Improving the Availability of Valuable Coconut Germplasm using Tissue Culture Techniques

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Abstract

Coconut cultivation faces a number of acute problems that reduce its productivity and competitiveness. These problems include various biotic and abiotic challenges as well as an unstable market for its traditional oil-based products. Around 10 million small-holder farmers cultivate coconut palms worldwide on c. 12 million hectares of land, and many more people own a few coconut palms that contribute to their livelihoods. Inefficiency in the production and supply of appropriate seedlings for replanting a generally ageing resource remains an issue, especially where there is uncertainty about the ability of local populations to resist potential disease threats. However, tissue culture in such cases is expected to provide pragmatic solutions. Over the past 60 years much research has been directed towards developing and improving protocols for (i) embryo culture; (ii) clonal propagation via somatic embryogenesis and (iii) germplasm conservation via cryopreservation. Recent advances have provided new ways to improve these protocols, especially cryopreservation. Although effective embryo culture and cryopreservation are now possible, the low efficiency of conversion of somatic embryos to ex vitro seedlings still restrains the large-scale clonal propagation of coconut. Although tissue culture in coconut has developed over the recent decades, further improvement of protocols and their application to a wider range of germplasm will boost their adoption for the breeding, conservation and propagation of coconut.

Keywords: Coconut, Cryopreservation, Embryo culture, Germplasm conservation, Somatic embryogenesis

Introduction

Together with the instability of the market for its traditional products, coconut field cultivation faces a multitude of challenges. In addition, productivity is affected by palm age, declining steadily after 35 years due to a decline in leaf area, by the rundown of soil nutrients, and through damage caused by cyclones, storms and tsunamis (Sisunandar et al. 2010a; Samosir and Adkins 2014). Speedy invasion of major pests, such as leaf beetles, and incurable diseases, such as phytoplasma-related lethal yellowing and viroid-caused cadang-cadang, have all resulted recently in a significant decline in the land area planted to coconut (Cordova et al. 2003; Harrison and Jones 2003; Lee 2013). Despite major efforts, through breeding programs aiming to increase oil yield in many countries, the general expectation for a higher, stable yield has not been realized (Samosir and Adkins 2004). A ‘conventional’ breeding approach to coconut improvement alone, involving multiple generations of inbreeding and finally hybridization, is unlikely to be a robust solution for increasing productivity (Thanh-Tuyen and De Guzman 1983; Batugal et al. 2009). The expectation that a plant adapted to the

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strand could be highly productive in a plantation structure, matching the oil palm for yield, for example, is perhaps misplaced.

It has been more than 60 years now since the first in vitro culture study was carried out on coconut (Cutter and Wilson 1954). Since then the landmark research achievements in coconut tissue culture have not been attained as much as they have for many other plant species. Some of the reasons often refer to the slow development of tissues in culture, and the varied response of the diverse range of coconut explant tissues used. Dominant features are the slow growth of these explant tissues in vitro, and their further lack of vigor when planted ex vitro (Fernando et al. 2010). Nonetheless, tissue culture and associated biotechnological interventions for coconut have been achieved in these three major aspects: (i) embryo culture; (ii) clonal propagation via somatic embryogenesis (SE); and (iii) germplasm conservation via cryopreservation. Significant achievements in zygotic embryo culture have now enabled the collection of rare germplasm, and the rapid production of tissue culture-derived seedlings (Rillo 1998). The technique has been improved recently to meet a greater expectation across a wider range of cultivars (Samosir and Adkins 2014), while zygotic plumular tissue can now be used to achieve clonal propagation via SE (Pérez-Núñez et al. 2006). However, difficulties in this process still hinder the establishment of a feasible protocol for the production of plantlets on a commercial scale.

This paper reviews the advances to date in tissue culture and the associated biotechnological approaches applied to coconut, a physiologically recalcitrant species. Through an analysis of past notable achievements, we hope the paper can assist researchers to refine and discover novel approaches for improving the quality and resilience of the ‘tree of life’.

**Germplasm rescue and sterile movement via embryo culture**

The most commonly used culture medium for coconut embryo culture is the Y3 medium developed by Eeuwens (1976), even though different ones have been shown to support zygotic embryo germination and growth. A high level of sucrose (> 4%) has been reported as an essential supplement for in vitro embryo germination. Activated charcoal has been used in many cases to help prevent tissue browning (Ashburner et al. 1993; Triques et al. 1997). Medium solidifiers, such as agar (1.5 to 0.8%), are often used to create a gelled substrate for the early stages of germination; however, recent studies report the use of a two-stage system including embryo culture in a liquid medium to obtain germination. This is followed by transfer to a gelled medium (Rillo 1998) or to nutrient-saturated vermiculite (Samosir and Adkins 2014) for seedling growth. More recently, other gelling agents such as Gelrite (Pech y Aké et al. 2004; Pech y Aké et al. 2007) and the addition of plant growth regulators including gibberellic acid (0.5 μM) have been reported to promote the rate and number of embryos germinating. Furthermore, synthetic auxin analogues such as naphthalene acetic acid (NAA) or indole-3-butyric acid (IBA) have been shown to promote root growth in the later stages of germination and during early seedling growth (Ashburner et al. 1993; Rillo 1998). The acclimatization of embryo-cultured plantlets has been achieved in many coconut cultivars using a number of potting soils and nursery conditions. A photoautotrophic and sucrose-free protocol, using CO2-enrichment (1,600 μmol mol−1) during the light phase, was found to improve seedling health and growth, and also the percentage of seedlings established from culture (Samosir and Adkins 2014).

Embryo culture has become an indispensable method for the collection of coconut germplasm from remote areas, and allowing long-distance delivery to the laboratory. An early-modified form of coconut germplasm collection involved the isolation of the mature embryo in the field, and its placement in a vial filled with sterile water or coconut water before being transported back to the laboratory (Rillo and Paloma 1991). A more proficient protocol was then developed which retained the embryos in a sterile state, embedded in a plug of solid endosperm recovered using a 2.5-cm diameter cork borer. This technique was further improved by the on-site surface sterilizing of the
endosperm plugs, placement in an ascorbic acid solution and retention at a cool temperature (ca. 5°C) during transport back to the laboratory (Adkins and Samosir 2002).

Even though embryo culture has been successfully achieved with many coconut cultivars, and can serve as a reliable tool for germplasm collection and exchange, the success rate of mature plants flourishing in soil can be low. There is also the risk of pathogenic viruses being present in embryo-cultured materials when they are intended to be moved and replanted. Therefore, the applicability of this technique to all coconut cultivars is still to be assessed and optimised as well as developing a mode of delivery to the smallholder.

**Clonal propagation via somatic embryogenesis**

Coconut SE research commenced over 30 years ago at Wye College, UK (Eeuwens and Blake 1977), and was continued by ORSTOM, France (Pannetier and Buffard-Morel 1982). These studies made use of a number of plant somatic tissues as initial explants (i.e. young leaves, stem slices from young seedlings, sections taken from rachillae of young inflorescences) to form embryogenic calli (Branton and Blake 1983; Gupta et al. 1984). However, more recently, there has been a shift to use either somatic tissues (e.g. immature inflorescences, ovaries) or to the easier to manipulate zygotic tissues (e.g. immature or mature embryos and embryo-derived plumules) to achieve SE in coconut (Samosir et al. 1998; Pérez-Núñez et al. 2006). More recently, recognising that only true somatic tissues can be used to produce true-to-type clones, some attention has returned to tissue explants taken from young inflorescence tissues (Antonova 2009).

The Y3 (Eeuwens 1976) and BM72 (Karunaratne and Periyapperuma 1989) media have been the most frequently used for callus culture, while MS (Murashige and Skoog 1962) and B5 (Gamborg et al. 1968) have been found to be less effective (Branton and Blake 1983; Bhallasarin et al. 1986). The inclusion of sucrose (3 to 4%) has proved to be important for coconut SE to take place, with activated charcoal (0.1 to 0.3%) typically applied to prevent explanted tissues and callus from browning, which is a stress-related response caused by the release of secondary plant products such as phenols, or ethylene (Samosir 1999). Dissimilarity in particle size, and the potency of the various activated charcoal types, has been shown to influence the frequency of somatic embryogenic callus formation (Sáenz et al. 2009). Polyvinylpyrrolidone (PVP), another common toxin-absorbing agent, was tested in coconut leaf-derived cell suspension cultures, but without any significant effect (Basu et al. 1988). However, polyvinylpolypyrrolidone (PVPP), used in zygotic embryo–derived callus culture, was found to have some positive effect in promoting the rate of SE (Samosir 1999).

Similar to other species, the sequential development of clonally propagated coconut plantlets is typically divided into three stages: firstly the production of callus and its proliferation; secondly the formation, maturation and germination of somatic embryos; and thirdly the acclimatisation of the plantlets to ex vitro conditions. Apart from 2,4-D, other synthetic auxins such as NAA (27 μM) in combination with 2,4-D (452 μM) have been used to promote callus formation on rachillae explants (Gupta et al. 1984). In addition, a study of the ultrastructural changes that take place during the acquisition of SE potential suggests that the gametophyte-like conditions produced by 2,4-D are required for a successful transition from the vegetative into the embryogenic state (Verdeil et al. 2001).

Supplementation of the callus proliferation and maturation medium with a cytokinin such as 6-benzylaminopurine (BAP), thidiazuron (TDZ), kinetin (Kin) or 2-isopentyl adenine (2iP), at 5 to 10 μM is also common place (Perera et al. 2009b). Callus formation is often best achieved in the dark for at least 1 month after culture initiation, and at 28 ± 1 °C (Adkins et al. 1998). However, in one study, dark incubation was extended to 3 months to achieve greater callus production (Pérez-Núñez et al. 2006). Further improvement in the timely production of embryogenic callus has been achieved by
applying into the medium one of the multifunctional polyamines, particularly putrescine (7.5 mM) or spermine (1.0 µM), to protect the explanted tissue from ethylene damage and/or to promote the rate of SE (Adkins et al. 1998). Inhibitors of ethylene production, such as aminoethoxyvinylglycine (AVG) and ethylene-action inhibitors such as silver thiosulphate (STS), have also been shown to provide a beneficial environment for callus multiplication, and the formation of somatic embryos (Adkins et al. 1998).

Abscisic acid (ABA), when applied at a moderate concentration (ca. 5 µM), has been shown to enhance the formation and the maturation of somatic embryos (Samosir et al. 1999; Fernando and Gamage 2000; Fernando et al. 2003). In addition, the use of osmotically-active agents such as polyethyleneglycol (PEG 3%) in combination with ABA (45 µM) has been shown to be beneficial, not only for the production of somatic embryos but also for their subsequent maturation and germination (Samosir et al. 1998). In a more recent study using immature inflorescence explants, Antonova (2009) demonstrated the benefits of using a specific growth retardant ancymidol (30 µM) to elevate the somatic embryo germination frequency from a low proportion to 56%.

It is worth noting that cell suspension culture systems have been successful in raising the rate of SE for some other members of the Arecaceae, including oil palm (Elaeis guineensis Jacq.; Teixeira et al. 1995). Further improvements may come from using a photoautotrophic culture system to raise plantlets (Samosir and Adkins 2014), and through the incorporation of fatty acids, notably lauric acid, into the plantlet maturation medium (López-Villalobos et al. 2001, 2011).

Germplasm conservation via cryopreservation

Over the past 30 years, scientists have been trying to develop a method for the safe and long term conservation of coconut germplasm. In the 1980s, the first attempt to cryopreserve coconut tissues was undertaken with immature zygotic embryos using a chemical dehydration and slow freezing technique (Bajaj 1984). However, more recently attention has shifted towards using mature (11 months post pollination) zygotic embryos (Sisunandar et al. 2014) and using a physical dehydration method; or using plumular tissues excised from mature zygotic embryos and employing a chemical dehydration method. As with most species, the cryopreservation protocol for coconut consists of four steps: firstly the pre-culture of the explanted tissues in preparation for drying; secondly tissue dehydration; thirdly tissue freezing; and finally tissue recovery involving thawing and plantlet production. Three tissue dehydration methods have been attempted: chemical dehydration, slow physical dehydration (desiccation taking place in a laminar air flow hood), and fast physical dehydration (fan forced drying using silica gel). For rapid physical dehydration a special apparatus has been developed to dehydrate embryos by exposure to dry air by fan-forcing the air over silica gel (Sisunandar et al. 2010b). By following the water loss during the physical drying of embryos (using differential scanning calorimetry) it was found that drying to 20% moisture content in a period of 8 hours gave the embryos to best chance of surviving cryopreservation. Upon recovery of embryos, this approach gave the higher proportion of plants growing in soil (up to 40%), a level that had not been achieved using any previous method. It was also shown that this cryopreservation method did not induce any measurable genetic change in the recovered plants (Sisunandar et al. 2010a).

The MS (Murashige and Skoog 1962), MW (Morel and Wetmore 1951) and Y3 (Eeuwens 1976) media formulations have all been commonly used in the tissue recovery stage, after cryopreservation, with the latter medium preferred in most studies (Sisunandar et al. 2010b; Sajini et al. 2011; Sisunandar et al. 2012). It is noteworthy that the application of auxins (2,4-D, NAA) or kinetin, either alone or in combination, did not significantly help embryo germination or plantlet recovery (Bajaj 1984; Chin et al. 1989). On the other hand, the addition of a high dose of sucrose (4 to 6%) has been shown to improve the germination of the
recovered embryos (N’Nan et al. 2008; Sisunandar et al. 2010b; Sajini et al. 2011). Successful establishment of plant in soil following cryopreservation of the embryo has been reported twice, using the chemical dehydration approach of Sajini et al. (2011) and the physical dehydration approach of Sisunandar et al. (2010b).

Up until now the majority of coconut cryopreservation work has focused on the use of zygotic embryos or isolated plumular tissues, the availability of which can be limited. Therefore, an interesting field for future research would be the application of cryopreservation to somatic embryogenic cell cultures. The successful preservation of such cultures would enable the production of many more coconut plants from one initial explant, as well as providing a new way to transfer germplasm around the globe.

Conclusion and future prospects

Ineffective plantlet conversion from in vitro culture systems to field-growing palms, remains a major bottleneck for many coconut research groups around the world. This is the result of unresolved or partly resolved problems which relate to the variable response of explanted tissues and their slow growth in vitro, and their further lack of vigour when planted ex vitro. As a result, achievements in coconut tissue have been attained less rapidly than for many other plant species. It is essential to reflect on, and then trial, procedures that work for other species to identify future improvements in coconut in vitro culture. The assembled information suggests that it may be possible to generate highly efficient embryogenic cell suspension cultures, derived from suitable callus lines, to help overcome contemporary challenges, and to develop a rapid clonal propagation system for coconut. Therefore, future research should be focused on the optimization of in vitro conditions to increase the production of somatic embryos using media additives, combined with a cell suspension culture system. Subsequent development and acclimatization could be further improved by using a temporary immersion approach together with a photoautotrophic system to promote rapid plantlet growth. As the coconut seed possesses a substantial source of natural plant nutrients and growth factors within its own liquid endosperm, further investigation may identify a role for coconut water in promoting SE in this generally recalcitrant species. Other possible improvements in the rate of SE may come from the application of molecular techniques that can identify the regulatory genes involved, and then promote their activity. Indeed, novel molecular tools might become available to further examine the regulation of the relevant genes, which can be selectively switched on during the acquisition of embryogenic competence.

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