Cell Biology & Development

| Cell Biol Dev | vol. 6 | no. 1 | June 2022| | E-ISSN 2580-4499|

Papaya seeds photo by Macro Cosmos Microscopy

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Published semiannually **PRINTED IN INDONESIA**

9 772580 449111

Cell Biology & Development

| Cell Biol Dev | vol. 6 | no. 1 | June 2022 |

ONLINE
http://smujo.id/cbd

e-ISSN
2580-4499

PUBLISHER
Society for Indonesian Biodiversity

CO-PUBLISHER
Indonesian Legumes and Tuber Crops Research Institute, Malang, Indonesia

OFFICE ADDRESS
Indonesian Legumes and Tuber Crops Research Institute. Jl. Raya Kendalpayak Km 8, Po. Box 66, Malang 65101, East Java, Indonesia. Tel.: +62-341-801468, Fax.: +62-341-801496, email: editors@smujo.id

PERIOD OF ISSUANCE
June, December

Society for Indonesian Biodiversity

Indonesian Legumes and Tuber Crops Research Institute, Malang, Indonesia

Published by Smujo International for The Society for Indonesian Biodiversity and Indonesian Legumes and Tuber Crops Research Institute, Malang, Indonesia

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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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Saharjo BH, Nurhayati AD. 2006. Domination and composition structure change at hemic peat natural regeneration following burning; a case study in Pelalawan, Riau Province. Biodiversitas 7: 154-158. DOI: 10.13057/biodiv/d070213.

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

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

Book:

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

Chapter in the book:

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

Abstract:

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

Proceeding:

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

Thesis, Dissertation:

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

Information from the internet:

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

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CELL BIOLOGY & DEVELOPMENT
Volume 6, Number 1, June 2022 E-ISSN: 2580-4499
Pages: 1-5 DOI: 10.13057/cellbioldev/t060101

Short Communication: Papaya (*Carica papaya*) seed extract test againts *Spodoptera litura* mortality

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Manuscript received: 19 November 2021. Revision accepted: 30 January 2022.

Abstract. *Bahuwa IC, Lamondo D, Katili AS. 2022. Short Communication: Papaya (*Carica papaya*) seed extract test against* Spodoptera litura *mortality. Cell Biol Dev 6: 1-5.* This study aims to determine the effect of papaya (*Carica papaya* L.) seed extract on mortality, the most influential concentration of papaya seed extract, Lethal Concentration (LC_{50}) 24 hours, and Lethal Time (LT_{50}) 24 hours armyworm (*Spodoptera litura* Fabricius, 1775) larvae instar III. The research was carried out at the Biology Department, Universitas Negeri Gorontalo, Indonesia, in June 2021. The method used was an experiment with a Completely Randomized Design consisting of 9 treatments and 3 replications, there are A (aquadest control), B (CMC control), C (1 ppm), D (50 ppm), E (100 ppm), F (200 ppm), G (400 ppm), H (800 ppm), and I (1600 ppm). The data were analyzed by probit analysis LC_{50}, LT_{50}, and One Way Anova. The results showed that the LC_{50} value was at a dose of 489 ppm. The value of LT_{50} 24 hours at a concentration of 1600 ppm is 10.61 hours, with the fastest time to kill 50% of armyworm larvae. Papaya seed extract affects the mortality of armyworm larvae; the higher the concentration, the less time it takes to kill 50% of the larvae.

Keywords: *Carica papaya,* caterpillar, mortality, papaya seeds, *Spodoptera litura*

INTRODUCTION

Insects are animal species belonging to the phylum Arthropoda that have habitats almost everywhere. One that belongs to the insect group is the armyworm or grayak caterpillar (*Spodoptera litura* Fabricius, 1775). That follows Rusdy (2009), who said that *S. litura* belongs to the order Lepidoptera, a polyphagous pest that causes damage to cultivated plants. Armyworm (*S. litura*) is a pest that often causes decreased productivity and reduced yields or crop failure in food crops. Plants commonly attacked by armyworm pests are corn, tomatoes, chilies, kale, cabbage, eggplant, spinach, soybeans, and mustard greens.

Farmers have made efforts to overcome the problem of armyworms that attack plants are controlled by using synthetic pesticides. However, the negative impact of synthetic pesticides is that pests become resistant, fertilizing chemical residues and killing natural enemies of pests. Therefore, it is necessary to control the armyworm that is friendly to the environment (Bedjo 2017; Saputra 2019). Asmaliyah et al. (2010) reported various types of plants that contain vegetable pesticides that can be used in pest control, namely, neem, papaya, duku, durian, tobacco, jatropha, cloves, garlic, belimbingwuluh, brotowali.

The community still considers papaya seeds waste and are not optimally used. Besides, the community still considers papaya seeds waste and have not been used optimally. According to research by Utomo et al. (2010), papaya seeds contain alkaloids that are toxic to larvae when used in large quantities and can result in nerve paralysis, cessation of the nervous system, and heart disease suppression, causing death in larvae.

The use of botanical pesticides is strongly recommended to replace the action of synthetic pesticides. Vegetable insecticides are insecticides whose basic ingredients are plant or natural ingredients. For example, the papaya plant (*Carica papaya* L.) has the potential as a vegetable insecticide because it contains alkaloids, terpenoids, and flavonoids that are highly toxic to insects (Julaily et al. 2013).

MATERIALS AND METHODS

Procedure

This study was conducted from June to July 2021 to manufacture extracts at the Chemistry Laboratory. The treatment was carried out at the Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Negeri Gorontalo, Indonesia. The tools used are a blender, measuring cup, beaker, Erlenmeyer, glass beaker, funnel, jar, aluminum foil, rotary evaporator, hand sprayer, filter paper, label paper, and writing utensils. The materials used were papaya seeds (*C. papaya*), 96% ethanol, and instar III larvae of armyworm (*S. litura*). Instar III larvae of armyworms were obtained from the Germination Center in Malang, East Java, Indonesia, then reared for acclimatization to the laboratory environment for 2 days and fed cabbage leaves before the treatment.

Papaya seeds were washed, cleaned, and dried until the water content was reduced. Next, dried papaya seeds were mashed using a blender and weighed 500 g. Then the smooth papaya seeds were extracted according to the Harborne method in Rasyid (2012). First, the smooth papaya seeds were macerated in a jar using 96% ethanol solvent and stirred using a stirring rod, then covered with aluminum foil and allowed to stand for 24 hours. Next, the maceration results were filtered using a funnel lined with filter paper. Then the pulp from the fine powder of papaya seeds from the results of the first maceration was macerated again using 96% ethanol. Finally, the results of filtering the papaya seed filtrate are put into a rotary evaporator at a temperature of 60^0C so that the ethanol evaporates and gets a thick extract. Furthermore, the extract was measured according to the concentration of 1 ppm, 50 ppm, 100 ppm, 200 ppm, 400 ppm, 800 ppm, and 1600 ppm.

The test animal was placed in a glass container or covered with gauze containing cabbage (cabbage) leaves. Each container contains one concentration of papaya seed extract with concentrations of 1 ppm, 50 ppm, 100 ppm, 200 ppm, 400 ppm, 800 ppm, and 1600 ppm; each contains 5 mL of papaya seed extract concentration. Each container contains 10 armyworms instar III and is sprayed on glass walls using a hand-held spray bottle containing papaya seed extract (*C. papaya*). Spraying was carried out 2 times, the first spraying and the second for 12 hours.

Data analysis

This study used experimental methods and a Completely Randomized Design with treatment determined by the formula t (n-1) 15 (Hanafiah and Nanang 2009) obtained 7 treatments and 3 replications. The papaya seed extract treatment concentration consisted of 0.1 ppm, 50 ppm, 100 ppm, 200 ppm, 400 ppm, 800 ppm, and 1600 ppm.

The collected data were analyzed using probit analysis to determine Lethal Concentration (LC_{50}) and Lethal Time (LT_{50}) values. ANOVA analysis was used to determine the effect of papaya seed extract and continued with Duncan's test to determine the significant difference in papaya seed filtrate concentration on the mortality of armyworm larvae effect. The percent mortality value is obtained from the formula:

$$\text{Mortality} = \frac{a}{b} x100\%$$ (Nurhudiman et al. 2018)

Where:
a= Number of dead larvae
b= Total Number of larvae tested

RESULTS AND DISCUSSION

The mortality percentage value of *Spodoptera litura* larva in each treatment

The results showed that the administration of papaya (*C. papaya*) seed extract affected the mortality of armyworm larvae (*S. litura*). The results of the calculation of the mortality percentage of armyworm larvae applied with papaya seed extract are described in Table 1.

The results of the study Table 1 obtained the mortality rate in each treatment within 24 hours. The details of the mortality control treatment were 0 individuals of armyworm larvae, a concentration of 1 ppm obtained a mortality percentage of 3%, a concentration of 50 ppm obtained a mortality percentage of 7%, a concentration of 100 ppm obtained a mortality percentage of 10%, a concentration of 200 ppm obtained a mortality percentage of 17%, a concentration of 400 ppm obtained a mortality percentage of 40%, a concentration of 800 ppm obtained a mortality percentage of 60%, a concentration of 1600 ppm obtained a mortality percentage of 87%. Based on this, the higher the dose of papaya seed extract given, the higher the mortality rate obtained.

Analysis of probit LC_{50} and LT_{50} on *Spodoptera litura* larvae in each treatment for 24 hours

Probit analysis aims to see at what concentration can kill armyworm larvae (*S. litura*) as much as 50%. The LC_{50} value using probit analysis obtained values of x and y= 1.5727+0.7675 with a regression value of 0.863; the results obtained LC_{50}= 489 ppm within 24 hours after application. Based on probit analysis that at a concentration of 489 ppm, it can kill 50% of larvae. The results of the LT_{50} value in the probit analysis in each treatment can be seen in Figure 2.

Table 1. The concentration of papaya seed extract on mortality of caterpillar larvae

Treatment	Total Dead Individuals	Mortality Percentage
A: Aquadest control	0	0 %
B: CMC control	0	0 %
C: 1 ppm	1	3 %
D: 50 ppm	2	7 %
E: 100 ppm	3	10 %
F: 200 ppm	5	17 %
G: 400 ppm	12	40 %
H: 800 ppm	18	60 %
I: 1600 ppm	26	87 %

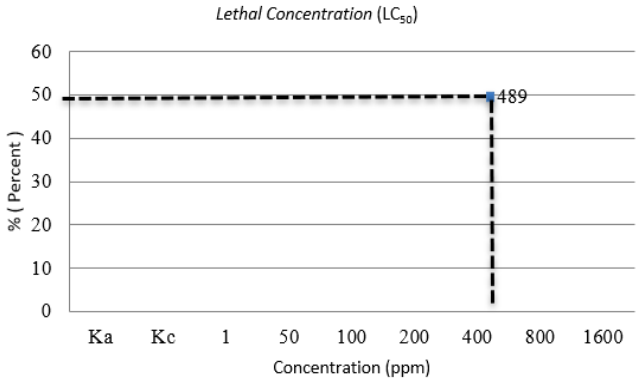

Figure 1. Graph of LC_{50} mortality of *Spodoptera litura* larvae

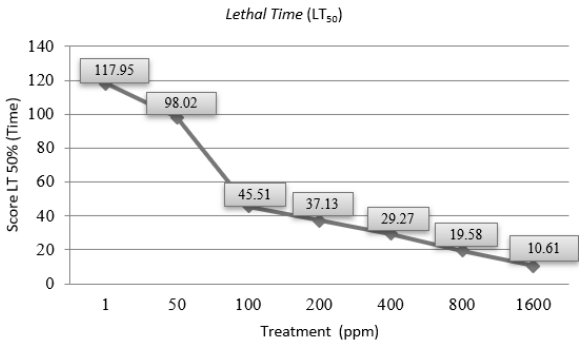

Figure 2. Graph of LT_{50} value in each treatment

The results obtained in the control showed no mortality activity, so the LT_{50} value was 0 because the x and y equation values were 0. The 1 ppm treatment had the highest LT_{50} value compared to other treatments, at 117.95 hours, while the lowest LT_{50} value was in the treatment of 1600 ppm at 10.61 hours. So it can be concluded that the most effective on the mortality of *S. litura* larvae is the treatment of 1600 ppm at 10.61 hours can kill the larvae as much as 50%. Based on the above, the higher the dose of papaya seed extract given, the faster the time used to kill armyworm larvae.

Statistic analysis

Based on Table 2, the One Way ANOVA statistical analysis results, the F count value is greater than the F table, namely 27.109>2.39 with a value of sig = 0.00 < = 0.05, which means H0 is rejected, and H1 is accepted. That shows the effect of giving papaya seed extract on the mortality of armyworm larvae and continued with the Duncan test.

Duncan's test aims to see a significant difference between papaya seed extract treatments on the mortality of armyworm larvae; the results of Duncan's test are shown in Table 3. Table 3 Duncan's analysis results show that treatment A was not significantly different from treatments B, C, D, E, and F but significantly different from treatments G, H, and I. Treatment B was not significantly different from treatments A, C, D, E, and F but significantly different from treatments G, H, and I. Likewise, treatments C, D, E, and F. Treatment G, was significantly different or significantly different from treatments A, B, C, D, E, F, and H, I. Treatment H was not significantly different from treatment I, but significantly different from treatment A, B, C, D, E, F, and G. The highest percent mortality value was found in the treatment. The value of I (1600 ppm) is 87%. This event differed significantly from all treatments, but treatment H differed from treatment I.

Discussion

The results showed the effect of giving papaya (C. papaya) seed extract on armyworm larvae (S. litura) instar III mortality. The highest armyworm mortality was found at a concentration of 1600 ppm, with as many as 26 individuals with a percentage value of 87%, while the lowest mortality was found in the 1 ppm treatment with 1 individual with a percentage value of 3%. The Aquadest and CMC control treatments did not show any deaths. The concentration that can kill 50% of the instar III armyworm (*S. litura*) is 489 ppm. Therefore, the mortality of *S. litura* instar III larvae was influenced by concentration; the higher the concentration of papaya seed extract (*C. papaya*), the death toll of the instar III armyworm larvae have increased.

Table 2. One-way ANOVA statistical analysis

Anova					
Mortality	**No. of squares**	**Free degrees**	**Average square**	**F Count**	**Sig.**
Between groups	116,202	7	16,600	27.109	.000
In grub	9.798	16	.612		
Total	126.000	23			

Table 3. Duncan's mortality test of caterpillar larvae given papaya seed extract

Treatment	Mortality percentage	Notation
(A) Aquadest control	0%	a
(B) CMC control	0%	a
(C) 1 ppm	3%	a
(D) 50 ppm	7%	a
(E) 100 ppm	10%	a
(F) 200 ppm	17%	a
(G) 400 ppm	40%	b
(H) 800 ppm	60%	c
(I) 1600 ppm	87%	c

Note: Different letter notations show significant differences between Duncan's test treatments = 0.05

Figure 3. *Spodoptera litura* infected with papaya seed extract (*Carica papaya*): A. The larvae's body shrinks and is black; B. The larvae' legs are black (Primary data 2021)

The results of observing the time required for the death of the armyworm showed that 1 hour after the application, the larva's body rolled up and moved very fast. After 4 hours of observation, some larvae were far away from the affected area and smeared with papaya seed extract. Observed 8-12 hours after use, the size of the larvae seems to be shrinking, moving slowly, and not so active. Under the treatment of 1600 ppm, the fastest time to kill the armyworm larvae was 10.61 hours, so it also showed that the higher the concentration, the faster the death time.

Larvae mortality in this study was also influenced by condition factors where the larvae used were instar III larvae. Haryono (2015) said that the digestive system is fully formed in this instar III larvae. Therefore, the saponins and alkaloids in papaya seeds can work optimally as poisons in the digestive tract.

The mortality of *S. litura* larvae obtained from each treatment showed no larval death in the control treatment, so the larvae continued to experience the development process. The characteristics of non-poisoned larvae are blackish-brown bodies marked by yellow spots, yellowish legs, and a body with fine hairs, and they do not emit a foul odor. Treatment of papaya seed extract on *S. litura* larvae showed mortality activity at 1 ppm, 50 ppm, 100 ppm, 200 ppm, 400 ppm, 800 ppm, and 1600 ppm. The larval mortality activity of each treatment was seen at 8 hours of observation, and the mortality value increased until the observation was 24 hours.

Changes in body shape characterize larval mortality, and the larva's body color becomes black and shrinks (Figure 3 A&B). The death of armyworm larvae indicated the influence of the chemicals in the applied papaya seed extract. Based on research by Arismawati et al. (2017), papaya seed extract (*C. papaya*) has a larvicidal effect characterized by the death of the larvae. The results of the phytochemical test of papaya seed extract contain secondary metabolic compounds of flavonoids, alkaloids, saponins, and tannins. These secondary metabolic compounds can inhibit larval growth. Taufiq et al. (2015) explained that the saponin content in papaya seeds works as a stomach poison where the substance enters the larva's body through the digestive system (mouth) and then poisons the larvae. Aside from that, Kartina et al. (2019) explained that the saponin content in papaya seeds works as a stomach poison where these substances enter the larva's body through the digestive system (mouth) and then poison the larvae. In addition, saponins are very influential as a contact poison seen in the external physical disorder (cuticle) in larvae, namely washing the waxy layer that protects the larval body parts so that it can cause death to the loss of a lot of body fluids.

Tannins are plant components that are phenols with a bitter taste. Tannins can interfere with the process of digesting food because tannins will bind to proteins in the digestive system (Utami et al. 2010). According to Azlansah et al. (2019), tannins work on the larval body to bind to proteins in the digestive system so that the process of protein absorption in the digestive system is disrupted.

According to Cania and Setyaningrum (2013), alkaloid compounds can be used as larvicides by working as contact poisons (contact poisoning). Alkaloids enter the body through absorption, degrade skin cell membranes, damage cells, and interfere with larval nerve work. Alkaloid compounds in the form of salts can degrade cell membranes. In comparison, the workings of flavonoid compounds influence the work of the respiratory system or act as respiratory poisons (fumigants).

Ahmad and Adriyanto (2019) reported that the use of papaya seed extract (*C. papaya*) has great potential as a bio larvicide because it contains secondary metabolic compounds in the form of alkaloids, saponins, flavonoids, and tannins that can inhibit and kill larvae in 3 ways, namely as (contact poisoning), stomach poison (stomach poisoning), and as a respiratory poison (fumigant).

In conclusion, based on the analysis test results, papaya seed extract (*C. papaya*) significantly affected the mortality of armyworm larvae (*S. litura*) with a calculated F value of 27.109. The dose that can kill the armyworm larvae (*S. litura*) is as much as 50%, namely at a dose of 489 ppm. The best time needed to kill 50% of larvae is at 1600 ppm treatment, which is 10.61 hours.

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CELL BIOLOGY & DEVELOPMENT
Volume 6, Number 1, June 2022 E-ISSN: 2580-4499
Pages: 6-12 DOI: 10.13057/cellbioldev/t060102

Lipid profiles, hematological parameters and histopathological analysis of CCl_4-intoxicated wistar albino rats treated with n-butanol extract of *Ficus glumosa* leaves

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Manuscript received: 6 January 2022. Revision accepted: 8 March 2022.

Abstract. *Abu MS, Yakubu OE, Onuche JI, Okpe O. 2022. Lipid profiles, hematological parameters and histopathological analysis of CCl_4-intoxicated wistar albino rats treated with n-butanol extract of* Ficus glumosa *leaves. Cell Biol Dev 6: 6-12.* This research critically assessed the effect of the n-butanol fraction of methanol extract of *Ficus glumosa* Delile leaves on serum lipid profile, hematological parameters, and some organ architecture of experimental albino rats intoxicated with carbon tetrachloride. The crude methanol extract was re-dissolved in 300 mL of distilled water and repeatedly partitioned in a separating funnel with 400 mL of n-hexane with vigorous shaking. This process was repeated using other solvents (ethyl acetate, n-butanol, and distilled water), and the n-butanol was selected based on antioxidant potency. A total of 35 albino rats were used. The rats were divided into 7 groups of 5 animals each. Lipid profile, hematological indices, and histopathological analysis were carried out. With the administration of the extract, triacylglycerol and total cholesterol levels in the Carbon tetrachloride-induced but treated rats were significantly ($P<0.05$) reduced compared to the normal levels, 1.84±0.27 mmol/L, and 0.54±0.11 mmol/L, respectively. In contrast, high-density lipoprotein was relatively increased compared to the normal level, 1.44±0.43 mg/dL. Similarly, packed cell volume, hemoglobin, and white blood cell levels were significantly ($P<0.05$) reversed to near normal in the extract-treated rats. On the other hand, the histopathological examinations of a liver section of the normal control group showed normal hepatocellular architecture with distinct hepatic cells with a well-conserved central vein. Carbo tetrachloride-induced control group liver showed intense hepatic necrosis with vascular congestion, kupffer cells hyperplasia, vacuolation, and degeneration of normal hepatic cells (hepatic necrosis). However, the induced but treated groups almost normalized the hepatic cells (moderate hepatic necrosis). The findings showed that the n-butanol fraction of *F. glumosa* could reverse the deleterious effects of CCl_4 on lipid profile and hematological parameters with the restoration of the architectural integrities of the liver and kidney of the treated rats.

Keywords: *Ficus glumosa,* hematological parameters, histopathology, lipid profile, n-butanol fraction

INTRODUCTION

Ficus glumosa Delile is commonly known as the fig tree or African rock fig (a sacred Fig tree of religious importance in ancient times documented in holy books such as the Bible and Qur'an). In Nigeria, it is more distributed in the southern region. It is referred to as "Kawuri" in the Hausa language (Paul 2013), "Obata" in Yoruba, "Obadan" in the Edo language (Aigbokhan 2014), "Akpuru" in the Igbo language, and "okoklodu" in the Idoma language (tribe found in the southern part of Benue state in Nigeria). *Ficus glumosa* is indigenous to tropical and sub-tropical Africa, including Nigeria, with few species being found in south Asia and the Mediterranean zone, where they usually inhabit dried river beds, fringe forests, savannah areas, and swamp forests in the coastal regions (Umar et al. 2013). In Cote d'Ivoire, the Central Africa Republic, and Zimbabwe, the latex is used to ameliorate pains from sprains and treat diarrhea and sore eyes, whereas in Central Africa, Senegal, East Africa, and Tanzania, the stem bark is used as mouthwash agents to alleviate toothache, to prevent conjunctivitis, treatment of jaundice, dysentery, typhoid fever and stomach disorders (Kwazo et al. 2015). In addition, this plant is used in traditional medicine in East Africa, Cameroon, and Senegal to treat edema, hypertension, diabetes, hemorrhoids, rheumatism, skin diseases, and stomatitis (Orwa et al. 2009).

Lipids have been noted to perform important bodily functions but may cause various health problems if present in excess amounts (Ankur et al. 2012). Hyperlipidemia is a medical condition characterized by elevated lipid/lipoproteins levels in the body, including high cholesterol and triglyceride levels (Guo et al. 2011; Braamskamp et al. 2012; Bassam 2013). Lipids are considered "fats" in the bloodstream, commonly divided into cholesterol and triglycerides. Cholesterol circulates in the bloodstream and is involved in the structure and function of cells, whereas triglycerides are either used immediately or stored in the fat cells (Lughetti et al. 2010). High cholesterol levels in the body have been considered a modifiable risk factor which is evident by the fact that plasma cholesterol at levels >200 mg/dL causes 4.4 million deaths in a year (Brouwers et al. 2012; Gosh and Gosh

2012). Various types of cholesterol have been reported that include total cholesterol (TC), consisting of all the cholesterols combined; HDL cholesterol, often referred to as good cholesterol that carries cholesterol from the peripheral cells to the liver; and LDL cholesterol often called bad cholesterol that carries cholesterol from the liver to the peripheral cells (Sacks and Katan 2002; Priskila et al. 2008; Siri-Tarino et al. 2010). The abnormal lipid levels result from an alteration in lipid metabolism, an unhealthy lifestyle, including a high-fat diet, and other lifestyle factors like being overweight, heavy alcohol use, and lack of exercise (Bassam 2013).

Carbon tetrachloride (CCl_4) is one of the rats' most used experimental models for hepatic and renal toxicity induction (Tsuchiya et al. 2007). Within the body, CCl_4 is metabolized by the liver enzyme CYTP450 to produce highly toxic trichloromethyl free radical ($CCl_3^{\cdot}$), which in turn reacts with oxygen to generate trichloromethyl peroxyl ($CCl_3O_2^{\cdot}$) free radicals that cause damage to hepatocytes and renal cells (Knockaert et al. 2012). Trichloromethyl and its peroxy radical can bind to proteins or lipids or abstract a hydrogen atom from an unsaturated lipid, initiating lipid peroxidation and, consequently, liver and kidney damage (Mariam et al. 2015). Treatment of carbon tetrachloride-induced hepatotoxicity in experimental rats with methanol leaves extract of *Jatropha tanjorensis* significantly ($P<0.05$) reduced the elevated liver function parameters, total cholesterol, triacylglycerol, low-density lipoprotein cholesterol and increased high-density lipoprotein cholesterol in rats (Madubuike et al. 2015). Equally, Saba et al. (2010) demonstrated the hepatoprotective potential of *Cnidoscolus aconitifolius* leaves in rats. They were found to be potent against hepatotoxicity induced by carbon tetrachloride by reversing liver enzymes to a near normal. In order to increase the horizon of traditional therapeutic molecules against chemical intoxicants, this research was designed to assess the effect of the n-butanol fraction of methanol extract of *F. glumosa* leaves on serum lipid profile, hematological parameters and some organs architecture of experimental albino rats intoxicated with carbon tetrachloride.

MATERIALS AND METHODS

Fractionation of crude methanol extract of *F. glumosa* leaves

Exactly 20 g of crude methanol extract was re-dissolved in 300 mL of distilled water and repeatedly partitioned in a separating funnel with 400 mL of n-hexane three times with vigorous shaking (Abu et al. 2020). At each portioning, the mixture was allowed to stand for 30 minutes to separate into distinct layers of hexane and aqueous. The n-hexane fraction was then collected and concentrated using a water bath. Next, the aqueous layer was partitioned with 400 mL of ethyl acetate to obtain an ethyl acetate fraction repeatedly. The above-aqueous layer was then saturated with distilled water and repeatedly partitioned with 400 mL of n-butanol solution. Finally, the n-butanol fraction and the aqueous residue were separated. That was followed by the evaporation of moisture content from the n-butanol fraction using a water bath maintained at 45ºC until the residues were obtained. The residual fraction was kept in a sealed container and refrigerated at 2-4ºC for further use.

Animal grouping

A total of 35 albino rats were used. The rats were divided into 7 groups of 5 animals each, Table 1.

Table 1. Animal grouping

Group	Group description	Treatment
1	Negative control	No treatment
2	Vehicle control	1 mL/kg b.w olive oil
3	Positive control	1 mL/kg b.w 50% carbon tetrachloride (CCl_4) in olive oil
4	100 mg extract	1 mL/kg 50% CCl_4 in olive oil + 100 mg/kg b.w *n*-butanol fraction
5	300 mg extract	1 mL/kg 50% CCl_4 in olive oil + 300 mg/kg b.w *n*-butanol fraction
6	500 mg extract	1 mL/kg 50% CCl_4 in olive oil + 500 mg/kg b.w *n*-butanol fraction.
7	100 mg silymarin	1 m/kg 50% CCl_4 in olive oil + 100 mg/kg b.w silymarin

Figure 1. *Ficus glumosa*

Toxicity and oxidative stress induction using CCl_4 and treatment with the n-butanol fraction of methanol extract of F. glumosa *leaves*

The animals were pre-treated on the first day of the experiment with 1 mL/kg body weight from 50% solution of CCl_4 in olive oil (IP), followed by oral administration of the extract after 24 hours of intoxication with CCl_4. Then, the administration of the *n*-butanol fraction was continued for 21 days with a once-weekly challenge with 1 mL/kg body weight 50% solution of CCl_4. Finally, the animals were fasted for 24 hours after the last administration of the extract and sacrificed at the end of the experiment for sample collection and subsequent analysis (Abu et al. 2020).

Collection and preparation of animal samples

Collection and preparation of sera samples

At the end of 21 days of treatment, the animals were sacrificed by decapitation using chloroform anesthesia. Blood samples were collected from the throat in plain bottles (for biochemical parameters) and EDTA bottles for hematological analysis. The Blood samples collected in plain tubes were allowed to clot, and the sera were separated by centrifugation using Labofuge 300 centrifuge (Heraeus) at 3000 rpm for 10 minutes. The sera collected were then subjected to biochemical analysis.

Collection of liver and kidney for histopathological analysis

After the rats were sacrificed and the blood samples were collected, the liver and the kidneys were quickly excised, trimmed of connective tissues, rinsed with saline to eliminate blood contamination, dried by blotting with filter paper, and weighed (to calculate the relative weight) and were placed in freshly prepared 10% formalin for histopathological studies.

Estimation of some lipid profiles of experimental animals

The total serum cholesterol was quantified by the method described by Stein (1987). Approximately 1000 µL of the cholesterol reagent made up of 4-amino antipyrine, phenol, peroxidase, cholesterol esterase, cholesterol oxidase, and buffer was added into a clean test tube containing 10 µL of serum, mixed, and incubated for 5 minutes at 37ºC. The absorbance was read against the reagent blank at 500nm within 60 minutes.

The concentration of cholesterol in the sample is given by standard:

Conc. of cholesterol (mg/dL) = ΔA_{sample} / $\Delta A_{standard}$ X Conc. of Standard

ΔA_{sample} = Change in absorbance of the sample

$\Delta A_{standard}$ = Change in absorbance of the standard

The serum triacylglycerol level was estimated by the enzymatic method described by Stein (1987). Approximately 1000 µL of the cholesterol reagent comprised of 4-aminophenazone, peroxidase, glycerol kinase, Glycerol-3-phosphate oxidase, and buffer was added into a clean test tube containing 10 µL of serum, mixed, and incubated for 5 minutes at 37ºC. The absorbance was read against the reagent blank at 500nm.

Conc. of triacylglycerol (mg/dL) = ΔA_{sample} / $\Delta A_{standard}$ X Conc. of Standard

ΔA_{sample} = Change in absorbance of the sample

$\Delta A_{standard}$ = Change in absorbance of the standard

The serum levels of HDL-c were determined by the enzymatic method described by Stein (1987). Exactly 0.5 µL of reagent A made up of phosphotungstate and magnesium chloride was added into a clean test tube containing 0.2 mL of serum, mixed thoroughly, and allowed to stand for 10 minutes at room temperature, centrifuged at 4000 rpm for 10 minutes and supernatant was collected. Exactly 0.1 mL of reagent B, made up of 4-amino antipyrine, sodium cholate, and dichlorophenol sulfonate, was added into a clean test tube containing 50 µL of sample supernatant, mixed thoroughly, and incubated for 30 minutes at room temperature. The absorbance was read against the reagent blank at 500 nm within 60 minutes.

HDL-c += ΔA_{sample} / $\Delta A_{standard}$ X Conc. of Standard

ΔA_{sample} = Change in absorbance of the sample

$\Delta A_{standard}$ = Change in absorbance of the standard

Assessment of some hematological parameters of experimental animals

The PCV is the volume of red blood cells (RBC) expressed as a fraction of the total volume of the blood. The microhaematocrit method was used (Cheesbrough 2000). Blood samples from the rats were filled into a heparinized capillary tube, after which one end of the tube was sealed by flaming. It was then centrifuged at a speed of 7,000 rpm for 5 minutes. The PCV was estimated using a microhaematocrit reader and expressed as a percentage of the blood's erythrocytes.

Hemoglobin concentration (Hb) was determined using the cyanmethemoglobin of Alexander and Griffins (1993a,b), respectively.

Sample solutions and standard solutions were prepared as follows.

Blank: Exactly 5000 µL of Drabkin reagent was mixed with 20 µL of distilled water

Standard: Exactly 5000 µL of Drabkin reagent was mixed with 20 µL of a standard hemoglobin solution.

Test sample: Exactly 5000 µL of Drabkin reagent was mixed with 20 µL of blood.

The concentration of hemoglobin was marked with Drabkin's method, with the use of a spectrophotometer. Once the Drabkin reagent was mixed with the blood, the solution was incubated at room temperature for 5 mins, and absorbance was measured at 540 nm against distilled water.

The concentration of hemoglobin was calculated according to the following formula:

$$Hb\ concn.\ \left(\frac{g}{dL}\right) = \frac{absorbance\ of\ tested\ sample}{absorbance\ of\ standard} X concn.\ of\ standard\ \left(\frac{g}{dL}\right)$$

The total white blood count was determined using the counting chamber method. First, exactly 20 µL of the blood sample was added to 0.4 mL of diluting fluid which consists of 2% acetic acid lightly colored with 1% crystal violet (1:21 dilution of the blood). The counter chamber was then filled with the mixture from above and allowed to stand for 3-5 minutes, after which it was placed on a microscope stage and observed using an X25 objective to count the number of cells seen a sufficient number of $1mm^2$ areas to obtain at least 100 cells.

Cell count (/L) = N x (D/D) x 10 x 10^6

Where: N = total number of cell counted; D = dilution of blood; A = total area counted (in mm^2); 10 = factor to convert area to volume (in µL); 10^6 = factor to convert count per µL to litre.

Histological study of the liver and kidney of experimental animals

A portion of the liver and kidneys of the animals were cut into two to three pieces and fixed in 10% formalin (Lillie 1965). The paraffin sections were prepared and stained with hematoxylin and eosin. The thin sections of the liver and kidneys were made into permanent slides and examined under a high (X250) resolution microscope with a photographic facility and photomicrographs.

Statistical analysis

The data were analyzed by the analysis of variance (ANOVA) using the SPSS program (version 20 SPSS Inc., Chicago, IL, USA). In addition, the differences in parameters between the various animal groups were compared using the Bonferroni multiple comparison test (post-hoc test). The results were expressed as mean ± standard deviation (SD). P-value less than 0.05 was considered as significant ($P< 0.05$). Results were presented in tables, charts, and graphs using Microsoft Word and Excel.

RESULTS AND DISCUSSIONS

Effect of *n*-butanol fraction of methanolic leaf extract of *F. glumosa* on lipid profile

The effects of daily oral administration of *n*-butanol fraction of methanolic leaf extract of *F. glumosa* for 21 days on the lipid profile (total cholesterol, TCH, triacylglyceride, TAG, and high-density lipoprotein, HDL) of CCl_4-induced liver damaged rats is represented in Table 2. There was a significant ($P<0.05$) increase in the level of TCH and TAG with a significant ($P<0.05$) decrease in the level of HDL of the CCl_4-induced control group compared with the induced but treated groups. However, there was no significant ($P>0.05$) difference in the HDL and TCH levels of the induced but treated groups compared with the normal control group.

Effect of n-butanol fraction of methanolic leaf extract of *F. glumosa* on hematological parameters

Some hematological parameters (packed cell volume PCV and hemoglobin HB) found in CCl_4-intoxicated rats are presented in Table 3. There was a significant ($P<0.05$) decrease in the level of packed cell volume and hemoglobin of the CCl_4-induced control group compared with the induced but treated groups. However, compared with the normal control group, there was no significant ($P>0.05$) difference in the levels of PCV and HB of the induced but treated groups. There was a significant increase ($P<0.05$) in WBC in the CCl4-induced but not treated group compared to the normal and CCl_4-induced but treated groups.

Histopathological effect of n-butanol fraction of methanol extract of *F. glumosa* leaves on liver and kidney of CCl_4 intoxicated albino rats

The histological section of the liver and kidneys of CCl_4-induced oxidative damage rats treated with an *n*-butanol fraction of methanol of *F. glumosa* leaves extract for 21 days is shown in Figures 2 and 3. The histopathological examinations of a liver section of the normal control group showed normal hepatocellular architecture with distinct hepatic cells with the well-conserved central vein. On the contrary, the CCl_4-induced control group liver showed intense hepatic necrosis with vascular congestion, kupffer cells hyperplasia, vacuolation, and degeneration of normal hepatic cells (hepatic necrosis). However, the induced but treated groups almost normalized the hepatic cells (moderate hepatic necrosis).

Discussion

The lowered levels of high-density lipoprotein (HDLc) and increased total cholesterol (TC) and triacylglyceride (TAG) in the CCl_4-induced untreated group were in agreement with earlier findings by Adejo et al. (2014), where CCl_4 was able to significantly ($P<0.05$) caused similar effects in rats. These observations could indicate metabolic distortion in the liver due to the severity of hepatic injury inflicted by CCl_4 (Abu et al. 2021). However, treatments with the n-butanol fraction of methanol extract of *F. glumosa* leaves and silymarin reversed the status to near normal. This ameliorative effect of the extract could be attributed to its bioactive compounds, possibly increasing lipase activity that hydrolyses TAG (Adejo et al. 2014). Again, the extract may have possibly acted as an inhibitor to hydroxyl-methyl-glutaryl-CoA reductase, a key enzyme in the *de novo* biosynthetic pathway of cholesterol (Gebhardt and Beck, 1996). On the other hand, the extract probably chelated the by-products of CCl_4 metabolism, thereby stabilizing the lipid regulatory roles of the liver (Dawood et al. 2014).

Table 2. Lipid profile of CCl_4-induced liver-damaged albino rats treated orally with an n-butanol fraction of methanolic leaf extract of *F. glumosa*

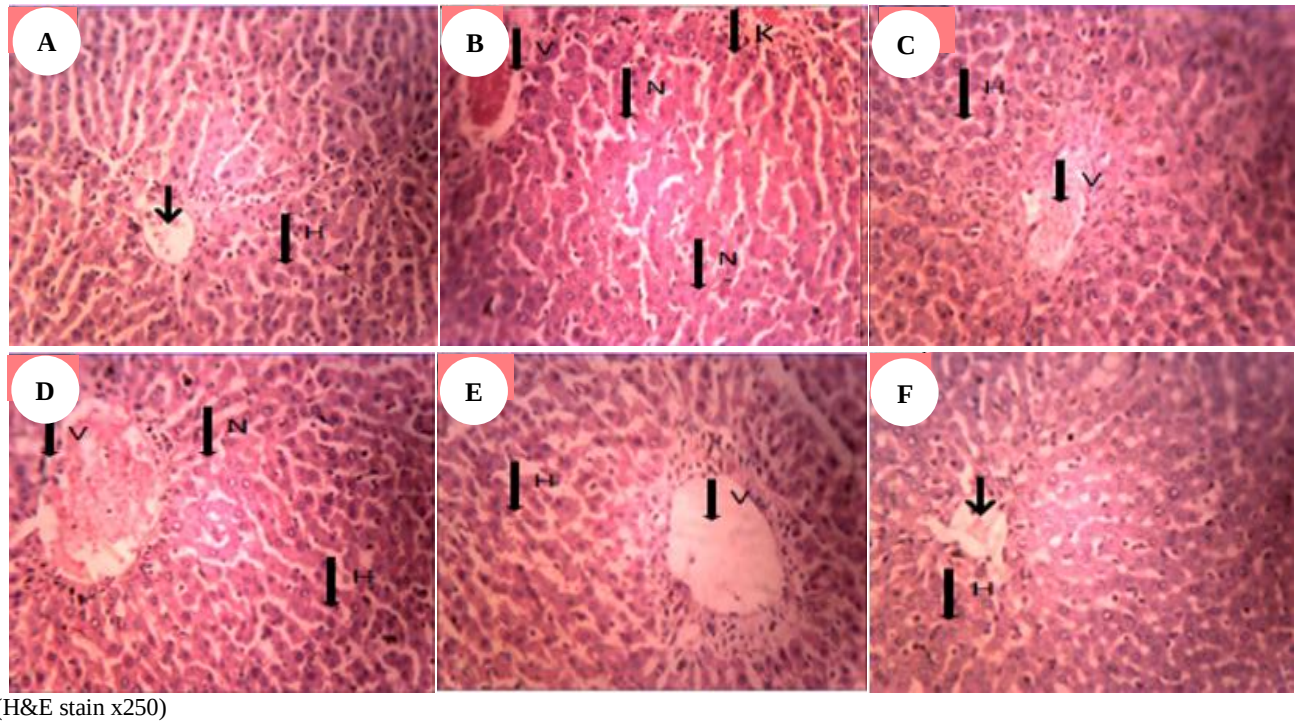
Group	TCH (mmol/L)	TAG (mmol/L)	HDL (mg/dL)
Normal control	1.84±0.27^{b}	0.54±0.11^{b}	1.44±0.43^{b}
Olive oil only	2.26±0.18^{b}	0.62±0.08^{b}	1.04±0.43^{b}
CCl_4 only	9.04±0.83^{ac}	1.26±0.11^{ac}	0.42±0.08^{a}
CCl_4 + 100mg extract	2.52±0.28^{b}	0.88±0.19^{ab}	0.64±0.17^{a}
CCl_4 + 300mg extract	2.30±0.16^{b}	0.80±0.07^{b}	0.92±0.13*
CCl_4 + 500mg extract	2.18±0.20^{b}	0.78±0.19^{b}	1.14±0.11^{b}
CCl_4 + 100mg sylimarin	2.28±0.23^{b}	0.78±0.15^{b}	0.94±0.18*

Note: n=5; values are in mean±standard deviation; values with different superscripts down the columns are significantly different at P<0.05; a= significantly different from the normal control group (P<0.05); b= significantly different from the group treated with CCl_4 without extract or standard drug treatment (P<0.05); c= significantly different from the group treated with CCl_4 and standard drug treatment (P<0.05); *= not significantly different from any of the three groups

Table 3. Hematological indices of CCl_4-induced liver-damaged albino rats treated orally with an n-butanol fraction of methanolic leaf extract of *F. Glumosa*

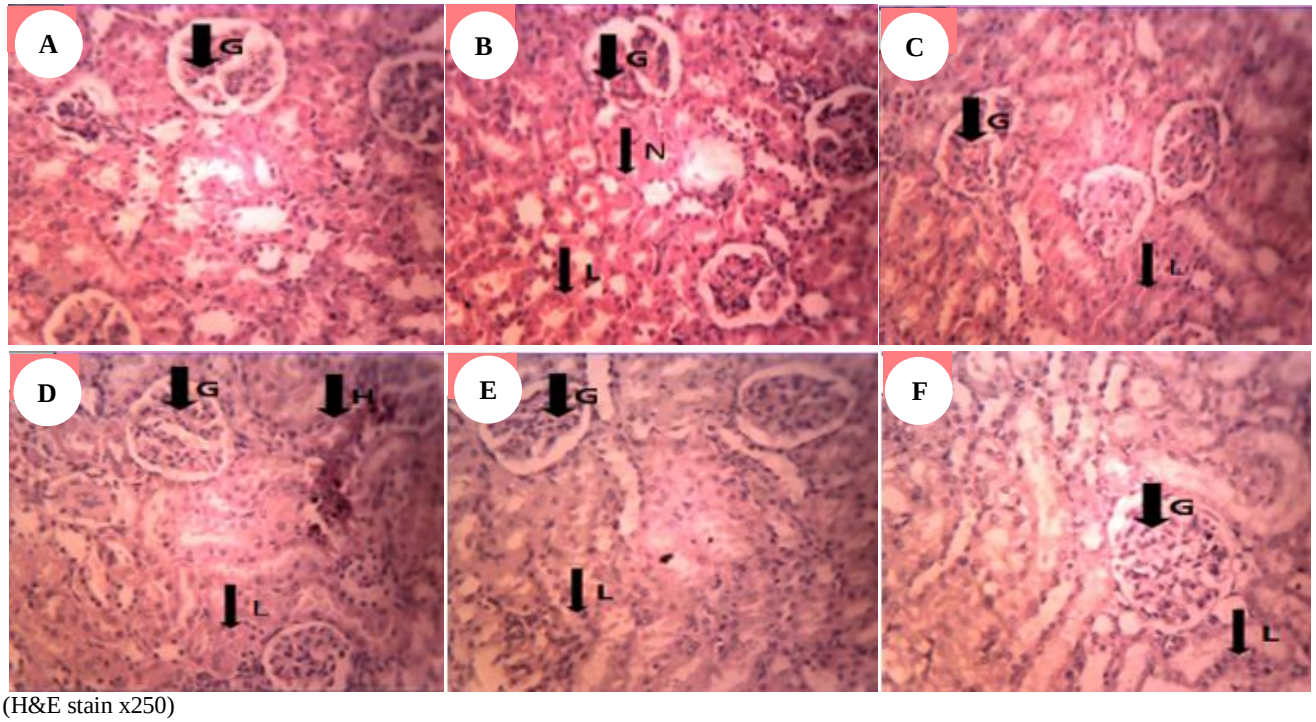
Group	PCV (%)	Hb (g/dL)	WBC (10^3)
Normal control	40.40±2.41^{b}	11.42±0.24^{b}	08.22±1.00^{b}
Olive oil only	41.00±4.30^{b}	11.16±1.72^{b}	07.00±0.43^{b}
CCL4 only	20.20±1.92^{ac}	05.42±0.41^{ac}	13.68±1.87^{ac}
CCL4 + 100mg extract	26.20±6.01^{ac}	08.98±1.06^{b}	08.90±0.54^{b}
CCL4 + 300mg extract	34.20±1.30^{ab}	09.00±2.09^{b}	08.98±1.77^{b}
CCL4 + 500mg extract	37.40±1.14^{b}	09.36±0.93^{b}	08.64±1.39^{b}
CCL4 + 100mg sylimarin	39.20±2.45^{b}	09.38±1.32^{b}	07.86±1.07^{b}

Note: n=5; values are in mean±standard deviation; values with different superscripts down the columns are significantly different at P<0.05; a. significantly different from the normal control group (P<0.05); b. significantly different from the group treated with CC_{l4} without extract or standard drug treatment (P<0.05); c. significantly different from the group treated with CCl_4 and standard drug treatment (P<0.05)



(H&E stain x250)

Figure 2. Representative photomicrograph of liver of CCl_4-induced liver-damaged albino rats treated with an n-butanol fraction of methanol extract of *F. glumosa* leaves. A. Normal control group, B. 1 mL/kg CCl_4-induced but not treated, C. 100 mg/kg of Sylimarin group, D. 100 mg/kg of *F. glumosa* extract, E. 300 mg/kg of *F. glumosa* extract, F. 500 mg/kg of *F. glumosa.* V: vascular congestion, N: necrosis, K: kupffer cells, H: hepatocytes



(H&E stain x250)

Figure 3. Representative photomicrograph of kidney of CCl_4-induced liver damaged albino rats treated with an n-butanol fraction of methanol extract of *F. glumosa* leaves. A. Normal control group, B. CCl_4-induced (1 mL/kg), C. 100 mg/kg of Sylimarin group, D. 100mg/kg of *F. glumosa* extract., E. 300 mg/kg of *F. glumosa* extract, F. 500 mg/kg of *F. glumosa* extract. G: glomerulus, N: Necrosis, L: lymphocyte

The release of CCl_4 reactive species [trichloromethyl ($CCl_3^{\cdot}$) and trichloromethyl peroxy ($CCl_3OO^{\cdot}$)] might have possibly caused the significant ($P<0.05$) transient decrease in the Hb concentration and PCV level due to hemolytic anemia caused by oxidation of sulphydryl groups of the erythrocyte membrane in addition to disturbing hematopoiesis, destruction of erythrocytes, reduction in the rate of their formation and/or their enhanced removal from circulation (Khalid et al. 2013; Maduka et al. 2014; Mariam et al. 2015). On the other hand, the CCl_4 treatment significantly ($P<0.05$) increased WBCs count, which may be attributed to lymphocyte infiltration of poisoned cells, a clear case of immune response to a chemical antigen by the body's defensive mechanism of the immune system (Saba et al. 2010). Meanwhile, treatment with an n-butanol fraction of methanol extract of *F. glumosa* leaves showed significant ($P<0.05$) reversal effects of these indices comparable with silymarin and normal control rats. The consequent reduction in red blood cells hemolysis and enhanced hematopoiesis with the decrease in the WBCs count may be ascribed to the stabilization of the free radicals by some antioxidants present in the *n*-butanol fraction of methanol extract of *F. glumosa* leaves, an effect that was in agreement with the findings of Yakubu et al. (2020) where an aqueous extract of *C. aconitifolius* leaves produced similar action against CCl_4-induced hepatotoxicity and haemotoxicity in rats.

The histopathological findings of the liver and kidney in the CCl_4-induced control group showed that CCl_4 caused intense vascular congestion, vacuolation, necrosis, and lymphocyte infiltrations in both organs (liver and kidney). These results agree with Akram et al. (2010) and Venkatanarayana et al. (2012), that reported similar effects on rats intoxicated with carbon tetrachloride. However, following the administration of the *n*-butanol fraction of methanol extract of *F. glumosa* leaves, the organs showed excellent recovery from both hepatotoxicity and nephrotoxicity to normal cellular architecture. That may be attributed to the ability of the extract to stabilize cellular structural components such as lipids, protein, and carbohydrates or a wound healing effect of the extract.

In conclusion, it was obvious from the result obtained that the induced but treated groups showed almost normalization of the hepatic cells (moderate hepatic necrosis). The extract could reverse the deleterious effects on lipid profile, hematological parameters, and liver and kidney architectural integrities. Those findings suggest that the extract can be used to manage anemia, hyperlipidemia, and liver and kidney disorders owing to its ameliorative effect on these cells, thereby returning biochemical parameters and the architectural integrity to near normal.

ACKNOWLEDGEMENTS

We appreciate the entire Biochemistry Department Laboratory of A.B.U, Zaria crew, for their support. This research is self-sponsored, and there was no financial donor involved.

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CELL BIOLOGY & DEVELOPMENT
Volume 6, Number 1, June 2022 E-ISSN: 2580-4499
Pages: 13-19 DOI: 10.13057/cellbioldev/t060103

Effect of giving nanochitosan preparations ethanol extract of neem leaves (*Azadirachta indica*) against pancreatic histology of white rat male (*Rattus norvegicus*) Sprague Dawley

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Manuscript received: 30 March 2022. Revision accepted: 2 June 2022.

Abstract. *Handayani S, Sitasiwi AJ, Isdadiyanto S, Mardiati SM. 2022. Effect of giving nanochitosan preparations ethanol extract of neem leaves (*Azadirachta indica*) against pancreatic histology of white rat male (*Rattus norvegicus*) Sprague Dawley. Cell Biol Dev 6: 13-19.* The selection of herbal plants as a treatment in the community is considered safer, more practical, and cheaper than synthetic drugs. Neem (*Azadirachta indica* A.Juss.) is one of the herbal plants that have the potential as an antioxidant. However, the low bioavailability of drugs and the distribution of active compounds in herbal plants are constraints in administering drugs orally. The solution to overcome this problem is to prepare the test material in the form of nanochitosan. This study aimed to analyze the effect of nanochitosan ethanol extract of neem *(A. indica)* leaf extract on the pancreas histology of male Sprague Dawley rats. This study used a completely randomized design (CRD) with 32 rats aged 2 months divided into 4 treatments, and each treatment consisted of 6 replications. P0 (normal rat group treated with 2 mL distilled water, P1 (normal rats induced with *Natrium tripolifosfat "*NaTPP" and 2 mL chitosan), P2 (normal rat group induced by nanochitosan neem leaf ethanol extract 1:0.5), P3 (normal rat group induced nanochitosan ethanol extract of neem leaves 1:1). Data were analyzed by ANOVA test with a significance level of 5%. Data that were not normally distributed were tested by the *Kruskal Wallis* test and Duncan's test. The results showed that the administration of nanochitosan ethanol extract of neem leaves 1:0.5 and 1:1 had no significant effect on the diameter parameter of the islets of Langerhans ($P \geq 0.05$) but had a significant effect on the parameters of pancreatic weight and damage scoring of the islets of Langerhans ($P \leq 0.05$). The administration of nanochitosan preparations of ethanolic extract of neem leaves can deliver bioactive compounds neem to the pancreas and minimize damage to the cells that make up the islets of Langerhans due to toxic neem compounds.

Keywords: Islet of Langerhans, nanoparticles, neem plant, pancreas

INTRODUCTION

The selection of herbal plants as a treatment in the community is considered safer, more practical, and cheaper than synthetic drugs, so they have become an alternative treatment for various diseases. The neem plant (*Azadirachta indica* A.Juss.) is one of the most studied herbal plants because of its properties that can treat various diseases (Pristiani and Astuti 2005; Fathoni et al. 2013). The benefits of the neem plant can be used as an antirheumatic, antioxidant, anti-inflammatory, immuno-potentiation, antifertility, antiviral, anticancer, and antipyretic (Ambarwati 2011). However, the side effects of neem are thought to cause kidney and pancreas damage. Wowiling's research (2013) stated that the advantages of neem include easy cultivation, its use as herbal medicine, relatively safe for humans, and the prevention of various diseases. However, the deficiency of the neem plant can cause liver and pancreas damage. That is presumably due to toxic compounds in neem and the low distribution of the active compound content of neem.

Oral administration of drugs is the most widely used method of administering drugs to test animals because the process is easy, safe, inexpensive, and convenient. However, obstacles in oral administering the drug are the low bioavailability and the low distribution of neem compounds to the body. According to research by Ajazuddin and Saraf (2010), the solution to overcome the oral administration problem is to prepare drugs in the form of nanochitosan to facilitate the absorption and distribution of drugs into the body to increase the bioavailability of neem plants. Prasetiowati et al. (2018) stated that the excess use of nanochitosan in medicinal plant extracts can facilitate the absorption and distribution of drugs into the body and reduce the toxic effects of drugs. The polymer used to manufacture nanoparticles is chitosan with a soluble compound in sodium tripolyphosphate (NaTPP) (Kafshgari et al. 2011). Martien et al. (2012) stated that chitosan could reduce the toxic effects of neem exposure, so it is very efficient to be developed as the main ingredient for making nanoparticles. Therefore, nanoparticles can provide an effective solution to overcome the difficulty of drug delivery into the body, facilitate the distribution of bioactive compounds from neem plants into the pancreas, and reduce the toxic effects of neem plants. This study aimed to analyze the effect of neem leaf extracts chitosan nanoparticles on the histological structure of the pancreas of male Sprague Dawley rats.

MATERIALS AND METHODS

This research was conducted for 8 months from March-October 2021 at the Laboratory of Animal Structure and Function Biology, Department of Biology, Faculty of Science and Mathematics (FSM), Universitas Diponegoro (UNDIP), Semarang, Central Java, Indonesia.

Tools and materials

The tools used in this study were 32 sets of rat cages, rat feed, rat drinking bottles, an oven, a grinder, a rotary evaporator, a refrigerator, an analytical balance, a heater, a measuring cup, *thermohygrometer*, 3 mL injection syringe, sonde, a set of surgical instruments, plastic container, gloves, paraffin bath, pins, petri dish, a digital scale with 0.01 g accuracy, sample vial, millimeter block paper, rotary microtome, dropper, object-glass, cover glass, microscopes, photomicrographs, cameras, and stationery.

The materials used were 70% ethanol, aquadest, A594 type chicken feed, drinking water, rice husks, label paper, tissue, latex, masks, cotton, chloroform, physiological saline solution, 10% Neutral Formalin Buffer (BNF) solution, water faucet, NaTPP solution, neem leaves, entellan, paraffin solution, xylol and hematoxylin-eosin (H&E). The test animals used in this study were 32 white male rats *(Rattus norvegicus* Berkenhout, 1769) Sprague Dawley, with no anatomical defects, obtained from the *Unit Pelayayanan Hewan Percobaan (UPHP)*, Universitas Gadjah Mada, Yogyakarta, Indonesia.

Research design

This study used a completely randomized design (CRD) which consisted of 4 treatments, and each treatment consisted of 6 replications. The determination of the dose was carried out according to the study of Sitasiwi et al. (2019). Furthermore, the test animals were grouped into 4 treatment groups, including: (i) P0: Control, normal rats were given 2 mL of distilled water. (ii) P1: Normal rats were given 2 mL of NaTPP + chitosan solution. (iii) P2: Normal rats were given nanochitosan ethanol extract of neem leaves 1:0.5. (iv) P3: Normal rats were given nanochitosan ethanol extract of neem leaves 1:1.

Preparation of neem leaf ethanol extract *(A. indica)*

Neem leaves were obtained from the UNDIP area, Semarang, Central Java, Indonesia. One kilogram of neem leaves was dried in an oven at a temperature of ±45-50°C for 3 days. The dried neem leaves were crushed and ground using a grinder and then sieved to obtain a powder form. According to Abror et al. (2018), the neem extraction step was done by soaking neem leaf powder in 70% ethanol for 3x24 hours. The extract obtained was then filtered. The filtering results were evaporated using a rotary evaporator at a temperature of 50°C to obtain a powdery extract; then, the neem leaf extract was stored in the refrigerator. The making of neem leaf extract was carried out at the Laboratory of the Universitas Semarang, Semarang, Central Java, Indonesia, for ± 2 weeks.

Nanoparticle preparation

The manufacture of chitosan nanoparticles from neem leaf ethanol extract *(A. indica)* by dissolving 1 mL of neem leaf extract into 35 mL of ethanol and adding 15 mL of distilled water. Then dissolved into 100 mL of chitosan solution using a magnetic stirrer and dissolved in 40 mL of 0.1% sodium tripolyphosphate (NaTPP) solution at room temperature at 3000 rpm for ± 2 hours to form a nanoparticle suspension. Furthermore, measurements were carried out using a Malvern particle size analyzer (Naela et al. 2019). Finally, the characterization of nanoparticles was carried out at the UNDIP Integrated Laboratory.

Test animal preparation

Rats were kept for 28 days in a rat cage and fed and watered ad libitum. The environmental conditions of the cages were checked using a thermohygrometer to measure the temperature and humidity of the cage conditions. Temperature and humidity measurements are carried out every 09.00 (morning) and 16.00 (afternoon). Replacement of husks (bed cage) is done every 3 days.

The final stage of acclimation was weighing the body weight of Sprague Dawley rats. Mice with uniform body weight were then grouped into 4 treatment groups. The lottery method determined the division of rats into treatment groups. Each treatment group had 6 rats as the replication unit.

Administration of test materials to test animals

The administration of neem leaf extract was carried out orally for 28 days every afternoon according to the dose in each treatment, namely P0 (Control) normal rats were given 2 mL of distilled water; P1, normal rats were given a solution of NaTPP and chitosan ethanol extract of neem leaves as much as 2 mL; P2, normal rats were given 2 mL of neem leaf ethanol extract nanochitosan with a ratio of 1:0.5; and P3, normal rats were given 2 mL of neem leaf ethanol extract nanochitosan with a ratio of 1:1.

Animal dissection and tissue collection

The treatment was administered using a cannula syringe or a probe attached to a 3 mL syringe. The provision of test materials begins with handling rats using the scrubbing technique (Darusman et al. 2018).

Pancreatic preparation

The histological preparations of the pancreas were carried out concerning the Berata and Samsuri (2017) method using the paraffin method and hematoxylin-eosin dye with an incision thickness of ± 5-7 microns.

Microscopic observation

Observations of the histology of pancreatic organs were performed using an Olympus BX51 microscope and photomicrograph with magnifications of 200x, 400x, and 1000x randomly in one preparation. In addition, the diameter and damage score calculation was carried out in one preparation with 200x and 400x magnification for each preparation.

Data analysis

Histological observation data in the form of the diameter of the islets of Langerhans, the area of the islets of Langerhans, the volume of the islets of Langerhans, the scoring of damage to the islets of Langerhans, and the weight of the pancreas were tested for normality. The Data is said to be a normal distribution if P≥0.05. Pancreatic weight, diameter, and area of the islets of Langerhans were tested using the One Way ANOVA statistical test at a 95% confidence level. Pancreatic volume data and scoring description of the histological structure of the islets of Langerhans were tested by the *Kruskal Wallis* test, followed by Duncan's test. Statistical testing was carried out using the SPSS version 26 application. The histological structure data of the pancreas were presented descriptively, and the measurement of the islets of the Langerhans area, the volume of the islets of Langerhans, and the weight of the pancreas were analyzed quantitatively.

RESULTS AND DISCUSSION

The morphology of the pancreas of *R. norvegicus* macroscopically looks pale red, elongated vertically with a length ranging from 5 cm from head to tail, has a pancreas weight of 100-200 mg, and visible blood vessels accompanied by indistinct hoops. These results are to the research of Treuting et al. (2018) that the morphology of the pancreas of *R. norvegicus* looks like white to pink grapes and visible blood vessels. A comparison of the morphology of the pancreas of *R. norvegicus* after administration of the test material for 28 days can be seen in Figure 1.

Based on Table 1, the results of the pancreatic ANOVA test with a significance level of 5% showed a significant difference in the pancreatic weight of rats *(R. norvegicus)* given nanochitosan preparations of neem leaf ethanol extract for 28 days. That is because the administration of the test material in the form of nanochitosan ethanol extract of neem leaves has a smaller size, so it is suspected that it can provide a more effective effect in delivering neem compounds to the target location and can increase the bioavailability of the test material in the body. On the other hand, the P0 and P1 groups experienced a decrease in pancreatic weight which was not significantly different (P≥0.05) in the test animals, which is presumably due to the low bioavailability of the test material. Furthermore, the research of Kakkar et al. (2011) showed that the administration of the test material in the form of nanoparticles could reduce the weight of the pancreas and increase the bioavailability of herbal medicines. Presumably, the test material suppresses the decrease in pancreatic weight compared to the positive control group, induced only by aquadest.

The preparation of nanochitosan was thought to deliver the active compound of the neem plant, as evidenced by the addition of pancreatic weight in groups P2 and P3. That is presumably because the active compound in the neem can protect the cells that make up the islets of Langerhans due to the Azadirachtin compound. Furthermore, Juanda and Jayadi (2015) stated that the higher the concentration of neem leaf extract, the higher the ability of pancreatic cells to minimize damage to the islets of Langerhans. The research of Septiana et al. (2012) showed that pancreatic samples could experience a decrease in the size and weight of the pancreas after being induced by the papaya leaf extract nanoparticle preparation test material. However, the morphology of the pancreas was still relatively the same, and the weight of the pancreas of rats was still relatively normal.

The success of making neem leaf nanoparticles using PSA shows that the smallest nanoparticle size in NaTPP: Chitosan is 202.3 nm, and the largest in NaTPP - Chitosan: SEEDM 1:0.5, which is 324.9 nm (Sitasiwi et al. 2021). The results showed that the particle size formed was still in the nano-size range and effectively delivered the drug to the target location, namely the pancreas, following the opinion of Rawat et al. (2006) that the nanoparticle size is < 300 nm. That proves that the nanoparticle preparation of neem leaf ethanol extract is said to be effective with a nanoparticle size ranging from 300 nm, which can deliver the bioactive content of neem to the pancreas.

Based on the results of the phytochemical test of the neem *(A. indica)* plant showed that the ethanolic extract of neem leaves contained alkaloids, terpenoids, flavonoids, phenolics, saponins, and tannins with positive test results. But negative in the test for steroid content. This result is different from the research of Soraya (2021) explained that neem leaf samples contained tannins, phenols, triterpenoids, saponins, and steroids but were negative in the alkaloid and flavonoid tests. That is because the flavonoid content of neem is not optimally distributed into the pancreas. That is presumably because the compound size is too large, so the compound that enters the body is damaged, which causes neem bioactive compounds cannot to enter the pancreas. Therefore, the administration of nanochitosan can assist in delivering the active compound neem to the cells that make up the pancreas and minimize the damage to the islets of Langerhans, as evidenced by the histological structure of the pancreas, which is getting better in the P3 treatment group. On the other hand, the research of Nugroho et al. (2020) showed that giving the test material in the form of nanoparticles could increase the bioactive content distribution of the binahong plant and repair the damaged cells that make up the islets of the Langerhans. This study proved that the damage to cells that make up the islets of Langerhans in group P1 could be suppressed by the presence of active neem compounds in groups P2 and P3, which showed a decrease in cell damage.

The results of the average diameter of the islets of Langerhans in white rats *(R. norvegicus*) after administration of nanochitosan preparations of neem leaf ethanol extract for 28 days are presented in Table 1. Based on the ANOVA test on the diameter parameters of the islets of Langerhans, the results were not significantly different (P≥ 0.05). The mean value of the diameter of the islets of Langerhans in the treatment groups P0, P1, P2, and P3 was 116.80 m, respectively; 128.05 m; 124.82 m; and 117.44 m. The average diameter of the islets of Langerhans is still

relatively normal, ranging from 100 m to 150 m (Mescher 2016). The results of the analysis showed that the ethanol extract of neem *(A. indica)* leaves in a ratio of 1:0.5 and 1:1 in groups P2 and P3 did not affect the structure of the cells that make up the islets of Langerhans so that the diameters of the cells of the islets of Langerhans were not significantly different. That is presumably because the preparation dosage is still a safe dose or dose. Following the research of Suhendro et al. (2018), the administration of ethanolic extract of neem leaves at a dose of 50-200 mg/g BW in Sprague Dawley rats was still a safe dose. The research of Azmi et al. (2022) showed that the administration of the test material for neem leaf ethanol extract did not affect the diameter of the islets of Langerhans but could improve the cell structure of the islets of Langerhans. That is presumably due to the antioxidant content of the neem plant, which can minimize damage to the structure of the islets of Langerhans.

Histological observations of the pancreas in the P0 group showed a normal histological structure as indicated by the clear boundaries of the islets of Langerhans, round cells, no voids in the islets, no severe damage in the P0 group, and no necrotic and cell degeneration were seen. According to Walean et al. (2020), the aquadest-induced negative control group in normal mice saw the islets of Langerhans under normal conditions. The cell nucleus was clearly visible, surrounded by normal acinar cells. In contrast to the histological observations of the pancreas in the P1 treatment group, the damage was quite severe, indicated by the presence of quite a lot of cell degeneration, necrotic cells started to appear, and parts of the islets of Langerhans cells were not visible, the shape of the cells was abnormal, and the cells underwent degeneration and necrosis. Damage to the P1 group was suspected because the P1 group was not induced by the active compound neem and the low level of solubility of the test material so that to stabilize it, it could be offset by giving a surfactant in the form of neem leaf ethanol extract which can regenerate the cells that make up the islets of Langerhans.

Histological observations of the pancreas in the P2 group showed that the damage was not severe compared to the P1 group, as indicated by the reduced level of necrotic cells. In addition, the distribution of cells looked more regular, and there was a reduction in the empty space in the islets. The following research by Setiadi et al. (2020) showed that the administration of the test material for the ethanol extract of aloe vera leaf at a dose of 120 mg/kgBB was able to improve the structure of the islets of Langerhans but still not like the normal state of the islets of Langerhans. Histological observations of the pancreatic organs of the P3 group showed that the islets of Langerhans in the test animals of the P3 group looked normal with clear boundary characteristics of the islets of Langerhans, parts of the islets of Langerhans cells were seen, the number of cells that were necrotic was slightly, and the shape of the cells was normal. In addition, the islets of Langerhans were more colorful and lighter than the exocrine pancreatic tissue, which is darker in color. The description of the histological structure of the P3 treatment group can be seen in Figure 2.

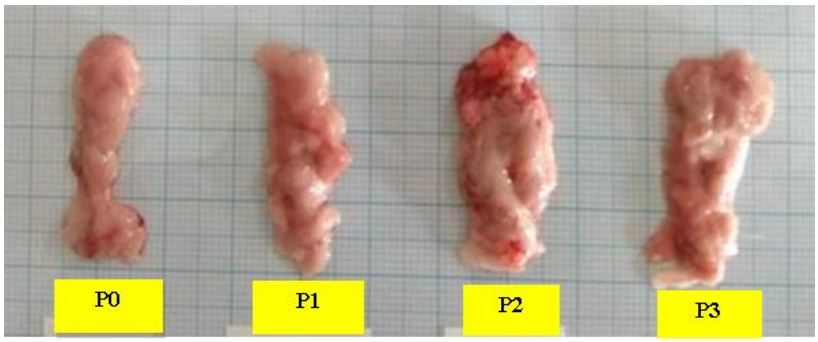

Figure 1. The pancreas of *Rattus norvegicus* Sprague Dawley Male

Table 1. ANOVA test of pancreatic weight, the diameter of the islets of Langerhans, and Duncan's test, the average score of damage to the islets of Langerhans

	Parameter		
Treatment	**Pancreas weight (mg) (Mean ± SD)**	**Diameter of the islets Langerhans (μm²) (Mean ± SD)**	**Average damage score (Mean ± SD)**
P0	101^{a} ± 47,273	$116,80^{a}$ ± 12,740	0^{a} ± 0
P1	104^{a} ± 35,265	$128,05^{b}$ ± 16,599	$3,12^{b}$ ± 0,94
P2	132^{b} ± 31,365	$124,82^{c}$ ± 15,515	$1,45^{c}$ ± 0,83
P3	168^{c} ± 33,391	$117,44^{a}$ ± 8,647	$1,29^{c}$ ± 0,85

Note: Different superscripts showed significant differences (P≤0.05). P0 = Normal control (normal rats were given 2 mL of distilled water), P1 Positive control normal rats were induced with 2 mL NaTPP and chitosan solution), P2 (Normal mice were induced with nanochitosan ethanol extract of neem leaves 1:0.5), P3 (Normal mice were induced with nanochitosan ethanol extract of neem leaves 1:1)

Table 1 shows the average value of the Langerhans Island damage score. The negative control treatment group (P0) got an average score of 0 with score of 0, which showed the normal pancreatic histological structure in the form of clear islets of Langerhans, the structure and size of the cells looked normal, and there was no hypertrophy of the cells or cell degeneration. That indicates that the islets of Langerhans are in normal condition or there is no necrosis. According to Tandi et al. (2017), normal islets of Langerhans were given a score of 0 with normal cell shape, absence of necrotic cells and cell degeneration, and normal cell shape. The P1 group rats induced with NaTPP and chitosan had an average score of 3.12 with a score of 3, meaning that the damage was quite severe. The damage shown in the histological preparations was pancreatic cells that underwent necrosis, abnormal cell shape, and degenerated cell nuclei, which causes the structure and shape of the islet of Langerhans to be irregular and different from the shape of the islet of Langerhans in general. The P2 group had an average damage score of 1.45 (Table 1), with some of the islets of Langerhans being given a score of 2.

The state of the islets of Langerhans, which was given a score of 2, was the boundary condition of the islets of Langerhans that started to become unclear, the number of cells was reduced, and some of the cells degenerated. There are abnormal cell shapes, and there are no necrotic cells visible. This damage is a characteristic of reversible damage. Tandi et al. (2017) stated that the characteristics of the islets of Langerhans, which were given a score of 2, were the boundaries of the islets of Langerhans, which looked unclear; the cells were irregular in shape. Some had cell degeneration, and the cells did not appear necrotic. The P3 group had an average damage score of 1.29 (Table 1) with a score of 1. The condition of the islets of Langerhans, which was given a score of 1, showed clear boundaries on the islets of Langerhans; no necrotic cells were seen, normal cell shape, and only degeneration. A comparison of the histological structure of the pancreas in all treatments can be seen in Figure 3.

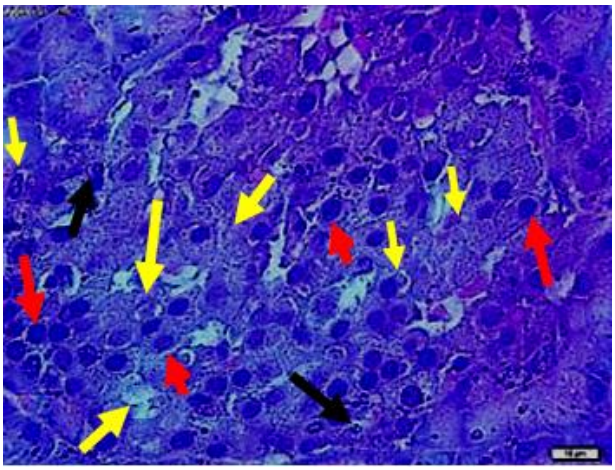

Figure 2. Histology of the islets of Langerhans *Rattus norvegicus* in group P3 at 1000x magnification with *Hematoxylin-Eosin* staining. Description: red arrows: normal cells, yellow arrows: cell degeneration, abnormal cell shape, and black arrows: necrotic cells

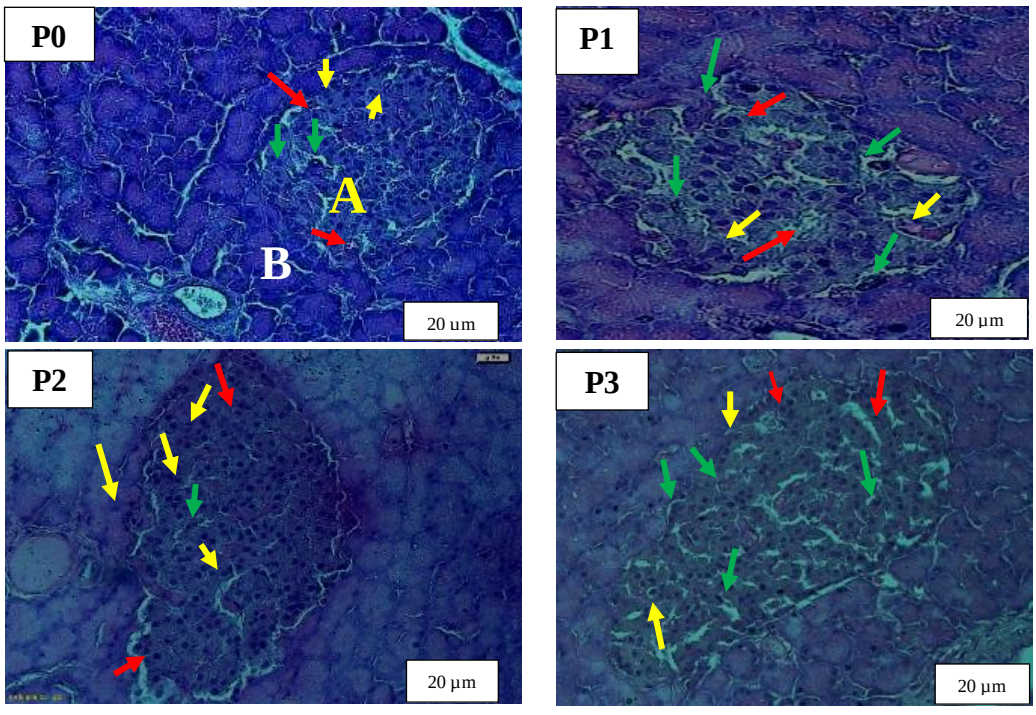

Figure 3. Histological structure of the islets of Langerhans *Rattus norvegicus* (HE,400x). A. Islets of Langerhans, B. Exocrine pancreas. Red arrows: unclear boundaries, yellow arrows: cell degeneration and abnormal cell shape, green arrows: necrotic cells

Islets of Langerhans improved when ethanol extract of neem leaf nanochitosan was given in a ratio of 1:0.5 and 1:1, which was able to repair the damage to the pancreas of rats, indicated by the level of pancreatic cell damage that was seen to be reduced compared to the P1 group. Furthermore, the pancreatic structure in groups P2 and P3 improved in the islets of Langerhans space. The distribution of cells looked more regular than in group P1, with normal cell shape, and the histological structure of the islets Langerhans began to improve. That improved the histological structure of the islets of Langerhans in the P3 group, followed by cell regeneration in the islets of Langerhans. The Prameswari and Widjanarko (2014) research showed that the absence of cell degeneration characterized the regeneration of Langerhans islet cells, and the normal cell shape and structure of Langerhans islets looked close to normal groups. Improvements in the histological structure of the islets of Langerhans in the P3 treatment group were thought to be influenced by the increased bioavailability of neem and the distribution of the antioxidant content of neem, which was able to be well absorbed by the pancreas along with the increase in the dose given in nano size so that an increase in the dose given was thought to cause an increase in the number of bioactive compounds contained in the ethanolic extract of neem leaves that reach the target organs.

In conclusion, neem leaf ethanol extract in the form of nanochitosan has the potential to deliver neem bioactive compounds to the pancreas and minimize damage to cells that make up the islets of Langerhans due to toxic neem compounds.

ACKNOWLEDGEMENTS

The author would like to thank the Faculty of Science and Mathematics, Universitas Diponegoro, Semarang, Central Java, Indonesia, which has funded this research with DIPA funds from the Faculty of Science and Mathematics of Universitas Diponegoro Number: 1970/UN.7.5.8/PP/2020.

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CELL BIOLOGY & DEVELOPMENT
Volume 6, Number 1, June 2022 E-ISSN: 2580-4499
Pages: 20-31 DOI: 10.13057/cellbioldev/v060104

Introgression of anthracnose resistance gene(s) into common bean (*Phaseolus vulgaris*)

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Manuscript received: 21 March 2022. Revision accepted: 6 June 2022.

Abstract. *Kazimoto GK, Nchimbi-Msolla SF, Mabagala RB. 2022. Introgression of anthracnose resistance gene(s) into common bean (*Phaseolus vulgaris*). Cell Biol Dev 6: 20-31.* Common bean anthracnose disease caused by the fungus *Colletotrichum lindemuthianum* causes significant yield losses. It is most destructive in areas with cool temperatures and high humidity (90-100%). The study aimed to introduce resistance genes into adapted but susceptible local cultivars of Masai Red and Soya Njano using conventional breeding methods. Five races of *C. lindemuthianum* were isolated and named from thirty-two common bean-diseased plant samples collected from Northern Tanzania. The sources of resistant genes were bean cultivars G2333 and AB136. Early developed populations were evaluated under field conditions in high altitude and humid environments at Bashnet in the Manyara region, in the Northern highlands of Tanzania. Both F2 and F3 populations of Soya Njano x G2333 were segregated for *C. lindemuthianum* resistance at a 9:7 ratio. Such segregation implied that two dominant epistatic genes conferred from G2333, the resistance being in the mode of epistatic gene interaction. The crosses between Masai Red x G2333 and F2 and F3 populations segregation ratio was 10:6, implying two dominant resistant genes were transferred to developed populations. The F2 and F3 progenies obtained from crossing Soya Njano and AB136 showed a ratio of 3:1. The F2 progenies from a cross between Masai Red and AB136 were segregated at a ratio of 3:1 and F3 progenies were 3:1. The 3:1 ratio confirmed single dominant gene inheritance conferred to developed progenies. The heritability (h^2) from populations of Soya Njano x G2333 and Masai Red x G2333 was between 0.41 and 0.45. While Soya Njano, Masai Red, and A136 were between 0.2 and 0.53, which implied moderate heritability. F2 and F3 populations developed need further testing using MAS to confirm the presence of resistant genes. Multi-location testing should be done to verify the resistance levels of the developed bean population in later generations.

Keywords: *Colletotrichum*, Morogoro, *Phaseolus*, resistance gene

INTRODUCTION

The common bean (*Phaseolus vulgaris* L.) is an annual crop that belongs to the family Fabaceae. The genus *Phaseolus* comprises 55 species and is an important grain legume grown within the boundary between two climatic zones, the tropics, and subtropics, with its primary center of diversity in Mexico, Southern Peru, Bolivia, and Argentina (Debouck 1994). The Portuguese later introduced it in East Africa and Brazil (Jones and Mejia 1999).

The domesticated bean species individually constitute a primary gene pool with its wild ancestral form. The wild bean's distribution northwards and southwards led to the formation of two geographically distinct gene pools in Meso America and the Andean (Broughton et al. 2003). Domestication of the common bean gave rise to several domesticated races of beans, and each of the two gene pools became the origin of races. Meso-American gene pool races were in Durango, Guatemala, and Jalisco, while the Andean gene pool was in New Granada, Peru, and Chile (Hillocks et al. 2006). A wide diversity of common bean cultivars is available in developing countries for production and crop improvement for adaptation to biotic and abiotic stress. The crop expresses wide variability in maturity ranging from 60-150 days (Blair et al. 2007). According to CIAT (2013), the common bean is the most vital grain legume in human diets. In sub-Saharan Africa, over 200 million people grow beans as a primary staple food and the most crucial source of calories after maize (Beebe et al. 2012). It enhances health-promoting aspects of the diet, thus vital in mitigating health risks for diseases such as obesity, cancer, diabetes, and heart disease (Raatz 2013).

The world's largest common bean producers are India, Brazil, Myanmar, and Mexico (FAOSTAT 2014). In Africa, Tanzania is the leading producer contributing 4.9% of the production (FAOSTAT 2014). However, the production of common beans in various parts of the world faces a few major biotic and abiotic constraints. Biotic stresses are caused by fungi, bacteria, viruses, and insect pests. The abiotic bean production constraints include macronutrients such as nitrogen [N] and phosphorus [P], micronutrients deficiency, such as excessive rain/flooding, drought, heat, and cold stress factors, each of which causes yield loss significantly (Beebe et al. 2012).

All agricultural zones in Tanzania are constrained by incidences of diseases and insect pests, both in the field and in storage. Major diseases are angular leaf spot (*Pseudocercospora griseola*) and anthracnose (*Colletotrichum lindemuthianum*), and Common bacterial blight (*Xanthomonas phaseoli).* Insect pests include bean stem maggot (*Ophiomyia* spp.), bean aphids (*Aphis fabae*),

bean leaf beetle (*Ootheca benningseni* (*Acanthosceli*), bean bruchids (*Acanthoscelides obtectus*) (Nyambo 2009).

Under favorable climatic conditions, anthracnose is a devastating common bean seed-borne disease. It causes significant yield loss in susceptible bean cultivars worldwide, resulting in 80-100% yield losses (Sharma et al. 2007). Infections can be quite destructive when climatic conditions are favorable to the pathogen. Economic yield losses can be as high as 100% (Davide and de Souza 2009). The yields are about three times as high in developed countries, such as U.S.A and Canada, compared to the developing countries (Porch et al. 2013). According to FAOSTAT (2015) estimates for 2013, the world bean production was 1235 kg/ha, while the yield for Africa was 799 kg/ha and 885 kg/ha for Tanzania. The yield potential is 1500 to 3000 kg/ha under reliable rainfall (Hillocks et al. 2006).

Several anthracnose management strategies, including planting mixtures of bean cultivars (Mwesigwa 2009), have been advocated to alleviate the deleterious effects of anthracnose disease on bean productivity. However, the success remains low due to smallholder producers' unaffordable cost of practices and labor constraints. Genetic resistance is the most cost-effective means of controlling the disease (Miklas et al. 2006; Tryphone et al. 2013). Developing well-adapted resistant bean cultivars is an effective alternative management option to control anthracnose (Kelly and Vallejo 2009).

This study aimed at introducing genes conferring resistance against anthracnose into popular local bean cultivars' Masai Red' and 'Soya Njano' to improve their productivity.

MATERIALS AND METHODS

Study location

This work was conducted in Long village, about 62 km west of Babati Town, Tanzania. The field experiment was conducted in a farmer's field located at S 04°13.815; E35° 27.090, at an elevation of 2,187 meters above sea level. The village experiences a bimodal rainfall pattern. Mid-November to mid-January is the predominant season for growing common beans in Babati rural areas.

Bean plant materials

Two locally adapted cultivars,' Masai Red' and 'Soya Njano,' were used as female parents. They were collected from farmers at Upper Kitete Village, Arusha Region, Tanzania. Unfortunately, both varieties succumb to bean anthracnose infection. The G2333 and AB136 are resistant to bean anthracnose disease and were used as donor parents. G2333 and AB 136 cultivars were used as male parents. The seed types of these parental genotypes are shown in Figure 1. Both donor-parent bean cultivars were obtained from the Sokoine University of Agriculture (SUA), Tanzania, bean breeding program. Some of their characteristics are given in Table 1. The segregating bean seeds in F1, F2, and F3 generations obtained by crossing Masai Red x G2333 are shown in Figure 2, representing other closes such as Soya Njano x G2333, Soya Njano x AB136, Masai Red x AB136, which also had to segregate bean seeds.

Generation of breeding lines

Hybridization

Purifying "Masai Red" and "Soya Njano" was done by planting 5 seeds of each variety in plastic pots. Two seeds harvested from a single plant were used in making crosses. Two seeds were sown per pot filled with sterilized forest soil, and thinning was done two weeks after planting. Six pots were used per variety for crossing establishment. Recipients, as well as donor parents, were planted in a staggered mode at an interval of seven to fourteen days. First, donor lines were planted seven to fourteen days before planting recipient parents. Diammonium Phosphate (DAP) at a rate of 60 kg/ha was used during sowing. Urea was top dressed at a rate of 20 kg N/ha. Watering was done throughout the time of the experiment.

Crosses were made between adapted local cultivars (Masai Red and Soya Njano) susceptible female parents with donor lines G2333 containing complementary genes Co-4², Co-5, and Co-7 and AB 136, carrying complementary genes Co-6, co-8. Crosses were as follows: Masai Red x G2333, Masai Red x AB136, Soya Njano x G2333, and Soya Njano x AB136 to get F1 populations (Figure 3).

Pollination was performed by rubbing the pollinated stigma of the male flower on the female flower. The hooking technique removed the donor parent's pollinated stigma using forceps and hooked it against the recipient parent flower (CIAT 1989). Before new emasculation, forceps were sterilized by dipping in alcohol to avoid contamination. The F1 progenies obtained by crossing were harvested from each cross separately and then grown to advance them to F2 and F3 populations.

Table 1. Bean cultivars used for the current study and some of their key phenotypic characteristics (Pastor-Corrales et al. 1994)

Genotype	Growth habit	Source of seed	Seed size	Seed color	Flower color	Reaction to anthracnose
Soya-Njano 1	Determinate Erect, bush	Upper Kitete	Medium	Yellow	Pale pink	Susceptible
Soya-Njano 2	Indeterminate	Upper Kitete and Slahamo	Small- round	Pale- Yellow	Pale pink	susceptible
Masai Red	Indeterminate	Upper Kitete	Small	Deep red	White	Susceptible
G2333	Indeterminate	SUA-Morogoro	Small	Maroon	White	Resistant
AB136	Indeterminate	SUA-Morogoro	Small	Red	White	Resistant

The recipient parent cultivars Masai Red and Soya Njano

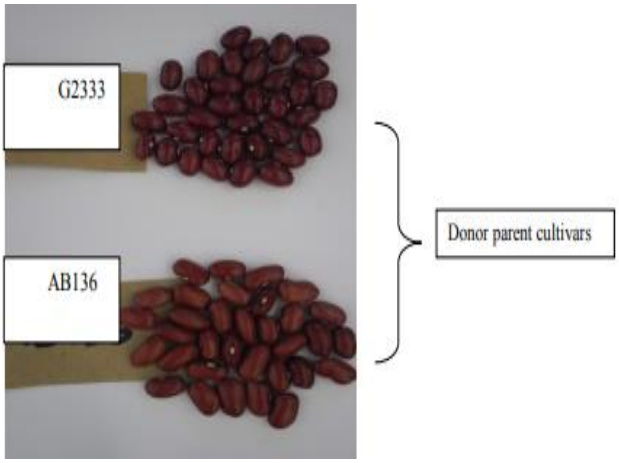

Figure 1. Bean cultivars used as donor parents G2333 and AB36 in hybridization

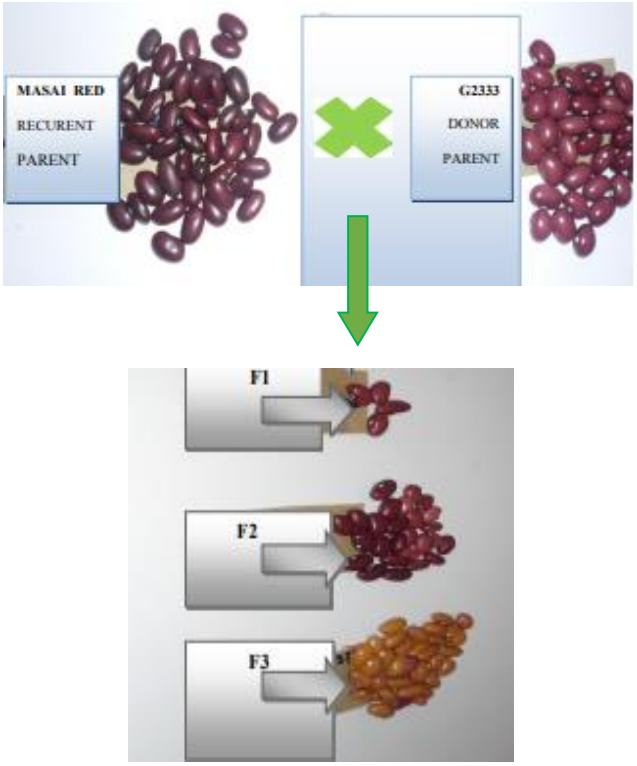

Figure 2. Crosses between Masai Red and G2333, F_1, F_2, and F_3 populations developed from them

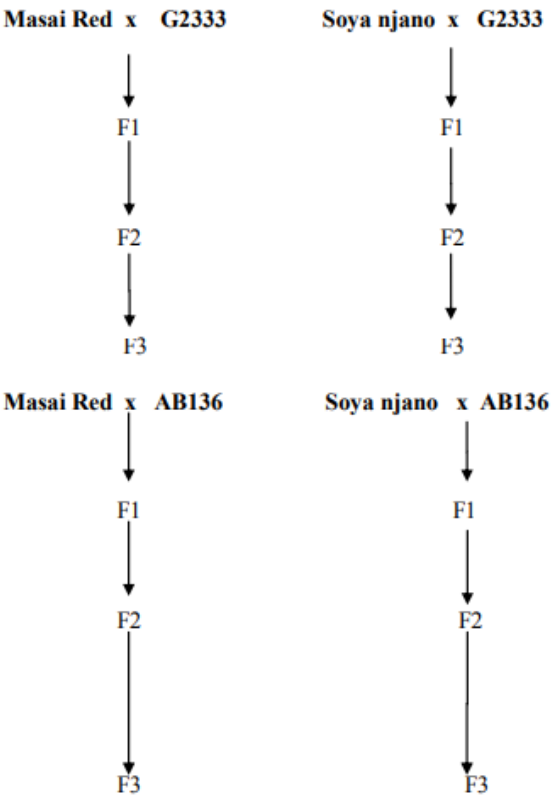

Figure 3. Crossing design for transferring anthracnose resistance genes to *Colletotrichum lindemuthianum* to susceptible bean parent cultivars

Screening for disease resistance

Collection of Colletotrichum lindemuthianum *isolates*

Stem, leaf, and pod samples bearing fresh symptoms of anthracnose infection were collected from farmers' fields in areas with natural infections across different agro-ecologies in Arumeru, Karatu, and Babati rural districts in northern Tanzania and Mvomero District in the Morogoro region.

The collected samples were placed between folds of paper bags and stored under normal room temperature at 24°C. Paper bags with diseased samples were kept open, unpiled, and separated to control decay. Each sample was labeled carefully to depict the names of varieties from which samples were picked, sites, villages, districts, and the region's name. GPS was used to mark each site's altitude, latitude, and longitude coordinates.

Medium and inoculum preparation

The fungal isolates were grown in the Petri plates at 24°C on a V8 medium composed of V8 juice (200 mL), $CaCO_3$ (3.0 g), Bato Agar (15 g), streptomycin (10 mg), and distilled autoclaved H_2O (1000 mL). Single spore isolates were established employing a standard procedure with modification according to Munda et al. (2009). First, the pathogens were isolated from well-developed and fresh lesions of bean pods and stems. Then, small pieces of infected tissue were cut between diseased and healthy tissue, at least five pieces, for a single culture. Next, the pieces were surface sterilized in alcohol 7% for less than 1 min. Next, the pieces were dipped with sterile forceps into 2% NaOCl (Sodium hypochlorite) for 3 minutes. Next, the

pieces were transferred aseptically into sterile distilled water and washed 3 times before being blotted dry on a sterile paper towel. Lastly, the pieces were transferred into prepared media within a petri dish and arranged, leaving isolation space between each other. The Petri dishes containing V8 medium containing the pieces were sealed and incubated at 24°C (Pastor-Corrales et al. 1994).

Inoculation

Seed inoculation procedure

Six single spore isolates of the coded isolates 2CRHOT, 3ASLAHMO, 6ASAR14, 1C-BASH-L, and 2D-1 collected from Long Village in Bashnet, were used. Each isolate was stored in V8 media at 4°C. Eight Petri plates with V8 medium without antibiotics were planted with a single spore of each isolate for multiplication. Inoculum-containing spore suspensions of 1.2 x 10^6 spores/mL for inoculating test plants were prepared from ten-day-old spore cultures according to the procedure described by Mahuku et al. (2002).

Seed inoculation

Four seeds of each differential cultivar were germinated by being placed in humid plates with more than 92% relative humidity at 25°C for 5 to 7 days. Germinated seeds were dip inoculated in a calibrated spore suspension of 1.2 x 10^6 spores/ml for 5 min in 200 mL (Bigirimana and Höfte 2001). The inoculated seeds were placed on humid plates and incubated in a dark room. After 2 days, the seedlings were at the emergence stage. They were transferred to trays, covered by a thin layer of sterilized soil, and incubated at 19-22°C with relative humidity above 92%.

Seedling inoculation

Planting parent cultivars and new populations

The experiment consisted of parent cultivars, derived F1, F2, and F3 populations, and bean differential cultivars planted in non-replicated and unrandomized plots. The plot size was 2 rows, each 2.75 m in length. The inter-row spacing was 50 cm, and intra hill spacing was 20 cm. Plots were planted with a donor, recipient cultivars, F1, F2, and F3 populations. Each row was a plot with 12 plants for a parent, spreader cultivars, and F1 populations. The F2 and F3 populations were planted in two rows per plot. The tested plant populations were irrigated every evening on rain-free days to provide high relative humidity conditions for 7 days after inoculation.

Seedling inoculation

The inoculum was prepared from spore suspension derived from 2D-1 Long Ayt. isolate cultures, raised on V8 medium, and kept in the darkness for ten days at 24°C. The ten days old sporulated cultures were flooded with 0 mL of sterile distilled water and scraped from the plates using a toothbrush. The spore suspensions were filtered through a four-layered gauze cloth. A hemocytometer was used to calibrate spore concentration to 1.2 x 106 spores/mL (Mahuku et al. 2002). Seedling inoculation was made by spraying with the aqueous conidial suspension on 14-day-old seedlings in the field. During the inoculation and incubation period, temperatures and relative humidity ranged between 20 to 21°C and 96 to 100%, respectively.

Disease score

The reaction of plants to *C. lindemuthianum* was evaluated 7 to 10 days post-inoculation. The disease score was done using a scale of 1-9 where (Van Schoonhoven and Pastor-Corrales 1987) seedlings with no visible symptoms (severity value 1) or showing limited necrotic lesions (severity values 2 to 3) were considered resistant. Seedlings with large sporulating lesions (severity values 4 to 8) or dead (severity value 9) were considered susceptible. A set of 12 common bean anthracnose standard differential cultivars was used to confirm the pathogenic identity of the *C. lindemuthianum* isolates.

Data analysis

GenStat statistical package was used to compute means, variance, standard deviation**,** standard error, and regression coefficient of variation between variables. The disease means scores of parents, F1, F2, and F3 populations were generated and used in estimating the narrow sense heritability of parents using regression analysis. Chi-square was used to compare the segregation of F2, and F3, populations to Mendelian ratios. Genetic gain for disease resistance was computed using a procedure proposed by Zobel and Talbert (1991), cited by Abengmeneng et al. (2015).

The selection differential (S) was estimated as below.

(1) $S = Xs - X\mu$

(2) Mid- parent = $\frac{P1 + P2}{2}$

(3) Deviation = grand mean score – mid-parent disease score

(4) % age deviation = $\frac{\text{Deviation x 100 \% Percentage deviation}}{\text{Mean}}$

(5) % age gained = % deviation x h^2

(6) Genetic gain G = % age gain x h^2 (percentage gain x heritability)

Where,

h^2: Narrow sense heritability.

S: Selection differential (difference between the mean of selected individual and the population mean

Xμ: Mean of population,

Xs: Mean phenotypic value after selection (sample mean),

Genetic gain (ΔG) was estimated as:

G = Percentage gain;

Where,

h^2: Narrow sense heritability;

Deviation x 100% percentage gain = percentage deviation x heritability

Naming of *Colletotrichum lindemuthianum* races

Four seeds of each differential cultivar were germinated and dipped into inoculate suspension of six different isolates. Six sets of differential cultivars were used. The germinated inoculated seeds were placed in a plastic tray and then covered with a thin layer of sterilized soil. Trays were placed in a growth chamber and watered daily for 14 days. Disease scoring was performed 10 days after sowing

(Bigirimana and Höfte 2001). The disease score data was used to determine susceptible and resistant cultivars. The races derived from different *C. lindemuthianum* isolates were distinguished by using a set of differential cultivars. This set consisted of 12 cultivars, each with a designated binary number as follows: Michelite, 1; Michigan Dark Red Kidney, 2; Perry Marrow, 4; Cornell 49-242, 8; Widusa, 16; Kaboon, 32; Mexico 222, 64; PI 207262, 128; To, 256; Tu, 512; AB136, 1024; and G2333, 2048. The sum of the numbers assigned to each infected cultivar of the differential set determined race designation (Pastor-Corales et al. 1994).

RESULTS AND DISCUSSION

Pathogen in Isolates

Thirty-two isolates of *C. lindemuthianum* were isolated from different common bean samples of infected bean cultivars from 26 sites within an elevation ranging between 1390-2197 meters above sea level (Table 2).

In Babati Rural, Karatu, and Arumeru districts, the samples were drawn from bush types (Lyamungo 90, JESCA), semi-climber (Canadian wonder), and climbing bean types (Masai Red). The common bean cultivars collected, such as Lyamungo 90, Soya Njano, JESCA, Canadian wonder, and farmer, were all infected with *C. lindemuthianum.* The common bean-diseased plant samples collected were as indicated in Figure 4.

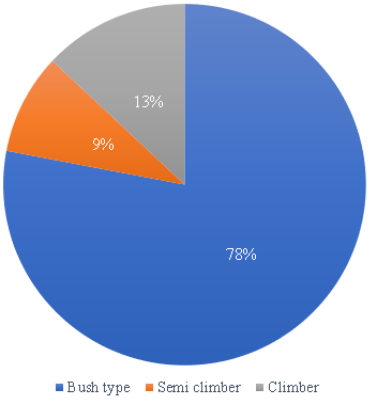

Figure 4. Common bean cultivars diseased samples percentage infected by *Colletotrichum lindemuthianum*, their growth habit and distribution as collected from Babati, Karatu, Mbulu, Arumeru, and Mvomero in Morogoro

Table 2. Collected isolates of common bean anthracnose disease from Babati, Karatu, Mbulu, Arumeru, and Mvomero Districts, Tanzania

Name of site	Sample code name	Variety	Growth habit	Gene pool	Altitude in m.absl
Bashnet-Manyara	1ABASH	JESCA (Punda)	Bush type	Andean	2189
Long /Endaw	1BASH	Farmer's variety	Bush type	Andean	2193
Long	1CBASH	Experimental variety	Climber	Mesoamerican	2195
Long	2A	Masai Red	Climber	Mesoamerican	2197
Endaw	2B	Masai Red	Climber	Mesoamerican	2186
Endaw	2C	Lyamungo 90	Bush type	Andean	2187
Endaw	2D	Farmer's var.	Bush	Andean	2183
Endaw	3A	Farmer's var.	Bush type	Andean	2146
Bony	3B	Farmer's var.	Bush type	Andean	2156
Bony	3D	Farmer's var.	Bush type	Andean	2166
Bony	3E	Farmer's var. (black grains)	Semi climb**er**	Mesoamerica	2170
Masquaroda-Mbulu	1AMASQ	Lyamungo 90	Bush	Andean	1895
Mbulu	1BMBLU	Lyamungo 90	Bush	Andean	1942
Simba	1A1KMS	Farmer's variety	Semiclimber	Mesoamerica	1498
Kambiya Simba	1BKMS	Farmer's variety	Climber	Mesoamerica	1523
Kainam	1A-2KAI	Farmer's variety	Bush	Andean	1544
Kambi ya samba	1CKMS	Soya Njano	Bush type	Andean	1533
Kambi ya Simba	1DKMBS14	Farmer'svariety	Semiclimber	Mesoamerica	1534
Kambi ya Simba	1EKMBS	Farmer's variety	Bush	Mesoamerican	1537
Rhotia	1ARhotia	Soya Njano	Bush	Andean	1613
Rhotia	2BRHOT	Soya Njano	Bush	Andean	1624
Rhotia	2CRHOT-KR	Soya Njano	Bush	Andean	1621
Slahhamo	3ASLAMO	Soya Njano	Bush	Andean	1532
Slahhamo	3BSLAMO	"Bwana shamba" Canadian wonder	Bush	Andean	1548
Upper Kitete	4AUPKIT	Soya Njano	Bush	Andean	1534
Upper Kitete	4BUPKIT	Soya Njano	Bush	Andean	1717
Upper Kitete	4CUPKIT	Soya Njano	Bush	Andean	1720
Upper Kitete	4DUPKIT	Soya Njano	Bush	Andean	1720
KITETE	5AKIT	Soya Njano	Bush	Andean	1749
SARI	6ASAR	Lyamungo 90	Bush	Andean	1411
SARI	6BSAR	Lyamungo 90	Bush	Andean	1399
Mgeta	ANyd-	Lyamungo 90	Bush	Andean	1645
Mgeta	ANyd-	Farmer's variety	Bush	Andean	1645

Key: m. a.s.l: meters above sea level

Pathogenicity test

Pathogenicity test and race naming of Colletotrichum lindemuthianum *under growth chamber condition*

A total of nine pure single spores were obtained from 32 diseased samples collected. Six out of nine isolated single spores of *C. lindemuthianum* were named using twelve standard differential cultivars.

Pathogenicity and race classification of Colletotrichum lindemuthianum *isolates*

Six *C. lindemuthianum* isolates showed pathogenicity undergrowth chamber conditions on 12 differential bean cultivars, as indicated in Table 3. Six of the 12 bean differential cultivars showed susceptibility to at least one of the collected *C. lindemuthianum* isolates.

The reactions of a set of common bean differential cultivars to 6 isolates of *C. lindemuthianum* allowed the identification of the races 21, 37, 55, 161, and 533 from Karatu, SARI (Arumeru) and Babati districts. The bean cultivar Michelite was susceptible to all *C. lindemuthianum* isolates collected from Arumeru, Karatu, Mbulu, and Babati rural.

In general, isolates collected around the study infected both Andean and Mesoamerica cultivars MDRK, Perry marrow, Widusa, and Kaboon; on the Mesoamerican cultivar Michelite, Mexico222, PI 207262, TO, and TU. None of the isolates infected cultivars Cornel 49242, Mexique 222, AB136, and G2333. According to Mahuku et al. (2002), susceptibility to *C. lindemuthianum* of G2333 and AB136 was not frequently reported. In addition, they were resistant under screen house and field conditions when the pathogenicity test was conducted. The isolate found in Northern Tanzania differed from those reported by (Ansari et al. 2004) and those reported by Drone and Bailey (1999) regarding bean differential cultivars it infected.

Pathogenicity testing of Colletotrichum lindemuthianum *under field conditions at Bashnet Manyara*

The results in Table 4 showed the compatibility of 2D-1 Long Ayt isolate of bean *C. lindemuthianum* to a set of 12 bean differential cultivars which allowed naming race 161. Isolate 2D-1 Long Ayt (161) was compatible with bean cultivars Michelite, Kaboon, and PI 207262; the other nine differential cultivars were not infected. However, this race exhibited compatibility with both indeterminate and determinate bean cultivars. The differential cultivars not infected by race 161 were MDRK, Perry Marrow, Widusa, G2333, AB136, Cornell 49242, Mexico 222, TO, and TU. However, an isolate designated 161 was not among the listed isolates collected from Africa and other parts of the world, as reported by (Ansari et al. 2004). It was also not in the list of the previous isolates collected in the southern highlands of Tanzania (Dron and Bailey 1999). These results implied that isolates collected at Bashnet consisted of *C. lindemuthianum* named race 161, which was not previously reported.

Introgression of anthracnose resistance in preferred varieties

Four genotypes were grown and crossed under screen house conditions during the 2014-2015 growing seasons at SUA. Two genotypes were resistant donors, and two were recipient cultivars. The general observation in Table 5 showed the cross performances between Soya Njano x G2333, which gave 37 seeds, and Masai Red x G2333 had the lowest number of seeds (15). The inadaptability of G2333 could have contributed to the environment during crossing work. The number of seeds obtained for F1 ranged from 15-37, and for F2, were from 45 to 138. Masai Red x AB136 gave the highest number of seeds (138). Both recurrent and donor parents were climbers; compared to Soya Njano x G2333 had the lowest (45). The F3 ranged from 34 to 61 seeds, as indicated (Table 5). These results comply with the reported results on common bean plants by (Porch and Jahn 2001). Therefore, the Soya Njano, Masai x AB136, crosses in F2 and F3 produced many seeds due to AB136 good environmental adaptability and successful crosses.

Table 3. Pathogenicity test of *Colletotrichum lindemuthianum* under growth chamber conditions

Gene pool	Isolate reaction on common bean differentials												Race
	A	B	C	D	E	F	G	H	I	J	K	L	
	M	A	A	M	M	A	M	M	M	M	M	M	
Isolate Codes													Designation
2CRHOT	S	R	S	R	S	R	R	R	R	R	R	R	21
3ASLAHMO	S	R	S	R	S	R	R	R	R	S	R	R	533
6ASAR14	S	R	S	R	R	S	R	R	R	R	R	R	37
1C-BASH-L	S	R	R	R	R	S	R	R	R	R	R	R	161
2D-1 Long Ayt	S	R	R	R	R	S	R	S	R	R	R	R	161
1b-Bashnet-Bony	S	S	S	R	S	S	R	R	R	R	R	R	55

Note: Differential cultivars of common bean and their binary values (in parentheses): A, Michelite (1); B, Michigan dark red, kidney (2); C, Perry marrow (4); D, Cornell 49242, (8); E, Widusa (16); F, Kaboon,(32); G, Mexique 222 (64); H, PI 207262 (128); I, TO (256); J, TU (512); K, AB136 (1024); and L, G2333 (2048). M=Mesoamerica, A=Andean gene pools, R=resistant, S=Susceptible. 2CRHOT: RHOT- Karatu Isolate; 3ASLAHMO: Slahmo-Karatu Isolate; 6ASAR14 SARI-Arusha Isolate; 1C-BASH-L;2D-1 Long Ayt. and 1b Bashnet-Bony were all from Bashnet

Table 4. Pathogenicity of *Colletotrichum lindemuthianum* under field conditions at Bashnet Manyara, Tanzania

Differential cultivars	Genes conferring resistance	Place of cultivar	Binary number	Gene pool	disease score *	Isolate reaction*
Michelite (A)	-	0	1	MA	5	S
MDRK (B)	Co – 1	1	2	A	1	R
Perry Marrow (C)	Co – 1^3	2	4	A	1	R
Cornel 49242 (D)	Co – 2	3	8	MA	1	R
Widusa (E)	Co – 9	4	16	MA	3	R
Kaboon (F)	Co – 1^2	5	32	A	5.5	S
Mexico 222 (G)	Co – 3	6	64	MA	3	R
PI 207262 (H)	Co – 4^3, Co – 9,	7	128	MA	4	S
TO (I)	Co – 4	8	256	MA	3	R
TU (J)	Co – 5	9	512	MA	1	R
AB 136 (K)	Co – 6, Co-8	10	1024	MA	1	R
G 2333 (L)	Co – 4^2, Co-5, Co-7	11	2048	MA	1	R
Race designation			161			

Note: Binary number of a specific race was computed by summating susceptible cultivars' binary numbers. MA; Middle American gene pool; A: Andean gene pool of *Phaseolus vulgaris*. Binary number; 2^n, where n is equivalent to the place of the cultivar within the series (0-11). Growth habit: I = Determinate; II = Indeterminate bush; III = Indeterminate bush with weak main stems and prostrate branches; IV = Indeterminate climbing habit.* Disease score and *bean differential cultivars reaction on isolates

Table 5. Number of seeds of F_1, F_2, F_3obtained from crossing the donor for anthracnose disease resistance and the adapted parents of common bean local cultivars

Parent material	No. of crosses	F_1 seeds	F_2 seeds	F_3 seeds
Soya Njano x G2333	58	37	45	40
Soya Njano x AB136	42	25	89	61
Masai Red x G2333	74	15	57	42
Masai Red x AB 136	42	22	138	34

Note: Inheritance pattern of anthracnose resistance in early populations of crosses of Soya Njano, Masai Red, and G2333, AB136 segregation ratios

Crosses made between Soya Njano, Masai Red and G2333

The parental cultivars Soya Njano, Masai Red, G2333, and AB136, developed populations (F1, F2, and F3) were tested for bean anthracnose disease resistance under field conditions. Results showed that Soya Njano plants were all susceptible, as indicated in plate 1d, while all plants in the donor parent cultivar G2333 population were resistant. Plants in the F1 generation were all resistant to bean anthracnose disease. The F2 generation segregation ratio of Soya Njano x G2333 was 9R: 7S Resistant: Susceptible (χ^2 = 0.01, P= 0.872) and F3 generation plants of had segregation ratio of 9R : 7S (χ^2 = 0.001, P = 0.979) (Table 6). The segregation data from F2 and F3 populations indicated that G2333 carried two dominant resistance genes. The results of bean cultivar G2333 conferring two were reported by Young and Kelly (1996). Similar results were reported by (Campa et al. 2011). However, the segregation ratio of 9: 7 implied that two pairs of genes with duplicate recessive epistasis were expressed by the heterozygous dominant individuals phenotypically distinguishable from other possible genotypes obtained from the studied populations (Burns 1980). González et al. (2015) reported that either additive main, epistatic, or both effects function concurrently; those are responsible for controlling anthracnose disease resistance in beans.

The F1 plant population was all resistant to *C. lindemuthianum*, indicating that a dominant gene was responsible for resistance. The F2 plant population segregation was fitted to 10R: 6S ratio (5R) Resistant and (3S) susceptible (χ^2 = 0.714; P > 0.05) and F3 population segregation ratio was 10R : 6S ratio (χ^2 = 0.002; P > 0.05) in (Table 6). These results revealed that two dominant genes conferred resistance to the developed populations of Masai Red and G2333. Similar inheritance was reported by Pastor-Corrales et al. (1994) on the presence of two dominant independent genes in G2333 controlling resistance to *C. lindemuthianum*. However, the segregation of 9: 7, 10:6 (5:3) ratios expressed two epistatic genes relationships that could correspond to the ratios found in this study, as reported by (Diering and Tomas 2001). Other findings showed that G2333 was a three-gene pyramided cultivar, with genes at different loci conferring resistance independently (Vallejo and Kelly 2009). According to Mahuku et al. (2002), genetic resistance to some pathotypes of *C. lindemuthianum* is conferred by various single, duplicate, or complementary dominant genes. However, bean cultivar G2333 was reported to be capable of controlling more than 380 races in different areas (Pastor-Corrales et al. 1994).

Crosses made between Soya Njano, Masai Red and AB136

The donor parent AB136, Soya Njano, Masai Red recipient bean cultivars, and F1, F2, and F3 populations derived from crosses made between Soya Njano, Masai

Red, and AB136 were inoculated with race 161. Soya Njano and Masai Red plants were all susceptible to *C. lindemuthianum*, as indicated in Figures 5A and 5B. The spreader row of Lyamungo 90 was susceptible, as shown in Figures 5C and 5D. The donor parent AB136 plants were all resistant. The F1 plants were all resistant to common bean anthracnose disease; this indicated that the dominant gene controlled resistance in AB136. The F2 plants segregation fitted to 3: 1 ratio ($X^2 = 3.56$, $P = 0.04$) (Table 7). The F3 population showed a significant difference from the F2 population at 0.05 probability. It exhibited that AB136 conferred a single dominant gene, similar to other studies (Gonçalves-Vidigal et al. 2001). The F3 population segregation ratio for resistance of *C. lindemuthianum* was also 3: 1 ($\chi^2 = 0.44$; $P < 0.50$). In the crosses between Masai Red and AB136, the F2 population segregation was 3R:1S ratio ($\chi^2 = 4.19$; $P < 0.05$) and F3 population segregated at a ratio of 3R: 1S ($\chi^2 = 0.55$; $P < 0.50$) (Table 8). The results conformed with other studies (Alzate-Marin et al. 1997) on the resistance of *C. lindemuthianum* races 89 and 64. All F1 populations developed from the crosses exhibited resistance, implying that resistance was due to dominant genes transferred into the derived generation. This study reported the presence of two independent resistant genes of AB136 to pathogen race 73 of *C. lindemuthianum*, where the (Co-6) gene was the dominant gene and a recessive gene assigned with the genetic symbol co-8 (Alzate- Marin et al. 1997).

Heritability estimation

The estimated narrow sense heritability ranged between 0.42 and 0.46 in populations derived from crosses between Soya Njano, Masai Red with G2333, and Masai Red with AB136 was 0.22 (Table 8 and Figure 3). These results implied the presence of moderate heritability in developed populations. Similar results were reported by (Poletine et al. 2006), that medium magnitude narrow sense heritability value, even at that moderate magnitude, indicated the possibility of success in obtaining resistant genotypes in derived populations. For example, the population derived from Soya Njano with AB136 had a heritability of 0.53 (53%) that showed moderate heritability, $R^2 = 0.29$ coefficient of determination (Figure 6).

Populations of Masai Red x AB136, Soya Njano x G2333, and Masai Red x G2333 were 5%-15%. The moderate narrow sense heritability in developed F2 from Soya Njano x AB136 implied that the mean performance of the developed populations has regressed at 53% towards the mean of the previous resistant generation (Stanfield 1991). When heritability for a trait is high, selection using phenotypic traits is effective (Campa et al. 2014).

Table 6. Segregation ratios for resistance and susceptible progenies in parental cultivars and their developed populations to *Colletotrichum lindemuthianum* under field conditions

Pedigree	Number of plants			Segregation		
	Generation	Resistant	Susceptible	Ratio (R:S)	χ^2	Probability
Soya Njano	P1	0	12	…….	…….	…….
G2333	P3	12	0	…….	…….	…….
Soya Njano x G2333	F1	12	0	…….	…….	…….
Soya Njano x G2333	F2	12	10	9:7	0.016	0.872
Soya Njano x G2333	F3	13	10	9:7	0.001	0.979
Masai Red	P2	0	12	…….	…….	…….
G2333	P3	12	0	…….	…….	…….
Masai Red x G2333	F1	12	0	…….	…….	…….
Masai Red x G2333	F2	15	6	10 : 6	0.714	0.296
Masai Red x G2333	F3	13	8	10 : 6	0.002	0.955

Note: χ^2: Chi test, P1= Soya Njano, P2: Masai Red, P3: G2333, P3: G2333; F1- F3: Soya Njano x G2333 and Masai Red x ABG2333

Table 7. Segregation ratios for resistance and susceptibility in parental cultivars and their derived populations to *Colletotrichum lindemuthianum* under field conditions

Pedigree	Number of plants			Segregation		
	Generation	Resistant	Susceptible	Ratio	χ^2	Probability
Soya Njano	P1	0	12	…….	……	…….
AB136	P4	12	0	…….	…….	…….
Soya Njano x AB136	F1	12	0	…….	…….	…….
Soya Njano x AB136	F2	14	10	3:1	3.56	0.04
Soya Njano x AB136	F3	13	6	3:1	0.44	0.50
Masai Red	P2	0	11	…….	…….	…….
AB136	P4	12	0	…..	…..	…….
Masai Red x AB136	F1	12	0	……..	……	…..
Masai Red x AB136	F2	13	10	3:1	4.19	0.05
Masai Red x AB136	F3	15	7	3:1	0.55	0.50

Note: χ^2: Chi test, P1: Soya Njano, P2: Masai Red, P3: G2333, P4: AB136, F1-F3: Soya Njano x AB136 and Masai Red x AB136

Table 8. Heritability in narrow sense estimation for *Colletotrichum lindemuthianum* in derived F2 and F3 populations

Generations (F2& F3)	Mean disease Score							
	F2	F3	SDEVF2	SDEF3	P-value	b (h^2)	A	R^2
P1 X P3	3.55	4.05	1.37	1.73	0.13	0.42	2.57	0.12
P2 X P3	2.67	3.62	1.65	1.96	0.07	0.46	2.38	0.15
P1 X P4	3.53	2.84	1.6	1.63	0.02	0.53	0.92	0.29
P2 x P4	4.17	3.22	1.93	1.61	0.73	0.22	2.18	0.05

Note: P1= Soya Njano, P2: Masai Red, P3: G2333, P4: AB136, R^2 Regression determination, F2, F3: Filial generation 2 and 3, SDEV: Standard deviation of F2 and F3, b: Coefficient of X slope; stands for (h^2) heritability in the narrow sense. P: P value; A: y-intercept

Figure 5. Pictures (A-D) show diseased bean plants with common bean anthracnose symptoms on different parts of the plants. A. Masai Red- pod symptoms; B. Soya Njano – pod symptom; C. Lyamungo 90 (diseases spreader variety); D. Lyamungo at vegetative stage

Comparisons of parents, F1, F2, and F3 for common bean anthracnose disease resistance

The mean disease scores of crosses of F1, F2, and F3 generated populations from Soya Njano, Masai Red with G2333 and mid-parent are presented in Figures 7A and 7B. The results show a reduction of disease reaction in the F_1 populations based on recipients and mid-parent resistance performance. The F2 populations of Masai Red x G2333 mean score were 2.7 more resistant than the mid-parent mean score of 3.8. The susceptible bean cultivars mean disease scores were between 6.0-6.5, which implied high susceptibility to common bean anthracnose. Abengmeneng et al. (2015) reported that genotypes with mean disease resistance above the population's mean performance are recommended for selection and use as seed. According to the results in Figures 7C and 7D, Soya Njano x, AB136 (F3) and Masai Red x AB136 (F3) exhibited improvement of resistance in developed populations to *C. lindemuthianum* and Soya Njano xG2333, the Masai Red x G2333 (F2 and F3) showed equal performance as mid-parent. Abengmeneng et al. (2015) reported that only genotypes whose phenotypes were approximate to the population mean were good for selection as resistant plants and fit in the Northern zone environment.

Estimation of genetic gain

The results in Table 9 showed that the mean anthracnose disease score was 6.0 for Masai Red and Soya Njano was between 6.0 to 6.5. The donor bean cultivar G2333's mean disease score was 1.5, and AB136 was 1.5. The maximum genetic gain through selection depends on the phenotypic variations present in the base population and maintained in the following cycles through selection (Janick 2010). However, the genetic gain in the F2 and F3

populations of Soya Njano and G2333 genetic gain is between 0.2 and 2.0. The F2 populations derived from Soya Njano, Masai Red, and AB136 genetic gain were absolute and ranged between (-0.1 to 0.8). Negative succession per season represented the stabilization of the genetic gain in disease resistance, as displayed by the figures close to zero (Chiorato et al. 2010). These results showed different levels of resistance to common bean anthracnose disease, hence, high potential genotypes resistant to *C. lindemuthianum* for selection.

Genetic gain results in Table 9 showed increased genetic gain through moderate narrow sense heritability. The results concur with the findings reported by Souza et al. (2014) that genetic gain depends on the availability of moderate to high heritability and a useful amount of genetic variation. Population breeding methods such as line development by standard backcrossing, pedigree, or bulk and recurrent selection were suggested as most perfectly suited to long-term genetic gains. However, these methods require sufficient time (Cowling 1996).

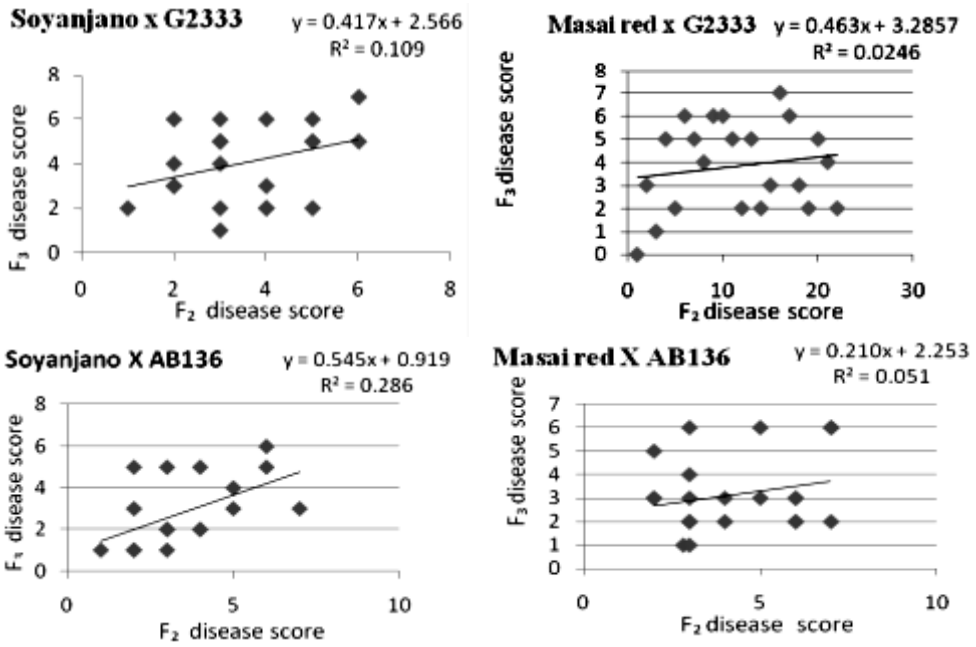

Figure 6. Heritability determination of the *resistance of C. lindemuthianum* using F3 populations to F2. P1: Soya Njano, P2: Masai Red, P3: G2333, P4 AB136, Regression graphs on the inheritance of F2 and F3 populations

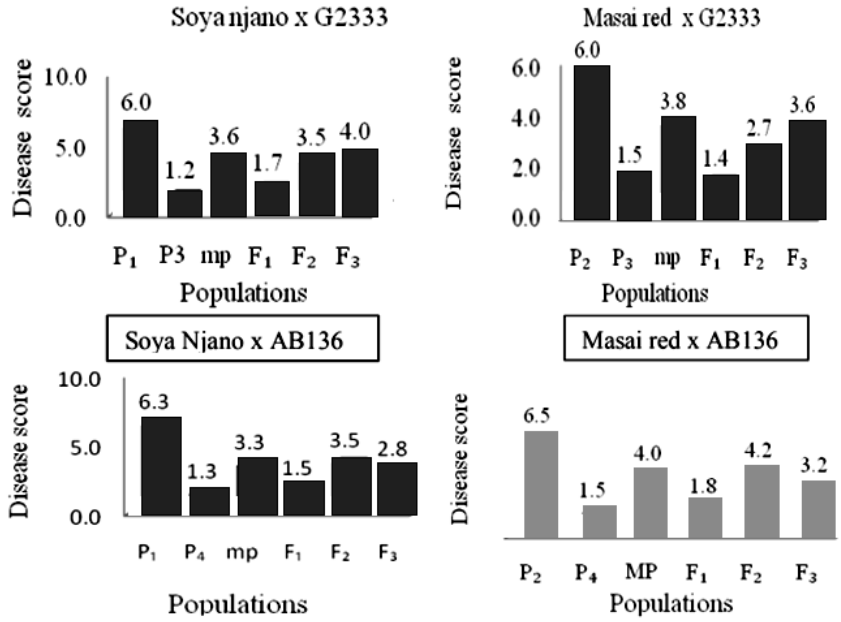

Figure 7. Comparison of mid-parents F1, F2, and F2 generations for resistance of Common-bean Anthracnose disease. Key: P1: Soya Njano, P2: Masai Red, P3: G2333, P4: AB136

Table 9. Genetic gain for *Colletotrichum lindemuthianum* resistance estimation in F2 and F3 derived populations

Parent material		Mpt	Mean disease score (1-9)	deviation/F-mid parent	Grand mean disease scores	Percentage deviation	Heritability in h^2	% age gained	Genetic gain (scores)
Soya Njano			6.0						
Masai Red			6.0						
G2333			1.5		1.5				
Soya Njano x G2333	F2	3.6	3.5	-0.1	3.5	-2.9	0.42	1.2	0.5
Masai Red x G2333	F2	3.8	2.7	-0.3	3.5	-8.6	0.46	3.9	1.8
Soya Njano x G2333	F3	3.6	4.0	-0.1	3.5	-2.9	0.26	0.7	0.2
Masai Red x G2333	F3	3.8	3.6	-0.3	3.5	-8.6	0.48	4.1	2.0
Grand mean			3.5						
Soya Njano			6.3						
Masai Red			6.5						
AB136			1.5		1.5				
Soya Njano xAB136	F2	3.3	3.5	0.1	3.4	2.9	0.53	1.6	0.8
Masai Red x AB136	F2	4	4.2	-0.6	3.4	-17.6	0.22	-3.9	-0.9
Soya Njano xAB136	F3	3.3	2.8	0.1	3.4	2.9	0.52	1.5	0.8
Masai Red x AB136	F3	4	3.2	-0.6	3.4	-17.6	0.24	-4.2	-1.0
Grand mean			3.4						

Note: Mpt: mid-parent, F: Filial generation

In conclusion, Race 161 was determined from diseased bean samples collected at Bashnet, with pathogenicity testing conducted under field conditions. Donor parents of bean cultivars G2333 and AB136 exhibited resistance to *C. lindemuthianum* pathogens when tested under field conditions, hence are potential donor parents. Other potential donor bean cultivars were MDRK, Perry Marrow, Widusa, Cornell 49 242, Mexico 222, TO, and TU, which exhibited resistance under field testing at Bashnet. The genes for resistance against common bean anthracnose disease were successfully introgressed into adapted bean cultivars Soya Njano and Masai Red using conventional breeding. The results showed two genes introgressed from resistant parents G2333 and one dominant gene from AB136. Heritability in the narrow sense of common bean anthracnose disease resistance was moderate in the developed populations in this study. However, the results showed that selecting resistant genotypes from the derived populations was possible.

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CELL BIOLOGY & DEVELOPMENT
Volume 6, Number 1, June 2022 E-ISSN: 2580-4499
Pages: 32-40 DOI: 10.13057/cellbioldev/v060105

Effect of the media type and auxin concentration on the growth of cuttings seedlings of pepper (*Piper nigrum*)

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Manuscript received: 4 March 2022. Revision accepted: 30 May 2022.

Abstract. *Amanah S, Budiastuti MTHS, Sulistyo A. 2022. Effect of the media type and auxin concentration on the growth of cuttings seedlings of pepper (*Piper nigrum*). Cell Biol Dev 6: 32-40.* Pepper (*Piper nigrum* L.) is an export commodity and has become one of the Indonesian sources of income. It is necessary to develop the best cultivation for increasing pepper production. Vegetative propagation has been chosen in this research, using manure as part of the media and auxin as a growth hormone. The research aims to find the best media and auxin concentration for the ideal growth of pepper grafting. The research conducted in Manyaran Wonogiri, Indonesia, with RCB design consists of two factors arranged in the factorial method. The first factor is the kind of media (soil, soil + manure, soil + manure + husk), and the second is auxin's concentration (0 g/L, 12.5 g/L, 25 g/L, 37.5 g/L; 50 g/L). Observation variables are bud growth time, number of buds, length of bud, number of leaves, leaf width, number of roots, length of root, and life percentage of cuttings. Data were analyzed by F test at 1% and 5% and continued with DMRT at 5% if the treatment had a significant effect. It is also continued with regression if there was an interaction between treatments. The longest bud was found on soil + manure + husk (12.28 cm) and followed by soil + manure (10.57 cm) and soil (6.15 cm) in the low level, respectively. The study revealed that the bud length and the number of roots were influenced by auxin concentration. The longest bud was found using 12.5 g/L of auxin (15.14 cm), followed by 50 g/L, 25 g/L, 0 g/L, and 37.5 g/L in the low level, respectively (12.05 cm, 7.72 cm, 7.67 cm, 5.75 cm). The highest number of roots was found on 0 g/L and 12.5 g/L of auxin (7.78), followed by 50 g/L, 25 g/L, and 37.5 g/L in the low level, respectively (4, 3.43, 2.33). The number of roots and percentage of life cuttings showed an interaction between the two factors. The number of roots on the soil shows a parabolic response with auxin. In the beginning, the number of roots decreased and then increased with increasing auxin concentration. The number of roots on soil + manure and soil + manure + husk showed the same pattern with auxin concentration. Almost 100% of life cuttings have been found on all media and auxin concentrations. Soil + manure with 25 g/L auxins and soil + manure + husk with 50 g/L auxins caused 33.33% and 66.67% life cuttings, respectively.

Keywords: Auxin, growth, media type, pepper, *Piper nigrum*

INTRODUCTION

Pepper (*Piper nigrum* L.) is an important crop in Indonesia because the yield of this commodity (pepper drupes) is a source of foreign exchange (Sopialena et al. 2018). Pepper is an export commodity that in 2000 had reached 68,727 tons and was valued at 221 million US$. Pepper exports rank sixth after rubber, oil palm, coffee, cocoa, and coconut. However, pepper productivity in Indonesia is still low compared to India and Malaysia (Azahari et al. 2021).

Given the very good prospects for this plant, pepper production needs to be developed with good cultivation efforts. It allows pepper farmers to increase their income and ultimately support the country's foreign exchange earnings. However, the reality is that farmers cultivate pepper plants very simply, as was done by pepper farmers in Manyaran Wonogiri. Pepper farmers in Manyaran only use soil media without adding manure (if manure is used, the dosage is not clear) or use PGR (growth regulators) in the nursery process, which results in poor plant growth because even though there are enough leaves, the roots have not grown perfectly (weak roots), so that when planted in the field, the opportunity to grow is very low.

Nurseries are indispensable as a way to provide planting material in large quantities. It is known that pepper plants can be planted directly vegetatively with the planting material in the form of stems with 7-9 segments. It is an obstacle to increasing crop production because planting material is limited, which is different if pepper plants are propagated vegetatively by seedlings in the form of stems with only 2-3 segments. Therefore, it becomes an opportunity for the availability of planting material quickly to support increased production.

The availability of healthy seedlings in large quantities is the key to the success of pepper production. Therefore, it is necessary to make nursery efforts that support the formation of healthy roots. The trick is to use a good planting medium for roots, which can provide nutrients and support root development (porous soil structure). Planting media with such conditions can be made by adding organic fertilizer (cow manure, husk) and a concrete step to utilize the available organic waste. Using mixed media of manure and husk can increase plant growth and yield (Zulia and Batubara 2020) because manure can provide organic matter and nutrients, improve soil's physical properties, and prevent water loss in the soil (Adugna 2016). In addition, husk plays a role in improving soil structure (better

drainage system), binding water, not easy to rot, K source, and not easy to compact.

In addition, the provision of auxin as a growth regulator (PGR), which can stimulate root growth, also could be applied. There is a lot of evidence that auxins affect stem growth and root formation. People know PGR (auxin) by Biooton and Rootone F (Artanti 2007). Jayusman's research (2005) showed that the concentration of Rootone F 1.5 g/40 mL gave the best results on the experimental parameters, namely the percentage of cuttings, the number of leaves, and the robustness of the seedlings. This study was used at 0 g/40 mL; 0.5 g/40 mL; 1 g/40 mL; 1.5 g/40 mL; and 2 g/40 mL concentrations.

The objectives of this study were: (i) to find a good planting medium for the growth of pepper cuttings seedlings, (ii) to obtain the administration of auxin that affected the growth of pepper cuttings, (iii) to reveal the relationship between planting media and auxin administration on the growth of pepper cuttings seedlings.

MATERIALS AND METHODS

Research place

This research was conducted in Demangan Village, Manyaran Sub-district, Wonogiri District, Central Java, Indonesia, at an altitude of 310 m above sea level.

Research design

This research was carried out in a factorial manner using a completely randomized design, with 2 treatment factors as follows:

Planting media consists of 3 kinds, namely:

M0: soil (control)

M1 : soil + manure (1:1)

M2 : soil + manure + husk (1:1:1)

Auxin concentration consists of 4 levels, namely: R0: control; R1 : 12.5 g/L; R2: 25 g/L; R3: 37.5 g/L; R4 : 50 g/L.

Thus, there are 15 treatment combinations. Each treatment was repeated 3 times so that there would be 45 treatment combinations.

Research procedure

Media and venue preparation

(i) Install paranet before starting the nursery to reduce sunlight, accelerating evaporation. (ii) Prepare and mix the media according to the treatment, then put it in a polybag measuring 14 x 20 cm. (iii) Prepare plastic and dry coconut leaves to cover the nursery.

Picking up the cutting material

(i) The cutting material was taken from the pepper plant in the Manyaran Wonogiri area in the afternoon. (ii) A sharp cutter was used to cut the cutting material so as not to be damaged. (iv) The cuttings used are taken from healthy and growing plants but not in flowering or fruiting conditions. (v) Cutting samples are taken from primary or climbing stems (not fruit branches).

Preparation of auxin treatment

(i) Dissolving auxin in water according to treatment. (ii) Stir the mixture first before dipping the cuttings. (iii) Dipping the cutting samples into the auxin mixture for 2 minutes before planting.

Planting

After taking the cutting samples, planting was carried out at 05.00 pm by inserting the cuttings into the media that had been prepared according to the treatment. The planting method is as follows: (i) Some of the media in the polybag is taken to be used as a place to insert cuttings that have been treated with auxin. (ii) The media is returned to the polybag carefully so that the position of the cuttings is right in the middle. (iii) The cuttings in polybags are watered sufficiently so that the media is tighter. (iv) The cutting samples are immediately placed in a nursery (concrete pipe culvert) with an air humidity of 70% and a temperature of 31°C. (iv) The concrete pipe culvert is covered with plastic trimmed with rubber tires, then given with dry coconut leaves

Upkeep

The upkeep includes watering with watering intensity according to media conditions and removing weeds or others that interfere with the growth of these pepper cuttings.

Observation variable

Sprouting time

The sprouting period is calculated when new shoots grow dark red (purple-red), then the shoots were observed every 2 weeks, beginning from 2 Weeks After Planting (WAP) to 12 WAP.

Number of shoots

The number of shoots was observed every 2 weeks, and the number of new shoots was counted on-site.

Shoot length

Shoot length was measured every 2 weeks from the stem where the shoots grew to the tip of the highest shoot.

Number of leaves

The number of leaves was calculated every 2 weeks, namely the leaves that have opened completely.

Leaf width

Leaf width was measured at the end of the study using millimeter paper. The procedure is that the leaves are drawn on millimeter paper which can be done by placing the leaves on millimeter paper, and the leaf pattern is followed. Leaf width is estimated based on the number of squares contained in the leaf pattern with the formula:

$$LD = n \times Lk \quad (1)$$

Where: LD: leaf width, n: number of squares; Lk: box width (cm^2)

Moreover, boxes with a size greater than or equal to half the reference size (cm^2) are selected to be considered in the above formula (Sitompul and Guritno 1995).

Number of roots

The number of roots calculated at the end of the study was the total number of roots per plant. The calculated roots are roots that emerge from the root neck and have root fibers that absorb nutrients in the soil.

Root length

Root length was measured at the end of the study from the root neck to the longest root tip.

Percentage of live cuttings

The percentage of live cuttings was calculated at the end of the study with the formula:

$$PSH = \frac{X \times 100\%}{T}$$

Where: PSH: percentage of live cuttings (%), X: number of live cuttings per treatment, T: number of replications in treatment (3)

Data analysis

Data were analyzed by analysis of variance based on the F test of 5% and 1%. It was continued with the 5% DMRT test if it had a significant effect. In addition, a regression analysis was performed to determine if there was an interaction.

RESULTS AND DISCUSSION

Seedling is one of the important stages in pepper cultivation, although pepper plants can be planted directly without going through the nursery process. The rate of photosynthesis will increase as the plant ages. The results of photosynthesis (photosynthate) are used for plant growth processes. Although growth occurs due to cell division and elongation processes, these processes require large amounts of carbohydrates.

The results showed that the media type significantly affected shoot length, while auxin concentration significantly affected shoot length and root number. Furthermore, the number of roots, media type, and auxin concentration showed a very significant interaction, and the percentage of live cuttings had significant interaction, too.

In detail, the discussion of each observation variable is as follows:

Time of shoot growing

One of the reasons for using cuttings to propagate plants vegetatively is that the time required to grow is faster. When shoot growth is an indicator of plant growth, the faster the shoot grows, the faster the time is needed for the plant to grow and develop. Shoots are formed from meristem cells that divide and form a bump/swelling at the end of the stem. The bump/swelling extends and encircles the tip (Gardner et al. 2017).

The type of media, the concentration of auxin, and their combination had no significant effect on shoot growth, which is one form of plant growth. Salisbury and Ross (2006) stated that growth in plants is limited to certain parts, which consist of several cells that have just been produced through the process of meristem cell division. It is the product of cell division that grows and causes growth. For example, the tip of the canopy has meristems that could form shoots. So when growing, shoots are more influenced by meristems.

The fastest shoot growth was in soil media with an auxin concentration of 50 g/L, and the longest shoot growth was in soil media with 12.5 g/L (Figure 1). Different types of growing media with different auxin concentrations did not significantly affect shoot growth, except for a slightly prominent auxin concentration of 12.5 g/L. Maybe auxin is a growth hormone inseparable from plant growth and development, and one of the roles of auxin is stimulating cell elongation in shoots (Artanti 2007). Gardner et al. (2017) also stated that added auxin could significantly affect shoot growth.

Number of shoots

Pepper plants have nodes as a place for roots or shoots to come out and internodes that separate one node from another. The type of media, the concentration of auxin, and the combination of the two had no significant effect on the number of shoots. It is because the number of shoots that are part of plant growth is more influenced by the meristems present in the cutting material used. Plant development could be explained in that the meristem cells will divide to produce new cells, and then new cells will grow and develop, which causes growth (Salisbury and Ross 2006).

The highest number of shoots was in the treatment of media of soil + manure + husk with an auxin concentration of 25 g/L (Figure 2). The combination of soil media gave the highest number of shoots at an auxin concentration of 37.5 g/L, while the combination of soil + manure media gave the highest number of shoots in the treatment without auxin.

The number of shoots in soil + manure + husk was more than in other media. Moreover, media is also more profitable because it is lighter and is one way of utilizing agricultural waste. Soil + manure + husk was the best-growing medium for the number of shoots because the growing media composition was complete by adding manure and husks. In addition, manures can provide additional organic matter in the growing media. Meanwhile, besides being an organic material, the husk can also improve the drainage of the growing media.

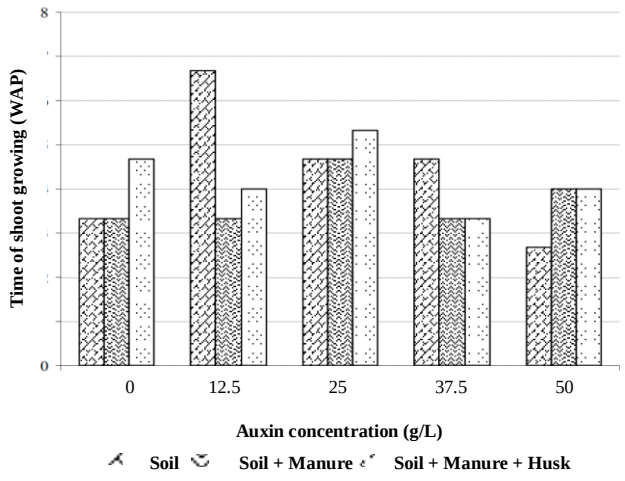

Figure 1. Average growth of pepper shoots on various media and auxin concentrations

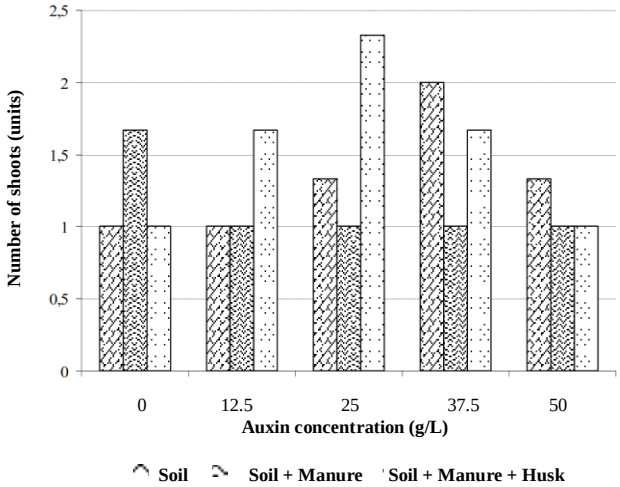

Figure 2. The average number of pepper shoots on various media and auxin concentration

According to Adnan et al. (2021), providing organic matter increases soil humus, reduces environmental pollution, and reduces nutrient depletion. Thus, applying organic matter can improve the physical properties and health of the soil. Meanwhile, husks play a very important role in improving soil structure so that the drainage system for planting media becomes better. Therefore, using mixed media of manure and husk can increase plant growth and yield (Zulia and Batubara 2020).

Shoots will grow if the concentration of cytokinin hormones is higher than auxin (Rineksane 2005). Auxin concentrations in plants can be high due to the accumulation of endogenous and exogenous auxins (Sairanen et al. 2012; Tian et al. 2013; Olatunji et al. 2017). It is suspected to be the cause of the best concentration of auxin in the number of shoots with soil + manure + husk media was 25 g/L. The administration of auxin at more than 25 g/L caused the accumulation of endogenous and exogenous auxin to be higher than the concentration of cytokinins, which could inhibit the shoot growth.

Shoot length

Planting media is a place to live for seedlings and find food that a suitable planting medium will improve plant growth and development. The growth that can be seen directly from the plant is the growth of shoot length.

Growth in stem height occurs in the intercalary meristems of the internodes. The internodes lengthen due to the increase in the number of cells and mainly due to the expansion of the cells. Growth due to cell division occurs at the base of the internode (intercalary), not the tip meristem. The amount of hormone in the intercalary meristem is limited because it is not produced by itself as occurs in the tip meristem, so growth regulators must be supplied from outside (Gardner et al. 2017).

The results of the data analysis showed that the type of media treatment and auxin concentration significantly affected shoot length (Table 1). However, these two treatments did not show any interaction with shoot length.

The longest shoot length produced was from a combination of soil + manure + husk treatment with an auxin concentration of 50 g/L, which was 24 cm. Thus it can be said that the right medium has a good effect on administering auxin. That is because the media supports plant growth outside (environment), while auxin is a growth regulator from within. So if these treatments are combined, it will have a better effect on plant growth.

The best planting media to maximize shoot length are soil media + manure + husk, soil media + manure, and soil media. It is because the more diverse the composition of the media, the better the growth. Soil media is the primary medium and is a control treatment. However, adding manure can give better yields on shoot length because manure is very good for supplying nutrients and improving soil quality. Manure is an organic fertilizer that can provide organic matter and nutrients, improve soil physical properties and restore lost nutrients. In addition, it can prevent water loss in the soil and the rate of water infiltration into the soil (Adugna 2016).

Moreover, adding husks to the planting media can also give better results because the husks can improve the drainage system by being easy to bind water, not easy to rot, and not easy to compact. It follows Dewi et al. (2007), who state that pepper plants require soil conditions that have good aeration and drainage.

Table 1. Average shoot length of pepper (cm) on various media and concentration of auxin (12 WAP)

Media type	Auksin concentration (g/L)					
	0	**12.5**	**25**	**37.5**	**50**	**Average**
Soil	6.5	12.83	3	2.08	6.33	6.15a
Soil + manure	6.33	18.67	16	6	5.83	10.57ab
Soil + manure + husk	10.17	13.91	4.17	9.17	24	12.28b
Average	7.67a	15.14b	7.72a	5.75a	12.05ab	

Note: Numbers followed by the same letter in one row or column are not significantly different in the Duncan test at the 5% level

In soil media, the longest shoot was 12.83 cm. In soil + manure media was 18.67 cm, while in soil + manure + husk medium, it was 24 cm (Table 1). So it can be said that the media treatment affected shoot length (the treatment was better than the control). Therefore, per the results of the F test, the media had a significant effect on shoot length.

The best auxin concentration at shoot length was 12.5 g/L (15.14 cm), then 50 g/L (12.05 cm). It follows Gardner et al. (2017), who stated that stems respond to auxin concentrations in a fairly wide range. Meanwhile, the auxin concentration of 37.5 g/L caused the shoot length to be lower than the control. It indicates that the growth of pepper-cutting seedlings requires the right concentration of auxin. Improper concentration will not stimulate the growth of pepper cuttings seedlings but will inhibit their growth.

The appropriate concentration of auxin for pepper-cutting seedlings on shoot length is 12.5 g/L. It follows the research of Olatunji et al. (2017) that plants require an appropriate concentration of auxin for their growth. An inappropriate concentration will not promote growth; on the contrary, it can even inhibit it. Artanti (2007) also stated that auxin was very influential on stem growth, but Dewi and Sabhara (2022) stated that the proper use of PGR would positively affect growth.

Number of leaves

The function of the leaves is to produce photosynthate, which plants need as a source of energy in growth and development (Dewi and Sabhara, 2022). Therefore, the highest number of leaves produced indicates the plant is experiencing better growth and development.

The type of media, the concentration of auxin, and the combination of the two had no significant effect on the number of shoots. According to Gardner et al. (2017), the number of nodes and internodes is the same as the number of leaves. All three have the same origin in the phytomer. The cutting material used in this study had the same nodes and internodes.

However, there were differences in the effect of the media type and auxin concentration on the number of leaves (Figure 3) because, according to Gardner et al. (2017), the number of leaves is also influenced by genetic and environmental factors. For example, leaf growth will be encouraged if sufficient water is available in the growing media.

The highest number of leaves produced was on soil + manure + husk with an auxin concentration of 50 g/L, while the second-highest number of leaves was in the same medium with an auxin concentration of 12.5 g/L. Therefore, it shows that soil + manure + husk with auxin was the proper medium with the ability to give the highest number of leaves for pepper cuttings.

Soil analysis showed that the exchanged K content of the soil was 0.23%. According to Sutedjo (1990), a fairly high K (no need to fertilize) is 0.3%, so adding husks in this medium can meet the K needs because husks are a source of K needed by plants.

Especially on soil + manure + husk treatment, adding auxin has a good effect on the number of leaves. However, the auxin concentration must be appropriate to obtain the optimal number of leaves; in this study, the highest number of leaves on the planting medium of soil + manure + husk was with an auxin concentration of 50 g/L. In comparison, with auxin concentrations of 25 g/L and 37.5 g/L, the number of leaves was less than that of 12.5 g/L. It shows that the effect of auxin absorption is observed from the concentration of auxin and the sensitivity of the recipient tissue (plant protein) (Salisbury and Ross 2006).

Leaf width

Leaves are generally considered the main photosynthetic-producing organs, so leaf observations are necessary to explain the growth processes that occur in the formation of plants. In addition, observation of leaves can be based on their function as light receivers and photosynthetic tools (Ren et al. 2018). On this basis, leaf width is the preferred parameter because the rate of photosynthesis per unit plant, in most cases, is determined largely by leaf width (Sitompul and Guritno 1995).

Large leaf width affects the growth of other plant organs. Increased leaf width is a form of plant growth resulting from cell division and elongation activity influenced by the availability of nutrients. Nitrogen is a nutrient that strongly supports the vegetative growth of plants, especially leaves.

Leaves are vegetative organs. Therefore, the N content influences their growth in the media. Soil analysis showed that the soil contained N (0.21%), and the manure contained N (1.44%), so combining these two growing media could increase the N content in the growing media used; thus, leaf growth can be increased.

The media type, the auxin concentration, and the combination of the two had no significant effect on leaf width. Because the leaves are part of the plant whose growth structure is certain (it will be dead to some extent), therefore media type and the concentration of auxin have no significant effect on the growth of leaf width (Salisbury and Ross 2006).

The best-growing media for leaf width is soil + manure + husk (Figure 4) because the planting medium has a complete composition. Manure adds organic matter, which means adding nutrients (especially N). Meanwhile, the husk improves the condition of the growing media by providing more air and water space (aeration and drainage).

The soil + manure media gave a smaller leaf width than the soil media. It is due to the addition of manure; even though it increased the N content needed for leaf growth, manure made water absorption greater than the soil media, so the planting medium was too much watering. This planting medium is also not accompanied by adding air space, while pepper plants require good aeration and drainage conditions (Dewi et al. 2007).

Leaf width on pepper cuttings seedlings with auxin treatment gave better leaf width than pepper cuttings without auxin treatment (control) (Figure 5). It is not following Rineksane's (2005) research that auxin could not increase leaf width. According to Marlin (2005), plants' growth and development (morphogenesis) treated with PGR are controlled by the balance and interaction of endogenous and exogenous PGR. So giving PGR (auxin) can affect the growth of pepper cuttings seedlings, especially leaf width.

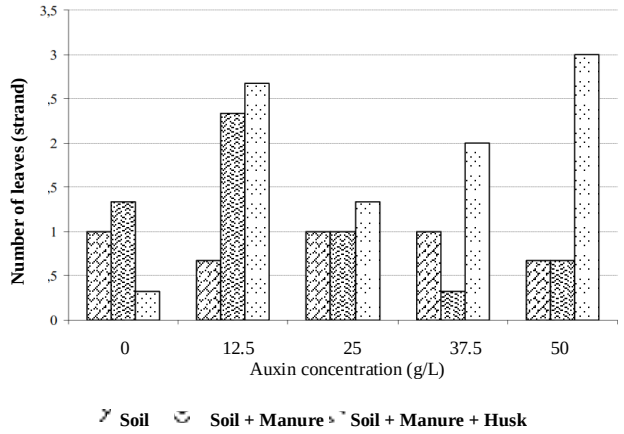

Figure 3. The average number of pepper leaves on various media and auxin concentrations

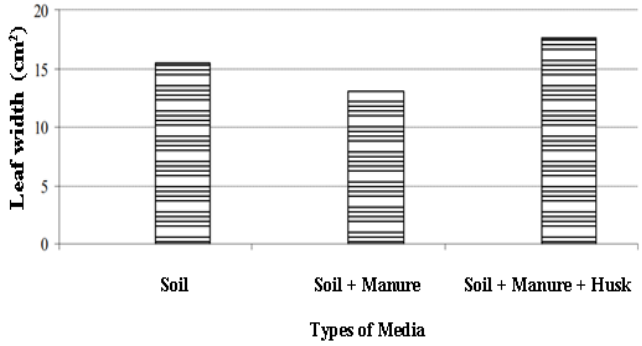

Figure 4. Average pepper leaf width on various media

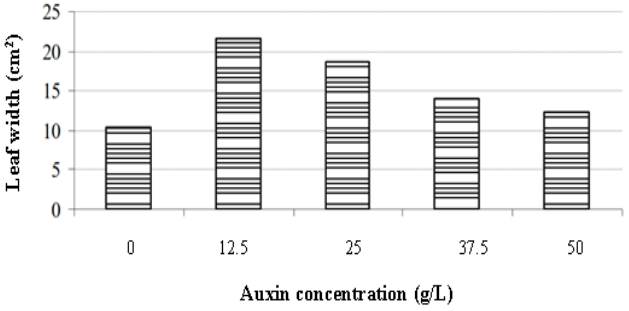

Figure 5. Average pepper leaf width at various concentrations of auxin

Table 2. The average number of pepper roots at various concentrations of auxin (3 months)

Auxin concentration treatment	Number of roots
Without auxin	7.78b
Auxin 12.5 g/L	7.78b
Auxin 25 g/L	3.43ab
Auxin 37.5 g/L	2.33a
Auxin 50 g/L	4ab

Note: Numbers followed by the same letter in one row or column are not significantly different in the Duncan test at the 5% level

Number of roots

Gardner et al. (2017) stated that roots are the main vegetative organs that supply water, minerals, and materials important for plant growth and development. Strong root growth is necessary for shoot strength and growth. Shoot growth will be disrupted if the roots are damaged due to biological, physical, or mechanical disturbances.

Filipović (2020) states that roots function in sucking water and salt-loaded liquids. Another function is to absorb plant nutrients and circulate them to all parts of the plant through the wood network. In addition, it also functions as a plant reinforcement so that its growth is strong. That is why it needs to observe the number of roots in this study.

The media type had no significant effect on the number of roots because the periclinal division determines the number of roots. As stated by Salisbury and Ross (2006), periclinal division, followed by the growth of offspring cells, causes protrusions, namely root primordia.

The data analysis showed that the auxin concentration significantly affected the number of roots (Table 2) because auxin is a growth regulator that stimulates root growth. Although the plant produces natural auxin, providing synthetic auxin from outside can stimulate root growth.

The interaction of media type and auxin concentration based on the results of data analysis showed a very significant effect on the number of roots (Table 2 and Figure 6). Because a planting medium is a place for roots to grow and auxin is a root growth regulator, the combination of the two greatly affects the number of roots. According to Wasito and Nuryani (2005), planting media in soil and organic matter combination provides two advantages: a root growth medium and a source of nutrients and water for root growth.

The highest number of roots was found in soil media treatment with an auxin concentration of 0 g/L (17.67) (Table 3). The second-highest number of roots was found in soil + manure + husk treatment with an auxin concentration of 12.5 g/L (11). The third-highest number of roots was found in soil + manure treatment with an auxin concentration of 12.5 g/L (9). The smallest number of roots was found in the treatment of soil media + manure + husk with an auxin concentration of 25 g/L and soil + manure media with an auxin concentration of 37.5 g/L.

Table 3. The average number of pepper roots on various media and auxin concentrations (3 months)

Media treatment	Auxin concentration (g/L)				
	0	12.5	25	37.5	50
Soil	17.67c	3.33ab	5.67ab	2.33a	4.67ab
Soil+manure	1.67a	9ab	4ab	1a	1.33a
Soil+manure+husk	4ab	11bc	1a	3.67ab	7ab

Note: Numbers followed by the same letter are not significantly different on Duncan's test level of 5%

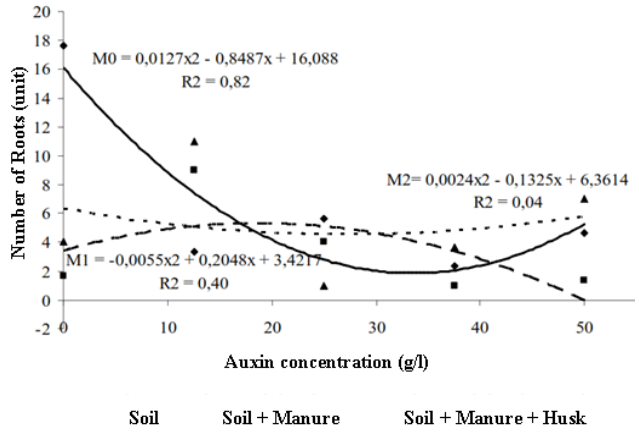

Figure 6. Relationship of the media type and auxin concentration on the number of pepper roots

The number of roots in soil media with auxin concentration of more than 37.5 g/L tends to increase. The determinant coefficient (R2) value is 0.82, meaning that adding auxin affects the number of roots. In soil + manure media, the highest number of roots was found in the treatment with an auxin concentration of 25 g/L. The determinant coefficient (R2) value is 0.40, which means that the effect of giving auxin is weak. On the other hand, in soil + manure + husk media, the least number of roots was found in the treatment with an auxin concentration of 25 g/L. The determinant coefficient (R2) value is 0.04, which means the effect of giving auxin is very weak.

The media type and auxin provision affect the number of roots. In soil media, adding auxin will again increase the number of roots at a concentration of 37.5 g/L (after previously decreasing with the administration of auxin). In soil + manure media, auxin with 12.5 g/L and 25 g/L concentrations will increase the number of roots. However, the higher the auxin concentration applied, the number of roots will decrease. In the medium of soil + manure + husk, adding auxin 25 g/L will increase the number of roots. It indicates an interaction between the type of media and the concentration of auxin on the number of leaves of pepper cuttings seedlings.

The number of roots in soil media showed a parabolic response to the auxin concentration. The number of roots decreased and increased again with increasing auxin concentration. The number of roots in soil + manure and soil + manure + husk with auxin concentration gave the same pattern (close to linear).

The planting medium is an external factor that determines plant growth. For example, the media in the cutting technique functions as a support for cuttings during the root growth period, maintains moisture, and facilitates air penetration. In addition, media with better availability of water and nutrients can stimulate plants to carry out photosynthesis more quickly, producing more photosynthesis, such as increasing the number of roots (Gardner et al. 2017).

Adding auxin increases the number of roots optimally if the concentration is appropriate. Rineksane (2005) stated that using Rootone F (auxin) increases the number of roots. Marlin (2005) states that auxin plays an important role in activating enzymes that make cell components so that once cell division begins, auxin will stimulate the formation of cells quickly. Artanti (2007) stated that auxin has several roles in supporting plant life, one of which is to encourage root primordial.

Root length

Root length results from the elongation of the cells behind the tip meristem. Strong root growth is commonly required for strength and growth (Gardner et al. 2017), while root length is a form of root growth. Genetically, rather than environmentally, mechanisms mainly control the overall shape of the root system, and soil affects it as well (Salisbury and Ross 2006).

The results of the F test showed that the type of media, the concentration of auxin, and the combination of the two had no significant effect on root length. It is because the root is a part of the plant where cell growth and development stages are mostly cell enlargement. In the enlargement of these cells, most water absorption can stretch the walls. The material for the new walls is synthesized so that the walls are not thin. As a result, the wall is widened at the tip at the root, so root growth is more elongated. The supply of external hormones with low concentrations stimulates plant physiological processes, but the response depends on the endogenous hormone level (Salisbury and Ross 2006). So the root length is influenced by the nature of the root, which is more dominant for the increase in root length.

The best root length was found in soil + manure + husk treatment with an auxin concentration of 50 g/L, followed by the same medium with an auxin concentration of 37.5 g/L (Figure 7). It shows that soil + manure + husk media had the best effect on root length because the mixed media of manure and husk can increase plant growth and yield (Zulia and Batubara 2020).

Gardner et al. (2017) also stated that better water availability and nutrients could stimulate plants to make photosynthesis more quickly, producing more photosynthate for roots. Media affects root length because the media is a place where roots grow. Media soil + manure + husk can meet the nutrient needs of plants and provide sufficient space for good root growth. Soil is a natural medium (general), manure provides additional nutrients, and husks provide sufficient space for roots and can bind water.

Salisbury and Ross (2006) added that temperature, aeration, and availability of water and minerals are important factors in root growth. These factors were available in soil + manure + husk media, so this planting medium gave the best results on the growth of pepper cuttings, especially root length. The addition of manures can add nutrients to the growing media while adding husks can improve the structure of the media to make it better (aeration and drainage).

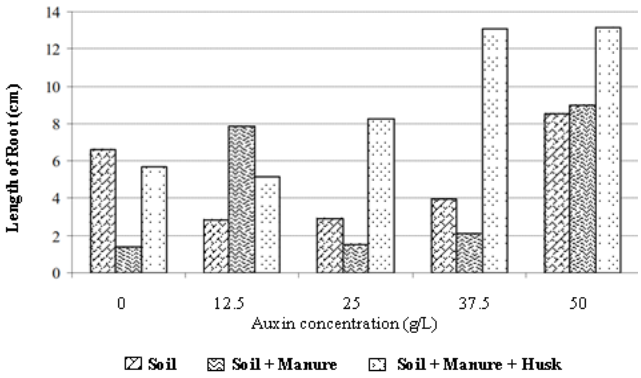

Figure 7. Average root length of pepper on various media and concentration of auxin

Giving auxin can provide better root length because auxin is a growth regulator that stimulates root growth. Especially in soil media, the application of auxin, which increased the number of roots, was a concentration of 50 g/L. In soil + manure media, the addition of auxin was able to increase the number of roots. It is in accordance with Rineksane's study (2005), which states that auxin plays a role in promoting root growth because auxin is a hormone that plays an important role in stimulating root growth. The Rootone F auxin used was formulated by several root growth hormones, so its use is more effective in stimulating roots (Puspitorini 2016).

Percentage of live cuttings

The results of the F test showed that the percentage of live cuttings on several types of media and the concentration of auxin had no significant effect because these seedlings die due to unfavorable weather changes. At the beginning of the nursery, the seedlings experienced good growth (70% humidity). Pepper plants require air humidity between 60-80% (Dewi et al. 2007). In the middle of the study, there were frequent rains, so the humidity of the nursery increased (presumed to be more than 90%) because, in the rainy season, air humidity can reach more than 90%.

In addition, this simple tool in the nursery is a bit annoying during the rainy season. The rainwater that fell wet the paranet, then hit the dried coconut leaves used to cover the plastic which covered the seedlings. As a result, coconut leaves as a cover become heavier, and a lot of water is poured on the plastic, which causes the surface of the plastic to lower and touch the already tall seedlings.

Then the pepper cuttings are left exposed but still in paranet shade to reduce moisture and further damage. However, after the seedlings are left open, environmental influences also affect the growth of the seedlings, including disease. Plants that had been disturbed due to the disruption of the covering apparatus slowly began to improve with the emergence of new shoots. However, some still cannot improve because of the damages that cause the stems to rot.

The type of media treatment and auxin concentration interacted with the percentage of live cuttings (Table 4). The success of pepper seedlings is quite high, as seen from the number of cuttings that live at the end of the observation. Overall there were 93.33% live cuttings, namely 42 of 45 seedlings.

Control media gave better results in the percentage of live cuttings than other media. The use of soil media was able to produce an average percentage of live cuttings of 100% at all auxin concentrations. Soil media + manure + husk at a concentration of 50 g/L auxins gave the percentage of live cuttings of 66.67%. Soil media + manure at a concentration of auxin 25 g/L gave the percentage of live cuttings of 33.33%. This result shows that soil media has the highest percentage of live cuttings.

Soil media-only is better than other combined media, such as soil media + manure or soil media + manure + husk. It is presumably due to the influence of the external/surrounding environment that affects the media and ultimately affects the pepper-cutting seedlings. However, this external environment can also directly affect the pepper cuttings seedlings' condition. So the pepper cuttings get two influences, namely from the outside environment and the planting medium.

Soil media is a commonly used and natural medium for all plants. However, if this media is exposed to continuous/large-intensity rainwater, it will cause the media to become saturated with water. Meanwhile, the soil + manure, which contains a lot of organic matter, will become very humid if continuously exposed to rainwater and can grow unexpected things such as pathogens. The same applies to soil + manure + husk, but there is also a husk with less manure. Therefore, it causes the percentage of live cuttings in soil + manure + husk media to be higher than soil + manure media, although it is still lower than soil media.

The percentage of live cuttings reached 93.33%, indicating that the ability of the cutting material to grow and develop is high. It means that the cutting material contains meristematic tissue that is actively dividing. Marlin (2005) stated that meristematic tissue that is actively dividing could grow and develop quite high (83.33-100%). So even though the percentage of auxin in this study did not reach 100%, it could be said to be quite high. Although the percentage of live cuttings was more influenced by the external environment than the effect of auxin administration, the analysis showed an interaction between the media and auxin treatment.

Table 4. The average percentage of live pepper cuttings on various media and auxin concentration (%)

Type of media	Auxin concentration (g/L)				
Soil	0	12.5	25	37.5	50
Soil+manure	100a	100a	33.33b	100a	100a
Soil+manure+husk	100a	100a	100a	100a	66.67ab

Note: Numbers followed by the same letter in one row or column are not significantly different in the Duncan test at the 5% level

The percentage of live cuttings in the auxin treatment was lower than the control (without auxin). It differed from Amirudin et al. (2004), who stated that synthetic PGR increased the percentage of live cuttings of pepper shrubs. It happened because the influence of the environment was at large on the percentage of live cuttings, so the unfavorable environment caused the percentage of live cuttings to be low.

The following conclusions can be drawn based on the results: (i) The best growth of pepper cuttings on shoot length was obtained in soil + manure + husk (12.28 cm) media. (ii) The growth of pepper cuttings was affected by PGR (auxin). The best concentration of auxin at shoot length (15.14 cm) and several roots (7.78) was 12.5 g/L. (iii) The interaction between the planting medium and the provision of auxin on the growth of pepper cuttings seedlings occurred in the number of roots and the percentage of live cuttings. The number of roots in soil media showed a parabolic response to the auxin concentration. The number of roots decreased and increased again by increasing auxin concentration. The number of roots in soil + manure and soil + manure + husk with auxin concentration gave the same pattern (close to linear). Most of the media produced a percentage of 100% live cuttings with the auxin addition. Still, in soil + manure with an auxin concentration of 25 g/L and soil + manure + husk with an auxin concentration of 50 g/L, the live cuttings were equal to 33.33% 66.67%, respectively.

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