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ORIGINAL ARTICLE

STUDY OF *IN VITRO* MULTIPLICATION SYSTEM IN *SIMAROUBA GLAUCA D. C*

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ABSTRACT: "*In vitro* regeneration studies in *Simarouba glauca*" was undertaken with the objective to see the possibility of developing faster and reliable *in-vitro* regeneration for *Simarouba glauca* using shoot tips and axillary buds as explants. For surface sterilization of shoot tip explants, treatment of mercuric chloride (0.1%) for 4 minutes duration resulted in 80 per cent aseptic culture establishment, while for axillary buds, treatment of mercuric chloride (0.1%) for 5 minutes duration proved superior with 80 per cent of aseptic culture establishment. MS + 2,4-D (5 mg/L) proved best for induction of callus which also had produced maximum average fresh weight and dry weight. The colour of callus changed finely to yellow from initial white, while the texture of callus remained same as compact. Regeneration was not observed in callus. MS + BAP 4 mg/L + IAA 0.5 mg/L proved superior and required least (36.80±0.23) days for establishment with very good growth of shoot tip explants, but axillary bud explants failed for establishment. MS + BAP 1.5 mg/L + IAA 0.5 mg/L + $AgNO_3$ 1.00 mg/L produced maximum average 6.2 ± 0.61 shoots, whereas axillary bud explants failed to produce multiple shoots. Charcoal and ascorbic acid proved well in managing the absorption and oxidation of phenolic substances. The profuse rooting to the shootlets was observed with MS + IAA 3 mg/L with 80 per cent rooting along with 4.2± 0.32 number of roots and required least 22.8±0.52 number of days for induction of roots with very good growth. Soil rite proved superior over pot mix 1:1:1, (fine sand: clay: FYM). During primary hardening 62.96 per cent survival of plantlets were observed with soil rite. During secondary hardening 72.00 per cent survival of plantlets was recorded with fair growth of plantlets. The results from present investigation clearly indicated the possibility of multiplying simarouba through *in vitro* regeneration.
KEYWORDS: *In vitro*, micropropagation, oil tree, S*imarouba glauca*

INTRODUCTION

Simarouba glauca is an exotic species belonging to family Simarubaceae. It is commonly found in North America and also known as "American bitter wood". The species is native to Salvador and in the countries on equator having rain forest i.e. in Mexico, Cuba and Central American states. It was first introduced in India in 1966 from Brazil for it's edible oil Aceituno or Acetone oil under the plant introduction scheme of Indian Council of Agricultural Research (ICAR), New Delhi, in National Bureau of Plant Genetic Resources (NBPGR) Station at Amravati, Maharashtra and in Dr. Panjabrao Deshmukh Agricultural University, Akola in 1970 (Joshi and Hiremath, 2000). *Simarouba glauca* was introduced in Orissa long back in 1980 as fast growing soil binding tree species (Munde, 2001) and in Bihar as an exotic species in the year 1987 (Jaipuria, 1996). The species *Simarouba glauca* is included in the list of rare plants of the world and it has got very limited distribution in wild and in the tropical American forests (Belousova and Denisora, 1992). Looking at the importance of the species, it's conservation and mass multiplication is needed. *Simarouba glauca*, an exotic tropical American oil yielding tree was found growing luxuriantly under coastal climate of Orissa, but no attempt has been made to grow it commercially as a plantation crop. The plant is not browsed by animals and can be propagated by seeds. It is medium size tree growing up to 15 to 20 meter in height and 50 to 80 cm in diameter. The importance of *Simarouba glauca* as an edible oil yielding tree is now being widely recognized. In the face of the dwindling edible oil yield from crops like groundnut, mustard and sesame and substantial quality of edible vegetable oil being diverted for industrial purpose, the significance of edible oil yielding trees like *Simarouba glauca* assumes added importance. The oil obtained from the seeds is widely used in the treatment of skin diseases, intestinal disorders and also used as an edible oil (60%) (Satpathy and Al-Sattar, 1984), in soap making, as high grade lubricant and in cosmetics. There is a clear indication that *Simarouba* can make a significant contribution

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to the edible oil and fat economy of the country by rising the production as well as productivity of oilseeds. The bark and leaf of tree is known to have anti-malarial properties. The wood of the species is white and soft and can be used in cheap furniture, match sticks and also have antidysentric properties. The seed cake is rich source of NPK and can be used as a manure in long duration perennial crop like coconut, cashew nut etc. This multipurpose tree crop with high degree of variability, wide range of adaptability to different agroclimatic regions, easy maintenance and with an inbuilt genetic potential to give high oil yield, is going to become one of the very important oilseed crops in the country. The said tree has got the potential of plantation in the waste land area, as it requires very less water and does not require any high quality supervision. In general, *Simarouba* is propagated by seed, budding or grafting but the availability of seed is very less and also require 5-6 years for bearing of fruits. Being a polygammodioecious in nature, genetic uniformity cannot be maintained in next generation. Availability of planting material is very less. Very few studies have been carried out in tissue culture of *Simarouba glauca*. High multiplication ratio in such cases can be achieved by tissue culture techniques, which enables to manifold increase of desired plants in a short time. Therefore, the present investigation is proposed to be undertaken with following objectives.

MATERIAL AND METHODS

2.1. Sterilization of explants

The locally available culture of *Simarouba glauca* was taken as source for explants. Axillary buds and shoot tips were used as explants. Shoot tips and axillary buds of *Simarouba* were washed thoroughly in running tap water for 30 min to remove all the dirt, treated with tween 20 for 10 minutes and washed with distilled water for 3 minutes. The explants were then soaked in Bavistin (0.1%) for 10 minutes and washed with sterilized distilled water thoroughly. The explants were surface sterilized with $HgCl_2$ (0.1%) and (0.2%) for 1 to 6 minutes and 3-4 washings with double distilled water.

2.2. Standardization of medium for callus induction

The axillary buds and shoot tips were surface sterilized and transferred to callus induction media. Callus initiation and development was observed carefully in the different treatments as given in Table 1. Ten test tubes were used for each treatment. The observations on fresh weight and dry weight, colour and texture of callus were recorded after 20 days, 40 days and 60 days of callus induction. For dry weight, tissues were dried in hot air oven at 60^0 - 70^0C for three hours. The callus cultures were transferred on differentiation media containing MS media with different concentrations and combinations of BAP and NAA (Table 1). Ten bottles were used for each combination. The observations on regeneration were recorded 75 days after incubation. The cultures were incubated in dark at 25 ± 2^0C temperature with photoperiod of 16 hrs. Shoots were separated individually from the base and transferred to rooting medium (Table 1).

Table 1: different treatment used for induction of callus, regeneration of callus and rooting

Sr. No.	Treatment combinations for callus induction	Sr. No.	Treatment combinations for regeneration of callus	Sr. No.	Treatment combination for rooting of shoots
1	MS + 2,4-D 0.1 mg/l	1	MS + BAP 0.5 mg/L + NAA 5.0 mg/l	1	MS + IAA 0.5 mg/l
2	MS + 2,4-D 0.5 mg/l	2	MS + BAP 1.0 mg/l + NAA 5.0 mg/l	2	MS + IAA 1.0 mg/l
3	MS + 2,4-D 1 mg/l	3	MS + BAP 1.5 mg/L + NAA 5.0 mg/l	3	MS + IAA 1.5 mg/l
4	MS + 2,4-D 2.5 mg/l	4	MS + BAP 2.5 mg/L + NAA 5.0 mg/l	4	MS + IAA 2.0 mg/l
5	MS + 2,4-D 5 mg/l	5	MS + BAP 3.0 mg/L + NAA 5.0 mg/l	5	MS + IAA 3.0 mg/l
6	MS + 2,4-D 7.5 mg/l	6	MS + BAP 0.5 mg/L + NAA 1.0 mg/l	6	MS + IAA 4.0 mg/l
7	MS + 2,4-D 10 mg/l	7	MS + BAP 1.0 mg/l + NAA 1.0 mg/l	7	MS + IBA 0.5 mg/l
		8	MS + BAP 1.5 mg/L + NAA 1.0 mg/l	8	MS + IBA 1.0 mg/l
		9	MS + BAP 2.5 mg/L + NAA 1.0 mg/l	9	MS + IBA 1.5 mg/l
		10	MS + BAP 3.0 mg/L + NAA 1.0 mg/l	10	MS + IBA 2.0 mg/l
				11	MS + IBA 3.0 mg/l
				12	MS + IBA 4.0 mg/l
				13	MS+ NAA 0.5 mg/l
				14	MS+ NAA 1.0 mg/l
				15	MS+ NAA 1.5 mg/l
				16	MS+ NAA 2.0 mg/l
				17	MS+ NAA 3.0 mg/l
				18	MS+ NAA 4.0 mg/l

2.3. Standardization of multiplication medium

Well established cultures were then transferred to proliferation media having different concentrations of growth hormones to know their response for multiple shoot induction.

Table 2: Treatment combinations used for multiplication of established explant

Sr. No.	Composition mg/l
1	MS + BAP 1.0 + IAA 0.5
2	MS + BAP 2.0 + IAA 0.5
3	MS + BAP 3.0 + IAA 0.5
4	MS + BAP 4.0 + IAA 0.5
5	MS + BAP 5.0 + IAA 0.5
6	MS + BAP 0.1 + IAA 0.5 + $AgNO_3$1.0
7	MS + BAP 0.25 + IAA 0.5 + $AgNO_3$1.0
8	MS + BAP 0.50 + IAA 0.5 + $AgNO_3$1.0
9	MS + BAP 1.0 + IAA 0.5 + $AgNO_3$1.0
10	MS + BAP 1.5 + IAA 0.5 + $AgNO_3$1.0
11	MS + BAP 2.0 + IAA 0.5 + $AgNO_3$1.0
12	MS + BAP 0.1 + IAA 0.5 + $AgNO_3$2.0
13	MS + BAP 0.25 + IAA 0.5 + $AgNO_3$2.0
14	MS + BAP 0.50 + IAA 0.5 + $AgNO_3$2.0
15	MS + BAP 1.0 + IAA 0.5 + $AgNO_3$2.0
16	MS + BAP 1.5 + IAA 0.5 + $AgNO_3$2.0
17	MS + BAP 2.0 + IAA 0.5 + $AgNO_3$2.0
18	MS + BAP 0.1 + IAA 0.5 + $AgNO_3$3.0
19	MS + BAP 0.25 + IAA 0.5 + $AgNO_3$3.0
20	MS + BAP 0.50 + IAA 0.5 + $AgNO_3$3.0
21	MS + BAP 1.0 + IAA 0.5 + $AgNO_3$3.0
22	MS + BAP 1.5 + IAA 0.5 + $AgNO_3$3.0
23	MS + BAP 2.0 + IAA 0.5 + $AgNO_3$3.0

MT – Multiplication treatment

Ten bottles were used for each treatment. After 45 days of inoculation average numbers of shoots per explant were recorded.

2.4. Acclimatization and transfer to soil

Plantlets with a well developed root system were washed carefully to remove agar and then transferred to the pots containing sterile soilrite and potmix made up of fine sand : clay : FYM (1:1:1) to study their effect on acclimatization of plantlets.

2.5. Data recording and statistical analysis

The observations recorded during the experimentation were subjected to statistical analysis. The means and standard errors were calculated as per procedure given in Panse and Sukhatme.

RESULT AND DISCUSSION

3.1. Sterilization of explants

Treatments of explants with 0.1 per cent $HgCl_2$ and 0.2 per cent $HgCl_2$ for 1 to 6 minutes duration were tried (Table 3). For shoot tip explants excised from locally available plants, treatment for 4 minutes duration was found to be the best with maximum 80 per cent survival of explants (10%) contamination and (10%) mortality. The treatments for 3 min and 5 minutes duration were also found effective for surface sterilization of shoot tip explants while the treatment for 1 min, 2 min and 6 minutes duration were found ineffective. In case of axillary buds explants treatment for 5 minutes duration should best result with 80 per cent survival of explants, 10 per cent contamination and 10 per cent death of culture. Rout and Das (1994) reported that disinfection with 0.1% (W/v) aqueous $HgCl_2$ solution for 25 min of *Simarouba* seeds were found effective. Raut *et al.*, (1999) reported that surface sterilization of nodal explants with $HgCl_2$ 0.1% for 3 minutes duration proved best. Razdan (2000) reported that $HgCl_2$ (0.1 to 1.0%) for 2 to 10 minutes duration was very effective for surface sterilization of varieties of explants in various species.

Table 3: Effect of different surface sterilization treatments for shoot tips and axillary bud explants

Sr. No.	Sterilants used	Duration (min.)	Contamination (%)		Death of culture (%)		Culture establishment (%)	
			ST	AB	ST	AB	ST	AB
1	$HgCl_2$ 0.1%	1	100	100	00	00	00	00
2	$HgCl_2$ 0.1%	2	60	80	00	00	40	20
3	$HgCl_2$ 0.1%	3	30	50	10	00	60	50
4	$HgCl_2$ 0.1%	4	10	20	10	10	80	70
5	$HgCl_2$ 0.1%	5	10	10	40	10	50	80
6	$HgCl_2$ 0.1%	6	00	00	70	50	30	50
7	$HgCl_2$ 0.2%	1	60	70	00	00	40	30
8	$HgCl_2$ 0.2%	2	50	50	20	00	30	50
9	$HgCl_2$ 0.2%	3	30	30	50	30	20	40
10	$HgCl_2$ 0.2%	4	10	20	60	50	30	30
11	$HgCl_2$ 0.2%	5	00	10	80	70	20	20
12	$HgCl_2$ 0.2%	6	00	00	100	100	00	00

ST = Shoot tip ; AB= Axillary bud

3.2. Callus induction and its growth

MS basal media supplemented with 2,4-D was used for induction of callus from two explants namely axillary bud and shoot tip (Table 4). After 15 days of inoculation of explants in media, it was observed that axillary bud explants established in all the treatment except for MS + 2,4-D 0.1 mg/L treatment combination. Among all the treatments, MS basal media was found to be better over all other establishment media. For induction of callus in axillary bud explants used, MS + 2,4-D 5.0 mg/L media was found to be best treatment, which resulted in early induction of callus and highest average per cent of callus formed. The least number of days (10.0± 0.28) were required to produce callus from axillary

bud explant, with very good growth of callus (Figure 1). For observing, the growth of callus, the fresh weight and dry weight of callus was measured at the fix time interval (Table 5). The maximum fresh weight measured by 50.5 ± 0.32 mg which turned to 7.65 ± 0.5 mg /L treatment for 20 days after callus induction. The same treatment i.e. MS + 2,4-D 5.0 mg/L had produced 76.25±0.16 mg fresh weight and 9.7±0.06 mg dry weight, after 40 days of callus induction while after 60 days of callus induction the maximum 98.75±0.53 mg fresh weight and 11.6±0.28 mg dry weight was produced by the same MS + 2,4-D 5 ml/L treatments. According to Rout and Das, (1994), the maximum callus induction was found in MS + BA 11.1 µM and MS + 13.42 µM NAA but our investigation does not confirm their findings as we achieved maximum callus induction with MS + 2,4-D.

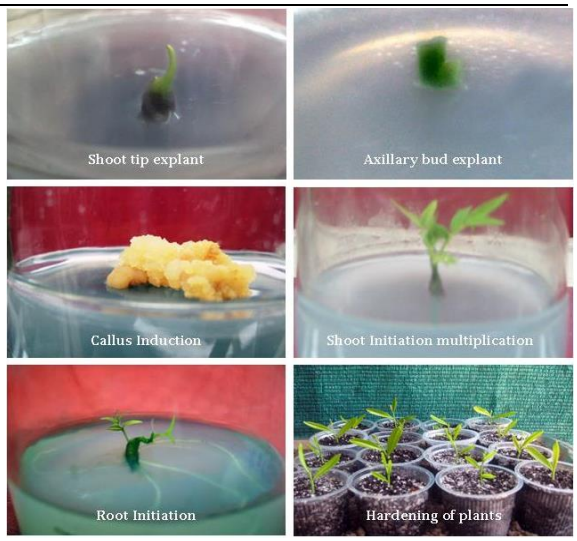

Figure 1: Various stages of plant regeneration of Simarouba glauca D. C

Table 4: Effect of different treatment combinations on induction of callus

Sr. No.	Treatment combinations	Mean no. of days required for callus induction	Growth up to 20 days from inoculation of explants.
1	MS + 2,4-D 0.1 mg/L	Nil	
2	MS + 2,4-D 0.5 mg/L	12.0±0.53	+++
3	MS + 2,4-D 1 mg/L	11.4±0.45	+++
4	MS + 2,4-D 2.5 mg/L	13.2±0.58	++
5	MS + 2,4-D 5 mg/L	10.0±0.28	++++
6	MS + 2,4-D 7.5 mg/L	11.8±0.50	+++
7	MS + 2,4-D 10 mg/L	13.0±0.63	+

Growth: + poor, ++ fair, +++ good, ++++ very good

Table 5: Effect of different treatments on growth of callus from axillary bud explants

Sr. No.	Treatment composition	20 days after culturing		40 days after culturing		60 days after culturing	
		Fresh weight (mg)	Dry weight (mg)	Fresh weight (mg)	Dry weight (mg)	Fresh weight (mg)	Dry weight (mg)
1	MS + 2,4-D 0.5 mg/L	27.0 ±0.28	7.34 ± 0.14	38.8 ±0.19	7.66 ± 0.05	45.6 ±0.21	8.31± 0.07
2	MS + 2,4-D 1 mg/L	36.5 ± 0.18	7.45 ±0.15	38.9 ±0.50	7.8 ±0.07	52.3 ± 0.50	8.49 ± 0.23
3	MS + 2,4-D 2.5 mg/L	34.5 ±0.18	7.4 ± 0.07	53.0 ± 0.28	8.34 ± 0.06	66.5 ±0.91	08.58 ±0.03
4	MS + 2,4-D 5 mg/L	50.5 ± 0.32	7.65 ± 0.05	76.25 ±0.16	9.70 ± 0.06	98.75 ±0.53	11.6 ± 0.28
5	MS + 2,4-D 7.5 mg/L	46.2±0.30	7.51 ± 0.05	68.25 ± 0.82	9.10 ± 0.05	84.20 ±0.13	10.06 ± 0.16
6	MS + 2,4-D 10 mg/L	42.5 ±0.49	7.48 ± 0.07	61.00 ±0.70	8.50 ±0.04	79.4 ±0.18	8.52 ± 0.11

3.3. Influence of media on callus morphology

Initially the colour of callus was white which turned yellow 60 days after callus induction (Table 6). The observations confirm the findings of Rout and Das, (1994) as they also found the yellow colour of callus. However, the texture of callus was constant as compare to compact, according to Rout *et al.*, (1999) it was friable. Out of the two explants used, only axillary bud explant has given the response to callus induction. Sixty days old callus was transferred to the various regeneration media, but the regeneration was not observed in a single case. This is in contradiction with the observations reported by Rout and Das, (1994) good amount of regeneration in *Simarouba* via somatic embryogenisis to the various combinations of BA and NAA was observed.

Table 6: Influence of media on colour and texture of callus

Sr. No.	Treatment combinations	Color and texture of callus					
		20 days after inoculation		40 days after inoculation		60 days after inoculation	
1	MS + 2,4-D 0.5 mg/L	White	Compact	Yellowish	Compact	Yellow	Compact
2	MS + 2,4-D 1 mg/L	White	Compact	Yellowish	Compact	Yellow	Compact
3	MS + 2,4-D 2.5 mg/L	White	Compact	Yellowish	Compact	Yellow	Compact
4	MS + 2,4-D 5 mg/L	Whitish yellow	Compact	Yellow	Compact	Yellow	Compact
5	MS + 2,4-D 7.5 mg/L	White	Compact	Yellow	Compact	Yellow	Compact
6	MS + 2,4-D 10 mg/L	White	Compact	Changing to yellow	Compact	Yellow	Compact

3.4. Establishment of explants

The shoot tip explant 80 per cent establishment was observed in MS + BAP 4.0 mg/L + IAA 0.5 mg/L followed by MS + BAP 3.0 mg/L + IAA 0.5 mg/L which produced 50 per cent establishment (Table 7). The shoot tip explants required least of 36.80±0.23 number of days for establishment and the growth observed was very good. Followed by MS + BAP 3.0 mg/L + IAA 0.5 mg/L produced 50 per cent establishment and good growth. While the response of axillary bud explants to establishment was not satisfactory it was almost nil. Rout *et al.*, (1999) had reported that good amount of establishment of explants was found in MS basal nutrient media supplemented with BA. Present investigation confirms the findings of Rout and Das upto some extent as we achieved 80 per cent establishment in MS basal nutrient media supplemented with BAP and IAA.

Table 7: Effect of different levels of cytokinins alone or in combination with auxin on establishment of shoot tips explants of *Simarouba*

Sr. No.	Composition	Establishment percentage (%)(Shoot tips)	No. of days required for establishment	Growth
1	MS + BAP (0.1- 5.0 mg/L)	-	-	-
2	MS + Kinetin (0.1 – 5.0 mg/L)	-	-	-
3	MS + IAA (0.1 – 5.0 mg/L)	-	-	-
4	MS + BAP (0.1 mg/L + IAA 0.5 mg/L)	10	67.60±0.36	+
5	MS + BAP (0.5 mg/L + IAA 0.5 mg/L)	30	58.34±0.27	++
6	MS + BAP (1.0 mg/L + IAA 0.5 mg/L)	40	47.50±0.25	++
7	MS + BAP (2.0 mg/L + IAA 0.5 mg/L)	40	42.08±0.22	+++
8	MS + BAP (3.0 mg/L + IAA 0.5 mg/L)	70	39.41±0.21	+++
9	MS + BAP (4.0 mg/L + IAA 0.5 mg/L)	80	36.80±0.21	++++
10	MS + BAP (5.0-mg/L) + IAA 0.5 mg/L)	50	52.20±0.34	++
11	MS+BAP (0.1-5.0mg/L)+ IAA 1.0 mg/L)	-	-	-

Growth: + poor; ++ fair; +++ good; ++++ very good

3.5. Induction of multiple shoots

The shoot induction from the established explants occurred upto 45 days of their culturing on test media (Table 8). For the established shoot tip explants the average maximum number of shoots produced were 6.2±0.61 with MS + BAP 1.0 mg/L + IAA 0.5 mg/L + $AgNO_3$ 1 mg/L followed by MS + BAP 1.00 mg/L + IAA 0.5 mg/L + $AgNO_3$ 1 mg/l which produced 4.0±0.56 average number of multiple shoots. While both treatment produced 3.8±0.57 and 3.8±0.56 with 60 per cent and 40 per cent multiple shoots respectively. Axillary bud explants failed to produce multiple shoots (Figure 1). The use of charcoal and ascorbic acid reduced browning and provided better condition for the development of multiple shoots.

Table 8 Effect of cytokinin alone and in combination with other growth regulators on multiple shoots formation in established shoot tip explants

Sr. No.	Composition MS +			Mean no. of shoots
	BAP (mg/L)	IAA (mg/L)	$AgNO_3$ (mg/L)	
1	1.0	0.5	-	00
2	2.0	0.5	-	00
3	3.0	0.5	-	00
4	4.0	0.5	-	00
5	5.0	0.5	-	00
6	0.1	0.5	1.0	00
7	0.25	0.5	1.0	2.16±0.25
8	0.5	0.5	1.0	2.94±0.28
9	1.0	0.5	1.0	3.8±0.57
10	1.5	0.5	1.0	6.22±0.61
11	2.0	0.5	1.0	3.43±0.46
12	0.1	0.5	2.0	2.82±0.31
13	0.25	0.5	2.0	2.34±0.24
14	0.50	0.5	2.0	3.82±0.28
15	1.0	0.5	2.0	3.91±0.53
16	1.5	0.5	2.0	4.0±0.56
17	2.0	0.5	2.0	3.73±0.55
18	0.1	0.5	3.0	2.97±0.41
19	0.25	0.5	3.0	2.43±0.38
20	0.50	0.5	3.0	2.12±0.27
21	1.0	0.5	3.0	1.97±0.21
22	1.5	0.5	3.0	1.12±0.19
23	2.0	0.5	3.0	00

3.6. Induction of roots from multiple shoots

MS nutrient medium supplemented with IAA 3.0 mg/L proved best with 80 per cent rooted shoots which required minimum 22.8 ± 0.52 number of days for root induction to shoot and produced 4.2± 0.33 number of primary roots per shoot with very good growth of roots (Table 9). This was followed by MS + IBA 2.0 mg/L which had produced 70 per cent rooting and required 27.4±0.60 number of days for root induction but produced only 2.2± 0.33 roots per shoot and growth observed was also good.

Table 9: Effect of different concentrations of auxins for induction of roots to multiple shoots of *Simarouba*

Sr. No.	Composition MS+			Percentage of rooted shoots	No. of days required for root induction to shoot	No. of primary roots per explant	Growth up to 45 days of culturing
	IAA (mg/L)	IBA (mg/L)	NAA (mg/L)				
1	0.5	-	-	10	31.63±0.69	1.61±0.23	++
2	1.0	-	-	30	27.42±0.56	1.97±0.29	++
3	1.5	-	-	50	28.8±0.58	2.4±0.52	+++
4	2.0	-	-	60	29.4±0.67	3.2±0.35	+++
5	2.5	-	-	60	25.6±0.54	2.01±0.31	++
6	3.0	-	--	80	22.8±0.52	2.2±0.33	+++
7	-	0.5	-	20	29.26±0.65	2.3±0.35	+++
8	-	1.0	-	30	28.45±0.57	3.1±0.37	+++
9	-	1.5	-	40	26.2±0.86	4.4±0.39	+++
10	-	2.0	-	70	27.4±0.60	2.2±0.33	++++
11	-	2.5	-	40	26.83±0.84	1.14±0.18	++
12	-	3.0	-	50	28.42±0.56	1.09±0.08	+
13	-	-	0.5	0	0	0	0
14	-	-	1.0	0	0	0	0
15	-	-	1.5	0	0	0	0
16	-	-	2.0	0	0	0	0
17	-	-	2.5	0	0	0	0
18	-	-	3.0	0	0	0	0

Growth - +: Poor; ++: Fair; +++: Good; ++++: Very good

3.7. Hardening of plantlets

In the process of primary hardening the plantlets were transferred to different hardening media and the pots were kept in mist chamber at very high humidity (90%; Figure 1). The humidity level was decreased slowly to acclimatize the plantlets. During primary hardening highest survival of 62.96 per cent was observed for soil rite with fair growth, while for pot mix 1:1:1 (fine sand: clay: FYM), 40.74 per cent survival of plantlets for soil rite pot mixture with fair growth. Whereas, in secondary hardening 72.00 per cent hardening was observed with fair growth of plantlets. The overall study revealed that *Simarouba* being a woody species, its *in vitro* response for multiplication was not comparatively good. These tissue cultured plant showed superior growth over seed derived stock. However, considering the fact that *Simarouba* is cultivated at very wide spacing (6 x 6 m), numbers of plants required /ha is less and average life of plant is 10 to 15 years. One time expenditure on quality planting material may be affordable. So, it can be suggested to make use of *in vitro* multiplication in this non conventional but economically important crop species, for production of true to type quality planting material.

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