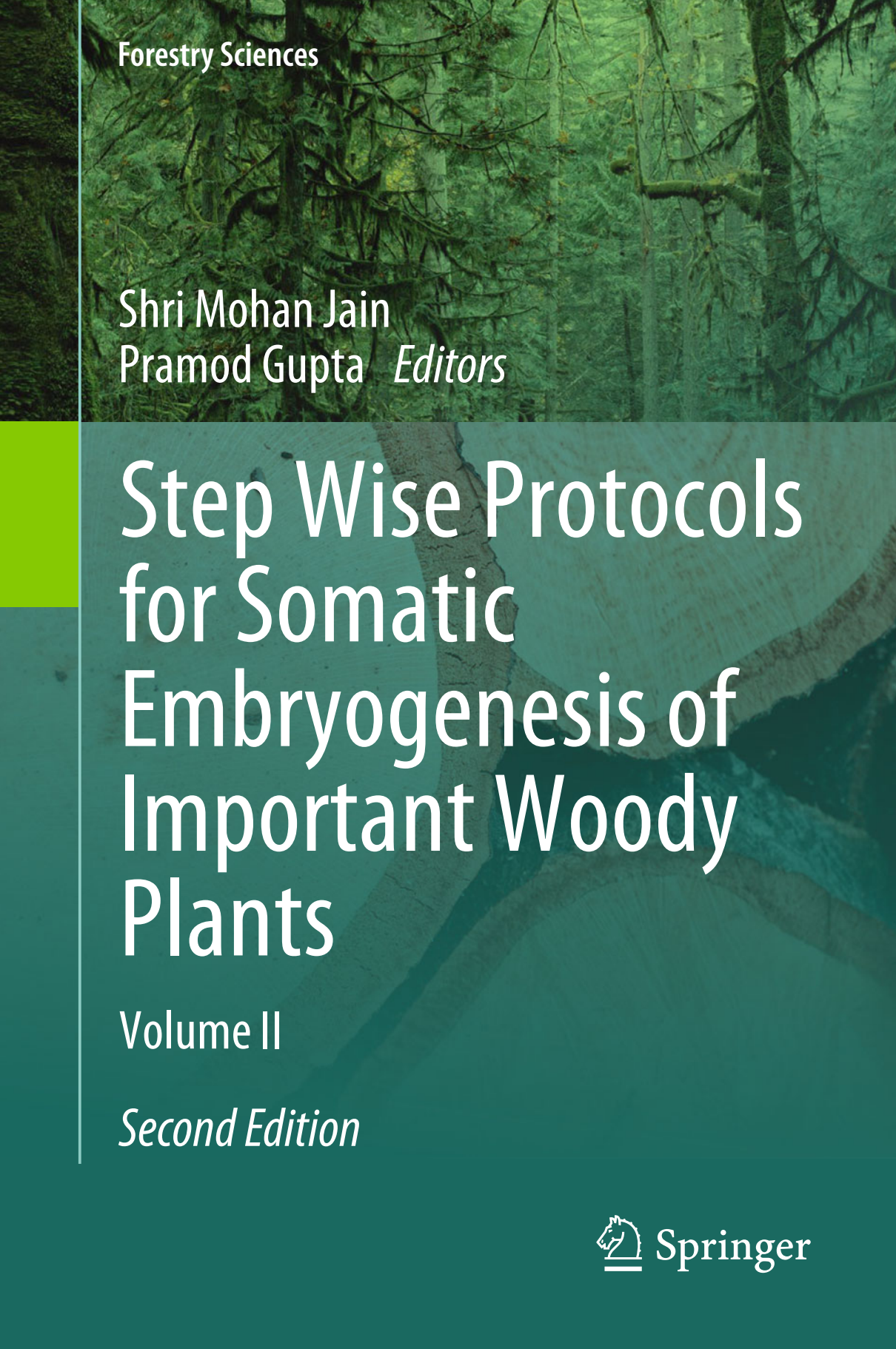
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# Chapter 27
# Somatic Embryogenesis in Neem


**Vartika Srivastava and Rakhi Chaturvedi**

## 27.1 Introduction

Neem, *Azadirachta indica* A. Juss, beholded as a potential and versatile medicinal tree, possessing exclusive therapeutic properties and is deep rooted in the field of medicine, forestry, agriculture and industry. The word '*Neem*' originates from Sanskrit word '*Nimba*' means to bestow health. It possesses most promising healing properties against cancer, diabetes, HIV, malaria, cardiovascular diseases, bacterial infections, skin diseases, allergies, and other ailments. It is well-known in Ayurvedic, Unani and Homeopathic forms of medicine. It affects the physiology and behavior of the insects and acts as the most effective insect-repellent. Its wood is termite resistant and is a valuable resource of timber. The auspicious attributes of the plant corresponds to its secondary metabolites. The most active constituent of neem is Azadirachtin (Koul 2004). Hence, the commercial importance of neem is always on a hike.

On the contrary, neem is accompanied with certain limitations. It constitutes high genetic variability due to cross-pollinating nature. The seeds are highly heterozygous and their germination capacity is lost soon after maturity. Moreover, neem is considered as recalcitrant species with long reproductive cycle (Puri 2003). Additionally, heterozygosity in neem causes variations in the production of secondary metabolites, consequently, it affects the commercial market in the field of medicine and agriculture. These attributes have been challenging and have attracted the biotechnologist for scientific research worldwide. Therefore, genetic diversity studies have also been performed via Amplified Foreign Length Polymorphism (AFLP; Singh et al. 1999) and Rapid Amplified Polymorphic DNA (RAPD) marker analysis (Deshwal et al. 2005).

V. Srivastava · R. Chaturvedi (✉)
Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, Guwahati 781039, Assam, India
e-mail: rakhi_chaturvedi@iitg.ernet.in



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Biotechnology perspectives, especially plant tissue culture, offers substantial methodologies for genetic improvement, production of homozygous lines and large-scale propagation of neem. Somatic embryogenesis is one of the way to achieve large scale plant propagation with an added advantage of increasing the number of somatic embryos through secondary embryogenesis, their enhanced automated production in bioreactor and serving as material for synthetic seed production, providing material for physiological, genetic and biochemical research (Singh and Chaturvedi 2013).

Somatic embryogenesis in neem is governed by several factors, such as effect of basal medium constituents, plant growth regulators (PGRs) and culture conditions. Their requirements may vary from explant to explant on the initiation and expression of somatic embryos, their multiplication and complete plant development. The chapter describes the methodology and morphogenic development of somatic embryos in Neem (*Azadirachta india*). Ontogenic analysis is rewarding at this level for proper differentiation of the observed structures and their conversion into complete plantlets. Successful generation of somatic embryos, consecutively via cyclic embryogenesis, have been added-on perspective of our study.

## 27.2 Somatic Embryogenesis: The General Aspect

The era of somatic embryogenesis began after 60 years of theory of totipotency by Haberlandt, is now one of the progressive areas of research in plant science (Loyola-Vargas 2016). The first successful generation of somatic embryos was reported in *Oenanthe aquatica* in 1958 (Miettinen and Waris 1958; Waris 1959). Fostering somatic cells to develop totipotency and walk through the pathway of embryogenesis is a complex coordinated process and requires skilled and specific approach.

Somatic embryogenesis is defined in two stages: Induction and Expression. The key ingredient in this process is endogenous auxin content, which is affected by the changes in genetic regulation and external application of exogenous PGRs. Developmental changes related to morphology and ultrastructural modifications are essential part of the process. Reviewing the molecular behavior of embryo development, SERK (SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE) gene is mostly upregulated in the regions where there is enhanced auxin content but the later do not induce the former (Hecht et al. 2001). Embryo development could be associated with or without callus development depending upon the plant species, therefore, expression of WUS (WUSCHEL) gene is increased in pro-embryogenic mass of callus (PEMs) (Zuo et al. 2002). Interestingly, callus cells being diverse in nature, are not the composite of embryogenic origin alike zygote or differentiated parts of the plant. They undergo induction process and reprogramming for attaining embryo development.

## 27.3 Somatic Embryogenesis in Neem: Key Factors

### *27.3.1 Explant Type*

Explant is congregation of diversity of cells and tissues and only a few of them have the potential for embryogenic induction. These tissues are more competent and specific for embryogenic potential and responds well to the induction treatment than the non-competent ones. Numerous changes occur at the molecular and physiological level of the explant at various stages of somatic embryogenesis. Upon PGR treatment, the differentiated competent cells of the explant undergo reprogramming. The entire process channelizes the cells to halt and re-activate the cell cycle. The target somatic cells are now destined for embryogenesis directly from the explants or indirectly via an intervening callus development. The type of explant is a critical factor for obtaining maximum embryogenic response. The immature zygotic embryos are served to be the best explant and the advanced stages of it showed considerable somatic embryogenesis as compared to the younger embryos; globular stage embryos exhibited browning after a few days of culture initiation. Hypocotyl region have also proved to be an alternative explant for better embryogenic response. Additionally, leaf, stem and root segments were also utilized for induction of somatic embryos but response was not satisfactory.

In neem, the most appropriate explant utilized for the study is immature zygotic embryos, which showed maximum embryogenic response in least number of days (Table 27.1) followed by immature cotyledons. Immature zygotic embryos at early dicotyledonous stage were effective in inducing somatic embryos on the explant directly (Chaturvedi et al. 2004) or indirectly via callus formation (Rout 2005). Immature zygotic embryos were efficiently used in other plants to obtain somatic embryogenesis, such as *Castanea sativa* Mill. (Sezgin and Dumanoğlu 2014), *Fraxinus mandshurica* Rupr. (Kong et al. 2012), *Acacia senegal* (L.) Willd. (Rathore et al. 2012), *Cassia angustifolia* (Parveen and Shahzad 2014). These explants are more proficient for somatic embryo induction, metabolically and biochemically, due to presence of milky white content in immature cotyledons of neem (Gairi and Rashid 2005). Chaturvedi et al. (2004) reported the differentiation of neomorphs (embryo-like organized shiny structures) from torpedo stage of zygotic embryos (Fig. 27.1).

### *27.3.2 Nutrient Requirements*

An optimum nutritional requirement in association with suitable environment is crucial for the growth and development of the explant that considerably mimics the natural growth of the plant. Nutrient medium is supplied with both inorganic and organic form of nitrogen, carbon source and growth regulators, and retained at an optimum pH of 5.8.

**Table 27.1** Type of explant and the percent response for embryo induction from various explants

| S. No. | Explant | Media | % Response | Days required for embryo induction | Remarks | References |
|---|---|---|---|---|---|---|
| 1. | Immature zygotic embryo (Early dicotyledonous) | MS + BAP (5 μM) | 57 | 28 | – | Chaturvedi et al. (2004) |
| 2. | Immature zygotic embryo (Early dicotyledonous) | MS + TDZ (0.1 μM) + ABA (4 μM) | 76.16 | 28 | – | Singh and Chaturvedi (2009) |
| | | MS + BAP (1 μM) + IAA (0.5 μM) | 100 | 56 | Secondary embryos preceded by callusing | |
| 3. | Mature seeds | MS + TDZ (10 μM) | 75 | 35 | – | Murthy and Saxena (1998) |
| 4. | Immature zygotic embryo | MS + BAP (1.11 μM) + 2,4-D (0.45 μM) | 82.8 | 56 | Intervening callus formation | Rout (2005) |
| 5. | Immature Cotyledons | MS + TDZ (0.5 μM) | 90–100 | 7 | – | Gairi and Rashid (2005) |
| 6. | Leaflets (Juvenile) | MS + IAA (1.5 mg/L) + Kinetin (1.5 mg/L) | – | 28 | Initially formation of embryogenic calli occurred | Shekhawat et al. (2009) |
| 7. | Nodal segments | MS + CH (1000 mg/L) | 66.2 | 56–70 | – | Akula et al. (2003) |
| | Root segments | MS + CH (1000 mg/L) | 72 | 56–70 | – | |
| | Leaf | MS + TDZ (4.5 μM) | 44 | 84 | Via callusing | |
| 8. | Stem | MS + BAP (1 mg/L) | – | 20–25 | – | Phukan et al. (2017) |
| 9. | Cotyledons and Hypocotyl | MS + NAA (0.5 mg/L + BAP (1 mg/L) + CH (1 g/L) + Sucrose (5%) | – | 28–35 | Formation of embryogenic callus | Su et al. (1997) |
| | | MS + CH (1 g/L) + Zeatin (0.2 mg/L) + Sucrose (5%) | 67 | 14 | Somatic embryos | |

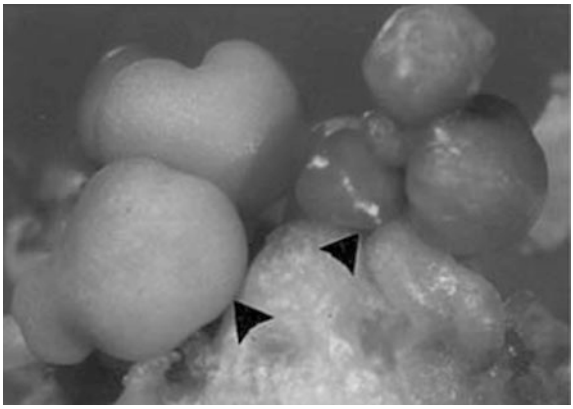


**Fig. 27.1** Development of neomorphs directly on explant on MS + 2,4-D (5 μM)

#### 27.3.2.1 Effect of Inorganic Nitrogen Source

Generally, ammonium and nitrate are the major sources of inorganic form of nitrogen, of which, ammonium being readily utilized by the plant. However, it causes decrease in pH of the medium leading to poor growth and development of the plant. Somatic embryogenesis in neem is achieved mostly in full strength Murashige and Skoog (1962, MS) medium. Differentiation of somatic embryos, neomorphs and shoots were tested in both MS and Gamborg et al. (1968, B5) medium but the MS medium provided a commendable response than B5 medium. This could be due to the presence of high amount of nitrogen in MS medium (in the form of ammonium and nitrate) as compared to B5 medium. Therefore, MS basal medium was chosen for all the experiments. Collateral effect could be observed in reports of Shekhawat et al. (2009), where additional supplementation of ammonium sulphate (50 mg/L) and potassium nitrate (100 mg/L) produced maximum number of somatic embryos. During the process of germination of somatic embryos, half-strength MS medium was proclaimed to have an alike effect as full strength MS (Shekhawat et al. 2009; Su et al. 1997). Effect of ammonium and nitrate have been reported in several plant systems portraying a varied response.

#### 27.3.2.2 Effect of Carbon Source

Apart from the nitrogen source, emphasis of carbon source is also crucial for playing a dual role of carbon source and osmoticum (Biahoua and Bonneau 1999). In general 3% sucrose is used for embryonic development, plant regeneration and cyclic embryogenesis (Chaturvedi et al. 2004; Singh and Chaturvedi 2009). However, low sucrose level (2%) also supported the growth of SE (Gairi and Rashid 2004, 2005) and even 1% sucrose was sufficient for plantlet regeneration.

Su et al. (1997) observed somatic embryogenesis in Neem at higher concentration of sucrose (5%). Maltose at 3% concentration also proved an efficient alternative for conversion of somatic embryos into plantlets (Akula et al. 2003).

### 27.3.3 *Effect of Plant Growth Regulators on Initiation of Somatic Embryos*

During embryogenesis, exogenous auxin treatments are provided to the explants. These inducers affect the regulatory processes of somatic embryos by causing changes in the endogenous indole acetic acid (IAA) content. Cytokinins also favors in somatic embryo initiation processes but majorly contributes to the cell division and reactivation of cell cycle in competent cells. During the stages of embryo development, embryos attain a bilateral symmetry. To achieve this state, endogenous auxin content plays a major role.

Chaturvedi et al. (2004) investigated on effects of different plant growth regulators (PGRs) on induction of somatic embryogenesis in Neem. Among all, role of cytokinins was found to be more influential where benzylaminopurine (BAP) at 5 μM concentration gave maximum embryogenic induction when early dicotyledonous and torpedo stages of embryos were used as explants. The sole presence of auxin (2,4-dichlorophenoxyacetic acid; 2,4-D) in the medium was observed ineffective in inducing embryogenesis. However, combined presence of auxins (2,4-D, IAA) or 1-naphthaleneacetic acid (NAA) and indole butyric acid (IBA) along with BAP had cumulative effect on embryogenesis but was preceded by callus.

In addition to the frequently used PGRs, the effect of Thidiazuron (TDZ), a substituted urea compound, was also explored on induction of somatic embryogenesis. TDZ alone was favorable for embryogenic induction but its combined presence with $GA_3$ promoted enhanced embryogenic responses (100%) but the number of somatic embryos per culture were low ($\sim$9 embryos/culture). However, the combined presence of TDZ and ABA in the medium promoted maximum number of somatic embryos ($\sim$32 embryos/culture) though percentage cultures showing embryogenesis was low (37%). The somatic embryos were induced on hypocotyl and plumular regions when immature zygotic embryos at early dicotyledonous and torpedo stages were used as explants (Fig. 27.2a).

A few other reports have emphasized the use of TDZ to induce somatic embryogenesis in Neem (Murthy and Saxena 1998, Gairi and Rashid 2004, 2005). Studies revealed that the effects of TDZ has two-way response: major cytokinin and minor auxin-like effect. It promotes induction of embryos at low concentration in lesser time compared to BAP as is seen in peanuts (Victor et al. 1999). The growth regulators, ABA and $GA_3$, promotes the regulatory process of embryogenesis, hence, augments the rate of embryo induction (Jiménez 2005) as observed in the author's laboratory. Apart from induction of somatic embryos, direct shoot organogenesis was also observed from the explants (Fig. 27.2b) along with appearance of somatic embryos at respective stages of development (Fig. 27.2c, d).

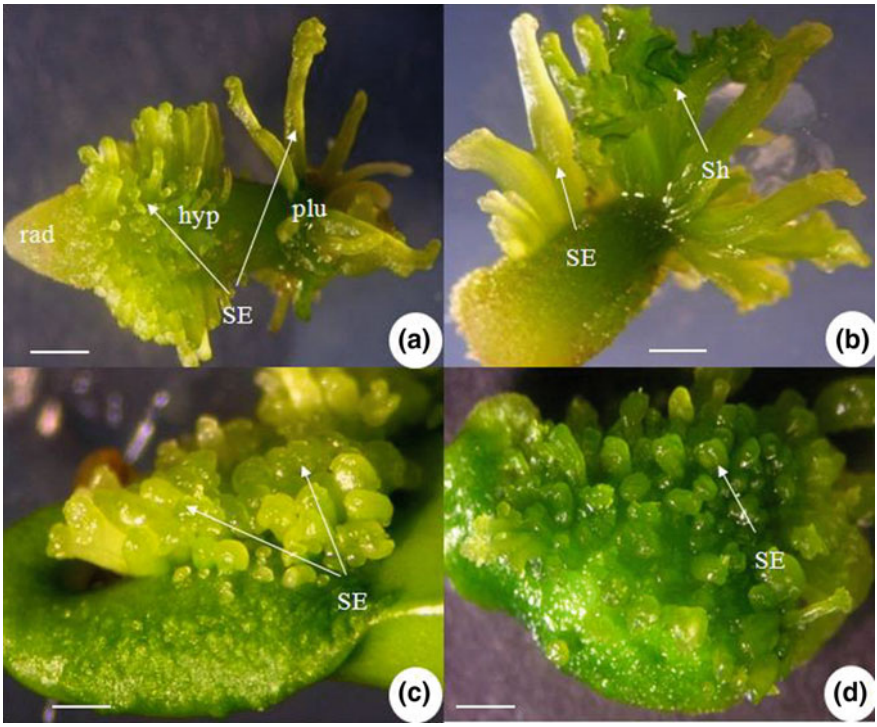



**Fig. 27.2** Induction of somatic embryos (SEs) on immature zygotic embryo explants, **a** 4-week-old culture, showing SEs from hypocotyl and plumular regions of explants, **b** Shoot induction along with induction of somatic embryos, **c** and **d** Appearance of different stages of SEs on explants

### *27.3.4 Effect of PGRs on Expression of Somatic Embryos*

The inductive stage of somatic embryogenesis involves the activation of embryogenic competence of the explant by providing the appropriate blend of growth hormones, medium constituents and culture conditions. Subsequently, expression of somatic embryos takes place and is marked by the reduced level of endogenous auxin content in several plant species. The transition from induction to the maturation period is usually moderate as the cells adapt themselves for further development and undergo the stages of zygotic embryo development, viz, globular, heart, torpedo and cotyledonary stages (Fig. 27.3). Studies on the removal of auxin or exogenous addition of cytokinins, ABA and $GA_3$, for the maturation and germination of somatic embryos are unclear which vary with the plant species. Certainly, these somatic embryos must pass through the maturation phase or else they failed to germinate to form complete plantlets.

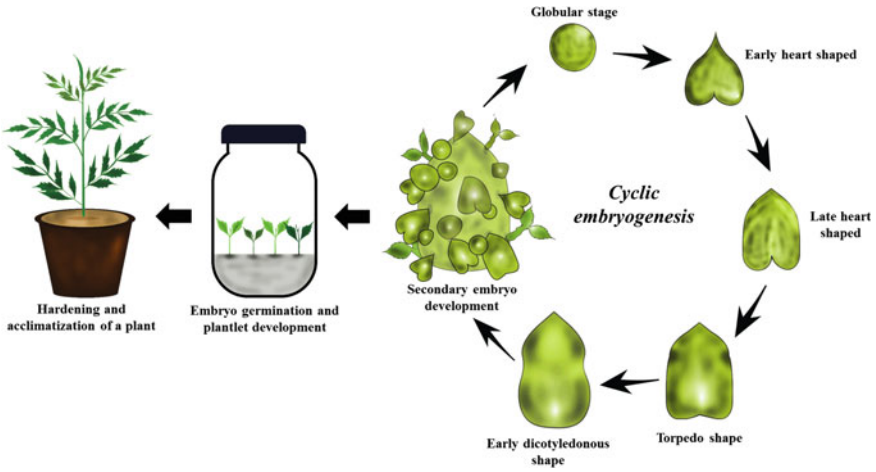



**Fig. 27.3** A progressive view of different stages of embryo development and cyclic embryogenesis

### *27.3.5 Cyclic Embryogenesis*

The process begins with the onset of generation of primary somatic embryos whose superficial tissues, such as cotyledon or hypocotyl regions serve as a bedrock for the generation of secondary and tertiary embryos (Fig. 27.3). Formulation of the medium composition for cyclic embryogenesis should focus on cyclic production of embryos rather than progressing them for further germination. In the author's laboratory, initially, primary somatic embryogenesis was obtained on MS + TDZ + ABA/$GA_3$. Thereafter, secondary embryos developed on the surface of hypocotyl regions of the primary embryos. Cyclic embryogenesis was successfully achieved on MS + IAA + $GA_3$ either directly or preceded by callus formation. Embryogenic potential of the cultures was observed to remain stable with consecutive passages (Singh and Chaturvedi 2009). Repetitive embryogenesis did not occur on the basal medium rather it caused germination in maximum cultures. Akula et al. (2003) reported repetitive embryogenesis exhibiting 4–7 fold multiplication within two to three cycles. Cyclic embryogenesis demonstrates a reproducible methodology for constant production of somatic embryos at large scale, thus, providing fundamental material for scale-up, metabolic and downstream processing studies.

## 27.4 Ontogenic Analysis of Somatic Embryos

The entire developmental process of somatic embryogenesis is accompanied with morphological, cellular and ultrastructural changes and accumulation of proteins and storage lipids serve as energy source. Histological analysis of the embryos at

different stages of embryonic development provided an insight into the structural and cellular details of the embryo, pro-embryogenic masses (PEMs) and callus. The staining techniques solely depends upon the cell content, such as Astra blue stains the cellulosic wall and the cytoplasm while safranin stains the cutinized cell wall and lignified membrane of vascular bundles (Cutler et al. 2007). Scanning electron microscopy provided ultrastructural studies of different stages of the developed embryos (Fig. 27.4a, b). Certain morphological deformity in somatic embryos, including presence of cotyledons or no cotyledons or fused cotyledons or presence of more than two cotyledons, were also observed. In a few cases, the radicular end of the somatic embryos were poorly differentiated and the hypocotyl was elongated consisting of cotyledons and provascular strands (Chaturvedi et al. 2004; Singh and Chaturvedi 2013). Histological studies differentiated the normal and abnormal development of somatic embryos successfully.

Apart from embryos, in the author's laboratory, occasionally, neomorphs were also developed on the explants (as shown in Sect. 27.3.1). These neomorphs, which appeared to be suppressed embryos with epidermal origin and closed provascular strands, were germinated in a monopolar fashion and gave rise to only shoots. The morphological differences with respect to the neomorphs and embryos were clearly understood by histological analysis. These structures differentiated mostly from torpedo stage embryo explants on MS + 2,4-D (5 μM) (Table 27.2). They are green, with smooth shiny surface and solid interior. Some exhibited spherical structure with visible appendages while others showed notches like heart-shaped embryos, some population developed foliar protuberances at the tip.

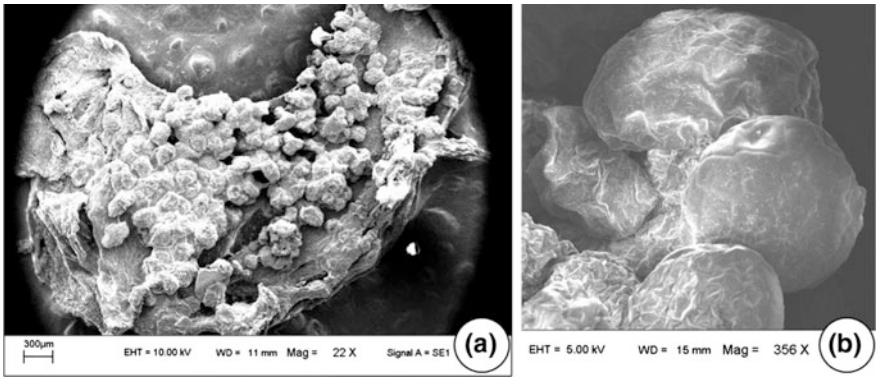



**Fig. 27.4** **a** Scanning electron microscopic analysis showing various stages of somatic embryos, **b** Same as **a**, depicting enlarged view of globular-shaped embryos

**Table 27.2** Stage of zygotic embryzygotic embryo at culture

| Regenerants ► Treatment (μM) | Globular embryo | | | Heart shape embryo | | | Torpedo shape embryo | | | Early dicot embryo | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Shoots | SE | NEO | Shoots | SE | NEO | Shoots | SE | NEO | Shoots | SE | NEO |
| MS | – | – | – | – | – | – | – | – | – | – | – | – |
| CH (1000 mgl−1) | – | – | – | – | – | – | – | – | – | – | – | – |
| 2,4-D (1) | – | – | 20 ± 1.0 (3) | – | – | 20 ± 1.0 (2) | – | – | 20 ± 1.0 (4) | – | – | 25 ± 3.0 (4) |
| 2,4-D (5) | – | – | 20 ± 1.0 (3) | – | – | 40 ± 1.0 (4) | – | – | 66 ± 1.0 (6) | – | – | 25 ± 1.0 (2) |
| BAP (5) | – | – | – | – | 20 ± 1.0 (20) | – | 25 ± 1.0 (6) | 40 ± 1.0 (10) | 40 ± 1.0 (5) | 57 ± 1 (18) | 57 ± 3.0 (25) | – |
| BAP (10) | – | – | – | – | – | – | – | 8 ± 1.0 (6) | 40 ± 1.0 (3) | 24 ± 1.0 (6) | 22 ± 1.0 (9) | 40 ± 1.0 (5) |
| TDZ (0.1) | – | – | – | – | 25 ± 2.0 (10) | – | 25 ± 1.0 (10) | 25 ± 1.0 (20) | – | – | 22 ± 1.0 (10) | – |
| BAP (5) + 2,4-D (1) | – | – | – | 20 ± 1.0 (5)** | – | – | 25 ± 3.0 (5)** | – | – | 30 ± 1.0(5)** | 50 ± 1.0 (6)** | 50 ± 1.0 (2)** |
| BAP (5) + IAA (2) | – | – | – | – | – | 33 ± 1.0 (5) | 35 ± 1.0 (5) | – | 30 ± 1.0 (4) | – | 16 ± 1. (8) | – |
| BAP (5) + NAANaphthalenacetic acid (NAA) (2) | – | – | – | – | – | – | 33 ± 1.0 (7) | – | 33 ± 1.0 (5) | – | – | 20 ± 1.0 (5) |
| BAP (5) +IAA (2) +NAANaphthalenacetic acid (NAA)(2) | – | – | – | – | – | – | 25 ± 0.0 (6) | – | 25 ± 1.0 (4) | 33 ± 1.0 (7) | – | – |
| BAP (5) +Kn (5) +IBA (0.5) | – | – | – | 20 ± 1.0 (3) | – | – | 16 ± 1.0 (5)** | – | – | – | 50 ± 4.0 (8)** | 50 ± 4.0 (2)** |
| BAP (5) + CH (1000 mgl−1) | – | – | – | – | – | – | – | – | – | – | – | – |
| BAP (10) + CH (1000 mgl −1) | – | – | – | – | – | – | 33 ± 1.0 (5) | – | – | 21 ± 0.5 (5) | 42 ± 3.0 (20) | – |
| 2,4-D (5) + CH (1000 mgl −1) | – | – | – | – | – | – | – | – | 55 ± 2.0 (5) | – | – | – |
| TDZ (0.1) + CH (1000 mgl −1) | – | – | – | – | – | – | – | – | – | – | – | – |
| IAA (3) + BAP (3) | | | | | | | | | | | | |
| +CH (1000mgl-1) | – | – | – | – | – | – | – | 28 ± 3.0 (8)** | – | 33 ± 1.0 (6) | 33 ± 1.0 (4) | – |

± = Significant error

*Note Control* MS; *Growth Period* 4 weeks

*SE* Somatic embryos; *NEO* Neomorphs

*The figures in parenthesis represent the number of regenerants per explant

**Indirect differentiation, from callused explant

## 27.5 Methodology

### *27.5.1 Establishment of Cultures*

Immature fruits from experimental neem plant were cleaned with 1% savlon (v/v) followed by washing with sterile distilled water (SDW). Under aseptic conditions in the laminar airflow, they were quick rinsed with 90% ethanol for 30 s followed by washing with SDW thrice. The fruits were finally surface sterilized with 0.1% $HgCl_2$ for 10 min and then washed thrice with SDW. The sterilized immature fruits were dissected under stereo-microscope for isolation of the embryos at its different stages of development. They were inoculated on basal media, MS and B5, fortified with growth regulators at various concentrations and combinations, 3% sucrose and 0.8% agar, to obtain the best responsive stage of zygotic embryo. The cultures were kept in diffused light (1000–2000 lx) at optimum temperature of 25 °C and 50–60% humidity. They were observed periodically, however, the data was recorded after four weeks.

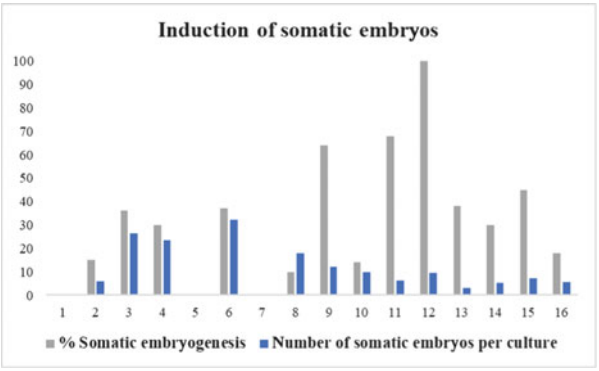
### *27.5.2 Induction Process*

Various forms of structures, like shoots, somatic embryos and neomorphs, were observed directly from the inoculated immature zygotic embryo explants or indirectly via callusing of explants (Table 27.2). The frequency of differentiation of these structures varied with the stage of the zygotic embryo and the culture medium. With BAP (5 μM), the heart shape, torpedo shape and early dicotyledonous embryos differentiated SEs but shoot differentiation was exhibited only by torpedo shape and early dicot embryos. The latter showed differentiation of shoots and SEs with higher frequencies (57%) than the former. Maximum population of neomorphs (66%) were observed on MS with 2,4-D (5 μM), when torpedo shape zygotic embryos were used as an explant.

Another set of experiment displayed somatic embryogenesis on plumular and hypocotyl regions of zygotic embryos on MS supplemented with ABA and $GA_3$ at various concentrations (Fig. 27.5).

### *27.5.3 Cyclic Embryogenesis*

It refers to a swift repetitive process of somatic embryogenesis on a large scale. During the process, secondary embryos were developed on the surface of primary embryos. The repetitive embryogenesis was obtained on MS + BAP (1 μM) + IAA (0.5 μM) where 100% cultures showed embryogenesis with an average of 14 embryos per explant, however, it is preceded by callusing of




| Media Type | TDZ (μM) | ABA (μM) | $GA_3$ (μM) |
|---|---|---|---|
| 1 | - | - | - |
| 2 | 0.5 | - | - |
| 3 | 1.0 | - | - |
| 4 | 2.0 | - | - |
| 5 | 0.5 | 1.0 | - |
| 6 | 1.0 | 1.0 | - |
| 7 | 2.0 | 1.0 | - |
| 8 | 0.5 | 4.0 | - |
| 9 | 1.0 | 4.0 | - |
| 10 | 2.0 | 4.0 | - |
| 11 | 0.5 | - | 1.0 |
| 12 | 1.0 | - | 1.0 |
| 13 | 2.0 | - | 1.0 |
| 14 | 0.5 | - | 4.0 |
| 15 | 1.0 | - | 4.0 |
| 16 | 2.0 | - | 4.0 |

**Fig. 27.5** Graphical representation of embryonic induction with respect to TDZ, ABA and $GA_3$

explants. The number of secondary somatic embryos increased until the end of 8 weeks of culture initiation. The somatic embryos was also utilized for artificial seed production as mentioned in Sect. 27.6.

### *27.5.4 Shoot Elongation, Multiplication, Rooting and Acclimatization*

Individual regenerated shoots (~2 cm) were detached from the explant and transferred on MS medium supplemented with BAP (0.5 μM), which resulted into better elongation of shoot in 4 weeks (Fig. 27.6a). Shoot multiplication via axillary bud proliferation in nodal segment culture was obtained on MS medium fortified with BAP (1 μM) and casein hydrolysate (250 mg/L) (Fig. 27.6b). Around 4 cm long shoot was transferred for rooting on ¼ MS (major) supplemented with IBA (0.5 μM) (Fig. 27.6c). Transplantation of rooted plants was performed in soilrite followed by their transfer to soil with 0.1% urea and Bavistin (1:1) for hardening (Fig. 27.6d). The plant was completely acclimatized within 8 months.

### *27.5.5 Ontogenic Analysis*

Somatic embryos at different stages were fixed in glutaraldehyde and dehydrated with ethyl-alcohol series gradually, followed by complete drying of the material. The dried material were finally gold-coated for SEM analysis as shown in Fig. 27.4a, b. Histological studies were also performed with these regenerants. They were fixed in FAA (Formalin: acetic acid: 70% ethanol) followed by sequential dehydration through tertiary-butyl-alcohol series. The section was


**Fig. 27.6** **a** A 4-week-old elongated shoot on MS + BAP (0.5 μM), **b** A 4-week old shoot on MS + BAP (1 μM) + CH (250 mg/L), **c** A 4-cm long shoot from **b**, exhibiting rooting on ¼ MS + IBA (0.5 μM), **d** An elongated mature plantlet transferred for hardening

embedded in paraffin wax for microtome sectioning. The sections were double stained with Astra blue (1%) and Safranin (1%), and visualized under stereomicroscope.

## 27.6 Applications and Future Endeavors

The worldwide usage of neem for healthcare, industry or research have always uplifted the economical value of the plant. Despite of the fact that it undergo limitations for natural propagation, scientists have flourished neem plantations via plant tissue culture technology successfully. Generation of somatic embryos aids in providing a common platform for other tissue culture methodologies, hence, it is the most influential tool for research and industry. An overview of the applications of somatic embryogenesis is presented in Fig. 27.7.

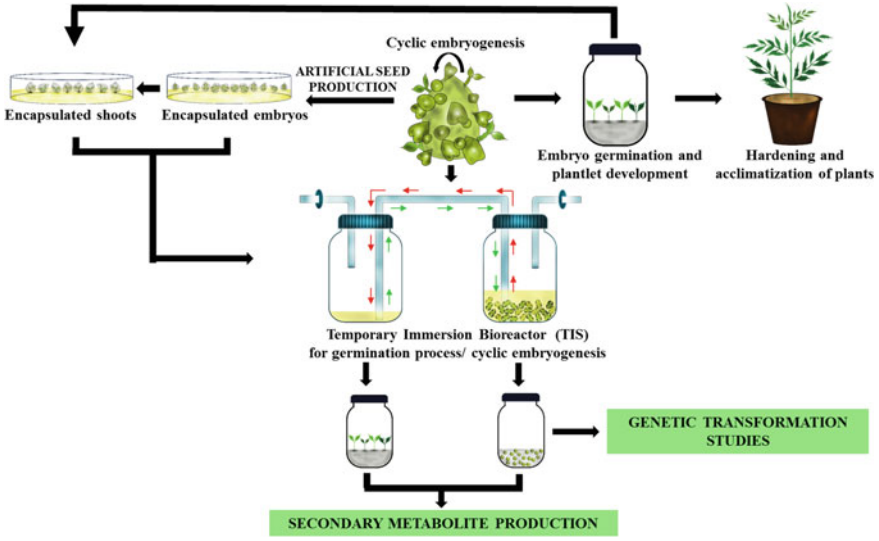



**Fig. 27.7** Overview of applications of somatic embryogenesis

### *27.6.1 Synthetic Seed Production*

Synthetic seeds are considered as an efficient way of plant propagation especially in case of recalcitrant species, and is regarded to be better than vegetative and micropropagation techniques because it requires low input area, can be directly transplanted to soil, hence, reducing the total cost. Moreover, somatic embryos are one of the prerequisite for the production of artificial seeds but latter is again a better choice as they remain viable for long duration and can be easily stored or transported (Saiprasad 2001). In neem, cyclic somatic embryogenesis have been successfully established in the author's laboratory along with production of synthetic seeds (Fig. 27.8), which could serve as a platform for efficient scale-up studies. Germplasm conservation via synthesis of artificial seeds technology is also advantageous.

### *27.6.2 Scale-up Via Bioreactor*

Bioreactor studies assist in large-scale production of secondary metabolites from in vitro cell suspension cultures. According to Prakash and Srivastava (2007), scale-up studies in stirred tank bioreactor have been performed in *Azadirachta indica* and evaluated scale-up parameters for maximum production of biomass and azadirachtin content. The recent advances, such as temporary immersion system opens up more ways for mass propagation of plant and natural metabolites. Apart

**Fig. 27.8** Synthetic seeds of somatic embryos of neem

from in vitro cell suspension cultures, somatic embryos (Etienne et al. 1999; Steinmacher et al. 2011), artificial seeds as well as in vitro shoots (Pérez-Alonso et al. 2012) could be propagated on a wide scale via temporary immersion system.

### *27.6.3 Secondary Metabolite Production*

Neem offers several advantages and is a reservoir of secondary products of which azadirachtin is the most potent metabolite among all. Neem seed oil have also been utilized as biodiesel production (Sekhar et al. 2009) as well as biopesticide (Weathersbee et al. 2005). Neem, being recalcitrant and cross-pollinating in nature, is diversified in case of its natural way of propagation and secondary metabolite production. Plant tissue culture technologies, such as somatic embryogenesis offers advantages for constant and enhanced production of bioactive compounds. Statistical media optimization studies (Singh and Chaturvedi 2012), haploid production (Srivastava and Chaturvedi 2011) and hairy root culture (Allan et al. 2002) provide enhanced benefits for increased production of the compounds.

### *27.6.4 Genetic Transformation Studies*

Somatic embryogenesis aids in proficient usage for genetic transformation studies. The plant species, which are recalcitrant, possess long reproductive cycle and breeding limitations are the important targets for transformation studies. Regeneration of transformed plants is often a setback (Giri et al. 2004), which can be overcome by somatic embryogenesis and other plant tissue culture techniques. Somatic embryogenesis have now become a common platform for transferring

genetic material and enhances the target trait. Tuominen et al. (1995) overexpressed iaaM and iaaH by *Agrobacterium* mediated transformation in *Populus* and demonstrated changes in wood formation properties and overall development of the plant. Allan et al. (2002) developed hairy root cultures through *Agrobacterium* mediated transformation via *Agrobacterium rhizogenes* in *Azadirachta indica* and found improvement in the developmental pattern of the plant. According to the recent advances in research, the molecular depth of somatic embryogenesis of neem and its genetic manipulation remains a topic of research.

## 27.7 Conclusion

Somatic embryogenesis is adapted as a promising plant tissue culture approach for high yield of elite clones of plant species. It is multi-target methodology for substantial propagation of plant, genetic transformation studies, and germplasm conservation. Somatic embryos are rich source of bioactive metabolites, paving a smooth path for pharmaceutical industries. Somatic embryogenesis in neem have been investigated thoroughly, however, proper germination and plantlet development from somatic embryos remains the area of interest along with scale-up studies. Effect of nutritional requirements on the initiation and expression of somatic embryos was critically analyzed. Methodology for cyclic embryogenesis have been speculated that can harbor large-scale production of plant species and constant production of target metabolites.

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