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Chapter in book:

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Thesis, Dissertation:

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

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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 study's objective is to find the best combination among concentrations of coconut water on 0%, 21%, 23%, 25%, 27%, and 29%, respectively, combined with 5% of glycerol for maintaining good spermatozoa motility, viability and minimizing spermatozoa abnormality. One part of semen/sperm was mixed with three parts of solvent (5% glycerol + fish ringer + coconut water) and equilibrated at 4°C for 45 min. The diluted sperm were then frozen at -34 °C for 48 h. Finally, cryopreserved sperms were thawed at 30 °C for 3-5 min. Spermatozoa quality was evaluated before and after sub-zero freezing. Based on the Kruskal-Wallis test, spermatozoa motility and viability were higher than control ($P<0.05$), while the spermatozoa abnormality was not significantly different compared to control ($P>0.05$). Twenty-five percent of coconut water combined with 5% of glycerol was 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 Ministry of Maritime Affairs and Fisheries, Republic of Indonesia data, Indonesian fish consumption increased by 5% in 2013 (KKP 2013). *Osphronemus goramy* (Lacepede 1801) is Indonesia's most popular and commercial freshwater fish (Saparinto 2008). Goramy fish production in Indonesia was about 280,079,000 kg in 2000 (Dunia Ikan 2008). However, nowadays, goramy fish is cultured conventionally because of the limited male and female parents. Therefore, they usually put male and female parents together in one pond for a long time, which will be caused inbreeding. Hence, the genetic materials' quality was reduced (Alam et al. 2002).

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

Cryoprotectants and extenders are two important solutions to protect cells from ice crystals during freezing (Muchlisin 2005). Based on Jamieson (1991), glycerol, methanol, and dimethyl sulphoxide (DMSO) are common intracellular cryoprotectants used for cryopreserved fish spermatozoa. On the other hand, milk, egg yolk, and sugar were used as extracellular cryoprotectants (Jamieson 1991). According to Routray et al. (2007), the extender is also needed to support cells with additional nutrition and maintain osmotic pressure during cryopreservation. Saline solution and fish Ringer solution are two extenders usually used 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), *Osteochiius hasseltii* (Sunarma et al. 2007), *Cyprinus carpio* (Akcay 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 the 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 a cryoprotectant for goramy spermatozoa. However, the effect of coconut water in many variations 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 a one-4,000-L square concrete cement pond until they attained 2.5-3.5 kg in the indoor Aquatic Biology laboratory, Biology Department, Faculty of Mathematics and Natural Sciences, Universitas Indonesia, Depok, West Java Province. The fishes were grouped into 6 fishes and were stocked in a 7-square concrete cement fish pond (1,000-L). The fish pond was equipped with a closed re-circulation system and a black plastic mesh lid to minimize disturbances and prevent fish from jumping out. Six experimental groups based on coconut water concentration were assigned four replications in a completely randomized design (Table 1). Fishes were fed with the commercial diet and leaf of *Allocasia macrorrhiza* two times daily *ad libitum* at 08:00 and 17:00.

Fish ringer preparation

Fish ringer solution was prepared according to the method of Ginzburg (1972). First, a fish ringer stock solution was prepared by dissolving 3.25 g NaCl, 0.125 g KCl, 0.175 g $CaCl_2.2H_2O$, and 0.1 g $NaHCO_3$ 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 $Na_2HPO_4.2H_2O$ with aquabidest up to 200 mLl, and by dissolving 4.08 g KH_2PO_4 with aquabidest up to 200 mLl. $Na_2HPO_4.2H_2O$ solution was then added to KH_2PO_4 solution until the pH reached 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 at 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 8-10 hours after being injected intra-muscularly with GnRH-analog (Ovaprim Syndel) at a single dose of 0.2 ml/kg body weight according to the modification method of Sunarma et al. (2007) and was put in 1.5 mL of cryotube.

Table 1. Composition of Semen/sperm, Fish ringer, Coconut Water (CW), and glycerol (G) of the experimental group

Composition	Experimental group					
	C	21% CW	23% CW	25% CW	27% CW	29% CW
Semen/sperm (µL)	50	50	50	50	50	50
Fish finger solution (µL)	140	98	94	90	86	82
Coconut water/ CW (µL)	0	42	46	50	54	58
5% glycerol/G (µL)	10	10	10	10	10	10

Note: * C= control group (0% CW); ** 21% CW, 23% CW, 25% CW, 27% CW, 29% CW = treatment group

Semen/sperm dilution

According to Akcay (2004), the ratio between the ejaculated semen/sperm and the solvent was 1:3. The sperm was added to the solvent solution in the cryotube. The composition of each component of the solvent solution and the ejaculated sperm is seen in Table 1.

Semen/sperm equilibration

The diluted sperm was then equilibrated at 4 °C for 45 minutes (Akcay et al., 2004; Bozkurt et al., 2005).

Semen/sperm freezing

The diluted sperm was frozen at -34°C for 48 h (Huang et al. 2004).

Semen/sperm thawing

The frozen sperm was incubated at 30°C for 3-5 min. (Akcay et al. 2004 and Bozkurt et al. 2005).

Semen/sperm evaluation and spermatozoa analysis

Visual observation was carried out on the fresh semen/sperm color, and the cryotube with a scale measured sperm volume. The standardized pH paper (pH range 5-10) measured sperm/semen pH. The spermatozoa viability, abnormality, and motility were observed under a trinocular microscope (Boeco) equipped with the digital eyepiece camera (MDCE-5a). This microscope was also connected to the computer equipped with 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 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 a 0.05 level of significance.

RESULTS AND DISCUSSION

Results

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

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

The percentage of spermatozoa motiliy 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 the Kruskal-Wallis test, there was a significant effect ($P<0.05$) of various concentrations of coconut water on spermatozoa viability and motility, respectively, to days after sub-zero freezing. On the other hand, there was no significant effect of coconut water on reducing spermatozoa abnormality ($P>0.05$) compared to the control (Table 3). According to the Dunnet test, 25% coconut water concentration showed the highest percentage of spermatozoa viability (82±1.86)% and motility (80.36±1.54)%, respectively.

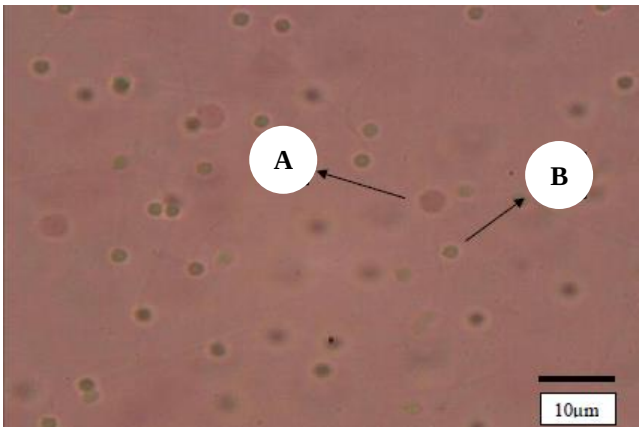

Figure 1. Spermatozoa non-viable (A); spermatozoa viable (B); 10x40 162x95mm (96 x 96 DPI)

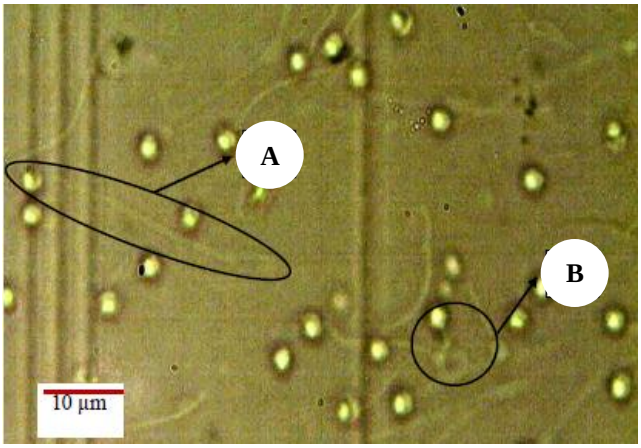

Figure 2. Normal spermatozoa (A); Round tail spermatozoa (B); 10x40 162x204 mm^2 (96 x 96 DPI)

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^a	75±3.36^b	79±2.22^{bc}	82±1.86^c	80±1.47^{bc}	77±1.96^{bc}
Abnormality (%)	11±1.83^a	11±1.41^a	12±1.04^a	10±1.03^a	11±0.83^a	11±0.75^a
Motility (%)	71.42±4.01^a	72.22±2.96^a	77.39±2.26^{bc}	80.36±1.54^b	77.13±2.59^c	74.09±1.98^c

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 to have the highest percentage of spermatozoa motility (80.36±1.54)%, two days after sub-zero freezing. This finding was similar to the previous study (Horvarth 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%; Akcay et al. 2004), *Osteochiius 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 statistically significantly different compared with other treatment groups and control. However, this finding was higher than previously 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). On the other hand, post-thaw viability in this study was lower than in 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 another treatment group of glycerol concentration and control group, although those results were not significantly different statistically. Our previous study showed a higher spermatozoa abnormality (29%; Abinawanto et al. 2011) when using the combination of 13% DMSO + 189M extender. Post-thaw abnormality in *Barbonymus gonionotus* spermatozoa was higher (45%) when preserved in the combination of 6% glucose + 10% methanol (Abinawanto et al. 2009). However, the post-thaw abnormality showed nearly similar (14%) when the spermatozoa of *Barbonymus gonionotus were* protected by the combination of 13% of egg yolk + 10% of methanol (Abinawanto et al. 2013).

Either treatment groups or control was visually shown as viable spermatozoa, motile spermatozoa, and abnormal spermatozoa. However, the percentage of spermatozoa viability, motility, and abnormality differed among treatment groups or between control and treatment groups. Therefore, 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 is also an indicator of the optimum condition of a percentage of glycerol as the cryoprotectant.

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

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REFERENCES

Abinawanto A, Fadhillah, Lestari R. 2009. The effect of glucose in various concentrations on sperm quality of *Barbonymus gonionotus* (Bleeker, 1850) 24 hours post-cryopreservation. J Rep Dev. 55 Suppl. DOI: 10.14882/jrds.102.0.224.0

Abinawanto, Bayu MD, Lestari R., Sunarma A. 2011. Spermatozoa quality of goramy fish, *Osphronemus goramy* Lacepede, 1801 twenty four hours post-cryopreservation: The role of dimethyl sulfoxide (DMSO) as a cryoprotectant. Biota 16:10-15.

Abinawanto, Anindita I, Lestari R. 2012b. Cryopreservation of spermatozoa of *Osphronemus goramy* fish using skim milk. Inter J Eng Innov Tech 2 (5):62-64.

Abinawanto, Nurman K, Lestari R. 2012a. The effect of sucrose on sperm quality of goramy fish, *Osphronemus goramy* Lacepede, 1801 two days post-cryopreservation. J Agric Sci Tech B2: 204-207.

Abinawanto, Rahayu S, Lestari R. 2013. Cryopreservation of Java Barb (*Barbonymus gonionotus*) spermatozoa using egg yolk as a cryoprotectant. Global Vet 10 (3): 318-321.

Akcay E, Bozkurt Y, Secer S, Tekin N. 2004. Cryopreservation of mirror carp semen. Turkey J Vet Animal Sci 28:837-843.

Alam MA, Akanda MSA, Alam S. 2002. Comparison of genetic variability between a hatchery and a river population of Rohu (*Labeo rohita*) by allozyme electrophoresis. Pakistan J Biol Sci 5 (9):959-961.

Bozkurt Y, Akcay E, Tekin N, Secer S. 2005. Effect of freezing techniques, extenders, and cryoprotectants on the fertilization rate of frozen rainbow trout (*Oncorhyncus mykiss*) sperm. Israeli J Aqua-Bamidgeh 57: 1-6.

Chao NH, Chao WC, Liu KC, Liao IC. 1987 The properties of tilapia sperm and its cryopreservation. J Fish Biol 30 (2):107-118.

Christensen JM, Tiersch TR. 2005. Cryopreservation of channel catfish sperm: effects of Cryoprotectant exposure time, cooling rate, thawing conditions, and male-to-male variation. Theriogenology 63 (8): 2103-2112.

Fickle J, Asja W, Arne L. 2007. Semen cryopreservation and the conservation of endangered species. Europ J Wild Res 53 (2): 81-89.

Ginzburg AS. 1972. Fertilization in fishes and the problem of polyspermy. Israel Program for Scientific Translation, Jerusalem.

Harvey B, Kelley NR, Ashwood-Smith M.J. 1982 Cryopreservation of zebrafish spermatozoa using methanol. Canada J Zool 60 (8): 1867-1870.

Harvey B. 1983 Cryopreservation of *Sarotherodon mossambicus* spermatozoa. Aquaculture 32: 313-320.

Horton HF, OttoAG. 1976 Cryopreservation of fish spermatozoa and ova. J Fish Res Board Canadian 32 (3-4): 995-1000.

Horvath A, Miskolczi E., Urbanyi B. 2003. Cryopreservation of common carp sperm. Aqua Liv Res 16: 457-460.

Horvath A. Urbanyi B. 2000. The effect of cryoprotectans on the motility and fertilizing capacity of cryopreserved African catfish, *Clarias gariepinus* (Burchell 1882) sperm. Aqua Res 31: 317-324.

Huang C, Dong Q, Walter RB, Tiersch TR. 2004. Sperm cryopreservation of green swordtail *Xiphophorus helleri*, a fish internal fertilization. Cryobiology 48 (3): 295-308.

Kang KH, Kho KH, Chen ZT, Kim JM., Zhang ZF. 2004. Cryopreservation of file fish (*Thamnaconus septentrionalis* Gunther, 1877) sperm. Aquacult Res 35: 1429-1433.

Kusuda S, Koide N, Kawamula H., Teranishi JT, Nakajima I., Yamaha E, Arai K., Ohta H. 2005 Cryopreservation diluents for spermatozoa of Sakhalin taimen *Hucho perryi*. Fish Sci 71: 293-298.

Muchlisin ZA, Hashim R., Chong ASC. 2004. Preliminary study on cryopreservation of tropical bagrid catfish (*Mystus* nemurus)

spermatozoa: the effect of extender and cryoprotectant on the motility after short-term storage. Theriogenology 62 (1-2): 25-34..

Park C, Chapman FA.. 2005. An extender solution for the short-term storage of Sturgeon semen. North Amer J Aqua 67 (1): 52-57.

Perchec G, Jeulin C, Cosson J, Andre F, Billard R. 1995. Relationship between sperm ATP content and motility of carp spermatozoa. J Cell Sci 108: 747-753.

Rurangwa E, Kime DE, Ollevier F, Nash JP. 2004. Review article: The measurement of sperm motility and factors affecting sperm quality in cultured fish. Aquaculture 234: 1-28.

Salisbury GW, Van De Mark NL. 1985. Reproductive physiology and induced breeding. W.H. Freeman and Co., San Francisco

Sunarma A., Hastuti DW, Sistina Y. 2007 The use of honey extender combined with different cryoprotectants on the preservation of sperm nilem fish (Indonesian Sharkminnow, *Osteochiius hasseltii* Valenciennes, 1842). Konferensi Aquaculture Indonesia 2007, Surabaya, 5-7 Juni, 2007. Masyarakat Akuakultur Indonesia, Surabaya [Indonesian]

Urbanyi B, Horvarth A, Varga Z, Horvarth L. 1999. Effect of extenders on sperm cryopreservation of African catfish, *Clarias gariepinus* (Burchell 1882). Aqua Res 30: 145-151.

WHO [World Health Organization]. 2010. WHO laboratory manual for the examination of human semen and sperm-cervical mucus interaction. 5th ed. World Health Organization, Geneva, Switzerland

Withler FC. 1982 Cryopreservation of spermatozoa of some freshwater fishes cultured in South and Southeast Asia. Aquaculture 26 (3-4): 395-398.

Zar JH. 1974. Biostatiscal analysis. Prentice-Hall Inc. London.

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

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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 an electrical conductivity test method for seed vigor test in sorghum, to recognize the relationship between the electrical conductivity test and potassium leakage, and to recognize the relationship between the electrical conductivity test and another variable on seed vigor. This study has two-step experiments. The objective of experiment I was to determine accurate combinations of water volume and amount of seed of the electrical conductivity test for seed vigor test in sorghum. A completely Randomized Design of 15 seed lots with 3 replications was used to determine the electrical conductivity method with different vigor. Experiment II was to test the electrical conductivity method of 21 seed lots of sorghum without an accelerated aging test, and 21 seed lots of sorghum were given an accelerated aging test. A completely Randomized Design of 21 seed lots with 3 replications was used in Experiment II. The variable observed was; 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 of seed sorghum (50 and 75 seed count). The result of these experiments showed that the electrical conductivity test method with 150 ml water volume and 75 seed count was accurate and suitable for the sorghum seed vigor test; the electrical conductivity test showed a positive correlation with potassium leakage; and the 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 vigor test

INTRODUCTION

The seed vigor test is a more sensitive index of seed quality than the germination test; any events that precede germination loss could serve as vigor tests. Therefore, seed vigor testing has increased the importance of ranking seed lots according to their physiological potential. One of the main concerns regarding seed vigor evaluation is obtaining reliable results 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 measure 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 seeds' development before physiological maturity, seed desiccation before harvest, and imbibition before germination (ISTA 1995). As a seed rehydrates during early imbibition, the ability of its cellular membranes to reorganize and repair any damage that may have occurred will influence the extent 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 damage to a greater extend than lower vigor seeds.

Consequently, electrolyte leakages measured from high vigor seeds are less than those from low vigor seeds. In addition, low vigor seeds have been shown to have decreased membrane integrity due to storage deterioration and mechanical injury. However, no suggested or recommended procedures for electrical conductivity sorghum seed vigor tests are available in the handbooks of vigor tests from the International Seed Testing Association (AOSA 1983; ISTA 1995).

The research reported here is aimed at; (i).obtaining an electrical conductivity test method for seed vigor test in sorghum. (ii) recognizing the relationship between the electrical conductivity test and potassium leakage, and (iii) recognizing the relationship between the electrical conductivity test and another variable on seed vigor.

MATERIALS AND METHODS

The experiments were carried out from February to July 2015. All the laboratory and field emergence tests were conducted at the Indonesian Center for Seed Testing and Quality of Food Crops and Horticulture Research and Development, Cimanggis, Depok, West Java, Indonesia. In addition, potassium leakage was conducted at the Laboratory of Soil Department, Bogor Agriculture University, West Java, Indonesia.

Seed water content

Seed water content was determined at 130°C for 2 hours in duplicate samples of ground 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 four and teen days after seeding, evaluated for normal development, and results were expressed as percent normal seedlings for each lot.

Germination first count

The seeds were performed simultaneously with the germination test, and the percent of normal seedlings was recorded four days after seeding.

Speed of Germination

The normal seedlings are evaluated daily, starting from the 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 methods (Table 1) and held in a germinator at 20°C. After 24 hours, the electrical conductivity of leachates was determined. In addition, each replication's electrical conductivity of leachates was measured using a conductivity meter (Type Cond 330i), and conductivity per gram of seed weight was calculated ($\mu S\ cm^{-1}g^{-1}$) and recorded.

Potassium leakage

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

Table 1. Eight combinations of 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 a 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

A completely Randomized Design of 15 seed lots with 3 replications were used to determine the electrical conductivity method with different vigor levels ranging from 39-94% as determined by standard germination. In addition, a completely Randomized Design of 21 seed lots with 3 replications was used to explore the relationship between the electrical conductivity test and other variable tests on the physiological quality of seed. The quality of selected seed lots was determined by standard germination, field emergence, first count, germination speed, conductivity, and potassium leachate. Analysis of variances was 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 has 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 lots of commercially accepted germination.

Although germination and vigor are closely associated, seed vigor is highly complex compared to standard germination. It provides additional information to assist in the differentiation of the physiological potential of seed lots, seed storability, and potential field performance. Practical vigor tests should 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 in most seed testing laboratories with minimum equipment and personnel training expenditure. Physically injured and mechanically damaged seeds can influence the results.

Furthermore, 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 methods, and several vigor tests. Correlation coefficients among all the tests were observed.

A highly significant correlation ($r = 0.90$) between standard germination and field emergence 21 days test is

shown in Table 2. A recent study (Baskin et al. 1993) showed a relationship between standard germination tests 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 conditions, the field emergence percent ranged from 69% to 97%, with a mean of 86.5%, and a highly significant correlation (r = 0.825**) was observed with the standard germination test. Under unfavorable field conditions (cold wet 69 soil condition), the mean-field emergence percent decreased to 65.9%. A low correlation coefficient (r = 0.501**) was observed between the 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 combinations of the electrical conductivity method, the highest correlations with field emergence at 21 days (r = -0.78), first count (r = -0.79), and standard germination (r = -0.89) determine by the electrical conductivity method with 150 ml deionized water and 75 seed count. According to the conductivity test with 150 ml deionized water and 75 seed count, potassium leakage also showed the 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 a preliminary recommendation for sorghum seed vigor test.

Negative correlations were always observed between conductivity, standard germination, and other seed vigor tests. That is because low germination and vigor seeds give a high amount of leakage of electrolytes (measured in μS cm-1g-1); in contrast, high vigor seeds give a 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). Furthermore, the conductivity test is rapid and simple and does not need personal skills 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, respiratory germination metabolism, and leaching in cotton seeds. Electron microscopy confirmed the deterioration of membranes due to weathering by cotyledon's lipids and proteins bodies and correlated well with conductivity measurements.

The electrical conductivity test is based on the measurement of resistance to the flow of an electric current imposed upon the seed steep water. Resistance is a function of the number of electrolytes in the 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 presumably indicates 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 combinations electrical conductivity method of 15 seed lots of sorghum

	IV	*DB*	*DT-7*	*DT-14*	*DT-21*	*50 50ml*	*75 50ml*	*50 100ml*	*75 100ml*	*50 150ml*	*75 150ml*	*50 200ml*	*75 200ml*	*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	-0.76	-0.85	-0.70	-0.72	-0.73	0.98	0.99	0.99	0.99	1				
75-150ml	**-0.79**	**-0.89**	-0.74	-0.77	**-0.78**	0.96	0.98	0.97	0.99	0.99	1			
50-200ml	-0.75	-0.88	-0.74	-0.77	-0.77	0.96	0.98	0.97	0.99	0.99	0.99	1		
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	1	
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	1

Potassium is 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 as the electrical conductivity test, producing results in a considerably shorter time. In addition, it focuses on a specific ion, while the electrical conductivity test evaluates a set of electrolytes released (Miguel and Filho 2002. Potassium leachate and electrical conductivity tests yielded similar results in ranking seed lots' physiological potential. However, based on the same principle, the electrical conductivity test provides results only after a 24 h imbibitions period when performed under the current procedure.

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

Our results showed that the electrical conductivity method with 150 ml deionized water and 75 seed count could predict standard germination and field emergence and could be used as a seed vigor test is generally recommended for sorghum seeds. But before seed agencies or sorghum seed centers standardize the conductivity test, the electrical conductivity method must be verified, especially for different sorghum types.

The highest correlation coefficients (r) showed among standard germination, first count, field emergence of 21 days obtained, potassium leakage, and electrical conductivity method 150 ml deionized water and 75 seed count, therefore electrical conductivity method 150 ml deionized water and 75 seed count were recommended for seed vigor tests in sorghum. Moreover, 21seed lots of 15 sorghum varieties were used in the experiment to verify the recommendations made from the results.

Seed sources and quality of 21lots determined by standard germination, first count, field emergence of 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 a mean of 75.39 %. The standard germination showed very highly significant differences among seed lots. Highly significant differences among 21 seed lots were also observed in the first count, field emergence, germination speed, 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

Lot Benih		Lama simpan	DB	IV	DT 21	KCT	DHL	ION K
Varietas	No	(bulan)	(%)	(%)	(%)	(%/etmal)	(µS/cm.g)	(ppm)
BMR P-3-5	21	0 - 6	56.83	44.83	38.67	16.05	12.27	889.47
BMR P-3-4	20	0 - 6	60.33	54.83	49.33	17.02	13.56	1,226.21
BMR P-3-3	19	0 - 6	64.00	50.83	37.33	17.83	10.72	684.61
KD4	15	0 - 6	73.00	66.50	82.00	18.39	17.04	886.50
BMR P-3-2	18	0 - 6	77.83	75.50	49.33	24.36	10.92	691.96
Samurai 1	4	7 - 12	39.83	36.67	61.33	11.49	24.69	1,640.65
Kawali 2014	14	7 - 12	72.50	68.00	92.00	24.42	14.30	620.73
Numbu 2014	10	7 - 12	73.83	72.17	88.67	17.83	9.48	471.28
Samurai 2	5	7 - 12	81.83	67.17	90.67	19.89	11.39	687.58
Numbu Freezer 2014	6	7 - 12	83.50	61.00	88.00	24.05	10.14	441.95
Super 2-2014	12	7 - 12	88.17	87.67	97.33	29.12	10.12	652.60
Pahat 2014	3	7 - 12	88.83	70.00	92.67	18.42	10.36	796.52
Super 1-2014	2	7 - 12	89.67	85.00	92.00	28.29	7.24	482.70
Tongkol Jantung	8	7 - 12	93.50	77.33	98.67	26.29	11.47	648.78
Telaga Bodas	7	7 - 12	94.00	74.83	100.00	23.98	3.87	227.46
Kawali 2013	13	13 - 18	84.50	66.83	87.33	23.29	9.88	438.73
Numbu 2013	9	13 - 18	86.00	81.67	82.67	25.11	6.78	423.50
Super 1-2013	1	13 - 18	89.00	86.50	94.00	27.80	7.33	443.13
Super 2-2013	11	13 - 18	90.50	88.17	94.00	28.73	8.89	555.96
Pahat 2010	16	> 24	44.17	29.67	44.67	17.78	24.76	2,191.98
Durra 2010	17	> 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	2191.98
Min			39.83	29.67	37.33	11.49	3.87	227.46
F test			**	**	**	**	**	**
CV (%)			3.92	5.29	8.08	3.64	7.31	11.04

Note: ** = Significant difference at $p < 0.01$.

Similar correlations among all tests found in the above experiment to determine the electrical conductivity method were also observed to verify the recommendations from the above results. Correlation coefficients among the first count, standard germination, field emergence of 21 days, germination speed, 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 a lower correlation was observed between the electrical conductivity test with field emergence (r = -0.53) and speed of germination (r = -0.66).

All test results were compared in an accelerated aging treatment test to survey the possibility of alternative sorghum seed vigor tests. In addition, this test provided information comparable to the other vigor tests performed between sorghum seed given accelerated aging treatment and seed sorghum as control.

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

After the accelerated aging test, the germination percentages ranged from 4.50 to 92.33%, and the germination percentage mean was 61.71%. After the accelerated aging test, the germination percentages showed highly significant differences among seed lots (Table 6). Highly significant differences among 21 seed lots were also observed in the first count, field emergence, germination speed, conductivity test and potassium leakage results.

An accelerated aging test was developed by (Delouche and Baskin 1973) to measure seed storability and evaluate vigor. The technique involved the exposure of seeds to adverse temperatures (40-45ºC) and 100% RH for varying lengths of time, followed by a regular germination test. As a result, 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, the decline in seed vigor, respiration rate, phosphatase activity, and sugar content accompanied by a complete decline of alpha-amylase activity are noticeable. The concentration, number of amino acids, and RNA and DNA contents also show a similar reduction with higher RNAse activity. Moreover, a rise in respiration rate, phosphatase activity, and sugar content accompanied by a complete decline in alpha-amylase activity and RNA, DNA, and protein content were noticeable in rice during seed deterioration (Zhoe et al. 2002).

Conductivity tests can predict field emergence and standard germination. The electrical conductivity test has been proven to indicate seed vigor in various crop species. In addition, it has been successfully related to field emergence and stands establishment. Analysis of linear regression was used to estimate field emergence and standard germination (Table 6).

In conclusion, based on the experiments and data collected from all tests, the following conclusions can be drawn: Electrical conductivity test method with 150 ml water volume and 75 seeds count was accurate and suitable for the sorghum seed vigor test; the electrical conductivity test showed a 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 by storage period and minimum to maximum percentages of standard germination test

Lot Benih		Lama simpan	DBAA	IVAA	DT 21AA	KCTAA	DHLAA	ION KAA
Varietas	No	(bulan)	(%)	(%)	(%)	(%/etmal)	(µS/cm.g)	(ppm)
BMR P-3-4	20	0 - 6	38.33	23.17	34.67	9.11	13.55	783.81
BMR P-3-5	21	0 - 6	38.67	30.17	44.00	9.49	11.95	674.78
BMR P-3-2	18	0 - 6	46.83	32.83	49.67	11.21	12.50	669.87
BMR P-3-3	19	0 - 6	48.33	33.83	48.00	11.80	9.13	553.44
KD4	15	0 - 6	56.33	53.67	48.67	17.89	16.07	979.29
Samurai 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
Kawali 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
Samurai 2	5	7 - 12	71.17	56.83	48.00	21.32	8.96	723.14
Super 2-2014	12	7 - 12	79.17	63.00	86.00	26.22	9.38	560.81
Super 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
Kawali 2013	13	13 - 18	74.67	66.33	28.00	20.32	7.12	551.95
Super 2-2013	11	13 - 18	81.33	79.00	77.33	22.70	9.64	587.39
Super 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

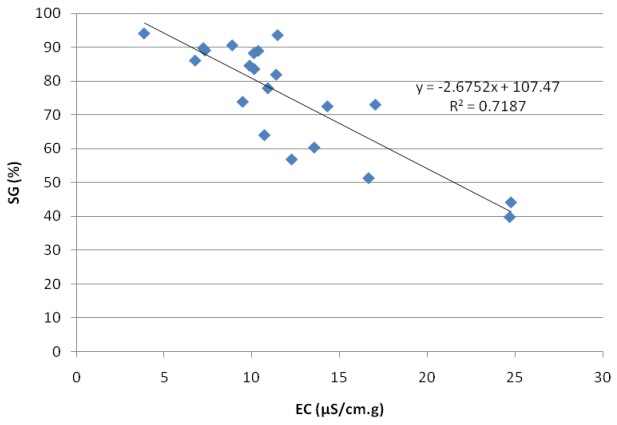

Figure 1. Linear regression between electrical conductivity (EC) and standard germination (SG)

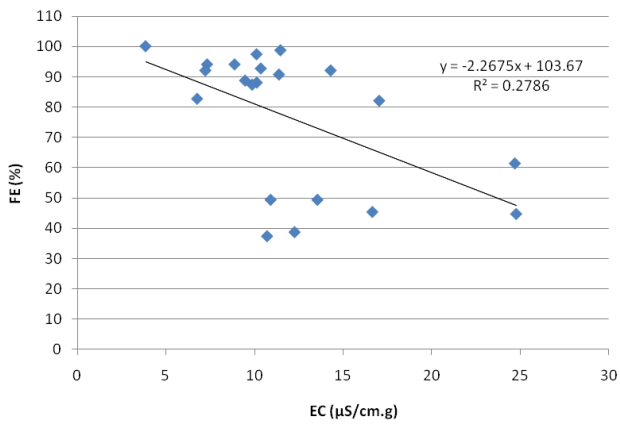

Figure 2. Linear regression between electrical conductivity (EC) and field emergence (FE)

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

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REFERENCES

Abdul-Baki, AA, Baker JE. 1973. Are changes in cellular organelles or membranes related to vigor loss in seeds. Seed Sci Technol 1: 89-125.

AOSA [Association of Official Seed Analysts]. 1983. Seed Vigor Testing Handbook. Contribution No. 32. Association of Official Seed Analysts. Lincon, NE., USA.

AOSA [Association of Official Seed Analysts]. 2014. AOSA Rules for Testing Seeds. Vol. 1-4. Association of Official Seed Analysts, Washington, DC.

Baskin CC, Paliwal S, Delouch JC. 1993. Estimating Field Emergence of Grain Sorghum. MS. Bulletin No. 996. Office of Agricultural Communications, Division of Agriculture Forestry and Veterinary Medicine,Mississippi Agricultural & Forestry Experiment Station. Mississippi State University, USA.

Delouche JC, Baskin CC. 1973. Accelerated aging techniques for predicting the relative storability of seed lots. Seed Sci Technol 1: 427-452.

Hampton JG, TeKrony DM. 1995. Handbook of Vigour Test Methods. International Seed Testing Association, Zurich, Switzerland. 117p.

ISTA [International Seed Testing Association]. 1995. Handbook of Vigour Test Methods. 3rd edition. Internaitonal Seed Testing Association. Zurich, Switzerland.

ISTA [International Seed Testing Association]. 2011. Seed Science and Technology. International Rules for Seed Testing. Zurich, Switzerland.

Lamarca EV, Barbedo CJ. 2014. Methodology of the tetrazolium test for assessing the viability of seeds of *Eugenia brasiliensis* Lam., *Eugenia uniflora* L. and *Eugenia pyriformis* Cambess. J Seed Sci 36 (4): 427-434.

McDonald MB. 1999. Seed deterioration: physiology, repair and assessment. Seed Sci Technol 27: 177-237.

Miguel MVC, Filho M. 2002. Potassium leakage and maize seed physiological potential. Sci Agric 59 (2): 315-319.

Pandey DK. 1992. Conductivity testing of seeds. In Modern Methods of Plant Analyses. New Series 14:273-299.

Ramos KMO, Matos JMM, Martins RCC, Martins IS. 2012. Electrical conductivity testing as applied to the assessment of freshly collected *Kielmeyera coriacea* Mart. Seeds. ISRN Agron. DOI: 10.5402/2012/378139

Szemruch C, Del Longo O, Ferrari L, Renteria S, Murcia M, Cantamutto M, Rondanini D. 2015. Ranges of vigor based on the electrical conductivity test in dehulled sunflower seeds. Res J Seed Sci 8 (1): 12-21.

Woodstock LW, Furman K, Leffler HR. 1985. Relationship between weathering deterioration and germination, respiratory metabolism, and mineral leaching from cotton seeds. Crop Sci 25: 459-466

Zhoe Z, Robards K, Helliwell S, Blanchard C. 2002. Ageing of stored rice: Change in chemical and physical attributes. J Cereal Sci 35: 65-68.

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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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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 reached 20 tons ha^{-1}; 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 without pruning (P0), prunings of the shoots 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), also lower 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 the P1 treatment, with a 4.93% difference compared to the 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 the P1 treatment (11.39%) was higher than the 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 the Cucurbitaceae family commonly planted by farmers in Indonesia. Cucumber cultivation is widespread worldwide, both in tropical and subtropical areas. Cucumber plants in Indonesia are grown in lowlands to highlands. The central regions of the cucumber cultivation area in Indonesia are West Java, East Java, Central Java, Aceh, and Bengkulu. DNA sequencing found that melon and cucumber are of 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 yearly. From 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 conditions affect plant performance (Crawley 2009). Therefore, the proper cultivation techniques as a cultural practice must be done in Indonesia to increase cucumber production.

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

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

Cucumber cultivation needs to be developed appropriately and sustainably. Therefore, pruning is needed to increase the growth and yield of cucumbers. Therefore, the pruning treatment experiment is expected to increase the growth and yield of cucumbers. Therefore, 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 without pruning (P0), the 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 were replicated five times so that there were 20 units of the 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 using a hoe. The planting process was done using a dough tool with a depth of 3-5 cm at a 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 soil. Routine watering was done every morning, especially in the early growth phase at 7-14 DAP. Watering was done every two days when the cucumber flowers emerged and were not done when it rains. Stitching was done when the plant was at the age of 14 DAP by replacing the plant that died or grew 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 made when cucumber was planted at the age of a week after planting. This study used bamboo or wood as a stoke marker for each plant and connected each other with ropes.

Basic fertilization used manure with a fertilizer dose of 20 tons ha^{-1} (429 g $plant^{-1}$). Further fertilization was done at the planting time and when the plants were 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 were at 10 DAP. Fertilizer was inserted into the soil at 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 the diameter was more than 2.5 cm. Cucumbers were harvested until the productive harvest was complete. Harvesting was done at 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 under the applied treatment. Pruning was done when the plant was 33 DAP using pruning shears to obtain good results.

Data analysis

Data of observation results were analyzed using Variance Analysis (F test) to determine the treatment's effect. If a significantly different result was found, the Least Significance Different (LSD) test with a 95% confidence level was conducted**.**

RESULTS AND DISCUSSION

Based on the analysis of variance, there is an effect of pruning in cucumbers. The pruning highly affects the plant length, number of leaves, fruit diameter and weight, and flowering age. However, the pruning did not affect the fruit length or 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 at 50 DAP (Figure 1). For example, 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 from the treatment of P0 and P2. On the other hand, treatment of shoot pruning on the main stem (P1) significantly resulted in a shorter plant than other treatments with no shoot pruning, but visually it was seen that the branch was longer than that without pruning.

Treatment of shoot pruning on the main stem (P1) resulted in the highest number of leaves per plant (59.90 pieces) compared to other treatments. In addition, treatment of shoot pruning on the main stem could increase the number of leaves by 16.19% compared to that 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. Pruning treatments (P1, P2, and P3) can generally accelerate the flowering times rather than without pruning treatment (P0). P1 and P3 could shorten the flowering time by 2.93% compared to without pruning treatment (P0). The average flowering age of cucumbers due to pruning in detail is shown in Figure 3.

The result of variance analysis showed that all cucumber pruning treatments had no significant effect on fruit length and the 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 several fruit parameters is the harvestable fruit data. All treatments were suspected of producing optimal assimilates, so they were insufficient to increase the number of harvestable fruit.

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), the 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) increased 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 that was prunings on the main stem increased the fruit diameter percentage by 4.92% compared to the treatment without pruning. The addition of fruit diameter is closely related to the number of leaves. Therefore, the optimal leaf surface area can produce assimilate, increasing the fruit diameter.

The result of the statistical test showed that the pruning treatment had a significant effect on the production per plant. For example, 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 those without pruning (P0). However, the best result was obtained by the P1 treatment, which showed an increase of 11.38% compared to without pruning treatment (P0).

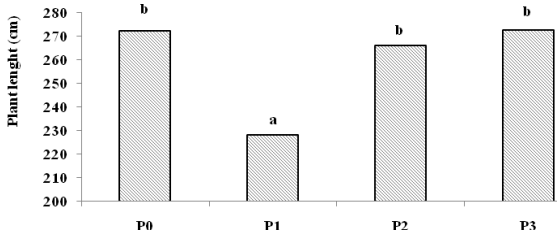

Figure 1. Effect of pruning on plant length

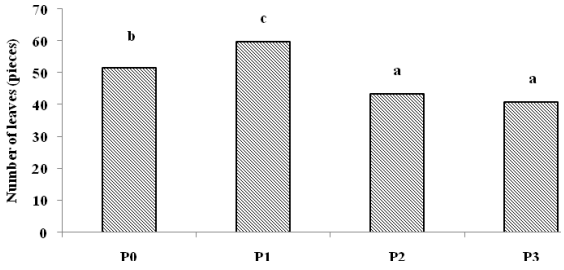

Figure 2. Effect of pruning on number of leaves

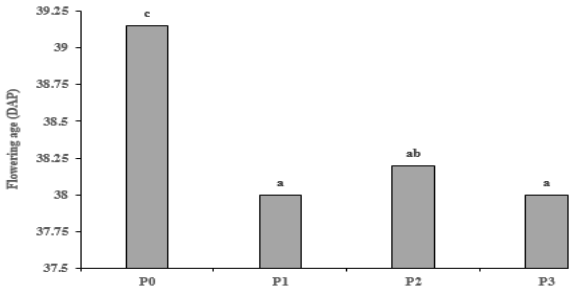

Figure 3. Effect of pruning on flowering age

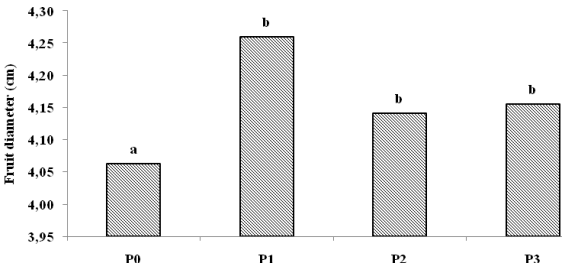

Figure 4. Effect of pruning on fruit diameter

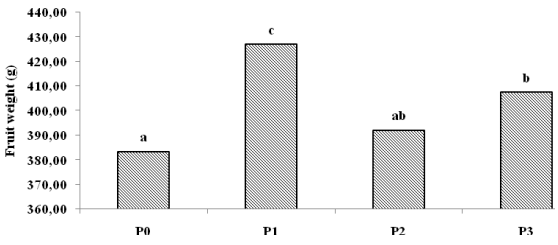

Figure 5. Effect of pruning on fruit weight.

Discussion

The plants 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 increases and further affects the 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. Therefore, IAA triggers change in plant development (Vanneste and Friml 2009).

The production data proved that the cucumber length was not positively correlated with the yield. 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 because the leaf is a plant organ with stomata associated with photosynthesis (Xu and Zhou 2008). Fischer et al. (2012) said that increasing the leaf-fruit ratio generally increases fruit growth and carbohydrate content.

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 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, leading to the availability of photosynthesis. However, the excessive vegetative growth caused a suboptimal use of photosynthesis results and decreased yield production (Coggins Jr and Lovatt. 2014).

The shoots of pruning on the main stem might inhibit the production of auxin in the main stem and increase the cytokinin hormone. That affects the extension of the lateral branches; the longer lateral branches produce a larger number of sections, as the increasing number of sections 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 nitrogen supply. Nitrogen affects photosynthesis and photoprotection in leaves (Pompelli et al. 2010), and the results of these photosynthates, which plants will use 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 prunings, which is certainly not expected by farmers because it will make an apical dominance that results in a longer vegetative phase and inhibition of the flowering time of the plant. That 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 is allocated for cell enlargement in the fruit tissue since the meristematic cells in the fruit will increase 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 assimilating the photosynthesis process is more widely allocated to enhance other plant growth processes, such as cell enlargement. Photosynthate positively affects fruit as a photosynthesis result (Yu et al., 2013).

Cucumber pruning can produce a better fruit weight. It is proven by the fruit weight produced by a shoot of pruning on the main stem (P1), which can gain a weigher fruit in the cucumber Mercy variety 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 the cucumber Mercy variety. Based on the data, the cucumber Mercy variety can produce 3.5-5 kg plant^{-1}, while this study only reached 1.28 kg. That might be caused by the cucumber being 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 new information that shooting prunings on the main stem could increase cucumber Mercy variety's quantity (fruit weight) in acid soils in Tarakan, North Kalimantan.

REFERENCES

Beadle C, Barry K, Hardiyanto E, Irianto R, Mohammed C, Rimbawanto A. 2007. Effect of pruning *Acacia mangium* on growth, form and heart rot. For Ecol Manage 238(1): 261-267.

Benner BL. 1988. Effects of apex removal and nutrient supplementation on branching and seed production in *Thlaspi arvense* (Brassicaceae). Am J Bot 75(5): 645-651.

Coggins Jr CW, Lovatt CJ. 2014. Plant Growth Regulators. In: Ferguson L, Grafton-Cardwell EE (eds) Citrus Production Manual. UC ANR, California.

Crawley MJ. 2009. Life history and environment. In: Crawley MJ (eds) Plant Ecology, Second Edition. Wiley-Blackwell, Victoria.

Dun EA, Brewer PB, Beveridge CA. 2009. Strigolactones: discovery of the elusive shoot branching hormone. Trends in Plant Sci 14(7): 364-372.

Farquhar GD, Sharkey TD. 1982. Stomatal conductance and photosynthesis. Ann Rev Plant Physiol 33(1): 317-345.

Feng S, Martinez C, Gusmaroli G, Wang Y, Zhou J, Wang F,Chen L, Yu L, Iglesias-Pedraz JM, Kircher S, Schäfer E, Fu X, Fan LM, Deng XW. 2008. Coordinated regulation of *Arabidopsis thaliana* development by light and gibberellins. Nature 451(7177): 475-479.

Fischer G, Almanza-Merchán PJ, Ramírez F. 2012. Source-sink relationships in fruit species: A review. Revista Colombiana de Ciencias Hortícolas 6(2): 238-253.

Ghosh A, Chikara J, Chaudhary D. 2011. Diminution of economic yield as affected by pruning and chemical manipulation of *Jatropha curcas* L. Biomass and Bioenergy 35(3): 1021-1029.

Hansen P. 1967. 14C-studies on apple trees. I. The effect of the fruit on the translocation and distribution of photosynthatess. Physiologia Plantarum 20(2): 382-391.

Hibberd JM, Sheehy JE, Langdale JA. 2008. Using C 4 photosynthesis to increase the yield of rice—rationale and feasibility. Cur Opin Plant Biol 11(2): 228-231.

Meier IC, Leuschner C. 2008. Leaf size and leaf area index in *Fagus sylvatica* forests: competing effects of precipitation, temperature, and nitrogen availability. Ecosystems 11(5): 655-669.

Pompelli MF, Martins SC, Antunes WC, Chaves AR, DaMatta FM. 2010. Photosynthesis and photoprotection in coffee leaves is affected by nitrogen and light availabilities in winter conditions. J Plant Physiol 167(13): 1052-1060.

Sebastian P, Schaefe H, Telford IR, Renner SS. 2010. Cucumber (*Cucumis sativus*) and melon (*C. melo*) have numerous wild relatives in Asia and Australia, and the sister species of melon is from Australia. Proc Nat Acad Sci USA 107(32): 14269-14273.

Usenik V, Solar A, Meolic D, Stampar F. 2008. Effects of summer pruning on vegetative growth, fruit quality and carbohydrates of 'Regina' and 'Kordia' sweet cherry trees on 'Gisela 5'. Europ J Hort Sci 73 (2): 62–68.

Vanneste S, Friml J. 2009. Auxin: a trigger for change in plant development. Cell 136(6): 1005-1016.

Xu Z, Zhou G. 2008. Responses of leaf stomatal density to water status and its relationship with photosynthesis in a grass. J Exp Bot 59(12): 3317-3325.

Yu K, Fan Q, Wang Y, Wei J, Ma Q, Yu D, Li J. 2013. Function of leafy sepals in *Paris polyphylla*: photosynthate allocation and partitioning to the fruit and rhizome. Funct Plant Biol 40(4): 393-399.

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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 out after three weeks of 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 Duncan's multiple separated the means rang test. The results obtained showed the progressive improvement of all Sorghum tested characters. There were significant differences in plant height, stem thickness, and shoot fresh and dry weight. In addition, 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 wide cultivated varieties, such as grain genotypes, fodder, fiber and sugar genotypes, and dual-purpose genotypes. Sorghum belongs to the C4 plant characteristic for tolerating biotic stresses more than many crops Gnansounou et al. 2005. Recently, Sorghum has received significant attention because of its newer use as a Biofuel feedstock (Paterson 2008). Assessment of the genetic variability within cultivated crops and varieties has a strong impact on plant breeding strategies and conservation of genetic resources (Dean et al. 1999; Simioniuc 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 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 the 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 the loss of an estimated 42 billion dollars and 5 million hectares of productive land each year. In addition, most farmlands 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 household food insecurity, especially among the poor peasants who are the most numerous in agriculture in the Sudan region of Mali. According to Sime and Aune (2014), the fallow, the traditional way to restore the fertility of the land, has almost disappeared in some places. In others, its duration was significantly reduced because of demographic pressure. The technical packages to sustainably increase production are not within their reach. Since 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 improved agricultural productivity by creating agricultural extension services. But these creations have not fulfilled farmers' expectations, mainly for 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 the farmers more space to develop 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). However, there are a few contradictory results on technology diffusion 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). As reported by Agricultural technologies of Borkina Faso (2010), the advantages of this technology are; (i) Location of the fertilizer near the root, thus obtaining a high concentration area which makes assimilation of nutrients easier. (ii) To limit 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 with this technology, which include: (i) The technology is time-consuming, laborious, and difficult to ensure each plant gets the right dose. (ii) Access to fertilizer, access to credit, insufficient information flow, and appropriate training policies for the farmers. (iii) Adopting the technology requires supportive and complementary institutional innovation and 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 the USA. Yet, the African crops are so starved of nutrients such as phosphorus, potassium, and nitrogen that the 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 farmers 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 an increase in vegetative yield and, finally, the seed. (iii) To minimize the cost of fertilizer application by the plant's minimum dose of fertilizer with maximum utilization.

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 low relative humidity, an annual rainfall rate of 150 mm, and a mean temperature of (20.3 C-36.1ºC) and clay soil Celtic pH 7.5-8.7 Abdulha Feez (2001).

Plant material and treatments

The plant material was a local variety of Sorghum (Sorghum bicolor L.) obtained from the 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 randomized complete block design (RCBD).

Cultural practice

The experimental site was disc plow and harrowed, followed by harrowing, leveling, and 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 date was in December 2015, and the seeds were sown in holes, each 40 cm apart, and the seed was sown at a depth of 20 cm. With fertilizer in the same hole. Weeding was done two times after, three weeks from sowing and one month from the first-hand weeding. After that, the plants were watered according to their need.

Data collection

Observations were conducted on agronomical traits. Every agronomical trait was observed by selecting five Sorghum plants randomly from each plot except 50% days to flowering. The 50% days to flowering was observed by recording the duration from planting to 50% of the plant population was blooming. Plant height was measured from the soil surface to the tip of the flag leaf using a measuring tape. The number of leaves per plant was observed by counting the average number per plant. The length of the inter-node was measured from one node to another 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 weighing the plant. Forage dry yield per plant was measured by drying the plant in the oven (80ºC) for 48 hours and then weighing the plant.

Statistical analysis

The data were analyzed according to the standard statistical procedure for a randomized complete block design described by Gomez and Gomez (1984) using the MSTAT-C computer package. 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, and shoot fresh and dry weights. At the same time, there is a significant difference in 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 a higher plant height for 3M followed by 2M, but generally, the difference was not big. The highest node length was recorded for a 4g microdose (16 cm), while the control resulted in the lowest value (12.5 cm). Figure 2 showed a consistent node length for 4M and 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 the number of leaves favored was 4M, followed by 2M and 3M. The 4 g microdose treatment resulted in the best stem thickness (4.36 cm), and the lowest value was recorded for the control (2.63cm). Figure 4 represents the stem thickness 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 the 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. Figures 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	Degree of freedom	Plant height (cm)	Node length (g)	Number of leaves	Stem thickness (cm)	Shoot fresh weight (g)	Shoot dry 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%),

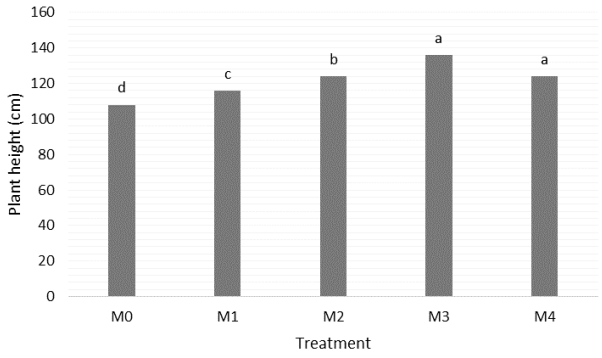

Figure 1. Effect of microdosing on sorghum plant height

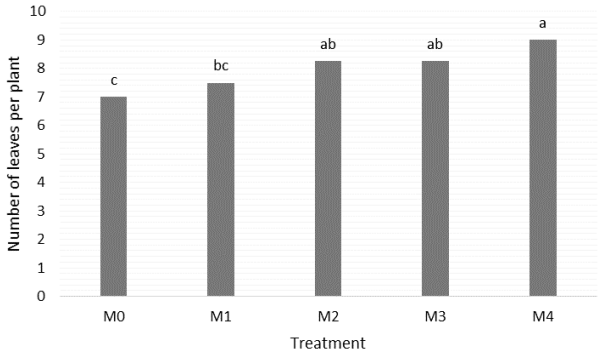

Figure 3. Effect of microdosing on number of leaves per plant of Sorghum

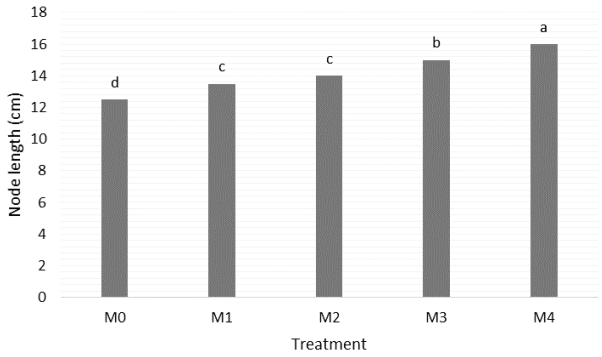

Figure 2. Effect of microdosing on sorghum node length

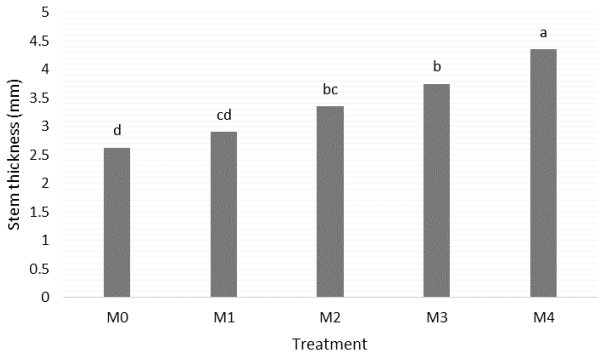

Figure 4. Effect of microdosing on sorghum stem thickness

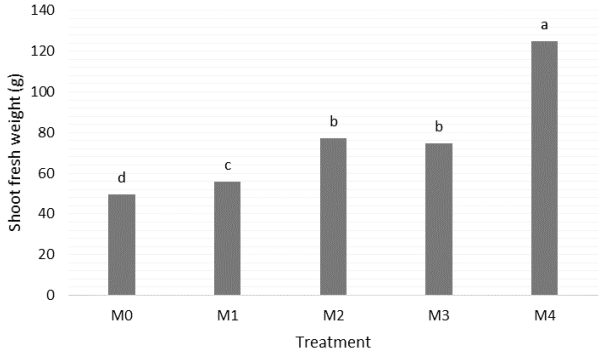

Figure 5. Effect of microdosing on fresh shoot weight of Sorghum

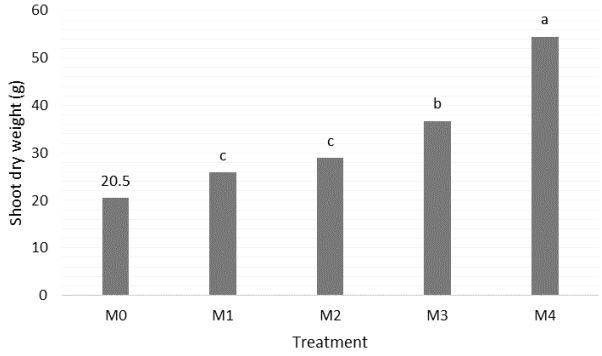

Figure 6. Effect of microdosing on dry shoot weight of Sorghum

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. That shows a need for fertilizer in Sorghum production in most soils of our country. An efficient fertilizer application method with a smaller fertilizer is the most important for marginal farmers in central Sudan. Such a method will potentially increase farmers' interest, economic viability, and sustainability concerning applying fertilizer to Sorghum. In this respect, the results of this study showed that the microdosing method of fertilizer application was found to improve Sorghum yields with smaller quantities of fertilizer. The study results strongly agree with 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. In as much 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 a 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 fertilizer levels were found to depress pocket seed germination and lower plant population at harvest. These negative effects on maize performance 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), and Coulibaly et al. (2000).

Therefore, if the farmers practice microdosing, they can obtain a good yield at a low fertilizer application rate. Yet, a further study based on long-term data is required to rectify optimum fertilizer rates for the different sites of sub-Sahelian countries depending on soil quality and other governing agroecological 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. That might be because placing fertilizer close to the seed in soils increases crop uptake, as reported by (FAOSTAT 2011). That indicates that under a better soil management system and favorable seasonal rainfall conditions, farmers can still get reasonable crop yields through microdose technology. However, the labor demand in microdosing (4.8 person-days ha−1) is nearly twice that in banding (2.3 days ha−1) for applying 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 is more productive and profitable. However, generally, microdosing followed the normal pattern from the high to the low. Therefore it is recommended to use 4M microdosing.

REFERENCES

Agricultural Technologies in Burkina Faso. 2010. Management of Natural Resources in Burkina Faso. UA-SAFGRAD. Burkina Faso.

Ayana A, Bekele E. 1999. Multivariate analysis of morphological variation in Sorghum (*Sorghum bicolor* (L.) Moench) germplasm from Ethiopia and Eritrea. Genet Res Crop 46: 24-36.

Azoulay G, Saizal R. 1994. Réforme de politique économique en Afrique subsaharienne dans les années 80 et performance du secteur agricole. Etude FAO/ Développement économique et social 129/202, Rome.

Baah F. 2007. Meeting the information needs of Ghanaian cocoa farmers: are farmer field schools the answer? J Sci Technol 27 (3): 163-173.

Bagayoko M, Mason SC, Traore S, Eskridge KM. 1996. Pearl millet /cowpea cropping system yields and soil nutrient levels. Afr Crop Sci J 4 (4): 453-462.

Bationo A, Sogodogo D, Mamado G, Taonada JS, Mahaman S. 2015. The fertilizer microdose Technology. Burkina Faso.

Bationo A. 1998. Research on nutrient flows and alleviation of soil fertility of dry lands. African J Agric Res 6 (14):41-50.

Bielder C. 2015. Microdose Fertilizer: a step on the ladder towards crop intensification in the Sahel. Earth and Life Institute, Louvre, Belgum.

Braun A, Jiggins J, Röling N, Berg H. van den, Snijders P. 2006. A Global Survey and Review of Farmer Field School Experiences. Report

prepared for the International Livestock Research Institute (ILRI). Endelea, Wageningen, The Netherlands.
Coulibaly A, Bagayoko M, Traore S, Mason SC. 2000. Effect of cropping cowpea and Sorghum on a West African soil. J Agric Sci 135: 399-401.
Davila JA, Sanchez de la Hoz MO, Loarce Y, Ferrer E. 1998. DNA and coefficients of parentage to determine genetic relationships in barley. Genome 41: 477-486.
Davis K. 2006. Farmer Field Schools: A Boon or Bust for Extension in Africa? J Intl Agric Exten Educ 13 (1): 91-97.
Dean RE, Dahlberg JA, Hopkins MS, Mitchell CV, Kresovich S. 1999. Genetic redundancy and diversity among 'orange' accessions in the U.S. national sorghum collection as assessed with simple sequence repeat (SSR) markers. Crop Sci 39: 1215-1221.
Druilhe Z, Jesús BH. 2012. Fertilizer subsidies in sub-Saharan Africa. FAO, Rome.
FAO. 1995. Politique d'ajustement structurel et de stabilisation et sécurité alimentaire. Intl J Curr Microbiol App Sci 5 (4): 698-704.
FAO. 2008. L'accès des femmes à la terre en Afrique de l'Ouest: problématique et pistes de solutions au Sénégal et au Burkina Faso. Mbour, 2-4 Juillet 2008.
FAO. 2011. L'approche 'champs écoles' permet aux petits producteurs du Mali de s'adapter aux changements climatiques/ Journal New Agriculturist. Cas d'études publiés pour la journée de l'Agriculture et du développement rural-Durban-Afrique du Sud/3 décembre 2011.
FAOSTAT. 2011. Top production-West Africa (Total)-2008. FAO, Rome.
Feder G, Murgai R, Quizon JB. 2004. The acquisition and diffusion of knowledge: the case of pest management training in farmer field schools, Indonesia. J Agric Econ 55 (2): 221-243.
Gnansounou E, Dauriat A, Wyman C. 2005. Refining sweet sorghum to ethanol and sugar: economic trade-offs in the context of North China. Bioresour Technol 96: 985-1002.
ICRISAT. 2009. International Crops Research Institute for the Semi Arid Tropics. Fertilizer Microdosing Boosting Production in unproductive lands. ICRISAT, India.
Khatam A, Muhammad S, Ashraf I. 2013. Farmers' field school: a strategy for benefiting resource poor farmers in Khyber Pakhtunkhwa, Pakistan. J Anim Plant Sci 23 (6): 1749-1753.
Morris M, Kelley VA, Kopicki RJ, Byerlee D. 2007. Fertilizer use in sub-Sahelian regions. J Agric Sci 88: 12-16.
Palé S, Mason SC, Taonda SJB. 2009. Water and fertilizer influence and residue management of cropping systems for pearl millet and cowpea yield. Afr Crop Sci J 8 (4): 1-8.
Paterson AH. 2008. Genomics of Sorghum. Intl J Plant Genom DOI:10.1155/2008/362451.
Piyadasa M, Karltun E, Olsson M. 2005. Soil organic matter dynamics after deforestation along a farm field chronosequence in southern highlands of Ethiopia. Agric Ecosyst Environ 109: 9-19.
Ribaut JM, Hoisington D. 1998. Marker assisted selection: New tools and strategies. Trends Plant Sci. 3: 236-239.
Sime DF, Aune KR. 2014. Response of Zea maize to microdose application levels. Ethiopian Agric J 25:24-28.
Simioniuc D, Uptmoor R, Friedt W, Ordon F. 2002. Genetic diversity and relationships among pea cultivars (*Pisum sativum* L.) revealed by RAPDs and AFLPs. Plant Breed 121: 429-435.
Singh KB, Singh S. 1991. Evaluation of exotic germplasm in lentil. J Agric Res 6 (2): 304-306.
Smith JSC, Smith OS. 1992. Finger printing crop varieties. Small holder production and processing of bioenergy as a strategy for an empirical law describing heterogeneity in the yield. Adv Agron 47: 85-140.
Togola A, Agbaka A, Agunbiade TA, Anato F, Chougourou DC, Nwilene FE. 2010. Connaissance paysanne des insectes foreurs de tiges du riz et leurs dégâts dans différentes zones écologiques du Bénin (Afrique de l'Ouest). Cah Agric 19: 262-266.
Vitale JD, Sanders JH. 2005. New markets and technological change in agriculture. Afr J Plant Soil 26 (2): 91-97.
Waddington H, Snilstveit B, Hombrados J, Vojtkova M, Philips D, Davies P, White H. 2014. Farmer Field Schools for improving Farming Practices and farmer Outcomes: A Systematic Review. Campbell Syst Rev 2014: 6. DOI: 10.4073/csr.2014.6.

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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 Biol Dev 1 (1): 23-30.* The aqueous filtered and unfiltered extract of the fruits of *Randia nilotica* (locally named 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*, and the results showed that unfiltered extracts were found to be relatively more potent than filtered ones (i.e., 100% was attained at 90 ppm and 80 ppm), respectively. The filtered extract was tested on uninfected *B. pfeifferi* and *B. truncatus* (100% was attained at 100 ppm and 90 ppm), respectively. The effect of the unfiltered extract on infected *B. pfeifferi* produced 100% mortality in a concentration of 70 ppm. The activity of the plant on cercariae and miracidia revealed that cercariae were more resistant 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 in the field.

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

INTRODUCTION

Schistosomes are digenetic trematodes that 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 near and around slow-moving water bodies in the tropics and subtropics. It is estimated that over 200 million people in 73 countries are infected (McCullough and Mott 1983), while a further 500-600 million or 4-5% of the world's population are at risk of being infected (Basch 1991; Brown 1994). Africa, China, and the new tropics are the most extensively infected areas. Man participated in the spread of schistosomiasis by creating habitats suitable for the snail hosts and the dispersal of the parasites through the traveling of infected people (Brown 1994).

In Africa, schistosomiasis is found in most countries, and the number of infected persons was estimated to be 90 million, while 180 million persons are at risk (Korte and Mott, 1980; Inobaya et al., 2014). There are five major species of schistosomes affecting humans. These species differ not only biologically but also in their geographical distribution and the types of infection they produce. Intestinal schistosomiasis, caused by *Schistosoma mansoni*, occurs 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). Finally, urinary schistosomiasis is caused by *S. haematobium*, which occurs in Africa and Eastern Mediterranean.

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

This study is intended to determine the efficiency of the plant *Randia nilotica* on the adult snail hosts, on cercaria, and the 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 the Kordofan area in Western Sudan. Plant Extraction was performed at the Department of Pathology, Faculty of Veterinary Medicine, University

of Khartoum. In the laboratory, the fruit was 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). Deep scooping collected the snails from the El-Seleit irrigated area on the Eastern part of the Blue Nile. 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 with boiling water, and allowed to dry. The water used for snail breeding was tested using a flame spectrophotometer to determine its chemical composition. The same water was then used to determine the laboratory's rate of development and hatching these snails. The egg masses produced by these snails were deposited on small pieces of cellophane materials, which were placed on the top surface of the water in the tanks to collect eggs. The cellophane materials contaminated with egg masses were removed daily and placed on water in other tanks to maintain the hatching of eggs and the growth of embryos. Finally, the juvenile snails were fed on algae or dried lettuce, and the range of growth and hatching periods were observed.

Extract preparation

The method of extract preparation described by Brackenbury et al. 1997) and Brackenbury (1999) was adopted in the experiment. The sum 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 test procedure, which was applied to 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. First, the number of dead snails was recorded. Then, 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 a 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 a lack the reaction to any external stimulus from the surrounding water. Therefore, the numbers of dead and living snails were recorded after 24 hours of exposure, followed by 24 hours of a recovery period.

The molluscicidal activity of *Randia nilotica* on *Biomphalaria pfeifferi* and *Bulinus truncatus*

Different dilutions were prepared from the stock solution prepared from R. nilotica fruits 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 each were put in one liter of the extract solution of known concentration in a container and exposed for 24 hours. First, 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%; the experiment was repeated three times, and the average obtained from the three readings was taken. Control results were similarly taken. Two types of extract were used, the first one the filtered extract and the other unfiltered. The extract was used against uninfected *Bulinus*, infected and uninfected *Biomphalaria*

Miracidicidal activity test

Production of miracidia

Miracidia were obtained using stool samples from people infected with *Schistosoma mansoni* in El-Seraha village in Gezira State. Samples were examined by the 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 a one-liter conical flask. Warm dechlorinated water was 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). First, 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 three hours. Next, ten miracidia were transferred to one ml of distilled water to serve as a control. The death of miracidia was determined by low 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.

The procedure for snail infection

The snail used in this procedure was *B. pfeifferi*. Each snail was placed in a microtiter plate containing 5ml of fresh water. Then, 3-5 miracidia were added to each microtiter 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. Then, they were put in a beaker containing distilled water (10 ml/snail). Finally, they were exposed to strong artificial light at 25 ºC. Shedding of cercariae started after about half an hour under these conditions.

The beaker was held against a light source to detect cercariae. 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, stained with lougols 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 microscope for over five hours after the activity decreased. The time required to kill all cercariae was recorded. Cercariae were considered dead if they became immotile and/or their oral and ventral suckers were extended. Twenty cercariae were transferred to one ml of distilled water on the same plate to serve as acontrol.

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*, *B. truncatus*, and cercariae and miracidia of *S. mansoni* using the SPSS program. The correlation coefficient that shows the relationship between the plant concentrations and the effect of this plant on the snails and cercariae and miracidia of *S. mansoni* was calculated. It was then drawn into a graph for filtered and unfiltered extracts against *B. pfeifferi*, *B. truncatus*, and cercariae and miracidia of *Schistosoma mansoni*. The potencies of filtered and unfiltered extracts on *B. pfeifferi* and *B. truncatus* and the potency of the unfiltered extract on infected and uninfected *B. pfeifferi* T-test were compared. 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 the unfiltered extract on infected *B. pfeifferi* by cercaria of *S. mansoni*. The probit values were calculated using the log values of the concentrations of the extract and the percentage of mortalities corresponding to them.

$$Y_1 = (Y\text{-}bx) + bx_1$$

Where

(Y1) 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 at 20 ppm resulted in 3%. In contrast, at 30 ppm produced 13% mortality, at 40 ppm resulted in 23% mortality, at 50 ppm resulted in 36% mortality, at 60 ppm resulted in 46%, concentration of 70 ppm produced 70% mortality, at 80 ppm produced 83% mortality, at 90 ppm resulted in 93% mortality. Finally, 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 at 20 ppm resulted in 6%. In comparison, at 30 ppm produced 16%mortality, at 40 ppm resulted in 30% mortality, at 50 ppm resulted in 53% mortality, at 60 ppm resulted in 70%, at 70 ppm produced 83% mortality, at 80 ppm produced 90% mortality. In comparison, a concentration of 90 ppm resulted in 100% mortality (Figure 2).

The effect of the filtered extract on the snail was highly significant ($p \leq 0.001$) (Table 1). At the same time, the correlation was positive between the filtered extract concentrations and the mortality; the correlation was positive ($r = 0.960$), and it was highly significant ($p \leq 0.01$). The effect of the other unfiltered extract was also highly significant ($p \leq 0.001$, Table 1), and the correlation was positive ($r = 0.969$) and highly significant ($p \leq 0.01$).

There was no significant difference in potency of the filtered and unfiltered extract on *B. pfeifferi*. In the concentrations of 10 ppm and 100 ppm, there were no significant differences (p =--). In comparison, a difference was found at 20 ppm ($p = 1.000$), and at 30 ppm, no significant difference was recorded between filtered and unfiltered extract ($p = 0.519$). At 40 ppm, the result showed no significant differences ($p = 0.491$). In the concentrations of 50 ppm and at 60 ppm the results were 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%. In comparison, at 30 ppm produced 13%mortality, at 40 ppm resulted in 30% mortality, and at 50 ppm resulted in 52% mortality. In comparison, at 60 ppm resulted in 70%, a concentration of 70 ppm produced 83% mortality, at 80 ppm produced 91% mortality, and at 90 ppm resulted in 100% mortality (Figure 4). On the other side, the activity of unfiltered extract on the snail showed that at 20 ppm resulted in 7%. In comparison, at 30 ppm produced 23%mortality, at 40 ppm resulted in 41% mortality, and at 50 ppm resulted in 70% mortality. In comparison, at 60 ppm resulted in 86%, at 70 ppm produced 93% mortality, while at 80 ppm produced 100% mortality (Figure 5).

The effect of the 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 the unfiltered extract on *B. truncatus* was also highly significant (p≤0.001) (Table 1). In addition, 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. At 70 ppm, the significance was (p = 0.101), while at the final concentration of 80 ppm, the significance was (p = 0.158) (Figure 6).

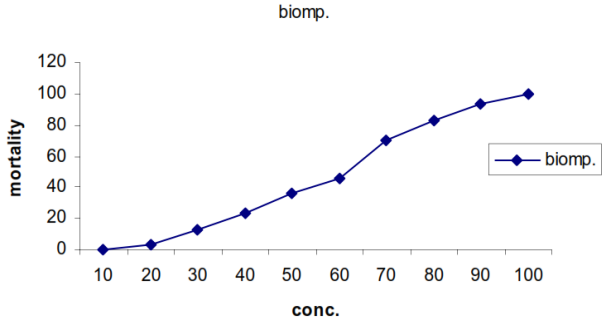

Figure 1. The effect of *R. nilotica* filtered extract concentration on *Biomphalaria pfeifferi*. Correlation Coefficient = 0.960

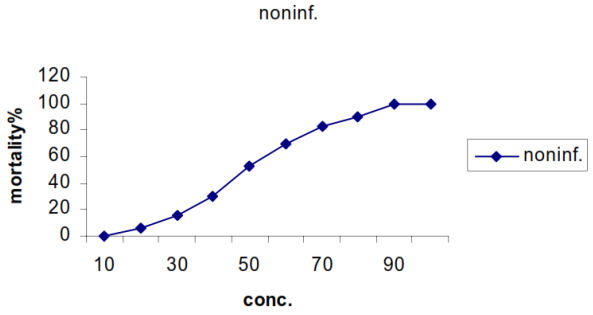

Figure 2. The effect of *R. nilotica* unfiltered extract concentration on *Biomphalaria pfeifferi*. Correlation Coefficient = 0.969

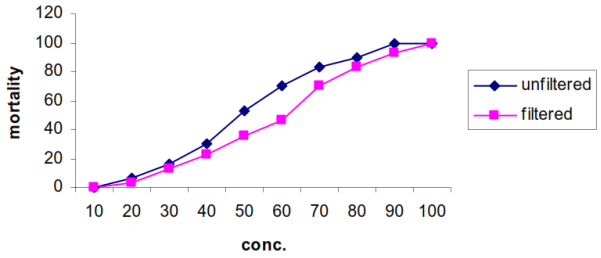

Figure 3. The effect of *R. nilotica* filtered and unfiltered extract on *Biomphalaria pfeifferi*.

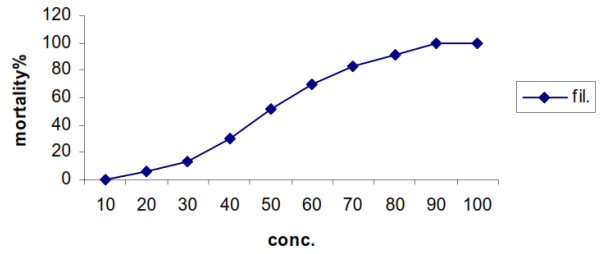

Figure 4. The effect of *R. nilotica* filtered extract concentration on *Bulinus truncatus*. Correlation Coefficient = 0.966

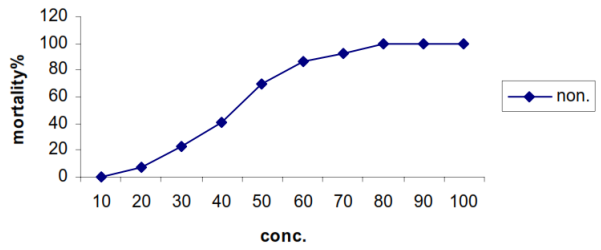

Figure 5. The effect of *R. nilotica* unfiltered extract concentration on *Bulinus truncatus*. Correlation Coefficient = 0.945

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 a concentration of 20 ppm resulted in 3% while at 30 ppm produced 23%mortality, at 40 ppm resulted in 33% mortality, at 50 ppm resulted in 70% mortality, at 60 ppm resulted in 90% mortality, at 70 ppm produced 100% mortality (Figure 7).

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

There were no significant differences in potency of the unfiltered extract on infected and uninfected *Biomphalaria* snails; in a concentration of 10 ppm, there were 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 at 500 ppm killed all cercariae in a few minutes (15 minutes), and at 250 killed the cercariae at 30 minutes. At 50 ppm, the cercariae were killed in about 3 hours, while at 25 ppm did not affect cercariae for about five hours (Figure 9).

The effect of the plant on cercariae was highly significant ($p \leq 0.001$) (Table 1). Furthermore, the time to kill the cercariae was decreased when the used concentration increased; this means the correlation was negative, the correlation coefficient was ($r = -0.751$), and 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 at 500 ppm killed the miracidia just in 10 minutes, and the concentrations of 250 ppm and 100 ppm killed all miracidia in 20 minutes and 45 minutes, respectively. While at 50 ppm killed all miracidia in 2 hours, at 25 ppm produced no effect on miracidia for up to 3 hours (Figure 9).

The plant was very effective on the miracidia of *S. mansoni* and was highly significant ($p \leq 0.001$) (Table 1). Although it was a negative correlation, the correlation coefficient ($r = -0.773$) was highly significant ($p \leq 0.01$).

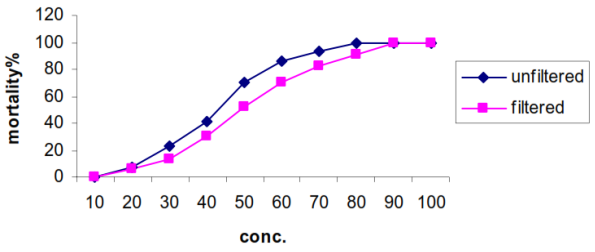

Figure 6. The effect of filtered and unfiltered extract of *R. nilotica* on *Bulinus truncatus*.

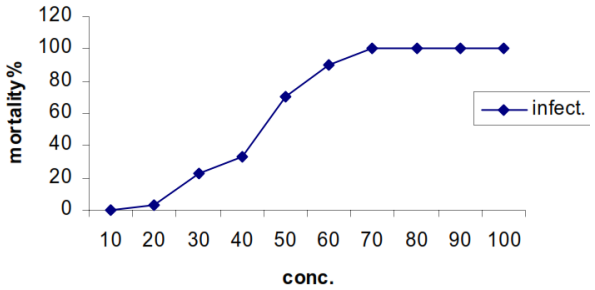

Figure 7. The effect of unfiltered extract of *R. nilotica* on infected *Biomphalaria pfeifferi*. Correlation Coefficient = 0.934

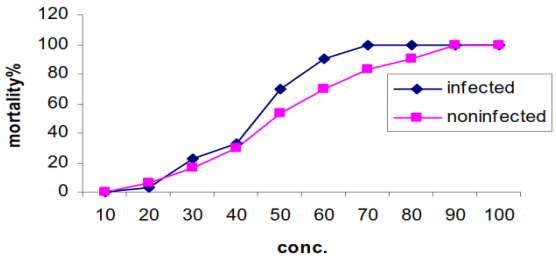

Figure 8. The effect of unfiltered extract of *R. nilotica* on infected and uninfected *Biomphalaria pfeifferi*

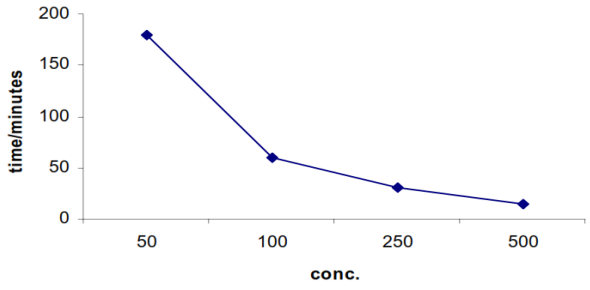

Figure 9. The effect of filtered extract concentration on Cercariae of *S. mansoni*. Correlation Coefficient =-0.751

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	49.570	0.893	114.393***

Note: ***P≤0.001

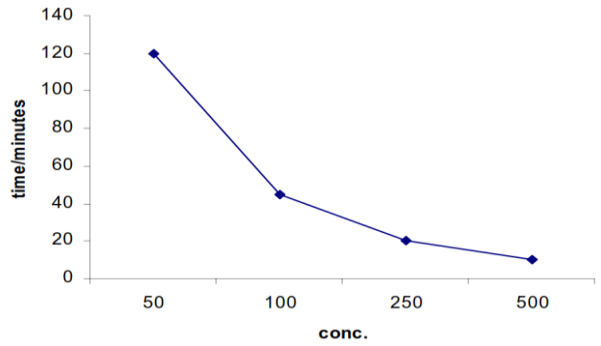

Figure 10. The effect of *R. nilotica* filtered extract concentration on Miracidia of *S. mansoni*. Correlation Coefficient = -0.773

Discussion

Aqueous extract of the plant *R. nilotica* filtered and unfiltered extract has been used in this study to assess 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 several 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.

This study revealed that the plant extract was highly effective against *B. pfeifferi*. The filtered extract produced LD50 (probit 5.00) at 53.33 ppm and LD90 (probit 6.28) at 97.95 ppm, while 100% mortality (probit 7.33) was produced at 100 ppm. On the other side, the unfiltered extract produced LD50 and LD90 at the concentrations of 46.24 ppm and 85.31 ppm, respectively. In comparison, 100% mortality was produced at 90 ppm and 100 ppm. That means unfiltered extract was more potent than filtered one; that effect may be due to the debris on an unfiltered extract that snails fed. At the same time, at 10 ppm, there was no effect on filtered and unfiltered extracts.

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

R. nilotica on B. truncatus revealed that filtered extract produced LD50 at 46.56 ppm. In comparison, it produced LD90 when the concentration increased to 84.33 ppm, indicating that 100% mortality was reached when 90 ppm and 100 ppm were used. On the other hand, the effect of the unfiltered extract on *B. truncatus* revealed that LD 50 (probit 5.00) was 39.63 ppm, while LD90 (probit 6.28) was produced at 67.61 ppm. In comparison, 100% mortality was produced when the 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 ones 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 the unfiltered extract on *B. pfeifferi* and *Bulinus truncates*, respectively.

The concentrations of filtered extract were relatively low compared to 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 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 also used against infected *B. pfeifferi* by miracidia of *S. mansoni*; the unfiltered extract was highly effective, LD50 with 40.83 ppm, while 90% mortality was produced at 65.16 ppm. The concentrations that produced 100% mortality were 70 ppm, 80 ppm,90 ppm, and 100 ppm.

Table 9. Means (±SE) of the effect of *Randia nilotica* filtered and unfiltered extract on infected *Biomphalaria pfeifferi* uninfected *B. pfeifferi* and *Bulinus truncatus*

Extract conc. (ppm)	Uninfected				Infected *B. pfeifferi*
	Filtered on *B. pfeifferi*	Filtered on *B. truncatus*	Unfiltered on *B. pfeifferi*	Unfiltered on *B. truncatus*	
10	0.00 ±0.00	0.00± 0.00	0.00±0.00	0.00±0.00	0.00±0.00
20	0.33±0.30	0.67±0.33	0.67±0.30	0.67±0.60	0.33±0.30
30	1.33±0.33	1.33±0.67	1.67±0.33	2.33±0.33	2.33±0.33
40	2.33±0.67	3.00±0.58	3.00±0.58	4.00±0.58	3.33±0.67
50	3.67±0.80	5.33±0.88	5.33±0.33	7.00±0.58	7.00±0.58
60	4.67±0.88	7.00±0.58	7.00±0.58	8.67±0.33	9.00±0.50
70	7.00±1.15	8.33±0.33	8.33±0.88	9.33±0.33	10.00±0.00
80	8.33±0.67	9.00±0.58	9.00±0.58	10.00±0.00	10.00±0.00
90	9.33±0.33	10.00±0.00	10.00±0.00	10.00±0.00	10.00±0.00
100	10.00±0.00	10.00±0.00	10.00±0.00	10.00±0.00	10.00±0.00

Table 10. Means (±SE) of the effect of time (minutes) of mortality of Miracidia and Cercariae due to the effect of *Randia nilotica* filtered extract.

Extract conc. (ppm)	Miracidia	Cercariae
50	120±2.89	180±2.89
100	45±2.89	60±1.53
250	20±1.73	30±1.00
500	10±1.15	15±1.00

These results revealed that uninfected snail was more resistant than the infected one; 100% mortality was given at 90 ppm on uninfected snails and 70 ppm on infected ones (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 on the miracidia of S. mansoni was observed in 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. The concentration of 250 ppm killed miracidia in about 20 minutes, while at 500 ppm killed all miracidia in a fewer time (10 minutes) (Tabel 3).

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

The plant was used against the cercariae of *S. mansoni*; at 50 ppm killed the cercariae within 180 minutes. The 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 the cercariae of *S. mansoni.*

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

Forty Sudanese plants 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 50 ppm within 3 hours and 5 hours, respectively. However, the correlation coefficient (r) was negative. That means that when at 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 subspecies *nilotica* and *adansoni* against cercariae and miracidia of *S. mansoni*. They found that high concentrations of both plant subspecies killed both cercariae and miracidia, as a concentration of 5,000 ppm of *A.nilotica* with subspecies *nilotica* killed cercariae and miracidia within 80 minutes and 35 minutes, respectively. Similar concentrations in the other subspecies (*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. truncates*, and showed the activity of the plant against cercariae and miracidia of *S. mansoni*. The plant killed the snails at high concentrations, with 100% mortality reached when concentrations of 1,200 ppm and 1,300 ppm were used on *B. pfeifferi* and *B. truncatus*, respectively. On the other hand, the 100% mortality of cercariae and miracidia of S. mansoni was taken in concentrations of 100 ppm and 50 ppm, respectively. These differences may be due to the plant's part (s) used, and 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. All experiments also showed that at 10 ppm did not produce any activity against the snails. Furthermore, the cercaricidal and miracidicidal activity of the plant revealed that it was very effective on the cercariae and miracidia of *S. mansoni* as it gave 100% mortality in a short time at lower concentrations. Also, in these experiments, at 25 ppm did not give any activity on the cercariae and/or miracidia of *S. mansoni*. Therefore, based on the results of the present study, it seems reasonable to conclude that the plant can be used to control schistosomiasis in Sudan, as it has numerous molluscicidal, cercaricidal, and miracidicidal activities. In addition, the plant grows naturally in different parts of Sudan, and its application requires simple technology.

REFERENCES

Adewenmi CO. 1980. Water extract of tetra pleura: An effective molluscicide for the control of Schistosomiasis and Fascioliasis in Nigeria. J Anim Prod 3: 7-15.

Ahmed MM, Hady, HM, Salama, MM, EL-Ghazali, S. 1994. Laboratory study on the molluscicidal effect of Earth Tec: an environmentally responsible copper sulfate product. J Egypt Soc Parasitol 24: 317-322.

Al-Sayed E, Hamid HA, Abu El Einin HM. 2014. Molluscicidal and antischistosomal activities of methanol extracts and isolated compounds from Eucalyptus globulus and Melaleuca styphelioides. Pharm Biol 52 (6):698-705.

Basch PF. 1991. Schistosomes. Development, Reproduction and Host Relations. Oxford University press, New York.

Bashir AK, Sulaiman SM, EL-Sheikh SH, EL-Kheir YM. 1987. Molluscicidal, Cercaricidal and Miracidicidal activity of *Acacia nilotica* SSP. nilotica and adansoni. Fitoterapia 108: 51-55.

Brackenbury TD. 1999. The molluscicidal properties of Apodytes dimidiata (Icacinaceae): geographical variation in molluscicidal potency. Ann Trop Med Parasitol 93 (5):511-518.

Brackenbury TD, Apleton CC, Kayonga L, Drewes SE. 1997. Molluscicidal properties of *Apodytes dimidiata* (Icacinaceae): Geographic variation in molluscicidal potency. J Med Appl Malacol 14: 299-302.

Brown DS. 1994. Fresh water snails of Africa and their medical importance. Taylor and Francis Ltd, London.

Doumenge JP, Mott KE, Cheung C, Villenava D, Chapuis O, Perrin MF, Reuad-Thomas G. 1987. Atlas of the global distribution of Schistosomiasis. Press Universitaires de Bordeaux, Talence.

El-Sheikh SH. 1994. Biological and Chemical Studies on Molluscicidal and Schistosomicidal Compounds. [Dissertation]. Faculty of Science, University of Khartoum, Khartoum.

Hotez PJ, Fenwick A. 2009. Schistosomiasis in Africa: An emerging tragedy in our new global health decade. PLoS Negl Trop Dis 3 (9): e485. DOI: 10.1371/journal.pntd.0000485

Hotez PJ, Kamath A. 2009. Neglected tropical diseases in sub-Saharan Africa: review of their prevalence, distribution, and disease burden. PLoS Negl Trop Dis. 3: e412. DOI: 10.1371/journal.pntd.0000412.

Hussein MF. 1973. Animal schistosomiasis in Africa, A review of *Schistosoma bovis* and *Schistosoma matteei*. Veterinary Bulletin 43: 341-347.

Ibrahim AM, Abdel-Gawad MM, El-Nahas HA, Osman NS. 2015. Studies on the molluscicidal activity of Agave angustifolia and Pittosporum tobira on schistosomiasis transmitting snails. J Egypt Soc Parasitol. 45 (1):133-41.

Ibrahim AB. 1998. The molluscicidal activity of *Catunaregam nilotica* and *Vangueria venosa*. [Thesis]. Faculty of Veterinary Medicine, University of Khartoum, Khartoum.

Inobaya MT, Olveda RM, Chau TNP, Olveda DU, Ross AGP. 2014. Prevention and control of schistosomiasis: a current perspective. Res Rep Trop Med 17 (5): 65-75.

Jordan P, Webbe G, Sturrock RF. 1993. Human Schistosomiasis, CAB International, Wallingford, UK.

Korte R, Mott K.E. 1980. Maintenance of Schistosomiasis control-an overview. Trop Med Parasitol 40: 130-131.

Lai YS, Biedermann P, Ekpo UF, et al. 2015. Spatial distribution of schistosomiasis and treatment needs in sub-Saharan Africa: a systematic review and geostatistical analysis. Infec Dis 15 (8): 927-940.

McCullough FS, Mott KE. 1983. The role of molluscicides in schistosomiasis control. WHO document. WHO/SHISTO/83.72.

Steinmann P, Keiser J, Bos R, Tanner M, Utzinger J. 2006. Schistosomiasis and water resources development: systematic review, meta-analysis, and estimates of people at risk. Lancet Infect Dis 6: 411-425.

Sulaiman SM, Ibrahim SM. 1985. *Schistosoma mansoni* (Gezira) infection in *Biomphalaria pfeifferi* snails from Gezira and Jebel Marra. J Trop Med Hyg 88:91-93.

Teesdale CH, Amin MA. 1976. Comparison of the bell technique, a modified kato thick smear technique and the digestion method for the field diagnosis of *Schistosoma mansoni*. J Helminthol 50: 17-20.

Wains GJP, McManus DP. 1997. Schistosomiasis vaccine development. The current picture. Bioassay. 19: 435-443.

WHO. 1965. Molluscicides screening and evaluation. Bulletin of the World Health Organization 33: 567-581.

WHO. 1973. Schistosomiasis control. WHO Expert Committee on Schistosomiasis. World Health Organization: technical Report. Series No. 515: 47.

WHO. 1994. The Control of Schistosomiasis. WHO Tech. Rep. Ser. 830.

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

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Abstract. *Herlina L, Pukan KK, Mustikaningtyas D. 2017. The endophytic bacteria producing IAA* (*Indole Acetic Acid) in* Arachis hypogaea. *Cell Biol Dev 1: 31-35.* Endophytic bacteria live in plant tissue and form colonies without harming the host. Every cormophyte with a stem and root may contain some endophytic bacteria that 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, to test in vitro the ability of endophytic bacteria isolates to produce 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 understand the ability of IAA production. IAA assay was measured using a spectrophotometer with a wavelength of 535 nm; 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 lowest IAA amounts were 69.68 (mg L^{-1}) and 8.50 (mg L^{-1}), respectively. Isolates that produce high IAA levels are applied to mungbean; it affects the number of lateral roots but does not affect 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 that form colonies in plant tissues without harming the host. Each high-level plant may contain several endophytic bacteria 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 their 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 have been used as biofertilizers to increase crop production, significantly reducing the chemical input to the environment (Luo et al., 2012; Ahemad et al., 2014).

Endophytic bacteria is 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 auxin, can help plants 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 kururiensis* in peanut plants causes plant growth to be better with the number of roots, increasing the plant's lateral roots. As a result, plant growth is rapid, giving 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 of 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 to synthesize the auxin hormone from microorganisms in their tissues. Therefore, IAA-producing bacteria potentially join the physiological process of plants by entering IAA-generated crops. The effect on the plant is that it is more sensitive to altering its IAA concentrations, thus helping to form 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 and lateral root development and stimulate density and root hair length, leading 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 tumefaciens and *Azotobacter vinelandii* (Khan and Doty 2009). Different bacterial groups were reported to produce IAA (indole-3-acetic acid) and 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 hormone that can stimulate plant growth by increasing the elongation process, stem extension, and 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. Suitable precursors are a primary factor in the 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 are expected to influence green beans' growth and development.

MATERIALS AND METHODS

The isolation of endophytic bacteria from *Arachis hypogaea*

The roots and leaves of the peanut were cleaned for about 20 minutes using running water. Next, 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 (RaduandKqueen, 2002). After sterilizing, the roots and leaves were aseptically mashed in a mortar, then put into a test tube containing sterile distilled water in a ratio of 1: 10 and diluted to 103. Next, 1 ml of the roots and leaves were spread on a nutrient medium for sterilizing and incubating at room temperature for 24 hours. Next, the growing bacteria colonies were subcultured into the same medium to obtain pure cultures. Finally, the characterization of colony morphology gram stain and some biochemical tests were conducted to distinguish bacterial isolates from one another.

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) inoculated into 30 ml of Luria-Bertani Tryptophan solution. At room temperature, bacterial cultures were incubated and shaken at 150 rpm for 7 days. The IAA level generated during cultivation was measured every two days a week. The IAA level was measured in colorimetry with a spectrophotometer at 535 nm wavelength. Culture fluid was centrifuged for 25 minutes at 5,000 rpm. The obtained filtrate was mixed with the Salkowski reagent (150 ml concentrated H_2SO4, 250ml of distilled water, 7.5 ml of 0.5M$FeCl_3$•$6H_2O$) with a ratio of 2:1. The mixture was then incubated in room temperature for an hour before the absorbance was measured at a wavelength of 535 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

The positive bacterial culture produced IAA was tested in liquid and solid to grow the mungbean plant. The introduction of endophytic bacteria is done on a sterile mungbean sprout. To get sterile sprouts then, mungbean seeds were grown in sterile media. First, the surface of mungbean seeds is washed under running water. Then, 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. Next, the seeds were washed with sterile distilled water, soaked with 10% chlorox solution with a shaker for 15 minutes, and washed with sterile distilled water three times. Next, the seeds were soaked with 5% chlorox solution with a shaker for 15 minutes, and then they were washed with sterile distilled three times. In the last stage, the seeds were soaked in 70% alcohol for one minute and rinsed with sterile distilled. Seeds of sterile mungbean were grown in an 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 an Mc. Farland solution (10^8 cells/ml) for one hour with 50% dilution. Sprouts soaked with aquades were used as controls. Each treatment was repeated six times. Any sprouts 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 locations, Gunungpati, Pakintelan, and Klipang, earned 22 endophytic bacterial isolates. After examining the bacteria producing IAA, 16 isolates have the ability to produce IAA (Table. 1). Based on the characterization result in table 1, it could describe that the isolates have a variation of colony morphology. Most of them are Gram-negative bacteria, and the cell

morphology is coccus and bacillus. It means that the 16 isolates may be different species or different genera. Further research is needed to identify the isolates bacteria species.

The ability of endophytic bacteria to produce IAA with the 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. This figure shows 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 the 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, availability of amino acids, and other N sources (Yurnaliza. 2010). The decrease in IAA levels on the fourth or sixth day is due to the available nutrients (tryptophan), which have begun to decrease.

The use of nutrients in every bacterium varies. In some isolates, it increases in line with incubation time because the enzyme that converts tryptophan to IAA is still low at the time of incubation on the second day. In line with bacterial growth rate, the enzyme used to convert 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 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 various pathogenesis and phytostimulation. IAA is a major auxin in plants involved in cell enlargement and division, tissue differentiation, and 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	The colony morphology						Gram	Cells morphology
	Size	Optical characteristic	Shape	Elevation	Texture	Margins		
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	Irregular	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

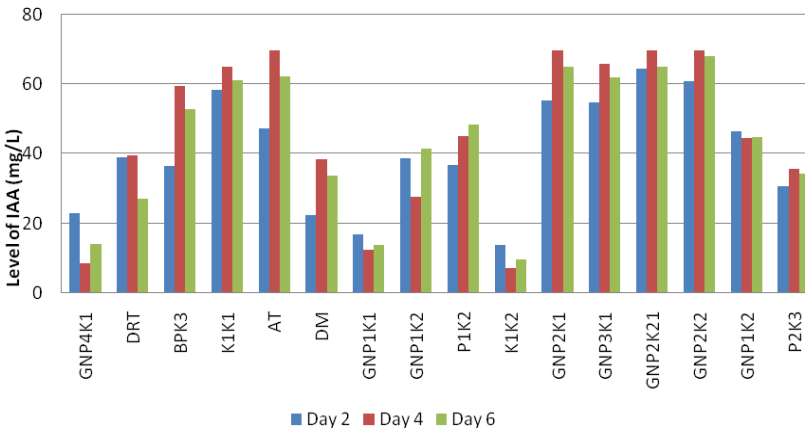

Figure 1. The production of IAA from various endophytic bacteria isolates

The synthesis of IAA by microbes depends on the pathway of tryptophan, where tryptophan is used as a precursor and diverse taxonomic and metabolic tissue tissues. Some endophytic microorganisms have the potential to synthesize IAA to increase or stimulate growth when colonization occurs with endophytes (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, cell division, and differentiation (Hasan et al., 2015). IAA-producing bacteria potentially affect the growth process from the amount of IAA in 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 were selected for 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 affects the increase of IAA concentration in plants causing stunt length inhibition. In contrast to the number of roots, IAA concentrations in plants stimulate lateral root formation. IAA bacteria can loosen the cell wall of plants and consequently increase the number of roots that increase exudation, providing additional nutrients to support the growth of the bacterial rhizosphere. IAA bacteria stimulate the development of the root system of host plants. IAA production of isolates can improve the fitness of microbial plant interactions (Hasan et al., 2015).

Endophytic bacteria not only generate IAA but also increase the availability of plant nutrients such as nitrogen, phosphate, and other minerals, so that plant growth increases. The root is one of the most sensitive organs to IAA fluctuations and is responsible for increasing the number of exogenous IAA useful for the 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. In addition, many important physiological processes include cell enlargement and differentiation. Therefore, when administered to the plant, the IAA produced by the bacteria will affect the plant tissue's sensitivity.

In conclusion, there were 16 isolates of endophytic bacteria having the ability to produce IAA. The incubation time affected the IAA content produced by bacteria, and IAA-producing endophytic bacteria isolate affected the number of lateral roots. Still, 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.

Table 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
GNP2K2I	15.425	19.5 ab
K1K1	14.20	23.75 a
P2K3	14.75	19.75 ab
Control	14.65	13.75 c

REFERENCES

Ahemad M, Kibret M. 2014. Mechanisms and applications of plant growth promoting rhizobacteria: current perspective. J King Saud Univ Sci 26: 11-20.

Ambawade MS, Pathade GR. 2015. Production of Indole Acetic Acid (IAA) by *Stenotrophomonas maltophilia* BE25 isolated from roots of banana (*Musa* spp.). Intl J Sci Res 4 (1): 2644-2650

Duan J, Jiang W, Cheng Z, Heikkila JJ, Glick BR. 2013. The complete genome sequence of the plant growth-promoting bacterium Pseudomonas sp. UW4. PLoS ONE 8: e58640. DOI: 10.1371/journal.pone.0058640.

Hassan E, Alikhani HA, Hosseini HM. 2015. Indole-3-acetic acid (IAA) production trait, a useful screening to select endophyte and rhizosphere competent bacteria for rice growth promoting agents. MethodsX 2 (2015) 72-78

Jasim B, Joseph AA, John CJ, Mathew J, Radhakrishnan EK. 2013. Isolation and characterization of plant growth promoting endophyt bacteria from the rhizome of *Zingiber officinale*. 3 Biotech 4 (2): 197-204.

Khan Z, Doty SL. 2009. Characterization of bacterial endophytes of sweet potato plants. Plant Soil 322: 197-207.

Lestari, P, Susilowati D N, Riyanti E I. 2007. Pengaruh hormon asam indol asetat yang dihasilkan oleh *Azospirillum* sp. terhadap perkembangan akar padi. Jurnal Agro Biogen 3 (2): 66-71. [Indonesian]

Luo SL, Xu TY, Chen L, Chen JL, Rao C, Xiao X, Wan Y, Zeng GM, Long F, Liu CB, Liu YT. 2012. Endophyte-assisted promotion of biomass production and metal-uptake of energy crop sweet sorghum by plant-growth-promoting endophyte *Bacillus*sp. SLS18. Appl Microbiol Biotechnol 93: 1745-1753.

Mattos KA, Padua VLM, Romerio A, Hallack LF, Neves BC, Ulisses TMU, Barros CJ, Todeschin AR, Previat JO, Mendoca Previato L. 2008. Endophytic colonization of rice (*Oryza sativa* L) by the diazaotrophic bacteriu *Burkholderia kururiensis* and its ability. Ann Acad Bras Cienc 80 (3): 477-493.

Moore TC. 1989. Biochemistry and Physiology of Plant Hormones. Spinger-Verlag, Berlin.

Nath R, Sharma GD, Barooah M. 2013. Screening of endophyt bacterial isolates of tea (*Camellia sinensis* L.) roots for their multiple plant growth promoting activities, Intl J Agric Environ Biotechnol 6 (3): 371-376.

Pandey PK, Yadav SK, Singh A, Sarma BK, Mishra A, Singh HB. 2012. Cross-species alleviation of biotic and abiotic stresses by the endophyte Pseudomonas aeruginosa PW09. J Phytopathol 160 (10): 532-539.

Rana A, Saharan B, Joshi M, Prasanna R, Kumar K, Lata N. 2011. Identification of multi-traits PGPR isolates and evaluating their potential as inoculants for wheat. Ann Microbiol 61: 893-900.

Ryan RP, Germaine K, Franks AF, Ryan DJ, Dowling DN. 2008. Bacterial endophytes: recent developments and applications. FEMS Microbiol Lett 278 (1): 1-9.

Saepen S, Jos S, Roseline R. 2007. Indole-3-Acetic acid in microbial and microorganism and microorganism plant signaling. Department of Microbial and Moleculer Systems, Centre of Microbial and Plant Genetics, Belgium

Shi Y, Lou K, Li C. 2009. Isolation, quantity distribution and characterization of endophyt microorganisms within sugar beet. Afr J Biotechnol 8: 835-840.

Taghavi S, Garafola C, Monchy S, Newman L, Hoffman A, Weyens N. 2009. Genome survey and characterization of endophyt bacteria exhibiting a beneficial effect on growth and development of poplar. Appl Environ Microbiol 75: 748-757.

Tarabily KA. 2008. Promotion of tomato (*Lycopersicon esculentum* Mill.) plant growth by rhizospherecompetent 1-aminocyclopropane-1-carboxylic aciddeaminase-producing streptomyceteactinomycetes. Plant Soil 308: P 161-174.

Yarnaliza, Siregar MW, Priyanti N. 2010. Peran bakteri endofit penghasil IAA terseleksi terhadap pertumbuhan tanaman padi. Prosiding Seminar Nasional Biologi FMIPA USU, 219-228. [Indonesian]

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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 Biol 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 lack endosperm. These plants commonly need an in vitro culture for mass propagation and seed germination. Their undeveloped embryos developed globular mass cells, a protocorm after germination. Occasionally, a structure similar to protocorm arises from tissue other than an embryo; thereby, the term protocorm-like body (*plb*) was introduced. Here, we develop synthetic seed hydrogel beads encapsulated *in G. scriptum* plbs, possibly germinating the seed and growing their embedded tissue. The objective of the research was to study the effects of the proportion of G scriptum Bl encapsulation matrix. Synthetic seed made by complexing sodium alginate with $CaCl_2$ 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/$CaCl_2$ratios.The result showed that $CaCl_2$ in all concentrations except 25 mM formed spherical hydrogel beads in all levels of Na-alginate. Alginate in concentrations of 2% and 3% gave the optimum result represented by a maximum germination index of 100%. The formation of the new plbs varied among different explants, even in a single explant. The germination time of each synthetic seed varied from 2 weeks to 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 plants comprising more than 24,000 species and categorized in 800 genera (Fay and Chase 2009). They occupy a wide range of ecological habitats from tropic to temperate climatic regions but exclude sea water and extremely cold environments (Tan et al. 1998). The amazing flower morphology has put them in a dilemma where their benefit faced conservation issues. Numerous orchid species are economically well-known plants in floriculture industries. Unfortunately, a collection of their native species for illegal trading and habitat destruction has made them in a thread situation (Kull et al. 2006).

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

Effective propagation and ex-situ conservation are the key factors that must be seriously managed to save orchids in nature (Fay 1994; Sarasan et al. 2006). However, their reproductive nature was dependent on their association with other organisms. This phenomenon has been considered the consequence of their flower structure influencing their pollination biology. On the other hand, the seed produced from successful fertilization is lacking in the endosperm, so there must be a co-relation with mycorrhiza to acquire a nutrient from environmental surroundings for the 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 a small number of new individuals 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 reviews. The medium in axenic condition discovers a symbiotic germination technique of Cymbidium orchid seeds and subsequent successful attempts (Yam and Arditti 2009). That *in vitro* technique was also visible for fixation of elite genotype by multiplying somatic tissue and generating their derivative through organogenesis as well as embryogenesis to become plantlets. Some combination with cryopreservation technique *in vitro* culture over an efficient tool for germplasm conservation for future benefits (Engelmann 2010).

Products of tissue culture such as plantlets, somatic embryos, callus, or protocorm-like bodies (*plbs*) could not easily introduce to the greenhouse or field because of the different environments inside and outside the bottle. The situation remains problematic for consumers with insufficient background in tissue culture. Thus, we have developed a synthetic seed, a *plb* encapsulated calcium-alginate hydrogel, to help the plantlet survive in the field or greenhouse successfully. Moreover, *Plbs* might be representatives of somatic embryos in Orchidaceae. Thus successful attempt would highlight the possibility of direct transfer of somatic embryos of orchids into the plantation.

MATERIALS AND METHODS

Plant material

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

Plbs induction medium

The basal MS (Murashige and Skoog) medium plus a vitamin from Phyto Technology Lab. contained 3% (w/v) sucrose and was used to induce *plbs* formation. The medium formulation would be called basal-MS medium in the 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 solidified 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 $CaCl_2.2H_2O$ solution; each drop containing a single plb was incubated in a $CaCl_2.2H_2O$ solution for 30 minutes. The solid hydrogel beads formed by the complexation of the two encapsulation matrices were recovered by decanting the $CaCl_2.2H_2O$ 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 a 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 to compare regeneration capabilities of different sources of explant on the induction of *plbs* formation. The result suggests that young shoots and previous *plb*, except leaf segments, have been successfully regenerated as new *plbs* in basal MS medium lacking plant growth regulator (PGR) (Table 1). However, *plb* formed by previous *plb* explants are more numerous than young shoot-derived *plbs*. The number of the new *plb* formed by the previous *plb* explant was around five compared to 1-2 *plbs* from the shoot explant. The limitation of shoot explant-producing *plbs* confirmed similar results from previous studies in the 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 a single explant. The first *plb* starts visibly 2 weeks after subculture and continues developing new plbs in the next days. Thus, four weeks after subculture, there got at least three types of *plbs* based on their development stages, i.e., globular *plb*,*plb* with new shoot meristem, and *plb* with developing shoot. The latter explicitly indicates 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 to larger than 4 mm. There would be *plb* with globular stage and 2-3 mm in diameter as candidates for encapsulation to become the 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

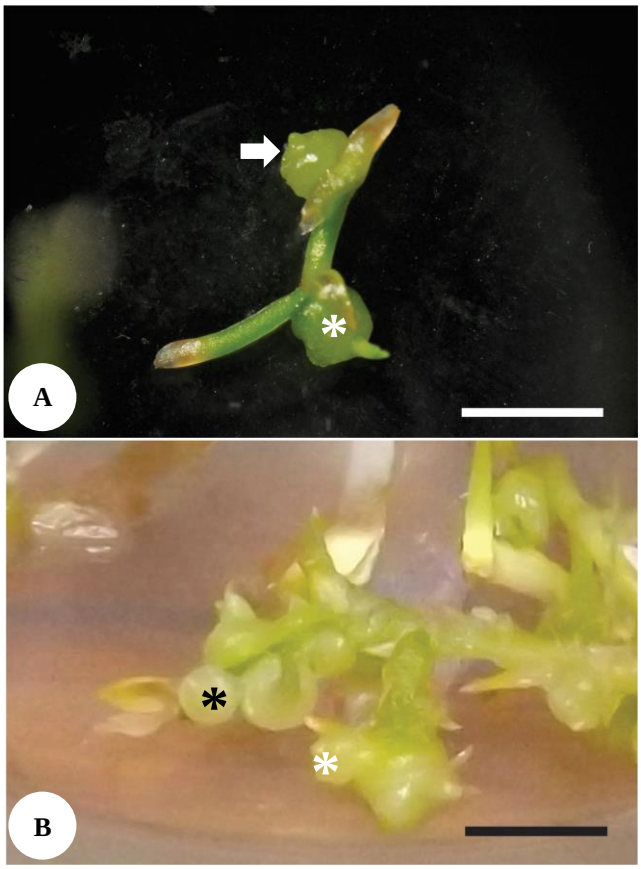

Figure 1. Formation of new *plbs* derived from varied explants cultured in MS basal medium. A. Shoot-derived *plbs*; B. *plb*-derived *plb*. White asterisk: *plb* with young developing shoot; Black asterisk: *plb* in the globular state; white arrow: *plb* with emergence shoot meristem. Bar = 1 cm.

Table 2. Physical properties of a calcium-alginate hydrogel of synthetic seed

Alginate	$CaCl_2.2H_2O$	Gel properties	Color	Shape
2%	25	+	Transparent	Unshape
	50	++	Less transparent	Spheric
	75	+++	Milky white	Spheric
	100	++++	Milky white	Spheric
3%	25	+	Transparent	Spheric
	50	++	Less transparent	Spheric
	75	+++	Milky white	Spheric
	100	++++	Milky white	Spheric
4%	25	+	Transparent	Spheric
	50	++	Less transparent	Spheric
	75	++++	Milky white	Spheric
	100	++++	Milky white	Spheric
5%	25	+	Transparent	Spheric
	50	+++	Less transparent	Spheric
	75	++++	Milky white	Spheric
	100	++++	Milky white	Spheric

Assessment of encapsulation matrix on hydrogel properties and development of encapsulated *plb*

The developed synthetic seed was represented by the structure of solid hydrogel encapsulated *plb* of *G. scriptum.* Alginate-based hydrogel is used 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 the rigidity of the hydrogel bead, thus enhancing better protection of the embedded *plb* from mechanical damage (Saiprasad 2001). The hydrogel coating-*plbs* were formed by cross-linking calcium ions with alginate ions by an ion exchange mechanism. In this study, the complexation of alginate anion from sodium-alginate solution with divalent calcium cation from $CaCl_2.2H_2O$ solution in the different contraction levels provides various degrees of physical properties of hydrogel capsules. Based on Figure 3, the concentration of calcium chloride below 25 mM results in an unspherish structure of the hydrogel. Concomitantly, all levels of sodium alginate solution in combination with appropriate calcium chloride (at least 50 mM) successfully formed solid globular hydrogel calcium alginate.

Since the hydrogel's physical barrier is considered to influence the coating plbs significantly, the stiffness of the hydrogel qualitatively also be tested by pushing the hydrogel with fingers and scored as described in Table 2. The qualitative result found that the hydrogel's stiffness would increase, coinciding with an increase in alginate concentration.

Germination of synthetic seed *G. scriptum*

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

Germination percentages of all treatments from various degrees of the ratio encapsulation matrix were summarized in Figure 3. The chart shows variations in germination percentage of 8 weeks old synthetic seed of *G. scriptum.* The sodium-alginate solution in a concentration of 2% and 3% (w/v) reached maximum capacity to germinate in all levels of calcium chloride ranging from 25 mM to 100 mM. However, germinations were reduced in treatment of 4% and 5% sodium-alginate solution in combination with

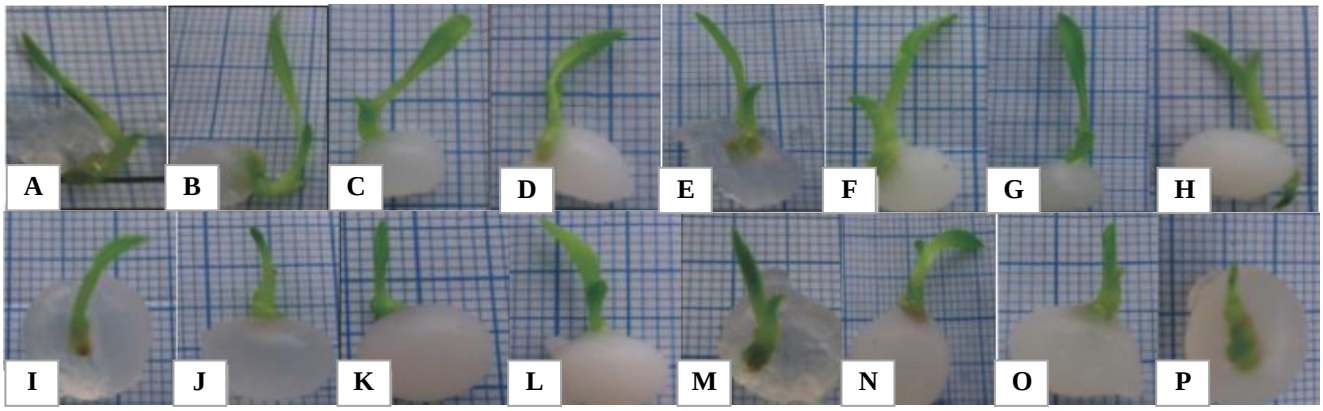

Figure 2. Germination of synthetic seed *G.scriptum*. All combinations of encapsulation matrix gave positive germination. The combination are: A. Alg 2% Cl 25; B. Alg 2% Cl 50; C. Alg 2% Cl 75; D. Alg 2% Cl 100; E. Alg 3% Cl 25; F. Alg 3% Cl 50; G. Alg 3% Cl 75; H. Alg 3% Cl 100; I. Alg 4% Cl 25; J. 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.

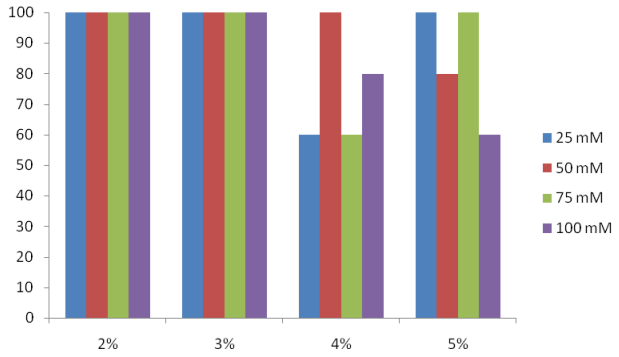

Figure 3. Germination percentage of 8 weeks old synthetic seed with variation in the ratio of encapsulation matrix. The horizontal axis is representative of the concentration of the sodium-alginate solution. Bar legends are representative of $CaCl_2.2H_2O$ concentration.

Calcium chloride in several level concentrations. These results suggest that sodium-alginate in a concentration of 2-3% was recommended for synthetic seed formation. Previous reports supported our finding that the concentration superior for developing synthetic seeds of *three orchid genera* (Saiprasad and Polisetty 2003) was 3% sodium-alginate in combination with 75 or 100 mM $CaCl_2$.

Furthermore, there found that the germination time of each synthetic seed varied from 2 weeks until 8 weeks after encapsulation. This phenomenon was predicted due to variation in the developmental state despite our use of the plbs, which is in uniform size on the *plb*. Previous reviews (Sharma et al. 2013) emphasize the importance of synchronizing high-quality explants to produce synthetic seeds for industrial applications. Somatic embryos represented by plbs in Orchidaceae apparently remained problematic in synchronous their developmental state to achieve the industrial application of the synthetic seed.

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

REFERENCES

Engelmann F. 2010. Use of biotechnologies for the conservation of plant biodiversity. In Vitro Cell Dev Biol-Plant 47 (1): 5-16.

Fay MF. 1994. In what situations is in vitro culture appropriate to plant conservations? Biodiv Conserv 3 (2):176-183.

Fay MF, Chase MW. 2009. Orchid biology: from Linnaeus via Darwin to the 21st century. Ann Bot 104 (3): 359-364.

Kakita H. Kamishima H. 2008. Some properties of alginate gels derived from algal sodium alginate. J App Phycol 20 (5): 543-549.

Kull T, Kindlmann P, Hutchings MJ, Primack RB. 2006. Conservation biology of orchids: introduction to the special issue. Biol Conserv 129 (1): 1-3.

Lee YI, Hsu ST, Yeung EC. 2013. Orchid protocorm-like bodies are somatic embryos. Amer J Bot 100 (11): 2121-2131.

Lestari DA, Santoso W. 2011. Inventory and habitat study of orchids species in Lamedai Nature Reserve, Kolaka, Southeast Sulawesi. Biodiversitas 12 (1): 28-33.

Nge KL, New M, Chandrkrachang S, Stevens WF. 2006. Chitosan as a growth stimulator in orchid tissue culture. Plant Sci 170 (6): 1185-1190.

Saiprasad GVS. 2001. Artificial seeds and their applications. Resonance 6 (5): 39-47.

Saiprasad GVS, Polisetty R. 2003. Propagation of three orchid genera using encapsulated protocorm-like bodies. In Vitro Cell Dev Biol-Plant 39 (1): 42-48.

Sarasan V, Cripps R, Ramsay MM, Atherton C, Mc Michen M, Prendergast G, RowntreeJK. 2006. Conservation In vitro of threatened plants-Progress in the past decade. In Vitro Cell Dev Biol-Plant 42 (3): 206-214.

Sharma S, Shahzad A, Teixeira da Silva J. 2013. Synseed technology-a complete synthesis. Biotech Adv 31 (2): 186-207.

Tan TK, Loon WS, Khor E, Loh CS. 1998. Infection of *Spathoglottis plicata* (Orchidaceae) seeds by mycorrhizal fungus. Plant Cell Rep 18 (1-2): 14-19.

Teixeira da Silva J, Tanaka M. 2006. Multiple regeneration pathways via thin cell layers in hybrid *Cymbidium* (Orchidaceae). J Plant Growth Reg 25 (3): 203-210.

Yam TW, Arditti J. 2009. History of orchid propagation: a mirror of the history of biotechnology. Plant Biotech Rep 3 (1): 1-56.