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In vitro regeneration of some diploid and triploid mulberry (*Morus* spp.) varieties using shoot tips as explant

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Abstract
The experiment was conducted to study the of micropropagation efficiency in diploid and triploid mulberry (*Morus* spp.) varieties i.e S13, RFS135, TR10, Vishala, M5 and G2, using shoot tip as explants. MS medium fortified with different concentrations and combinations of growth hormones were used. Alone BAP and kinetin were used in initiation media. BAP 2.0 mg/l was found to be most effective in inducing sprouting in most of the varieties. Initiated shoots were transferred to the multiplication medium containing BAP (0.5 mg/l-2.5 mg/l) + NAA (0.5 mg/l and 1.0 mg/l) and BAP (0.5 mg/l-2.5 mg/l) + kinetin (0.5 mg/l and 1.0 mg/l). BAP 2.0 mg/l in combination with kinetin 1.0 mg/l was efficient in multiple shoots formation. The micro shootlets were subcultured to obtain sufficient growth of the multipleshoots. The results indicated that explants inoculated in alone BAP has showed high percent of shoot initiation. For multiple shoot formation BAP in combination with kinetin was found to be effective in inducing efficient multiple shoots. Two auxins tested for root initiation, NAA showed best response in Vishala and TR10 varieties and average number of roots/shootlet was found to be maximum in triploid varieties.

Keywords: *In vitro* efficiency, mulberry varieties, shoot tip explants, diploid varieties, triploid varieties

Introduction
Mulberry is a vital tree having great importance in silk industry as its leaves plays a key role as sole food for silkworms (*Bombyx* mori.L). Besides the industrial and economic importance of mulberry, it is also considered as a valuable plant in having medicinal properties. Efforts on propagation of mulberry using seeds are time consuming due to poor seed germination and climatic conditions and the plant propagation through stem cuttings produced a few number of plants. Propagation for the production of true to type seedlings is achievable through *In vitro* cultures and is an advantage especially for plants which are highly valuable and which are difficult to propagate through conventional technique. Due to the perennial nature of the plant combined with prolonged generation period slows down the process of mulberry improvement. Further, mulberry is a cross pollinated crop and highly heterozygous in nature (Kavyashree *et al*, 2001) [4].
It normally takes 4 to 5 years for raising the saplings of this variety through conventional root grafting techniques. Therefore, for quick propagation of this poor rooting popular mulberry variety, a one step *In vitro* protocol was developed by culturing nodal explants from 2 year old plants on Murashige & Skoog (MS) media supplemented with individual as well as combination of phytohormones. The maximum shoot bud proliferation (6.3±0.71 in cm) and rooting (14.7±0.53 in cm) was observed when nodal explants were cultured on the combinational media of BAP (1.0 mg/L) and IBA (1.0 mg/L) after 14 days of culture. These *In vitro* raised plantlets were hardened by using the sterile soil and vermiculite in 2:1 ratio. Only 25 days were required for the micropropagation and hardening of raised plantlets of Goshoerami through this single step protocol. The hardened plantlets were successfully established in the field with 83% survival rate. The developed one step protocol can be used efficiently for the mass propagation of this elite mulberry variety throughout the year with in short span of 25 days.
If such cultivars developed through tissue culture was found to be good and encouraging. Hence to improve and stabilize the few newly developed mulberry cultivars to the local conditions, micro propagation provide a foundation for the future work in *In vitro* regeneration. In this regard an attempt has been made to evaluate the performance of few diploid and triploid mulberry varieties i.e S13, RF135, Tr10, Vishala, M5 and G2 using micro propagtion technique. Among the selected cultivars S13 and RFS135 are rainfed diploid mulberry varieties, Tr10 and Vishala are triploid irrigated varieties. Whereas M5 and G2 were diploid irrigated mulberry varieties.

Maetrials and Methods
Collection of explants
The plant material used in this study was collected from shoots of 35-40 days pruned mulberry varieties. Healthy apical shoots were excised from the mulberry plant. The leaves were removed and the shoot tip region measuring about 1.5 cm-2.0 cm each containing an apical bud was used as explants.

Explants surface sterilization
Shoot tips were washed thoroughly under running tap water for 15-20 minutes. These explants were treated with sterilants like two drops of tween 20 for 3-5 min, 1% Bavestin for 10 min and savlon for 3 min. A protocol was standardized for sterilization of shoot tip explants using mercuric chloride and sodium hypochlorite with different treatment durations which varies with the variety. These

explants were rinsed properly thrice after every treatment with double distilled water to remove traces of sterilants.

Table 1: The sterilization duration was presented

Varieties	$HgCl_2$ (%) + 70% Ethanol	NaOCl (%) + 70% Ethanol
S13	0.1 $HgCl_2$ for 3 min + ethanol for 30 sec	0.5 NaOCl for 7 min + ethanol for 30 sec
RFS 135	0.1 $HgCl_2$ for 3 min + ethanol for 30 sec	0.5 NaOCl for 5 min + ethanol for 30 sec
Tr 10	0.1 $HgCl_2$ for 3min + ethanol for 30 sec	0.5 NaOCl for 7 min + ethanol for 30 sec
Vishala	0.1 $HgCl_2$ for 3min + ethanol for 1 min	1.0 NaOCl for 5 min + ethanol for 1 min
M 5	0.1 $HgCl_2$ for 3min + ethanol for 1 min	0.5 NaOCl for 10 min + ethanol for 30 sec
G2	0.1 $HgCl_2$ for 3 min + ethanol for 1 min	0.5 NaOCl for 7 min + ethanol for 30 sec

MS media for initiation and multiple shoot formation

MS media supplemented with alone BAP (0.5 mg/l-3.0 mg/l) and Kinetin alone (0.5 mg/l-3.0 mg/l) for initiation from shoot tip explants. Observations were made daily on number of days for bud break, shoot length and number of shoots, and data was recorded. The shootlets developed in the initiation media were subcultured regularly in to the fresh media. For multiple shoot formation, MS medium fortified with BAP (0.5 mg/l-2.5 mg/l) + NAA (0.5 mg/l and 1.0 mg/l) and BAP (0.5 mg/l-2.5 mg/l) + kinetin (0.5 mg/l and 1.0 mg/l) were used.

***In vitro* rooting**

Shootlets having 3.0-5.0 cm length were selected and placed in the MS medium containing NAA (0.5 mg/l-3.0 mg/l) and IBA (0.5 mg/l-3.0 mg/l) for rooting. Data was recorded on rooting percentage and average number of roots/shootlets after four weeks of inoculation.

Acclimatization

The rooted shootlets were taken out from the media, washed with tap water to remove traces of agar and planted in a plastic cup containing autoclaved vermicultite and sand in 1:1 ratio. It is covered with a polythene sheet to maintain humidity and kept in the culture room for 10 days. This was directly supplied with ¼ strength MS salts for one week. Then the plants were transferred to the polybags containing soil and organic matter in 2:1 ration and kept in green house. Plants were watered for every 4 days for 2 weeks. Well established plants were transplanted to the earthen pots.

Results and Discussion

Initiation percentage from shoot tip explants

The percentage of initiation shoot tip explants was observed when the explants are inoculated in the medium containing BAP (0.5 mg/l-3.0 mg/l) alone and kinetin (0.5 mg/l-3.0 mg/l). The initiation frequency showed significant variation among the varieties and the initiation percentage from shoot tips explants of different varieties is presented in the table 2.

In BAP alone, the initiation percentage varied from 46.6-53.3. Maximum shoot initiation percentage was recorded in TR10 and Vishala 53.3 at 2.0 mg/l and 2.5 mg/l respectively. Whereas remaining varieties showed 46.6 percentage of initiation in different concentrations of BAP.

In kinetin alone the sprouting frequency varied from 33.3-39.9. Highest sprouting was recorded in S13, TR10, Vishala and M5 at 2.0 mg/l and lowest sprouting was recorded in RFS135 and G2 at 1.5 mg/l and 2.0 mg/l. When the kinetin concentration was increased, the frequency of sprouting was decreased. No sprouting was observed in kinetin above 3.0 mg/l concentration between two auxins tested. BAP was found to be superior over kinetin. The present reports are in accordance with the reported of Yadav *et al* (1990) [1], Patnaik and Chand (1997) [3] and Chitra and Padmaja (1999) [5] who stated that BAP was superior over kinetin in inducing sprouting from shoot tip explants.

Increased concentration of BAP, morethan 2.5 mg/l induced callus formation at the base of the explants. The present findings are supporting the reports of Attia-o-Attia *et al* (2014) [6]. Among six varieties tested TR10 and Vishala showed better performance than the remaining varieties.

Multiple shoot initiation percentage from shoot tip explants

The shootlets developed from the initiation media were transferred to the multiplication media containing BAP (0.5 mg/l-2.5 mg/l) in combination with NAA (0.5 mg/l and 1.0 mg/l) and BAP (0.5 mg/l-2.5 mg/l) with kinetin (0.5 mg/l and 1.0 mg/l) and observed for the percentage of multiple shoot formation. The percentage of multiple shoot formation from shoot tip explants is presented in the table 3.

In BAP+NAA (0.5 mg/l), the maximum multiple shoot formation varied from 26.6% to 39.9%. Highest multiple shoot initiation was observed in S13, TR10 and Vishala at 2.0 mg/l and 2.5 mg/l BAP and lowest percentage was observed in G2 2.0 mg/l. In BAP + NAA 1.0 mg/l maximum multiple shoot initiation was noticed in RFS135 (39.9) at 2.0 mg/l + 1.0 mg/l NAA. Whereas lowest multiple shoot initiation percentage was observed in S13 and M5 (26.6) at 2.0 mg/l + 1.0 mg/l NAA.

In BAP + kinetin (0.5 mg/l) combination best multiple shoot initiation was observed in S13, TR10 and Vishala at 1.5 mg/l and 2.0 mg/l. lowest multiple shoot initiation percentage was observed in M5 and RFS135 at 1.5 mg/l and 2.0 mg/l BAP. In BAP + Kinetin (1.0 mg/l), maximum multiple shoot initiation was noticed in TR10 (54.4) at 2.0 mg/l followed by S13 (53.30) at 1.5 mg/l. lowest 46.63 was observed in RFS 135, M5 and G2 at 2.0 mg/l + kinetin 1.0 mg/l.

The present study clearly shows that the presence of BAP in the multiplication media was found to be most essential for the shoot elongation. Among various combinations tested for multiple shoot initiation, the maximum percentage of multiple shoot formation was observed in BAP + kinetin combination. Increased concentration of NAA, reduced the multiple shoot formation percentage. In BAP + NAA, the percentage of multiple shoot formation was considerably reduced when the concentration of NAA was increased above 0.5 mg/l and BAP above 2.0 mg/l.

BAP was essential for high shoot multiplication rate as reported by Murashige (1974). The present findings are in acceptance with the reports of Sajeevan *et al*., (2011) [5] who reported that increase in BAP concentration in combination with NAA decreased the regeneration capacity. In contrast to the present findings Muhammad Akram and Fatheem Aftab (2012) [9] have reported that combined effect of BAP and NAA have great role in *In vitro* shoot multiplication.

In BAP + Kinetin combination has enhanced the percentage of multiple shoot formation. When the concentration of Kin was increased to 1.0 mg/l in combination with BAP above 1.0 mg/l, the percentage of multiple shoot was also increased. The shoot multiplication rate was enhanced in presence of two cytokines (BAP and Kinetin). In the present study BAP + Kinetin was found to be best combination for shoot multiplication. In contrast Chattapadhyay *et al.*, (2011) have suggested that MS medium with combined effect of auxin and cytokines is essential for shoot multiplication.

Percentage of root initiation from shoot tip expants (%)

The shootlets were transferred to the rooting media containing NAA (0.5 mg/l-3.0 mg/l) alone and IBA (0.5 mg/l-3.0 mg/l) alone. The Percentage of root initiation from shoot tip explants was presented in the table 4.

In NAA, the root initiation percentage varied from 73.3-33.3. The highest root initiation percentage was observed in Vishala (73.3) followed by Tr10 (70.0) at 1.5 mg/l. Lowest root initiation percentage was observed in RFS 135 (33.33) at 1.5 mg/l.

In IBA, the root initiation percentage varied from 30.0-40.0. Highest root initiation percentage was observed in Tr10 (40.0) followed by Vishala (36.6) at 1.5 mg/l. Lowest initiation percentage (30.00) was observed in the remaining varieties at different concentrations.

Between the two auxins tested for root initiation, NAA showed best response in Vishala (73.3) followed by Tr10 (70.6) varieties and average number of roots/shootlet was found to be maximum in triploid varieties Vishala and Tr10. Findings of present study were corroborated with the previous results of Kim *et al*., (1985) [11], Jain and Dutta (1992) [12] and Chattopadhyay (2011) [10] who have reported NAA was more responsive in root induction.

In contrast, to the present studies Ponchia and Gardiman (1996), Sajeevan *et al.*, (2011) [15] and Yasinta ratna *et al*., (2017) [16] noticed IBA was more responsive in root induction than NAA.

Table 2: Effect of different concentration and combinations of growth hormones on initiation from mulberry from shoot tip explants

Plant growth regulators (mg/l)		Initiation (%)					
BAP	KIN	S 13	RFS 135	TR 10	Vishala	M 5	G 2
0.5	-	39.97±6.65	33.30±6.70	39.97±6.65	8.83±3.87	33.30±6.70	11.07±3.87
1.0	-	39.97±6.65	39.97±6.65	46.63±6.65	33.30±6.70	46.63±6.65	33.30±6.70
1.5	-	46.63±6.65	39.97±6.65	46.63±6.65	33.30±6.70	39.97±6.65	39.97±6.65
2.0	-	36.63±3.35	46.63±6.65	53.30±6.70	46.63±6.65	39.97±6.65	46.63±6.65
2.5	-	26.63±6.65	39.97±6.65	39.97±6.65	53.30±6.70	33.30±6.70	39.97±6.65
3.0	-	19.97±6.65	33.30±6.70	33.30±6.70	46.63±6.65	33.30±6.70	33.30±6.70
-	0.5	11.07±3.87	13.30±0.00	13.30±6.70	8.87±7.68	13.30±6.70	11.07±3.87
-	1.0	13.30±0.00	19.97±6.65	13.30±6.70	13.30±6.70	33.30±6.70	11.07±3.87
-	1.5	33.30±6.70	33.30±6.70	33.30±6.70	33.30±6.70	33.30±6.70	13.30±0.00
-	2.0	39.97±6.65	26.63±6.65	39.97±6.65	39.97±6.65	39.97±6.65	33.30±6.70
-	2.5	33.30±6.70	19.97±6.65	33.30±6.70	39.97±6.65	33.30±6.70	11.07±3.87
-	3.0	31.07±3.87	13.30±0.00	13.30±6.70	33.30±6.70	33.30±6.70	6.63±6.65

Each value represents the average of 3 replications (n=3): ex plants treated with different concentrations of plant growth regulators±indicates the standard error values

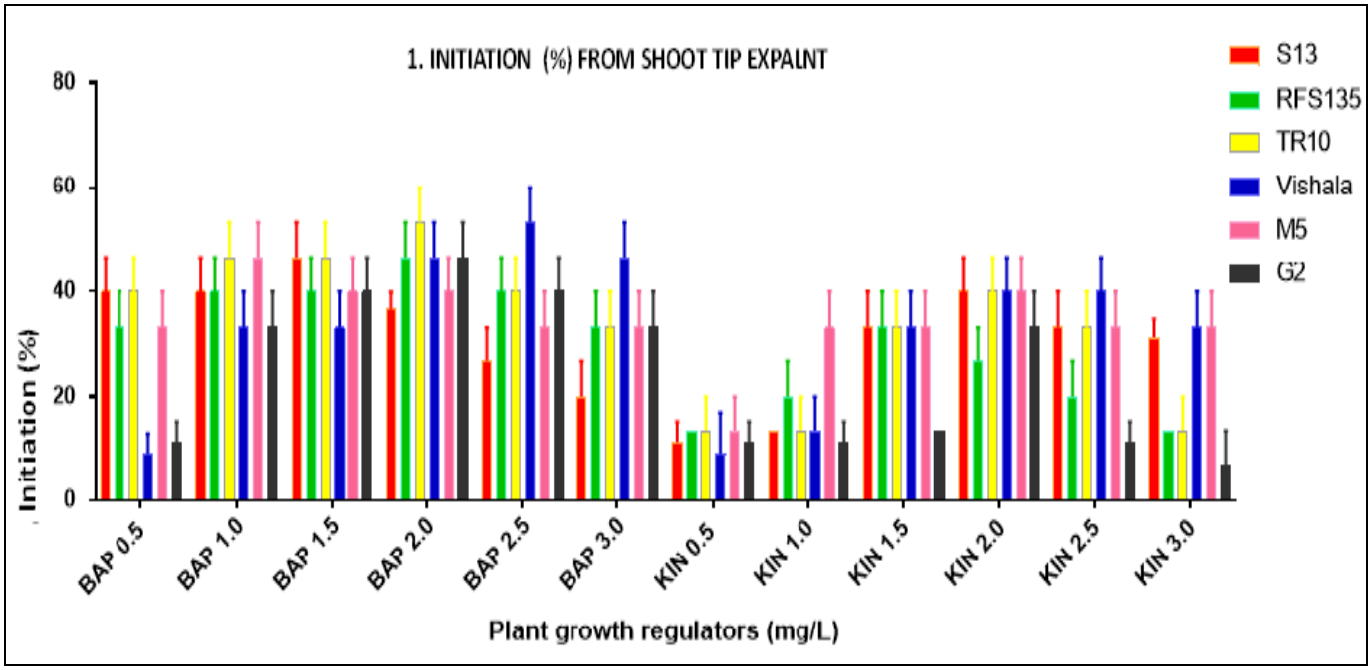

Fig 1

Table 3: Effect of different concentration and combinations of growth hormones on multiple shoot initiation from shoot tip explants

Plant growth regulators (mg/l)			Multiple shoot initiation (%)					
BAP	NAA	KIN	S 13	RFS 135	TR10	Vishala	M 5	G 2
0.5	0.5	-	26.63±6.65	13.30±6.70	26.63±6.65	8.83±3.87	13.30±6.70	8.87±7.68
1.0	0.5	-	33.30±6.70	19.97±6.65	26.63±6.65	19.97±6.65	19.97±6.65	13.30±6.70
1.5	0.5	-	33.30±6.70	19.97±6.65	33.30±6.70	26.63±6.65	26.63±6.65	19.97±6.65
2.0	0.5	-	39.97±6.65	26.63±6.65	39.97±6.65	33.30±6.70	26.63±6.65	26.63±6.65
2.5	0.5	-	33.30±6.70	33.30±6.70	33.30±6.70	39.97±6.65	33.30±6.70	19.97±6.65
0.5	1.0	-	13.30±6.70	13.30±6.70	13.30±0.00	8.87±7.68	8.87±7.68	8.83±3.87
1.0	1.0	-	13.30±6.70	26.63±6.65	19.97±6.65	19.97±6.65	13.30±0.00	13.30±0.00
1.5	1.0	-	19.97±6.65	33.30±6.70	19.97±6.65	26.63±6.65	19.97±6.65	26.63±6.65
2.0	1.0	-	26.63±6.65	39.97±6.65	33.30±6.70	26.63±6.65	26.63±6.65	33.30±6.70
2.5	1.0	-	19.97±6.65	33.30±6.70	26.63±6.65	33.30±6.70	19.97±6.65	26.63±6.65
0.5	-	0.5	13.30±6.70	13.30±0.00	13.30±6.70	26.63±6.65	19.97±6.65	13.30±6.70
1.0	-	0.5	39.97±6.65	19.97±6.65	33.30±6.70	33.30±6.70	26.63±6.65	26.63±6.65
1.5	-	0.5	46.63±6.65	26.63±6.65	39.90±6.65	39.97±6.65	33.30±6.70	33.30±6.70
2.0	-	0.5	33.30±6.70	33.30±6.70	46.63±6.65	46.63±6.65	26.63±6.65	39.97±6.65
2.5	-	0.5	33.30±6.70	19.97±6.65	39.97±6.65	46.63±6.65	19.97±6.65	33.30±6.70
0.5	-	1.0	33.30±6.70	26.63±6.65	33.30±6.70	33.30±6.70	26.63±6.65	26.63±6.65
1.0	-	1.0	46.63±6.65	33.30±6.70	39.97±6.65	39.97±6.65	31.07±3.81	33.30±6.70
1.5	-	1.0	53.30±6.70	39.97±6.65	46.63±6.65	46.63±6.65	39.97±6.65	39.97±6.65
2.0	-	1.0	39.97±6.65	46.63±6.65	54.40±8.40	53.30±6.70	46.63±6.65	46.63±6.65
2.5	-	1.0	33.30±6.70	33.30±6.70	46.63±6.65	53.30±6.70	39.97±6.65	39.97±6.65

Each value represents the average of 3 replications (n=3); ex plants treated with different concentrations of plant growth regulators±indicates the standard error values

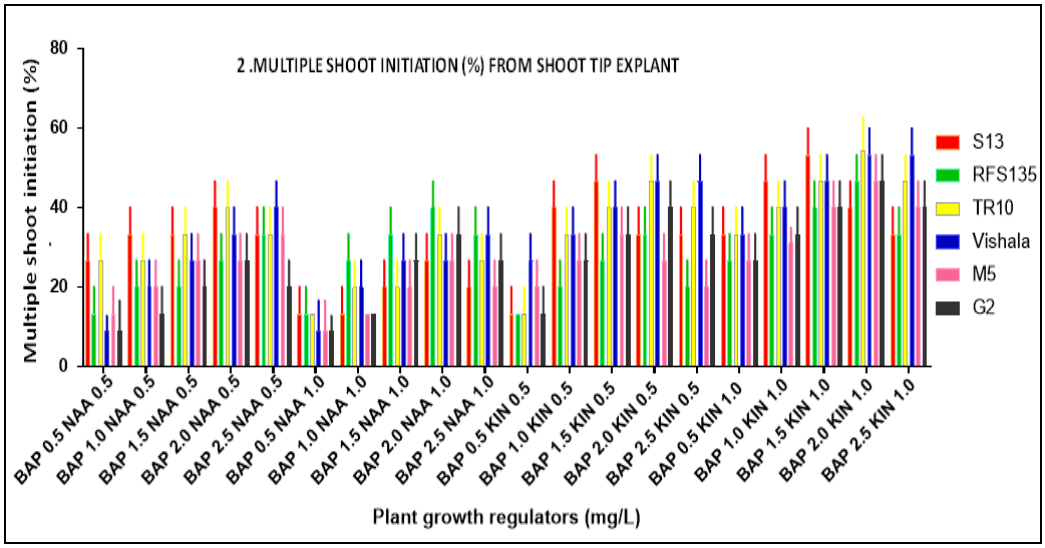

Fig 2

Table 4: Effect of different concentration and combinations of growth hormones on root initiation from shoot tip explants

Plant growth regulators (mg/l)		Root initiation (%)					
NAA	IBA	S 13	RFS 135	TR 10	Vishala	M 5	G 2
0.5	-	20.00±0.00	20.00±10.00	30.00±10.00	30.00±10.00	30.00±10.00	30.00±10.00
1.0	-	33.33±5.77	23.33±5.77	40.00±10.00	40.00±10.00	43.33±5.77	43.33±5.77
1.5	-	69.33±5.50	33.33±5.77	70.66±6.11	73.33±7.63	30.00±10.00	40.00±10.00
2.0	-	33.33±5.77	30.00±10.00	40.00±10.00	33.33±5.77	20.00±10.00	40.00±0.00
2.5	-	30.00±10.00	30.00±0.00	30.00±10.00	20.00±0.00	10.00±0.00	30.00±10.00
3.0	-	23.33±5.77	20.00±10.00	20.00±10.00	16.67±5.77	0.00±0.00	20.00±0.00
-	0.5	20.00±0.00	20.00±0.00	20.00±0.00	13.33±5.77	6.67±5.77	10.00±0.00
-	1.0	20.00±0.00	16.67±5.77	30.00±0.00	26.67±5.77	30.00±10.00	16.67±5.77
-	1.5	30.00±10.00	30.00±10.00	40.00±10.00	30.00±0.00	23.33±5.77	30.00±10.00
-	2.0	20.00±0.00	20.00±10.00	30.00±10.00	36.67±5.77	13.33±5.77	23.33±5.77
-	2.5	16.67±5.77	20.00±0.00	20.00±10.00	20.00±10.00	10.00±0.00	16.67±5.77
-	3.0	10.00±0.00	10.00±0.00	20.00±10.00	16.67±5.77	0.00±0.00	10.00±0.00

Each value represents the average of 3 replications (n=3); ex plants treated with different concentrations of plant growth regulators±indicates the standard error values

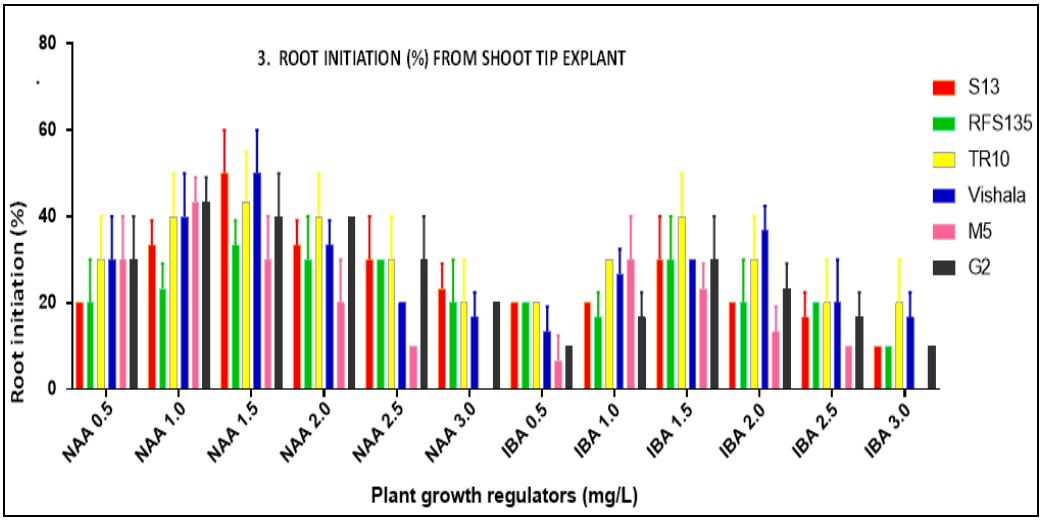

Fig 3

Initiation from shoot tip explants

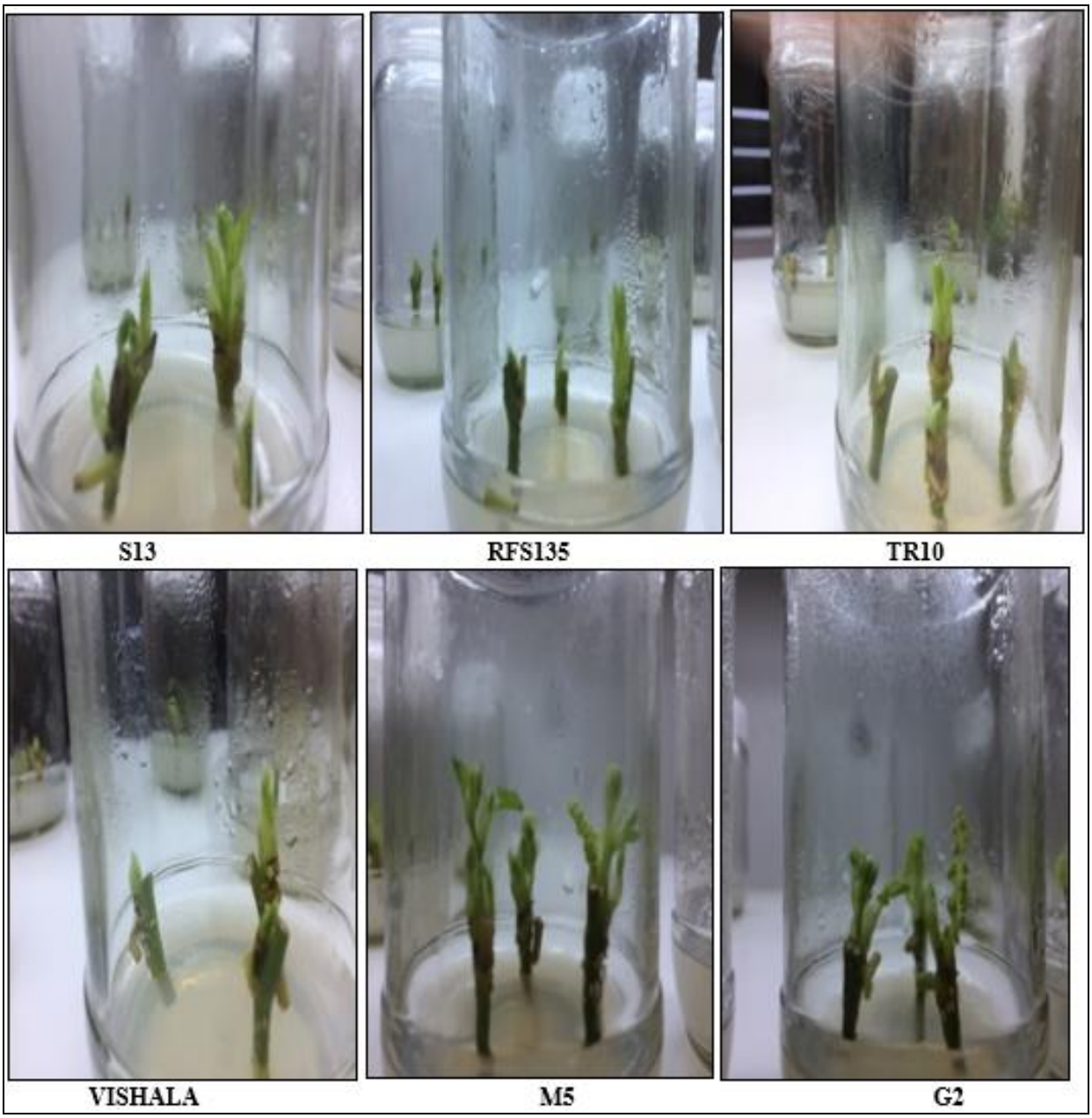

Fig 4

Multiple shoot initiation from shoot tip explants

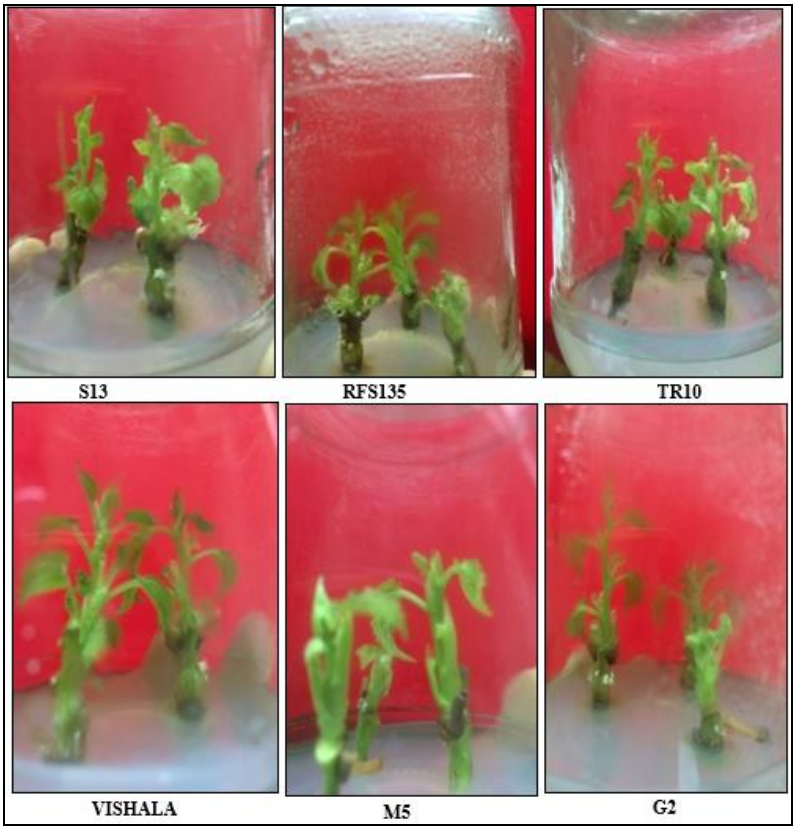

Fig 5

Root initiation from shoot tip explant

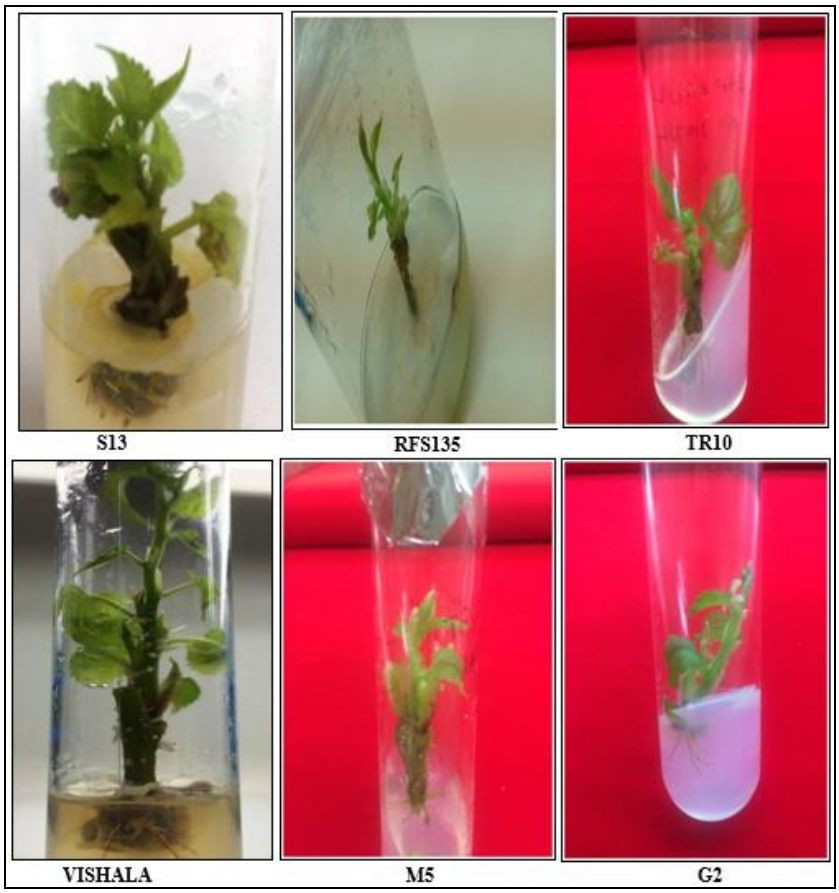

Fig 6

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