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Research review paper

In vitro androgenesis in tree species: An update and prospect for further research

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ABSTRACT

Most, if not all, trees are outbreeding, highly heterozygous and undergo a long developmental period before reaching their reproductive stage. Classical breeding and cross-pollinating procedures are both unpredictable and time-consuming. In vitro androgenesis is, thus, the most prolific and desirable approach of haploid production. But various attempts to induce androgenic potential in the trees have met with rather limited success, as they ought to be extremely recalcitrant in culture. The success rate in this case is nowhere close to that achieved for some model species like *Brassica* and *Nicotiana*.

Our review article intends to focus on the overview of androgenic process and all the major contributions till date on tree species with regard to this aspect. We wish to bring together in one place all the important variables used by different workers, that influence androgenic potential immensely like, stage of anther or microspore at culture, media composition, combinations and concentrations of growth regulators, and additives. This will prove to be a worthy guide to all the prospective workers in this area and in designing their experiments further.

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1. Introduction

In vitro androgenesis is the most efficient, quick and dependable technique to produce haploid plants. The technique becomes an indispensable tool for producing double haploids, more so for tree species, because the majority of the trees are outbreeding, highly

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heterozygous and have long generation cycle. 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. The perspective of raising haploid plants through in vitro androgenesis, thus, 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.

By definition, “androgenesis”, is the transition of pollen from gametophytic mode of development, normally manifested in pollen-tube growth, to the formation of a sporophyte, which is haploid. Thus, haploids are sporophytic plants carrying gametophytic chromosome number of their parents. Natural occurrence of sporophytic haploids was first described by Dorothy Bergner in the weed species *Datura stramonium* in 1922 (Blakeslee et al., 1922). This was followed by *Nicotiana tabacum* (Clausen and Mann, 1924), *Triticum aestivum* (Gains and Aase, 1926) and subsequently, in several other plant species (Kimber and Riley, 1963). However, the credit for producing the first haploids in lab, through anther culture, goes to Guha and Maheshwari (1964, 1966) in *Datura*, which led to the emergence of a very efficient technique for haploid production in large numbers.

The number and extent of studies on the induction of androgenesis in trees, however, are limited and results obtained to date are nowhere near those of model crops such as *Brassica* and *Nicotiana*. The major problem encountered is the extreme recalcitrance of tree species in culture. In the past decades, induction of callus from cultured anthers and the production of haploid plants from calli have been reported by researchers only in 13 tree species belonging to 12 families (Table 1). The most effective and popular technique to obtain haploids is by in vitro anther or microspore culture. Anther culture has been successfully applied to many plant species to produce haploids; its single biggest advantage is its simplicity. Large-scale anther culture operations can overcome the limitation of low per-anther yield. However, the presence of extraneous tissues (eg. The anther wall) makes this a messy system for cell biology and other precise studies (Forster et al., 2007). Isolated microspore culture in contrast to anther culture, has several important advantages (Bonga et al., 1997; Radojević 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 and studied using cell biology, microbiology and functional genomic techniques (Touraev et al., 2001). 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, but might be used in studies on gametophytic to sporophytic transition in pollen, for transformation and for in vitro selection systems (Kasha, 1989; Tuveesson and Ohland, 1992). So far, there is only a single woody species where androgenic haploids have been raised successfully by isolated microspore culture (Höfer et al., 1999; Höfer, 2004). 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.

Factors that affect androgenesis have been described elaborately by Atanassov et al. (1995) and Bhojwani and Razdan (1996). It has been observed that the genotype, environment, physiological status of the donor plant, developmental stage of microspores at culture, culture conditions, media, carbon source and its concentration play a

pivotal role in induction of androgenesis. High sugar concentration during induction phase tends to suppress the divisions of somatic cells and, thereby, promotes microspore callusing/embryogenesis. In most of the cases, incubation of anthers/microspores continuously in dark has been found essential or promotory for androgenesis. Likewise, anthers from the first flush of flowers in the season have been shown to be more responsive. 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 may be more crucial than the composition of the nutrient medium. The switch between normal gametophytic pollen development and direct sporophytic embryo development, which must be effective before the asymmetric division of the pollen cell, might be amplified by different abiotic stress (Barret et al., 2004), like heat in rapeseed (Lichter, 1982) and cold in corn (Brettel et al., 1981) and might be related to mechanisms described in somatic embryogenesis (Hecht et al., 2001). Alternatively, 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.

Significant biochemical and molecular genetic analyses have been performed by many authors to reveal the mechanism of embryogenic induction in microspores (Cordewener et al., 1996; Touraev et al., 1997). Formation of heat shock proteins, protein phosphorylation and reinitiation of DNA replication take place during the formation of embryogenic microspores/pollen (Kyo and Harada 1990; Zarsky et al., 1995; Cordewener et al., 1996). Several genes have been isolated which have been thought to play an important function in embryogenic tobacco, wheat or *Brassica napus* microspores (Garrido et al., 1993; Boutilier et al., 1994; Reynolds and Crawford 1996). The genes involved in the response to androgenesis has been approached with molecular mapping and QTL analysis where it was concluded that genetic control of androgenic induction in specific material might be oligogenic (Beaumont et al., 1995; Manninen 2000). Despite several efforts, the biological mechanisms involved in androgenesis induction are in its infancy. No protein or gene has been isolated so far which qualifies as an inducer, or at least specific marker, for embryogenic microspores.

Owing to remarkable importance of androgenesis, particularly in woody species, prime focus of this review is to include all the major contributions made in this area. Besides, the review will also discuss those reports where the success remains merely up to the development of microcalli. The effect of various parameters, including the media composition, stress pretreatments and culture conditions are also described here in this review. These details would lead to a better understanding of the process and more efficient protocols, for the production of haploid and double haploid plants in woody trees, which are thought to be recalcitrant.

2. *Aesculus hippocastanum* L. (Family: Hippocastanaceae; Common name: Horse chestnut)

A. hippocastanum is used in homeopathic medicine for reducing the swelling of hemorrhoids or veins (phlebitis). Androgenesis in this plant has a long history dating back to late 1970s (Radojević, 1978). In all the reports published till date embryogenesis is the only mode of haploid plant development in this genus (Radojević, 1978, 1991; Radojević, et al., 1998; Calić et al., 2003, 2003/4; Calić et al., 2005a). Calić et al. (2003/4) proposed a protocol for mass production of haploids in microspore suspension cultures of *A. hippocastanum*. They stressed the advantage of microspore culture over anther culture as embryogenesis from the latter runs the risk of being originated from filament, tapetum or anther wall. Cytological analysis revealed that all the regenerants originated from uninucleate microspores had a haploid number of chromosomes, while 50% of those derived from

Table 1
Androgenic response in tree species

S. no.	Taxa/family	Explant	Stage of microspore at culture	Medium Induction	Regeneration		Response	References
1.	<i>Aesculus hippocastanum</i> (Hippocastanaceae)	A, M	Uni	Mod. MS (2% Sucrose)+PA (10 mg/l)+NA (5 mg/l)+Vit B1 (2 mg/l)+AdS (10.86 µM)+CH (200 mg/l)+Kin (4.6 µM)+2,4-D (4.52 µM)	MS+Gln (400 mg/l)		E → PI	Calic et al. (2003/4, 2005a)
2.	<i>Albizia lebbek</i> (Leguminosae)	A	Uni/Bi	B ₅ +2,4-D (2.26 µM)+Kin (9.2 µM)	B ₅ +BAP (4.4 µM)+IAA (2.86 µM)		C → Sh/E → PI	Gharyal et al. (1983a)
3.	<i>Annona squamosa</i> (Annonaceae)	A	Uni	N (2% Sucrose)+IAA (28.55 µM)	N+BAP (8.8 µM)+NAA (5.37 µM)		C → PI	Nair et al. (1983)
4.	<i>Azadirachta indica</i> (Meliaceae)	A	Uni	MS (9% Sucrose)+2,4-D (1 µM)+NAA (1 µM)+BAP (5 µM)	N+BAP (8.8 µM)+IAA (0.57 µM) MS+BAP (5/7.5 µM) ^{MM-AI}		C → Sh C → Sh → PI	Chaturvedi et al. (2003)
5.	<i>Camellia sinensis</i> (Theaceae)	A	Uni	N6+Gln (800 mg/l)+Ser (100 mg/l)+Kin (9.2 µM)+2,4-D (2.26 µM)	N6+Ad (20 mg/l)+LAH (10 mg/l)+ZEA (9.12 µM)		C → Sh → PI (in 1 out of 9 cv.)	Chen & Liao (1982)
		M	?	MS+2,4-D (4.52 µM)+Kin (0.46 µM)	MS+BAP (2.2 µM)		EC	Pedroso and Pais (1994)
		A	?	N6 (7% Sucrose)+L-Gln (400 mg/l)+NAA (0.54 µM)+2,4-D (0.45 µM)+Kin (0.46 µM)			E → PI	Shimokado et al. (1986); Raina and Iyer (1992)
							Ed	Saha and Bhattacharya (1992)
6.	<i>Carica papaya</i> (Caricaceae)	A	Uni	MS+BAP (0.44 µM)+NAA (0.54 µM)			E → PI	Rimberia et al. (2005)
			Uni	MS+AC (1%)+BAP (2 µM)+NAA (0.5 µM)			E → PI	Litz and Conover (1979)
			Uni	MS BM			E → PI	Tsay and Su (1985)
			Uni/Bi	MS (half strength of salts & vitamins with full strength of Na-Fe EDTA; 6% Sucrose)+NAA (10.74 µM)+BAP (4.4 µM)			C	
7.	<i>Cassia siamea</i> (Leguminosae)	A	Uni/Bi	B ₅ +CM (15%)+2,4-D (9.04 µM)+Kin (2.3 µM)			C	Gharyal et al. (1983b)
8.	<i>Cerantonia siliqua</i> (Leguminosae)	A	Uni/Bi	MS+2,4-D (2.26 µM)+TDZ (18.16 µM)			C → PE	Custódio et al. (2005)
9.	<i>Citrus clementina</i> (Rutaceae)	A	Uni	N6 Chu with N & N Vit+Lac (18,000 mg/l)+Gal (9000 mg/l)+CW (5%)+CH (500 mg/l)+L-Gln (200 mg/l)+B (0.5 mg/l)+AA (500 mg/l)+NAA (0.11 µM)+2,4-D (0.09 µM)+Kin (4.6 µM)+BAP (2.2 µM)+ZEA (2.28 µM)+TDZ (0.45 µM)+GA ₃ (1.45 µM)	MS+GA ₃ (2.89 µM)+NAA (0.05 µM)MM-CC		C → E → PI	Germana & Chiancone (2003)
10.	<i>Eriobotrya japonica</i> (Rosaceae)	A	Uni	N6 Chu with MS vitamins+Lac (18,000 mg/l)+Gal (9000 mg/l)+CW (5%)+CH (500 mg/l)+L-Gln (200 mg/l)+B (0.5 mg/l)+AA (500 mg/l)+NAA (0.11 µM)+2,4-D (0.09 µM)+Kin (4.6 µM)+BAP (2.2 µM)+ZEA (2.28 µM)+TDZ (0.45 µM)+GA ₃ (1.45 µM)			E C	Germana et al. (2006)
11.	<i>Hevea brasiliensis</i> (Euphorbiaceae)	A	?	MC (7% Sucrose)+Kin (4.6 µM)+2,4-D (4.52 µM)+NAA (5.37 µM)+CM (5%)	MS (7–8% Sucrose)+Kin (2.3–4.6 µM)+NAA (1.07 µM)+GA ₃ (1.45 µM)		C → E → PI	Chen et al. (1982)
12.	<i>Malus domestica</i> (Rosaceae)	A, M	Uni	MN6mac KNO ₃ (1950 mg/l)+(NH ₄) ₂ SO ₄ (277 mg/l) KH ₂ PO ₄ (400 mg/l)+CaCl ₂ ·2H ₂ O (166 mg/l)+MgSO ₄ ·7H ₂ O (185 mg/l)+MS mic+B ₅ vit+Gln (1256 mg/l)+MES (1950 mg/l)+Mal (90,000 or 180,000 mg/l)	MS+TDZ (0.45 µM)		E → PI	Höfer et al. (1999), Höfer (2004)
13.	<i>Morus indica</i> (Moraceae)	A	Uni	MB (8% Sucrose)+NAA (2.69 µM)+BAP (4.4 µM)	MB (8% Sucrose)+PVP (600 mg/l)+I (75 mg/l)+NAA (2.69 µM)+BAP (2.2 µM)+2,4-D (4.52 µM)		C → E → PI	Jain et al. (1996)
14.	<i>Peltophorum pterocarpum</i> (Leguminosae)	A	Uni	Mod. MS (10% Sucrose)+ KNO ₃ (2500 mg/l)+NH ₄ NO ₃ (800 mg/l)+Th (10 mg/l)+I (1000 mg/l)+Gln (800 mg/l)+NAA (5.37 µM)+Kin (4.6 µM)	Mod. MS+NAA (0.54 µM)+Kin (2.3 µM)		C → E → PI	Rao & De (1987)
15.	<i>Populus sp.</i> (Salicaceae)	A	Tetrad/Uni	MS+2,4-D (2.26 µM)+Kin (0.46 µM)	MS+NAA (0.54 µM)+BAP (4.4 µM)		C → Ed → AdSh → PI	Stoehr & Zsuffa (1990)
				MS (2% Sucrose)+2,4-D (2.26 µM)+Kin (4.6 µM)	MS or WPM (2.5% Sucrose)+NAA (0.54 µM)+BAP (4.4 µM)		C → Sh → PI	Kiss et al. (2001)
16.	<i>Quercus sp.</i> (Fagaceae)	A	?	Smac+MS mic+cofac+AC (1% w/v)	Induction medium without AC+Gln (500 mg/l)		E → PI	Bueno et al. (1997)

Before being transferred to regeneration media, calli and embryos were multiplied on MM (Multiplication medium): AI MS+2,4-D (1)+Kn (10); CC MS (5% Sucrose)+NAA (0.11).

A – Anther; AA – Ascorbic acid; AC – Activated charcoal; Ad – Adenine; AdS – Adenine Sulfate; AdSh – Adventitious shoots; B – Biotin; BAP – 6-Benzylaminopurine; B₅ – Gamborg et al. (1968); BM – Basal medium; Bi – Binucleate; C – Callus; CH – Casein hydrolysate; CM – Coconut milk; CPPU – N-(2-chloro-4-pyridyl)-N-phenylurea; CW – Coconut water; 2,4-D-2,4 – Dichlorophenoxyacetic acid; E – Embryos; EC – Embryogenic calli; Ed – Embryoid; GA₃ – Gibberellic acid; Gal – Galactose; I – Inositol; IAA – Indole-3-acetic acid; Kin – Kinetin (N⁶-furfuryladenine); LAH – Lactalbumin hydrolysate; Lac – Lactose; L-Gln – L-Glutamine; M – Isolated microspore; Mal – Maltose; MB – Modified Bourgin; MC – Modified Chen et al. (1978); MES – 2-(N-morpholino)-ethanesulfonic acid; MN6 mac – Modified N6 macromineral; MS – Murashige & Skoog medium (1962); MS mic+cofac – MS micronutrients and cofactors; Nicotinic acid – NA; N – Nitsch & Nitsch (1969); N6 – Chu (1978), Chu et al. (1990); NAA – α-Naphthalene acetic acid; PA – Pantothenic acid; PE – Proembryos; PI – Plantlet; PVP – Polyvinylpyrrolidone; S mac – Sommer et al. (1975) macronutrients; Ser – Serine; Sh – Shoot; TDZ – Thidiazuron; Th – Thiamine; Uni – Uninucleate; Vit B1 – Vitamin B1; WPM – Woody Plant Medium; ZEA – Zeatin.

anther cultures were diploids. Modified MS basal medium with MS mineral salts, 2% sucrose, pantothenic acid (10 mg l^{-1}), nicotinic acid (5 mg l^{-1}), vitamin B1 (2 mg l^{-1}), adenine sulfate ($10.86 \text{ }\mu\text{M}$), myoinositol (100 mg l^{-1}) and casein hydrolysate (200 mg l^{-1}) was used to achieve androgenesis. Androgenic embryos were induced from uninucleate microspores in liquid medium of isolated microspore cultures and semi-solid medium of anther cultures. The induction of androgenic embryos occurred in the combined presence of equal concentrations of both 2,4-D ($4.52 \text{ }\mu\text{M}$) and Kinetin ($4.6 \text{ }\mu\text{M}$) in the modified MS basal medium. However, further embryo development and multiplication required a reduced concentration of 2,4-D ($0.05 \text{ }\mu\text{M}$) in the semi-solid medium where the microspore suspension was plated as a thin film. Embryos were germinated on glutamine enriched (400 mg l^{-1}) modified MS basal medium without the growth regulators. Low temperature ($6 \text{ }^{\circ}\text{C}$) treatment to androgenic embryos over a period of six months has improved the germination by increasing the elongation of roots and total growth of the plantlets (Calić et al., 2005a). This treatment was found more favourable for the germination of anther derived embryos than the microspore derived ones. However, there was no significant difference in the conversion of embryos derived from the two explants into plantlets. The same group also achieved secondary embryogenesis and plant development from primary androgenic embryos of this genus, in order to develop efficient in vitro regeneration methods for use in genetic transformation experiments applicable in the pharmaceutical industry. Calić et al. (2005b) obtained secondary embryogenesis on basal medium without growth regulators mostly on the cotyledons and radicular region of the embryos.

3. *Albizia lebbek* L. (Family: Leguminosae; Common name: Siris)

A. lebbek L. or the “East Indian Walnut” is an important leguminous drought-resistant tree suitable for arid regions. The only report on haploid production in this genus is through anther culture by Gharyal et al. (1983a). They used anthers at late uninucleate to early binucleate stages of microspores in the experiments and observed embryogenesis or organogenesis through a callus phase. Addition of either Kinetin or 2,4-D to the B₅ (Gamborg et al., 1968) basal medium favored delayed senescence but with no response. On the other hand, combined presence of both 2,4-D ($2.26 \text{ }\mu\text{M}$) and Kinetin ($9.2 \text{ }\mu\text{M}$) promoted callusing from the microspores. Transfer of callus to B₅ basal medium promoted more root differentiation than shoot differentiation. Addition of BAP ($4.4 \text{ }\mu\text{M}$) + IAA ($2.86 \text{ }\mu\text{M}$) to the basal medium promoted shoot bud differentiation in almost 100% callus cultures with occasional appearance of embryoids. Since the well-developed shoots were rarely formed, therefore, only two plantlets were achieved upon transfer of shoots to the basal medium.

4. *Annona squamosa* L. (Family: Annonaceae; Common name: Sugar apple or Custard apple)

Sugar apple is high in calories and a good source of iron. Seeds of *A. squamosa* are reservoir of various antimicrobial and cytotoxic constituents. Haploid plants were induced in this genus from anther callus by Nair et al. (1983). Flower buds, containing anthers at mid-late uninucleate stages of microspore development, were dissected out in a solution of 6% sucrose and activated charcoal (0.25%) to prevent the release of phenolic compounds directly into the medium. The anther cultures required an initial one week dark incubation followed by 3–4 days light exposure, and a group culture of anthers (10–12 anthers per group) to induce androgenesis. In initial experiments, single anthers and anther groups (20 groups per 80 mm Petriplate and 10 ml media) were cultured on Nitsch's medium (Nitsch and Nitsch 1969) with 6% sucrose containing glutamine (800 mg l^{-1}), L-serine (100 mg l^{-1}), inositol (1000 mg l^{-1}), 2,4-D ($13.56 \text{ }\mu\text{M}$), IAA ($11.42 \text{ }\mu\text{M}$) and Kinetin ($2.3 \text{ }\mu\text{M}$). Single anther cultures did not respond, anther groups showed swelling of individual

anthers in 50% cultures. However, bursting of swollen anthers and production of white to grey, granular, friable callus occurred in 80% cultures after 2 weeks when individual anther groups were transferred on Nitsch's medium supplemented with IAA ($28.55 \text{ }\mu\text{M}$) and incubated in light. For regeneration, anther calli were subcultured on Nitsch's medium supplemented with either BAP ($8.8 \text{ }\mu\text{M}$) and NAA ($5.37 \text{ }\mu\text{M}$) or BAP ($8.8 \text{ }\mu\text{M}$) and IAA ($0.57 \text{ }\mu\text{M}$); while the former medium showed whole plantlet regeneration in 5% callus cultures, latter medium showed multiple shoot induction in 10% cultures.

5. *Azadirachta indica* A. Juss. (Family: Meliaceae; Common name: Neem)

Neem is an evergreen, tropical, multipurpose tree and owes many important medicinal, agrochemical and economic uses to its credit. Today, due to its remarkable environment friendly, biopesticidal properties shown by azadirachtin and other related triterpenoids, the tree has attained global importance. Androgenesis is highly desirable in this tree species to overcome the prevalent self-incompatibility, heterozygosity and long reproductive cycle. However, a limited effort has been made for the improvement of this valuable tree through in vitro haploid production. Chaturvedi et al. (2003) for the first time achieved androgenic haploids of neem by anther culture at early-to-late uninucleate stage of pollen (Figs. 1 and 2). Callusing from anthers was induced on MS basal medium (with 9% sucrose) supplemented with 2,4-D ($1 \text{ }\mu\text{M}$), NAA ($1 \text{ }\mu\text{M}$) and BAP ($5 \text{ }\mu\text{M}$) while the calli multiplied best on 2,4-D ($1 \text{ }\mu\text{M}$) and Kinetin ($10 \text{ }\mu\text{M}$). These calli differentiated shoots when transferred to a medium containing only BAP; $5 \text{ }\mu\text{M}$ BAP being the optimum concentration for younger calli (75% cultures differentiated shoots) but older calli showed the best regeneration at $7.5 \text{ }\mu\text{M}$ BAP. Histological analysis revealed that initially the anther wall cells had divided while microspore divisions occurred only after 4 weeks. In 8-week-old cultures the entire anther locules were filled with microcalli. Of the plants that regenerated from anther callus, 60% were haploid, 20% were diploid and 20% were aneuploid. The haploid plantlets were later established in soil.

6. *Camellia sinensis* L. (Family: Theaceae; Common name: Tea)

Tea is one of the most important non-alcoholic, caffeine containing beverage in the world. The initial attempts for producing haploids through anther culture were pioneered by Katsuo (1969), Okano and Fuchinone (1970), who could produce roots from the anther derived calli. The first successful report on plant regeneration from anthers of tea were reported in one of the cultivars (Fuyun No 7) out of nine tested by Chen and Liao (1982). The calli continued to proliferate into either shiny masses or shoots when the anthers were cultured on N6 medium supplemented with Kinetin ($9.2 \text{ }\mu\text{M}$), 2,4-D ($2.26 \text{ }\mu\text{M}$), L-Glutamine (800 mg l^{-1}) and Serine (100 mg l^{-1}) followed by subculturing on N6 medium supplemented with Zeatin ($9.12 \text{ }\mu\text{M}$), adenine (20 mg l^{-1}) and lactoalbumin hydrolysate (10 mg l^{-1}). Later on shoots were rooted on MS medium containing IAA ($0.57 \text{ }\mu\text{M}$). Three out of four plants were haploids, the rest were aneuploids with a chromosome number $2n=18$. Saha and Bhattacharya (1992) used the medium with 7% sucrose, NAA ($0.54 \text{ }\mu\text{M}$), 2,4-D ($0.45 \text{ }\mu\text{M}$), Kinetin ($0.46 \text{ }\mu\text{M}$) and glutamine (400 mg l^{-1}) and observed only globular structures from anther cultures, which failed to develop further. Later on, Raina and Iyer (1992) and Shimokado et al. (1986) described the differentiation of true pollen embryos and regeneration of plantlets. Pedroso and Pais (1994) achieved embryogenic calli from isolated microspores cultured on MS medium (Murashige and Skoog, 1962) supplemented with 2,4-D ($4.52 \text{ }\mu\text{M}$) and Kinetin ($0.46 \text{ }\mu\text{M}$) and subsequently on MS with BAP ($2.2 \text{ }\mu\text{M}$) but further differentiation was not achieved. A correlation was established between the developmental stage of pollen and colour of the anthers for selecting appropriate stage of pollen for optimal response; uninucleate stage

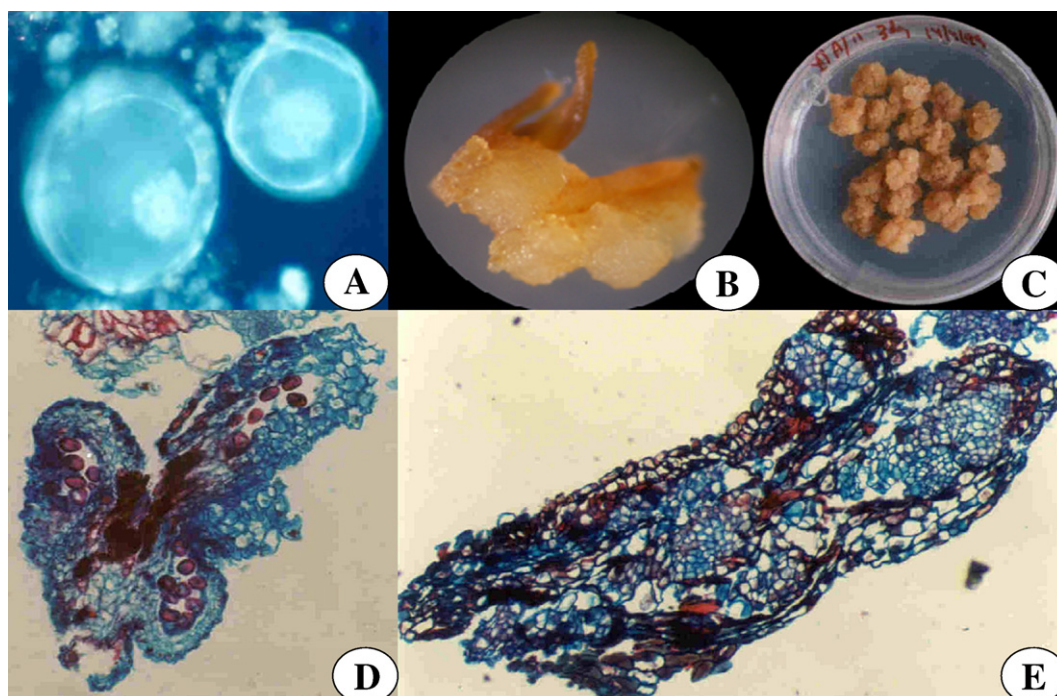


Fig. 1. In vitro anther culture in Neem: (A) Squash of an anther at culture, showing two microspores stained with 4',6-diamidino-2-phenylindole (DAPI). Whereas the larger grain is at the late uninucleate stage, the smaller one is at mid-uninucleate stage. (B) A 4-week-old culture of anther showing emergence of white calli. (C) As in B, anthers are completely covered by proliferating brown callus after 8 weeks. (D) Transverse section of an anther at culture, after 4 weeks, showing the origin of callus from the surface of anthers; microspores appear unchanged. (E) Same as D, microspores callused after 8 weeks and the microcalli can be seen inside the anther locules. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of microspores was found to be the best to induce androgenesis (see Mondal et al., 2004).

7. *Carica papaya* L. (Family: Caricaceae; Common name: Papaya)

Papaya is a popular tropical tree, fruits of which are consumed both raw (as vegetable) and ripened. Ripened papaya is rich in carotene and also aids in digestion. The unripe fruit is rich in 'papain' capable of digesting protein in acid, alkaline and neutral medium. It is a polygamous species with three sex types, male, female, and hermaphrodite (Storey, 1938; Hofmyer, 1938). There are two different breeding systems in papaya the Hawaiian system and the Yarwun (Queensland) system (Aquilizan, 1987; Manshardt, 1992). More than six generations are needed for homogenizing alleles for a particular trait in these breeding systems (Ray, 2002). Thus, methods are required to shorten the breeding period using techniques such as haploid induction via anther culture (Rimberia et al., 2005).

The initial report on development of haploids in anther cultures of papaya came from Litz and Conover (1979). They used stationary liquid MS medium with 3% sucrose, activated charcoal (1%), BAP (2 μ M) and NAA (0.5 μ M) and produced haploids at a success rate of 0.4%. Tsay and Su (1985) were unable to confirm the results of Litz and Conover (1979). However, they emphasized that growth regulator supplemented medium always induce undifferentiated callus from both microspores and somatic cells as well, while haploid embryos could be produced on basal medium without growth regulators. The high frequency (29%) of undifferentiated callus was induced from anthers cultured at uninucleate to first pollen mitosis microspore stages in dark on half strength MS salts and vitamins with full strength Na-Fe EDTA containing medium supplemented with NAA (10.74 μ M), BAP (4.4 μ M) and 6% sucrose. However, haploid plants were induced directly from anthers cultured on MS basal medium (with 3% sucrose) without growth regulators at the rate of 0.7%. Repeated large-scale

secondary embryogenesis was observed when these anther-derived embryoids were transferred on the fresh medium of same composition. Approximately 50 haploid plants were achieved from the secondary embryos. Rimberia et al. (2005) investigated the positive effect of high temperature pretreatment to anthers on induction of embryos from microspores. The embryo induction rate was 4.2% when anthers were cultured on MS semi-solid medium with BAP (0.44 μ M) and NAA (0.54 μ M) after pre-treating them in water for 1 day at 35 °C. These embryos were transferred to MS+CPPU (0.0025 mg l⁻¹) to form shoots which later developed roots on MS+IBA (7350 μ M) medium. By sex-diagnostic PCR assay, all plantlets derived from embryos were determined as female. Rimberia et al. (2006) further improved the embryo induction rate to 13.8% without any pretreatment but by including *N*-(2-chloro-4-pyridyl)-*N*-phenylurea (CPPU; 0.01 mg l⁻¹) in MS agar medium containing NAA (0.54 μ M). They used the anthers at uninucleate to binucleate microspore stages. This induction frequency is the highest ever reported for papaya anther culture so far. The induced embryos were transferred on MS+CPPU (0.0025 mg l⁻¹) for shoot growth. Rooting of the developed shoots was promoted by treating their basal part with IBA (7350 μ M) in a 50% ethanol solution for 10 min. Sex of the established plantlets was identified by a sex specific DNA molecular marker and determination was done by flow cytometry. It was found that 96.3% of these plants were triploids and 3.7% were tetraploids while no haploids or dihaploids were obtained in this study. All the embryo-derived plantlets were identified as female.

The occurrence of polyploids via anther culture had been reported to be related to the microspore developmental stages. Engvild et al. (1972) observed that anthers with young uninucleate microspores gave rise to haploid plants, anthers with older uninucleate microspores gave rise to diploid plants, and anthers with binucleate microspores gave rise to triploid plants. Engvild (1973) produced triploid plants of *Petunia axillaris* from anthers with binucleate microspores. Sunderland et al. (1974) also suggested that triploids

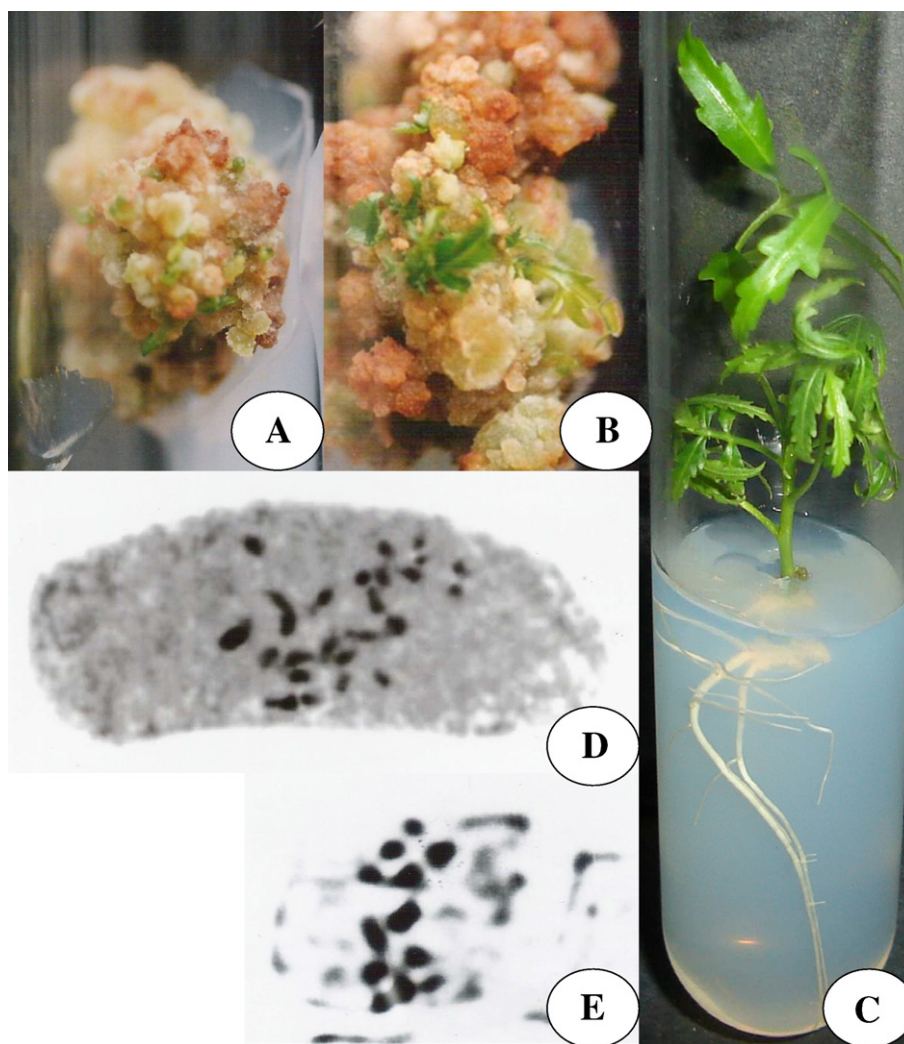


Fig. 2. Haploid plant development in Neem: (A) Anther callus from Fig. 1C, 6 weeks after subculture on MS+BAP; many green shoot-buds have differentiated. (B) Same, after 8 weeks, showing distinct shoots. (C) A shoot from (B), rooted on 1/4 MS+IBA (0.5 μ M). Healthy, branched roots have differentiated directly from the cut basal end of the shoot. (D) Root-tip cell of a seedling, showing diploid chromosome number $2n=2x=24$. (E) Shoot-tip cell of anther-derived plant, showing haploid chromosome number $2n=x=12$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

were predominantly induced in later developmental stages of microspores. Rimberia et al. (2006) further described that in an embryonic grain where a single nucleus divides asymmetrically into a generative and a vegetative nucleus, an endo-reduplicated generative nucleus fuses with a vegetative nucleus to form a triploid embryo, whereas the fusion of an endo-reduplicated generative nucleus and two vegetative daughter nuclei lead to the formation of a tetraploid embryos.

8. *Cassia siamea* Lam. (Family: Leguminosae; Common name: Bombay Blackwood or Siamese Cassia)

C. siamea is one of the most common trees cultivated in Southeast Asia. The plant has a great medicinal value, and is used for the treatment of fevers/malaria and jaundice/hepatitis. A decoction of bark is given in diabetes, while a paste is used as a dressing for ringworm and chilblains. The roots are used as antipyretic and leaves for curing constipation, hypertension and insomnia. The flowers are effective against insomnia and asthma; they are also commonly used in salads and curries in India and Myanmar (Kaur et al., 2006).

Gharyal et al. (1983b) investigated the proliferation of callus from microspores of *C. siamea*. The anthers at late uninucleate to early bi-

celled stages were implanted on B₅ medium containing coconut milk (15% v/v), 2,4-D (9.04 μ M) and Kinetin (2.3 μ M). The anthers split open after two weeks of inoculation in the medium and ejected white, friable calli from inside the anther locules along with the yellowish somatic callus from the wall and filament. Cytological squash preparation of the white calli showed the presence of haploid cells indicating its pollen origin.

9. *Ceratonia siliqua* L. (Family: Leguminosae; Common name: Carob tree)

C. siliqua L. is an evergreen, polygamous tree of slow growth and great longevity. The species is economically important because of the locust bean gum (E410, also called carubin) obtained from its pods. This gum is employed in a wide range of products in the food industry; among the most important of which are ice cream, baby foods and pet foods. Both its economical and ecological value makes carob one of the most important Mediterranean trees (Custódio et al., 2005).

There is a single report on anther culture in this species, which is limited to the formation of multicellular structures and proembryos from the androgenic calli (Custódio et al., 2005). They studied the androgenic response of anthers taken from different developmental stages of flowers. It was reported that to obtain androgenic calli,

anthers should be collected from flowers in the developmental phase-I, which correspond to the formation of pollen mother cells, the microspore tetrads, and uni-to-binucleate pollen grains. High frequency (100%) callogenesis was obtained when anthers were cultured on MS medium supplemented with 2,4-D (2.26 μM) combined with at least one cytokinin, TDZ (18.16 μM). The frequency of haploid cells was found to be 17.2%. However, these calli failed to produce shoots or embryos and the multicellular structures and proembryos showed no further development.

10. *Citrus sp.* (Family: Rutaceae; Common name: Citrus)

Citrus species represent the largest production of fruits worldwide. They are valued for their antioxidant properties and are one of the richest sources of vitamin C and essential oils. Haploid seedlings were first obtained by the application of gamma rays in *C. natsudaidai* (Karasawa, 1971). Thereafter, there have been reports on production of haploids via in vivo crosses (Oiyama and Kobayashi, 1993), gynogenesis induced by in vitro pollination with pollen from a triploid plant (Germana and Chiancone, 2001) and by in vivo parthenogenesis (Germana, 2006). Although anther culture technique has been used to obtain haploid calli, embryoids and plantlets with limited efforts in few *Citrus* species like *C. madurensis*, *C. limon*, *C. deliciosa* \times *C. paradisi* (Chen et al., 1980; Germana et al., 1991; Germana and Reforgiato, 1997), extensive research has been carried on *C. clementina*. Many studies have been conducted to induce embryogenic calli and haploid plantlet development in *C. clementina* Hort. Ex Tan. cv Nules (Chaturvedi and Sharma, 1985; Geraci and Starrantino 1990; Germana et al., 1994, 2000a,b, 2005). Germana and Chiancone (2003) proposed an improved and detailed protocol for haploid induction through anther culture of *C. clementina* Hort. Ex Tan. cv Nules by evaluating a number of factors that affect androgenesis. They observed that temperature pretreatment of flower buds at 4 °C and 25 °C for 14 days were more favourable to induce embryogenic callus and embryoids in anther cultures. Anthers were excised from pretreated flower buds and cultured on N6 medium (Chu, 1978) supplemented with Nitsch and Nitsch vitamins (Nitsch and Nitsch, 1969), galactose (9000 mg l^{-1}), lactose (18,000 mg l^{-1}), coconut water (5% v/v), casein hydrolysate (500 mg l^{-1}), L-glutamine (200 mg l^{-1}), biotin (0.5 mg l^{-1}), ascorbic acid (500 mg l^{-1}), NAA (0.11 μM), 2,4-D (0.09 μM), Kinetin (4.6 μM), BAP (2.2 μM), Zeatin (2.28 μM), TDZ (0.45 μM) and GA_3 (1.45 μM) in dark for 15 days before being shifted to diffuse light at 25 ± 2 °C. With this protocol, 1.9% anther cultures showed embryoid development and a total of 570 and 1000 embryoids developed in Nules and SRA 63 cultivars, respectively. Direct gametic embryogenesis without callus formation was observed in 7% responsive anther cultures of the cv Nules and in 45% of the responsive anther cultures of the cv SRA 63. The embryoids were later germinated on MS medium containing GA_3 (2.89 μM) and NAA (0.05 μM). Recently, Chiancone et al. (2006) studied the effect of polyamines, spermidine and putrescine, with an aim to further improve the rate of embryogenic callus and embryoid induction in anther cultures of *C. clementiana* cv Nules. The addition of 2 mM spermidine to the suggested medium of Germana and Chiancone (2003) stimulated gametic embryogenesis in 4% cultures whereas putrescine did not influence embryo production. Flow cytometric analysis revealed that the regenerants were mostly tri-haploids; few were doubled-haploids while none of them were haploids.

11. *Eriobotrya japonica* Lindl. (Family: Rosaceae; Common name: Loquat)

E. japonica is an important tree for its use as an ornamental and horticultural plant, especially in sub-tropical and Mediterranean countries. Not much biotechnological work has been done on *E. japonica*. Single preliminary report is available on anther culture

in this genus, which deals with the formation of multicellular pollen only (Germana et al., 2006). Flower buds were pretreated at 4 °C for 14 days before harvesting anthers from them. Anthers were cultured on N6 basal medium (Chu 1978), containing MS (Murashige and Skoog, 1962) vitamins, lactose, galactose, L-glutamine, biotin and plant growth regulators as suggested by Germana and Chiancone (2003) for *Citrus clementiana* Hort. ex Tan. The cultures were incubated in dark for 15 days before being transferred to light. Out of the nine cultivars tested, only four showed callusing.

12. *Hevea brasiliensis* (Muell.-Arg.) (Family: Euphorbiaceae; Common Name: Rubber)

H. brasiliensis is a perennial cross-pollinating tree. As the name indicates, its latex has been used to make rubber on a commercial scale since a long time. Chen et al. (1982) for the first time optimized the culture conditions for embryo and plantlet development in anther cultures of *H. brasiliensis*. They emphasized the requirements of Kinetin (4.6 μM), 2,4-D (4.52 μM), NAA (5.37 μM), 5% coconut milk and 7% sucrose concentration in the modified basal medium (Chen et al., 1978) and 50 days incubation of anthers in the culture medium for selective development of calli from the microspores. Calli could differentiate embryoids when subcultured on MS medium supplemented with Kinetin (2.3–4.6 μM), NAA (1.07 μM), GA_3 (1.45 μM) and 7–8% sucrose. They had also observed that a decrease in the total amount of nitrogen in the medium favours callus formation from the anthers while an increase in total nitrogen content is inhibitory. Thus, the balance of nitrate versus ammonium, the level of plant growth regulators and the length of time required for anthers on different media, play a crucial role in the origin of calli and embryoids from microspores. The chromosome count from calli, embryoids and plantlets revealed that they originate from (poly) haploid pollen grains mostly ($n=2x=18$) (somatic chromosome number in rubber is 36). A few haploid cells with 9 chromosomes were consistently observed.

13. *Malus domestica* Borkh. (Family: Rosaceae; Common Name: Apple)

M. domestica or apple is a very important temperate fruit tree. The fruit is a rich source of iron and is widely used in preparation of jams and jellies. The in vitro methods to induce androgenesis and to produce haploid plants in apple have met with rather limited success in comparison to other plant species (Höfer and Lespinasse, 1996). Fei and Xue (1981), Xue and Niu (1984), Höfer (1995) reported embryogenesis and limited plant formation in anther cultures of apple. Moreover, the induction of embryogenesis in cultured anthers was low and highly genotype dependent (Höfer 1995, 1997). Hidano et al. (1995) were able to induce calli and embryos in various cultivars of *M. pumila* but complete haploid plantlets were obtained only in one cultivar; activated charcoal and cold pretreatment were found favorable for embryogenesis. Kadota et al. (2002) studied the androgenic response from anthers of six apple cultivars and reported callogenesis, embryogenesis and complete plantlet development from anther cultures of all the cultivars but cytological analysis revealed that only one regenerated plantlet was haploid, while the rest were diploids. Höfer et al. (1999) for the first time used isolated microspores of *M. domestica* Borkh. at late uninucleate stage of development and, reported induction of embryogenesis and successful plantlet development from them. By isolated microspore culture, the formation of calli and embryos from somatic tissues of anther was avoided. A combination of starvation and a cold treatment was found important and the best condition for induction and development of microspore embryos was the starvation of buds or microspores for 1–2 days at 4 °C or 27 °C. Although a significant effect of medium composition was not observed but optimal concentration and type of carbohydrate was

important for embryo induction from anther and microspore cultures. The optimal concentration was higher (250 mM) for microspore cultures as compared to 146 mM for anther cultures (Höfer and Hanke, 1990) and the effective sugar for anther culture was sucrose rather than maltose. In one of the cultivars, Höfer et al. (1999) achieved 17% embryo induction from microspore cultures as compared to only 7% induction from anther cultures on modified N6 basal medium (Chu et al., 1990). Embryos were germinated on MS+TDZ (0.45 μ M). It was suggested that an increase in the frequency of embryo induction is possible up to ten times by microspore culture depending on the genotype, and various pretreatments can improve the embryo induction rate but is not necessary (Höfer 2004).

14. *Morus* sp. (Family: Moraceae; Common name: Mulberry)

Mulberry (*Morus* sp.) is a vital crop for the sericulture industry and a sole source of feed for the silkworms. Initial studies in mulberry androgenesis were performed on induction of division in pollen culture (Katagiri, 1989), effects of sugars and alcohols on pollen division (Katagiri and Modala, 1991), embryo differentiation (Sethi et al., 1992) and production of haploid plantlets from anther culture (Shoukang et al., 1987). However, detailed studies were conducted by Jain et al. (1996), who evaluated the effect of various physical and chemical pretreatments on induction of haploid plants in anther cultures. It was revealed that cold pretreatment to flower buds at 4 °C for 24 h increased the percentage of anther cultures showing callus induction from microspores (9.20%) while kinetin pretreatment was ineffective and high level of it at 18.4 μ M was toxic leading to aborted pollen grains. The anthers from pretreated floral buds were cultured on modified Bourgin medium (MB) (Qian et al., 1982) supplemented with NAA (2.69 μ M), BAP (4.4 μ M) and 8% sucrose to induce embryogenic callus from microspores. The calli developed embryoids when subcultured on modified MB medium with myoinositol (75 mg l⁻¹), 8% sucrose, NAA (2.69 μ M), BAP (2.2 μ M), 2,4-D (4.52 μ M) and PVP (600 mg l⁻¹). These embryoids developed into plantlets on removal of 2,4-D from the medium. Rhizogenesis from the callus was observed when subcultured on MB medium containing NAA (5.37 μ M) and BAP (2.2 μ M) with reduced myoinositol (75 mg l⁻¹). Cytological nature of induced roots confirmed the haploid nature of the calli.

15. *Peltophorum pterocarpum* (DC) K. Hayne (Family: Leguminosae; Common name: Copper pod)

P. pterocarpum is commonly grown as an ornamental plant. The aqueous and ethanolic extracts from the bark of the plant have been found active against enterohaemorrhagic *Escherichia coli* O157:H7 (Voravuthikunchai et al., 2004). It is one of the few leguminous trees where haploid production has been achieved. Rao and De (1987) reported proliferation of haploid calli, embryos and plantlets from anthers cultured at mid-late uninucleate stages of microspores. A pretreatment to flower buds at 14 °C for 8 days and a high sucrose concentration of 10% in the anther culture medium are the specific requirements to trigger androgenesis in this tree species. The colour of the anther is a reliable and efficient indicator for identification of suitable stage of microspores. The anthers from cold pretreated flower buds were cultured on modified MS medium (MMS-A) containing high nitrate KNO₃ (2500 mg l⁻¹) and low ammonia NH₄NO₃ (800 mg l⁻¹), thiamine HCl (10 mg l⁻¹), inositol (1000 mg l⁻¹), glutamine (800 mg l⁻¹), NAA (5.37 μ M) and Kinetin (4.6 μ M), to induce callus and proembryos from anthers. However, the proembryos did not develop further but shoot buds were differentiated on MMS-A medium supplemented with NAA (0.54 μ M) and Kinetin (2.3 μ M). The shoots were rooted on half strength MMS-A medium with NAA (0.54 μ M). The haploid nature of embryos, callus and regenerated plantlets were confirmed by chromosome count where only 12% of them were haploids and the rest were diploids.

16. *Populus* sp. (Family: Salicaceae; Common name: Poplar)

Poplars (*Populus* sp.) are economically important trees cultivated for their high wood quality that finds use in paper industry and energy production. First successful report on anther culture in the genus is by Wang et al. (1975), who described the formation of haploid plants via organogenesis from pollen calli. Subsequently, several reports were published on production of pollen plantlets from anther culture of Poplar (Zhu et al., 1980; Ho and Raj, 1985; Kim et al., 1986; Mofidabadi et al., 1995). Stoehr and Zsuffa (1990) induced haploids with higher success via embryogenesis in *P. maximowiczii* through anther culture. Anthers at uninucleate stage of microspores were taken from cold pretreated flower buds (at 4 °C for 4 days) and cultured on MS medium containing 2,4-D (2.26 μ M) and Kinetin (0.46 μ M). Globular calli developed from anthers after 4–8 weeks of dark incubation in the medium at 20 °C. However, microspore division and embryoidal structures resembling globular to heart shaped embryoids appeared only when the anthers with globular calli were transferred to MS medium containing NAA (0.54 μ M) and BAP (4.4 μ M). The embryoids germinated precociously without developing cotyledons. After transfer to MS medium with BAP (4.4 μ M), adventitious shoots developed mainly from the roots. Shoots were rooted on half strength MS medium supplemented with NAA (0.13 μ M). Out of 34 plants analyzed cytologically, 22 showed haploid chromosome number ($n=19$), one was aneuploid and the rest were diploids. Kiss et al. (2001) further improved the rate of haploid plant regeneration by increasing the rate of induction from the anthers and with sustained shoot regeneration frequency. They also analyzed the field grown plants of anther culture origin by morphological and molecular methods. The anthers were taken from floral buds collected from five different genotypes of two poplar species viz. *P. nigra* L. and *P. deltoides* Bartr. Flower buds were cold pretreated at 4 °C for 8–14 days. For callus induction and shoot regeneration they used the media suggested by Stoehr and Zsuffa (1990) with some modifications in the media composition and culture conditions. For callus induction from anthers, MS (Murashige and Skoog 1962) medium was used with 2,4-D, Kinetin and 2% sucrose and cultures were incubated in dark for 6–7 weeks at 25 °C. Further, callus proliferation was done after subculturing the 1 cm diameter calli on the same medium and kept in dark at 25 °C. Calli for shoot organogenesis were transferred on either MS or WPM medium (Lloyd and McCown 1980) supplemented with BAP (4.4 μ M), NAA (0.54 μ M) and 2.5% sucrose and incubated in light at 23 °C. Shoots were rooted on hormone-free WPM. They pointed out that frequency of callus induction, shoot regeneration and number of shoots per calli is highly genotype dependent. The callus induction in most responsive clones ranged from 59% to 75% with 4% to 79% shoot regeneration and 1–9 shoots per calli. From 208 rooted plants, 8 haploids, 179 diploids, 4 tetraploids and 17 aneuploid plants were obtained. In the field population, the haploid plants could be easily identified by their retarded development and morphological characteristics such as size and shape of leaves, strong branching etc. Six primers of the 48 primers tested were able to detect polymorphism among the field plants. Deutsch et al. (2004) reported plant regeneration via embryogenesis from isolated immature pollen culture of two poplar hybrids (*Populus nigra* L. \times hybrid 'Aue1' and 'Aue2'). Microsatellite marker analysis indicated that most of the regenerants were haploid.

17. *Quercus* sp. (Family: Fagaceae; Common name: Oak tree)

Oak tree is well known for its bark which is used to make cork that are utilized in bottle stoppers, cricket balls etc. Development of haploids in this plant is reported for the first time by Bueno et al. (1997) through anther culture. Anther cultures were raised on a medium containing macronutrients (Sommer et al., 1975), micronutrients and cofactors (Murashige and Skoog 1962), 3% sucrose, 1% (w/v) activated charcoal and 0.8% (w/v) agar (pH 5.6). Embryos were induced

directly from the anthers as white globular structures resembling heart-to-torpedo shaped embryos after a stress treatment of 5 days at 33 °C followed by incubation of cultures at 25 °C in dark. Embryos developed into complete plantlets when transferred to induction medium but without activated charcoal and with glutamine (500 mg l⁻¹). Much later, Bueno (2003) analyzed the ploidy level stability, genetic variability, morphology, developmental pattern and cellular organization of microspore derived embryos in long-term anther cultures (upto 10–12 months). It was found that 90.7% embryos were haploid, 7.4% were diploid and the rest 1.9% showed mix population of haploid and diploid nuclei. This frequency of haploid occurrence in 12-month-old cultures was similar to that obtained in only 20-day-old cultures. The results in this study indicated that ploidy changes are rare in this in vitro system, and do not significantly increase during long-term incubations.

18. Conclusion

The discussion in the foregoing sections has adequately revealed the merit of haploids. In spite of the regular attempts to induce androgenesis in tree species, success rate achieved has only been marginal, primarily due to their recalcitrance in cultures. Though in many cases, calli and embryos have been obtained, the main impediment lies in conversion of these calli and embryos into plantlets. Also, in addition, the cases where regeneration has been reported, it is mostly preceded by callusing, which increases the chances of variation. It is also obvious that several factors are responsible for regeneration of plantlets from the calli or embryos. Evidently, the stage of microspore at culture and temperature pretreatments plays a pivotal role in androgenic induction while the nutrient medium composition, permutation–combination and concentration of growth regulators are other decisive factors. Apart from these, the genotype, physiological status of the donor plant also play an imperative role in this process. Although, there is no single standard condition or protocol for induction of androgenesis in plants, these criteria help to narrow down the choices to an experimenter and design the experiment more precisely.

Today, haploids and double haploids have been reported in about 200 plant species belonging to almost all the families of the plant kingdom (Forster et al., 2007), not even 10% of these are the tree species. Thus, much scope of work still lies ahead in this area of plant tissue culture because several significant trees are still untouched. Further insight is needed to understand and improve the process of androgenesis in these woody genera. This may be attained by combining different essential disciplines like molecular biology, signal transduction research, cell biology and protocol design. Given to the importance and applications of haploidy and double haploidy, a better knowledge of this aspect becomes mandatory so that this area of plant biotechnology crosses the limits of laboratory and reaches on the industrial scale, thereby making it practically more viable. We expect that details of the review would lead to a better understanding of the processes and would arouse broad interest to scientists working in the related area.

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