Goramy spermatozoa quality after sub-zero freezing: The role of coconut water as the cryoprotectant

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Manuscript received: 14 January 2017. Revision accepted: 5 May 2017.

Abstract. Abinawanto, Putri PE. 2017. Goramy spermatozoa quality after sub-zero freezing: The role of coconut water as the cryoprotectant. Cell Biol Dev 1: 1-5. The coconut water effect combined with 5% of glycerol for preserving goramy spermatozoa at -34 °C for 48 hours has been studied. The objective of study is to find the best combination among 0%, 21%, 23%, 25%, 27%, and 29%, respectively, of coconut water combined with 5% of glycerol for maintaining the good spermatozoa motility and viability, and minimizing spermatozoa abnormality. One part of semen/sperm were mixed with three parts of solvent (5% of glycerol + fish ringer + coconut water), and were equilibrated at 4 °C for 45 min. The diluted sperm were then freeze at -34 °C for 48 h. Cryopreserved sperms were thawed at 30 °C for 3-5 min. Spermatozoa quality were evaluated before and after sub-zero freezing. Based on Kruskal-Wallis test, spermatozoa motility and viability were higher than control (P <0.05), while the spermatozoa abnormality were not significantly different compared to control (P>0.05). Twenty five percent of coconut water combined with 5% of glycerol were the best combination for preserving spermatozoa motility (80.36±1.54)% and spermatozoa viability (82±1.86)%, and also minimized spermatozoa abnormality (10±1.03)%.

Keywords: Coconut water, cryoprotectant, Osphronemus goramy, spermatozoa quality, sub-zero freezing

INTRODUCTION

According to the data of the Ministry of Maritime Affairs and Fisheries, Republic of Indonesia, Indonesian fish consumption increased 5% within 2013 (KKP 2013). Osphronemus goramy (Lacepede 1801) is the popular and commercial freshwater fish in Indonesia (Saparinto 2008). Goramy fish production in Indonesia was about 280,079,000 kg in 2000 (Dunia Ikan 2008). Nowadays, goramy fish was cultured, conventionally, because of the limited male and female parents. They usually put male and female parents together in one pond, in a long time, and it will be caused inbreeding. Hence, the quality of the genetic materials were then reduced (Alam et al. 2002).

The strategy to maintain the genetic materials for long period is by cryopreservation method. According to Bozkurt (2005), cryopreservation is the ex-situ conservation effort to preserve the genetic materials in sub-zero temperature for a certain time. The genetic materials cryopreserved, include spermatozoa, ovum, somatic cells, and embryo (Simione, 2003).

Cryoprotectant and extender are two important solutions needed for protecting cells from ice crystals during freezing (Muchlisin 2005). Based on Jamieson (1991), glycerol, methanol and dimethyl sulphoxide (DMSO) are common intracellular cryoprotectant used for cryopreserved fish spermatozoa. On the other hand, milk, egg yolk, and sugar were used as the extracellular cryoprotectant (Jamieson 1991). According to Routray et al. (2007), extender is also needed to support cells with additional nutrition, and maintain osmotic pressure, during cryopreservation. Saline solution and fish Ringer solution are two extender which usually use for fish spermatozoa cryopreservation (Muchlisin 2005).

The previous cryopreservation study has been reported related to the fish spermatozoa, such as in Barbonymus gonionotus, Java Barb (Abinawanto et al. 2013), Osphronemus goramy (Abinawanto et al. 2011, 2012a, 2012b), Osteochiis hasseltii (Sunarma et al. 2007), Cyprinus carpio (Akay et al. 2004), tilapia’s fish (Chao et al. 1987), rainbow trout (Stoss and Donaldson 1983), carp (Harvey 1983; Horvath et al. 2003; Withler 1982), and salmonid (Harvey et al. 1982).

Dimethyl Sulfoxide (DMSO) was used as the cryoprotectant during cryopreservation of goramy spermatozoa for 24 hours (Abinawanto et al. 2011). Besides, sucrose (Abinawanto et al. 2012a) and skim milk (Abinawanto et al. 2012b) were also used as the cryoprotectant for goramy spermatozoa. However, the effect of coconut water in many variation of concentration combined with 5% of glycerol on the spermatozoa quality of goramy was still unknown. Accordingly, the aim of the study was to find the suitable concentration of coconut water among 0%, 21%, 23%, 25%, 27%, or 29% in preserving spermatozoa motility and viability and in reducing the spermatozoa abnormality during cryopreservation.
MATERIALS AND METHODS

Preparation of fish

Fifteen mature males of Osphronemus goramy were brought from the private commercial hatchery, Parung-Bogor, West Java, Indonesia. All fishes were acclimatized for 14 d in one-4000-L square concrete cement fish pond till they attain 2.5-3.5 kg size in the indoor Aquatic Biology laboratory, Biology Department, Faculty of Mathematics and Natural Sciences, Universitas Indonesia, Depok, West Java Province. The fishes were grouped of 6 fishes and were stocked in 7 square concrete cement fish pond (1000-L). The fish pond were equipped with closed re-circulation system and a black plastic mesh lid to minimize disturbances and prevent fish from jumping out. Six experimental group based on coconut water concentration were assigned to four times replication in a completely randomized design (Table 1). Fishes were fed with the commercial diet and leaf of Allocaasia macrorrhiza two times daily ad libitum at 08:00 and 17:00.

Fish ringer solution preparation

Fish ringer solution was prepared according to the method of Ginzburg (1972). A fish ringer stock solution was prepared by dissolving 3.25 g NaCl; 0.125 g KCl; 0.175 g CaCl₂·2H₂O; and 0.1 g NaHCO₃ with aquabidest up to 500 mL. The extender fish ringer solution was then kept at 4 °C.

Activator solution preparation

The activator solution was prepared based on the method of Perchec et al. (1995) by diluting 45 mM NaCl, 5 mM KCl, and 30 mM Tris with aquabidest up to 100 mL.

0.5% Eosin-Y solution preparation

The 0.5% of Eosin-Y solution was prepared according to the method of WHO (2010), by diluting 0.5 g of the Eosin-Y with aquabidest up to 100 mL.

0.15M of Phosphate buffer solution pH 6.8 preparation

The phosphate buffer solution was prepared by dissolving 5.34 g Na₂HPO₄·2H₂O with aquabidest up to 200 mL, and by dissolving 4.08 g KH₂PO₄ with aquabidest up to 200 mL. Na₂HPO₄·2H₂O solution was then added to KH₂PO₄ solution until the pH reach 6.8. The Phosphate buffer solution was then kept at 4 °C.

Giemsa solution preparation

The Giemsa solution was prepared based on WHO (2010), by diluting one part of the Giemsa stock solution and 10 parts of the phosphate buffer solution pH 6.8. The mixed solution was then filtered by Whatman filter paper number one.

Collection of the ejaculated sperm/semen

The sperm was collected by hand stripping method, 8-10 hours after injected intra-muscularly with GnRH-analog (Ovaprim Syndel) at a single-dose of 0.2 ml/kg body weight according to modification method of Sunarma et al., (2007), and was put in 1.5 mL of cryotube.

Semen/sperm evaluation and spermatozoa analysis

Visual observation was carried out to the fresh semen/sperm color and sperm volume was measured by the cryotube with scale. The standardized pH paper (pH range 5-10) was used to measure sperm/semen pH. The spermatozoa viability, abnormality, and motility were observed under trinocular microscope (Boeco) equipped with the digital eye piece camera (MDCE-5a). This microscope was also connected to the computer equipped by the image driving software (Scopephoto 2.0.4). The Rurangwa et al. (2004) method was used to analyze the spermatozoa motility, whereas the spermatozoa viability and spermatozoa motility were analyzed by Salisbury and Van De Mark method (1985).

Data analysis

All data were analyzed by Kruskal-Wallis and Dunnet’s multiple comparison test (Zar 1974) using a statistic program of SPSS version 13 for Windows. All probability values were set at 0.05 level of significance.
RESULTS AND DISCUSSION

Results
Fresh semen (FS) were milky white, pH 8.06±0.05, and 0.57±0.10 mL of volume per ejaculate (Table 2).

Figure 1 showed non-viable spermatozoa showed red color on the sperm head (A), while the viable spermatozoa showed green color on the sperm head (B). Both of viable and non-viable spermatozoa were found in all treatment groups and control. However, percentage of spermatozoa viability were different quantitatively either among treatment groups or between control and treatment groups. Either the normal or abnormal spermatozoa morphologically were also found in all treatment groups and control (Figure 2). However, the percentage of spermatozoa normal and abnormal were different, among treatment groups or between control and treatment groups. Table 3 showed the percentage of spermatozoa abnormality, viability, and motility two days after sub-zero freezing.

The percentage of spermatozoa motility after freezing in control; and in various coconut water concentration of 21%, 23%, 25%, 27%, and 29%; were: (71.42±4.01)%, (72.22±2.96)%, (77.39±2.26)%, (80.36±1.54)%, (77.13±2.59)% and (74.09±1.98)%, respectively (Table 3). The spermatozoa viability in control; and in various coconut water concentration of 21%, 23%, 25%, 27%, and 29%, two days after sub-zero freezing, were: (72±3.42)%, (75±3.36)%, (79±2.22)%, (82±1.86)%, (80±1.47)% and (77±1.96)% respectively (Table 3). On the other hand, the spermatozoa abnormality after freezing, in control; and in various coconut water concentration of 21%, 23%, 25%, 27%, and 29%, were: (11±1.83)%, (11±1.41)%; (12±1.04)%; (10±1.03)%; (11±0.83)% and (11±0.75)% respectively (Table 3).

Based on Kruskal-Wallis test, there were significant effect (P<0.05) of various concentration of coconut water on spermatozoa viability and motility, respectively, to days a after sub-zero freezing. On the other hand there was not significant effect of coconut water on reducing the spermatozoa abnormality (P>0.05) compared to control (Table 3). According to the Dunnet test, the concentration of 25% of coconut water showed the highest percentage of spermatozoa viability (82±1.86)% and motility (80.36±1.54)% respectively.

Table 2. Fresh semen/sperm evaluation and spermatozoa analyses of goramy spermatozoa, before freezing

| Parameter | Color | Volume | Parameter | Color | Volume |
|-----------|-------|--------|-----------|-------|--------|
| Semen/sperm | Milky white | 0.57±0.10 | 8.06±0.05 | 84±3.16 | 13±0.63 |

Note: Values are means ± SD of four replicates

Table 3. The percentage of the spermatozoa abnormality, viability, and motility of goramy, 48 hours after sub-zero freezing

| Parameter | Experimental group |
|-----------|-------------------|
|           | C | 21%CW | 23%CW | 25%CW | 27%CW | 29%CW |
| Viability (%) | 72±3.42<sup>a</sup> | 75±3.36<sup>a</sup> | 79±2.22<sup>a</sup> | 82±1.86<sup>a</sup> | 80±1.47<sup>b</sup> | 77±1.96<sup>b</sup> |
| Abnormality (%) | 11±1.83<sup>a</sup> | 11±1.41<sup>a</sup> | 12±1.04<sup>a</sup> | 10±1.03<sup>a</sup> | 11±0.83<sup>a</sup> | 11±0.75<sup>a</sup> |
| Motility (%) | 71.42±4.01<sup>a</sup> | 72.22±2.96<sup>a</sup> | 77.39±2.26<sup>b</sup> | 80.36±1.54<sup>b</sup> | 77.13±2.59<sup>c</sup> | 74.09±1.98<sup>c</sup> |

Note: Values are means ± SD of four replicates. Mean values having the same superscript are not significantly different (p > 0.05)
Discussion

The concentration of 25% coconut water was shown the highest percentage spermatozoa motility (80.38 ±1.54)%, two days after sub-zero freezing. This finding was similar with the previous study (Horvath and Urbanyi, 2000) when they preserved spermatozoa of Clarias gariepinus. On the other hand, post thaw motility in this study was lowered compared our previous study (80.98%; Abinawanto et al. 2012b; 96.10%; Abinawanto et al. 2013). However, post thaw motility in this study was higher than Brachydanio rerio (51%; Harvey et al. 1982), Oreochromis mossambicus (70%; Harvey 1983), tilapia’s fish (40%; Chao et al. 1987), Cyprinus carpio (55%; Akçay et al. 2004), Osteochiatus hasseltii (63.33%; Sunarma et al. 2007), and Osphronemus goramy (68.58%; Abinawanto et al. 2011). Post thaw viability in the treatment group of 5% glycerol was 75.5 ± 5.43%, although it was not significant different compared with other treatment groups and control, statistically. However, this finding was higher than previous reported in spermatozoa of Mystus nemurus (60%; Muchlisin et al. 2004), Cyprinus carpio (20%; Withler 1982; 58%; Horton and Otto 1976), and Osphronemus goramy (63.5%; Abinawanto et al. 2011). Post thaw viability in this study on the other hand was lowered than our previous work in Barbonymus gonionotus spermatozoa (77.25%; Abinawanto et al. 2009; 85.50; Abinawanto et al. 2013). The effect of 5% of glycerol can decline the post thaw abnormality (14.83 ± 2.79)%, better than other treatment group of glycerol concentration and control group, although those results were not significant different, statistically. Our previous study showed the higher spermatozoa abnormality (29%; Abinawanto et al. 2011) when using the combination of 13% of DMSO + 189M extender. Post thaw abnormality in Barbonymus gonionotus spermatozoa was higher (45%) when preserved in the combination of 6% of glucose + 10% of methanol (Abinawanto et al. 2009). However, the post thaw abnormality showed nearly similar (14%) when the spermatozoa of Barbonymus gonionotus protected by the combination of 13% of egg yolk + 10% of methanol (Abinawanto et al. 2013).

Either all of treatment groups or control were shown the viable spermatozoa, motile spermatozoa, and abnormal spermatozoa, visually. However, the percentage of spermatozoa viability, motility and abnormality were different among treatment groups, or between control and treatment groups. The optimum condition of the treatment group (percentage of glycerol) was shown by the lowest percentage of spermatozoa abnormality (by reduced percentage of spermatozoa abnormality) after sub-zero freezing for two days. Furthermore, the highest percentage of viability or motility of spermatozoa among the treatment groups or between control and treatment groups two days after sub-zero freezing also as the indicator of the optimum condition of percentage of glycerol as the cryoprotectant.

The data obtained in the present study indicate that 25% of coconut water combined with 5% of glycerol showed the highest spermatozoa motility and spermatozoa viability, and also can reduced spermatozoa abnormality two days after sub-zero freezing.

ACKNOWLEDGEMENTS

We would like to thank Directorate General for Higher Education, Department of National Education, Republic of Indonesia that had funded this study through the University of Indonesia Competitive Research Grant No. 240/AT/DRPM-UI/N1.4/2008 for the financial support.

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Electrical conductivity for seed vigor test in sorghum (*Sorghum bicolor*)

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Manuscript received: 20 April 2017. Revision accepted: 17 May 2017.

Abstract. Fatonah K., Suliansyah I., Rozen N. 2017. Electrical conductivity for seed vigor test in sorghum (*Sorghum bicolor*). Cell Biol Dev 1: 6-12. The objectives of this study were to obtain electrical conductivity test method for seed vigor test in sorghum, to recognize relationship between electrical conductivity test and potassium leakage, and to recognize relationship between electrical conductivity test and other variable on seed vigor. This study have two step experiments. The objective of Experiment I was to determine accurate combinations of water volume and amount seed of the electrical conductivity test for seed vigor test in sorghum. Completely Randomized Design of 15 seed lots with 3 replications were used in Experiment I. The variable were observed: standard germination, field emergence, speed of germination, first count, conductivity, potassium leakage and eight combination conductivity method of water volume (50, 100, 150 and 200 ml) and amount seed sorghum (50 and 75 seed count). The result of this experiments showed that electrical conductivity test method with 150 ml water volume and 75 seed count was accurate and suitable for sorghum seed vigor test; electrical conductivity test showed positive correlation with potassium leakage; and electrical conductivity test can be used for seed vigor test in sorghum and provided the potential of physiological seed were shown through: standard germination test, field emergence test, first count test and speed of germination with negative correlation.

**Keywords**: Sorghum seeds, electrical conductivity test, seed vigour test

**INTRODUCTION**

Seed vigor test is more sensitive index of seed quality than germination test, any of the events which precede loss of germination could serve as vigor tests. Seed vigor testing has reached increasing importance to rank seed lots according to their physiological potential. One of the main concerns regarding seed vigor evaluation is obtaining reliable result within a short time for quality control programs. Literature shows that available rapid seed vigor tests which produce consistent information on seed physiological potential are those associated with the determination of enzymatic and respiratory activities and cell membrane integrity such as the tetrazolium and electrical conductivity tests, respectively (Abdul-Baki and Baker 1973; Ramos et al. 2012; Lamarca and Barbedo 2014; Szemruch et al. 2015).

A vigor test can be a measurement of one or more of these events. The conductivity test is a measurement of electrolytes leaking from seeds. Changes in the organization of cell membranes occur during the development of seeds prior to physiological maturity, seed desiccation before harvest, and during imbibition prior to germination (ISTA 1995). As seed rehydrates during early imbibition, the ability of its cellular membranes to reorganize and repair any damage that may have occurred will influence the extend of electrolyte leakage from seeds. The greater the speed with which the seeds are able to re-establish their membrane integrity the lower the electrolyte leakage. Higher vigor seeds are able to reorganize their membranes more rapidly, and repair any damages to a greater extend, than lower vigor seeds. Consequently, electrolyte leakages measured from high vigor seeds are less than that measured from low vigor seeds. Low vigor seeds have been shown posses decreased membrane integrity as a result of storage deterioration and mechanical injury. However, there are no suggested or recommended procedures for electrical conductivity sorghum seed vigor tests are available in the handbooks of vigor test from the International Seed Testing Association (AOSA 1983; ISTA 1995).

The research reported here is aimed at: to obtain electrical conductivity test method for seed vigor test in sorghum, to recognize relationship between electrical conductivity test and potassium leakage, and to recognize relationship between electrical conductivity test and other variable on seed vigor.

**MATERIALS AND METHODS**

The experiments have been carried out from February to July 2015. All the laboratory and field emergence tests were conducted at Indonesian Center for Seed Testing and Quality of Food Crops and Horticulture Research and Development, Cimanggis, Depok, West Java, Indonesia. Potassium leakage were conducted at the Laboratory of Soil Department, Bogor Agriculture University, West Java, Indonesia.
Seed water content
Was determined at 130°C for 2 hours in duplicate samples of grinded seeds of fine scale as recommended by the AOSA Rules for Testing Seeds (AOSA 2014). The results were expressed as percentage water content (fresh weight basis).

Germination test:
Performed on three 100-seed replicates planted between rolled paper towels moistened and germinated at 25°C. Seedling counts were conducted at four and ten days after seeding, evaluated for normal development, and results were expressed as percent normal seedlings for each lot.

Germination first count
Was performed simultaneously to the germination test and percent normal seedlings was recorded four days after seeding.

Speed of Germination
The normal seedlings are evaluated on daily basis starting from first count till the final count

Accelerated aging
A single layer of seeds of each lot was placed on a wire mesh screen and suspended over 40 mL of distilled water inside a plastic accelerated aging box. Boxes were held at 43°C and 95% relative humidity for 72 hours in an incubator. Seed water content (oven method at 130°C/2 h) was also determined before and after the aging period to evaluate the accuracy of the accelerated aging results.

Electrical conductivity
The electrical conductivity of the leachate from whole imbibed seeds was determined by eight combination electrical conductivity method (Table 1) and held in a germinator at 20°C. After 24 hours, the electrical conductivity of leachates was determined. The electrical conductivity of leachates of each replication was measured by using conductivity meter (Type Cond 330i) and conductivity per gram of seed weight was calculated (μS cm⁻¹g⁻¹) and recorded.

Potassium leakage
Three replicates of 50 seeds per lot weighed and were placed in disposable plastic cups with 50 mL deionized water and held at 20°C. After 24 hours imbibition periods the amount of leached potassium was determined by a flame photometer Type Corning 405. Results were expressed in ppm.

Table 1. Eight combination electrical conductivity method

| Treatment | Amount of seed | Water (mL) |
|-----------|----------------|------------|
| 1         | 50             | 50         |
| 2         | 50             | 100        |
| 3         | 50             | 150        |
| 4         | 50             | 200        |
| 5         | 75             | 50         |
| 6         | 75             | 100        |
| 7         | 75             | 150        |
| 8         | 75             | 200        |

Field emergence
Three replicates of 50 seeds each per seed lot were distributed in plastic box 38 cm long, 30 cm wide and 12 cm deep, holding sand sufficiently wet for germination. Emerged seedlings were counted 7, 14 and 21 days after planting and the mean percentage determined for each lot.

Statistical analysis
Completely Randomized Design of 15 seed lots with 3 replications were used to determine electrical conductivity method with different vigor levels ranging from 39-94% as determined by standard germination. Completely Randomized Design of 21 seed lots with 3 replications were used to explore relationship between electrical conductivity test and other variable test on the physiological quality of seed. The quality of selected seed lots were determined by standard germination, field emergence, first count, speed of germination, conductivity and potassium leachate. Analysis of variances were performed on the data with the Statistical Analysis System (SAS version 9.0 for Windows). Correlation coefficients between all test results were calculated to observe the relationships of all tests.

RESULT AND DISCUSSION

Determine electrical conductivity method as sorghum seed vigor test
The electrical conductivity as seed vigor test in sorghum is not recommended electrical conductivity test method for sorghum seeds have been prescribed in international seed vigor testing handbooks. Seed vigor comprises those seed properties which determine the potential for rapid, uniform emergence and development of normal seedlings under a wide range of field conditions. Recently, Hampton and TeKrony (1995) emphasized that seed vigor testing must rank seed lots of commercially accepted germination.

Although germination and vigor are closely associated, seed vigor is highly complex compared to standard germination and provides additional information to assist in differentiation of the physiological potential of seed lots, seed storability and potential field performance. It is desirable that practical vigor tests consider rapidity, simplicity, objectivity, reproducibility of test results, and relationship with seedling emergence. The conductivity test offers a quick (24 hrs), objective vigor test that can be conducted easily on most seed testing laboratories with minimum expenditure for equipment and training of personnel. Physically injured and mechanical damaged seeds can influence the results.

In order to identify the electrical conductivity method for seed vigor test in sorghum, 15 seed lots of different vigor levels were tested for standard germination test, field emergence, 8 electrical conductivity method and several vigor tests. Correlation coefficients among all the tests were observed.

Highly significant correlation (r = 0.90) was observed between standard germination and field emergence 21 days...
test are shown in Table 2. Recent studies (Baskin et al. 1993) of the relationship between standard germination test and field emergence of sorghum under favorable and unfavorable field conditions. Standard germination percent of seed lots ranged from 63% to 99% with a mean of 89.5%. Under favorable condition the field emergence percent ranged from 69% to 97% with a mean of 86.5% and highly significant correlation (r = 0.825**) was observed with standard germination test. Under unfavorable field condition (cold wet 69 soil condition) the mean field emergence percent decreased to 65.9% and low correlation coefficient (r = 0. 501**) was observed between standard germination test and field emergence.

All seed vigor tests, first count (r = 0.83), potassium leakage (r = -0.83) and conductivity test with 150 ml deionized water and 75 seed count (r = -0.89) provided highly significant correlations with standard germination. Among eight combination the electrical conductivity method, the highest correlations with field emergence 21 days (r = -0.78), first count (r = -0.79) and standard germination (r = -0.89) determine by electrical conductivity method with 150 ml deionized water and 75 seed count. According to conductivity test with 150 ml deionized water and 75 seed count, potassium leakage also showed highest correlation (r = 0.93).

From the results of maximum correlation coefficients (r) of electrical conductivity method with 150 ml deionized water and 75 seed count with standard germination, first count, field emergence 21 days obtained, and potassium leakage; electrical conductivity method with 150 ml deionized water and 75 seed count is recommended as preliminary recommendation for sorghum seed vigor test. Negative correlations were always observed between conductivity test and standard germination test and other seed vigor tests. This is because low germination and vigor seeds give high amount of leakage of electrolytes (measured in μS cm-1g-1), in contrast high vigor seeds give low amount of leakage of electrolytes.

The electrical conductivity test is acknowledged as one of the best tests for the evaluation of the loss of cell membrane integrity by the concentration of electrolytes released by seeds during imbibition such as inorganic ions; cell membrane integrity is considered one of the primary physiological events of seed deterioration process (Delouche and Baskin 1973). Conductivity test is rapid, simple and do not need personal skill for result analysis.

The lower the membrane integrity, the greater the electrolyte leakage in the steep water, thus the greater the conductivity measurement (ISTA 2011; Woodstock et al. 1985) found relationships between weathering deterioration, germination respiratory metabolism, and leaching in cotton seeds. The deterioration of membranes due to weathering was confirmed by electron microscopy of cotyledon’s lipids and proteins bodies and correlated well with conductivity measurements.

The electrical conductivity test is based on measurement of resistance to flow of an electric current imposed upon the seed steep water. Resistance is a function of the amount of electrolytes in solution. Pure water has a great electrical resistance, but solutions of electrolytes, which are ionic substances, allow electric currents to flow. Many cellular constituents are acids, bases, or their salts, i. e., electrolytes. Electrolyte efflux from seeds during imbibition is presumably an indication of seed cell membrane condition. Weak seeds generally possess poorer membrane structure, which results in greater electrolyte loss and higher conductivity measurements (Pandey 1992).

### Table 2. Correlation coefficients (r) of standard germination (DB), field emergence 7, 14, 21 days after planting (DT-7, 14, 21), first count (IV), potassium leakage (Ion K) and 8 combination electrical conductivity method of 15 seed lots of sorghum

| IV  | DB  | DT-7 | DT-14 | DT-21 | 50ml | 75ml | 50ml | 75ml | 50ml | 75ml | 50ml | 75ml | 50ml | 75ml | 50ml | 75ml | Ion K |
|-----|-----|------|-------|-------|------|------|------|------|------|------|------|------|------|------|------|------|------|
| IV  | 1   |      |       |       |      |      |      |      |      |      |      |      |      |      |      |      |      |
| DB  | 0.83| 1    |       |       |      |      |      |      |      |      |      |      |      |      |      |      |      |
| DT-7| 0.76| 0.87 | 1     |       |      |      |      |      |      |      |      |      |      |      |      |      |      |
| DT-14| 0.77 | 0.89 | 1.00 | 1     |      |      |      |      |      |      |      |      |      |      |      |      |      |
| DT-21| 0.77 | 0.90 | 0.99 | 1.00 | 1    |      |      |      |      |      |      |      |      |      |      |      |      |
| 50-50ml | -0.69 | -0.81 | -0.64 | -0.67 | -0.67 | 1 |      |      |      |      |      |      |      |      |      |      |      |
| 75-50ml | -0.76 | -0.84 | -0.68 | -0.71 | -0.71 | 0.98 | 1    |      |      |      |      |      |      |      |      |      |      |
| 50-100ml | -0.70 | -0.81 | -0.65 | -0.68 | -0.68 | 0.99 | 0.99 | 1    |      |      |      |      |      |      |      |      |      |
| 75-100ml | -0.77 | -0.86 | -0.72 | -0.75 | -0.75 | 0.98 | 0.99 | 0.98 | 1    |      |      |      |      |      |      |      |      |
| 50-150ml | -0.79 | -0.89 | -0.74 | -0.77 | -0.78 | 0.96 | 0.98 | 0.97 | 0.99 | 0.99 | 1    |      |      |      |      |      |      |
| 75-150ml | -0.75 | -0.88 | -0.74 | -0.77 | -0.77 | 0.96 | 0.98 | 0.97 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 |
| 50-200ml | -0.75 | -0.88 | -0.74 | -0.77 | -0.77 | 0.96 | 0.98 | 0.97 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 |
| 75-200ml | -0.75 | -0.87 | -0.71 | -0.74 | -0.74 | 0.96 | 0.98 | 0.97 | 0.99 | 0.98 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 |
| Ion K | -0.71 | -0.83 | -0.74 | -0.76 | -0.77 | 0.84 | 0.90 | 0.87 | 0.91 | 0.89 | 0.93 | 0.92 | 0.93 | 0.92 | 0.93 | 0.92 | 0.93 |
Potassium has been shown to be the main ion leached by seeds during imbibition, followed by sodium and calcium, and may be used as an indicator of cell membrane integrity. The potassium leachate test is based on the same principle of the electrical conductivity test with the additional advantage of producing results in a considerable shorter period of time. In addition, it focuses on a specific ion, while the electrical conductivity test evaluates a set of electrolytes release (Miguel and Filho 2002. Potassium leachate and electrical conductivity tests yielded similar results in the ranking of physiological potential of seed lots. The electrical conductivity test, based on the same principle, provides results only after a 24 h imbibition period when performed under current procedure.

Relationship between electrical conductivity test and other variable test on the physiological quality of seed

Our results showed that electrical conductivity method with 150 ml deionized water and 75 seed count could predict standard germination and field emergence and could be used as seed vigor test in generally recommended sorghum seeds. But before the conductivity test is being standardized by seed agencies or sorghum seed centers, verifications of electrical conductivity method needs to be done especially for different sorghum types.

According to the highest correlation coefficients (r) showed among standard germination, first count, field emergence 21 days, speed of germination, conductivity test and potassium leakage are shown in Table 3. Standard germination percentages ranged from 39.83 to 94.00% with mean of 75.39 %. The standard germination showed very highly significant differences among seed lots. Highly significant differences among twenty one seed lots were also observed in first count, field emergence, speed of germination, conductivity test and potassium leakage results.

Seed sources and quality of twenty one lots determined by standard germination, first count, field emergence 21 days, speed of germination, conductivity test and potassium leakage are shown in Table 3. Standard germination percentages ranged from 39.83 to 94.00% with mean of 75.39 %. The standard germination showed very highly significant differences among seed lots. Highly significant differences among twenty one seed lots were also observed in first count, field emergence, speed of germination, conductivity test and potassium leakage results.

**Table 3.** Standard germination and vigor tests of 21 seed lots of sorghum without accelerated aging treatment, data sorted according by storage period and minimum to maximum percentages of standard germination test

| Varietas | Lama simpan (bulan) | DB (%) | IV (%) | DT 21 (%) | KCT (%/etal) | DHL (µS/cm.g) | ION K (ppm) |
|----------|---------------------|--------|--------|------------|--------------|---------------|--------------|
| BMR P-3-5 | 0 - 6 | 56.83 | 44.83 | 38.67 | 16.05 | 12.27 | 889.47 |
| BMR P-3-4 | 0 - 6 | 60.33 | 54.83 | 49.33 | 17.02 | 13.56 | 1,226.21 |
| BMR P-3-3 | 0 - 6 | 64.00 | 50.83 | 37.33 | 17.83 | 10.72 | 684.61 |
| KD4 | 0 - 6 | 73.00 | 66.50 | 82.00 | 18.39 | 17.04 | 886.50 |
| BMR P-3-2 | 0 - 6 | 77.83 | 75.50 | 49.33 | 24.36 | 10.92 | 691.96 |
| Samurai 1 | 7 - 12 | 39.83 | 36.67 | 61.33 | 11.49 | 24.69 | 1,640.65 |
| Kawali 2014 | 7 - 12 | 72.50 | 68.00 | 92.00 | 24.42 | 14.30 | 620.73 |
| Numbu 2014 | 7 - 12 | 73.83 | 72.17 | 88.67 | 17.83 | 9.48 | 471.28 |
| Samurai 2 | 7 - 12 | 81.83 | 67.17 | 90.67 | 19.89 | 11.39 | 687.58 |
| Numbu Freezer 2014 | 7 - 12 | 83.50 | 61.00 | 88.00 | 24.05 | 10.14 | 441.95 |
| Super 2-2014 | 7 - 12 | 88.17 | 87.67 | 97.33 | 29.12 | 10.12 | 652.60 |
| Pahat 2014 | 7 - 12 | 88.83 | 70.00 | 92.67 | 18.42 | 10.36 | 796.52 |
| Super 1-2014 | 7 - 12 | 89.67 | 85.00 | 92.00 | 28.29 | 7.24 | 482.70 |
| Tongkol Jantung | 7 - 12 | 93.50 | 77.33 | 98.67 | 26.29 | 11.47 | 648.78 |
| Telaga Bodas | 7 - 12 | 94.00 | 74.83 | 100.00 | 23.98 | 9.88 | 438.73 |
| Kawali 2013 | 13 - 18 | 84.50 | 66.83 | 87.33 | 23.29 | 8.89 | 555.96 |
| Numbu 2013 | 13 - 18 | 86.00 | 81.67 | 82.67 | 25.11 | 6.78 | 423.50 |
| Super 1-2013 | 13 - 18 | 89.00 | 86.50 | 94.00 | 27.80 | 7.33 | 443.13 |
| Super 2-2013 | 13 - 18 | 90.50 | 88.17 | 94.00 | 28.73 | 8.89 | 555.96 |
| Pahat 2010 | > 24 | 44.17 | 29.67 | 44.67 | 17.78 | 24.76 | 2,191.98 |
| Durra 2010 | > 24 | 51.33 | 40.67 | 45.33 | 12.29 | 16.66 | 1,430.58 |

**Mean** | 75.39 | 65.99 | 76.48 | 21.54 | 11.99 | 787.28 |

**Max** | 94.00 | 88.17 | 100.00 | 29.11 | 24.76 | 2,191.98 |

**Min** | 39.83 | 29.67 | 37.33 | 11.49 | 3.87 | 227.46 |

**F test** | **** | **** | **** | **** | **** | **** |

**CV (%)** | 5.92 | 5.29 | 8.08 | 3.64 | 7.31 | 11.04 |

Note: **** = Significant difference at p < 0.01.
Similar correlations among all tests as found in above experiment to determine electrical conductivity method were also observed in this experiment to verify the recommendations made from the results above. Correlation coefficients among first count, standard germination, field emergence 21 days, speed of germination, potassium leakage, and conductivity test with 150 ml water volume and 75 seed count, of 21 seed lots of 15 sorghum varieties are shown in Table 4.

The results showed highly significant correlations between the electrical conductivity method with 150 ml deionized water and 75 seed count and the standard germination (r = -0.85), first count (r = -0.79) and potassium leakage (r = 0.92). But lower correlation was observed between electrical conductivity test with field emergence (r = -0.53) and speed of germination (r = -0.66).

All test result compared in accelerate aging treatment test to survey for possibility of alternative sorghum seed vigor tests. This test provided information comparable to the other vigor tests performed between sorghum seed given accelerated aging treatment and seed sorghum as control.

Twenty one seed lots of the fifteen sorghum varieties also were used in this experiment to compare between seed control and seed after accelerate aging treatment. Each seed lots after accelerate aging treatment were conducted by standard germination, first count, field emergence 21 days, speed of germination, conductivity test and potassium leakage. Correlation coefficients among first count, standard germination, field emergence 21 days, speed of germination, potassium leakage, and conductivity test with 150 ml water volume and 75 seed count, of 21 seed lots of 15 sorghum varieties also observed in this experiment and shown in Table 5.

The germination percentages after accelerated aging test ranged from 4.50 to 92.33% and germination percent mean was 61.71%. The germination percentages after accelerated aging test showed very highly significant differences among seed lots (Table 6). Highly significant differences among twenty one seed lots were also observed in first count, field emergence, speed of germination, conductivity test and potassium leakage results.

Accelerated aging test developed by (Delouche and Baskin 1973) to measure seed storability and evaluate vigor. The technique involved the exposure of seeds to adverse levels of temperature (40-45°C) and 100% R.H. for varying length of time followed by regular germination test. The seeds absorbed moisture from the humid atmosphere and aged rapidly due to high temperature. The basis for this test is that higher vigor seeds tolerate the high temperature-high humidity treatment and thus retain their capability to produce normal seedlings in the germination test (AOSA 1983).

During aging declined in seed vigor, respiration rate, phosphatase activity and sugar content accompanied by a complete decline of alpha amylase activity and RNA, DNA and protein content were noticeable in rice during seed deterioration (Zhoe et al. 2002).

Conductivity test can predicting the field emergence and standard germination. The electrical conductivity test has been proved as indicator of seed vigor in wide range of crop species and has been successfully related to field emergence and stand establishment. Analysis of linier regression was used to estimate field emergence and standard germination (Table 6).

In conclusion, based on the experiments and data collected from all test, the following conclusions can be drawn: Electrical conductivity test method with 150 ml water volume and 75 seeds count was accurate and suitable for sorghum seed vigor test; electrical conductivity test showed positive correlation with potassium leakage; and electrical conductivity test can be used for seed vigor test in sorghum and provided the potential of physiological seed were shown through: standard germination test, field emergence test, first count test and speed of germination with negative correlation.

Table 4. Correlation coefficients (r) of standard germination (DB), field emergence 21 days after planting (DT), first count (IV), potassium leakage (Ion K) and electrical conductivity method 150 mL water volume and 75 seed count of 21 seed lots of sorghum without accelerated aging treatment

|        | IV  | DB  | DT  | KCT | DHL | Ion K |
|--------|-----|-----|-----|-----|-----|------|
| IV     | 1   |     |     |     |     |      |
| DB     | 0.92| 1   |     |     |     |      |
| DT     | 0.87| 0.82| 1   |     |     |      |
| KCT    | 0.86| 0.83| 0.67| 1   |     |      |
| DHL    | -0.79| -0.85| -0.53| -0.66| 1   |
| Ion K  | -0.81| -0.86| -0.65| -0.67| 0.92| 1    |

Table 5. Correlation coefficients (r) of standard germination (DBaa), field emergence 21 days after planting (DTaa), first count (IVaa), potassium leakage (Ion Kaa) and electrical conductivity method 150 mL water volume and 75 seed count of 21 seed lots of sorghum After accelerated aging treatment

|        | IVaa | DBaa | DTaa | KCTaa | DHLaa | Ion Kaa |
|--------|------|------|------|-------|-------|---------|
| IVaa   | 1    |      |      |       |       |         |
| DBaa   | 0.98| 1    |      |       |       |         |
| DTaa   | 0.84| 0.86| 1    |       |       |         |
| KCTaa  | 0.94| 0.95| 0.87| 1     |       |         |
| DHLaa  | -0.76| -0.82| -0.73| -0.71| 1     |
| Ion Kaa| -0.76| -0.81| -0.73| -0.72| 0.91  | 1       |
Table 6. Standard germination and vigor tests of 21 seed lots of sorghum after accelerated aging treatment, data sorted according to storage period and minimum to maximum percentages of standard germination test

| Varietas         | Lot Benih Lambar (bulan) | DBAA (%) | IVAA (%) | DT 21AA (%) | KCTAA (%/etmal) | DHLAA (µS/cm.g) | ION KAA (ppm) |
|------------------|--------------------------|-----------|-----------|-------------|-----------------|-----------------|----------------|
| BMR P-3-4        | 20                       | 38.33     | 23.17     | 34.67       | 9.11            | 13.55           | 783.81         |
| BMR P-3-5        | 21                       | 38.67     | 30.17     | 44.00       | 9.49            | 11.95           | 674.78         |
| BMR P-3-2        | 18                       | 46.83     | 32.83     | 49.67       | 11.21           | 12.50           | 669.87         |
| BMR P-3-3        | 19                       | 48.33     | 33.83     | 48.00       | 11.80           | 9.13            | 553.44         |
| KD4              | 15                       | 56.33     | 53.67     | 48.67       | 17.89           | 16.07           | 979.29         |
| Samari 1         | 4                        | 7 - 12    | 30.33     | 28.50       | 15.33           | 10.64           | 23.36          | 1,246.27       |
| Numbu 2014       | 10                       | 7 - 12    | 55.33     | 53.67       | 42.67           | 12.97           | 9.23           | 534.36         |
| Pahat 2014       | 3                        | 7 - 12    | 59.67     | 58.50       | 68.67           | 17.35           | 9.96           | 566.04         |
| Kawaii 2014      | 14                       | 7 - 12    | 68.83     | 56.83       | 67.33           | 17.02           | 8.80           | 805.05         |
| Numbu Freezer 2014 | 6                      | 7 - 12    | 69.00     | 66.00       | 49.33           | 13.39           | 10.15          | 580.58         |
| Samari 2         | 5                        | 7 - 12    | 71.17     | 56.83       | 48.00           | 21.32           | 8.96           | 723.14         |
| Saper 2-2014     | 12                       | 7 - 12    | 79.17     | 63.00       | 86.00           | 26.22           | 9.38           | 560.81         |
| Saper 1-2014     | 2                        | 7 - 12    | 83.83     | 80.00       | 87.33           | 26.73           | 6.28           | 456.11         |
| Telaga Bodas     | 7                        | 7 - 12    | 85.17     | 79.50       | 74.67           | 22.83           | 3.75           | 276.69         |
| Tongkol Jantung  | 8                        | 7 - 12    | 92.33     | 88.17       | 89.33           | 28.05           | 10.14          | 693.05         |
| Kawaii 2013      | 13                       | 13 - 18   | 74.67     | 66.33       | 28.00           | 20.32           | 7.12           | 551.95         |
| Saper 2-2013     | 11                       | 13 - 18   | 81.33     | 79.00       | 77.33           | 22.70           | 9.64           | 587.39         |
| Saper 1-2013     | 1                        | 13 - 18   | 86.83     | 84.67       | 85.33           | 28.08           | 6.02           | 337.23         |
| Numbu 2013       | 9                        | 13 - 18   | 91.33     | 83.67       | 84.67           | 29.48           | 5.20           | 249.90         |
| Pahat 2010       | 16                       | > 24      | 4.50      | 3.00        | 7.33            | 0.70            | 23.35          | 1,702.95       |
| Durra 2010       | 17                       | > 24      | 34.00     | 27.83       | 33.33           | 8.48            | 12.13          | 1,163.55       |

| Mean              | 61.71                    | 54.72     | 55.70     | 17.42       | 10.79           | 699.82         |
| Max               | 92.33                    | 88.17     | 89.33     | 29.48       | 23.36           | 1702.95        |
| Min               | 4.50                     | 3.00      | 7.33      | 0.70        | 3.75            | 249.90         |
| F test            | **                       | **        | **        | **          | **              | **             |
| CV (%)            | 6.07                     | 6.63      | 15.10     | 5.92        | 7.68            | 11.76          |

Figure 1. Linier regression between electrical conductivity (EC) and standard germination (SG)

Figure 2. Linier regression between electrical conductivity (EC) and field emergence (FE)
Table 6. Prediction of standard germination value and Field emergence value by electrical conductivity test

| EC (µS/cm·g) | Prediction of SG (%) | Prediction of FE (%) |
|--------------|-----------------------|----------------------|
| ≤ 5.0        | ≥ 94.09               | ≥ 92.33              |
| 5.1 - 7.5    | 87.41 - 93.83         | 86.66 - 92.11        |
| 7.6 - 10.0   | 80.72 - 87.14         | 81.00 - 86.44        |
| 10.1 - 12.5  | 74.03 - 80.45         | 75.33 - 80.77        |
| 12.6 - 15.0  | 67.34 - 73.76         | 69.66 - 75.10        |
| 15.1 - 17.5  | 60.65 - 67.07         | 63.99 - 69.43        |
| 17.6 - 20.0  | 53.97 - 60.39         | 58.32 - 63.76        |
| 20.1 - 22.5  | 47.28 - 53.70         | 52.65 - 58.09        |
| 22.6 - 25.0  | 40.59 - 47.01         | 46.98 - 52.42        |
| 25.1 - 27.5  | 33.90 - 40.32         | 41.31 - 46.76        |
| 27.6 - 30.0  | 27.21 - 33.63         | 35.65 - 41.09        |
| 30.1 - 32.5  | 20.53 - 26.95         | 29.98 - 35.42        |
| 32.6 - 35.0  | 13.84 - 20.26         | 24.31 - 29.75        |
| 35.1 - 37.5  | 7.15 - 13.57          | 18.64 - 24.08        |
| > 37.6       | < 6.88                | < 18.41              |

ACKNOWLEDGMENTS

We would like to thank the Directorate General of Human Resources Development, Indonesian Ministry of Agriculture, Jakarta for granting us the research fund. I also thank the staff of Indonesian Center for Seed Testing and Quality of Food Crops and Horticulture Research and Development, Cimanggis, Indonesia for their support and help rendered to me in carrying out my laboratory work, Amiyarsi Mukti and Endang Murwantini for helping me in my farm work, all my classmates in Andalas University and Bogor Agriculture University for making class friendly and these activities is also acknowledged.

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Effects of pruning on growth and yield of cucumber (*Cucumis sativus*) Mercy variety in The acid soil of North Kalimantan, Indonesia

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Manuscript received: 31 May 2017. Revision accepted: 19 June 2017.

Abstract. Mardhiana, Pradana AP, Adiwena M, Kartina, Santoso D, Wijaya R, Maliki A. 2017. Effects of pruning on growth and yield of cucumber (*Cucumis sativus*) Mercy variety in The acid soil of North Kalimantan, Indonesia. Cell Biol Dev 1: 13-17. In recent years, cucumber production in Tarakan, North Kalimantan only reaches 20 tons ha⁻¹. In fact, cucumber production potential could reach 49 tons ha⁻¹. Several factors that limit the low productivity of cucumbers in Tarakan are acid soil and cultivation techniques which are still limited. This study aimed to determine the effect of pruning on the growth and yield of cucumbers in acid soil in Tarakan. The study was conducted using Randomized Complete Block Design with the treatment of without pruning (P0), shoot of prunings on the main stem (P1), pruning of whole lateral branches above the third section (P2), and pruning of 2 lateral branches that emerged first above the third section (P4). The results showed that plant height was 16.17% (P1) and 2.26% (P2) lower also 0.13% higher (P3) than the control (P0). The highest number of leaves was found in treatment P1 (16.19%) compared to P0. The best fruit diameter was also found in P1 treatment with 4.93% difference compared to P0. Furthermore, a highly significant and the best result on weight per fruit were also obtained by P1 treatment. The results showed that the fruit weight of P1 treatment (11.39%) was higher than P0. This study provided new information that the pruning treatment of shoots on the main stem of cucumber variety Mercy in acid soil could increase the diameter and weight of cucumber.

Keywords: Fruit diameter, lateral branches, leaves, low pH, main stem

INTRODUCTION

Cucumber (*Cucumis sativus* L.) is one of the vegetable crops from Cucurbitaceae family commonly planted by farmers in Indonesia. Cucumber cultivation is widespread throughout the world, both tropical and sub-tropical area. Cucumber plants in Indonesia are grown in lowlands to highlands. The central regions of cucumber cultivation area in Indonesia are West Java, East Java, Central Java, Aceh, and Bengkulu. DNA sequencing found that melon and cucumber are Asian origin and have numerous previously overlooked species-level relatives in Australia and around the Indian Ocean (Sebastian et al. 2010).

Cucumber production in Indonesia is still relatively low. Based on data from the Central Bureau of Statistics, cucumber production in Indonesia continues to decline from year to year. In 2011 to 2015, the cucumber productions were 521,535 tons, 511,525 tons, 491,636 tons, 477,989 tons and 447,696 tons respectively. Besides genetic factors, environmental factors such as climate, cultural practices, and post-harvest condition affect on plant performance (Crawley 2009). The proper cultivation techniques as one of cultural practice need to do in Indonesia to increase the cucumber production.

Increasing cucumber production requires appropriate cultivation techniques. One possible action taken to increase the cucumber production is by improving the cultivation techniques through a proper pruning. According to Usenik et al. (2008), pruning influenced vegetative growth, fruit quality and had no negative effect on peach. Cucumber plants of age about 21 Days After Planting (DAP) usually grow with very dense branch and leaves which lead to producing a vegetative growth only, so the formed flowers and fruits tend to decrease.

Some cucumber farmers in Tarakan City have not done the intensive cultivation techniques such as pruning. Whereas, pruning can affect plants. According to Beadle et al. (2007), pruning in *Acacia* trees can decrease the total number of branches and improved stem straightness. With few branches, the plant will get an optimized light availability. Light gives an effect to plant developmental processes (Feng et al. 2008). The application of pruning technique is still a few due to the limited knowledge and poor information obtained by farmers.

Cucumber cultivation needs to be developed appropriately and sustainably. Pruning is needed as the effort to increase the growth and yield of cucumbers. Therefore, the pruning treatment experiment is expected to increase the growth and yield of cucumber. This study aimed to determine the effect of pruning on the growth and yield of cucumber variety Mercy in acid soils in Tarakan, North Kalimantan, Indonesia.
MATERIALS AND METHODS

Time and site of study
The study was conducted in Experimental Farm of Faculty of Agriculture, Universitas Borneo Tarakan, Tarakan, North Kalimantan, Indonesia from December 2016 to February 2017.

Experimental design
The study was conducted using Randomized Complete Block Design with the treatment of without pruning (P0), shoot of prunings on the main stem (P1), pruning of whole lateral branches above the third section (P2) and pruning of two lateral branches that emerged first above the third section (P3). These treatments replicated five times so that there are 20 units of experimental unit. Each experimental unit consists of four plant samples, so the total sample is 80.

Cultural practice
The study area was measured and cleared from weeds and other plants grown. The land was dug with a depth of ± 20 cm by using a hoe. Planting process was done using a dough tool with a depth of 3-5 cm at the distance of 60 cm between the rows and 30 cm within the rows. Two seeds are inserted into the planting hole, then covered with a little of soil. A routine watering was done every day in the morning especially in the early phase of growth at the age of 7-14 DAP. Watering was done every two days when the cucumber flowers were emerged and were not done when it rains. Stitching was done when the plant is at the age of 14 DAP by replacing the plant that died or grows abnormally with new plants and thinning was done by leaving one best plant per planting hole at the plant age of 21 DAP. Application of stake was done when cucumber plant at the age of a week after planting. This study used bamboo or wood as a stoke marker of each plant and connected each other with ropes.

Basic fertilization used manure with fertilizer dose of 20 tons ha⁻¹ (429 g plant⁻¹). Further fertilization was done at the planting time and when the plants at the age of 10 days after planting. The fertilizers applied were urea 280 kg ha⁻¹ (6 g plant⁻¹), SP-36 260 kg ha⁻¹ (5 g plant⁻¹) and KCl 525 kg ha⁻¹ (9.5 g plant⁻¹). The half dose was applied at the planting time and when the plants at the age of 10 DAP. Fertilizer was inserted into the soil with a distance of ± 15 cm from the stem. The cucumber fruits that were ready to be harvested followed the criteria: the fruit was green and diameter was more than 2.5 cm. Cucumbers were harvested until the productive harvest was complete. Harvesting was done at the age of 47, 48, and 49 DAP.

Pruning
Pruning of maintenance was done by removing leaves and branches that grow before the third section. Treatment of cucumber pruning was done in accordance with the applied treatment. Pruning was done when the plant was 33 DAP using a pruning shears to obtain a good pruning results.

Data analysis
Data of observation result were analyzed by using Variance Analysis (F test) to know the effect of treatment given. If the significant different result found the Least Significance Different (LSD) test with 95% confidence level then conducted.

RESULTS AND DISCUSSION

Based on the analysis of variance, there is an effect of pruning in cucumber. Pruning highly affected the plant length, number of leaves, fruit diameter and fruit weight and affect on flowering age. However, the pruning did not affect the fruit length and the number of fruits per plant. The recapitulation of the effects of pruning on the growth and yield of cucumber is described in Table 1.

Based on the result of the variance, it is known that the pruning treatment has a very significant effect on the cucumber length of at the age of 50 DAP (Figure 1). Figure 1 showed the treatment of pruning of two lateral branches that emerged first above the third section (P3) showed the highest yield compared to other treatments of 272.45 cm, but not significantly different with the treatment of P0 and P2. Treatment of shoot pruning on the main stem (P1) significantly resulted in the shorter plant than other treatments with no shoot pruning, but visually it was seen that the branch was longer than that of without pruning.

Treatment of shoot pruning on the main stem (P1) resulted in the highest number of leaves per plant (59.90 pieces of leaves) compared to other treatments. Treatment of shoot pruning on the main stem could increase the number of leaves by 16.19% compared to that of without pruning (P0). The number of cucumber leaves due to the effect of pruning in detail is shown in Figure 2.

Based on the 5% LSD test, it is known that the shoot pruning treatment on the main stem (P1) and the pruning of the two lateral branches that emerged first above the third section (P3) yielded the fastest flowering time rate compared to other treatments, which is 38 DAP. In general, pruning treatments (P1, P2, and P3) can accelerate the flowering times rather than without pruning treatment (P0). P1 and P3 could shorten the flowering time of 2.93% compared to without pruning treatment (P0). The average flowering age of cucumber due to pruning in detail is shown in Figure 3.

The result of variance analysis showed that all cucumber pruning treatment had no significant effect on fruit length and number of fruit per plant (Table 2). Based on the results, the average fruit length was 19.98 cm, and the average number of fruit that is harvestable in each sample was 3.11 pieces. The data in a number of fruit parameter is the data for harvestable fruit. All of the treatments were suspected to be incapable of producing optimal assimilates so that they were insufficient to increase the number of harvestable fruit.
MARDHIANA et al. – Pruning effects on growth and yield of cucumber

Table 1. Recapitulation of the results of the effects of pruning on the growth and yield of cucumbers

| Parameters          | F test result |
|---------------------|---------------|
| Plant length        | **            |
| Number of leaves    | **            |
| Flowering age       | *             |
| Fruit length        | ns            |
| Fruit diameter      | **            |
| Number of fruit per plant | ns          |
| Fruit weight        | **            |

Note: ns = not significantly different; * = significantly different; ** = highly significantly different

Table 2. Effect of pruning on fruit length and number of fruit per plant

| Treatment | Fruit length (cm) | Number of fruit per plant |
|-----------|-------------------|----------------------------|
| P0        | 19.68 ns          | 3.15 ns                    |
| P1        | 20.05 ns          | 3.00 ns                    |
| P2        | 20.20 ns          | 3.20 ns                    |
| P3        | 19.99 ns          | 3.10 ns                    |
| Average   | 19.98             | 3.11                       |

Note: ns = not significantly different; P0: treatment of without pruning (P0), shoot of prunings on the main stem (P1), pruning of whole lateral branches above the third section (P2), and pruning of 2 lateral branches that emerged first above the third section (P3).

The result of the statistical test showed that the pruning treatment had a significant effect on fruit diameter. The general pruning treatment (P1, P2, and P3) was able to increase the fruit diameter compared to the treatment without pruning (P0). The effect of pruning on average fruit diameter in detail is shown in Figure 4.

The highest average fruit diameter was obtained by the pruning treatment of shoots on the main stem (P1) of 4.26 cm. The shoot of prunings on the main stem was able to increase the percentage of fruit diameter by 4.92% compared to the treatment without pruning. The addition of fruit diameter is closely related to the number of leaves. The optimal leaf surface area can produce assimilate which is able to increase the fruit diameter.

The result of the statistical test showed that the pruning treatment had a significant effect on the production per plant. Treatment of shoot pruning on the main stem (P1) yielded the highest average weight per fruit of 427.11 g plant⁻¹ compared to other treatments (P0, P2, and P3). The effect of pruning on weight per fruit is shown in Figure 5.

In general, pruning treatments (P1, P2, and P3) had a better effect on average weight per fruit than that of without pruning (P0). The best result was obtained by P1 treatment which showed an increase of 11.38% compared to without pruning treatment (P0).
Discussion

The plants are pruned on the main stem are shorter than other treatments. It might be related to the flow of auxin in the plants, the synthesis of auxin on the shoot of the main stem is stopped due to the shoot pruning, while the synthesis of cytokinin increased and further affect the growth of branch growth. Ghosh et al. (2011) said that pruning suppresses apical dominance. The apical meristem and the young leaves are the centers of TAA and IAA (Indole Acetic Acid) synthesis which are then transported to the stems thus inhibiting the development of lateral shoots. IAA is one type of auxin that causes apical dominance. IAA is a trigger for change in plant development (Vanneste and Friml 2009).

The cucumber length was not positively correlated with the yield. This was proved by the production data. The plant stems are not a major contributor to photosynthesis, but it is more affected by the number of leaves. The optimal number of leaves is the largest contributor to photosynthesis result because the leaf is a plant organ which have stomata associated with photosynthesis (Xu and Zhou 2008). Fischer et al. (2012) said that increasing the leaf-fruit ratio generally increases fruit growth and carbohydrates content.

Basically pruning is intended to control the optimal number of leaves thereby improving the yield. Pruning is an attempt to create a better state of the plant, so that sunlight can enter to the whole parts of the plant, increase interception of light into the canopy of plants and increase the availability of air circulation and CO2 in the canopy. The sufficient light and CO2 and other supporting factors will increase the photosynthesis rate which leads to increase the availability of photosynthesis. The excessive vegetative growth caused a suboptimal use of photosynthesis results and led to decrease the yield production (Coggins Jr and Lovatt. 2014).

The shoots of pruning on the main stem might be able to inhibit the production of auxin in the main stem and increase cytokinin hormone. This affects the extension of the lateral branches, the longer lateral branches produce a larger number of section, as the increasing number of the section will also increase the number of leaves. Meier and Leuschner (2008) said that leaf expansion and stand leaf area of beech are controlled by several abiotic factors including spring temperature and possibly also nitrogen supply. Nitrogen affects photosynthesis and photoprotection in leaves (Pompelli et al. 2010) and the results of these photosynthates which will be used by plants to support an increase in yields (Hibberd et al. 2010).

The production of auxin in the main stem continues to proceed without the shoot of pruning. This is certainly not expected by farmers because it will make an apical dominance that results in a longer vegetative phase and inhibition of flowering time of the plant. This was in line with Dun et al. (2009) statement that the production of auxin might trigger the production of a second hormone to inhibit bud outgrowth.

The shoot of pruning on the main stem (P1) is expected to create an optimal growing space for the leaves which perform photosynthesis. The result of the photosynthesis allocated for cell enlargement in the fruit tissue since the meristematic cells in the fruit will result in increasing the volume size so that the cell growth is in line with the increase of fruit diameter. Pruning essentially reduces unproductive parts of the plant so that the assimilate of the photosynthesis process is more widely allocated to enhance other plant growth processes such as cell enlargement. Photosynthesize as a photosynthesis result has a positive effect on fruit (Yu et al. 2013).

Cucumber pruning is able to produce a better fruit weight. It is proven by the fruit weight that is produced by shoot of pruning on the main stem (P1) which is able to gain a weigher fruit in the cucumber Mercy variety is between 350-400 g per fruit. Although the average fruit weight shows an improvement, it has not been able to achieve the average yield of cucumber Mercy variety. Based on the data, cucumber Mercy variety is able to produce 3.5-5 kg plant⁻¹, while the study conducted only reached 1.28 kg. This might be caused by the cucumber harvested only 3 times because after the 3rd harvest time the plant suffered a pest attack of fruit fly (Bactrocera sp.) causing rotten cucumber fruit and not feasible to harvest. This study provided a new information that shoot of prunings on the main stem could increase the quantity (fruit weight) of cucumber Mercy variety in acid soils in Tarakan, North Kalimantan.

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Microdosing technology of fertilizer for sorghum production at Shambat, Sudan

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Abstract. Arbab MBM, Dagash YMI. 2017. Microdosing technology of fertilizer for sorghum production at Shambat, Sudan. Cell Biol Dev 1: 18-22. The study was conducted at the experimental farm of Sudan University of Science and Technology, College of Agricultural Studies; Khartoum North-Shambat, to investigate the agronomic response and efficiency of fertilizer microdosing in Sorghum. An experiment with the following treatments was achieved: control without fertilizer, microdosing treatments with the rates of 1, 2, 3 and 4 g NPK per plant hole at sowing. The treatments were arranged in a completely randomized block design with four replications. The experiment was conducted during the growing season of 2015. Weeding was carried once after three weeks from seed germination and irrigated weekly. The following parameters were considered during experimentation; the number of leaves, plant height, node length and stem thickness, while the shoot fresh and dry weights were recorded at termination. The data collected were subjected to analysis of variance and the means were separated by Duncan’s multiple range test. The results obtained showed the progressive improvement of all Sorghum tested characters. There were highly significant differences in plant height, stem thickness, shoot fresh and dry weight. The number of leaves and the node length showed significant differences. The four gram microdose gave the best results.

Keywords: Microdosing, technology, fertilizer, sorghum

INTRODUCTION

Sorghum (Sorghum bicolor L.) Moench; is the world’s fifth most commonly grown cereal crop after wheat, rice, maize and barley Poehlman (1994). Sorghum has many types of cultivated varieties, such as grain genotypes, fodder, fiber and sugar genotypes and dual purpose genotypes. Sorghum belongs to C4 plant characteristic for tolerate a biotic stresses more than many crops Gnansounou et al. 2005. Recently, sorghum had received significant attention because of the newer use as a Biofuel feedstock (Paterson 2008). Assessment of the genetic variation within cultivated crops and varieties has a strong impact on plant breeding strategies and conservation of genetic resources (Dean et al. 1999; Simioniu et al. 2002) and is particularly useful in the characterization of individuals, accessions and cultivars in germplasm collections and for the choice of parental genotypes in breeding programs (Davila et al. 1998; Ribaut et al. 1998). In the past, indirect estimates of similarity based on morphological information have been widely used in many species including sorghum (Ayana 1999). However, morphological variation does not reliably reflect the real genetic variation because of genotype environment interactions and the largely unknown genetic control of poly-genetically inherited morphological and agronomic traits (Smith and Smith 1992).

Molecular analyses in conjunction with morphological and agronomic evaluation of germplasm are recommended, because these provide complementary information and increase the resolving power of genetic diversity analyses (Singh et al. 1991). Land degradation affects more than half of Africa, leading to loss of an estimated 42 billion dollars and 5 million hectares of productive land each year. The majority of farm lands produce poor yields due to poor farming techniques (nutrient deficiency and irregular watering) (ICRISAT 2009).

The decline in fertility of croplands is the basis of food insecurity in households especially the poor peasants are the most numerous in agriculture in the Sudan region of Mali. According to Sime and Aune (2014), the fallow which was the traditional way to restore the fertility of the land has almost disappeared in some places and in others its duration was significantly reduced because of demographic pressure. The technical packages to sustainably increase production are not within their reach. From the 1980s, there has been a decline in public funding in agriculture and paralysis of the sector of small producers in developing countries because of the structural adjustment policies of the IMF and the World Bank (Azoulay and Saizal 1994; FAO 1995; World Bank 2007).

Many governments in sub-Saharan Africa have made efforts in improving agricultural productivity through the creation of agricultural extension services. But these creations have not fulfilled the expectations of farmers mainly rural women (FAO 2008). The development of sub-Saharan agriculture took from that moment an approach for the identification of technical innovation and communication giving more space to the farmers in the development of appropriate strategies for development. The farmer field school is one of these strategies lying in the extension approach of bottom up allowing farmers to...
join the basis for understanding what to achieve in finding appropriate solutions to their development issue. It was piloted in 90 countries and reached 10 to 15 million farmers worldwide (Waddington et al. 2014). There are a lot of results on the evaluation of farmer field schools: Togola et al. (2010), FAO (2011), Braun et al. (2006), Feder et al. (2004), and Piyadasa (2005). There is, against few results on the diffusion of technology from a farmer field school in sub-Saharan Africa (Davis 2006; Baah 2007).

The microdose technology is the application of small mineral fertilizer doses in the seed hole during sowing or next to the seedling after emergence (10 days after sowing). The advantages of this technology as reported by Agricultural technologies of Borkina Faso (2010) are: (i) Location of the fertilizer near the root, thus obtaining a high concentration area which makes assimilation of nutrients easier. (ii) To limits phosphorus fixation phenomena by the soil. (iii) To reduce loss of Potassium (K) and Nitrogen (N) through leaching. (iv) To achieve an early start of plant growth. (v) To increase the efficiency of fertilizer used. (vi) To minimize production cost. (vii) To improve small producers income. (viii) To increase the number of mineral fertilizer users.

However, ICRISAT (2009) mentioned some difficulties accompanied with this technology, which include: (i) The technology is time consuming, or laborites and difficult to ensure each plant gets the right dose. (ii) Access to fertilizer, access to credit, insufficient flow of information and in appropriate training polices to the farmers. (iii) The adoption of the technology requires supportive and complementary institutional innovation as well as input and output market linkage.

As mentioned by many researchers, the technology uses only about one-tenth of the amount typically used on wheat and one-twentieth of the amount used on corn in USA. Yet, the African crops are so starved of nutrients such as phosphorus; potassium and nitrogen even that micro amount often doubles crop yields (Bationo et al. 2015; Bielder 2015).

This study also investigates if people are more likely to adopt the technology if they receive it free of charge and how knowledge passes from farmer via social networks. Thus, the study aimed to fulfill the following objectives: (i) To test the response of sorghum to microdosing practices under Shambat clay soils. (ii) To determine suitable microdosing levels that lead to increase in vegetative yield and consequently the seed. (iii) To minimize the cost of fertilizer application by the minimum dose of fertilizer with maximum utilization by the plant.

MATERIALS AND METHODS

Experimental site and treatment

A field experiment was conducted at the demonstration farm of the College of Agricultural Studies, Sudan University of Science and Technology, Shambat Khartoum North, (Latitude 15.40 N., 32, 32 E., elevation 380 m above Sea level). The climate is semi-desert with a low relative humidity and annual rainfall rate 150 mm and a mean temperature of (20.3 C-36.1 C) and clay soil celtic pH 7.5-8.7 Abdulla Feez (2001).

Plant material and treatments

The plant material was local variety of sorghum (Sorghum bicolor L.) that obtained from College of Agricultural Studies, Sudan University of Science and Technology (Shambat). This plant was treated by using four level treatment, i.e. control (without fertilizer; M0), 1 g compound fertilizer microdosing (M1), 2 g compound fertilizer microdosing (M2), 2 g compound fertilizer microdosing (M3) and 4 g compound fertilizer microdosing (M4) in a randomize complete block design (RCBD).

Cultural practice

The experimental site was disc ploughed and harrowed, then followed by harrowing and leveling, riding up North-South. The spacing between ridged was70 cm. Five replications were divided into four plots, each plot was 3×3, consisting of five rows. The sowing data was in December 2015. The seeds were sown in holes each 40 cm a part, the seed were sown at the depth of 20 cm. With fertilizer in the same hole. Weeding was done two time after three weeks from sowing and after one month from the first hand weeding. The plants were watered according to the need.

Data collection

Observations were conducted on agronomical traits. Every agronomical trait was observed by selecting five plants of sorghum randomly from each plot except 50% days to flowering. The 50% days to flowering was observed by recording the duration from planting to 50% the plant population were blooming. Plant height was measured from soil surface to the tip of the flag leaf using a measuring tape. Number of leaves per plant was observed by counting the average number of leaves per plant. Length of inter node were measured form one node to other node. Stem diameter was measured using a strip and a ruler and then the mean stem diameter per plant was estimated. Forage fresh yield per plant was measured by weighting the plant. Forage dry yield per plant was measured by drying the plant at the oven (80°C) for 48 hours and then weighed plant.

Statistical analysis

The data was analyzed according to the standard statistical procedure for a randomized complete block design as described by Gomez and Gomez (1984) using MSTAT-C computer package, and the means were separated by Duncan Multiple Range Test (DMRT).

RESULTS AND DISCUSSION

Results

The results of the study for all tested parameters are indicated in (Table 1) and separate detailed figures from (1-
6). According to (Table 1), the results revealed that, there are highly significant differences among the treatments for the plant height, stem thickness, shoot fresh and dry weights; while there is a significant difference for the node length and number of leaves. The coefficient of variation for all tested parameters ranged between 3.73-14.47%. The plant height was higher for the 3 g microdose treatment (136 cm) and the lowest for the control (108 cm). Figure 1 revealed higher plant height for 3M followed by 2M but generally the difference was not big. The highest node length was recorded for 4g microdose (16 cm) while the control resulted in the lowest value (12.5 cm). Figure 2 showed a consistent node length for 4M, 3M but 1M was higher than 2M.

The best number of leaves was obtained from the 4 g microdose treatment and the lowest was recorded for the control. Figure 3 for number of leaves favored 4M followed by 2M and then 3M. The 4 g microdose treatment resulted in the best stem thickness (4.36 cm) and the lowest value was recorded for the control (2.63 cm). Figure 4 represent the stem thickness which was following the normal distribution from the high to the low 4M,3M,2M,1M and 0M. The highest values of shoot fresh and dry weights were recorded from 4 g microdose treatment (125 and 54.50 g) while the lowest values were obtained from the control (49.5 and 20.5 g) respectively. Figure 5 and 6) for the fresh and dry weights favored 4M followed by 3M.

**Table 1.** Summary of ANOVA (F. value) of *Sorghum bicolor* on micro dose experiment.

| Source of variation | ofDegree freedom | ofPlant height (cm) | Node length (g) | Number of leaves | ofStem thickness (cm) | fresh Shoot weight (g) | dry Shoot weight (g) |
|---------------------|-----------------|---------------------|-----------------|-----------------|-----------------------|------------------------|---------------------|
| Replication         | 3               | 0.4996              | 3.2727          | 0.9057          | 1.1211                | 1.2496                 | 0.6128              |
| Fertilizer          | 4               | 36.9939**          | 4.9773*         | 5.3774*         | 15.1966**             | 237.4937**             | 57.1195**           |
| Error               | 12              | -                   | -               | -               | -                     | -                      | -                   |
| Total               | 19              | -                   | -               | -               | -                     | -                      | -                   |
| LSD 5%              | -               | 5.94                | 1.80            | 0.98            | 0.51                  | 5.73                   | 5.18                |
| CV (%)              | -               | 3.73                | 8.53            | 8.31            | 10.08                 | 9.41                   | 14.47               |

Ns= not significant, * Significant (5%), ** highly significant (1%),

**Figure 1.** Effect of microdosing on sorghum plant height

**Figure 2.** Effect of microdosing on sorghum node length

**Figure 3.** Effect of microdosing on number of leaves per plant of sorghum

**Figure 4.** Effect of microdosing on sorghum stem thickness
Discussion
Irrespective of different adverse conditions in the study site during the experimentation, all of the fertilizer rates (microdosing) increased yields compared to the control. This shows that there is a need for applying fertilizer in Sorghum production at most soils of our country. A fertilizer application method that is efficient with a smaller amount of fertilizer is to be the most important for marginal farmers in the central Sudan. Such a method will have high potential to increase farmers’ interest, economic viability and sustainability with respect to applying fertilizer in Sorghum. In this respect, results of this study showed that the microdosing method of fertilizer application was found to improve Sorghum yields with smaller quantities of fertilizer. The results of the study are strongly agreed with those obtained by Khatam et al. 2013; Morris et al. (2007).

Previous studies on the response of Sorghum and pearl millet reported by Palé et al. (2009); Vitale and Sanders (2005), had also shown similar effects that lower fertilizer rates increased crop yields more than the higher rates in microdosing in sub-Saharan countries. Inasmuch as, the results of the study concerning the adoption of microdose technology reported by Agricultural Technologies in Burkina Faso (2010), showed that, the lowest fertilizer rate in microdosing was able to improve sorghum yield more than that of broadcasting in sub-Saharan countries. Similar results were also reported by Bationo (1998); Bagayoko et al. (1996) as they concluded that, the cereals in general revealed lower yield response to the highest fertilizer rate in microdosing and this can be owed that, there is a limit to the dose of fertilizer that can be applied through microdosing. They also noticed that, the high levels of fertilizer found to depress pocket seed germination and lower plant population at harvest and these negative effects on maize performances might be attributed to the burning effects of high doses of fertilizer in the microdosing method of application. Such remarks were also reported by Druilhe and Jesús (2012); FAOSTAT (2011); Coulibaly et al. (2000).

Therefore, if the farmers are practicing microdosing, they can obtain a good yield at a low rate of fertilizer application. Yet, further study based on long-term data is required to rectifying optimum fertilizer rates for the different sites of sub-Saharan countries depending on soil quality and other governing agro-ecological conditions.

As a result, the microdosing method of fertilizer application becomes more efficient in increasing the yield of cereals than the banding and broadcasting method of fertilizer application. This might be due to the fact that placing fertilizer close to the seed in soils increases fertilizer uptake by crops as reported by (FAOSTAT 2011).

This indicates that under a better soil management system and favorable seasonal rainfall conditions, farmers can still get reasonable yields from crops through the application of microdose technology. Although the labor demand in microdosing (4.8 man-days ha\(^{-1}\)) is nearly twice that in banding (2.3 days ha\(^{-1}\)) for the application of fertilizers, the microdosing method still appears attractive and viable. Like in several other areas in Ethiopia, the opportunity cost for labor is low in the central rift valley.

In conclusion, 4M (4 g microdosing) revealed the best results for most parameters and are more productive and profitable. In general microdosing followed the normal pattern from the high to the low. Therefore it is recommended to use 4M microdosing.

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Toxicity of Randia nilotica fruit extract on Schistosoma mansoni, Biomphalaria pfeifferi and Bulinus truncatus

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Abstract. Ebodi AYE, Ahmed MM. 2017. Toxicity of Randia nilotica fruit extract on Schistosoma mansoni, Biomphalaria pfeifferi and Bulinus truncatus. Cell Bio Dev 1 (1): 23-30. The aqueous filtered and unfiltered extract of the fruits of Randia nilotica (locally name as Shagarat El-Murfaein) were assessed as molluscicides against Biomphalaria pfeifferi and Bulinus truncatus as well as their effect on cercariae and miracidia of Schistosoma mansoni. The plant was tested on uninfected B. pfeifferi and B. truncatus, the results showed that unfiltered extracts were found relatively more potent than filtered one (i.e. 100% was attained at 90 ppm and 80 ppm) respectively. While, filtered extract tested on uninfected B. pfeifferi and B. truncatus (100% was attained at 100 ppm and 90 ppm) respectively. The effect of unfiltered extract on infected B. pfeifferi produced 100% mortality in concentration of 70 ppm. The activity of the plant on cercariae and miracidia revealed that cercariae was more resistance than miracidia (i.e.50 ppm killed all cercariae within 3 hours while killed miracidia within 2 hours. The results were statistically analyzed and discussed, and the findings were promising and could open new avenues for the practical use of the plant at the field.

Keywords: Toxicity, Randia nilotica, fruit extract, Schistosoma mansoni, Biomphalaria pfeifferi, Bulinus truncatus

INTRODUCTION

Schistosomes are digenetic trematodes, which belong to the family Schistosomatidae. They inhabit the blood vessels of their hosts and are therefore known as blood flukes. Schistosomiasis (bilharziasis), is an important public health issue for rural communities located near and around slow-moving water-bodies in the tropics and sub tropics. It is estimated that over 200 million people in 73 countries are infected (McCullough and Mott 1983) while further 500-600 millions or 4-5% of the world populations are at risk of infection they produce. Intestinal schistosomiasis, caused by Schistosoma mansoni, occur in Africa, Eastern Mediterranean, Caribbean Islands and South America. S. japonicum also known as Asian schistosome, is present in southeast Asia, China and the Philippines and only in small foci in other countries (Wains and McManus 1997). Another form of intestinal schistosomiasis, caused by S. intercalatum, has been reported in Africa in parts of Cameroon, Gabon and northeast Zaire (Doumenge et al. 1987; Lai et al. 2015). Urinary schistosomiasis is caused by S. haematobium, occurs in Africa and Eastern Mediterranean.

In Sudan the history of schistosomiasis began after building the Sennar Dam in 1925 and the establishment of the Gezira Agriculture Scheme. Integrated schistosomiasis control programmes were recommended by the World Health Organization (WHO) in 1994. The snail control is an important preventive strategy associated with the treatment of infected people together with environmental and socio-economic improvements and health education with community participation. Although chemical molluscicides are the most used approach of snail control (WHO 1973, 1994), yet chemicals have their hazards. In addition to their high costs, they may be toxic to aquatic fauna and source to pollution. All factors make it imperative to consider using molluscicides of plant origin either naturally growing or locally cultivated.

This study is intended to determine the efficiency of the plant Randia nilotica on the adult snail hosts, on cercaria and miracidia of Schistosoma mansoni.

MATERIALS AND METHODS

The plant

Randia nilotica is a plant that belongs to the family Rubiaceae. The plant is locally known as Shagarat El-Murfaein. The fruits of this plant are the parts used in this study. It was collected from Kordofan area in Western Sudan. Extraction of the plant was performed at the Department of Pathology, Faculty of Veterinary Medicine, University of Khartoum. In the laboratory the fruit were air dried under-shade before they were coarsely-powdered.
The snails

Snail collections

The snails used throughout the experiments were Biomphalaria pfeifferi and Bulinus truncatus, which are the most important vectors for the transmission of human and animal schistosomiasis in Sudan (Hussein 1973; Sulaiman and Ibrahim 1985; Jordan et al. 1993), as well as sub Sahara Africa (Hotez and Fenwick 2009; Hotez and Kamath 2009; Steinmann et al. 2006). The snails were collected from El-Seleit irrigated area on the Eastern part of the Blue Nile by deep scooping. The scoops were constructed from the kitchen sieves, supported by an iron frame and mounted on a handle (1-2 meters long). The snails were then maintained and bred in the laboratory of bilharziasis at the Department of Pathology, Faculty of Veterinary Medicine, University of Khartoum.

Snail breeding

About 10-20 snails were put in plastic tanks, in which the water was changed even three days by normal dechlorinated water and was cleaned periodically from faecal debris. The temperature in the laboratory was kept at 25-30°C. The snails were fed dried lettuce leaves, prepared as follows, by washing green leaves of lettuce by boiling water, and allowed to dry. The water used for snail breeding was tested to determine its chemical composition using flame spectrophotometer. The same water was then used to determine the rate of development and hatching of these snails in the laboratory. The egg-masses produced by these snails were deposited on small pieces of celophane materials which were placed on the top surface of water in the tanks to collect eggs. The celophane materials contaminated with egg-masses were removed daily and placed on water in other tanks to maintain hatching of eggs. The juvenile snails were fed on algae or dried lettuce, the range of growth and periods of hatching were observed.

Extract preparation

The method of the extract preparation described by Brackenbury et al. (1997) and Brackenbury (1999) was adopted in the experiment. An amount of 2.5 grams of coarsely powdered materials of R. nilotica fruits was soaked in 200 ml of distilled water in a flask for 24 hours. The contents of the flask were then filtered, and the volume was adjusted up to 250 ml, using distilled water. A stock solution was then prepared for future use.

Test for molluscicidal activity

The procedure of the test which was applied for the materials obtained from the fruits of R. nilotica was carried out according to the method recommended by WHO (1965). Ten viable snails were put in one liter of the extract concentration and left for another 24 hours. They were then removed and put in dechlorinated water for another 24 hours as a recovery period. The number of dead snails was recorded. The control was prepared by putting another group of ten snails in one liter of dechlorinated water devoid of the extract. The snails are usually considered dead when they show lack of movements, retraction or hanging out of their shells; in such cases, the snail bodies and shells will be discolored. Death is also confirmed by lack the reaction to any external stimulus from the surrounding water. The numbers of dead and living snails were recorded after 24 hours of exposure followed by 24 hours of recovery period.

Molluscicidal activity of Randia nilotica on Biomphalaria pfeifferi and Bulinus truncatus

From the stock solution which was prepared from R. nilotica fruits, different dilutions were prepared to study their molluscicidal activity on B. pfeifferi and B. truncatus. A group of ten viable B. pfeifferi and another group of ten B. truncatus snails were put each in one liter of the extract solution of known concentration in a container and exposed for 24 hours. First, a titration was used at the rate of 100, 200, 300, 400, 500, and 600 ppm. According to the results obtained from the titration, different other concentrations were used for further screening to achieve mortality percentages ranging from zero to 100% from each concentration; the experiment was repeated three times and the average obtained from the three readings was taken. Control results were similarly taken. Two types of the extract were used, the first one was the filtered extract and the other one was unfiltered. The extract was used against uninfected Bulinus, infected and uninfected Biomphalaria

Miracidicidal activity test

Production of miracidia

Miracidia were obtained using stool samples obtained from people infected with Schistosoma mansoni in El-Seraha village in Gezira State. Samples were examined by locally developed direct thick smear method described by Teesdale and Amin (1976). The positive samples containing eggs were put in normal saline and mixed in a conical flask. The stool sample was sieved in a wire mesh and then filtered. The filtrate was collected into one-liter conical flask. Warm dechlorinated water was then added and the flask was put under artificial light for about one hour to induce hatching.

Test for toxicity of Randia nilotica on miracidia

The test was carried out according to the method recommended by WHO (1965). A group of ten miracidia suspended in 0.5 ml distilled water, were transferred to micro-titer plates each containing one ml of the extract of a known concentration of 25, 50, 100, 250 and 500 ppm of Randia nilotica. The miracidia were examined under a dissecting microscope over a period of three hours. Ten miracidia were transferred to one ml of distilled water to serve as control. Death of miracidia was determined by low of motility and by exhibiting granular shape. Each experiment was repeated three times for each concentration, and the average of three results was then taken. The time taken to kill all miracidia was observed and recorded.
Procedure of snail infection

The snails used in this procedure was *B. pfeifferi*. Each snail was placed in a micro-titer plate containing 5ml of fresh water. 3-5 miracidia were added to each micro-titer plate. The snails were exposed to schistosome miracidia under light for 24 hours. Screening for schistosome infection to detect transmission of infection in the snails started 30 days after exposure to miracidia.

Detection of the infection in snails

Firstly, the snails were washed with dechlorinated tap water 2-3 times to wash out tissue debris. They were put in a beaker containing distilled water (10 ml/snail). They were exposed to strong artificial light at a temperature of 25C. Shedding of cercariae started after about half an hour under these conditions.

For the detection of cercariae, the beaker was held against a source of light. Cercariae of *Schistosoma* species were identified under the microscope by biforked tails and the absence of eyespots. Based on the procedure mentioned above, the snails were screened and the infected ones were isolated for use in the experiment. They were then exposed to artificial light for half an hour to produce the cercariae. A volume of 0.5 ml solution containing the cercariae was randomly taken, and spread on a petri dish. The cercariae were then fixed and stained with loughols iodine and counted under a dissecting microscope. The required number for the test was then taken from the sample.

Cercaricidal activity test

The test was carried out according to the method recommended by WHO (1965). Twenty cercariae were suspended in 0.1 ml distilled water and incubated with one ml of different concentrations of the plant *R. nilotica* extract in micro-titer plates, the concentrations used were 25.50,100,250 and 500ppm. The micro-titer plates were examined under a dissecting microscope over a period of five hours after with the activity decreased. The time required to kill all cercariae was recorded. Cercariae were considered dead if they become immotile and/or their oral and ventral suckers are extended. Twenty cercariae were transferred to one ml of distilled water on the same plate to serve as control.

Statistical analysis

ANOVA, t-test and probit procedure were carried out to analyze. ANOVA test was used to assess the activity of filtered and unfiltered extracts of the plant *R. nilotica* on *B. pfeifferi* and *B. truncatus* cercariae and miracidia of *S. mansoni* using SPSS program. The correlation coefficient that shows the relationship between the plant concentrations and the effect of this plant of the snails and cercariae and miracidia of *S. mansoni* was calculated. It was then drawn into graph for filtered and unfiltered extracts against *B. pfeifferi*, *B. truncatus*; cercariae and miracidia of *Schistosoma mansoni*. To compare the potencies of filtered and unfiltered extracts on *B. pfeifferi* and *B. truncatus*, and to compare the potency of unfiltered extract on infected and uninfected *B. pfeifferi* T-test was used. Furthermore, the probit analysis was used to confirm the potency of *R. nilotica* filtered and unfiltered extract against *B. pfeifferi* and *B. truncatus*, and to confirm the potency of unfiltered extract on infected *B. pfeifferi* by cercaria of *S. mansoni*. The probit values were calculated using the log values of the extracts concentrations and the percentage mortalities corresponding to them.

\[
Y_1 = (Y - bX) + bx_1
\]

Where

- \((Y_1)\) is the calculated (predictable) probit value.
- \((Y)\) = average of the% mortalities.
- \((X)\) = average of the extract concentrations. \(b = \) is a constant (least square estimate).
- \(x = \) is the log of the concentration used.

RESULTS AND DISCUSSION

The activity of *Randia nilotica* filtered and unfiltered extract on * Biomphalaria pfeifferi*

The titration of the activity of filtered extract on *B. pfeifferi* revealed that concentration of 20 ppm resulted in 3% while concentration of 30% produced 13% mortality, concentration of 40 ppm resulted in 23% mortality, concentration of 50 ppm resulted in 36% mortality, concentration of 60 ppm resulted in 46%, concentration of 70 ppm produced 70% mortality, concentration of 80 ppm produced 83% mortality, concentration of 90 ppm resulted in 93% mortality, while the highest concentration of 100 ppm produced 100% mortality (Figure 1).

On the other side the activity of unfiltered extract on the snails showed that concentration of 20 ppm resulted in 6% while concentration of 30 ppm produced 16%mortality, concentration of 40 ppm resulted in 30% mortality, concentration of 50 ppm resulted in 53% mortality, concentration of 60 ppm resulted in 70%, concentration of 70 ppm produced 83% mortality, concentration of 80 ppm produced 90% mortality, while concentration of 90 ppm resulted in 100% mortality (Figure 2).

The effect of filtered extract on the snail was highly significant (p≤0.001) (Table 1), while the correlation was positive between the filtered extract concentrations and the mortality, the correlation was positive (r = 0.960) and it was highly significant (p≤0.01). The effect of the other unfiltered extract, was also highly significant (p≤0.001, Table 1), and the correlation was positive (r = 0.969), and was highly significant (p≤0.01).

There was no significant difference in potency of filtered and unfiltered extract on *B. pfeifferi*. In concentration of 10 ppm and 100 ppm there was no significant differences (p = --), while in concentration of 20 ppm the difference was found (p = 1.000), in concentration of 30 ppm recorded no significant difference between filtered and unfiltered extract (p = 0.519), in concentration of 40 ppm the result showed no significant differences (p = 0.491), in concentrations of 50 ppm and of 60 ppm the results was recorded as (p = 0.152) and as (p = 0.091),
respectively. In concentration of 70 ppm (p = 0.411), in concentration of 80 ppm (p = 0.492), and in concentration of 90 ppm no significant difference was shown (p = 0.116) (Figure 3)

**The activity of *Randia nilotica* filtered and unfiltered extract on *Bulinus truncatus***

The titration of the activity of filtered extract revealed that concentration of 20 ppm resulted in 6% while concentration of 30 ppm produced 13% mortality, concentration of 40 ppm resulted in 30% mortality, concentration of 50 ppm resulted in 52% mortality, while concentration of 60 ppm resulted in 70%, concentration of 70 ppm produced 83% mortality, concentration of 80 ppm produced 91% mortality, concentration of 90 ppm resulted in 100% mortality (Figure 4). On the other side the activity of unfiltered extract on the snail showed that concentration of 20 ppm resulted in 7% while concentration of 30 ppm produced 23% mortality, concentration of 40 ppm resulted in 41% mortality, concentration of 50 ppm resulted in 70% mortality, while concentration of 60 ppm resulted in 86%, concentration of 70 ppm produced 93% mortality, while concentration of 80 ppm produced 100% mortality (Figure 5).

The effect of filtered extract on *B. truncatus* was highly significant (p≤0.001, Table 1). The correlation coefficient was positive (r = 0.966), and was highly significant (p≤0.01). The effect of unfiltered extract on *B. truncatus* was also highly significant (p≤0.001) (Table 1). The correlation between extract concentration and the percentage mortality was positive (r = 0.945), and was highly significant (p≤0.01).

The concentration of 10 ppm was not significant (p = -), and the same result was produced in concentrations of 90 ppm and 100 ppm. In concentration of 20 ppm the significance was (p = 1.000), while in concentration of 30 ppm, it was (p = 0.230), the significance in concentration of 40 ppm was (p = 0.288), in concentrations 50 of ppm and 60 ppm the effect showed no significance (p = 0.189) and (p = 0.067), respectively. In concentration of 70 ppm the significance was (p = 0.101), while in the last concentration of 80 ppm, the significance was (p = 0.158) (Figure 6).
The activity of *Randia nilotica* unfiltered extract on infected and uninfected *Biomphalaria pfeifferi*

The titration of the activity of unfiltered extract of *R. nilotica* on infected *B. pfeifferi* revealed that concentration of 20 ppm resulted in 3% while concentration of 30 ppm produced 23% mortality, concentration of 40 ppm resulted in 33% mortality, concentration of 50 ppm resulted in 70% mortality, concentration of 60 ppm resulted in 90% mortality, in concentration of 70 ppm produced 100% mortality (Figure 7).

The effect of unfiltered extract on infected *B. pfeifferi* snail was highly significant ($p \leq 0.001$) (Table 1). It gave a positive correlation. The correlation coefficient was ($r = 0.934$), this correlation was highly significant ($p \leq 0.01$).

There was no significant differences in potency of unfiltered extract on infected and uninfected *Biomphalaria* snail, in concentration of 10 ppm there was no significant differences ($p = -$). The same result was produced in concentrations of 90 ppm and 100 ppm. There was no significant difference in concentration of 20 ppm ($p = 0.519$), while in concentration of 30 ppm the significance was ($p = 0.230$), in concentrations of 40 ppm and 50 ppm there was no significant difference ($p = 0.725$ and $p = 0.189$), respectively. In concentration of 60 ppm gave no significance ($p = 0.070$). the significance in concentration of 70 ppm was ($p = 0.132$), while in concentration of 80 ppm was ($p = 0.158$) (Figure 8).

The activity of *Randia nilotica* filtered extract on cercariae of *Schistosoma mansoni*

The activity of *R. nilotica* filtered extract on cercariae showed that the concentration of 500 ppm killed all cercariae in a few minutes (15 minutes), and the concentration of 250 killed the cercariae at 30 minutes. In concentration of 50 ppm, the cercariae killed in about 3 hours, while concentration of 25 ppm gave no effect on cercariae for about five hours (Figure 9).

The effect of the plant on cercariae was highly significant ($p \leq 0.001$), (Table 1). The time to kill the cercariae was decreased when the used concentration increased, this means correlation was negative, the correlation coefficient was ($r = -0.751$), this correlation was highly significant ($p \leq 0.01$).

The activity of *Randia nilotica* filtered extract on miracidia of *Schistosoma mansoni*

The activity of *R. nilotica* filtered extract on miracidia showed that concentration of 500 ppm killed the miracidia just in 10 minutes, concentrations of 250 ppm and 100 ppm, killed all miracidia in 20 minutes and 45 minutes, respectively. While the concentration of 50 ppm killed all miracidia in 2 hours, the concentration of 25 ppm produced no effect on miracidia for up to 3 hours (Figure 9).

The plant was very effective on miracidia of *S. mansoni*, and was highly significant ($p \leq 0.001$) (Table 1). It was negative correlation, the correlation coefficient ($r = -0.773$), it was highly significant ($p \leq 0.01$).
Table 1. ANOVA degree of freedom (df), mean squares (MS), R-square (R) and F value (F) for infected and uninfected Biomphalaria, Bulinus, cercariae and miracidia treated by filtered and unfiltered extract of Randia nilotica

| Extract Source | df | MS   | R     | F       |
|----------------|----|------|-------|---------|
| Filtered extract |    |      |       |         |
| Biomphalaria pfeifferi | 9  | 42.404 | 0.922 | 34.381*** |
| Bulinus truncatus | 9  | 46.756 | 0.932 | 63.758*** |
| Cercariae | 3  | 16818.750 | 0.564 | 1770.395*** |
| Miracidia | 3  | 7418.750 | 0.598 | 471.032*** |
| Unfiltered extract |    |      |       |         |
| (Infected Biomphalaria pfeifferi) | 9  | 54.089 | 0.873 | 135.222*** |
| (Uninfected Biomphalaria pfeifferi) | 9  | 45.870 | 0.910 | 72.427*** |
| Bulinus truncatus | 9  | 49.870 | 0.893 | 114.393*** |

Note: ***P ≤ 0.001

Discussion

Aqueous extract of the plant R. nilotica filtered and unfiltered extract, has been used in this study for the assessment of their activities. Filtered and unfiltered extracts were used on the snails B. pfeifferi and B. truncatus, while filtered extract was used against cercariae and miracidia of Schistosoma mansoni. Some researchers have reported high molluscicidal activities in the alcoholic extracts of different parts of a number of species belonging to the family Rubiaceae (Adewenmi 1980; Ahmed et al. 1994). One of these plants was R. nilotica. The stem of this plant was found very active against both snails.

The results of this study revealed that the plant extract was highly effective against B. pfeifferi. Filtered extract produced LD50 (probit 5.00) at concentration of 46.56 ppm, while it produced LD90 when the concentration increased to 84.33 ppm. This indicates that 100% mortality was reached when 90 ppm and 100 ppm were used. On the other hand, the effect of unfiltered extract on B. truncatus revealed that LD 50 (probit 5.00) was 39.63 ppm, while LD90 (probit 6.28) produced at concentration of 67.61 ppm. While 100% mortality was produced when concentrations 80 ppm, 90 ppm and 100 ppm were used.

The effect of each concentration revealed no significant difference between filtered and unfiltered extract on Bulinus truncatus.

The above results indicate that unfiltered extracts of R. nilotica were more potent than filtered one on both B. pfeifferi and B. truncatus. However, high molluscicidal activities were attained on both snails only when higher concentrations were used i.e 100% resulted in concentrations of 100 ppm and 90 ppm of filtered extract and 90 ppm and 80 ppm of unfiltered extract on B. pfeifferi and Bulinus truncatus respectively.

The effect of two extracts revealed no significant differences in each concentrations.

The effect of R. nilotica on B. truncatus revealed that filtered extract produced LD50 at concentration of 46.56 ppm, while it produced LD90 when the concentration increased to 84.33 ppm. It indicates that 100% mortality was reached when 90 ppm and 100 ppm were used. On the other hand, the effect of unfiltered extract on B. truncatus revealed that LD 50 (probit 5.00) was 39.63 ppm, while LD90 (probit 6.28) produced at concentration of 67.61 ppm. While 100% mortality was produced when concentrations 80 ppm, 90 ppm and 100 ppm were used.

The effect of each concentration revealed no significant difference between filtered and unfiltered extract on Bulinus truncatus.

The plant was used also against infected B. pfeifferi by miracidia of S. mansoni, unfiltered extract was highly effective, LD50 with 40.83 ppm, while 90% mortality was produced at concentration of 65.16 ppm. Concentrations that produced 100% mortality were 70 ppm, 80 ppm, 90 ppm and 100 ppm.

The above results indicate that unfiltered extracts of R. nilotica were more potent than filtered one on both B. pfeifferi and B. truncatus. However, high molluscicidal activities were attained on both snails only when higher concentrations were used i.e 100% resulted in concentrations of 100 ppm and 90 ppm of filtered extract and 90 ppm and 80 ppm of unfiltered extract on B. pfeifferi and Bulinus truncatus respectively.

Concentrations of filtered extract were relatively very low as compared with the stem extracts of R. nilotica which produced 100% mortality when 1150 ppm and 1000 ppm were used on B. pfeifferi and B. truncatus respectively (Ibrahim 1998). The results above also revealed that the snail B. pfeifferi was more resistant than B. truncatus in both extracts, however 100% mortality was attained when 100 ppm and 90 ppm were used on B. pfeifferi and 90 ppm and 80 ppm on B. truncatus of filtered and unfiltered extracts respectively.

The plant was used also against infected B. pfeifferi by miracidia of S. mansoni, unfiltered extract was highly effective, LD50 with 40.83 ppm, while 90% mortality was produced at concentration of 65.16 ppm. Concentrations that produced 100% mortality were 70 ppm, 80 ppm, 90 ppm and 100 ppm.
The time taken to kill cercariae and miracidia was only lower concentration was used i.e in concentration of resistant than miracidia. These results were attained when minutes respectively (Table 2). The effect of the plant was 500 ppm killed all cercariae within 30 minutes and 15 minutes. Concentration of 250 ppm killed the cercariae within 120 minutes and 45 minutes respectively. Similar concentration in the other sub species (adansoni) killed cercariae and miracidia within 80 minutes and 35 minutes respectively. Simlar correlation coefficient (r) was negative. This means that when the concentration of the plant extract was increased, the time taken to kill all cercariae and/or miracidia decreased.

These results revealed that uninfected snail was more resistant than the infected one; 100% mortality was given at concentration of 90 ppm on uninfected snails, and at concentration of 70 ppm on infected one (Table 2). This difference may be due to the morbidity of infected snails by miracidia. The effect of unfiltered extract of the plant revealed no significant difference between infected and uninfected B. pfeifferi.

The activity of the plant used on miracidia of S. mansoni was observed the time taken to kill all miracidia. In concentrations of 50 ppm and 100 ppm miracidia were killed in about 120 minutes and 45 minutes respectively. Concentration of 250 ppm killed miracidia in about 20 minutes, while concentration of 500 ppm killed all miracidia in a fewer time (10 minutes) (Table 3).

The effect of the plant on miracidia of S. mansoni was highly significant.

The plant was used against cercariae of S. mansoni, in concentration of 50 ppm killed the cercariae within 180 minutes. Concentration of 100 ppm killed the cercariae within 60 minutes, while concentrations of 250 ppm and 500 ppm killed all cercariae within 30 minutes and 15 minutes respectively (Table 2). The effect of the plant was highly significant when used against cercariae of S. mansoni.

These results revealed that cercariae were more resistant than miracidia. These results were attained when only lower concentration was used i.e in concentration of 50 ppm the time taken to kill cercariae and miracidia was 180 minutes and 120 minutes respectively. It was observed that concentration of 25 ppm was not active on both miracidia and cercariae within 3 hours and 5 hours respectively (Table 3).

Forty Sudanese plant were tested by El-Shiekh (1994) for their miracidicidal and cercaricidal activity against S. mansoni. In these plants R. nilotica was found highly effective, by killing all miracidia and cercariae of S. mansoni at concentration of 50 ppm within 3 hours and 5 hours respectively. The correlation coefficient (r) was negative. This means that when the concentration of the plant extract was increased, the time taken to kill all cercariae and/or miracidia decreased.

Bashir et al. (1987) reported the activity of the plant Acacia nilotica, with sub species nilotica and adansoni against cercaria and miracidia of S. mansoni. They found that high concentrations of both sub species of the plant killed both cercariae and miracidia, as concentration of 5000 ppm of A. nilotica with sub species nilotica killed cercariae and miracidia within 80 minutes and 35 minutes respectively. Similar concentration in the other sub species (adansoni) killed cercariae and miracidia within 100 minutes and 80 minutes respectively. Al-Sayed et al. (2014) reported that Eucalyptus globulus has a potential source for biocidal compounds against S. mansoni and its snail host. Ibrahim et al. (2015) stated that Agave angustifolia and Pittosporum tobira have cercaricidal and miracidicidal potencies against S. mansoni.

Ibrahim (1998) tested the potency of the stem of the plant Randia nilotica on B. pfeifferi and B. truncatus, and showed the activity of the plant against cercariae and miracidia of S. mansoni. The plant killed the snails at high concentrations, 100% mortality was reached when concentrations of 1200 ppm and 1300 ppm were used on B. pfeifferi and B. truncatus respectively. Where the 100% mortality of cercariae and miracidia of S. mansoni was taken in concentration of 100 ppm and 50 ppm respectively. These differences may be due to the part (s) used of the plant, and that the plant used in that study was obtained from Sudan National Garden in Khartoum where this plant was cultivated.
In conclusion, from molluscicidal examinations of the plant _R. nilotica_ against _B. pfeifferi_ and _B. truncatus_ it could be concluded that the activity of this plant was high in the form of both filtered and unfiltered extract against both snails, and it gave good results even in lower concentrations. The experiments also showed that the concentration of 10 ppm did not produce any activity against the snails in all experiments. The cercaricidal and miracidicidal activity of the plant revealed that it was very effective on cercariae and miracidia of _S. mansoni_. As it gave 100% mortality in short period of time at lower concentrations. Also in these experiments the concentration of 25 ppm did not give any activity on cercariae and/or miracidia of _S. mansoni_. Based on the results of the present study it seems reasonable to conclude that the plant can be used for the control of schistosomiasis in Sudan, as it has considerable molluscicidal, cercaricidal and miracidicidal activities. In addition the plant grows naturally in different parts of the Sudan and its application requires simple technology.

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The endophytic bacteria producing IAA (Indole Acetic Acid) in *Arachis hypogaea*

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Manuscript received: 6 November 2016. Revision accepted: 25 June 2017.

**Abstract.** Herlina L, Pukan KK, Mustikaningtyas D. 2017. The endophytic bacteria producing IAA (Indole Acetic Acid) in *Arachis hypogaea*. Cell Bio Dev 1: 31-35. Endophytic bacteria are bacteria living in plant tissue and forming colony without harms the host. Every cormophyte, plants that have a stem and root, may contain some endophytic bacteria which can produce biological compounds or secondary metabolites. The objective of the study was to obtain endophytic bacteria isolates from peanut plants (*Arachis hypogaea*) at three locations, and to test in vitro the ability of endophytic bacteria isolates for producing IAA, and also to analyze IAA on the growth and development of mungbean plants. The study was carried out in three stages; the first was the isolation of endophytic bacteria from the leaves, stems, and roots; the second was the test of in vitro endophytic bacteria isolates to know the ability of IAA production. IAA assay was measured by using a spectrophotometer with a wavelength of 535 nm, and the third was the introduction of IAA-producing endophytic bacteria in mungbean. The parameters observed were the length of sprouts and the number of lateral roots. The results showed that 16 isolates were selected based on IAA-producing ability. The isolates could produce different IAA with different morphological characteristics. After the fourth day of incubation, the highest and the lowest of IAA amount were 69.68 (mg L⁻¹) and 8.50 (mg L⁻¹) respectively. Isolates that produce high IAA levels are applied to mungbeans, it affects the number of lateral roots but it does not have effect on the length of the sprouts. DM and K1K1 isolates have the effect of increasing lateral root formation and are expected to be potential sources of bioactive metabolites.

**Keywords:** Endophytic bacteria, IAA, *Arachis hypogaea*

**INTRODUCTION**

Endophytic bacteria are microbes that live in tissues which form colonies in plant tissues without harming the host. Each high-level plant may contain several endophytic bacteria which are capable of producing biological compounds or secondary metabolites suspected as a result of coevolution or transfer genetics from host plants to endophytic microbes (Duan et al. 2013). The biological association between endophytic microbes and host plants varies from neutral, commensalism, to symbiosis. Plants are a food source for endophytic microbes in completing its life cycle. Endophytic bacteria can be isolated from the surfaces of sterile plant tissue or extracted from inner plant tissues (Pandey et al. 2012; Ryan et al. 2008). In particular, bacteria enter the tissues through germinated tissue, roots, stomata, and damaged tissue. In recent years, endophytic bacteria are used as biofertilizers to increase crop production as it significantly reduces the chemical input to the environment (Luo et al. 2012; Ahemad et al. 2014).

Endophytic bacteria are one of the microorganisms that are now beginning to develop its role in increasing plant growth through its ability to produce growth hormone and N2 retardation from the air. The ability of endophytic microorganisms to produce plant hormones such as IAA (*Indole Acetic Acid*) or better known as auxin can help plants to grow better as in some food crops such as peanuts, corn, wheat and sugarcane (Mattos et al. 2008). Auxin-producing endophytic bacteria can help plants to grow and develop in addition to endogenous auxin possessed by plants. Auxin in plants is usually present in meristem tissues (Spaepen et al. 2007). Auxin produced by endophytic bacteria *Burkholderia kururienisi* in peanut plants cause plant growth to be better with the number of roots, and it makes lateral roots of the plant increases. Plant growth is rapid, and it gives high yielding products (Mattos et al. 2008).

The mechanism of increasing plant growth by endophytic bacteria can occur in several ways including folic, nitrogen fixation, stimulation lateral root growth and production of growth hormones such as auxin, ethylene, and cytokines (Ahemad et al. 2014). Plants meet the needs of hormones through their ability in synthesizing the auxin hormone from microorganisms in their tissues. IAA-producing bacteria potentially join the physiological process of plants by entering IAA generated crops. The effect on the plant itself is that the plant is more sensitive in altering its IAA concentrations thus helping in the formation of lateral roots, adventitious roots and primary root elongation (Ryan et al. 2008).

Various research results reported that some groups of microbes are capable of producing compounds that can accelerate plant growth. Some soil microorganisms that produce IAA such as *Stenotrophomonas maltophilia* from the banana root can promote plant growth (Ambawade and Pathade, 2015). *Azospirillum* which produces IAA can
accelerate plant growth, lateral root development, stimulate density and root hair length, which in turn leads to increased nutrient uptake in peanut crops to increase peanut plant height and make this bacteria function as bacterial fertilizer (Lestari et al. 2007). The effect of *Azotobacter* in increasing root biomass is due to the income of (*Indol Acetic Acid*) in the root zone. IAA-producing endophytic bacteria successfully isolated from plant roots are Agrobacterium tumafaciens and *Azotobacter vinelandii* (Khan and Doty 2009). Different bacterial groups were reported to produce IAA (indole-3-acetic acid), the most important auxin that regulates plant development such as cell extension, cleavage, differentiation, gene regulation, and other tropical responses (Nath et al. 2013).

Auxin is one type of hormone that can stimulate plant growth by increasing elongation process and stem extension as well as cell differentiation (Tarably et al. 2008). In the IAA plant, the tissue is synthesized in various parts of the plant body. Generally, IAA was mostly produced in the growing parts of plants. Tryptophan is a precursor in auxin biosynthesis both in plants and in microorganisms. Tryptophan contains active compounds that spur the growth of rhizosphere microbes and endophytes. The availability of suitable precursors is a primary factor of microbial secretion of secondary metabolites. IAA microbial biosynthesis in soil may be driven by the presence of tryptophan originating from root exudates or damaged cells (Spaepen et al. 2007). The purpose of this research is to isolate and identify IAA-producing endophytic bacteria, which is expected to also influence the growth and development of green beans.

**MATERIALS AND METHODS**

The isolation of endophytic bacteria from *Arachis hypogaea*

The roots and leaves of peanut were cleaned for about 20 minutes using running water. Roots and leaves were sterilized by soaking them in alcohol solution 70% for 2 minutes, hypochlorite solution 5% for 5 minutes, and alcohol solution 70% for 30 seconds, and then were rinsed with sterile distilled water twice (RadauandKqueen, 2002). After sterilizing, the roots and leaves were aseptically mashed in a mortar, and then put into a test tube which contained sterile distilled water in a ratio of 1: 10 and made dilution to 103. 1 ml of the roots and leaves were spread on a nutrient medium for sterilizing and incubating at room temperature for 24 hours. To obtain pure cultures, the colonies of growing bacteria were subcultured into the same medium. To distinguish bacterial isolates from one another, the characterization of colony morphology gram stain and some biochemical tests were conducted.

The in vitro process for producing IAA of endophytic bacteria

The in vitro process for producing IAA of endophytic bacteria was done by generating the bacteria in media containing tryptophan 3 ml bacterial suspension, with some cells of $10^8$ CFU/ml/ equal to McFarland (Bresson and Borges, 2004) was inoculated into 30 ml of Luria-Bertani Tryptophan solution. At room temperature, bacterial cultures were incubated and shaken at 150 rpm for 7 days. Every two days in a week, the IAA level generated during cultivation was measured. The measuring of IAA level was done in colorimetry way with a spectrophotometer at 353 nm wavelength. Culture fluid was centrifuged for 25 minutes at 5000 rpm. The obtained filtrate was mixed with the Salkowski reagent (150 ml concentrated H$_2$SO$_4$, 250ml of distilled water, 7.5 ml of 0.5MFeCl$_3$•6H$_2$O) with a ratio of 2:1. The mixture was then incubated at room temperature for an hour before the absorbance was measured at a wavelength of 353 nm. IAA level produced by endophytic bacteria was determined from the linear plot of the absorbance value of a standard IAA.

**Introduction of IAA-producing endophytic bacteria in mungbean plants**

Positive of bacterial culture which produced IAA were tested in liquid and solid forms to mungbean plant growth. Introduction of endophytic bacteria is done on sterile mungbean sprout. To get sterile sprouts then mungbean seeds were grown in sterile media. The surface of mungbean seeds is washed under running water. The seeds are soaked in a mixture of Agrep (fungicide) solution with two drops of 80% tween solution, and incubated for 30 minutes at 120 rpm. The seeds were washed with sterile distilled water, then soaked with 10% chlorox solution with shaker for 15 minutes and washed again with sterile distilled water for three times. The seeds were soaked with 5% chlorox solution with a shaker for 15 minutes, then they were washed with sterile distilled for three times. The last stage, the seeds were soaked in 70% alcohol for one minute and rinsed with sterile distilled. Seeds of sterile mungbeans were grown in agar medium. The seeds were grown for one week and placed in a room with less light, and then the young sprouts were transferred into a sterile container.

The sprouts are immersed into a production suspension that has equalized the turbidity with a Mc. Farland solution ($10^8$ cells/ml) for one hour with 50% dilution. Sprouts soaked with aquades were used as controls. Each treatment repeated six times. Any sprouts that have been immersed in a production suspension are grown on sterile soil media in polybags. The growing sprouts are observed after one week. The parameters observed were the length of sprouts, the number of lateral roots and the wet weight of the plant.

**RESULTS AND DISCUSSION**

The isolation of endophytic bacteria from *Arachis hypogaea*

The isolation results from three location, that is Gunungpati, Pakintelan, and Klipang, earned 22 endophytic bacterial isolates. After examining the bacteria producing IAA, there are 16isolates that have the ability to produce IAA (Table. 1). Based on the characterization result at table 1, describe that the isolates have variation of colony morphology. Most of them are Gram negative
bacteria and the cells morphology are coccus and bacillus. It means that the 16 isolates may be different species or different genus. The further research needed to identify the isolate species.

The ability of endophytic bacteria in producing IAA with invitro process

Variation was found in the abilities of endophytic bacteria in producing IAA, depending on its isolates and the age of cultures as presented in Figure 1.

From the Figure 1, it can be seen that the production of IAA by bacteria is mostly (10 isolates) on the fourth day after incubation. While on isolate GNP1K2 and P1K2 IAA, production increased with increasing culture time. 4 isolates i.e. GNP4K1, K1K2, GNP1K1 and GNP2K2 experienced highest IAA production on the second day. This difference is thought to be due to variations in the type of bacteria and location. The production of IAA by bacteria varies due to environmental factors, growth rates and availability of amino acids and other N sources (Yurnaliza. 2010). The decrease of IAA levels on the fourth or sixth day due to the available nutrients (tryptophan) which has begun to decrease. The use of nutrients in every bacterium varies. In some isolates, it increases in line with incubation time because at the time of incubation in the second day, the enzyme that converts tryptophan to IAA is still low. In line with bacterial growth rate, the enzyme used in conversion of tryptophan to IAA is active enough to produce high IAA (Taghavi et al. 2009). It has been reported that endophytic bacteria produce significant IAA, in B. cereus (Rana et al. 2011.) and P. putida (Jasim et al. 2013). The production rate was found to be maximal in the case of P. putida (ECL5) and the minimum in C. michiganensis (ECL6) in the presence of tryptophan. IAA is the most common plant hormone, which stimulates plant growth and reproduction (Taghavi et al. 2009). IAA produced by bacteria interacts with plants in a variety of pathogenesis and phytocontrol. IAA is a major auxin in plants involved in cell enlargement and division, tissue differentiation, physiological processes (Spaepen et al. 2007). The amount of IAA produced by bacteria plays an important role in the interaction of microbial plants. Plant growth modulation was performed with optimal IAA concentration range.

Table 1. Characteristics of bacteria producing IAA

| Isolate | Size     | Optical characteristic | The colony morphology | Gram | Cells Morphology |
|---------|----------|-------------------------|-----------------------|------|-----------------|
| K1K1    | Moderate | Translucent             | Circular              | Raised | Glisten        | Serrate | - Coccus          |
| AT      | Pinpoint | Translucent             | Circular              | Convex | Smoothly glisten| Entire  | - Bacillus        |
| P1K2    | Moderate | Translucent             | Circular              | Raised | Smoothly glisten| Undulate | - Coccus          |
| DM      | Moderate | Translucent             | Circular              | Raised | Glisten        | Undulate | - Bacillus        |
| GNP2K22 | Moderate | Translucent             | Circular              | Raised | Glisten        | Undulate | - Coccus          |
| DTR     | Small    | Translucent             | Circular              | Raised | Glisten        | Undulate | - Bacillus        |
| P2K3    | Small    | Translucent             | Circular              | Raised | Glisten        | Undulate | + Bacillus        |
| K1K2    | Small    | Translucent             | Circular              | Raised | Glisten        | Undulate | - Coccus          |
| GNP2K2  | Moderate | Translucent             | Circular              | Raised | Glisten        | Undulate | - Bacillus        |
| GNP2K21 | Small    | Translucent             | Circular              | Convex | Smoothly glisten| Entire  | - Bacillus        |
| GNP1K1  | Pinpoint | Translucent             | Circular              | Raised | Glisten        | Undulate | - Bacillus        |
| BPK3    | Moderate | Translucent             | Circular              | Raised | Glisten        | Serrate  | - Coccus          |
| GNP1K2  | Large    | Translucent             | Circular              | Raised | Glisten        | Entire   | - Bacillus        |
| GNP2K1  | Small    | Translucent             | Circular              | Raised | Glisten        | Entire   | - Bacillus        |
| GNP3K1  | Small    | Translucent             | Circular              | Raised | Glisten        | Entire   | - Bacillus        |
| GNP4K1  | Pinpoint | Opaque                  | Circular              | Raised | Glisten        | Undulate | - Bacillus        |

Figure 1. The production of IAA from various endophytic bacteria isolates
The synthesis of IAA by microbes is dependent on the pathway of tryptophan where tryptophan is used as a precursor and different diverse taxonomic and metabolic tissue tissues. Some endophytic microorganisms have the potential to synthesize IAA to increase or stimulate growth when colonization occurs with endophytics (Shi et al. 2009). One of the main contributions of these microorganisms to plant growth is the production of molecules such as auxin (Spaepen et al. 2007). Indole 3 acetic acid (IAA) to auxin can stimulate growth such as cell lengthening and cell division and differentiation (Hasan et al. 2015). IAA-producing bacteria potentially affect the growth process from the amount of IAA into production and tissue sensitivity to IAA concentration changes.

Introduction of IAA-producing endophytic bacteria to mungbean plants
Isolates applied to mungbean plants with high IAA content of 16 isolates obtained were selected 5 isolates, application results to plants can be seen in Table 2.

One way ANOVA analysis results show that IAA-producing bacterial isolates did not affect the length of sprouts but influenced the number of roots. In low concentrations, IAA causes root and shoot elongation, if IAA concentrations are higher, the elongation of shoots and roots becomes inhibited (Moore, 1989). The addition of exogenous IAA has an effect on the increase of IAA concentration in plants causing stunt length inhibition. In contrast to the number of roots, IAA concentrations that exist in plants actually stimulate the formation of lateral roots. IAA bacteria can loosen the cell wall of plants and consequently increase the number of roots that increase exudation that provide additional nutrients to support the growth of bacterial rhizosphere. IAA bacteria stimulate the development of root system of host plants. IAA production of isolates can improve the fitness of microbial plant interactions (Hasan et al. 2015).

Endophytic bacteria do not only generate IAA but also increase the availability of plant nutrients such as nitrogen, phosphate, and other minerals so that plant growth increases. Root is one of the most sensitive organ to IAA fluctuations and is responsible for increasing the number of exogenous IAA useful for primary root elongation process, lateral root formation and adventive root (Ryan et al. 2008) IAA is the major auxin hormone in plants that controls plant growth. Many important physiological processes take place including cell enlargement and cell differentiation. The IAA produced by the bacteria when administered to the plant will have an effect on the sensitivity of the plant tissue.

In conclusion, there were 16 isolates of endophytic bacteria having the ability to produce IAA. The incubation time had an effect on the IAA content produced by bacteria and IAA-producing endophytic bacteria isolate affected the number of lateral roots but it did not affect the length of the sprouts. The largest bacterial isolates stimulating the formation of lateral roots are isolates of DM and K1K1.

### Tabel 2. The effect of some isolates of IAA producers on the growth of root length and number of roots

| Isolat   | Length of sprout | Number of roots |
|----------|------------------|-----------------|
| AT       | 16.025           | 15 bc           |
| DM       | 15.025           | 24.75 a         |
| GNP2K21  | 15.425           | 19.5 ab         |
| AT1K1    | 14.20            | 23.75 a         |
| P2K3     | 14.75            | 19.75 ab        |
| Control  | 14.65            | 13.75 c         |

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Effects of encapsulation matrix on physical properties and germination viability of calcium-alginate encapsulated plbs of *Grammatophyllum scriptum*

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Abstract. Pitoyo A, Anggarwulan E, Ariza I. 2017. Effects of encapsulation matrix on physical properties and germination viability of calcium-alginate encapsulated plbs of *Grammatophyllum scriptum*. Cell Bio Dev 1: 36-40. *Grammatophyllum* is a tropical epiphytic orchid commonly found in the moist areas of South-East Asia. Like most orchid species, the genus comprises species with a very small, micro-size seed mass and lacked endosperm. These plants commonly need an in vitro culture for mass propagation and seed germination. Upon germination, their undeveloped-embryos developed a globular mass cells, a protocorm. Occasionally, a structure similar to protocorm arises from tissue other than an embryo, thereby the term protocorm-like body (plb) were introduced. Here, we develop synthetic seed, hydrogel beads encapsulated *G. scriptum* plbs and possibility to germinate the seed and growth their embedded tissue. The objective of the research was to study the effects of the proportion of encapsulation matrix of *G. scriptum*. B. Synthetic seed made by complexation of sodium alginate with CaCl2 on physical properties and germination of protocorm-like bodies (plbs) embedded inside the hydrogel. The experiment was designed by a single factor-completely randomized design with the treatments of several combinations of Na-alginate/CaCl2 ratios. The result showed that CaCl2 in all concentration except 25 mM formed spherical hydrogel beads in all level concentration of Na-alginate. Alginate in concentration 2% and 3% gave the optimum result represented by maximum germination index 100%. The formation of the new plbs varied among different explants even in single explant. The time of germination of each synthetic seeds was varied from 2 weeks until eight weeks after encapsulation. In conclusion, physical properties have no significant barrier for developing plbs to emergencies through penetration encapsulation matrix.

Keywords: CaCl2, *Grammatophyllum scriptum*, Na-alginate, synseed

INTRODUCTION

Orchidaceae is one of the diverse and widespread families of flowering plant comprised more than 24,000 species and categorized in 800 genera (Fay and Chase 2009). They occupy a wide range of ecological habitat from tropic to the temperate climatic region but exclude sea water and extreme cold environment (Tan et al. 1998). The amazing of their flowers morphology have made them in a dilemmatic position where their benefit was faced with their conservation issues. Numerous orchid species are economically well-known plants in floriculture industries. Unfortunately, over a collection of their native species for illegal trading and their habitat destruction has absolutely made them in thread situation (Kull et al. 2006).

The genus *Grammatophyllum* is a large or giant, tropical epiphytic orchids commonly found in the moist areas of South-East Asia. It members have two distinctive types pseudobulb, that is some species with very large, long stem-like structure and the others a short conical one. *G. scriptum* is a species with sort conical pseudobulbs with two or three oblanceolate leaves placed near the apex. It relative species, *G. speciosum* and *G. papuanum*, are orchid with long stem-like pseudobulb with a linear, acute leaves spread in two rows along the length. The inflorescence about 2 meters, bearing the first apical half insert closely complete flowers and the bottom half occasionally placed some distorted flowers with a wider in position. The flowers 10 cm or more broad. Previous reports indicated that *G. scriptum* was found in many areas in Indonesia, such as Lamedai Nature Reserve, Kolaka, Southeast Sulawesi (Lestari and Santoso 2011). Despite the members of *G. scriptum* easily found in cultivation area or nurseries, their position in the wild has now be classified as rare.

Effective propagation and ex-situ conservation is the two key factors are must be seriously managed to save orchid in nature (Fay 1994; Sarasan et al. 2006). However, their reproductive nature was dependent in association with other organisms. This phenomenon has considered as consequences of their flower structure that influence their pollination biology. In the other hand, seed produced from successful fertilization lacked in the endosperm, so their must co-relation with mycorrhiza to acquisition a nutrient from environmental surrounding for development of small immature embryos.

Plant tissue culture has been familiar in orchids mass propagation because of the low preference of the seeds to germinate and only small number new individual formed through conventional vegetative propagation. Symbiotic dependency with fungal mycorrhiza for germination of their micro-size and lacked endosperm seeds were ignored by culturing them in the aseptic rich-nutrition medium.
Numerous successful attempts in orchid mass propagation via tissue culture have been recorded in some review. A symbiotic germination technique of Cymbidium orchid seeds is discovered by the medium in axenic condition, and subsequent successful attempts (Yam and Arditti 2009). This in vitro technique was also visible for fixation of elite genotype by multiplying somatic tissue and generating embryogenesis to become plantlets. In combination with cryopreservation technique of via tissue culture have been recorded in some review. A

Numerous successful attempted in orchid mass propagation

plbs embryo, callus, or protocorm-like bodies (plbs) could not easily introduce to greenhouse or field because of the different environment inside and outside the bottle. The situation remains problematic for consumer that has no enough background in tissue culture. Thus, we have developed synthetic seed, a plb encapsulated calcium-alginate hydrogel to aid the plantlet to be successful survive in field or greenhouse. Plbs might representatives of somatic embryos in Orchidaceae. Thus successful attempt would highlight the possibility of direct transfer of somatic embryos of orchid into plantation.

MATERIALS AND METHODS

Plant material
Sterile plantlets derived from a symbiotic germination of G. scriptum seeds were used as materials for plbs production. Leaf segments, young shoots, and primary plb were isolated and subcultured in plb induction media. Next fractionated plb aggregates obtained from the induction medium would be used as ‘artificial embryo’ for synseed (synthetic seed).

Plbs induction medium
The basal MS (Murashige and Skoog) medium plus vitamin from Phyto Technology Lab., containing 3% (w/v) sucrose and was used to induced plbs formation. The medium formulation would be called basal-MS medium in next discussions. The pH of the medium was adjusted to 5.6-5.8 using 0.1 N HCl and 0.1 N NaOH prior solified by agar 8 g and sterilized at 121°C at 1 atm for 20 min in an autoclave.

Optimization of encapsulation matrix
Na-alginate (PhytoTechnology Lab.) was used with calcium chloride dihydrate for cross-linked hydrogel formation. Alginate solutions were prepared in various levels by dissolving sodium alginate (2%, 3%, 4%, and 5%) w/v in basal MS plus vitamin and sucrose 3% solution. Calcium chloride solution in various level (25 mM, 50 mM, 75 mM, and 100 mM) were prepared in distilled water.

Encapsulation procedure
Plbs aggregates formed in induction cultures were separated, blot dried, and embedded in the sodium alginate solution. Pipetting dropped each alginate-layering plb into CaCl$_2$.H$_2$O solution. The drops, each containing a single plb, were incubated in a CaCl$_2$.H$_2$O solution for 30 minutes. The solid hydrogel beads formed by complexation of the two encapsulation matrix were recovered by decanting the CaCl$_2$.H$_2$O solution and washing with sterilized de-ionized water. Beads were placed in a glass bottle (5 beads per bottle) with moist cotton, sealed with aluminum foil, and stored at 25°C for 15 days for evaluation.

Data collection and analysis
Germination percentage (%) and the time required for germination were recorded and evaluated for 5 encapsulated plbs each treatment. Statistical analysis was made with completely randomized design (CRD; for a single factor) and factorial CRD (for more than one factor, gelling agents data). Means were evaluated at P ≤ 0.05 level of significance using Duncan’s New Multiple Range Test (DMRT).

RESULTS AND DISCUSSION

plb formation
We used all parts of the plantlet for possibility comparing regeneration capabilities of different sources of explant on the induction of plbs formation. The result suggests that young shoot and previous plb except leaf segments have been successfully regenerated new plbs in basal MS medium lacking plant growth regulator (PGR) (Table 1). However, plb formed by previous plb explant have more numerous compared by young shoot-derived plbs. The number of the new plb formed by previous plb explant were around five compared to 1-2 plbs from shoot explant. The limitation of shoot explant producing plbs confirmed similar resulted from previous studies in same species, even with the supplement of PGR (Lysnandar 2012).

Furthermore, we found that the development stage of the new plbs cannot be synchronized (Figure 1). The formation of the new plbs varied among different explants even in single explant. The first plb start visibly by 2 weeks after subculture and subsequently continue developing new plbs in next days. Thus, four weeks after subculture we got at least three type of plbs based on their development stages i.e. globular plb, plb with new shoot meristem, and plb with developing shoot. The latter explicitly indicate that the capacity of plb to regenerate into plantlet in basal MS medium without exogenously plant hormone. The new plb also apparently varied in size ranging from less than 1mm until larger than 4 mm in diameter. We chose plb with globular stage and 2-3 mm in diameter as candidates for encapsulation to become synthetic seed.

Table 1. Formation of new plbs from different explants

| Sources of explant | Number of plbs | Time of first emerged (as*) |
|--------------------|----------------|----------------------------|
| Young shoot segments | < 2 | 4 weeks |
| Leaf segments | - | - |
| Primary plbs | > 5 | 2 weeks |

Note: *as: after subculture
Assessment of encapsulation matrix on hydrogel properties and development of encapsulated \( plb \)

Synthetic seed which has developed were represented by structure of solid hydrogel encapsulated \( plb \) of \( G. \) scriptum. We used alginate-based hydrogel because of several characteristics of alginic acid which are (i) ability to form gels in the presence of divalent (or multivalent) cations, particularly calcium ions; (ii) biocompatible properties, the matrix has well known in medical and life science researchers; and (iii) ability to fix other materials in the gel (Kakita and Kamishima 2008). This matrix also provides rigidity of the hydrogel bead thus enhanced better protection to the embedded \( plb \) from mechanical damage (Saiprasad 2001). The hydrogel coating-\( plbs \) were formed by cross-linking of calcium ions with alginate ions by mechanism of ion exchange. In this study, complexation of alginate anion from sodium-alginate solution with divalent calcium cation from \( \text{CaCl}_2.2\text{H}_2\text{O} \) solution in the different contraction level provide various degree of physical properties of hydrogel capsules. Based on Figure 3, the concentration of calcium chloride below 25 mM results in unspherish structure of the hydrogel. Concomitantly, All level of sodium alginate solution in combination with appropriate calcium chloride (at least 50 mM) were successful in the formation of solid globular hydrogel calcium alginate.

Since the physical barrier of the hydrogel considered to become significantly influence of the coating \( plbs \), we also test qualitatively the stiffness of the hydrogel by pushing the hydrogel by fingers and scored as described in Table 2. We qualitatively found that the stiffness of the hydrogel would increase coincide with increase in alginate concentration.

**Germination of synthetic seed \( G. \) scriptum**

Germination of the \( plb \) encapsulated-beads, synthetic seed of \( G. \) scriptum (Figure 2), were represented by the emerging of shoot or root penetrating the calcium-alginate capsule (Machii, 1992). Two weeks after encapsulation, several \( plb \) grew and developed shoot which emerged preceded root in penetrating hydrogel layer. Root was formed later after eight weeks of encapsulation. As hydrogel layer rich with micro and macronutrient as well as vitamin and sucrose, this layer representing artificial endosperm which aids embryo during growth and development. Our results suggested the capacity of \( plb \) as superior explant for synthetic seed embryo. Some authors (Lee et al. 2013; Teixeira da Silva and Tanaka 2006) explained that \( plb \) is a representative of somatic embryo among member of Orchidaceae.

Germination percentages of all treatments from various degree of the ratio encapsulation matrix were summarized in Figure 3. Base on the chart, there are variations on germination percentage of 8 weeks old synthetic seed of \( G. \) scriptum. The sodium-alginate solution in concentration of 2% and 3% (w/v) reached maximum capacity to germinate in all level calcium chloride ranging from 25 mM up to 100 mM. However, germinations were reduced in treatment of 4% and 5% sodium-alginate solution in combination with
Figure 2. Germination of synthetic seed *G. scriptum*. All combination of encapsulation matrix gave positive germination. The combination are: A. Alg 2% Cl 25; B. Alg 2% Cl 50; C. Alg 2% Cl 75; D. Alg 2% Cl 100; E. Alg 3% Cl 25; F. Alg 3% Cl 50; G. Alg 3% Cl 75; H. Alg 3% Cl 100; I. Alg 4% Cl 25; J. Alg 4% Cl 50; K. Alg 4% Cl 75; L. Alg 4% Cl 100; M. Alg 5% Cl 25; N. Alg 5% Cl 50; O. Alg 5% Cl 75; P. Alg 5% Cl 100.

Figure 3. Germination percentage of 8 weeks old synthetic seed with variation in the ratio of encapsulation matrix. Horizontal axis is representative of concentration sodium-alginate solution. Bar legends is representative of CaCl₂·2H₂O concentration.

calcium-chloride in several level concentrations. These results suggest that sodium-alginate in concentration ranging 2-3% were recommended for synthetic seed formation. Previous reports supported our finding that concentration which superior for developing synthetic seed of *three orchid genera* (Saiprasad and Polisetty 2003) was 3% sodium-alginate in combination with 75 or 100 mM CaCl₂.

Furthermore, we found that the time of germination of each synthetic seeds was varied from 2 weeks until 8 weeks after encapsulation. This phenomenon was predicted due to variation in developmental state of the *plb*. Previous reviewed (Sharma et al. 2013) emphasize the importance to synchronize high-quality explant for mass production synthetic seed for industrial application. Somatic embryos which represented by *plb* in Orchidaceae were apparently remained problematic in synchronous their developmental state to achieved industrial application of the synthetic seed.

In conclusion, our research indicated that ratio of encapsulation matrix gave varied physical properties of hydrogel beads calcium-alginate, but they have no significant barrier for developing *plb* to emergencies through penetration encapsulation matrix.

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Goramy spermatozoa quality after sub-zero freezing: The role of coconut water as the cryoprotectant
ABINAWANTO, PRAMITA EKA PUTRI

1-5

Electrical conductivity for seed vigor test in sorghum (Sorghum bicolor)
KHODRATIEN FATONAH, IRFAN SULIANSYAH, NALWIDA ROZEN

6-12

Effects of pruning on growth and yield of cucumber (Cucumis sativus) Mercy variety in the acid soil of North Kalimantan, Indonesia
MARDHIANA, ANKARDIANSYAH PANDU PRADANA, MUH. ADIWENA, KARTINA, DWI SANTOSO, RIZZA WIJAYA, ANAS MALIKI

13-17

Microdosing technology of fertilizer for sorghum production at Shambat, Sudan
MONIRAH BABIKER MOHAMED ARBAB, YASSIN MOHMAD IBRAHIM DAGASH

18-22

Toxicity of Randia nilotica fruit extract on Schistosoma mansoni, Biomphalaria pfeifferi and Bulinus truncatus
ALADDIN YOUSIF ELTEIB EBODI, MOHAMMED MAGZOUB AHMED

23-30

The endophytic bacteria producing IAA (indole acetic acid) in Arachis hypogaea
LINA HERLINA, KRISPINUS KEDATI PUKAN, DEWI MUSTIKANINGTYAS

31-35

Effects of encapsulation matrix on physical properties and germination viability of calcium-alginate encapsulated plbs of Grammatophyllum scriptum
ARI PITAYO, ENDANG ANGGARWULAN, IKA ARIZA

36-40