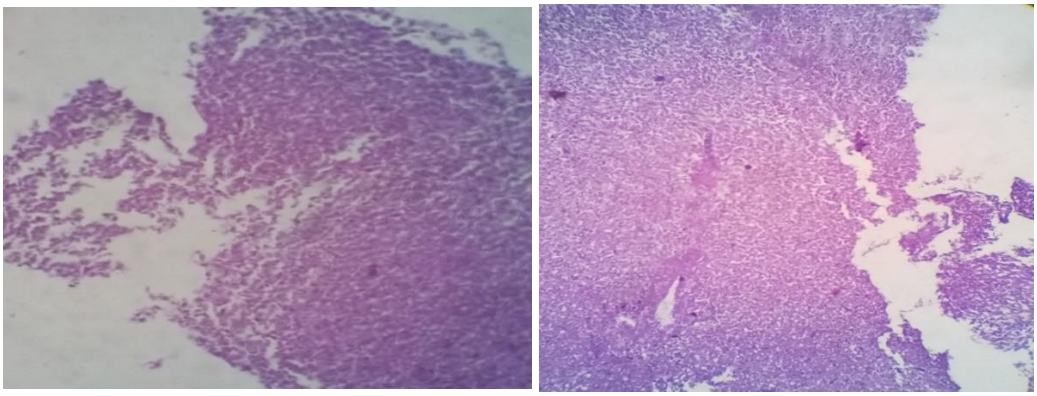
Figure 1. Histological appearance of the gills tissue of Nile tilapia exposed to ½ LC_{50} 96-h for fenitrothion (0.1 mg/L) and thiobencarb (0.16 mg/L) compared to control (H and E stained, X40, X100). The control showed bronchial arch, gills lamellae, epithelial layer, and normal cells. Fenitrothion treatment showed hyperplasia and necrosis. Thiobencarb treatment showed bronchial arch, gills lamellae, epithelial lifting, necrosis, and desquamations

Histological examination of the gills of the control group showed no microscopical abnormalities. The gills were observed to be made up of double rows of filaments, which arise perpendicularly to the lamellae. A squamous epithelium lined the lamellae; below that epithelium were lamellar blood sinuses. Between the lamellae, the filament is lined by a thick stratified epithelium (Figure 1-the Control). The gills of fish subjected to fenitrothion; showed hyperplasia, necrosis, cell lysis, and hyperemia (Figure 1-Fenitrothion). The gills subjected to thiobencarb showed articulated the bronchial arch, which carries gills lamellae, epithelial lifting, necrosis in the fusion of some lamellae, and desquamations (Figure 1-Thiobencarb). Similar to our findings, thickening of the lamellar epithelium (fusion) was also reported after glyphosate exposure in Nile tilapia (Jiraungkoorskul et al. 2003). The first target organ of pollutants was the gills because of their large interface area between the external and internal fish environment, vital functions such as gas exchange and ion osmoregulation; particularly, the gills are sensitive to adverse environmental conditions (Abbas et al. 2007).

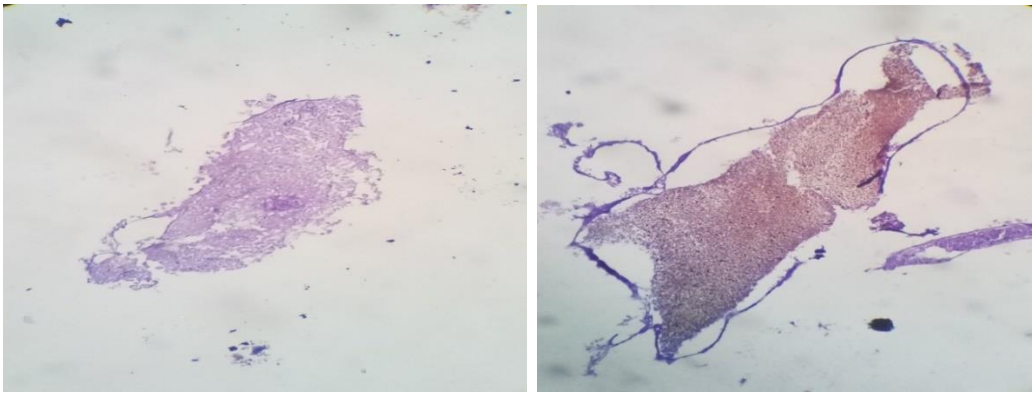
In addition, histological investigations of fish organs, especially the liver, repeatedly proved to be an extraordinarily sensitive tool to reveal both adaptive processes and detrimental effects in fish induced by organic pollutants. Since the fish liver is regarded as a major site of

storage, biotransformation, and excretion of pesticides and since the gut is the first organ to come into contact with foodborne contaminants, histological changes of these organs were chosen as criteria for the sublethal action of two pesticides. The liver of the treated fish showed hepatic lesion, necrosis, and pycnotic nuclei for insecticide fenitrothion. Side effects on fish by herbicide thiobencarb were congestion, degeneration of many hepatocytes, nuclease, hepatic lesions, vacuole pyknotic nuclei, necrosis degeneration, and dilatation of sinusoids in Figure 2.

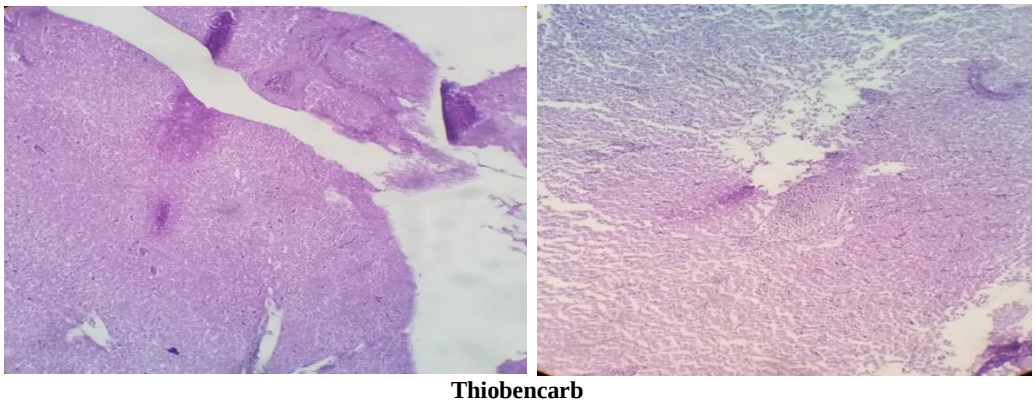
Previous studies investigating the side effects of pesticides have also shown similar alterations. For example, hypertrophy, vacuolization, and degeneration of hepatocytes, widespread nuclear pycnosis, focal necrosis have been reported in the *Puntius conchonius* exposed to dimethoate and carbaryl (Gill et al. 1988). The same alterations (hypertrophy, vacuolization, nuclear pycnosis, karyolysis, and fatty degeneration of hepatocytes) have also been recorded by Gill et al. (1990). In addition, they investigated the effects of aldicarb, phosphamidon, and endosulfan on the liver.



Control



Fenitrothion



Thiobencarb

Figure 2. Histological appearance of the liver tissue of Nile tilapia exposed to ½ LC_{50} 96-h for fenitrothion (0.1 mg/L) and thiobencarb (0.16 mg/L) compared to control (H and E stained, X40, X100). The control showed normal architecture of the liver tissue showing a continuous mass of polygonal cells called hepatocytes, distinct nuclei, sinusoid vessels, and blood vessels. Fenitrothion treatment showed hepatic lesion, necrosis, pycnotic of nuclei (left), and necrosis, pycnotic of nuclei (right). Thiobencarb treatment showed congestion, degeneration of many hepatocytes, nuclease (left), hepatic lesions, vacuole, pycnotic nuclei, necrosis degeneration, many hepatocytes degeneration, and dilatation of sinusoids (right)

In another research study, Cengiz et al. (2001) found hepatic lesions, including hypertrophy, degeneration, sinusoid enlargement, hemorrhage, pycnosis position of nuclei, vacuolization of the cell cytoplasm, infiltration of mononuclear lymphocyte. Also, Cengiz and Unlu (2006) found hepatic lesions in the liver tissues of fish exposed to deltamethrin pyrethroid, characterized by hypertrophy of hepatocytes, circulatory disturbances, and a significant increase of kupffer cells, focal necrosis, fatty degeneration, narrowing of sinusoids and nuclear pycnosis. In addition, roundup concentration corresponded to the 96-h LC50 value for adult fish tilapia, induced histopathological alterations in the liver, nuclear pyknosis, and vacuolation of hepatocytes (Jiraungkoorskul et al. 2002).

The fish kidney comprises three distinct systems: hematopoietic, endocrine, and excretory. Lesions developed in the kidney may involve one or all three tissue systems. Thus it is essential to examine the changes that may occur in different kidney cell types (Abbas et al. 2007). It can be seen in Figure 3 the effect of tested pesticides on the histological kidney examination. The effect of fenitrothion on the posterior kidney of Nile tilapia shows Malpighian corpuscles (bowman's capsules and glomerulus), epithelial hypertrophy of bowman capsules, elongation of bowman capsules and broader of B-C, necrosis, and atrophy in the glomerulus, necrosis in epithelial cells of B-C for fenitrothion.

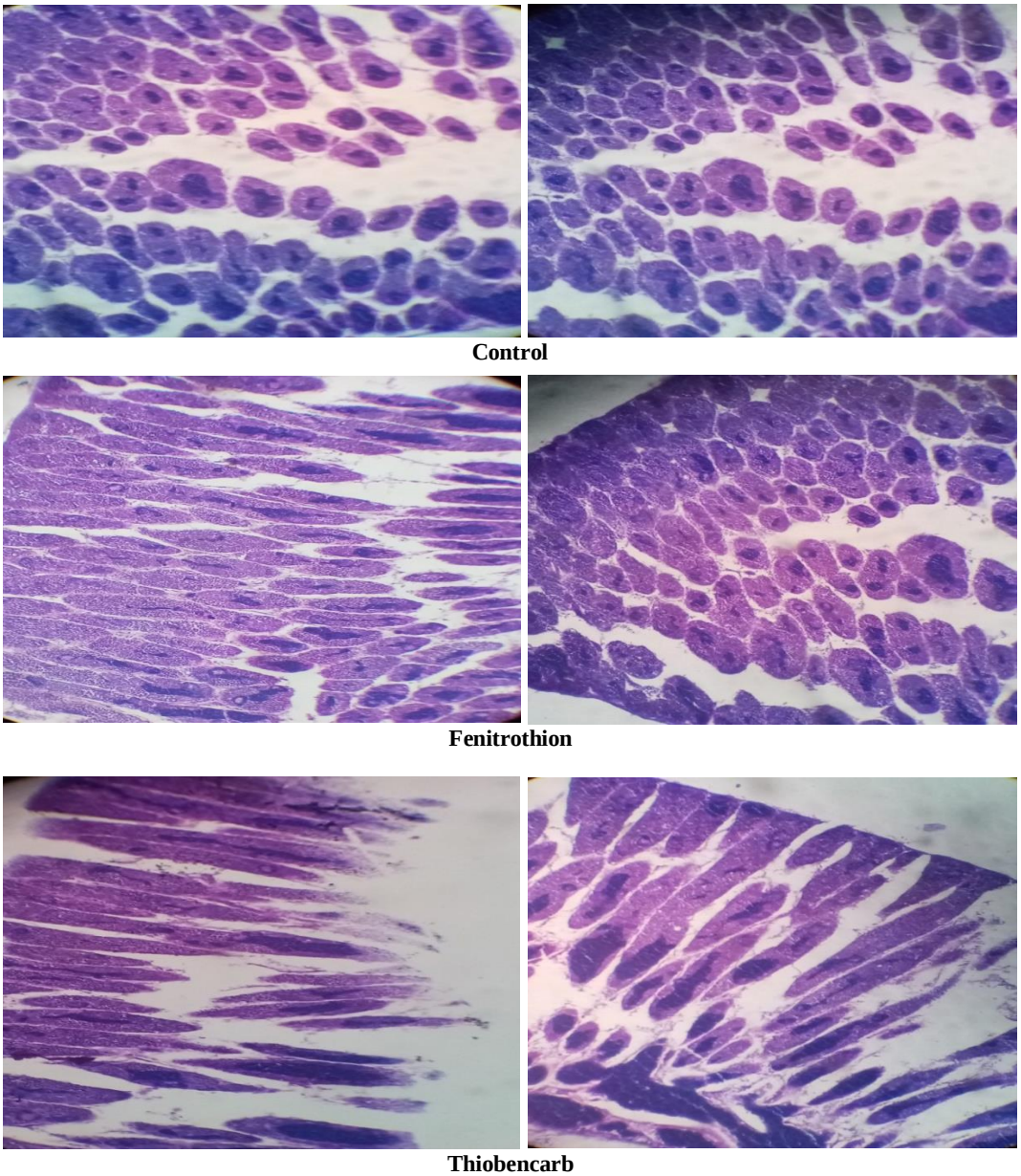


Figure 3. Histological appearance of the kidney tissue of Nile tilapia exposed to ½ LC_{50} 96-h for fenitrothion (0.1 mg/L) and thiobencarb (0.16 mg/L) compared to control (H and E stained, X40, X100). The control showed normal histological structure and normal cells. Fenitrothion treatment showed Malpighian corpuscles (bowman's capsules and glomerulus), epithelial hypertrophy of bowman capsules, elongation of bowman capsules and broader of B-C, necrosis, and atrophy in the glomerulus necrosis in epithelial cells of B-C. Thiobencarb treatment showed broader B-Cs, elongation of B-Cs, necrosis, and atrophy of glomeruli (blood capillaries)

Also, the effects of thiobencarb were epithelial hypertrophy of bowman capsules with broader B-Cs, elongation of B-Cs, necrosis, and atrophy of glomeruli (blood capillaries). In a similar; effect of the herbicide glyphosate on mosquito fish, Gambusia affinis; showed in kidney tissue, necrosis, and degeneration of epithelial cells of tubules, increasing the size of Bowman's capsule, congestion and atrophy, and disappearance of the glomerulus, separation of tubules, increasing of size and congestion of blood vessels, bleeding between tubules and bilirubin pigment diffusing around blood vessels (Al-Kawaz 2019). Histological changes in the kidney associated with pesticides in fish have been studied by many authors (Mostakim et al. 2015).

It was recorded that herbicide thiobencarb affects gills, kidneys, and liver more than insecticide fenitrothion. These results may be attributed to the lipophilic nature of the herbicides; this observation was supported by El-Sayd and Radwan (2004). Furthermore, the highest concentration of thiobencarb was in the liver, while the lowest was found in the fish brain (Abumourad et al. 2010). Our results are also compatible with the results obtained by Abbas et al. (2007) and Eissa et al. (2015), who studied the side effects of Nile tilapia *O. niloticus* exposed to thiobencarb. In addition, the results of fenitrothion are in agreement with the results of other studies that investigated the effects of sublethal fenitrothion on Nile tilapia; it showed histopathological alterations in the gills, liver, and kidney (Benli and Özkul 2010), the effect of organophosphorus insecticides malathion and fenitrothion on fish tilapia (Ohaida and Akrawee 2010), the effect of fipronil on Nile tilapia (El-Murr et al. 2015), and histological effects of deltamethrin on tissues of gills, liver, and kidney of Nile tilapia (Yildirim et al. 2006), mosquitofish (Cengiz and Unlu 2006). In general, our study shows a relation between the type of the pesticide and biochemical changes, as well as the severity of expression of the histological alteration in the Nile tilapia tissues. Due to humans' high consumption of Nile tilapia and considering the pesticides used in agriculture, the possible toxic effects of these pesticides in fish tissues for commercial interest have become a great concern. These side effects may be tissue alteration, detected by the histological examination. Overall, such experiments could be successfully used in research and applied in monitoring programs to monitor the side effects of pesticides on fish. Furthermore, this research supports earlier findings that highlight the value of scientific inquiry in the natural world (Zaki et al. 2018; Zaki et al. 2019; Saber et al. 2020; Saber et al. 2021).

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Multivariate discrimination of selected taxa of the Fabaceae family

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Abstract. *Abdulrahman MD. 2022. Multivariate discrimination of selected taxa of the Fabaceae family. Nusantara Bioscience 15: 227-232.* Nigeria is among the most interesting and diversified countries globally regarding tropical vegetation and medicinal plants. The taxonomy of the Fabaceae family is not entirely clear, to organize species into manageable groups that are helpful for taxonomical, conservational, or pharmacognostic study. This study aimed to discriminate the leaves of *Dialium guineense* Willd., *Detarium microcarpum* Guill. & Perr, *Tamarindus indica* L, *Acacia nilotica* (L.) Willd. ex Delile, *Abrus precatorius* L., *Senna occidentalis* (L.) Link, *Erythrina senegalensis* DC. and *Pterocarpus erinaceus* Poir. based on the mineral elements contents coupled with multivariate analysis. Three samples of each wild-growing species were collected. Unsupervised multivariate analysis using SIMCA-P (V.14.1, Umetrics Sweden) was employed. Five model groups were formed based on their mineral element contents. The species were fully discriminated along the PC1, accounting for 39.3% of the variation. Evidence from this study showed that a combination of mineral element analysis and chemometrics yielded a powerful classification method. Taxonomic identification of plants through biological research combined with chemometrics is an excellent method for preventing the adulteration or consumption of plants with excessive contents or harmful ingredients. However, a mix of molecular and developmental datasets is still necessary to explicitly examine their connections.

Keywords: Chemometrics, genera, medicinal plants, SIMCA, taxonomy

INTRODUCTION

The science of classifying, identifying, and describing different plant species into groups or classes is known as plant taxonomy (Cope et al. 2012). Although morpho-anatomical characteristics are fundamental to understanding the evolutionary relationship between plants, chemical and molecular identification are also necessary to classify plants correctly (Abdulrahman et al. 2018). Taxonomists have developed and employed biomarkers to help identify and categorize plants at the molecular and chemical levels (Abdulrahman et al. 2019). However, there is still a great deal of debate around the classification of plants concerning taxonomic identification of plants because of the significant variation in the chemical and molecular content of the plant, even among comparable species of plant. Therefore, the outcomes of the chemical studies on the plants will be a crucial piece of evidence to support the characterization and identification of these plants (Yunusa et al. 2018). Since the dawn of time, humanity has relied solely on plants to provide them with food, medicine, and oxygen for themselves and their domesticated animals (Abdulrahman and Abba 2021). Medicinal plants also serve food and contribute greatly to human health since they include all the important nutrients humans require. Without a doubt, the great civilizations of the ancient Chinese, Indians, and North Africans left written evidence of man's ingenuity in using plants to treat a wide range of ailments (Kankara et al. 2015). Furthermore, ethnobotany is becoming more prominent worldwide to satisfy the curiosity and willingness to understand how the environment and plants interact to help man survive (Abdulrahman and Abba 2021).

Due to extensive variation in plant chemical and molecular content, even among closely related species, there is still much controversy regarding the classification of plant species (Abdulrahman et al. 2018). Classification schemes were made because there is a lot of diversity between species and people wanted to learn more about it. As a result, numerous plant species exist, but the methods used to identify and categorize them are time-consuming and often inaccurate. Furthermore, the number of knowledgeable plant taxonomists is currently low and declining. This issue is so significant that it has been given the moniker "taxonomic obstacle" worldwide. Conversely, chemometrics can examine many plant samples simultaneously (Abdulrahman et al. 2021a).

The innovative methods developed in chemometrics have benefitted plant identification investigations for enhancing herbal and pharmaceutical compositions. Chemometrics is a useful method for identifying and classifying various plant parts (Yunusa et al. 2018). Multivariate analysis is used in computational systems to process numerical or metabolite data statistically. Finding the proper one will be simpler if they are grouped. Only a few of the approaches that fall under the category of "discrimination analysis" are Principal Component Analysis (PCA), Orthogonal Projections to Latent Structure Discriminant Analysis (OPLS-DA), and Hierarchical Cluster Analysis (HCA) (Yunusa et al. 2018). Score plots, loading plots, and even discrimination maps are produced by multivariate research for improved visualization and understanding of smaller datasets. In recent years,

numerical taxonomy has substantially assisted taxonomic studies (Saric et al. 2009; Ningrum and Chasani 2021).

The earth's second most popular medicinal plant belongs to the Fabaceae family (Da et al. 2018), with reports of over 490 species (Da et al. 2018). It is well known that the family has therapeutic benefits. More than 20 genera have been established within the family. Members of this family utilize a variety of environments. The food crops in the family are important economically because they are high in protein and other micronutrients that are good for livelihoods and health, especially in developing nations. However, because the Fabaceae family contains organisms well suited to the initial colonization and exploration of a variety of settings, such adaptations are brought about by the family's interaction with ectomycorrhizal fungi or with nitrogen-fixing bacteria (Da et al. 2018). Morphological characteristics will not provide sufficient data for the taxonomic characterization of Fabaceae species (Vižintin et al. 2012). Furthermore, due to flaws in traditional taxonomy, this technique cannot meet the complex demands of this species identification. The chemical components of all medicinal plants need to be assessed for identification and pharmacological research purposes to ensure dependability and repeatability. As a result, a reliable method of Fabaceae authentication is required (Al-Dabbagh and Fathulla 2022).

Trace elements are essential in the biosynthesis of active chemical compounds found in medicinal plants, which are responsible for their therapeutic and toxic properties. Furthermore, plants have both helpful and negative impacts on the human body due to their chemical components (Mat et al. 2006). Therefore, morphological observations and chemical content analysis are critical for proper authentication.

Moreover, Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA) were used to distinguish between the species. The present study aims to determine the mineral content of the leaves of *Dialium guineense* Willd., *Detarium microcarpum* Guill. & Perr, *Tamarindus indica* L., *Acacia nilotica* (L.) Willd. ex Delile, *Abrus precatorius* L., *Senna occidentalis* (L.) Link, *Erythrina senegalensis* DC., and *Pterocarpus erinaceus* Poir. to be taxonomically identified. The plants mentioned above are known for their medicinal value in the northern part of Nigeria. The plant leaves, bark, roots, and fruits treat many ailments, including toothache, fever, cough, typhoid, and other diseases (Abdulrahman et al. 2020).

MATERIALS AND METHODS

Herbarium deposition and taxonomic identification

Each of the species under study was collected in triplicate in Kaduna State, Nigeria. For the collected samples, herbarium specimens were prepared. A botanist from Ahmadu Bello University Zaria (ABU), Sanusi Namadi, identified the collected plants which were later placed at the ABU herbarium (Table 1).

Table 1. Voucher number of the studied plant species

Species	Voucher number
Abrus precatorius L.	0917
Acacia nilotica (L.) Willd.ex Delile	0900266
Detarium microcarpum Guill. & Perr.	002346
Dialium guineense Willd.	007429
Erythrina senegalensis DC.	07095
Pterocarpus erinaceus Poir.	0751
Senna occidentalis (L.) Link	060125
Tamarindus indica L.	08961

Mineral element analysis

Samples of leaves were collected and dried in an oven at 60ºC for 24 hours before being ground into powder using a grinding machine. Then, between 0.15 and 0.20 grams of each sample and 100 microliters of standard solution were weighed into vials (polyethylene) and immediately subjected to neutron irradiation in a Triga MK-II reactor. Instrumental Neutron Activation Analysis (INAA) is used to identify the elements in the leaf. Short-lived radionuclides were used for element detection; for elements like V, Al, Ca, Mg, and V, this meant an irradiation time of 1 minute, followed by 20 minutes of cooling and 5 minutes of counting time. For elements like Na and K, this meant an irradiation time of 1 minute, followed by 24 hours of cooling and 20 minutes of counting time. Case in point elemental long-lived radionuclides for as, the process involved 7 hours of irradiation, followed by 3-5 days of cooling, and 2-4 hours of counting time; for Ba, Cr, Fe, Co, and Zn, the process involved 6 hours of irradiation, followed by 20-30 days of cooling, and 1-2 hours of counting time. Three identical sets of experiments were performed (Mat et al. 2006).

Multivariate analyses

Multivariate analyses with imputed data were performed using a SIMCA-P (V.14.1, Umetrics Sweden) to perform Hierarchical Cluster analysis (HCA) and Principal Component Analysis (PCA) (Morais et al. 2020).

Principal Component Analysis (PCA)

PCA focus on examining pairs of variables that are linearly connected. Also, the PCA can be used to tell apart closely related species and to pinpoint the specific states of characters that are responsible for the observed relationship (Nuez et al. 2004).

Hierarchical cluster analysis

Using the information that has been given, hierarchical cluster analysis attempts to classify the data. Hierarchical Cluster Analysis (HCA) creates a similarity matrix between the plant species under study, then highly similar clusters, highlighting similarities and contrasts between and among the clusters (Abdulrahman et al. 2021c). The cophenetic correlation coefficient between the distance matrix and the tree matrix was calculated to assess the closeness of the cluster analysis to the distance matrix.

RESULTS AND DISCUSSION

In many regions of the world, the use of medicinal plants in conventional medicine is becoming more widespread. However, a well-established and strict quality evaluation system must be implemented before medicinal plants can be trusted for their alleged benefits, authenticity, and safety. Computational techniques are used in metabolomics, specifically, multivariate analysis to statistically process the chemical content based on numerical values to assign known metabolites to specific species for identification (Okada et al. 2010; Saito and Matsuda 2010; Tokalıoğlu 2012; Xia and Wishart 2016). Through multivariate analysis, the concept of mineral element analysis combined with chemometrics shows potential as a rapid method to discriminate between plant species. The concept was employed in studying some medicinal or cultivated plant species (Tokalıoğlu 2012; Kumar et al. 2019). An unsupervised analysis was used to discriminate and classify plant species based on multivariate analysis. A summary of the fit plot for the Principal Component Analysis (PCA) is shown in Figure 1.

The R2 value represents the percentage of variation in the training data set with the PCA. R2 is a fit metric that indicates how well the model fits the data. A large R2 (nearing 1) is required to show how well the data set fits the model. According to cross-validation, Q2 is the percentage of the variance of the data set predicted by the model. The model's ability to forecast fresh data is measured in Q2. A high Q2 shows a high level of predictability of 0.998 (Figure 1). The model is, therefore, fit for further analysis of the PCA and HCA models. A similar pattern of fitness and predictive model (FT-NIR: R^2X (cum): 0.956, Q^2 (cum) = 0.952. FT-NIR: R^2X (cum): 0.753, Q^2 (cum) = 0.731) (Maree and Viljoen 2011). PCA offers a wide variety of methods for better-representing data categorization and grouping instances into clusters with common characteristics. For any two groups to be truly distinguishable from one another, they must share certain similarities along the same dimensions that set them apart (Tokalıoğlu 2012).

Multivariate statistical analysis can significantly augment more conventional ways of investigating mineral chemistry by uncovering connections between elements and grouping geochemical results into pertinent and understandable groupings (Dmitrijeva et al. 2020). Principal Component Analysis (PCA) is a window that shows how the observations are related. These graphics display similarities, dissimilarities, and other patterns in the data. The score plot is a map of the observations.

Along the PC 1 X axis, there was clear, distinct discrimination of the examined species (Figure 2). The *D. guineense, D. microcarpum, T. indica, A. nilotica*, and *A. precatorius* were found along the left side of the PC1 while along the right side of the PC1 *S. occidentalis, E. senegalensis* and *P. erinaceus* were found with the variation accounting to 39. 3% (Figure 2 to 6). Along the Y-axis PC2, had no clear discrimination of *T. indica* and *P. erinaceus* (Figure 2). However, intra-species variation was seen in *T. indica* and *P. erinaceus* in the X-axis of the PC2 (Figures 2-6). The variation in PC 2 accounts for 21.5 % (Figures 2-6). The PCA revealed *D. guineense, D. microcarpum, T. indica, A. nilotica*, and *A. precatorius* are closely related in terms of calcium, potassium, magnesium, and sodium. Calcium is solely responsible for nerve and muscle maintenance. It is also reported to serve as an engine for activating enzymes in the body, absorption of dietary vitamin B, and synthesis of the neurotransmitter acetylcholine (Mat et al. 2006). Potassium is also necessary for activating enzymes, particularly coenzymes, which are necessary for muscle function and appropriate body growth (Abdulrahman et al. 2021b). Magnesium is a key cofactor in transporting glucose and enzymes involved in carbohydrate oxidation mechanisms in cell membranes (Mat et al. 2006). Sodium is one of the most important elements for maintaining life, and a lack of it causes physiological function to be impaired.

The relationship of the score plots resulted from the fingerprint (Figure 3). The score loading plot represents the weights combining variables to form the score units. They are proportional to the correlations between the scores and the assessed variables. For example, the scores on the X axis result from the following variables (Figures 3 and 4) while the Y axis (Figures 3-5).

The Biplot goal is to co-chart scores and loadings so that they may be displayed and interpreted simultaneously (Abdulrahman et al. 2021c). As a result, this plot better displays similarities and differences between observations to better understand the observations in relation to the study variables.

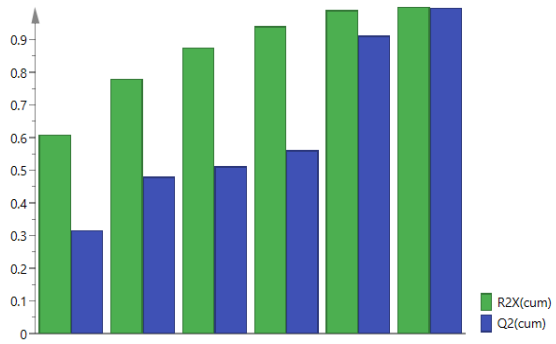

Figure 1. Summary of fit model

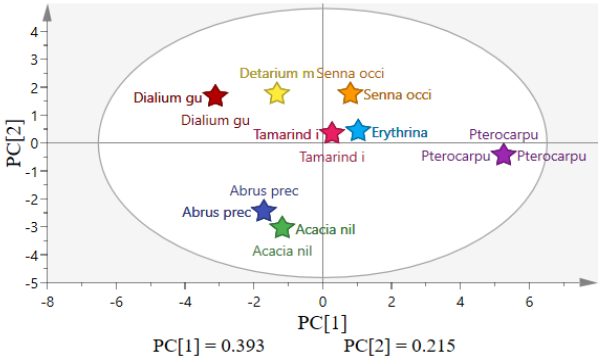

Figure 2. PCA score plot of *D. guineense, D. microcarpum, T. indica, A. nilotica, A. precatorius, S. occidentalis, E. senegalensis* and *P. erinaceus*

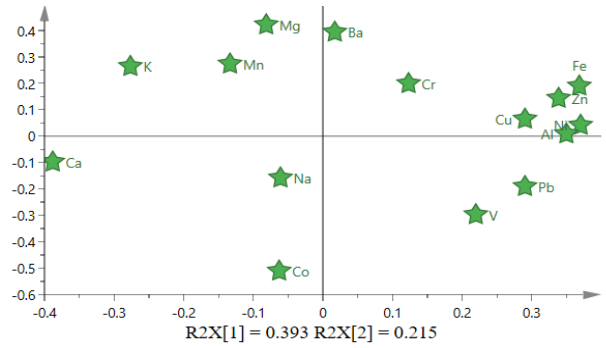

Figure 3. PCA score plot of the mineral analysis responsible for the discrimination

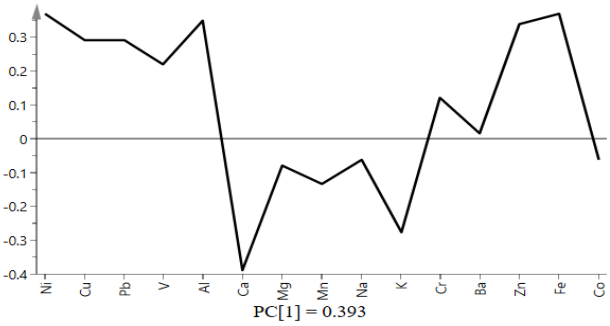

Figure 4. Loading score plot of the mineral analysis responsible for the discrimination of the PC1

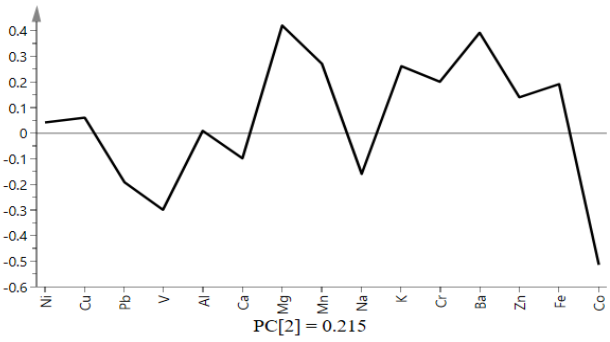

Figure 5. Loading score plot of the mineral analysis responsible for the discrimination of the PC1

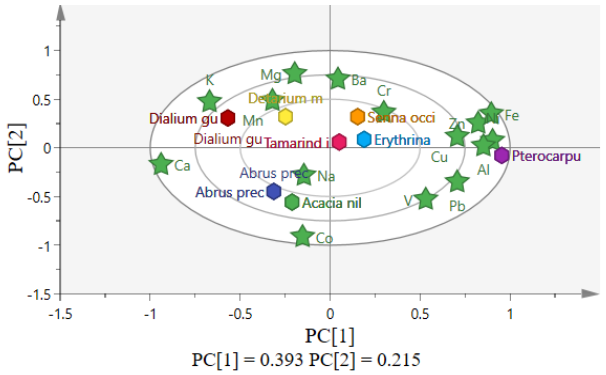

Figure 6. Bi-plots of the score and loading plots showing variables responsible for the formation of scores

Species near the variables have high levels of these variables and low levels of variables located opposite. Variables near the plot origin do not affect the formation of the scores in the plots. The bi-plots along the Y-axis revealed that the species are high in terms of calcium, potassium, cobalt, magnesium, manganese, and sodium. While along with the X-axis, Aluminum, Iron, Zinc, and Vanadium (Figure 6). The PCA score plot of the mineral analysis also shows a high relation in terms of baron, cranium, iron, copper, aluminum, nickel, and vanadium from *S. occidentalis*, *E. senegalensis*, and *P. erinaceus*. Principal Component Analysis (PCA) score plots are a good model for identifying species based on their chemical contents in their studies, quality control, and discrimination of three *Curcuma* species with the aid of metabolomics (Xiang et al. 2011). Moreover, in agreement with the findings on *Ficus deltoidea* discrimination in Peninsular Malaysia, they report variation (Intra and inter) among similar species of the same cultivar (Fatihah et al. 2014).

The HCA was constructed to further determine the relationships and dissimilarities and categorize them into various classes (Fatihah et al. 2013). The model was used to create Hierarchical Cluster Analysis (HCA) to classify them into various groups. The Dendrogram (Tree plot) displays the results of HCA. The number of clusters formed is shown in the tree plot (Figure 7). The tree plots are divided into two major groups, with the first group having only species of *P. erinaceus* with *a* 92% bootstrap value. While the other main groups divide into two main groups, with the first group from the left side accommodating *S. occidentalis*, *T. indica, and E. senegalensis* and the other subgroups accommodating *D. guineense, D. microcarpum, A. nilotica*, and *A. precatorius* at 81% bootstrap value (Figure 7).

The HCA dendrogram further confirmed the relationship (similarities and differences) that the PCA had already established. The HCA model showed a similar pattern in the score plot. The dendrogram was split into different groups based on the trace element contents. Interestingly, the correlation table revealed that the parameters used to distinguish plant species were very good and accurate in identifying them based on their mineral contents. The mean and standard deviation of the variable is shown in Table 2.

The correlation table shows that all variables used in the study positively impact the formation of scores along the Y and X axes of the score plot (Table 3). Table 3 reveals the correlation matrix of the variables used to discriminate the medicinal-importance plants from the family Fabaceae. The models showed a clear separation of the species concerning their mineral contents. The findings of the studies using mineral element analysis combined with chemometrics have provided an efficient way of discriminating plant species that are closely related. Biological investigations combined with chemometrics are a great tool for the taxonomic identification of plants to avoid adulteration and consumption of plants with high contents and dangerous ingredients.

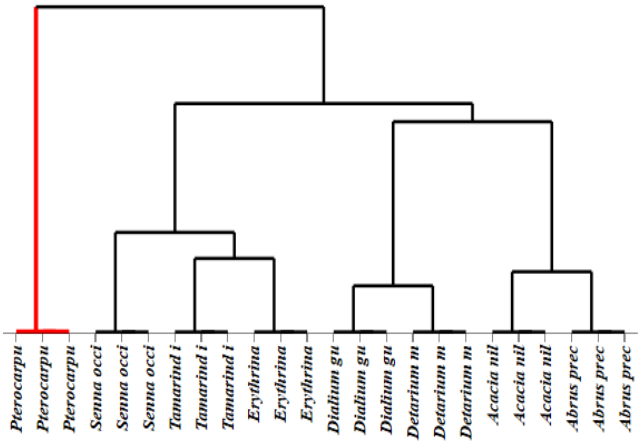

Figure 7. Hierarchical cluster analysis of *D. guineense, D. microcarpum, T. indica, A. nilotica, A. precatorius, S. occidentalis*, *E. senegalensis,* and *P. erinaceus*

Table 2. Mean and standard deviation of the study variables

Variables	Mean	Standard deviation
Ni	3.36	2.44
Cu	47.51	45.79
Pb	2.357	1.24
V	0.19	0.08
Al	97.01	111.18
Ca	0.76	0.20
Mg	0.10	0.02
Mn	27.93	10.61
Na	343.64	206.84
K	0.83	0.39
Cr	1.18	0.98
Ba	22.66	11.70
Zn	39.43	25.75
Fe	98.88	64.73
Co	0.37	0.35

Table 3. Correlation matrix of the study variable

	Ni	Cu	Pb	V	Al	Ca	Mg	Mn	Na	K	Cr	Ba	Zn	Fe	Co
Ni	1	0.4308	0.6194	0.6704	0.9759	-0.7810	0.0497	0.0538	0.0941	-0.5376	0.2130	0.1189	0.6479	0.9112	-0.1224
Cu		1	0.4627	-0.1008	0.4022	-0.8178	-0.3256	-0.5551	-0.4409	-0.5148	-0.0220	0.1857	0.6552	0.4718	-0.3925
Pb			1	0.5449	0.6703	-0.5545	-0.1967	-0.4359	0.3725	-0.3471	-0.1901	-0.3439	0.5526	0.5130	0.0586
V				1	0.6599	-0.3258	-0.2313	-0.1028	0.4773	-0.5610	0.1074	-0.2401	0.1275	0.4546	0.5818
Al					1	-0.7045	0.0938	0.1127	0.1903	-0.5145	0.0287	0.0342	0.5773	0.8331	-0.0996
Ca						1	0.1313	0.4047	0.2626	0.6352	-0.3391	-0.2973	-0.7686	-0.8424	0.3284
Mg							1	0.7048	0.3129	0.5994	0.0540	0.5092	-0.1011	0.1701	-0.6441
Mn								1	0.1401	0.4542	-0.0216	0.1824	-0.2298	0.0223	-0.2506
Na									1	0.2575	-0.4676	-0.0274	-0.4398	-0.1220	0.2679
K										1	-0.0931	0.1592	-0.3147	-0.3827	-0.4113
Cr											1	0.2047	0.4759	0.5167	-0.1746
Ba												1	-0.0861	0.2357	-0.6075
Zn													1	0.8221	-0.4163
Fe														1	-0.3771

Note**:** Ni: Nickel, Cu: Copper, Pb: Iron, V: Vanadium, Ca: Calcium, Mg: Magnesium, Na: Sodium, K: Potassium, Cr: Cranium, Ba: Baron, Zn: Zinc Fe: Iron, Co: Cobalt

For the first time, we have attempted to propose a chemometrics phylogenetic framework for a subset of the Nigerian Fabaceae family, which will serve as a foundation for future phytochemical and pharmacological studies. Moreover, gaining a more comprehensive knowledge of the systematic links between the varieties will facilitate these plants' rapid exploitation and long-term sustainability.

The common bean, pea, and legume are among the edible and economically significant species of flowering plants in the Fabaceae family. In all, they comprise the second-largest family of plants. Despite being tamed by humans, they are significant commercially. Differentiating *D. guineense*, *D. microcarpum, T. indica*, *A. nilotica, A. precatorius*, *S. occidentalis*, *E. senegalensis,* and *P. erinaceus* from one another was done using the fingerprints left by mineral elements in the leaves. The formation of five models based on mineral composition led to the discovery of a significant relationship between the species. Furthermore, it is discovered that species from the same region differ. The discrimination was explained using PCA, and HCA supported the produced dendrogram. The multivariate analysis will be crucial for the delimitation and authentication of plant species. The results would be of great value to support the classical taxonomy.

However, a mix of molecular and developmental datasets is still necessary to explicitly examine their connections. That is the first attempt at proposing a chemometrics phylogenetic framework for a subset of the Nigerian Fabaceae family; it will provide the basis for future taxonomic research. Understanding the systematic relationships between the variations is crucial for quick commercialization and long-term viability.

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The impacts of *Hevea brasiliensis* (rubber tree) plantation on soil nutrients in Southern Nigeria

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Abstract. *Ndakara OE, Ohwo O. 2022. The impacts of* Hevea brasiliensis *(rubber tree) plantation on soil nutrients in Southern Nigeria. Nusantara Bioscience 14: 234-239.* This study investigated how *Hevea brasiliensis* (Willd. ex A.Juss.) Müll.Arg. impact on the soils within the humid rainforest ecosystem of Nigeria. The study used quasi-experimental and stratified random sampling techniques to select sampling units. Samples of soils under *H. brasiliensis* and rainforest (the control) were collected using an auger, and their laboratory analyses for total organic matter (TOM), total nitrogen (N), available phosphorus (P), exchangeable potassium (K), and pH were carried out using standard methods. Data generated were statistically analyzed using the mean, standard deviation, standard error of mean, and t-test. Findings showed that the soils under rainforest have higher nutrient properties than plantations of *H. brasiliensis*. Soil pH values were lower under rainforest than under plantations of *H. brasiliensis*. While TOM, N, and K differed significantly between rainforest and *H. brasiliensis* at a 5% confidence level, available phosphorus and pH were insignificant at a 5% confidence level. Tree species' capability to improve soil nutrition reflects its positive impact on the ecosystem. Since soil nutrients under *H. brasiliensis* are lower than soil nutrients under rainforest, efficient application of organic manure is required to improve the soil nutrient status for sustainable ecosystem functioning and management of the degraded rainforest environment.

Keywords: Agro-ecosystem, biogeochemical cycling, rubber plantation, soil nutrient quality, tree influence circle

INTRODUCTION

The rainforest has undergone increasing amounts and intensity of changes in land use, ranging from selective logging to widespread shifting cultivation agricultural practice, plantation, and intensive agriculture. This land uses impact forest soils together with the proper functioning and structure of the ecosystem (Aweto 2001; Ndakara 2012a). The alteration of land uses from the forest into non-forest requires assessment at a regional scale to adequate knowledge of how such changes affect the biogeochemical processes (Ndakara 2012b) while emphasizing the possible potentials of human-dominated landscape to sustain continued human use (Tsujino et al. 2016).

After a forested area is cleared for agriculture, plantations, or agroforestry, a series of changes occur (Phil-Eze 2010; Amiolemen et al. 2012; Ndakara and Ofuoku 2020). The most conspicuous changes are observed in vegetation compositions, structure, and soil properties (Gruba and Mulder 2015; Augusto et al. 2017; Suzuki et al. 2021). When a rainforest is converted into plantations and assemblages of single species of exotic trees, the role of the tree community in maintaining soil quality is reduced. Similarly, agroforestry practice, centered on integrating trees into farming for several beneficial purposes within the humid rainforest zone, should have exerted impacts on the soils within the rainforest environment. While it has been established that not all species of trees can return nutrient elements to soils effectively (Aweto and Ekiugbo 1994; Londe et al. 2016; Ndakara 2016), this gap can be filled by adding rainforest trees which can return nutrients to the soil to balance the nutrient availability within the ecosystem (Ndakara 2012b).

The agroecosystems, which are fast replacing the natural rainforest ecosystem, are less diverse floristically and structurally less complex than the original rainforest (Barrios et al. 2012). Although some tree plantations seem superficially similar and alike to forest cover, they are less efficient than the rainforest in nutrient cycling and soil management (Aweto 2001; Ndakara 2012a). The problems of nutrient impoverishment under tree plantations within the rainforest ecosystem are yet to be effectively resolved (Ndakara and Ofuoku 2020). In addition, plants exert a high impact on soil properties. When a forest is converted into plantations and agroforestry, the capacity of tree cover to protect soil surfaces is affected, exposing the soil to degradation. As the agroforestry trees grow, soil deteriorations at different levels occur, and soil's physical and nutrient properties are altered (Kazumichi et al. 2018).

Some studies investigating the impact of cultivated plants on soils within rainforests observed that most soil nutrient properties were significantly lower under the cultivated plants than under the adjacent forest (Phil-Eze 2010; Ndakara and Ofuoku 2020). Barrios et al. (2012) observed that soil nutrient levels during cultivation within rainforest zone were depleted, with the depletion increasing with the age of cropping the land. Although numerous

studies on nutrient cycling in a rainforest (Liu et al. 2015) and fallow land (Saimo et al. 2019) are now available, little is known about the changes in nutrient cycling when fallow is in the form of tree plantations such as the plantation of *Hevea brasiliensis* (Willd. ex A.Juss.) Müll. Arg. or rubber. This knowledge gap emphasizes the necessity of studies that assess the main changes in the rainforest soil nutrient qualities when replaced with tree plantations. Although few studies have investigated this (e.g., Liu et al. 2015 in the context of erosion sites), replication is needed to enrich the variation of soil catena in a different climatic region.

In southern Nigeria, the natural rainforest has largely disappeared owing to centuries of agricultural activities, settlement development, fuel wood exploitation, and logging. These activities resulted in converting the natural rainforest ecosystem into agroecosystems and savanna landscapes (Aweto 2001). As a result, the relics now feature only as island habitats, and the timber tree species which characterize rainforests are now hardly found, while some species are extinct (Ndakara 2016). In some cases, plantations of crops that are non-indigenous to the rainforest are cultivated based on the environmental requirements of such exotic tree species. However, their implications on the rainforest soils concerning nutrient cycling and sustainable soil nutrient management must be adequately documented.

Moreover, examining the soil under exotic trees in the rainforest becomes necessary. Therefore, this research aimed to assess the ecological impacts of *H. brasiliensis* stands on soil nutrient elements in the context of rainforests in southern Nigeria. We expect the results of this study to inform whether the agroforestry practice of *H. brasiliensis* plantation in the region has implications on rainforest soil nutrient quality.

MATERIALS AND METHODS

Study area

The study was carried out in Ughelli North Local Government Area of Delta State, within the humid rainforest belt of Southern Nigeria (Figure 1). This region which covers a land area of 818 km^2, is geographically located at 4042'-5036' N and 5000'-6006' E. The climate is humid sub-equatorial based on the Af Koppens classification. The regional climate is influenced by two air masses: Tropical Maritime (MT) and Tropical Continental (CT). This region extends from the coast to roughly inland, and it falls within areas with an annual rainfall of 2,000-4,000 mm (which increases the rate of soil nutrient leaching), with a mean annual temperature of approximately 31.5^{0}C (Ndakara 2012a; Ndakara and Eyefia 2021; Ukoji and Ndakara 2021).

The natural vegetation is lowland rainforest of the moist evergreen forest type, with riparian vegetation within the water-logged area. The natural vegetation covers have been highly degraded, while the landscape is now dominated by a mosaic of different stages of savanna enclaves and patches of rainforest remnants (Ndakara 2016). The soils within this study area are mainly products of coastal deposits, which consist of well-drained sandy loam over coarse sandy clay loam subsoil thus, classified under the Alfisols, Ultisols, Oxisols, and Psalments based on the United States Department of Agriculture (USDA) classification. The soils are ideal for supporting the growth of tree plants, making it possible to grow plantations of exotic trees such as *H. brasiliensis*. As part of the agro-based economic activities, the plantations of exotic tree crops replaced the natural rainforest cover by cutting down the indigenous tree species.

Data collection procedure

The choice of the study area was based on the existing practice of *H. brasiliensis* plantation within the rainforest region of southern Nigeria. The quasi-experimental method was used. A stratified random sampling technique was employed to divide the study area into seven quarters in line with areas with the existing large extent of *H. brasiliensis* plantations. In each of the quarters, a plot of rubber plantation measuring 30×30 m was established, together with an adjoining rainforest of the same measurement from the same quarters, which served as study control following the studies by Aweto and Ekiugbo (1994), Ndakara (2012a), Ndakara and Ofuoku (2020). Samples of soil were collected once from both the plantation and adjacent rainforest using augers from 0-15 cm and 15-30 cm depths (Ndakara 2012a; Ndakara and Ofuoku 2020), making a total of 14 soil samples collected. The 14 soil samples obtained were based on the areas with soil, relief, and climate homogeneity where mature and undisturbed rainforest relics were found.

The samples collected were then analyzed in the laboratory for properties that directly affect soil fertility status and crop productivity to biogeochemical cycling (Ndakara 2012b). The soil properties analyzed were total organic matter (TOM), total nitrogen (N), available phosphorus (P), exchangeable potassium (K), and pH. Standard methods were adopted during the laboratory analysis exercise. The method of Walkley-Black wet oxidation was applied to determine organic carbon before conversion to TOM; Auto-analyzer was used to determine total N; available P was ascertained using a Spectrophotometer; a Flame photometer was used to determine exchangeable K; while soil pH determination was adopted the electrometric approach.

Data analysis

Data generated were further analyzed statistically using SPSS 15.0 version. The descriptive statistics were used to ascertain the mean, standard deviation, and standard error of mean values for the concentrations obtained for each nutrient element. In addition, T-test statistics were used to ascertain the variations in soil properties between topsoil and subsoil under plantations of *H. brasiliensis*, soil properties between topsoil and subsoil under adjoining rainforest, topsoil properties between adjoining rainforest and *H. brasiliensis* plantation, and subsoil properties between adjoining rainforest and *H. brasiliensis* plantation at 5% level of confidence.

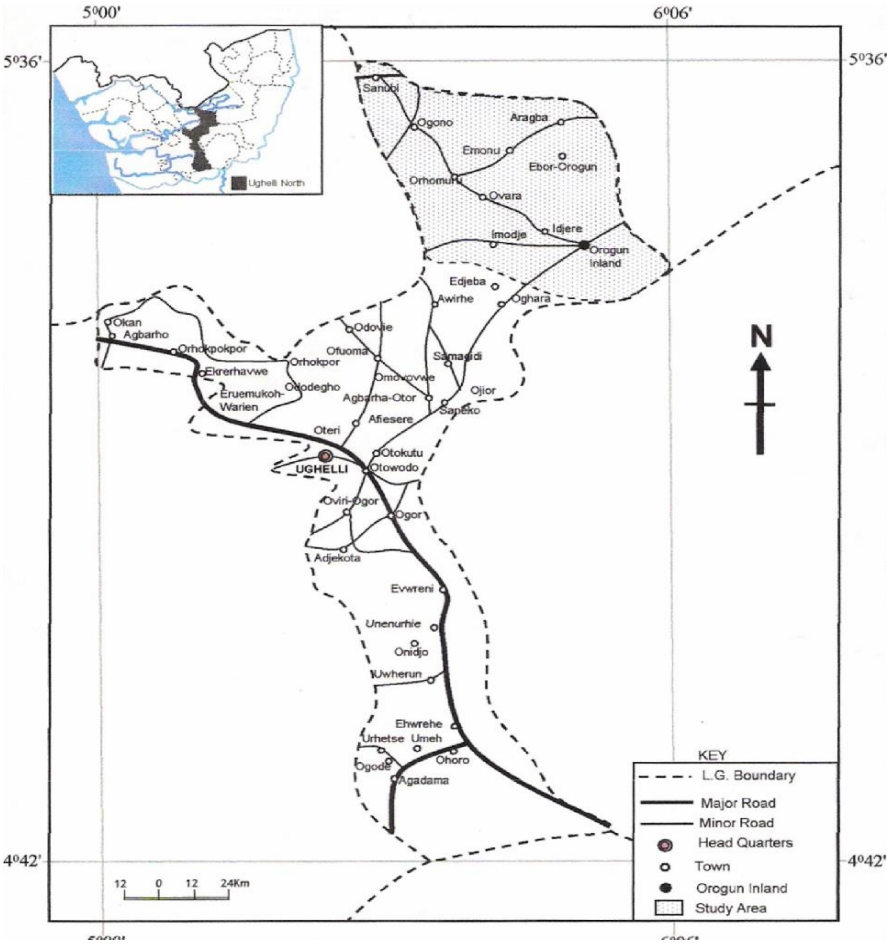

Figure 1. Map of the study area in Ughelli North Local Government Area, Delta State, Southern Nigeria

RESULTS AND DISCUSSION

Soil TOM (total organic matter) represents an important plant nutrient source. As mentioned earlier, the build-up of TOM is one of the significant and major changes in soils under tree stands. That is because organic matter influences the concentration and status of nutrients that accumulate under tree stands (Aweto 2001).

Table 1 presents the results of descriptive statistics for the concentrations of soil properties under plantations of *H. brasiliensis* and rainforest. In general, soils under the rainforest have higher nutrient properties than soils under the plantations of *H. brasiliensis*. That shows that the build-up of nutrients is higher under the rainforest, a vegetation community with various species, in contrast with the pure stand plantation of *H. brasiliensis*. The mean (μ), standard deviation (S2), and standard error of mean (SEM) values for the concentrations of TOM in both topsoil and subsoil are higher under the rainforest than under the *H. brasiliensis* plantation. However, the concentrations of TOM are higher in the topsoil under both rainforest and *H. brasiliensis* in the topsoil and subsoil layers, respectively, which shows that trees can accumulate TOM in the topsoil under their stands, whether in cultivated or natural habitats.

Similarly, the μ, S2, and SEM values for the N concentrations in both topsoil and subsoil are higher under rainforest than under *H. brasiliensis*. However, the concentrations of N are higher in the topsoil under both rainforest and *H. brasiliensis* in the topsoil and subsoil layers, respectively. Higher topsoil nutrients were reported in studies by Fabricio et al. (2018), Liu et al. (2015), and Ndakara (2016). However, the SEM values for the concentrations of N under the rainforest and *H. brasiliensis* are the same, which shows that trees can accumulate more N in the topsoil under their stands than in the subsoil.

Like the other nutrient elements, the μ values for the concentrations of P in both topsoil and subsoil are higher under the rainforest than under *H. brasiliensis.* However, the concentrations of P are higher in the topsoil under both rainforest and *H. brasiliensis.* The implication of the mean concentrations is that nutrient elements are higher in the topsoil layer under trees than in the subsoil layer. The S2 and SEM values for the concentration of P in the topsoil are higher under *H. brasiliensis* than under rainforest but higher under rainforest than the *H. brasiliensis* in the subsoil layer, respectively. That shows that soil nutrients are dynamic, as shown in studies by Fabricio et al. (2018) and Ndakara and Ofuoku (2020). The mean concentration of nutrient elements may vary in proportion to its standard deviation and standard error of mean values. Trees can

accumulate more P in the topsoil under their stands than in the subsoil. The μ, S2, and SEM values for the K in both topsoil and subsoil are higher under rainforest than under *H. brasiliensis*. However, K contents are higher in the topsoil under both rainforest and *H. brasiliensis* in the topsoil and subsoil layers, respectively. The SEM values for the concentrations of K under the rainforest and *H. brasiliensis* are the same, which shows that trees can accumulate more K in the topsoil under their stands than in the subsoil. Higher K content in topsoils was reported in studies by Ndakara (2012b) and Suzuki et al. (2021).

Soil acidity varied strikingly between the topsoil and subsoil, as well as between the rainforest and the plantation of *H. brasiliensis*. Within both soil layers, the μ values for the soil pH are higher under *H. brasiliensis* than under the rainforest. That implies that soils under rainforests are more acidic than those under *H. brasiliensis*. However, the S2 and SEM values for the pH contents are higher in the rainforest than in the soils under *H. brasiliensis*. Therefore, the stand of *H. brasiliensis* does not increase soil acidity within the rainforest environment. From this finding, it could be deduced that improvement in soil quality is not solely dependent on reduced acidity in the soil. Within the rainforest cover, tree species contained are numerous. Some of the tree species are more acidic than others, but the aspect of nutrients return to the soil will not be in the same direction. For instance, the lower pH content of the soils under *H. brasiliensis* than the soils under the rainforest would have meant that the soils under *H. brasiliensis* contained more nutrient elements than the soils under the rainforest. The species variant within the rainforest was earlier identified by Ndakara (2012a) to account for higher concentrations of nutrients in the native rainforest cover within the tropical environment.

The much lower nutrient contents under plantations of *H. brasiliensis* could be attributed to a factor of *H. brasiliensis* stands not being capable of improving soil nutrient elements through corresponding and adequate replacement of soil nutrient use over time. This finding is similar to results reported in studies by Phil-Eze (2010), Barrios et al. (2012), Ndakara (2012a), Liu et al. (2015), Ndakara (2016), Fabricio et al. (2018), Ndakara and Ofuoku (2020), and Suzuki et al. (2021), where soil nutrient properties were higher under native rainforest. However, the much lower nutrient properties under plantations could indicate that species of trees in the rainforest have a higher ability to return and enhance soil nutrient properties than *H. brasiliensis*. Soil pH values are lower under rainforests than under plantations of *H. brasiliensis*. The observed variation in the soil pH values reflects equal acidity for soils under both rainforest and *H. brasiliensis*, which corroborates the findings by Ndakara (2012a) and Ndakara and Ofuoku (2020).

Table 2 presents the results of t-test statistics for the differences in soil nutrient properties between rainforests and plantations of *H. brasiliensis*. Generally, soil nutrient properties of TOM, N, and K are significantly different between rainforests and plantations of *H. brasiliensis* at the 5% confidence level. At the same time, the observed differences in mean values of available phosphorus (P) and pH are not significant. However, the t-test statistical results for the differences in the μ values of TOM content of the topsoil between the rainforest and *H. brasiliensis* are significant at the 5% level, indicating that rainforest topsoils contained more TOM than the soil under *H. brasiliensis*. While from the t-test statistical results for the differences in the μ values of TOM content of the subsoil between the rainforest and *H. brasiliensis* is significant at the 5% level, affirming that subsoils under the rainforest contained more TOM than the subsoil under *H. brasiliensis*.

Table 1. Descriptive statistics of the soil properties under *Hevea brasiliensis* plantation and rainforest

Soil properties	Statistics	Topsoil		Subsoil	
		***H. brasiliensis* plantation**	**Rain-forest**	***H.brasiliensis* plantation**	**Rain-forest**
TOM (%)	Mean	4.02	6.20	1.10	2.74
	SD	0.79	0.72	0.22	0.82
	SE	±0.31	±0.28	±0.09	±0.32
N (%)	Mean	0.43	0.64	0.21	0.28
	SD	0.08	0.13	0.03	0.04
	SE	±0.03	±0.05	±0.01	±0.01
P (mg/kg)	Mean	12.88	14.82	6.13	7.65
	S.D	6.03	1.94	1.29	1.72
	S.E	±2.28	±0.73	±0.49	±0.65
K (mg/kg)	Mean	56.29	114.57	18.71	31.00
	S.D	6.18	21.40	3.04	10.20
	S.E	±2.34	±8.09	±1.15	±3.86
pH	Mean	6.09	6.06	5.76	5.63
	SD	0.64	0.74	0.68	0.80
	SE	±0.24	±0.28	±0.26	±0.30

Table 2. T-test Statistical results of the differences in soil properties between *H. brasiliensis* plantation and rainforest

Soil properties	Soil layer	F-value	df	T-value	Sig. 2-tailed value	Remark
TOM (%)	Topsoil	0.444	12	5.448	0.000	Significant
	Subsoil	11.843	12	5.204	0.011	Significant
N (%)	Topsoil	4.950	12	3.875	0.002	Significant
	Subsoil	0.424	12	3.784	0.003	Significant
P (mg/kg)	Topsoil	2.065	12	0.805	0.436	Not Sig.
	Subsoil	1.270	12	1.872	0.086	Not Sig.
K (mg/kg)	Topsoil	6.517	12	6.923	0.000	Significant
	Subsoil	2.760	12	3.055	0.010	Significant
pH	Topsoil	0.103	12	0.077	0.940	Not Sig.
	Subsoil	0.001	12	0.324	0.751	Not Sig.

The t-test statistical results for the differences in the μ values of N content of the topsoil between the rainforest and *H. brasiliensis* is significant at the 5% level, showing that rainforest topsoils contained more N than the soil under *H. brasiliensis*. Similarly, the t-test statistical results for the differences in the μ values of N content of the subsoil between the rainforest and *H. brasiliensis* is significant at the 5% level, emphasizing that subsoils under the rainforest contained more N than the subsoil under *H. brasiliensis*. In contrast, the t-test statistical results for the differences in the μ values of P content of the topsoil between the rainforest and *H. brasiliensis* are not significant at the 5% level, although a difference exists. Similarly, the t-test statistical results for the differences in the μ values of P content of the subsoil between the rainforest and *H. brasiliensis* are insignificant at the 5% level.

There is a significant difference in the μ values of K content of the topsoil between the rainforest and *H. brasiliensis* at the 5% confidence level, implying that rainforest topsoils contained more K than the soil under *H. brasiliensis*. In the subsoils, there are significant differences in μ values of K content under the rainforest and *H. brasiliensis* at the 5% confidence level, affirming that subsoils under the rainforest contained more K than the subsoil under *H. brasiliensis*. The t-test statistical results for the differences in the μ values of pH content of the topsoil between the rainforest and *H. brasiliensis* are not significant at the 5% level since the acidity of topsoil under rainforest is at the same range as that under *H. brasiliensis*. A similar result for soil acidity is also found in the subsoil.

As presented earlier, the higher nutrient contents under rainforest reflect a higher capability of built-up soil nutrients. Significant differences between soils under rainforest and cultivated exotic tree species were reported by Aweto and Ekiugbo (1994), Aweto (2001), Phil-Eze (2010), Ndakara (2012b), Liu et al. (2015), Fabricio et al. (2018), Ndakara and Ofuoku (2020), and Suzuki et al. (2021). The capability of trees to improve soil nutrients reflects the positive impact on the rainforest ecosystem. However, the observed insignificance in soil pH shows that stands of *H. brasiliensis* do not effectively alter the acid-base content of soils within rainforest environments.

In conclusion, this study investigated how *H. brasiliensis* plantation impacts the soils within Nigeria's humid rainforest ecosystem. Regarding the implications of cultivating *H. brasiliensis* on the functioning of the rainforest and the soils underneath, by which the plants grown on such soils could either negatively or positively impact them. The true reflection of such impact is determined and predicted by the functional contributions of plants to soils in the environment they are grown. However, the investigation of *H. brasiliensis* as a non-indigenous tree grown within a rainforest environment revealed reduced soil nutrient properties. Therefore, this study is of paramount importance to the quest to manage the degraded rainforest ecosystem effectively. The results from this research show that soils in the rainforest have higher nutrient properties than plantations of *H. brasiliensis*, except for soil pH values which were lower under the rainforest. While TOM, N, and K differed significantly between rainforest and *H. brasiliensis* at a 5% level of confidence, available phosphorus and pH were insignificant. Since soil nutrients under *H. brasiliensis* are lower than soil nutrients under rainforest, efficient application of organic manure is required to improve the soil nutrient status for sustainable ecosystem functioning and management of the degraded rainforest environment.

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Phenotypically and genotypically estimation of virulence factors in *Salmonella* serovar *typhi* isolated from patients with enteric fever in Al-Najaf, Iraq

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Abstract. *Zghair LS, Motaweq ZY, Lafta HC. 2022. Phenotypically and genotypically estimation of virulence factors in* Salmonella serovar typhi *isolated from patients with enteric fever in Al-Najaf, Iraq. Nusantara Bioscience 14: 128-133. Salmonella* serovar *typhi*, often known as enteric fever, causes typhoid fever and has been a major human infectious disease for centuries, surviving in poor sanitation and overcrowding. Only 64 (58.1%) Gram-negative bacteria were found from the 110 total specimens, with 46 (41.8%) Gram-positive bacteria. The 64 samples were divided into 42 (65.6%) males and 22 (34.4%) females. This work presents the isolation and identification of 64 *Salmonella typhi* isolates obtained from specimens. In addition, the flagellin gene was found in 64 isolated probable typhoid fever patients (*fliC-d).* In this study, phenotypic techniques were used to detect several virulence factors. The results showed that small colonies of L-form bacteria grow on the edges of a petri dish when one of the β-lactam antibiotics (a class of antibiotics that includes penicillin) is given to wild-type bacteria, showing 52 (81.3%) of isolates could produce L-form and observed in 44 (68.75%). The ability to generate CFA/I and CFA/II were found in 68.7% of isolates. The large percentage of CFA produced showed CFA/III production, 64 (100%).

Keywords: CFA, L-form, *Salmonella* serovar *typhi*, typhoid fever, virulence factors

INTRODUCTION

Salmonella is a Gram-negative bacterium that belongs to the Enterobacteriaceae family. Having rod-shaped bacteria. The genus *Salmonella* flagellated (flagella peritrichous-found everywhere around the cell body) is facultative-anaerobes and non-spore formation predominantly motile with cell diameters ranging from: 0.7 to 1.5 μm, lengths from 2.0 to 5.0 μm. Two genus exist, *S. enterica* and *S. bongori* and there are divided into six subspecies that include over 2,600 serotypes (Fàbrega and Vila 2013; Gal-Mor et al. 2014; LeLièvre et al. 2019).

As a result, increasing the chances of discovering carriers is crucial to limit the harm they bring to populations. A sensitive, specific, and quick diagnostic technique for identifying typhoid patients and carriers would be ideal. *Salmonella* species that are pathogenic attack non-phagocytic gut epithelium by delivering a specific set of effectors via finely tuned hardware involving the Type 3 secretion system (T3SS), which plays a key role in *Salmonella* pathogenesis (Que et al. 2013). The *S. typhi* utilizes two T3SSs: *Salmonella* pathogenicity island 1 (SPI-1) and *Salmonella* pathogenicity island 2 (SPI-2) (SPI-2). SPI-1 is a gene cluster with a 40-kb district that includes 39 genes encoding T3SS-1, its chaperones, effector proteins, and transcriptional controllers that regulate the expression of multiple destructiveness genes both inside and outside SPI-1 (Zhang et al. 2018).

Surface K antigens are the smallest common antigens discovered in *Salmonella* species and are heat-sensitive polysaccharides located on the bacterial capsule surface. Antigens of virulence (Vi) are a subclass of K antigen, Dublin, *Paratyphi* C, and *Typhi* are the only three pathogenic serovars (Wattiau et al. 2011). The capsular Vi antigen is a linear homopolymer of alpha 1-4 coupled to galactose aminouronic acid, which is variably acetylated at the C3 site. One of the main traits that differentiate *S. typhi* from nontyphoid *Salmonella* (NTS) is the production of a polysaccharide capsule named the Vi antigen. The Vi capsule reduces phagocytosis while promoting serum resistance, most likely by preventing antibodies from attacking the O-antigen (Hart et al. 2016). This work aimed to identify the phenotype of pathogenicity factors in *Salmonella* serovar *typhi* represented by capsule, L-form bacterium, and Colonization Factor Antigen type.

MATERIALS AND METHODS

The research was carried out at the Bacteriology and Molecular Laboratories, Department of Biology, Faculty of Sciences, Kufa University, Iraq.

Clinical specimens and patients

Blood samples (110) were taken from patients suffering from enteric fever at AL-Sadder Medical City and AL-Furat General Hospital/Al-Najaf-Iraq for three months, from August 2021 to November 2021. Four milliliters of fresh venous blood were taken and separated into two halves using sterile syringes. For the Widal test, one milliliter of blood was used, with three milliliters of blood delivered into a special screw placed in bact/alert 3D

apparatus incubated at 37°C for a week. If positive sample, each specimen was inoculated using a direct method of inoculation on a culture of selective media, namely MacConkey, XLD, and SS agar. The inoculation cultivation dishes were directly incubated overnight at 37°C for 18 to 24 hours, then stored until they were needed (Cheesbrough 2010). Identification of *S. typhi* isolates by Microscopic Properties, Cultural Characteristics, and Biochemical Tests. GN ID cards were used to confirm *S. typhi* isolates using the automated VITEK-2 compact system. To complete the final identification, it was performed on each bacterial isolate. The GN ID card is based on well-established biochemical (64 reactions) methodologies and newly created substrates that measure many metabolic processes (German BioMerieux Company).

Molecular diagnostic methods

Purification and extraction of DNA

The extracted *S. typhi* DNA was prepared using the boiling technique. Briefly, colonies were suspended in 100 microliters of sterile distilled water and boiled at 100°C for 15 minutes in the water bath, then immediately frozen at -20°C for one hour, then centrifugation at 14000 xg for 10 min and the supernatant was conserved for the used in the amplification-operation (Yang et al. 2008). The concentration and purity of DNA can be determined by Williams et al. (2007).

Polymerase Chain Reaction (PCR) assay

Thermo cycle PCR was used to re-confirm *S. typhi* diagnosis, this technique requires specific primers for the *flic-d* gene, including sequence information. The primer of flagellin gene *fliC-d*-F:5'-ACTCAGGCTTCCCGTAA CGC-3' and *fliC-d*-R: 5'-GGCTAGTATTGTCCTTATCGG-3', in the product size 763 bp (Levy et al. 2008). Five µl of master mix, 5 µl of template DNA mixed with 2.5µl each set of primers in a suitable PCR tube, the rest of the total volume was attained to 25 µl by sterile nuclease-free water, the mixture vortexing well. The PCR for the *flic-d* gene was planned to include a primary denaturation step, denaturation, annealing, and extension at 94°C for 4 minutes, 40 cycles at 94°C for 45 seconds, 56°C for 30 seconds, and 72°C for 45 seconds, respectively. The reaction mixture was kept at 4°C until employed after the last extension step at 72°C for roughly 10 minutes (Levy et al. 2008). Then, using 1% agarose gel electrophoresis and 3µl of ethidium bromide dye, all PCR products were examined. The particular cover on the electrophoresis tank was closed, and the electric current was matched (70 volts for 1.5-2 h). Finally, the gel documentation system was used to identify the electrophoresis data.

Phenotype detection of some virulence factors

Detection of capsule (vi antigen) production

A loopful of suspected culture was mixed with a loopful of nigrosin stain on a clean and dry slide by allowing it to air dry at room temperature. The slide was gently cleaned with water before being stained for 2 minutes by methylene blue stain and left to air dry at room temperature. Next, the slide was softly washed with water and viewed using an oil laboratory microscope. The nigrosin stain gives the unstained capsule a dark backdrop, while the methylene blue stain gives the cells a blue tint (Harley and Prescott 2002).

L-form detection of S. typhi

According to multiple studies, when one of the β -lactam antibiotics (which includes penicillin) is administered to wild-type bacteria in a petri dish, tiny colonies of L-form bacteria grow on the plate's edges. Penicillin treatment not only selects L-forms (which are penicillin-resistant) but also stimulates L-form growth (Casadesús 2007). Penicillin discs (10µ) were used according to the Kirby-Bauer disc diffusion method. The method included the following steps : (i) A isolate of previously discovered bacteria was improved by mixing a growth from an isolated colony with 5 ml of sterile normal saline at a cell density comparable to the turbidity of McFarland tube No. (0.5), which is about equivalent to 1.5x108 cells/ml of bacteria. Inoculums were obtained using a sterile cotton swab and streaked over Muller Hinton agar medium. With heated, sterilized forceps, the antibiotic discs were placed on the surface of the medium at evenly spaced intervals. Incubation the plate at 37°C for 18 hours (Perilla et al. 2003). (ii) According to the Domingue methods, the agar containing the bacteria was cut out from the edges of the plate as discs, then transported to the variant broth incubate at 35°C for 7-10 days. Then by using a cotton swab immersed in bacterial suspension, spread the bacteria on variant agar, after incubate for 18 hours at 35°C, the L-form or cell wall deficient bacteria were grown as fried egg colonies on variant agar. (iii) After staining with Gram stain, the bacterial colonies appear as spherical or ovoid shapes and agglutinated with each other (Domingue et al. 1979).

Haemagglutination

This was detected via the presence of clumping of erythrocytes, caused via fimbriae of *S. typhi* when D-mannose is present. The assay was carried out using the mannose-sensitive and mannose-resistant haemagglutination assays, as well as the bacterial Haemagglutination assay-slide method. The *S. typhi* was inoculated on nutrient broth at 37°C for 48 hrs. Blood from human (O) and different animal blood types was collected under sterile conditions into Alsever's solution at a ratio of 2 volumes: 1 volume of blood and stored at the refrigerator. Each type of blood was taken and N.S. washed three times and formed up to a 3% suspension in N.S. (AL-Khafagee 2018). The slide was rocked at room temperature for 5 minutes after one drop of RBC suspension was introduced to a drop of broth culture. The presence of clumping was viewed as a sign of haemagglutination. The absence of haemagglutination in a comparable series of tests in which a drop of 2% W/V D-mannose and a drop of broth culture were added to the red cells, revealed mannose-sensitive haemagglutination. The presence of 3% haemagglutination in the presence of 2% W/V D- mannose was used to detect mannose-resistant haemagglutination. This technique was also used to identify the type of fimbria in crude oil (Vagarali et al. 2008).

RESULTS AND DISCUSSION

Salmonella typhi isolates identification

During the present study period, 110 blood samples from typhoid patients were collected. The first identification of *S. typhi* isolates has been based on morphological, biochemical, and microscopical studies. Gram-negative bacilli, alone or in pairs, peritrichous flagellated, motile and non-spore producing bacteria were identified microscopically as *S. typhi*. Although the morphological of *S. typhi* isolates utilized special media such as XLD (Xylose Lysine Deoxycholate) agar, SS (*Salmonella* Shigella) agar, and MacConkey agar, appeared on culture medium once appeared the typical characteristics at 37°C after 18-24h, *S. typhi* colonies appeared smooth, rounded, convex, non-hemolytic, and grey-white color on blood agar. However, *S. typhi* colonies on MacConkey agar looked pale yellow (non-lactose ferment), 1-3 mm in diameter, and after 18-24 hours at 37°C, as well as good development of *S. typhi* colonies on XLD agar emerged gray hue with black center colonies due to its ability to create H_2S.

The TSI, Sugars, Oxidase, Indole, Ureases, and simmone citrate assays indicated the biochemical results of *S. typhi* isolates. In the TSI slants test, the slant and butt turned AKL/ACID red and yellow, suggesting non-fermentation of glucose on the slant and acid generation with H_2S in the bottom. Further tests of *S. typhi* isolates yielded negative results for oxidase, indole generation, urease generation, and citrate use. Identifying of *S. typhi* isolates using the VITEK-2 GN ID Cards System comprised various biochemical assays. The results indicated *S. typhi* with cards IDing a wide range of good isolates (percentage from 95 to 99%). On MacConkey agar, there are 64 *S. typhi* colonies.

Typhoid fever is caused by *S. enterica* serovar *Typhi*, which is an acute systemic sickness that causes a significant proportion of illness and mortality, especially in impoverished nations. The *S. enterica* serovar *Typhi* infections are common among travelers returning from disease-endemic areas in Europe (Fabrizio et al. 2009).

Confirmation of *S. typhi* by PCR amplification of *FliC-d*

Salmonella typhi clinical isolates were tested using the polymerase chain reaction technique was another method for confirming the identification of *S. typhi* with a specific gene with 763 bp. The observations are that most *S. typhi* isolates carry the *fliC-d* gene, which is typical of *S. typhi*. Furthermore, the PCR showed a total of 64 (100%) positive results from blood (Figure 1). This finding is in agreement with Khan et al. (2012), who found that out of 80 suspected typhoid fever cases, the flagellin gene (*fliC-d)* was detected by PCR in 56 (70%) cases, which matches the results of a previous study in Bangladesh, where PCR was positive in 88.7% of suspected typhoid fever cases.

A result obtained by the VITEK-2 system is the same obtained by the PCR technique. The results are comparable to those of Ali (2015), who discovered that the positive result from the Vitek 2 compact system and the PCR technique was 65 (32.5%).

In 64 patients, 42 (65.6%) were male and 22 (34.4%) were female. Males were estimated to be infected at a higher rate than females. This result is compatible with study results in the Al-Musaib District (Al-Khafaji et al. 2006) and Diyala District (Saleh 2013). This could be because most males were out-doored, and they could be seen as food-eating and handling or contact with other patients from this perspective (Flayyih 2017). The patient's age rate is from (1-60) years old, the patients' ages are distributed as follows in Figure 2.

The lowest incidence was among the (31-40) and (41-50) were 4.7% and 6.3%, respectively, while the percentage of age group (21-30) was 7.8% while the highest incidence was among the (0-10) 46.9 %. The disease affects all ages with 12.5% for (51-60) and >60. These findings are dissimilar to Al-Sultany (2003) previous research, which obtained the most infective age between (16-20) at 23.5%. The results also correlated with Flayyih (2017). The age range (11-20) had the highest occurrence, according to the researchers (54%), while Ali (2015) also found that majority of participants in the instances were between the ages of (51-60) years. These results also compare with Prince (2002), who found the ages (35-10) was the most infective, as in Tabel 1.

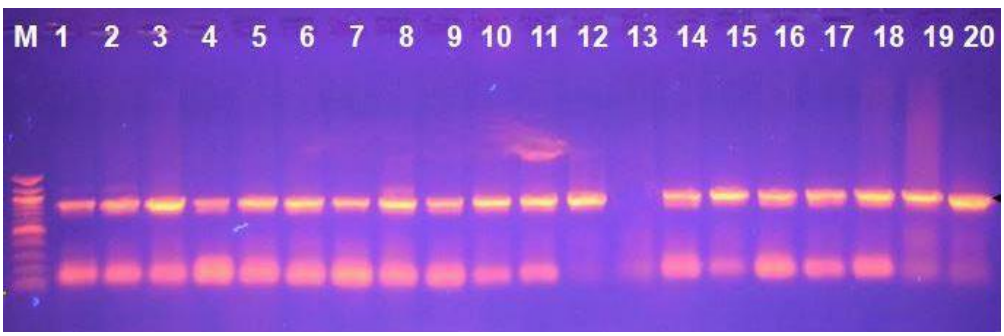


Figure 1. PCR product of *fliC-d* gene primers with product 763 bp gel electrophoresis Lane (M): DNA molecular size marker (100-bp ladder), Lanes (1-20): positive *fliC-d* gene results

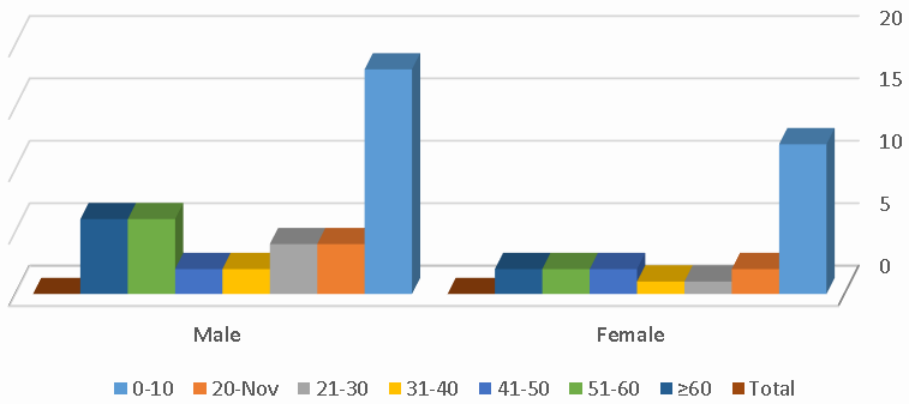


Figure 2. Age rate from female and male of *S. typhi* patients

Table 1. Prevalence of *Salmonella* serovar *typhi* according to the age groups

Age group	Total no. (%)	Female	Male
0-10	30 (46.9)	12	18
11-20	6 (9.4)	2	4
21-30	5 (7.8)	1	4
31-40	3 (4.7)	1	2
41-50	4 (6.3)	2	2
51-60	8 (12.5)	2	6
≥60	8 (12.5)	2	6
Total	64 (100)	22 (34.4)	42 (65.6)

Salmonella typhi virulence factors detection

All *S. typhi's* ability to create a large number of virulence factors.

Capsule detection

The encapsulated isolates of *S. typhi* were detected using the nigrosin stain. The findings revealed that 56 isolates (87.5%) were encapsulated isolates with tiny polysaccharide capsules**.**

The *S. typhi*, unlike most other *S. enterica* serovars, may produce a carbohydrate capsule known as Vi-CPS antigen. The production of this antigen, which is influenced by environmental inputs, is critical for extracellular survival and protection against neutrophil oxidative bursts. TNF-αresponse in human macrophages is likewise reduced following absorption. According to current thinking, Vi-CPS is implicated in immune evasion during infection in the human host, and hence is critical during infection (Eed et al. 2011).

Hemolysin production

The results of *S. typhi* isolates' virulence factors revealed that none of the *S. typhi* isolates produced hemolysin in a blood agar medium**.** This results agree with the findings of Flayyih (2017).

Colonization Factor Antigen (CFA):

Figure 3 shows 44 (68.75%) of isolates have CFA/I and CFA/II. Because of the significant production of CFA, 64 (100%) of *S. typhi* isolates were found to produce this component. CFA/I and CFA/II were also identified in *S. typhi* isolates, albeit in lower percentages than CFA/III. These results disagree with the findings of Ali (2015). According to the findings, 31% of isolates could produce CFA/III, 15% could produce CFA/II, and 92% of *S. typhi* isolates could produce CFA I.

Fimbriae are thought to play an important function in epithelial cell adhesion. Fimbriae mediate bacterial colonization and host cell communication by binding to specific host receptors. Fimbrial adhesins control the bacterial pathogen's fate in the host as well as the progression of the disease process. Type-1 fimbriae are also significant in determining the organism's pathogenicity. Jaroni's (2014) experiments revealed that a mannose-resistant haemagglutinin was required for *Salmonella* to attach to target cells. Fimbriae 1 is thought to play a key function in adhesion to epithelial cell surfaces (which facilitates bacterial colonization) and determining *S. typhi* pathogenicity.

The adherence of bacteria in mucous surfaces or epithelial cells of the gastrointestinal tract revealed a link between mannose-sensitive hemagglutinin (MSHA) or type 1 fimbriae and bacterial pathogenicity. Among the isolates, CFA/II exhibited the lowest prevalence. This factor produces agglutination in chicken blood and helps bacteria bind to unique and complex carbohydrate receptors on small intestinal epithelial cells (Hamid and Jain 2008).

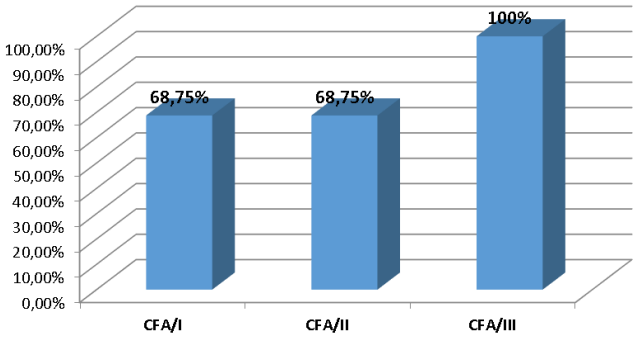
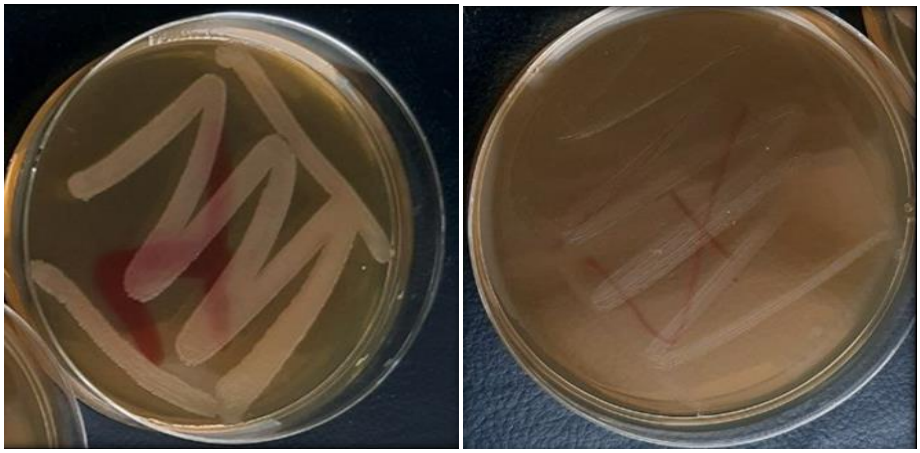


Figure 3. Types of colonization factor antigen in *Salmonella* serovar *typhi*



A B

Figure 4. L. form of *Salmonella typhi* isolates after incubation at 37°C for 24 hrs. A. L. form cell on variant agar B. control cell on variant agar

Table 2. Showed isolates bacteria to produce L. form

Num	L-form	Total
1-64	positive	52
	negative	12
total		64

L. form detection

According to multiple studies, when one of the β - lactam antibiotics is administered to wild-type bacteria in a petri dish, tiny colonies of L-form bacteria grow on the plate's edges. Likewise, penicillin treatment not only selects for L-forms (which are penicillin-resistant) but also causes L-form growth (Casadesús 2007).

Table 2 showed that 52 (81.3%) isolates could produce L-form. This result agrees with Al-Sultany's (2003) findings, which found that 82.3% of isolates could lose their cell wall and produce L-form after culturing on special media was prepared for this target. After staining with Gram stain and examining with a light microscope, the bacterial colonies appear as spherical or ovoid shapes and agglutinated (Kalaivani et al. 2014), as in Figure 4.

In conclusion, this study found that about 100% of *S.* serovars *Typhi* isolated from the blood of enteric fever patients had many virulence factors by phenotypic tests.

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