Laporan Akhir Projek Penyelidikan Jangka Pendek bertajuk "Propagasi In Vitro Nangka (*Artocarpus heterophyllus*) cv Mastura"

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Introduction

Market value of tropical fruits has increased significantly due to population growth, better living conditions and promotion of international tourism. Increased attention is being focused upon the promotion of both wild edible fruits and introduced cultivated species. Tropical fruits exported by Malaysia are bananas, limes, pomelos, citrus fruit, mangoes, pineapples and papaya. Every year tropical fruits are gaining more and more attention from tropical fruit producing and importing countries, with the respective governments agree to cooperate, to improve earnings, nutrition, consumer choice and food security. However several countries in the tropical region are not able to meet the grower's demand for certified healthy planting material. Tropical fruit varieties are often infected by virus diseases and the traditional methods of propagation of these fruits species are not adequate to meet the demand. A number of the fruit growing countries which are located in Asia, Africa and Latin America are attempting to overcome the shortage of planting material through the establishment of tissue culture laboratories for supplying clonal plant materials. Jackfruit (*Artocarpus heterophyllus* Lamk) is an important tropical tree values for its nutritious fruits and durable timber (Amin and Jaiswal, 1993). It was considered as one of the potential new crops that attracted increased interest in the world. Since the jackfruit tree is able to out-crossing, new hybrid can be easily bred. Mastura is one of a local new hybrid produce through the cooperation between USM and Inter Crystal Agritech (ICA) in 2000.

Generally, jackfruit is grown from seeds but the seeds are difficult to germinate even just after a short period of storage (Singh 1986; Samaddar, 1990). Furthermore, seed-propagated plants have high variation in plant progenies and

rarely identical to the parent plants. Breeding the desirable genotypes using conventional vegetative propagation methods such as grafting and air layering are time consuming and difficult (Amin and Jaiswal, 1993). Besides, these techniques required skills, suitable plants source, good condition of rootstock and favorable environment. Tissue culture technique can hence be used as an alternative method for clonal propagation of selected *A. heterophyllus* genotypes to produce healthy and virus free plant materials.

3 Materials and Methods

3.1 Establishment of Aseptic Explants

The apical shoots of jackfruit with two to three axillary buds (about 3 -5 cm long) were collected and placed in distilled water to maintain the freshness. The explants were brushed gently, using a soft brush, with a mixture liquid detergent and Clorox® (2:1) which contained 5.3% sodium hypochlorite. The explants were then immersed in the same mixture with continuous agitation for one minute. This was followed by rinsing three times with sterile distilled water. The explants were then dipped into 70% ethanol for one minute to remove the latex secreted from the cut edges of the explants. The shoot explants were then surface-sterilized with 20% (v/v) Clorox® added with two drops of Teepol, for 10, 15 or 20 minutes respectively on the first stage followed by rinsing three times with sterile distilled water. The explants were again surface-sterilized using 10% Clorox® (v/v) added with two drops of Teepol, also for 10, 15 or 20 minutes for the second stage and followed by rinsing three times with sterile distilled water. After the two-stage surface-sterilization procedure, the outer covering of the green stipules was removed and the inner shoots were cut and used as explants.

3.2 Induction of Multiple Shoots Formation of *A. heterophyllus*

Aseptic shoots explants with an average height of 5 mm were cultured on basic MS medium supplemented with a combination of 6-Benzylaminopurine (BA) and Indole-3-butyric acid (IBA), both at the concentration of 0, 2.0, 4.0, 6.0, 8.0 and 10.0 mg/L using 6^2 factorial designs. Culture media were prepared in 50

ml conical flask and closed with rubber stopper. Two explants were cultured in each flask and six flasks were used for each culture medium treatment using factorial block design. An explant that formed two shoots or more with a shoot height of at least 3 mm was considered as forming multiple shoots. The survival rate, percentage of explant forming multiple shoots and the number of shoots formed from each explant were recorded after nine weeks of culture. The data were analyzed using two-way Analysis of Variance (ANOVA) and comparison of means using Tukey Test (HSD) at $p = 0.05$.

3.3 Effect of Reduced BA and IBA in the MS Medium on Multiple Shoots Formation of *A. heterophyllus*

The shoot explants were cultured on MS medium supplemented with BA at 3.0, 3.5, 4.0, 4.5 and 5.0 mg/L and IBA at 0, 0.5, 1.0, 1.5 and 2.0 mg/L using 5^2 factorial designs. Culture media were prepared and placed in 50 ml conical flask. Three explants were cultured in each flask and six flasks were used for each culture medium treatment using complete randomized block design. The survival rate, percentage of explant forming multiple shoots, number of shoots formed from each explant and each shoot height were recorded after nine weeks of culture. The data were analyzed using two-way ANOVA and comparison of means by Tukey Test (HSD) at $p = 0.05$.

3.4 Rooting of *In Vitro* Shoots

The *in vitro* shoots produced on MS medium supplemented with 4.5 mg/L BA with at least 2 cm height were used for rooting purposes. The leaves were trimmed down to one or two leaves. Hard calluses formed at the shoot base were removed before rooting procedures. The *in vitro* shoots were inoculated on ½ MS medium with 3% sucrose and supplemented with 0.5 mg/L α-naphthalene acetic acid (NAA) and 0.5 mg/L IBA. The cultures were kept in total darkness (place in closed box) for 10 days. Then these cultures were transferred to basic ½ MS salt medium with 3.0 % sucrose and incubated in light (2000 – 2500 lux). The

experiment was repeated three times. The rooting responses were observed after four weeks.

3.5 Acclimatization of the *In Vitro* Plantlets

Rooted plantlets were removed carefully (without breaking the delicate roots) from the test-tube and rinsed with distilled water to remove traces of rooting medium. Each plantlet was trimmed to two to three leaves and planted in pot containing mixture of organic soil and top soil (1:1) and all the pots were placed in a basin covered with transparent plastic which was punch with small holes for aeration. The basin was placed in the growth chamber regulated with 24 hours photoperiod of 660 lux and temperature at 25 ± 1°C for two to three weeks. Soil was kept at good draining condition at all times. After two to three weeks, the plastic cover was removed and the plantlets still placed in the growth chamber for another one to two weeks. The plantlets were removed from the growth chamber and kept in the plant house. The survival rate and condition of the plantlets were recorded after four weeks in the plant house.

Results and Discussion

Establishment of Aseptic Explants

The surface-sterilization of apical shoot plus axillary buds using two stage sterilization with 20 % (v/v) Clorox® for 10 minutes on the first stage followed by 10 % (v/v) Clorox® for 10 minutes on the second stage produced 85.3 ± 5.3% aseptic explants. Longer sterilization period reduced the percentage of establishing aseptic explants (Table 1). For shoot explants, the cap-like stipule acted as a protective shield to protect the young inner shoot from contaminates and the corrosive effect of the surface sterilization agents. Sterilization of shoot explants with 20% (v/v) Clorox® for 10 minutes on the first stage followed by 10% (v/v) Clorox® for 10 minutes on the second stage produced 100% aseptic explants and all the shoot explants survived the sterilization process. In the

surface sterilization process, Teepol was added as a wetting agent for a better contact between the sterilization agent and the surface of explant

Table 1: The percentage of aseptic explants of *A. heterophyllus* obtained from the two-stage surface sterilization method.

	Sterilization period (min) for 1st stage surface sterilization using 20% (v/v) Clorox®		
Sterilization period (min) for 2nd stage surface sterilization using 10% (v/v) Clorox®	10 minutes	15 minutes	20 minutes
10 minutes	85.3 ± 5.3%	48.1 ± 1.9%	51.9 ± 1.9%
15 minutes	47.5 ± 2.5%	55.1 ± 8.1%	59.3 ± 0.7%
20 minutes	57.8 ± 2.2%	47.5 ± 2.5%	32.3 ± 2.3%

Induction of Multiple Shoots Formation of *A. heterophyllus*

After inoculation of shoot explants on MS medium supplemented with 0 – 10 mg/L BA and 0 – 10 mg/L IBA for one to two weeks, the cap-like stipules of the shoot explants ruptured and young leaf and shoots emerged. Compact and friable calluses were formed at the basal end of the shoot explant (Figure 1). After three weeks, when the shoot explants with the removal of stipule and callus, were sub-cultured to fresh medium, the explants started to respond to the treatment of BA and IBA.

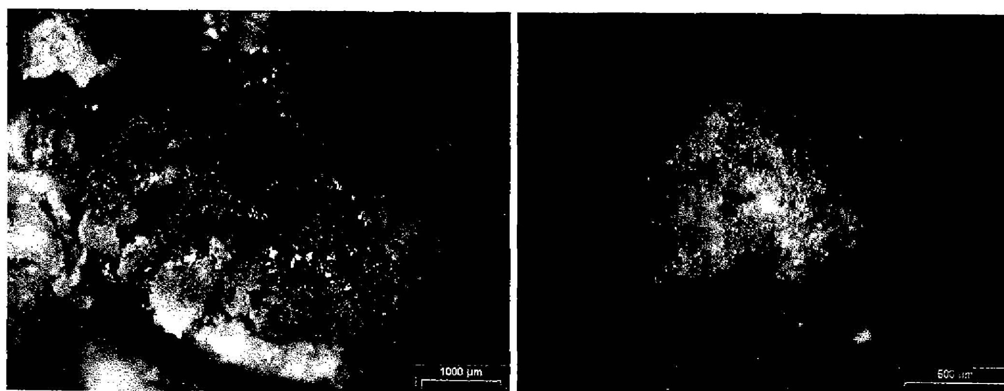

Figure 1: Hard calluses (Left) and friable calluses (Right) formed at the basal end of the shoot explant.

An average of 50% - 100% of the explants survived when cultured on MS medium supplemented with BA and IBA (both at 0, 2.0, 4.0, 6.0, 8.0 and 10.0 mg/L). MS medium supplemented with 4 mg/L BA induced most of the explants (83.3 %) to form multiple shoots with an average of 4.0 ± 0.4 shoots per explant. All the shoots survived (100%) when maintained on MS medium supplemented with 4 mg/L BA while the rest of the tested media induced only 1 – 2.5 shoots per explant. As the amount of BA and IBA supplemented into the MS medium increased, the percentage of the shoot explants forming multiple shoots decreased even though their survival rate remained relatively high (Table 2).

One or two weeks after inoculation into MS medium supplemented with 4.0 mg/L BA, the stipule of the apical shoot broke and young multiple shoots formed. After two to three sub-culture cycles with three weeks interval for each cycle, the number of shoots formed from each explants increased by two to three folds. It was observed that when the shoots were further sub-cultured up to six to seven cycles, new shoots formed more easily compared to first and second sub-cultured cycle, with an average of 5 mm shoot height. The newly formed shoots were healthy with fresh green leaves. Some of the newly formed leaves were deeply looped, the same type of leaves that could be seen on the mother plant. The newly formed shoots were normal even after several times of sub-cultures for one year.

For *A. heterophyllus,* the presence of only cytokinin in the MS medium was sufficient to induce multiple shoots formation. The most number of shoots were induced when the shoot explants were cultured on medium MS medium + 4.5 mg/L BA, where 7 shoots per explant were obtained. Present of IBA in the medium (with of without BA) only produced 3 shoots or less from each explant. Hence the additional of 4.5 mg/L of BA was sufficient for multiple shoot formation of *A. heterophyllus*. Similar observation was reported by Amin and Jaiswal (1993) and Roy *et al.* (1990). Amin and Jaiswal reported that the shoot multiplication medium for *A. heterophyllus* cultivar of India, MS medium supplemented with BA and kinetin (1.0 – 2 mg/L) was able to induce 6 – 10 new shoots per explant. Roy *et al.* (1990) was able to induce 5 – 7 shoots from each explant also from the *A. heterophyllus* of India cultivar using MS medium supplemented with 1.0 mg/L BAP and 0.5 mg/L kinetin. The amount of BA added into the MS medium for the Malaysian cultivar was about twice the amount reported by these two groups of researchers. This could be due to the different cultivar and age of *A. heterophyllus* used in the experiment.

Table 2: The effect of BA and IBA (0.0 – 10.0 mg/L) in MS medium on the multiple shoots formation and survival rate of *A. heterophyllus* shoot explants.

BA (mg/L)	IBA (mg/L)	Survival rate (%) ± s.e.		Percentage explants forming multiple shoot (%) ± s.e.		Mean no. of shoots/explant (mean ± s.e)	
0	0	83.3 ± 10.5	ab	33.3 ± 10.5	fgh	1.2 ± 0.1	rs
	2	100.0 ± 0.0	a	33.3 ± 16.7	fgh	1.3 ± 0.2	qrs
	4	100.0 ± 0.0	a	66.7 ± 16.7	fg	1.7 ± 0.2	qrs
	6	100.0 ± 0.0	a	33.3 ± 16.7	fgh	1.3 ± 0.2	qrs
	8	100.0 ± 0.0	a	0.0 ± 0.0	h	1.0 ± 0.0	rs
	10	100.0 ± 0.0	a	16.7 ± 10.5	gh	1.2 ± 0.1	rs
2	0	66.7 ± 10.5	ab	33.3 ± 10.5	fgh	1.3 ± 0.3	qrs
	2	100.0 ± 0.0	a	66.7 ± 10.5	fg	2.2 ± 0.3	qr
	4	100.0 ± 0.0	a	50.0 ± 12.9	fgh	1.7 ± 0.2	qrs
	6	100.0 ± 0.0	a	66.7 ± 10.5	fg	1.8 ± 0.2	qrs
	8	83.3 ± 10.5	ab	50.0 ± 12.9	fgh	1.3 ± 0.2	qrs
	10	83.3 ± 10.5	ab	66.7 ± 16.7	fg	1.5 ± 0.2	qrs
4	0	100.0 ± 0.0	a	83.3 ± 10.5	f	4.0 ± 0.4	p
	2	100.0 ± 0.0	a	66.7 ± 10.5	fg	2.2 ± 0.2	qr
	4	83.3 ± 10.5	ab	33.3 ± 16.7	fgh	1.2 ± 0.3	rs
	6	100.0 ± 0.0	a	83.3 ± 10.5	f	2.0 ± 0.2	qrs
	8	100.0 ± 0.0	a	50.0 ± 0.0	fgh	1.5 ± 0.3	qrs
	10	83.3 ± 10.5	ab	33.3 ± 10.5	fgh	1.2 ± 0.1	rs
6	0	83.3 ± 10.5	ab	66.7 ± 21.1	fg	2.2 ± 0.6	qr
	2	83.3 ± 10.5	ab	33.3 ± 10.5	fgh	1.5 ± 0.3	qrs
	4	100.0 ± 0.0	a	83.3 ± 10.5	f	2.2 ± 0.1	qr
	6	83.3 ± 10.5	ab	33.3 ± 10.5	fgh	1.3 ± 0.3	qrs
	8	83.3 ± 10.5	ab	33.3 ± 0.0	fgh	1.2 ± 0.3	rs
	10	83.3 ± 10.5	ab	33.3 ± 10.5	fgh	1.2 ± 0.1	rs
8	0	100.0 ± 0.0	a	66.7 ± 10.5	fg	2.5 ± 0.2	q
	2	83.3 ± 10.5	ab	33.3 ± 10.5	fgh	1.5 ± 0.3	qrs
	4	50.0 ± 12.9	b	16.7 ± 10.5	gh	1.0 ± 0.3	s
	6	100.0 ± 0.0	a	33.3 ± 10.5	fgh	1.3 ± 0.1	qrs
	8	83.3 ± 10.5	ab	33.3 ± 10.5	fgh	1.5 ± 0.3	qrs
	10	66.7 ± 10.5	ab	16.7 ± 10.5	gh	1.0 ± 0.2	s
10	0	83.3 ± 10.5	ab	50.0 ± 12.9	fgh	2.2 ± 0.3	qr
	2	66.7 ± 10.5	ab	33.3 ± 10.5	fgh	1.3 ± 0.3	qrs
	4	66.7 ± 10.5	ab	33.3 ± 10.5	fgh	1.2 ± 0.3	rs
	6	83.3 ± 10.5	ab	66.7 ± 10.5	fg	1.5 ± 0.2	qrs
	8	100.0 ± 0.0	a	50.0 ± 12.9	fgh	1.5 ± 0.1	qrs
	10	66.7 ± 10.5	ab	16.7 ± 10.5	gh	1.0 ± 0.2	s

(Means values within the same column followed by the same alphabet are not significantly difference based on Tukey test, p = 0.05)

Effect of Reduced BA and IBA in MS Medium on Multiple Shoots Formation of *A. heterophyllus*

The survival rate of the shoot explants decreased as the concentration of BA in the culture medium increased from 3.0 to 5.0 mg/L and IBA increased from 0.5 to 2.0 mg/L. The shoot explants cultured on MS medium supplemented with BA (3.0 – 5.0 mg/L) without IBA had higher survival rate (67% - 100%) compared to the shoot explants cultured on MS medium supplemented with BA (3.0 – 5.0 mg/L) and IBA (0.5 – 2.0 mg/L).

The percentage of the shoot explants formed multiple shoot were also higher (66.7% - 100%) on MS medium supplemented with BA (3.0 – 5.0 mg/L) only, compared to shoot explants on MS medium supplemented with BA (3.0 – 5.0 mg/L) and IBA (0.5 – 2.0 mg/L) with less then 77.8% of shoot explants forming multiple shoots.

The number of shoots formed from each explant was high (4.2 – 7.0 shoot per explant) when the shoot explants were cultured on MS medium supplemented with BA only. With the presence of IBA, the average number of shoots formed from each explant was three shoots per explant and below. The multiplication of shoots decreased when the concentration of IBA increased from 0.5 to 2.0 mg/L with the presence of BA. The shoot explants cultured on MS medium supplemented with BA 4.5 mg/L induced the highest number of multiple shoot formed with the formation of 7.0 ± 1.0 shoots per explant (Table 3). The multiple shoots formed from each of the shoot explant were normal and healthy and usually consisted of younger shoots at the base of the shoot explant (Figure 2).

Table 3: Effect of reduced concentration of BA and IBA in MS medium on the multiple shoots formation of *A. heterophyllus*.

BA (mg/L)	IBA (mg/L)	Percentage of Survival Rate (%) ± s.e.		Percentage of The explants Formed Multiple Shoots (%) ± s.e.		Mean Number of Shoots per Explants (mean ± s.e.)		Mean of Shoots' Height (mm ± s.e.)	
3.0	0.0	100.0 ± 0.0	a	100.0 ± 0.0	f	5.0 ± 0.9	lmn	4.6 ± 0.6	st
	0.5	44.5 ± 14.1	abc	22.2 ± 14.1	ijk	0.9 ± 0.4	q	3.5 ± 1.1	stuv
	1.0	77.8 ± 14.1	abc	66.7 ± 12.2	fghi	3.0 ± 1.2	mnopq	2.7 ± 0.1	tuv
	1.5	100.0 ± 0.0	a	77.8 ± 7.0	fgh	3.0 ± 0.1	mnopq	5.2 ± 0.4	st
	2.0	77.8 ± 7.0	abc	33.3 ± 0.0	hijk	1.1 ± 0.1	pq	4.0 ± 0.4	stu
3.5	0.0	88.9 ± 7.0	ab	77.8 ± 14.1	fgh	5.4 ± 1.1	lm	4.8 ± 0.5	st
	0.5	77.8 ± 7.0	abc	55.6 ± 7.0	fghij	2.4 ± 0.4	nopq	4.3 ± 0.7	st
	1.0	77.8 ± 7.0	abc	66.7 ± 0.0	fghi	2.9 ± 0.4	mnopq	4.8 ± 0.2	st
	1.5	77.8 ± 14.1	abc	44.4 ± 18.6	ghijk	2.0 ± 0.6	nopq	3.3 ± 0.6	stuv
	2.0	55.6 ± 18.6	abc	33.3 ± 12.2	hijk	1.6 ± 0.5	pq	2.8 ± 0.9	tuv
4.0	0.0	66.7 ± 12.2	abc	66.7 ± 12.2	fghi	4.2 ± 1.1	mnop	5.9 ± 0.8	s
	0.5	77.8 ± 7.0	abc	44.4 ± 7.0	ghijk	2.3 ± 0.5	nopq	4.4 ± 0.3	st
	1.0	55.6 ± 18.6	abc	33.3 ± 12.2	hijk	1.6 ± 0.5	pq	2.4 ± 0.9	tuv
	1.5	55.5 ± 14.1	abc	55.5 ± 14.1	fghij	2.5 ± 0.5	mnopq	5.2 ± 0.4	st
	2.0	44.5 ± 14.1	abc	44.5 ± 14.1	ghijk	2.1 ± 0.8	nopq	2.6 ± 0.8	tuv
4.5	0.0	88.9 ± 7.0	ab	88.9 ± 7.0	fg	7.0 ± 1.0	l	3.9 ± 0.2	stuv
	0.5	55.5 ± 14.1	abc	33.3 ± 0.0	hijk	1.8 ± 0.3	opq	2.8 ± 0.1	tuv
	1.0	66.7 ± 0.0	abc	44.5 ± 14.1	ghijk	1.2 ± 0.2	pq	4.1 ± 0.3	stu
	1.5	55.5 ± 14.1	abc	22.2 ± 14.1	ijk	0.9 ± 0.4	q	3.6 ± 0.7	stuv
	2.0	44.5 ± 14.1	abc	22.2 ± 14.1	ijk	0.8 ± 0.3	q	2.7 ± 1.0	tuv
5.0	0.0	77.8 ± 14.1	abc	66.7 ± 12.2	fghi	4.9 ± 1.3	lmno	4.1 ± 0.2	stu
	0.5	66.7 ± 12.2	abc	55.5 ± 14.1	fghij	2.3 ± 0.9	nopq	3.7 ± 0.1	stuv
	1.0	22.2 ± 14.1	c	0.0 ± 0.0	k	0.2 ± 0.1	q	1.2 ± 0.7	uv
	1.5	22.2 ± 7.0	c	0.0 ± 0.0	k	0.2 ± 0.1	q	1.0 ± 0.4	uv
	2.0	33.3 ± 12.2	bc	11.1 ± 7.0	jk	0.7 ± 0.3	q	1.2 ± 0.5	uv

(Means values within the same column followed by the same alphabet are not significantly difference based on Turkey test, p=0.05)

Figure 2: Normal and healthy multiple shoots of *A. heterophyllus* on MS medium supplemented with 4.5 mg/L BA.

The height of each induced multiple shoot ranged from 1 to 6 mm. Shoot height was slightly reduced with the present of IBA (0.5 mg/L – 2.0 mg/L) in the culture medium. The multiple shoots grew well with the formation of young shoots that had succulent stems, long internodes, and light-green leaves (some of the leaves were lobed). The culture medium turned brown when the shoot explants were cultured on the medium for more then three weeks. The browning was more intense when the explants were cultured for more than five weeks or when the concentrations of both BA and IBA in the MS medium were increased. When the explants were cultured for a period of more then six weeks, the shoot growth became retarded and most of the shoots became necrotic and dead.

Rooting of *In Vitro* Shoots

Only 12.5% of the *in vitro* shoots cultured on solid ½ MS medium supplement with 0.5 mg/L IBA produced roots after three to four weeks. The same result was obtained for the shoots cultured on MS solid medium supplement with 4.0 mg/L IBA. The number of roots formed was few (one to two roots) and thin. All the *in vitro* shoots did not produced roots in ½ MS liquid medium supplemented with 0, 0.5, 1.0, 2.0, 4.0, and 8.0 mg/L IBA in the culture pack. Herbaceous plants sometimes could root readily *in vitro*, but not for woody plant. This was most likely due to woody plant lack of root initiator as a result of

imbalance growth regulators (especially lack of auxin) (Hamish and Sue, 1998). Therefore rooting protocol was developed to root the *in vitro* shoots of *A. heterophyllus.*

Based on the results obtained from the previous experiments, the best rooting method was established by culturing the *in vitro* shoots on ½ MS (1.5% sucrose) + 0.5 mg/L NAA + 0.5 mg/L IBA and incubated in total darkness for ten days. The shoots were then sub-cultured to fresh rooting medium and exposed to light for three weeks. The percentage of rooting was found to be consistent and repeatable (40 %) and the roots produced were normal and healthy. The length of the roots were long (5 – 6 cm) with lateral roots. The plantlets grew healthily even up to six weeks on the rooting medium (Fig. 3).

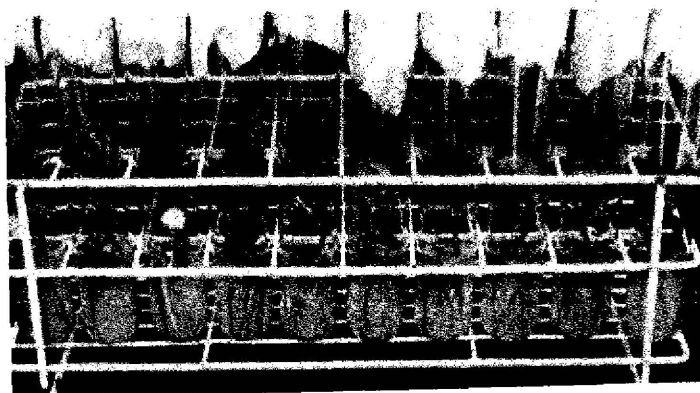

Figure 3: *In vitro* shoots produced normal, long and healthy roots with abundant lateral roots. These shoots were incubated in darkness for ten days on ½ MS + 0.5 mg/L NAA + 0.5 mg/L IBA rooting medium followed by sub-culturing to fresh rooting medium and three weeks light exposure

4.5 Acclimatization of *In Vitro* Plantlets

Acclimatization of the plantlet was successful following the acclimatization procedure. About 70 – 80% of the explants survived with this procedure. The procedure consisted of the following steps:

1) Rooted plantlets were removed from test-tube and rinsed with distilled water to removed traces of rooting medium.

2) Plantlets were trimmed to two to three leaves and planted in pot containing 1:1 organic soil and top soil.
3) Plantlets were covered with transparent plastic (punched with small holes for aeration).
4) Thw plastic covered plantlets were kept in the growth chamber (24 hours photoperiod with 600 lux light intensity, temperature 25 ± 1°C) for two to three weeks.
5) Plastic cover was removed and the plantlets were kept in chamber for another one to two weeks.
6) The plantlets were transferred from growth chamber to plant house.

The acclimatized plantlets grew normally and healthy as that of the normal seedlings (Figure 4).

Figure 4: Successfully acclimatized *A. heterophyllus* plantlet in the pot.

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