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*Review*

# The status of temporary immersion system (TIS) technology for plant micropropagation

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**The development of temporary immersion systems (TIS) for *in vitro* culture, in the late 1990s heralded new and semi-automated means of micropropagation of plants for agricultural and conservation purposes. By 2005, various systems had been developed and tested and their benefits were proven with a variety of commercially-important crops such as sugar-cane, coffee, banana, and yam. Progress in the field and the adoption of these systems in subsequent years has been reviewed. Improvements on previously-developed TIS protocols for economically-important species have been reported, and this continues to attest to their benefits when compared with protocols that employ semi-solid or liquid media. Though TIS protocols have recently been developed for other species, this list is limited. On the other hand, there appears to be an increasingly great interest in TIS technology for the production of secondary metabolites and for physiological studies.**

**Key words:** Bioreactor**,** *in vitro* culture, temporary immersion systems (TIS), micropropagation.

## INTRODUCTION

Plant tissue culture is a general term that encompasses a variety of *in vitro* manipulations of plant cells, tissues and organs that direct the de-differentiation of the parental cells into meristematic (or embryogenic) cells, which then divide and differentiate into plant organs and/or whole plants. As the piece of the parent plant utilized to initiate the culture (the explant) is typically small and, theoretically, every one of its cells has the potential to produce a plant, the end result is the mass multiplication of the parent genotype, known as micropropagation. The morphogenic routes through which cells regenerate into plants are organogenesis (the formation of shoots followed by rooting or of particular organs of interest) and embryogenesis (the formation of somatic embryos and their germination into complete plants), and both can occur via an intervening callus stage. In practice, all micropropagation protocols are established empirically, on a case by case basis, by determining the medium components (for example, plant growth regulators, nutrients) and environmental conditions (for example, light, temperature) for each stage of morphogenesis. Typically, the cultures are supported by a semi-solid substrate which is comprised of a gelling agent. This technique is, therefore, ideal for the clonal multiplication of commercially-important elite genotypes, threatened species and those difficult to propagate through other means. In addition, *in vitro* culture manipulations are now exploited in numerous and ever expanding ways, including germplasm conservation, genetic modification via mutagenesis or genetic engineering, virus elimination, production of secondary metabolites, etc. Details of these techniques and their applications can be found in many books and reviews (George and Debergh, 2008).

As discussed by Mehrotra et al. (2007) and Akin-Idowu et al. (2009), the advantages of micropropagation over the conventional propagation methods have been accepted decades ago and are now routinely employed

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**Abbreviations: BIT®,** Twin-Flask system; **BTBB,** balloon-type bubble**; BIB®,** bioreactor of immersion by bubbles; **RITA®,** recipient for automated temporary immersion; **TIS,** temporary immersion system.

by many research laboratories and commercial agriculture (Hamill et al., 2009; Snyman et al., 2011a), horticulture (Akin-Idowu et al., 2009), forestry (Watt et al., 1997) and other enterprises. However, it has long been recognised that one of the major constraints of such protocols is the need for regular sub-culturing (every 4 to 6 weeks), due to nutrient depletion from the relatively small volumes (10 to 30 ml, depending on container) of semi-solid media utilized, and the associated running and labour expenditure. Scaled-up and automated systems are, therefore, desirable to overcome and/or minimize production costs, increase multiplication rates and reduce the amount of handling during the steps required for micropropagation. For this purpose, gelling agents are not ideal as, aside from not being inert, they do not allow for easy automation. Liquid media, on the other hand, provide close contact and uniform access of nutrients to the cultures, they can be renewed without changing the culture vessel, sterilisation is possible by ultrafiltration and containers can be larger than those utilized for semi-solid media. However, liquid culture has its disadvantages, including asphyxia and physiological disorders exhibited by the plants. As discussed below, many strategies have been investigated and proposed to overcome the constraints of the protocols that rely on semi-solid media.

## BIOREACTORS AND TEMPORARY IMMERSION SYSTEMS

A bioreactor is a self-contained sterile environment which capitalises on liquid nutrient or liquid/air inflow and outflow systems. It is designed for intensive and often scaled-up cultures, and affords maximum opportunity for monitoring and control over micro-environmental conditions (for example, agitation, aeration and temperature). The first report on the use of a bioreactor for micropropagation was by Takayama and Misawa (1981) who multiplied Begonia using shake cultures. Today, a large number of different types of reactors have been designed and can be reproduced in-house or can be purchased. Their main differences lie in the types of vessels and the mechanisms that provide culture agitation (non-agitated, mechanical or pneumatically). Most of these were illustrated and described in detail by Etienne and Berthouly (2002) and Paek et al. (2001; 2005). The former review presented the automated plant culture system (APCS) with full immersion (Tisserat and Vandercook, 1985) and four partial immersions systems, namely those of Aitken-Christie and Davies (1988), Simonton et al. (1991), the RITA® (Alvard et al., 1993) and the Twin Flasks (BIT®) (Escalona et al., 1999) systems. Paek et al. described the airlift and bubble column-type, balloon-type bubble (BTBB), stirred tank and ebb and flood bioreactors. Although successes have been reported with all of them, it appears that the choice of bioreactor type lies on the required end product (for example, shoot multiplication, somatic embryos) and, in some cases, the expertise and resources to build or purchase it. More recently, Mehrotra et al. (2007) discussed the various options for the large-scale plant propagation in bioreactors focusing on those involving submerged cultures but concluded that the ebb and flood types have several advantages, including the independent control of each culture stage.

Of all of the available systems, those involving temporary immersion have a variety of characteristics that make them highly suitable for use in semi-automated micropropagation. First and foremost, and as the name implies, the plant cultures are not constantly immersed in liquid media, which often affects plant growth and morphogenesis negatively. Temporary immersion systems (TIS) allow for control of contamination, adequate nutrient and oxygen supply and mixing, relatively infrequent subculturing, ease of medium changes and limited shear damage. For these reasons, Ziv (2000, 2005) and Arencibia et al. (2008), amongst others, have described TIS as the “most natural tissue-culture approach”.

A number of different TIS have been utilized successfully in the last seven years for the micropropagation of a variety of plant species of agricultural, medicinal and conservation value (Table 1). Some of the TIS are patented and others are the result of the ingenuity of researchers who may not have the funds to purchase them. The Twin-Flask system (BIT®) of Escalona et al. (1999) consists of a container for growing plants and a reservoir for the liquid medium. When a solenoid valve is opened and compressed air is turned on, the medium is forced into the first flask, immersing the plants. The process is reversed when another solenoid valve is opened and air pressure forces the medium back into the original reservoir. The RITA® system (recipient for automated temporary immersion) (Alvard et al., 1993) is an apparatus made up of an upper compartment containing the explants and lower compartment which contains the liquid culture medium. They are linked together so that when an overpressure is applied to the lower compartment, the medium is pushed into the upper one; when the overpressure drops, the liquid medium returns to the lower compartment. Consequently, the cultures in the upper compartment are temporarily immersed when the upper compartment is flooded with the liquid medium, the frequency and time period of which can be regulated. Stanly et al. (2010) applied this principle to a reusable Nagene® polysulfone filtration system by connecting each of the two compartments with a tube fitted with a 22 µm filter, through which pressure was applied to push the culture medium onto the upper compartment that housed the explants. The outlet on the top of the vessel allowed for pressure to escape and the air entering the vessel was filtered through a sterile syringe filter. A similar set-up to

that of the RITA® that uses Plantima containers has recently become available (Yan et al., 2010). The bioreactor of immersion by bubbles (BIB®) system developed by Soccol et al. (2008) has an interlinked system with tubes of flexible rubber that provide the cultures with air and nutrient solution by bubbling. The apparatus has two glass compartments divided transversally by a porous plate. On the top (larger compartment), stainless steel tripods and mesh are inserted to support the cultures.

The successes and benefits of the described semi-automated TIS, for the mass multiplication of plants, were comprehensively reviewed by Etienne and Berthouly (2002) and Paek et al. (2001; 2005). At that time, it was abundantly clear that the technology offered new ways to achieve high plantlets yields *in vitro*, at low cost, suitable for research and commercial activities. The aim of the present review is to assess the subsequent progress on the uses of TIS, and the realisation of such promise, since those publications.

## RECENT PUBLICATIONS ON TEMPORARY IMMERSION SYSTEMS (TIS) (2005 TO 2012)

### New protocols

Since 2005, TIS have been employed for the mass propagation of a wide range of species (Table 1). However, only fewer than 10% of those publications are on regeneration via somatic embryogenesis, even though this was previously an area of intensive and productive research on economically-important crops such as rubber (Etienne and Berthouly, 2002), coffee (Etienne et al., 2006) and banana (Haq and Dahot, 2007). The recent publications on somatic embryogenesis include those of Albarran et al. (2005) and Gatica-Arias (2008) on coffee, the latter exploring TIS for large scale production of somatic embryos for genetic transformation. A protocol for a twin flask bioreactor was also devised for *Theobroma cacao*, resulting in a 13-fold yield of somatic embryos after 3 months in culture, compared with 10.2 ± 1.9 embryos per explants in 12 months on semi-solid medium (Niemenak et al., 2008). The RITA® and a twin-flask systems were successfully employed for oil (Sumaryono et al., 2008) and peach (Steinmacher et al., 2011) palms. The only other report found in the literature on somatic embryogenesis using TIS in the last seven years concerns the endangered medicinal plant *Camptotheca acuminata* (Sankar-Thomas et al., 2008). It describes a high yielding protocol with an immersion regime of 1 min every 6 h and 0.5 mg $L^{-1}$ BAP in the medium for embryo conversion.

The other recent studies focused on micropropagation via the organogenesis pathway (Table 1). Some reported on the continuation of efforts to improve yields of certain species, including plantain (Roels et al. 2005, 2006), pineapple (Scheidt et al., 2009), date palm (Fki et al., 2011) and sugarcane (Yang et al., 2010; Snyman et al., 2011b). Others provided TIS protocols for species that had not been previously investigated, including some of pharmacological and medicinal value (Debnath, 2009; Stanly et al., 2010; Yan et al., 2011; Malosso et al., 2012).

Of note is that each research group has adopted a particular type of apparatus, the most popular being the BIT® (or variations of this twin-flask system), BIB® and RITA® systems (Table 1). Consequently, although the aim of many of the reported studies was to test the validity of one type of TIS in increasing plantlet yields, as compared with semi-solid media or permanent immersion (Jova et al., 2011), few compared the efficacy of different TIS types. Regarding the former, most authors reported increased multiplication rates with their TIS of choice as compared with semi-solid, although some reported no differences (Hanhineva et al., 2005; Jo et al., 2008; Stanly et al., 2010) (Table 1). A few found permanent immersion bioreactors to be the most adequate for multiplication, for example Wu et al. (2007) and Ross and Castillo (2009) working with jewel orchid and blueberry, respectively. In terms of the efficacy of different systems, Scheidt et al. (2009, 2011) found BIB® to be superior to RITA® for the propagation of pineapple and tea tree seedlings and Sankar-Thomas et al. (2008) provided evidence for the use of RITA®, instead of a twin vessel system, for somatic embryogenesis in *C. acuminata*. The reality is that, although different TIS have been available for over 10 years, there still have not been enough comparisons of the systems for newcomers to the field to make informed decisions as to which system to adopt for their purposes.

### Factors that affect organ and plantlet yield in temporary immersion systems (TIS)

In most published reports, the strategy was to utilize *in vitro* explants to initiate TIS so as to eliminate the problem of microbial contamination and consequent culture losses. However, particularly in woody species, the explants (nodal explants, buds) carry endogenous bacteria or fungi that proliferate very quickly once exposed to the liquid medium. This together with the relatively large number of explants placed in each vessel is the main cause of TIS *Eucalyptus* culture losses (McAlister et al., 2005; Watt et al., 2006). Hence, in large commercial activities, such as forestry, the cost implications of such incidents can be extremely serious.

There are a number of approaches (for example, serological techniques, protein and genetic profiling) to dealing with endogenous and latent contamination, but the majority require specialised instrumentation and labour as reviewed by Herman (2004). The most commonly- employed tactic is treating the explants with and/or incorporating antibiotics and biocides such as PPM™ (Plant Cell Technology, Washington, DC) in the

**Table 1.** Summary of reported work undertaken using temporary immersion systems (TIS) since 2005.

| Species | Explant | Type of TIS | Product of multiplication stage | Immersion Regime/day | Yield/explant* | Multiplication (compared with semi-solid unless specified) | References |
|---|---|---|---|---|---|---|---|
| *Alocasia mazonica* (elephant's ear) | Corms | Ebb and flood, balloon type | Shoots | 30 min / 8 h | 7 | No difference | Jo et al. (2008) |
| *Ananas comosus* (pineapple) | Axillary buds | BIT®* | Shoots | 3 min / 2 h | 19 | 3 | Da Silva et al. (2007) |
| *Ananas comosus* (pineapple) | *In vitro* plantlets | RITA®** | Shoots | 15 min / 2 h | Not reported | 1.3 (liquid) | Scheidt et al. (2009) |
| | | B.I.B®*** | Shoots | 15 min / 2 h | Not reported | 3 (liquid)<br>2.3 (RITA®) | |
| *Anoectochilus formosanus* (jewel orchid) | Shoot tips | Ebb and flood | Shoots | 15 min / 1 h | 19.5 | Not investigated | Wu et al. (2007) |
| Apple rootstock M26 | *In vitro* shoots | RITA® | Shoots | 3- 5 min / 1.5 h | 9 | Not investigated | Zhu et al. (2005) |
| Apple rootstock M9 EMLA | Single node cuttings | RITA® | Multiplication | 15 min / 8h | 7 | Higher dry mass than liquid | Chakrabarty et al. (2007) |
| *Camptotheca acuminata* | Hypocotyl segments | RITA® | Embryos | 1 min / 6 h | 5.8 | Not investigated | Sankar-Thomas et al. (2008) |
| | | Dual vessel system | Embryos | 1 min very 6 h | 3 | Not investigated | |
| *Charybdis* sp. (squill) | Meristematic nodules | Flasks in series | shoots | 5 min | 38 | 0.25 | Wawrosch et al. (2005) |
| *Coffea arabica* | Embryogenic suspensions | RITA® | Embryos | 1 min / 4 h | 2 250 embryos | Not investigated | Alabarrán et al. (2005) |
| *Curcuma zedoaria* | Longitudinally-halved *in vitro* shoots | Modified Nalgene® polysulfone filtration**** | Shoots | 15 min | 4.7 | No difference | Stanly et al. (2010) |
| *Cymbopogon citratus* (lemon grass) | *In vitro* plants | Dual glass flasks | Shoots | 4 (length of time not reported) | Not reported | 12.3 | Quiala et al. (2006) |

**Table 1.** Contd.

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *Discorea* spp. (yam) | Nodal segments | Glass flasks | Shoots<br>Tubers | 10 min / 6 h | 4.5 | 4 | Jova et al. (2005) |
| *Discorea* spp. (yam) | Nodal segments | TIS and constant immersiom | Microtubers | 15 min / 6 h | 2.8 Microtubers/plant | Not investigated | Jova et al. (2012) |
| *Discorea fordii* (chinese yam) | Single node leafy cuttings from *in vitro* shoots | Plantima containers***** | Shoots<br>Tubers | 3 min / 4 h | Not reported | 2 (shoots)<br>8 (tubers) | Yan et al. (2011) |
| *D. alata* (chinese yam) | Single node leafy cuttings from *in vitro* shoots | Plantima containers | Shoots<br>Tubers | 3 min / 4 h | Not reported | 2.1 (shoots) | |
| *Elaeis guineensis* (oil palm) | Immature leaves callus | Similar to RITA® | Somatic embryos | 3 min / 6 h | 10-16 embryos | Not investigated | Sumaryono et al. (2008) |
| *Eucalyptus globulus* | *In vitro* shoots | BIT® | Shoots | 2 min / 12 h | 7 | Not stated | Gonzáles et al. (2011) |
| *Eucalyptus grandis*, and *hybrids* | *In vitro* shoots | RITA® | Shoots | 30 s / 10 min | 4.7 (subtropical clones)<br>2.4 (cold-tolerant clones) | 4 - 6 (in ½ time) | McAlister et al. (2005) |
| *Fragaria* x *ananassa* (strawberry) | Leaf pieces | RITA® | Shoots | 5 min / 4 h | Not reported | No difference | Hanhineva et al. (2005) |
| *Hydrastis canadensis* | shoots | RITA® | Shoots | 3 min / 1 h | Not reported | 5.6 | He et al. (2007) |
| *Hippeastrum* x *chmielli* | Pieces of *in vitro* bulblets | BIT® | Bulblets | 15 min / 4 h | 6.5 | 2 | Ilczuk et al. (2005) |
| *Jacaranda decurrens* | Nodal segments | RITA® | Shoots | 15 min / 4 h | 9.6 | 1.4 | Malosso et al., 2012 |
| *Lessertia frutescens* | Single node explants | B.I.B® | Shoots | 30 min / 4h | 12.9 | 1.2 | Shaik et al. (2010) |

**Table 1.** Contd.

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *Musa* (plantain) | Longitudinally-halved *in vitro* shoots | BIT® | Shoots | 4 min / 3 h | Not reported | Not reported | Aragón et al., 2005 |
| | Longitudinally-halved *in vitro* shoots | BIT®; Nalgene containers | Shoots | 4 min / 3 h | Not reported | 2.5 | Roels et al. (2005) |
| *Panax quinquefolius* (american ginseng) | Nodal segments | Liquid Lab™ Rocker | Shoots | 30 s / 1-2 min | 12 | Not investigated | Uchendu et al. (2011) |
| *Phoenix dactylifera* (date palm) | Intact and fragmented juvenile leaves | RITA® | Shoots | 15 min | 8.4 | 2 | Fki et al. (2011) |
| *Saccharum officinarum* | *In vitro* plants | Erlenmeyer flasks | Shoots | 3 min / 3 h | Not reported | Not reported | Yang et al. (2010) |
| *Saccharum* spp. | Leaf disks | RITA® | Embryos | 1 min / 72 h | 330 | 9 | Snyman et al. (2011b) |
| *Siraitia grosvenorii* | Nodal segments | Plantima containers | Shoots | 4 min / 4 h | 8.75 | 2.6 | Yan et al. (2010) |
| *Theobroma cacao* | Embryogenic callus | BIT® | Embryos | 1 min / 6h | 159 | 13 | Niemenak et al. (2008) |
| *Vaccinium angustifolium* (lowbush blueberry) | 3 node stem sections with intact leaves | RITA® | Shoots | 15 min / 4 h | 9.1 | 3 | Debnath (2009) |
| *Zinziber zerumbet* | Longitudinally-halved *in vitro* shoots | Modified reusable Nalgene® polysulfone filtration | Shoots | 15 min | 4.6 | 1.5 | Stanly et al. (2010) |

Twin-flasks system (BIT®); **, Reactor of Automatized Temporary Immersion (RITA®); ***, Temporary Bioreactor of Immersion by bubbles (B.I.B.®); ****, NalgeNunc International, USA; *****, A-Tech Bioscientific Company Limited., Taipei, Tawain

culture media. Although often successful (McAlister et al., 2005; Luna et al., 2008), antibiotics are expensive, they have optimum pH conditions and degrade quickly. Another option, particularly when dealing with explants from parent plants grown in the field, is to screen them on a semi-selective microbial medium such as that formulated by Viss et al. (1991). This has proven effective for the production of axenic start-up cultures of eucalyptus buds in RITA® vessels (Watt et al., 2006). In a similar approach, Mordocco et al. (2009) employed semi-solid SmartSett® shoot induction medium to obtain contaminant-free sugarcane explants for multiplication in RITA®.

Micropropagation success in TIS is dependent on (1) The volumes of the culture container and liquid medium in relation to explant biomass at initiation and subsequent culture stages; (2) the immersion regimes to which the cultures are subjected; and (3) the effect that each of these parameters has on the others. Given the various types and sizes of TIS, as well as types of cultures, there is a wide range of published inoculum densities, for example, 10 pineapple buds per 300 ml medium in 1 L flasks (da Silva et al., 2007), 30 elephant's ear corms per 1 L medium in 3 L flasks and 5 micro-shoots of *E. globulus* per 200 ml medium in 600 ml flasks (Gonzáles et al., 2011). Although biomass of the explants is usually reported as part of the description of the protocol used, only a few authors have reported on the optimisation of the explant biomass for TIS initiation. In this regard, the highest multiplication rate for *Eucalyptus* was obtained when cultures were started with 50 buds per RITA® vessel (McAlister et al., 2005). For the production of potato microtubers, the best ratio was 60 explants in 3.5 L media (Perez-Alonso et al., 2007) and for the multiplication of plantain it was 5 intact shoots and 150 ml media (Cejas et al., 2011).

A commonly-encountered problem in tissue culture is hyperhydricity of the cultured material. This is a physiological disorder that results in morphological and physiological alteration of plants, often giving them a 'glassy' (formerly described as vitrified) appearance, due to apoplastic water accumulation. Although hyperhydricity may occur under semi-solid conditions, it is more prevalent in liquid culture as a result of the constant, partial or temporary immersion of the explants and often leads to necrosis (Berthouly and Etienne, 2005). Consequently, in TIS the duration and frequency of the immersion are the most decisive parameter for successful micropropagation, as they influence nutrient and water uptake and consequently hyperhydricity of the cultured material. As shown in Table 1, immersion regimes vary greatly depending on the species under study, the TIS employed and the route of morphogenesis. Regarding the latter, evidence has accumulated in support of the proposal by Teisson and Alvard (1995) that frequent but short immersion duration cycles stimulate somatic embryogenesis and eliminate embryo hyperhydricity. For example, Albarrán et al. (2005) showed that changing from 15 min every 4 h to 1 min every 4 h decreased hyperhydricity and increased embryo conversion of coffee somatic embryos and Gatica-Arias et al. (2008) employed an even longer resting time (1 min every 8 h). Similar results were reported for cacao when that same immersion regime was utilized (Niemenak et al., 2008).

Shoot hyperhydration is usually found to be lower in TIS than in semi-solid and liquid media and most of the recent publications continue to uphold this notion (Wawrosch et al., 2005; Stanly et al., 2010; Yan et al., 2010). On the other hand, Shaik et al. (2010) found 50% of the shoots of *Lessertia frutescens* produced in a balloon-bubble bioreactor to have symptoms of hyperhydricity not encountered on semi-solid medium. It is feasible that this was caused by the long and frequent immersion regime utilized in that study (30 min immersionevery 4 h), as most other workers tend not to apply immersion periods longer than 15 min. Increasing the resting time between immersions from 1 min/12 h to 1 min/72 h, combined with reduced nutrient supply, decreased hyperhydricity significantly in sugarcane plantlets produced in RITA® (Snyman et al., 2011b). Similarly, lower nutrient supply and sucrose levels, combined with a 2 min/12 h immersion regime successfully diminished hyperhydration in *E. globulus* shoots produced in a twin-flask system (González et al., 2011).

Various researchers attribute the beneficial effect of TIS on the elimination of hyperhydricity and increased propagation yields, compared with semi-solid and liquid protocols, to the renewal of its headspace with the surrounding air (Zobayed, 2005). This, according to Roels et al. (2005, 2006) prevents the accumulation of $CO_2$ and $C_2H_4$ that occurs above the semi-solid medium and has detrimental effects on the shoots in culture. Jova et al. (2011) also contend that the improved physiological parameters (for example, chlorophyll, net photosynthesis, transpiration, stomatal conductance) of yam plants produced in TIS, compared with those from continuous liquid culture, are caused by the sporadic renovation of the container's internal atmosphere and the intermittent contact of the plants with the liquid nutrient medium. In addition, in TIS the headspace can be renewed by $CO_2$ enrichment. Aragón et al. (2010) found that 1 200 µmol $mol^{-1}$ $CO_2$ supplied with 30 mg $l^{-1}$ sucrose at low light intensity (80 µmol $m^{-2}$ $s^{-1}$) resulted in improved leaf and root development, and reduced respiration of *Musa* cultures in the TIS.

## Acclimatization, field performance and genetic fidelity

Most of the early studies reported that TIS-produced plants were more successful in surviving the *ex-vitro* acclimation stage than those produced on semi-solid and liquid media (Etienne and Berthouly, 2002). These differences have been attributed to the physiological status of the plants *in vitro* resulting from the propagation method applied, and there is continued evidence that the TIS environment prepares the plantlets for the stress of acclimatization. For example, Yang and Yeh (2008) reported that during *ex-vitro* acclimatization, *Calathea orbifolia* plants (used in landscaping) produced in TIS had much higher photosynthetic rates and subsequently higher leaf area, fresh and dry weights than those from semi-solid media. Aragón et al. (2010) reported that TIS-produced sugarcane plants exhibit an activated anti-

oxidative system that allows them to cope with the stress of the *ex-vitro* environment.

There is, however, scarce information on the performance of plants regenerated via TIS once they are transferred to field conditions. It is possible that this omission is based on the presumption that somaclonal variation events are rare in TIS since most of the cultures are not produced via a callus stage, the levels of plant growth regulators are usually not high and the culture period is relatively short. The literature reviewed indicates that there are two reports that confirm this view and both are on sugarcane. One is the early work by Lorenzo et al. (2001) validating the use of TIS in micropropagation with phenotypic data from a two year- long field trial. Those authors found that, although there were some differences among propagation systems (conventional versus TIS) in the first six months of field growth, regarding stem diameter and length, they disappeared with time. The more recent study employed AFLP, in addition to phenotypic analyses, and the field trial lasted 6 months (Snyman et al., 2011b). The phenotypic data confirmed those of Lorenzo et al. (2001), and only 0 to 0.9% of polymorphic bands were scored compared with the conventionally-propagated control. There is also a recent report on date palm, were the authors (Fki et al., 2011) stated that they did not observe any phenotypic differences amongst 400 plants produced in RITA®, but no further details were provided. Similarly, Uchengu et al. (2011) reported that american ginseng plants produced in a TIS "appeared phenotypically normal".

On the other hand, somaclonal variation is a major concern in certain crops and needs to be controlled by choice of explant, media type, types and concentrations of plant growth regulators, time in culture, number of subcultures, etc. For example, Debnath (2011) has argued recently that clonal fidelity is a major concern in berry micropropagation that employs bioreactors and, aside from culture conditions and monitoring of phenotypes, molecular markers should be utilized for the genetic identification of variants.

## Novel applications

Temporary Immersions Systems result in increased biomass with less labour and faster than conventional (semi-solid) techniques. Consequently, they have been adopted by many propagation and breeding programmes to save costs and to accelerate the production of plantation units of elite clones and/or the release of new cultivars. In South Africa, plants produced in RITA® have been used by the forestry industry to produce *Eucalyptus* mother plants for hedges as a source of cuttings for the nurseries, and by the sugar industry to supply disease-free seedcane to growers (Meyer et al, 2009; Snyman et al., 2011a). In Australia, propagation via RITA® has been integrated into the SmartSett® sugarcane programme (Mordocco et al., 2009). TIS have even been employed successfully in the rejuvenation (and subsequent micropropagation) of the spanish red cedar (*Cedrela odorata L*.) (Peña-Ramires et al., 2010). Another suitable application of the technology is the regeneration (and bulking-up) of transgenic plants, such as demonstratedfor strawberry (Hanhineva and Käarenlampi, 2007).

TIS have also attracted attention for applications other than just clonal multiplication for planting units. For example, Hajari et al. (2006) used RITA® to fast-track the bulking-up of *in vitro* shoots to be used as explants for callus initiation, and to produce adventitious buds from root explants for multiplication, before and after cryopreservation (Hajari et al., 2009; 2011). Similarly, Niemenak et al. (2008) utilized TIS to produce the required large quantities of callus and somatic embryos, at different stages of morphogenesis, to analyse their amino acid content and composition.

Due to the size of the culture vessels and the relative ease in which the environmental culture conditions can be controlled, TIS are also being explored for secondary metabolite production by differentiated plant tissues and organs. This strategy has been shown to be suitable for biomass production of specific organs, from which the products of interest can then be extracted. Examples include buds of lemon grass for citral (Quiala et al., 2006), hairy roots of *Beta vulgaris* for betalains (Pavlov and Bley, 2006), shoots of *Digitalis* for digoxin and digitoxin (Pérez-Alonso et al., 2009) and roots of *Panax ginseng* for saponin (Langhansova et al., 2012). For the same reasons, TIS are also proving a useful tool to investigate cellular pathways and processes. Using RITA® vessels, Ivanov et al. (2012) found that temperature played a critical role in the patterns of alkaloid production by *Leucojum aestivum* (Amaryllidacea) and Arencibia et al. (2008) used a similar approach to study the differential genomic responses of whole sugarcane plants in response to phenylpropanol compounds. In another study, sugarcane shoots were induced to produce phenolic compounds, which were then sprayed on tomato seedlings and found to have some success in inducing resistance against *R. solanacearum*, the causal agent of bacterial wilt (Yang et al., 2010).

## CONCLUSION

In 2002, Etienne and Berthouly predicted that TIS would become simpler and less costly, and would increasingly find favour with researchers and commercial enterprises. However, since then, the most popular TIS are still the twin-flasks, immersion by bubbles and the RITA® systems, seemingly without major modifications. The major progress in terms of these systems is an enhanced understanding on how to eliminate hyperhydricity, particularly by the use of short immersions and long resting times. In contrast, there is hardly any information on the field performance of plants produced by temporary

immersion protocols. In terms of popularity, various authors indicate that this technology has been incorporated in research and commercial propagation programmes but details are not available in the scientific literature. This is not entirely surprising as commercial companies tend not to divulge specific details of their activities. An exception is the report by Mordocco et al. (2009) which illustrates a TIS-based strategy for the release of sugarcane cultivars. Other promising fields for the use of TIS are the bulking-up organs for the production and extraction of secondary metabolites, and as tools to investigate metabolic and genomic studies under controlled conditions.

Considering the proven successes and benefits of TIS for clonal multiplication, it is surprising that there are relatively few species propagated with these systems. In this regard, an area where TIS can have a significant impact is as a driver for research and associated commercial activities on 'orphan crops' - species which, although essential to the livelihoods of millions of poor people throughout the world, are being threatened and facing genetic erosion (Bhattacharjee, 2009; Dubois, 2009).

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