EVALUATION OF DIFFERENT ACCLIMATIZATION METHODS OF *IN VITRO* BANANA (*Musa acuminata)* PLANTLETS

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EVALUATION OF DIFFERENT ACCLIMATIZATION METHODS OF *IN VITRO* BANANA (*Musa acuminata*) PLANTLETS

A thesis presented by

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to

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The thesis attached hereto, entitled **"Evaluation of Different Acclimatization Methods of *In Vitro* Banana (*Musa acuminata*) Plantlets"** was prepared under the direction of chairperson of the candidate's supervisory committee and has been approved by all members of that committee and board of examiners as a partial fulfillment of the requirements for the degree of **MASTER OF AGRICULTURAL SCIENCE (HORTICULTURE AND AGRICULTURAL BIOTECHNOLOGY).**

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DECLARATION OF ORIGINALITY

This thesis represents the original work of the author, except where otherwise stated. It has not been submitted previously for a degree at any University.

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DEDICATED TO MY BELOVED PARENTS,

U SHANE LINN AND DAW TIN HTWE

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Evaluation of Different Acclimatization Methods of *In Vitro* Banana (*Musa acuminata*) Plantlets

ABSTRACT

Three sets of experiments were involved in this study. They were carried out at the Plant Tissue Culture Laboratory, Department of Horticulture and Agricultural Biotechnology, Yezin Agricultural University from December, 2012 to July, 2014.

Experiment I was aimed at investigating the effect of different substrates in acclimatization stage on micropropagated banana plantlets. Randomized Complete Block (RCB) design was employed with three replications. The treatments were sand:garden soil, sand:compost, sand:burnt rice husk, sand:vermicompost, sand only, sand:garden soil+arbuscular mycorrhizal fungi (AMF), sand:compost+ AMF, sand:burnt rice husk+AMF, sand:vermicompost +AMF and sand+AMF. In Experiment II, effects of sugar concentrations and agar levels on the *in vitro* acclimatization were studied on the *ex vitro* acclimatization of micropropagated banana plantlets. Two factors factorial experiment in RCB design was used with 30 replications. Three levels of sugar concentrations (10, 20, 30 $g.L^{-1}$) and three levels of agar medium (3, 5, 7 $g.L^{-1}$) were studied. In Experiment III, the effect of pre-treatments on acclimatization of *in vitro* grown banana plantlets were studied. RCB design with three replications was used. The treatments were control (water), homai, ferti-start, atonik, moralmone, ferti-start+ homai, atonik+ homai, moralmone +homai.

Among different substrates in acclimatization stage, the combination of sand + burnt rice husk +AMF provided the highest values in number of leaves, leaf area, fresh weight and dry weight of micropropagated banana plantlets. Sand only substrate gave the highest survival percentage. Among the sugar concentrations and agar levels in *in vitro* hardening, 30 $g.L^{-1}$ of sugar and 3 $g.L^{-1}$ of agar gave the maximum values in most parameters studied. The maximum survival percentage was observed on 30 $g.L^{-1}$ sugar + 3 $g.L^{-1}$ agar and 30 $g.L^{-1}$ sugar + 5 $g.L^{-1}$ agar in *in vitro* acclimatization to *ex vitro* condition. According to pre-treatments in acclimatization, the plantlets treated with homai gave the highest results in most parameters.

Thus, it can be concluded that substrates which have good aeration are more important for micropropagated plantlets during the acclimatization period. 30 $g.L^{-1}$ of sugar and 3 $g.L^{-1}$ of agar are the best sugar concentration and agar level for micropropagated banana plantlets during *in vitro* acclimatization. Homai should be used as a pre-treatment on acclimatization stage of *in vitro* grown banana plantlets.

Key words: Acclimatization, Banana, Substrates, Arbuscular mycorrhizal fungi, Sugar, Agar, Pre-treatments

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Ministry of Agriculture and Irrigation

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Evaluation of Different Acclimatization Methods of *In Vitro* Banana (*Musa acuminata*) Plantlets

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Key words: Acclimatization, Banana, Substrates, Arbuscular mycorrhizal fungi, Sugar, Agar Pre-treatments

1. INTRODUCTION

Bananas and plantains are among the most important crops in the world representing a major staple food for millions of people in developing countries. They contribute to food security by producing fruit year-around and provide incomes to rural populations (Roux et al. 2008). Banana globally ranks as the fourth major crop after rice, wheat and maize and is considered as a poor man's crop in tropical and subtropical countries (Jain and Swennen 2004). The top five banana-producing countries are India, Equador, Brazil, Uganda, and the Philippines. According to FAO (2011), banana is grown in around 150 countries across the world approximately 5 million hectares producing 95 million tons.

In Myanmar, Banana is one of most important and common fruits and it can be grown throughout the country. The total banana production area is 179, 946 acres in Myanmar. It accounts for 11.64 % of total fruit sown area. The productivity of banana is 1, 059 bunches per acre (DOA 2011). Banana can be propagated by suckers and tissue culture techniques. *In vitro* propagation is a fastest and more efficient way of production than that by suckers. I*n vitro* banana can be multiplied the whole year-round with a large scale of virus free plantlets. However, one of the greatest challenges faced by this technique is to overcome the factors affecting growth and survival of the plantlets following the *in vitro* to the *ex vitro* conditions. An acclimatization process before transfer to the nursery is required to improve survival and growth of the plantlets (Schultz 2001). Perhaps the most widespread and certainly significant mutualism between plants and fungi is the root symbiosis, termed arbuscular mycorrhiza (AM). These fungal endosymbionts are nearly universal in their association with flowering plants including agriculturally important crop species (banana and plantain, papaya, tomato, grapes, avocado, tropical pineapple, etc.) (Jeffries and Dodd 1991). Mycorrhizal association enhances plant growth and productivity by increasing nutrient element uptake (Al-Karadi 2002). *In vitro* hardening is the important step and it can prevent transplantation loss and increase survival percentage (Short et al. 1987). The growth and multiplication of shoots *in vitro* are affected by many factors, one of which is the type of exogenous carbon source added to the medium (ILL-Wan and Korban 1998). The carbon sources serve as energy and osmotic agents to support the growth of plant tissues (Lipavska and Konradova 2004).

2. MATERIALS AND METHODS

2.1 Experimental Site and Procedure

The experiment was carried out at the Plant Tissue Culture Laboratory, Department of Horticulture and Agricultural Biotechnology, Yezin Agricultural University (YAU) from December, 2012 to July, 2014. Micropropagated banana plantlets (*Musa acuminata* cv. Thi-hmwe (khon-war)) obtained from VFRDC (Vegetables and Fruits Research and Development Center), Department of Agriculture were used as test cultivars.

2.1.a Experiment 1: Effect of Different Substrates on Acclimatization of *In Vitro* Propagated Banana Plantlets

The experiment was laid out in Randomized Complete Block (RCB) design with three replications. Fifteen plantlets were used in each treatment. Different types of substrates were used in this experiment. The treatments were as follows: T_1- Sand : Garden soil (1:1) (v/v), T_2 - Sand : Compost (1:1) (v/v), T_3 - Sand : Burnt rice husk (1:1) (v/v), T_4 - Sand : Vermicompost (1:1) (v/v), T_5 - Only sand (v/v), T_6 - Sand : Garden soil (1:1) + Arbuscular mycorrhizal fungi (AMF) (v/v), T_7 - Sand : Compost (1:1) + AMF (v/v), T_8 - Sand : Burnt rice husk (1:1) + AMF (v/v), T_9 - Sand : Vermicompost (1:1) + AMF (v/v) and T_{10} - Sand + AMF (v/v). Healthy and uniform rooted plantlets with 3 to 4 leaves were selected from *in vitro* cultures and washed with tap water to remove residues of agar from the roots. Plantlets were transplanted to plastic box containing sterile substrate mixture for two weeks as the primary hardening under the shade house. Then the plantlets were shifted to the individual plastic bag and covered with a thin plastic film to maintain the moisture for one week. After that, plastic films were removed and plantlets were kept under the greenhouse as a secondary hardening. The plantlets were treated with arbuscular mycorrhizal fungi (AMF) during the secondary hardening. The AMF was used at the rate of 5 g per 1 kg of potting substrates.

2.1.b Experiment 2: Effect of Sugar Concentrations and Agar Levels in *In Vitro* Acclimatization of Banana Plantlets

This experiment was carried out to determine the effect of sugar concentrations and agar levels in *in vitro* acclimatization of banana plantlets. The Murashige and Skoog's medium was used as a basal medium. The experimental design was Randomized Complete Block (RCB) design with two factors factorial arrangement. The Factor (A) - Three levels of sugar concentration (10, 20, 30 $g.L^{-1}$) and the Factor (B) - Three levels of agar concentration

(3, 5, 7 $g.L^{-1}$) were used. The pH of the medium was adjusted to 5.8 before autoclaving and 40 ml of media was dispended in each culture bottle and these were autoclaved at 121°C for 15 minutes. Two healthy and uniform plantlets were placed in each bottle and these culture bottle were kept at 20 ± 2 °C temperature for four weeks as *in vitro* hardening. After four weeks in *in vitro* hardening, the plantlets were removed from culture bottles and washed thoroughly with tap water to remove agar. For the primary hardening, the plantlets were transferred to the plastic box containing sterile substrate mixture for two weeks. After that, the plantlets were transplanted individually in each plastic bag and covered with plastic film under the shade house for one week. For the secondary hardening, the plantlets were transfered to the greenhouse condition with the relative humidity of 65 --85 percent and the temperature range of 24 35°C.

2.1.c. Experiment 3: Effect of Various Pre-treatments on *In Vitro* Propagated Banana Plantlets during Acclimatization

Potting mixture (sand: garden soil: burnt rice ash, 1:1:1 by volume) was used as the hardening medium. Randomized Complete Block (RCB) design with three replications was used. The treatments were as follows: T_1 - Control (water), T_2 - Homai (1%), T_3 - Ferti-start (1%), T_4 - Atonik (1%), T_5 - Moralmone (1%), T_6 - Ferti-start (1%) + Homai (1%), T_7 - Atonik (1%) + Homai (1%) and T_8 - Moralmone (1%) + Homai (1%). Well rooted plantlets were carefully removed from culture vessels and they were washed with tap water to remove agar. Then, the plantlets were dipped quickly in each solution according to the treatments. For the control treatment, the plantlets were treated with distilled water only.

2.2 Data Collection

The following data were collected for all experiments: plant height (cm), number of leaves, Leaf area (cm^2), Root length (cm), Pseudo-stem diameter (cm), Shoot fresh weight (g), Root fresh weight (g), total fresh weight (g), shoot dry weight (g), root dry weight (g), total dry weight (g) and survival (%).

2.3. Statistical analysis

Analysis of variance was performed using Gemstat Version 9.0. Treatment means were compared by LSD at 5 %.

3. RESULTS AND DISCUSSION

3.1. Experiment 1: Effect of Different Substrates on Acclimatization of *In Vitro* Propagated Banana Plantlets

3.1.a Plant height

According to the results in Table 4.1, it was observed that substrate (sand only) and substrate (sand+ compost) showed maximum plant height (17.00 cm and 16.80 cm, respectively) followed by sand + burnt rice husk + AMF (16.40 cm) which was not statistically different from other substrates. The minimum plant height (13.00 cm) was observed from substrate (sand + vermicompost + AMF). Sand + compost and sand only substrates were significantly different from sand + vermicompost + AMF except other substrates. These data indicated that different types of substrates have a strong influence on plant height of micropropagated banana plantlets. Plantlets derived from micropropagation cannot absorb nutrients from the soil substrates in the early stage of acclimatization because they try to be adapted to the surrounding environment. In this study the reason why plant height was lower might depend on the type of substrates and the amount of AM fungi application. The more pore space and better aeration of substrate can give the better plant performance. The findings are in line with the following researchers.

3.1.b Number of leaves

Number of leaves per plantlet as affected by different substrates is described in Table 4.1. The highest number of leaves (5.67) was observed from substrate (sand + AMF) which was not statistically different from sand + burnt rice husk + AMF (5.33) and sand + garden soil + AMF (5.33) and followed by sand only (5.00), sand + burnt rice husk (5.00), and sand + compost (4.67). The lowest number of leaves (3.00) was observed from substrate (sand + garden soil), which was not statistically different from sand + vermicompost (3.00). Sand + garden soil + AMF, sand + burnt rice husk + AMF and sand + AMF were significantly different from sand + garden soil, sand + vermicompost and sand + vermicompost + AMF except other substrates. These results indicated that the number of leaves was influenced by the different types of substrates. Substrates containing AMF increased the number of leaves of micropropagated banana plantlets than the ones without AMF.

3.1.c Root length

As described in Table 4.1, significant effect of different substrates was observed in root length. The highest root length (15.87 cm) was observed in sand substrate followed by sand + garden soil (8.83 cm) and sand + compost (8.77 cm) which were not statistically different from other substrates. The lowest root length (6.33 cm) was observed from substrate (sand + vermicompost + AMF). Sand only substrate was significantly different from other substrates. These results stated that the root length of micropropagated banana plantlets was strongly influenced by different types of substrates during acclimatization period.

3.1.d Pseudo-stem diameter

The effect of different substrates on pseudo-stem diameter is shown in Table 4.1. The only sand substrate showed the highest pseudo-stem diameter (0.47 cm) followed by sand + garden soil + AM fungi (0.43 cm), sand + burnt rice husk + AM fungi (0.42 cm) and sand + burnt rice husk (0.40 cm). The lowest pseudo-stem diameter (0.13 cm) was observed in substrate (sand + vermicompost). Sand only substrate was not significantly different from sand + burnt rice husk, sand + garden soil + AMF and sand + burnt rice husk + AMF except other substrates. The results showed that the pseudo-stem diameter of micropropagated banana plantlets was highly influenced by different substrates and the positive effect of AM fungi in the acclimatization stage could be seen.

3.1.e Survival percentage

The survival percent of *in vitro* grown banana plantlets as affected by different substrates is summarized in Figure 4.1. The highest survival percent (87.5 %) was observed from the treatment (sand without AM fungi) and the second highest survival percent (75 %) was found in sand + burnt rice husk (1:1) without AM fungi, sand + garden soil (1:1) with AM fungi and sand + vermicompost (1:1) with AM fungi. The lowest value (37.5 %) was resulted from sand + compost (1:1) with AM fungi. These results indicated that different substrates and AMF application influenced the survival percentage of micropropagated banana plantlets at 45 days after deflasking during acclimatization stage.

3.2 Experiment 2: Effect of Sugar Concentrations and Agar Levels in *In Vitro* Acclimatization of Banana Plantlets

3.2.a Plant height

Table 4.2 demonstrates the effect of sugar concentrations and agar levels on plant height of *in vitro* acclimatization of micropropagated banana plantlets. The results indicated that different sugar concentrations showed a significant difference on plant height at 1% level. The highest plant height (36.99 cm) was resulted from 30 $g.L^{-1}$ and the lowest plant height (32.19 cm) from 20 $g.L^{-1}$. The treatment 30 $g.L^{-1}$ of sugar was statistically different from 20 $g.L^{-1}$ and 10 $g.L^{-1}$ of sugar. These results indicated that sugar concentrations have a strong influence on plant height of micropropagated banana plantlets. The higher the sugar concentration, the greater the plant height. Significant difference of plant height was observed among the agar levels (Table 4.2). According to different agar levels, 3 $g.L^{-1}$ treatment had the highest plant height (37.66 cm) and the lowest plant height (30.93 cm) was recorded from treatment (7 $g.L^{-1}$), which was not significantly different from treatment (5 $g.L^{-1}$). These results indicated that the plant height of micropropagated banana plantlets was highly influenced by agar levels. As the agar level was increased in the medium (from 3 $g.L^{-1}$ to 7 $g.L^{-1}$), there was a decrease in plant height (from 37.66 cm to 30.93 cm).

3.2.b Number of leaves

The effect of different sugar concentration on number of leaves was not significant (Table 4.5). The maximum number of leaves (8.33) was obtained from the treatment (30 $g.L^{-1}$) and the minimum number of leaves (7.67) was found in treatments 10 $g.L^{-1}$ and 20 $g.L^{-1}$. According to this finding, the number of leaves of micropropagated plantlets was not highly influenced by increasing or decreasing sugar concentrations in the medium. Number of leaves per plantlet was not significantly affected by different agar levels. Although the treatment (3 $g.L^{-1}$) gave the maximum result (8.33), it was not significantly higher than those of 5 $g.L^{-1}$ and 7 $g.L^{-1}$. It also means that the number of leaves of micropropagated banana plantlets was not altered by the effect of different agar levels from *in vitro* to *ex vitro* condition.

3.2.c Shoot fresh weight

Shoot fresh weights (g) from different sugar concentrations are shown in Table 4.2. Shoot fresh weight (g) was significantly different at 1 % level. The highest shoot fresh weight (16.92 g) resulted from treatment (30 $g.L^{-1}$) and the lowest (11.38 g) from treatment (20 $g.L^{-1}$). Treatment 30 $g.L^{-1}$ of sugar was significantly different from 10 $g.L^{-1}$ and 20 $g.L^{-1}$ of sugar. It was found that different sugar concentrations had a strong influence on shoot fresh weight of micropropagated banana plantlets from *in vitro* to *ex vitro* condition.

No significant difference in shoot fresh weight (g) was observed in different agar levels. Maximum shoot fresh weight (14.53 g) was obtained from treatment (3 $g.L^{-1}$) and the minimum shoot fresh weight (12.74 g) from treatment (5 $g.L^{-1}$). These results indicate that the shoot fresh weight was not influenced by the different agar levels.

3.2.d Root fresh weight

Results of root fresh weight (g) are presented in Table 4.2. Although the effect of different sugar concentrations on root fresh weight was not statistically different, the maximum root fresh weight (4.39 g) was found in treatment (10 $g.L^{-1}$) and the minimum (3.57 g) from treatment (20 $g.L^{-1}$). These results showed that sugar concentrations did not influence root fresh weight of micropropagated banana plantlets during in *in vitro* acclimatization.

Among the different agar levels, there was no significant difference in root fresh weight (g). Maximum root fresh weight (4.9 g) was observed from treatment (7 $g.L^{-1}$) and minimum fresh shoot weight (3.26 g) was collected from treatment (5 $g.L^{-1}$). These results also indicated that there was no influence of different agar levels on root fresh weight of banana plantlets from *in vitro* to *ex vitro* condition.

3.2.e Survival percentage

Figure 4.2 shows the effect of different sugar concentrations and agar levels on survival percentage of *in vitro* grown banana plantlets. The maximum survival percentage was observed from 30 $g.L^{-1}$ sugar + 3 $g.L^{-1}$ agar and 30 $g.L^{-1}$ sugar + 5 $g.L^{-1}$ agar from *in vitro* acclimatization to *ex vitro* condition. It can be clearly seen that the survival percentage

of micropropagated banana plantlets was influenced by different sugar concentrations and agar levels of micropropagated banana plantlets from *in vitro* to *ex vitro* condition. The higher external sucrose concentrations in culture media could promote survival and better growth performance of micropropagated plantlets through *in vitro* acclimatization to *ex vitro* condition. According to this finding, the minimum agar level (3 $g.L^{-1}$) gave the maximum survival percentage. High survival percentage was due to concomitant hardening along with rooting and no damage to the root system. It may be concluded that the combination of high sugar concentration and low level of agar gelled medium can be more preferable for *in vitro* hardening of banana plantlets and could be used for other economically important species, when high levels of agar are suspected to have inhibitory effects.

3.3 Experiment 3: Effect of Various Pre-treatments on *In Vitro* Propagated Banana Plantlets during Acclimatization

3.3.a Plant height

Table 4.3 shows the effect of various pre-treatments on plant height of *in vitro* banana plantlets. There was significant effect of various pre-treatments on plant height of micropropagated banana plantlets at 5 % level. The maximum plant height (35.20 cm) was observed from Homai, followed by Moralmone (31.80 cm), Ferti-start + Homai (30.20 cm) and Control (water) (21.60 cm). The minimum plant height (22.20 cm) was found in Atonik, which was followed by Ferti-start (22.40 cm) and Moralmone + Homai (22.60 cm). Homai was significantly different from Atonik + Homai, Moralmone + Homai, Ferti-start and Atonik except Moralmone, Ferti-start + Homai and Control. The other pre-treatments except Homai did not show their effects in most parameters during the acclimatization period because the roots of *in vitro* derived plantlets are non-functional and they cannot absorb nutrients. The plantlets have to try to be adapted to the surrounding environment. Homai can prevent the infection of diseases caused by adhering agar (even after thorough washing with water) around the roots and it can make the strengthening of banana plantlets during short duration. The banana plantlets derived from *in vitro* were delicate because of poorly developed cuticle, poor stomatal activity, limited mesophyll and plenty of intracellular cavities which could lead excessive transpiration and poor plant development.

3.3.b Number of leaves

Table 4.3 shows the effect of various pre-treatments on number of leaves of *in vitro* banana plantlets. There was a significant difference in number of leaves of micropropagated banana plantlets among various pre-treatments at 1 % level. The highest number of leaves (8.00) was obtained from Homai followed by the treatment Control (water) (7.60) and the lowest number of leaves (5.33) was found in Atonik. Homai was significantly different from other pre-treatments except Control. It can be seen that the pre-treatment (Homai) influence the number of leaves of micropropagated banana plantlets during the acclimatization period.

3.3.c Survival percentage

The effect of various pre-treatments on survival percentage of *in vitro* grown banana plantlets was shown in Figure 4.3. Among the various pre-treatments, Homai and Control (water) gave the highest survival percentage (92 %) at 45 days after transplanting during the acclimatization stage. The second highest result (84 %) was obtained from Moralmone and Moralmone + Homai. The third highest result (80 %) was observed from Ferti-start + Homai and Atonik + Homai. The lower survival percentage (76 %) was found from Atonik. The lowest survival percentage is 72 % from the treatment of Ferti-start. According to these findings, the survival percentage of micropropagated banana plantlets was influenced by different pre-treatments at 45 days after transplanting during the acclimatization period.

Table 4.1 Effect of different substrates on plant height and number of leaves of *in vitro* banana plantlets at 45 days after deflasking

Treatments	Plant height(cm)	Number of leaves	Root length(cm)	Pseudo-stem dia.(cm)
Sand+garden soil	14.93 ab	3.00 c	8.83 b	0.18 de
Sand+compost	16.80 a	4.67 ab	8.77 b	0.27 cd
Sand+burnt rice husk	15.67 ab	5.00 ab	6.60 b	0.40 ab
Sand+vermicompost	13.60 ab	3.00 c	7.67 b	0.13 e
Sand only	17.00 a	5.00 ab	15.87 a	0.47 a
Sand+gardensoil+AMF	15.10 ab	5.33 a	8.33 b	0.43 ab
Sand+compost+AMF	16.00 ab	4.33 abc	8.33 b	0.27 cd
Sand+Burntricehusk+AMF	16.40 ab	5.33 a	7.13 b	0.42 ab
Sand+vermicompost+AMF	13.00 b	3.67 bc	6.33 b	0.25 cd
Sand+AMF	14.00 ab	5.67 a	8.13 b	0.33 bc
$LSD_{0.05}$	3.54	1.59	4.26	0.11
Pr>F	0.29	<0.02	<0.01	<0.001
CV%	32.00	20.60	28.90	20.40

Means followed by the same letter in each column are not significantly different at 5% LSD.

Table 4.2 Effect of different sugar concentrations and agar levels on plant height and number of leaves of micropropagated banana plantlets at 45 days after deflasking

Treatments	Plant height (cm)	Number of leaves	Shoot fresh weight (g)	Root fresh weight (g)
Sugar				
10 $g.L^{-1}$	30.47 b	7.67	12.34 b	4.39 a
20 $g.L^{-1}$	32.19 b	7.67	11.38 b	3.57 a
30 $g.L^{-1}$	36.99 a	8.33	16.92 a	4.32 a
$LSD_{0.05}$	3.92	1.18	2.97	1.47
Agar				
3 $g.L^{-1}$	37.66 a	8.33	14.53 a	4.12 ab
5 $g.L^{-1}$	31.06 b	8.11	12.74 a	3.26 b
7 $g.L^{-1}$	30.93 b	7.22	13.37 a	4.90 a
$LSD_{0.05}$	3.92	1.18	2.97	1.47
Pr>F				
Sugar	<0.01	0.41	<0.002	0.44
Agar	<0.003	0.14	0.45	0.09
Sugar × Agar	0.09	0.94	0.05	0.79
CV%	11.80	15.00	21.90	35.90

Means followed by the same letter in each column are not significantly different at 5% LSD.

Table 4.3 Effect of pre-treatments on plant height and number of leaves of *in vitro* banana plantlets at 45 days after deflasking

Treatments	Plant height (cm)	Number of leaves
Control	27.60 abc	7.60 ab
Homai	35.20 a	8.00 a
Ferti-start	22.40 c	5.47 cd
Atonik	22.20 c	5.33 d
Moramone	31.80 ab	5.93 cd
Ferti-start+Homai	30.20 abc	6.60 bc
Atonik+Homai	25.10 bc	6.07 cd
Moramone+Homai	22.60 c	6.40 cd
$LSD_{0.05}$	8.88	1.14
Pr>F	<0.05	<0.002
CV%	18.70	10.10

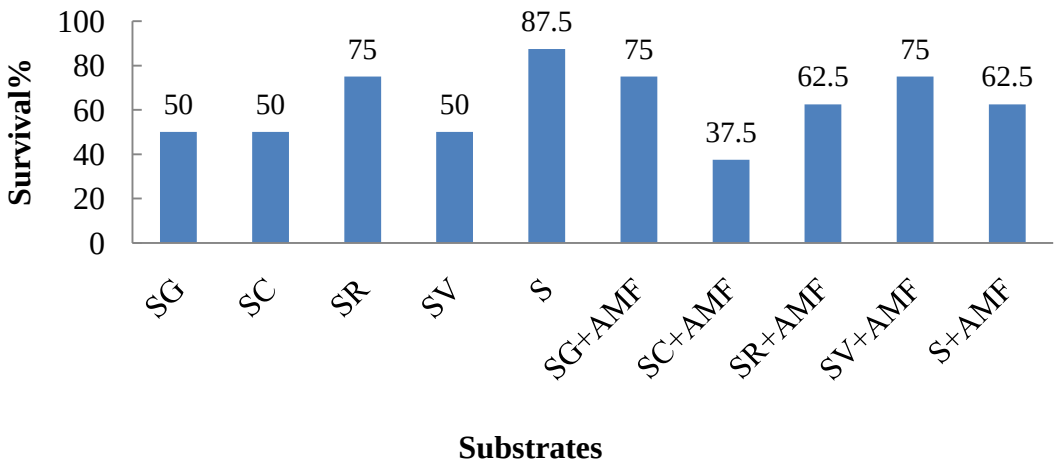

Figure 4.1 Effect of different substrates on survival percentage of *in vitro* banana plantlets at 45 days after deflasking during acclimatization period (SG = sand+garden soil, SC = sand+compost, SR = sand+burnt rice husk, SV = sand+vermicompost, S = sand only, AMF = arbuscular mycorrhizal fungi

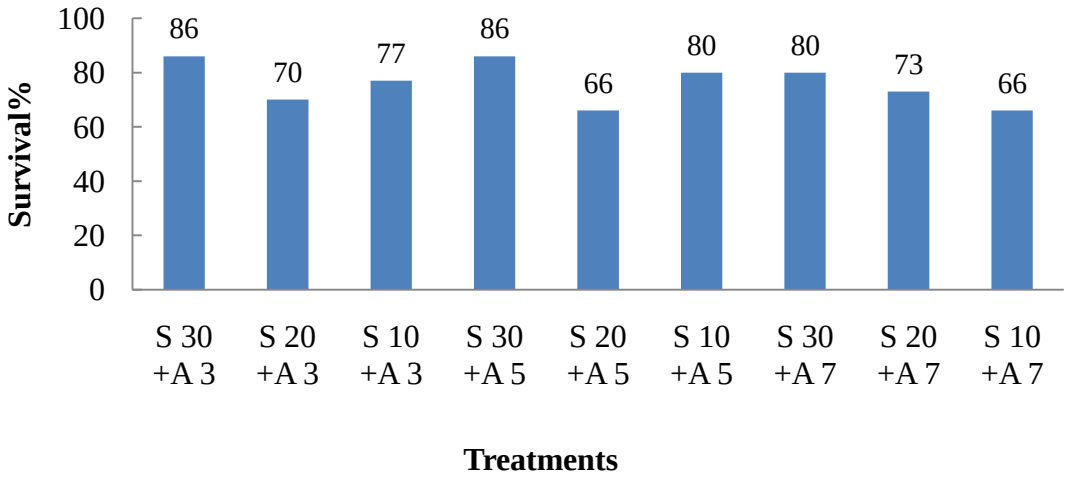

Figure 4.2 Effect of different sugar concentrations and agar levels on survival percentage of micropropagated banana plantlets at 45 days after deflasking during acclimatization period (S = sugar concentration, A = agar levels, $g.L^{-1}$)

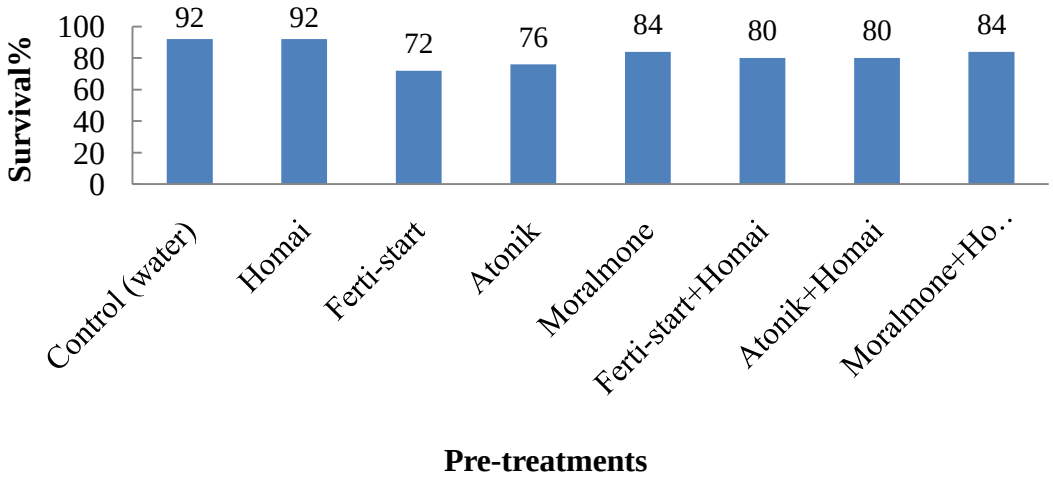

Figure 4.3 Effect of different pre-treatments on survival percentage of micropropagated banana plantlets at 45 days after deflasking during acclimatization period

4. CONCLUSIONS

According to the results of experiment I, the plantlets treated with AM fungi gave good growth performance in most parameters. Among the different substrates in the acclimatization stage, the combination of sand + burnt rice husk + AMF provided the highest values in number of leaves and leaf area of micropropagated banana plantlets. Sand only substrate gave the highest survival percentage at 45 days after deflasking during the acclimatization period.

According to the experiment II, among the sugar concentrations and agar levels in *in vitro* hardening, the treatment (30 $g.L^{-1}$ of sugar) gave the best results in most parameters studied. Maximum survival percentage was observed from (30 $g.L^{-1}$ of sugar + 3 $g.L^{-1}$ of agar) and (30 $g.L^{-1}$ of sugar + 5 $g.L^{-1}$ of agar) at 45 days after deflasking from *in vitro* to *ex vitro* condition. According to experiment III, among the different pre-treatments during the acclimatization period, the plantlets treated with Homai gave the highest results in most parameters.

According to the findings of this study, the substrates which have good aeration and pore spaces are more important for micropropagated plantlets during the acclimatization period. Among the different sugar concentrations and agar levels, 30 $g.L^{-1}$ of sugar and 3 $g.L^{-1}$ of agar are the best sugar concentration and agar level for micropropagated banana plantlets during *in vitro* acclimatization. Homai should be used as a pre-treatment on acclimatization stage of *in vitro* grown banana plantlets.

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INTRODUCTION

Bananas and plantains are among the most important crops in the world representing a major staple food for millions of people in developing countries. They contribute to food security by producing fruit year-around and provide incomes to rural populations (Roux et al. 2008). The banana belongs to the family *Musa* and is grown in most tropical countries. Banana originated in Assam-Burma-Indo-China region (Chakravorty 1951). It is an important food consumed world-wide and it can be eaten unripe or ripe, raw or processed (cooked, fried and roasted, etc). The fruit contains vitamins (A, B and C), minerals (calcium, potassium and iron) and has few calories (90 to 120 kcal/100 g). The fruit is approximately 70% water; the solid material is principally carbohydrates (23 to 32 g/100 g) with little protein (1.0 to 1.3 g/100 g) and fat (0.37 to 0.48 g /100 g) (FAO 2006).

Banana globally ranks as the fourth major crop after rice, wheat and maize and is considered as a poor man's crop in tropical and subtropical countries (Jain and Swennen 2004). The top five banana-producing countries are India, Equador, Brazil, Uganda, and the Philippines. According to FAO (2011), banana is grown in around 150 countries across the world approximately 5 million hectares producing 95 million tons.

In Myanmar, Banana is one of most important and common fruits and it can be grown throughout the country. The total banana production area is 179, 946 acres in Myanmar. It accounts for 11.64 % of total fruit sown area. The productivity of banana is 1, 059 bunches per acre (DOA 2011). In Myanmar, banana is used in various ways such as dessert, dehydrated banana, banana chips, banana steamed with sugar, sticky rice and coconut, fried banana and baked banana. The tip of inflorescence after fruit set (mostly male flower) is also used as a salad. In some areas, green banana is used as a traditional dish. Banana stem pith of two varieties Rakhine nget-pyaw and Hpi-gyan nget-pyaw (*Musa balbisiana*) can be used in soup for rice noodle, Myanmar's traditional favorable food. Banana is also used customarily in religious ceremonies (Sein Hla Bo 2004).

Banana can be propagated by suckers and tissue culture techniques. *In vitro* propagation is a fastest and more efficient way of production than that by suckers. I*n vitro* banana can be multiplied the whole year-round with a large scale of virus free

plantlets. However, one of the greatest challenges faced by this technique is to overcome the factors affecting growth and survival of the plantlets following the *in vitro* to the *ex vitro* conditions. Tissue-cultured plantlets have very divergent leaf anatomy and physiology (Pierik 1987) and therefore, require an acclimatization period during transition from the culture vessel to a greenhouse or field conditions.

Acclimatization process is the physiological and anatomical adjustment of the *in vitro*-raised plantlets after their transplantation into natural environment (Kumar et al. 2009). The factors controlling acclimatization are temperature, light intensity, humidity, shading, soil medium, containers, misting and fertigation etc.

Souza et al. (1997) and Hoffmann (2002) reported that banana plantlet acclimatization can be divided into two phases. In phase I, *in vitro* plantlets are transferred to controlled environments (greenhouse or box shade, under 20 to 28 ºC, 80 to 90% RH and 70% shade cloth) for a three to six-week period. In phase II, plantlets are shifted to trays, pots or bags under 50% shade, in a temperature range from 18 ºC to 34 ºC and a relative humidity higher than 75%, for gradual hardening.

During plant acclimatization under gradual high light intensity, leaves present a darker green color due to mesophyll cell differentiation and increases in pigment content (Sandoval et al. 1994). Increases in chlorophyll content were observed depending on the environmental condition during acclimatization (Pospisilova` et al. 1999). He also stated that Donnelly and Vidaver (1984), Zaffari et al. (1998) and Israeli et al. (1995) observed increasing chlorophyll with the reduction of sun light from alteration in synthesis and degradation of pigments.

In vitro hardening is the important step and it can prevent transplantation loss and increase survival percentage (Short et al. 1987). Reduced inorganic salts and sucrose promote rooting in a flowering plant *Omithogalum thyrsoides Jacq* (Nayak and Sen 1987). In *Saintpaulia ionantha*, rooting was better on low sugar containing media. Root formation is the energy-demanding process and thus exogenous supply of carbohydrate is required. However, this being the last stage of *in vitro* culture, it is important to transform the plant from heterotrophic to autotrophic mode of nutrition. The supply of exogenous sugars should be ideally reduced at this stage.

The growth and multiplication of shoots *in vitro* are affected by many factors, one of which is the type of exogenous carbon source added to the medium (ILL-Wan and Korban 1998). The carbon sources serve as energy and osmotic agents to support the growth of plant tissues (Lipavska and Konradova 2004). There have been various

opinions on the beneficial effects of various carbon sources (sucrose, fructose, glucose, table sugar, etc) on the growth of plant *in vitro*.

Heterotrophic plant growth depends on the uptake of sugar, water, and nutrients from the medium. Agar or other organic gelling agents are frequently used despite problems of mineral impurities, limited hydraulic conductance, and limited availability of solutes to the tissue and binding of toxic exudates near the tissue interface (Smith and Spomer 1995; Williams 1995; Leifert et al. 1995). Solute movement through gelled media and transfer to the plant is primarily by diffusion (Williams 1993). Uptake at the interface surface may proceed against concentration gradients at latter stages of the culture cycle when active uptake by roots and callus is likely to occur. The sealed culture vessel with high humidity limits transpiration, restricting mass flow of dissolved solutes through the xylem and intercellular space.

Plant tissue culture has become an important and advantageous tool for rapid propagation for several plant species. Although this technique has got several successful applications, there are still hurdles which limit its widespread use. One of the most critical problems is the transfer of *in vitro* plantlets to *ex vitro* conditions. This often results in high mortality rates, since plantlets have limitations to resist transplant stress (Puthur et al. 1998). Micropropagated plants have been continuously exposed to an unique microenvironment that has been selected to provide minimal stress and nearly optimal conditions for plant multiplication. These conditions lead to a phenotype which is more fragile than greenhouse-grown plants due to different anatomical, morphological and physiological factors. High mortality is often observed upon transfer to *ex vitro* conditions because the cultured plants have functionally impaired stomata and a poorly developed cuticle and root system (Wang et al. 1993). After transfer to *ex vitro* conditions, the plantlets have to correct the above mentioned abnormalities to become adapted to a natural environment. An acclimatization process before transfer to the nursery is required to improve survival and growth of the plantlets (Schultz 2001). One of the approaches to reduce the stress of acclimatization and enhance the growth of micropropagated plantlets is to inoculate with arbuscular mycorrhizal (AM) fungi (Lovato et al. 1996). The term ‘mycorrhiza’, coined by Frank (1885), refers to an association between fungi (myco) and root (rhiza) as a mutualistic symbiosis where the host plant receives mineral nutrients via fungal mycelium (mycotrophism) while the heterotrophic fungus obtains carbon compounds from the photosynthesis of the host (Mukerji et al. 2000). Perhaps the most widespread and

certainly significant mutualism between plants and fungi is the root symbiosis, termed arbuscular mycorrhiza (AM). These fungal endosymbionts are nearly universal in their association with flowering plants including agriculturally important crop species (banana and plantain, papaya, tomato, grapes, avocado, tropical pineapple, etc.) (Jeffries and Dodd 1991). Mycorrhizal association enhances plant growth and productivity by increasing nutrient element uptake (Al-Karadi 2002). They also impart other benefits to plants including enhanced enzymatic production (Adriano-Anaya et al. 2006), increased rate of photosynthesis (Wu and Xia 2006), enhancement of nitrogen fixation by symbiotic or associative N_2-fixation bacteria (Javaid et al. 1994; Antunes et al. 2006), osmotic adjustment under drought stress (Ruiz-Lozano 2003), increased resistance to pests (Whipps 2004), tolerance to various abiotic stress factors (Javaid and Bajwa 1999; Takeda et al. 2007) and improving soil aggregation (Rillig and Mummey 2006) and thus improved soil physical properties and stability.

After a period of *ex vitro* acclimatization, the weaned plants should be raised in pots in the greenhouse to obtain plants large enough to be grown in the field. As in any potted plant, the quality of transplants depends mainly on the substrate utilized. In this regards, Deoe Silva et al. (1998) stated that, for the best growth of tissue cultured banana plantlets, a potting substrate should have a good balance between its water-holding capacity and drainage characteristics, which allows the roots to obtain sufficient water without drowning. Traditionally, fully acclimatized banana plantlets are raised in sand plus peat moss (Murali and Duncan 1995). It was reported by Lee (2007) that a mix of peat, rice hull and sand increased growth rate of banana plantlet while sawdust could support their growth for two months.

Therefore, according to the above reasons, the experiments were carried out with the following objectives:

(1) To observe the effect of suitable substrates on adaptation of *in vitro* propagated banana plantlets

(2) To assess the effect of pre-treatment and

(3) the effect of *in vitro* hardening on acclimatization of banana plantlets

LITERATURE REVIEW

2.1 Banana Propagation

Banana is one of the most important fruit crop grown all over the world. It provides a valuable source of income through local and international trade (Frison et al. 1997). Banana is a crop with dual propagation abilities, sexual through seeds and asexual through suckers. Seed propagation is common in wild species, which are diploid and undergo normal meiosis, fertilatization and seed-set. The extend of seed set, germinability and dormancy depends on species. Cultivated banana were derived from two diploid (2n) parent genomes of the genus *Musa acuminata* (Malaysia) and *M. balbisiana* (India) (Stover and Simmonds 1987; Simmonds 1962; Georget et al. 2000). All cultivated commercial bananas are triploid and sterile, except a few parthenocarpic AA and AB diploids. Sucker propagation is the only natural means of their perpetuation; artificial methods of propagation include macropropagation (Singh et al. 2011). Micropropagation is the cost-effective way that can be done locally. It can rapidly multiply plantlets to distribute a new variety or replace plants in disease-affected fields.

However, expansion of banana production is limited, because of the shortage of healthy plant material availabile to the farmers. The transmission of harmful insects, nematodes and viral disease by field-grown suckers has prompted interest in the use of aseptic culture techniques. With the increasing demand and vast export potential coupled with the farmers' desire to grow *in vitro* propagated banana on a large area are becoming increasingly important (Roux et al. 2001; Ray et al. 2006). So, *in vitro* propagation appears to be an attractive system for banana, which makes it possible to get plantlets free from insects, bacteria and other microorganisms to fulfill farmers' demands (Krikorian and Cronauer 1984; Ma and Shii 1972; Vuylsteke 1998).

2.2 Micropropagation of Banana

Micropropagation is the practice of rapid multiplication of stock plant material to produce a large number of progeny plants under aseptic conditions using modern plant tissue culture methods. Application of micropropagation in banana has the following advantages: rapid multiplication, requirement of limited mother stock,

product uniformity, season independent production, agronomic advantages such as crop rotation and intercropping, etc., production of disease free planting material and high return, etc. In case of banana, micropropagation is usually carried out by the following methods:

Shoot Tip culture: Shoot tip (meristem plus a few attached leaf primordial) culture of *Musa* may be considered simple, easy, and applicable to a wide range of *Musa* genotypes. Multiplication rates range from 2 to 10 or more shoot or bud propagules per month, resulting in potential propagation rates of several thousands or millions of plants per year. Such rates are several orders of magnitude greater than those achievable through conventional propagation and the resulted plants are disease-free (Krikorian and Cronauer 1984).

Cell Suspension Culture: In banana, plant regeneration from cell suspension via somatic embryogenesis was reported (Novak et al. 1982). Somaclonal variation, which is the most serious problem in micropropagation through somatic embryogenesis, is quite low in some banana varieties. The micropropagation by cell suspension culture and somatic embryogenesis is, therefore, one of the most promising techniques for obtaining large amounts of quality plantlets with low production costs.

2.3 Characteristics of *In Vitro* derived plantlets

The micropropagated plants have been continuously exposed to a unique microenvironment that had been selected to provide minimal stress and nearly optimal conditions for plant multiplication. Plantlets grow heterotrophic, and develop within culture vessels under conditions which are characterized by a saturated atmosphere, relative low light intensity averaging of photosynthetic photon flux 12 – 70 mmol $m^{-2}s^{-1}$, relatively high and constant temperature (20 - 28 ºC), low rate of gas exchange inside the containers and high concentration of carbonhydrate and exogenous growth regulators in the medium. These factors often induce physiological, anatomical and morphological abnormalities which interfere with the acclimatization subsequent to transplanting resulting in low survival rates *ex vitro* (Ziv 1991; Puthur et al. 1998).

In vitro plantlets are invariably diminutive and much smaller than greenhouse grown plants. The percentage of water content is increased and the dry matter

accumulation per unit area is reduced compared to greenhouse grown plants. This is reflected in fragile organs with reduced mechanical support tissue and thin cell walls (Brainerd and Fuchigami 1981; Donelly et al. 1985).

The relatively low light levels and a saturated internal atmosphere promote leaves *in vitro* that anatomically resemble shade leaves (Brained and Fuchigami 1981; Lee et al. 1988; Marin and Gella 1987) and hydrophytic plant leaves (Grout and Ashton 1977). They often have reduced or no epicuticular or cuticular wax, which lack the characteristic crystalline structure and differ in chemical composition from the control plants (Grout 1975; Grout and Ashton 1977; Sutter 1985; Short et al. 1987). *In vitro* stomata have slow response time and due to an impaired function they do not close in response to stimuli such as darkness, abscisic acid application or when exposed to high levels of carbon dioxide (Brained and Fuchigami 1981; Wardle et al. 1983; Ziv et al. 1987).

In vitro plants principally require sugar as a carbon source (Conner and Thomas 1982), and the CO_2 uptake capability is low (Donelly et al. 1985). Consequently when transplanted out of the culture vessel, plantlets suffer from severe environmental stresses and substantial losses may occur (Van Huylenbroeck and Debergh 1996).

2.4 Acclimatization of micropropagated plantlets

The term acclimatization is defined as the climatic adaptation of an organism, especially a plant, which has been moved to a new environment (Conouer and Poole 1984).

To promote *ex vitro* survival and physiological competence, especially to protect them against water stress and encourage autotrophy, a transitional environment is usually supplied for the acclimatization interval, ranging from one to several weeks (Brained and Fuchigami 1981; Grout and Millam 1985; Fabbri et al. 1986). In this transitional environment the relative humidity is kept in the range of 70 – 100 % and the light level can be increased towards ambient conditions. The stage of acclimatization is the beginning of the autotrophic existence of the plant, including the initiation of the physiological processes necessary for survival.

Difficulties in transplanting tissue-cultured plantlets to soil are well documented (Puthur et al. 1998; Subhan et al. 1998). They appear to be as a direct result of the culture-induced phenotype which reflects adaptation to *in vitro* conditions but have a harmful effect when plantlets are transferred to the greenhouse or field where the relative humidity tends to be less than 100 %, the ambient light levels are much higher than in culture, the temperatures are fluctuating and the substrate has a much higher water potential.

The special conditions during *in vitro* culture result in the formation of plantlets of abnormal morphology, anatomy and physiology. After *ex vitro* transfer, these plantlets might easily be impaired by sudden changes in environmental conditions, and so need a period of acclimatization to correct the abnormalities (Pospisilova` et al. 1999).

2.5 Hardening of Plantlets *In Vitro*

The hardening of plantlets in vitro by 1) decreasing air humidity, e.g, by using lids permeable for water vapor or by bottom cooling, 2) increasing irradiance, or 3) increasing CO_2 concentration by forced ventilation can ameliorate wilting of plants after transplantation (Kanechi et al. 1998). However, these procedures might lead to a quick drying out of the cultivation medium and to an impairment in plantlet growth (Solarova et al. 1996). Acclimatization can be improved by hormonal stimulation of root development (Van Telgen et al. 1992; Diaz-Perez et al. 1995). Concentrations of saccharose and agar in the medium can also affect subsequent acclimatization to *ex vitro* conditions, e.g, *Brassica napus, Eucalyptus camaldulensis, Triticum aestivum*, and *Vitis vinifera* (Fila et al. 1998; Vorackova et al. 1998).

Sucrose is the solute supplied in the largest quantity in most tissue culture media, having both nutritive and osmotic effects on plant growth. Ibaraki and Kurata (1993) described the movement of sucrose in their adjacent model as a series of three resistance components: a) diffusion across the medium with the diffusion coefficient specific to solute/solvent, b) boundary layer resistance at the interface surface of the plant and medium, and c) resistance in the plant tissue corresponding to the biochemical sink strength and the plant's transport properties. Sucrose moves approximately 4-times faster in stationary water than agar gel. The boundary resistance at the plant/medium interface was approximately 6000 times greater in agar

than liquid media per unit surface area. It is easily envisioned that a plantlet impinged on the surface of an agar gel has a much smaller surface area for exchange at its base than a similar plantlet wet with nutrient across its entire surface. Ibaraki and Kurata (1998) further developed a heterotrophic growth model that simulated fresh and dry weight based on water and sugar uptake. Dry matter accumulation was determined by the difference between sugar levels in medium and plant at the interface surface and the area of that surface. Fresh weight gain is related to the plants relative water content, the water content of the medium, and the interface surface area.

The composition of culture medium is a major determinant of *in vitro* growth of plants. The mineral salts, sugar as carbon source and water are the main components for most tissue culture media (Gamborg and Phillips 1995). Sugar is an important component in medium and its addition is essential for *in vitro* growth and development of plants because photosynthesis is insufficient, due to the growth taking place in conditions unsuitable for photosynthesis or without photosynthesis (Pierik 1997). The sugar concentration chosen is dependent on the type and age of growth material; very young embryos require a relatively high sugar concentration. Generally the growth and development increases with sugar concentration, until an optimum is reached and then decreases at high concentrations. The most commonly used source of carbon is sucrose at a concentration of 20 – 50 $g.L^{-1}$ (Bhojwani and Razdan 2004). Koroch et al. (1997) reported that preconditioning at different sucrose concentration prior to acclimatization had no effect on plant establishment, but influenced plant quality. In some cases, independent growth could not be achieved on a medium without sucrose during rooting (Grout and Price 1987). George and Sherrington (1984) had drawn the conclusion that for optimal growth and multiplication, 20 – 40 $g.L^{-1}$ sucrose was found to be optimum. Not only biomass formation, but photosynthesis was also positively affected by exogenous sucrose. Apparently, high sucrose levels were more stressful for the shoots, which exhibited reduced green leaves and poor development. In banana, after completing rooting, the total carbohydrates and proteins have shown slight increase over multiplication stage. Tissue type had significant effect on total sugar, amino acid, protein and lipids. During dehardening (the period after hardening), level of soluble sugar and sucrose decreased in all temperature treatments and there was a highly significant positive

correlation between sucrose level and level of threshold (LT_{50}) values (Hazarika et al. 2001).

The almost universal application of agar powder, a mixture of polysaccharides derived from red algae as the gelling material in culture media for all the aforesaid branches of plant tissue culture is due to its following advantages: high clarity, stability (after autoclaving), non toxic nature (MacLachlan 1985; Henderson and Kinnersley 1988). However, wide-spread use of agar in plant tissue culture media has certain limitations in many plant systems, which in the long run affects plant survival after transfer to soil from culture vessels. Agar is by far the best support system to keep plant cultures from being submerged in the medium. From the physical and chemical point of view, it is convenient to work with agar since it melts at about 100ºC and solidify at about 45ºC. In addition, agar does not react with media components nor is it digested by plant enzymes.

On the other side, different brands of agar vary in their purity and gelling strength. Concentrations generally range between 5 $g.L^{-1}$ and 10 $g.L^{-1}$. Furthermore, agar does not gel well under acidic conditions and thus may not be appropriate for situations in which a low pH (< 4.5) is required. The concentration of agar may be critical to plant growth in culture. Media that is too soft may produce hyperhydricity in tissue culture-raised plants whereas media that is too hard may cause reduced plant growth (Gangopadhyay et al. 2009). These shortcomings become compounded later and manifested in the physiology status of tissue culture-raised plantlets, especially during stages of rooting and hardening. The survival percentage during transition from test tube to soil is often become very limited since the *in vitro* raised plantlets in agar-gelled media are very seldom adapted readily to *in vivo* condition (Pierik 1996).

2.6 Factors affecting on Acclimatization of Micropropagated Plantlets

The factors affecting on acclimatization of micropropagated plantlets are light intensity, humidity, temperature, substrates and so on.

2.6.1 Light intensity

In vitro plantlets are generally grown under low light intensity (1, 200 - 3, 000 lux) and temperature (25 ± 2ºC), hence direct transfer to broad spectrum sunlight

(4, 000 - 12, 000 lux) and temperature (26 - 36ºC) might cause charring of leaves and wilting of plantlets (Chandra et al. 2010). It is, therefore, necessary to accustom the plant in the natural conditions by a process of hardening or acclimatization (Lavanya et al. 2009). The culture containers could be kept in the greenhouse with loose lids. Micropropagated plantlets can be left in shade for 3 - 6 days under diffused natural light to make them adjust to the conditions of new environment. This helps in semi-hardening of plants and leads to shoot elongation (Lavanya et al. 2009). Transfer of microshoots from *in vitro* to *ex vitro* conditions under direct sunlight might cause photoinhibition and chlorophyll (Chl) photobleaching. The exposure of *Calathea louisae* and *Spathiphyllum floribundum* plantlets to high irradiance immediately after transplantation caused photoinhibition and even Chl photobleaching (Van Huylenbroeck et al. 1995). No photoinhibition was found during growth in the greenhouse when *Nicotiana tabacum* plantlets were acclimatized in two phases, first in the greenhouse (low irradiance of 30 - 90 μmol m^{-2} s^{-1}) and then in the open air under light intensity of 200 - 1,400 μmol m^{-2} s^{-1}. *In vitro* to *ex vitro* transfer might lead to a transient decrease in photosynthetic parameters. Net photosynthetic rate in *Solanum tuberosum* and *Spathiphyllum floribundum* plants decreased in the first week after transplantation and increased thereafter (Pospisilova` et al. 1999). High sucrose and salt containing media, low light level and low carbon dioxide concentration in culture vessel are some of the important limiting factors among various physical micro environmental factors which influence photosynthesis of *in vitro* cultured plants. For *in vitro* growth, a continuous supply of exogenous sucrose is required (20 - 30 $g.L^{-1}$) as a carbon source (Hazarika 2004). This is because of the exogenous supply of sucrose, which does not necessitate the normal development of photosynthetic apparatus. Therefore, *in vitro* cultured plants are poor in chlorophyll content and the enzymes responsible for photosynthesis i.e. ribulose biphoshate carboxylase (RubPcase) are inactive or absent. The low RubPcase activity may be due to the presence of sucrose during the development of leaves (Donnelly and Vidaver 1984).

2.6.2 Humidity

Normally, the environments in *in vivo* are quite different when compared to those *in vitro* conditions, in terms of relative humidity (RH), constant temperature, air ventilation, nutrient levels, etc (Kozai et al. 1997; Chen 2004; Hazarika 2006). Relative humidity (RH) control of *in vitro* acclimatization is a major factor in

enhancing the biochemical, physiological and morphological characters of plantlets when transplanted to *in vivo* (Cha-um et al. 2003; Talbott et al. 2003). There are many techniques for controlling the RH in the culture vessel of plant tissue culture, such as, saturated salt addition to the culture chamber and increasing the air ventilation rate (Cui et al. 2000; Cha-um et al. 2003; Shim et al. 2003). Acclimatized plantlet adaptation is an important mechanism in the transplanting process of plant micropropagation, relating to survival percentage, growth and development (Van Huylenbroeck et al. 1998; Van Huylenbroeck et al. 2000; Kadleček et al. 2001; Fila et al. 2006). Development of photosynthetic properties are very important for *in vitro* plantlets, which could be improved by altering their environmental growth conditions, such as increasing light intensity, humidity, air temperature or CO_2 concentration. Variations of humidity between 1 and 0.2 kPa vapour pressure deficit (VPD) have small effects on the physiology and development of horticultural crops (Grange and Hand 1987). Nevertheless, some authors observed that low VPD and temperature positively affects dry matter accumulation, but they did not record any physiological disorders in plantlets (Bakker 1984; Yoshida 1981). Humidity and temperature play an important role in the growth and development of plantlet metabolism during acclimatization. High humidity often causes shoot elongation, increase fresh weight, leaf area but may cause diseases such as mildew and botrytis as well as bacterial infection where moisture forms on the leaf surfaces (Kranz 1996; Remigio et al. 2003). High humidity level also reduces transpiration rate. Since transpiration is essential not only to cool leaf surface temperature but creates a suction effect resulting in water and mineral uptake and transportation within the plantlets. Thus, the deficiency in water supply could induce physiological and morphological changes in plantlets affecting the growth and survival rate (Tenhunnen et al. 1987).

2.6.3 Temperature

Temperature is one of the most important factors affecting plant growth and physiology (Berry and Bjorkman 1980). Increasing temperature may substantially increase carbon assimilation in some plant assuming other environmental factors do not limit assimilation. However, low temperature induces stomata closure and inactivation of photosynthetic cycle enzymes, which reduced utilization of light energy in carbon metabolism and induced photoinhibition. The inactivation of photosynthesis is associated with photosystem II and is very sensitive to heat stress and humidity (Bjorkman et al. 1980), which decreased stomatal conductance (Menzel

and Simpson 1986). The amount of inactive photosynthetic enzymes increases under heat stress paralleling the loss of photosynthetic activity. This loss of photosynthetic enzymes activation in response to high temperature occurs before any other plant process is adversely affected (Crafts-Brandner and Salvucci 2000). High temperatures can also damage the internal photosynthetic process directly. Thus, the heat inhibition of photosynthesis may consist of stomatal limitation. The description of the mechanisms of acclimation to high temperature and humidity along with the morphological and physiological responses is necessary to maximize crop production.

2.6.4 Substrate

Good plant development depends to a large part on the growing medium used. If a plant develops a good root system in a well-balanced substrate, this does not mean that the plant is pampered and will not adapt to the harsh life outside a nursery. In fact, the opposite applies. To survive in the harsh environment of a field, often without additional watering and fertilizing, a plant needs a well-developed and strong root system. The development of a healthy root system depends not only on the genetic properties of the plant but to a large extent on the physical and chemical properties of the substrate used. The substrate properties that influence seedling growth can be divided into physical properties (water-holding capacity, porosity, plasticity and bulk density) and chemical properties (fertility, acidity and buffer capacity) (Bilderback 1982).

Garden soil

A soil is composed of materials in the solid, liquid and gaseous states. These materials must exist in the proper proportions for satisfactory plant growth. Soil-based growing media were the norm prior to the advent of soilless media based on peat. The major problem with soil is to supply consistent quality and the risk of pathogens including weed seeds (Handreck and Black 1994). Potting mixes with more than 30% soil by volume usually have poor aeration in pots. These mixes also have a high bulk density and can have a low level of available water if there is too much clay in the soil. But, clay soils can increase cation exchange capacity and can contribute micro-organisms and nutrients, especially iron and other trace elements.

Sand

Sand is sometimes included as an ingredient in growing media substrates. Substrates with medium to very coarse particle sizes are generally preferred as

rounded particles can separate out during mixing. Sand provides ballast and helps overcome re-wetting problem (Handreck and Black 1994).

Compost

Compost is generally defined as an aerobically decomposed product of organic wastes such as cow dung, and animal droppings, farm and forest wastes and municipal solid wastes. Composts show high porosity, air capacity and water holding capacity (Abad et al. 2001). It increases organic matter content of the soil including the humic substances that affect nutrient accumulation and promote plant root growth. And it supplies balanced nutrients to plant roots and stimulates growth (Canellas et al. 2000; Singleton et al. 2003).

Vermicompost

The ability of some species of earthworms to consume and break down a wide range of organic residues such as sewage sludge, animal wastes, crop residues and industrial refuse is well known (Mitchell et al. 1980; Edwards et al. 1985; Chan and Griffiths 1988; Hartenstein and Bisesi 1989). In the process of feeding, earthworms fragment the waste, enhance microbial activity and accelerate rates of decomposition, leading to a humification effect through which the unstable organic matter is oxidized and stabilized, as in composting but by a non-thermophilic process (Elvira et al. 1998). The end product, commonly termed vermicompost, is quite different from the parent waste material. Vermicompost is finely divided peat-like material with high porosity, aeration, drainage, water-holding capacity (Edwards and Burrows 1988). They have greatly increased surface areas, providing more microsites for microbial decomposing organisms, and strong adsorption and retention of nutrients (Shi-wei and Fu-zhen 1991). Vermicompost is usually more stable than their parent materials with increased availability of nutrients and improved physicochemical and microbiological properties (Albanell et al. 1988; Edwards and Burrows 1988; Shi-wei and Fu-zhen 1991; Orozco et al. 1996).

Burnt Rice Husk

Rice husk is major byproduct obtained from rice. When burned, rice husk help to build up of soil structure and aeration as they hold shape for a long time, have good water holding capacity, and are bacteria and fungus-free making them a good potting material (Aspinall 2003). Rice husk ash contains high percentage of potassium and phosphorus than nitrogen. Potassium and phosphorus contents of paddy husk were 0.01 - 2.69 % P_2O_5 and 0.1 - 2.54 % K_2O respectively. Decomposition of materials

would provide additional nutrients to the growing medium which may lead to higher uptake of nutrient by the crop and subsequently high yield.

2.7 The role of mycorrhizal symbiosis

The ability of the root systems to establish beneficial symbiotic relationships with soil microorganisms represents one of the most successful strategies that land plants have developed to cope with abiotic and biotic stresses imposed during the colonization of terrestrial ecosystems (Allen 1996). These beneficial components of soil biota include mycorrhizae, which are mutualistic associations occurring between the roots of most plant species and certain groups of fungi. Seven types of mycorrhizal associations are namely known, (vesicular-) arbuscular mycorrhiza, ectomycorrhiza, ectenmycorrhiza, arbutoid-, monotropoid-, ericoid- and orchidoid mycorrhiza. Types of mycorrhizas are categorized on the basis of taxonomic groups of fungi and plants involved and the alteration in the morphogenesis of fungi and roots, which occurs during the development of the new structure that is mycorrhizal (Harley and Smith 1983). The (vesicular-) arbuscular mycorrhiza or (V)AM are the most intensively studied types of mycorrhizae because they are present in most agricultural and natural ecosystems and play an important role in plant growth, health and productivity (Harley and Smith 1983; Ginaninazzi et al. 1990; Lovato et al. 1995).

There are only a few genera belonging to *Cruciferae*; *Chenopodiaceae* and *Cyperaceae* where they are not found due to the presence of glucosinolates and their hydrolysis products such as isothiocyanates in and around the roots (Glenn et al. 1988), which are toxic to the growth of fungi. (V)AM fungi belong to the class Zygomycotina, order Glomales (Walker 1992; Morton 1995) whose origin has been dated 353 - 452 million years ago (Simon et al. 1993). About 150 species of the genera *Acaulospora, Enthrophospora, Gigaspora, Glomus, Sclerocystis* and *Scutellospora* have up to now been recognized as forming symbiotic associations with plants. These fungi form morphologically distinct resting spores in the soil and can be multiplied in the presence of a host plant. Some of these spores can be surface sterilized and used to produce new spores in axenic seedlings or root organ culture (Mosse and Hepper 1975). The close relationship of (V)AM fungi with their host plants is mirrored by their obligatory biotrophic status. In absence of a host, their growth is limited to a relatively short time (20 - 30 days) after which many modifications in fungal morphology point to a cessation of hyphal growth. Presence

of the root allows development of vegetative mycelium, which, under favorable conditions, can colonize 60 – 90 % of the length of the root system (Becard and Piche 1989; Bonfante et al. 1995). Mycorrhizal colonization begins with the hyphae that arise from soil-borne propagules, large resting spores of the (V)AMF or mycorrhizal root fragments.

The fungal hyphae penetrate the root between the epidermal cells and form an appressorium in the first cell layers. This stage marks the autotrophic growth of the fungus. The colonizing hyphae pass through the intercellular spaces and then enter the root tissues spreading between and through cells of the cortical root layers. Once the hyphae have reached the inner cortex they grow into the cells and form tree-like structures called 'arbuscules'. These branched hyphae are closely surrounded by the intact host plasma lemma and represent a large surface of cellular contact between both symbionts. These facilitate the exchange of metabolites between host and fungus. The arbuscules are probably the main transfer site of mineral nutrients, mainly phosphorus, from the fungus to the plant and of carbon compounds to the fungus (Smith and Smith 1991; Bonfante et al. 1995). As internal colonization spreads, the extraradical hyphae ramify, and grow along the root surface forming more penetration points. They also grow outwards into the surrounding soil, thus developing an extensive tri-dimensional network of mycelium which interfaces with soil particles. Smith and Gianinazzi-Pearson (1990) indicate that the length of the external hyphae growing in soil associated with mycorrhizal roots reaches an average of 1 m cm^{-1} root, but values of up to 10 - 14 m cm^{-1} root have also been recorded. These mycelial network can extend several centimeters outwards from the root surface, bridging over the zone of nutrient depletion around roots to absorb low-mobile ions from the bulk soil (mineral nutrients). In return the plant provides the fungus with sugars, amino acids and vitamins essential for its growth (Harley and Smith 1983).

The colonized plant is better nourished and better adapted to its environment. It obtains increased protection against environmental stresses (Sylvia and Williams 1992), cold (Charest et al. 1993; Paradis et al. 1995), salinity (Davis and Young 1985) and pollution (Leyval et al. 1994; Shetty et al. 1995). In addition, the symbiosis tends to reduce the incidence of root diseases and minimizes the harmful effect of certain pathogenic agents (St- Arnaud et al. 1995). In agriculture, the increased uptake of soil minerals by colonized plants means that it is possible to consider reducing substantially the application of fertilizers and pesticides, and at the same time obtain

equivalent or even higher crop yields (Abbott and Robson 1992). The fact that colonized plants are better able to obtain their nourishment from the soil and resist environmental stresses gives fungal symbionts a biofertilizing and crop protecting role. Maximum benefits will only be obtained from inoculation with efficient mycorrhizal fungi and careful selection of compatible host/fungus/substrate combinations. The performance of micropropagated plants may be greatly improved by ensuring a suitable mycorrhizal establishment at outplanting.

2.8 Mycorrhizae and Micropropagation

The technique of micropropagation eliminates all microorganisms, including mycorrhizal fungi, which are naturally an integral part of the plant, assuring satisfactory growth and development in microbial-rich and nutrient-poor environments. The *ex vitro* acclimatization is a critical step in the micropropagation cycle and the lack of microorganisms can affect survival and growth of *in vitro* produced plantlets. Previous studies have shown that inoculation with endomycorrhizal fungi at the time of transplanting the micropropagated plantlets to *ex vitro* conditions significantly improves survival and growth due to improved absorption of nutrients and water, and to increased stress tolerance (Azcon-Aguilar and Barea 1997; Jaizme-Vega et al. 1997). Therefore, there is a high potential for introducing (V)AMF into the micropropagation system of high valued plants during acclimatization. When the AM symbiosis is established the fungus receives carbon molecules from the plant, and the plant receives nutrients (especially phosphorus) and water from the fungus (Harrison and Van Buuren 1995; Gosling et al. 2006). In this way, AM plants are usually more tolerant to several stresses, including drought, than non-AM plants (Auge 2004; Ruiz-Lozano 2003; Aroca et al. 2008).

MATERIALS AND METHODS

Experimental site

The experiment was carried out at the Plant Tissue Culture Laboratory, Department of Horticulture and Agricultural Biotechnology, Yezin Agricultural University (YAU) from December, 2012 to July, 2014.

Experimental Material

Micropropagated banana plantlets (*Musa acuminata* cv. Thi-hmwe (khon-war)) obtained from VFRDC (Vegetables and Fruits Research and Development Center), Department of Agriculture were used as test cultivars.

3.1 Experiment 1: Effect of Different Substrates on Acclimatization of *In Vitro* Propagated Banana Plantlets

The experiment was laid out in Randomized Complete Block (RCB) design with three replications. Fifteen plantlets were used in each treatment. Different types of substrates were used in this experiment.

The treatments were as follows:

T_1- Sand : Garden soil (1:1) (v/v)

T_2 - Sand : Compost (1:1) (v/v)

T_3 - Sand : Burnt rice husk (1:1) (v/v)

T_4 - Sand : Vermicompost (1:1) (v/v)

T_5 - Only sand (v/v)

T_6 - Sand : Garden soil (1:1) + Arbuscular mycorrhizal fungi (AMF) (v/v)

T_7 - Sand : Compost (1:1) + AMF (v/v)

T_8 - Sand : Burnt rice husk (1:1) + AMF (v/v)

T_9 - Sand : Vermicompost (1:1) + AMF (v/v)

T_{10} - Sand + AMF (v/v)

Micropropagated plantlets of banana, Thi-hmwe (khon-war) cultivar, were used as test cultivars. Healthy and uniform rooted plantlets with 3 to 4 leaves were selected from *in vitro* cultures and washed with tap water to remove residues of agar from the roots. Plantlets were transplanted to plastic box containing sterile substrate mixture for two weeks as the primary hardening under the shade house. Then the plantlets were shifted to the individual plastic bag and covered with a thin plastic film to maintain the moisture for one week. After that, plastic films were removed and plantlets were kept under the greenhouse as a secondary hardening. The plantlets were treated with arbuscular mycorrhizal fungi (AMF) during the secondary hardening. The AMF was used at the rate of 5 g per 1 kg of potting substrates. Garden soil and compost were obtained from Department of Horticulture and Agricultural Biotechnology, YAU. Burnt rice husk and sand were obtained from Yezin village. Vermicompost was obtained from Department of Agricultural Research (DAR).

3.2 Experiment 2: Effect of Sugar Concentrations and Agar Levels in *In Vitro* Acclimatization of Banana Plantlets

This experiment was carried out to determine the effect of sugar concentrations and agar levels in *in vitro* acclimatization of banana plantlets. The Murashige and Skoog's medium was used as a basal medium. The experimental design was Randomized Complete Block (RCB) design with two factors factorial arrangement.

The Factor (A) - Three levels of sugar concentration

S_1 - 10 $g.L^{-1}$

S_2 - 20 $g.L^{-1}$

S_3 - 30 $g.L^{-1}$

The Factor (B) - Three levels of agar concentration

A_1 - 3 $g.L^{-1}$

A_2 - 5 $g.L^{-1}$

A_3 - 7 $g.L^{-1}$

The pH of the medium was adjusted to 5.8 before autoclaving and 40 ml of media was dispended in each culture bottle and these were autoclaved at 121°C for 15 minutes. Two healthy and uniform plantlets were placed in each bottle and these culture bottle were kept at 20 ± 2 °C temperature for four weeks as *in vitro* hardening.

-- After four weeks in *in vitro* hardening, the plantlets were removed from culture bottles and washed thoroughly with tap water to remove agar. For the primary hardening, the plantlets were transferred to the plastic box containing sterile substrate mixture for two weeks. After that, the plantlets were transplanted individually in each plastic bag and covered with plastic film under the shade house for one week. For the secondary hardening, the plantlets were transfered to the greenhouse condition with the relative humidity of 65 85 percent and the temperature range of 24 35°C.

3.3 Experiment 3: Effect of Various Pre-treatments on *In Vitro* Propagated Banana Plantlets during Acclimatization

Experimental material

Healthy and uniform tissue cultured banana plantlets of cultivar, Thi-hmwe (khon-war), were used as experimental material.

Hardening media

Potting mixture (sand: garden soil: burnt rice ash, 1:1:1 by volume) was used as the hardening medium.

Method and Preparation

Randomized Complete Block (RCB) design with three replications was used. Twelve plantlets were used for each treatment.

The treatments were as follows:

T_1 - Control (water)

T_2 - Homai (1%)

T_3 - Ferti-start (1%)

T_4 - Atonik (1%)

T_5 - Moralmone (1%)

T_6 - Ferti-start (1%) + Homai (1%)

T_7 - Atonik (1%) + Homai (1%)

T_8 - Moralmone (1%) + Homai (1%)

Well rooted plantlets were carefully removed from culture vessels and they were washed with tap water to remove agar. Then, the plantlets were dipped quickly in each solution according to the treatments. For the control treatment, the plantlets were treated with distilled water only.

The plantlets were hardened in the plastic box containing sterile substrate mixture at the primary stage for a period of two weeks under the shade house. After two weeks, the plantlets were transferred individually into each plastic bag and covered with plastic film to retain moisture content for one week. The secondary hardening was done in the greenhouse which provided the relative humidity of 65 - 90 percent and the temperature range from 24 - 35°C.

3.4 Data Collection

The following data were collected for all experiments.

(1) Plant height (cm)

The height of the plantlet was recorded by measuring the length from the base of corm to the tip of the leaf.

(2) Number of leaves

The number of fully opened leaves in each plantlet was counted and recorded.

(3) Leaf area (cm^2)

The leaf area was measured by using the formula for banana plantlets.

Leaf area (cm^2) = leaf length (cm) × leaf width (cm) × 0.8

(Obiefuna and Ndubizo 1979)

(4) Root length (cm)

It was measured from the base of corm to the tip of root of banana plantlet.

(5) Pseudo-stem diameter (cm)

Its diameter was recorded by using vannier clipper.

(6) Shoot fresh weight (g)

The aerial part of the plantlet including pseudo-stem and leaves were weighed.

(7) Root fresh weight (g)

All the roots from the plantlet were separated and weighed.

(8) Total fresh weight (g)

It includes all the fresh weight of leaves, stems and roots.

(9) Shoot dry weight (g)

The samples observed for fresh weight were dehydrated in a hot air oven at 70ºC for 24 hr.

(10) Root dry weight (g)

The roots observed for fresh weight were oven-dried at 70ºC for 24 hr.

(11) Total Dry weight (g)

It includes all the dry weight of leaves, stem and roots.

(12) Survival %

The number of plantlets which had survived at 45 days after transplanting was counted and recorded as percentage.

(13) SPAD reading (Chlorophyll content)

It was measured by using SPAD meter to determine the chlorophyll content of micropropagated banana plantlets. The youngest fully developed leaf from each plantlet was used to measure the chlorophyll content.

(14) Number of stomata

Using clear nail varnish method is a traditional method to measure the stomata density by making the impression with nail-varnish on the leaf and viewing it under a microscope. The youngest fully-developed leaf from each plantlet was used to measure the number of stomata.

(15) Quantum yield of primary photochemistry

Quantum yield of primary photochemistry was measured by using Flurpen FP 100 to determine photosynthesis ability among the micropropagated banana plantlets. The youngest fully developed leaf from each plantlet was used to

measure the quantum yield. The plants were well watered and they were kept under the sun for 1 hr before the measurement.

SPAD reading, number of stomata and quantum yield of primary photochemistry were measured only in Experiment II.

All data were collected at the end of the experiment.

3.5 Statistical Analysis

Analysis of variance was performed using Gemstat Version 9.0. Treatment means were compared by LSD at 5 %.

RESULTS AND DISCUSSION

4.1 Experiment I: Effect of different substrates on acclimatization of *in vitro* propagated banana plantlets

4.1.1 Plant height

According to the results in Table 4.1, it was observed that substrate (sand only) and substrate (sand+ compost) showed maximum plant height (17.00 cm and 16.80 cm, respectively) followed by sand + burnt rice husk + AMF (16.40 cm) which was not statistically different from other substrates. The minimum plant height (13.00 cm) was observed from substrate (sand + vermicompost + AMF). Sand + compost and sand only substrates were significantly different from sand + vermicompost + AMF except other substrates. These data indicated that different types of substrates have a strong influence on plant height of micropropagated banana plantlets. Plantlets derived from micropropagation cannot absorb nutrients from the soil substrates in the early stage of acclimatization because they try to be adapted to the surrounding environment. In this study the reason why plant height was lower might depend on the type of substrates and the amount of AM fungi application. The more pore space and better aeration of substrate can give the better plant performance. The findings are in line with the following researchers.

Deoe Silva et al. (1998) stated that, for the best growth of tissue-cultured banana plantlets, a potting substrate should have a good balance between its water holding capacity and drainage characteristics, which allows the roots to obtain sufficient water without drowning. As in any potted plant, the quality of transplant depends mainly on the substrate utilized.

Yano-Melo et al. (1999) reported that the plant height of mycorrhiza inoculated micropropagated banana plantlets were significantly higher than those of control plants.

4.1.2 Number of leaves

Number of leaves per plantlet as affected by different substrates is described in Table 4.1. The highest number of leaves (5.67) was observed from substrate (sand + AMF) which was not statistically different from sand + burnt rice husk + AMF (5.33)

and sand + garden soil + AMF (5.33) and followed by sand only (5.00), sand + burnt rice husk (5.00), and sand + compost (4.67). The lowest number of leaves (3.00) was observed from substrate (sand + garden soil), which was not statistically different from sand + vermicompost (3.00). Sand + garden soil + AMF, sand + burnt rice husk + AMF and sand + AMF were significantly different from sand + garden soil, sand + vermicompost and sand + vermicompost + AMF except other substrates. These results indicated that the number of leaves was influenced by the different types of substrates. Substrates containing AMF increased the number of leaves of micropropagated banana plantlets than the ones without AMF. Estrada-Luna et al. (2000) also described that the presence of mycorrhiza resulted in the development of stronger and more vigorous shoots than in non-mycorrhizal plantlets in micropropagated plantlets. Mycorrhizal plants also had an increased number of fully expanded leaves. Subhan et al. (1998) reported that the ex *vitro* growth in terms of shoot height and number of leaves of AMF-inoculated *Sesbania sesban* plantlets was superior to that of non-inoculated control plants. It may be caused by different nutrient contents of the growing substrates. Bitar and Mohamed (2009) also reported that burnt rice husk could not be assessed as potting medium for banana, being only utilized in one treatment (sand + burnt rice husk). However, plantlets grown in sand + burnt rice husk had significantly more height, number of leaves, leaf area, shoot fresh weight and root dry weight of banana plantlets.

Table 4.1 Effect of different substrates on plant height and number of leaves of *in vitro* banana plantlets at 45 days after deflasking

Treatments	Plant height(cm)	Number of leaves
Sand+garden soil	14.93 ab	3.00 c
Sand+compost	16.80 a	4.67 ab
Sand+burnt rice husk	15.67 ab	5.00 ab
Sand+vermicompost	13.60 ab	3.00 c
Sand only	17.00 a	5.00 ab
Sand+gardensoil+AMF	15.10 ab	5.33 a
Sand+compost+AMF	16.00 ab	4.33 abc
Sand+Burnt rice husk+AMF	16.40 ab	5.33 a
Sand+vermicompost+AMF	13.00 b	3.67 bc
Sand+AMF	14.00 ab	5.67 a
$LSD_{0.05}$	3.54	1.59
Pr>F	0.29	<0.02
CV%	32.00	20.60

Means followed by the same letter in each column are not significantly different at 5% LSD.

4.1.3 Leaf area

Results indicated that different types of substrates have a significant influence on leaf area of micropropagated banana plantlets (Table 4.2). The substrate (sand + burnt rice husk + AMF) gave the highest leaf area (18.25 cm^2) followed by sand + burnt rice husk (14.48 cm^2), sand only (13.56 cm^2), sand + compost + AMF (13.27 cm^2), sand + compost (12.80 cm^2) and sand + garden soil (12.60 cm^2). The lowest leaf area (6.49 cm^2) was observed from substrate (sand + vermicompost). Sand + burnt rice husk + AMF was significantly different from sand + vermicompost + AMF, sand + AMF, sand + garden soil + AMF and sand + vermicompost except other substrates. According to this study, it was observed that substrates highly affected the leaf area of *in vitro* banana plantlets during acclimatization period. Substrates containing AMF increased the leaf area more than the ones without AMF.

The result is in agreement with Pedraza-Santos et al. (2001) who stated that the greater leaf area in mycorrhizal-inoculated plantlets than in the non-inoculated plantlets. However, Estrada-Luna et al. (2000) and Hunt (1982) concluded that mycorrhizal plants had a lower leaf area ratio and this indicated a more efficient photosynthesizing surface, since the leaf area to plant dry matter was reduced. Bitar and Mohamed (2009) stated that substrate (sand + burnt rice husk) gave better leaf area of micropropagated banana plantlets but the substrate (sand + vermicompost) gave the highest result.

4.1.4 Root length

As described in Table 4.2, significant effect of different substrates was observed in root length. The highest root length (15.87 cm) was observed in sand substrate followed by sand + garden soil (8.83 cm) and sand + compost (8.77 cm) which were not statistically different from other substrates. The lowest root length (6.33 cm) was observed from substrate (sand + vermicompost + AMF). Sand only substrate was significantly different from other substrates. These results stated that the root length of micropropagated banana plantlets was strongly influenced by different types of substrates during acclimatization period. The fine texture substrate with good aeration and well drainage can help the root to penetrate in the substrate and absorb water and nutrients. The micropropagated plantlets cannot stand for the stresses especially drought and flood condition. The reason why the lower root length in all

substrates except from sand only substrate was may be due to different structure of substrates. However, Bitar and Mohamed (2009) reported that the least transplant growth for most growth characters was noticed in banana plantlets grown in 100% sand. Murali and Duncan (1995) reported that traditionally fully acclimatized banana plantlets are raised in sand plus peatmoss. Morales-Payan and Stall (2003) also concluded that sand should not be considered as a substrate component in a sustainable system and Neal and Wagner (1983) reported that it has the disadvantage of being heavy and difficult to transport when moving transplants to the field.

According to the statistical analysis, the substrates containing AM fungi were not significantly different from each other. But the substrate (sand + burnt rice husk + AM fungi) gave longer root. The effect of AM fungi on root development might be the result of better mineral nutrition in the colonized plants. But in this study, the effect of AM fungi on root length was not significantly found in the substrates except sand + burnt rice husk + AMF. The AMF caused increased root ramification and reduced adventitious root length but an increased number of these roots per plant. These transformations result in denser roots more capable of absorbing nutrients exploring laterally as well as anchoring the plant more firmly in the soil. However, Sato et al. (1999) reported that increased root length was observed in micropropagated plants that received multiple inoculums with the mycorrhizal fungi. The root system of micropropagated plants consist of a series of adventitious roots that develop directly from the corm or the subterranean stem. These roots branch forming primary and secondary roots. Berta et al. 1990 also concluded that it had been observed in other monocotyledons that when mycorrhizal fungi were present, adventitious root length was reduced while their number increased. To evaluate morphological and topological changes that the mycorrhization induces in micropropagated banana root system, the plantlets were inoculated with an AM forming fungi and roots were studied during the first phases of development. The first differences between the roots infested with mycorrhizal fungi and the control plants were seen at 40 days after inoculation (Jaizme-Vega et al. 1994; Garcia Perez and Jaizme-Vega 1997).

4.1.5 Pseudo-stem diameter

The effect of different substrates on pseudo-stem diameter is shown in Table 4.2. The only sand substrate showed the highest pseudo-stem diameter (0.47 cm) followed by sand + garden soil + AM fungi (0.43 cm), sand + burnt rice husk + AM fungi (0.42 cm) and sand + burnt rice husk (0.40 cm). The lowest pseudo-stem diameter (0.13 cm) was observed in substrate (sand + vermicompost). Sand only substrate was not significantly different from sand + burnt rice husk, sand + garden soil + AMF and sand + burnt rice husk + AMF except other substrates. The results showed that the pseudo-stem diameter of micropropagated banana plantlets was highly influenced by different substrates and the positive effect of AM fungi in the acclimatization stage could be seen. It was possibly caused by the effect of different physical structure of growing substrates on nutrient uptake and water absorption of the plantlets. However, Bitar and Mohamed (2009) also reported that, better pseudo-stem diameter resulted from the substrate (sand + vermicompost). Sato et al. (1999) demonstrated that mycorrhizal fungi are well known to enhance the vegetative growth of micropropagated plantlets in terms of enhanced pseudo-stem girth.

Table 4.2 Effect of different substrates on leaf area, root length and pseudo-stem diameter of *in vitro* banana plantlets at 45 days after deflasking

Treatments	Leaf area(cm^2)	Root length(cm)	Pseudo-stem diameter(cm)
Sand+garden soil	12.60 ab	8.83 b	0.18 de
Sand+compost	12.80 ab	8.77 b	0.27 cd
Sand+burnt rice husk	14.48 ab	6.60 b	0.40 ab
Sand+vermicompost	6.49 c	7.67 b	0.13 e
Sand only	13.56 ab	15.87 a	0.47 a
Sand+gardensoil+AMF	9.36 bc	8.33 b	0.43 ab
Sand+compost+AMF	13.27 ab	8.33 b	0.27 cd
Sand+burnt rice husk+AMF	18.25 a	7.13 b	0.42 ab
Sand+vermicompost+AMF	10.85 bc	6.33 b	0.25 cd
Sand+AMF	12.17 bc	8.13 b	0.33 bc
$LSD_{0.05}$	5.72	4.26	0.11
Pr>F	<0.04	<0.01	<0.001
CV%	26.90	28.90	20.40

Means followed by the same letter in each column are not significantly different at 5% LSD.

4.1.6 Shoot fresh weight

Table 4.3 describes the effect of different substrates on shoot fresh weight of micropropagated banana plantlets. The substrate (sand + burnt rice husk + AM fungi) gave the highest shoot fresh weight (2.33 g) followed by sand+ burnt rice husk (2.03 g). The lowest shoot fresh weight (0.67 g) was observed from substrate (sand + vermicompost). Sand + burnt rice husk + AMF was significantly different from sand + vermicompost, sand + garden soil, sand + garden soil + AMF and sand + compost but sand + burnt rice husk was not statistically different from sand only, sand + compost + AMF and sand + AMF. The results revealed that shoot fresh weight of micropropagated banana plantlets strongly fluctuated by different substrates and higher shoot fresh weight can be obtained by adding the AM fungi to the substrates. The performance of micropropagated plantlets may be greatly improved by ensuring a suitable mycorrhizal establishment at planting. The maximum benefit from AM fungi may be obtained from a careful selection of compatible host/ fungus/ substrate combinations. Bitar and Mohamed (2009) also reported that the substrate (sand+burnt rice husk) gave significantly more shoot fresh weight of micropropagated banana plantlets during acclimatization stage. Yano-Melo et al. (1999) stated that banana plantlets inoculated with arbuscular mycorrhizal fungi showed an increment in shoot fresh weight over non-inoculated plantlets.

4.1.7 Root fresh weight

Results stated that different types of substrates had a strong influence on root fresh weight of micropropagated banana plantlets (Table 4.3). The sand only and sand + compost substrates gave the maximum results (1.00 g) and (0.97 g) followed by sand + burnt rice husk + AMF (0.77 g), sand + compost + AMF (0.67 g), sand + garden soil + AMF (0.60 g) and sand + burnt rice husk (0.57 g). The minimum root fresh weight (0.27 g) was observed from substrate (sand + garden soil). There was no significant effect of different substrates on root fresh weight of micropropagated banana plantlets. The positive effect of AM fungi on root fresh weight could be seen. The effect of different substrates on root fresh weight is partly due to the architectural changes in the root development. Since growth media are different in terms of nutrient content, it is possible for plants grown on different substrates to have a differential response. The plants forming mycorrhizae tend to have a lower root and shoot ratio,

which means the greater biomass efficiency because less energy is directed to root formation. In the study of Bitar and Mohamed (2009), the substrate (cocofiber + vermicompost) gave significantly more root fresh weight of micropropagated banana plantlets. Yano-Melo et al. (1999) also reported that banana plantlets inoculated with arbuscular mycorrhizal fungi showed an increment in the root fresh weight but it was not different from non-inoculated plantlets.

4.1.8 Total fresh weight

The effect of different substrates on total fresh weight is stated in Table 4.3. The substrates (sand + burnt rice husk + AMF and sand only) gave the highest total fresh weight (3.10 g and 2.77 g) followed by sand + burnt rice husk (2.60 g) and although the substrate (sand + burnt rice husk) showed the maximum result (2.43 g), it was not statistically different from sand + compost, sand + compost + AMF and sand + AMF (2.43 g, 2.23 g, and 2.13 g). The lowest total fresh weight (1.00 g) was obtained from substrate (sand + vermicompost). Results indicated that the total fresh weight was highly influenced by different substrates and there was a positive effect of AM fungi. The higher total fresh weight was observed when AM fungi were added. The burnt rice husk helps to build up of soil structure and aeration and contains high percentage of potassium and phosphorus than nitrogen. Decomposition of materials would provide additional nutrients to the growing medium which may lead to higher uptake of nutrient by the crop and subsequently high yield. AMF was also found to play an important role in the growth of various micropropagated plantlets, such as avocado (Vidal et al. 1992), pine apple (Guillemin et al. 1992), pear and peach (Raprarini et al. 1994), citrus (Camprubi and Calvet 1996) and guava (Estrada-luna et al. 2000). Similarly, the effect of AMF on Shrimanti variety of banana had been positive on growth and yield (Phirke et al. 2002). Singh and Singh (2004) observed a marked increase in the uptake of P, Ca, Mg Zn and Cu in the mycorrhizal plants in banana. Aneesa Rani et al. (2004) too noted that application of 2 g Azotobacter, PSBs and 10 g VAM gave maximum height, girth, root length and number of roots/root fibres in cashew seedlings. Thus, the above findings on the effect of different substrates and AMF are in concurrence with the observations made on other horticultural crops, besides substantiating earlier findings.

Table 4.3 Effect of different substrates on shoot fresh weight, root fresh weight and total fresh weight of *in vitro* banana plantlets at 45 days after deflasking

Treatments	Shoot fresh wt.(g)	Root fresh wt.(g)	Total fresh wt.(g)
Sand+garden soil	0.80 de	0.27 b	1.07 d
Sand+compost	1.47 bcd	0.97 a	2.43 abc
Sand+burnt rice husk	2.03 ab	0.57 ab	2.60 ab
Sand+vermicompost	0.67 e	0.33 b	1.00 d
Sand only	1.77 abc	1.00 a	2.77 a
Sand+gardensoil+AMF	1.03 cde	0.60 ab	1.63 bcd
Sand+compost+AMF	1.57 abcd	0.67 ab	2.23 abc
Sand+burnt rice husk+AMF	2.33 a	0.77 ab	3.10 a
Sand+vermicompost+AMF	1.10 cde	0.37 b	1.47 cd
Sand only+AMF	1.63 abc	0.50 ab	2.13 abc
$LSD_{0.05}$	0.79	0.58	1.05
Pr>F	<0.005	0.16	<0.005
CV%	32.00	55.70	29.80

Means followed by the same letter in each column are not significantly different at 5% LSD.

4.1.9 Shoot dry weight

Results showed that different types of substrate had a strong influence on shoot dry weight of micropropagated banana plantlets (Table 4.4). The substrate (sand + burnt rice husk + AMF) gave the maximum shoot dry weight (0.16 g) followed by the substrate (sand only, 0.11 g), which was not statistically different from sand + burnt rice husk (0.10 g), sand + compost + AMF (0.10 g), sand + compost (0.09 g), sand + vermicompost + AMF (0.09 g) and sand + AMF (0.09 g). The minimum shoot dry weight (0.05 g) was observed from substrate (sand + vermicompost). Sand + burnt rice husk + AMF was significantly different from sand + garden soil, sand + vermicompost and sand + garden soil + AMF except other substrates. These results showed that the shoot dry weight of micropropagated banana plantlets was fluctuated by different types of substrates during the acclimatization period. The positive effect of AM fungi was found in shoot dry weight of micropropagated banana plantlets. The increase in dry shoot weight of mycorrhiza-inoculated plantlets could be attributed to increasing availability of phosphorus to plants which can play a regulatory role for photosynthesis through chlorophyll production. De Lima et al. (2006) reported that the greater shoot dry weight of mycorrhiza-inoculated *Anthurium andreanum* plantlets was observed. Mwajita et al. (2007) also reported that micropropagated bananas were highly dependent on mycorrhizal association with plants inoculated with the three *Glomus* species, having higher dry shoot weight than those inoculated with *Gigaspora albida* and the control.

4.1.10 Root dry weight

Table 4.4 describes the effect of different substrates on root dry weight of micropropagated banana plantlets. The substrates (sand only and sand + compost) showed the maximum results (both 0.08 g) followed by sand + vermicompost + AMF (0.05 g) and the other substrates which were not significantly different from each other. The minimum root dry weight (0.03 g) was observed from substrate (sand + garden soil). The substrates (sand + compost and sand only) were significantly different from other substrates except sand + vermicompost + AMF. These results indicated that the root dry weight was altered by different types of substrates and the positive effect of AM fungi can be seen when AM fungi was added to the substrates. Although some substrates produced greater root dry weight, no apparent and

consistent patterns were observed. These results also showed that the micropropagated plantlets had differential preference for growing substrates. The root development was expressed by an increase in root length of the primary root which persisted throughout the study period.

Kacar et al. (2010) reported that the higher root dry weight of cherry rootstocks was observed from the substrate (andesitic tuff: soil: compost) and the rootstocks treated with *Glomus mosseae* gave the more root dry weight than non-treated plantlets during the acclimatization period. The results suggest that AM fungi not only have a preference for some micropropagated plantlets, but also for the substrates in which the plantlets were grown.

4.1.11 Total dry weight

Results indicated that the different types of substrates had a strong influence on the total dry weight of micropropagated banana plantlets (Table 4.4). The substrate (sand + burnt rice husk+ AMF) showed the maximum result (0.19 g) followed by sand only substrate (0.19 g), sand + compost (0.17 g), sand + vermicompost + AMF (0.14 g), sand + burnt rice husk (0.14 g), sand + compost + AMF (0.14 g) and sand + AMF(0.13 g). The minimum total dry weight (0.09 g) was observed from substrate (sand + vermicompost). Sand + burnt rice husk + AMF was significantly different from sand + garden soil and sand + vermicompost but sand only substrate was not statistically different from sand + garden soil + AMF, sand + compost + AMF, sand + burnt rice husk, sand + compost + AMF, sand + vermicompost + AMF and sand + AMF. The total dry weight of micropropagated banana plantlets was influenced by different types of substrates during acclimatization period. The positive effect of AM fungi can be seen by adding AMF into the substrates and it might be the result of better mineral nutrition in colonized plants.

According to Kacar et al. (2010), growth substrates significantly affected dry weight of cherry rootstocks and the maximum total dry weight was observed from the substrate (andesitic tuff: soil: compost). Tenoury (1996) reported the effect of several different AM fungi on the development and nutrition of banana varieties of local interest, during the rooting and acclimatization phases. Every study inoculating with any of the mycorrhizal forming fungi (*Glomus* spp.) has shown an increase in dry

weight, fresh weight and other plant development of micropropagated banana plantlets.

Table 4.4 Effect of different substrates on shoot dry weight, root dry weight and total dry weight of *in vitro* banana plantlets at 45 days after deflasking

Treatments	Shoot dry wt.(g)	Root dry wt.(g)	Total dry wt.(g)
Sand+garden soil	0.07 b	0.03 b	0.10 c
Sand+compost	0.09 ab	0.08 a	0.17 abc
Sand+burnt rice husk	0.10 ab	0.04 b	0.14 abc
Sand+vermicompost	0.05 b	0.04 b	0.09 c
Sand only	0.11 ab	0.08 a	0.19 ab
Sand+garden soil+AMF	0.07 b	0.04 b	0.11 bc
Sand+compost+AMF	0.10 ab	0.04 b	0.14 abc
Sand+burnt rice husk+AMF	0.16 a	0.04 b	0.21 a
Sand+vermicompost+AMF	0.09 ab	0.05 ab	0.14 abc
Sand only+AMF	0.09 ab	0.04 b	0.13 abc
$LSD_{0.05}$	0.07	0.04	0.09
Pr>F	0.23	0.06	0.19
CV%	42.70	41.40	35.20

Means followed by the same letter in each column are not significantly different at 5% LSD.

4.1.12 Survival percentage

The survival percent of *in vitro* grown banana plantlets as affected by different substrates is summarized in Figure 4.1. The highest survival percent (87.5 %) was observed from the treatment (sand without AM fungi) and the second highest survival percent (75 %) was found in sand + burnt rice husk (1:1) without AM fungi, sand + garden soil (1:1) with AM fungi and sand + vermicompost (1:1) with AM fungi. The lowest value (37.5 %) was resulted from sand + compost (1:1) with AM fungi. These results indicated that different substrates and AMF application influenced the survival percentage of micropropagated banana plantlets at 45 days after deflasking during acclimatization stage.

Several researchers revealed many substrate combinations for micropropagated plantlets. Vasane and Kothari (2008) stated that soil: press mud cake (PMC): vermicompost (1:1:1) and soil: press mud cake: vermicompost (2:1:1) gave better performance in terms of plant growth and survival percentage of micropropagated banana var. Grand Naine plants than soil alone, soil: press mud cake: vermicompost (3:1:0) and soil: press mud cake: vermicompost (3:0:1) during the secondary hardening. Sathaiamoorthy et al. (2001) suggested various combinations: (i) peat soil: FYM, sand: top soil: vermicompost and sand: FYM: vermicompost: red soil, along with neem seed cake (50 g/kg of matrix). Similarly Chandre Gowda et al. (2002) suggested the use of soil, compost, coir pith and sand in equal proportions for secondary hardening. Pedraza-Santos et al. 2001 suggested that mycorrhizal colonization did not affect plantlet survival percentage during acclimatization stage. Behind all these observations, the common denominator is to avail locally available matrix seemingly such as burnt rice husk, garden soil, etc. in our case, besides sand to provide porous texture to the medium.

The differences in substrate effects may be due to differences in their physical and chemical characters. Results of Meerow (1994) indicated that higher substrate water holding capacity was consistently associated with better growth in potted plants. The less bulk density indicated less substrate compactness and more pore spaces which allowed better root aeration, nutrients and water uptake for subsequent growth enhancement. Drzal et al. (1999) also reported that water is mainly held by the micropore space of a growth medium, while rapid drainage and air entry is facilitated

by macropores. Therefore, an adequate distribution of large and small pores is essential for a good medium.

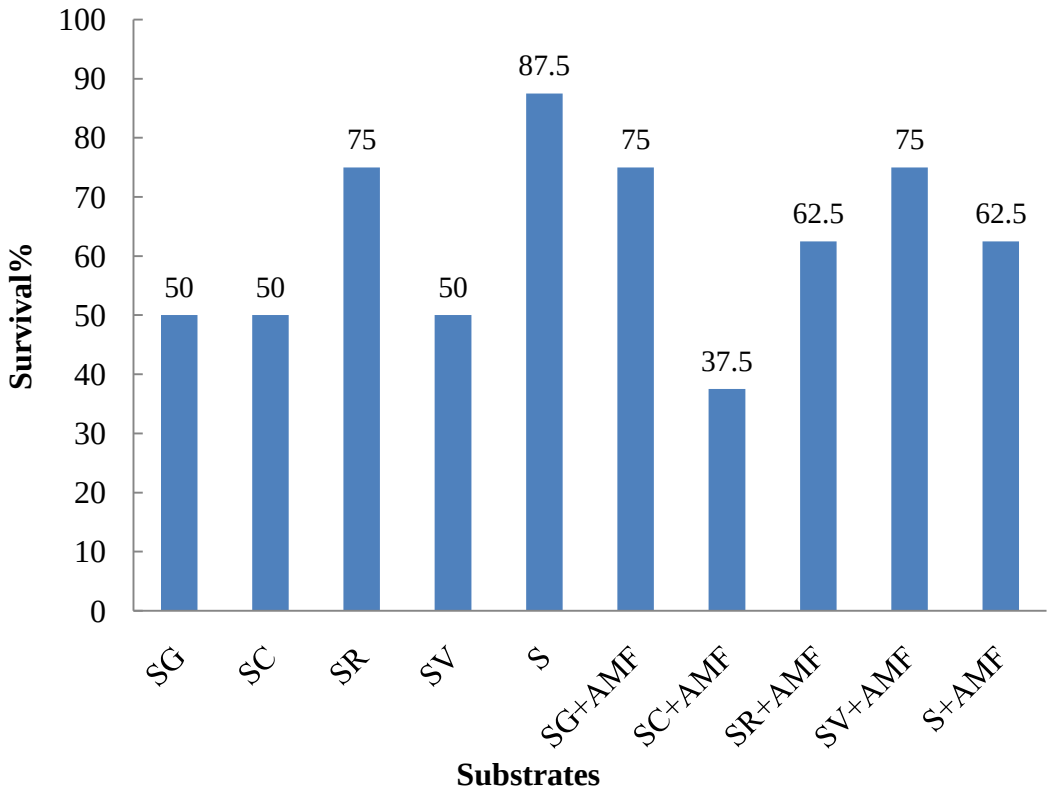

Figure 4.1 Effect of different substrates on survival percentage of *in vitro* banana plantlets at 45 days after deflasking during acclimatization period

SG = sand+garden soil, SC = sand+compost, SR = sand+burnt rice husk

SV = sand+vermicompost, S = sand only, AMF = arbuscular mycorrhizal fungi

4.2 Experiment 2: Effect of sugar concentrations and agar levels in *in vitro* acclimatization of micropropagated banana plantlets

4.2.1 Plant height

Table 4.5 demonstrates the effect of sugar concentrations and agar levels on plant height of *in vitro* acclimatization of micropropagated banana plantlets. The results indicated that different sugar concentrations showed a significant difference on plant height at 1% level. The highest plant height (36.99 cm) was resulted from 30 $g.L^{-1}$ and the lowest plant height (32.19 cm) from 20 $g.L^{-1}$. The treatment 30 $g.L^{-1}$ of sugar was statistically different from 20 $g.L^{-1}$ and 10 $g.L^{-1}$ of sugar. These results indicated that sugar concentrations have a strong influence on plant height of micropropagated banana plantlets. The higher the sugar concentration, the greater the plant height. Mohamed and Alsadon (2009) demonstrated that the tallest micropropagated potato plantlets were found from the culture medium containing 30 $g.L^{-1}$ sucrose. Wainwright and Scrace (1989) reported that maximum value for shoot height of micropropagated plantlets of *Potentilla fruticosa* and *Ficus lyrata* were obtained *in vivo* when previously conditioned with 20 g or 40 $g.L^{-1}$ sucrose. George and Sherrington (1984) also indicated 20 – 40 $g.L^{-1}$ sucrose was optimal for plant growth and multiplication. Hazarika et al. (1999) also reported that *in vitro* preconditioning of citrus microshoots with sucrose concentration of 30 $g.L^{-1}$ was found optimum for subsequent *ex vitro* survival and growth.

Significant difference of plant height was observed among the agar levels (Table 4.5). According to different agar levels, 3 $g.L^{-1}$ treatment had the highest plant height (37.66 cm) and the lowest plant height (30.93 cm) was recorded from treatment (7 $g.L^{-1}$), which was not significantly different from treatment (5 $g.L^{-1}$). These results indicated that the plant height of micropropagated banana plantlets was highly influenced by agar levels. As the agar level was increased in the medium (from 3 $g.L^{-1}$ to 7 $g.L^{-1}$), there was a decrease in plant height (from 37.66 cm to 30.93 cm). Similar finding was obtained by Short et al. (1987), who observed that *ex vitro* shoot height, number of leaves and root length of cauliflower and chrysanthemum decreased with an increasing agar concentrations (from 12 to 20 $g.L^{-1}$). Debergh (1983) reported that both reduced water and mineral availability in media occurred through the decreased pore size and increased matrix potential at high agar concentrations. Debergh (1983)

also stated that the point at which the agar concentration negatively affects plant growth varied with different brands of agar. Ikram and Muhammad (2007) reported that maximum plant height of micropropagated banana plantlets was obtained from the solidified medium with 3.60 $g.L^{-1}$ phytagel. Mohamed and Alsadon (2009) also reported that the 2 $g.L^{-1}$ of agar gave the more plant height of micropropagated potato plantlet than that of 7 $g.L^{-1}$ of agar gelled medium. Au et al. (2012) also stated that 7 $g.L^{-1}$ of agar gelled medium gave the minimum plantlet height of micropropagated banana plantlets (Pisang Awak cultivar). Similar results were achieved by Lucyszyn et al. (2006 and 2007), who observed that tobacco and strawberry plantlets developed on media with 6 $g.L^{-1}$ of agar were shorter.

According to the statistical analysis, there was no interaction between different sugar concentrations and agar levels for plant height of micropropagated banana plantlets indicating that the effect of sugar concentrations on plant height of micropropagated banana plantlets did not depend on the supply of agar levels.

4.2.2 Number of leaves

The effect of different sugar concentration on number of leaves was not significant (Table 4.5). The maximum number of leaves (8.33) was obtained from the treatment (30 $g.L^{-1}$) and the minimum number of leaves (7.67) was found in treatments 10 $g.L^{-1}$ and 20 $g.L^{-1}$. According to this finding, the number of leaves of micropropagated plantlets was not highly influenced by increasing or decreasing sugar concentrations in the medium. Fowler (1983) and Merillon et al. (1984) reported that sucrose may also influence secondary metabolism in cell and organ culture and it could be assumed that larger persistent leaves packed with greater amounts of storage compounds would contribute more after transplantation. Gorut and Millam (1885) also stated that increasing the concentration of sugar in the medium might maximize the nutrient function of persistent leaves.

Number of leaves per plantlet was not significantly affected by different agar levels. Although the treatment (3 $g.L^{-1}$) gave the maximum result (8.33), it was not significantly higher than those of 5 $g.L^{-1}$ and 7 $g.L^{-1}$. It also means that the number of leaves of micropropagated banana plantlets was not altered by the effect of different agar levels from *in vitro* to *ex vitro* condition.

Interaction between different sugar concentrations and agar levels was not found in number of leaves per plantlet. It means that effect of sugar concentrations on number of leaves of micropropagated banana plantlets was not influenced by supply of different agar levels in *in vitro* to *ex vitro* condition.

Table 4.5 Effect of different sugar concentrations and agar levels on plant height and number of leaves of micropropagated banana plantlets at 45 days after deflasking

Treatments	Plant height (cm)	Number of leaves
Sugar		
10 $g.L^{-1}$	30.47 b	7.67
20 $g.L^{-1}$	32.19 b	7.67
30 $g.L^{-1}$	36.99 a	8.33
$LSD_{0.05}$	3.92	1.18
Agar		
3 $g.L^{-1}$	37.66 a	8.33
5 $g.L^{-1}$	31.06 b	8.11
7 $g.L^{-1}$	30.93 b	7.22
$LSD_{0.05}$	3.92	1.18
Pr>F		
Sugar	<0.01	0.41
Agar	<0.003	0.14
Sugar × Agar	0.09	0.94
CV%	11.80	15.00

Means followed by the same letter in each column are not significantly different at 5% LSD.

4.2.3 Leaf area

The effect of different sugar concentrations on leaf area (cm^2) per plantlet is given in Table 4.6. In this experiment, there was highly significant difference among the different sugar concentrations at 1 % level. The highest leaf area (128.70 cm^2) was recorded from the treatment (30 $g.L^{-1}$). The lowest leaf area (86.40 cm^2) was observed from the treatment (10 $g.L^{-1}$). The treatment 30 $g.L^{-1}$ of sugar was significantly different from 10 $g.L^{-1}$ and 20 $g.L^{-1}$ of sugar. According to this study, the different sugar concentrations have a strong influence on leaf area of micropropagated banana plantlets. The higher the sugar concentrations gave the more increasing the leaf area. Kadlecek et al. (2001) reported that the tobacco plantlets with 30 $g.L^{-1}$ sucrose *in vitro* gave maximum total leaf area after acclimatization period. Tadesse et al. (2000) also indicated that leaf area at the beginning of the acclimatization phase was found to be important characteristic for achieving a high leaf area at the end of acclimatization.

Similar results among the agar levels were obtained in leaf area development. No significant difference of leaf area per plantlet was observed among the agar levels. The maximum leaf area (118.70 cm^2) per plantlet was obtained in treatment with 3 $g.L^{-1}$ of agar, followed by 7 $g.L^{-1}$ (96.90 cm^2) and the minimum leaf area (93.20 cm^2) in 5 $g.L^{-1}$ of agar.

No interaction effect was observed between different sugar concentrations and agar levels on leaf area of micropropagated banana plantlets. It is indicating that the effect of sugar concentrations on leaf area of micropropagated banana plantlet did not depend on the supply of agar levels.

4.2.4 Root length

Table 4.6 demonstrates the effect of different sugar concentrations on root length (cm). Root length (cm) was strongly affected by different sugar concentrations at 1 % level. The longest root length (24.23 cm) was obtained in treatment (30 $g.L^{-1}$) and the shortest root length (16.59 cm) from treatment (20 $g.L^{-1}$). The treatment 30 $g.L^{-1}$ of sugar was significantly different from 10 $g.L^{-1}$ and 20 $g.L^{-1}$ of sugar. Gauchan (2011) reported that, in their experiment with maize plantlets, the maximum root length was obtained from 2.5 $g.L^{-1}$ to 7.5 $g.L^{-1}$ sucrose. Singh and Shymal (2001) reported that the microshoots of hybrid tea rose cv. Sonia gave maximum rooting with

40 $g.L^{-1}$ sucrose, while for Super-star the best rooting was recorded with sucrose at 25 $g.L^{-1}$. Lane (1978) also concluded that, like any other morphogenetic process, rooting is the energy-consuming process and hence level of carbon source is desired. The difference in their specific requirement for media might be due to genotypic effect. Root initiation in apple decreased proportionately with decreasing sucrose level and Zimmerman (1983) also revealed that shoots without sucrose did not survive after transferring to greenhouse.

Among the agar levels, there was no significant difference in root length (cm) of micropropagated banana plantlet. Maximum root length (22.06 cm) was collected from treatment (3 $g.L^{-1}$) and the minimum root length (17.14 cm) was observed from 5 $g.L^{-1}$. The treatment 3 $g.L^{-1}$ of agar was statistically different from 5 $g.L^{-1}$ of agar which was not statistically different from 7 $g.L^{-1}$ of agar. These results show that the root length was not affected by different agar levels. It is possible that increased *in vitro* root absorption might counteract the reduced water and nutrient availability in such cases. Barrett-Lennard and Dracup (1988) reported that excellent root growth of *Trifolium subterraneum* was observed from agar gelled medium.

There was no interaction between different sugar concentrations and agar levels in root length (cm). It means that the effect of sugar concentrations on root length of micropropagated banana plantlet was not affected by the supply of different agar levels.

4.2.5 Pseudo-stem diameter

The statistical result of pseudo-stem diameter (cm) per plantlet is presented in Table 4.6. Pseudo-stem diameter (cm) had a highly significant response to different sugar concentrations at 1 % level. Among the different sugar concentrations, the highest pseudo-stem diameter (0.94 cm) per plantlet was observed from the treatment (30 $g.L^{-1}$). The lowest result (0.78 cm) was found in treatment (10 $g.L^{-1}$). The treatment 30 $g.L^{-1}$ of sugar was significantly different from 10 $g.L^{-1}$ and 20 $g.L^{-1}$ of sugar. These results show that pseudo-stem diameter was strongly influenced by different sugar concentrations. The higher the sugar concentration, the larger the pseudo-stem diameter. Ikram and Muhammad (2007) reported that the highest pseudo-stem diameter of micropropagated banana plantlets was obtained from 30 $g.L^{-1}$ sucrose with 30 $mg.L^{-1}$ L-cystein and appropriate hormone combination.

The pseudo-stem diameter (cm) per plantlet was also significantly affected by different agar levels at 5 % level. Among the agar levels, the highest pseudo-stem diameter (0.89 cm) was resulted from treatment (3 $g.L^{-1}$) but was not statistically different from treatment (7 $g.L^{-1}$). The lowest pseudo-stem diameter (0.76 cm) was obtained from (5 $g.L^{-1}$). The treatment 3 $g.L^{-1}$ and 7 $g.L^{-1}$ of agar was significantly different from 5 $g.L^{-1}$ of agar. These results show that the pseudo-stem diameter was affected by different agar levels. Ikram and Muhammad (2007) also reported that maximum pseudo-stem diameter of micropropagated banana plantlets was obtained from the solidified medium with 3.60 $g.L^{-1}$ phytagel. However, Au et al. (2012) revealed that 7 $g.L^{-1}$ agar gelled medium gave minimum pseudo-stem diameter in the micropropagated banana plantlets.

The interaction effect was found between different sugar concentrations and agar levels on pseudo-stem diameter (cm). It means that the effect of sugar concentrations on pseudo-stem diameter of micropropagated banana plantlets might change depending on different agar levels.

Table 4.6 Effect of different sugar concentrations and agar levels on leaf area, root length and pseudo-stem diameter of micropropagated banana plantlets at 45 days after deflasking

Treatments	Leaf area (cm^2)	Root length (cm)	Pseudo-stem diameter (cm)
Sugar			
10 $g.L^{-1}$	86.40 b	18.00 b	0.78 b
20 $g.L^{-1}$	93.70 b	16.59 b	0.80 b
30 $g.L^{-1}$	128.70 a	24.23 a	0.94 a
$LSD_{0.05}$	24.64	4.67	0.09
Agar			
3 $g.L^{-1}$	118.70 a	22.06 a	0.89 a
5 $g.L^{-1}$	93.20 b	17.14 b	0.76 b
7 $g.L^{-1}$	96.90 ab	19.62 ab	0.89 a
$LSD_{0.05}$	24.64	4.67	0.09
Pr>F			
Sugar	<0.005	<0.007	<0.002
Agar	0.09	0.11	<0.01
Sugar × Agar	0.15	0.96	<0.01
CV%	23.90	23.80	10.60

Means followed by the same letter in each column are not significantly different at 5% LSD.

4.2.6 Shoot fresh weight

Shoot fresh weights (g) from different sugar concentrations are shown in Table 4.7. Shoot fresh weight (g) was significantly different at 1 % level. The highest shoot fresh weight (16.92 g) resulted from treatment (30 $g.L^{-1}$) and the lowest (11.38 g) from treatment (20 $g.L^{-1}$). Treatment 30 $g.L^{-1}$ of sugar was significantly different from 10 $g.L^{-1}$ and 20 $g.L^{-1}$ of sugar. It was found that different sugar concentrations had a strong influence on shoot fresh weight of micropropagated banana plantlets from *in vitro* to *ex vitro* condition.

No significant difference in shoot fresh weight (g) was observed in different agar levels. Maximum shoot fresh weight (14.53 g) was obtained from treatment (3 $g.L^{-1}$) and the minimum shoot fresh weight (12.74 g) from treatment (5 $g.L^{-1}$). These results indicate that the shoot fresh weight was not influenced by the different agar levels. Mohamed and Alsadon (2009) stated that maximum shoot fresh weight of micropropagated potato plantlets was observed from 2 $g.L^{-1}$ of agar gelled medium.

There was no interaction effect between different sugar concentrations and agar levels in shoot fresh weight (g) of *in vitro* culture on acclimatization of banana plantlets. This indicates that the effect of sugar concentrations on shoot fresh weight cannot fluctuate depending on the different agar levels of micropropagated banana plantlets in *in vitro* acclimatization.

4.2.7 Root fresh weight

Results of root fresh weight (g) are presented in Table 4.7. Although the effect of different sugar concentrations on root fresh weight was not statistically different, the maximum root fresh weight (4.39 g) was found in treatment (10 $g.L^{-1}$) and the minimum (3.57 g) from treatment (20 $g.L^{-1}$). These results showed that sugar concentrations did not influence root fresh weight of micropropagated banana plantlets during in *in vitro* acclimatization.

Among the different agar levels, there was no significant difference in root fresh weight (g). Maximum root fresh weight (4.9 g) was observed from treatment (7 $g.L^{-1}$) and minimum fresh shoot weight (3.26 g) was collected from treatment (5 $g.L^{-1}$). These results also indicated that there was no influence of different agar levels on root fresh weight of banana plantlets from *in vitro* to *ex vitro* condition.

There was no interaction effect between different sugar concentrations and agar levels for root fresh weight (g). It means that the effect of sugar concentrations on root fresh weight of micropropagated banana plantlet was not affected by the supply of different agar levels.

4.2.8 Total fresh weight

Table 4.7 describes the effect of different sugar concentrations on total fresh weight (g). In this study, total fresh weight (g) was significantly different at 1 % level. The highest total fresh weight (21.24 g) was resulted from treatment (30 $g.L^{-1}$) and the lowest total fresh weight (14.89 g) was from treatment (20 $g.L^{-1}$). Treatment 30 $g.L^{-1}$ of sugar was significantly different from 10 $g.L^{-1}$ and 20 $g.L^{-1}$ of sugar. According to these results, the total fresh weight was strongly influenced by different sugar concentrations during from *in vitro* acclimatization to *ex vitro* condition.

There was no significant difference in total fresh weight (g) in different agar levels. Maximum total fresh weight (18.66 g) was obtained from treatment (3 $g.L^{-1}$) and minimum total fresh weight (16.01 g) was recorded from treatment (5 $g.L^{-1}$). These results indicated that agar levels have no influence on total fresh weight of micropropagated banana plantlets from *in vitro* to *ex vitro* condition.

No interaction effect was observed between different sugar concentrations and agar level on total fresh weight (g). It means that the effect of sugar concentrations on total fresh weight of micropropagated banana plantlet was not affected by the supply of different agar levels.

Table 4.7 Effect of different sugar concentrations and agar levels on shoot fresh weight, root fresh weight and total fresh weight of micropropagated banana plantlets at 45 days after deflasking

Treatments	Shoot fresh weight (g)	Root fresh weight (g)	Total fresh weight (g)
Sugar			
10 $g.L^{-1}$	12.34 b	4.39 a	16.73 b
20 $g.L^{-1}$	11.38 b	3.57 a	14.89 b
30 $g.L^{-1}$	16.92 a	4.32 a	21.24 a
$LSD_{0.05}$	2.97	1.47	3.77
Agar			
3 $g.L^{-1}$	14.53 a	4.12 ab	18.66 a
5 $g.L^{-1}$	12.74 a	3.26 b	16.01 a
7 $g.L^{-1}$	13.37 a	4.90 a	18.21 a
$LSD_{0.05}$	2.97	1.47	3.77
Pr>F			
Sugar	<0.002	0.44	<0.007
Agar	0.45	0.09	0.31
Sugar × Agar	0.05	0.79	0.16
CV%	21.90	35.90	21.40

Means followed by the same letter in each column are not significantly different at 5% level.

4.2.9 Shoot dry weight

Different sugar concentrations had a significant effect on shoot dry weight (g) at 5 % level (Table 4.8). The highest shoot dry weight (1.23 g) was resulted from treatment (30 $g.L^{-1}$) and the lowest shoot dry weight (0.78 g) from treatment (20 $g.L^{-1}$). The treatment 30 $g.L^{-1}$ of sugar was significantly different from 20 $g.L^{-1}$ of sugar which was not statistically different from 10 $g.L^{-1}$ of sugar. These results showed that sugar concentrations had a strong influence on shoot dry weight of micropropagated banana plantlets. Wainwright and Marsh (1986) reported that sucrose concentration of 40 $g.L^{-1}$ prior to transferring watercress microcutting to *in vivo* conditions showed maximum dry weight of established plantlets.

There was a significant difference in shoot dry weight (g) due to different agar levels at 5 % level. The highest shoot dry weight (1.27 g) was recorded in treatment (7 $g.L^{-1}$) and the lowest shoot dry weight (0.73 g) from treatment (5 $g.L^{-1}$). The treatment 7 $g.L^{-1}$ of agar was significantly different from 5 $g.L^{-1}$ of agar which was not statistically different from 3 $g.L^{-1}$ of agar gelled medium. These results indicated that agar levels have a strong influence on shoot dry weight of micropropagated banana plantlets during from *in vitro* to *ex vitro* condition. The reduction of plantlet growth which was achieved on media containing 3 $g.L^{-1}$ of agar could be due to the reduction of plant water potential below threshold level for cell expansion, which is a prerequisite for shoot formation and growth (Owens and Wozniak 1991). Mohamed and Alsadon (2009) stated that minimum shoot dry weight of micropropagated potato plantlets was observed from agar gelled medium of 2 $g.L^{-1}$.

The interaction between different sugar concentrations and different agar levels was not observed in shoot dry weight (g) of *in vitro* hardening of micropropagated banana plantlet. It means that the effect of sugar concentrations on shoot dry weight of micropropagated banana plantlet was not affected by the supply of different agar levels.

4.2.10 Root dry weight

The statistical results of root dry weight (g) as affected by different sugar concentrations and agar levels are presented in Table 4.8. Among the different sugar concentrations, there was no significant difference in root dry weight (g). The maximum root dry weight (0.32 g) was recorded from treatment (30 $g.L^{-1}$) and the minimum (0.26 g) from treatment (20 $g.L^{-1}$), which was not significantly different

from treatment (10 $g.L^{-1}$). These results showed that the root dry weight was not influenced by different sugar concentrations during *in vitro* condition.

In different agar levels, there was no significant effect on root dry weight (g). The maximum root dry weight (0.31 g) was collected from treatment (7 $g.L^{-1}$) and the minimum (0.27 g) from treatment (3 $g.L^{-1}$). It can be seen that the root dry weight was not changed by different agar levels from *in vitro* to *ex vitro* condition.

Interaction effect between different sugar concentrations and agar levels was not found on root dry weight (g) per plantlet. It means that the effect of sugar concentrations on root dry weight of micropropagated banana plantlet was not affected by the supply of different agar levels.

4.2.11 Total dry weight

The response of sugar concentrations and agar levels on total dry weight (g) is presented in Table 4.8. It can be clearly seen that there was a significant difference in total dry weight (g) due to different sugar concentrations at 5 % level. Among the different sugar concentrations, the highest total dry weight (1.56 g) resulted from treatment (30 $g.L^{-1}$) and the lowest (1.04 g) from treatment (20 $g.L^{-1}$). Treatment 30 $g.L^{-1}$ of sugar was significantly different from 20 $g.L^{-1}$ which was not significantly different from 10 $g.L^{-1}$ of sugar. These results indicated that sugar concentrations strongly influenced total dry weight of micropropagated banana plantlets. Kadlecek et al. 2001 reported that, after acclimatization period, the maximum total dry matter was observed in tobacco plantlets originally cultured *in vitro* on MS medium with 3 $g.L^{-1}$ of sucrose.

There was also significant difference on total dry weight (g) among different agar levels. The highest total dry weight (1.58 g) was observed from treatment (7 $g.L^{-1}$) and the lowest total dry weight (1.01 g) from treatment (5 $g.L^{-1}$). The treatment 7 $g.L^{-1}$ of agar was significantly different from 5 $g.L^{-1}$ which was not statistically different from 3 $g.L^{-1}$ of agar. These results also showed that the total dry weight was strongly influenced by different agar levels.

No interaction effect of different sugar concentrations and agar levels was recorded on total dry weight (g) of *in vitro* hardening of micropropagated banana plantlets. It means that the effect of sugar concentrations on total dry weight of

micropropagated banana plantlet was not affected by the supply of different agar levels.

Treatments	Shoot dry weight (g)	Root dry weight (g)	Total dry weight (g)

Sugar			
10 $g.L^{-1}$	0.94 ab	0.27	1.21 ab
20 $g.L^{-1}$	0.78 b	0.26	1.04 b
30 $g.L^{-1}$	1.23 a	0.32	1.56 a
$LSD_{0.05}$	0.34	0.09	0.40
Agar			
3 $g.L^{-1}$	0.96 ab	0.27	1.22 ab
5 $g.L^{-1}$	0.73 b	0.28	1.01 b
7 $g.L^{-1}$	1.27 a	0.31	1.58 a
$LSD_{0.05}$	0.34	0.09	0.40
Pr>F			
Sugar	<0.04	0.36	<0.05
Agar	<0.01	0.59	<0.03
Sugar × Agar	0.19	0.98	0.35
CV%	34.30	32.80	31.70

Table 4.8 Effect of different sugar concentrations and agar levels on shoot dry weight, root dry weight and total dry weight of *in vitro* grown banana plantlets at 45 days after deflasking

Means followed by the same letter in each column are not significantly different at 5% LSD.

4.2.12 SPAD Reading (Chlorophyll content)

The response of the different sugar concentrations and agar levels on SPAD reading is presented in Table 4.9. No significant difference in SPAD reading was

observed among different sugar concentrations. Sugar (30 $g.L^{-1}$) gave the maximum value (36.26). Da Silva Costa et al. (2009) reported that, with micropropagated 'Caipira' (AAA) banana plantlets, the maximum chlorophyll content was found in the treatment containing 30 $g.L^{-1}$.

In this study, there was also no significant difference in SPAD reading among different agar levels. The maximum value (35.34) was recorded from the treatment (3 $g.L^{-1}$) and the minimum value (32.00) from treatment (5 $g.L^{-1}$).

There was no interaction effect between different sugar concentrations and agar levels on SPAD reading of *in vitro* hardening of micropropagated banana plantlets. It means that the effect of sugar concentrations on SPAD reading of micropropagated banana plantlet was not affected by the supply of different agar levels.

4.2.13 Number of stomata

Table 4.9 shows the effect of different sugar concentrations and agar levels on number of stomata (mm^{-2}) of the leaves of micropropagated banana plantlets. There was no significant difference in number of stomata among different sugar concentrations. The number of stomata per square millimeter varies from 11.53 to 12.01 among the sugar concentrations. The maximum number of stomata (12.01) was counted from treatment (30 $g.L^{-1}$) and the minimum number of stomata (11.53) was from treatment (20 $g.L^{-1}$). Da Silva Costa et al. (2009) reported that greater number of stomata per square millimeter of micropropagated 'Caipira' (AAA) banana plantlets was observed in the lower hypodermis in the natural environment with only 30 $g.L^{-1}$ of sugar. Similar results were obtained by Rocha (2005) for the 'Prata-Ana' banana cultivar, which showed greater stomata density on the lower surface in the natural environment with 15 $g.L^{-1}$ or 30 $g.L^{-1}$ sucrose. Mohamed and Alsadon (2009) also reported that the highest stomata density of micropropagated potatao plantelts was observed on the media containing 30 $g.L^{-1}$. Kundu & Tigerstedt (1998) also concluded that the increase in stomata density could provide the plant with increased gas conductance, thus avoiding photosynthesis to be limited under different environmental conditions. Nevertheless, Da Silva Costa (2009) revealed that the occurrence of stomata and difference in the number of stomata depends on the species and the cultivation condition.

Number of stomata was not affected by different agar levels. The maximum number of stomata (12.41) was produced by treatment (7 $g.L^{-1}$).

Interaction effect between different sugar concentrations and agar levels was not observed on number of stomata of *in vitro* hardening of micropropagated banana plantlet. It means that the effect of sugar concentrations on number of stomata of micropropagated banana plantlet was not affected by the supply of different agar levels.

4.2.14 Quantum yield of primary photochemistry

Table 4.9 shows the effect of different sugar concentrations and agar levels on quantum yield of primary photochemistry (f_v/f_m) of micropropagated banana plantlet. No significant difference in quantum yield of primary photochemistry was observed among the different sugar concentrations. The maximum quantum yield of primary photochemistry (0.66) was recorded from treatment (10 $g.L^{-1}$), which was not statistically different from 20 $g.L^{-1}$ and 30 $g.L^{-1}$ of sugar. Kadleck et al. (2001) reported that photosynthetic capacity was high on the media containing 30 $g.L^{-1}$ sucrose in the acclimatization of micropropagated tobacco plantlets to ex vitro environment and during the second acclimatization stage and after some rapid transient peaks, photosynthetic parameters either slowly or considerably increased or stabilized to a certain level. Pospisilova` et al. (1999) also revealed that net photosynthetic rate in *Solanum tuberosum* and *Spathiphyllum floribundum* plants decreased in the first week after acclimatization and increased thereafter. Hazarika (2004) stated that high sucrose- and salt-containing media, low light level and the carbon dioxide concentration in culture vessel were some of the important limiting factors among various physical micro environmental factors which influence photosynthesis of *in vitro* cultured plants. For *in vitro* growth, a continuous growth supply of exogenous supply of sucrose is required 20 – 30 $g.L^{-1}$ as a carbon source. Donnelly and Vidaver (1984) demonstrated that *in vitro* cultured plants are either poor in chlorophyll content or enzymes activity for photosynthesis (RubPcase) and the low RubPcase activity may be due to the presence of sucrose during the development of leaves.

There was a significant difference on quantum yield of primary photochemistry of micropropagated banana plantlets among different agar levels. The

maximum quantum yield of primary photochemistry (0.68) was observed from treatment (5 $g.L^{-1}$) which was significantly different from 3 $g.L^{-1}$ (0.63) and 7 $g.L^{-1}$ (0.65) of agar.

No interaction effect between different sugar concentrations and agar levels was found on quantum yield of primary photochemistry of *in vitro* hardening of micropropagated banana plantlets. It means that the effect of sugar concentrations on quantum yield of primary photochemistry of micropropagated banana plantlet was not affected by the supply of different agar levels.

Table 4.9 Effect of different sugar concentrations and agar levels on quantum yield of primary photochemistry, SPAD reading and number of stomata of micropropagated plantlets at 45 days after deflasking

Means followed by the same letter in each column are not significantly different at 5% LSD.

Treatments	Quantum yield of primary photochemistry	SPAD Reading	Number of Stomata(mm^{-2})
Sugar			
10 $g.L^{-1}$	0.66	31.91	11.60
20 $g.L^{-1}$	0.65	32.22	11.53
30 $g.L^{-1}$	0.64	36.26	12.01
$LSD_{0.05}$	0.03	5.13	1.38
Agar			
3 $g.L^{-1}$	0.63 b	35.34	11.27
5 $g.L^{-1}$	0.68 a	32.00	11.45
7 $g.L^{-1}$	0.65 b	33.04	12.41
$LSD_{0.05}$	0.03	5.13	1.38
Pr>F			
Sugar	0.20	0.17	0.74
Agar	<0.01	0.39	0.20
Sugar × Agar	0.69	0.47	0.66
CV%	4.60	15.30	11.80

4.2.15 Survival percentage

Figure 4.2 shows the effect of different sugar concentrations and agar levels on survival percentage of *in vitro* grown banana plantlets. The maximum survival

percentage was observed from 30 $g.L^{-1}$ sugar + 3 $g.L^{-1}$ agar and 30 $g.L^{-1}$ sugar + 5 $g.L^{-1}$ agar from *in vitro* acclimatization to *ex vitro* condition. It can be clearly seen that the survival percentage of micropropagated banana plantlets was influenced by different sugar concentrations and agar levels of micropropagated banana plantlets from *in vitro* to *ex vitro* condition. The higher external sucrose concentrations in culture media could promote survival and better growth performance of micropropagated plantlets through *in vitro* acclimatization to *ex vitro* condition. It is in line with Da Silva Costa et al. (2009), who reported that, at 30 days after transplanting, 100 % survival was recorded in plants from natural environment in both 15 $g.L^{-1}$ and 30 $g.L^{-1}$. Possibly, the exposure of the shoots to greater irradiance and temperature amplitudes promoted the hardening of the plants still *in vitro*, since the greenhouse conditions were more similar to that in which the plants were acclimatized, thus favoring lower stress in the transplanting and the *ex vitro* reestablishment. Folliot and Marchal (1992), Skrebsky et al. (2004) and Pacheco et al. (2006) also reported that the effect of sucrose on the success of plant hardening for *Musa spp*., *Pfaffica glomerata* Spreng. Pedersen and *Arachis retusa*, using between 15 $g.L^{-1}$ and 60 $g.L^{-1}$. Skrebsky et al. (2004), Fuentes et al. (2005) and Pacheco et al. (2006) also stated that the negative effects were obtained from the partial or total removal of sucrose on the *ex vitro* development of micropropagated plants. Fuentes et al. (2005) also concluded the low survival and the slow development of *Cocos nucifera* L. cultivated under the absence or low sucrose concentration was a result of the deficiency in the formation of carbon backbone and the allocation of the stocks from the leaves formed *in vitro* to sustain the *ex vitro* reestablishment. Kadleck et al. (2001) reported that survival rates at 46 days after transfer in originally photomixotropically (30 $g.L^{-1}$ sucrose) grown plants under high light and low light were 100 %. De La Vina et al. (1999) stated that survival rates in other plant species were described to be lower and the survival rates of avocado plants grown in a smaller sucrose concentration than in a large one were 100 and 70 %, and 90 and 70 %, respectively with proceeding *ex vitro* growth (2 and 6 months). The higher survival rates corresponded to higher sucrose concentration.

The gelling agent such as agar, which is usually added to increase media viscosity, contributes the survival of the plantlets. The media which is too soft can give hyperhydricity and the media that is too hard can cause the reduction of plant

growth and survival in *ex vitro* condition. According to this finding, the minimum agar level (3 $g.L^{-1}$) gave the maximum survival percentage. High survival percentage was due to concomitant hardening along with rooting and no damage to the root system. It may be concluded that the combination of high sugar concentration and low level of agar gelled medium can be more preferable for *in vitro* hardening of banana plantlets and could be used for other economically important species, when high levels of agar are suspected to have inhibitory effects. Mohamed and Alsadon (2009) also reported that gelling agents had no significant effect on potato plantlets acclimatization which was successfully adapted to the greenhouse conditions with a survival rate varying between 92 and 98 %. However, Au et al. (2012) reported that 7 $g.L^{-1}$ of agar gelled medium compared with shake flask system and temporary immersion system (TIP) using other plant growth regulators gave higher survival percent of micropropagated banana plantlets (Pisang Awak cultivar) during acclimatization.

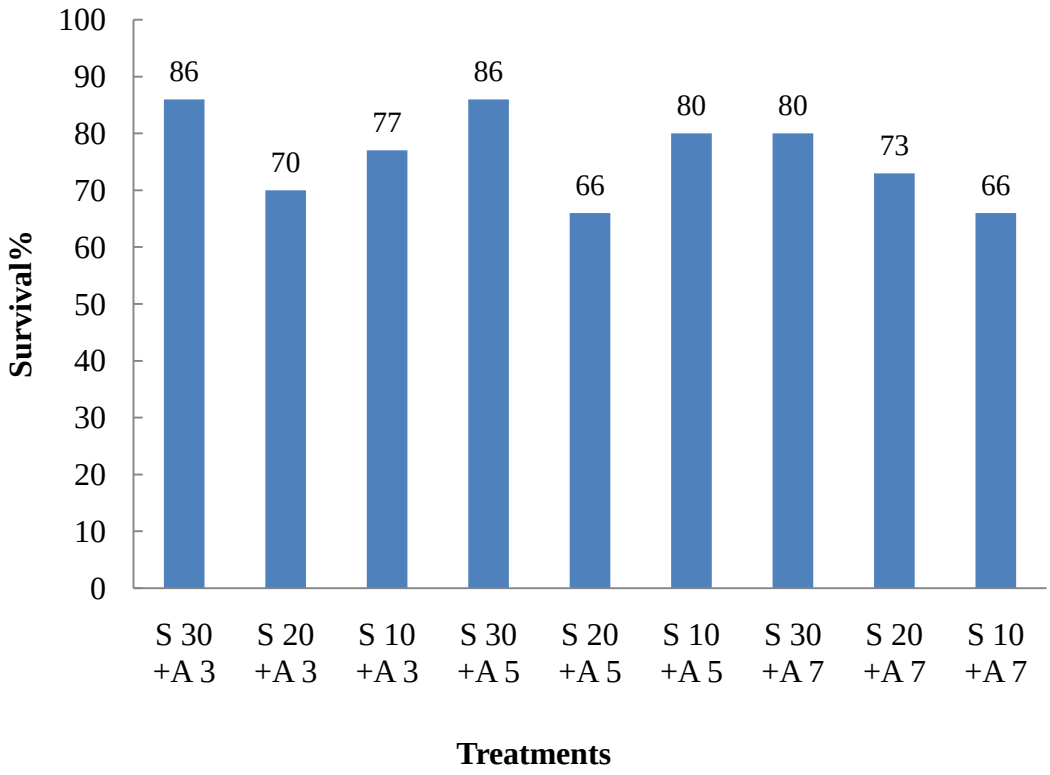

Figure 4.2 Effect of different sugar concentrations and agar levels on survival percentage of micropropagated banana plantlets at 45 days after deflasking during acclimatization period

S = sugar concentration, A = agar levels, $g.L^{-1}$

4.3 Experiment 3: Effect of various pre-treatments on *in vitro* propagated banana plantlets in acclimatization

4.3.1 Plant height

Table 4.10 shows the effect of various pre-treatments on plant height of *in vitro* banana plantlets. There was significant effect of various pre-treatments on plant height of micropropagated banana plantlets at 5 % level. The maximum plant height (35.20 cm) was observed from Homai, followed by Moralmone (31.80 cm), Ferti-start + Homai (30.20 cm) and Control (water) (21.60 cm). The minimum plant height (22.20 cm) was found in Atonik, which was followed by Ferti-start (22.40 cm) and Moralmone + Homai (22.60 cm). Homai was significantly different from Atonik + Homai, Moralmone + Homai, Ferti-start and Atonik except Moralmone, Ferti-start + Homai and Control. The other pre-treatments except Homai did not show their effects in most parameters during the acclimatization period because the roots of *in vitro* derived plantlets are non-functional and they cannot absorb nutrients. The plantlets have to try to be adapted to the surrounding environment. Homai can prevent the infection of diseases caused by adhering agar (even after thorough washing with water) around the roots and it can make the strengthening of banana plantlets during short duration. The banana plantlets derived from *in vitro* were delicate because of poorly developed cuticle, poor stomatal activity, limited mesophyll and plenty of intracellular cavities which could lead excessive transpiration and poor plant development. This is in confirmation with the earlier workers. Vasane and Kothari (2008) reported that fungicidal treatments applied on micropropagated banana cv. Grand Naine gave maximum plantlet height during the secondary hardening.

4.3.2 Number of leaves

Table 4.10 shows the effect of various pre-treatments on number of leaves of *in vitro* banana plantlets. There was a significant difference in number of leaves of micropropagated banana plantlets among various pre-treatments at 1 % level. The highest number of leaves (8.00) was obtained from Homai followed by the treatment Control (water) (7.60) and the lowest number of leaves (5.33) was found in Atonik. Homai was significantly different from other pre-treatments except Control. It can be seen that the pre-treatment (Homai) influence the number of leaves of micropropagated banana plantlets during the acclimatization period. Vasane and Kothari (2008) stated that, during the secondary hardening, the maximum number of leaves was also observed from fungicidal treatments.

Table 4.10 Effect of pre-treatments on plant height and number of leaves of *in vitro* banana plantlets at 45 days after deflasking

Treatments	Plant height (cm)	Number of leaves
Control	27.60 abc	7.60 ab
Homai	35.20 a	8.00 a
Ferti-start	22.40 c	5.47 cd
Atonik	22.20 c	5.33 d
Moramone	31.80 ab	5.93 cd
Ferti-start+Homai	30.20 abc	6.60 bc
Atonik+Homai	25.10 bc	6.07 cd
Moramone+Homai	22.60 c	6.40 cd
$LSD_{0.05}$	8.88	1.14
Pr>F	<0.05	<0.002
CV%	18.70	10.10
Control,Ferti-start,Atonik, Moralmone vs Homai, Ferti-start+Homai, Atonik+Homai, Moramone+Homai [x]	0.29 [y]	0.02

Means followed by the same letter in each column are not significantly different at 5% LSD.

[y] values for contrast represent P values from F test (Pr>F). [X] contrast for different pre-treatments.

4.3.3 Leaf area

Table 4.11 shows the effect of different pre-treatments on leaf area of *in vitro* banana plantlets. There was significant effect of different pre-treatments on leaf area

of micropropagated banana plantlets at 5 % level. Among the treatments, the highest leaf area (88.20 cm^2) was resulted from Homai but was not statistically different from Moralmone (66.60 cm^2) and Ferti-start + Homai (66.90 cm^2). The lowest leaf area (36.50 cm^2) was found from Atonik. Homai was significantly different from Control, Ferti-start, Atonik + Homai, Moralmone + Homai and Atonik. According to this finding, it can be clearly seen that leaf area of micropropagated banana plantlet was influenced by the pre-treatment (Homai). Vasane and Kothari (2008) stated that, during the secondary hardening, the maximum leaf width and leaf length (cm) of micropropagated cv. Grand Naine was observed from fungicidal treatments.

4.3.4 Root length

Table 4.11 shows the effect of various pre-treatments on root length of *in vitro* banana plantlets. There was not significant effect of various pre-treatments on root length of micropropagated banana plantlets among the treatments. The maximum root length (20.04 cm) was observed from Homai followed by Moralmone (19.07 cm) and Atonik + Homai (15.86 cm). The minimum root length (13.45 cm) was observed from Atonik. These results indicated that the effect of different pre-treatments could not significantly influence root length of micropropagated banana plantlets during the acclimatization period. Vasane and Kothari (2008) also reported that fungicidal treatments applied on micropropagated banana cv. Grand Naine gave better root length during the secondary hardening. It may be due to the fact that the *in vitro* formed roots do not function properly (with fewer root hairs) *in vivo*, are rather weak, and often die. The *in vitro* formed roots often have to be replaced by newly formed subterranean roots. As a consequence of the non-functional roots, transpiration outside the glass vessels is too high and can result in loss of many plants.

4.3.5 Pseudo-stem Diameter

Table 4.11 shows the effect of various pre-treatments on pseudo-stem diameter of *in vitro* banana plantlets. No significant effect of them on pseudo-stem diameter of micropropagated banana plantlets was observed. The maximum pseudo-stem diameter (0.72 cm) was found from Homai, followed by Ferti-start + Homai (0.59 cm), Control (water) (0.58 cm), Moralmone (0.57 cm), Atonik + Homai (0.51 cm) and Ferti-start (0.46 cm). The minimum pseudo-stem diameter (0.34 cm) was found from Atonik. Homai was significantly different from Atonik and

Moralmone + Homai. These results indicated that pseudo-stem diameter was not influenced by different pre-treatments during the acclimatization period. However, Vasane and Kothari (2008) as well reported that fungicidal treatments applied on micropropagated banana cv. Grand Naine gave maximum pseudo-stem girth during the secondary hardening.

Table 4.11 Effect of pre-treatments on leaf area, root length and pseudo-stem diameter of *in vitro* banana plantlets at 45 days

Treatments	Leaf area(cm^2)	Root length(cm)	Pseudo-stem

			diameter(cm)
Control	52.70 bc	14.88	0.58 ab
Homai	88.20 a	20.04	0.72 a
Ferti-start	51.20 bc	15.53	0.46 ab
Atonik	36.50 c	13.45	0.34 b
Moralmone	66.60 ab	19.07	0.57 ab
Ferti-start+Homai	66.90 ab	15.63	0.59 ab
Atonik+Homai	47.90 bc	15.86	0.51 ab
Moralmone+Homai	43.90 bc	13.69	0.41 b
$LSD_{0.05}$	29.86	6.68	0.26
Pr>F	<0.05	0.39	0.14
CV%	30.00	23.80	28.10
Control,Ferti-start,Atonik, Moralmone vs Homai, Ferti-start+Homai, Atonik+Homai, Moralmone+Homai [x]	0.17 [y]	0.72	0.27

Means followed by the same letter in each column are not significantly different at 5% level.

[y] values for contrast represent P values from F test (Pr>F). [X] contrast for different pre-treatments.

4.3.6 Shoot fresh weight

Table 4.12 shows the effect of various pre-treatments on shoot fresh weight of *in vitro* banana plantlets. There was significant effect of various pre-treatments on shoot fresh weight of micropropagated banana plantlets at 5 % level. The maximum shoot fresh weight (10.55 g) was observed from Homai followed by Moralmone (7.91 g) and the minimum shoot fresh weight (3.91 g) from Atonik. Homai was significantly different from Control, Ferti-start, Atonik, Atonik + Homai and Moralmone + Homai except Moralmone and Ferti-start + Homai. These results indicated that the shoot fresh weight of micropropagated banana plantlets was influenced by different pre-treatments during the acclimatization period. It is in line with the finding of Haque and Ghosh (2013) who stated that, fungicide application on *in vitro* derived *Aloe vera* plantlets gave better shoot fresh weight during *ex vitro* condition.

4.3.7 Root fresh weight

Table 4.12 shows the effect of various pre-treatments on root fresh weight of *in vitro* banana plantlets. There was significant effect of various pre-treatments on root fresh weight of micropropagated banana plantlets at 1 % level. The highest root fresh weight (2.75 g) was observed from Homai followed by Moralmone (1.77 g), Ferti-start + Homai (1.46 g) and Ferti-start (1.42 g). The lowest root fresh weight (0.87 g) was found from Atonik. Homai was significantly different from other pre-treatments. These results indicated that the root fresh weight of micropropagated banana plantlets was influenced by different pre-treatments during acclimatization period. The result from this study was consistent with that of Vasane and Kothari (2008), who pointed out that the fungicidal treatment without biofertilizer application on banana micropropagated plantlets obtained maximum root fresh weight during the secondary hardening.

4.3.8 Total fresh weight

Table 4.12 shows the effect of various pre-treatments on total fresh weight of *in vitro* banana plantlets. There was significant effect of various pre-treatments on total fresh weight of micropropagated banana plantlets at 5 % level. The maximum total fresh weight (13.30 g) was recorded from Homai followed by Moralmone (9.67 g) and Ferti-start + Homai (9.29 g) and the minimum total fresh weight (4.78 g) was occurred from Atonik. Homai was significantly different from Control, Ferti-

start, Atonik + Homai, Moralmone + Homai and Atonik. These results indicated that the total fresh weight of micropropagated banana plantlets was influenced by different pre-treatments during the acclimatization period. Haque and Ghosh (2013) reported that, fungicide application on *in vitro* derived *Aloe vera* plantlets gave maximum total shoot weight during *ex vitro* condition.

Table 4.12 Effect of pre-treatments on shoot fresh weight, root fresh weight and total fresh weight of *in vitro* banana plantlets at 45 days after deflasking

Treatments	Shoot fresh weight(g)	Root fresh weight(g)	Total fresh weight(g)
Control	6.10 bc	0.95 b	7.05 bc
Homai	10.55 a	2.75 a	13.30 a
Ferti-start	6.26 bc	1.42 b	7.68 bc
Atonik	3.91 c	0.87 b	4.78 c
Moralmone	7.91 ab	1.77 b	9.67 ab
Ferti-start+Homai	7.82 abc	1.46 b	9.29 abc
Atonik+Homai	5.89 bc	1.06 b	6.95 bc
Moralmone+Homai	4.45 bc	1.08 b	5.53 bc
$LSD_{0.05}$	3.94	0.92	4.68
Pr>F	0.06	<0.01	<0.04
CV%	34.00	36.80	33.30
Control,Ferti-start,Atonik, Moralmone vs Homai, Ferti-start+Homai, Atonik+Homai, Moralmone+Homai [x]	0.24 [y]	0.14	0.19

Means followed by the same letter in each column are not significantly different at 5% level.

[y] values for contrast represent P values from F test (Pr>F). [X] contrast for different pre-treatments.

4.3.9 Shoot dry weight

Table 4.13 shows the effect of various pre-treatments on shoot dry weight of *in vitro* banana plantlets. There was no significant effect of fungicide and plant growth regulators application on dry shoot weight of micropropagated banana plantlets. The maximum shoot dry weight (0.57 g) was observed from Homai, followed by Homai + Ferti-start (0.46 g), Moralmone (0.43 g) and the minimum shoot dry weight (0.23 g) was found from Atonik. Homai was significantly different from other pre-treatments except Moralmone and Ferti-start + Homai. These results indicated that the shoot dry weight of micropropagated banana plantlets was not influenced by different pre-treatments during the acclimatization period. Scaranari et al. (2009) reported that the micropropagated banana plantlets treated with fungicide under black 50 % shade cloth for nine weeks gave maximum shoot dry weight during acclimatization period.

4.3.10 Root dry weight

Table 4.13 shows the effect of various pre-treatments on root dry weight of *in vitro* banana plantlets. There was significant effect of various pre-treatments on root dry weight of micropropagated banana plantlets at 5 % level. The highest root dry weight (0.28 g) was found from Homai followed by Moralmone (0.22 g), Homai + Ferti-start (0.16 g) and the lowest root dry weight (0.09 g) was observed from Atonik. Homai was significantly different from others except Moralmone. These results indicated that the root dry weight was influenced by different pre-treatments during the acclimatization period. Annette et al. (1994) also stated that the micropropagated flowering plants of *Purshia tridentata* (Bitterbrush) gave better root dry weight when the plants were applied with fungicide treatment during acclimatization stage.

4.3.11 Total dry weight

Table 4.13 shows the effect of various pre-treatments on total dry weight of *in vitro* banana plantlets. There was significant effect of various pre-treatments on total dry weight of micropropagated banana plantlets at 5 % level. The highest total dry weight (0.85 g) was found from Homai followed by Moralmone (0.64 g) and Homai + Ferti-start (0.62 g). The lowest total dry weight (0.33 g) was obtained from Atonik. Homai was significantly different from other pre-treatments except Moralmone and Ferti-start + Homai. These results indicated that the total dry weight of micropropagated banana plantlets was influenced by different pre-treatments during

the acclimatization period. Scararari et al. (2009) also stated that micropropagated banana plantlets treated with fungicide gave maximum total dry weight during the acclimatization period.

Table 4.13 Effect of pre-treatments on shoot dry weight, root dry weight and total dry weight of *in vitro* banana plantlets at 45 days after deflasking

Treatments	Shoot dry weight(g)	Root dry weight(g)	Total dry weight(g)
control	0.32 bc	0.14 bc	0.45 bc
Homai	0.57 a	0.28 a	0.85 a
Ferti-start	0.36 bc	0.14 bc	0.50 bc
Atonik	0.23 c	0.09 c	0.33 c
Moralmone	0.43 abc	0.22 ab	0.64 ab
Ferti-start+Homai	0.46 ab	0.16 bc	0.62 ab
Atonik+Homai	0.34 bc	0.11 bc	0.47 bc
Moralmone+Homai	0.29 bc	0.12 bc	0.41 bc
$LSD_{0.05}$	0.21	0.11	0.29
Pr>F	0.07	0.06	0.04
CV%	31.50	41.10	30.90
Control,Ferti-start,Atonik, Moralmone vs Homai, Ferti-start+Homai, Atonik+Homai, Moralmone+Homai [x]	0.10	0.54	0.14

Means followed by the same letter in each column are not significantly different at 5% level.

[y] values for contrast represent P values from F test (Pr>F). [X] contrast for different pre-treatments.

4.3.12 Survival percent

The effect of various pre-treatments on survival percentage of *in vitro* grown banana plantlets was shown in Figure 4.3. Among the various pre-treatments, Homai and Control (water) gave the highest survival percentage (92 %) at 45 days after transplanting during the acclimatization stage. The second highest result (84 %) was obtained from Moralmone and Moralmone + Homai. The third highest result (80 %) was observed from Ferti-start + Homai and Atonik + Homai. The lower survival percentage (76 %) was found from Atonik. The lowest survival percentage is 72 % from the treatment of Ferti-start. According to these findings, the survival percentage of micropropagated banana plantlets was influenced by different pre-treatments at 45 days after transplanting during the acclimatization period. Vasane and Kothari (2008) also revealed that fungicidal treatments applied on micropropagated banana cv. Grand Naine gave maximum survival percentage (90 %) during the secondary hardening.

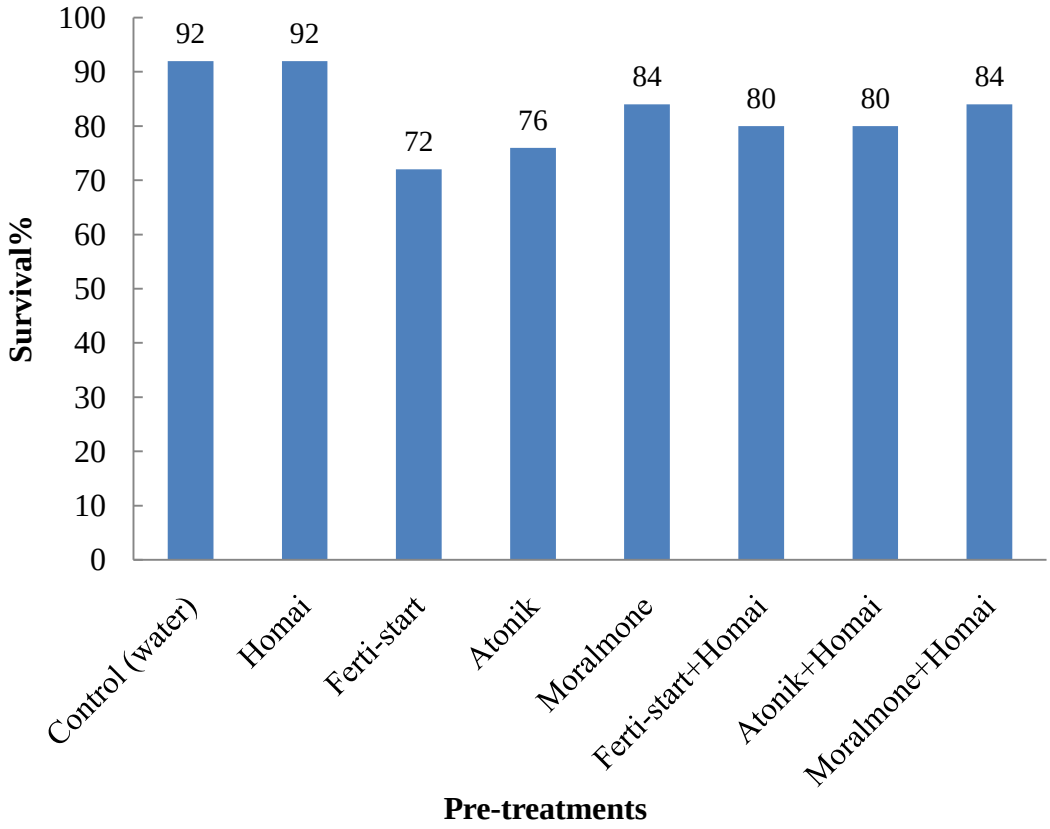

Figure 4.3 Effect of different pre-treatments on survival percentage of micropropagated banana plantlets at 45 days after deflasking during acclimatization period

CONCLUSION

According to the results of experiment I, the plantlets treated with AM fungi gave good growth performance in most parameters. Among the different substrates in the acclimatization stage, the combination of sand + burnt rice husk + AMF provided the highest values in number of leaves, leaf area, fresh weight and dry weight of micropropagated banana plantlets. Sand only substrate gave the highest survival percentage at 45 days after deflasking during the acclimatization period.

According to the experiment II, among the sugar concentrations and agar levels in *in vitro* hardening, the treatment (30 $g.L^{-1}$ of sugar) gave the best results in most parameters studied. Agar (3 $g.L^{-1}$) showed the maximum plant height, number of leaves, leaf area, root length, pseudo-stem diameter, shoot fresh weight and total fresh weight. Maximum survival percentage was observed from (30 $g.L^{-1}$ of sugar + 3 $g.L^{-1}$ of agar) and (30 $g.L^{-1}$ of sugar + 5 $g.L^{-1}$ of agar) at 45 days after deflasking from *in vitro* to *ex vitro* condition. According to experiment III, among the different pre-treatments during the acclimatization period, the plantlets treated with Homai gave the highest results in most parameters.

According to the findings of this study, the substrates which have good aeration and pore spaces are more important for micropropagated plantlets during the acclimatization period. Among the different sugar concentrations and agar levels, 30 $g.L^{-1}$ of sugar and 3 $g.L^{-1}$ of agar are the best sugar concentration and agar level for micropropagated banana plantlets during *in vitro* acclimatization. Homai should be used as a pre-treatment on acclimatization stage of *in vitro* grown banana plantlets.

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APPENDICES

Appendix 1. Physical and chemical properties of the tested substrates

Substrates	Bulk density(gcm^{-3})	Porosity (%)	pH
Sand+ garden soil	0.684	-0.810	6.17
Sand+ compost	0.640	-0.696	7.28
Sand+ burnt rice ash	0.850	-1.25	6.16
Sand+ vermicompost	0.620	-0.643	6.81
Sand only	0.830	-1.19	6.28
Sand+ garden soil+ burnt rice ash	0.640	-0.696	5.93

Bulk density = W/V (gcm^{-3}), Porosity (%) = 1- (Bulk density × 2.65)

Appendix 2. Homai

Homai	1,2-bis (3-methoxycarbonyi-Zthioureido) benzene
Thiophanate methyl	50 %
Thiram	30 %
Other additives	20%
Broad-spectrum fungicide	- both systemic and contact fungicidal functions excellent for control of "damping off" in vegetable and fruit nurseries - unique combination fungicide provides excellent disease control through its translaminar and systemic movement - low toxicity to mammals, aquatic organisms and wildlife and does not show adverse effect to beneficial creatures

Appendix 3. Atonik (aromatic nitro phenolic compound)

Name of Chemicals	Sodium 5-nitroguaiacolate	Sodium ortho-nitrophenolate	Sodium para-nitrophenolate
Molecular formula	$C_7H_6NO_2Na$	$C_6H_4NO_3Na$	$C_6H_6NO_3Na$
Its properties	liquid form	- stimulates plant activity without causing malformation or toxicity - accelerates the plasma streaming of the cells by increase in the endogenous auxin level - enhances growth and some essential metabolic processes of treated plants	- stimulates flowering process, - adapt to unfavorable environment - enhances yields, nutrients uptake from the soil, accumulation of photosynthesis products and development of soil bacteria

Appendix 4. Moralmone

Name of Chemicals	Sodium O-nitrophenol	Sodium P-nitrophenol	Sodium 2,4-dinitrophenol	Sodium 5-nitroguaiacol
Molecular Formula	$C_6H_5NO_3Na$	$C_6H_5NO_3Na$	$C_6H_4N_2O_5Na$	$C_7H_6NNaO_4$
Its properties	Liquid form	- enhance plant growth - stimulate root formation	- stimulate flowering process	- enhance yield and quality products

Appendix 5. Ferti-start

Name of chemicals	Chelated Iron	Thiamine Hydrochloride (Vitamin B_1)
Molecular formula	EDTA-$FeNH_4$	$C_{12}H_{17}ClN_4O_2S.HCl$
Its properties	- liquid, powder or granulated form	- give the strength of root growth - to reduce the shock of transplanting - effective solution for orchids, rose, bedding plant, trees, vegetables, shrubs and other ornaments according to the instructions on the chemical

Appendix 6. Materials used in the experiments

a. Arbuscular Mycorrhizal Fungi (AMF)

b. Homai

c. Atonik

d. Ferti-start

e. Moralmone

Appendix 7

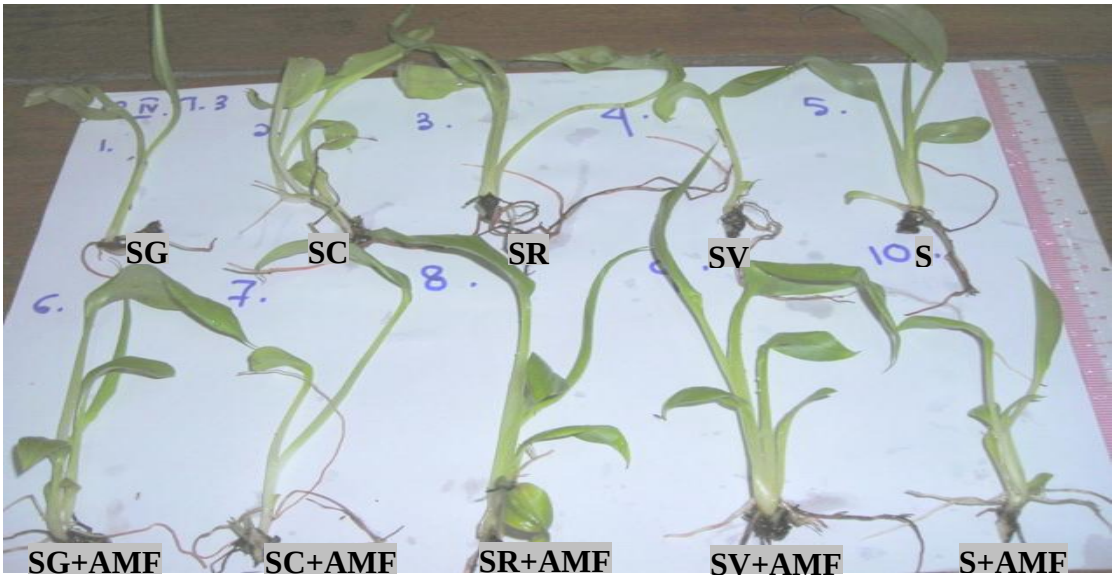
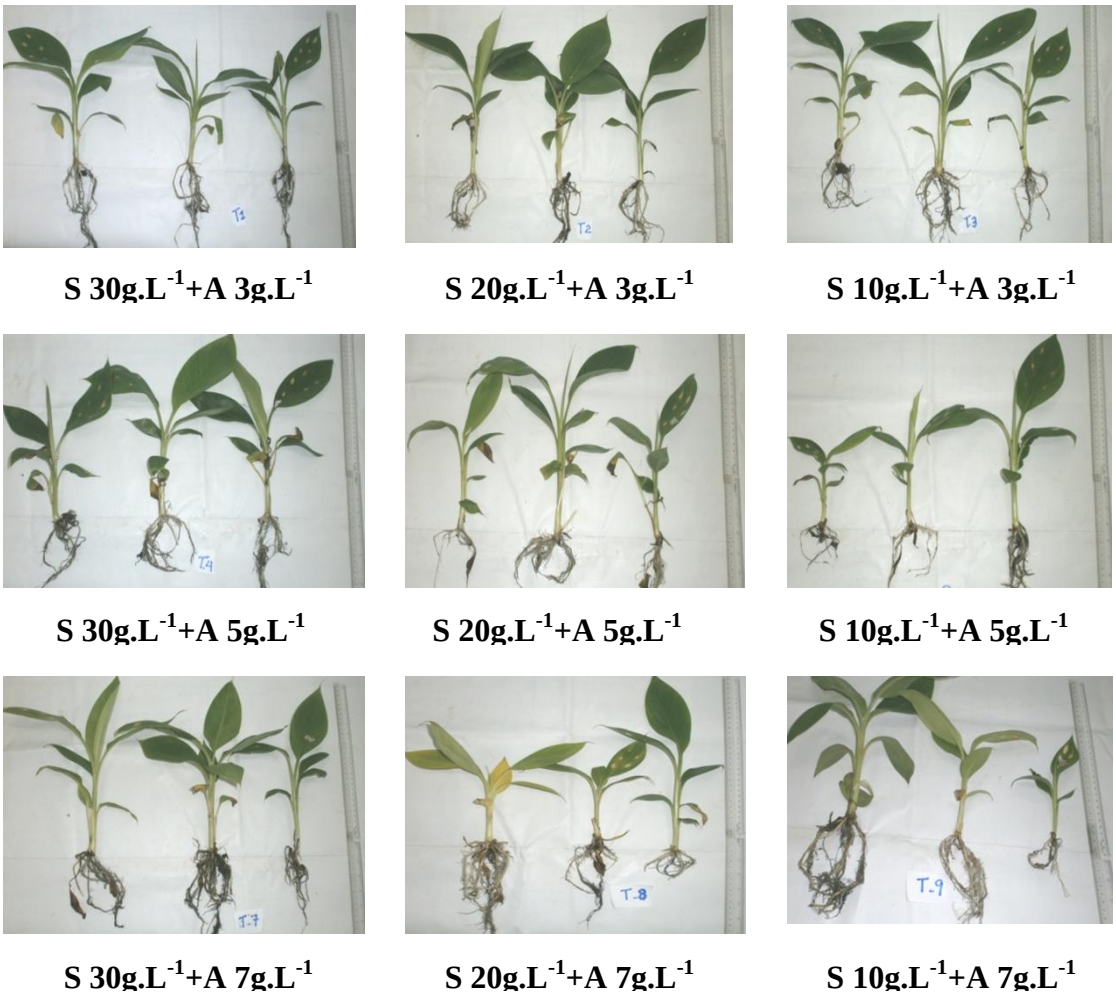


Plate 1. Micropropagated Banana Plantlets treated with different Substrates at 45 days after deflasking during Acclimatization Stage (SG=sand+garden soil, SC=sand+compost, SR=sand+burnt rice husk, SV=sand+vermicompost, S=sand only, AMF=arbuscular mycorrhizal fungi)

Appendix 8



S 30g.L^{-1}+A 3g.L^{-1} **S 20g.L^{-1}+A 3g.L^{-1}** **S 10g.L^{-1}+A 3g.L^{-1}**

S 30g.L^{-1}+A 5g.L^{-1} **S 20g.L^{-1}+A 5g.L^{-1}** **S 10g.L^{-1}+A 5g.L^{-1}**

S 30g.L^{-1}+A 7g.L^{-1} **S 20g.L^{-1}+A 7g.L^{-1}** **S 10g.L^{-1}+A 7g.L^{-1}**

Plate 2. Micropropagated banana plantlets treated with different sugar concentrations and agar levels in *in vitro* to *ex vitro* acclimatization at 45 days after deflasking. S = sugar, A= agar

Appendix 9

Control (water)	Homai	Ferti-start	Atonik	Moral-mone	Ferti-start+ Homai	Atonik +Homai	Moral mone+ Homai

Plate 3. Micropropagated banana plantlets treated with pre-treatments at 45 days after deflasking during the acclimatization stage