Induction of Callus and Plant Regeneration in *Momordica dioica* (*Momordica dioica* Roxb Ex. Willd)

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ABSTRACT

Genetic improvement of *Momordica dioica* has now become important due to its nutritive and medicinal value. In this respect, *in vitro* methods of propagule production help to reduce the time taken for breeding cycle. The general objective of this study was to develop a protocol for micro propagation of *M. dioica* for large-scale plant production. Different plant parts were used (leaves, tendrils, nodal segments, excised embryos) for callus induction. Excised embryos of mature fruits were better than other plant parts for callus induction and shoot regeneration. Surface sterilization of mature fruits with 10% 'Clorox' resulted in lowering contamination and retaining viability of embryos. Surface sterilization of seeds lowered embryo viability. Callus was induced in excised embryo in a basal MS medium with IAA (0.1, 0.5, 1.0 mgl^{-1}) or GA_3 (0.1, 0.5, 1.0 mgl^{-1}) combined with TDZ (0.1, 0.5, 1.0 mgl^{-1}) in dark condition. Shoots were regenerated on transfer to light in the same medium or at lower level of IAA, GA_3 or TDZ or in the absence of growth regulators. However, TDZ at a lower level of 0.1 mgl^{-1} alone or in combination gave rise to a higher number of regenerated shoots. Single nodal segments of well-developed regenerated shoots were used to induce roots in a MS basal medium of lower strength (1/4, 1/3, 1/2) either in liquid or agar solidified form. The presence of charcoal (0.3%) and IBA (0.1 mgl^{-1}) were also incorporated to enhance rooting. A solid basal MS of 1/3 strength with 0.5 mgl^{-1} IBA gave a higher number of explants that rooted. A constraint was the development of callus at the base of the shoot. Rooted plantlets were hardened and transferred to soil. Improvement of rooting and acclimatization of plantlets is recommended.

KEY WORDS; *Momordica dioica*, micro propagation, callus, plant regeneration, thidiazuron, gibberellic acid, indole-3-acitic acid, indole-3-butyric acid, root induction

1. INTRODUCTION

Momordica dioica (Thumbakaravila) belongs to the family Curcurbitaceae. It is a dioecious perennial with a tuberous root system and grows in the wild. It can be cultivated in the Low Country Dry Zone (LCDZ), where most of the other crops would not perform well, *i.e.* Hambanthota district, Ampara district, *etc.*

The immature fruit of the plant is a delicious vegetable of high nutritional value. All parts of the plant are used in ayurvedic medicine (Gunarathna, 2004).

The plants are grown from seed, shoot cuttings or tuberous roots. The tubers remain dormant in drought and unfavorable period. The seeds germinate and grow into seedlings in May-June. Shoot cuttings take more than six weeks to come to transplanting stage from planting. The plant flowers once a year in *Maha* season and seed production is seasonal. Plants raised from seeds take more time to flowering. Further, seeds have a 6-7 months dormant period and tuberous roots have a 2 months dormant period (Gunarathna, 2004). Commercial propagation is thus hindered by these drawbacks. Investigations on *in vitro* plant regeneration from excised mature embryos of the species were carried out to overcome these problems.

General objective of this study was to develop a protocol for micro propagation of *M. dioica* for large-scale plant production.

From the total expenditure of any plant propagation technique, 35-75% is spent for adventitious root formation (Debergh and Maene, 1981, Klerk *et al.*, 1999). Therefore, due to the economic importance, many researchers have tried to improve rooting and have investigated the effects of different plant growth regulators (Haissig and Davis, 1993) and the influence of non-hormonal compounds *in vitro* (Ricci *et al.*, 2001). Among these compounds Thidiazuron[1–Phenyl–3–(1,2,3–thidiazol–5–yl)urea, TDZ] play a major role due to its high cytokinin activity, extreme stability of the compound in plant tissue. TDZ exposed tissues retain TDZ either free or conjugated form and affects efficient transport and translocation of auxin.

2. MATERIALS AND METHODS

This study was carried out at the Plant Biotechnology Project of the Institute of Fundamental Studies, Hantana Road, Kandy.

2.1. Culture Media Preparation

The basal medium for all culture was MS (Murashige and Skoog's (1962)) with sucrose 2% for seed germination and 3% for callus induction and shoot regeneration. The media were supplemented with agargel and growth regulators details of which are given in respective sections. The culture vessels with medium after autoclaving at 106°C for 6 minutes were kept overnight before use.

2.2. In-vivo Germination of Tubers and Seeds

Eight *M. dioica* tubers were individually planted in clay pots filled with a potting mixture containing equal parts of compost, river sand, top soil and coir dust.

Fresh seeds extracted from ripe fruits collected from Aralaganwila were planted after removing the

mucilage cover by rinsing in tap water. Seeds were placed in a circular manner with 25 seeds per pot. A total of 100 seeds were planted in 4 pots. It was used as the control to compare germination of seeds that were scarified by rubbing with sand paper. Hundred scarified seeds were planted in 4 pots as described for the control. Labeling was done by using plastic tags and pots were placed on the wire mesh under shelter. Watering was done early in the morning and seedling emergence was observed at weekly interval over a period of 6 months.

2.3. In-vitro Germination of Excised Embryos

Embryos were excised from seeds that were surface sterilized with 5, 8, and 10% 'Clorox' (active ingredient sodium hypochlorite (NaOCl)) only or with 10% 'Clorox' followed by 0.3% mercuric chloride ($HgCl_2$) solution as described in Annex 1.1. Embryos were also excised from seeds, after surface sterilization of fruits (Annex 1.2). Embryos were placed singly in culture tubes containing germination medium MS with 0.2 mgl^{-1} gibberellic acid A_3 (GA_3) or 0.2 mgl^{-1} indole-3-acitic acid (IAA) or without GA_3 or IAA. Each germination medium was tested with the five different surface sterilization methods with ten replicates per sterilization method. A total of 150 embryos in 150 tubes were cultured. They were kept in the growth room at a temperature 24 ± 4 °C and 12 hour photoperiod illuminated by fluorescent tubes (Osram 'Daylight' 40W). Observations on microbial (fungal/ bacterial) contamination and germination were recorded.

2.4. Selection of Explant for Callus Induction

Leaves, tendrils, nodal segments from field plant and excised embryos of seeds were used as explants after surface sterilization as described in Annex 1.2. Aseptically extracted excised embryos from the sterilized fruits were also used. MS medium (25 ml) with two levels of TDZ (0.1 mgl^{-1} and 0.2 mgl^{-1}) was prepared in baby food jars (Sigma Co. Ltd.). Five excised embryos were cultured in a jar with 5 replicates per each TDZ level. Those embryos were kept dark in a growth room at 24 ± 4 °C for 60 days. Observation of microbial contamination and number of explants that induced callus were recorded.

2.5. Callus Induction from Excised Embryos of M. dioica

Excised embryos from aseptically extracted seeds from surface sterilized fruits were used for callus induction experiment. Thirty two treatment combinations of TDZ, GA_3 and IAA (Table 01) were tested.

Each treatment was replicated three times and each replicate consisted of 5 embryos in a jar containing 25 ml of the medium. Those jars were kept at 24 ± 4 °C in dark condition.

Observations on microbial contaminations, number of explants that induced callus, days to callus initiation and callus quantity were recorded. The callus quantity was visually scored as 1 (no callus), 2 (low callus quantity), 3 (medium callus quantity) and 4 (high callus quantity) (Plate 01). The data were statistically analyzed using the Statistical Analysis Software (SAS) package. The medium that gave the highest number of explants that induced the highest callus quantity was selected for further experiments.

Table1: Treatment combinations of the growth regulators

		TDZ levels (mgl^{-1})			
		0.0	**0.1**	**0.5**	**1.0**
IAA levels (mgl^{-1})	0.0	0.0,0.0	0.1,0.0	0.5,0.0	1.0,0.0
	0.1	0.0,0.1	0.1,0.1	0.5,0.1	1.0,0.1
	0.5	0.0,0.5	0.1,0.5	0.5,0.5	1.0,0.5
	1.0	0.0,1.0	0.1,1.0	0.5,1.0	1.0,1.0
GA_3 levels (mgl^{-1})	0.0	0.0,0.0	0.1,0.0	0.5,0.0	1.0,0.0
	0.1	0.0,0.1	0.1,0.1	0.5,0.1	1.0,0.1
	0.5	0.0,0.5	0.1,0.5	0.5,0.5	1.0,0.5
	1.0	0.0,1.0	0.1,1.0	0.5,1.0	1.0,1.0

2.6. Regeneration of Shoots from Callus

Callus that developed from embryos cultured in the selected medium with GA_3 and TDZ or IAA and TDZ combinations were transferred to the same medium, medium with a low level of GA_3 or IAA (0.1 mgl^{-1}), medium with low level of TDZ (0.1 mgl^{-1}) and to a growth regulator free MS medium. All culture media (pH 5.6 – 5.8) were solidified with agar (0.47%, w/v) and cultures were incubated under florescent white light at 12-hour photoperiod.

2.7. Root Induction

2.7.1 Determination of Rooting in Liquid and Agar Solidified Medium

Nodal segments of *in-vitro* regenerated shoots were transferred to root initiation medium. Liquid and solidified (0.47% agargel) half strength basal media with 2% sucrose, 0.1mgl^{-1} IBA were tested with 5 replicates per treatment. They were kept at temperature 24 ± 4 °C with 12 hour photoperiod. Observations of microbial contamination, formation of callus with root, root with no callus, callus quantity and axiliary bud development into shoot were recorded.

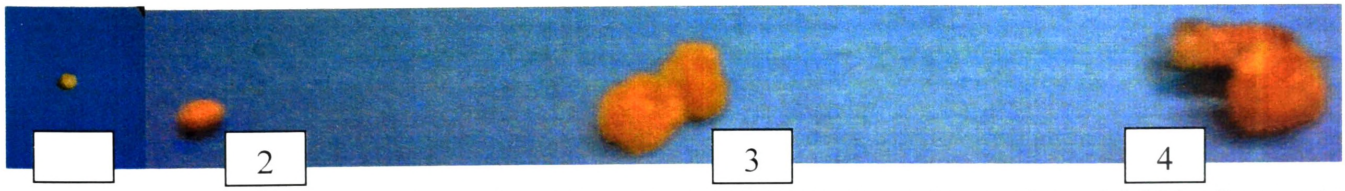

Plate 01: Various scores given to callus, 1 (no callus), 2 (low callus quantity), 3 (medium callus quantity) and 4 (high callus quantity)

2.7.2 Determination of Basal Medium Strength and Presence of Charcoal for Rooting

Nodal segments of *in-vitro* regenerated shoots were transferred to root initiation medium with 1/2, 1/3 and 1/4 MS medium with 2% sucrose, 0.1 mgl^{-1} IBA with or without 0.3% charcoal (BDH chemical co. ltd.) and solidified with 0.47% agargel. Ten baby food jars with 5 nodal segments per jar were prepared for each of the 12 treatments. They were cultured at temperature 24 ± 4 °C with 12 hour photoperiod. Observations mentioned in section 2.7.1 were recorded.

2.7.3 Determination of IBA Level Required for Root Induction

Nodal segments of *in-vitro* regenerated shoots were cultured in 1/3 MS medium with 2% sucrose with 0.5 or 1.0 mgl^{-1} IBA, solidified with 0.47% agargel with 0.3% charcoal or without charcoal. Five baby food jars with 5 nodal segments were prepared for each of the 4 treatments. Culture conditions and observations were the same as described earlier.

2.8 Acclimatization of Rooted Plants

Rooted plantlets in different culture media were first transferred to hormone free, 1/2 MS liquid medium with 2% sucrose. Plantlets were supported on filter paper bridges. They were transferred to jars with sterilized tap water. After a week the lids were opened. Plantlets that did not wilt after opening the lid, were potted in hardening trays containing coir dust. The tray was covered with a glass box to maintain high relative humidity (RH). Plantlets were exposed after a four days.

3. RESULTS

3.1 In-vivo Germination of Tubers and Seeds

Only 2 tubers, out of 8 planted, grew into plants after 4 months.

No germination took place in unscarified seeds (control) while only 2 seeds germinated out of 100 scarified seeds after one month.

3.2 In-vitro Germination and Microbial Contaminations in Excised Embryos

Percentage contamination and germination of excised embryos were given in Table 02. Lowest contamination was observed in treatment IV and V (Table 02). No seeds were germinated in treatment IV whereas in treatment V 2% seed germination was recorded.

3.3 Selection of Explant for Callus Induction

Percentage of explants that induced callus was given in Figure 01. Leaves, nodal segments and tendrils took more than 20 days to initiate callus. Highest callus induction was in excised embryos. Lowest callus induction was in tendrils and nodal segments (Figure 01). Shoot regeneration was not observed from any of the explants tested.

3.4 Callus Induction in Excised Embryos in the Presence of IAA and GA_3 with TDZ

The effect of different IAA and TDZ treatment combinations on callus development in excised embryos of *M. dioica* were given in Table 03. The contamination level of 16.7% was observed and these explants did not germinate or induce callus. There was a significant variation in the mean number of days to callus initiation ranging from 16.6 – 51.0 days, percentage explants that initiated callus (13.3 – 100%) and the mean callus quantity (7.7 -15.7). The highest callus quantity (15.7) was obtained from treatment 16 and 7 and callus has initiated early (18 – 19 days). The earliest callus initiation in 16 days was observed in treatment 1 in the absence of any growth regulators which was not significantly different from the treatments 6, 7, 15 and 16. However, the callus quantity developed in treatment 1 was low (7.7). Highest number of callus producing explants was given in treatment 4, which took more than 21 days to callus initiation. Treatment 7 having 93.3% callus producing explants with early initiation of callus (19 – 20 days), which gave the highest callus quantity, was selected for shoot regeneration.

The effects of GA_3 and TDZ treatment combinations on callus development in excised embryos of *M. dioica* were given in Table 04.

A contamination level of 8.3% of the embryos was observed and these embryos did not germinate or induce callus. There was no significant difference in the mean number of days to callus initiation (17.3 – 29.5 days), percentage explants that initiated callus (53.3 – 90.0) and mean callus quantity (11.3 – 17.7) among the treatments of callus induction medium with different combinations of GA_3 and TDZ (Table 04). The earliest time taken for callus initiation was at 17 days in treatment 10 (0.5 mgl^{-1} GA_3, 0.1 mgl^{-1} TDZ). However, this treatment resulted a low callus quantity (14.7). The highest callus quantity (17.7) was observed from treatment 4 (0.0 mgl^{-1} GA_3, 1.0 mgl^{-1} TDZ) and 8 (0.1 mgl^{-1} GA_3, 1.0 mgl^{-1} TDZ), and with the callus initiated times within 20 – 23 days respectively. Calli, which were induced in treatment 8 were used for plant regeneration.

Table 02: Percentage contamination and germination of excised embryos

	Surface sterilization method	% of contamination	% of germination
I	Seed sterilization with 5% 'Clorox' solution	100	—
II	Seed sterilization with 8% 'Clorox' solution	93.3	0%
III	Seed sterilization with 10% 'Clorox' solution	43.3	0%
IV	Seed sterilization with 10% 'Clorox' solution and 0.3% $HgCl_2$	6.6	0%
V	Sterilization of fruits with 10% 'Clorox' solution	6.6	2%

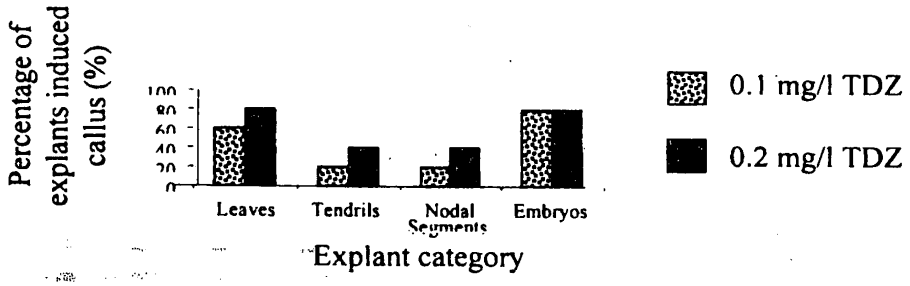

Figure 01: Callus induction in different explants

Table 03: Effect of TDZ and IAA on callus development in excised embryos of *M. dioica*

Treatment No.	IAA (mg/l)	TDZ (mg/l)	Mean days * to callus initiation	Percentage* of explants with callus (%)	Mean callus* quantity after 60 days
1	0.0	0.0	16.6 ± 3.6 c	33.3 a	7.7 ± 23.3 a
2	0.0	0.1	51.0 ± 14.4 a	40.0 a	8.7 ± 23.3 a
3	0.0	0.5	31.9 ± 21.7 bc	80.0 a	10.7 ± 23.3 a
4	0.0	1.0	24.5 ± 8.0 bc	100.0 a	13.0 ± 23.3 a
5	0.1	0.0	40.5 ± 16.3 a	13.3 a	6.3 ± 23.3 a
6	0.1	0.1	19.2 ± 6.0 c	80.0 a	14.3 ± 23.3 a
7	0.1	0.5	19.8 ± 8.0 c	93.3 a	15.7 ± 23.3 a
8	0.1	1.0	30.4 ± 14.7 bc	86.7 a	14.7 ± 23.3 a
9	0.5	0.0	32.6 ± 13.6 bc	80.0 a	13.0 ± 23.3 a
10	0.5	0.1	26.8 ± 14.3 bc	86.7 a	11.7 ± 23.3 a
11	0.5	0.5	32.1 ± 8.7 bc	86.7 a	10.0 ± 23.3 a
12	0.5	1.0	24.3 ± 14.0 bc	80.0 a	13.3 ± 23.3 a
13	1.0	0.0	27.1 ± 14.8 bc	86.7 a	14.7 ± 23.3 a
14	1.0	0.1	21.2 ± 7.7 c	60.0 a	11.0 ± 23.3 a
15	1.0	0.5	18.0 ± 10.7 c	80.0 a	12.3 ± 23.3 a
16	1.0	1.0	18.6 ± 11.6 c	86.7 a	15.7 ± 23.3 a

Treatment means with different letters along a column are significantly different
***Treatment effects are significantly different at P = 0.05**

Table 04: Effect of TDZ and GA_3 on callus development in excised embryos of *M. dioica*

Treatment No.	GA3 (mg/l)	TDZ (mg/l)	Mean days to callus initiation [NS]	Percentage of explants with callus (%) [NS]	Mean callus quantity after 60 days [NS]
1	0.0	0.0	22.6 ± 16.4	73.3	14.0 ± 21.8
2	0.0	0.1	27.0 ± 12.9	66.7	14.0 ± 21.8
3	0.0	0.5	20.2 ± 12.9	86.7	14.0 ± 21.8
4	0.0	1.0	23.5 ± 11.4	86.7	17.7 ± 21.8
5	0.1	0.0	27.9 ± 17.5	53.3	11.3 ± 21.8
6	0.1	0.1	18.5 ± 10.6	53.3	12.3 ± 21.8
7	0.1	0.5	22.4 ± 18.1	90.0	17.0 ± 18.0
8	0.1	1.0	20.1 ± 12.6	86.7	17.7 ± 21.8
9	0.5	0.0	29.5 ± 16.2	66.7	12.3 ± 21.8
10	0.5	0.1	17.3 ± 11.5	73.3	14.7 ± 21.8
11	0.5	0.5	26.0 ± 14.8	66.7	13.0 ± 21.8
12	0.5	1.0	19.0 ± 19.3	73.3	13.7 ± 21.8
13	1.0	0.0	23.9 ± 14.5	70.0	14.0 ± 18.0
14	1.0	0.1	22.9 ± 9.0	80.0	15.0 ± 18.0
15	1.0	0.5	21.8 ± 14.7	86.7	15.7 ± 21.8
16	1.0	1.0	20.2 ± 11.9	73.3	13.0 ± 21.8

[NS]Treatment means are not significantly different at P = 0.05

3.5 Regeneration of Shoots from Callus

Shoots were regenerated from the callus transferred to different shoot regeneration media (Table 05). The highest numbers of shoots were regenerated in medium with 0.1mgl^{-1} TDZ either alone or in combination with IAA or GA_3. 0.1 mg l^{-1} IAA also regenerated 9.8 shoots which was ranked among the high mean number category (Table 05). In the absence of growth regulators there was a significant reduction in shoot regeneration.

3.6 Root Induction

3.6.1 Determination of Rooting in Liquid and Agar Solidified Medium

The effect of liquid and solid medium for rooting was given in Table 06. Both liquid and solid media gave an appreciable level of callus and root formation at the basal end of the nodal segments. The development of axiliary bud into a shoot was significantly higher in solid than in liquid medium

(Table 06). Agar solidified medium was selected for further investigation on rooting as less explants (80%) developed callus at the base.

3.6.2 Determination of Basal Medium Strength and Presence of IBA and Charcoal for Rooting

The effect of basal medium strength and presence of IBA and charcoal for rooting was given in Table 08. A high percentage of shoots (74%) did not develop roots in 1/2 MS medium with 0.1 mgl^{-1} IBA and charcoal (Table 07). In 1/4 MS medium without IBA and with charcoal, the percentage of rooted shoots was high (66%), (Table 07). However, all rooted shoots developed basal callus. The highest percentage of shoots (20%) that rooted, without callus formation at base was resulted in 1/3 MS medium with 0.1 mgl^{-1} IBA and without charcoal. Even though mean number of roots per shoot and root length were low, that medium was used for further studies on rooting.

3.6.3 Determination of IBA Level Required for Root Induction

The effect of different IBA levels with or without charcoal on root induction was shown in Table 08. Increasing the IBA level to 1 mgl^{-1} and presence of charcoal reduced root induction (Table 08). The highest percentage of rooted shoots (26.0%) was found in 1 mgl^{-1} IBA without charcoal. However, the highest number of rooted shoots (with or without callus formation) was obtained (22.0% + 24.0%) in the presence of 0.5 mgl^{-1} IBA.

Table 05: Shoot regeneration from callus in four different media, at 1 month of transferring

Callus initiated medium	Shoot regeneration medium	Mean number of shoots * per callus (after 1month)	
0.1 mgl^{-1} IAA	MS	1.8 ± 0.9	b
+	0.1 mgl^{-1} TDZ	12.4 ± 2.6	a
0.5 mgl^{-1} TDZ	0.1 mgl^{-1} IAA	9.8 ± 1.5	a
	0.1 mgl^{-1} IAA + 1.0 mgl^{-1} TDZ	10.2 ± 1.1	a
0.1 mgl^{-1} GA_3	MS	3.0 ± 0.7	b
+	0.1 mgl^{-1} TDZ	10.4 ± 0.9	a
1.0 mgl^{-1} TDZ	0.1 mgl^{-1} GA_3	0.8 ± 1.3	b
	0.1 mgl^{-1} GA_3 + 1.0 mgl^{-1} TDZ	8.0 ± 2.0	a

Treatment means with different letters along a column are significantly different
*Treatment effects are significantly different at P = 0.05

Table 06: Effect of liquid and solid medium for rooting of nodal segments of *M. dioica*

Treatment (medium)	% Explants with			
	Callus	Roots	High callus quantity	Axiliary bud growth
Liquid	100	80	60	60
Solid	80	80	100	100

Table 07: Effect of basal medium strength, IBA level and presence of charcoal on rooting

Treatment	% of Shoots without root	% Rooted explants		Mean * number of roots per shoot			Mean root * length (cm)			
		With callus	Without callus							
1/2 MS	58.0	24.0	18.0	2.4 ±	1.4	ab	2.0 ±	1.1	a
1/2 MS + C	54.0	30.0	16.0	1.6 ±	1.5	abc	0.6 ±	0.9	bcd
1/2 MS + IBA (0.1 mg/l)	56.0	34.0	10.0	3.0 ±	0.9	a	1.6 ±	0.6	ab
1/2 MS + C + IBA(0.1 mg/l)	74.0	18.0	8.0	0.5 ±	0.6	c	0.3 ±	0.4	d
1/3 MS	70.0	22.0	8.0	0.4 ±	0.6	abc	0.4 ±	0.9	abcd
1/3 MS + C	64.8	16.7	18.5	1.9 ±	1.6	c	1.0 ±	0.9	cd
1/3 MS + IBA (0.1 mg/l)	67.0	16.0	20.0	0.6 ±	0.7	c	0.4 ±	0.6	cd
1/3 MS + C + IBA(0.1mg/l)	70.0	16.0	14.0	0.6 ±	0.8	c	0.1 ±	0.2	d
1/4 MS	48.0	34.0	18.0	1.5 ±	1.3	cb	1.1 ±	0.9	abcd
1/4 MS + C	34.0	66.0	0.0	1.7 ±	1.4	abc	1.5 ±	1.1	abc
1/4 MS + IBA (0.1 mg/l)	38.0	44.0	18.0	2.4 ±	1.2	ab	1.2 ±	0.6	abcd
1/4 MS + C + IBA(0.1 mg/l)	68.0	26.0	6.0	1.0 ±	1.2	cb	0.7 ±	1.2	bcd

Treatment means with different letters along a column are significantly different
*Treatment effects are significantly different at P = 0.05

Table 08: Effect of different IBA levels with or without charcoal on root induction

Treatment	% of Shoots without roots	% Rooted Explants		Mean * number of roots per shoot	Mean root * length (cm)
		With callus	Without callus		
1/3 MS + IBA (0.1 mg/l)	67.0	16.0	20.0	0.6 ± 0.7 b	0.4 ± 0.6 b
1/3 MS + IBA (0.1 mg/l) + C	70.0	16.0	14.0	0.6 ± 0.8 b	0.1 ± 0.1 b
1/3 MS + IBA (0.5 mg/l)	54.0	22.0	24.0	2.0 ± 1.2 a	1.7 ± 1.0 a
1/3 MS + IBA (0.5 mg/l) + C	66.0	20.0	14.0	1.4 ± 0.9 b	0.4 ± 0.3 b
1/3 MS + IBA (1.0 mg/l)	74.0	0.0	26.0	2.7 ± 1.8 a	1.5 ± 0.9 a
1/3 MS + IBA (1.0 mg/l) + C	80.0	0.0	20.0	1.3 ± 1.5 b	0.9 ± 1.5 b

Treatment means with different letters along a column are significantly different
*Treatment effects are significantly different at P = 0.05

3.7. Acclimatization of Regenerated Plants

Comparison of vitrified and healthy plants during acclimatization was shown in Figure 02. Even though 200 plantlets were transferred to liquid 1/2 MS medium, only 113 (56.5%) plantlets survived and were transferred to sterilized tap water jars for further hardening. Out of those plantlets, only 63 (55.75%) survived and were potted in coir dust in the nursery. However, only 12 plants survived in the nursery.

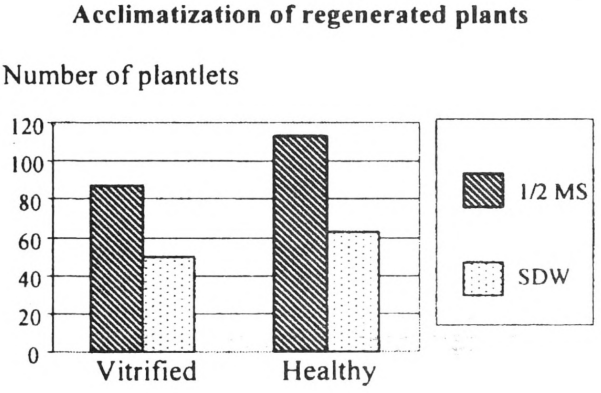

Figure 02: Comparison of vitrified and healthy plants during acclimatization

4. DISCUSSION

Momordica dioica showed a low percentage of seed germination (2%) as well as tuber growth (25%) in soil. It took more than a month for seed germination and tuber growth. *In-vitro* method of plant propagation could therefore be used for an alternative source of propagule production. Therefore, it is important to exploit the *in-vitro* methods of plant propagation to overcome these limitations.

Even though percentage of contamination decreased with increasing concentration of 'Clorox' (NaOCl) during surface sterilization, it increased browning of explants. With 10% 'Clorox' solution alone, there was 43.3% contamination. When 10% 'Clorox' solution, followed by 0.3 mgl^{-1} $HgCl_2$ was used in surface sterilization, the lowest percentage of contamination (6.6%) and browning could be observed (Table 01). However, germination did not take place even after 3 months. This may be due to the toxic effect of $HgCl_2$. Surface sterilization of fruit with 10% 'Clorox' solution before extracting seeds resulted in low (6.6%) contamination and better (2%) seed germination (Table 01). Therefore, surface sterilization of fruits may be recommended for *in-vitro* studies. Although leaves, nodal segments and tendrils induced callus, only a few shoots were regenerated from those calli. Excised embryos developed more callus and regenerated more shoots, thus could be used for callus induction and plant regeneration of *M. dioica*.

In callus induction media with combinations of IAA and TDZ, significant differences in the mean number of days to callus initiation, percentage of explants that initiated callus and mean callus quantity after 60 days were observed. However, media with combinations of GA_3 and TDZ did not show significant variation. Medium with the combination of lowest level of GA_3 (0.1 mgl^{-1}) and highest level of TDZ (1.0 mgl^{-1}) and medium with 0.1 mgl^{-1} IAA in combination with 0.5 mgl^{-1} TDZ were considered as the optimum treatment combinations for callus induction .

TDZ, a urea-derived cytokinin, and a major defoliant used in cotton (Thomas and Katterman 1986, Malik and Saxena 1992), is a potent cytokinin for woody plant tissue culture (Huetteman and Preece, 1993). In excised embryos of *M. dioica*, the presence of TDZ (0.1, 0.5 and 1.0 mgl^{-1}) in combination with IAA (0.1, 0.5 and 1.0 mgl^{-1}) in the initial culture medium appeared to play an important role in callus induction leading to subsequent shoot regeneration. Lowering the level of TDZ 0.1 mgl^{-1} brought about plant regeneration from callus. Similar result was obtained from previous experiments by Meemaduma and Ramanayake (2002) who used TDZ and IAA combinations for callus induction and GA_3 and TDZ combinations for shoot multiplication. TDZ has also been used to enhance somatic embryogenesis and germination of Bamboo (*Bambusa edulis*) (Lin *et al.*, 2004). In *M. dioica* TDZ brought about shoot regeneration from callus but not somatic embryogenesis. Further, it was reported that (Lin *et al.*, 2004) a relatively low TDZ concentration (0.01–0.1 mgl^{-1}) increased somatic embryo germination up to 81-84% and TDZ was more potent than the other cytokinin treatments like, N-benzyl-9-(2-tetrahydropyranyl)-adenine(BPA), benzyladenine (BA), Kinetin (KN) and Zeatin (Zn). The present study resulted a similar observation when a high concentration of TDZ (1.0mgl^{-1}) was used for callus induction and a low concentration of TDZ (0.1 mgl^{-1}) for shoot regeneration. Aparna and Rashid (2004) reported that transfer of caryopses of rice

(*Oryza sativa*) to the medium containing TDZ or BA after a short treatment with 2, 4-dichlorophenoxyacetic acid (2,4-D) resulted in regeneration of somatic embryos. TDZ gave rise to 50% of somatic embryo germination when used at a relatively high concentration of 2.2 mgl^{-1} compared to 0.1 – 1.0 mgl^{-1} used in this experiment. Furthermore, 100% shoot regeneration from callus was observed in this experiment.

For root initiation and axiliary bud development into shoot, agar solidified medium was better than liquid medium. However, callus development at basal end of the nodal segments was a problem. This problem could be overcome to some extent by using reduced strength of basal medium (1/3 MS) and addition of charcoal. Finely divided activated charcoal has been added to rooting medium with the aim of reducing callus formation at basal end of the nodal segments. Charcoal has been reported to have four advantageous effects depending on the type of culture. They absorb toxic compounds secreted from cultured tissues or present in agar that would otherwise inhibit growth, prevent unwanted callus growth, promote morphogenesis, particularly embryogenesis, and promote root formation (George and Sherrington, 1984).

6. CONCLUSIONS

The *in-vitro* germination of excised embryos was low. The most effective surface sterilization technique was sterilization of ripe fruit using 10% 'Clorox'. Suitable explant for the callus induction and shoot regeneration was excised embryo from seed. Callus induction in *Momordica dioica* embryos took place in basal MS medium with IAA or GA_3 (0.1 mgl^{-1}) in combination with TDZ (0.5 or 1.0 mgl^{-1}). For shoot regeneration, medium with lower TDZ level 0.1 mgl^{-1} alone or in combination with IAA was suitable whereas, Agargel solidified medium was better than liquid medium for root induction. Root induction was better in 1/3 MS basal medium with 0.5 mgl^{-1} IBA. Further investigations on rooting and acclimatization are recommended in order to use this method for propagule production.

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ANNEX 1: Surface sterilization of explant

1.1. Seeds were separated from ripe fruits and washed. They were air dried and stored in a refrigerator. Stored seeds were scarified by rubbing on sand paper. About 30 – 50 seeds were placed in a 250 ml filter flask with water and 2 drops of Teepol and shaken for 15 minutes and kept under running water for 20 minutes. Seeds were surface sterilized using 5, 8 or 10% 'Clorox' (Sodium hypochlorite (NaOCl)) for 20 minutes or 10% 'Clorox' followed with 0.3% Mercuric chloride ($HgCl_2$) for 5 minutes after which they were rinsed thoroughly in sterilized distilled water.

1.2. Tender leaves, tendrils and nodal segments harvested from potted plants and fruits were rinsed and surface sterilized as for seeds.