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Micropropagation and Microtuber Induction in *Dioscorea wallichii* Hook.f.

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Abstract

An *in vitro* protocol was developed for the micropropagation and microtuber induction in *Dioscorea wallichii* for the first time using nodal explant at Central Tuber Crops Research Institute, Thiruvananthapuram, India. MS medium with Benzyl Amino Purine (BAP) at 2 µM recorded the best response for shooting with the development of 2.6 shoots with an average shoot length of 8.6 cm and 11.8 nodes on each shoot which occurred within 5.6 days of inoculation. Kinetin (Kin) was found to be less effective for shooting. The addition of Naphthalene Acetic Acid (NAA) (1 µM) along with cytokinin (1-6 µM) also reduced shooting. Among the different levels of sucrose tested, MS medium with 8% sucrose was the best for shooting with the initiation of a single shoot of 11.8 cm length and with 9.6 nodes each within 17 days of inoculation. Microtuber induction was recorded in the presence of BAP (1-2 µM) and Kin (2 - 4 µM) in the medium. Addition of 8% sucrose produced the biggest microtubers with 5.28 cm length from the basal region in 4.4 months. Hence MS medium with BAP (2 µM) was adjudged as the best medium for shooting, whereas, MS basal medium with 8% sucrose was found to be the best for shooting and microtuber induction in *D. wallichii*.

Key words: *Dioscorea*, *in vitro* propagation, *in vitro* tuber induction, BAP, Kin, NAA, sucrose

Introduction

Dioscorea wallichii belonging to the family Dioscoreaceae is a climbing herb with edible tubers and vines twining right. In the tribal belts of Wayanad in Kerala it is used as a food known as 'Narakalasu' or 'Narakizhangu' (Narayanan et al., 2011). The tubers of this species are also eaten in Raipur, Puri, Cuttack, Dhenkanal, Jhansi, Ranchi and Chota Nagpur in Eastern India. In South India, the species is known to occur in different places at different elevations in Wayanad, Mangalore, Nilgiri, Quilon, Gudulur and Malayattur (Prain and Burkill, 1938). Mature stems of the species are 1.0-2.5 cm thick, green with purple tinge and armed with small brown spines of 0.3 - 0.5 cm on the basal and middle regions of the vines whereas, the upper regions are without spines. The average number of spines on a node is about 20 and on the internodes they range from 80-100. The young leaves are of different shades, varying from purplish green, light green or dark purple, depending on clones. Mature leaves are simple, cordate long and alternate in arrangement with pink margin in certain accessions. The petioles are purple with cream lines in the middle and dark purple at both ends. The petiole length varies from 5-9 cm, leaf width from 8-12 cm, leaf length from 7-14 cm and internodal length about 8 cm (Fig.1). Bulbils are never seen to occur on *D. wallichii*.

The tubers of *D. wallichii* are very long and thin, reaching about 80 cm in length and 1 cm in diameter. They are highly branched with brownish skin, bearing dark spots and numerous dark branched hairs (Fig.1 inset). The flesh of the tuber is light yellow in colour and highly fibrous. The tuber is sweet, non sticky and used as curry/side dish and consumed as a source of carbohydrate. Cooked tubers could be chewed and juice consumed for treatment of jaundice (Edison et al., 2006). Conventional vegetative propagation of this species is by

tuber setts, but this method is limited in its potential for large scale multiplication. This species is dioecious with shy flowering or non-synchronized flowering or non-flowering by nature. Hence chances of finding fertile seeds are highly reduced. So an efficient clonal propagation for rapid multiplication of this species is sought. As *D. wallichii* holds considerable potential for improvement and utilization, collections of the species were made from forest areas of South India for detailed studies. This paper reports the development of a protocol for the micropropagation and microtuber induction of the species from nodal explants, for the first time.

Materials and Methods

Accessions *of Dioscorea wallichii* were collected from the forests of Wayanad, Kerala, namely Chempra, Choorani, Manikunnumala, Sugandhagiri, Soochipara, Kanthanpara, Attamala and Chulliyode. The collected accessions were grown in pots in the shade net house of Central Tuber Crops Research Institute (CTCRI), Thiruvananthapuram, India, with the vines trailed on coir ropes. Farmyard manure (FYM) was mixed with the potting mixture as manure and vermiculite was spread on top of the potting mixture to hold moisture. The vines from 5-6 month old accessions grown as above were used as the source material for the present study.

Nodes were used as the explants in all the experiments. The nodes were washed in tap water to remove the surface contaminants and soaked in 1% teepol for 30 min and again washed in tap water. They were then disinfected with 0.1% mercuric chloride for 10 min and washed with sterile distilled water several times to remove the mercuric chloride completely.

For micropropagation, Murashige and Skoog medium (MS medium) (Murashige and Skoog, 1962) supplemented with various concentrations of plant growth hormones, viz., Benzyl Amino Purine (BAP) (1-10 µM) and Kinetin (Kin) (1-10 µM) individually as well as combinations of Kin (1-6 µM) or BAP (1-6 µM) with NAA (1 µM), sucrose 30 g l^{-1} and activated charcoal 1 g l^{-1} were used (Table 1). For microtuber induction, MS basal medium with different concentrations of sucrose (1-10%) were used. The pH of the medium was adjusted to 5.8 and autoclaving was done at 120 ^{o}C for 15 min. The cultures were grown at 26 ± 2^{o}C, under an 16: 8 hr light:dark regime (3000 lux). For hardening of cultures, the rooted plantlets were washed to remove the adhering medium and transferred to plastic cups filled with vermiculite. After 60 days, the plantlets were transferred to pots until the development of 5-6 new leaves, after which they were transplanted to field.

Table 1. Range of concentrations of additives used in MS medium for micropropagation and microtuber induction

BAP (µM)	Kin (µM)	NAA (µM)	Sucrose (%)
1-10	-	-	-
-	1-10	-	-
1-6	-	1	-
-	1-6	1	-
-	-	-	1-10

The experiments were conducted with five replicates for each treatment using a Completely Randomized Design. Response variation in terms of number of nodes developed, roots and shoots as well as shoot length after 16 weeks were recorded. Number of days for shoot initiation was also recorded. The variation among means were statistically analyzed using SAS 9.3 (SAS, 2010).

Results and Discussion

Micropropagation

Effect of BAP and kinetin on shooting in nodal cultures

In MS medium containing BAP (1-5 µM) sprouting was recorded within 5.6-7.6 days of culture, and the sprouting ranged from 60-80%. Of the different concentrations, BAP @ 2 µM elicited the best response with 80% sprouting and the production of 2.6 shoots with an average shoot length of 8.6 cm and 11.8 nodes on each shoot (Fig. 2). In the presence of BAP (3-5 µM), shooting was present but the response was less than that of 2 µM (Fig. 3). Rooting occurred from the nodes and base of developing shoots. When the concentration of BAP was increased further (6-7 µM) callusing was observed and beyond 7 µM there was no response of shooting.

In the medium containing Kin, sprouting was delayed and occurred after 12.4 – 13.6 days of inoculation and the sprouting ranged from 70-80%. Shooting was observed only up to a concentration of 5 µM of Kin. Kin at 4 µM induced a maximum of 1.8 shoots per explant, with a shoot length of 6 cm containing 6.8 nodes

each (Fig. 4). At higher concentrations of Kin (6-10 µM) either callusing or absence of shooting was observed (Table 2).

The present study showed that MS medium supplemented with BAP @ 2 µM produced better results in terms of percentage of development, number of shoots per explant, average shoot length and average number of nodes in *D. wallichii*. Similar response of BAP was observed in the case of other wild *Dioscorea* species also. In *D. oppositifolia* and *D. pentaphylla* shooting was more in the presence of BAP than Kin (Poornima and Ravishankar, 2007). The promotive effects of BAP (2 mg l^{-1}) on shoot bud induction was observed by Chaturvedi (1975) in *D. floribunda* nodal cultures. The presence of cytokinins in the MS medium was found to increase the shooting potentiality. Growth and morphogenesis *in vitro* are regulated by the interaction and balance between the growth regulators supplied in

Table 2. Effect of different concentrations of BAP/Kin alone and in combination with NAA and sucrose on shooting in nodal cultures of *D. wallichii*

BAP (µM)	Kin (µM)	NAA (µM)	Sucrose (%)	Sprouting (%)	No. of days for initial sprouting*	No. of shoots*	No. of nodes*	No. of leaves*	Shoot length (cm)*
1	-	-	-	70	5.6 ± 0.13	1.8 ± 0.11	6.8 ± 0.11	7.8 ± 0.11	6.4 ± 0.13
2	-	-	-	80	5.6 ± 0.13	2.6 ± 0.13	11.8 ± 0.96	12.4 ± 0.13	8.6 ± 0.13
3	-	-	-	60	5.8 ± 0.11	2.2 ± 0.11	4.8 ± 0.11	7.4 ± 0.13	5.6 ± 0.13
4	-	-	-	60	6.6 ± 0.13	1 ± 0	4.8 ± 0.11	4.8 ± 0.11	5.6 ± 0.13
5	-	-	-	70	7.6 ± 0.13	1 ± 0	4.6 ± 0.13	4.8 ± 0.11	4.6 ± 0.13
-	1	-	-	70	13.6 ± 0.13	1 ± 0	2.6 ± 0.13	3 ± 0.17	2.4 ± 0.13
-	2	-	-	70	13.6 ± 0.13	1 ± 0	2.6 ± 0.13	4.4 ± 0.13	3 ± 0.17
-	3	-	-	80	12.6 ± 0.13	1 ± 0	4 ± 0.17	4.4 ± 0.13	3.2 ± 0.11
-	4	-	-	80	12.4 ± 0.13	1.8 ± 0.11	6.8 ± 0.11	9 ± 0.17	6 ± 0.17
-	5	-	-	70	13.6 ± 0.13	1 ± 0	3.8 ± 0.11	4.4 ± 0.13	4 ± 0.17
1	-	1	-	65	20.4 ± 0.13	1 ± 0	1.8 ± 0.11	3 ± 0.17	2.4 ± 0.13
2	-	1	-	65	17.4 ± 0.13	1 ± 0	1.8 ± 0.11	2.2 ± 0.11	3.2 ± 0.11
3	-	1	-	70	17.4 ± 0.13	1 ± 0	1 ± 0	3 ± 0.17	4 ± 0.17
4	-	1	-	70	15.4 ± 0.13	1 ± 0	3.6 ± 0.13	8.8 ± 0.11	4 ± 0.17
5	-	1	-	65	17.6 ± 0.13	1 ± 0	2.8 ± 0.11	3 ± 0.17	3.2 ± 0.11
-	1	1	-	50	22 ± 0.17	1 ± 0	1.8 ± 0.11	3 ± 0.17	3.2 ± 0.11
-	2	1	-	60	22 ± 0.17	1 ± 0	2.8 ± 0.11	4.4 ± 0.13	4 ± 0.17
-	3	1	-	60	20 ± 0.17	1 ± 0	2.8 ± 0.11	6.6 ± 0.13	3.2 ± 0.11
-	4	1	-	50	22 ± 0.17	1 ± 0	1.8 ± 0.11	3.6 ± 0.13	4 ± 0.17
-	-	-	1	60	18 ± 0.17	1 ± 0	0 ± 0	1 ± 0	1 ± 0
-	-	-	2	60	17 ± 0.17	1 ± 0	1.8 ± 0.11	2.2 ± 0.11	2.6 ± 0.13
-	-	-	3	70	17.6 ± 0.21	1 ± 0	1.8 ± 0.11	3 ± 0.17	4.4 ± 0.13
-	-	-	4	70	15.4 ± 0.27	1 ± 0	1.8 ± 0.11	4.4 ± 0.13	4.6 ± 0.13
-	-	-	5	80	14.6 ± 0.21	1 ± 0	2.8 ± 0.11	4.8 ± 0.11	4.6 ± 0.13
-	-	-	6	80	15 ± 0.17	1 ± 0	4.6 ± 0.13	4.8 ± 0.11	7.4 ± 0.21
-	-	-	7	80	16.2 ± 0.11	1 ± 0	4.6 ± 0.13	5.6 ± 0.13	7.4 ± 0.21
-	-	-	8	80	17 ± 0.17	1 ± 0	9.6 ± 0.13	6.6 ± 0.13	11.8 ± 0.11
-	-	-	9	60	17 ± 0.17	1 ± 0	1.8 ± 0.11	4.4 ± 0.13	4.6 ± 0.13

*Each value is Mean ± SE of five replications per treatment. LSD of number of days for initial sprouting = 0.825; LSD of number of shoots = 0.228; LSD of number of nodes = 1.114; LSD of number of leaves = 0.709; LSD of shoot length = 0.727

the medium and the growth substances produced endogenously by cultured cells. In tissue culture, cytokinins are necessary for plant cell division. They regulate the synthesis of proteins involved in the formation and function of mitotic spindle apparatus. To encourage the growth of axillary buds and to reduce apical dominance in shoot cultures, cytokinins are incorporated into the medium (George et al., 2008).

Effect of cytokinin and auxin on shooting

In the medium containing BAP (1-6 µM) and NAA (1 µM) a single shoot was formed, which was longer than those with the addition of Kin and NAA, but with less number of leaves and nodes, except in the combination of BAP (4 µM) and NAA (1 µM) (Fig. 5), which produced healthy single shoot having a length of 4 cm with 8.8 leaves. Shooting was present up to a concentration of 5 µM of BAP with NAA with 65-70% sprouting. Higher concentrations of BAP along with NAA recorded no response in explants. Rooting was also high in the presence of BAP and NAA (Table 2).

The shoots produced in the medium containing Kin and NAA were small and very weak with very few nodes in all the combinations (Fig. 6) with 50-60% sprouting. The medium containing Kin (3 µM) and NAA (1 µM) induced the development of a single shoot with a few nodes and 6.6 leaves. Higher concentration of Kin (5 µM) induced darkening of explants without shooting. The presence of both the auxin and cytokinin (NAA and BAP/Kin) in the medium was not found to be suitable for the *in vitro* regeneration in *D. wallichii*. This is in contrast to the synergism of auxin and cytokinin reported in the micropropagation of *D. nipponica* (Chen et al., 2007).

Effect of sucrose levels on shooting

Among the different levels of sucrose tested in MS basal medium without any hormones, only single shoot initiation was observed. Sucrose at 8% was found to be the best concentration in which shoot bud initiation occurred within 17 days of inoculation. There was 80% shoot sprouting with the development of single shoot of greatest shoot length (11.8 cm), number of nodes (9.6) and leaves (6.6). Sucrose at 3% was used as the control in which shoots of 4.4 cm length with 1.8 nodes and 3 leaves were induced. Only the shooting response in 4 and 5% sucrose agreed with the results of control. Sucrose at 6 and 7% in the medium produced lengthy shoots of 7.4 cm but the number of nodes were reduced to 4.6. The lower concentration of sucrose at 1% in the medium produced only a single weak shoot of 1 cm length and one leaf. At higher concentrations of sucrose (9%) in the medium, a single shoot of 4.6 cm with 1.8 nodes and 4.4 small leaves was induced. At 10% sucrose level, there was no response of shoot development (Table 2, Fig.7).

The number of roots and root length increased with increasing concentrations of sucrose up to 8%. At 1 and 2% sucrose, only 1-2.2 small thin roots were produced which increased to 9.2 long roots from the base and nodes at 8% sucrose concentration. With 9% sucrose, very few thin roots were produced. Similar response in *D. alata* was reported wherein increase in the sucrose concentration from 2 to 5% enhanced the shoot and root development about two folds (Acedo, 2003). Sucrose is an essential carbon source under *in vitro* conditions wherein photosynthesis alone could not sufficiently support growth and development of the explants.

Microtuber induction

Effect of BAP and Kin on microtuber induction

Microtuber induction was noticed in the presence of BAP (1 and 2 µM) with 3% sucrose. A single microtuber was formed which was white in colour initially and later changed to brown. BAP (1 µM) induced the formation of a single, small round microtuber of 0.23 cm diameter from the middle node after 11.8 months of culture while BAP (2 µM) induced a single, oval microtuber of 0.42 cm length and 0.23 cm width with 8-10 roots after 8.6 months.

Of the different concentrations of Kin with 3% sucrose, Kin at 2- 4 µM induced single microtuber. Kin @ 2 µM and 3 µM induced small round microtubers after 8.4 and 6.2 months respectively with 0.3 cm diameter. With the addition of Kin @ 4 µM, oval microtubers of 0.92 cm length and 0.53 cm width were produced from the middle nodes after 4.4 months (Table 3, Fig. 8).

Similar induction of large-sized microtubers in the presence of Kin rather than BAP was reported in *D. bulbifera* shoot cultures, wherein MS medium supplemented with Kin was found to enhance

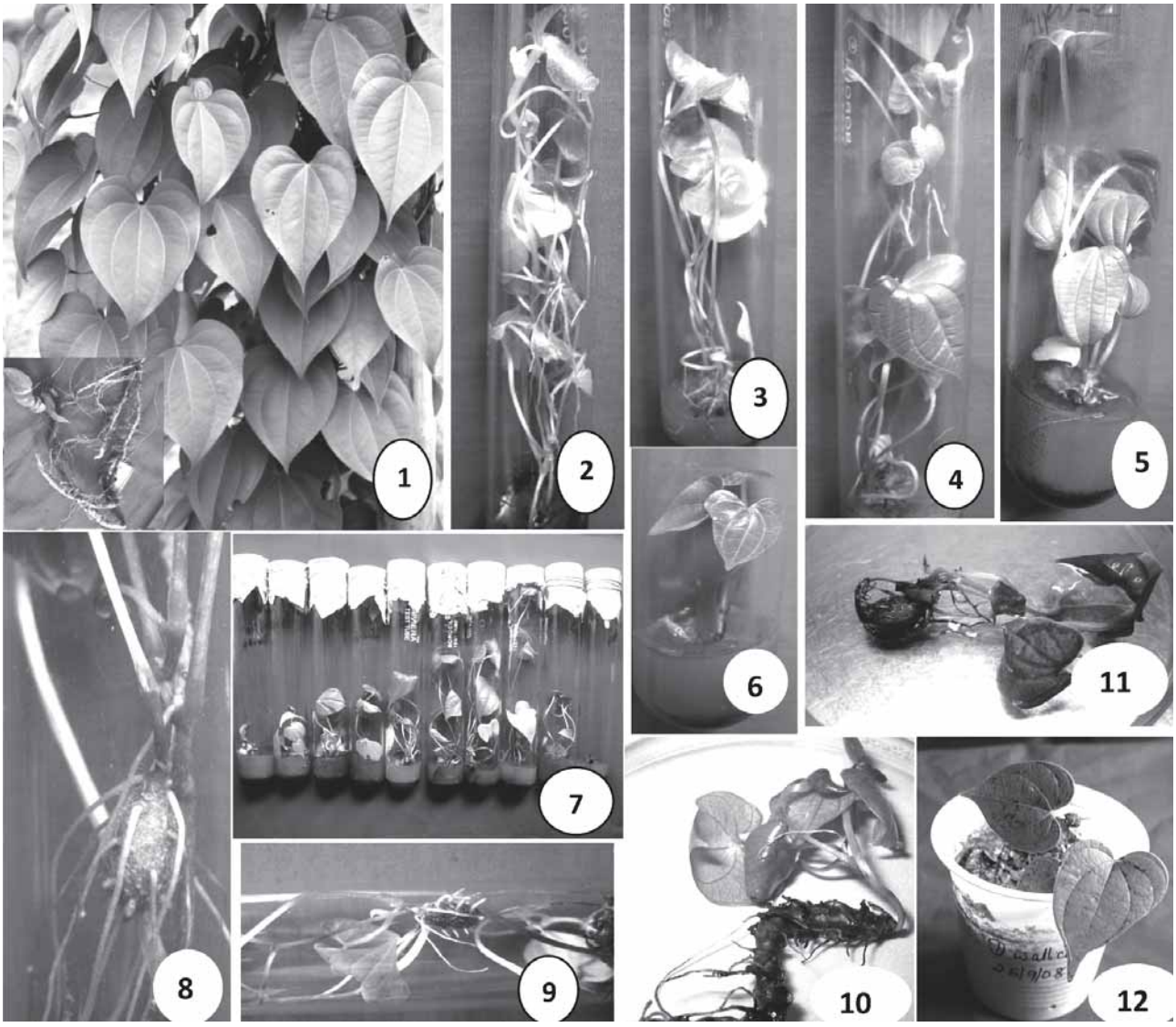

Fig.1. *D. wallichii* plant with tubers in the inset. Fig. 2. The best response of BAP (2 µM) in MS medium on shooting. Fig. 3. Reduced response of higher concentration of BAP (4 µM) in MS medium. Fig. 4. Shooting in presence of Kin (4 µM) MS medium. Fig.5. Effect of BAP (4 µM) and NAA (1 µM) in MS medium. Fig.6. Effect of Kin and NAA in MS medium showing small shoots and less leaves. Fig.7. Shooting in different concentrations of sucrose (1-10%) in MS medium. Fig.8. Oval microtuber from middle node in MS medium with Kin (4 µM). Fig.9. Oval microtuber from basal node in MS medium with 5% sucrose. Fig.10. Microtuber of 5 cm length in MS medium with 8% sucrose. Fig.11. Oval microtuber in MS medium with 6 and 7% sucrose. Fig.12. Hardened plant

microtuberization (Mantell and Hugo, 1989). However BAP (2 mg l^{-1}) also produced a single, oval bulbil from the lowest node in the same species (Asha and Nair, 2007). On the other hand, in *D. composita*, BAP decreased the percentage of microtuberisation at least by half (Alizadeh et al., 1998).

Effect of sucrose levels on microtuber induction

Single microtuber with plenty of roots on the surface were induced *in vitro* with the addition of 3 - 9% sucrose. In all the combinations, one microtuber/culture was induced. The incorporation of 3 - 4% sucrose produced oval microtubers from the basal node of the developed shoots. In the presence of 5% sucrose, oval microtubers were produced from the nodes or base (Fig. 9). Sucrose at 8% produced the biggest microtubers with 5.28 cm length from the basal region after 4.4 months (Fig. 10). Addition of 6 and 7% sucrose produced light brown oval microtubers from the basal nodes (Fig. 11). In the

presence of 9% sucrose small round tubers were produced from the nodal region after 7.8 months (Table 3).

Sucrose at 8% concentration in the medium was the single most significant combination for the induction of big microtubers within the shortest period of four months. Similar effect was reported in *D. composita* in which 8% sucrose was the best concentration for microtuber induction (Alizadeh et al., 1998). There are several reports which indicate the need to increase or decrease the sucrose concentrations for the induction of microtubers in yams. In *D. cayenensis*, greater number and size of microtubers were achieved when 4% sucrose was used (Jasik and Mantell, 2000) whereas enhancing sucrose concentration from 3 to 8% inhibited tuberization rate in *D. fordii* and *D. cayenensis-D. rotundata* complex (Yan et al., 2011; Ovono et al., 2007). Microtubers offer several advantages over *in vitro* plants in yams. They can be stored for several months and are less bulky and hence handling and shipping are easier, thus facilitating commercialization and international exchange of germplasm (Ng, 1988).

Hardening of shoots

In *D. wallichii* the individual microshoots developed roots in MS basal medium without any hormones. Similar results were reported in *D. oppositifolia* and *D. pentaphylla* (Poornima and Ravishankar, 2007). The shoots along with roots developed in MS medium, were transferred

Table 3. Effect of different concentrations of BAP/Kin and sucrose concentrations on microtuber induction

BAP (µM)	Kin (µM)	Sucrose (%)	No. of micro tubers	Size of microtuber		Duration for initiation of microtuber* (months)	Nature of microtuber
				Length* (cm)	Width* (cm)		
1			1	0.23 ± 0.01	0.23 ± 0.01	11.8 ± 0.11	Small, brown, round from the middle node
2			1	0.42 ± 0.01	0.23 ± 0.01	8.6 ± 0.13	Brown, oval from the basal node
	2		1	0.32 ± 0	0.32 ± 0	8.4 ± 0.13	Small, round, brown from the middle node
	3		1	0.31 ± 0	0.31 ± 0	6.2 ± 0.11	Small, round, black from the basal node
	4		1	0.92 ± 0.01	0.53 ± 0	4.4 ± 0.13	Oval, brown from the middle node
		3	1	0.42 ± 0.01	0.12 ± 0	7.8 ± 0.11	Brown, oval from basal node
		4	1	0.42 ± 0	0.12 ± 0	7.8 ± 0.11	Dark brown, oval from basal node
		5	1	0.63 ± 0	0.31 ± 0	7.4 ± 0.13	Black, oval from nodal or basal region
		6	1	1.1 ± 0.02	0.53 ± 0.01	5.8 ± 0.11	Light brown, oval from basal node
		7	1	1.15 ± 0.02	0.52 ± 0.01	6.4 ± 0.13	Light brown, oval from basal node
		8	1	5.28 ± 0.06	0.52 ± 0	4.4 ± 0.13	Black, elongated from basal region
		9	1	0.53 ± 0	0.53 ± 0	7.8 ± 0.11	Black, round from middle node

*Each value is Mean ± SE of five replications. LSD of microtuber length = 0.107; LSD of microtuber width = 0.0234; LSD of duration for microtuber induction = 0.621

to plastic cups with vermiculite and the microtubers were used for further shoot regeneration *in vitro*. In 60 days the transferred cultures showed normal growth and development of new leaves (Fig. 12). After 60 days, the plantlets were transplanted to pots and kept in shade house for establishment. The plantlets grew into normal plants, as from tuber cuttings, with underground tuber formation and their survival was 90%.

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