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ONE- STEP DIRECT REGENERATION OF *GYMNEMA SYLVESTRE* (RETZ) R. BR. EX ROEMER & SCHULTES FROM LEAF EXPLANTS INDUCED BY THREE CYTOKININS

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A current study is underway to investigate the possibility of developing a one-step direct plant let regeneration in *Gymnema sylvestre* (Retz) R. Br. For this, the effect of leaf explants was cultured in MS medium containing different concentrations of three cytokinins TDZ / BAP / Kn (0.5–3.0 mg / L). The maximum number of shoot bud expansion was observed at (2.0mg / L). TDZ, compared to all other concentrations of Kn / BAP. Shoot bud stimulation in TDZ / BAP / Kn gradually decreased as the concentration increased above (2.0mg / L). The high frequency of shoots (2.0mg / L) was induced at TDZ. In vitro regenerated shoots produced the largest number of IBA-containing roots in the MS medium (1.0mg / L). Thus, the plant developed *in vitro* using leaf cultures was established in pots containing garden soil outside in the shade at room temperature and mild conditions. These plants bloom 8 weeks after transfer to pots. The established protocol can be used to quickly multiply the actual product to type plants.

Keywords: *Gymnema sylvestre* (Retz) R. Br, multiplication, Leaf explants, TDZ, BAP, Kn and IBA. IAA

Abbreviations: TDZ- Thidiazuron BAP- 6-Benzylaminopurin, Kn-Kinetin and IBA- Indole-3 butyric acid. IAA- Indole-3-Acetic Acid.

Introduction:

Gymnema sylvestre (Retz) R. Br. It belongs to the family Asclepiadaceae, which is a harmful species. It is a perennial slow-growing timber climber of tropical and subtropical regions (Anonymous, 1997). It is a powerful anti-diabetic plant and is used in folk, homeopathic and ayurvedic medicine (Mitra *et. al*., 1995).

It is also used in treating of asthma, eye complaints, inflammations, family planning and snake bite (Anonymous, 1956; Selvanayagam *et al*., 1995). In addition, it possesses antimicrobial, anti hypercholesterolemic (Bishayee and Chatterjee, 1994), hepatoprotective (Rana and Avadhoot, 1992) and sweet suppressing (Kurihara, 1992) activities. It also acts as feeding deterrents to caterpillar, *Prodenia eridania* (Granich *et al*., 1974), prevent dental caries caused by *Streptococcus mutans* (Hiji Yasutake, 1990) and in skin cosmetics (Maeda *et al*., 1996). There is a growing demand for leaves of *G. sylvestre* in the pharmaceutical trade due to its use as a remedy for diabetes and also as a tonic of the nerves and as a laxative (Shanmugasundaram *et al*., 1983), as an anti-sweetner (Kurihara, 1992) and as an antihypercholesterolemic (Bishayee and Chatterjee,1994). It also has stomatic, diuretic and cough suppressant property (Kapoor, 1990). Increasing awareness of the side effects of Western drugs have made general public turn towards the herbal medicine, thus the demands for medicinal plants have drastically increased. Due to over exploitation, this plant species has become threatened and is listed in International Union for Conservation of Nature (IUCN) red data book (Shailasree *et al*., 2012).

G. sylvestre is a slow growing, perennial woody climber (Shrivastava and Singh, 2011). Seeds lose viability in a short period of storage (Reddy *et al*., l998). Conventional propagation methods are hampered due to its poor seed viability, low rate of germination and poor rooting ability of vegetative cuttings (Komavali and Rao, 2000). The propagation of plant through seed results in less survivability under natural conditions (Anonymous, 1950). Therefore, to fulfill the increasing demand of this potent medicinal plant and population, *in vitro* culture and micropropagation could be an alternative method to aid in its conservation. Therefore, the propagation of this plant species by alternative methods is needed. According to them, a maximum number of roots could be obtained on MS medium supplemented with NAA at 1.0 mg/L. (Karthic and Seshadri 2009) used young stem cuttings with an actively growing side branch as explants in their hydroponic experiment conducted with plastic tubes. MS medium containing 1/10 strength of MS salts supplemented with IBA at different concentrations (0.5, 1.0, 2.5 mg/L) and IBA (0.5 mg/L) resulted in the highest rooting (66%) and survival (96%). Based on the findings, they claimed that their protocol can serve as an alternative to the existing *in vitro* and clonal multiplication protocols for *G. sylvestre*. (Subathra Devi and Srinivasan 2008) further stressed that *in vitro* propagation of *G. sylvestre* is not very different from that of *G. elegans* and may be applicable for other economically important woody Climbers as well. Though direct regeneration studies have been conducted so far, this paper deals with the direct plant regeneration system with large number of shoots within a short period from leaf explants of *G. sylvestre* induced by /TDZ/BAP/Kin.

Methodology.

Plant seeds of *G. Sylvester* from a single plant grown in Forest Range Officer Waddepalli Hanankonda. The seeds are washed in tap water for 5 minutes, treated with 2-3 drops- 40 to 10 minutes and finally, the seeds are washed three times with clean distilled water. The seeds are then disinfected with 0.1% $HgCl_2$ for 5 minutes and rinsed three times with clean distilled water. Surface-sterilized seeds in Murashige and Scoog (1962) medium were replaced with 2% sucrose and fortified with 0.8% agar (Himidia) (Ashok *et.al.*, 2002) at 5.5 medium pH 5.8 samples increased 40-60 mol m-2 s Photoperiod of photoperiod under white fluorescent light of -1 intensity. Leaf (0.8–1.0 cm^2) was described from 8-week-old seedlings for Callis induction, these descriptions were given by vaccinating the MS medium with different concentrations of cytokinins (1.0– 3.0 mg / L) such as TDZ / BAP / Kn. (Table-1) All descriptive growth regulators have been used only as cytokines in the culture medium. 0.8% agar agar for 25 min cultures in 25 x 150 mm cultures tubes and all media was adjusted to pH 5.8 before autoclaving at 121^0C and 103kpa.

Culture Media and Culture Conditions: The leaf explains an area of about 0.5–10 cm from the seeds of *G. sylvester*, which contains MS basal salts for the regeneration medium, which are replaced with different concentrations of TZD / BAP / Kn. The pH of the media was adjusted to 5.8 with 0.1 N HCL or 0.1N Na OH, fortified with 0.8% difco-bacterial agar, and autoclaved for 15–20 min at 121^0C under 15 PSI. All cultures were incubated at 25^0C with 16hours photoperiod under white fluorescent light of 40–60 m m-2 $^{s-1}$ intensity.

Shoots extending from the leaf were excluded and cultured in different (0.5–2.0 mg / L) IAA / IBA-associated MS-mediated rooting medium. To remove traces of agar the rooted plants are gently removed from the flasks and the roots are washed in tap water.

Acclimatization

After washing the agar with distilled water and with a mixture of soil ritual (1: 1) the rooted plants were transferred to the pots for two weeks. The potted plants should be covered with a layer of transparent polythene to maintain high humidity and the plants should be accustomed to field conditions for two weeks with a solution of half-strength MS salts every three days. Two weeks later the accustomed plants were transferred to pots containing ordinary garden soil and placed in a greenhouse under natural day length conditions.

Results: -

The results of the Leaf explants cultures on the development of multiple shoots and roots are shown in (Table-1 Fig-I.II and III). The Leaf explants of *G. sylvestre* cultured on different hormonal combinations showed varied results.

Table – 1

Influence of various concentrations of MS+ TDZ/BAP/Kin on induction of shoot bud's proliferation from Leaf explants cultures of *G. sylvestre*.

Hormone concentration (mg/L)	% of plantlet production	Average No. of shoots / Explants ± (SE)*	Average length of shoots (cms) ± (SE)*
TDZ			
0.5	50	4.9 ± 0.42	1.0 ± 0.28
1.0	60	5.3 ± 0.07	2.4 ± 0.33
1.5	64	8.5 ± 0.62	3.4 ± 0.62
2.0	74	9.3 ± 0.43	3.6 ± 0.42
2.5	52	6.0 ± 0.25	2.3 ± 0.35
3.0	50	2.0 ± 0.25	2.0 ± 0.25
BAP			
0.5	48	3.2 ± 0.32	1.3 ± 0.38
1.0	53	4.2 ± 0.27	2.0 ± 0.23
1.5	58	4.5 ± 0.32	2.4 ± 0.42
2.0	60	8.3 ± 0.23	2.8 ± 0l.22
2.5	50	4.2 ± 0.23	2.0 ± 0.25
3.0	42	2.0 ± 0.34	1.2 ± 0.35
Kin			
0.5	43	2.0 ± 0.52	1.2 ± 0.22
1.0	47	4.3 ± 0.37	1.8 ± 0.23
1.5	52	4.5 ± 0.42	2.0 ± 0.42
2.0	58	6.0 ± 0.23	2.2 ± 0.32
2.5	48	3.4 ± 0.65	1.8 ± 0.25
3.0	40	2.0 ± 0.45	1.1 ± 0.35

*** Mean ± Standard Error**

The Leaf explants became active with in weak after inoculation and new shoots became distinct by the seconds and third weak with leaves and internodes. The explants survival from leaf explants of nature plant of *G. sylvestre* varied with season. According to the present observations, the explants were collected from field grown plants thought out the year to determine the ideal season for culture established.

Fig- I Influence of various concentrations of MS+ TDZ on induction of shoot bud's proliferation from Leaf explants cultures of *G. sylvestre*.

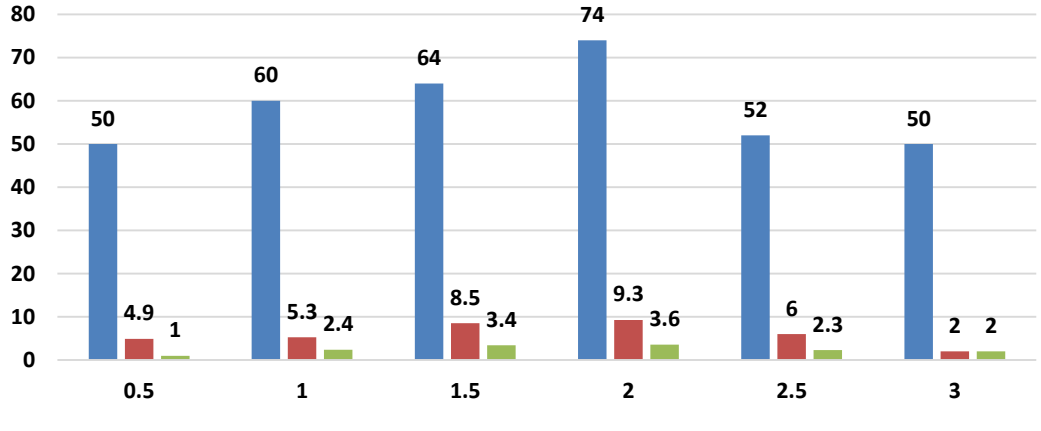
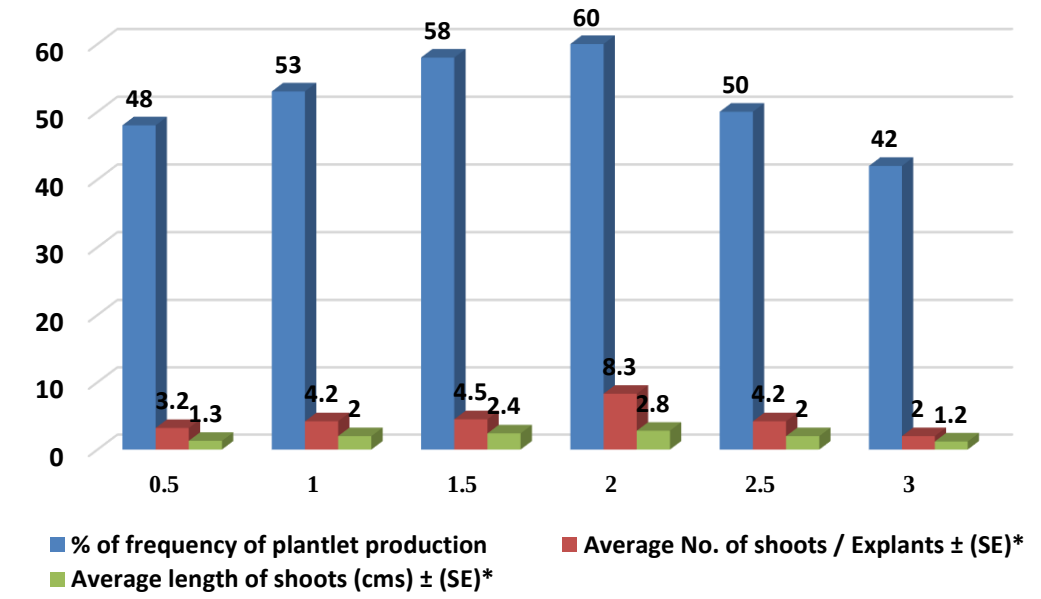


Fig-II Influence of various concentrations of MS+ BAP on induction of shoot bud's proliferation from Leaf explants cultures of *G. sylvestre*.



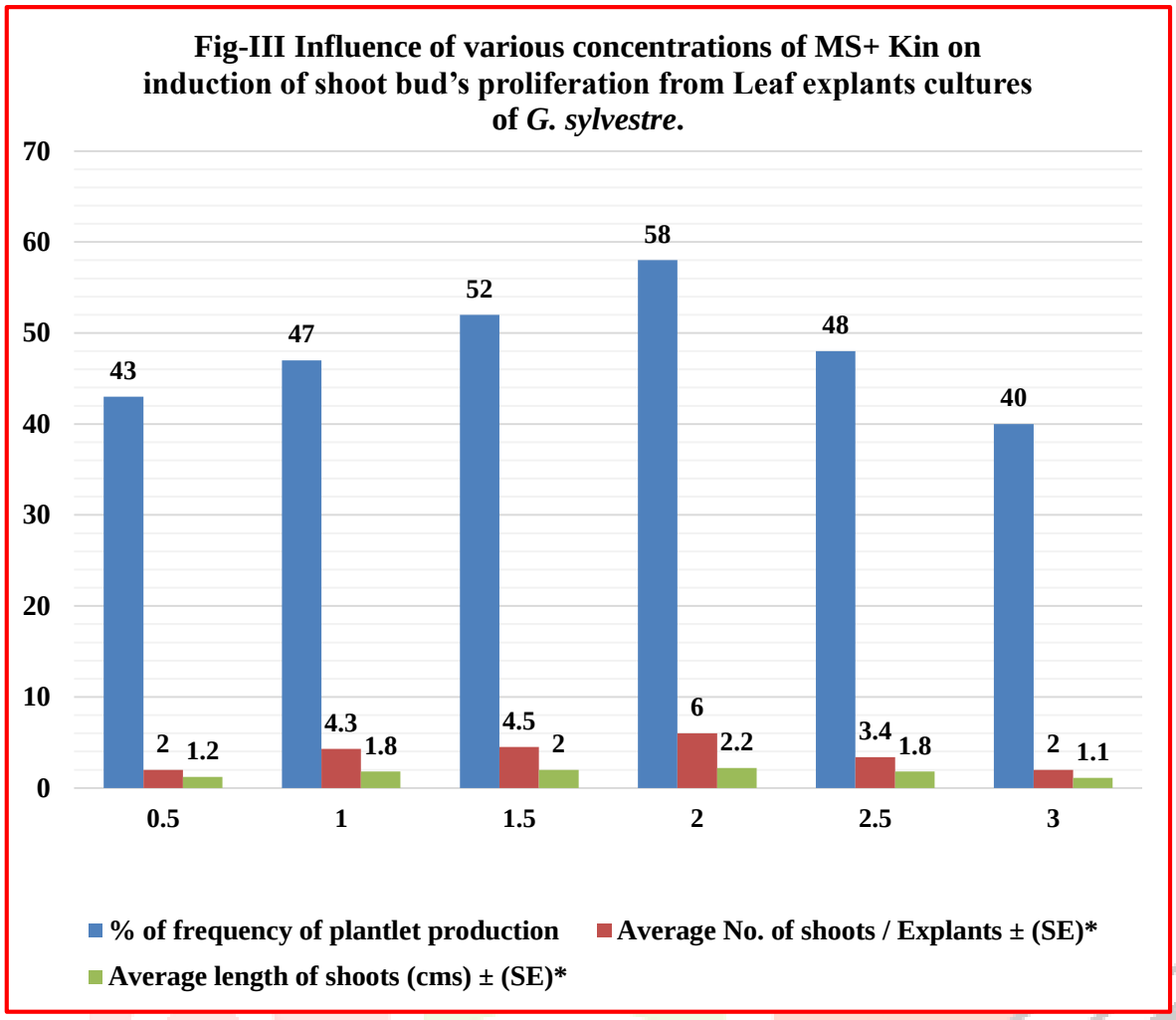


Table- 2 Influence of various concentrations of IAA/IBA on rooting of *in vitro* shoots of *G. sylvestre* after 8 weeks of incubation

Hormone concentration (mg/L)	% of Response plantlet production	Average no of roots (S.E) *
IAA		
0.5	45	1.0 ± 0.13
1.0	60	2.3 ± 0.32
1.5	65	3.8 ± 0.22
2.0	54	3.0 ± 0.32
IBA		
0.5	47	1.4 ± 0.43
1.0	73	2.5 ± 0.32
1.5	54	4.0 ± 0.42
2.0	50	3.4 ± 0.32

*** Mean ± Standard Error**

Effect of TDZ

The leaf explants were excised from the surface sterilized, *in vitro* grown, 30-d old seedlings and cultured on MS medium augmented with TDZ (1.0–3.0 mg/L) for multiple shoot induction of all the different concentrations of TDZ tested, (2.0 mg/L) TDZ was found to be more effective in inducing (15.0 ± 0.3 shoots/explants). But at high concentration of TDZ (3.0 mg/L) considerably the number of shoot induction was

found to be reduced. As the concentration of TDZ was increased up to 2.0mg/L the multiple shoots number was increased but as the concentration of TDZ (2.0mg/L) to (3.0 mg/L) TDZ resulted the number of shoots were reduced. (Fig-I a).

Effect of BAP

Leaf explants were cultured on MS medium amended with various concentrations of BAP (1.0-3.0 mg/L) as role growth regulators showed the direct organogenesis (Table -1) Maximum number of shoot bud proliferation (8.3 ± 0.23 shoots /explant) was found at (2.0 mg/L) BAP. At 1.0, and 2.0 mg/L BAP induced (3.2 ± 0.32 and 4.2 ± 0.27) shoots/explant with 48 and 53% of cultures responded. When the concentration was increased up to (3.0 mg/L) BAP gradually induction of multiple shoots was reduced. At (2.5 and 3.0 mg/L) BAP induced (4.2 ± 0.23 and 2.0 ± 0.34) shoots/explants with 50 and 42 percentage of cultures were responded. The number of shoot bud induction was found to be decreased as the concentration of BAP increased. At high concentration of BAP showed a smaller number of shoots per explants. (Fig-I b).

Effect of Kn

Leaf explants were cultured on MS medium containing different concentrations of cytokinin Kn (1.0- 3.0 mg/L) as role growth regulators showed the direct organogenesis/ shoot formation (Table -1) to find out the difference between TDZ, BAP and Kn in inducing the direct plant regeneration from Leaf explants in *G. sylvestre.* Maximum number of shoot bud proliferation (06.0 ± 0.23 shoots/ explant) was found at (2.0 mg/L) compared to all other concentrations of Kn at (1.0 and 2.0 mg/L) Induced (2.0 ± 0.52 and 4.3 ± 0.37 mg/L) shoots / explant with 438 and 47 percentage of cultures responded. Percentage of response was gradually increased up to (2.0 mg/L) Kn. At above (2.0 mg/L) concentration a smaller number of shoots was recorded. At (2.5 and 3.0 mg/L) Kn induced (3.4 ± 0.65 and 2.0 ± 0.45) shoots/explants with 48 and 40 percentage of cultures were also recorded on MS + Kn. However high induction of ability was found in all the concentrations of TDZ, Kn compared to BAP.

***In vitro* rooting**

Fully elongated healthy shoots were transferred on to full strength MS root induction medium (RIM) (Murashige and Skoog 1962) fortified with different concentration of IAA/IBA (0.5 – 2.0 mg/L). Profuse rhizogenesis was observed on (1.0 mg/L) IBA, compared to (0.5 -2.0 mg/L) IAA on MS medium containing (1.0 mg/L) IBA whereas 73% of plants produced roots with (8.3 ± 0.87) roots/ explant. (Fig-I -c).

Acclimatization

Rooted plantlets were removed from the culture medium and the roots were washed under running tap water to remove agar. Then the plantlets were transferred to polypots containing pre- soaked vermiculite and maintained inside a growth chamber set at 28^{o} C and 70 – 80 % relative humidity. After three weeks they were transplanted to poly bags containing mixture of soil + s and + manure in 1: 1: 1 ratio and kept under shade house for a period of three weeks. The potted plantlets were irrigated with Hogland's soulation every 3 days for a period of 3 weeks. (Fig-I d).

Fig-I Plantlet regeneration from Leaf explants of *Gymnema sylvestre* a), multiple shoot production on MS+ (2.0mg/L) TDZ after six weeks (b) Plantlet proliferation on MS (2.0mg/L) BAP (c) root induction from leaf regenerate shoots on MS+(2.0mg/L) IBA (d) Hardening of plantlet.

Discussion:

We were successful in direct regenerating plants from leaf explants of *G. sylvestre* cultures on MS medium fortified with different concentrations of cytokinins i.e. TDZ/BAP/Kn (1.0-3.0 mg/L). Maximum number of shoot buds was induced at (2.0 mg/L) TDZ in comparison to Kn/BAP as role growth regulators. It was interesting find out that the shoots induction was enhanced in all the concentrations of cytokinins. However, the shoot bud proliferation was found to more on (2.0 mg/L) with TDZ compared to (2.0 mg/L) in with Kn/BAP but the concentration of TDZ induced highest number of plantlet regeneration among all hormonal combinations and concentrations were used in *G. sylvestre*.

The effect of TDZ was found to be highest the concentration of (2.0 mg/L) recording shoot buds/explants (9.3 ± 0.43). At lowest concentration of (0.5 mg/L) recording shoot buds/explants (4.9 ± 0.42) (Table-1). The average length of shoots and number of roots were found to be significantly higher in MS medium, supplemented with hormones. For Leaf explants *G. sylvestre*, the best growth regulator concentration for

direct shoot induction in MS was observed in the presence of (2.0 mg/L) TDZ which gave the highest shoot elongation (2.8 ± 0.22).

The presence of (0.1 mg/L) NAA and (2.0 mg/L) TDZ was best medium for shoot induction from nodal explants (Amarasinghe *et.al.*, 2011). Which conform the results of the present study of Leaf explants. The presence of higher concentrations of BAP (2.0 mg/L) in the medium suppresses the shoot development (Komalavalli and Rao 2000) confirming the results of the present study, where the mean shoot length was drastically reduced in the presence of ≥ (1.5 mg/L) BAP. However, the growth regulator free MS (control) also showed high shoot elongation (14.2 ± 0.37) and the difference between those two treatments is not significant.

The results are summarized in Table -2. Regenerated shoots were rooted on MS medium without supplementing any growth regulator (Reddy *et. al.*, 1998). However, in contrast present study indicated that the growth regulator frees MS (Control) did not induce roots *in vitro.* ANOVA using Kruskal - Wallis Test indicated that all the treatments were significantly different om each other.

High frequency of rooting (58%) was obtained in leaf explants derived shoots on MS medium supplemented with IBA (2.0 mg/L) (Komalavalli and Rao 2000). In the present study the best growth regulator concentration for *in vitro* root induction was observed in MS supplemented with (1.0 mg/L) IBA giving 73% success in root induction (Fig-1). This indicates that the decreased levels of basal salt concentrations may have affected the percentage rooting (Komalavalli and Rao 2000). Presence of lower levels of IBA (≤ 2.0 mg/l) did not induce roots in *in vitro* propagated shoots. Increased concentrations of higher than (1.0 mg/L) IBA not only retarded the root induction but also induced callus at the base of the shoot. Thus, it could be suggested that MS supplemented with (1.0 mg/L) IBA in MS would be the best medium for rooting of *in vitro* shoots of *G. sylvestre.*

The highest percentage of survival (73) was observed in the substrate with a high proportion of sand (soil: sand: 1: 2) (Table-2). (Reddy *et. al.*, 1998) reported that for acclimation, plantlets were removed from the rooting medium 8 weeks after root initiation and transferred to fresh tubes containing autoclaved tap water. After 8- 10 days, plantlets were subsequently transferred to plastic pots (9 × 9 cm) containing autoclaved soil rite covered with perforated polythene bags to maintain humidity; and were kept under culture room conditions for about 7 days. After three weeks, polythene bags were removed, and pots were transferred to the garden and placed under the shade till the new leaves appeared. Then they were planted under normal garden conditions. The transplantation success was about 75%. In the present study, substrate mixture with soil: sand (1: 3) enhanced the surviving ability (Fig-1).

An increasing portion of sand in the mixture enhances the surviving ability as smaller amount of water was retained in the substrate (Senarath *et al.* 2007). Plantlets are unable to compete with soil microbes and to cope with the harsh environmental conditions especially immediately after removal of the plantlets from *in vitro* conditions. After removal of the plantlets from the propagator, they were exposed to natural environment gradually. Yet, it was observed that especially during the rainy season, the survival ability of the plantlets was

poor thus making it essential to keep the plantlets in a propagator for a long period if the rainy season persisted.

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