Seed Progeny Population of Wild Banana *Musa acuminata* ssp.*malaccensis* for Fusarium Screening

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Abstract : The purpose of this study was to produce wild banana plant *Musa acuminata* ssp. *malaccensis* from seeds which were resistant to Fusarium wilt. Experiments were carried out on four samples of different banana seeds from three populations taken from various locations. Seeds were germinated through *in vitro* and *in vivo* methods. *In vitro* method was used for embryo rescue technique and *in vivo* method was used for seed germination in a greenhouse. In order to identify which method is able to produce a large number of wild banana plants within a short period of time, a comparison was made between *in vitro* and *in vivo* methods. Result showed that germination rate of embryo observed from *in vitro* (embryo culture) was higher (60%) and faster compared to *in vivo* method (seed germination) which was 3%. Plantlets produced by *in vitro* method were better than those which were generated by *in vivo* method. This is because they could live longer, more than 90 days. Contaminations in culture revealed that Fungi and Bacteria are the main polluters. It was found that scarification and small crack in banana seed coat and mixture of soil and sand (2:1) increased the germination rate of seed, but immersion of banana seed with the use of water bath technique did not rise the germination rate.

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1. Introduction

Banana is the main fruit in international trade which represents the fifth most important agricultural crop in world trade (Aurore et al., 2009). *Musa acuminata*is is one of the most imperative crops which prepare source of income and food in many countries (Jones, 2000). Banana production is under threat because of Fusarium wilt caused by *Fusarium oxysporum*. sp. *Cubense* (*Foc*) (Ploetz, 2005), and this disease is first recognized in Australia in 1876 (Ploetz, 2000). *Foc* is a soil borne pathogen that produces chlamydo spores which enable the fungus to persist in soil in the absence of the host (Dita et al., 2010). *Foc* can also be effectively spread in soil through being attached to implements or vehicles. Fusarium wilt or Panama disease is the most imperative lethal disease of banana (Moore et al., 2001) which has collapsed many hectares of Cavendish bananas in tropical nations including Malaysia and Indonesia (Hwang and Ko, 2004). Symptoms of fusarium wilt are wilting of the older leaves and their yellowish color which progresses to the younger leaves until the entire plant dies. Crops with improved infection show discoloration of the rhizome and necrosis of xylem vessels in the pseudostem (Dita et al., 2010). In general, several factors influence the development of the disease, such as type of cultivar itself, soil, drainage, and environmental conditions (Rowe, 1990). The most commonly used evaluation method for Fusarium wilt is a pot system (Subramaniam et al., 2006; Weber et al., 2007; Smith et al., 2008), followed by a hydroponic method (Groenewald et al., 2006; Van den Berg et al., 2007). Hwang and Tang (1996) studied Fusarium wilt screening for Cavendish bananas in Taiwan, using the unconventional development method for screening and generating soma-clonal variants. Two clones, GCTCV-215-1 and 217, with good tolerance to *Foc* race 4 (VCG 0121) were detected (Hwang, 1999). However, a field selection from Giant Cavendish, known as GCTCV-218, finally rescued the banana industry in Taiwan from disruption by Fusarium wilt (Hwang and Ko, 2004). Smith et al. (2008) claimed that 8 week-old crops with10 to 15 cm height were more suitable for consistent infection than the plants less than 10 cm in the pot system, while plants were much smaller in the hydroponic system (Groenewald et al., 2006). In some studies, disease improvement was estimated 7 to 8 weeks (Smith et al., 2008) and 6 weeks (Groenewald et al., 2006; Van den Berg et al., 2007) after inoculation in the pot system and hydroponic system, respectively. Youmbi et al. (2011) used different temperature and pH values for the evaluation of germination rates in *Musa accuminata* and found that the optimum temperature was 30°C and the optimum pH values were 6.5 for *Calcutta* and 6.8 for *M53* and *Zebrina*. Jafari et al. (2011) found that the highest concentration of BAP (33

µM) simultaneously increased the formation of abnormal shoots in *Musa acuminata*cv. *berangan*. They also detected that proliferation media supplemented with IAA showed enhanced shoot multiplication and elongation but did not help to reduce the abnormality index that occurred.Waite (1963) detected that three races (1, 2, and 4) of *Foc*impacted banana crops, while race 3 onlyinfluencedHeliconia. Race 4 harms Cavendish cultivars and those that are sensitive to race 1 and 2. Race 4 strains are secluded into subtropical and tropical race 4. Tropical race 4 invadescrops in the tropic environments, but subtropical race 4 influencescrops in the regions with pronounced winters (Ploetz, 2006). At present, there are no economic biological, chemical or cultural measures of controlling Fusarium wilt in an infected field (Ploetz, 2006; Buddenhagen, 2009). It is accepted that the breeding and selection for disease resistance is the most impressive and sustainable management option (Buddenhagen, 2009). Field screening for resistance to Fusarium wilt depends on the presence of environmental conditions conducive to disease development, and is time-consuming and expensive (Vakili, 1965). Traditional breeding actions to find a resistant replacement for Cavendish bananas have had limited success, often because of the reluctance by consumers to accept the new hybrids (Daniells et al., 1995). Conventional breeding strategies are also hindered by the fact that Cavendish bananas are sterile and do not produce seed (Robinson, 1996). Therefore, non-conventional strategies such as transformation are more realistic and could be more successful.

2. Materials and Methods

The research was performed at Institute of Biological Sciences Laboratory, University of Malaya, Kuala Lumpur, Malaysia. Mature seed samples of a diploid *Musa acuminate* ssp. *malaccensis* that were produced by open pollination were used. The fruits were washed by distilled water, skins were removed, and seeds were extracted under axenic conditions. Seeds were planted in soil mixture (sand + leaf compost) and maintained in the greenhouse. Four populations were used as sources of seeds for *in vitro* and *in vivo* study. The populations were FT, KK (KK1 and KK2) and KL8. FT population was gained from Negeri Sembilan, KL8 population was taken from the University of Malaya, while KK was prepared from Kuala Kangsar, Perak. For *in vitro* culture, seeds were soaked in distilled water followed by quick wash with 70% (v/v) ethanol. Embryos were removed and cultured in glass jar (60 × 80cm) with each jar containing 10 embryos. The culture medium consisted of Murashige and Skoog salts (1962) supplemented with nicotinic acid (0.125 mg l^{-1}), ascorbic acid (0.2 mg l^{-1}), thiamine HCl (0.5 mg l^{-1}), pyridoxine HCl (0.125 mg l^{-1}), myo-inositol (2.5 mg l^{-1}), glutamine (150 mg l^{-1}), sucrose 5% and PH of the media was adjusted to 5.8 with 1 N HCl. 30 ml medium were dispensed into each 150 ml glass jar before autoclaving at 121˚C for 30 minutes. Cultures were maintained at 16 hour light with a temperature of 28 ± 2˚C. Different concentrations of BAP (6-benzylaminopurine) were used for different type of embryos according to their age. The BAP concentrations were 0, 0.2 and 0.4 µM. No BAP were used for the new germinated embryo in MS_0 jar. 0.2 µM BAP were used for the embryos that have been transferred from MS_0 jar, whereas 0.4 µM BAP for the embryos transferred from MS medium with 0.2 µM BAP. Cultures were observed at least 4 days a week to make sure that the embryo received an adequate light and to maintain the room temperature. All jars contaminated with bacteria and fungus were removed to make the culture room clean. Germinated seeds were counted and recorded in a proper schedule. For *in vivo* method, seeds were gained from fruits and washed with distilled water. Then, seeds were immersed in a beaker filled with water to determine which seeds were capable to germinate. Flouting seeds were separated as they did not have endosperm to germinate. After this, seeds were planted in soil mixture to be cultivated in greenhouse. Three kinds of medium (soil, sand, and soil + sand) were used to germinate seeds and different rates of germinations were compared and observed. For enhancing germination rate, two additional methods were used. Before planting the seeds, they were bathed with water for 48 hours. Then, they were scratched to give a small crack for revealing the inner parts. In the next stage, germination rate was observed in the greenhouse. Fertilizers were used after plants reached 5 cm tall. Plants were inspected 3 days a week to evaluate the germination rate. In order to kill the microorganisms, instruments were soaked overnight in Teepol, rinsed with tap water and distilled water subsequently followed by drying in the oven at 50˚C. Some other instruments were autoclaved at 121˚C, 1.05 kg/cm^2 for 20 minutes to ensure sterility. A laminar air flow (LAF) was exposed to UV light for 10 minutes to sterilize the surface area in the LAF. The fan blower of the LAF was switched on and the inside surface was swabbed with 80 per cent alcohol. The LAF was kept empty for at least 15 minutes prior to use. The forceps and scalpels were dipped into hot bead sterilizer for 10 minutes before used followed by 80 per cent alcohol after used. Two-month-old plantlets (10-15cm tall) were suitable for disease symptom expression. Test plantlets with vigorous root systems were removed from the growth tray and roots were immersed in micro conidial suspensions with a wide range of concentrations in spores/ml such as 5×10, 5×10^2, 5×10^3, 5×10^4, 5×10^5, 5×10^6. Roots were immersed in pathogen suspension for two hours. All susceptible

plants would produce foliage and rhizome symptoms within 10 to 28 days.

3. Results

3.1. *In vitro* analysis of germination rate

From the experiment conducted on embryo rescue of *Musa acuminata* ssp. *malaccensis*,embryos were observed and classified into three groups according to their conditions. All of them were identified whether they germinate, swell, or dead and the percentage was observed.

The percentage of germinated embryos, swell, and dead in FT population are showed (Fig. 1). For this population, the germination percentage was 46%, swell 14%, and dead 40%. The percentage of germination and dead were not varied too much and the difference was only 6%. The 46% of germination could be considered as low for embryo culture of *Musa acuminata*, whereas the dead rate was high (40%).

For the KK population (KK1), the results showed 63% for germination, 9% and 28% for swell and dead respectively. Germination rate was so high (63%) compared to dead which was only 28%. Among other populations (FT, KK2, and KL8), germination rate of this population was the highest (Fig. 2).

Fig. 3 demonstrated the percentage for germination, dead, and swellwhich were 57%, 35%, and 8% respectively. In this population, germination gained higher percentage compared to swell (8%). It's was observed that the embryos which were not germinated did not produce any rooting or even swelling.

In KL8 population, germination, dead, and swell were devoted 55%, 43%, and 2%. KL8 embryo germination was average but compared to KK2 population the dead percentage was higher. All data from embryo culture in all populations are showed in graph below (Fig. 4).

Germination rate in KK1 was the highest (63.54%), followed by KK2 (57.14%), KL8 (55%), and the lowest one was FT (45.70%). For swell embryo, FT got the highest rate among others, while KK1 was 8.96% and KK2 was 8.29%. The lowest percentage was belonged to KL8 (2.25%). Dead embryos showed different percentage in various populations. For instance, KL8 had the highest rate (42.75%) followed by FT (40.44%), KK2 (34.57%), and KK1 (27.5%). Comparison of the populations showed that in all populations swell embryo had a low amount and germinations demonstrated high rates. The result of germination in FT population was not good compared to other ones. This is because it did not reach to half of the cultured embryos. Dead embryo had also the highest amount in FT. The same trend was occurred in KL8 as germination was good but dead embryo rate was extremely higher. KK1 and KK2 populations exhibited the very good results as the germination rates were high and dead embryos had low rates. The best outcome went to KK1 with the highest germination and the lowest dead embryos (Fig. 5).

Fig. 1 Percentage of germinated embryo in FT population

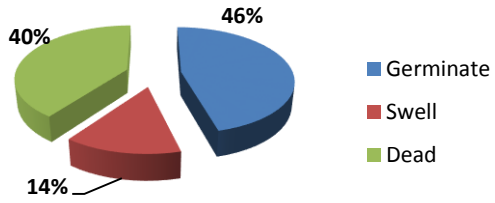


Fig. 2 Percentage of germinated embryo in KK1 from KK population

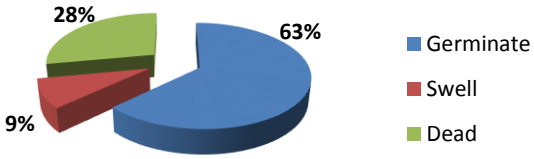


Fig. 3 Percentage of germinated embryo in KK2 from KK population

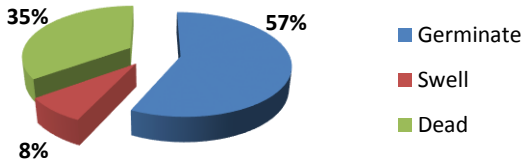


Fig. 4 Percentage of germinated embryo in KL8 population

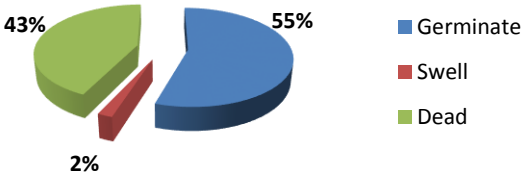


Fig. 5 Percentage of germinate, swell, and dead embryos

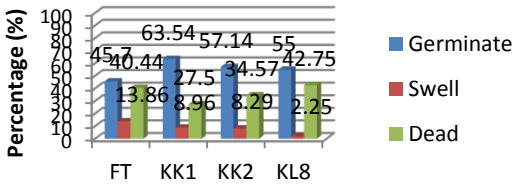
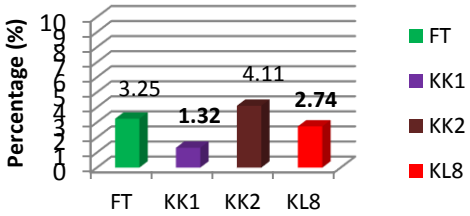


Fig. 6Percentage of seed germination



3.2. *In vivo* analysis of germination rate

From the study of wild banana (*Musa acuminata* ssp. *malaccensis*) seeds the germination rate was observed and compared among all populations. According to the figure 6, KK2 and KK1 had the highest (4.11%) and lowest (1.32%) germination rates, respectively. Considering overall results, germination did not even reach 5% and this was not suitable because of the weak germination rate.

3.3. Analysis of Multiplication Rate

Multiplication means the ability of a plantlet that is germinated from embryo or seed to multiply. For *in vitro* plantlet multiplication, the number of multiplication observed in plantlets in each population is shown in the Table 1.FT had the highest number of plantlets (12) in its first time multiplication, followed by 10 for KK1, 8 for KK2, and 8 for KL8. In the second time and third time multiplication, FT had, again, the highest rates. As a whole, all populations showed higher plantlets in their first multiplications.

Table 1. Multiplication rate of FT, KK1, KK2, and KL8 population

	FT	KK1	KK2	KL8
V1	12	10	8	8
V2	6	5	4	3
V3	4	3	2	0

V1- First multiplication, V2- Second multiplication, V3- Third multiplication

3.4. Jar Contamination

In order to find germination and multiplication rate many jars were contaminated through embryo rescue technique. Only two bacteria and fungi contaminants were detected. Fungi were the major contaminant in FT and KK populations which polluted 85% in FT, followed by 80% in KK1 and 89% in KK2. In KL8, the major pollutant was bacteria (52%) but fungi was lower (48%) (Table 2).For FT and KK populations, bacteria were minor polluter compared to fungi. In jars polluted by fungi, there were white layers on the surface on agar, white colonies, and mycelium structures. In jars with bacteria, there were many small creamy and yellow colonies which were seen 2 to 3 days faster than fungal pollution.

Table 2. Percentage of contamination in FT, KK1, KK2, and KL8 population

	FT	KK1	KK2	KL8
Fungi (%)	85	80	89	48
Bacteria (%)	15	20	11	52

3.5. Growth rate of Banana seeds using different soil structures

In *in vivo* study, different soil (Soil, Sand, Soil + Sand) were used to increase the germination rate of banana seeds. Seeds were planted in trays with different soil in greenhouse.Mixture of soil + sand had the highest number of germination (152 seeds from 2000) that devoted 7.6% and the lowest rate occurred when seeds were cultured in sand which had 1.1% germination (22 seeds from 2000) (Fig.7).

In *in vivo* study, two approaches were conducted to increase germination rate, including: Water Bath and Scratching or Cracking to the seeds coat.

Fig. 7 Germination rate of seeds in different soil structures

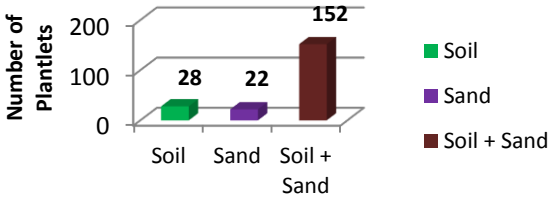


Figure 8. Jars contain *in vitro* plantlets

Figure 9. Example of *in vitro* plantlets roots and shoots

Figure 10. Fungal contaminations in embryo culture

Figure 11. Example of wild banana *Musa acuminata ssp. malaccensis*

Figure 12. Seeds gained from the fruit

Figure 13. Mixture of soil and sand

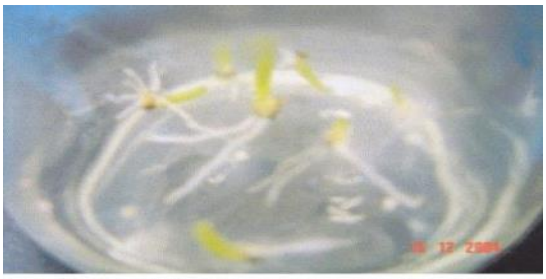


Figure 14. Embryos germinated in a jar, produced roots and shoots

Figure 15. Seeds germinated in a tray in greenhouse

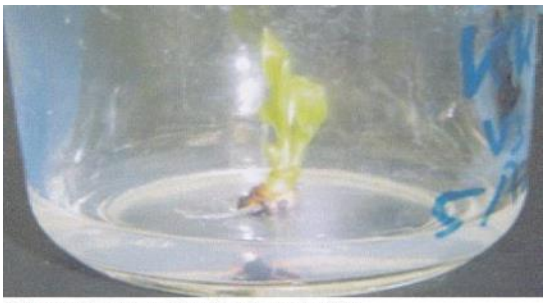


Figure 16. Example of *in vitro* plantlet

Figure 17. Example of *in vivo* plantlets

Figure 18. Plantlets are ready to be sub-cultured

Figure 19. Died *in vivo* plantlets due to external factors

Figure 20. Height measurement of *in vitro* plantlet

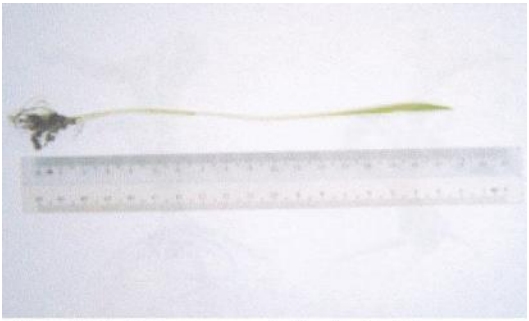


Figure 21. Height measurement of *in vivo* plantlet

Table 3. Growth rate of *in vitro* and *in vivo* plantlets

Day(s)	*In vitro*	*In vivo*
0	0	0
10	65	0
20	84	0
30	100	0
40	100	45
50	98	92
60	97	100
70	95	85
80	94	64
90	94	28

3.6. Growth rate comparison between *in vivo* and *in vitro* plantlet

Average of 100 plantlets that were germinated from seeds were observed and recorded from 0 to 90 days. The growth rate of 100 wild banana plantlets in 90 days is shown (Table 3). Results demonstrated that embryos germinated faster in *in vitro* than those of *in vivo*. In 10 days, 65 plantlets were produced through embryo rescue method while no germination occurred in *in vivo* technique. This trend was also the same on the day of 20 for *in vivo* method as no germination was seen, whereas, 84 plantlets were observed in jars. Until the day of 30, there was no germination for *in vivo* method yet, but after 40 days, 45 seed were germinated. It took 60 days for seeds to germinate thoroughly (100 plantlets) in *in vivo* method. On the day of 70, 5 plantlets were died in *in vitro*, but in *in vivo* 15 plantlets were lost. After 90 days, plantlets in tray decreased to 28 but in *in vitro*, they had 94 plantlets. As a whole, plantlets in *in vitro* were more stable than those in *in vivo*. Figures 8 to 21show various details about materials, methods, and estimations in this study.

4. Discussions

4.1. *In vitro* Seed Germination

Germination means the embryos have germinated and produced roots and shoots. Swell is a state occurred in certain embryos, after the swelling state the embryos developed into plantlet and produced roots and shoots. Observation of the experiment showed that the embryos can be classified into three states whether they were germinated, swell, or death. From overall result of all *in vitro* seeds germination experiments, for every population, the percentage of germination was higher than the swell and dead embryos. This was due to the good media and technique in culturing embryos. From the results gained, the highest germination rate was observed in KK1 population which was 63%. However, according to earlier studies by Asif et al. (2001), *in vitro* embryo culture increased the germination rate up to 90% and above. Youmbi et al. (2011) claimed that with every increase in concentration of saccharose, germination rate was decreased. Furthermore, they found that the ideal temperature for germination of *Musa acuminata* was 30^0 C. In this study, the low percentage of germination rate was due to contamination, improper sterilization and inexperience in performing embryo culture technique. The germination and growth of the *in vitro* grown embryos were affected by the media

composition and culture condition. Contaminations were observed in each population of embryo culture jars. Fungi and bacteria were the main contaminants in embryo culture of wild banana *Musa acuminata* ssp. *malaccensis*. About more than 30% of contaminated jars were observed from *in vitro* experiments and pollutions affected embryo rescue results badly. From the experiment, in FT and KK populations, fungi were the major contaminant compared to KL population which was bacteria. Fungi contaminated 85% of all jars in FT population, 80% in KK1 and 89% in KK2. Bacteria were found in 52% of all contaminated KL8 jars while fungi were 48%.

4.2. *In vivo* Seed Germination

Germination rate of seeds observed in 60 days was low, 5%. This result was quite the same as reported byAsif et al. (2001). Seed germination of wild*Musa* was found to be difficult to achieve under natural conditions. Vuylsteke and Swennen (1993) reported that low seed germination percentage of wild *Musa* was due to malformed and shriveled embryos, absence of endosperm, seed coat to be softer, and embryo was missing even if the endosperm and chalaza mass were fully developed and the testa had normal hardness. Result from the seeds in water bath experiment showed that only 30 seeds were germinated in about 2 months. In the control experiment only 23 seeds were germinated. The results were low and water bath technique was ineffective in increasing germination rate or less effective in shortening the germination time. This result was different from the findings of Afele and De Langhe (1991) who observed increased embryo germination after soaking the seeds in water for different periods of time. However, Simmonds (1952) and Stotzkyet al. (1962) reported that seed soaking was either deleterious or inefficient for wild *Musa* germination and there was no evidence of embryo dormancy in *Musa acuminata*. However, the low seed germination under natural conditions could be affected by several other factors. Seed coat has been reported to be a major barrier in seed germination (Bhat et al., 1994; Graven et al., 1996). Therefore, experiment has been done in banana seeds coat in order to prevent it from affecting germination of seeds. Labadie (1978) suggested that lightly cracking the seeds before planting can increase the germination. When seeds were given scratch, the result was interesting because more germination was observed and germination process was fast. It can be concluded that seed scarification can promote the number of seeds germinated up to 6 times compared to normal germination. Experiments also conducted in different soil mixture which had effects on germination. The result showed that there was agermination increment in seeds in soil + sand mixture compared to soil or sand. This was because the mixture of soil and sand produced deep and well-drained soil. Light sandy soil required considerable mulching to improve water retention and nutrients quickly launched from this type of soil (Butterfield, 1967).

4.3. Multiplication Rate

Only plantlets produced from *in vitro* technique can multiply whereas *in vivo* plantlets cannot multiply and remain as a single plantlet. Therefore, experiment was only done in *in vitro* plantlets. Plantlets with many branches or more than one stem were used for multiplication with the use of sub-culturing method. FT population had the highest number of plantlets that were able to multiply for the first time multiplication (V1), second time (V2), and third time (V3). This happened because the first experiment of embryo culture was conducted on FT and followed by KK1, KK2, and KL8. Sub-culturing process was first done in FT population and the number of V1, V2, and V3 plantlets was more than those of KK1, KK2, and KL8.

4.4. Growth Rate of *in vitro* and *in vivo* Plant

Embryo in *in vitro* culture was germinated faster than *in vivo* seeds to produce plantlets. Results showed that within 10 days, 65 embryos were germinated in *in vitro* culture compared to *in vivo* seeds which were germinated after 30 days. Seed coat has been reported to be a major barrier in seed germination (Bhat et al., 1994; Graven et al., 1996). Wild banana *Musa acuminata* ssp.*malaccensis* seeds were very hard and took long time to be broken and this issue made germination very slow. On 90 th day observation, there were still 94 *in vitro* plantlets and 28 *in vivo* plantlets remained. It can be concluded that survival rate of *in vivo* plantlets were lower than *in vitro*. The death of *in vivo* plantlets was because of sunlight, wind, and water. They died because of poor sunlight. They also had very thin stem and weak roots compared to *in vitro*. This made them to die when they were exposed to wind and the lack of water easily disrupted them.

4.5. Fusarium Screening

In this study, experiment on Fusarium screening could not be done completely due to the culture contaminations and the lack of time to grow wild banana plant *Musa acuminata* ssp.*malaccensis* with the height of 10 to 15 cm. Some of the plants produced from *in vivo* and *in vitro* methods died before they reach 10 cm height and this was insufficient for differential disease symptom expression. Fusarium wilt disease usually takes 4 to 5 months to be expressed on banana plants.

5. Conclusions

Banana is an important food and income source for local and international market. Local banana production mainly comprises Cavendish bananas, PisangBerangan, Pisang Mas, PisangRastali, PisangNangka, Pisang Abu, and Pisang Tandok. However, banana production and area found to be decreasing for the past few years due to outbreak of diseases (Masdek et al., 2003). The most important disease threatening banana industry is Fusarium wilt caused by *Fusarium oxysporum f.* sp.*Cubense*. Fusarium wilt is widely distributed in Malaysia and majority of cultivars are reported to be sensitive (Ho et al., 1994). There is no chemical and cultural control and the only way is to produce tolerant cultivars. The wild diploid progenitor *Musa acuminata* is highly fertile and reported to be highly resistant to Fusarium wilt (Vakili, 1965). Therefore, embryo rescue of wild banana seeds should be useful in producing resistant plants. For the germination rate, *in vitro* plants were better than *in vivo* method. Multiplication can only be seen in experiment on germination of wild banana plant using *in vitro* method. Plantlets produced from embryo culture method were better than those created from greenhouse as they were more stable and lived longer. Seed scarification increased germination up to 6 times compared to non-scarification in germination. The mixture of soil + sand gave the best germination rate with the use of *in vivo* method. In contrast, water bath technique was not effective in germination of banana seeds. Fusarium screening is an important way to select resistant plants to Fusarium wilt. For the future studies, screening method can be carried out in other plants and other fungi species to detect tolerance to various types of fungi species.

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