

IN VITRO PLANT REGENERATION IN A MALE STERILE LINE OF CHILLI (*Capsicum annuum* L.)

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ABSTRACT

An efficient method for *in vitro* plant regeneration of a male sterile line of chilli (*Capsicum annuum* L.) was developed. Cotyledons, cotyledonary nodes and shoot apex explants were excised from 12, 18, 24 and 30 day old *in vitro* grown seedlings and cultured on Murashige and Skoog (MS) basal medium supplemented with different concentrations and combinations of Benzyl aminopurine (BAP), Kinetin (KIN) and Indole acetic acid (IAA). The highest number of adventitious shoots were formed directly, without any intervening callus, on the explants on MS medium containing 9.0 mg/l BAP + 2.0 mg/l KIN + 1.0 mg/l IAA after 5 weeks of culture. The process of induction, proliferation and elongation of shoots was completed on the medium containing high concentration of BAP. Explants from 18 and 24 day-old seedlings produced more multiple shoots than explants from 12 and 30 day-old seedlings. Cotyledon explants (either wounded or un-wounded) exhibited better regeneration capacity and produced more multiple shoots than cotyledonary nodes and shoot apex explants. The axillary shoots with few leaf primordia were subsequently cultured in medium containing 9.0 mg/l BAP + 2.0 mg/l KIN + 1.0 mg/l IAA for shoot multiplication. By incorporating Indole buteric acid (IBA) into the ½ MS rooting medium, 100% of the shoots produced roots. The rate of survival of plants during hardening was 86%. The tissue culture approach appears highly relevant to multiplication of male sterile line for further use in hybrid seed production.

KEYWORDS: *Capsicum annuum* L., Cotyledons, *In vitro*, Male sterile, Micropropagation, Plant regeneration, Shoot multiplication.

INTRODUCTION

Chilli (*Capsicum annuum* L.) is an important commercial spice grown as an annual vegetable all over the world. The pods are gaining popularity in houses not only for their uniqueness but also because they are low in sodium, cholesterol free, low in calories and high in vitamins A and C (Danise, 2003). Though India stands first in chilli cultivation covering 45 per cent of the world hectareage, its productivity is quite low (less than 1 t/ha) as compared to USA, China, South Korea, Taiwan and some other countries (Peter, 1998 and Madhavi *et al.*, 2002). The main reason for low productivity is the use of open pollinated varieties with minimal use of hybrid varieties. Recent experience provides the superiority of F1 hybrids in chillies for earliness, high productivity, high fruit weight and dry recovery. Development of hybrids by manual emasculation and hand pollination is a tedious process that involves high cost. Exploitation of heterosis economically depends on the

development of new techniques such as genetic male sterility (GMS), which leads to cheap hybrid seed production. The use of genetic male sterility controlled by a recessive gene can greatly help in making F1 hybrid, because tedious and costly hand emasculating of individual flower bud can be avoided (Patel *et al.*, 2001). This technique also increases the purity of F1 seed, as there is no self-pollination (Madhavi *et al.*, 2002). However, the major bottleneck in the seed production of GMS based hybrids is the female parent of genetic male sterile line, having 1:1 ratio of male fertile: male sterile plants which suggests currently practiced rouging of male fertile plants at the anthesis by observing each and every individual.

Tissue culture technology has contributed immensely to the advancement of agriculture for more than five decades. *In vitro* techniques for different crops e.g. tobacco, petunia, potato, sugar cane, carrot, rice, citrus and strawberry etc., have already been standardized primarily due to the ease of these crops to manipulate. However, chilli spp. have remained recalcitrant to *in vitro* manipulation and have lagged far behind in the application of tissue culture techniques. If the production of male sterile plants can be done through tissue culture, the tedious rouging out can be avoided and seed yield per unit area can be increased. Clonal propagation of male sterile lines may ensure the requirement of male sterile plants in the field. It may facilitate 100% genetically pure hybrid plant stand as well as utilization of male sterile plants in other hybridization programs. The tissue culture approach appears highly relevant to multiplication of male sterile lines for further use in hybrid seed production (Gupta *et al.*, 1998 and Gupta *et al.*, 2002).

MATERIALS AND METHODS

A study was conducted in the Department of Vegetable Crops in collaboration with the Department of Plant Breeding, Genetics and Biotechnology, Punjab Agricultural University during 2004-2005 to develop a protocol for *in vitro* plant regeneration in a genetic male sterile line of chilli, ms 12.

Plant material

Seeds of chilli male sterile line, "ms12", obtained from the Department of Vegetable Crops, PAU, were sterilized in 75% commercial bleach, Ala (5% sodium hypochlorite) for 20 minutes and then rinsed three times in sterile distilled water under a laminar flow hood. Sterilized seeds were cultured in culture tubes (25 x 150 mm, Borosil) containing 15 ml of half-strength MS basal medium (Murashige and Skoog, 1962) supplemented with 3% sucrose adjusted to pH 5.8 before adding 0.8% (W/V) agar and autoclaved at 121°C for 20 min. They were placed at 25±2°C and incubated with a photoperiod of 16hrs. After germination cotyledons, wounded

cotyledons, cotyledonary nodes and shoot apices excised from 12, 18, 24 and 30 day old, *in vitro*- grown seedlings were used as explants.

Culture media and conditions

The explants were cultured on medium containing 3% (W/V) sucrose and supplemented with growth regulators. The media were adjusted to pH 5.8 by using 1 N NaOH and 1 N HCl before adding 0.8% (W/V) agar. About 15 ml and 50 ml of the media were then dispensed in culture tubes (25 x 150 mm, Borosil) or 200ml jars respectively and closed with cotton plugs before autoclaving at 121°C for 20 min. Different combinations and concentrations of BAP, Kinetin and IAA were tested for shoot initiation. Different treatments used for establishment of cultures (both liquid and solid) were as follows.

Medium	Abbreviation
MS + BAP 1.0 mg/l + KIN 1.0 mg/l + IAA 0.5 mg/l	- MS1
MS + BAP 3.0 mg/l + KIN 2.0 mg/l + IAA 1.0 mg/l	- MS2
MS + BAP 6.0 mg/l + KIN 4.0 mg/l + IAA 1.0 mg/l	- MS3
MS + BAP 7.5 mg/l + KIN 3.0 mg/l + IAA 1.0 mg/l	- MS4
MS + BAP 9.0 mg/l + KIN 2.0 mg/l + IAA .0 mg/l	- MS5
MS + BAP 10.0 mg/l + KIN 5.0 mg/l + IAA 1.0 mg/l	- MS6
MS + BAP 10.0 mg/l + KIN 7.5 mg/l + IAA 1.0 mg/l	- MS7

There were 7 treatments replicated thrice in a completely randomized design. The number of shoot buds developing on the explants were recorded after 5 weeks of culture and the effect of shoot induction media was evaluated.

Shoot proliferation and elongation of shoots

After induction of shoots from the initial cultures, shoots were excised separately and transferred to solid and liquid MS media supplemented with various concentrations of BAP, KIN, IAA and GA₃ for shoot proliferation and elongation.

Medium	Abbreviation
MS + BAP 3.0 mg/l + KIN 2.0 mg/l + IAA 1.0 mg/l	-MM1
MS + BAP 5.0 mg/l + KIN 3.0 mg/l + IAA 1.0 mg/l	- MM2
MS + BAP 6.0 mg/l + KIN 4.0 mg/l + IAA .0 mg/l	- MM3
MS + BAP 7.5 mg/l + KIN 3.0 mg/l + IAA 1.0 mg/l	- MM4
MS + BAP 9.0 mg/l + KIN 2.0 mg/l + IAA 1.0 mg/l	- MM5
MS + GA ₃ 5.0 mg/l	- MM6

There were 6 treatments replicated thrice in a completely randomized design. The concentration of cytokinins in the shooting medium was gradually reduced before transferring the shoots to the rooting medium. The number of shoots developing on the explants were recorded after 2 and 3 weeks of culture and the effect of shoot multiplication media was evaluated.

Induction of rooting

The *in vitro* derived shoots were separated and transferred to the rooting medium for root initiation. The half strength MS medium supplemented with IAA, NAA and IBA were tested in following combinations.

Medium	Abbreviation
½ MS (Solid)	- MR1
½ MS (Liquid)	- MR2
½ MS + IAA 1.0 mg/l + NAA 1.0 mg/l (Solid)	- MR3
½ MS + IAA 1.0 mg/l + NAA 1.0 mg/l (Liquid)	- MR4
½ MS + IBA 1.0 mg/l + NAA 1.0 mg/l (Solid)	- MR5
½ MS + IBA 1.0 mg/l + NAA 1.0 mg/l (Liquid)	- MR6
½ MS + NAA 2.0 mg/l (Solid)	- MR7
½ MS + NAA 2.0 mg/l (Liquid)	- MR8
½ MS + IBA 2.0 mg/l (Solid)	- MR9
½ MS + IBA 2.0 mg/l (Liquid)	- MR10
½ MS + IBA 2.0 mg/l + 2.0 g/l Charcoal (Solid)	- MR11
½ MS + IBA 2.0 mg/l (one week) - ½ MS (Solid)	- MR12

The experiment was conducted in a completely randomized design with two replications. Data on percentage of rooting and the length of roots were recorded after 3 weeks.

Hardening and transplanting of rooted plantlets

Plantlets with well developed roots were carefully transferred from the culture tubes into a trough of running water for two hours to remove the traces of agar and other constituents of the culture medium adhering to plantlets. These plantlets were hardened by placing the root system on moist cotton with ½ MS salts for three days under high light condition in side the incubation room. The plantlets were then transferred to plastic pots (5 cm in diameter) containing surface soil. The pots were kept covered with polythene sheet to maintain a high relative humidity for six days in a glass house before exposing them to ambient temperature for ten days. Plants were then transferred to the 25-30 cm diameter clay pots containing top soil and farm yard manure at the ratio of 1:1.

Statistical analysis

All the shoot induction and shoot proliferation experiments were set up in a completely randomized design and each treatment had 3 replicates. The data on the shoot regeneration percentage, number of days taken to shoot initiation and number of multiple shoots regenerated on explants were analyzed using 2 factor factorial experiment in Complete Randomized Design (CRD). Root induction experiments were set up in a

completely randomized design and each treatment had 2 replicates. Data on the percent shoots responded, number of days to rooting and number of roots per shoot were analyzed using one-way analysis of variance.

RESULTS AND DISCUSSION

Response of different explants to establishing/initial culture media

Plant regeneration via organogenesis involves removing plant tissues such as hypocotyls or cotyledons from germinating seeds or seedlings and placing them on tissue culture media to induce differentiation and development of organs and plantlets. Except anther culture, it is generally shown that chilli is recalcitrant to *in vitro* plant regeneration from somatic explants and genetic transformation has been limited (Kang *et al.*, 1998). Establishment of initial cultures on liquid media completely failed. Callus formation was observed initially (7-10 days after culturing) at the base of the explants but all the explants turned brown later on and no shoot initiation was observed in liquid cultures at all the levels of BAP and KIN. Distorted and shriveled explants were seen in cultures with 12 and 18 day old explants. Shoot initiation and regeneration were the only success of the explants cultured on solid media.

As illustrated in Table 1, significantly higher rate of shoot regeneration was shown in 24 day old cotyledonary nodes followed by 18 day old cotyledonary nodes. There is not much difference for shoot regeneration among the wounded and un-wounded cotyledons but 24 day old explants of both wounded and un-wounded cotyledons showed better response followed by 18 day old explants. It was observed that all these explants cultured on 9.0 mg/l BAP concentration performed well followed by 7.5 mg/l BAP and 6.0 mg/l BAP respectively. The best interaction of media X age of the explant was observed in MS5 media with 24 day old both wounded and un-wounded cotyledons followed by MS5 media with 18 day old cotyledonary nodes and shoot apices, respectively. There have been several reports of successful plant regeneration from adventitious buds in capsicum. Embryos, seedlings and vegetative plant parts have been used as explants, where cotyledons and hypocotyls were the most effective for organogenesis (Sanatombi and Sharma, 2006).

Response of wounded and un-wounded cotyledon explants was very poor on the lowest and the highest BAP concentrations. Except cotyledonary nodes, other explants did not respond to the MS7 medium which was having the highest BAP and KIN concentrations and most of the cultures were browned and finally died. The regeneration protocols consisted of adenine type cytokinins, commonly Benzyl aminopurine (BAP), with or without auxin (Pundeva *et al.*, 1998).

Table 2. Number of days taken for shoot regeneration from wounded cotyledons, un-wounded cotyledons, cotyledonary nodes and shoot apex explants cultured on different media.

Medium	Age of the explant (days)																			
	Wounded cotyledons					Un-wounded cotyledons					Cotyledonary nodes					Shoot apices				
	12	18	24	30	mean	12	18	24	30	mean	12	18	24	30	mean	12	18	24	30	mean
MS1	35.00	33.00	32.00	35.00	33.75	35.00	33.00	32.00	35.00	33.75	14.00	12.33	11.50	13.33	12.71	12.00	11.33	11.33	12.64	11.83
MS2	27.00	29.00	25.67	28.5	27.54	29.00	29.67	27.33	30.00	29.00	12.33	12.00	12.33	12.33	12.25	11.67	11.33	10.67	11.83	11.38
MS3	25.00	28.67	26.00	28.00	26.92	27.33	29.33	27.33	30.00	28.50	12.00	11.33	11.50	11.83	11.67	12.33	9.33	10.33	11.67	10.92
MS4	27.00	28.33	24.67	26.33	26.51	28.00	28.67	28.00	29.33	28.50	10.33	10.50	9.67	11.33	10.46	10.00	9.00	9.00	10.00	9.50
MS5	28.00	26.33	24.67	26.00	26.25	27.67	28.00	26.33	28.00	27.50	8.67	7.33	8.00	9.17	8.29	10.00	8.33	8.67	8.67	8.92
MS6	29.00	23.33	22.67	25.33	25.08	27.00	24.67	25.33	26.67	25.92	7.83	7.33	7.67	8.33	7.79	10.00	7.83	8.14	8.83	8.71
MS7	35.00	33.00	32.00	35.00	33.75	35.00	33.00	32.00	35.00	33.75	7.83	6.33	7.33	7.83	7.33	14.00	13.33	13.00	13.33	13.42
Mean	29.43	28.81	26.81	29.17		29.86	29.48	28.33	30.57		10.43	9.60	9.71	10.57		11.43	10.07	10.17	11.00	
LSD																				
(p=0.05)					1.05					0.67					0.78					0.64
Media					0.79					0.51					0.59					0.48
Age					2.10					1.35					NS					NS
M X A					4.49					2.79					9.51					7.32
CV																				

Days taken for shoot initiation

With regard to the days taken for shoot bud initiation from the different explants, wounded and unwounded cotyledonary explants showed a similar pattern that of more than 4 weeks for shoot initiation. According to the results obtained cotyledonary nodes were found to be the best explant for early shoot initiation. Days taken for shoot bud initiation was less with the increase of cytokinin in the medium (Table 2). Induction of shoot buds and their subsequent proliferation is a function of cytokinins such as 2ip, kinetin and BAP of which BAP has been found the most effective (Chen and Evans, 1990). When considering the age of the explants, the least number of days were taken by the 18 and 24 days old cotyledonary nodes respectively. Shoot initiation in shoot apices showed a similar pattern to that of cotyledonary nodes. Also, it was observed that the high concentration of cytokinins was detrimental to the explants irrespective of their age.

Number of shoots regenerated per explant

The MS medium supplemented with 9.0 mg/l BAP + 2.0 mg/l KIN + 1.0 mg/l IAA gave rise to the highest number of shoots/explant in wounded cotyledons (Plate 1) followed by MS medium supplemented with BAP 7.5 mg/l + KIN 3.0 mg/l + 1.0 mg/l IAA in wounded cotyledons and unwounded cotyledons. But, the numbers of shoots per explant were reduced when increasing the concentration of above cytokinins in all the explants. The results clearly showed that 24 days old wounded cotyledons were the best explant generating more number of shoots per explant followed by 18 days old wounded cotyledonary explants, 24 days old un-wounded cotyledonary explants and 18 days old un-wounded cotyledonary explants respectively. The number of shoots regenerated was increased with the increase in BAP and KIN concentrations in the media up to a level and beyond that it was detrimental to all the types of explants. The increase in BAP concentration up to optimum level results in increase in mean shoot number due to increase in the activity of cell division (Skoog and Armstrong, 1970). It was observed that there were more number of shoots per explant in MS5 medium (BAP 9.0 mg/l + KIN 2.0 mg/l) followed by MS4 (BAP 7.5 mg/l + KIN 3.0 mg/l + 1.0 mg/l IAA) and MS3 (BAP 6.0 mg/l + KIN 4.0 mg/l + 1.0 mg/l IAA) respectively (Table 3). Sanatombi and Sharma (2006) observed up to a maximum of 7 buds and 5 buds from shoot apex explants cultured on BAP 44.4 μ M in chilli cultivars of Meitemorok and Haomorok, respectively.

Shoot multiplication and elongation

BAP in the culture medium has been known to cause proliferation of adventitious in many plants (Murashige, 1974). The elongation growth of leafy buds was severely inhibited in the continuous

presence of high concentrations of cytokinins, and frequently the buds became quite thick, ill-defined and vitreous (Binzel *et al.*, 1996).

Table 4. Shoot multiplication in different media after 14 days and 21 days of culture.

Medium	Number of shoots per explant						
	14 days after sub-culturing				21 days after sub-culturing		
	1 st Sub-	2 nd Sub-	3 rd Sub-	Average	1 st Sub-	2 nd Sub-	Average
MM1	1.58	1.75	1.50	1.61			
MM2	2.33	2.75	2.58	2.55	1.65	1.25	1.45
MM3	3.25	3.06	2.88	3.06	2.30	2.00	2.15
MM4	4.08	3.88	4.00	3.99	2.20	1.80	2.00
MM5	4.23	4.00	3.88	4.04	1.80	1.20	1.50
Mean	3.09	3.09	2.97	3.05	2.03	1.81	1.65

Average number of shoots per explant in sub-culturing was showed decreasing trend in subsequent culturing. It was observed that there were 3.09 shoots/explant during first and second sub-culturing and 2.97 shoots/explant in third sub-culturing irrespective of the media used (Table 4). Maximum number of shoots were obtained in MM5 medium (4.04 shoots/explant) (Plate 2.) followed by MM4 medium (3.99 shoots/explant). Fourteen days interval of sub-culturing was found to be better than the twenty one days interval. Dying of shoots was observed in 21 days duration of sub-culturing which led to the low number of shoots per explant cultured. All the cultures failed in MM6 media which were having GA₃ 5 mg/l.

Rooting of *in vitro* raised shoots

It is evident from the data presented in Table 5, that percent shootlets responding to root initiation on ½ MS medium varied with the addition of different auxins. Hundred percent root initiation was observed in all the shootlets cultured on MR12 solid medium for one week and subsequently transferred to the ½ MS hormone free medium 2.0 mg/l of IBA indicates that it is the best treatment for root initiation.

The time taken for root initiation was minimum (11.66 days) in (MR12) medium followed by MR11 medium (16.00 days). Delay in rooting and callus formation at the base of the explant was observed in media supplemented with IAA.

Number of roots per shoot

The highest number of roots per shoot was observed in MR12 medium (7.50 roots/shoot) followed by MR9, MR11 (5.00 roots/shoot) and MR5, MR7 (4.00 roots/shoot). It was clearly observed with the present studies that those rooting media supplemented with IBA were responsible for the increase of number of roots per shoot.



Plate 1. Clusters of shoot buds regenerated from cotyledons cultured on MS medium supplemented with BAP 9.0 mg/l + KIN 2.0 mg/l + IAA 1.0 mg/l. (Magnification 1 X 1).



Plate 2. Shoot multiplication 14 days after sub-culturing on MS medium supplemented with BAP 9.0 mg/l + KIN 2.0 mg/l + IAA 1.0 mg/l. (Magnification 1 X 1).

Table 5. Effect of different rooting media on rooting of *in vitro* generated shoots.

<i>Medium</i>	<i>Per cent responded</i>	<i>Days to rooting</i>	<i>Number of roots/shoot</i>	<i>Root length* size</i>
MR1	27.77	20.30	2.50	Short
MR3	27.80	20.00	3.00	Short
MR5	41.67	19.66	4.00	Medium
MR7	36.10	20.00	4.00	Short-Medium
MR9	63.90	17.00	5.00	Medium
MR11	55.57	16.00	5.00	Medium-Long
MR12	100.00	11.66	7.50	Long

* Length of the roots Size category of roots
 Less than 3 cm Short
 3 cm to 6 cm Medium
 Above 6 cm Long

Length of the roots

It was observed that NAA and IAA caused callusing at the cut end. Culturing the shoots on half strength MS medium supplemented IBA 2.0 g/l for one week and then transferring to half MS medium without growth hormones (MR12) was found to be the best medium for root elongation. More than 6cm long roots obtained from the same medium followed by MR11 medium (½ MS Supplemented with IBA 2.0 mg/l + charcoal 2.0 g/l) were having medium-long (3-6 cm in length) size roots.

Hardening and field establishment of rooted plantlets

Hardening the plantlets 3 days prior to the transfer to soil on moist cotton with ½ MS salts was found to be more effective than that of hardening plantlets on moist cotton with water. Eighty six per cent survivals were observed with ½ MS salts and 75% survivals were observed with simple water treatments. Ninety two percent survivals were observed three weeks after planting the hardened plantlets in soil. Keeping the potted plants under high humidity during initial six days helped a lot to obtain the high survival after planting (Plate 3). Plantlets having 4-5 well developed leaves performed well after putting them on soil. Plants showed vigorous growth, well developed branches and attained maturity early and displayed the features of male sterility.

Table 6. Per cent survival after hardening and field establishment.

Hardening			
<i>Hardening treatment</i>	<i>No. of plantlets hardened</i>	<i>Number of plantlets survived</i>	<i>Percent survival</i>
½ MS Salt	30	26	86.66
Water	24	18	75
Plant establishment			
<i>Number of plantlets established</i>	<i>Number of plantlets survived</i>	<i>Per cent survival</i>	
26	24	92	

The technique of micropropagation has witnessed a major success in its application for large scale propagation of the rare and elite germplasm. The tissue culture derived plants are usually true to type, vigorous and free from diseases. At the same time, the *in vitro* multiplication can be carried out throughout the year, irrespective of the season. Thus, increasing the rate of propagation manifolds as compared to conventional methods. In the present investigation, an attempt has been made to solve the problem associated with maintenance and multiplication of male sterile line via micropropagation

In micropropagation studies, explants of genetic male sterile line were cultured on MS medium supplemented with different combination and concentration of phytohormones. Explants from younger seedlings (18 - 24 days old) responded the best on MS medium supplemented with BAP 9.0 mg/l + KIN 2.0 mg/l + IAA 1.0 mg/l followed by MS medium supplemented with BAP 7.5 mg/l + KIN 3.0 mg/l + IAA 1.0 mg/l.

It was observed that 24 days old wounded cotyledons were the best explants generating multiple shoots per explant followed by 18 days old wounded cotyledonary explants, 24 days old unwounded cotyledonary explants and 18 days old unwounded cotyledonary explants respectively.

More shoot proliferation was obtained on MS medium supplemented with BAP 9 mg/l + KIN 2 mg/l + IAA 1 mg/l. The number of days kept for shoot proliferation also was found to be an important factor in shoot multiplication. It was observed that number of shoots per sub-culturing cycle decreasing with the increasing of time duration. Sub-culturing after every fortnight period was observed to be more successful than extending the period for three weeks because dying of shoots was observed when extending the culturing period. There was decreasing trend in number of shoots per explant in successive sub-culturing.

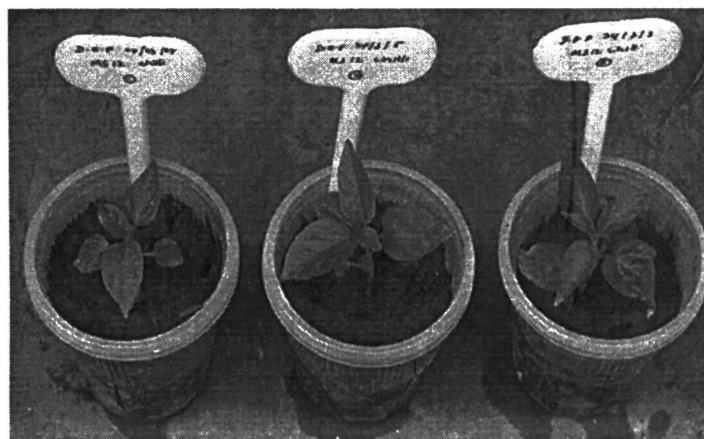


Plate 3. Properly hardened male sterile chilli plants ready for field planting.

Hundred percent shootlets responded to the root initiation, less number for rooting and lengthy roots resulted with the MR12 solid medium ($\frac{1}{2}$ MS medium supplemented with IBA 2.0 mg/l one week and subsequently transferred to $\frac{1}{2}$ MS hormone free medium). Therefore, IBA 2.0 mg/l has been found to be the best for root initiation.

Hardening the plantlets 3 days prior to the transfer to soil on moist cotton with $\frac{1}{2}$ MS salts was found to be more effective than that of hardening plantlets on moist cotton with water. Eighty six per cent survivals were observed with $\frac{1}{2}$ MS salts and 75% survivals were observed with simple water treatments.

CONCLUSIONS

The present investigation indicated that the technique of micro-propagation can be utilized for rapid and true to type multiplication of male sterile lines. However, long term experiments covering a large number of media with different concentrations of growth regulators (BAP and KIN) etc. are required to measure the rate of multiplication up to the level when it can be used for commercial purpose. The media used to elongate the multiple shoot buds developed from cotyledons should be further standardized to obtain more number of shoots than the number of shoots obtained.

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