Chapter 8
Somatic Embryogenesis in Cashew (*Anacardium Occidentale* L.)

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Abstract Somatic embryogenesis has seen many advances. However, many aspects are not fully understood especially on cashew (*Anacardium occidentale*) despite several studies conducted for the technique improvement. Regenerate in vitro viable embryos through asexual cells (haploid or diploid) is the target of somatic embryogenesis. This process leads to the production of bipolar structures with root/shoot axis well defined. The in vitro culture of cashew is brought out with emphasis on the critical factors that influence the explants response and plantlet regeneration. The recalcitrant nature of cashew has been attributed to abnormal development observed in the calli derived from its explants in some cases and to the limited success recorded up to here in tissue culture of the plant. This review highlights advances, challenges, and future prospects in somatic embryogenesis research of cashew.

Keywords Cashew · Somatic embryogenesis · True-to-type plants · Explant Browning · Growth Regulators

Abbreviations

ABA	Abscisic acid
BA	6-Benzyl adenine
BAP	6-Benzylaminopurine
B5	Gamborg media
Ca	Calcium

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CE	Corn extract
CW	Coconut water
DSE	Directly somatic embryos
2,4-D	2,4-Dichlorophenoxyacetic acid
GA_3	Gibberellic acid
GRS	Growth regulators substances
H_2O_2	Hydrogen peroxide
IAA	Indole-3-acetic acid
ISE	Indirect somatic embryos
LS	Linsmaier and Skoog, 1975
M	Molar
μM	Micromolar
MS	Murashige and Skoog, 1962
N6	Chu et al., 1975
NAA	α-Naphthaleneacetic acid
ORG	Organogenesis
O_2	Oxygen
PAE	Papaya extract
%	Percentage
PGRs	Plant growth regulators
pH	Potential of hydrogen
PE	Potato extract
PVP	Polyvinylpyrrolidone
PPO	Polyphenol oxidase
POD	Peroxidase
PAL	Phenylalanine ammonia lyase
SH	Schenk and Hildebrandt, 1972
SE	Somatic embryogenesis
TE	Taro extract

1 Introduction

Somatic embryogenesis is the greatest expression of cell totipotence in plant cells. In short, totipotence is the ability of a plant cell to undergo a chain of complex coordinated metabolic and morphological steps to generate a complete and normal plant or sporophyte without the participation of sexual techniques. The in vitro technique by which any somatic cell develops into a zygotic structure that finally produces a plant theoretically is somatic embryogenesis (Rao 1996; Jiménez 2005). Somatic embryos have a single cellular origin like zygotic embryos (Rao 1996). Several authors have come to this conclusion, especially in *Agave tequilana* (Blue agave), with the unicellular origin of somatic embryos that have been reported (Gutiérrez-Mora et al. 2004; Portillo et al. 2007). The somatic embryogenesis

process consists in general of two main steps, the induction and the expression of embryos obtained (Rodríguez-Garay et al. 2000; Gutiérrez-Mora et al. 2004; Jiménez 2005). Theoretically, any part of the plant can be used to initiate somatic embryogenesis with somatic cells. However, significant differences in competence were found in practice. Generally, the cells coming from young tissues including immature zygotic embryos that are more competent for somatic embryogenesis (Gutiérrez-Mora et al. 2012). Somatic embryos induction requires simple manipulation of in vitro culture conditions. The growth regulators substances (GRS) such as auxins, cytokinins, abscisic acid, and gibberellins are one of the principal components in the culture medium. Moreover, auxins are the most important elements in the induction of the process (Rao 1996; Dodeman et al. 1997; Jiménez 2005; Fehér 2006; Jiménez and Thomas 2006). Also, it is important to notify that endogenous hormone play important roles in somatic embryogenesis. The signal for the cell polarization and the asymmetric division given by auxins as it may happen in the zygotic counterparts is a necessity to somatic cells (Gutiérrez-Mora et al. 2004; Pagnussat et al. 2009). Regarding, the initial steps of the development of a somatic embryo, the induction is usually initiated by the action of selected auxins [the most used auxin for most species is 2,4-Dichlorophenoxiacetic acid (2,4-D)] (Nomura and Komamine 1986; Jiménez 2005). In vitro somatic embryogenesis is practiced in many tissue culture laboratories with many species, genotypes, and explants, but the biological background of the process is still largely unknown.

The cashew tree is highly valued for its edible nuts and cashewnut shell liquid. It has attracted interest from conventional plant breeders and biotechnology programs with the goal of improving productivity. It is the most widely cultivated tree fruit in the world. Its culture has spread to several countries and has become a staple of cooking, especially in India. In 2019, world production is 3,960,680 tonnes (FAOSTAT 2019). The high demand for cashew kernels in the confectionery industry has led to increased cultivation of the crop. It is an achene rich in proteins, vitamins, trace elements, and monounsaturated fatty acids that regulate cholesterol levels. In addition, cashew fruits have high vitamin C levels with approximately 200 mg/100 g of juice, four times higher than that of orange juice (Trevisan et al. 2006). However, current propagation methods have become a limiting factor in supplying adequate planting material (Gogate and Nadgauda 2003). Planting material production request the use of technology such as in vitro tissue or cells culture in order to produce a large quantity of clone in a short time with low cost. In addition, micropropagation techniques essentially take two ways: organogenesis (micropropagation) and somatic embryogenesis (Margara 1989; Saadi 1991). The successful micropropagation method in woody plants is somatic embryogenesis. This method makes it possible to produce whole plants in a very short time without going through the constraints usually experienced by micro-cuttings (caulogenesis and rhizogenesis steps) (Daikh and Demarly 1987; Roguet 1989).

The aim of this review is to summarize the vast amount of information published so far on somatic embryogenesis in cashew (*Anacardium occidentale* L.) that can be utilized for the commercial cultivation of in vitro plants.

2 General Characteristics of Somatic Embryogenesis (SE)

Shoot organogenesis (ORG) or somatic embryogenesis (SE) are the two alternative morphogenetic pathways that in vitro culture techniques are taking to enable plant regeneration (Villalobos and Engelmann 1995). Both SE and ORG may be induced at the same time under the same tissue culture conditions (Fiore et al. 1997; Pasternak et al. 1999; Castillo et al. 2000). So, the difference between somatic embryogenesis and organogenesis can sometimes be difficult, and even a detailed comparative histological analysis of the morphogenic process can only insinuate an embryo-like origin of developing structures (Bakos et al. 2000). However, they can be separated in space and time (Ma and Xu 2002; Mithila et al. 2003; Singh et al. 2003; Vikrant and Rashid 2003) with the use of appropriate medium composition, principally type or concentration of plant growth regulators (PGRs). Somatic embryos are distinguished by bipolar structure presenting shoot and root meristems, with a closed tracheal system separated from the maternal tissue and, frequently single-cell origin and production of specific proteins. Although genetic components determine the potential of species/genotypes to form somatic embryos, the expression of embryogenic competency at the cellular level is defined by developmental and physiological cues. Somatic embryos can be developed indirectly, through callus tissue (ISE) or directly from explant tissue (DSE). Somatic embryos developing via DSE are formed from competent explant cells which, contrary to ISE, are able to undergo embryogenesis without dedifferentiation, i.e., callus formation. It is believed that both processes are extremes of one continuous developmental pathway (Carman 1990). The distinction between DSE and ISE can be difficult (Emons 1994), and both methods have been observed to occur simultaneously in the same tissue culture conditions (Turgut et al. 1998). Somatic embryogenesis can be induced directly or through callus in the culture of somatic embryos. This last process is called secondary somatic embryogenesis in contrast to primary somatic embryogenesis induced from explant cells. It has been found that secondary somatic embryogenesis has much higher efficiency compared to primary somatic embryogenesis for many plant species (Raemakers et al. 1995; Akula et al. 2000; Vasic et al. 2001). Some cultures are able to maintain their competence for secondary embryogenesis for many years and thus provide useful material for various studies, as demonstrated for *Vitis rupestris* (Sand Grape) (Martinelli et al. 2001). The most frequent mode of embryogenesis is the indirect type of regeneration.

In cultures of *Anacardium* explants, indirect somatic embryo formation was also the most frequently observed mode, since, in the majority of the protocols, callus development preceded somatic embryo formation.

3 Factors Crucial for SE Induction

Physiological state of an explant-donor plant, genotype, age, the external environment which includes composition of media and physical culture conditions (temperature, light), type of plant and developmental stage of an explant are some factors that determine the in vitro development of cells and tissues. Somatic cells from an explant, callus, or suspension cells produce embryogenic cells which turn into somatic embryos. Just a few somatic cells are responsive to embryogenesis induction factors and capable of enduring somatic embryogenesis. Such cells, which represent an intermediate state between somatic and embryogenic cells, are called competent. Competent cells exhibit sensitivity to physical and chemical stimuli that initiate the embryogenic pathway of their development, while embryogenic cells are already established during embryogenesis. Embryogenic cells undergo embryogenesis without stimulation by external stimuli (De Jong et al. 1993). A prerequisite for the successful establishment of somatic embryogenesis is a proper choice of plant material. The experiments establish in the special conditions required successful embryonic induction. This is without knowing why a given genotype/explant has embryogenic potential and how and why competence or commitment is reached or what is the real initiator of the development of the embryo.

4 Effects of Media Composition on Growth and Development of Cashew

The components of plant tissue culture media can be classified into inorganic salts, organic compounds, complex natural preparations, and inert support materials (Huang and Murashige 1977). The nutrient media determines the success of plant cell and organ cultures. The recalcitrance of certain species can rise above by manipulating other media components (Birhman et al. 1994). The study of Samson et al. (2006) has shown that two- or four-fold dilution of the MS salts increased the development of coffee embryogenic callus rate by 2.6 and 5.7, respectively, in comparison to full strength MS salts. This clearly shows that modifications of the medium, mainly manipulating the doses of inorganic salts and vitamins, can have a significant effect on somatic embryogenesis possibly by altering the osmotic potential of the medium. The ions of different types are the active factor in the medium rather than the compounds. One type of ion can be provided by more than one compound. Levels developed by Murashige and Skoog (1962) for tobacco tissue culture (Nassar 2004) "MS medium" (Murashige and Skoog 1962) is the most widely used plant culture medium (Vuylsteke 1989) where inorganic salts levels used in most plant tissue culture media are based. In cashew culture, formulations media were multiple with modifications of MS media (MS/2; MS/4) (Browning et al. 1987). Other popular media include B5 (Gamborg et al. 1968), SH (Schenk and Hildebrant 1972), N6 (Chu et al. 1975), and LS (Linsmaier and Skoog 1975) media

(Hussein 2012; Saad and Elshahed 2012). The proximal part of the cotyledons produced somatic embryos when they were initially cultured on SH medium with NAA and BA (Sy et al. 1991). Hegde et al. (1994) observed that pieces of cotyledons produced somatic embryos on LS medium with Ca pantothenate.

5 Carbon Source

Sugars are an essential energy source in all tissue culture media and create the appropriate osmotic conditions for cell growth in vitro. The type and concentration of sugar added in the media influence somatic embryogenesis. The carbohydrate of choice as a carbon source usually is sucrose probably because it is the most common carbohydrate in plant phloem (Murashige and Skoog 1962; Thorpe 1980; Lemos and Baker 1998; Fuentes et al. 2000; Ahmad et al. 2007). Sucrose has been used most often to induce somatic embryos in different plant species including cultures of *Asparagus officinalis* (asparagus) (Levi and Sink 1990). It has been showed that carbohydrates added to the culture medium may play several roles, including the histo-differentiation of somatic embryos through the direct regulation of gene expression (Lipavská et al. 2000).

In the cashew case, sucrose is an important element in the induction of somatic embryos and has been most frequently employed for their induction. Kembo and Hornung (1999), Gogte and Nadgauda (2000), and Martin (2003) induced embryogenic cultures by using 3% of sucrose while Cardoza and D'Souza 2002 used 2% of sucrose. Gogte and Nadgauda (2000) induced somatic embryos by using 4% of sucrose whereas Ananthakrishnan et al. (1999) and Shirly and Thimmappaiah 2005 used 6% of sucrose to obtain somatic embryos.

6 Light Conditions

One of the most important environmental signals is light, and its numerous effects on plant growth and development are so much known. Despite the fact that in every protocol on somatic embryogenesis induction light requirements were described, systematic research on the light effect on in vitro response of cultured explants was limited. Morphogenic response and its efficiency can be influenced by the spectrum, intensity, and duration of the light supplied for in vitro cultures. To minimize the production of inhibitory compounds from tissues in the culture medium, it has been suggested that cultures should be maintained in reduced light intensity or in darkness (Evans et al. 1983). 49% and 44% of the surveyed protocols for SE induction showed the necessity of mostly photoperiod or darkness requirement, respectively (Gaj 2004). Several works in cotyledon culture like *Helianthus annuus* (common sunflower) (Fiore et al. 1997), *Malus domestica* (apple) (Paul et al. 1994), leaf culture of *Camellia reticulata* (Camellia) and *Camellia japonica* (Camellia)

(San-Jose and Vieitez 1993) have revealed the beneficial effect of darkness on somatic embryogenesis induction.

Generally, *Anacardium occidentale* experiments were done in dark because of the richness of phenolic compounds (Aliyu 2005). Studies in the nucellar culture of cashew revealed a gainful effect of darkness on somatic embryogenesis induction (Shirly and Thimmappaiah (2005)).

7 Explant Type

The most important factor which determines the embryogenic capacity of the culture seems to be the type of explant and its well-defined developmental stage. Efforts to select a responsive explant were focused on finding plant tissues containing competent cells, i.e., capable of undergoing somatic embryogenesis following stimulation by applied external factors (Gaj 2004).

The explants are a source of competent cells, and, on the other hand, determination of physical and chemical factors which switch on their embryogenic pathway of development.

Different types of explants used to induce somatic embryogenesis showed variable responses involving the effect of several factors such as tissue or organ type, harvest time, age, light effect and genotype as found by Fiore et al. 1997 in the cotyledon culture of *Helianthus annuus*. Limited embryogenic capacity at a short developmental stage has been frequently observed in zygotic embryos used for SE induction. In the case of zygotic embryos used for somatic embryogenesis induction, embryogenic capacity restricted to a short-lasting stage of development was often noticed. The greater embryogenic potential is most frequently presented by younger zygotic embryos (Maheswaran and Williams 1984; Eady et al. 1998; Garin et al. 1998). However, between embryogenic ability and the degree of zygotic embryo maturity, a reverse correlation was reported in some plants (Turgut et al. 1998; Choi et al. 1998) including Arabidopsis (Gaj 2001).

Cashewnut was found difficult to propagate in vitro from mature plant tissues (nodal segments or shoot apices) due to its recalcitrant nature, microbial contaminations, and high phenolic exudation (Aliyu 2005). Therefore, efforts have been made to find a suitable alternative explant source. In this effort, nucellus tissues from developing seeds were selected because the embryogenic ability of the nucellus was well established in *Vitis vinifera* (European grape) (Mullins and Srinivasan 1976), *Ribes rubrum* (Red currant) (Zatyko et al. 1975), *Myrciaria cauliflora* (Jaboticaba) (Litz 1984), and *Mangifera indica* (Mango) (Litz et al. 1984). Although the morphogenetic potential of the nucellus has been found apparent in the past several years, there have been only a few successful attempts to exploit this tissue for in vitro studies involving woody plants (Bonneau et al. 1994). The first attempts to induce embryogenic cashew cultures involved seed explants (Jha 1988; Lakshmi Sita 1989; Hegde et al. 1990, 1991; Sy et al. 1991). Somatic embryos were obtained from cotyledon pieces of 6–8-week-old germinated seedlings (Lakshmi Sita 1989),

cotyledons from mature seeds (Sy et al. 1991), and sections of immature cotyledons (Hegde et al. 1994). Jha (1988) reported morphogenesis in callus cultures derived from zygotic embryos and the occurrence of globular protuberances which developed into embryo-like structures while Hegde et al. (1992) observed embryogenesis in cotyledonary segments. However, the obtained embryos could not be germinated. Cardoza and D'Souza (2000) reported induction of direct somatic embryos from the radicular end of zygotic embryos. Secondary embryos developed from the primary embryos. However, conversion of embryos to the whole plant was not achieved. Kembo and Hornung (1999) explored the possibility of developing a simple but efficient method of optimizing cultural conditions necessary for the induction of callus using plumular tissue excised from mature zygotic embryos of cashew as explants and comparing with the response of cotyledonary tissue. Responses extended from swelling of embryos and cotyledons to the proliferation of adventitious roots and white callus-like structures. A number of combinations produced abnormal plants with curled leaves and multiple shoots. Ananthakrishnan et al. (1999) and Gogte and Nadgauda (2000) induced embryogenic cultures from (elite) nucellar cultures. The mature embryos germinated but not convert into complete plantlets. Plant regeneration through direct somatic embryogenesis was established on cashew using seed coat explants (Martin 2003). Induction of somatic embryos on different explants excised from immature zygotic embryos like excised cotyledons and excised hypocotyl with radicle, as well as on intact zygotic embryos as small as 1–2 mm has been obtained.

8 Stress Factors

Nowadays, it is known that somatic cells can acquire embryogenic potential as a result of various external chemical and physical stimuli, generally referred to as stressors. Dudits et al. (1995) claimed that stress is an essential component of embryogenesis where the development of somatic embryos is induced. Its action in the induction of embryogenesis of microspore cultures has been demonstrated by the work of Touraev et al. (1996) and Dunwell (1996). Various factors, such as osmotic pressure, heavy metal chlorides, pH, high or low temperatures, starvation, mechanical injury of explants, or high level of auxin can stimulate the embryogenic competence of somatic cells cultured in vitro. This observation is corroborated by the work of Kiyosue et al. (1993). The importance of the interaction between auxin and stress signaling which results in the acquisition of the embryogenic competence of the somatic cell by a broad cellular reprogramming manifested at different levels (Feher et al. 2003) is one of the hypotheses highlighted on the mechanisms involved in stress-induced embryogenesis. Regardless of the precision of the mechanism, stress treatment triggers the expression of factors that affect cell cycle regulation, gene expression and thus induce somatic embryogenesis. As several experimental observations show, the differentiated fate of plant cells, depending on positional information and developmental signals, can be easily modified under in vitro

conditions. Exposure of injured cells or tissues to suboptimal nutrients or hormones supply (e.g. under in vitro culture conditions) constitutes a radical change in the cellular environment, which generates significant stress effects. The response to stressful conditions depends on two main parameters: the stress level and the physiological state of the cells. If the stress level exceeds cellular tolerance, the cells die. In contrast, lower stress levels improve metabolism and induce coping mechanisms (Lichtenthaler 1998). Adaptations include reprogramming of gene expression, as well as changes in the physiology and metabolism of cells. Stress alters source/sink regulation by activating sink-specific enzyme genes alongside the defense against stress (Roitsch 1999).

9 Endogenous Hormones

Endogenous hormone levels can be considered as major factors in determining the specificity of cellular responses to rather general stress stimuli. In recent years, a large number of experimental observations have focused on the central roles of endogenous levels of indoleacetic acid (IAA) and abscisic acid (ABA) during the early stages of embryogenesis (Feher et al. 2003). The different levels of endogenous phytohormones in various explant tissues could be a factor influencing the requirements of exogenous growth regulators. The effect of genotype on somatic embryogenic competence has been clearly demonstrated. The presence of varying levels of endogenous phytohormones, in particular cytokinins, in different genotypes could influence their response to somatic embryogenesis. Wenck et al. (1988) observed that orchard grass genotypes in which embryogenesis was difficult to induce contained significantly higher levels of endogenous cytokinins than embryogenic genotypes.

Plants are sessile organisms that have endogenous signals to cope with biotic and abiotic challenges (Gilroy and Trewavas 2001). Phytohormones are chemical signals that produce low concentrations and travel around the plant, triggering various responses in tissues and cells. The level of endogenous phytohormones is considered to be one of the crucial factors influencing the embryogenic potential of explants. The quantity and quality of endogenous hormones (auxins, cytokinins, and ABA) were found to be different in the petioles of *Actinidia deliciosa* (kiwifruit) (Centeno et al. 1996), in the zygotic embryos of *Corylus avellana* (Hazel) (Centeno et al. 1997), *Triticum aestivum* (Wheat) (Hess and Carman 1998), and the cotyledons of *Panax ginseng* (Chinese Ginseng) (Choi et al. 1997) show different embryogenic potential. In *Arabidopsis*, an elevated level of auxins has been reported in cotyledons primordia (Ni et al. 2001), which correlates with the embryogenic competence displayed by cotyledonary parts of zygotic embryos (Luo and Koop 1997; Gaj 2001).

10 Role of Plant Growth Regulators (Auxins and Cytokinins) on Embryogenesis

Plant growth regulators play a critical role in determining the development and developmental pathway of the plant cells in tissue culture. This may be due to the accumulation of specific biochemical compounds. Addition of one or more growth regulators to the medium resulted in the maintenance of specific and balanced inorganic and organic compounds in the growing tissue. This leads to the development of cells or tissues into shoots or roots, or even death. Dahot (2007) underlined this well in these works. The high efficiency of 2,4-D for the induction of the embryogenic response found in different in vitro systems and plant species indicates a specific and unique character of this plant growth regulator. It is an auxin herbicide, synthetic growth regulator which appears to act not only as an exogenous auxin analog, but also as an effective stress agent. Several works including that of Feher et al. (2003) prove how 2,4-D causes various changes in the physiology and gene expression of cells, implying its possible role as a stress factor triggering the model of embryogenic development in plant cells in culture. The stress-like action of plant growth regulators should also be taken into account when very high concentrations of exogenous auxins for induction of somatic embryogenesis were required to induce somatic embryos in certain plant systems. For example, 452 M of 2, 4-D was effective in the culture of *Serenoa repens* (saw palmetto) (Gallo-Meagher and Gerrn 2002) and more than 200 M of NAA in *Pisum sativum* (Pea) (Özcan et al. 1993).

Studies on cashew have revealed the importance of PGR_s. According to Lakshmi Sita (1989), cotyledon explants on medium containing Naphthalene Acetic Acid (NAA), 2,4-D, and Benzyl Adenine (BA) developed embryogenic cultures. Then, somatic embryos formed in the presence of NAA, BA, or kinetin. Sy et al. (1991) showed that the proximal part of the cotyledons produced somatic embryos when they were initially cultured on Schenk and Hildebrandt (SH) (1972) medium with NAA and BA, in the presence of casein hydrolysate and adenine sulfate. Hegde et al. (1994) noticed that pieces of cotyledons formed somatic embryos on LS medium with pantothenate of Ca, IAA, and BA under a photoperiod of 20 h. It was also observed that leafy shoots and roots develop on the medium supplemented with activated charcoal. Despite this, there was in general poor organization of shoot meristems.

Somatic embryogenesis potentially offers many possibilities for large-scale plant propagation. Biotechnological applications such as genetic modification of trees to select desired stress tolerance traits and gene transfer are uses of somatic embryogenesis. Recently, the use of nucellus tissues from developing seeds for the induction of somatic embryogenesis has been investigated by Ananthakrishnan et al. (1999) and Cardoza and D'Souza (2000). Ananthakrishnan et al. (1999) observed callus induction from nucellar explants excised from 1-month-old developing cashew fruits on MS medium containing 6.78 μM 2,4-D. The differentiation of the calli into somatic embryos was noticed when calli were transferred to a liquid MS medium

supplemented with 4.52 μM 2,4-D. Different stages of development of the somatic embryos were recorded but there was no further development of the torpedo stage in this liquid medium containing 2,4-D. The conversion of somatic embryos to whole plants has not been achieved. In 2000, Gogte and Nadgauda have mentioned early cotyledonary somatic embryos with a combination of 2,4-D and GA_3 from cashew nucellar tissue. But these embryos did not fully develop. Studies using nucellar calluses in the presence of picloram were conducted by Cardoza and D'Souza (2000) who revealed the development of globular somatic embryos. The maturation of these globular somatic embryos was carried out in the presence of picloram and putrescine and germination was obtained in basal MS medium. Moreover, somatic embryogenesis has also been reported using the immature zygotic embryo as an explant (Cardoza and D'Souza 2000; Gogate and Nadgauda 2003). In both cases, somatic embryos were formed directly from the tip of the radicle or the end of the radicle of the immature zygotic embryo. Therefore, the presence of picloram (2.07 μM) (Cardoza and D'Souza 2000) or 5 μM 2,4-D + 5 μM BAP + GA3 (3 μM) (Gogate and Nadgauda 2003) favored the formation of somatic embryos. A medium with 20 μM of ABA and 3% maltose was used for the maturation. Gogate and Nadgauda (2003) and Cardoza and D'Souza (2000) reported the germination of somatic embryos on a medium lacking growth regulator.

11 Effect of Other Biochemical Factors on Somatic Embryogenesis

Some researchers: Ichihashi and Islam (1999), Islam et al. (2003), and Rahman et al. (2004) have pointed out that the addition of complex organic extracts, such as coconut water, the extract of taro, potato extract, corn extract, and papaya extract are essential for somatic embryogenesis in some species. In addition, it has also been noted that these organic extracts were either non-mutagenic or less mutagenic compared to conventional growth regulators. As such, Lam et al. (1991) asserted that their incorporation into culture media can minimize somaclonal variations. However, because it was not possible to determine which particular constituent of the extract promotes somatic embryogenesis and ensure the consistency of the actual extract each time it was prepared, it can also be said that organic extracts were undefined components.

12 In Vitro Recalcitrance, Phenolic Exudation, and Tissue Browning

Several studies including that of Krishna and Singh (2007) on woody species showed that some of the major problems in the culture of plant tissues of woody species include in vitro recalcitrance of plant tissues and associated phenolic exudation on browning of the media and explants. These factors are closely related, and their interactions are not fully understood. Recalcitrance occurs when plant cells, tissues, or organs do not respond to in vitro culture manipulations that would otherwise induce somatic embryogenesis or organogenesis. This is a major obstacle in plant tissue culture applications (Benson 2000). In particular, *Anacardiaceae* species are well known to be recalcitrant to in vitro treatments. Several authors (Boggetti et al. (2001); Ananthakrishnan et al. (2002)) reported the in vitro recalcitrance of *Anacardium occidentale*. Benson (2000) demonstrated that physiology of the donor plant, in vitro manipulations, and in vitro plant stress physiology are a number of factors that influence or trigger recalcitrant responses. Thorough knowledge of the life cycle of the donor plant, such as the phases of reproduction, rejuvenation, and dormancy, is therefore necessary to minimize the effects of recalcitrant plant tissues as shown by the work of McCown (2000). Careful handling of the in vitro environment can help overcome the problem of recalcitrant plant tissue. According to Benson (2000), this can be achieved by having an optimal balance of auxins and exogenous cytokinins in the growth medium. Further, he suggested applying compounds that are not strictly plant growth regulators, such as polyamines and antioxidants, as a means of alleviating in vitro recalcitrance. Kumar et al. (1998) have shown that in vitro stress physiology can induce an accumulation of ethylene in culture vessels, which ultimately reduces cell proliferation. Many plants are rich in phenolic compounds. Therefore, after tissue damage, these compounds are oxidized by polyphenol oxidases and the tissue turns brown. Evans et al. (1983) demonstrated that oxidation products are known not only to darken tissue but also inhibit the activity of various proteins which may have an inhibitory effect on somatic embryogenesis. According to Gannoun et al. (1995), Raghuvanshi and Srivastava (1995), Thimmappaiah et al. (2002a), Tabiyeh et al. (2006), and Krishna et al. (2008), browning of excised explants and the resulting discoloration of culture media remains one of the main challenges in plant tissue culture systems of *Anacardiaceae* species. The browning encountered in plant tissue culture was caused by the oxidation of phenolic compounds which are released into the medium during the excision of explants. Raghuvanshi and Srivastava (1995) reported that exudation of phenolic compounds from the wound site of the explant impairs the regenerative capacity of plant cells in vitro. Krishna et al. (2008) showed that when phenolic compounds are released from vacuoles due to excision of explants, they are released into the cytoplasm where the oxidative enzyme system is activated, resulting in tissue browning. As the work of Robards et al. (1999) has shown, one of the key enzymes involved in the oxidation process is polyphenol oxidase (PPO), a copper-containing oxidase responsible for the catalysis of O_2 dependent oxidation of

catechols to quinones. The optimum pH of 5.8 used in plant tissue culture is conducive to PPO activity, the optimum pH range of which is 5.0–7.0. Other enzymes in the wound-induced browning process include peroxidase (POD) and phenylalanine ammonia lyase (PAL). Tabiyeh et al. (2006) demonstrated that the activity of PAL, a key enzyme in the phenylpropanoid pathway, triggers the synthesis of phenylpropanoid compounds responsible for browning. In addition, PPO has been shown in research works of Robards et al. (1999) and Krishna et al. (2008) to synergistically stimulate the activity of POD through the generation of its substrate (H_2O_2) during the oxidation of phenolic compounds. To mitigate the negative effects of tissue browning in the micropropagation of *Anacardiaceae s*pecies, various pretreatments targeting inhibition of key oxidative enzymes have been used. Gannoun et al. (1995), Thimmappaiah et al. (2002a), Onay et al. (2004), Tabiyeh et al. (2006), and Krishna et al. (2008) had shown that several antioxidant compounds, used alone or in combination, such as ascorbic acid, citric acid, and salicylic acid have provided effective levels of browning control in in vitro culture of *Anacardiaceae* species. Additionally, adsorbent materials such as polyvinylpyrrolidone (PVP) and activated charcoal have been used to control browning effects in vitro. Success in controlling browning using PVP has been reported in the in vitro culture of *Pistacia vera* (pistachio) (Gannoun et al. 1995), *Mangifera indica* (Raghuvanshi and Srivastava 1995), and *Anacardium occidentale* (Ananthakrishnan et al. 2002). Likewise, in several cashew studies Das et al. (1996, 1999), Ananthakrishnan et al. (1999), Gogte and Nadgauda (2000), and Thimmappaiah et al. (2002b, c) the control of browning in vitro has been controlled or minimized by the application of activated charcoal. Activated carbon was often used in plant tissue culture to enhance cell growth and development (Pan and Van 1998). Its influence on growth and development can be attributed mainly to the adsorption of inhibitory substances in the culture medium (Fridborg et al. 1978; Horner et al. 1977; Theander and Nelson 1988; Weatherhead et al. 1978, 1979), drastically reducing phenolic oxidation or accumulating brown exudates (Carlberg et al. 1983; Liu 1993; Teixeria et al. 1994), changing the pH of the medium to a level optimal for morphogenesis (Owen et al. 1991) and establishing a darkened environment in the medium and thus simulating soil conditions (Dumas and Monteuuis 1995). Other anti-browning techniques described by Krishna et al. (2008), such as etiolating of mother plants and frequent subcultures in fresh medium, have had limited success in the in vitro culture of *Anacardiaceae* species. The exudation of phenolic compounds and the subsequent browning of the explants were reduced to some extent by frequently (initially every 2 days for the first week, followed by weekly subcultures during the induction phase) subculturing the explants, by using activated charcoal (0.3%) and PVP (0.5%) in the culture media, and incubation in the dark. Explant necrosis has also been attributed to the effect of strong disinfection. The use of explants from seedlings germinated in vitro or young shoots treated with a fungicide has been shown to improve the success rate considerably.

13 Conversion of Somatic Embryos into Plants

Further development and completion of maturation are induced by abscisic acid applications and the imposition of a drying period as well as the initiation of embryo development is stimulated by the removal of PGRs. Joy IV et al. (1991) showed that both treatments are necessary for the production of morphologically and physiologically mature embryos. Under a dissecting microscope, embryos appear as yellow nodules surrounded by translucent embryogenic tissue. After 2 weeks, the size of the embryos increases and they become larger in relation to the tissue mass, due to the increase in mitotic activity. A well-defined protoderm and organized root and shoot tips are visible at this time. Then, the apical poles of the embryos take the shape of a dome due to the formation of functional shoot apical meristem. At the root pole, the root cap begins to form. It is only after 3 weeks of culture that the apical meristems are completely different. At this stage, the embryos have developed a visible procambium and the cotyledons (4–6) that begin to emerge from the apical pole. The axis of the embryo lengthens, and the embryos acquire a creamy yellow coloration indicating the deposition of storage products, namely starch and later proteins. Cotyledonary embryos are produced after 4 weeks of maturation. They have reached a length of about 2–3 mm and are characterized by the presence of fully expanded cotyledons. At this time, embryos have accumulated a large amount of storage products, including proteins and lipids. According to Etienne et al. (1993), slow desiccation improved germination and was more effective in stimulating conversion into plantlets. Slow desiccation resulted in a substantial accumulation of starch and protein reserves necessary for the continued development of immature embryos compared to rapid dehydration. Therefore, desiccation could be used to improve germination as the embryo approaches physiological maturity. In large-seeded species, the sequential events during the formation of a fully mature zygotic embryo are very complex. Wang and Janick (1984) asserted that the lack of complete understanding of these processes underlying maturation, therefore, poses difficulties in reproducing the normal development of somatic embryos in vitro in these species. This may be one of the reasons for the problems encountered in somatic embryo maturation experiments in the current system, as the cashewnut is a large-seeded species whose cotyledons make up the majority of the seed mass. The development of cotyledons and the accumulation of storage products surely play an important role in the physiological maturation of the embryo in vivo. The application of in vitro methods based on somatic embryogenesis for plant regeneration is not only by high efficiency of somatic embryos formation but frequently depends on the capacity of embryos for plant development. Redenbaugh et al. (1986) explained that the process of developmental changes undergone by a somatic embryo, called conversion, involves the formation of primary roots, a shoot meristem with a leaf primordium, and the greening of hypocotyls and cotyledons. Despite the high number of somatic embryos produced, it has often been noticed, problems of lack or low frequency of conversion of embryos into plants.

Gogte et al. (2000) observed developmental abnormalities including fused embryos, lack of cotyledon formation, fused cotyledons, multiple cotyledons, and asymmetric cotyledon development in *Anacardium occidentale*. Jha and Das (2004) found that a period of preconditioning or post-maturation was essential for the germination of somatic embryos and germination was obtained after 4 to 5 weeks of preconditioning on MS media containing BA (1.0 mg/l). However, only 8.05 to 23.2% of cases were somatic embryos induced on different explants. Somatic embryogenesis from immature embryos via the callus phase had been attempted in cashew by Jha (1988) but resulted in the development of neomorph-like structures with disproportionate root/shoot ratios. Direct embryogenesis from pieces of immature cotyledons was attempted by Hegde et al. (1994), and embryoids were obtained without an intermediate callus phase. These embryoids were unable to develop further into complete plantlets. The studies by Lazzeri et al. (1987) and Sofiari et al. (1997) revealed a detrimental effect of 2,4-D on the regeneration capacity of somatic embryos. Frequently, ÖZcan et al. (1993) and Rodriguez and Wetzstein (1994, 1998) observed the development of malformed embryos, including multicotyledon or "fan-shaped" embryos, when 2,4-D was included in an induction medium. Choi et al. (1997) and Yasuda et al. (2000) extrapolated that such morphological abnormalities could result from disturbances in the polar transport of endogenous auxins caused by external PGRs. This has been noticed by Fischer et al. (1997) and Hadfi et al. (1998) in the treatment of developing zygotic embryos in vitro with polar auxin transport inhibitors, or exogenously applied auxins, which led to a wide range of morphogenetic alterations, including polyembryos and others strictly resembling malformed somatic embryos.

14 Defiance in Getting Somatic Embryos

Several advances were reached until now about the development of protocols for somatic embryogenesis of *Anacardium occidentale*. Yet, converting somatic cells into somatic embryos is not an easy task, and among the advances, some problems remain. Considering the several aspects involved in the initiation and permanence of the SE process, several variables may be investigated.

The development of cryopreservation protocols is an important step towards promoting the conservation of genetic material for *A. occidentale*. In 2001, Guerra et al. demonstrated that the mechanical strength of the alginate capsule combined with low-temperature storage prevents the germination of encapsulated somatic embryos of *Acca sellowiana (*Pineapple Guava*)*. Likewise, Cangahuala-Inocente et al. (2007) observed a significant increase in the survival of plantlets compared to the direct conversion of encapsulated somatic embryos. Gogte and Nadgauda (2000) showed that the rate of abnormal somatic embryos in cashewnuts is high. Therefore, conversion to temporary immersion systems, adjustment of plant growth regulator concentrations, duration of induction phase, and conditions for somatic embryos are also fundamental issues for future studies towards an efficient protocol

of somatic embryogenesis of *Anacardium occidentale*. Finally, Aguilar et al. (2006) and Aizen and Vazquez 2006 have concluded that global environmental change has and will have several effects on the reproductive biology of plant populations. The capacity of plant populations to reproduce successfully, with consequences for their demography evolution, and long-term persistence will be all directly impacted by climate change, habitat fragmentation, and pollinator decline. Further research on the biology of plant reproduction will therefore be of crucial importance for transaction with these environmental challenges and for maintaining biodiversity, genetic resources, and human well-being. Considering all these findings led us to envisage that in the worst scenario zygotic embryonic and seed development would be limiting. Hence, the development of trustworthy in vitro regenerative methods based on somatic embryogenesis should be a possible alternative to be considered.

15 Conclusion

Somatic embryogenesis, one of the most important tools in plant biotechnology, can be used to clone an individual plant at a relatively low cost. Under specific conditions, a single cell can reprogram itself from its original specialization to an embryogenic state, especially in plant species considered to be recalcitrant in somatic embryogenesis. Establishing detailed culture conditions, including the sequence and periodicity of the media applied, can be effective. So far, many advances have been made in the somatic embryogenesis of *Anacardium occidentale*, encouraging the use of this technology in new perspectives, in addition to genetic improvement and production of clonal forests. Our growing knowledge of somatic embryogenesis and other in vitro micropropagation procedures gives hope for a bright future, thus allowing progress in the conservation and sustainable use of plant genetic resources around the world.

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