PLANT TISSUE CULTURE AND APPLIED PLANT BIOTECHNOLOGY

Prof. Ashwani Kumar
M.Sc. (Gold Medal), Ph.D., Dipl. (German Language)
Alexander von Humboldt Fellow (Germany)
FBS, FPSI, FISMPP
Fellow Assoc. Biotechnology and Pharmacology
Former Head of the Department of Botany
And Director Life Sciences
University of Rajasthan
Jaipur-302 055, India

Dr. Shikha Roy
Department of Botany
University of Rajasthan
Jaipur - 302 055 India

Aavishkar Publishers, Distributors
Jaipur 302 003 (Raj.) India

First Published in 2011 by
Prem C. Bakliwal for
Aavishkar Publishers, Distributors
807, Vyas Building, Chaura Rasta
Jaipur 302 003 (Raj.) India
Phone : 0141-2578159
e-mail : aavishkarbooks@hotmail.com

ISBN -

PLANT TISSUE CULTURE AND APPLIED PLANT BIOTECHNOLOGY

Printed at
Sheetal Printers
Jaipur (Raj.) India

Contents

Chapter 8

Biotechnological Improvement of Neem

PRIYANKA SRIVASTAVA, MITHILESH SINGH AND RAKHI CHATURVEDI

*[a]*Department of Biotechnology, Indian Institute of Technology- Guwahati, Guwahati - 781039, Assam, India*

SUMMARY

Azadirachta indica A. Juss or Neem (Meliaceae) is a versatile tropical, evergreen tree, which has attained worldwide prominence in recent years due to its therapeutic and insecticidal properties. Its use in folklore has been documented since long where it has been utilized to cure and treat various ailments. The antifeedant and biopesticidal properties of the tree have further enhanced its global value. These diverse biological activities are attributed to several limonoids like Azadirachtin, present in various parts, particularly the seed kernels. However, these assets of neem have been underutilized due to limitations put forth by its long generation cycle and strict out-breeding nature that cause enormous variability in physical traits and chemical profile. Conventional breeding programmes for qualitative and quantitative improvements have been rendered inefficient due to prevalent heterozygosity and perennial nature of the tree. In this regard, plant tissue culture offers a lucrative alternative for quick propagation of plus neem trees. Till date, various explants ranging from nodes, leaves, zygotic embryos, endosperm, nucellus, cotyledons, hypocotyls, protoplasts, anthers and ovaries have been used for the micropropagation of this tree. Also, the in vitro raised cultures from many of the above stated explants have tested positive for presence of important metabolites like Azadirachtin. However, more strategic and intensive efforts are required to channelize laboratory outputs to a commercial scale. This chapter deals with the recent developments in tissue culture of neem and focuses on potential areas where biotechnological intervention may play a key role in improvement of this important tree species.

INTRODUCTION

Mahogany's botanical cousin, Neem or *Azadirachta indica* A. Juss. is an adaptable, tropical, evergreen tree of the family Meliaceae. Referred to as "Wonder Tree" by many, the plant is native to South and Southeast Asia and grows well in tropical and subtropical areas around the world. The history of Neem is inextricably linked to the history of Indian way of life. It is an integral part of Indian ayurveda since ages. This robust looking tree has numerous important medicinal, agrochemical and economic uses to its credit. Almost each and every part of this tree, particularly the leaves, bark and seeds, has multiple uses. Its deep root system is well adapted to retrieving water and nutrients from the soil profile and is very sensitive to water logging. It thrives best in hot, dry climates where shade temperatures often reach 50 degree celsius and annual rainfall ranges from 400 to 1,200 millimeters. Apart from this, neem tree is a renewable source of various useful products. Besides being a popular avenue tree, with a large crown, the wood of neem has been used as timber for house building, furniture and other domestic and agriculture tools. The wood of neem resembles teak wood in its strength, and is more resistant to shock, fungi, and insect attack. It is immune to termites and is durable even outdoors (Thengane *et al.*, 1995).

As far as commercial appeal is concerned, the major demand of neem is for its seed-oil, which contains several bioactive compounds. Azadirachtin, a highly oxidized limonoid (triterpenoid) present prominently in neem seed kernels, is mainly responsible for diverse biological activities. It possesses insect repellent, antifeedant, larvicidal, growth inhibiting properties against a wide range of pests and, thus, has been well recognized as an environment friendly, biodegradable biopesticide. In neem, the reproductive phase normally begins after five years and the tree yields an average of 20 kg of fruits per year with maximum production reaching around 50 kg per year in a fully grown adult tree (>10 year old) (National Research Council, 1992). Of the fruit yield, only about 10% is attributed to seed kernels, and desired biological active compounds comprise only 10 grams per kilograms of kernel weight. Thus, an adult neem tree produces only about 20 grams of pesticidal compounds in a season (Schmutterer, 1990). Besides availability, the quality of seeds in terms of azadirachtin content and absence of aflatoxin is a major concern for the neem based industry. Fruits, in neem, are available from June-August which coincides with the rainy season in India. Thus, only about one-third of the fruits are collected due to operational problems and quality considerations (Jayaraj, 1993; Vyas and Mistry, 1996; Venkateswarlu and Mukhopadhyay, 1999). Keeping quality of the seeds is also poor due to high moisture (30-35%) and oil (40-45%) content.

Neem trees show wide genetic variation in terms of tree size, morphology, fruit size, and fruit production within the natural population (Ermel *et al.*, 1984, 1987; Benge, 1989; Schmutterer, 1990; Ketker and Ketker, 1993; Kumaran *et al.*, 1993; Srivasuki *et al.*, 1993; Ermel, 1995; Wewetzer, 1998). Considerable variability in the azadirachtin content of their seeds, irrespective of the habitat, is also observed (Ermel, 1995; Sidhu *et al.*, 2003). Improvement in azadirachtin production can be achieved by clonal propagation of elite trees. Vegetative propagation of neem by the conventional methods is possible but difficult (Dogra and Thapliyal, 1996). Therefore, it is normally grown from seeds, which yield a

TABLE 1

Summarized data on regeneration in tissue cultures of Azadirachta indica

S. No.	Mode of Propagation	Explant used	Adult/ Juvenile	Observations	References
1	**Axillary Shoot**	Nodal	J	Axillary shoots→ Plantlets	Drew, 1993; Quraishi *et al.* 2004
	Proliferation	segments	A	Axillary shoots→ Plantlets	Joarder *et al.*, 1993; Gill *et al.*, 1996; Joshi & Thengane, 1996; Islam *et al.*, 1997; Sarker *et al.*, 1997; Venkateswarlu & Mukhopadhyaya, 1999; Sharma *et al.*, 1999; Chaturvedi *et al.*, 2004a
		Apical & axillary shoot bud	A	Axillary shoots→ Plantlets	Roy *et al.*, 1996
		Nodal segments from crown branches	A	Axillary shoots→ did not survive	Quraishi *et al.*, 2004
		Nodal segments from basal sprouts	A	Axillary shoots→ Plantlets	Quraishi *et al.*, 2004
2.	**Adventitious**	Leaf	J	Callus→ Shoots→ Plantlets	Sarker *et al.*, 1997
	Shoot			Adv shoots→ Plantlets	Eeswara *et al.*, 1998
	Proliferation			Adv shoots→ Plantlets	Salvi *et al.*, 2001
		Leaf discs	A	Callus→ Shoots→ Plantlets	Narayan & Jaiswal, 1985; Ramesh & Padhya, l990
				Adv shoots→ Plantlets	Singh & Chaturvedi, 2009

Contd...

...Contd.

S. No.	Mode of Propagation	Explant used	Adult/ Juvenile	Observations	References
		Leaf tip	?	Callus→ Shoots	Abubacker & Alagumanian, 1999
		Zygotic embryo	J	Calluses→ Shoots	Rangaswamy & Promila, 1972
			A	Shoots→ Plantlets	Chaturvedi *et al.*, 2004b
		Cotyledonary node	J	Adv shoots→ Plantlets	Salvi *et al.*, 2001
		Cotyledons	J	Adv shoots→ Plantlets	Salvi *et al.*, 2001
				Shoots→ Plantlets	Nirmalakumari *et al.*, 1993
				Callus→ Shoots	Abubacker and Alagumanian, 1999
		Roots	J	Adv shoots→ Plantlets	Salvi *et al.*, 2001
3.	**Somatic Embryogenesis**	Cotyledons	J	Nodules→ Plantlets Calli→ Embryos→ Shoots	Muralidharan & Mascarenhas, 1989 Islam *et al.*, 1993; Su *et al.*, 1997
				Calli→ Embryos→ Plantlets	Shrikhande *et al.*, 1993
					Murthy & Saxena, 1998
				Embryos→ Plantlets	Murthy & Saxena, 1998
					Gairi & Rashid, 2004
		Hypocotyl	J	Calli→ Embryos→ Shoots	Su *et al.*, 1997
				Embryos→ Plantlets	Gairi & Rashid, 2004
		Epicotyl	J	Embryos→ Plantlets	Gairi & Rashid, 2004
		Cotyledonary node	J	Embryos→ Plantlets	Gairi & Rashid, 2004
		Leaf	J	Calli→ Embryos→ Plantlets	Akula *et al.*, 2003
				Embryos→ Plantlets	Gairi & Rashid, 2004

Contd...

...Contd.

S. No.	Mode of Propagation	Explant used	Adult/ Juvenile	Observations	References
		Nodes	J	Calli→ Embryos→ Plantlets	Drew, 1993; Akula *et al.*, 2003
				Embryos→ Plantlets	Akula *et al.*, 2003
		Roots	J	Calli→ Embryos→ Pantlets	Akula *et al.*, 2003
				Embryos→ Plantlets	Akula *et al.*, 2003
		Immature zygotic	A	Calli→ Embryos	Chaturvedi *et al.*, 2004b
		embryos		Calli→ Neotnorphs→	Chaturvedi *et al.*, 2004b
				Shoot→ Plantlets	
				Calli→ Embryos→ Plantlets	Rout, 2005
4.	**Haploid production**	Anthers (uninucleate microspore)	A	Calli→ Plantlets (Diploid)	Gautam *et al.*, 1993
		Anthers (early-late uninucleate microspore)		Calli→ Shoots→ Plantlets (Haploid)	Chaturvedi *et al.*, 2003b
		Ovaries	A	Calli→ Shoots→ Plantlets (Diploid)	Srivastava *et al.*, 2009
5.	**Triploid production**	Immature endosperm	A	Callus→ Shoots→ Plantlets (Triploid)	Chaturvedi *et al.*, 2003a
6.	**Protoplast culture**	Protoplast	A	Cell Division and multiplication	Chaturvedi, 2003

A= Explants from Adult Plants; J= Explants from Juvenile Plants; ?= **Not mentioned, Adv=Adventitious**

heterogeneous population due to strict cross-pollinating nature of the plant. Moreover, the seeds loose viability within two weeks (Mohan Ram and Nair, 1996). Plant cell and tissue culture would not only surmount these limitations but also hasten the production of clonal material for field planting. It has been suggested as an alternative means for year-round production of azadirachtin and other neem metabolites with the added potential of increasing yield by culture selection and manipulation, irrespective of the season (Allan *et al.,* 1999).

Of late, numerous papers were published on *in vitro* plant regeneration from various somatic tissues of neem (Table 1). A large proportion of them deal with embryonal or seedling explants (Muralidharan and Mascarenhas, 1989; Nirmalakumari *et al.*, 1993; Shrikhande *et al.*, 1993; Sarker *et al.*, 1997; Chaturvedi *et al.*, 2004a; Rout, 2005; Singh *et al.*, 2009 a,b). However, a few papers have reported shoot regeneration from endosperm tissues (Chaturvedi *et al.*, 2003a) and anther / microspores (Chaturvedi *et al.*, 2003b) of adult tree origin.

To channelize the output obtained on laboratory scale, more efforts are required in right direction to make the results more viable and of use to general public. This chapter deals with the recent developments in tissue culture of neem and focuses on potential areas where biotechnological intervention may play a key role in improvement of this important tree.

IN VITRO STUDIES IN NEEM

Axillary Shoot Proliferation

Shoot proliferation from axillary buds is desirable where generation of true-to-type or clonal plants is of interest, like in case of propagation of plus trees of neem. The best explant for this purpose is the nodal segments. Micropropagation by induction of shoots from pre-existing meristems is a most popular approach to clonal propagation of plants because the cells of the shoots-apex are uniformly diploid and least susceptible to genotypic changes under culture conditions. Thus, it guarantees that the characteristics of the source plant are conserved (Rao and Venkateswara, 1985). It offers many advantages over the conventional methods of vegetative propagation: (1) The rate of multiplication is extremely rapid and can continue round the year, independent of the season. Thus, over a million plants can be produced in a year starting from a small piece of tissue. (2) The enhanced rate of multiplication can considerably reduce the period between the selections of plus trees and raising enough planting material for field trials.

Consequently, several investigators have attempted clonal propagation of neem tree. Drew (1993) cultured apical and nodal segments from 6-12 months old seedlings of neem. The axillary shoots, developed *in vitro*, were cut into one and two node segments for further multiplication. Two node cuttings gave better response than single node cuttings. On the best medium, MS (Murashige and Skoog, 1962) with 0.1 µM 6- benzylaminopurine (BAP), 100% cultures produced two shoots (probably one from each node) with a mean height of 10.8 mm. Recurrent multiplication of shoots was not attempted. Gill *et al.* (1996) cultured 0.5-1 cm long nodal segments and found that 4.4 µM BAP alone induced shoot proliferation in 72% cultures (5-7 shoots per culture), which was slightly improved (80%) by further addition of 2.5 µM indole-3-butyric acid (IBA) to the medium. The auxin did

not affect the number of shoots per explant. Roy *et al.* (1996) also found a combination of an auxin (α-naphthalene acetic acid; NAA) and a cytokinin (BAP) to be better than BAP alone for shoot proliferation in the cultures of nodal segments from adult trees. However, the shoots produced on a medium containing BAP and NAA were very small and unsuitable for rooting. Good multiplication and growth of shoots (6 cm long) occurred with the addition of 10% Coconut Milk (CM) and 0.15% Casein Hydrolysate (CH) to MS + 4.4 μM BAP and 0.5 μM NAA. Islam *et al.* (1997) reported synergism between BAP and kinetin (Kn) for shoot proliferation. Sarker *et al.* (1997) reported nodal explants to be the best for micropropagation of neem among various explants like hypocotyl, leaf and shoot tip. He observed that the nodal explants responded best on MS + 8.8 μM BAP + 1.0 μM NAA medium. Clonal multiplication of elite neem trees via axillary shoot proliferation has also been reported by Venkateswarlu and Mukhopadhyaya (1999). The micropropagated plants flowered after 25 months of transplantation and appeared true-to-type in terms of morphology, growth habit and azadirachtin content.

In most of the above studies, either the juvenile material or material of unspecified age was used, which is of little significance in clonal propagation of elite trees. The few other reports lack crucial information such as the rate of shoot multiplication in recurrent subcultures. Initiation and *in vitro* propagation of mature trees in general is difficult due to various problems, mainly recalcitrance of the tissue, contamination and field establishment. Joarder *et al.* (1993) found that the pre-culture of nodal segments, taken from 30-year-old neem tree, on MS basal medium for 2 weeks followed by 4-weeks on MS + 6.6 μM BAP medium was essential / beneficial for bud-break to occur. Although the paper lacks details regarding the rate of shoot multiplication in subsequent subcultures, initially two shoots developed per node and the number of shoots increased with the increasing number of subcultures and then declined after five to six subcultures. Joshi and Thengane (1996) cultured nodal segments from 2-5-year-old juvenile trees as those from 15-20-year-old mature trees showed negligible bud-break. Sharma *et al.* (1999) cultured nodal segments from 3-, 7- and 40-year-old trees and found the explants from younger trees to be more responsive. Shoot multiplication at a rate of five-fold in 30 days was achieved, generally, after the fifth passage on MS medium supplemented with 1.1 μM BAP and 0.29 μM IAA and 81 μM adenine sulphate. However, in the explants taken from 3 and 7-year-old trees, this rate of shoot multiplication was achieved even by the third subculture. Islam *et al.* (1997) recorded 4.5 shoots per explant upto fourth subculture from nodal segments of 25-year-old tree on MS medium supplemented with 4.4 μM BAP and 4.7 μM Kn. Quraishi *et al.* (2004) accomplished the micropropagation of neem by culture of nodal segments from crown branches of a mature tree, basal-sprouts of another mature tree and a single juvenile plant. A significant variation was observed in *in vitro* response of explants from these three sources. In case of crown and basal-sprouts explants, addition of 12.5 μM polyvinylpyrrolidone (PVP-40) in the establishment medium was required to control the leaching of phenols into the medium; juvenile explants did not show phenolic leaching. DKW medium (Driver and Kuniyuki, 1984) with 0.22 μM BAP was found to be significantly better than MS medium for shoot proliferation. Shoot cultures of crown branch origin did not survive and eventually died after the third subculture. In the presence of 4.9 μM IBA in half strength DKW, 90% of the shoots from basal sprout and 100 % of the shoots of juvenile origin formed roots, and plantlets survived transplantation.

Chaturvedi *et al.* (2004a) reported a recurrent method of clonal propagation of a 50-yearold neem tree through axillary shoot proliferation. In this case, multiple shoots were formed on ½ MS medium (major inorganic salts reduced to half strength) supplemented with 1 µM BAP and 0.5 µM gibberellic acid (GA3) (Fig. 1A). The number of shoots enhanced further when cultures were transferred to ½ MS + 1 µM BAP + 500 mg l-1 CH (Fig. 1B). However, both the media did not support shoot growth and the shoots remained stunted. Therefore, further elongation and multiplication of shoots were achieved on full MS + 1 µM BAP + 250 mg l-1 CH through single node segment cultures, at a rate of 7-8-fold every 5 weeks, on fresh medium of the same composition (Fig. 1C). This rate of shoot multiplication was maintained for almost 5 years. The shoots were readily rooted on ¼ MS (major inorganic salts reduced to quarter strength) supplemented with 0.5 µM IBA, with a frequency as high as 82% (Fig. 1D). Finally, the micropropagated plants were established in soil with more than 87% survival rate (Fig. 1E).

Adventitious Shoot Proliferation

Adventitious shoot proliferation in cell and tissue cultures, in response to hormonal manipulation of the culture medium, require *de novo* differentiation of meristematic region, randomly, all over the tissue other than the pre-existing meristem. It is a multistep process and a series of intracellular events, collectively called induction, occur before the appearance of morphologically recognizable organs. Micropropagation via adventitious shoot regeneration may occur directly or indirectly via an intervening callus phase. Indirect regeneration often results in somaclonal variations, making this strategy less desirable for large-scale clonal multiplication (Marcotrigiano and Jagannathan, 1988; Thorpe *et al.*, 1991). Therefore, regeneration of shoots directly from the explants is regarded as the most reliable method for clonal propagation.

Shoot regeneration from leaf explants derived from adult Neem trees was first reported by Narayan and Jaiswal (1985). Leaf discs formed unorganised callus on a medium containing 2,4-D and BAP. Transfer of callus to a medium containing 0.44 µM BAP induced shoot-bud differentiation from them. On this medium, 5-8 shoots appeared after 6-7 weeks. Addition of 0.3 µM NAA in conjunction with BAP enhanced the frequency of shoot differentiation from 54% to 62.5%. Ramesh and Padhya (1990) cultured leaf discs from an "elite" tree. The explants formed green nodular callus on Wood and Braun's medium (Wood and Braun, 1961) supplemented with 4 µM each of Kn and BAP. After weeks on the same medium, the callus differentiated 10-12 shoot-buds adventitiously. Further, addition of 16 µM adenine sulphate to the medium raised the number of buds per culture to 18-20. Healthy shoots were obtained when the shoot-buds were transferred to GA_3 containing medium. Origin of the leaf discs affected the regeneration frequency. Discs from middle of the leaf were more regenerative than those from the basal and apical portions. Eeswara *et al.* (1998) tested the regeneration potential of leaf explants from 18-month-old seedlings raised from seeds obtained from Niger, Ghana and Srilanka. Direct differentiation of adventitious shoot-buds occurred on MS + 4.4 µM BAP + 3.7 µM Kn + 32.6 µM adenine sulphate. These authors have highlighted the importance of dark incubation for shoot-bud differentiation. Cultures incubated in light, right from the beginning, did not form any shoot-bud. Two weeks of dark incubation was necessary for shoot-bud induction in most of the clones tested. For recurrent multiplication of shoots,

it was essential to reduce the concentration of cytokinin ten times the original concentration. These authors also observed synergism between BAP and Kn for growth and multiplication of shoots. Individually, BAP or Kn in the medium caused increased abnormalities and vitrification of shoots. The medium recommended by the authors for recurrent shoot multiplication is MS + 0.44 µM BAP + 0.4 µM Kn + 3.3 µM adenine sulphate. Singh and Chaturvedi (2009c) reported organogenesis from leaf explants and found the presence of at least one auxin or one cytokinin to be obligatory for the induction of organogenic calli from explants. Whereas BAP was found to be most favorable for shoot organogenesis in these calli (Fig. 2A), root organogenesis could be achieved on NAA supplemented medium (Fig. 2B). Thus, the kind of growth regulator had a significant effect on shoot/root organogenesis in these differentiating calli. On MS + 5 µM BAP, compact calli developed at the cut end of the explants, which after 5 weeks turned into nodulated calli in more than 76% cultures. Subsequently, an average of 7 green shoots per explant were developed from these nodules, after 10 weeks. Shoots were elongated at a lower concentration of BAP (0.5 µM) and multiplied by forced axillary branching on MS + 1 µM BAP + CH 250 mgl^{-1}. For rooting, ¼ strength MS medium supplemented with 0.5 µM IBA was tested where 79% of the shoots developed roots directly at the base, within 4 weeks. Salvi *et al.* (2001) observed shoot regeneration from various seedling explants (cotyledon, cotyledonary node, epicotyl, hypocotyl, leaf, root-shoot zone, root) on MS + 8.9 µM BAP + 0.6 µM IAA. However, leaf explants produced the highest numbers of shoot buds per explant and the roots the least.

Abubacker and Alagumanian (1999) found cotyledons to be the best juvenile explant for regeneration. All cultures of cotyledon explants developed green, compact and nodular callus on MS + 6.8 µM 2,4-D + 2.9 µM IAA + 6.6 µM BAP and on the same medium shoots were differentiated from the callus. Chaturvedi *et al.* (2004b) obtained plantlets from immature zygotic embryo cultures via neomorph formation and adventitious shoot bud formation. Maximum direct shoot bud differentiation (57%) occurred from early dicotyledonous stage of embryo on MS + 5 µM BAP whereas maximum neomorph formation (66%) occurred in cultures of torpedo shape embryos on MS + 5 µM 2,4-D. It was possible to regenerate full plants from the neomorphs via organogenesis on MS + 5 µM BAP.

The limitation associated with immature zygotic embryo culture or other juvenile tissues of seedling origin is that the plants regenerated by this approach are derived from unselected bulked seeds, representing different genotypes. At this stage it is difficult to predict which of these seeds will eventually give rise to an elite tree. In contrast to this, micropropagation by *de novo* differentiation of shoot buds from excised leaves is more suitable for large scale propagation of selected elite mature trees. Again, the initial explant will not be a limiting factor because the leaves are available in abundant numbers throughout the year as compared to zygotic embryos, hypocotyl and cotyledons in case of short viable seeds.

Somatic Embryogenesis

In tissue cultures, plant regeneration may occur via organogenesis or somatic embryogenesis. The latter offers many advantages over the former. Single cell origin of embryos favor the use of this process for more efficient plant transformation. Embryo is

a bipolar structure (rather than monopolar) and requires single hormonal signal to develop complete plantlet, while organogenesis requires two different hormonal signals to induce first a shoot organ and then a root organ. Thus, somatic embryogenesis favors the possibility to automate large scale production of embryos in bioreactors and their field planting as synthetic seeds. It can also be used for the production of metabolites in species where embryos are the reservoir of important biochemical compounds. This prompted many scientists to achieve regeneration in Neem via somatic embryogenesis and the most frequently used explants are the juvenile material such as excised cotyledons, hypocotyls or immature zygotic embryos (Table 1). Although, majority of the publications have described regeneration via somatic embryogenesis, in many cases the structures described as embryos do not appear and/or behave like embryos. Muralidharan and Mascarenhas (1989) were probably the first to describe embryo-like structures in the cultures of cotyledon segments of Neem. On B_5 medium containing 4.4 µM BAP, the explants formed nodular structures, which appeared bipolar. Upon transfer to hormone-free B_5 medium, the nodular structures separated from the parent explant and developed leafy structures and a tap root. The nodular structures never showed bipolar germination.

Shrikhande *et al.* (1993) reported *in vitro* plant regeneration via somatic embryogenesis in the cultures of immature cotyledons on MS (with 5% sucrose) + 4.4 µM BAP + 2.9 µM IAA + 1000 mgl-l CH. Although these authors have furnished histological data in support of embryogenesis, the structures labelled as somatic embryo appear more like shoots, with their vascular traces running into the callus. None of the structures resembles a bipolar embryo with closed radicular and plumular poles. Moreover, on germination medium the so called "embryos" developed only a shoot, which had to be transferred to hormone-free ½ MS medium for rooting. Most surprisingly, the authors have described mature embryos with suspensor. Mature embryos generally lack a suspensor and at no stage can the suspensor be seen in macrophotographs as depicted in this paper. Su *et al.* (1997) were unable to confirm the results of Shrikhande *et al.* (1993). Under the culture conditions Shrikhande *et al.* (1993) got somatic embryos, Su *et al.* observed only shoot bud formation. The calli initiated on MS + 4.4 µM BAP + 2.7 µM NAA + 1000 mgl^{-1} CH, differentiated greenish, globular detachable structures when transferred to MS + BAP + IAA + CH, in suspension cultures. In further subcultures on the same medium, the globular structures differentiated shoots and roots. However, if transferred to MS (5% sucrose) supplemented with only CH (1000 mgl^{-1}) the globular structures produced embryos on the spherical head. Maximum embryo differentiation (73%) occurred on MS (10 % sucrose) + Zeatin (0.2 mgl^{-1}). Germination of embryos occurred on ½ MS (1% Sucrose).

Islam *et al.* (1993) reported very high incidence of embryogenesis in the cultures of cotyledons, on MS + 8 µM NAA. However, the germination frequency of the embryos on MS + 0.9 µM BAP + 0.03 µM GA_3, was very poor (maximum 11.7%) and germination was monopolar. The embryos formed only shoots. Murthy and Saxena (1998) observed somatic embryo formation in the cultures of mature seeds directly as well as via callusing. The cotyledonary callus differentiated nodular structures in suspension cultures on MS medium with or without TDZ (1 µM). When planted on hormone-free, semi-solid medium, these structures differentiated embryos, which germinated (60%-70%) on the same medium.

Akula *et al.* (2003) reported induction of somatic embryogenesis in four out of seven

selected clones of neem. Direct induction of somatic embryogenesis was achieved from both nodal and root explants within eight weeks of culture on MS medium without growth regulators. When these embryos were left on the induction medium, approximately 15% of the somatic embryos developed into whole plantlets after passing through a series of developmental stages. Indirect somatic embryogenesis was obtained from leaf explants grown on MS medium supplemented with a combination of 2.3-4.4 µM Thiodiazuron (TDZ) and 0.5 µM 2,4-D. Gairi and Rashid (2004) reported direct differentiation of somatic embryos on different regions of hypocotyl, epicotyl, cotyledonary-node, cotyledons and leaves of intact seedlings of *Azadirachta*. Individual embryos on transfer to hormone-free medium regenerated readily into plantlets. The developmental stage of embryo at the time of culture is an important determinant of their morphogenic response. In many tree species, including *Juglans* (Tulecke and McGranahan, 1985), *Liriodendron tuipifera* (Sotak *et al.*, 1991) and *Prunus avium* (DeMarch *et al.*, 1993), somatic embryogenesis was strongly affected by the developmental stage of the zygotic embryo. To identify the most responsive stage of embryos for morphogenesis, Chaturvedi *et al.* (2004b) cultured immature embryos at different stages of development- globular, heart-shape, torpedo-shape and early dicotyledonous stage. (Early dicotyledonous embryos were 2.5 times smaller than fully developed dicotyledonous embryos (Fig. 3A) on MS medium supplemented with a range of growth regulators. Morphogenic responses varied considerably with the stage of embryo at culture. Globular embryos generally turned brown without showing any morphogenic response. Older embryos germinated, formed calli or differentiated three types of organized structures: shoots, somatic embryos and neomorphs (abnormal structures with varied morphology). The same explant often differentiated more than one kind of regenerants. Maximum somatic embryogenesis and shoot-bud differentiation occurred directly from the explant on MS + 5 µM BAP medium, and the most responsive embryo stage was early dicotyledonous, followed by torpedo shape. The former showed differentiation of shoots and somatic embryos at higher frequency (57%) (Fig. 3B). In the combined presence of 5 µM BAP and 1 µM 2,4-D in MS medium, early dicotyledonous embryos showed a fairly high degree of somatic embryogenesis and neomorph formation (50%) but these structures were differentiated from the callused explants (Fig. 3C). However, regeneration involving a callus phase runs the risk of introducing variability due to genetic instability of the callus cells. Therefore, only directly differentiated regenerants were utilized by them for micropropagation.

Irrespective of the treatment and the stage of zygotic embryo, somatic embryos exhibited considerable morphological abnormalities, such as pluricotyledony (Fig. 4A i), fusion of cotyledons (Fig. 4A ii) and absence of cotyledons (Fig. 4A iii). The occurrence of normal dicotyledonous embryos was extremely rare. On MS basal medium, 3% of the embryos developed a long tap root in 4 weeks (Fig. 4B) but plumular shoots did not appear. Although somatic embryos never germinated to form complete plantlets on any of the treatments tested, secondary embryogenesis occurred on MS basal medium supplemented with BAP + IAA, or GA_3 + IAA. On MS + 1 µM BAP + 0.5 µM IAA, 100% somatic embryos exhibited secondary embryogenesis with a mean number of 14 embryos per parent embryo. Secondary embryogenesis was preceded by callusing of the primary somatic embryos (Fig. 4C). However, on 5.0 µM GA_3 + 2.5 µM IAA, secondary embryos differentiated directly from the primary somatic embryos (Fig. 4D, E). Like primary somatic embryos, secondary

somatic embryos exhibited morphological abnormalities, and failed to germinate on any of the treatments tested.

Rout (2005) reported somatic embryogenesis from immature zygotic embryos that were cultured 40 days after anthesis, on MS medium supplemented with 1.11 µM BAP and 4.52-6.78 µM 2,4-D. Best embyogenic callus proliferated in the medium fortified with 1.11 µM BAP and 4.52 µM 2,4-D. Embyogenic callus when transferred on same medium containing reduced auxin (0.45 µM 2,4-D), numerous embryos proliferated from the surface of callus. Maturation and germination of the somatic embryos occurred on half strength MS salts and vitamins supplemented with ABA and 2% sucrose; the maximum percentage (64.2%) of germination was observed with 0.94 µM ABA within 2 weeks of culture.

Haploid Production

Anther culture

The most effective and popular technique to obtain haploids is by *in vitro* anther or microspore culture. The perspective of raising haploid plants through *in vitro* androgenesis offers scores of foreseeable advantages like, shortening of breeding period, production of homozygous diploid lines in a single step through chromosome doubling and isolation of valuable recessive traits at sporophytic level which otherwise, remain accumulated and unexpressed in natural heterozygous diploid population. The prevalent heterozygosity and absence of pure lines in woody plants make selection and genetic studies rather difficult to conduct. Conventional breeding programmes are both unpredictable and time consuming where several generations of selfing is required to obtain homozygous pure lines, which is difficult to realize in woody plants due to long generation cycle.

Anther culture has been successfully applied to many plant species to produce haploids; its single biggest advantage is its simplicity. Isolated microspore culture in contrast to anther culture, has several important advantages (Bonga *et al.*, 1997; Radojevic *et al.*, 2002). By removing the anther wall, proliferation of diploid sporophytic tissues are avoided. More importantly, homogenous population of microspores at the developmental stage, most suitable for androgenesis can be obtained. In addition, as the development of microspores is independent of the sporophytic tissues, the media components and culture treatments have direct access to the microspores. Thus, under optimal conditions, more microspores can be induced to convert into embryos. In spite of these positive attributes, microspore culture cannot yet compete with anther culture for double haploid production, especially in tree species. Pelletier and Ilami (1972) introduced the concept of "Wall Factor", according to which the somatic tissues of the anther play an important role in the induction of sporophytic divisions in pollen.

Androgenesis is highly desirable in Neem trees to overcome the prevalent self incompatibility, heterozygosity and long reproductive cycle. In spite of this, a limited effort has been made for the improvement of this valuable tree through *in vitro* haploid production. Gautam *et al.*, (1993) cultured anthers at the uninucleate stage of microspores and observed some multicellular pollen on a medium containing NB (Nitsch, 1969) with 10 µM BAP or MS + 10 µM BAP + 10 µM NAA / IAA. However, all the plants regenerated from anther callus were diploid. Chaturvedi *et al.*, (2003b) for the first time achieved androgenic haploids of neem by anther culture at early-to-late uninucleate stage of pollen

(Fig. 5A). It has been postulated that androgenic induction is only possible with immature anthers containing immature pollen at early-to-late uninucleate stage of development. In fact, the stage of microspore development at which the anthers are cultured is more crucial than the composition of the nutrient medium. The competence of a microspore to enter embryogenic development is governed by its ability to mount a stress response. Later stages in pollen development are unable to mount such a response (Cordewener *et al.*, 1995), thereafter, becoming incompetent for embryogenic induction.

Chaturvedi *et al.* (2003b) obtained haploid formation in Neem via callusing. The best medium for inducing callusing in the anther cultures was MS basal medium (9% sucrose) supplemented with 1 µM 2,4-D, 1 µM NAA and 5 µM BAP (Fig. 5B). Histological sections revealed that in 4-week-old cultures the anther-wall cells had started dividing while the microspores appeared to be unchanged (Fig. 5C). However, in 8-week-old cultures the anther locules were filled with the callus (Fig. 5D). The anther callus multiplied best on MS medium supplemented with 1 µM 2,4-D and 10 µM Kn (Fig. 5E). These calli differentiated shoots when transferred to a medium containing BAP (Fig. 5F,G); 5 µM BAP was optimum for young calli (75% cultures differentiated shoots), but older calli showed the best regeneration with 7.5 µM BAP. The composition of the callus maintenance medium had a definite effect on the regeneration potential of calli. Calli maintained on medium containing 2,4-D exhibited good regeneration initially, but calli multiplied on a medium containing 1 µM 2,4-D and 10 µM Kn retained the regeneration potential for a longer period. Shoots elongated at a lower concentration of BAP at 0.5 µM (Fig. 5H). These shoots were multiplied by forced axillary branching and rooted *in vitro* (Fig. 5I). The plants were subsequently established in soil. Of the plants that regenerated from anther callus 60% were haploid (2n=x=12) (Fig. 5J), 20% were diploid (2n=2x=24) and 20% were aneuploid (2n=2x-2=22).

Ovary culture

Gynogenic development of plants from unfertilized cells of female gametophyte (embryo sac) in ovary / ovule cultures is an alternative to anther / pollen culture for haploid production. Gynogenesis was first reported in barley by San Noeum (1976). This method of haploid production is more tedious than androgenesis. Whereas there are indefinite numbers of microspores (male gametes) within the anther wall for androgenic haploid production, there is single egg cell (female gamete) per flower for gynogenic haploid production. The female gamete is deep seated within the embryo sac (female gametophyte), thus making the entire process very cumbersome. However, the technique is useful where anther culture has been unsuccessful, plants are male sterile or androgenesis is confronted with the problem of albino or non-haploid formation. To date, there is single report on *in vitro* ovary culture of Neem by Srivastava *et al.* (2009). Unfertilized ovaries, obtained from closed flower buds of an adult 54-year-old neem tree were used as explants. Ovaries were excised from flower buds of four sizes (2, 3, 4 and 5 mm) and the corresponding developmental stage of ovary was determined by paraffin sections (Fig. 6A-D). The best medium for inducing calli from unfertilized ovaries was MS medium with 9% sucrose, 1 µM 2,4-D and 5 µM BAP; calli were further maintained and multiplied on MS medium supplemented with 0.5 µM 2,4-D either alone or in combination with 4.5 µM Kinetin. The callus maintenance medium, concentration of BAP in the regeneration medium and stage

of ovary at culture were found to be critical factors for shoot differentiation from callus. Maximum shoot regeneration (78%) was observed when calli, induced from ovaries of 4 mm size flower buds and proliferating on MS + 0.5 µM 2,4-D, were subcultured to MS containing 5 µM BAP. Histological analysis revealed that 4 mm sized flower buds correspond to a 2-nucleate stage of embryo sac. The shoots were multiplied by forced axillary branching on MS medium supplemented with 1.0 µM BAP and 250 mgl^{-1} CH. Later, the shoots were rooted on ¼ strength MS medium supplemented with 0.5 µM IBA at a frequency of 79%. The plants were subsequently hardened with transplantation rate of 81.8%. The cytological analysis by root-tip squash preparation revealed that all the plantlets were diploid.

Plant regeneration via ovary culture has also been reported in several plants such as lily (Van Tuyl *et al.*, 1991), sweet potato (Ruth *et al.*, 1993), onion (Bohanec *et al.*, 1995; Luthar and Bohanec, 1999), sugar beet (Gurel *et al.*, 2000), maize (Tang *et al.*, 2006), coconut (Perera *et al.*, 2007) and *Psoralea corylifolia* (Chand and Sahrawat, 2007). Although the main objective of the above studies, including the Neem was to obtain haploid plants, regeneration of diploid plants revealed the potential use of ovary explants for adventitious shoot proliferation. Occurrence of uniformly diploid plants indicated the feasibility of utilizing this juvenile unfertilized ovary explants for large scale micropropagation of neem. In contrast, the leaves, nodal and intermodal segments from mature trees show recalcitrance due to accumulation of secondary metabolites.

Triploid Production

Endosperm is a unique tissue in its origin, development and ploidy level. It is a product of double fertilization but unlike the embryo it is triploid and develops into a formless tissue (Bhojwani and Bhatnagar, 1999). It is, therefore, an interesting tissue for morphogenesis. The earliest attempt to grow endosperm tissue in cultures was made by Lampe and Mills 1933 (cited in LaRue, 1936). However, the first tissue cultures of immature maize endosperm were raised in 1949 by LaRue. Since then, immature and mature endosperm of several species has been shown to form continuously growing calli (Bhojwani and Razdan, 1996). Totipotency of endosperm cells was first demonstrated by Johri and Bhojwani (1965). To date, differentiation of shoots/ embryos/ plantlets from endosperm tissue has been reported for at least 45 species belonging to 21 families (Thomas and Chaturvedi, 2000). In many of these reports the regenerants were shown to be triploid. A key factor in the induction of cell divisions in mature endosperm cultures is the association of the embryo. The embryo factor is required only to trigger cell divisions; further growth occurs independent of the embryo. Production of triploids from endosperm cultures of neem was reported for the first time by Chaturvedi *et al.* (2003a).

Triploid plants are usually seed-sterile. However, there are many examples where seedlessness caused by triploidy is of no serious concern or, at times, even advantageous. Some of the crops where triploids are already in commercial use include several varieties of apple, banana, mulberry, sugar beet and watermelon (Elliott, 1958). Natural triploids of tomato produced larger and tastier fruits than their diploid counterparts (Kagan-Zur *et al.*, 1990). In neem, azadirachtin is obtained from the kernels and, therefore, triploids may not be good for the elites selected for this tetratriterpenoid. However, the elites of neem selected for other secondary metabolites such as nimbin, nimbinin, nimbidin,

nimbindiol and tannins, produced in the bark, and quercetin, nimbosterol and gedunin obtained from leaves (Thengane *et al.*, 1995), triploidy may offer advantages. Traditionally, triploids are produced by crossing induced superior tetraploids and diploids. This approach is not only tedious and lengthy (especially for tree species) but in many cases it may not be possible due to high sterility of autotetraploids (Esen and Soost, 1973; Gupta, 1982). In contrast, regeneration of plants from endosperm, a naturally occurring triploid tissue, offers a direct, single step approach to triploid production. This is particularly useful for tree species like neem. The selected triploids, expected to be sexually sterile, can be bulked up by micropropagation.

There has been a single published report on *in vitro* production of triploid plants from endosperm tissues of Neem by Chaturvedi *et al.* (2003a). Neem bears non-endospermous seeds. Therefore, only immature endosperm could be cultured. The seed stage corresponding to early-dicot stage was found convenient to excise the endosperm. In older seeds, the endocarp becomes hard rendering dissections difficult. Generally, it has been found that mature endosperm requires the initial association of embryo to form callus but immature endosperm proliferates independent of the embryo. However, in neem the association of embryo proved essential to induce callusing of immature endosperm; the best explant was immature seed (Fig. 7A). Endosperm callusing generally requires an auxin, a cytokinin and a rich source of organic nitrogen, such as yeast extract (YE) or CH (Nakajima, 1962; Rangaswamy and Rao, 1963; Bhojwani and Johri, 1971; Srivastava, 1971; Wang and Chang, 1978; Gmitter *et al.*, 1990). Neem endosperm showed good proliferation on MS + 5 μM 2,4-D (45%) but best callusing (53%) occurred on MS + 5 μM NAA + 2 μM BAP + 500 mgl^{-1} CH. The responding cultures exhibited bursting of seed after 2 weeks of culture initiation, releasing the green embryo and callusing endosperm (Fig. 7B). After another week, the seed had wide open and a white fluffy callus had emerged (Fig. 7C).

Regeneration in mature endosperm cultures may occur directly from the endosperm (Johri and Bhojwani, 1965; Bhojwani and Johri, 1970; Nag and Johri, 1971) or after callusing (Lakshmi Sita *et al.*, 1980). However, in immature endosperm cultures, regeneration is invariably preceded by callusing (Gmitter *et al.*, 1990; Garg *et al.*, 1996; Thomas *et al.*, 2000). Regeneration from endosperm tissue generally occurs via organogenesis. The two exceptions are *Citrus* (Wang and Chang, 1978) and *Santalum* (Lakshmi Sita *et al.*, 1980) where somatic embryogenesis was observed. On MS + NAA + BAP + CH, the compact and nodular calli of neem endosperm differentiated green loci (Fig. 7D). When these calli were transferred to a medium containing a cytokinin (BAP/Kinetin), shoot-buds differentiated from all over the callus. Maximum regeneration, in terms of number of cultures showing shoot-buds and number of buds per callus, occurred in the presence of 5 μM BAP (Fig. 7E). A characteristic feature of the regenerants from neem endosperm callus was the preponderance of multicellular glands on the surface of the shoots (Fig. 7F). These glands, occasionally stalked, were found in large numbers on younger leaves close to the shoot apex. Shoots were multiplied by forced axillary branching and rooted *in vitro*. Cytologial analysis revealed that over 66% of the plants were triploid with 2n=3x=36 (Fig. 7G) and rest were diploids with 2n=2x=24 (Fig. 7H).

Protoplast Culture

The application of protoplast technology for the improvement of woody plants offers new option to complement conventional breeding programs. The ability of isolated

protoplasts to undergo fusion and take up macromolecules and cell organelles offers many possibilities in genetic engineering and crop improvement (Bhojwani *et al.*, 1977). However, to fully explore the potentials for protoplasts-technology on woody crops, efficient and reproducible methods for protoplast isolation and purification must first be established. There is single report on protoplast isolation and culture in Neem by Chaturvedi (2003).

Since leaf tissue is a readily accessible source of genetically uniform cells, it is often desirable to use mesophyll protoplasts in somatic hybridization studies, but, in Neem, leaf tissues do not yield large number of protoplasts owing to the difficulty in removing the lower epidermis (Chaturvedi, 2003). An alternative, therefore, is the cultured cell material where protoplasts can show greater potential to divide (Bhojwani and Rajdan, 1996). Chaturvedi (2003) isolated protoplasts from Neem callus raised from anthers on MS + 5 µM pCPA, MS + 1 µM 2,4-D, MS + 1 µM 2,4-D + 1 µM Kn and MS + 1 µM 2,4,5-T. The soft and friable callus, obtained after four subcultures, was found to yield more protoplasts than earlier subcultures. Maximum yield (5.8×10^6) protoplasts / g fresh weight) of viable protoplasts was obtained from granular, friable, light brown callus grown on MS + 5 µM pCPA (Fig. 8A). Besides cycles of subcultures, the age of the callus also appeared to influence protoplast production. Calli from 3-week-old subcultures consistently gave maximum yields of protoplasts than the calli from 2- or 5- week-old subcultures. The protoplasts were characteristically spherical but varied considerably in size (Fig. 8B). As compared to mesophyll protoplasts, those isolated from cultured tissues exhibit a great variation in their size, which the author suggested is chiefly due to heterogeneity of the cultured cell population. The optimum duration for enzyme treatment, containing a mixture of 2% cellulase R-10 and 0.2% Macerozyme R-10, was 4 h. Longer incubations in the enzyme solution resulted in clumping of the protoplast. Further, the author observed that protoplast cultured as thin layer were better in terms of sustained division than as flattened drops. Most of the protoplasts cultured on MS + 5 µM pCPA liquid medium became oval after 4 days, indicating new cell wall regeneration. Approximately 10% of the protoplasts entered division after 11 days (Fig. 8C). However, considerable work needs to be done to optimize the various parameters for isolation and culture of protoplasts.

Genetic Transformation

Genetic transformation of plants is becoming an indispensable aid to plant tissue culturists in understanding the role of individual genes in the life of a plant. This technique offers several advantages over the traditional methods as it allows introduction of genes from variable sources unlike the traditional ones where the movement of genes is restricted between closely related plants only. Furthermore, addition of a useful trait by molecular techniques would not disrupt an elite phenotype which is often a problem associated with conventional breeding where whole genomes are transferred from the donor to the recipient organism.

Very few attempts have been made to genetically engineer neem. Naina *et al.* (1989) reported that wounded aseptic seedlings infected with *Agrobacterium tumefaciens* developed tumours that differentiated shoots on MS basal medium. The shoots synthesized octopine and were kanamycin resistant, suggesting their transformed nature. Allan *et al.* (2002)

established hairy root cultures from stem and leaf explants of Neem, with an aim to investigate their production of insect antifeedant compounds in general and azadirachtin in particular. They found that hairy root cultures had a relatively fast growth rate (0.134 g dry weight per day) and showed a 100-fold increase in biomass over a 4-week culture period. Both roots and cultivation medium showed antifeedant activity against desert locust *Schistocerca gregaria*.

Recently, Satdive *et al.* (2007) conducted a study where the seedlings of Neem were infected by *Agrobacterium rhizogenes* for obtaining hairy root cultures. They also studied the effect of different culture media and elicitation, on growth and production of azadirachtin by hairy root cultures of *Azadirachta indica.* Out of the three media tested, Ohyama and *Nitsch* (1972), Gamborg's (1968) and MS basal media, hairy roots cultured on Ohyama and Nitsch's basal medium produced maximum yield of azadirachtin (0.017% DCW). Further, addition of biotic elicitor and signal compounds enhanced the production of azadirachtin as compared to control cultures on Ohyama and Nitsch medium. Additionally, extracts from hairy roots were found to be superior against the larvae of *Spodoptera litura* for antifeedant activity, as compared to those obtained from leaves.

Secondary Metabolite Production

The order Rutales that include family Rutaceae, Meliaceae, Simaroubaceae and Cneoraceae is amongst the richest and most diverse sources of secondary metabolites in the Angiospermae. Almost every part of the tree, leaves, bark and seeds have long been used for medicinal purposes. The most distinguishing metabolites of the Rutales are limonoids, which are tetranortriterpenoids derived from tirucallane furanyl/steroidal skeleton. They have also attracted considerable interest because of their fascinating structural diversity and their wide range of biological activity. In particular they characterize members of the family Meliaceae, where they are abundant and varied.

A. indica which belongs to the same family contains numerous compounds that exhibit a wide range of bioactivity. The spectrum of compounds chiefly comprise of nimbin, nimbidin, nimbolide, salanin and azadirachtin. Among these the most significant compound is Azadirachtin, a complex tetranortriterpenoid that makes up the major proportion of the seed kernels. It is a highly oxidised tetranortriterpenoid which boasts a plethora of oxygen functionality, comprising enol ether, acetal, hemiacetal, and tetrasubstituted oxirane as well as a variety of carboxylic esters. It has been the focal point of research for the last several years. This is one compound that is responsible for broad range of bioactivity including biopesticidal and antimicrobial properties. Azadirachtin has a complex molecular structure, and as a result the first synthesis was not published for over 22 years after the compound's discovery. Recently, Veitch *et al.* (2007) realized a major breakthrough in first total synthesis of this compound. Both secondary and tertiary hydroxyl groups and tetrahydrofuran ether are present and the molecular structure reveals 16 stereogenic centres, 7 of which are tetrasubstituted. These characteristics explain the great difficulty encountered when trying to produce it by a synthetic approach.

Biotechnologically, azadirachtin is abundant in the seed kernels but as discussed in introductory section of the chapter, the seeds show a lot of variation in azadirachtin content owing to the enormous heterozygosity prevalent in the genus. Moreover, the

storage quality of seeds is poor due to high moisture content and they lose viability within a few weeks. Sidhu *et al.* (2003) reported that the variability in azadirachtin content and synthesis of other triperpenoids like nimbin and salanin is due to the individual genetic differences between the trees and not due to environmental factors. Keeping all this in mind and the hardships associated with the chemical synthesis of the molecule, researchers all over the world are focusing on tissue culture and scale up studies to harvest this important compound from *in vitro* raised cultures. These aseptic cultures offer an advantage over conventional sources by providing consistent supply of chemicals all the year round unaffected by environmental fluctuations and geographical barriers, once an elite source is selected.

Raval *et al.* (2003) studied the effect of basal media on growth and production of azadirachtin-related limonoids in plant cell culture of neem with an aim to enhance their yield as they form major group of pesticidal compounds in the neem trees. While maximum biomass was obtained on MS medium, maximum production of azadirachtin related limonoids was observed on White's medium (White, 1963). The results obtained indicated toward non-growth associated production characteristic of azadirachtin related compounds. Two years later, Prakash and Srivastava (2005) came out with statistically optimized media for cell growth and azadirachtin production in neem suspension cultures, obtained from the seed kernels. A maximum of 15.02 g/l biomass and 2.98 mg/g azadirachtin was produced using optimum nutrient concentrations. The studies were extended further to establish the kinetics of cell growth/ azadirachtin formation and substrate consumption of *A. indica* suspension culture in low shear steric impeller bioreactor with statistically optimized conditions. The culture marked a growth of 15.5 and 0.05 g/l azadirachtin in 10 days of cultivation after which azadirachtin concentration showed a decline (Prakash and Srivastava 2006). Further, Prakash and Srivastava (2007) compared azadirachtin production in stirred tank bioreactor with two different impellers. The maximum cell mass (18.7 g/l) and azadirachtin yields (0.071 g/l) were observed with centrifugal impeller as compared to setric impeller bioreactor. Prakash and Srivastava (2008) also studied the role of elicitors like salicylic acid, chitosan, jasmonic acid, methyl jasmonate, and yeast extract at different concentrations in shake flask suspension culture of *A. indica*. Chitosan, salicylic acid and jasmonic acid stimulated the highest increase in azadirachtin content, which ranged from 2 to 3-fold greater than the control. The combined effect of these elicitors on azadirachtin content studied by Response Surface Methodology resulted in 5-fold higher azadirachtin production (15.9 mg/g DCW versus 3.2 mg/g in control cultures) due to synergistic effect of these elicitors.

Recently, Singh and Chaturvedi (2009d) studied the effect of morphogenesis and explant source on azadirachtin production. They established cultures from various explants of neem (zygotic embryo, leaf, anther and ovary), and screened calli on the best culture medium for the highest azadirachtin production. Eight cell lines (four dedifferentiated and four redifferentiated) were eventually selected on the basis of cell differentiation response. In all *in vitro* cell lines, the highest azadirachtin content (2395.7 Kg/g DCW) was found in redifferentiated callus originated from immature zygotic embryo. A positive correlation between organogenesis and azadirachtin production in neem has been observed.

Colour Page

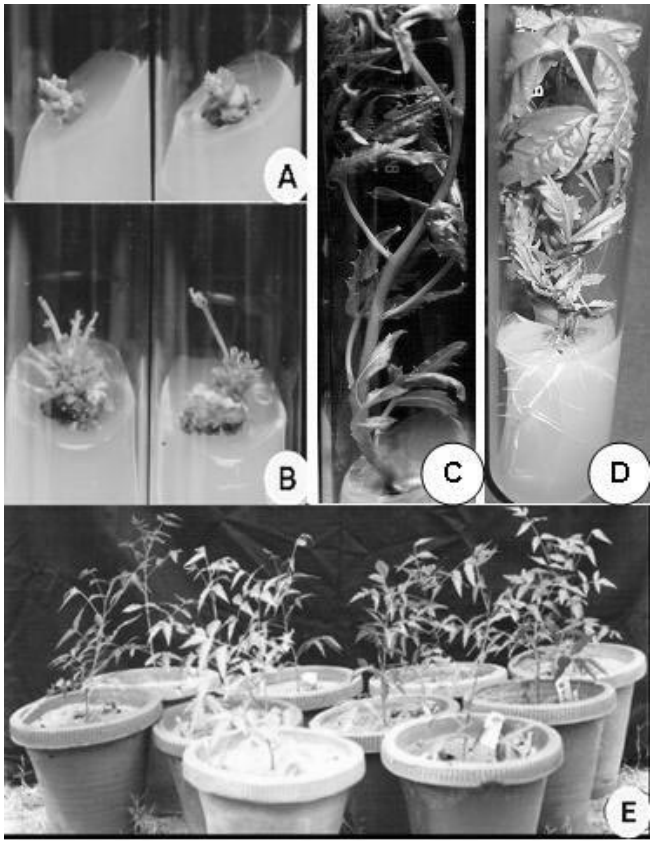

Fig. 1 : Nodal Segment Culture

(A) Five-week-old single node segment cultures on ½ MS + 1 µM BAP + 0.5 µM GA_3, showing the emergence of multiple shoots at the node and basal callusing; the shoots did not elongate.

(B) Cultures transferred from A to ½ MS + 1 µM BAP + 500 mg l^{-1}CH. Some of the shoots have elongated and some new shoots have differentiated after 5 weeks.

(C) A nodal segment from B, 5 weeks after transfer to MS + 1 µM BAP + 250 mg l^{-1}CH. The axillary shoot has elongated considerably.

(D) 4-week-old culture on ¼ MS + 0.5 µM IBA. The shoot has developed healthy roots directly from the cut, basal end.

(E) Hardened micropropagated plants, 11 months after transfer to soil.

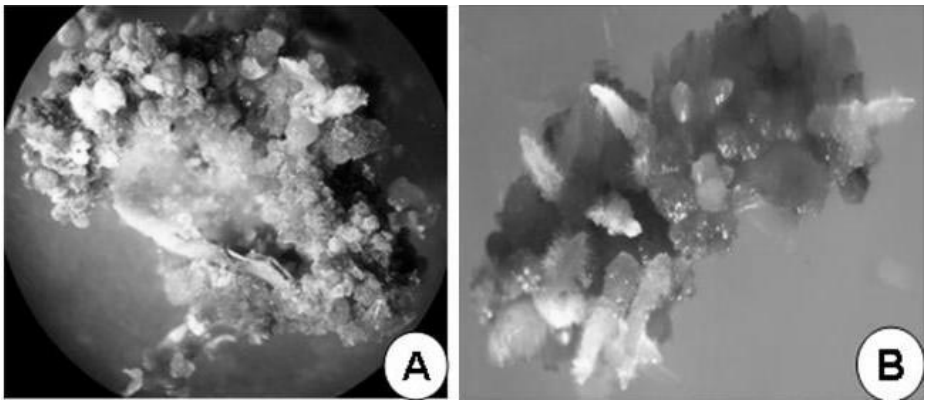

Fig. 2 : Leaf Culture

(A) 8-week-old culture of leaf disc on MS + 5.0 µM BAP. Brownish green, nodulated callus has developed which later differentiated into green shoot buds.

(B) 5-week-old culture of leaf disc on MS + 5.0 µM NAA, showing root proliferation from nodulated callus.

Colour Page

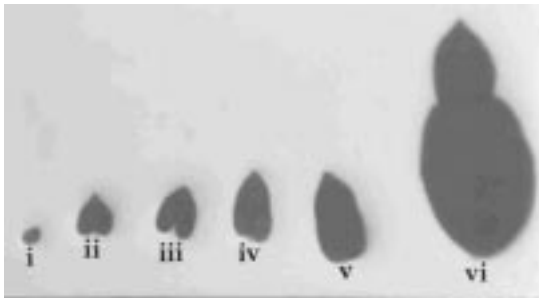

Fig. 3 : Somatic Embryogenesis

(A) Different stages of zygotic embryos (i) Globular, (ii) early heart shape, (iii) late heart shape, (iv) torpedo shape, (v) early dicotyledonous, (vi) fully developed dicotyledonous embryo.

(B) Four-week-old culture of an early dicotyledonous embryo on MS + 5 µM BAP, showing the differentiation of somatic embryos (arrows) on one side and shoots (arrowheads) on the other side of the explant.

(C) Four-week-old culture of an early dicotyledonous stage embryo on MS + 5 µM BAP + 1 µM 2,4-D, showing differentiation of somatic embryos (SE) and neomorphs (NEO) from the callused explant.

Fig. 4 : Somatic Embryogenesis

(A) Somatic embryos excised from cultures showing: (i) pluricotyledony, (ii) fusion of cotyledons, and (iii) absence of cotyledons.

(B) Somatic embryos cultured on MS basal medium for 4 weeks; very long tap root has developed in both the embryos but the plumular end has not formed a shoot.

(C) Eight-week-old somatic embryos culture on MS + 1 µM BAP + 0.5 µM IAA, showing callusing of the explant and differentiation of secondary embryos from the callus.

(D, E) Cultures of somatic embryos (5- and 8-week-old) on MS + 5.0 µM GA_3 + 2.5 µM IAA, showing secondary embryogenesis directly without any callus formation.

Colour Page

Fig. 5 : Anther Culture

(A) Squash of an anther at culture, showing two microspores stained with DAPI. Whereas the larger grain is at the late uninucleate stage, the smaller one is at the mid-uninucleate stage.

(B) 8-week-old anther cultures on MS + 5 µM BAP + 1 µM 2,4-D + 1 µM NAA. Anthers were completely covered by proliferating brown callus.

(C) Transverse section of an anther, after 4 weeks of culture, showing the origin of callus from the surface of anthers; microspores appear unchanged.

(D) Same as C, microspores callused after 8 weeks and the microcalli can be seen inside the anther locules.

(E) 8-week-old anther callus multiplied on MS + 10 µM Kn + 1 µM 2,4-D.

(F) Anther callus from E, 6 weeks after subculture on MS + BAP; many green shoot-buds have differentiated.

(G) Same as F, after 8 weeks, showing distinct green shoots.

(H) Individual elongated shoots on MS + 0.5 µM BAP after 8 weeks. Shoot has elongated well.

(I) A shoot from H, rooted on ¼ MS + 0.5 µM IBA. Healthy, branched roots have differentiated directly from the cut basal end of the shoot.

(J) Shoot-tip cell of anther derived plant, showing haploid chromosome number 2n=x=12.

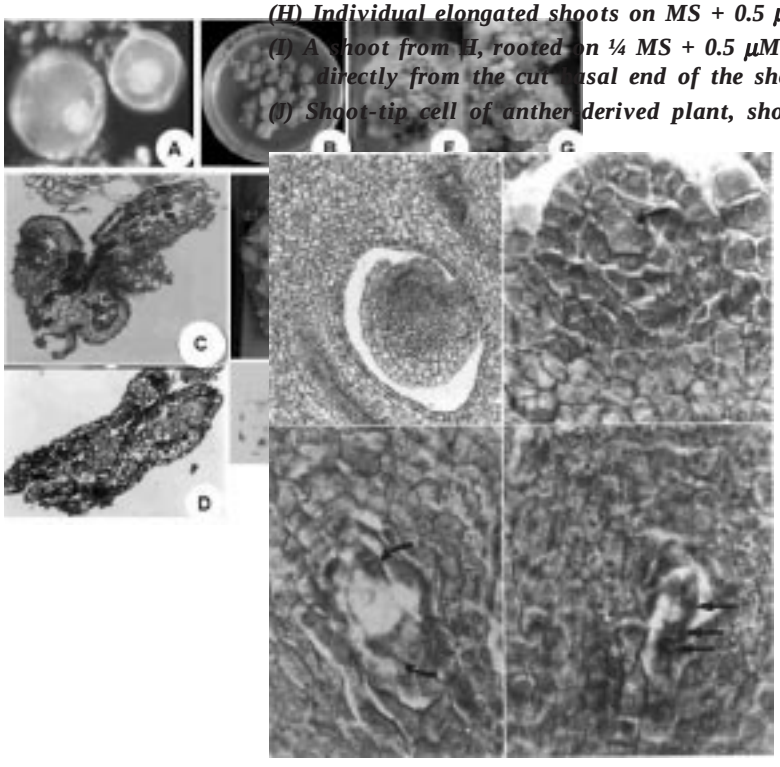

Fig. 6 : Ovary Culture

(A) A section of an ovary from 2 mm flower bud, showing the presence of immature ovule at the integumentary primordia stage.

(B) A section of an ovary from 3 mm flower bud, showing megaspore-mother-cell (arrow marked).

(C) A section of an ovary from 4 mm flower bud, showing 2-nucleate (arrow marked) embryo sac.

(D) A section of an ovary from a 5 mm flower bud, showing 4-nucleate embryo sac. Three of the nuclei are clearly seen in the picture (arrow marked).

Colour Page

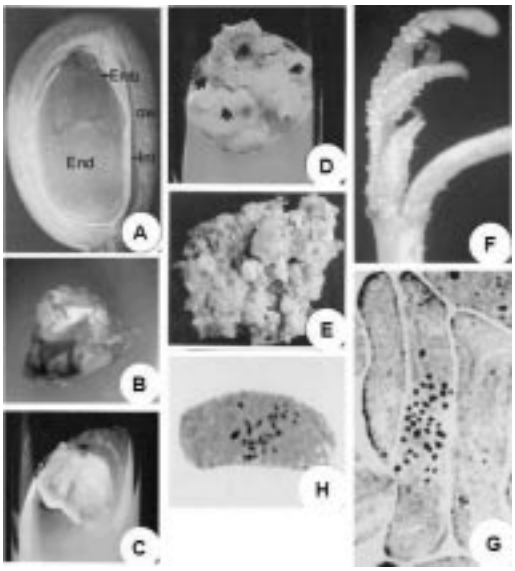

Fig. 7 : Endosperm Culture

(A) Longitudinal half of a neem fruit at the early dicotyledonous stage of the embryo. At this stage it has a massive endosperm. (Emb, Embryo; End, Endosperm; OW, Ovary Wall; Int, integument)

(B) 2-week-old culture of an immature seed on MS + 5 µM NAA + 2 µM BAP +500 mgl^{-1} CH. The seed has burst, releasing the green embryo and callusing endosperm.

(C) Same, after 3 weeks; a white fluffy endosperm callus has emerged from the burst seed. The green embryo can be seen at one end.

(D) 8-week-old subculture of endosperm callus on MS + 5 µM NAA + 2 µM BAP + 500 mgl^{-1} CH, showing callus proliferation. Note the appearance of green loci (arrowhead marked).

(E) 5-week-old subcultures of endosperm callus on MS + 5 µM BAP, showing differentiation of numerous shoots.

(F) A regenerant showing multicellular glands on its surface.

(G) Shoot tip cell of an endosperm-derived plant, showing triploid number of chromosomes (2n = 3× = 36).

(H) Root tip cell of a seedling, showing diploid number of chromosomes (2n = 2x= 24).

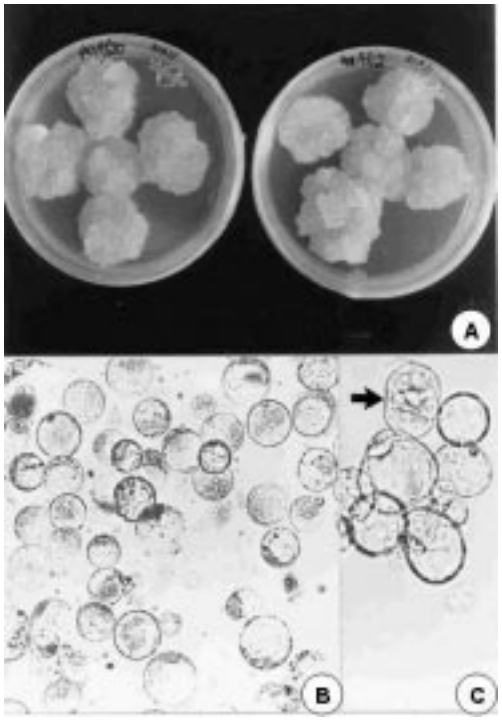

Fig. 8 : Protoplast Culture

(A) Three-week-old proliferating granular, friable, light brown anther callus on MS + 5 µM pCPA.

(B) Freshly isolated protoplasts from calli as in A, showing considerable variation in size.

(C) Eleven-day-old culture of protoplast in liquid MS medium supplemented with 5 µM pCPA. The protoplasts have regenerated walls as indicated by the change in their shape. One of the protoplast has also divided (arrow).

CONCLUSIONS

The success stories achieved in various laboratories throughout the world indicate towards the immense popularity, importance and demand of this medicinal tree species. Though numerous breakthroughs have been achieved in the area of micropropagation, secondary metabolite analysis and bioassays of neem using different explants, few important areas like haploid production and genetic transformation are still at infancy. Moreover, the commercial viability of the tree and its products like azadirachtin, has not been very promising since demand to supply ratio is very high. Somehow, there is an important link missing that will step up the magnitude of fruits obtained in laboratory to a commercial scenario. Cumulative utilization of biotechnological tools and concerted unidirectional efforts are required, so that the results obtained on laboratory scale can be made more accessible and brought to the benefit of public in general.

REFERENCES

Abubacker MN, Alagumanian S. 1999. In vitro organogenesis of various explants of *Azadirachta indica* A. Juss. (Neem). Plant Tissue Cult. 9: 177-180.

Akula C, Akula A, Drew R. 2003. Somatic embryogenesis in clonal neem, *Azadirachta indica* A. Juss. and analysis for *in vitro* azadirachtin production. In Vitro Cell Dev Biol.- Plant 39: 304–310.

Allan EJ, Eeswara JP, Jarvis AP, Mordue (Luntz) AJ, Morgan ED, Stuchbury T. 2002. Induction of hairy root cultures of *Azadirachta indica* A. Juss. and their production of azadirachtin and other important insect bioactive metabolites. Plant Cell Rep. 21: 374–379.

Benge MD. 1989. Cultivation and propagation of the neem tree. In: Jacobson M, editor. Focus on Phytochemical Pesticides. Vol. I. The Neem Tree. Boca Raton: CRC Press, pp. 1-18.

Bhojwani SS. 1984. Culture of endosperm. In: Vasil IK, editor. Cell Culture and Somatic Cell Genetics. Vol. I. Laboratory Procedures and their Applications. Orlando: Academic press, pp. 258-268.

Bhojwani SS, Bhatnagar SP. 1999. The Embryology of Angiosperms. New Delhi, India: Vikas Publishing House Pvt. Ltd., pp. 359.

Bhojwani SS, Johri BM. 1970. Cytokinin-induced shoot bud differentiation in mature endosperm of *Scurrula pulverulenta*. Z Pflanzenphysiol. 63: 269-275.

Bhojwani SS, Johri BM. 1971. Morphogenetic studies on cultured mature endosperm of *Croton bonplandianum*. New Phytol. 70: 761-766.

Bhojwani SS, Power JB, Cocking EC. 1977. Isolation, culture and division of cotton callus protoplast. Plant Sci Lett. 8: 85-89.

Bhojwani SS, Razdan MK. 1996. Plant Tissue Culture: Theory and Practice. Amsterdam: Elsevier, pp. 502.

Bohanec B, Marijana J, Alojz I, Branka J. 1995. Studies of gynogenesis in onion (Allium cepa L.) induction procedures and genetic analysis of regenerants. Plant Sci. 104: 215-224.

Bonga JM, Park YS, Cameron S, Charest PJ. 1997. Applications of *in vitro* techniques in the preservation of conifer germplasm and in conifer tree improvement. In: Razdan MK, and Cocking EC, editors, Conservation of Plant Genetic Resources *in vitro*. Florida: Scientific Publishers Inc., pp. 107–122.

Chand S, Sahrawat AK. 2007. Embryogenesis and plant regeneration from unpollinated ovary culture of Psoralea corylifolia. Biol Plant. 51: 223-228.

Chaturvedi R. 2003. Isolation and culture of Neem (*Azadirachta indica A.* Juss.) callus protoplast. Phytomorphology 53: 57-61.

Chaturvedi R, Razdan MK, Bhojwani SS. 2003a. An efficient protocol for the production of triploid plants from endosperm callus of neem, *Azadirachta indica* A. Juss. J Plant Physiol. 160: 557-564.

Chaturvedi R, Razdan MK, Bhojwani SS. 2003b. Production of haploids of neem (*Azardirachta indica* A. Jurs.) by anther culture. Plant Cell Rep. 21: 531-537.

Chaturvedi R, Razdan MK, Bhojwani SS. 2004a. In vitro clonal propagation of an adult tree of neem (*Azadirachta indica* A. Juss.) by forced axillary branching. Plant Sci. 166: 501-506.

Chaturvedi R, Razdan MK, Bhojwani SS. 2004b. In vitro morphogenesis in zygotic embryo cultures of neem (*Azadirachta indica* A. Juss.). Plant Cell Rep. 22: 801-809.

Cordewener JHG, Haus G, Görgen E, Busink R, Hause B, Dons HJM, Van Lammeren AAM, Van Lookeren Campagne MM, Pechan P. 1995. Changes in synthesis and localization of members of the 70-kDa class of heat-shock proteins accompany the induction of embryogenesis in Brassica napus L. microspores. Planta 196: 747–755

DeMarch, Grenier E, Mianay N, Sulmont G, David H, David A. 1993. Potential of somatic embryogenesis in *Prunus avium* immature zygotic embryos. Plant Cell Tissue Organ Cult. 34: 209-215

Dogra PD, Thapliyal RC. 1996. Gene resources and breeding potential, In: Randhawa NS, Parmar BS, editors. Neem. New Delhi: New Age International Pvt. Ltd., pp. 27-32.

Drew RA. 1993. Clonal propagation of neem by tissue culture. In: Proc. World Neem Conf., Bangalore, pp. 999-1005.

Driver D, Kuniyuki, A. 1984. *In vitro* propagation of Paradox Walnut rootstock. Hort. Sci. 19: 507–509.

Eeswara JP, Stuchbury T, Allan EJ, Mordue AJ. 1998. A standard procedure for the micropropagation of the neem tree (*Azadirachta indica* A. Juss.). Plant Cell Rep. 17: 215-219.

Elliott FC. 1958. Plant Breeding and Cytogenetic. McGraw-Hill, NewYork, USA.

Ermel K, Pahlich E, Schmutterer H. 1984. Comparison of the azadirachtin content of neem seeds from ecotypes of Asian and African origin. In: Proc. 2nd Int. Neem Conf., Rauischholzhausen, pp. 91-93.

Ermel K, Pahlich E, Schmutterer H. 1987 Azadirachtin content of neem kernels from different geographical locations, and its dependence on temperature, relative humidity and light. In: Proc. 3rd Int. Neem Conf., Nairobi, pp. 171-184.

Ermel K. 1995. Azadirachtin content of neem seed kernels from different regions of the world. In: Schmutterer H, editor. The Neem Tree: Source of Unique Natural Products for Integrated Pest Management, Medicine, Industry and Other Purposes. Weinheim: VCH, pp. 222-230.

Esen A, Soost RK. 1973. Seed development in Citrus with special reference to 2x, 4x crosses. Am J Bot. 60: 448-462.

Gairi A, Rashid A. 2004. Direct differentiation of somatic embryos on different regions of intact seedlings of *Azadirachta* in response to thidiazuron. J Plant Physiol. 161: 1073–1077.

Gamborg OL. Miller RA, Ojima K. 1968. Nutrient requirements of suspension cultures of soyabeen root cells. Exp Cell Res. 50: 151-158.

Garg L, Bhandari NN, Rani V, Bhojwani SS. 1996. Somatic embryogenesis and regeneration of triploid plants in endosperm cultures of *Acacia nilotica*. Plant Cell Rep. 15: 855-858.

Gautam VK, Nanda K, Gupta SC. 1993. Development of shoots and roots in antherderived callus of *Azadirachta indica* A. Juss. – A medicinal tree. Plant Cell Tissue Organ Cult. 34: 13-18.

Gill RIS, Gill SS, Gosal SS. 1996. Protocol for *in vitro* asexual multiplication of *A. indica*. In: Islam AS, editor. Plant Tissue Culture. Bangladesh, pp. 155-160.

Gmitter FG. Jr, Ling XB, Deng, XX. 1990. Induction of triploid Citrus plants from endosperm calli *in vitro*. Theor Appl Genet. 80: 785-790.

Gurel S, Gurel E, Kaya Z. 2000. Double haploid plant production from unpollinated ovules of sugar beet (*Beta vulgaris* L.). Plant Cell Rep. 19: 1155-1159.

Gupta PP. 1982. Genesis of microspore-derived triploid petunias. Theor Appl Genet. 61: 327-331.

Islam R, Joarder N, Joarder OI, Hossain M, Rahman SM, Naderuzzaman. 1993. Somatic embryogenesis and organogenesis in neem. In: Proc. World Neem Conf., Bangalore, pp. 955-960.

Islam R, Hoque A, Khalekuzzaman M, Joarder OI. 1997. Micropropagation of *Azadirachta indica* A. Juss. from explants of mature plants. Plant Tissue Cult. 7: 41-46.

Jayaraj S. 1993. Neem in pest control: Progress and perspectives. In: Proc. World Neem Conf., Bangalore, pp. 37-43.

Joarder OI. Naderuzzaman ATM, Islam R, Hossain M, Joarder N, Biswas BK. 1993. Micropropagation of neem through axillary bud culture. In: Proc. World Neem Conf., Bangalore, pp. 961-966.

Johri BM, Bhojwani SS. 1965. Growth response of mature endosperm in cultures. Nature. 298: 1345-1347.

Joshi MS, Thengane SR. 1996. In vitro propagation of *Azadirachta indica* A. Juss. (Neem) by shoot proliferation. Indian J Exp Biol. 34: 480-482.

Kagan-Zur V, Mills D, Mizrahi Y. 1990. Callus formation from tomato endosperm. Acta Hort. 280: 139-142.

Ketkar CM, Ketkar MS. 1993. Azadirachtin content of neem and its by-products from different parts of India. World Neem Conf., Bangalore, pp. 54.

Koul O, Isman MB, Ketkar CM. 1990. Properties and uses of neem, *Azadirachta* indica. Can J Bot. 68: 1-11.

Kumaran K, Surendran C, Rai RSV. 1993. Variation studies and heritable components of seed parameters in neem (*Azadirachta indica* A. Juss.). In: Proc. World Neem Conf., Bangalore, pp. 167-172.

Laibach F. 1925. Das Taubwerden von Bastardsamen und die kunstliche Aufzucht fruh absterbender Bastardembryonen. Z Bot. 17: 417-459.

Laibach F. 1929. Ectogenesis in plants. Methods and genetic possibilities of propagating embryos otherwise dying in the seed. J Hered. 20: 201-208.

Lakshmi Sita G, Raghva Ram NV, Vaidyanathan CS. 1980. Triploid plants from endosperm cultures of sandalwood by experimental embryogenesis. Plant Sci Lett. 20: 63-69.

Lampe L, Mills CO. 1933. Growth and development of isolated endosperm and embryo of maize. Abs Papers Bot Soc., Boston.

La Rue CD. 1949. Culture of the endosperm of maize. Am J Bot. 36: 798.

Luthar Z, Bohanec B. 1999. Induction of direct somaticembryogenesis in onion (*Allium cepa* L.) using a two step flower or ovary culture. Plant Cell Rep. 18: 797-802.

Marcotrigiano M, Jagannathan L. 1988. Paulownia tomentosa Steud. 'Somaclonal Snowstorm'. Hort Sci. 23: 226-227.

Mohan Ram HY, Nair MNB. 1996. Botany, In: Randhawa NS, Parmar BS, editors, Neem. New Delhi: New Age International Pvt. Ltd., pp. 6-26.

Murashige T, Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco cultures. Physiol Plant. 15: 473-497.

Muralidharan EM, Mascarenhas AF. 1989. In vitro Morphogenesis in *Azadirachta indica* A. Juss. and Eucalyptus citriodera Hook F. Tissue Culture and Biotechnology of Medicinal and Aromatic Plants, CIMAP, Lucknow, pp. 49-55.

Murthy BNS, Saxena PK. 1998. Somatic embryogenesis and plant regeneration of neem (*Azadirachta indica* A. Juss.). Plant Cell Rep. 17: 469-475.

Nag KK, Johri BM. 1971. Morphogenetic studies on endosperm of some parasitic angiosperms. Phytomorphology. 21: 202-218.

Naina NS, Gupta PK, Mascarenhas AF. 1989. Genetic transformation and regeneration of transgenic neem (*Azadirachta* indica) plants using *Agrobacterium tumefaciens*. Curr Sci. 58: 184-187.

Nakajima T. 1962. Physiological studies of seed development, especially embryonic growth and endosperm development. Bull. Univ. Osaka Perfect. Ser. B. 13: 13-48.

Narayan P, Jaiswal VS. 1985. Plantlet regeneration from leaflet callus of *Azadirachta indica* A. Juss. J Tree Sci. 4: 65-68.

National Research Council. 1992. Neem: a tree for solving global problems. National Academy Press, Washington, D.C.

Nirmalakumari A, Ramaswamy NM, Sree Rangaswamy SR. 1993. Tissue culture studies in neem (*Azadirachta indica* A. Juss.). In: Proc. World Neem Conf., Bangalore, pp. 981-992.

Nitsch JP. 1969. Experimental androgenesis in *Nicotiana*. Phytomorphology 19: 389-404.

Ohyama K, Nitsch JP. 1972. Flowering haploid plants obtained from protoplasts of tobacco leaves. Plant Cell Physiol. 13: 229-236.

Pelletier G, Ilami M. 1972. Les facteurs de làndrogenese *in vitro* chez *Nicotiana tabacum*. Z Pflanzenphysiol. 68: 97-114.

Perera PIP, Hocher V, Verdeil JL, Doulbeau S, Yakandawala DMD, Werrakoon LK. 2007. Unfertilized ovary: a novel explant for coconut (*Cocos nucifera* L.) somatic embryogenesis. Plant Cell Rep. 26: 21-28.

Prakash G, Srivastava AK. 2005. Statistical media optimization for cell growth and azadirachtin production in *Azadirachta indica* (A. Juss) suspension cultures. Process Biochem. 40: 3795–3800.

Prakash G, Srivastava AK. 2006. Modeling of azadirachtin production by *Azadirachta indica* and its use for feed forward optimization studies. Biochem Eng J. 29: 62–68.

Prakash G, Srivastava AK. 2007. Azadirachtin production in stirred tank reactors by *Azadirachta indica* suspension culture. Process Biochem. 42: 93-97.

Prakash G, Srivastava AK. 2008. Statistical elicitor optimization studies for the enhancement of azadirachtin production in bioreactor *Azadirachta indica* cell cultivation. Biochem Eng J. 40: 218–226.

Quraishi A, Koche V, Sharma P. Mishra SK. 2004. In vitro clonal propagation of neem (*Azadirachta indica*). Plant Cell Tissue Organ Cult. 46: 1-4.

Radojevic L, Rodriguez R, Zdravkovic-Korac S, Jevremovic S. 2002. The role of biotechnology in multiplication and improvement of woody plants: present and perspectives. In: Quarrie S, Krstic B, Janic V, editors. Plant Physiology in the New Millenium. Belgrade: Yugoslav Society of Plant Physiology, pp. 2.

Ramesh K, Padhya MA. 1990. *In vitro* propagation of neem, *Azadirachta indica* (A. Juss.), from leaf discs. Indian J Exp Biol. 28: 932-935.

Rangaswamy NS, Promila. 1972. Morphogenesis of the adult embryo of *A. indica* A. Juss. Z Pflanzenphysiol. 67: 377-379.

Rangaswamy NS, Rao PS. 1963. Experimental studies on *Santalum album* L. Establishment of tissue culture of endosperm. Phytomorphology 13: 450-454.

Rao KS, Venkateswara R. 1985. Tissue culture of forest trees: Clonal Multiplication of *Eucalyptus grandis* L. Plant Sci. 40: 51-55.

Raval KN, Hellwig S, Prakash G, Ramos-Plasencia A, Srivastava A, and Buchs J. 2003. Necessity of a Two-Stage Process for the Production of Azadirachtin-Related Limonoids in Suspension Cultures of *Azadirachta* indica. J Biosci Bioeng. 96: 16-22.

Rout GR. 2005. *In vitro* somatic embryogenesis in callus cultures of *Azadirachta indica* A. Juss.-a multipurpose tree. J For Res. 10: 263–267.

Roy SK, Assaduzzaman M, Hossain Z. 1996. Clonal propagation of *Azadirachta indica* by *in vitro* culture. In: Islam AS, editor. Plant Tissue Culture. New Delhi: Oxford and IBH, pp. 149-154.

Ruth SK, Stephen LS, John CB. 1993. Ovule culture of sweet potato (*Ipomoea batatas*) and closely related species. Plant Cell Tissue Organ Cult. 32: 77-82.

Salvi ND, Singh H, Tivarekar S, Eapen S. 2001. Plant regeneration from different explants of neem. Plant Cell Tissue Organ Cult. 65: 159-162.

San Noeum LH. 1976. Haploides d'Hordeum vulgare L. par culture *in vitro* d'ovaires non fécondés. Ann Amélior Plantes 26: 751–754.

Sarker RH, Islam MR, Hoque MI. 1997. In vitro propagation of Neem (*Azadirachta indica* A. Juss.) plants from seedling explants. Plant Tissue Cult. 7: 125-133.

Satdive RK, Fulzele DP, Eapen S. 2007. Enhanced production of azadirachtin by hairy root cultures of *Azadirachta indica* A. Juss by elicitation and media optimization. J Biotechnol. 128 (2): 281-289.

Schmutterer H. 1990. Properties and potential of natural pesticides from the neem tree. Ann Rev Entomol. 35: 271-297.

Schmutterer H. 1995. The tree and its characteristics, In: Schmutterer H, editor. The Neem Tree: Source of Unique Natural Products for Integrated Pest Management, Medicine, Industry and Other Purposes. Weinheim: VCH, pp. 1-29.

Sharma AK, Sharma M, Chaturvedi HC. 1999. Conservation of phytodiversity of *Azadirachta indica* A. Juss. through *in vitro* strategies. In: National Symposium on Role of Plant Tissue Culture in Biodiversity Conservation and Economic Development. G.B. Pant Institute of Himalayan Environment and Development, Almora, pp. 1-13.

Shrikhande M, Thengane SR, Mascarenhas AF. 1993. Somatic embryogenesis and plant regeneration in *Azadirachta indica* A. Juss. *In Vitro* Cell Dev Biol. 29P: 38-42.

Sidhu OP, Kumar V, Behl HM. 2003. Variability in neem (*Azadirachta* indica) with respect to azadirachtin content. J Agric Food Chem. 51: 910–915.

Singh M, Chaturvedi R, 2009a. An efficient protocol for cyclic somatic embryogenesis in neem (*Azadirachta indica* A. Juss.). In: Proc. Int. Conf. on Energy and Environment. National Institute of Technology, Kurukshetra. pp. 656-658.

Singh M, Chaturvedi R. 2009b. An efficient protocol for cyclic somatic embryogenesis in neem (*Azadirachta indica* A. Juss.). Int J Environ Sci Engg. 1: 49-51.

Singh M, Chaturvedi R. 2009c. De novo shoot and root organogenesis in leaf disc cultures of *Azadirachta indica* A.Juss. In:

Singh M, Chaturvedi R. 2009d. Screening of *in vitro* cell lines for enhanced azadirachtin production. In: Proc. Int. Conf. on Food security and Environmental Sustainability. Indian Institute of Technology, Kharagpur.

Sotak RJ, Sommer HE, Merkle SA. 1991. Relation of the developmental stage of zygotic embryos of yellow-poplar to their somatic embryogenic potential. Plant Cell Rep. 10: 175-178.

Srivastava PS. 1971. *In vitro* induction of triploid roots and shoots from mature endosperm of *Jatropha panduraefolia*. Z Pflanzenphysiol. 66: 93-96.

Srivastava P, Singh M, Mathur P, Chaturvedi R. 2009. *In vitro* organogenesis and plant regeneration from unpollinated ovary: a novel explant of neem (*Azadirachta indica* A. Juss.). Biol Plant. 53: 360-364.

Srivasuki KP, Philomina NS, Vijaya KR. 1993. Vegetative propagation of *Azadirachta indica* A. Juss. In: Proc. World Neem Conf., Bangalore, pp. 1013-1016.

Su WW, Hwang W.I., Kim SY, Sagawa Y. 1997. Induction of somatic embryogenesis in *Azadirachta indica*. Plant Cell Tissue Organ Cult. 50: 91–95.

Tang F, Tao Y, Zhao T, Wang G. 2006. In vitro production of haploid and double haploid plants from pollinated ovaries of maize (*Zea mays*). Plant Cell Tissue Organ Cult. 84: 233- 237.

Thengane S, Joshi M, Mascarenhas AF. 1995. Somatic embryogenesis in neem (*Azadirachta* indica). In: Jain SM, Gupta P, Newton R, editors. Somatic Embryogenesis in Woody Plants. Vol. II. Important Selected Plants. Dordrecht: Kluwer Academic Publishers, pp. 357-374.

Thomas TD, Bhatnagar AK, Bhojwani SS. 2000. Production of triploid plants of mulberry (*Morus alba* L.) by endosperm culture. Plant Cell Rep. 19: 395-399.

Thomas TD, Chaturvedi R. 2008. Endosperm culture: a novel method for triploid plant production. Plant Cell Tissue Organ Cult. 93: 1-14.

Thorpe TA, Harry IS, Kumar PP. 1991. Application of micropropagation to forestry. In: Debergj PC, Zimmerman RH, editors. Micropropagation, technology and application. Dordrecht, The Netherlands: Kluwer Academic Publisher, pp. 311-336.

Tulecke W, McGranahan G. 1985. Somatic embryogenesis and plant regeneration from cotyledons of walnut, *Juglans regia* L. Plant Sci Lett. 40: 57-63.

Van Tuyl JM, Van Dien MP, Van Creij MGM, Van Kleinwee TCM, Franken J, Bino RJ. 1991. Application of *in vitro* pollination, ovary culture, ovule culture and embryo rescue for overcoming incongruity barriers in interspecific *Lilium* crosses. Plant Sci. 74: 115-126.

Veitch GE, Beckmann E, Burke BJ, Boyer A, Maslen SL, Ley SV. 2007. Synthesis of azadirachtin: a long but successful journey. Angew Chem Int Ed. 46: 6385-6395.

Venkateswarlu B, Mukhopadhyay K. 1999. Azadirachtin content in the seeds of micropropagated neem plants in relation to its mother tree. Curr Sci. 76: 626-627.

Vyas BN, Mistry KB. 1996. Processing and Standardisation. In: Randhawa NS, Parmar BS, editors. Neem. New Delhi: New Age International (P) Ltd., pp. 111-120.

Wewetzer A. 1998. Callus cultures of *Azadirachta indica* and their potential for the production of azadirachtin. Phytoparasitica 26: 47-52.

White PR. 1963. The cultivation of animal and plant cells. (2nd ed.), New York: Ronald Press.

Wang T, Chang C. 1978. Triploid *Citrus* plantlets from endosperm culture. In: Proc. Symp. Plant Tissue Culture. Peking: Science Press, pp. 463-467.

Wood HN, Braun AC. 1961. Studies on the regulation of certain essential biosynthetic systems in normal and crown-gall tumor cells. Proc. Natl. Acad. Sci. USA, 47: 1907-1913.

❐❐❐