Plant Regeneration by Somatic Embryogenesis in Azadirachta indica A.Juss. (Neem)

Homen Phukan, *Ravindra Kumar, Pradip Kr Mitra

Institutional Biotech Hub, North Lakhimpur College (Autonomous), Lakhimpur-787031, Assam, India
**Tissue culture laboratory, Dept. of Botany, North Lakhimpur College (Autonomous), Lakhimpur-787031, Assam, India.*

Abstract – From the earliest period people using plant based medicine for their primary health care. Out of them neem tree (Azadirachta indica A. Juss.) belongs to the family Meliaceae is one of the important medicinal plant using from ancient Vedic period in different ailments and now it has a great demand in commercial purpose due to its active medicinal compounds (e.g. Azatin, Azadirachtin).The conventional method of propagation is not sufficient to overcome the requirements of neem tree. Somatic embryogenesis is a potential tissue culture method which can be utilized in regeneration of neem tree for high rate of multiplication. The present observation revealed that the concentration of BA (0.5mg/l and 1.0mg/l) and 2, 4-D 0.1mg/l in individual MS medium were responded to best callus from the inoculated explants i.e. leaf and stem. The MS medium supplemented with 0.5mg/l BA was showed earlier response to callus formation in both leaf (in 8 days) and stem (in 11 days). Whereas combination of IAA and BA was not resulted good effects. The callus grown on MS medium with 2, 4-D showed white color instead of normal green color. Out of the various growth regulators treatment only BA supplemented MS medium stimulated to Somatic embryos (SEs) and 0.5mg/l concentration responded earlier than 1.0mg/l BA. It was resulted that MS medium supplemented with BA as growth regulator is suitable for germination of SEs of Azadirachta indica and 3rd subculture of callus is optimum for maximal SEs germination.

Keywords: Somatic embryogenesis, BA, Azadirachta indica, Azadirachtin.

INTRODUCTION

From the earliest period people has been dependent on the plants for medicine, wood, food, flavors and many other uses. The conventional Indian System of medicine mainly Ayurveda which is involved dispensing of the herbal and plant products in various form such as powder's, extracts, decoctions etc. The WHO reported that 80% of the people in the world use medicinal plants for their primary health care [1]. The use of herbal medicines is growing in developing countries, presently 25% of UK population use plant based medicine. About 40% compounds used in pharmaceutical industries or directly or indirectly derived from plant because the chemical synthesis of such compounds is either not possible and/or economically not viable.

The neem tree, Azadirachta indica A. Juss., an evergreen tropical tree belonging to the family Meliaceae, is native to the ride regions of the Indian Subcontinent [10] [5]. It is high valued multipurpose tree, the wood and products of which have traditionally been used for timber, insecticidal, fertilizers, antiparasitic, contraceptic, antipyretic and antiseptic properties. The fruit produce oil which is used in the manufacture of soaps and detergents. It has been reported in Ayurvedic medicine, interested in neem mainly due to its biocontrol properties [2]. The first commercial neem insecticide, Margosan-o, was registered by the Environmental Protection Agency (EPA) in 1998 [3] and since then the number of commercial and experimental neem insectisides has increased markedly with Azatin (Agridyne Technology, USA) recently receiving EPA approval for use on food crops [3].The commercial potential of this tree has renewed worldwide research interest in neem. Neem seeds are main source of neem oil and Azadirachtin, the principle active constituent. A single tree will yield on an average of about 20.5 kg of fruit per year and average yield of Azadirachtin from seed being around 2-4 mg/g dry weight of seed kernels (National Research Council). The development of simple and rapid method for production of uniform neem population with high yields is desirable. It seems appropriate to employ tissue culture technique for its production.

In vitro cultivation of woody species was not successful until the 1970s, when also tissue culture of neem was first reported [4]. The term tissue culture is commonly used in a very vide sense to include in vitro culture of plant cells, tissues as well as organs. But in a strict sense, tissue culture denotes the in vitro culture of plant cells into an unorganized mass, e.g., callus cultures. Propagation of neem tree via tissue culture has been investigated in several studies. In the majority of these studies, the culture protocols were based on organogenesis. In spite of having such valued properties, improvement of neem by

conventional methods is very limited owing to its highly heterozygous nature, long reproductive cycle and poor seed yield. In this regard, somatic embryogenesis may offer an effective system for plant regeneration and improvement [5].
It was reported on successful neem regeneration via somatic embryogenesis (Indirect somatic embryogenesis) were induced from leaf and stem explant and experiment were conducted using solid medium [6]. Somatic embryogenesis has many advantages over Micropropagation. It is high density propagation method and easily accessible for automation. The propagules produced by somatic embryogenesis have fewer variations [10]. A new culture protocol is discussed here for induction of somatic embryogenesis, plantlets regeneration via callus and somatic embryo.

MATERIAL AND METHODS

Seed sterilization & inoculation

Fruit of matured seeds of neem were surface sterilized with 70% v/v ethanol (E-Merck, India) for 2-3 min and freshly prepared 0.1% w/v solution of $HgCl_2$ for 15 min, washed five times with sterilized distilled water. Seeds were removed from fruits under aseptic condition, inoculated on MS [8, Murashige and Skoog, 1962] medium with 0.8% agar and grown in vitro on MS medium.

Explant collection & inoculation

The leaf and stem explants were collected from aseptically grown 2-3 month old plants, and inoculated on MS medium, with various concentration of IAA (E- Merck India), BA (E- Merck India), 2, 4-D (Loba chemie, Mumbai, India) [8].The callus was subcultured on MS medium with either same or different GRs (Table-1) and modified medium (MS medium with 100mg/l Myo-Inositol) for SEs production. The globular shaped SE was transferred on MS medium with 0.5/1.0 mg/l BA and in combination of 2.0mg/l BA and 0.1mg/l IAA. The 2.0mg/l BA regenerated shoots were inoculated on MS medium within combination of 1.0mg/l BA and 0.1mg/l IAA, and the shoots from SE were inoculated on 1.0mg/l IBA. The rooted shoot was inoculated on MS medium. For maturation of SEs were transferred on modified MS medium, in which 2X macronutrients added. All media were solidified with 0.7% agar and pH5.8. Cultures were incubated under 25umol $m^{-2}s^{-1}$quantum flask density of light provided by cool white fluorescent lamp (3 tubes X 40W, Philips, India) for 16h light/8h dark [9]. Temperature and relative humidity (RH) of culture room were maintained at 22 ± 1^0C and 70 ± 2% respectively.

RESULT AND DISCUSSION

Seeds were germinated on MS medium within 2-3days. The initiation of callus (Figure-1) were varied on leaf and stem in each case of growth regulators (GRs) (Table1). Both concentration of BA (0.5 and 1.0mg/l) and 0.1 mg/l 2, 4-D were responded to best callus. The MS medium supplemented with 0.5mg/l BA showed earlier in initiation of callus in both leaf (in 8 days) and stem (in 11 days). The 0.5/1.0 mg/l IAA stimulated good callusing in leaf, not in stem. The combination of IAA and BA were not resulted good effects. The color of all calluses was green but 2, 4-D showed white callus (Fig. - 2).

Only BA stimulated SEs formation in which 0.5mg/l responded first in both leaf and stem, but 1.0mg/l delayed by 10-13 days (Fig – 4-6). The maximum number of SEs (180) was obtained in 1.0mg/l BA from stem explants. The data on number of SEs per explant formed and number of days took after subculture on MS medium with different concentration of GRs are presented in table-1. After 1^{st}, 2^{nd}, 3^{rd}, or 4^{th} subculture, globular shiny masses is frequently developed into SEs (Table 2.1). In this study, number of SEs were observed over a period of 5^{th} subculture of callus, because the callus gradually turned brown and become very hard [6] under similar culture condition. It was observed that during 1^{st}, 2^{nd} and 3^{rd} subculture of callus, the germination rate of SEs were significantly increasing, where after 4^{th} and 5^{th} subculture it was significantly decreased and finally no SEs was shown on the inoculated medium(Table-2.2). The all stage of somatic embryogenesis was formed on MS medium with 1.0mg/l BA within 20-25 days.

The shoot buds were stimulated at much number from callus on MS medium with 2.0 mg/l BA within 35-42days (figure-3).The formation of multiple shoots from shoot buds were presented on MS medium with 0.5mg/l BA and 0.1mg/l IAA (25-30 days). The 3-4 roots formed from shoots as well as shoot elongation (≈ 0.8 cm) presented within 30days on MS medium with 1.0mg/l IBA and 0.1mg/l IAA (figure-7).

During subculture of globular shaped SEs, multiple shoots were initiated on MS medium with 2.0mg/l BA and 0.1mg/l IAA. When sub-cultured on MS medium with 0.1mg/l IAA the shoot elongation occurred in length of 2.0-2.5 cm together many shoot stimulation (figure - 8) and roots formed from shoots within 30-35 days. The elongation of shoots is achieved better on 0.1mg/l IAA, in comparison to combination of (0.5 mg/l BA and 0.1mg/l IAA) and (1.0mg/l IBA and 0.1mg/l IAA).

On continuous sub cultured (0.5mg/l BA) SEs formed only a large leaf. The callus obtained from 2, 4-D medium were subcultured two times with 1.0mg/l BA, they showed very low number of SEs but formed only leaves from maximum SEs.
The result of the culture protocol has good potential for mass propagation of neem trees in batch culture for probing the somatic embryo in neem. The present experiments have shown that it is a possible to induce somatic embryo and shoot bud, for complete plantlet development from stem and leaf of the neem.

Table 1- Callusing of Neem explant**.**

Explant	Leaf								Stem							
Concentration of GRs (mg/l)	0.5		1.0		2.0		4.0		0.5		1.0		2.0		4.0	
	Average number of days of															
	SC	MC	SC	MC	SC	MC	SC	MC	SC	MC	SC	MC	SC	MC	SC	MC
BA	08	19*	10	20*	15	28	18	29	11	18*	13	21*	16	25	17	35
IBA	No	-	No	-	26	47#	No	-	No	-	30	50#	28	47#	29	47#
IAA	14	25	16	30	No	-	24	45#	23	41#	16	27	26	48#	13	22
Concentration Of GRs (mg/l) in combination	0.5				1.0				0.5				1.0			
IAA and BA	SC		MC		SC		MC		SC		MC		SC		MC	
	25		60		38		69#		28		58		35		70#	
Concentration of 2, 4-D(mg/l)	0.1			0.2			0.5		0.1			0.2			0.5	
	SC	MC	SC	MC	SC	MC			SC	MC	SC	MC	SC	MC		
	20	28*	23	28	No	-			21	27*	21	29	No	-		

* Best callus # callus at both ends of stem/only side of leaf
SC- Callus initiated MC- maximum callus

Table 2.1- Number of Somatic Embryos (SEs) formed in BA at a single explant**.**

allus		Leaf				Stem			
Concentration of BA (mg/l)		0.5		1.0		0.5		1.0	
No of		SEs	Day	SEs	Day	SEs	Day	SEs	Day
subculture	1st	12	46	14	58	28	48	35	59
	2nd	21	49	20	54	100	48	150	60
	3rd	22	41	23	49	105	35	180	45
	4th	06	32	08	32	20	25	25	28
	5th	No	-	No	-	No	-	No	-

Table 2.2- Rate of somatic embryo formation of rapid subculture of callus

Callus		Leaf		Stem	
Conc. of BA (mg/l)		0.5	1.0	0.5	1.0
Rate (No of SEs/ No of Days) of SEs production after rapid subculture of callus	1st	0.26	0.24	0.58	0.59
	2nd	0.43	0.37	2.08	2.50
	3rd	0.54	0.46	3.00	4.00
	4th	0.18	0.25	0.8	0.89
	5th	0.0	0.0	0.0	0.0

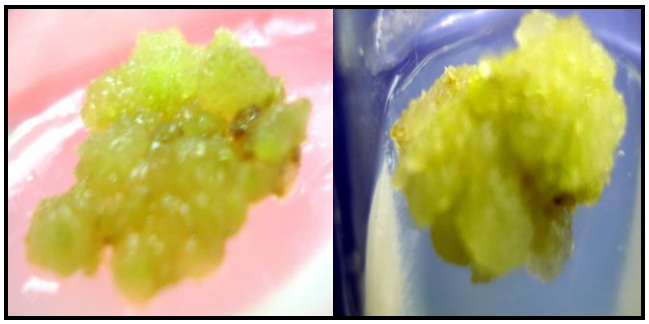

Fig- 1: Leaf & Stem callus at growing stage

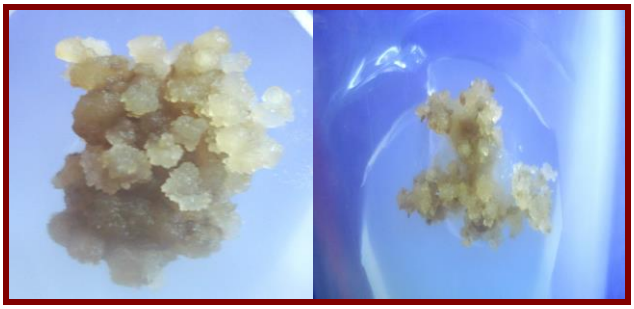

Fig -2: Leaf & stem callus on 2, 4-D.

Fig -3: Direct shoot buds regeneration from callus.

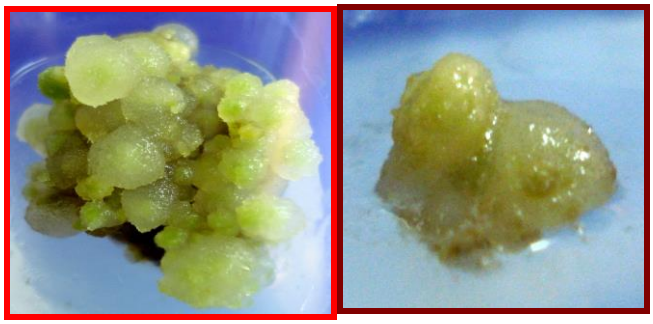

Fig- 4: Globular & Heart shaped somatic embryos (SEs).

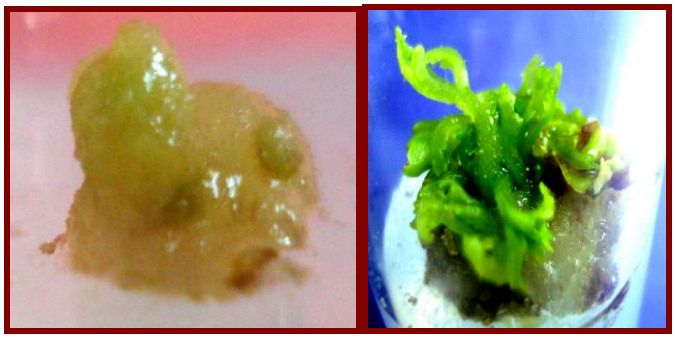

Fig - 5: Torpedo -shaped SE.

Fig - 6: Multiple shoots regeneration from cotyledonary shaped SE.

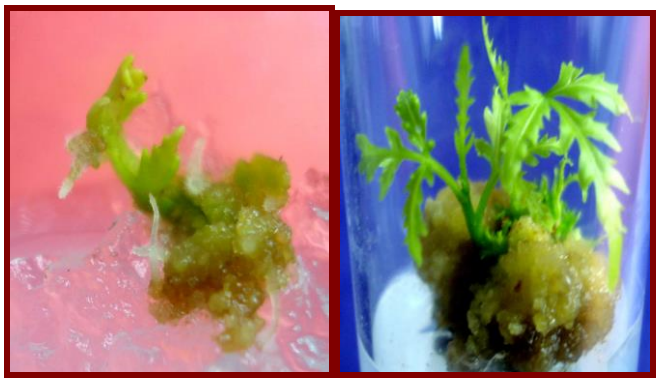

Fig -7: Roots regeneration from plantlet.

Fig - 8: Shoot differentiation from subcultured SE.

CONCLUSION

The present experiments have shown that it is possible to induce multiple shoots differentiation and complete plantlet development from callus and SE. It can be conclude that MS medium supplemented with BA as growth regulator is suitable for germination of SEs of Azadirachta indica and 3rd subculture of callus is optimum for maximal SEs germination.

ACKNOWLEDGEMENT

The authors are very much grateful to Department of Biotechnology, Ministry of Science and Technology Govt. of India for providing financial support to establish the Institutional Biotech hub at North Lakhimpur College (Autonomous), Assam.

REFERENCES

1. Geneva: World Health Organization; 2002. WHO Report. WHO/EDM/TRM/2002; p. 19.
2. Ramarethinam, S., Marimuthu, S and Murugesan, N.v. neem as an effective biocontrol agent for tea pests. 2004.
3. J. P. Eeswara, T. Stuchbury, E. J. Allan, A. J. Mordue (Luntz). A standard procedure for the micropropagation of the neem tree (Azadirachta indica A. Juss), Plant Cell Reports, January 1998, 215–219.
4. Antje Wewetzer. Callus Cultures of Azadirachta indica and Their Potential for the Production of Azadirachtin, Phytoparasitica 26(1), 1998, 47-52.
5. Mithilesh Singh, Rakhi Chaturvedi. An efficient protocol for cyclic somatic embryogenesis in neem (Azadirachta indica A.juss.), International Journal of Biological, Biomolecular, Agricultural, Food and Biotechnological Engineering Vol: 3, No: 3, 2009, 133-135.
6. Wei Wen Su, Wen-Ing Hwang, Se Young Kim, Yoneo Sagawa. Induction of somatic embryogenesis in Azadirachta indica, Plant Cell, Tissue and Organ Culture, August 1997, Volume 50, 91–95.
7. Lingaraj Sahoo.Plant biotechnology lab manual.Department of Biotechnology Indian Institute of Technology Guwahati. 2-35.
8. Murashige T & Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant 15: 473-497.
9. Suresh Khadke and Aniket Kuvalekar. Direct Somatic Embryogenesis and Plant Regeneration from Leaf and Stem Explants of Nothapodytes Foetida: A Critically Endangered Plant Species. International Journal of Plant, Animal and Environmental Sciences. Vol 3. Jan-March 2013.
10. Chakradher Akula, Anisha Akula and Rod Drew. Somatic embryogenesis in clonal neem, Azadirachta indica A. juss. and Analysis for in vitro Azadirachtin production. In vitro cell developmental Biol. –plant 39, 2003: 304-310.