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Soybean spouts during early growth photo by United Soybean Board

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Book:

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

Chapter in book:

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

Abstract:

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

Proceeding:

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

Thesis, Dissertation:

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

Information from internet:

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

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Effects of mycorrhizal and *rhizobium* inoculation on soybean growth in acidic soils of Gatanga, Kenya

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Abstract. *Kamau NN, Kungu JB, Mugendi D. 2020. Effects of mycorrhizal and* rhizobium *inoculation on soybean growth in acidic soils of Gatanga, Kenya. Cell Biol Dev 4: 1-16*. Central Kenya's farmers have found it challenging to appropriately conserve and replace soil nutrients due to small landholdings and poverty. The inevitable result has been soil erosion and nutrient leaching, resulting in soil acidity. The purpose of this study was to see how inoculating soybeans (*Glycine max* Merr.) with both *mycorrhiza* and *rhizobium* as a biological approach to enhancing soil fertility in acidic soils in Gatanga, Thika District, affected soil fertility. Field studies on sterilized and non-sterilized soils taken from Gatanga were conducted at Gatanga and Kenyatta University (on-station). The field studies used a complete randomized block design, whereas the on-station experiments used a complete randomized design. The Genestat for Windows Version 8.11 was used to analyze variance (ANOVA) on the data, with means separated using LSD at a 5% significance difference. As a result of the dual inoculation with *mycorrhiza* and *Rhizobium*, the growth parameters of height, root collar diameter, shoots, and root dry weight all increased. Higher nitrogen fixation by soybeans, as demonstrated by increased nodulation and grain yields, was also a result of dual inoculation. On the germination of soybeans, dual inoculation with mycorrhiza and *rhizobium* had no significant effect ($p<0.05$). In the long rains, the height of soybeans increased greatly over the control by 88 %, but in the short rains, the growth was not significant. In the on-station experiments, there was no significant difference in height between sterilized and non-sterilized soil. Dual inoculation improved root collar diameter in the long and short rains by 80% and 8.6%, respectively. Dual inoculation raised the dry weight of the shoots by 140 % in the on-farm long rains 2005 season, whereas the changes were not significant in the short rains season and on-station experiments. In the on-farm long rains 2005 season, dual inoculation improved grain yields by 356 %, while on-station experiments saw grain yields increase by 76 % and 107 % in sterilized and non-sterilized soils, respectively. Even though nodulation was poor in all the experiments, the number of nodules increased by 676 % over the control during the long rains of 2005. The control (S) had no nodules in the on-station experiments; maybe low precipitation caused the short rain crop to perform worse than the long rain. Finally, mycorrhiza and *rhizobium* biological organisms could boost the productivity of the legume soybean in acidic soils. However, technologies to make microorganisms available to farmers must be developed. The obligatory nature of mycorrhizal fungi makes cultivation and commercialization difficult, and the short shelf life of *rhizobium* at room temperature precludes its usage by resource-limited farmers.

Keywords: Acidic soils, growth performance, mycorrhiza, *rhizobium*, soybean

INTRODUCTION

Under continuous cultivation and in degraded soils, crop productivity in tropical countries is limited by the availability of one or more nutrients, with nitrogen and phosphorus being the most limiting (Giller 2002). Fertilization is thus required to boost crop yields. In Kenya, 7.5 million hectares (about one-third of the country) are acidic (Kanyanjua et al. 2002). These are deep, well-drained soils with low activity clay typical of an older environment with a lot of rain; because they are severely leached, they have a limited cation exchange capacity due to base leaching. The major clay fraction is kaolinite, which has a 1:1 silica:aluminum layer structure and has a lower negative charge than the 2:1 clay minerals (Giller 2002). When soils have been leached of other bases, aluminum could become the dominating cation if the original material has much of it. The proportion of cation exchange capacity occupied by cations that predominate in most soils, notably calcium, magnesium, and Potassium, can be as high as 80-90 %, and the base saturation, that is, the proportion of cation exchange capacity occupied by cations that predominate in most soils, is low (Giller 2002). For legume grain crop production, biological nitrogen fixation (BNF) by legumes provides an alternative and less expensive supply of nitrogen. Soil acidity, particularly in sub-humid countries, impedes nitrogen fixation by reducing phosphorus availability, limiting plant root development, and exacerbating the problem of insufficient nutrient input from the soil (Harter 2002). In acidic soils, a considerable part of applied phosphorus is bound to iron and aluminum oxides, rendering it unavailable to plants (Schroth et al. 2003).

In biological nitrogen fixation, phosphorus is essential for energy supply. Using arbuscular mycorrhiza symbiosis can solve the problem of plants' phosphorus (P) availability. First, AM (arbuscular mycorrhiza) inoculation boosts phosphorus uptake and nitrogen-fixing (Young 1997). Mycorrhiza is a fungus that feeds on carbohydrates from the host plant. In exchange, they boost nutrient extraction from the soil, causing the plant's root system to expand in size and surface area (Sieverding 1991). Next,

legume plants have a shallow root system and benefit substantially from this connection.

In deteriorated acidic soils, nitrogen availability to plants is also a limiting factor (Giller 2002). However, rhizobium, a soil bacteria, can create a symbiotic relationship with legumes. *Rhizobium* bacteria provide ammonia or amino acids to the plant through nitrogen fixation in exchange for organic acids as a carbon and energy source (Leigh 2002).

Most legumes simultaneously have symbiotic relationships with rhizobium and arbuscular mycorrhizae fungi (Barea et al. 2005). *Rhizobium* fixes nitrogen, which helps plants grow. The fungus helps the host by improving the efficiency of mineral and water uptake from the soil and changing host metabolisms and other physiological factors. Plant development is often significantly stronger in this tripartite interaction than when the plant is in a symbiotic relationship with just one bacteria (Arora et al. 1991). Inoculated legumes with mycorrhiza and rhizobia will reduce the requirement for mineral fertilizers, saving energy. It has been determined that the manufacture and use of nitrogen fertilizers absorb 50% of the total energy consumed in agricultural production in tropical regions (Chikowo 2004).

Plants with two mutualistic symbionts, such as *rhizobium* nodulated and mycorrhizal plants, are well adapted to environments with low nitrogen and phosphate availability (Bagyraj 1996). Unlike mineral fertilizers, both mycorrhiza and *Rhizobium* symbionts are likely to be self-replenishing once established. In highly cultivated areas, nitrates' presence in groundwater is a major health concern, as it can cause methemoglobinemia in babies, cancer, and respiratory sickness (Comly 1987). It is also a major source of pollution, resulting in issues such as eutrophication and stratospheric ozone depletion (Bohlool et al. 1992). BNF will provide a cost-effective and environmentally friendly method of crop production by minimizing external inputs while increasing internal inputs.

Glycine max is a legume that grows well in acidic soils; the general goal of this study was to investigate the effects of simultaneous inoculation with AM fungus and *rhizobium* on the growth performance of this legume. The research sought to determine (i) the effect of inoculating Glycine max with AM and *Rhizobium* bacteria on the establishment and germination of the plant in acidic soils, (ii) the effect of inoculating Glycine max with AM and *Rhizobium* bacteria on grain yield and biomass production in acidic soils, and (iii) the quantitative impact of inoculating Glycine max with AM and *Rhizobium* bacteria on nitrogen fixation in acidic soils.

MATERIALS AND METHODS

Site description

The on-farm experiment was carried out in the Gatanga division, Thika district, in the Central Province of Kenya. At an altitude of 1,680 meters above sea level, the region is located at 38º 58' 0" E and 0º 55' 59" S. The location is on the eastern slopes of the Nyandarua Ranges, and it receives 1,000 mm of rainfall yearly, which is split into two seasons that begin in mid-March and end in mid-October. The annual average temperature is 25ºC. According to FAO/UNESCO (1974), the soils are well-drained, extraordinarily deep dusky red friable clays with humic acid topsoil, characteristic humic Nitisols produced on tertiary basic igneous rocks. The soils are acidic and leached (Jaetzold and Schmidt 1983). The topography of the area is undulating and rolling. Gatanga has a population of 103,048 people, with a density of 410 people per square kilometer, according to the 1999 census. The average size of an agricultural holding is 0.25 hectares (Ministry of Agriculture, Thika district).

Kenyatta University is located in the Upper Midlands 4 district (UM4). It is located at 37º 10' 0" E, 0º 34' 0" S, and is 1650 meters above sea level. The annual average temperature is 25ºC. It is located in a semi-humid climatic zone, receiving an annual total of 750 mm of rainfall in two separate seasons: long rains (LR) from mid-March to June and short rains (SR) from mid-October to December.

Treatments and design of the study

The experiment took place on-farm in Gatanga as well as on-station at Kenyatta university. Soybean was used as a test crop (Glycine max). The studies in the on-farm study were designed using a complete randomized block design (CRBD). The plots were 7 m by 3.5 m in size and were divided into two blocks. The crop was cultivated for two seasons, 2005 long drops of rain and 2005 short drops of rain. Within the block, treatments included the following: (i) Soybeans inoculated with *Rhizobium* (S+R). (ii) Soybeans inoculated with both *rhizobium* and mycorrhiza (S+R+M). (iii) Soybeans inoculated with *Rhizobium* plus P fertilizer (S+P+R). (iv) Soybeans inoculated with mycorrhiza plus P fertilizers (S+P+M). (v) Soybeans inoculated with mycorrhiza (S+M). (vi) Soybeans Plus P fertilizers (S+P). (vii) Soybeans on their own (S) (Control).

Each treatment comprised a row of 25 plants, with each plant serving as a replication of the treatment. Planting occurred at the specified 45 x 15 cm spacing. Random sampling was used to decide the treatments assigned to the rows. A mixture of three AM species, *Glomus etunicatum*, *Glomus intraradices*, and *Gigaspora albida*, was used to inoculate the treatments S+R+M, S+P+M, and S+M by inserting 10 grams of it below the seeds in the planting hole. At a rate of 250 kg per hectare, triple super phosphate fertilizer containing 46 kg of P2O5 per 100 kg was given to the treatments S+P+R, S+P+M, and S+P. The *Rhizobium* inoculant for soybeans was obtained from the Kabete campus and applied at a rate of 50 grams per 15 kg of soybeans to the treatments S+R, S+R+M, and S+P+M. The control treatment consisted of soybeans grown in their natural state.

The on-station study employed a completely randomized design for the experiments (CRD). Each treatment comprised three 20 cm diameter half litter planting pots, one filled with sterilized soil and the other with non-sterilized soil gathered from Gatanga. For 48 hours, the soil was disinfected in an oven with hot air at 100°C. Seven treatments were used, the same as those used in on-farm

testing. Three times for each treatment, a total of 42 plots were created. Three soybeans seeds per pot were planted and then trimmed to one plant per pot. For four months, the plants were nurtured in a greenhouse. When germination occurred, plants were watered once daily for eight days and then once a week for the next three months.

Data collection

The pH, soil organic matter, accessible phosphorus, total nitrogen, exchangeable Potassium, magnesium, calcium, and cation exchange capacity of topsoil (0-20cm) obtained from Gatanga were all determined in a laboratory. At eight days, the germination percentage was assessed by counting the number of plants that had emerged. The height growth of the plants was determined every 15 days using a systematic sampling procedure by measuring the distance from the soil level to the growing apex of each plant, beginning one month after planting and ending at the onset of flowering, when plant height growth ended. The diameter of the root collar was measured three months after planting. Plants were picked and measured using a simple random sampling procedure with the help of vernier calipers. Five and one plants in the on-field and on-station studies were selected using destructive sampling with a hoe at the flowering and seed laying stages, respectively. Soil that had adhered to the root was rinsed away with a gentle trickle of tap water. After that, the nodules were separated and numbered. Four plants were randomly chosen at harvest, and their grain yields were determined following hand threshing. The identical plants' above- and below-ground biomass was dried in an oven at 50 °C until it reached a consistent weight.

Analyses of soils

Before planting, topsoil was collected from the Gatanga study site and evaluated for pH, organic matter content, total nitrogen, Potassium, and phosphorus. Five cores (diagonally and centrally) were sampled to a depth of 0-20 cm. After thoroughly mixing the soil and removing all visible plant debris to ensure homogeneity, it was wrapped in polythene sheets and sent to the University of Nairobi Kabete campus laboratory for analysis.

Soil pH was determined using a pH meter and a glass electrode in a 2.5:1 water-to-soil suspension ratio. Next, a dispenser was used to add 25 mL of deionized water to 10 mg of dirt obtained from Gatanga in a 60 mL bottle. After 10 minutes of stirring, the solution was allowed to stand for 20 minutes. Finally, a pH electrode was immersed after allowing the soil to settle, and readings were collected after pH stability.

Organic matter determination in soils

The Walkley-Black method (Okalebo et al. 2002) acquired soil organic matter. First, the organic carbon was oxidized using potassium dichromate in the presence of strong sulphuric acid. Next, to promote homogeneity and facilitate oxidation, a soil sample passed through a 2 mm sieve to remove the coarse fraction was ground to pass through a 0.5 mm sieve. Two (2) grams of this soil were placed in a conical flask, and 10 ml of 1 N potassium dichromate was added using a pipette and spun to oxidize the carbon in the soil, followed by the addition of 20 ml of concentrated sulphuric acid (36 N) in a constant stream. The heat of dilution produced by adding sulphuric acid provided a constant amount of heat to aid in the oxidation process. After that, the mixture was allowed to cool for 20 minutes. After adding 200ml of distilled water, 5.0 ml of 85 % orthophosphoric acid and 5.0 ml of diphenylamine sulphonate indicator were added.

Furthermore, the mixture was titrated with 0.1 N ferrous sulfates to decrease the residual dichromate. When the combination reached the endpoint, it changed color from turbid dark blue to light green. Carbon content in soil was determined using the following formula, which considers that 1 ml of dichromate oxidizes 0.39 mg of carbon (the average recovery rate of 77 % is taken into account).

$$\% \text{ Carbon} = \frac{(\text{m.e dichromate} - \text{m.e } FeSO4) \times 0.39}{\text{Weight of soil in grams}}$$

The amount of carbon obtained was then multiplied by 2 to obtain the percentage of soil organic matter (C forms an average of 58 % of soil organic matter).

Determination of available phosphorus

The Olsen technique was used to determine phosphorus levels (Okalebo et al. 2002). A polyethylene shaking container was filled with 2.5 grams of sieved soil and 50 ml Olsen extraction solution (0.5 M $NAHCO_3$ pH 8.5). The mixture was agitated for 5 minutes on a mechanical shaker before filtering through Whatman No 5 paper. Flasks were then filled with 10 ml of P standard solution, 10 ml of the sample, and 2 reagent blanks, with 5 ml of 0.8 boric acid added to each flask. After that, 10 mL of ascorbic acid reagent was added to each flask, followed by 50 mL of distilled water. The contents were sealed and thoroughly shaken. The absorbance of the solution was measured at a wavelength of 880 nm after one hour. The standard P calibration curve was then used to calculate the solution's parts per million (ppm). The phosphorus concentration in the sample was estimated as P mg kg^{-1} using the formula:

$$P = \frac{(a-b) \times v \times f \times 1000}{1000 \times w}$$

Where:

a = concentration of P in the sample b = concentration of P in the blank

v = volume of the extracting solution f = dilution factor

w = weight of the sample.

Determination of total nitrogen

Total nitrogen was quantified using a wet oxidation method based on Kjeldahl digestion with sulphuric acid and a catalyst (Anderson and Ingram 1993). First, a piece of the soil sample was crushed and sieved at 0.5 mm. Then, 0.3g of it was consumed and digested for 1 hour at 110ºC using a 2.5 ml digestion mixture (dissolved 3.2 g salicylic acid in 100 ml sulphuric acid – selenium mixture.) After

that, the solution was cooled, hydrogen peroxide was added, and the mixture was heated at 330ºC until colorless. Finally, 25 mL of distilled water was added until the sediments were completely dissolved. Total nitrogen was determined by calorimetry, with absorbency measured at 650nm and the following equation calculated:

$$\% \text{ N in soil sample} = \frac{(a - b) \times v \times 100}{1000 \times w \times al \times 1000}$$

Where:

a = Concentration of N in the solution b= Concentration of N in the blank

v = Total volume at the end of analysis procedure w = Weight of the dried soil sample taken

al = Aliquot of the solution taken for analysis.

Determination of exchangeable Potassium, sodium, calcium and magnesium, and cation exchange capacity

The exchangeable Potassium, sodium, calcium, and magnesium were removed by leaching the soil sample with neutral normal ammonium acetate. First, 5 grams of acid-washed sand was placed in numbered plastic funnels with an absorbent cotton wool stopper, followed by 5 grams of soil mixed with 5 ml sand. On top of the funnel, another layer of sand was added. Next, a funnel was filled with a Whatman filter paper No. 42 and inserted into the neck of a 250 ml flask. The funnel was then filled with a 10 aliquot of 20 mL ammonium acetate, which was allowed to drain through. The flask was then removed after being repeated several times. The ammonium ions (NH_4+) took the place of the soil's exchangeable cations. Titration with EDTA was used to quantify the exchangeable calcium and magnesium ions' presence in the ammonium acetate leachate, whereas flame photometry was used to determine Potassium and sodium.

The same soil and sand funnels were used to determine cation exchange capacity. The sample was repeatedly washed with methyl alcohol to eliminate leftover ammonium acetate. After transferring the alcohol-washed soil to a round-bottomed flask, 500 mL of water was added and connected to a Liebig condenser to create a 500 mL conical flask containing 20 mL of 2% boric acid and a few drops of mixed indicator (methyl red and methyl blue in methanol). The sample was then treated with three spatulas of magnesium oxide (to displace the NH_4 trapped in the soil). The contents of the flask were heated until 300 ml was distilled over and collected in the receiver. The liquid in the receiver turned green instead of blue. Next, to assess the amount of NH_4 in the distillate, the contents of the receiver were titrated with 0.1 N HCl to a pink endpoint. Because the initial soil sample weighed 5.0 g, each milliliter of 0.1 N HCl employed in the titration was equivalent to 2 m.e. per 100 g exchangeable capacity.

Determination of mycorrhiza spore count

The wet sieving and decanting procedure (Gerdemann and Nicolson 1963) were used first, followed by sucrose centrifugation (Daniels and Skipper 1982). The soil sample was suspended in water before being decanted using sieves (with 0.350 mm, 0.125 mm, and 0.045 mm). The contents of the medium and finest sieves were transferred separately with some water to 100 ml centrifuge tubes. Next, a gradient was produced by injecting a 40 ml sugar solution (70 g dissolved in 100 ml water) into the bottom of the tube. The sample was centrifuged for 2 minutes at 2000 rotations per minute. Soil particles settled to the bottom, and spores stayed on the surface during this process. Spores were removed with a syringe and deposited on a clean sieve with a 0.045 mm mesh hole, then rinsed in water for 3 minutes before being transferred to a petri dish. A stereomicroscope with a magnification of 40X was used to examine the sample. After that, the spores were counted.

Data analysis

Analysis of variance was performed on the data using the computer software Genstat for Windows version 8.11 (Genstat for Windows) (Genstat 2005). Next, the standard error of differences in means was employed to distinguish treatment means at a 95% confidence interval. Finally, mycorrhizal reliance was determined as the yield of inoculated plants minus the yields of non-inoculated plants divided by the yield of non-inoculated plants multiplied by 100.

RESULTS AND DISCUSSION

Soil chemical properties

The soil chemical properties for the Gatanga study site are shown in Table 1.

Soil pH

The soil in the area had a mean pH of 5.4 and was classed as mildly acidic (Table 1). That could have happened because most of the base cations were leached off, leaving clay colloids dominated by aluminum and hydrogen ions (Giller 2001). When large amounts of aluminum ions (Al3+) in the soil combine with water molecules at pH levels below 5.5, H+ is produced (aluminum hydrolysis). Aluminum ions may be concentrated to the point where root development is hindered or delayed, preventing plants from absorbing water or nutrients. As a result, they grow stunted and develop nutritional shortage syndromes (Ball 1999). One of the most critical soil variables that affect nutrient availability is pH. Macronutrients such as magnesium, calcium, and phosphorus are often less available in acidic soils. Still, micronutrients such as manganese, iron, boron, zinc, and copper are typically more available in high-pH soils but become less available as the pH increases above 8. (Muriuki and Quareshi 2001). The availability of molybdenum, on the other hand, diminishes as the pH of the soil decreases, which is especially true in the case of legume production. Nitrogenase, a molybdenum-rich enzyme, is found in the nodules of leguminous crops. As a result, if the soil pH is low and the available molybdenum is low, legumes will seem N strained, and leguminous crop output will decline. Because phosphorus mineralization is greater in near-neutral soils than in more acidic soils, the release of key elements from soil organic matter through mineralization could have been governed indirectly by soil pH through its influence on microbial activity (Muriuki and Quareshi 2001).

Table 1. Soil chemical properties for Gatanga site, Kenya

Soil parameter	Level of nutrient	Optimum level	remarks
pH 1:2.5 (soil: water)	5.4	5.5-7.0	Acidic
Phosphorus	8.0 ppm	15-25	Low
% Nitrogen	0.1%	0.2-0.5	Low
%Carbon	0.5%	10-30	Low
% Soil organic matter	1.0%	20-60	Low
Potassium	0.3 Cmol/kg	0.2-0.6	Adequate
Magnesium	0.4 Cmol/Kg	0.5-4	Adequate
Calcium	2.9 Cmol/kg	4-10	Low
Sodium	0.1 Cmol/kg	0.2-0.5	Low
CEC	15.5 Cmol/kg	15-25	Low

Available phosphorus

Compared with the optimal plant growth level, the available soil phosphorus level was low (8.0 ppm), which might be explained by its chemical linkage to iron and aluminum as a somewhat insoluble precipitate that makes it unavailable to plants (Giller 2001). Soil pH has a big impact on phosphorus availability. After reacting with free aluminum and iron ions in the soil at a low pH (pH 5.5), phosphates of limited solubility are generated, effectively "tying" it up (Wieldetholt and Johnson 2005). In addition, excess calcium in the soil solution due to basic soil conditions (pH > 7.5) might precipitate with P, reducing its availability. At pH 5.5-7.5, phosphorus could be the most accessible for plant uptake (Muriuki and Quareshi 2001). Phosphorus may have existed in organically or inorganically formed soils of Gatanga. Moreover, the soils of the Gatanga site contain little organic matter, an essential source of labile or quickly mineralized phosphorus; changes in soil organic matter (SOM) are likely to be accompanied by changes in plant availability of P (Frizano 1999).

Total nitrogen

Table 1 shows that the total nitrogen concentration of the soil was low (0.1 %). The low organic matter condition of the Gatanga site's soils could have contributed to this. The mineralization of soil organic matter is one of the sources of nitrogen. Nitrogen mineralization rates in the field are known to be affected by differences in the quantity and quality of soil organic nitrogen (Schroth et al., 2003). Microorganisms multiply and contribute to mineralization when they have enough food in the form of soil organic matter (Sande et al. 2001). Soil microorganisms play a key role in decomposing soil organic matter (SOM) and releasing inorganic nutrients into the soil by acting as a catalyst. The nutrients are then available for plant consumption (Smith 1994). Therefore, soil microorganisms must have been low in the Gatanga soils due to the low organic matter concentration, resulting in low organic nitrogen mineralization and, thus, a low total nitrogen level.

Organic matter in the soil

Table 1 shows that the soils at the Gatanga location have little soil organic matter (1 %). Living bacteria, partially decomposed plant debris, and hummus make up the organic matter in the soil (Griffin 2006). The soil's low level of organic matter may have been caused by crop removal and a lack of organic material in the form of manure in the area. Low crop yields, as a result of insufficient or non-use of fertilizers, could have resulted in little residue being returned to the soil, adding to the low levels of organic matter. Through the binding of microscopic soil particles into bigger aggregates by crop leftovers and some microorganisms like mycorrhiza, soil organic matter works as a revolving bank account for nutrients, improves soil structure, and reduces soil erosion (Perucci et al. 2000). The cation exchange capacity, and thus the soil's ability to retain nutrients in a plant-available form, in soils dominated by low activity clay, as is the situation at the Gatanga site, is highly influenced by its organic matter concentration (Schroth et al. 2003)

Potassium

Table 1 shows that the soils at the Gatanga site have appropriate potassium levels, which could be explained by the fact that the rocks that produced Gatanga's soil contained sufficient Potassium. The findings are consistent with those of other researchers who have found that Potassium is not a key nutrient-limiting factor in Central Kenya (Gikonyo et al. 2000). Potassium is a macronutrient and one of the most important minerals for plant growth (Muriuki and Quareshi 2001), which involves water, nutrient, and carbohydrate transport in plant tissue. Although Potassium (K) is not found in any major plant component, it is important for various physiological activities essential for plant growth, such as protein synthesis and maintaining plant water balance (Beegle 1990).

Magnesium

Magnesium levels in the soil were low but not insufficient, as indicated in Table 1, at 0.4 Cmol/kg, compared to the optimum for plant growth, which is 0.5-4 Cmol/kg. The poor organic matter content of the soil may have exacerbated the condition. As a result, the soil's ability to hold cations such as magnesium is limited, and the nutrient is easily leached. Magnesium shortage causes poor and stunted plant growth since it is the primary core of the chlorophyll molecule in plant tissue (Mayland 1983).

Calcium

Table 1 demonstrates that calcium levels in the soil were low. That could be due to a lack of organic matter in the soil, causing the soil to be unable to retain the cation and, as a result, leaching. Calcium is a necessary component of plant cell walls (Muriuki and Quareshi 2001).

Sodium

Sodium levels were low, at 0.1 %, compared to the ideal values for crop growth (see Table 1). Soil dispersion, poor water infiltration, and probable salt toxicity in plants would occur from sodium levels exceeding 0.5 % (Muriuki and Quareshi 2001).

Cation exchange capacity

The ability of a soil to hold cation nutrients is measured by its cation exchange capacity. Cations are elements that have a positive charge (Muriuki and Quareshi 2001). Calcium (Ca2+), magnesium (Mg2+), potassium (K+), sodium (Na+), and aluminum (Al3+) are the most prevalent exchangeable cations in soil. Clay and humus colloids have negatively charged particles that hold cations (Sachs, 1999). Table 1 demonstrates that the soils at the Gatanga experimental site had a poor cation exchange capacity (15.5 Cmol/kg). That could be due to the soil's poor organic matter content. The consequence is that the soil's nutrient and water storage capacity is insufficient. Because the cation reservoir (humus and clay) is low, soluble elements like potassium sulphate cannot be retained efficiently. Lack of water inhibits microorganisms like mycorrhiza, which are obligatory symbionts that rely on plant roots for existence. Because the cation nutrients are in the soil water, these soils are also prone to leaching. CEC can be improved by adding lime, which raises the pH, or by adding organic matter, which lowers the pH.

Mycorrhiza inoculant's spore count

The spore count was determined using a mycorrhiza inoculant obtained from the Kenya Forestry Research Institute (KEFRI) and utilized in on-station and on-field experiments. Table 2 summarizes the findings. *Glomus intraradices*, *Glomus etunicatum*, and *Gigaspora albida*, three mycorrhiza species, were employed to inoculate the soybeans. *Glomus etunicatum* had the largest spore count (205) in the long rain experiments, but *Glomus intraradices* and *Gigaspora albida* had the same number (140) of spores per 30 grams of inoculant. *Glomus etunicatum* had the largest number of spores (220) in short rains and the experiments at the screen house, followed by *Glomus intraradices* (180) and *Gigaspora albida* (135) per 30 grams, as indicated in Table 2. Mycorrhiza fungi species and strains have been shown to differ in their ability to improve nitrogen uptake and plant growth (Gracy and Bagyaraj 2005), necessitating the use of three different species in the trial.

Glomus leptotichum and *Glomus intraradices* were the best AM symbionts in boosting plant biomass compared to the others in an experiment to examine the efficacy of eleven mycorrhiza fungus on Kalmegh (*Andrographis paniculata*) (Chiramel et al. 2006). In addition, root colonization and sporulation were higher with the two symbionts, allowing for increased fungal-host contact and nutrient exchange and enhancing plant growth.

Effect of mycorrhiza and *Rhizobium* inoculation on soybeans germination

Table 3 demonstrates that the germination of soybeans was significantly different for the various treatments ($p<0.05$) in both the on-farm and on-station experiments. Only the dual inoculated, and mycorrhiza treated plants (S+R+M and S+M) demonstrated significantly greater germination rates than the control plants during the LR 2005 season (S). Soybeans infected with mycorrhiza (S+M) had the maximum germination percentage of 97.5%, whereas those inoculated with *rhizobium* and planted with phosphatic fertilizers (S+P+R) had the lowest germination percentage of 70%. The performance in decreasing order of the germination rate was S+M>S+R+M> S> S+P+M> S+R> S+P>S+P+R.

In the on-farm experiments 2005 LR, pairwise comparisons between the mycorrhizal plants and the control (soybeans alone with 80% germination) found that inoculation considerably enhanced germination in the S+M (97.5%) and S+R+M (87.5%) treatments, but not in the S+P+M treatment, where the rate of germination declined slightly to 77.5 %.

Table 2. Mycorrhiza spore count in the inoculant sourced from KEFRI used in the on-field and on-station experiments

Mycorrhiza species	Number of spores per 30 grams pure inoculum	
	Long rains 2005	Short rains 2005 and on-station
Glomus intraradices	140	180
Glomus etunicatum	205	220
Gigaspora albida	140	135

Table 3. Germination of soybeans on-field in Gatanga and on-station at Kenyatta University, Kenya, under different treatments

Treatments	On-farm		On-station	
	Long rains (%)	Short rains (%)	sterile soil (%)	Non-sterile soil (%)
Soybeans + *Rhizobium*	75.0cd	79.5a	77.7c	100.0a
Soybeans + *Rhizobium* + Mycorrhiza	87.5b	67.0d	100.0a	89.0b
Soybeans + P fertilizer + *Rhizobium*	70.0d	79.0a	77.7c	89.0b
Soybeans + P fertilizer + Mycorrhiza	77.5c	54.0d	100.0a	100.0a
Soybeans	80.0c	73.0b	89.00b	67.0c
Soybeans + Mycorrhiza	97.5a	77.0a	67.0d	56.0d
Soybeans + P fertilizer	70.5d	77.0a	67.0d	67.0c
SED	2.7	1.5	0.22	0.31

Note: Numbers in each column followed by the same letter are not significantly different at p = 0.05

Dual inoculation of soybeans with mycorrhiza and *Rhizobium* (S+R+M) resulted in significantly better germination (87%) than *Rhizobium*-treated plants (75%) but significantly lower germination than mycorrhiza-inoculated plants (S+M) (97.5%).

S+R had the highest germination rate of 79.5% in the second season, with short rainfall in 2005, whereas soybeans planted with phosphatic fertilizers and inoculated with mycorrhiza (S+P+M) had the lowest (54%). S+R, S+P+R, S+M, S+P, S, S+R+M, and S+P+M had the highest germination rates, followed by S, S+R+M, and S+P+M.

Treatments S+R, S+P+R, S+M, and S+P showed considerably higher germination rates than the control (S), whereas mycorrhizal treatments S+R+M and S+P+M had significantly lower rates than the control (S) ($p<0.05$). Furthermore, the single inoculated plants S+R (79.5%) and S+M (77%) germination rates were much greater than the dual inoculation plants S+R+M (67 %).

S+R+M and S+P+M had the highest germination rates of 100 % in the on-station sterile soils experiment, whereas S+P and S+M had the lowest at 67%. S+P+M=S+R+M>S> was the performance in decreasing order of germination. S+M=S+P> S+R=S+P+R> S+M=S+P. Only S+P+M and S+R+M (both mycorrhizal) germination rates were considerably greater than the control ($p<0.05$).

Dual inoculation with mycorrhiza and *rhizobium* improved germination rate in sterile soils, demonstrating that singly treated plants S+M and S+R had significantly lower germination rates than dual injected plants (S+R+M). S+P+R had a considerably greater germination percentage than S+P, indicating that *Rhizobium* inoculation may have boosted the germination rate in P-applied plants, whereas S+R had the same rate. The germination rate of S+P+M was much greater than that of S+P and S+M, indicating that mycorrhiza inoculation may have aided germination.

S+R and S+P+M had the highest germination rates of 100 % in non-sterile soil, while S+M had the lowest at 56%. S+R and S+P+M, S+R+M and S+P+R, S and S+P, and S+M were the top performers in decreasing the germination rate. When comparing the treatments to the control (S) plants, it was discovered that the S+R, S+R+M, S+P+M, and S+P+R treatments had considerably greater germination rates than the S+M and S+P treatments ($p<0.05$). The germination rates of dual inoculated plants (S+R+M) were significantly lower than the *rhizobium* inoculated (S+R) treatments but significantly higher than the mycorrhiza inoculated (S+M) treatments. That indicated that mycorrhiza, but not *Rhizobium*, might have had a role in germination.

The trial results were inconclusive, indicating that inoculation with mycorrhizal fungi and *Rhizobium* bacteria could boost soybean germination. Furthermore, this is because the plants had not formed roots at the time of germination and hence had not begun the symbiotic interaction with the two bacteria. Mycorrhiza hyphae respond to the presence of a root by growing towards it, establishing contact, and growing along its surface to form an association (Brundret et al. 1994). Many investigations have revealed a time lag between mycorrhizal inoculation and the time it takes for its effects to develop in the plant (Brandon and Shelton 1993). The fungus is at the lag stage during germination and is autotrophic (Sieverding 1991)

Those findings contrast with Kikuchi et al. (2007), who revealed that flavonoids detected in root exudates during plant germination operate as signaling molecules in symbiotic ectomycorrhizal fungi-woody plant partnerships. Similarly, the *Rhizobium* bacteria are in their saprophytic phase. Therefore, they will only infect and gain admission into the root (infective phase) and subsequently participate in the creation of a functional root nodule (symbiotic phase) in response to chemical signals (flavanoids) released by the germinating plant. Brandon and Shelton (1997) reached the same conclusion while studying the factors impacting the early growth of *Leucaena leucocephala*.

As AM fungus progressively expanded root colonization, phosphorus extraction efficiency improved, implying that high rates of P would be required to compensate for early delayed colonization. Large impacts of mycorrhiza inoculation were observed 41 days after sowing in their trials.

Germination is a process that involves the mobilization and use of food reserves (Howell 1960), and it is influenced by environmental elements like temperature, soil moisture, nutrients, and oxygen availability (Fagena et al. 1997). In addition, internal seed physiology, such as seed vitality, genetic potential, and seed maturation, as well as hormonal or chemical changes that occur as the seed is building its food stores, affect seed germination (Bennet 2004). One or all of these factors could explain the discrepancies in germination rates across the three treatments.

Effect of inoculating soybeans with mycorrhiza and *rhizobium* on height increment

In the on-field experiments, significant differences in height were seen between the various treatments at all three phases of height assessment, namely 30, 45, and 60 days after planting (Table 4).

After the 30th day of the LR 2005 season, only the treatments S+R+M and S+P+M (both mycorrhizal) had considerably larger height increments than the control S. In contrast, on the 45th day, S+R+M, S+P+R, S+P+M, and S+M all had significantly higher height increments than the control. S+R, S+R+M, S+P+R, and S+P+M levels were considerably higher on the 60th day than in control. The increased height increment in mycorrhizal (S+R+M) plants treated with *rhizobium* could be attributable to increased nutrient absorption and photosynthetic rates. It is well established that mycorrhizal colonization enhances plant development by boosting nutrient uptake and use (Marschner and Dell 1994, Clark and Zeto 2000). AM fungus may have shortened the distance nutrients diffused through soils to the roots via their hyphae.

Table 4. Height of soybeans during the long and short rains 2005 under different treatments at the on-field experiment at Gatanga, Kenya

Treatment	Long rains 2005 (cm)			Short rains 2005 (cm)		
	30 days	45 days	60 days	30 days	45 days	60 days
Soybeans + *Rhizobium*	9.20b	10.80d	19.90b	7.70c	10.70cd	14.20a
Soybeans + *Rhizobium* + Mycorrhiza	14.90a	18.50a	26.90a	8.85a	12.65a	15.25a
Soybeans + P fertilizer + *Rhizobium*	10.75b	14.55bc	21.95b	8.65ab	12.20ab	15.15a
Soybeans + P fertilizer + Mycorrhiza	15.45a	16.00b	23.50ab	8.00ba	10.60d	14.95a
Soybeans	7.90b	10.45d	14.30c	8.25abc	11.65bc	14.10a
Soybeans + Mycorrhiza	10.55b	14.03c	16.30c	7.90bc	11.70ab	14.35a
Soybeans + P fertilizer	9.00b	10.35d	15.75c	7.95bc	11.55bcd	13.75a
SED	1.90	1.24	1.71	0.39	0.50	0.84

Note: Numbers in each column followed by the same letter are not significantly different at p= 0.05

In the on-field LR 2005 season experiment, dual inoculation with mycorrhiza and *Rhizobium* (S+R+M) boosted soybeans' height significantly ($p<0.05$) over the control (S) at all three phases. At all stages, dual inoculation with mycorrhiza and *Rhizobium* (S+R+M) resulted in a significantly greater height increment than S+R and S+M (singly inoculated plants). Furthermore, this could have occurred due to the benefits derived from the tripartite symbiosis of legume-AM fungi-*Rhizobium* through host nutrition stimulation (Barea et al. 1992). The extraradical mycelium of the AM fungus extends beyond the zone of phosphate depletion, establishing a new source of soluble phosphates (Smith and Read 1997). Rhizobia's nitrogenase enzyme fixes atmospheric nitrogen in nodules, while fungal hyphae aid in the uptake of ions, primarily phosphates, via mycorrhizal roots (Postgate 1998; Leigh 2002). As a result, the photosynthesis rate increases, so the height increases.

Rhizobium inoculation plus the application of phosphatic fertilizer (S+P+R) resulted in a considerably greater height increase than *Rhizobium* inoculation alone (S+R) on the 30th day. On the 45th and 60th days, inoculating with *rhizobium* and applying phosphatic fertilizer (S+P+R) resulted in a considerably greater height increment than plants treated with phosphatic fertilizer (S+P). Increased height growth was facilitated by the addition of P fertilizer to soils that were deficient in P.

At all stages, inoculating plants with mycorrhizas and applying phosphatic fertilizers (S+P+M) resulted in considerably greater height increase than mycorrhiza-singly inoculated plants (S+M) and phosphatic fertilizer-applied plants (S+P). At all phases, the mycorrhizal inoculated plants (S+M) had the maximum height, indicating the mycorrhiza fungi's efficacy in extracting nutrients, particularly P, from the soil. Mycorrhizas have been shown to boost growth by boosting nutrient intake, particularly P and other critical elements (Marschner and Dell 1994; Clark and Zeto 2000). However, this was accomplished by mycorrhizal fungus physically exploring the soil more extensively than the roots. The ratio of hyphae to root length has been determined to be between 300 and over 8000. (Read and Boyd 1986; Jones et al. 2001). The discrepancies between the three treatments were significantly different ($p<0.05$) at all stages during the short rains of the 2005 season, as well as during the long rains. At all stages, all treatments except the dual inoculation plants (S+R+M) on the 45th day did not perform substantially better than the control (S). On the 30th and 45th days, dual inoculation with mycorrhizal fungi and *Rhizobium* bacteria (S+R+M) resulted in a considerably greater growth increment than *Rhizobium* singly infected plants (S+R), but not on the 60th day. Inoculating with mycorrhiza and *Rhizobium* (S+R+M) resulted in a considerable height increase above (S+M) on the 30th day, but not at the other stages.

The data indicate that the soybeans-Rhizobium-mycorrhiza symbiosis did not benefit the short rains crop, which would have increased photosynthesis and, thus, height increment. During the 2005 long drops of rain, when moisture was not a constraint, the *Rhizobium* bacteria transformed atmospheric nitrogen to ammonia, which the plants might have ingested and converted to amino acids, resulting in a growth boost. Moisture stress during the 2005 season's short rains may have harmed these bacteria's ability to fix atmospheric nitrogen, as P and other nutrients can only be absorbed in solution. In field experiments, soybeans planted with P alone (S+P) grew at a non-significant rate relative to the control (S). Although P was supplied, plants' roots could not absorb it properly without the benefit of the increased surface area caused by the mycorrhizal connection. Inoculation with mycorrhiza and application of P fertilizer (S+P+M) resulted in a non-significant height increment above S+M and S+P at any stage during the brief rains. Inoculating soybeans with *rhizobium* and applying P fertilizer (S+P+R) resulted in a little height increase over planting with P alone (S+P) during the short rains.

In the on-station studies, the differences between the various treatments were significant ($p<0.05$) in both sterile and non-sterile soils, except on the 60th day in the sterile soils (Table 5). Dual inoculation with mycorrhiza and *Rhizobium* (S+R+M) resulted in a significantly greater height increase ($p<0.05$) than the control (S) on the 30th and 45th days, but not on the 60th day, in sterile soil. Only on the 30th day did the dual inoculation plants (S+R+M) significantly outgrow the control (S) in the non-sterile soil. Indigenous microbes could have contributed to increased nutrient absorption, resulting in height increment in non-sterile soils. On the 30th and 45th days, dual inoculation with *rhizobium* and mycorrhiza (S+R+M) resulted in a considerably greater height increment than inoculating with *rhizobium* alone (S+R), but not on the 60th day. In non-sterile conditions, dual inoculation (S+R+M) resulted in a considerably greater height increment than inoculating with

rhizobium alone (S+R) on the 30th day.

Dual inoculation with *rhizobium* and mycorrhiza (S+R+M) in sterile soils resulted in a much greater height than S+M on the 30th day. A considerable plant height increased on the 30th observed day in non-sterile soil but significantly reduced on the 45th and 60th days. The observation showed no increase in height above single-inoculated plants (S+R) and (S+M) due to the dual inoculation with mycorrhizal fungi and *rhizobium*. The benefits of legume *Rhizobium* mycorrhiza symbiosis were not reflected in these experiments. Without the benefits of mycorrhiza, phosphorus and other generally stable elements of the soil may not have been accessed by plants. The inadequate delivery of these nutrients impeded the BNF process.

The difference between the (S+P) and the mycorrhiza plants (S+P+M) was considerably higher in all tests (S+P+M) (sterile and non-sterile). Mycorrhizal fungi could have played a key role in acquiring additional P and other nutrients from distances beyond phosphorus depletion. Nutrients for BNF must eventually lead to greater photosynthesis.

In an experiment to assess rock phosphates and mycorrhizal impacts on growth and nutrient uptake of F albida seedlings in alkaline soil, even without rock phosphates, mycorrhiza inoculated plants achieved a higher biomass result. The dependency on mycorrhiza decreased as the phosphates increased (Ba and Guissou 1996).

In the case of most tropical soils, the soil at the experimental location was heavily weathered, acidic, and base-leached, resulting in low fertility (Giller 2001). Phosphorus is known to be rapidly reduced within a few millimeters of the growing root (Sieverding 1991). This zone cannot be fully refilled with P due to the exceedingly slow diffusion rate. External mycorrhizal mycelium extends far beyond this zone, increasing the amount of soil available for P absorption (Sieverding 1991). Acidic soils promote increased organic acid excretion, which increases the solubility and uptake of the mineral nutrient P and the micronutrients Zn, Fe, and Mn in particular (Marschner 1992). Phosphorus is a crucial nutrient that is deficient in nitrogen fixation. *Rhizobium* is extremely susceptible to the mycorrhizal association and requires it to meet the high P requirements for nodulation and nitrogen fixation (Bhatia et al. 1998). Dual inoculation with micorrhiza, *G. caledonius*, and *rhizobium* improved the performance of transplanted *P. juliflora* in a semiarid wasteland.

Similarly, *Leucaena leucocephala* injected with three *Rhizobium* strains greatly boosted height growth in a field experiment in Jammu, India (Dutt and Palhania 1983). Marques et al. (1999) concluded after inoculating *Cetrolobium tomentosum*, a woody legume, with both mycorrhizal and *rhizobium* bacteria. *Rhizobium*-inoculated plants increased in height only when connected with a mycorrhizal fungus.

Effect of mycorrhiza and *Rhizobium* inoculation of soybeans on root collar diameter

In the on-farm experiments, there was a significant difference ($p<0.05$) between the various treatments during the long and short rains of the 2005 seasons, but not in the on-station experiments, as shown in Table 6.

Table 6 indicates a significant increase in root collar diameter over the control and the other treatments in the on-farm LR 2005 studies. For example, inoculating soybeans with mycorrhiza (S+M) or *Rhizobium* (S+R) enhanced root collar diameter by 54.8% and 30%, respectively, above the control.

Table 5. Height of soybeans in the on-station sterile and non-sterile soils under different treatments at Kenyatta University, Kenya

Sterile soil (cm)				Non-sterile soil (cm)		
Treatment	**30 days**	**45 days**	**60 days**	**30 days**	**45 days**	**60 days**
Soybeans + *Rhizobium*	16.2cd	41.7bc	58.7	27.9c	51.0b	75.7b
Soybeans + *Rhizobium* + Mycorrhiza	25.4a	49.3a	61.7	34.7a	53.0b	73.7b
Soybeans + P fertilizer + *Rhizobium*	14.0de	20.0d	59.7	12.1d	25.0c	56.7c
Soybeans + P fertilizer + Mycorrhiza	23.7ab	53.0a	75.3	35.0a	54.0b	74.7b
Soybeans	15.7d	36.0c	56.7	28.9c	46.3b	62.bc
Soybeans + Mycorrhiza	21.7b	52.3a	68.3	30.3bc	65.7a	88.7a
Soybeans + *Rhizobium*	11.3e	41.3bc	62.7	8.9d	23.7c	62.0bc
SED	1.5	3.6	4.6	2.1	5.0	6.4

Note: Numbers in each column followed by the same letter are not significantly different at $p \leq 0.05$.

Table 6. Effect of mycorrhiza and *Rhizobium* inoculation of soybeans on root collar diameter in Gatanga and Kenyatta University, Kenya

Treatments	On-farm (cm)		On-station (cm)	
	Long rains	**Short rains**	**Sterile**	**Non-sterile soil**
Soybeans + *Rhizobium*	3.2d	2.3d	2	2
Soybeans + *Rhizobium* + Mycorrhiza	4.5ab	3.8ab	2	3
Soybeans + P fertilizer + *Rhizobium*	4.1abc	3.4c	1	2
Soybeans + P fertilizer + Mycorrhiza	4.6a	3.9a	2	2
Soybeans	2.5 e	3.5bc	2	2
Soybeans + Mycorrhiza	3.9acd	3.8ab	1	2
Soybeans + *Rhizobium*	3.5cd	3.5bc	2	2
SED	0.4	0.2	0	0

Note: Numbers in each column followed by the same letter are not significantly different at $p=0.05$.

Dual mycorrhizal inoculation of soybeans (S+R+M) resulted in a root collar diameter gain of 80% above the control (much more than S+R), which indicated that mycorrhizal fungus may have contributed to the root collar's growth.

Although the increase was not significant, the treatment S+R+M had a bigger root diameter than S+M. Thus, when soybeans were planted with only one microorganism inoculant (S+R and S+M), the dual inoculated plants (S+R+M) had a higher root collar diameter. The increased diameter of the root collar in mycorrhizal plants may result from increased inorganic nutrient absorption and photosynthesis rates (Marschner 1992). Increased photosynthetic rates resulting from the availability of P and other nutrients via wider mycorrhiza exploration of soils and provision of plant accessible N through biological nitrogen fixation by the *Rhizobium* bacteria could have resulted in larger diameters in the dual inoculation plants (S+R+M) (Allen et al. 1981). In the long rains, the treatment S+P+R showed an increase that was not substantially greater than S+P and similar to S+R. Providing P and inoculating with *rhizobium* may have resulted in increased photosynthesis rates and, thus, a bigger root collar diameter. Dual inoculated plants (S+R+M) had the same root collar diameter as mycorrhiza solely treated plants (S+M) during the short rain drops but were substantially larger than *rhizobium* inoculated plants (S+R). Dual inoculation improved the diameter of the root collar by 8.6% compared to the control S. Due to dryness, mycorrhizal fungi could not efficiently perform their nutrient acquisition duty.

Though the differences between treatments were not significant in the on-station experiments, soybeans inoculated with both mycorrhiza and *Rhizobium* (S+R+M) exhibited the largest root collar diameter (3 mm) in both the sterile and non-sterilized soil, as indicated in Table 6. Mycorrhiza inoculation has been found to increase plant root collar diameter. Ghosh and Verma (2006) inoculated *Acacia mangium* with three VA-mycorrhiza fungi (*Glomus occultum, Glomus aggregatum*, and *Glomus mosseae*). They discovered that all inoculations increased shoot, height, root collar diameter, chlorophyll, and biomass compared to uninoculated control seedlings. Inoculation with mycorrhizal fungus and rhizobial bacteria does not lead to steadily increased plant development. In a study of the effect of dual inoculation with *rhizobium* and mycorrhiza on the growth of *Calliandra calothyrsus*, it was discovered that, while plants inoculated with both symbionts grew better than controls, the results were not statistically significant. Inoculation did not have a long-term effect on tree growth, even when most nodules were inoculated (Leisueur and Sarr 2008).

Effect of mycorrhiza and *Rhizobium* inoculation of soybeans on root dry weight

Some significant differences among the treatments were shown in the root dry weight ($p<0.05$) on-farm 2005 long drops of rain season experiments. Table 7 reveals that the differences between the on-farm short rains 2005 season and the on-station experiments were not significant. The differences between control (S) and the other treatments were significant in the on-field long rains 2005 season experiment, except for S+R and S+P. Soybeans planted with phosphorus and mycorrhizal fungi (S+P+M) had the highest roots dry weight, followed by soybeans planted with phosphorus and *Rhizobium* fungi (S+P+R). As seen in Table 7, the control (S) had the least root mass. The higher weight in mycorrhizal plants could be related to the ability of mycorrhizal fungi to improve nutrient intake via increased surface area for absorption. Enhanced nutrient uptake must increase photosynthetic rate, resulting in increased plant growth. Therefore, the roots grew faster, implying an enhanced flow of photosynthates to the roots and fungal hyphae. The association of plant roots with mycorrhizal fungi may have increased the amount of phosphorus available to the plants, increasing root biomass.

The results of these experiments suggest that higher photosynthates to the roots increased mycorrhizal plants' root biomass due to increased phosphorus nutrition caused by mycorrhizal fungi. Gueye (1990) reached the same conclusion after inoculating Bambara groundnuts with *rhizobium* and mycorrhiza (*Glomulus mosseae*). Mycorrhizal infection was reported to always result in a considerable rise in root weight. In a greenhouse experiment in Belgium, micropropagated bananas inoculated with mycorrhizal *Glomus interradices* exhibited significantly larger shoots, dry root weight, and P content than non-mycorrhizal plants (Declerck et al. 2002).

Table 7. Effect of mycorrhiza and *Rhizobium* inoculation of soybeans on dry rot weight in On-farm and On-station Experiments in Gatanga, Kenya

Treatments	On-farm (g/plant)		On-station(g/plant)	
	Long rains	Short rains	Sterile	Non-sterile soil
Soybeans + *Rhizobium*	0.522d	0.345	0.04	0.09
Soybeans + *Rhizobium* + Mycorrhiza	1.146bc	0.761	0.5	0.21
Soybeans + P fertilizer + *Rhizobium*	1.44abc	0.459	0.05	0.08
Soybeans + P fertilizer + Mycorrhiza	1.96a	0.470	0.32	0.12
Soybeans	0.477d	0.414	0.07	0.11
Soybeans + Mycorrhiza	1.034c	0.705	0.16	0.2
Soybeans + *Rhizobium*	0.781d	0514	0.08	0.05
SED	0.3114	0.1693	0	0

Note: Numbers in each column followed by the same letter are not significantly different at p=0.05.

During the SR season, the discrepancies in root dry weight between treatments were not significant. Drought stress was applied to the crop during the flowering stage, resulting in growth retardation. Moreover, this is in contrast to an experiment in which drought stress raised the percentage of mycorrhizal infection in *G. sepium* and *Albizia lebbeck* by an average of 8-41 % (Awatonye et el. 1992).

The root dry weight differences across the four treatments were not significant ($p<0.05$) in the on-station studies. That was true for both sterilized and non-sterilized soils, albeit mycorrhizal plants had a larger diameter than non-mycorrhizal plants, as shown in Table 7. effect of Mycorrhiza and *Rhizobium* inoculation on soybeans.

Shoot dry weight

As Table 8 indicates, there was a significant difference in dry shoot weight (p0.05) between the different treatments only during the 2005 long drops of rain season on the farm, but not during the 2005 short drops of rain season or on-station experiments. During the 2005 long drops of the rain season, except for the S+R and S+P treatments, differences between the control and each of the other treatments were significant. The order of performance was S+P+R >S+M> S+R+M> S+P+M> S+R>S and S+P.

The dual inoculated plants (S+R+M) increased their shoot dry weight by 172 % compared to the control S, but the single inoculated plants (S+R and S+M) increased by 103 and 185 %, respectively. Furthermore, this could be attributable to increased inorganic nutrient absorption, particularly P, and increased photosynthetic rates caused by mycorrhiza and *Rhizobium* inoculation (Jia and Gray 2004). As a result, there was a little increase in the shoot dry weight of the dual inoculation plants (S+R+M) compared to S+R. However, the other mycorrhizal plants, S+M and S+R+M, performed better than the non-mycorrhizal plants, except for treatment S+P+M.

The dual inoculated plants could be attributed to increased inorganic nutrient absorption, particularly of P, and increased photosynthetic rates in inoculated plants. Extensive mycorrhizal hyphal networks may have enabled soybeans to collect phosphorus from sources outside the roots' nutrient depletion zone and to solubilize phosphorus from inaccessible sources (Marschner 1992). Mycorrhizal plants may also have obtained P from ordinarily unavailable sources, both inorganic and organic (Koide and Kabir 2000; Feng et al. 2003). The additional phosphorus may have been used as an energy source in biological nitrogen fixation, a process in which atmospheric nitrogen is converted to ammonia (NH3), which the plant absorbs and converts to amino acids, resulting in increased leaf weight (Hogberg 1986).

Increases in stem and leaf biomass were closely linked with increases in P uptake in an experiment to determine the effect of mycorrhiza inoculation in an alley cropping trial, indicating that the improvement was ascribed to mycorrhiza inoculation (Atayese et al. 1992). Without mycorrhizal colonization, *Faidherbia albida* seedlings developed slowly, producing more biomass when colonized (Ba and Guissoui 1996). At the end of drought stress, inoculating *Faidherbia albida* and *Acacia nilotica* with mycorrhiza and *rhizobium* in barren soil boosted the plant biomass of the two tree species (Onsubi et al. 1992).

Similar results were achieved in an experiment to determine the effect of dual inoculation of black locust (*Robinia pseudoacacia* L.) with rhizobia and glomus on desurfaced soil. Again, a synergistic impact was discovered, as dual inoculation resulted in a 93% increase in shoot mass compared to single inoculation (Ferrali et al. 2008). The authors concluded that mycorrhizal colonization enables nodulated plants to meet their P requirements in soils deficient in P.

Combining S+R+M was the most effective treatment in the 2005 short drops of rain season experiment, followed by S+P+R, S+P+M, S+P, S+R, S+M, and S. Dual inoculation (S+R+M) resulted in greater soybean dry weight. However, the difference was not statistically significant when each microbe was used alone (S+R and S+M). Due to the drought during this experiment, multiple treatments may have failed to demonstrate a meaningful difference over the control and among themselves. The number of rhizobia in the soil may have decreased significantly due to soil drying, as illustrated in Table 8. The rate of nitrogen fixation and the transfer of nitrogen fixation products to shoots may have been slowed down by lowering the soil water content (Giller 2001). Awatonye et al. (1992) concluded the same thing about *Acacia auriculiformis* in an experiment to assess the response of certain tropical nitrogen-fixing woody legumes to drought and mycorrhiza inoculation in sterile soil. They concluded that mycorrhiza-inoculated plants outlived uninoculated plants and had a higher dry matter, nutritional content, and larger leaf surface area.

Table 8. Effect of mycorrhiza and *Rhizobium* inoculation of soybeans on shoot dry weight in Gatanga and Kenyatta University, Kenya, Kenya

Treatments	On-farm (g/plant)		On-station (g/plant)	
	Long rains	Short rains	sterile	Non-sterile soil
Soybeans + *Rhizobium*	3.35bcd	0.7	0.76	0.6
Soybeans + *Rhizobium* + Mycorrhiza	4.49b	1.2	1.92	1.2
Soybeans + P fertilizer + *Rhizobium*	7.94a	1.0	0.65	0.6
Soybeans + P fertilizer + Mycorrhiza	3.92b	0.8	1.39	1.3
Soybeans	1.65cd	0.4	0.51	0.5
Soybeans + Mycorrhiza	4.71b	0.6	1.42	1.2
Soybeans + *Rhizobium*	1.17d	0.8	0.63	0.6
SED	1.097	0.2	0	0

Note: Numbers in each column followed by the same letter are not significantly different at p=0.05

Although the results were not statistically significant, the mycorrhizal plants had greater shoot weights than the non-mycorrhizal plants in the on-station experiments. The presence of indigenous rhizobia or mycorrhiza in unsterilized soil may account for the experiment's findings. Indigenous strains may have competed for nodule occupancy with introduced strains, reducing their efficacy (Marques et al. 1999).

Effects of mycorrhiza and *Rhizobium* inoculation on soybeans grain yields

Table 9 shows inoculating soybeans with mycorrhiza and *rhizobium* resulted in a significant difference ($p<0.05$) between the different treatments on-farm (long rains 2005 season) and on-station sterilized and non-sterilized soils. Due to dryness, no grain yields were realized in the second season (short rains 2005).

Soybeans inoculated with mycorrhiza and *Rhizobium* (S+R+M) produced the highest grain yields during the 2005 long drops of the rain season, followed by soybeans planted with phosphorus and inoculated with mycorrhiza (S+P+M). The remainder of the sequence was; S+P+R, S+M, S+P, and S+R and S in that order. Except for soybeans treated with *Rhizobium* (S+R), all treatments increased grain yields much more than the control (S).

Dual inoculation of soybeans with mycorrhiza and *Rhizobium* (S+R+M) resulted in a 356% increase in grain production compared to the control (S). By contrast, inoculation with either of the microorganisms alone resulted in a 71 percent rise in *Rhizobium* (S+R) and a 189 % increase in mycorrhiza (S+M) growth. Dual inoculation improved grain yield by 82 % and 166 % above singly inoculated plants S+M and S+R, respectively. Thus, mycorrhizal fungi and *rhizobium* operated synergistically since simultaneous inoculation increased grain yield more than either mycorrhizal fungus or *rhizobium* alone (Table 9). Inoculation with mycorrhiza may have expanded the volume of soil to be examined for nutrient intake, therefore improving the efficiency of soil nutrient absorption. Nutrients such as phosphorus, which were in short supply in the site soil, have a far slower diffusion rate in soil than the rate at which growing roots absorb them and, therefore, rapidly deplete the root zone (Busman et al. 2002). The extraradical mycelium of mycorrhizal fungi must have expanded well outside the depletion zone, establishing a new source of soluble phosphates (Smith and Read 1997). Thus, phosphorus was made available to plants and used in nodules for biological nitrogen fixation, where it provided the energy necessary to convert nitrogen to ammonia. As a result of the conversion of ammonia to amino acids and proteins, soybean grain yields were boosted.

Along with providing P, mycorrhiza fungus may have helped to improve nutrient uptake by increasing the absorbent surface (Marschner 1992; Marschner and Dell 1994). The overall consequence was an increase in photosynthetic rate and, as a result, in yields. Mridha et al. (1992) discovered that dual inoculation with *rhizobium* and micorrhiza (*Glomus clarum*) significantly enhanced the growth, yield, and nutrient content of yard-long beans (*Vigna unguiculata* sesquipedalis) compared to the non-inoculated control. Sieverding (1991) conducted over 50 field tests inoculating cassava cultivars with mycorrhiza in acidic soils of varying fertility levels and reported an increase in tuber yields of 20-25 %. Applying P fertilizer to mycorrhiza inoculated Soybeans (S+P+M) resulted in a grain production increase that was not statistically significantly greater than plants grown with fertilizer alone (S+P) or with mycorrhiza alone (S+M). Inoculating soybeans with *rhizobium* and fertilizing with P (S+P+R) increased grain production much more than *Rhizobium*-treated plants (S+R) but not mycorrhiza-treated plants (S+M). Mycorrhiza could have enhanced the volume of soils from which the plant roots obtained P.

Soybeans inoculated with mycorrhizal fungi (S+M) produced the maximum yields in sterilized soils. S+M> S+P+M> S+R+M> S+R> S>S+P and S+P+R, in decreasing order of grain yield. Each therapy was qualitatively distinct from the others. For example, in the 2005 long drops of rain experiment, plants treated with mycorrhiza produced more than non-mycorrhizal plants. In non-sterilized soils, grain yields decreased in the following order: S+R+M> S+P+M> S+M, S+P> S+P+R> S, and S+R. Mycorrhizal plants fared much better than non-mycorrhizal plants in both sets of on-station studies. Dual inoculation of soybeans with mycorrhiza and *Rhizobium* (S+R+M) increased grain yield statistically more than when soybeans were planted with *rhizobium* alone (S+R) but resulted in a gradual decrease when soybeans were planted with mycorrhiza alone (S+M). Dual inoculation (S+RM) increased grain production much more than S+R or S+M in non-sterile soils. The synergistic interactions between the elements of the tripartite symbiotic association (legume-*Rhizobium*-mycorrhiza) have been proven to increase legume productivity.

Table 9. Effect of mycorrhiza and *Rhizobium* inoculation on soybeans grain yield (g/plant) in on-farm and on-station experiments

Treatment	On-farm	On-station	
	Long rains 2005	Sterile	No-sterile soil
Soybeans + *Rhizobium*	0.84cd	0.52d	0.37g
Soybeans + *Rhizobium* + Mycorrhiza	2.24a	0.86c	0.89a
Soybeans + P fertilizer + *Rhizobium*	1.71ab	0.23g	0.62e
Soybeans + P fertilizer + Mycorrhiza	1.98ab	0.9b	0.76b
Soybeans	0.49d	0.49e	0.43f
Soybeans + Mycorrhiza	1.42bc	1.59a	0.74c
Soybeans + *Rhizobium*	1.39bc	0.33f	0.67d
SED	0.32	0.20	0.15

Note: Numbers in each column followed by the same letter are not significantly different at $p=0.05$.

Jia et al. (2004) discovered that plants having symbiotic associations with *rhizobium* and arbuscular mycorrhizal fungi have better photosynthetic rates per unit leaf area. Lizzy (1999) discovered that particular mycorrhizal fungi and *Rhizobium* combinations boosted plant growth and yield when used on peas and lentils. According to Xavier and Germida (2003), pea yield and nitrogen nutrition inoculated with mycorrhizal fungi and *rhizobium* differed depending on the mycorrhizal fungi-*Rhizobium* strain combination used. Inoculating peas with a superior *Rhizobium* strain and a suitable mycorrhiza fungal species increased yield and N nutrition.

Mycorrhiza dependency of soybeans inoculated with mycorrhiza and *rhizobium*

Mycorrhiza dependency, defined as a plant's reliance on mycorrhiza to achieve maximal growth or production at a given level of soil fertility (Brundett et al. 1994), was high (248.9 % to 312.3 %) for all plants treated with mycorrhiza in the on-farm experiment (Table 10). In on-station experiments, mycorrhizal dependence ranged from 75.5 to 224.5 % in sterilized treatments and from 72.1 to 107.6 % in non-sterilized treatments. The effect of competition in non-sterilized soil from indigenous mycorrhiza species implies the low mycorrhiza dependence in this experiment. In general, soybeans are legumes and hence have a coarse root system with a high phosphorus need for biological nitrogen fixation (Brundett et al. 1994).

Due to the low fertility level of the soils at the experimental site (Table 1), mycorrhizal fungi may have played a significant role in supporting the plant in obtaining nutrients, particularly phosphorus, by depleting their large hyphal network and making them available for plant absorption and utilization (Bagyara 1996; Arola et al. 2004), furthermore, this resulted in higher production. Ghosh and Verma (2006) discovered that when *Acacia mangium* was inoculated with three VA-mycorrhiza fungi (*Glomus occultum*, *G. aggregatum*, and *G. mosseae*), its growth was 57 % dependent on *G. occultum*, 47 % dependent on *G. mosseae*, and 46 % dependent on *G. aggregatum*. Ba and Gissou (1996) found that increasing the quantities of rock phosphate and mycorrhiza fungi had a negative effect on the growth and nutrient uptake of *Faidherbia albida* seedlings in alkaline soil. Mycorrhizal-inoculated plants absorbed significantly more P from the soil and rock phosphate than non-mycorrhizal plants.

Effect of inoculating soybeans with micorrhiza and *rhizobium* on root nodule numbers

As indicated in Table 11, the number of nodules in the various treatments differed significantly, that is, (P0.05) in both the on-farm experiments (LR and SR 2005 seasons) and the on-station experiments on sterile and non-sterile soils. In the on-farm experiment (LR), all treatments except S+R and S+P had considerably more root nodules than the control S. The following treatments were performed in decreasing order of the number of nodules: S+M>S+R+M>S+P+M>S+P>S+R=S+P+R>S.

Table 10. Effect of inoculating soybean with mycorrhiza and *rhizobium* on mycorrhiza dependency in on-farm and on-station experiments in Gatanga, Kenya

Treatment		Mean yield (Kg/Ha)	Mycorrhiza dependency (%)
Soybeans	Long rains 2005 (on-field)	299.3	312.3
+*Rhizobium*	Sterilized soil	127.4	75.5
+Mycorrhiza	Non-Sterilized soil	131.9	107.6
Soybeans + mycorrhiza	Long rains 2005 (on-field)	253.3	248.9
	Sterilized soil	235.6	224.5
	Non-Sterilized soil	109.6	72.1
Soybeans + P fertilizer + Mycorrhiza	Long rains 2005 (on-field)	293.3	303.9
	Sterilized soil	133.3	83.6
	Non-Sterilized soil	112.6	76.8
Soybeans (CONTROL)	Long rains 2005 (on-field)	72.6	
	Sterilized soil	72.6	
	Non-Sterilized soil	63.7	

Table 11. Number of soybean nodules under different treatments in the on-farm and on-station experiments in Gatanga, Kenya

Treatments	On-farm (no/plant) 2005		On-station (no/plant) 2005	
	Long rains	Short rains	Sterile soil	Non-sterile soil
Soybeans + *Rhizobium*	6.0b	3bc	3d	4a
Soybeans + *Rhizobium* + Mycorrhiza	26.4a	14.4a	7b	4a
Soybeans + P fertilizer + *Rhizobium*	19.5b	2.0bc	0f	1c
Soybeans + P fertilizer + Mycorrhiza	29.4a	6.3b	8a	4a
Soybeans	3.4b	0.4c	0f	0d
Soybeans + Mycorrhiza	16.6a	6.3b	5c	4a
Soybeans + P fertilizer	6.1b	1.6c	1e	2b
SED	6.40	2.20	0.32	0.40

Note: Numbers in each column followed by the same letter are not significantly different at p=0.05.

In comparison to the individually inoculated plants with *Rhizobium* (S+R) and mycorrhiza (S+M), dual inoculation (S+R+M) resulted in a substantially larger increase in the number of nodules than the control (S).

Dual inoculation with mycorrhiza and *Rhizobium* (S+R+M) significantly increased the number of nodules compared to *rhizobium* inoculated soybeans (S+R), but not to mycorrhiza inoculated soybeans (S+M). The number of nodules produced by *rhizobium* inoculated soybeans (S+P+R) did not differ significantly from *rhizobium* inoculated (S+R), or P applied soybeans (S+P).

This experiment demonstrated that plant roots could not properly acquire P from the soil without the advantage of mycorrhiza. Phosphorus application to mycorrhiza-inoculated soybeans (S+P+M) had no significant effect on the number of nodules compared to mycorrhiza-only inoculated soybeans (S+M). Only the mycorrhizal plants S+R+M, S+P+M, and S+M, exhibited significantly larger nodule numbers than the control in the short rains experiment. The following was the order of the performance in decreasing order of the number of nodules: S+R+M>S+P=M>S+M>S+R>S+P+R>S+P and S. Dual inoculation with both microorganisms (S+R+M) significantly enhanced root nodules compared to single inoculation with *Rhizobium* (S+R) and mycorrhiza (S+M). Phosphorus fertilization of *Rhizobium*-inoculated soybeans (S+P+R) had no significant effect on the number of nodules compared to soybeans inoculated with *rhizobium* alone (S+R). In contrast, P fertilization of mycorrhiza-inoculated plants (S+P+M) significantly increased the number of nodules compared to S+P but not S+M. The results of the LR and SR experiments on the number of nodules on mycorrhizal plants might be related to the role of mycorrhizal fungus in nutrient sourcing, which was observed in both experiments. Soybean roots that have been inoculated with mycorrhizae have been shown to increase nodulation and nitrogen fixation, especially in soils low in accessible nitrogen.

A similar conclusion was reached by P. Olsen and Habte (1995) when they investigated the influence of mycorrhizal infection on nodulation and nitrogen buildup in the plant *Cajanus cajan*. The incorporation of P explained the increased nodulation observed at low soil P concentrations by mycorrhizae. The application of phosphorus fertilizer to soybeans has drastically minimized mycorrhizal infection in the crop (Hicks and Loynachan 1987). Inoculated plants could meet the high P demand for nitrogen fixation by *rhizobium*, resulting in a rise in nodulation and, as a result, an increase in nitrogen fixation. An inoculation of mycorrhizal fungi and *rhizobium* produced similar results when *Leucaenia leucocephala* was inoculated with *Rhizobium* (Punj and Gupta 1988). Compared to single inoculation of either organism, dual inoculation resulted in better growth, nodulation, and nitrogen fixation. In a similar vein, inoculation of *Acacia auriculiformis* with both mycorrhiza (*Glomus fasciculatum*) and *rhizobium* resulted in the highest number of nodules and other growth parameters, as well as the highest number of nodules and other growth parameters (Chang et al. 1986). Nodules are where biological nitrogen fixation occurs, and a rise in their number indicates an increase in biological nitrogen fixation during the experiment. In their investigation of the interaction of *Cajanus cajan* with *Rhizobium* and vesicular-arbuscular mycorrhiza *Glomus aggregatum*, Olsen and Habte (1995) discovered that, at low soil P levels, mycorrhiza inoculation significantly increased nodule numbers and shoot dry weight. They concluded that the increased nodulation was caused by mycorrhiza-mediated P uptake. The same conclusion was reached by Kumar et al. (1998), who investigated the effects of mycorrhizal fungi, *rhizobium*, and phosphate on nodulation in chickpea and reached the same conclusion. Nodulation was dramatically higher in the dual inoculation plants compared to the non - inoculated plants. In the legume *Faidherbia albida*, inoculating the seedlings with *G. mosseae* and Brady *Rhizobium* resulted in profuse nodulation of the legume seedlings (Diop et al. 2002). Infected soybeans with Brady *Rhizobium* and *Glomulus clarum* produced 30 % more nodules than soybeans inoculated with *rhizobium* alone, according to the results (Antunes et al. 2006). Compared to single inoculation, Stanchaveva et al. (2006) found that dual inoculation of pea (*Pisum sativa*) with mycorrhiza and *rhizobium* boosted plant biomass, nodulation parameters, and nitrogen fixation activity.

Dual inoculation with rhizobia and mycorrhiza (S+R+M) produced the same number of nodules as when each of the microorganisms was treated alone (S+R and S+M) in the non-sterile soil treatments, indicating that there was no statistically significant difference. Inoculating with mycorrhiza and applying P fertilizers (S+P+M) resulted in a significant increase in nodule increment compared to S+P alone, but not to S+M alone.

Mycorrhizal plants (S+R+M, S+P+M, S+M) produced more nodules in sterile soils than non-mycorrhizal plants. When the number of nodules was counted in decreasing order, the results were as follows: S+1PM > S+1PM > S+1PM > S+1PM > S+1PM > S+1PM > R+1PM > R+1PM. S, the control, did not have any nodules.

All control trials (S) had reduced nodulation, which could have been caused by a lack of compatible and effective rhizobia, nutritional shortages, and an inadequate inoculum of mycorrhizal fungi, among other factors. Houngnandan et al. (2000) researched Mucuna pruriens as a fallow plant to restore soil fertility and manage the invasive grass *Imperata cylindrica* in the Beninese descended savanna. They came to the same conclusion as they did in their earlier research. Because of low quantities of functional rhizobia, the rate of nitrogen fixation by the plant was frequently limited, and this rate may be increased by rhizobial inoculation, except in extremely P-poor soils. The author concluded that farmer's management approaches that encourage the growth of mycorrhizal fungi would alleviate the P deficit and, as a result, boost nitrogen fixation.

In summary, with this study, the major goal was to determine the effect of dual inoculation of mycorrhizal fungi and *rhizobium* on the growth performance of the legume *Glycine max* grown in acidic soils. In particular, the study sought to determine the impact of inoculating

Glycine max with mycorrhizal and *rhizobium* bacteria on the germination, grain yield, and biomass production of the plant in acidic soils. A secondary goal of the study was to measure the influence of inoculating *Glycine max* with mycorrhizae and *rhizobium* on the amount of nitrogen fixed in acidic soils. The study's findings reveal that all growth parameters, except germination, are greatly improved when soybeans are inoculated with mycorrhizae and rhizobium in acidic soils. Plant height, root collar diameter, biomass (shoots and roots), and yields rose due to the dual inoculation of mycorrhiza and *Rhizobium* bacteria on the same plant, according to the results. The effect of inoculation differed depending on the crop's growth stage under investigation. According to the study, inoculating soybeans with mycorrhizae and *rhizobium* did not influence seed germination. However, it is possible because mycorrhizal fungi and *Rhizobium* bacteria colonization of the roots was still at a low level shortly after planting. At the time of planting, the mycorrhiza *Rhizobium* symbiosis in soybeans had not yet been established.

The study discovered that inoculating soybeans with mycorrhizal fungi and *rhizobium* enhanced plant height when moisture was not a constraint. Growth in height is not greatly increased when soybeans are planted with simply phosphate fertilizers and *rhizobium* or mycorrhizae. Mycorrhizal-inoculated plants' root collar diameter, roots, and shoot dry weights were greater than those of non-mycorrhizal plants during the long rains season when moisture was plentiful. During the short rains and the on-station experiments, this was not the case, however. A combination of mycorrhizal fungi and *Rhizobium* bacteria inoculated into soybeans resulted in a considerable increase in grain yield when growing circumstances were perfect. The number of soybean nodules was significantly higher in the treatments that contained mycorrhiza. Still, it was highest in the dual inoculated plants, indicating that dual inoculation with mycorrhiza and *rhizobium* increased the rate of nitrogen fixation, with nodules being the sites where this activity takes place. Dual inoculation with mycorrhiza and *rhizobium* increased the rate of nitrogen fixation, with nodules being the sites.

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Characterization of pollen development and female reproductive structure of *Phalaenopsis amabilis* after pollination

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Abstract. *Udkhiawati I, Solicahatun, Pitoyo A. 2020. Characterization of pollen development and female reproductive structure of Phalaenopsis amabilis after pollination. Cell Biol Dev 4: 17-25.* Orchid is a superior commodity in ornamental plants. Enthusiasts of orchids cause the demand for varieties diversity. Orchids often get obstacles in pollination, so it is necessary to characterize the development of post-pollination pollen and female gametophyte structure. Characterization was conducted through in vitro and in vivo methods. This research aims to determine the development of pollen and female gametophyte structure of *Phalaenopsis amabilis* (L.) Blume after pollination and detect the potential of in vitro pollen germination. This research was explorative. It involves methods were in vitro pollen germination and preparation of anatomy of pollen and ovule of the 1st DAP (Day After Pollination), 2nd DAP, 3rd DAP, 4th DAP, 7th DAP, 20th DAP, 30th DAP, 50th DAP, and 60th DAP. The observations included in vitro culture of pollen cultured in Brewbaker and Kwack (mBK) media using a phase-contrast microscope and observation of anatomical development of post-pollination pollen tube and ovule using a fluorescence microscope. The observed data consisted of in vivo female gametophytes and pollen development in vitro and in vivo methods. The data were analyzed descriptively. From observation of post-pollination pollen, it was known that the pollen tube started to germinate on the 2nd -3rd DAP. At the 7th DAP, all pollens had germinated and formed the pollen tube, and then the pollen tubes continued to grow closer to the ovule. Primordial ovule began to form on the 7th DAP, then continued to develop a seed on 60th DAP, and then an embryo pouch formed. The in vitro pollen germination did not show any change in pollen after 30th DAC (Day After Cultivating).

Keywords: Characterization, in vitro culture, ovule, *Phalaenopsis amabilis*, pollen

INTRODUCTION

Orchids are a group of angiosperm plants, the leading commodity in ornamental plants. Data from the Central Statistics Agency in 2015 shows that orchid production in Indonesia has increased. In 2006, the production of orchids was 10,903,444 stalks/year, while in 2015, the production of orchids reached 21,514,789 stalks/year. The more orchid lovers in the ornamental plant industry market, the demand for novelty orchid varieties is increasing, so it is necessary to increase new superior varieties of orchids, one of which is by crossing. Crosses require parents with outstanding traits, so a combination of these traits will appear in the cross results. (Widyastoety et al., 2010).

Orchids can be crossed interspecies (between species in one genus), intraspecies (one species in one genus), or intergeneric (between genera) (Sarwono, 2002). Orchids have a vast potential to produce new varieties. However, crosses on orchids often encounter obstacles, namely pollination failure. Immature reproductive organs could cause pollination failure, failure of pollen germination on foreign stigmas (Marwoto et al., 2012), and cross-compatibility. According to (Widyastoety et al., 2010), pollen from small orchids will usually fail if pollinated on large orchids because the pollen tube cannot reach the embryo sac.

Orchids have a different reproductive system from other angiosperms. The structure of the ovule in orchids is not fully developed before pollination and even partially has not been formed. Pollination will trigger the formation and differentiation of ovules, so pollination is essential for regulating the ovule and embryo sac development. Due to the time difference between pollination and the maturity of the embryo sac, orchid fertilization takes place over a long period. In addition, the pollen and pollen tube must survive in the ovary for a long time. Therefore, post-pollination characterization is necessary to determine the development of post-pollination pollen and the role of pollen in the development of female gametophytes.

The characterization carried out included the time of the emergence of the pollen tube and the growth of the ovary and ovule. Characterization can be done in vitro and in vivo. Efforts to grow pollen tubes in vitro are expected to be a solution to overcome the crossbreeding problem due to the inability of the pollen tube to reach the embryo sac in the ovule. Fertilization through in vitro culture with pollen tube formation has also been reported in Brassica rapa (Lorbiecke, 2012). Moreover, this in vitro pollen tube culture was carried out by treating the stigma tissue extract on growth media. According to Chen and Fang (2016), stigma tissue extract is needed to initiate pollen germination in vitro. There are indications that the stigma network has molecules as signals for pollen tube germination.

Post-pollination characterization can also be carried out in vivo to determine the development of the pollen tube and

post-pollination ovule in vivo by making anatomical preparations for each stage of ovarian development. Post-pollination characterization is expected to provide additional information to overcome problems in generative propagation and quality improvement of orchids such as crosses. Research on plant reproduction, especially in orchids, has not been widely carried out, so there is a need for further research on the reproduction of angiosperms, especially in orchids.

The aims of this research were (i) to know pollen tube development and female gametophyte structure in *Phalaenopsis amabilis* (L.) Blume post-pollination, (ii) to provide a chemical signal that stimulates pollen tube growth, female gametophyte development, and to know pollen tube grows towards the post-pollination development and differentiation of ovule.

MATERIALS AND METHODS

Plant material

Phalaenopsis amabilis flower (Anggrek Bulan or moon orchid) was collected from an ornamental market in Surakarta, Central Java, Indonesia.

Procedure

Pollination of Phalaenopsis amabilis

To implant the pollinia, one must first open the anther cap on the flower with a sterile toothpick before inserting it into the stigma cavity.

Pollen collection

Pollinia were sterilized by immersion in 0.05% NaClO and then rinsed using distilled water. After sterilization, the pollinia were placed in sterile Petri dishes and then squashed to obtain pollen grains which would later be cultured as explants.

Extraction of stigma tissue of Phalaenopsis amabilis

Orchid stigma was cut using a scalpel and then sterilized by immersion in 70% ethanol for 20 seconds. The stigma pieces were immersed in 3 ml of distilled water and vacuumed to obtain the stigma tissue extract. The stigma tissue extract was mixed in the pollen germination medium.

In vitro culture of Phalaenopsis amabilis

The in vitro culture stage includes sterilization activities using an autoclave at a temperature of 121ºC and a steam pressure of 1.5 atm for 20 minutes, making Brewbaker and Kwack (mBK) media consisting of 10% sucrose, 100 mg/L H3BO3, 100 mg/ L CaCl2.2H2O, 100 mg/L MgSO4.7H2O, 100 mg/L KNO3, and 5 g agar. Sucrose was dissolved in 500 ml of distilled water and heated on a hot plate; after dissolving, it was added with 100 mg of H3BO3, 100 mg of CaCl2.2H2O, 100 mg of MgSO4.7H2O, 100 mg of KNO3, and the obtained stigma extract while stirring until homogeneous. After boiling, it was removed and cooled. A total of 500 ml of distilled water was heated, added 5 g of agar powder, and stirred until it boiled. After boiling, remove it and cool to a temperature of 60ºC. The cold media solution was added to the agar solution and stirred until homogeneous. The explants were planted in LAFC (Laminar Air Flow Cabinet).

Preparation of fresh preparations of Phalaenopsis amabilis

Post-pollinated orchid pollen on the stigma or ovary was cut into thin strips and stained with aniline blue. The fresh preparations were observed under a fluorescence microscope with a UV-1A filter.

Preparation of anatomical preparations of ovule development

Preparation of anatomical preparations for the development of orchid ovules using the paraffin method includes the stages of fixation, dehydration, clearing, infiltration, embedding, and staining.

Observation

In the in-vitro, culture observations were started at one DAC (Day After Cultivating) using a phase-contrast microscope. Observations were made periodically to see the direction of growth of the pollen tube. In vivo pollen observations were preceded by staining with aniline blue and then observed under a fluorescence microscope using a UV-1A filter. Observation of female gametophyte in vivo was carried out by making preserved preparations for the post-pollination stage of ovule development consisting of 1 DAP (Day After Pollination), 2 DAP, 3 DAP, 4 DAP, 7 DAP, 20 DAP, 30 DAP, 50 DAP, and 60 DAP using a fluorescence microscope using a UV-1A filter.

Data analysis

Data on pollen development, female gametophyte, and pollen tube growth are presented descriptively.

RESULTS AND DISCUSSION

Observation of morphological changes in the flower of *Phalaenopsis amabilis* Post-pollination

The results of observations on the success of pollination were indicated by changes in flower morphology after pollination. These changes include wilting of flower ornaments, changes in pigmentation, maturation of ovaries, differentiation of ovules, and development of female gametophytes.

Figure 1 shows the morphological changes of *P. amabilis* from 0 DAP (Days After Pollination) to 60 DAP. At 0 DAP, perianthium (flower decoration), the orchid looks fresh and clean white, consisting of 3 sepals and three petals where one petal is modified into a labellum structure. The gynostemium (organ structure consisting of androecium and gynoecium) is white, and a pollinium is covered by an anther cap located at the tip of the gynostemium. In gynostemium, there is a stigma hole as a way for pollinia to enter at the time of pollination. The ovary looks like a short segment directly related to the flower stalk, located at the end of the flower stalk below the gynostemium, and there are longitudinal lines.

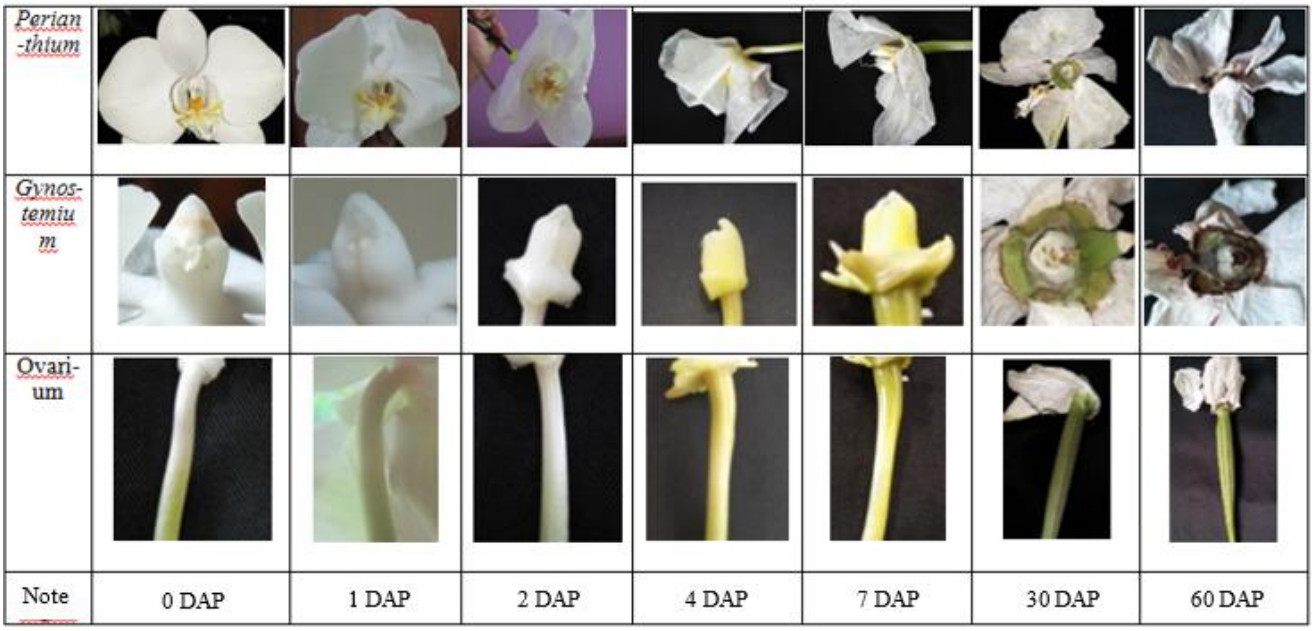

Figure 1. Morphological changes of *Phalaenopsis amabilis* post pollination; DAP (Days After Pollination)

At 1 DAP, the flower buds begin to wither; the stigma hole closes around the pollinia. The ovaries have not changed. At 2 DAP, the flower ornaments were wilting; the gynostemium started to swell. The swelling of the gynostemium is also thought to be due to cell division in gynostemium tissue. At this stage, there is stimulation of the development of the ovule, then the ovary enlarges and undergoes differentiation. At 4 DAP, the flower embellishments are wilting. Gynostemium continues to swell and begins to change color to yellow-green. The color change is thought to be due to the production of chlorophyll. The ovaries begin to swell and change color to yellow-green, as in gynostemium. Ovarian swelling is believed to be due to the accumulation of starch.

At 7 DAP, the withered flower buds became dry, the base of the corolla swelled, the gynostemium and ovary became swollen and enlarged due to the development of the female gametophyte structure. At 30 DAP, the flower and gynostemium adornment dried, and the ovary formed a fruit structure. At 60 DAP, the surface of the gynostemium was slightly blackened and dried; the fruit structure was growing. The results of post-pollination orchid flower morphology observations follow the explanation of O'neil et al. (1993), who stated that the success of pollination caused changes in orchid flowers. Curtis (1943) in Nadeau et al. (1993) explained that the gynostemium cells in orchids swell after pollination so that the gynostemium becomes prominent and the stigma hole closes around the pollinia. The wilting of floral ornaments are caused by the auxin-induced spike in ethylene from pollen during pollination (Hew and Young, 2004). According to Hsiang (1950), pollination causes swelling of gynostemium due to increased water absorption capacity, while most flower ornaments wither due to increased transpiration.

There were several morphological changes in post-pollination month orchid flowers in this observation. Changes in the morphology of orchids after pollination can be caused by several things, one of which is the interaction between hormones. Hew and Young (2004) stated that pollination causes the transfer of auxin from pollen to the stigma. As a result of this auxin transfer, growth hormone spreads into the stigma and induces ethylene production in the stigma tissue. Auxin induces the expression of genes encoding the enzymes ACC synthase and ACC oxidase. ACC synthase and ACC oxidase enzymes play a role in ethylene biosynthesis. The ethylene biosynthesis process begins with converting methionine to *S-adenosylmethionine* (SAM), then SAM is catalyzed by the ACC synthase enzyme to *1-aminocyclopropane-1-carboxylic acid* (ACC). The enzyme ACC oxidase catalyzes ACC to ethylene. Both enzymes are produced in the stigma, while in the flower jewelry, there is only ACC oxidase, so the ethylene synthesis in the flower jewelry depends on the transfer of ACC from the stigma. ACC stimulates ethylene production, causing wilting.

Growth of pollen tube flower of *Phalaenopsis amabilis* in vivo

Pollinia morphology of Phalaenopsis amabilis

Figure 2 is a morphological image of the *P. amabilis* pollinia observed under a fluorescence microscope at 100x magnification using a UV-1A filter.

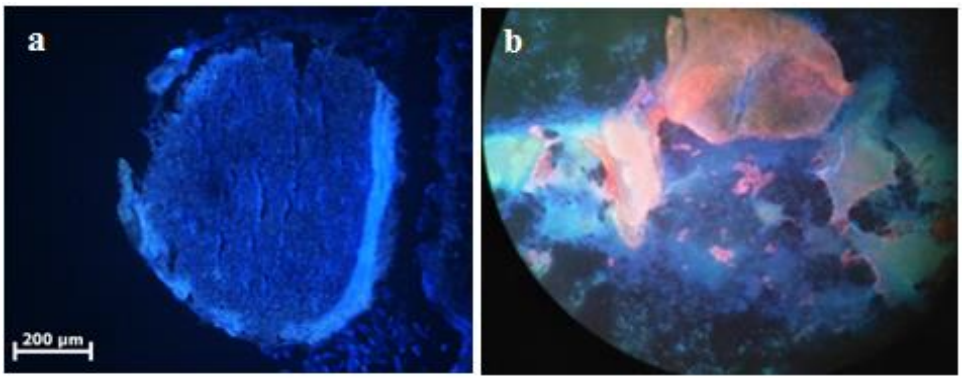

Figure 2. Morphology of *Phalaenopsis amabilis* pollinia. a. P.B. observations of *P. amabilis* pollinia on a fluorescence microscope using a UV-1A filter at 100x magnification; b. Observation of a fresh section of *P. amabilis* pollinia on a fluorescence microscope using a UV-1A filter at 100x magnification.

Figure 2. is a morphological image of the pollinia *P. amabilis*. Figure 2a is a cross-section of the *P. amabilis* pollinia observed under a fluorescence microscope with aniline blue staining. These observations indicate that the pollen of *P. amabilis* collected into a single structure called pollinia. In one pollinium, there are thousands of pollen. Figure 2b shows the color luminescence difference in *P. amabilis* pollinia after squash. *P. amabilis* pollinia preparations were viewed using a fluorescence microscope under a UV-1A filter. Pollen on the outer pollinia or surface glows with a red glow, while the pollen inside produces a blue glow. It happens because there is an exine on the surface of pollinia. In exine, there is elastoviscin which contains carotenoids. The exine will glow red when under a fluorescence microscope. The results of this observation indicate differences in the pollen constituents' walls on the pollinia's surface with the pollen inside the pollinia. In pollinia, an elastic material binds pollen together with pollinia called elastoviscin. Elastoviscin in the epidendrum is a lipid polymer. Elastoviscin in pollinia has the same role as pollenkitt in pollen. Paccini and Hesse (2005) explained that pollenkitt is an oily coat pollen layer. Pollenkitt's functions include keeping pollen from losing water, making it easier for pollen to spread, protecting it from U.V. filter radiation, protecting pollen from bacteria and fungi, and making it easier for pollen to reach the stigma.

Observation of Phalaenopsis amabilis

Figure 3 shows an image of the *P. amabilis* pollen grains observed under a fluorescence microscope at 100x magnification using three different filters. In Figure 3. it can be seen that the pollen grains that have been stained with aniline blue can be seen under a fluorescence microscope using a UV-1A filter. There is no visible presence of pollen grains in the emission of filters B-2A and G-2A. It happens because the excitation wavelength of the UV-1A filter is 360-370 nm, the B-2A filter is 450-490 nm, and the G-2A filter is 510-560 nm. In comparison, the maximum excitation wavelength can be absorbed by aniline blue is 370 nm. (Scordato and Schwartz, 2017). It also follows the statement of Vieira et al. (2015) that aniline blue has been used as a specific dye for Callose which can stain selectively and fluoresce under U.V. filters. A collection of substances called Callose is in the pollen wall and tube. Callose is one of the most dynamic components of the cell wall. Callose is synthesized and deposited on the outer surface of the plasma membrane by callose synthase present on the membrane. The function of Callose in the pollen wall is to control the balance of cell wall turgor pressure, resistance to pressure around the pollen tube wall, and water permeability (Parre and Geitmann, 2005). Kho and Baer (1968) explained that Callose is a polysaccharide in the form of β-1,3-glucans. Furthermore, Callose will brightly glow when stained with aniline blue and viewed under a U.V. filter (Jefferie and Belcher, 1974). Evans et al. (1984) stated that aniline blue could interact directly with the surface of β-1,3-glucans through the hydrophobic region.

Pollen tube growth

Figure 4 shows the growth of the *P. amabilis* pollen tube under a fluorescence microscope using a U.V. filter. Pollen tubes are formed after pollination as a sign of successful pollination. Pollination is the arrival of pollen on the stigma (Lorbiecke, 2012). No pollen tubes appeared at 1 DAP (Day After Pollination) observation; moreover, pollen is still in the form of granules attached to each other and gathered into one. At 2 DAP, the pollen grain size was more prominent, but the pollen tube was not visible. It indicates that hydration has occurred in the pollen. At 3 DAP, the pollen tube has started germinating, and even a pollen tube has begun to elongate. Following Bedinger's (1992) statement, the pollen tube begins to germinate through the pores on the exine surface, and then the pollen tube elongates rapidly. Therefore, pollen tube germination is suspected to begin at 2-3 DAP. According to Wihelmi and Preus (1997), after penetration of the pollen tube, it will begin to germinate and form a tube that grows between the stigma cells. Pollen tubes emerge from each pollen grain. At 4 DAP, the pollen tube progressively elongates within the stigma and begins to grow toward the ovary. At 7 DAP, all pollen tubes germinated and grew lengthwise into the ovary. In the observation results in Figure 4g, a collection of pollen tubes leads to the primordia of the ovule. At 20 DAP, several ovular primordia were formed in the ovary.

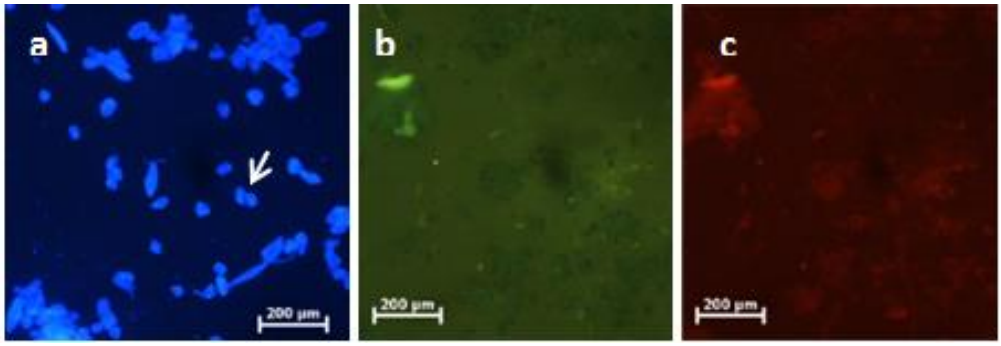

Figure 3. Observation of *Phalaenopsis amabilis* on a fluorescence microscope using different filters. Description: a. UV-1A filter; b. B-2A filters; c. G-2A filters. ; magnification 100x; arrows indicate pollen grains.

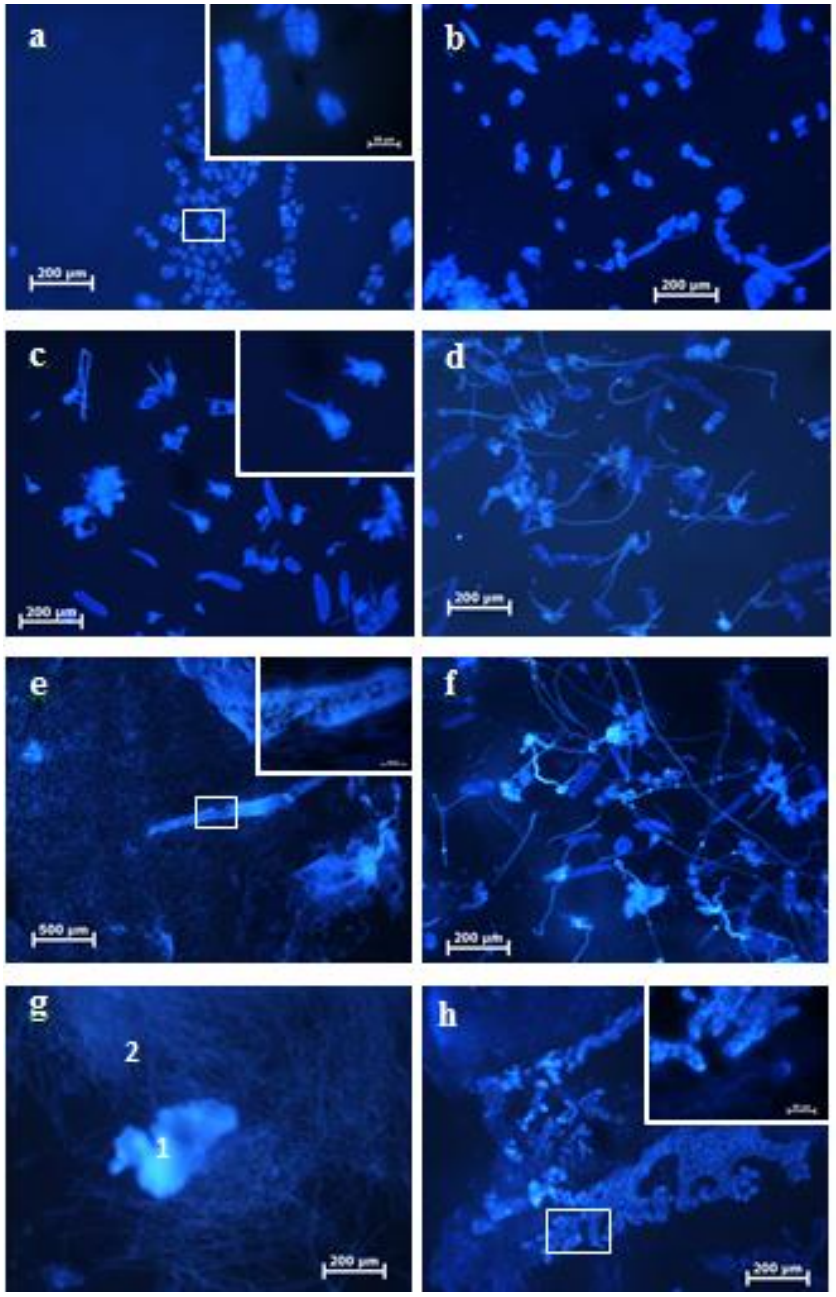

Figure 4. Observation of *Phalaenopsis amabilis* pollen tube growth using a fluorescence microscope at 100x magnification. Description: a. 1 DAP (Day After Pollination); b. 2 DAP; c. 3 DAP; d. 3 DAP; e. 4 DAP; f. 7 DAP; g. 7 DAP; h. 20 DAP; 1. Primordia ovule; 2. Pollen tube collection

After the interaction between pollen and stigma occurs, then pollen undergoes penetration. The pollen tube extends inside the stigma cell, whereas the pollen tube grows through the extracellular matrix in style. The extracellular matrix has hollow solid channels. It is supported by Wihelmi and Preus's (1997) statement that the matrix content includes lipids, proteins, carbohydrates, and small molecules. Presumably, these components provide signals to support the growth of pollen tubes. The pollen tube continues to grow in a nutrient-rich matrix where the female gamete secretes the nutrients. After the pollen tube leaves the style, it is suspected that it receives a signal from the ovule to arrive at the ovule. From this observation, it can be concluded that pollination is divided into several stages, namely (a) the arrival of pollen on the stigma, (b) penetration and germination of pollen, (c) growth of the pollen tube on the stigma and style (d) the pollen tube to the ovule and (d) the arrival of sperm, in the embryo sac.

The observations in this study showed the growth of pollen tubes after pollination. The growth of pollen tubes is thought to be stimulated by many factors. One of the factors that can stimulate the growth of pollen tubes is the molecules of the stigma. Wihelmi and Preus (1997) stated that stigma is also a factor that stimulates the growth of pollen tubes. The pollen tube germinates inside the stigma and grows towards the ovule via the style. During growth, the pollen tube receives several molecules from the female tissue (ovule). These molecules are ions, peptides, and glycoproteins. The movement of the pollen tube, along with style towards the ovule, is directed by a chemoattractant signal. Examples of signals that direct the pollen tube to the ovule has been found in several species, such as chemocyanin derived from *Lilium* and TTS protein derived from *Nicotiana tabaccum* (Higashiyama and Takeuchi 2015).

Ovule development of the Phalaenopsis amabilis *in vivo*

Figure 5 shows the development of the *P. amabilis* ovule post-pollination. Ovaries in orchids have not matured before pollination. The stages of ovule development in this study include (i) the development of the cell mass to form a primordial ovule, (ii) division of the placenta to form dichotomous branches, (iii) the formation of the integument, and (iv) the formation of the embryo sac. Figure 5a shows the mass cell development in the primordial ovule at 7 DAP. The primordial ovule is formed from the placenta, located in the innermost part of the ovary. The mass cell development forms a structure such as small protrusions grouped into a single unit. It is supported by the statement of Nadeau et al. (1996) that the first stage in the development of the orchid ovule is cell division in the cell wall area.

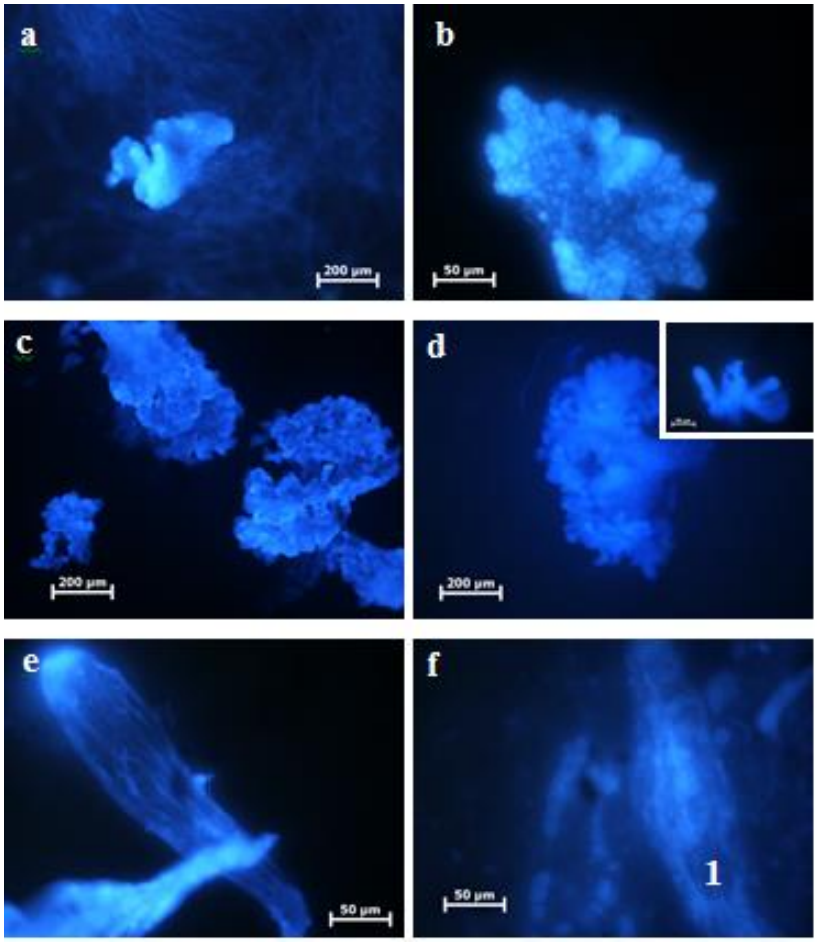

Figure 5. Observation of the growth of *Phalaenopsis amabilis* ovules using a fluorescence microscope. Description: a. 7 DAP (Days after Pollination) 100x magnification; b. 20 DAP 400x magnification; c. 30 DAP 100x magnification; d. 50 DAP 100x magnification; e. 60 DAP; f. 60 DAP 400x magnification; 1. Embryo sac

This area is elongated and forms dichotomous branches to produce thousands of ovular primordia. At this stage, the dermal and subdermal layers cells are densely packed with the cytoplasm. Figure 5b shows an ovule at the age of 20 DAP, and the appearance of the primordial ovule is growing into an elongated bulge-like structure. In Figure 5c, the ovule is 30 DAP, and it can be seen that the protrusion of the ovule is growing and looks lumpy and forms dichotomous branches. Duncan and Curtis (1942) stated that at 35 days after pollination, there was placental proliferation followed by elongation and swelling of the ovaries, supported by elongation of the pollen tube. The ovules differentiate between 30 and 40 days after pollination.

At 50 DAP, Figure 5d shows many ovule structures in the placenta in the form of dichotomous branches to form a finger-like structure. Nadeau et al. (1996) stated that the subdermal cells at the tip of the primordia enlarge to form archesporial cells. The deep integument begins to form a ring from periclinal cell division near the tip of the primordia. It is accompanied by asymmetric growth and cell division on one side of the primordia to form the anatrophic orientation of the ovule. The outer integument is formed after the integumentary and funiculus cells enlarge and turn into vacuolate. The archesporial cells enlarge to form megasporocytes directly. The meiotic division of the megasporocyte forms four cells, but the development of the megaspore crushes one cell near the chalaza. The vacuoles begin to unite in the surviving megaspores.

Further mitotic division occurs according to the development of the polygonum type, namely megaspore cells that divide three times in a row to produce eight nuclei. After the first division of the megagametophyte cell, the nuclei migrate to the ends of the coenocytic megagametophyte, where they divide twice to form three antipodes at the end of the chalaza, an egg cell, and two synergides at the end of the micropyle. The two remaining nuclei gather at the center of the cell to form the polar nucleus. After all mitotic divisions are complete, a cell wall forms between the nuclei at both ends of the megagametophyte but not around the polar nucleus. At 60 DAP, figure 5e shows the ovule has formed an integument, while Figure 5f shows a structure resembling an embryo sac. Niimoto and Sagawa (1962) stated that macrogametophyte maturation occurs at 60 DAP, and fertilization occurs at 65-70 DAP.

Morphological observation of pollen culture of the flower of *Phalaenopsis amabilis*

Figures 6A and 6B show observations of pollen tube cultures on two different media. Figure 6A is a pollen tube culture with Brewbaker and Kwack (mBK) media, while Figure 6B is a pollen tube culture with Brewbaker and Kwack (mBK) media and stigma extract. The observations for 30 days after planting concluded that pollen tube cultures performed on both media showed no pollen tubes growing. It is thought to be caused by several things, including the media and growing environment, that are not suitable for pollen germination and growth. Pacini and Hesse (2005) stated that pollen could only germinate if the media is mixed with stigma extract or lipids.

In this study, culture was carried out on two media, and one of the media was added with stigma extract, but there was no pollen tube growth in both media. This result is thought to have occurred because the stigmatic exudate mixed with the media did not meet the pollen needs to germinate. In Lorbiecke's research (2012), the pollen tube culture of Brassica rapa was successfully carried out using the semi-in Vivo method, where the pistil was cut and then cultured in pollen culture media after pollination that the need for stigma exudate was fulfilled.

The solid media use could also cause the failure of pollen tube growth in this study. Bar-Shalon and Mattson (1977) in Hodgkin (1987) stated that pollen from plants with a wet stigma type germinated more easily in liquid media. Pollen cultures in this study did not include ovules; furthermore, it is suspected that this also affects the germination of pollen tubes. Higashiyama and Takeuchi (2015) stated that several molecules from the female could stimulate pollen tube germination. These molecules could be ions, peptides, and glycoproteins. The nutrients contained in the media are also very influential in pollen tube growth.

In this study, the media used was Brewbaker and Kwack (mBK) media with a composition of 10% sucrose, 100 mg/L H_3BO_3, 100 mg/L $CaCl_2.2H_2O$, 100 mg/L $MgSO_4.7H_2O$, 100 mg/L KNO_3, 5 g instant agar, and distilled water. Johhri and Vasil (1961) stated that sucrose and boron influenced pollen tube germination. Sucrose plays a role in controlling the osmotic concentration in the media for pollen tube germination. In contrast, boron assists sugar absorption and metabolism, increasing oxygen absorption and being involved in synthesizing pectin to elongate pollen tube walls. It is suspected that the sucrose and boron in the media used did not meet the pollen needs to germinate. Several studies have used hormones, vitamins, carotenoids, antibiotics, and other organic salts in pollen tube cultures that play a role in pollen tube germination. This study did not use these components. Growing environmental factors are also very influential in pollen tube germination. The optimum temperature for pollen tube growth was 20º-30ºC, while the temperature used during the study was 18ºC; according to the temperature of the culture incubation room, it may be one of the causes of pollen failure to germinate and form a pollen tube.

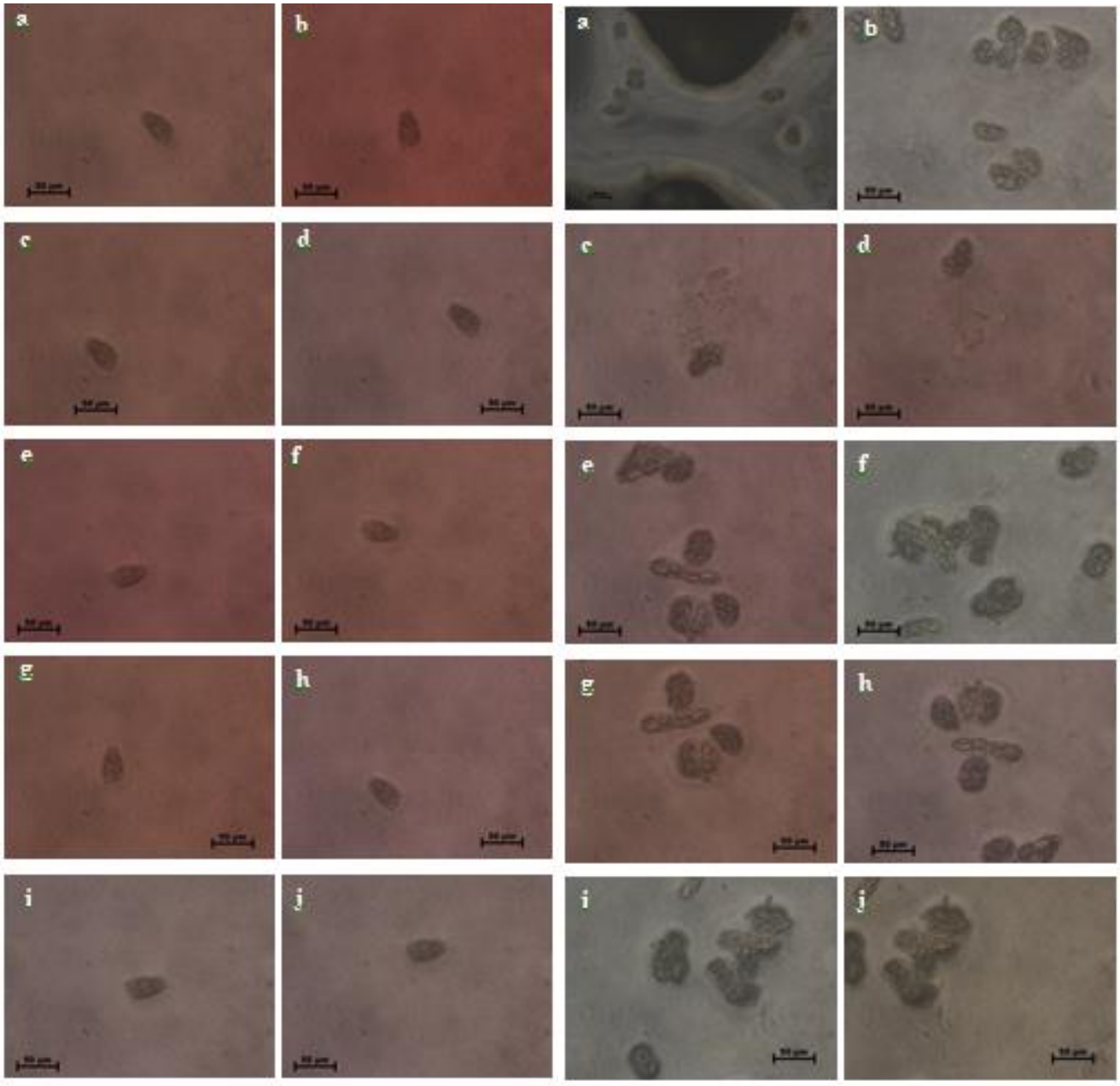

Figure 6. Observation of *Phalaenopsis amabilis* pollen culture on a phase-contrast microscope. **A**. On Brewbaker and Kwack Media (mBK). **B**. On Brewbaker and Kwack and stigma extracts. Description: a. 0 DAC (Day After Cultivating); b. 1 DAC; c. 2 DAC; d. 3 DAC; e. 4 DAC; f. 5 DAC; g. 6 DAC; h. 7 DAC; i. 20 DAC; j. 30 DAC; 400x magnification

In conclusion, pollen tube development and female gametophyte structure in *P. amabilis* post-pollination are interrelated. In addition to providing a chemical signal that stimulates pollen tube growth, female gametophyte development is also influenced by pollen tube growth. The pollen tube grows towards the post-pollination ovule while the ovule undergoes post-pollination development and differentiation. In vitro pollen tube culture has not been successful.

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Physiological and phytochemical characters of *Eleutherine palmifolia* affected by treatment of variation in light intensity and water capacity

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Abstract. *Firdaus NM, Mudyantini W, Sugiarto. 2020. Physiological and phytochemical characters of* Eleutherine palmifolia *affected by treatment of variation in light intensity and water capacity. Cell Biol Dev 4: 26-39.* Bawang dayak (*Eleutherine palmifolia* (L.) Merr.) is one of the tubers widely cultivated in Kalimantan with phytopharmacological potential, but its usage in traditional medicine is still limited. This study aims to ascertain the physiological and phytochemical characteristics of *E. palmifolia* following treatment with varying amounts of water and light intensity. The study used a completely randomized design (CRD) with two components: the treatment of light intensity 50% and 100% and water availability at 50%, 75%, and 100% concentrations, to create six treatment combinations. Flowering pace, leaf number, breadth, length, and blossom number were measured once every three days for one month. Wet weight, dry weight, stomata index, chlorophyll, carotene, vitamin C, flavonoids, and shoot ratio were assessed at harvest. The data were analyzed using ANOVA (Analysis of Variant). If there was a significant difference, a further test was carried out with the Duncan Multiple Range Test with a test level of 5%. The treatment effect on water availability of 75% and light intensity of 50% (K75I50) was significantly different and gave the highest value on leaf width of 0.28 cm, stomata index of 36.84 cm, carotenoid content of 39.70 g/ mL in *E. palmifolia*. The given treatments were not significantly different in the number of leaves, leaf length, the number of flowers, wet weight, dry weight, chlorophyll, vitamin C, flavonoids, and shoot ratio of Dayak roots. Water availability and 100% light intensity (K100I100) are the optimal treatments to accelerate the flowering of Dayak onion plants with a yield of 26.00 days.

Keywords: *Eleutherine palmifolia*, light intensity, physiological characters, phytochemicals, water availability

INTRODUCTION

Eleutherine palmifolia (L.) Merr., or *bawang dayak*, is one type of plant beneficial to health but is used infrequently in the community for treatment. The *E. palmifolia* is a native plant to Indonesia, more precisely to Kalimantan, and may be used as a phytopharmaceutical. The community makes extensive use of this plant on tubers. The tubers of *E. palmifolia* contain a variety of phytochemicals, including alkaloids, glycosides, flavonoids, phenolics, steroids, and tannins (Firdaus et al., 2013). The *E. palmifolia* has been used empirically by indigenous people to treat various diseases, including breast cancer (Sudarmawan et al. 2010), hypertension, diabetes mellitus, cholesterol, ulcers, and colon cancer, as well as to prevent stroke and alleviate abdominal pain following childbirth. Additionally, the leaves of this plant have been shown to increase breast milk production (Galingging 2009).

Water availability is one issue that arises during the long dry season. Groundwater levels will continue to decline in this condition due to high evaporation. Apart from wreaking havoc on the soil, a lack of water has a detrimental effect on plants, as water does not dissolve the nutrients required by plants. As a result, nutrient availability to plants is reduced, resulting in decreased productivity or even wilting. The requirement for water is quite different in plants whose life is sustained by tubers than in plants whose life is supported directly by roots. Tubers and dry matter rely highly on an adequate water supply (Kadayifci et al. 2005).

Light intensity is a critical factor that affects plant growth and development. Too much light has a detrimental effect on plants, specifically the occurrence of chlorosis, chlorophyll damage, and rapid transpiration. On the other hand, when light levels are low, plants consume existing food reserves rather than storing them (Forniawan et al., 2017). The *E. palmifolia*, on the other hand, prefer full sunlight to shaded conditions for growth (Yusuf 2009). Full light intensity results in an increase in photosynthate accumulation in *E. palmifolia* tubers. Some photosynthetic content stored in *E. palmifolia* is in the form of essential compounds such as flavonoids, primarily used by tubers due to their higher biomass production than other organs. Based on these issues, it is necessary to research the physiological and phytochemical characteristics of *E. palmifolia*, as well as the effect of light intensity and water availability variations on its growth. The *E. palmifolia* will be stressed by the combination of the two treatments. Therefore, it is necessary to investigate the most effective methods for increasing the cultivation and the content of beneficial phytochemicals, one of which is flavonoids. Unfortunately, there is no information on the combination of the two treatments. What has been done is to treat each separately without combining them.

The aims of this study were (i) to examine the effect of light intensity and water availability on the physiological and phytochemical characteristics of *E. palmifolia*, and (ii)

to determine the best combination of light intensity and water availability treatment for *E. palmifolia*

MATERIALS AND METHODS

The research was carried out from December 2019 to October 2020. The research was carried out at the Central Laboratory Greenhouse, the Integrated Mathematics and Natural Sciences Laboratory and Biology, Universitas Sebelas Maret, Surakarta, Indonesia.

Research design

This study used a completely randomized design (CRD) with two factors: variations in light intensity at two levels and variations in water administration concentration at three levels, to obtain six treatment combinations. Each treatment was replicated five times in this study. The following treatment parameters were determined: leaf number, leaf width, leaf length, midrib height, flower number, flowering speed, stomata index, crown to root ratio, chlorophyll content, carotenoid content, vitamin C content, and flavonoid content. Rolling once a week for one month was used to administer the combination of treatments. In this study, the following treatments were used in combination:

Water availability factor (A) with three levels, namely:
K100 = control (100% field capacity)
K75 = 75% field capacity
K50 = 50% field capacity
Light intensity factor with two levels, namely:
I100 = control (100%)
I50 = 50% field capacity

The six treatment combinations are shown in Table 1.

Procedure

Selection of tubers samples and preparation of e. palmifolia seeding

The material for this study was freshly harvested *E. palmifolia* tubers of uniform size from Surakarta, Indonesia. First, 60 tubers of *E. palmifolia* were selected in their native state. The tubers are picked based on their size. The tubers of *E. palmifolia* were then weighed using an analytical balance set to a weight range of 5-7 grams. Next, uniformly sized *E. palmifolia* tubers were planted in the prepared media. Tubers of *E. palmifolia* were planted by burying half of the tubers into a media mixture in 11x20 polybags consisting of compost, manure, soil, and husks in a ratio of 1:1:1:1. Seed preparation was carried out for 15 days.

Determination of field capacity

Before use as a planting medium, the gravimetric method is used to determine the soil's field capacity (weighing). The field capacity was determined by mixing soil, husks, manure, and compost in a 1:1:1:1 ratio until the mixture reached a dry weight of 1 kg. After confirming that the media mixture weighs 1 kg, it is placed in a polybag measuring 15 x 20 cm with a capacity of 1 kg and a perforated bottom, then saturated with water until no water drips. The following formula calculates field capacity (FC):

Field Capacity = (Weight of soil + Polybag + Water) – (Weight of soil + Polybag) (Patoni 2000).

Treatment determination

Variations in light intensity and water availability were considered in this study. The water availability treatment was applied when the *E. palmifolia* plants had 2-4 leaves. Water was sprayed at 100%, 75%, and 50% of the field capacity, respectively (Nasir et al. 1996). Watering is performed every four days (to ensure that treatment conditions remain consistent with the level of water availability being tested) (Haryati et al., 2010). In the Green House, polybags are placed at two different light intensities, 50%, and 100%. Wickramasinghe et al. (2015). Weeding is accomplished by removing weeds from the plant's immediate vicinity. Each treatment was repeated five times, focusing on field capacity. After exactly one month, the same water and light intensity volume was applied to *E. palmifolia* plants to be analyzed.

Daily observation

Daily observations were made using thread and a ruler to determine the height of the midrib, the number of leaves, the width of the leaves, the length of the leaves, the speed of flowering, and the number of flowers. In addition, all treatment polybags were observed every three days for one month. After exactly one month, *E. palmifolia* plants were treated with the same volume of water and light intensity to be analyzed.

Relative growth calculation

The Relative Growth Rate (RGR) is the essential crop strategy indicator in crop productivity under environmental stress. Since the beginning of growth at a specific time interval, the relative growth rate increases the relative size of the existing plant. The calculation of the relative growth rate is as follows.

$$RGR = \frac{Wfinal - Winitial}{Winitial}$$

Where:
RGR : Relative Growth Rate
Wfinal : final day growth parameters
Winitial : initial day growth parameters

Table 1. Combination of Treatment of water availability and light intensity on *E. Palmifolia*

	I100	**I50**
K100	K100I100	K100I50
K75	K75I100	K75I50
K50	K50I100	K50I50

Stomata index measurement

The stomata index was determined by incision of the epidermal leaf of the *E. palmifolia* plant. The lower epidermal leaves were sampled. The imprinting method is used to determine the stomatal index. From a total of 30 pots, one leaf each was selected and smeared on the underside of the leaf using clear nail polish. After applying, the polished area is left for 1 hour until it dries completely. When the nail polish dries, clear tape is applied to the area where the polish has been applied and then slowly removed until all the polish is removed and sticks to the clear tape. The clear tape containing the print is then placed on a glass object and observed under a digital microscope to observe the index. The formula for calculating the stomata index is as follows.

$$\text{stomata index} = \frac{\text{number of stomata}}{\text{number of epidermal cells - number of stomata}} x100$$

Wet and dry biomass measurement

Plant biomass measurements were carried out for wet and dry biomass. The crown and roots were measured separately, whereas the total biomass was determined by adding the shoot and root biomass, both dry and wet. After two months of maintenance, wet biomass was weighed. Dry biomass was prepared by drying it in an oven at 60°C until it reached a constant weight and weighed.

Measurement of the root-to-top ratio

The ratio of roots and shoots was obtained by comparing the biomass of roots and shoots when wet and dry.

Testing for photosynthetic pigment levels

Leaves weighing 0.5 grams were macerated using a mortar and pestle and dissolved in 5 mL of 80% PA (Pro Analysis) acetone intermittently while slowly mashed. Then the smooth leaves were filtered using a glass funnel in a test tube using Whatman 42 filter paper. The filtered filtrate was chlorophyll extract. All processes are carried out in conditions protected from sunlight (Prasetyo and Laili, 2015). First, the obtained filtrate is poured into the cuvette until 2/3 of the cuvette is filled. Next, the 80% PA (Pro analysis) acetone solution was poured into the cuvette until 2/3 of the cuvette was filled for the blank solution on the UV-Vis spectrophotometer. Furthermore, the content of chlorophyll and carotenoids was measured using a UV-Vis spectrophotometer at wavelengths of 480 nm, 645 nm, and 663 nm. After obtaining the absorbance value, the chlorophyll content can be calculated by the following formula:

Chlorophyll a mg/g leaf weight = (12.7 x A663) – (2.69 x A645 x 10^{-1})

Chlorophyll b mg/g leaf weight = (22.9 x A645) – (4.68 x A663 x 10^{-1})

Total chlorophyll mg/g leaf weight = Klorofil a + Klorofil b

$$\text{Carotenoids } \mu\text{mol/g leaf weight} = \frac{(A480 + 0.114 \text{ x } A663 - 0.638 \text{ x } A645) \text{ x } V \text{ x } 103}{112.5 \text{ x } 0.1 \text{ x } 10}$$

Photosynthetic pigment data processing was carried out in the last week of observation.

Measurement of vitamin C levels

Determination of pure vitamin c calibration curve

Pure ascorbic acid was weighed as much as 0.02 mg, 0.04 mg, 0.06 mg, 0.08 mg, 0.012 mg, and 0.016 mg and dissolved in 10 mL of aquabides to the mark so that the solution concentration was 2 ppm, 4 ppm, 6 ppm, 8 ppm, 12 ppm, and 16 ppm. Then the maximum absorption was measured at a wavelength of 265 nm with a UV-Vis spectrophotometer using an aquabides blank (Karinda and Citraningtyas 2013). After analyzing using a UV-Vis spectrophotometer, a calibration curve was made using excel.

Determination of vitamin C levels in samples

Tubers of *E. palmifolia* were weighed 5 g and crushed and smoothed; 10 mL of aquabides were added and homogenized. Next, the solution is filtered. The filtrate was put into a 10 mL measuring cup, then aquabides were added to the mark and homogenized. Finally, the absorption was measured at a wavelength of 265 nm (Karinda and Citraningtyas, 2013).

Measurement of flavonoid level

A total of 2 g samples of dried *E. palmifolia* tubers were put into a maceration container. Then added with ethanol PA (Pro Analysis) 96% 10 mL until the entire sample was submerged, closed, and left for 24 hours. Next, the macerate was filtered using Whatman 42 filter paper. The filtrate was obtained through filtering with a funnel, and then the pulp was macerated again with 96% PA (Pro Analysis) 10 mL ethanol so that the filtrate was almost colorless. Finally, all filtrates were combined and evaporated using a fan until there was no more liquid PA 96% ethanol to obtain an ethanolic extract of *E. palmifolia* tubers. The thick extract of *E. palmifolia* tuber obtained was used for further analysis by UV-Vis spectrophotometer.

Quercetin standard curve creation

In the making of quercetin solution concentration, weighed as much as 0.06 mg, 0.08 mg, 0.010 mg, 0.012 mg, and 0.014 mg of quercetin standard and dissolved in 10 mL of aquabides, so the quercetin solution concentration was 6 ppm, 8 ppm, 10 ppm, 12 ppm, 14 ppm. Then 1 mL of 2% AlCl3 and 1 mL of 120 mM potassium acetate were added. Samples were incubated for one hour at room temperature. Finally, the absorbance was determined using the UV-Vis spectrophotometric method at a maximum wavelength of 435 nm (Stankovic 2011).

Determination of total flavonoid content of Eleutherine palmifolia *tubers*

The extract *Eleutherine palmifolia* tubers were dissolved with 1 mL of 96% PA (Pro Analysis) ethanol to dissolve the remaining extract still attached to the porcelain cup. Then, according to the treatment, the solution was put into a test tube, and 1 mL of 2% $AlCl_3$ solution and 1 mL of 120 mM potassium acetate were added. Samples were

incubated for one hour at room temperature. Finally, the absorbance was determined using the UV-Vis spectrophotometric method at a maximum wavelength of 435 nm (Stankovic 2011).

Data analysis

Observational data of *Eleutherine palmifolia* tubers were analyzed by statistical analysis. In addition, quantitative data on daily observations (number of flowers, flowering speed, number of leaves, leaf width, stem height, and leaf length) and observations at harvest (dry and fresh biomass, flavonoid test, vitamin C test, photosynthetic pigment test, root shoot ratio, and index stomata) were analyzed by One Way ANOVA (Analysis of Varian). If there was a significant difference between treatments, proceed with DMRT at the 5% test level.

RESULTS AND DISCUSSION

Physiological character is closely related to the growth and productivity of its environment. The relationship shows that the physiological character formed can support plant growth needs with a larger sink so that the productivity of a plant can be adequately achieved. The ability of the source and sink will determine the potential yield of plants. The source is estimated as the total available energy and carbohydrates derived from the photosynthesis process after flowering and accumulation before flowering (Nurchayati et al., 2019). The sink results from photosynthesis stored as food reserves (Mastur 2015). Environmental factors also have a core role in influencing the formation of physiological characters. The environment plays a role in influencing plant behavior to regulate physiological processes to achieve comparable conditions between the environment and plant internals (Soverda 2012).

The phytochemical content in *E. palmifolia* plants has a vital role in plants defending themselves from stress, both drought and light stress. In addition, one of the secondary metabolites produced by plants is to defend themselves from unfavorable environmental conditions such as temperature, climate, pests, and plant diseases (Dwidjoseputro 1992).

Number of leaves

The number of leaves of *E. palmifolia* increased in the overall treatment for 30 days. The given interaction triggers the *E. palmifolia* plant to maintain the turgidity of plant cells, which impacts cell enlargement, stomata opening, and protoplasm formation. According to Felania (2017), water contributes to the turgidity of plant cells by acting as a constituent of protoplasmic cells and regulating plant temperature. Therefore, the increase in the relative growth of the number of leaves on *E. palmifolia* plants was triggered by the fact that the given water aided in the formation of protoplasmic cells, which could result in an increase in the formation of leaf organs on *E. palmifolia* plants subjected to all treatments. Additionally, the treatment interactions carry out photosynthesis, which results in the formation of tuber weight, which is useful for providing nutrients for growth (Figure 1).

The results of variance (Table 2) on the relative growth of the number of leaves of *E. palmifolia* showed that the overall effect of the treatments given was not significantly different. The relative growth of the number of effective leaves on 50% water availability and 50% light intensity treatment was 31.20 strands. This treatment resulted in fewer leaves because the chlorophyll in the leaves of *E. palmifolia* could absorb excess light, causing damage to the center of the chloroplast and resulting in decreased photosynthesis. The decrease in photosynthate production, especially the formation of vegetative organs, will also be hampered. According to Shao et al. (2014), plants exposed to excessive light damage photosynthetic equipment, particularly chloroplasts, resulting in decreased photosynthetic activity.

The combination given affects the response of *E. palmifolia* plants to drought stress which closes stomata to slow down water loss through the transpiration process. The slowing of water loss causes a tendency to produce materials (polysaccharides, lignins, proteins, lipids, amino acids, and other elements) widely used for canopy production. According to Mebrahtu et al. (2018), in the presence of water stress, the process of stomata closure to promote slow transpiration may occur in shallot plants. Plants directly suited to reward will respond by allocating dry matter for crown growth (Grime 1979).

Table 2. Relative growth of the number of the leaf of *E. palmifolia* after treatment with water availability and light intensity for 1 month (blades)

Treatment		Result
Water availability (%)	**Light intensity (%)**	**(blade)**
50	100	28.40
75	100	25.40
100	100	28.60
50	50	31.20
75	50	29.20
100	50	30.60

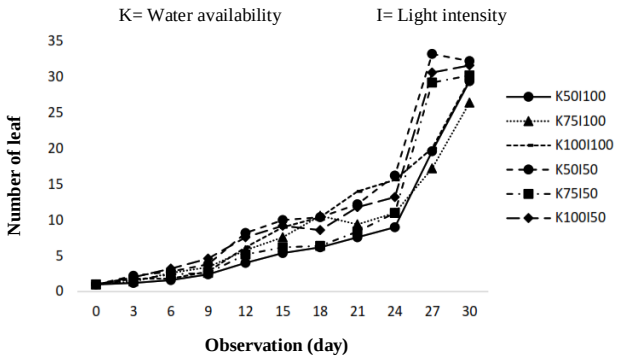

Figure 1. Daily leaf number growth of *E. palmifolia* after treatment with various light intensity and water availability combinations

The number of leaves in the formation correlates with the value of the root crown ratio. In addition, 50% water availability helps roots maximize nutrient absorption into the media to absorb the nutrients needed by *E. palmifolia* plants to make the necessary dry matter, and carotenoids become part of *E. palmifolia* plants to protect PSII from ROS during the process.

Leaf width

On *E. palmifolia* plants, the relative growth of leaf width increased from day 0 to day 18 and lowered from day 18 to day 30. When the water conditions in the planting media and the roots of *E. palmifolia* were reduced, photosynthate synthesis decreased, resulting in a gradual increase in leaf width. According to Bizuneh (2019), eliminating water leads the onion to grow until the root system's available water is fully depleted. All available nutrients generate photosynthate during the early phase of plant growth, which is later consumed and utilized by *E. palmifolia* plants to form new vegetative organs (Figure 2).

The variance analysis (Table 3) reveals a significant difference between water and light availability provision and the relative growth of leaves on *E. palmifolia* plants. The combination of 75% water and 50% light intensity resulted in the highest average value of 0.28 cm for *E. palmifolia* leaf width. The range of used treatments increased leaf surface area. The cells on the surface of the leaves grow more rapidly and expand more significantly as a result of maximizing light capture in shaded conditions for photosynthesis. According to Semida et al. (2017), its leaf surface increases when a plant is given shade. Due to photosynthesis, shade enables cells to grow in size and quantity. The best mixture facilitates carbon uptake by Dayak onion plants in *E. palmifolia* plants. As a result, *E. palmifolia* plants are capable of efficient photosynthesis, which enables the creation of vegetative organs to occur optimally. Shi et al. (2018) report that dryness can impede plant growth by lowering water loss and carbon assimilation. The interaction of the two (light intensity and water availability) leads to a photosynthetic cycle that generates, assimilates, and consumes plants to increase the size of *E. palmifolia* plant cells (cells that play a role in forming leaf width). Shade improves the chloroplasts' ability to capture light and assimilate it, which is advantageous for developing *E. palmifolia* plant cells (cells that support the formation of leaf width). Carotenoids have a critical function in the development of leaf width. Carotenoids absorb light that chlorophyll cannot absorb in darkened situations, speeding up the photosynthesis process. The condition of 75% water availability contributes to providing nutrients and light to *E. palmifolia* plants.

Leaf length

The graph depicts the relative increase in leaf length (strands) following the comprehensive treatment. The rise shows that *E. palmifolia* plants are becoming more tolerant of the overall treatment. When sufficient nutrients are available, photosynthesis occurs efficiently, and the produced photosynthate is also sufficient for leaf development. According to Manan and Machfudz (2015), optimal water availability is intimately tied to how plants absorb nutrients during their metabolic processes. Therefore, plants increase their leaf area in response to increased water availability. Furthermore, the combination of treatments resulted in an additional tolerance in increased chlorophyll content, indicating that this *E. palmifolia* plant got additional light energy that was subsequently employed optimally to generate photosynthate, specifically leaf length. According to Niinemets (2010), the low-light tolerant plant species in shadowed situations increase their chlorophyll content more than they do in full-light conditions (without shade) (Figure 3).

The variance analysis revealed that the effect of the combined treatment of water availability and light intensity on the relative leaf length growth of *E. palmifolia* plants was not substantially different (Table 4). Bozkurt and Keskin (2018) also found that the amount of water applied to cucumber plants had no significant effect on leaf length increase. Treatment with 75% water availability and 50% light intensity yielded a yield of 1.80 cm. The combination of these treatments was sufficient for *E. palmifolia* plants to avoid over-saturation due to the enormous volume of stagnant water in the root zone, allowing the *E. palmifolia* root system to function normally and grow rapidly. According to Boskurt and Keskin (2018), plants grown under stagnant water conditions could not perform regular respiration, consequently impeding plant growth.

Table 3. Relative growth of leaf width of *E. palmifolia* after treatment with water availability and light intensity for one month (cm)

Treatment		Result (cm)
Water availability (%)	Light intensity (%)	
50	100	0.05^{ab}
75	100	- 0.25^{a}
100	100	0.08^{ab}
50	50	0.03^{ab}
75	50	0.28^{b}
100	50	0.14^{b}

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

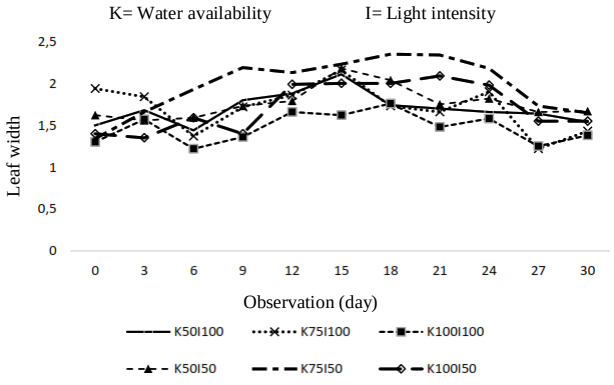

Figure 2. The relative growth of daily leaf width of *E. palmifolia* after the treatment of several combinations of light intensity and water availability

Optimal treatment can help reduce sun radiation exposure. Decreased exposure to solar radiation causes total chlorophyll (a and b) to be protected from excess damage (chlorophyll table) so that the formation of leaf organs can be optimal. Semida et al. (2017) stated that the shading effect could reduce radiation exposure and temperature around *E. palmifolia* plants. Lettuce that grew in the shade had a higher content of chlorophyll a and b than lettuce that was not exposed to shade. The shading effect also allows carotenoids to maximize light capture, which chlorophyll does not.

Midrib height

All treatments increased the relative growth of midrib. Even when subjected to various treatments, *E. palmifolia* plants exhibit great production and resistance to the creation of vegetative organs used for life support. The availability of water and the amount of light could signal that there are controls in place that promote the activity of photosynthesizing, which is required for plants to survive (Figure 4).

The variance analysis results (Table 5) show that when water availability and light intensity were combined, there was no significant effect on the relative development of midrib height in *E. palmifolia* plants. Moreover, 75% water availability and 100% light intensity were the ideal treatment for *E. palmifolia* plants to retain water potential and increase physiological and metabolic activities while maintaining a 7.50 cm yield. The interplay of the treatments aids in providing oxygen and carbon dioxide required for proper respiratory activity, ensuring that *E. palmifolia* plants continue to perform physiological and metabolic functions. According to Rachmawati and Retnaningrum (2013), when plants are submerged in water, their oxygen and carbon dioxide supply is lowered, interfering with photosynthesis and respiration.

The physiological process accelerated the growth of the midrib of *E. palmifolia* under conditions of 75% water availability and 100% light intensity. According to Sopandie (2014), light is involved in various physiological activities in plants, including photosynthesis, respiration, nutrition and assimilation transfer, growth and development, leaf opening and closure, and plant movement. When exposed to full sunshine intensity, the height of the midrib increased but was not accompanied by an increase in leaf number or width. Furthermore, it was determined that plants that received complete light treatment were more effective at stimulating midrib development acceleration than plants that received shade treatment. However, according to Hamdani et al. (2018), providing excessive shade to potato plants decreases growth rates. It is due to the low efficiency of photosynthesis caused by reduced CO_2, inhibited by a decrease in light intensity.

Table 4. Relative growth of *E. palmifolia* leaf length after treatment with water availability and light intensity for one month (cm)

Treatment		Result (cm)
Water availability (%)	Light intensity (%)	
50	100	1.24
75	100	1.04
100	100	1.48
50	50	1.07
75	50	1.80
100	50	1.56

Table 5. Relative growth of midrib height of *E. palmifolia* after treatment with water availability and light intensity for one month

Treatment		Result (cm)
Water availability (%)	Light intensity (%)	
50	100	5.51
75	100	7.50
100	100	2.88
50	50	3.87
75	50	3.94
100	50	4.83

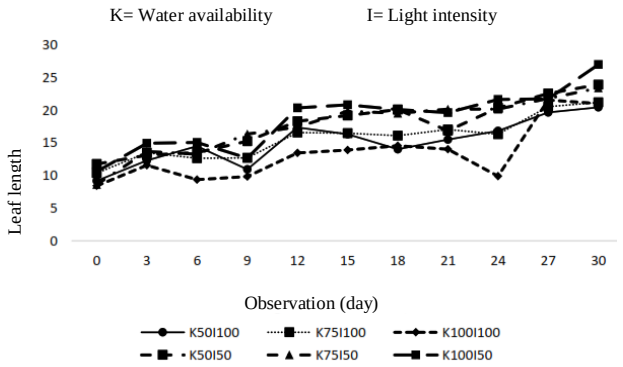

Figure 3. After treatment, the daily leaf length of *E. palmifolia* with several combinations of light intensity and water availability

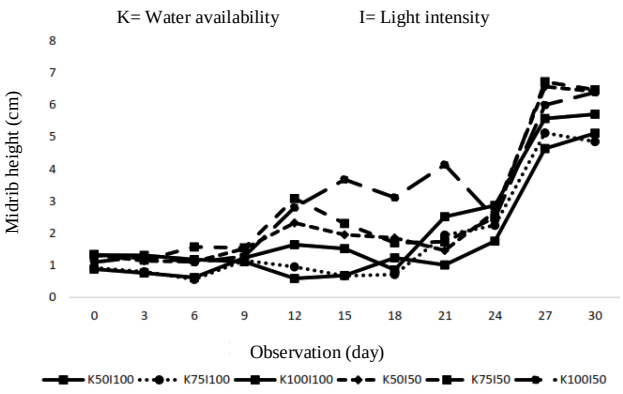

Figure 4. Daily midrib height of *E. palmifolia* after treatment with several combinations of light intensity and water availability

The wet weight of 22.50 grams (wet weight table) supported optimal growth of the midrib. The correlation between midrib height and wet weight indicates that potassium (a compound stored in *E. palmifolia* tubers and one of which affects the wet weight of tubers) is one of the nutrients absorbed by plants with tubers (Woldetsadik 2003). Potassium regulates the translocation of newly formed carbohydrates (Akhtar et al. 2002) and accelerates plant tissue growth (Hakim et al. 1986) so that the height of the midrib can be formed optimally.

Flowering speed

The study discovered that the interaction effect of water availability and light intensity on the rate of emergence of *E. palmifolia* flowers was not substantially different (Table 6). On the 26th day, the combination of water availability treatment and 100% light intensity produced optimal results for the flowering speed of *E. palmifolia* plants. The E. palmifolia plant completed its life cycle on that day by transitioning from the vegetative to the generative phase. This change happened due to the *E. palmifolia* plant utilizing certain nutrients (carbohydrates) to hasten flowering before waterlogging stress and extreme light intensity. In species that are not resistant to flooding, the interplay of treatment with water availability and 100% light intensity results in inundation and excess radiation, which can hinder the commencement of flower buds, flower blooms, fruit set, and fruit enlargement (Ezin et al. 2010). On the flooding stress, the carbohydrate content produced by photosynthesis drops. According to Ye et al. (2018), high waterlogging decreases carbohydrate content in *Arundinella anomala* plants. In contrast, it is well established that carbohydrates function in the flowering process (Fauzi et al., 2017).

The treatment interaction (K100I100) affected the plant's ability to survive in the presence of drought. Drought stress induces resistance in *E. palmifolia* plants. Therefore, *E. palmifolia* plants are categorized as escape plants, which means they finish their life cycle before being subjected to severe drought stress. Typically, this is accomplished by accelerating flowering and fruiting (Lestari 2006).

The considerable chlorophyll content combined with 100% light intensity optimally created carbohydrates, a necessary component for flower formation in *E. palmifolia* plants (chlorophyll table). As a significant element produced, Carbohydrates are kept as food reserves, and some are used to expedite the flowering process by *E. palmifolia* plants.

Amount of flower

The graph of the relative growth of flower rates increased from the 24th day to the 30th day. The *E. palmifolia* plants completed their vegetative phase on the 24th day and entered the generative phase on the same day. Flowering on the 24th day is influenced by the availability of water, light conditions, or the interaction of the two. The treatment given to *E. palmifolia* plants indicated they completed their reproductive cycle quickly on the 24th day by efficiently using and storing food reserves before the more severe drought and light stress. Barnabas et al. (2008) stated that this type of escape strategy is a strategy for plants to overcome drought by completing their reproductive cycle before severe drought stress occurs. Through a short life cycle, higher growth rate, efficient storage, and use of food reserves.

Research on the number of flowers showed that the effect of treatment on water availability and light intensity was not significantly different on the number of flowers. Treatment of 75% water availability and 50% light intensity showed the optimal interaction value in influencing the number of flowers compared to treatments with as many as 6.20 strands (Table 7). The interactions that occur produce products in the form of high carbohydrates to support flower initiation considering that flowering requires abundant energy. Increased carbohydrate status at the time of bud will stimulate the flowering process. These results follow the research of Pingping et al. (2017) on *A. carambola* plants, who reported that plants subjected to drought stress gave faster flowering results. Drought stress stimulated carbohydrate accumulation to initiate flower buds of *A. carambola*. Fauzi et al. (2017) also confirmed that in *Mangifera indica*, the canopy conditions with high carbohydrate accumulation supported the initiation of flowering, of course, in conditions that supported flowering. A supportive environment, such as sufficient water and light, stimulates carbohydrate accumulation in the shoots during the late vegetative phase to increase flower bud formation.

Table 6. The *E. palmifolia* flower emergence speed after treatment with variations in light intensity and water availability (days)

Treatment		Result (day)
Water availability (%)	Light intensity (%)	
50	100	26.67
75	100	27.00
100	100	26.00
50	50	27.00
75	50	27.00
100	50	27.00

Table 7. The number of *E. palmifolia* flowers after treatment with water availability and light intensity for one month (strands)

Treatment		Result (blade)
Water availability (%)	Light intensity (%)	
50	100	5.40
75	100	1.40
100	100	2.80
50	50	3.60
75	50	6.20
100	50	3.40

The optimal treatment altered the red light (Pr) received by *E. palmifolia* plants and converted it to long red light (Pfr) (660 nm) to accelerate generative development in *E. palmifolia* plants. According to Utami (2016), the generative phase in short-day plants begins with phytochromes receiving red light (Pr) and converting it to long red light (Pfr). Phytochromes are homodimers and polypeptide groups in *E. palmifolia*, each containing a prosthetic group called a chromophore. The phytochrome is physiologically affected by the light-absorbing chromophore. Phytochrome 2 is more active when plants are exposed to strong light. Pf and Pfr are formed from Phytochrome 2. Pfr is not generated in the absence of light. If Pfr exceeds Pr, apical dominance is lost, and the plant is induced into the generative phase. Phytochromes are found in the nucleus and throughout the cytoplasm of the cell. Shade circumstances maintain a healthy equilibrium between pf and pfr, producing good and optimal flower production.

Additionally, the quantity of blooms is proportional to the amount of carotenoids present. Carotenoids contribute to the absorption of light that is not absorbed by chlorophyll. Thus, the photosynthetic process can optimize the production of carbohydrates for future usage as flowering substrates.

Stomata index

The treatment of water availability and light intensity on the stomata index of *E. palmifolia* plants gave significantly different effects (Table 8). 100% water availability and 50% light intensity resulted in the best stomata index compared to other treatments, 36.89 cm. *E. palmifolia* plants responded by increasing the number of stomata on the epidermal surface. An increase in the stomata index indicates that the *E. palmifolia* plant is optimal in carrying out photosynthesis or metabolism and indicates that the plant is still surviving, which is indicated by the normal process of water loss and increased net CO_2 uptake in the leaves so that the photosynthesis process can take place optimally and increase productivity. According to Subantoro (2014), through his research, plants with drought stress environmental growth conditions will reduce the number of stomata, thereby reducing the rate of water loss followed by stomata closure and decreased net CO_2 uptake in leaves. MAPKs play an important role for *E. palmifolia* plants in maintaining drought stress conditions ranging from moderate to extreme levels. Plant resistance to drought stress due to MAPKs signaling activity. MAPKs consist of several sub-enzymes, namely MKK4/MKK5-MPK3/MPK6, which play an important role in controlling stomata development according to environmental conditions. The control carried out by several sub-enzymes of MAPKs is optimizing the ratio of differentiation between stomata and epidermal cells on the lower surface of the leaf according to environmental conditions. The lower the level of drought stress in an environment, the performance of MAPKs decreases. Research conducted using the plant Arabidopsis thaliana showed that MKK4/MKK5-MPK3/MPK6 had dual functions for stomata development and environmental stress response pathways. In stomata development, the function of this module is the transduction of endogenous and exogenous signals to target cells. Specifically, it optimizes plants' differentiation ratio of stomata and epidermal cells (Wang et al. 1998).

The interaction of the two treatments affects the genetic activity of SDD1 (Stomatal Density and Distribution). SDD1 is required for the beginning of stomata, the number of stomata, and the level of stomatal density per leaf area. Shade activates the SDD1 gene, resulting in the abaxial leaf's active production of stomata and epidermal cells. In addition, the SDD1 gene plays a role in developing stomata, producing protodermal cells that originate from stomata and epidermal cells (Berger and Thomas 2000).

Furthermore, the presence of a high stomata index in the presence of 100% water availability and 50% light intensity affects the gas exchange activity of *E. palmifolia* plants. As a result, *E. palmifolia* plants use the gas exchange to increase photosynthetic activity, which results in the assimilate formation process occurring properly. The assimilate is channeled to the phloem, one of which goes to the tubers to form the optimal wet weight under 100% water availability and 50% light intensity (wet weight table).

Wet and dry weight

The interaction impact of water availability and light intensity on the wet weight of *E. palmifolia* plants was not significantly different. The treatment with 75% water availability and 100% light intensity decreased the wet weight value for *E. palmifolia* plants. In contrast, the treatment with 100% water availability and 50% light intensity increased 29.77 grams in wet weight for *E. palmifolia* plants (Table 9). Ratri et al. (2015) showed that when turmeric plants were subjected to water stress, the intensity of the light had no discernible influence on fresh weight. It is believed that the plant has a tolerance for shade and water stress to maintain proper metabolic function. It does not affect the turgor pressure in plant cells, allowing plants to survive and avoid severe withering when light intensity is high and water availability in the soil drops. The absence of wilting shows that the *E. palmifolia* plant's primary productivity is in good health. A high wet weight indicates the presence of nutrients in tubers, roots, and other plant parts that *E. palmifolia* plants utilize to generate vegetative organs.

Table 8. Abaxial stomata index (lower leaf surface) of *E. palmifolia* after treatment with variations in water availability and light intensity for one month (cm)

Treatment		Result (cm)
Water availability (%)	Light intensity (%)	
50	100	32.52[a]
75	100	36.19[b]
100	100	36.30[b]
50	50	34.93[ab]
75	50	36.84[b]
100	50	36.89[b]

Note: The numbers followed by the same letter are not significantly different at the 5% DMRT test level

According to Table 10, the effect of the combination of water availability and light intensity on the dry weight of *E. palmifolia* plants was not significantly different. The combination of 100% water availability and 50% light intensity led to the dry weight of *E. palmifolia* plants aggregating to 7.56 grams. According to Ratri et al. (2015), the dry weight of turmeric plants had no discernible effect on shadow and drought stress. Therefore, it is believed that shading and water stress did not affect the photosynthetic activity or photosynthate translocation. Plants can also maintain a balance between water loss and absorption, thereby reducing the amount of water in plant cells.

Root canopy ratio

The interaction effect of water availability and light intensity on the root crown ratio of *E. palmifolia* was not significantly different (Table 9). Anggraini et al. (2015), in the study of black locust plants, stated that the root crown ratio increase occurred because the biomass allocation to the roots of *E. palmifolia* plants decreased and was shifted to crown growth. It is reinforced by Nejad et al. (2010), who noted that the water deficit decreased the root crown ratio in maize. In drought conditions, the allocation of biomass to roots is usually increased to be used as an effort to access water sources (Zlatev and Fernando 2012).

The optimal treatment interaction obtained played a role in increasing the root temperature (Table 11). An increase in root temperature causes an increase in root activity to absorb nutrients in the growing media. The application of treatment caused a decrease in temperature, which resulted in the allocation of biomass being directed towards the goal of crown growth. Increased root activity in absorbing nutrients would greatly impact photosynthate formation activity so that the canopy increase can be optimally carried out. Wilson (1988) stated that increasing root temperature increased root activity in absorbing nutrients so that it could accelerate root formation. Akmalia and Suharyanto (2017) said that the lowest light intensity would allocate the biomass towards the canopy so that the value of the root-crown ratio is smaller.

Chlorophyll content

The effect of light intensity and water availability on chlorophyll a, b, and total chlorophyll was not significantly different (Table 12). Chlorophyll a, b, and total were formed in *E. palmifolia* at a light intensity of 8.79 g/mL, 13.06 g/ml, and 21.85 g/mL, respectively, with 100% water availability. This condition implies that the *E. palmifolia* plant is still tolerant, allowing for good metabolism and photosynthesis. The treatment of water availability and 100% light intensity resulted in a blue color spectrum that accelerated the *E. palmifolia* plant's reaction to chlorophyll creation, resulting in increased chlorophyll production. The reaction results in chlorophyll synthesis from the glutamate molecule, which is deaminated to create α-ketoglutarate. α-ketoglutarate is transformed into the amino acid levulinate in the presence of sunlight via transaminases with the assistance of ATP and NADPH. The release of levulinate amino acids occurs sequentially into the water, NH_3, and CO_2, resulting in the formation of protoporphyrinogen. The chlorophyll synthesis process is continued with the production of Mg^+ to Mg^-protoporphyrin monomethylester. The presence of magnesium in protoporphyrin monomethylester affects chlorophyll's ability to absorb light. Chlorophyll a is formed when Mg-protoporphyrin monomethylester reacts with H+ ions. The creation of chlorophyll b begins with the synthesis of methyl oxidation in chlorophyll a. The enzyme CAO aids in the formation of chlorophyll b. (Chlorophyll and Oxygenase). This enzyme is responsible for transferring electrons from the methyl group in chlorophyll a to chlorophyll b (Tanaka et al. 2005) (Figure 5).

Table 9. Total wet weight of *E. palmifolia* after treatment with variations in water availability and light intensity for one month (grams)

Treatment		Result (gram)
Water availability (%)	Light intensity (%)	
50	100	26.44
75	100	22.50
100	100	26.17
50	50	26.36
75	50	25.42
100	50	29.77

Table 10. The total dry weight of *E. palmifolia* after treatment with variations in water availability and light intensity for one month (days)

Treatment		Result (gram)
Water availability (%)	Light intensity (%)	
50	100	6.52
75	100	6.12
100	100	6.68
50	50	6.64
75	50	6.63
100	50	7.56

Table 11. Root crown ratio of *E. palmifolia* after treatment with variations in water availability and light intensity for 1 month (cm)

Treatment		Result (cm)
Water availability (%)	Light intensity (%)	
50	100	1.65
75	100	0.96
100	100	1.84
50	50	1.23
75	50	1.33
100	50	1.18

Table 12. The content of chlorophyll a, b, and total of *E. palmifolia* after treatment with variations in water availability and light intensity for one month (g/mL)

Treatment		Chlorophyll a	Chlorophyll b	Total chlorophyll
Water availability (%)	Light intensity (%)			
50	100	6.14	9.84	15.98
75	100	5.05	9.07	14.12
100	100	8.79	13.06	21.85
50	50	7.26	8.78	16.04
75	50	8.29	9.68	17.97
100	50	6.92	9.85	16.80

The ideal treatment combination (100% water availability and 100% light intensity) (Table 12) enhanced the chloroplast's ability to avoid gene expression reprogramming, resulting in chlorosis or programmed cell death. Reduced H_2O_2 levels protect *E. palmifolia* plants from undergoing programmed cell death. According to Wei et al. (2015), the decrease in chlorophyll content under drought stress could result from ROS activity damaging the chloroplasts. ROS generation in chloroplasts in response to abiotic stress (such as drought) can also result in gene expression reprogramming, causing cells to enter chlorosis or programmed cell death (Lee et al. 2007).

The chlorophyll a, b, and total content in the treatment of water availability and 100% light intensity resulted in an increase in chlorophyll activity with the help of vitamin C, which was sufficient to reduce ROS levels in *E. palmifolia* plants, allowing photosynthesis to occur quickly and reliably. In addition, since chlorophyll b has a larger molecular weight (907.49 g/mol) than chlorophyll a (839.51 g/mol), it resulted in higher average content.

Carotenoid level

Based on the variance (Table 13), the interaction effect of water availability and light intensity on the carotenoid content of *E. palmifolia* plants was significantly different. The best treatment was 75% water availability and a 50% light intensity of 39.70 g/mL. This treatment was able to increase the value of the best carotenoid levels. The optimal interaction affects the activity of carotenoid formation and the extension of the antenna for light absorption. The function of carotenoids is to give plants the ability to reduce singlet oxygen (oxygen which has high energy and is more reactive to organic compounds) PSII which, if left unchecked, will trigger oxidative reactions that can damage the PSII reaction center complex and the core of PSII in the photosynthesis process. In addition, when the carotenoid content is high, there is a reduction in the content of ROS (Reactive Oxygen Species) which, when high, causes cell and tissue damage. According to Van Gorkom and Schelvis (1993), the carotenoids in the PSII reaction center in photosynthesis reduce singlet oxygen, which protects against oxidative damage. Uarrota's (2018) research also corroborates that carotenoids play an important role in converting singlet oxygen that can damage PSII into less reactive triplet oxygen.

The best treatment produced helps the light-harvesting activity increase due to the antenna activity performance of the carotenoids to increase energy in light absorption. Carotenoids help plants expand the light capture area, which will later function for the smooth process of photosynthesis. It can be seen that the result of photosynthesis involving carotenoids is flower formation which occurs more quickly in *E. palmifolia* when it is given shade treatment (Table 7). Carotenoids accelerate flowering and give color to flowers. Strazlka et al. (2003) suggested that carotenoids have an impact on accelerating the formation and coloring of flowers. Strazlka et al. (2003) stated that carotenoids could act as energetic antennae under low light conditions, harvesting light at wavelengths not absorbed by chlorophyll and transferring excited electrons to the phytochemical reaction center. Under these conditions, carotenoids play a role in expanding the absorption of light in photosynthetic activity.

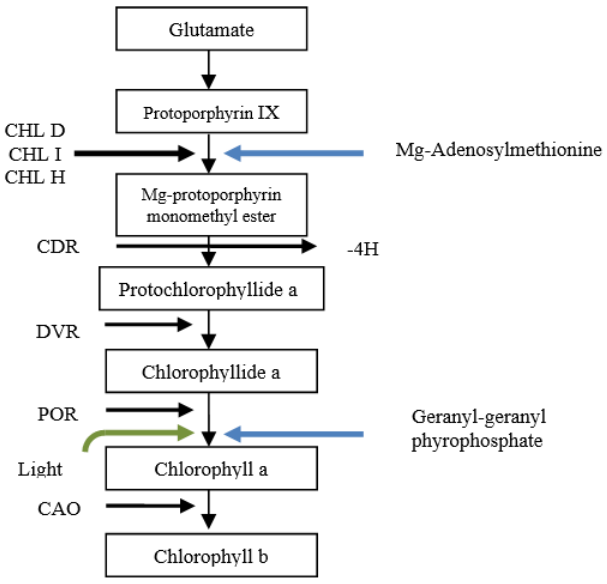

Figure 5. Mechanism of biosynthesis of chlorophyll a and b

Vitamin content

The linear regression line equation for ascorbic acid absorption is Y = 0.0004x + 0.0662 with an R2 value of 0.6773. The range of data obtained from validating the ascorbic acid level test method demonstrates a linear response. The test's regression coefficient was 0.6773 (67.73 %) (Figure 6).

This study (Table 14) demonstrated no significant difference in the effect of light intensity treatment and water availability on the vitamin C content of *E. palmifolia* plants. Vitamin C concentrations ranging from 0.06754 to 0.06760 g/mL in *E. palmifolia* tubers enabled *E. palmifolia* plants to survive under drought and light stress. Ascorbic acid protects *E. palmifolia* plants from various environmental stressors, including dryness and excessive light (Venkatesh and Se 2014).

The interaction between water availability and light intensity resulted in an optimal vitamin C concentration of 0.06760 g/mL in the 100% water availability and light intensity treatment (Table 14). These results are obtained because flooding causes hypoxia, a condition where the oxygen level (O^2) used for metabolic processes is too low. The oxygen content in the growing media is used by microorganisms more quickly than diffusion to the roots. Hypoxia triggers genetic activity in plants to prepare *E. palmifolia* plants to acclimatize to stressful environments. Hypoxia triggers ROS formation (Reactive Oxygen Species) as a signal that receives "information" from the stressed environment. The ROS produced in the *E. palmifolia* plant comes from the RBOH (Respiratory Burst Oxidase Homolog)/NADPH oxidase system as an important protein that produces ROS that functions as a signal for regulating growth, development, and stress response. If excessive, ROS produced by *E. palmifolia* plants will damage cells and tissues in *E. palmifolia* plants.

The coordination between RBOH, ROS, and ascorbic acid produced was able to carry out aerobic respiration without significant damage to plant cells and tissues due to inundation stress. Shasidaran et al. (2018) stated that during floods/excessive puddles, a signaling process occurs in the form of ROS (Reactive Oxygen Species) induction via RBOH (Respiratory Burst Oxygen Homolog). Therefore, to provide stimulation so plants can acclimate to stagnant conditions, antioxidants in ascorbic acid play an important role in controlling ROS so that cells and tissues during the acclimatization process to inundation stress are not damaged (Ullah et al., 2017).

The interactions that occur (Table 14) affect *E. palmifolia* plants' genetic activity. According to Massot et al. (2013), the activity of APX (ascorbate peroxidase) in symplast has about the same ability to regulate ascorbic acid synthesis. Moreover, GME, GPP1, and GLDH can synthesize ascorbic acid, which is greatly influenced by the quantity and quality of light. Full light conditions create an atmosphere with a temperature that is more conducive to enhancing the activity of the three genes. According to Massot et al. (2013), there was no discernible change in the ascorbic acid concentration of shaded and unshaded plants. This situation was created by a little rise in ascorbic acid level in the shade, induced by modestly enhanced expression of the GME, GPP1, and GLDH genes at 120C. The effective vitamin C content in the treatment of water availability and 100% light intensity suggested an interaction that resulted in a vitamin C content sufficient to lower ROS levels in *E. palmifolia* plants.

Glucose has a critical role as an energy source, a carbon supply, and a signaling molecule that regulates gene expression during the formation of secondary metabolites. Secondary metabolites can be formed in response to carbon sources, and secondary metabolites can be maintained at high carbohydrate concentrations. Carbohydrates influence the synthesis of secondary metabolites in plant cells via glycolysis and the Krebs cycle. The produced glucose will subsequently enter the vitamin C manufacturing process via the D-glucuronic acid and L-gulonic acid pathways, which will convert it to ascorbic acid. However, the phases of vitamin C production via the D-glucuronic and L-gulonic acid routes begin with the conversion of glucose-6-phosphate to glucose-1-phosphate and end with the formation of ascorbic acid, do not always proceed in the same direction or the same order. Because of D-glucuronate and L-gulonate, there are additional phases, one of which is an oxidation process, which results in vitamin C degradation. Oxidation is a chemical reaction in which a molecule, an atom, or an ion releases electrons (Syefanis et al., 2019).

Table 13. Carotenoid content of *E. palmifolia* after treatment with variations in water availability and light intensity for one month (g/mL)

Treatment		Result (g/mL)
Water availability (%)	Light intensity (%)	
50	100	19.24[ab]
75	100	7.39[a]
100	100	28.19[ab]
50	50	38.87[b]
75	50	39.70[b]
100	50	21.49[ab]

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

Table 14. After treatment, the vitamin C content of *E. palmifolia* with variations in water availability and light intensity for one month (g/mL)

Treatment		Result (g/mL)
Water availability (%)	Light intensity (%)	
50	100	0.06756
75	100	0.06754
100	100	0.06760
50	50	0.06756
75	50	0.06758
100	50	0.06758

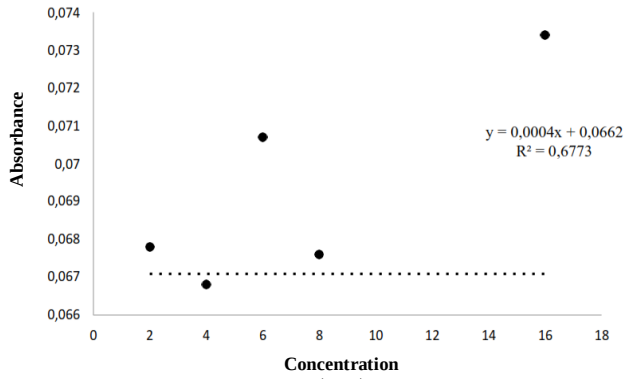

Figure 6. Graph of standard curve of vitamin C solution

Table 15. The flavonoid content of *E. palmifolia* after treatment with variations in water availability and light intensity for one month (g/mL)

Treatment		Result (g/mL)
Water availability (%)	Light intensity (%)	
50	100	2.517×10^{-1}
75	100	2.500×10^{-1}
100	100	2.515×10^{-1}
50	50	2.505×10^{-1}
75	50	2.508×10^{-1}
100	50	2.515×10^{-1}

Flavonoid content

The analysis of variance revealed that the combined effect of water availability and light intensity on the flavonoid content of *E. palmifolia* plants was not substantially different. Compared to other treatments, the combination of 50% water availability and 100% light intensity (Table 15) offered effective treatment with a yield of $2,517 \times 10^{-1}$ g/mL. It demonstrates that the *E. palmifolia* plant has an efficient photosynthetic and metabolic mechanism. Flavonoids produced in adequate amounts during drought and light stress could minimize the quantities of free radicals that can damage cell and tissue organelles, impairing the life of *E. palmifolia* plants. The Sufficient flavonoids enable Arabidopsis thaliana plants to survive by boosting their ability to minimize free radical levels (Shojaie et al. 2016). Drought stress affects increasing the flavonoid content in the tubers and roots of *E. palmifolia*, which plays a critical role in the control of ROS (Reactive Oxygen Species), which, if allowed to accumulate, will damage the cell organelles and plant tissues of *E. palmifolia*, impairing metabolic processes in *E. palmifolia* plants. According to Brown et al. (1998), plants generate flavonoids to combat oxidative damage to cells and tissues during drought, thereby reducing the generation of excess reactive oxygen species (ROS). Therefore, sufficient flavonoid content can aid in the response of plants to drought stress. Under drought stress, *E. palmifolia* plants execute phenolic (flavonoid) production more efficiently than under normal climatic conditions. Drought stress inhibits flavonoid biosynthesis pathways, hence protecting plants from harmful impacts. The function of PAL enzymes (enzymes involved in membrane integrity and canopy formation) and CHS in the phenylpropanoid pathway (the primary pathway for the synthesis of phenolic compounds (flavonoids)) is critical for the continuance of flavonoid formation under stressful circumstances. Under stressful conditions, a rise in the enzymes PAL (Phenylalanine ammonia-lyase) and CHS (Chalcone synthase) encouraged an increase in the synthesis of flavonoids, such as kaempferol and quercetin.

Additionally, an increasing the transcript level of genes encoding essential enzymes in phenolic biosyntheses such as F3H, CHI, FLS (Flavono Synthase), and FGT occurred in parallel with the increase in both enzymes (Flavonol glycosyltransferase). Both substances are categorized as flavonoids, which help prevent the oxidative stress response from increasing (Kurepa et al., 2019). According to Sharma et al. (2019), plants that grow under extreme stress have a greater capacity for flavonoid biosynthesis than plants that grow under normal conditions. The biosynthesis of phenolics, particularly flavonoids, enhanced PAL and CHS enzymes under stress conditions. Increases in these enzymes level were also accompanied by increases in the levels of gene transcripts encoding essential enzymes involved in phenolic biosynthesis, including F3H, CHI, FLS (Flavono Synthase), and FGT (Flavonol glycosyltransferase). The treatment with 50% water availability and 100% light intensity induced the roots of *E. palmifolia* plants to produce more primary photosynthate; this primary photosynthate was then employed as a substrate for *E. palmifolia* to produce flavonoids in the epidermal cells. *E. palmifolia* plants utilize antioxidants in the form of flavonoids to prevent excessive oxidative responses. It is consistent with the research of Warren et al. (2003), who found that increased light intensity increases the creation of primary photosynthate, and primary photosynthate produces phenolics. Antioxidants are believed to be created as a response to protect plants from oxidative stress. Numerous studies have demonstrated increased flavonoid content in various taxa that grow in bright sunshine rather than shade (Karimi et al., 2013).

The production of flavonoids begins with phenylalanine, which is formed when glucose is converted to pyruvic acid and then transferred to the acetoacetyl-CoA pathway via the Krebs cycle. Next, the acetoacetyl-CoA pathway generates phenylalanine, which is converted into flavonoid compounds such as quercetin and kaempferol via the phenylpropanoid biosynthesis pathway (Penuelas and Marc 1998).

This study can draw the following conclusions: (i) The interaction of light intensity treatment and water availability substantially affected leaf width, stomata index, and carotenoids. However, the quickest flowering time was 26 days in the treatment with 100% water availability and light intensity and was not substantially different in the other treatments. In addition, leaf quantity, length, midrib height, flower count, root crown ratio, wet and dry weight, chlorophyll content, vitamin C content, and flavonoids were not substantially different. (ii) The combination treatment of

75% water availability and 50% light intensity (K75I50) increased the leaf width, and carotenoid content of *E. palmifolia* plants the most. In contrast, the 100% water availability treatment and 50% light intensity (K100I50) combination treatment is the best treatment to increase the stomatal index.

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Effect of carrot leaf juice (*Daucus carota*) on the lung histology structure of white rats (*Rattus norvegicus*) induced by cigarette smoke

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Abstract. *Utami AS, Harini M, Handajani NS, Hidayat LN. 2020. Effect of carrot leaf juice (*Daucus carota*) on the lung histology structure of white rats (*Rattus norvegicus*) induced by cigarette smoke. Cell Biol Dev 4: 40-45.* Cigarette smoke is hazardous to both active and passive smokers. The impact results in oxidative stress, which results in cell damage across the lung's histological structure, including tissue dilation in the alveoli, epithelial abrasion, and other abnormalities. Carrot leaves contain beta carotene, an antioxidant that can help repair damaged cells caused by cigarette smoke's oxidative stress. The goal of this study was to assess the histological structure of the lungs following induction with cigarette smoke and the influence of carrot leaf juice (*Daucus carota* L.) on the lung structure following induction with cigarette smoke. A completely randomized design (CRD) with five replications was adopted in this investigation. The rats were divided into four groups: those induced by cigarette smoke and given 4 mL distilled water, those induced by cigarette smoke and given 4 mL carrot leaf juice (2 mL carrot leaf juice added to 2 mL distilled water), those induced by cigarette smoke and given 4 mL carrot leaf juice (3 mL carrot leaf juice added to 1 mL distilled water), and those induced by cigarette smoke and given 4 mL oat straw juice. For 14 days, white rats were provoked with three cigarettes every day. On the 31st day, rats were killed via cervical dislocation, their lungs were removed, and histological preparations using the paraffin method and Hematoxylin-Eosin staining were performed. Quantitative data were analyzed using non-parametric techniques Anova test, and qualitative data collection by describing the effect of cigarette smoke before and after carrot leaf juice administration.

Keywords: Beta carotene, carrot leaf, *Daucus carota*, histopathology, lung

INTRODUCTION

Cigarette smoke significantly negatively influences the health of active smokers, the environment, and passive smokers. Cigarettes and carcinogenic chemicals are inextricably linked. The consequences are severe, one of which is lung cancer. Several components can reduce the carcinogenic mechanism of the lungs due to smoking, such as anti-inflammatory drugs (budesonide, celecoxib, aspirin, naproxen, licofelone), antidiabetic drugs (Metformin, pioglitazone), antineoplastic agents (lapatinib, Bexaroterie, vorinostat), and other drugs and supplements (phenethyl isothiocyanate, Myo-inositol, N-acetylcysteine, ascorbic acid, berry extract). Pharmacological developments here are intended to prevent the impact of cigarette smoke on the body (Flora et al., 2016).

Oxidative stress that occurs in the lungs due to cigarette smoke has been shown to increase the levels of ceramides that accumulate in cells. The concentration of ceramide in the lungs will increase the release of superoxide (O^{2-}), which acts as a free radical. The accumulation of ceramide from cigarette smoke in the cells causes lung emphysema and cell death in the alveoli (Petrache et al. 2008).

The content of cigarettes, such as tar, nicotine, and carbon monoxide, has a harmful effect on the body. However, cigarettes also contain metal elements such as Al, As, Cd, Cr, Cu, Pb, Mn, Hg, Ni, Po-210, Se, and Zn, which have harmful effects on the organs of active and passive smokers. The content of these metals, if inhaled by passive smokers, will impact lung tissue and be deposited in alveolar tissue. Besides that, it is also toxic to the fetus. Carbon monoxide, tar, nicotine, and other metal elements in cigarettes can be carcinogenic in the body (Chiba and Masoroni 1992).

One of the effects of smoking on lung health is causing emphysema in the terminal bronchioles. Emphysema is a clinical condition with abnormalities in the anatomical structure of the lungs in the form of widening and destruction of the walls of the terminal bronchioles. Emphysema can also occur in the alveolar walls (Alsagaff et al. 1989).

Carrot leaves contain vitamin C, -carotene, fiber, and minerals such as Na, P, K, Ca, Mg, Mn, Zn, and Fe. Besides that, they also contain omega-3 fatty acids. Alpha-linolenic acid (LNA) is an essential fatty acid in omega-3, which plays an important role in human health (Almeida et al., 2009). Carrot leaf paste with a concentration of 5% can increase levels of antioxidants and omega-3 fatty acids. The compounds responsible for the antioxidant function in carrot leaves are flavonoids. The function of the antioxidant itself is to prevent cell damage such as cancer, inflammation, atherosclerosis, or other cell damage caused by free radicals (Boroski et al., 2011).

Omega-3 fatty acids and antioxidants in carrot leaves serve as nutritional enhancers for the body. Carrot leaf paste is made by extracting dried carrot leaves. The results

showed that carrot leaf paste with a concentration of 5% increased levels of antioxidants and omega-3 fatty acids. Flavonoids are one of the compounds responsible for the antioxidant function in carrot leaves. The function of antioxidants is to prevent cell damage such as cancer, inflammation, atherosclerosis, or other cell damage caused by free radicals (Boroski et al., 2011).

Beta carotene in carrot leaves is a group of carotenoids that act as antioxidants. In addition, carotenoids play an important role as a secondary defense system, warding off free radicals such as peroxyl radicals. Besides that, carotenoids act as pro-oxidants (Stahl 2016; Abidin et al. 2019). Beta carotene is important for cardioprotection or other organs and binds free radicals that enter the body. And it has a significant effect on preventing damage due to increased radicals, one of which is cigarette smoke (Csepanyi et al., 2015).

MATERIALS AND METHODS

Research time and location

The research was carried out from April to August 2017. First, it was carried out at the Biology Laboratory, Faculty of Mathematics and Natural Sciences; and Histology Laboratory, Faculty of Medicine at the Universitas Sebelas Maret, Surakarta; then Laboratory of Animal Anatomy, Faculty of Biology, Universitas Gadjah Mada, Yogyakarta, Indonesia.

Methods and materials

Carrot leaves, kretek cigarettes, 2 of 3-month-old male white rats weighing 200 grams, pellets as white rat feed, aquadest as a white rat drink, and aquadest, a package of ingredients for making sliced preparations using the paraffin method, were utilized in this investigation (70 percent ethanol, xylol, paraffin, hematoxylin-eosin staining kit).

The experimental design employed total randomization (CRD). Twenty-eight male white rats were divided into four treatment groups: group p1 was exposed to only cigarette smoke, group p2 was exposed to cigarette smoke and given 2 mL carrot leaf juice per day, group p3 was exposed to cigarette smoke and given 3 mL carrot leaf juice per day, and group p4 was exposed to cigarette smoke and given 4 mL carrot leaf juice per day.

White rats were exposed to cigarette smoke from three cigarettes daily for 14 days (Tohomi et al. 2014), beginning on day eight, in a rat cage enclosed in a cardboard lid measuring 50 x 35 x 20 cm with a ventilation hole measuring 20 x 10 cm. Carrot leaf juice was administered once daily to white rats exposed to cigarette smoke for seven days, beginning on day 22. On day 29, before surgery, the white rats were killed via cervical dislocation. The lungs of white rats were removed and preserved. The results of observations of damage to the histological structure of the lungs will be scored, namely:

0 = no changes in histological structure
1 = damage to less than 25% of the total field of view
2 = damage to 26-50% of the entire field of view
3 = damage to 51-75% of the total field of view
4 = damage to 51-75% of the entire field of view

The scoring results will be used as quantitative data for the ANOVA statistical test.

RESULTS AND DISCUSSION

Alveoli cross section

For each of the two preserved preparations, the degree of tissue damage was assessed using a scoring system (see Table 1). So, for the two preserved preparations that were seen in 50 fields of vision at a magnification of 100x, there were 100 scoring data points.

Bronchi cross-section

The degree of tissue damage was evaluated using a scoring system (Table 2). Each preparation was observed in 50 fields of view, resulting in 100 score data for the two preserved preparations at a magnification of 100x.

Cross-section of bronchioles

Scoring (Table 3) was used to determine the degree of tissue damage in each of the two conserved preparations. Using 50 fields of view for each preserved preparation, the total scoring data for the two preserved preparations was 100 with a magnification of 100x (Figure 4-11).

Table 1. Description of alveolar damage scoring from the One Way ANOVA Test

Treatments	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Control	2	3.0000	.00000	.00000	3.0000	3.0000	3.00	3.00
P1	2	3.0000	.00000	.00000	3.0000	3.0000	3.00	3.00
P2	2	3.0000	.00000	.00000	3.0000	3.0000	3.00	3.00
P3	2	3.0000	.00000	.00000	3.0000	3.0000	3.00	3.00
Total	8	3.0000	.00000	.00000	3.0000	3.0000	3.00	3.00

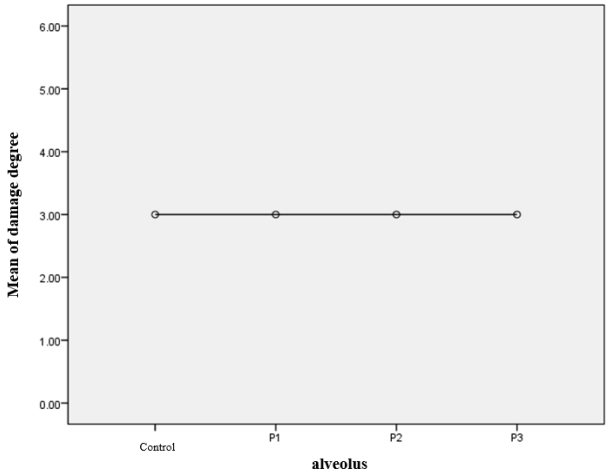

Figure 1. Means plots One Way Anova Test

Table 2. Description of bronchial damage scoring from One Way Anova Test

Treatments	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Control					4.0000	4.0000	4.00	4.00
P1	2	4.0000	.00000	.00000	4.0000	4.0000	4.00	4.00
P2	2	4.0000	.00000	.00000	4.0000	4.0000	4.00	4.00
P3	2	4.0000	.00000	.00000	4.0000	4.0000	4.00	4.00
Total	8	4.0000	.00000	.00000	4.0000	4.0000	4.00	4.00

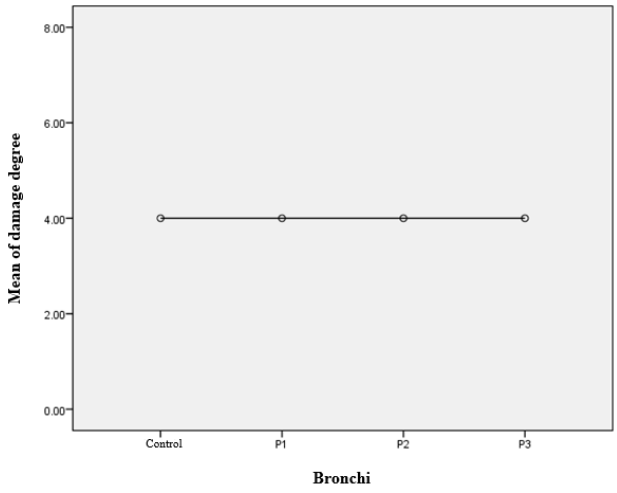

Figure 2. Means plots of One Way ANOVA Test

Table 3. Description of bronchiolar damage scoring from One Way ANOVA test

Treatments	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Control	2	3.5000	.70711	.50000	-2.8531	9.8531	3.00	4.00
P1	2	3.5000	.70711	.50000	-2.8531	9.8531	3.00	4.00
P2	2	3.0000	.00000	.00000	3.0000	3.0000	3.00	3.00
P3	1	3.0000	.	.	.	.	3.00	3.00
Total	7	3.2857	.48795	.18443	2.8344	3.7370	3.00	4.00

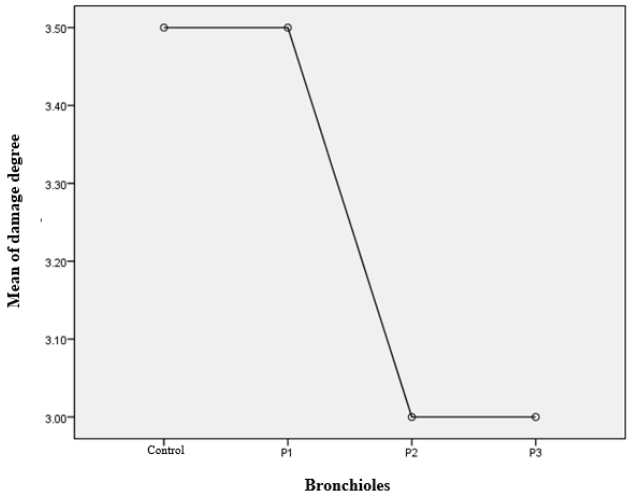

Figure 3. Means Plots of One Way ANOVA Test

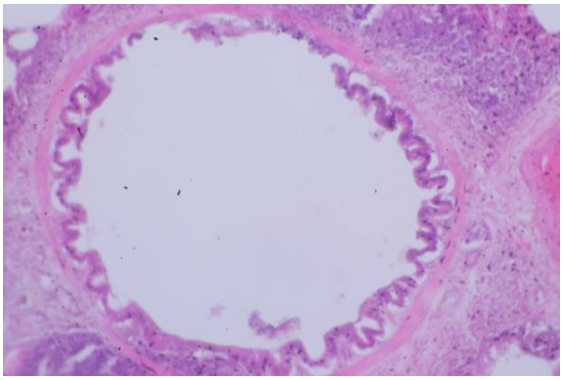

Figure 4. Cross-section of the bronchus control

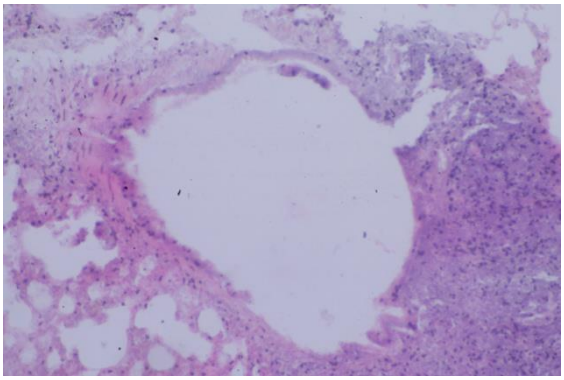

Figure 6. Cross-section of the bronchus P1

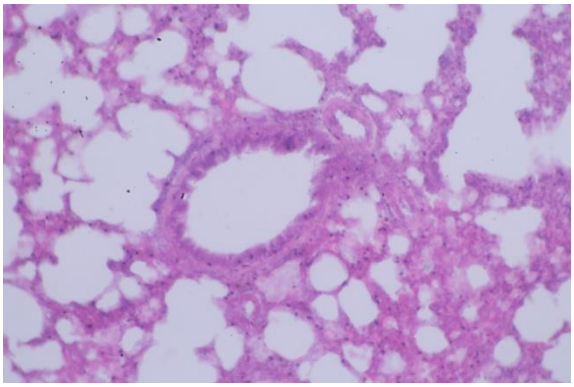

Figure 5. Cross-section of bronchioles control

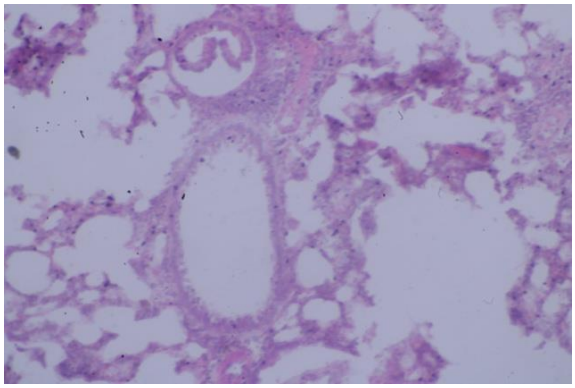

Figure 7. Cross section of bronchioles P1

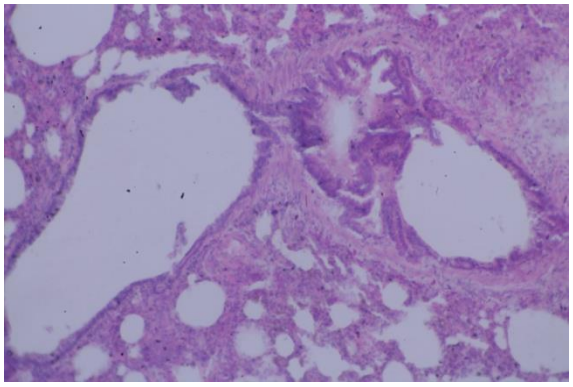

Figure 8. Cross-section of bronchus P2

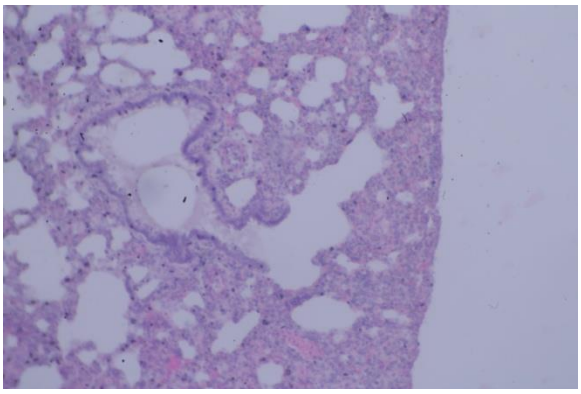

Figure 9. Cross section of bronchioles P2

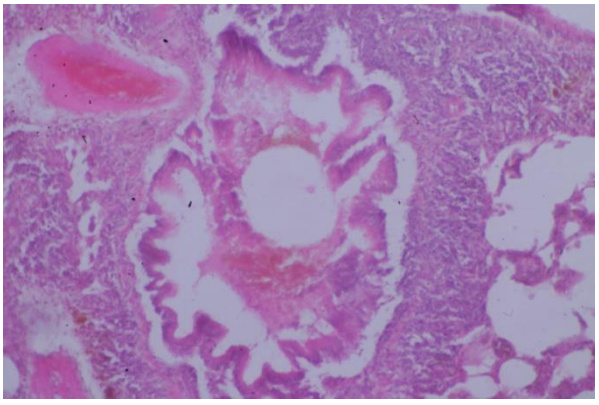

Figure 10. Cross-section of bronchus P3

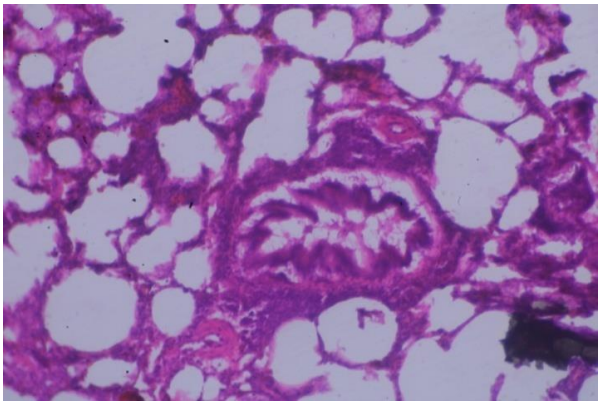

Figure 11. Cross-section of bronchioles P3

Discussion

Allevoluus histopathology

Observation of the alveolus on preserved preparations showed that the diameter of the alveolus experienced widening and lost its elasticity. Cigarette smoke causes fatty acid chains to break and produces various compounds, one of which is MDA (Malondialdehyde). These compounds are formed due to the reaction of lipid peroxides that react continuously. In addition to the peroxide reaction, cigarette smoke causes protein oxidation reactions, increasing the proteolytic enzyme reaction and inactivating the antiproteolytic enzyme. This condition causes the levels of the two enzymes to experience an imbalance. Antioxidants are one way to prevent a decrease in the percentage density per volume in the alveoli caused by the effects of free radicals from cigarette smoke. The content of phenol and ethanol from plants or fungi can prove this. Consumption of antioxidants can balance the levels of proteolytic and antiproteolytic enzymes (Rahimah et al., 2010).

In the control preparation (P1) with exposure to cigarette smoke for 14 days and seven days in oral distilled water, observations were made with a magnification of 100x. The tissue damage was scored, one of which was alveolar dilation. The average number of observations is 3, with damage between 51% and 75%. Likewise, for preparations, P2 with treatment exposed to cigarette smoke for 14 days and seven days in oral 2 mL carrot leaf juice, P3 with treatment exposed to cigarette smoke for 14 days and seven days in oral 3 mL carrot leaf juice, and P4 with treatment in exposure to cigarette smoke for 14 days and seven days in oral 4 mL of carrot leaf juice, all of them gave observations with an average score of 3. The scores obtained were then carried out with a one-way ANOVA test. The results showed that carrot leaf juice did not affect repairing damage to the lung alveoli.

Histopathology of the bronchi and bronchioles

Observations of the bronchi and bronchioles of the lungs revealed that the epithelium had been rubbed, leaving only smooth muscle tissue. ROS are molecular byproducts of oxygen metabolism. Superoxide anion radicals (O_2-), singlet oxygen (O_2), hydrogen peroxide (H_2O_2), and highly reactive hydroxyl radicals are all ROS derivatives (OH). Although reactive oxygen species (ROS) are found in all aerobic cells, an excess of ROS in a cell results in oxidative stress, which is toxic. Additionally, these molecules are produced as a byproduct of cellular metabolisms, such as mitochondrial respiration. ROS that accumulates in tissues will bind to lipids in cellular membranes, causing damage to nucleotides in DNA (Waris and Ahsan. 2006).

In the control preparation (P1), after 14 days of exposure to cigarette smoke and seven days of oral distilled water, observations were made at a magnification of 100x. Then the bronchi and bronchioles were scored for tissue damage. The average number of bronchial observations was three, with damage ranging from 76% to 100%. Similarly, preparations P2, P3, and P4 all produced observations with an average score of 4. The obtained scores were then analyzed using a one-way ANOVA test.

The findings indicated that carrot leaf juice did not affect repairing bronchial lung damage. In addition, while all treatments had an average of 3.2 on the bronchioles, the one-way ANOVA test revealed that carrot leaf juice had little effect on repairing tissue even when the damage was severe.

Beta carotene plays a critical role in the body, particularly in the scavenging of free radicals. Beta carotene deficiency can result in the formation of cleavage products (CP) and oxidative stress (Alija et al. 2006). Antioxidants play a critical role in preventing damage caused by oxidative stress caused by cigarette smoke because the oxidative effects of cigarette smoke can cause damage to microsomal proteins and increase proteolysis so that it can cause DNA damage. Therefore, beta carotene is an antioxidant that should be consumed in the recommended dose by individuals who smoke or are exposed to cigarette smoke. Due to its critical role in reducing oxidative stress caused by free radicals, consuming beta-carotene-containing fruits and vegetables is essential in binding free radicals that enter the body (Omenn 1998).

This study indicates that carrot leaf juice has no significant effect on the histopathology of white rat lung's alveolus, bronchi, and bronchioles, including loss of alveolar tissue elasticity and abrasion of epithelial tissue in the bronchi and bronchioles.

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Effect of antibiotics in eliminating bacterial wilt (*Ralstonia solanacearum*) from in vitro propagated ginger

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Abstract. *Markos T, Feyissa T. 2020. Effect of antibiotics in eliminating bacterial wilt (*Ralstonia solanacearum*) from in vitro propagated ginger. Cell Biol Dev 4: 46-52.* Ginger (*Zingiber officinale* Roscoe) is a herbaceous perennial. It is cultivated commercially in most tropical regions of the world and is a member of the Zingiberaceae family. In conventional ginger cultivation, underground rhizomes are used as planting material. However, it is afflicted by diseases that result in the senescence and degeneration of tissues. Due to *Ralstonia solanacearum* infection, substantial rhizome losses have been recorded. Therefore, disease-free planting material is required to cultivate ginger successfully. Plant tissue culture technology has been successfully used to commercialize pathogen-free plants and conserves the germplasm of rare and endangered species. In in-vitro, culture techniques offer an alternative method of plant multiplication and a method for crop enhancement. Frequently, ginger multiplication media are contaminated with *R. solanacearum*, which can survive endophytically in plantlets, rendering them useless for in vitro propagation. Therefore, an experiment was undertaken to determine the efficiency of antibiotics against the in vitro development of *R. solanacearum.* Four antibiotics, gentamicin, tetracycline, ampicillin, and streptomycin, at four concentrations, 130 mg/L, 160 mg/L, 200 mg/L, and 250 mg/L, were evaluated for their ability to eradicate bacteria from in-vitro propagated ginger. Gentamicin was proven superior to other therapies, with the maximum inhibition (22 mm) at 250 mg/L, followed by tetracycline at 200 mg/L (18 mm), streptomycin at 130 mg/L (13 mm), and ampicillin at 130 mg/L (12 mm). The *R. solanacearum* can be eradicated from in vitro propagated ginger by applying antibiotics in the micropropagation media.

Keywords: Identification, in vitro susceptibility test, inhibition zone, isolation

INTRODUCTION

Ginger is a perennial herbaceous, rhizomatous plant belonging to the Zingiberaceae family of the Scitaminae order. The genus Zingiber contains approximately 85 fragrant herb species. Ginger (*Zingiber officinale* Roscoe) is one of the most extensively produced fresh vegetables and dried spices from the Orient (George and Sivarana 2014). Ginger (Zingibil in Amharic) has been traded for longer than most other spices and may have been recognized in Ethiopia from ancient times; it is mostly farmed in the Southern Nations, Nationalities, and Peoples Regional State (SNNPRS). Some areas of Western Oromia and Northern Amhara have also begun producing ginger, albeit at a smaller level (Fikre and Kifle 2013).

Ginger thrives in warm, humid climates and is cultivated at altitudes between sea level and 1,500 meters above sea level. It can be grown in both rain-fed and irrigated environments. For the growth of ginger to be effective, there must be moderate precipitation from the time of seeding until the rhizomes sprout, fairly strong and evenly distributed rains during the growing period, and dry weather for about a month before harvesting. Ginger grows best in well-drained sandy loam, clay loam, red loam, or lateritic loam soils. A friable, humus-rich loam is suitable for growing ginger. However, because ginger is an exhaustible crop, it is not advisable to cultivate it annually on the same soil (Ravindran and Babu 2005). Conventionally propagated ginger rhizomes were contaminated with pathogens such as *Fusarium oxysporum*, *Pseudomonas solanacearum*, *Pythium* spp., and roundworms. As ginger is susceptible to soil-borne illnesses such as bacterial wilt, soft rot, and Fusarium wilt, the importation of fresh rhizomes for crop establishment, whether for study or commercial production, appears risky (Endrias and Kifle 2011).

In Ethiopia, pathogens are currently attacking ginger plants, and the disease's intensity is escalating (Hunduma et al. 2016). It is a result of the introduction of novel pathogen strains from different regions of the world via latently infected planting materials (George and Sivarana 2014). Because vegetative propagation is inefficient, several rhizomes are required for the typical vegetative propagation of ginger. In addition, rhizomes utilized for vegetative growth are sensitive to diseases that cause tissue senescence and degeneration during storage and cultivation. Heavy ginger losses have been seen due to *Ralstonia solanacearum*-caused bacterial wilt. The disease is transmitted primarily through rhizomes. Therefore, successful ginger gardening requires the production of disease-free clones. The most effective method for managing bacterial wilt is cultivating disease-free ginger from pathogen-free seed. In in-vitro, culture techniques offer an alternative method of plant multiplication and a method for crop enhancement. It has become an indispensable method for addressing the limitations of healthy plant material. Therefore, it is necessary to investigate ways to supply disease-free plant material. The

most effective methods for managing bacterial wilt involve the cultivation of disease-free ginger. Therefore, it must investigate the effect of drugs on eradicating *R. solanacearum* from ginger plantlets produced in vitro.

MATERIALS AND METHODS

Preparation of plant donors and stock solutions

Plant donor preparation: Ginger rhizomes were taken from the experimental site of the Areka Agricultural Research Center, located 300 kilometers southwest of Addis Ababa, Ethiopia, in the Wolayita Zone of the Southern Nations Nationalities and Peoples Regional state. Fresh rhizomes were properly cleansed with water and stored in sterile sand at 20 to 25 degrees Celsius in a greenhouse. The rhizomes were irrigated daily with distilled water for 15 to 20 days to induce sprouting. Explants derived from sprouted stem tips and auxiliary buds on these rhizomes were used in later tests.

MS stock preparation: As the basic components of the medium, Murashige and Skoog (MS) medium, with its complete macro and micronutrients, vitamins, sucrose, and agar, was utilized.

Preparation of plant growth regulators stock: The stock solutions of plant growth regulators (2.0 mg/L BAP and 1.0 mg/L kinetin) were made by weighing and dissolving the powder in double-distilled water at a concentration of 3 to 4 drops of 1 N HCl.

Culture medium preparation and culture condition

Proper amounts of MS stock solutions and 3% (w/w) sucrose were used to make the culture medium. The pH was adjusted to 5.8 with 1 N NaOH or 1N HCl, and a plant growth regulator was applied. Micro-oven was then utilized to dissolve 0.6% (w/v) agar. The medium was sterilized by autoclaving at 121°C for 15 minutes at 105 Kpa. Next, 40 ml of media was placed into a baby jar culture vessel for shoot initiation, multiplication, and rooting. Before closing, the culture vessel and its cap were flamed and sealed with a strip of Parafilm, and the vessels were properly identified. The maintaining cultures were 30 days, with a 12 h photoperiod and 3000 Lux of light intensity from cool white fluorescent lights at a temperature of 25+2°C.

Surface sterilization of explants and initiation of shoot

After storing new rhizomes in a greenhouse for 15 to 20 days at 20 to 25 degrees Celsius, the sprouting branch tips and auxiliary buds were employed as explants. First, the shoot tip and auxiliary bud explants were rinsed with sterile distilled water and then briefly submerged in 70% ethanol. Next, the explants were treated for 15 minutes under aseptic conditions with 5% active chlorine concentration local bleach (Clorox) containing two drops of Tween-20. The explants were then carefully cleaned (three to four times) with sterile distillate water. Around 0.7 to 1.5 cm long shoot tips were employed for shoot initiation. The explants were grown on MS medium supplemented with 2.0 mg/L BAP and 1.0 mg/L Kin.

Multiple shoot development, rooting, and acclimatization

Transferring the started shoots to MS media supplemented with 4.0 mg/L BAP for 30 days. Next, the multiplied shoots were cultured in MS Full strength medium for root formation with 1.0 mg/L NAA. The number of surviving plants was then recorded after the plantlets were planted in a sterilized standard potting mixture containing soil, sand, and coffee husk in a 3:1:2 ratio.

Isolation of bacterial contamination and characterization of the isolated bacterium

The lowest portions of well-regenerated plantlets were cut off, and the upper portions were suspended for ten minutes in five milliliters of sterile distilled water to extract detectable bacterial pollutants (*R. solanacearum*). The bacterial suspension was serially diluted with 9.0 mL of sterile water once the water in the test tube turned cloudy due to leaking bacterial cells from sick tissue. Bacterial ooze was obtained from pseudo-stems of infected plants by serially diluting the bacterial suspension in sterile distilled water and cultured on a TZC medium (Kelman 1954). The bacterial suspension was poured over the surface of Nutrient Agar (NA) using the serial dilution method and the streak plate method. The plates were then incubated for 24 hours at 28 degrees Celsius. Kelman (1954) described that *R. solanacearum* isolates were purified by streaking a single colony of each isolate on Triphenyl Tetrazolyl Chloride (TZC) medium and incubating the plates at 30°C for 36 hours.

Preparation of inoculums and Testing for Pathogenicity

For inoculation, a single colony of *R. solanacearum* exhibiting virulence, fluidity, irregularity, and creamy white with pink in the center was chosen and adjusted to 3.2 x 108 cfu mL^{-1}. Eight-leafed tissue-cultured Ginger plantlets were inoculated via stem piercing and leaf infection pinpricks. The bacterial suspension was sprayed on each test plant's two leaves (Stromberg et al. 2004). In a greenhouse, the virulence characteristics of *R. solanacearum* isolates were evaluated on healthy tissue culture plantlets. The seedlings were transferred into 9-centimeter-diameter plastic pots containing sterilized soil, sand, and coffee husk in a ratio of 1:1:2. They were then grown in the greenhouse. For inoculation, a single colony of *R. solanacearum* exhibiting virulence, fluidity, irregularity, and creamy white with pink in the center was chosen and adjusted to 3.2 x 108 cfu mL^{-1}. Ginger seedlings were inoculated with one milliliter of inoculums per plant via stem puncture and leaf infection pinpricks. The eighth-leaf stage plants were inoculated once by puncturing the stem's base with a needle soaked in inoculums. Each experiment was carried out thrice. The daytime and nighttime temperatures ranged from 25 to 35°C, with 12 hours of daylight and darkness. Symptoms of the disease were monitored for one week following vaccination. If the plant had symptoms of wilting, the Interaction was deemed pathogenic.

Isolates of bacteria in an antibiotic medium

Evaluation of antibacterial agents on the growth of R. solanacearum *in vitro*

Antibiotic sensitivity testing was conducted on bacterial isolates grown in Muller Hinton agar containing antibiotics (17.5 gm of Acid Hydrolysate of Casein, 2.0 gm of Beef Extract, 1.5 gm of Starch, 17.0 gm of agar). Four antibiotics, Gentamicin, Tetracycline, Ampicillin, and Streptomycin, were employed at various concentrations (130 mg/L, 160 mg/L, 200 mg/L, and 250 mg/L). Filter paper discs (Whatman no-44) measuring 6mm in diameter were soaked in the various chemical concentrations for 5 minutes before being transferred to the Muller Hinton medium in the Petri dishes. The inoculation plates were refrigerated for four hours at 4°C to permit the diffusion of chemicals into the medium. Then, the plates were incubated in the dark for 24 hours at 25°C and 25°C. The sizes of the inhibition zones surrounding the disks were measured and recorded.

Using antibiotics to treat plant material

The efficiency of Gentamicin, Tetracycline, Ampicillin, and Streptomycin in eradicating bacterial contamination was evaluated by this medicine. First, the 0.20-micrometer filter-sterilized membrane used was newly manufactured, and the antibiotics were added to the BAP-supplemented multiplication medium. Next, growing shoots were carefully excised along with the lower sheath base portion and transferred to a multiplication medium supplemented with 4.0 mg/L BAP and respective antibiotics. The medium was then incubated for 10 days under a 12 h photoperiod, 3000 Lux of light intensity from cool white fluorescent lamps, and a temperature of 25+0.5°C.

Data analysis

The experiment employs a CRD design in which one ginger variety (boziab) and four antibiotics with four concentration levels were utilized for four replicated treatments. The experimental data were analyzed using a one-way analysis of variance and a GLM comparison of means at the 0.05 significance level. Using Minitab 17.0, the mean, standard deviation, and standard error of the mean were evaluated. Compared to the average number of in-vitro-grown shoots, the growth components and elimination of pollutants status were studied.

RESULTS AND DISCUSSION

The initial surface sterilization experiment was effective when 0.7-1.5 cm shoot tips were treated with 70% ethanol for 5 minutes, followed by double sterilization with 5% active chlorine concentration of local bleach (Clorox) for 15 minutes. This procedure yielded 95% sterile explants and 2% dead explants 10 days after inoculation in shoot induction of MS media. When the shoots began to multiply, however, indicators of wilting emerged. Shoot tips were frequently contaminated after a few generations of culturing using plant material from the infected field.

MS medium contamination appeared as a hazy, yellowish-white color or was restricted to the soil surrounding the plant's root zone (Figure 1).

However, because the growth medium employed in tissue culture may not suit bacteria or inhibit their growth, pathogens may be undetected for lengthy periods (Isenegger et al. 2003).

On Tetrazolium Chloride (TZC) Agar selective media, isolates displayed a light to red color with a distinctive red center, similar to the pathogen *R. solanacearum.* All *R. solanacearum* isolates were shown to be virulent after 48 hours of incubation on TZC medium, yielding a pink or light red color or the unmistakable red core and white margin. On TZC media, virulent colonies appeared well-separated, irregular, fluid, and dull white with a small pink center (Figures 2.A and 2.B). In contrast, non-virulent colonies appeared dark red (Figure 2.C).

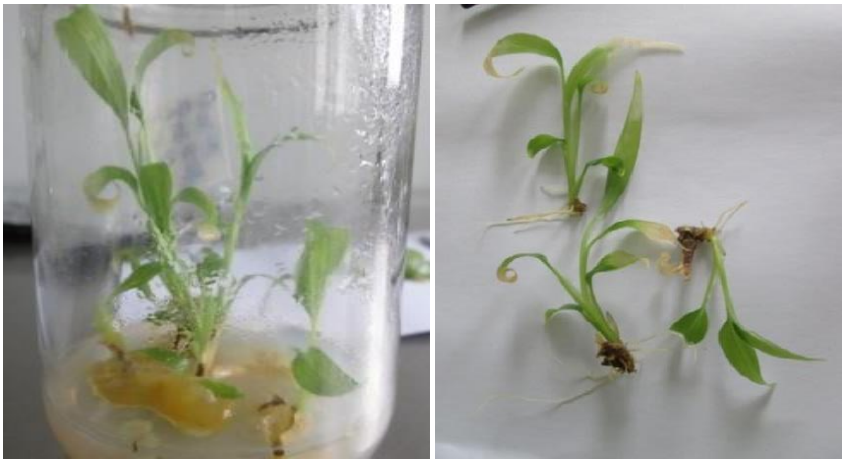

Figure 1. Diseased ginger plantlets in vitro

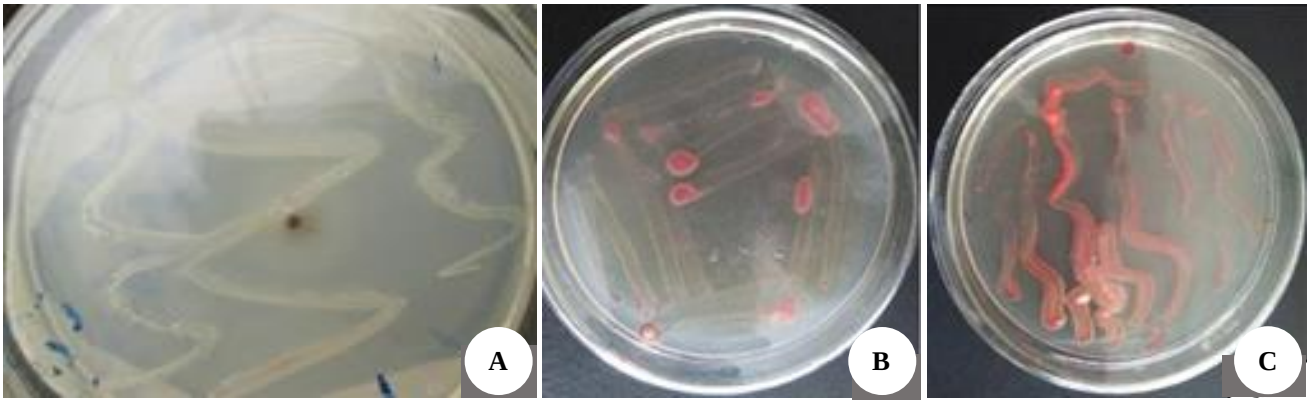

Figure 2. The *R. solanacearum* isolates on Tetrazolium Chloride (TZC) medium. Virulent colonies (A and B) and non-virulent colonies (C)

Pathogenicity for *Ralstonia solanacearum*

Pathogenicity test result for *R. solanacearum* within 5 to 15 days after inoculation, run isolation under artificial stem inoculation method caused ginger to wilt (Figure 3.A and 3B). The results of the pathogenicity study in India indicate that ginger wilt occurs between 5 and 7 days after inoculation (Kumar and Sarma 2004). Small, water-soaked lesions accompanied by leaf curling were observed as a symptom. The plants first exhibited daytime wilting and nighttime recovery, but after four to five days, they unexpectedly collapsed. The first wilt symptoms observed on the above-ground portion of the ginger plant were a mild yellowing and withering of the lower leaves. Then, the wilt spreads upward, affecting the younger leaves, followed by the yellowing and browning of the entire pseudo-steam. However, the plant dries out very quickly, and its leaves become yellow-brown within 5 to 10 days.

Young succulent shoots frequently become soft and rotting as pseudo-steam progresses, and diseased shoots easily separate from the underground rhizome at the soil line (Figures 3.A, 3.B, and 3.C). Kumar and Sarma (2004) described the identical ginger bacterial wilt symptom. The inoculated plant lost turgidity; leaves began falling, and the plant rapidly wilted (Figure 3.C). Initial developmental stage symptoms on leaves, pseudo-stems, and rhizomes of infected ginger plants were identical to those described in the literature (Trujillo 1964). Fine milky white filaments, composed of a mass of bacteria in extracellular slime, flow downward from the severed extremities of xylem arteries in vascular tissue. Bacterial exudates and leaf symptoms separate this wilt from fungus (Hayward 1964).

In vitro evaluation of antibacterial chemicals on the growth of *R. solanacearum*

This study was conducted to determine the efficacy of commercially available antibacterial agents against the growth of *R. solanacearum* under in vitro conditions. The findings of antibiotic susceptibility tests were derived from the data supplied by the disc diffusion method. In terms of zones of inhibition around the discs, the disc diffusion method for determining antibacterial activity revealed a considerable decrease in bacterial growth (Figure 4).

According to the activities of the four antibiotics on each bacteria, the inhibition zones grew as the antibiotic concentrations rose, indicating concentration-dependent activity. *R. solanacearum* isolates were highly susceptible to tetracycline, streptomycin, gentamycin, and ampicillin based on this study's antibiotic susceptibility testing of single antibiotic treatments. The *R. solanacearum*-inoculated Mueller-Hinton agar (MHA) medium is surrounded by discs carrying various antibiotics at varying doses.

Figure 3. After 6 to 8 days of inoculation with *R. solanacearum* (A and B), the inoculated plant exhibited wilting symptoms 15 days after inoculation (C)

Rooting and acclimatization

The experiment conducted in a greenhouse revealed that only 10% of plants survived when ginger plantlets were not treated with antibiotics. But ginger plantlets treated with antibiotics (antibiotics are added to the rooting medium before acclimation) decreased the incidence of bacterial wilt and enhanced its survival rate by 94% (Figure 5).

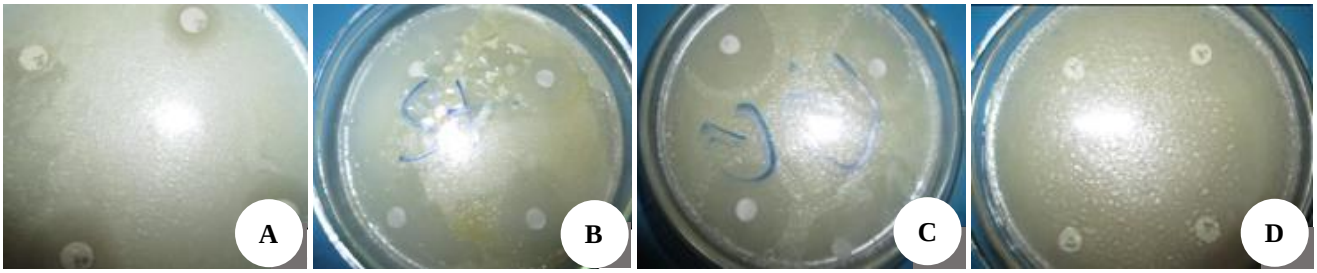

Figure 4. *Ralstonia solanacearum*-inoculated Mueller-HInternationalon agar (MHA) medium surrounded with discs containing various antibiotics at different doses. Streptomycin-containing discs (A and B), gentamicin-containing discs (C and D)

Figure 5. Acclimatized ginger plantlets treated with antibiotics (A), control (B)

Table 1. Inhibitory zones (mm) around discs containing different concentrations of various antibiotics placed on the surface of Mueller-Hinton agar (MHA) medium inoculated with the *Ralstonia solanacearum*

Anti-biotics	Concentration (mg/L)	Inhibitory zones (mm)
Ampicillin	130	10
	160	12
	200	5
	250	6
Tetracycline	130	12
	160	10
	200	10
	250	15
Gentamicin	130	13
	160	15
	200	18
	250	22
Streptomycin	130	6
	160	14
	200	12
	250	11
Antibiotics free	0	0

Table 2. Antibiotics treatment of plant material results in shoots free from *R. solanacearum*

Antibiotics	Antibiotics Concentration (mg/L)	Shoot number Mean±SD
Control	0	8.00±2.71abc
Ampicillin	130	11.51±1.29a
Ampicillin	160	8.75±1.70abc
Ampicillin	200	6.74±1.70bc
Ampicillin	250	7.00±1.40bc
Gentamaycine	130	6.00±1.40c
Gentamaycine	160	5.70±1.50c
Gentamaycine	200	5.75±1.70c
Gentamaycine	250	6.75±0.50bc
Streptomycin	130	6.75±1.25bc
Streptomycin	160	7.00±0.00bc
Streptomycin	200	6.60±0.57bc
Streptomycin	250	6.50±0.57bc
Tetracycline	130	8.00±1.5bc
Tetracycline	160	7.70±0.81bc
Tetracycline	200	7.20±0.50bc
Tetracycline	250	6.00±1.15c

Note: Numbers are mean and SD of Four replicates (four plants in each culture jar). Means followed by the same letter in a column are not significantly different by Tukey's test at $\alpha = 0.05\%$

Discussion

In the current investigation, diseased samples were gathered to identify the pathogens related to disease in in-vitro-produced ginger plantlets. The causative agent *R. solanacearum* was identified from the pseudo stem of ginger plantlets produced in vitro that exhibited the disease's distinctive symptoms. Following isolation, *R. solanacearum* isolates were purified by streaking a single colony of each isolate on a Tetrazolium chloride (TZC) agar medium. A single colony of *R. solanacearum* is virulent, fluid, uneven, and cream-colored with a pink center. In ginger plantlets produced in vitro, virulent isolates of *R. solanacearum* were found. On TZC medium, colonies of pink or light red color, or colonies with a red center and a whitish edge, were generated by *R. solanacearum* strains isolated from in vitro-cultivated ginger. After 24 hours of incubation, *R. solanacearum* formed colonies on TZC media that were fluid and pink or light red. These findings concur with those of Kelman (1954), Schaad et al. (1980), Suslow et al. (1982), French et al. (1995), and de Melo et al. (1999). The tentative identification of *R. solanacearum* was confirmed by the observation that none of the isolates preserved violet color, i.e., the isolates retained counter stain (pink color). These outcomes parallel those discovered by Suslow et al. (1982), who observed that the isolates of *R. solanacearum* did not preserve violet color, i.e., they retained counterstain (pink color).

The biochemical characterization demonstrated that the disease that threatens ginger under in vitro conditions is caused by *R. solanacearum* and that the disease was detected in the tissue of ginger plantlets. Furthermore, the etiology and pathogenicity tests confirmed that *R. solanacearum* causes bacterial wilt in ginger.

Pathogenicity tests conducted on an *R. solanacearum* isolate using artificial stem inoculation demonstrated that it caused wilt symptoms in ginger plants 7 to 15 days after inoculation. Several researchers have demonstrated the pathogenicity of *R. solanacearum*, which causes bacterial wilt (Winstead and Kelman 1952; Schell 2000; Williamson et al. 2002; Kumar and Sarma 2004; Umesha et al. 2005; Hikichi et al. 2007; Artal et al. 2012; Thomas and Upret 2014; Zulperi et al. 2014).

To recover healthy plants, knowledge of the effect of antibiotics on bacteria and plants is vital. By determining the minimal inhibitory concentration (MIC) and, subsequently, the minimal bactericidal concentration (MBC) of all stable antibiotics after 24 h of incubation following the antibiotic susceptibility test, the effect of the selected antibiotics on *R. solanacearum* isolates was determined. MBC reflects the cidal potential of the antibiotics on the isolates, whereas MIC reveals the inhibitory potential. According to Bonev et al. (2008), the efficiency of antibiotics can be measured by their ability to inhibit bacterial growth, as represented by the minimal inhibitory concentration (MIC), or by their ability to kill bacteria, as described by the minimal bactericidal concentration (MBC).

According to antibiotic sensitivity testing, gentamicin, tetracycline, streptomycin, and ampicillin were the most effective antibiotics against the growth of *R. solanacearum* in vitro. Bacterial growth was reduced when shoot tips were grown on an antibiotic-supplemented multiplication medium for three weeks. Intensive development of high-quality shoots also occurred. The shoot in antibiotic-grown cultures was truly devoid of bacterial contamination, and cultures have grown on MS medium without antibiotics. Thus, all of the tested antibiotics proved effective against *R. solanacearum*. These results are consistent with those previously reported by some researchers (Hidaka and Murano 1956; Dutta and Verma 1969; Indersenan et al. 1981; Khan et al. 1997; Singh et al. 2000; Devanath et al. 2002; Dubey 2005; Sunder et al. 2011; Gupta and Razdan 2013; Owoseni and Sangoyomi 2014).

Different quantities of streptocycline inhibited the growth of the pathogen, with a maximum inhibition zone (IZ) of greater than 25 mm at a concentration of 400 ppm. Paul (1998) observed that 250 and 500 ppm concentrations of ambistryn, oxytetracycline, and streptocycline inhibited *R. solanacearum* in vitro. Singh and Jagtap (2017) also documented the effectiveness of antibacterial chemicals and bioagents against the in vitro growth of *R. solanacearum.* The average inhibition ranged from 6.2 mm (copper hydroxide) to 20.05 mm (Streptocycline). However, the average inhibition was much greatest for streptocycline (20.05 mm). Then, the medicines gentamycin (17.5 mm), tetracycline (16.5 mm), and Streptocycline (11.95 mm) were administered.

In conclusion, according to the study's results, the R. solanacearum survived surface sterilization of ginger explants before their use in tissue culture laboratories. Antibiotics will lower the pollutants, increasing plantlets' survival rate as the prevalence of *R. solanacearum* in plant tissue culture continues to rise. The *R. solanacearum* continues to pose a concern to plant tissue culture. However, strategies for minimizing pollutants with antibiotics have shown that gentamicin is substantially more effective than other treatments, with the greatest inhibition, followed by tetracycline and streptomycin. Antibiotic therapy of plants could eradicate bacterial contamination from in vitro plantlets that have been infected. Therefore, the information here suggests that chemical control of the bacterial wilt of ginger may be possible. Under laboratory circumstances, the antibiotics could eradicate the bacterium in this experiment.

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