

# Towards automating micropropagation: from cells and shoots to plants in one step

by

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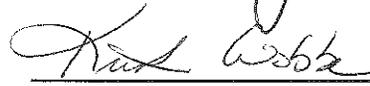
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## **Abstract**

A mist reactor was used to study plant growth and development under various environmental conditions towards the production of healthy plantlets ready for soil transplant in one step from inoculation. In addition, a 3D type of cultivation via surface attachment of explants to vertically hanging strips inside the mist reactor was also investigated to maximize productivity with minimal footprint. Using carrot as the model species, pre-embryogenic cell suspensions were successfully spray-inoculated onto hanging poly-L-lysine (PLL)-coated nylon mesh to which they then attached and remained for several weeks while they developed into rooted plantlets. To study single step micropropagation from shoot explants to fully acclimatized plantlets, *Artemisia annua* was used as the model species. Nodal cuttings of *A. annua* were inoculated onto PLL-coated mesh strips by briefly immersing the strips in the suspension of nodal cuttings. Investigation of medium, phytohormones, CO<sub>2</sub>, ventilation level and humidity ensued resulting in selection of a preferred final process that reduced physiological aberrations like hyperhydricity and was time efficient. The nodal cuttings that attached to the strips were first misted with half strength shooting medium for 7 days to develop new shoots. Then the new shoots were misted with the rooting medium supplemented with NAA for 12 days to develop roots. Rooted plantlets were acclimatized in the same rooting medium for 9 days to acquire fully functional stomata prior to planting into soil. Taken together this study suggested that fully developed plantlets ready for planting into soil could be obtained in a single step in a bioreactor from embryogenic cells or from nodal explants.

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# Chapter 1 Background<sup>a</sup>

## 1.1 Micropropagation introduction

Micropropagation is a modern plant propagation technology that uses plant tissue culture techniques to clonally and rapidly reproduce a large number of pathogen-free progeny plants from a small amount of initiating plant material (cells, tissues, and organs). The process is not subject to variation of season or weather. It generally consists of *in vitro* initiation, multiplication, elongation and rooting under controlled environment, acclimatization and *ex vitro* establishment in the soil. The regeneration of whole plants *in vitro* involves alteration of growth regulators (phytohormones) and other culture medium constituents. With the advances of tissue culture and genetic modification techniques, micropropagation has evolved into a flourishing industry and an important research tool (see section 1.2), and offers many advantages over traditional plant vegetative propagation. On the other hand, it is very labor intensity and therefore more expensive than other propagation methods. See Table 1.1 for a detailed description of pros and cons of micropropagation.

## 1.2 Commercial production and applications

After its establishment in the 1960s (Murashige 1974), micropropagation has grown into a large global commercial industry. Hundreds of companies throughout the world are engaged annually in producing billions of plants by micropropagation (Singh and Shetty 2011; Winkelmann *et al.* 2006). These companies are mostly located in European countries (e.g.

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<sup>a</sup> Portions of this chapter was published in Fei & Weathers (2015).

Netherland, Belgium, Germany and Italy), the USA, India, South Africa, Israel, China, Argentina, and Brazil. The variety of plants produced via micropropagation covers horticultural species, agricultural crops, and other economically important plants (Anderson *et al.* 2011; Lewandowski *et al.* 2003). With the advances in tissue culture technology, micropropagation has expanded its potential into plant production for biofuels (Cavallaro *et al.* 2014; Czako and Márton 2012), and environmental restoration (Giri *et al.* 2004; Merkle *et al.* 2012; Peña-Ramírez *et al.* 2010; Stukely *et al.* 2007; Willyams and Daws 2014).

**Table 1.1** Advantages and disadvantages of micropropagation

	Micropropagation
Advantage	<ul style="list-style-type: none"> <li>• Rapid mass propagation;</li> <li>• Clonal propagation;</li> <li>• Year round production;</li> <li>• Minimal need for elite stock plant material;</li> <li>• Can propagate recalcitrant plants and those difficult to propagate by seed;</li> <li>• Easy production of new variety by <i>in vitro</i> hybridization and transformation;</li> <li>• Minimal requirement on space and arable land;</li> <li>• Germ and pathogen free;</li> </ul>
Disadvantage	<ul style="list-style-type: none"> <li>• Labor intensive;</li> <li>• Abnormalities can occur due to typical <i>in vitro</i> environment in sealed sterile containers (high relative humidity; low light intensity; limited gas exchange; presence of sucrose and plant growth regulators);</li> <li>• Risk of contamination due to the presence of sugar in the culture medium.</li> </ul>

Besides rapid plant multiplication, *in vitro* techniques have many applications in genetic improvement, germplasm conservation, elimination of pathogens, and production of secondary metabolites, recombinant proteins and vaccines (Engelmann 2011; Huang and

McDonald 2012; Paz-Maldonado and González-Ramírez 2014). One of the most important arenas for tissue culture is agriculture. As world population expands, food scarcity becomes more urgent. Facing this challenge, tissue culture has an important role and offers great potential in agriculture by providing safe, high quality planting material of food crops to feed the world (Ashraf *et al.* 2012; Le 2005). Besides food, plant-derived secondary metabolites are also target products.

Compared to whole crop plants, *in vitro* cultures of plant cells or differentiated tissues offer a source of defined standard phytochemicals in large volumes produced via a more efficient, reliable and predictable manner (Kumar *et al.* 2014; Urbańska *et al.* 2014). Among secondary metabolites, those produced by medicinal plants have gained much attention (Hu and Jia 2012; Murthy *et al.* 2014; Rout *et al.* 2000). Large scale cultures of cells, adventitious and/or hairy roots and somatic embryos for medicinal compounds have been developed (Baque *et al.* 2012; Cui *et al.* 2013; Paek *et al.* 2009; Paek *et al.* 2005; Shohael *et al.* 2014).

## **1.3 Micropropagation methods and stages**

### **Methods of micropropagation**

The methods currently available for propagation of plants *in vitro* can be summarized as follows:

- 1) Multiplication of shoots from axillary buds.

This is by far the most common and reliable method of true-to-type *in vitro* propagation mainly due to the high genetic fidelity of axillary originated shoots (George *et al.* 2008a). It is also the method by which most micropropagated plants currently are produced (George, *et al.* 2008a). Explants for initiating *in vitro* cultures include shoot meristems (apical dome with 1-2 youngest leaf primordia), shoot tips (shoot apical meristem with a few primordia and unexpanded leaves), nodes, and floral meristems. New axillary shoots are induced from these explants by the addition of plant growth hormones in the medium. When these newly-developed shoots grow into microshoots, they are separated for further subculture or rooting. There is an exception in nodal culture because it does not need the addition of plant growth hormones to develop axillary shoots that arise from pre-existing axillary buds in the nodes.

2) Formation of adventitious shoots either directly on pieces of tissues or organs (direct organogenesis); or indirectly from undifferentiated callus (indirect organogenesis).

Direct adventitious shoot formation from a primary explant is used for a variety of ornamental, crop, cash and medicinal plants including *Achimenes*, *Saintpaulia*, *Sinningia*, *Streptocarpus*, *Anthurium*, *Begonias*, *Cactus*, *Epiphyllum*, *Gerbera*, *Hosta*, *Lilium*, *Dendrobium*, *Vanilla*, *Orchis*, *Musa*, *Annona*, *Ananas*, *Embelia*. (Annapurna and Rathore 2010; George, *et al.* 2008a; Nagori and Purohit 2004; Rout, *et al.* 2000). Explants for direct organogenesis can be a leaf ± the petiole, internode and fragmented shoot meristem (George, *et al.* 2008a). Induction of adventitious shoots from a primary explant requires cytokinins (Huetteman and Preece 1993; Lu 1993). The induced shoot initials may form a proliferating mass of shoots (bud cluster)

when transferred to liquid culture. Bud cluster culture is also suitable for large-scale production in bioreactors, but at some point is also likely to develop hyperhydric shoots (Alvard *et al.* 1993). To address this problem, Ziv proposed using growth retardants to minimize shoot size during bud cluster proliferation followed by a separate step with medium changes to encourage shoot elongation (Ziv *et al.* 1998; Ziv 2005). Compared to axillary shoot production, this technique is more prone to yielding off-types as a result of some cells reverting to the meristematic state during shoot regeneration (Miguel and Marum 2011; Skirvin *et al.* 1994). To minimize genetic variation, the initial adventitious shoots formed on primary explants are followed by only axillary shoot development during succeeding subcultures (George, *et al.* 2008a).

In contrast, indirect adventitious shoots arise from undifferentiated callus previously dedifferentiated from the mother explant. Callus growth can be induced with a high level of auxin in the medium, and can generally form on leaf, stem, root segments, pieces of storage tissue, embryos, and shoot tips (George, *et al.* 2008a). Adventitious shoots are then induced from callus by reducing the auxin to cytokinin ratio in the medium (George, *et al.* 2008a). These shoots derived from callus have even greater somaclonal variation compared to direct adventitious shoots and as a result are not as desirable in commercial production unless new genotypes are required for selection and plant breeding (Skirvin, *et al.* 1994).

- 3) Somatic embryogenesis either directly from pieces of tissues or organs, or indirectly from undifferentiated callus or cell suspensions.

Somatic embryogenesis is a process in which a bipolar structure, resembling a zygotic embryo, develops from a non-zygotic cell. Unlike unipolar growth of shoots and roots, bipolar somatic embryos can form whole plants once germinated without culturing on multiple types of medium. The bipolar structure can be formed directly on explants, and suitable explants for direct somatic embryogenesis include zygotic embryos of monocotyledons, dicotyledons and gymnosperms, parts of young seedlings, anthers, female gametophytes, ovules, nucellar embryos, nucellus tissues and even other somatic embryos (George, *et al.* 2008a). Some species, i.e. orchids, have protocorms or procorm-like bodies, and these structures can also give rise to whole plants similar to formation of somatic embryos (Lee *et al.* 2013). However, since direct somatic embryogenesis does not provide a mechanism for proliferation of embryogenic tissue, it is quantitatively disadvantageous as embryos directly initiated *in vitro* may be inadequate for large-scale cloning, except through shoot multiplication.

Similar to indirect adventitious shoots, indirectly formed somatic embryos also first must undergo a dedifferentiation step from mother explants to form callus and cell cultures, and then re-differentiate from them to form somatic embryos. Although induction of embryogenic callus requires auxin in the culture medium, subsequent embryo development requires absence of auxin in the culture medium (George, *et al.* 2008a). For plants with embryogenic competence, embryogenic callus is commonly formed from seed embryos, highly meristematic tissue, and in some cases from root sections (George, *et al.* 2008a). In embryogenic cell suspension cultures, plantlets can theoretically be produced in large numbers

using lower cost bioreactors because plantlets do not require individual handling. The derived somatic embryos are then either further germinated *in vitro* into plantlets or encapsulated as synthetic seeds to be sowed in soil (Etienne-Barry *et al.* 1999). Once initiated, embryogenic callus can continue producing embryos with each subculture. After successive subculture passage, callus and embryogenic cell suspensions may lose their embryogenic competence (Corredoira *et al.* 2003; Liu *et al.* 2009b; Reynolds 1986; Vasil 1985). Somatic embryos can also be used as “synthetic seeds” for direct sowing into *ex vitro* conditions, but this requires careful control of bioreactor conditions to synchronize embryo development (Shimazu and Kurata 2003). There is also species-dependent pretreatment, encapsulation or dehydration that may be required (Ducos *et al.* 2005; Haque and Ghosh 2014; Jayasankar *et al.* 2001; Sharma *et al.* 2013; Standardi and Micheli 2013). Also, somatic embryogenesis has a high frequency of genetic variation (Miguel and Marum 2011; Skirvin, *et al.* 1994); only a few mainly woody plant species (e.g. coffee, conifer, spruce, date palm, oil palm, ginseng) are currently propagated in large scale via embryogenesis (Ducos *et al.* 2009; Gupta and Timmis 2005; Othmani *et al.* 2011; Shohael *et al.* 2005; Shohael, *et al.* 2014; Soh *et al.* 2011; Yang *et al.* 2013).

#### 4) Formation of storage organs for only some species.

Species that naturally produce storage organs (bulbs, corms, tubers) can be induced to form small versions of these organs *in vitro* to provide a convenient and rapid means of micropropagation and/or genotype storage. Small bulbs can be produced from axillary buds,

adventitious buds developed on leaf pieces, inflorescence stalk, and detached bulb scales. Induction of small bulbs requires high sugar and auxin levels (Vishnevetsky *et al.* 2003). Examples of *in vitro* propagation via bulb formation include *Amaryllis* (De Bruyn *et al.* 1992), *Allium* (Kim *et al.* 2003), *Lilium* (Miwa 1991), *Narcissus* (Santos *et al.* 1998), and *Tulipa* (Rice *et al.* 1983). Small corms (cormlets) can form on buds, the basal end of callus and root primordia in the presence of high levels of sugar, auxins, cytokinins and sometimes growth retardants (Ziv *et al.* 1970; Ziv 1989). Some examples of *in vitro* propagation via cormlet formation include gladiolus (Ziv 1989), saffron (Devi *et al.* 2011), and banana (Venkatachalam *et al.* 2006). Miniature tubers (microtubers) also can be induced from buds/shoots *in vitro* under high levels of sucrose, cytokinins, auxins, and growth retardants (e.g. paclobutrazol) (Romanov *et al.* 2000; Vreugdenhil *et al.* 1994; Ziv 2005). Potato (Hussey and Stacey 1981), yam (Balogun 2009; Jova *et al.* 2005) and cocoyam (Omokolo *et al.* 2003) are some examples of propagation by *in vitro* microtuber formation.

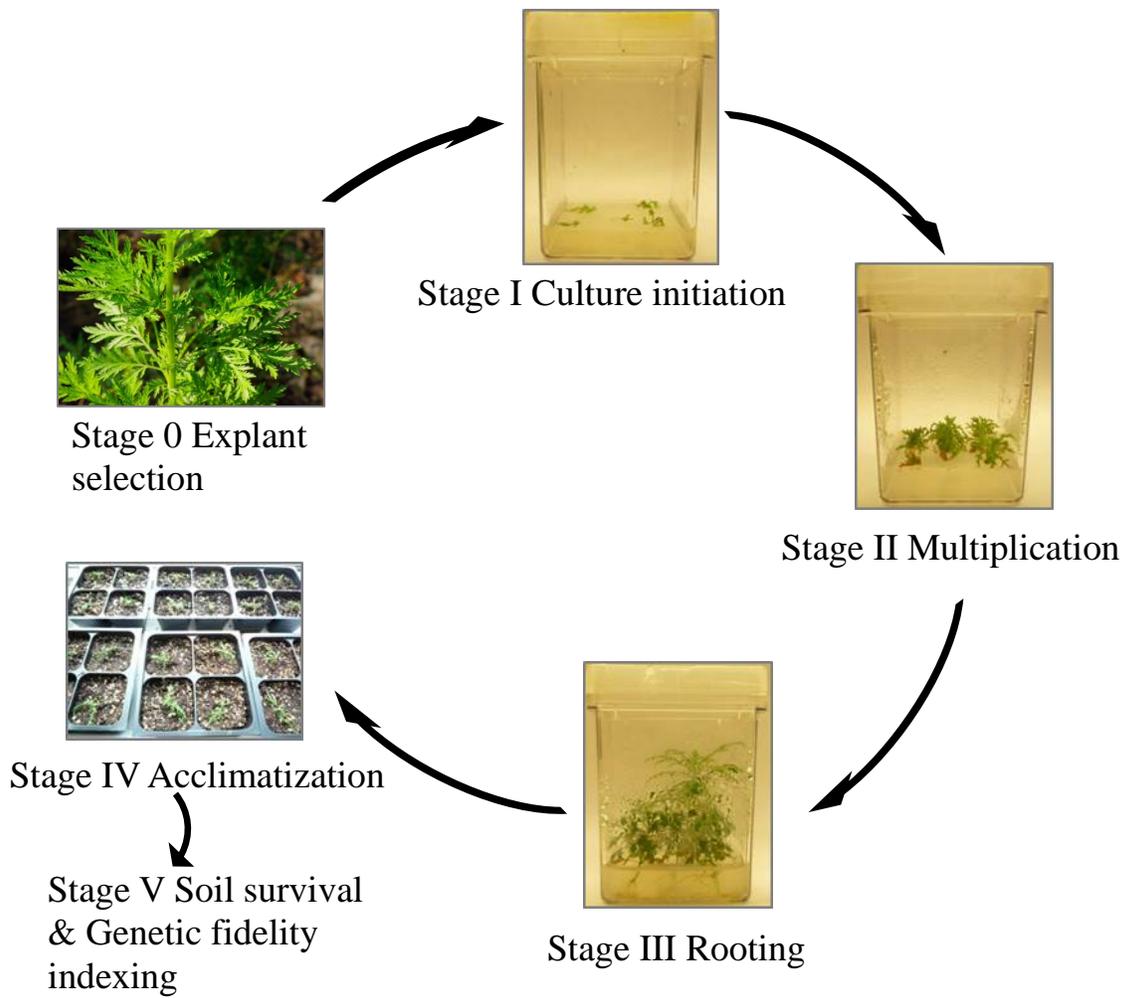
### **Stages of micropropagation**

The general protocol for micropropagation is composed of 6 stages (0-V) (Figure 1.1). While the practices in stage 0, IV and V are generally the same for different micropropagation methods, the strategy used for stages I-III may vary (see Table 1.2).

**Stage 0** Mother plant selection and preparation. This stage includes 1) careful selection of the mother plant, which must be typical of the variety or species, and free from any sign of disease; 2) some pre-treatments in an effort to reduce contamination level of explants and to improve

*in vitro* growth. Pretreatments may include moving chosen plants to sterile soil, using non-overhead watering, and chemical treatment to prevent systemic bacterial, fungal, or viral disease (Debergh and Maene 1981).

**Stage I** Initiation of aseptic culture. In this step, selected plant tissue, e.g. leaf, bud, stem (node), root, seed, or embryo, is first excised, sterilized by disinfectants to kill off any surface contaminants, and then rinsed repeatedly with sterile water prior to being placed in nutrient medium. Commonly used sterilizing solutions include hypochlorite, mercuric chloride, ethanol, silver nitrate, bromide water, and hydrogen peroxide. The explant is often sequentially soaked in more than one of these solutions before an aseptic culture is established. Usually a batch of explants is treated as described and transferred to culture at the same time. Containers having contaminated explants are discarded after the initiation of aseptic culture. Those remaining uncontaminated and also with vigorous growth are considered successfully established *in vitro* cultures, and can then be used in the next step. Stage I would be regarded as satisfactorily completed if an adequate number of aseptic *in vitro* cultures are successfully established. For indirect somatic embryogenesis, an auxin is often included in this stage to induce embryogenic cell suspensions or callus.



**Figure 1.1** Stages of micropropagation

**Table 1.2** Stages I, II and III for various micropropagation strategies

Propagation strategies	Stage I Initiation	Stage II Multiplication	Stage III Rooting
Multiplication of axillary shoots	Transfer of disinfected shoot meristems, shoot tips, lateral buds, nodes, aseptic floral meristem, aseptic seeds to semi-solid media.	Induce the formation of multiple shoots and allow for growth till large enough to be separated, either as new explant for subculture in this stage or passage to stage III. In node culture, the pre-existing axillary bud in each node grows into a single shoot. Subculture is indefinite.	Elongation of shoots from stage II and rooting <i>in vitro</i> or <i>ex vitro</i> .
Adventitious shoots from direct organogenesis	Establishing axenic culture of mother plant tissue (e.g. leaf, internode).	Shoots induced directly on the explant without prior formation of callus. Shoots then divided to be used either for subculture in stage II or passage to stage III.	Same as for shoot cultures.
Adventitious shoots from indirect organogenesis	Initiation and isolation of callus with superficial shoot meristems.	Subcultures of divided callus pieces following by transfer to shoot inducing medium for shoot development.	Same as for shoot cultures.
Direct embryogenesis	Establishment of embryogenic tissue explants or previous formed somatic embryos.	Direct induction of somatic embryos from explants without prior callus formation.	Growth of embryos into plantlets.
Indirect embryogenesis	Initiation and isolation of callus capable to forming embryos. Or obtaining embryogenic suspension cultures from callus.	Transfer of subcultures of callus or suspension culture to a media favoring embryo development.	Growth of embryos into plantlets; or encapsulated into synthetic seed for sowing.
Storage organ formation	Cultures of tissue/organs capable of storage organ development (e.g. bud cluster).	Induction of storage organ formation.	Growth of shoots/plantlets from storage organs; or growing storage organs to a size suitable for soil planting.

**Stage II** Multiplication. The aim of this stage is to produce new plant outgrowths or propagules that when separated from the cultures are capable of giving rise to whole plants. Depending on different micropropagation methods, tissues being multiplied can be newly-developed axillary or adventitious shoots, somatic embryos, or miniature storage or propagative organs. The multiplication of shoots is usually achieved through a higher ratio of cytokinins to auxins in the medium (Wickson and Thimann 1958). Cytokinins are extremely effective in removing the apical dominance of shoots and thus generate “bushy” shoot cultures (Mok 1994; Sachs and Thimann 1967). The multiplied shoots can also be used as the basis for further cycles of multiplication to increase their number. On the other hand, node culture does not require such a high level of cytokinins to stimulate boost of axillary buds but instead relies on repeated subculture of single nodes or nodal pieces with several nodes to obtain axillary shoots. The medium in node culture is generally good for shoot elongation and thus can be used after multiplication with exposure to cytokinins and before rooting. For indirect somatic embryogenesis, embryogenic cells from Stage I are transferred to auxin-free medium to develop somatic embryos.

**Stage III** Elongation and rooting. In this stage, newly developed shoots and somatic embryos are separated and further developed into complete plantlets. Stage III includes shoot rooting or germination of somatic embryos in cytokinin-free medium. Sometimes shoots may need an additional elongation period prior to rooting. Each separated new shoot from stage II is transferred to rooting medium, which usually contains a high concentration of auxin (e.g. IBA,

NAA) to stimulate *in vitro* rooting of many plants. This can be the most labor intensive step in micropropagation, comprising 35-75% of the total cost of production (Debergh and Maene 1981). Rooting may also occur *ex vitro* in conjunction with stage IV by removing unrooted shoots from the *in vitro* environment to soil or other potting mixtures (Hazarika 2003). Unrooted shoots can also be chopped into nodal segments and shoot tips (~5mm), encapsulated usually in alginate for storage, and then used as “seed” for greenhouse culture (Ahmad *et al.* 2012; Chand and Singh 2004; Preece and West 2006; Sarkar and Naik 1998; Singh *et al.* 2009; Singh *et al.* 2010).

**Stage IV** Acclimatization and transplant to the soil. In contrast to *ex vitro* conditions, the typical *in vitro* environment has high relative humidity, no pathogens, and low light intensity (Aitken-Christie *et al.* 1995a, also see Table 1.3). Because of these differences, plantlets developed *in vitro* differ with soil grown plants mainly in two aspects. First, *in vitro* cultured plants have dysfunctional leaf stomata that are incapable of complete closure under conditions of low relative humidity (Brainerd and Fuchigami 1982; Correll and Weathers 2001b; George *et al.* 2008c; Joshi *et al.* 2006). Plantlets *in vitro* also have less leaf epicuticular wax or wax with altered chemical composition than plants grown in soil (Correll and Weathers 2001b; George, *et al.* 2008c; Grout 1975; Sutter 1985). As a result, these tissue cultured plants lose water rapidly when moved to external conditions (Conner and Conner 1984; Grout and Aston 1977; Preece and Flickinger 2009; Sutter and Langhans 1982).

**Table 1.3** Environmental comparison of typical *in vitro* and *in vivo* condition

	<i>In vitro</i>	<i>Ex vitro</i> (soil grown)
Aerial physical environment	<ul style="list-style-type: none"> <li>• Controlled temperature between 20-25 °C</li> <li>• Cool white fluorescent or LED light bulb with limited light spectrum</li> <li>• Low light intensity 35-70 <math>\mu\text{mol m}^{-2} \cdot \text{s}^{-1}</math></li> <li>• Fluctuating CO<sub>2</sub> concentration between day and night</li> <li>• Accumulation of ethylene</li> <li>• &gt;95% relative humidity</li> <li>• Limited ventilation</li> </ul>	<ul style="list-style-type: none"> <li>• Temperature not controlled</li> <li>• Full spectrum sun light with intensity <math>\sim 2000 \mu\text{mol m}^{-2} \cdot \text{s}^{-1}</math></li> <li>• Consistent ambient air with relative humidity 20%~70%</li> </ul>
Chemical environment	Composition defined medium containing sugar, inorganics, and growth regulators	<ul style="list-style-type: none"> <li>• Chemically undefined soil;</li> <li>• Use of pesticides and fertilizers after seeding</li> </ul>
Biological environment	Axenic culture	<ul style="list-style-type: none"> <li>• Symbiotic microorganisms</li> <li>• Viral and bacterial infection</li> <li>• Pest attack</li> </ul>

Second, plantlets *in vitro* have lower photosynthetic ability than field plants (Triques *et al.* 1997). The reduced photosynthesis is a result of readily available sucrose (or other carbon hydrates) in the medium and low light intensity as well as CO<sub>2</sub> *in vitro* (Grout 1988). Moreover, plantlets *in vitro* can also develop lethal abnormalities with severe physio-morphological changes, i.e. hyperhydricity, as a result of *in vitro* culture conditions (see section 1.3). So there is a necessity for plants to undergo a period of acclimatization during which they develop functional stomata, normal epicuticular wax, and sufficient photosynthetic activity in persistent and new leaves to sustain their survival *ex vitro* (Chandra *et al.* 2010; Correll *et al.* 2001; Correll and Weathers 2001a; Correll and Weathers 2001b; Pospisilova *et al.* 1999).

In practice, plantlets are carefully removed from Stage III containers, potted into soil or some other potting mixture, and then kept for several days to weeks in high humidity and reduced light intensity. High humidity can be generated by automated intermittent fogging or misting (Okada *et al.* 1992; Preece and Flickinger 2009). Humidity is then gradually reduced together with gradually increasing light intensity to prepare plants for greenhouse or field conditions (Kirdmanee *et al.* 1995b; Pospisilova, *et al.* 1999).

Plantlets can also be acclimatized *in vitro*, which involves changing *in vitro* conditions by physical and chemical means (Ziv 1995a). Physical manipulation deals with alteration of the gaseous environment to resemble *ex vitro* condition, and includes reducing relative humidity (Cha-Um *et al.* 2010; Correll, *et al.* 2001; Correll and Weathers 2001a; Deng and Donnelly 1993) and increasing CO<sub>2</sub> availability (Correll and Weathers 2001b; da Silva *et al.* 2005; Deng and Donnelly 1993; McCartan *et al.* 2004). These treatments often may also include reduction or elimination of sugar in the medium to stimulate autotrophic growth (da Silva, *et al.* 2005; Deng and Donnelly 1993), addition of growth retardants such as paclobutrazol or uniconazole (Cha-um *et al.* 2009; Murali and Duncan 1995; Ritchie *et al.* 1991), addition of an osmotic pressure stressor, e.g. PEG (Zaid and Hughes 1995), and abscisic acid (ABA) (Hronková *et al.* 2003). Incorporation of these practices during stage II and III have not only improved plant quality *in vitro* but also shortened the time required for acclimatization (Shin *et al.* 2014). Indeed the practice of photoautotrophic micropropagation (see section 1.6) is an attempt to produce *in vitro* plants that are physiologically similar to those grown in a greenhouse.

**Stage V** Confirmation of genetic fidelity. In this stage the quality of micropropagated plants is confirmed by disease indexing and genetic fidelity testing. Pathogen and contamination diagnosis range from conventional microscopy to ELISA and genomic analysis (Cassells and Doyle 2006; Cassells 2012). Genetic fidelity tests nowadays use PCR-based DNA markers such as arbitrary (Random Amplified Polymorphic DNA, RAPD), semi-arbitrary (Inter-Simple Sequence Repeat, ISSR; Amplified Fragment Length Polymorphism, AFLP), and sequence-based (Simple Sequence Repeat, SSR) (Chavan *et al.* 2013; Fatima *et al.* 2013; Singh *et al.* 2013) methods.

## **1.4 Hyperhydricity**

Hyperhydricity is a physiological disorder in plant tissue cultures that causes a reduction of propagation and death of tissues when transferred to *ex vitro* conditions. It occurs in a wide range of plant species and develops mainly in leaves and stems. The hyperhydric tissue appears visibly as glassy, watery in appearance, and leaves are curly, rigid, and brittle (Dewir *et al.* 2006b; Ziv 1991c). Studies on leaf structure indicate that compared to normal leaves, hyperhydric leaves have round and elevated stomata, larger epidermal and mesophyll cells, bigger intercellular spaces, and thinner cuticles (Gaspar 1991; Ziv 1991c; Ziv and Ariel 1994). Compared to normal leaves, hyperhydric leaves also have a higher content of water in the apoplast, lower levels of chlorophyll, reduced lignin as a direct result of reduced phenylalanine ammonia lyase (PAL) activity and phenols, lower levels of cellulose, pectins

and cutin, as well as callose deposits (Debergh *et al.* 1992; Kevers 1985; Kevers and Gaspar 1986; Kevers *et al.* 1987; Olmos *et al.* 1997; Ziv 1991b; Ziv and Ariel 1994).

Biochemical analysis of hyperhydric leaves showed elevated lipoxygenase (LOX) activity and malondialdehyde content (MDA, the decomposition product of lipid by LOX), and increased solute leakage, suggesting lipid peroxidation due to oxidative stress (Dewir, *et al.* 2006b; Olmos, *et al.* 1997; Piqueras *et al.* 2002; Wu *et al.* 2009). The occurrence of oxidative stress was confirmed by direct evidence of increased hydrogen peroxide in hyperhydric shoots, i.e. epidermal cell walls and intercellular space, depletion of antioxidants, and elevated antioxidant enzyme activity (Dewir, *et al.* 2006b; Fernandez-García *et al.* 2008; Saher *et al.* 2004; Sreedhar *et al.* 2009; Wu, *et al.* 2009).

Oxidative stress in hyperhydric tissue may be caused by hypoxia that results from water over accumulating in the apoplast for the following reasons. First, excessive water, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and peroxidase activity co-localize in the intercellular space (apoplast) (Fernandez-García, *et al.* 2008; Gribble *et al.* 1998). Second, the air volume of the apoplast is dramatically reduced as a result of water accumulation and this significantly affects gas diffusion (Jackson 2008; van den Dries *et al.* 2013). Third, hyperhydric shoots showed alteration in carbohydrate metabolism in their oxidative pentose phosphate and fermentative pathways, suggesting adaptation to hypoxia stress conditions (Saher *et al.* 2005c).

Oxidative stress triggers a series of downstream events that ultimately result in hyperhydric symptom. For example, increased H<sub>2</sub>O<sub>2</sub> triggers over-production of ethylene by activating 1-aminocyclopropane-1-carboxylic oxidase (*ACO*) gene expression (Kim *et al.* 2008). Pectins are a group of heterologous polymers in the primary cell wall contributing to cell adhesion, cell wall architecture, and cell wall mechanical strength (Saher *et al.* 2005b). They are highly de-esterified in hyperhydric tissue due to significantly increased expression and activity of pectin methyl esterases (PMEs) induced by oxidative stress (Chandran *et al.* 2008; Qu *et al.* 2011; Saher, *et al.* 2005b). Less esterification of pectin of hyperhydric tissue yields a stiffer pectate gel via alteration of the Ca<sup>2+</sup> bridge, contributing to cell wall stiffening and rigidity of hyperhydric tissue (Fernandez-García, *et al.* 2008; Goldberg *et al.* 1996).

Although the complete mechanism underlying development of hyperhydric symptoms are yet to be elucidated, the major environmental factors that contribute to the process are known (Table 1.3). High relative humidity, limited gas exchange, high levels of cytokinins and ammonium in the medium, and soft-gelled media are all known to induce hyperhydricity (Dutta Gupta and Prasad 2010; Ivanova and van Staden 2008; Ivanova and Van Staden 2009; Kevers *et al.* 2004). Thus, hyperhydricity can theoretically be reduced and even 'reversed' by changing these environmental conditions. For example, hyperhydric shoots of *Rubus chamaemorus* produced normal shoots after transfer from submersion in liquid medium to gelled medium (Debnath 2007). Control of hyperhydricity has been achieved by reduction of relative humidity through elevated ventilation or bottom-cooling (Casanova *et al.* 2008;

Correll, *et al.* 2001; Correll and Weathers 2001a; Ivanova and Van Staden 2010; Majada *et al.* 1997; Saher *et al.* 2005a), by altering the type of cytokinin (Ivanova and Van Staden 2011; Kadota and Niimi 2003; Sandal *et al.* 2001), by increasing ion and/or magnesium concentration (Yadav *et al.* 2003), by altering gelling agent (Ivanova and Van Staden 2011; Whitehouse *et al.* 2002), by increasing agar concentration (but at the cost of reduced shoot multiplication) (Casanova, *et al.* 2008; Saher, *et al.* 2004), by using porous support material (Tascan *et al.* 2010), by addition of growth retardants to reduce shoot growth (Chen and Ziv 2001), and by adding anti-vitrification agents (Whitehouse, *et al.* 2002), osmotic stressors (Kadota *et al.* 2001), rare earth elements (Wang *et al.* 2007), and antioxidant agents, e.g. polyamines (Tabart *et al.* 2014). The antioxidant effect of polyamines results from their anion- and cation-binding properties during radical scavenging, and also from their capability to inhibit both lipid peroxidation and metal-catalysed oxidative reactions (Groppa and Benavides 2008).

## **1.5 Physical environmental factors that affect *in vitro* tissue cultures:**

The key to successful micropropagation is to produce high quality plantlets *in vitro* physiologically resembling *ex vitro* plants as much as possible. The quality of plant tissue *in vitro* is greatly affected by the microenvironment in which plant tissues are cultured. Understanding how plants *in vitro* respond to these environmental factors paves the way for production of plantlets with improved physiology (Figure 1.2).

### Micropropagation

Light: 15~100  $\mu\text{mol m}^{-2}\cdot\text{s}^{-1}$

RH: > 90%

O<sub>2</sub>: ambient level

CO<sub>2</sub>: 0.039% ~ 0.3% (v/v)

C<sub>2</sub>H<sub>4</sub>: minimal level for organogenesis

Gas exchange: < 0.05 vvm

Light: 50~300  $\mu\text{mol m}^{-2}\cdot\text{s}^{-1}$

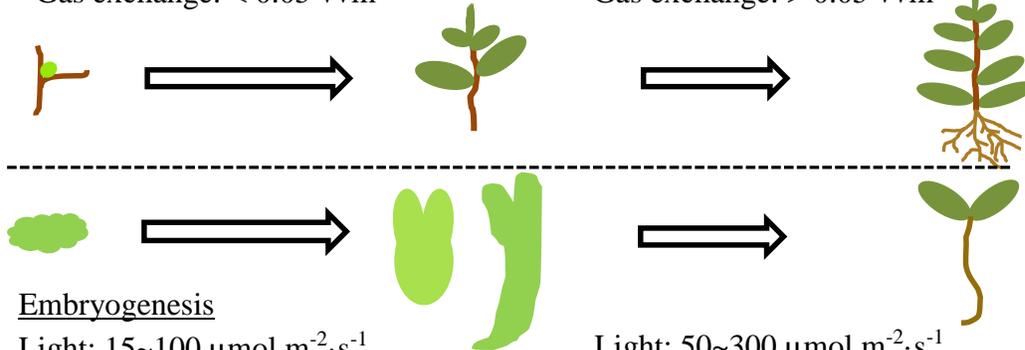
RH: 75 ~ 85%

O<sub>2</sub>: ambient level

CO<sub>2</sub>: 0.0039 ~ 0.3% (v/v)

C<sub>2</sub>H<sub>4</sub>: as low as possible

Gas exchange: > 0.05 vvm



### Embryogenesis

Light: 15~100  $\mu\text{mol m}^{-2}\cdot\text{s}^{-1}$

RH: > 90%

O<sub>2</sub>: ambient level

CO<sub>2</sub>: 0.3% ~ 5% (v/v)

C<sub>2</sub>H<sub>4</sub>: minimal level for organogenesis

Gas exchange: 0 ~ 0.005 vvm

Light: 50~300  $\mu\text{mol m}^{-2}\cdot\text{s}^{-1}$

RH: 75 ~ 90%

O<sub>2</sub>: ambient level

CO<sub>2</sub>: 0.3% ~ 5% (v/v)

C<sub>2</sub>H<sub>4</sub>: as low as possible

Gas exchange: >0.005 vvm

**Figure 1.2** Key factors for *in vitro* production of high quality plantlets

## 1.5.1 Light

White fluorescent lamps have been the primary light source used in micropropagation because their spectrum (400-700 nm) generally matches the requirements of *in vitro* cultures and they give a relatively uniform horizontal distribution of photosynthetic photon flux density over the entire culture shelf.

Besides fluorescent lamps, light emitting diodes (LED) with a single wavelength have been widely studied as an alternative light source. A mix of blue (450-480 nm) and red (640-660 nm) LED yielded better overall plant growth than that of fluorescent light (Kim *et al.* 2004b;

Li *et al.* 2010; Nhut *et al.* 2003). The preferred ratio of blue and red light is species and cultivar dependent. For example, the strawberry cultivar ‘Akihime’ showed better plantlet growth under 30% blue+70% red than other ratios with more red LED in the light mix (Nhut, *et al.* 2003). Among three blue:red ratios, 3:1, 1:1 and 1:3, plantlets of *Gossypium hirsutum* showed best growth under the 1:1 ratio (Li, *et al.* 2010). Plantlet growth of *Brassica napus*, however, was greater under a 3:1 blue to red light mix than 1:1 or 1:3 of blue to red light mix (Li *et al.* 2013). The protocorm-like-body of *Dendrobium officinale* yielded similar shoot number under blue:red ratios of 2:1 and 1:1, and shoot numbers under these two light conditions were more than those cultured under a 1:2 of a blue:red LED mix (Lin *et al.* 2011b). Integration of small amount of far-red LED light into a red and blue light mix enhanced leaf expansion, numbers of leaves and roots, chlorophyll contents, and biomass of *Oncidium* plantlets (Chung *et al.* 2010).

Increasing light intensity accompanied with CO<sub>2</sub> enrichment of the culture environment promotes accumulation of photosynthetic compounds and stimulates photoautotrophic growth of chlorophyllous tissues (Kozai *et al.* 1990; Xiao and Kozai 2006b). With increased light intensity, the morphology of *in vitro* tissues also resembles *ex vitro* tissues with increased thickness of leaves, larger palisade and spongy parenchyma and functional stomata (Fan *et al.* 2013). Light enhances biomass yield probably by enhancing photomixtrophic growth (Ahmed *et al.* 2008; Geipel *et al.* 2014). Light also activates and/or enhances major enzymes involved in secondary metabolite production, and thus also enhances the production of compounds like

phenolics (Victório *et al.* 2011), flavonoids (Koyama *et al.* 2012), and ginsenosides (Yu *et al.* 2005).

Besides light intensity, light quality also greatly affects growth, physio-morphological characteristics and even secondary metabolite production of plant tissues *in vitro*. Besides their photosynthetic importance, blue and red light also regulate photomorphogenesis, growth, and development of plantlets in micropropagation. Through cryptochrome, blue light can increase leaf number, and thickness, chlorophyll content in leaves and promote differentiation (Kurilčik *et al.* 2008; Li, *et al.* 2010; Lin, *et al.* 2011b; Liu *et al.* 2011; Macedo *et al.* 2011; Poudel *et al.* 2008). In contrast to stimulating effects, blue light may also inhibit shoot elongation and rooting (Kurilčik, *et al.* 2008; Lee *et al.* 2007; Moreira da Silva and Debergh 1997; Nhut, *et al.* 2003). Red and far red light signal through phytochrome regulation of plantlet elongation, starch accumulation in leaves, formation of storage organs, leaf expansion, rooting, as well as somatic embryogenesis (Appelgren 1991; Ascencio-Cabral *et al.* 2008; Chen *et al.* 2014; D'Onofrio *et al.* 1998; Hahn *et al.* 2000; Heo *et al.* 2006; Hunter and Burritt 2004; Jao *et al.* 2005; Li, *et al.* 2010; Macedo, *et al.* 2011; Nhut, *et al.* 2003; Poudel, *et al.* 2008; Rodríguez-Sahagún *et al.* 2011; Sæbø *et al.* 1995; Torné *et al.* 2001; Wu and Lin 2012). Far-red (FR) light counteracts the phytochrome activity of red light and it is the R:FR ratio that is critical in altering physiological responses. For example, the stem of chrysanthemum plantlets irradiated under the R:FR ratio of 0.5 was longer, but also more fragile than those grown under  $R:FR \geq 1$  (Kim, *et al.* 2004b), suggesting secondary effects on gibberellins and

lignin. Plants of *Ruppia maritima* grown under an R:FR ratio of 0.55 produced fewer nodes and branches than did plants grown under the ambient R:FR ratio of 0.95 (Rose and Durako 1994). While plantlet length and leaf area increased, the number of axillary shoots of *Azorina vidalii* (Wats.) Feer decreased when R:FR was reduced from 1.1 to 0.6 (Moreira da Silva and Debergh 1997). Compared to a R:FR ratio of 98, a lower ratio of 6 decreased in somatic embryogenesis of *Araujia sericifera* (Torné, *et al.* 2001)

Although seemingly sparsely studied, light quality can also play a role in embryogenesis. For example, compared to darkness, far-red, or red-far-red exposure, red light increased somatic embryos fourfold in quince (*Cydonia oblonga*) (D'Onofrio, *et al.* 1998). While initiation of somatic embryos from *Agave tequilana* showed no dependency on light quality, later development into the cotyledon stage was maximized after exposure to either red or white light (Rodríguez-Sahagún, *et al.* 2011). Red or white light stimulated development of cotyledons on China rose (*Rosa chinensis* Jacq.) somatic embryos, whereas most darkness-grown somatic embryos did not have any cotyledons (Chen, *et al.* 2014). Chen *et al.* (2014) also showed that in China rose, the calli from white light gradually lost their embryogenesis capability, while the calli from red light remained embryogenic. In carrot suspensions, darkness produced the most somatic embryos, which did not differ from cells exposed to red or green light (Michler and Lineberger 1987). On the other hand, both white and blue light inhibited somatic embryo formation (Michler and Lineberger 1987). Michler and Lineberger (1987) also showed that in carrot, red light enhanced development of the heart stage.

## 1.5.2 Relative humidity

The typical relative humidity (RH) inside a conventional tightly-sealed culture vessel ranges from 95% - 100% (Fujiwara and Kozai 1995). Such a high RH is beneficial for germination of somatic embryos *in vitro* (Roberts *et al.* 1990). However, compared to soil-grown plants, leafy tissues grown under high humidity usually developed a thinner layer of cuticle wax (Sutter and Langhans 1982) and dysfunctional stomata (Brainerd and Fuchigami 1982; Kozai and Zobayed 2003). Plantlets with these developmental abnormalities exhibited excessive water loss and death when exposed to ambient humidity *ex vitro*. When *in vitro* plantlets are moved into greenhouse or field environments, they have to develop fully functional stomata during acclimatization (Brainerd and Fuchigami 1982). The above mentioned developmental abnormalities are accentuated in hyperhydric plantlets, which usually do not survive acclimatization (Ziv 1991a).

Moderately reduced RH (75%-85%) improves plant growth, epicuticular wax deposition, stomatal function, and reduces hyperhydricity, and consequently results in improved *ex vitro* survival and resistance to desiccation (Correll and Weathers 2001b; Gribble 1999; Kozai *et al.* 1993; Maier and Post-Beittenmiller 1998; Sáez *et al.* 2012; Tanaka *et al.* 1992; Zobayed *et al.* 2001b). Reduced RH also promotes new bud formation (Ibrahim and Debergh 2001). On the other hand, for non-hyperhydric tissue *in vitro*, reduced RH also may result in slightly reduced net photosynthesis (Tanaka, *et al.* 1992). This reduction in photosynthesis under lower RH was attributed to smaller leaf area, reduced stomatal opening leading to reduced mesophyll diffusion of intercellular CO<sub>2</sub>, and decreased quantum yield and efficiency (Kozai, *et al.* 1993;

Tosens *et al.* 2012; Zhang *et al.* 1996). When RH was < 70%, *in vitro* growth and subsequent biomass yield were significantly decreased (Gribaudo *et al.* 2003; Yue *et al.* 1993). RH of *in vitro* cultures, therefore, should be kept around 75-85% to maintain vigorous plantlet growth without biomass loss (Kozai, *et al.* 1993). Reduction of RH in closed culture vessels has been achieved through bottom cooling and/or increased ventilation with ambient air (Gribble 1999; Saher, *et al.* 2005a).

### **1.5.3 Gas components**

The gas environment of the headspace of culture vessels directly affects photosynthesis and thus growth of *in vitro* plantlets. Main gases of concern for plant tissue culture include carbon dioxide (CO<sub>2</sub>), oxygen (O<sub>2</sub>) and ethylene (C<sub>2</sub>H<sub>4</sub>), as further discussed.

#### **1.5.3.1 Carbon dioxide (CO<sub>2</sub>)**

Availability of CO<sub>2</sub> in the headspace of the culture containers is important for photosynthesis of chlorophyllous tissue grown *in vitro*. Conventional culture containers with their poor ventilation are CO<sub>2</sub> limited, below the critical concentration of 35 μL L<sup>-1</sup>, so photosynthesis is also limited (De Proft *et al.* 1985; Fujiwara *et al.* 1987). Increasing the CO<sub>2</sub> concentration inside culture containers through increased ventilation or headspace enrichment promoted photosynthesis and autotrophic growth when accompanied with elevated light intensity (see section 1.6, Kozai, *et al.* 1990; Mosaleeyanon *et al.* 2004; Thongbai *et al.* 2011). To stimulate photosynthesis of *in vitro* plant tissues, CO<sub>2</sub> levels between 0.1-0.3% are needed (Kozai *et al.*

2006; Kozai 2010; Park *et al.* 2011; Radochová and Tichá 2008; Shim *et al.* 2003; Vyas and Purohit 2006; Zobayed *et al.* 1999a). CO<sub>2</sub> levels >1%, however, resulted in decreased chlorophyll content and net photosynthetic rate (Cournac *et al.* 1991; De Proft, *et al.* 1985; Norikane *et al.* 2010). For the C<sub>4</sub> plant *Actinidia deliciosa*, 0.2% CO<sub>2</sub> was adequate to inhibit photosynthesis (Arigita *et al.* 2002). Reduction of photosynthetic rate by excessive CO<sub>2</sub> is probably due to reduced Rubisco (the enzyme responsible for CO<sub>2</sub> fixation) activity (de la Viña *et al.* 1999; Fuentes *et al.* 2005; Hdider and Desjardins 1995; Norikane, *et al.* 2010).

As a result of increased photosynthesis, biosynthesis of secondary metabolite may also increase. For example, St. John's wort yielded more hypericin and pseudohypericin under 0.15% CO<sub>2</sub> than ambient controls (Mosaleeyanon *et al.* 2005). CO<sub>2</sub> enrichment of air to 0.08% increased biosynthesis of flavonoids and phenolic compounds in *in vitro* cultured *Zingiber officinale* Roscoe (Ghasemzadeh *et al.* 2010).

Elevated CO<sub>2</sub> levels in the culture container also increase somatic embryogenesis (Barbón *et al.* 2008b; Rosnow *et al.* 2011) and root biomass (Cha-um *et al.* 2011; Fisichella and Morini 2003; Jeong *et al.* 2006; Rosnow, *et al.* 2011). The mechanism by which CO<sub>2</sub> stimulates cell and tissue growth may arise not only from increased photosynthesis but also from non-photosynthetic CO<sub>2</sub> assimilation. For example, CO<sub>2</sub> enrichment increased biomass of heterologous cell and root cultures, suggesting CO<sub>2</sub> can affect the growth of cell and root tissue *per se* and independent of photosynthesis (Ducos *et al.* 1988; Jeong, *et al.* 2006; Maurel and Pareilleux 1986; Thanh *et al.* 2006). The mechanism whereby non-photosynthetic CO<sub>2</sub>

assimilation occurred was probably via phosphoenolpyruvate carboxylase activity (Bihzad and El-Shora 1996; Nagano *et al.* 1994; Ting and Osmond 1973).

CO<sub>2</sub> levels not tolerated by photosynthesis may be appropriate for other responses. For example, effective CO<sub>2</sub> levels for enhanced somatic embryogenesis varied with species and cultivars from 0.3 to 5% (Barbón *et al.* 2008a; Barbón, *et al.* 2008b; Buddendorfjoosten and Woltering 1994; Chung and Bae 2000; Huang *et al.* 2006; Rosnow, *et al.* 2011; Takamura *et al.* 2010). An extremely high CO<sub>2</sub> concentration (e.g. 10%), however, is toxic to embryo proliferation (Hohe *et al.* 1999a). For heterotrophic cell suspension cultures, high CO<sub>2</sub> concentrations (2.5%-5%) increased biosynthesis of anthraquinones, total phenolics and flavonoids in *Morinda citrifolia* (Jang *et al.* 2013). Cell suspensions of *Panax ginseng*, however, yielded less ginsenosides under such CO<sub>2</sub> conditions (Thanh, *et al.* 2006). Adventitious root cultures of *Panax ginseng* also yielded lower levels of ginsenosides under 2.5%-5% CO<sub>2</sub> enrichment than ambient controls (Jeong, *et al.* 2006).

### **1.5.3.2 Oxygen (O<sub>2</sub>)**

Oxygen is essential for aerobic respiration of plant tissue *in vitro*. In conventional culture vessels containing photosynthetic tissues, O<sub>2</sub> level progressively decreases during the dark period (Chen 2006), suggesting the necessity of improving gas exchange to keep a relatively stable gaseous environment. In photorespiration, oxygen competes with CO<sub>2</sub> for Rubisco binding in C<sub>3</sub> plants and, therefore, can inhibit growth of chlorophyllous explants (Bauwe 2001). Oxygen at 1% enhanced the net photosynthetic rate both *in vitro* and *ex vitro* (Fila *et*

*al.* 2006). Root development, however, requires adequate O<sub>2</sub> throughout the root zone (Drew 1997). For land plants, flooding the root zone generates hypoxic stress and triggers biosynthesis of endogenous ethylene, which then enhances the development of aerenchyma in the roots and adventitious roots on the stem to adapt to the flooding situation (Drew *et al.* 1979). Root development *in vitro* can be greatly improved by the use of porous supporting materials that increase O<sub>2</sub> diffusion around roots (see section 1.5.5).

Oxygen also affects differentiation of embryogenic cells. Although callus formation and explant viability is not affected, anoxia almost completely inhibits embryogenesis (Fisichella and Morini 2003), suggesting oxygen is required for embryogenesis. Low O<sub>2</sub> concentration may enhance embryo formation by simulating the *in ovule* environment normally encountered during zygotic embryo development (Carman 1988). The overall demand on O<sub>2</sub> then increases during subsequent maturation to the cotyledon stage (Jay *et al.* 1992; Shigeta *et al.* 1996; Shimazu and Kurata 1999).

Of course O<sub>2</sub> and CO<sub>2</sub> function in combinations that may fluctuate at different concentration optima depending on developmental stage of somatic embryos. Thus, while the early stage of embryogenesis may prefer relatively low O<sub>2</sub> and high CO<sub>2</sub> levels (de Feria *et al.* 2003; El Meskaoui and Tremblay 1999; Fisichella and Morini 2003; Kvaalen and Arnold 1991), there is considerable species and cultivar variation. For example, embryo initiation of celery is favored under 30% dissolved oxygen (DO) (ca. 5 mL L<sup>-1</sup>) plus 3% CO<sub>2</sub> (Huang, *et al.* 2006). On the other hand one cultivar of *Cyclamen persicum* had significant more embryos formed

at 40% DO (ca 7mL L<sup>-1</sup>) than another cultivar where there was better embryo formation at lower oxygen levels (Hohe *et al.* 1999b). Embryo differentiation is also affected as shown in *Coffea arabica* where a DO of 80% (ca. 14 mL L<sup>-1</sup>) generated more total embryos, but many fewer at the torpedo stage than at 50% DO (ca. 8.4 mL L<sup>-1</sup>) (de Feria, *et al.* 2003).

### 1.5.3.3 Ethylene (C<sub>2</sub>H<sub>4</sub>)

Ethylene is a volatile plant growth regulator produced by all living plant tissues. It has a wide range of developmental and physiological effects including fruit ripening, leaf epinasty with root anoxia, stem length reduction, lateral cell expansion, apical dominance and dormancy breakage, adventitious root and root hair formation, and stress response mediation (Abeles *et al.* 1992a; Abeles *et al.* 1992b; Bleecker and Kende 2000). During *in vitro* cultivation, biosynthesis of C<sub>2</sub>H<sub>4</sub> increases in response to environmental stress (George *et al.* 2008b; Saher, *et al.* 2004). Once produced and released into the headspace, C<sub>2</sub>H<sub>4</sub> cannot be absorbed by plants and can accumulate to high concentrations in sealed culture vessels (De Proft, *et al.* 1985; El Meskaoui and Tremblay 1999; Santamaria *et al.* 2000b), resulting in growth abnormalities (Biddington 1992; Fujiwara and Kozai 1995).

During organogenesis of *in vitro* explants, signaling by critical concentrations of ethylene seems beneficial to initiate differentiation in morphogenic responses including shoot regeneration (Chatfield and Raizada 2008; Kevers *et al.* 1992) and root initiation (Ivanchenko *et al.* 2008; Kim *et al.* 2010). For shoot morphogenesis, the critical concentration varies with species and cultivars (Al-Khayri and Al-Bahrany 2004; Kumar *et al.* 1998). For subsequent

growth, however, the concentration of C<sub>2</sub>H<sub>4</sub> in sealed vessels is generally toxic to shoots and roots (Jin Goh *et al.* 1997; Kevers, *et al.* 1992; Neto *et al.* 2009; Reis *et al.* 2003). Growth can be improved by decreasing ethylene concentration and use of inhibitors of the ethylene signal pathway (BuddendorfJoosten and Woltering 1994; BuddendorfJoosten and Woltering 1996; Kevers, *et al.* 1992; Kumar *et al.* 2009; Ma *et al.* 1998; Park *et al.* 2012; Santana-Buzzy *et al.* 2006; Zobayed *et al.* 2001a).

Ethylene also seems to be required for early differentiation during somatic embryogenesis (Huang, *et al.* 2006; Huang *et al.* 2001; Jha *et al.* 2007; Kępczyńska *et al.* 2009; Kępczyńska and Zielińska 2011; Liu *et al.* 2010; Lu *et al.* 2011; Nissen 1994). However, there are some conflicting reports on the effect of headspace C<sub>2</sub>H<sub>4</sub> on somatic embryogenesis (George, *et al.* 2008b; Jiménez 2005). It is thus likely that species and cultivars vary in their endogenous production of C<sub>2</sub>H<sub>4</sub> and optimal C<sub>2</sub>H<sub>4</sub> concentration for embryo development. Sub-optimal-producers may need an exogenous supply of C<sub>2</sub>H<sub>4</sub>, while over-producers may require removal of C<sub>2</sub>H<sub>4</sub> (El Meskaoui and Tremblay 1999; El Meskaoui and Tremblay 2001; Pérez-Jiménez *et al.* 2014). In sealed containers C<sub>2</sub>H<sub>4</sub> generally accumulates to toxic levels for subsequent embryo maturation (El Meskaoui *et al.* 2000). Embryo development can be improved, however, by increasing ventilation, using a C<sub>2</sub>H<sub>4</sub> trap (e.g. potassium permanganate), adding inhibitors of C<sub>2</sub>H<sub>4</sub> biosynthesis (e.g. aminoxyacetic acid, aminoethoxyvinylglycine), or action (e.g. silver nitrate, CO<sub>2</sub>) (Hosseini *et al.* 2009; Kumar, *et al.* 1998; Roustan *et al.* 1994; Roustan *et al.* 1989; Roustan *et al.* 1990; Wang *et al.* 2011).

Although the role of C<sub>2</sub>H<sub>4</sub> in development of hyperhydric symptoms is not clear, C<sub>2</sub>H<sub>4</sub> biosynthesis is stimulated in hyperhydric tissues (Franck *et al.* 2004; Kevers and Gaspar 1985; Kevers and Gaspar 1986; Saher, *et al.* 2004). C<sub>2</sub>H<sub>4</sub> may inhibit abscisic acid-mediated stomatal closure and reduce lignin synthesis (Tanaka *et al.* 2005). Application of C<sub>2</sub>H<sub>4</sub> inhibitors, however, does not necessarily reduce hyperhydricity (Fal *et al.* 1999; Kevers and Gaspar 1985; Mayor *et al.* 2003; Park *et al.* 2004).

#### **1.5.4 Gas exchange**

The gas environment inside a culture container is highly dependent on the gas exchange rate (ventilation) of the headspace gas. Rate of gas exchange is usually defined as the number of volumetric exchanges of headspace gas per unit time. In passive ventilation, the unit time is usually per hour due to low gas exchange and the gas exchange rate is described as N; whereas in forced ventilation, the unit time is usually per minute and the gas exchange rate is described as vvm, which is the number of volumetric headspace gas exchanges per minute. These two units of gas exchange rate are interconvertible:  $N = vvm \times 60$ . Increasing gas exchange benefits growth by increasing CO<sub>2</sub> level and reducing relative humidity as well as toxic volatiles (i.e. C<sub>2</sub>H<sub>4</sub>) in the headspace of a culture container (Thongbai *et al.* 2010; Xiao *et al.* 2003; Zobayed, *et al.* 2001a). As a result of improvement in the headspace environment, plantlets *in vitro* show increased photosynthesis and biomass (shooting and rooting), decreased hyperhydricity, more deposition of epi-cuticular wax, functional stomata, and enhanced contents of secondary metabolites (Hahn and Paek 2001; Ivanova and Van Staden 2010; Majada *et al.* 1998; Majada

*et al.* 2001; Majada, *et al.* 1997; Majada *et al.* 2002; Mohamed and Alsadon 2010; Mohamed and Ibrahim 2012; Shohael and Paek 2013; Wang *et al.* 2013; Zhao *et al.* 2012).

In practice, the gas exchange rate can be increased by increasing passive ventilation in culture containers or integrating a system of forced ventilation. In passive ventilated culture containers, the gas exchange rate is increased by using porous closures or gas permeable membranes on the closure (Chen *et al.* 2006b; Fujiwara and Kozai 1995; Mohamed and Alsadon 2010; Tsay *et al.* 2006). By using these strategies, the gas exchange rate can be elevated from 0.04 times h<sup>-1</sup> (0.00066 vvm) under non-ventilated conditions to around 5 times of headspace volumetric exchange h<sup>-1</sup> (0.083 vvm) (Chen and Chen 2002; Cui *et al.* 2000; Thongbai, *et al.* 2010). Except for small culture containers, the gas exchange rate under passive ventilation may still be limited, so even in spite of CO<sub>2</sub> enrichment, CO<sub>2</sub> concentration inside the container is challenging to maintain at ambient levels (0.039%) (Xiao, *et al.* 2003). Increased ventilation in gelled medium can desiccate the medium when gas exchange is increased even via passive ventilation and then alter osmotic potential of the medium, thereby limiting *in vitro* growth (Shim, *et al.* 2003; Yann *et al.* 2010).

Forced ventilation, on the other hand, is more effective than passive ventilation in terms of gas exchange rate for promoting photosynthesis of shoots, *in vitro* rooting and *ex vitro* survival (Gutiérrez *et al.* 2011; Kubota and Kozai 1992; Son *et al.* 2009; Zhao *et al.* ; Zobayed 2005; Zobayed *et al.* 2004). Forced ventilation is also more effective than passive ventilation in promoting photosynthesis of cotyledonary stage embryos and their subsequent germination as

well as conversion to plantlets in bioreactors (Afreen *et al.* 2005; Ducos *et al.* 2007b; Ducos *et al.* 2007c). Compared to passive ventilation, forced ventilation is a more reliable means of controlling the headspace environment (Zobayed, *et al.* 2004).

Forced ventilation is now essential for maintaining efficient gas exchange for photoautotrophic growth in large culture containers, i.e. bioreactors (Xiao *et al.* 2011). Forced ventilation is achieved by flushing humidified air into the culture container via an air pump connected to a sterile air filter (Kozai *et al.* 2000). The gas exchange rate under forced ventilation can be adjusted to more than 10 times  $\text{h}^{-1}$  (0.16 vvm), which efficiently replenishes  $\text{CO}_2$  for photosynthesis in bioreactors (Xiao and Kozai 2006b; Xiao, *et al.* 2003; Zobayed *et al.* 1999b; Zobayed 2000) and provides more uniform gas distribution in the headspace and usually, therefore, in the culture medium.

### **1.5.5 Cultivation substrates**

Conventional micropropagation uses agar mixed together with nutrients to form a gelled matrix on which plant tissues grow. However, agar is expensive and induces severe hyperhydricity, thus other gelling agents (e.g. gellan gum, guar gum, and starch) have been used (Babbar *et al.* 2005; Franck *et al.* 1998; Jain and Babbar 2002). Among these alternatives, gellan gum (Gelrite, Phytigel) has found wide acceptance. Compared to agar, it also gives rise to better quality plant tissues that have greater shoot multiplication, longer shoots, and larger

miniature storage organs (Babbar, *et al.* 2005; Kumar *et al.* 2003; Podwyszynska and Olszewski 1995; Veramendi *et al.* 1997).

Besides gelled medium, there are other options for porous supporting substrates depending on end goal, i.e. for plantlet rooting. Root development needs sufficient oxygen around the root zone (Drew 1997). Gelled medium is 100% water, and solubility of O<sub>2</sub> under typical *in vitro* culture conditions may not be adequate to support healthy root development (Atkinson and Mavituna 1991; Geankopolis 1993; Spomer and Smith 1996). Compared to gelled matrices, porous supporting materials soaked with liquid medium provide more oxygen throughout the root system, resulting in roots with improved physiological and anatomical features (Afreen *et al.* 1999; Fujiwara and Kozai 1995; Jay-Allemand *et al.* 1992; Newell *et al.* 2003; Prasad and Gupta 2006). In addition to benefiting root development, use of porous materials (e.g. polyester fiber) also reduced hyperhydricity in static liquid cultures (Tascan, *et al.* 2010). Common supporting materials include Florialite (a mixture of vermiculite and cellulose fibers) (Afreen, *et al.* 1999; Afreen *et al.* 2000; Kozai, *et al.* 2000; Marriott and Sarasan 2010; Nakamura 2006; Nguyen *et al.* 1999; Saldanha *et al.* 2014; Xiao and Kozai 2006a; Xiao, *et al.* 2003; Zobayed, *et al.* 1999b), vermiculite (Heo *et al.* 2001; Xiao and Kozai 2006b), sugarcane bagasse (Mohan *et al.* 2004; Rodríguez-Vázquez *et al.* 1999), perlite (Lucchesini *et al.* 2006; Xiao *et al.* 2000), ‘rockwool’ (Kubota and Kozai 1992; Norikane, *et al.* 2010), and mixed perlite and vermiculite (Oh *et al.* 2012). In commercial operations, Florialite and vermiculite are preferred mainly because of their relative ease of handling and quality of root

development (Kozai, *et al.* 2006; Zobayed, *et al.* 2004). Use of these porous materials enhances water and nutrient uptake and thereby promoting overall growth of plantlets and ultimately their *ex vitro* survival (Afreen, *et al.* 1999; Kirdmanee *et al.* 1995a; Nguyen, *et al.* 1999).

## 1.6 Photoautotrophic micropropagation

Photoautotrophic micropropagation was developed after the observation that shoots *in vitro* are photosynthetic so increased headspace CO<sub>2</sub> concurrent with increased light intensity promoted photosynthesis (Kozai, *et al.* 1990). Since one major difference between plantlets *in vitro* and those in the soil is photosynthetic ability, the quality of plantlets *in vitro* and their *ex vitro* survival can be greatly improved if they grow autotrophically *in vitro*. However, the typical *in vitro* environment is CO<sub>2</sub> and light limited, and has to be adjusted for photoautotrophic growth *in vitro* by increasing light intensity (100-300  $\mu\text{mol m}^{-2}\cdot\text{s}^{-1}$ ) and headspace CO<sub>2</sub> levels, while gradually eliminating sugar in the medium (Kozai, *et al.* 2006; Kozai 2010; Park, *et al.* 2011; Radochová and Tichá 2008; Serret *et al.* 1997; Shim, *et al.* 2003; Vyas and Purohit 2006; Zobayed, *et al.* 1999a). Light intensity beyond 300  $\mu\text{mol m}^{-2}\cdot\text{s}^{-1}$  does not yield a higher photosynthetic rate (Fan *et al.* 2013). Some C<sub>4</sub> plants, such as sea oats and kiwi, need sucrose to accumulate starch and start the photosynthetic machinery (Arigita *et al.* 2010; Valero-Aracama *et al.* 2007). To increase headspace CO<sub>2</sub>, ventilation is improved and RH is generally reduced resulting in plantlets not only capable of photoautotrophic growth but also having better physiological characteristics, i.e. stomata and cuticle, than those

cultured in conventional containers (Afreen 2005; Couceiro *et al.* 2006). Thus plantlets *in vitro* are well established for *ex vitro* survival and stage IV acclimatization can therefore be eliminated (Badr *et al.* 2011; Hahn and Paek 2001; Park, *et al.* 2011; Xiao and Kozai 2004; Zobayed *et al.* 2000). Besides physiological improvements, photoautotrophic micropropagation also reduces contamination risk by using sugar-free medium, and thus larger vessels can be used. As a result of these benefits, there are reductions both in plant loss and production costs compared to conventional propagation using small vessels and sugar-containing medium (Kozai, *et al.* 2006; Xiao and Kozai 2004; Zobayed, *et al.* 2004).

After studies on how *in vitro* plants responded to microenvironment conditions (see section 1.5), commercial scale photoautotrophic (sugar-free) micropropagation modules with large vessels/bioreactors (120L) and forced ventilation were developed (Kozai, *et al.* 2006; Zobayed, *et al.* 2004). Explants were cultured on porous supporting materials (e.g. vermiculite and Florialite) soaked with liquid sugar-free medium, light intensity between 100-300  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , and forced air ventilation with  $\pm \text{CO}_2$  (Kozai, *et al.* 2006; Xiao and Kozai 2004). Xiao *et al.* (2011) even raised the possibility of scaling up the culture system to an aseptic room culture where the whole room is the “vessel”. Hundreds of plant species have now been cultivated via photoautotrophic micropropagation (Kubota *et al.* 2005; Xiao, *et al.* 2011).

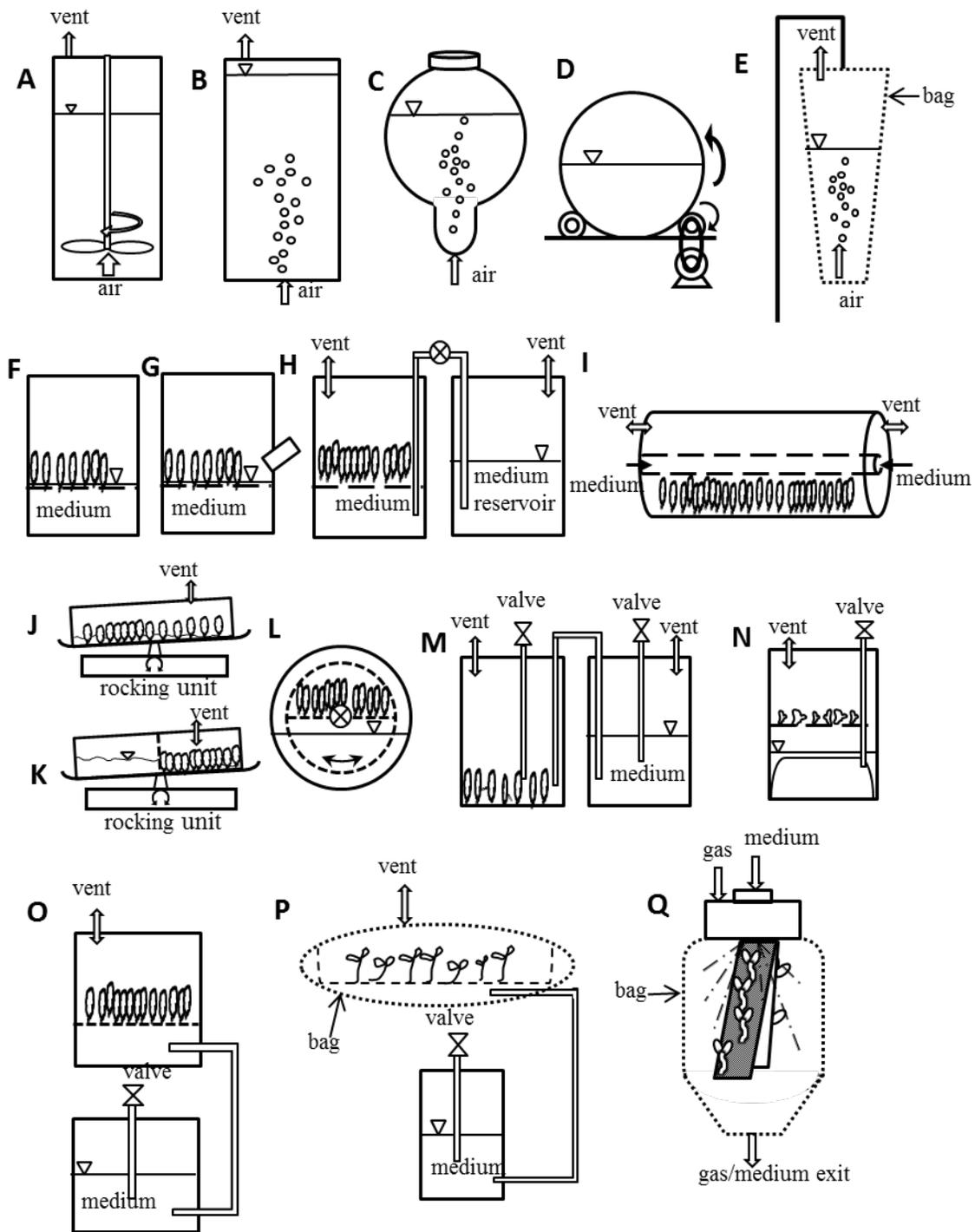
## **1.7 Bioreactors for micropropagation:**

The use of bioreactors for plant propagation originates from the limitations in traditional micropropagation production technology. Those limitations involve periodic transfer of plant

materials to fresh medium to sustain continuous tissue growth and proliferation as well as switching from one stage to the next. All of these operations require significant labor in handling each plantlet, which in turn increases production cost (Chu 1995; Florkowski 1988; Pachauri and Dhawan 1989). Traditional micropropagation uses gelled medium and a large number of small containers, which not only complicates automation but also provides an adverse culture environment which can ultimately result in plant abnormalities and losses during acclimatization (see section 1.4).

Use of a bioreactor to automate all or some of the various stages can reduce labor input and hence cost of plantlets since explants are no longer manually positioned in the culture container and medium can be readily renewed without manual transfer of plantlets (Aitken-Christie *et al.* 1995b; Alister *et al.* 2005; Lorenzo *et al.* 1998; Takayama and Akita 2006). Nutrient distribution is also more uniform in liquid than in gelled medium (Adelberg and Fári 2010; Smith and Spomer 1995; Williams 1992). However, plant species vary considerably in morphology as well as in their demand on nutrients and environmental conditions, so bioreactor design for micropropagation is challenging. As a result, many types of bioreactors have been designed and used for cultivating plant tissue and organ cultures as illustrated schematically in Figure 1.3. These include: A, the classic stirred tank (STR); B, the bubble column reactor (BCR); C, balloon-type bubble reactor (BTBR); D, rotating drum; E, life reactor; F, Lifeguard reactor; G, Growtek reactor; H, continuous partial immersion (CPI) reactor with medium replenishment; I, the artificial plant ovary (APO) reactor; J and K,

temporary immersion systems (TISs) riding on a rocker; L, the '3R' reactor; M-P, pneumatically driven TISs; and Q, the mist reactor. A number of these reactors also have disposable culture bags whereby both contamination risk and initial capital cost are reduced. These bioreactors differ by method of agitation and ventilation, vessel construction and type of immersion. In Figure 1.3 reactors with disposable culture bags include: E, the life reactor; P, the box-in-a-bag; and Q, the mist reactor. The focus here is not on cell suspensions, but rather on cultivation of fully differentiated plantlets.



**Figure 1.3** Various bioreactors used for plant tissue, organ and somatic embryos. Triangles in the figure indicate liquid level. A, the classic stirred tank (STR); B, the bubble column reactor (BCR); C, balloon-type bubble reactor (BTBR); D, rotating drum; E, life reactor; F, Lifeguard reactor; G, Growtek reactor; H, continuous partial immersion (CPI) reactor with medium replenishment; I, the artificial plant ovary (APO) reactor; J and K, temporary immersion systems (TISs) riding on a rocker; L, the '3R' reactor; M-P, pneumatically driven TISs; and Q, the mist reactor.

### **1.7.1 Liquid-phase (continuous complete submersion) reactors**

Plant tissues in liquid phase reactors (Figure 1.3A-E) are always immersed in the liquid medium. The stirred tank reactor is used for microbial cultures (Figure 1.3A). Its impellers for agitation of the medium create high hydrodynamic shear stress to differentiated plant tissues, and thus it is not a reasonable design. There are other stirred reactors that use a spinning filter or cell lift impeller and aeration tubes to provide low shear and bubble free aeration for somatic embryos (Moorhouse *et al.* 1996; Sorvari *et al.* 2005; Styer 1985). However, these reactors are not good options for plant tissues due to tissue adhesion to the spinning filter and aeration tubes (Sorvari, *et al.* 2005). Furthermore, capital and operation costs exceeded product yield and value (Moorhouse, *et al.* 1996). The rotatory drum reactor (Figure 1.3D) is another mechanical driven low shear mixing reactor; it has been used to culture bulblets (Paek *et al.* 1996).

Low-shear mixing and aeration in reactors can also be achieved by bubbling; these reactors include air-lift (Figure 1.3B), bubble column (BCR, Figure 1.3B), and balloon type bubble (BTBR, Figure 1.3C) reactors. The air-lift and BCR reactors are comprised of a cylinder of glass or plastic (autoclavable) with a bottom frit attached via tubing to a gas supply, e.g. air, which passes through the frit subsequently forming small bubbles that rise through the column of liquid medium thereby aerating and mixing the culture. Gas vents via a sterile filter at the top. Compared to BCR and BTBR, the air-lift reactor has an additional draft tube placed either inside or outside the growth chamber to provide greater nutrient circulation. The airlift and

bubble column reactors have been used to produce somatic embryos (Misra and Dey 2013; Tapia *et al.* 2009; Yang, *et al.* 2013). Unfortunately one of the problems with cylinder shaped reactors is foaming, so the BTBR was developed. The broad surface area of the culture liquid alleviates foaming and provides even better gas exchange than the cylinder shaped reactors (Shohael, *et al.* 2014). A variety of different plant species have produced somatic embryos in the BTBR at a variety of volumes (Table 1.5, (Chin *et al.* 2014; Ho *et al.* 2006; Kang *et al.* 2006; Shohael, *et al.* 2014; You *et al.* 2012).

The air-lift Life reactor (Figure 1.3E) used a disposable plastic bag to culture bud clusters of ferns, potato, banana, and gladiolus (Ziv, *et al.* 1998). However, microtubers produced in the Life reactor were malformed and had a low sprouting rate, and the microshoots showed significantly lower *ex vitro* survival compared to those developed on gelled medium (Grigoriadou and Leventakis 2003).

One common problem of submerged liquid cultures is their limited gas exchange (Curtis 2005). They also have a high risk of developing hyperhydricity, i.e. for woody trees and dicotyledonous species (Debnath 2007; Paek, *et al.* 2005; Preil 2005). Shoots may therefore be difficult to cultivate in liquid-phase reactors. To avoid shoot hyperhydricity, Ziv *et al.* (1998) added growth retardants to the medium in the reactor to minimize shoot development. This method, however, requires subsequent transfer of plant materials onto hormone-free medium prior to soil transplant (Ziv, *et al.* 1998).

Table 1.4 Examples of species cultivated as plantlets in different types of bioreactors shown in Figure 1.3

Reactor type		Culture type and plant species	Ref.
Liquid-phase	Stirred reactors	<b>Somatic embryos</b> of <i>Picea sitchensis</i> <b>Shoots/plantlets</b> of <i>Stevia rebaudiana</i>	(Moorhouse, <i>et al.</i> 1996) (Takayama and Akita 2006)
	Rotary drum	<b>Bulblets</b>	(Paek, <i>et al.</i> 1996)
	Air-lift, BCR, BTBR	<b>Microtubers</b> of <i>Solanum tuberosum</i> <b>Protocorms and Protocorm-like-bodies</b> <i>Dendrobium candidum</i> <i>Cymbidium sinense</i> <i>Oncidium</i> <b>Shoots/plantlets</b> <i>Chrysanthemum</i> <i>Alocasia amazonica</i> <i>Anoectochilus formosanus</i> Hayata <b>Somatic embryos</b> of a variety of species	(Akita and Takayama 1994)  (Cui <i>et al.</i> 2014) (Gao <i>et al.</i> 2014) (Yang <i>et al.</i> 2010)  (Sivakumar <i>et al.</i> 2005) (Jo <i>et al.</i> 2008) (Yoon <i>et al.</i> 2007) see Table 1.5
Gas/liquid gas-phase: CPI	Lifeguard Growtek	<b>Shoots/plantlets</b> <i>Santalum album</i> , <i>Dendranthema grandiflora</i> , <i>Ananas comosus</i> , <i>Solanum tuberosum</i> , <i>Ananas comosus</i> <i>Chrysanthemum</i> , <i>Hypericum perforatum</i> <b>Somatic embryos</b> of <i>Catharanthus roseus</i>	(Dey 2005; Goel <i>et al.</i> 2009)  (Mujib <i>et al.</i> 2014)
	Modified BCR or BTBR reactors for CPI	<b>Bulblets</b> <i>Allium sativum</i> <i>Lilium spp</i> <b>Microtubers</b> of <i>Solanum tuberosum</i> <b>Shoots/plantlets</b> <i>Allium sativum</i> <i>Spathiphyllum cannifolium</i> <i>Vitis spp</i> <i>Alocasia amazonica</i>	(Kim <i>et al.</i> 2004a) (Nesi <i>et al.</i> 2014) (Piao <i>et al.</i> 2003)  (Kim, <i>et al.</i> 2004a) (Dewir <i>et al.</i> 2006a) (Jin <i>et al.</i> 2013) (Jo, <i>et al.</i> 2008)
	Rotary CPI reactor	<b>Microtubers</b> <i>Solanum tuberosum</i> <i>Dioscorea opposita</i>	(Akita and Ohta 1998) (Akita and Ohta 2002)
	APO	<b>Plantlets</b> of <i>Hosta</i> , <i>Gerbra</i> , <i>Arundo</i> , <i>Eucalyptus</i>	(Adelberg and Fári 2010)
Liquid/gas-phase: TIS	Thin-film liquid rocker, 3R, BioMINT	<b>Microtubers</b> of <i>Solanum tuberosum</i> <b>Minirhizome</b> of <i>Curcuma longa</i> <b>Shoots/plantlets</b> <i>Alocasia amazonica</i> <i>Hosta</i> <i>Hemerocallis</i> <i>Cedrela odorata</i> <i>Capsicum chinense</i> <i>Ananas comosus</i> and tobacco	(Kämäräinen-Karppinen <i>et al.</i> 2010), (Adelberg and Cousins 2007) (Adelberg and Toler 2004) (Adelberg 2005) (Adelberg <i>et al.</i> 2007) (Peña-Ramírez, <i>et al.</i> 2010) (Bello-Bello <i>et al.</i> 2010) (Adelberg and Fári 2010)
	RITA®, Twin flask system and their variations	Somatic embryos, microtubers and plantlets of a wide variety of ornamental plants, fruits, cash crops, tree species etc.	Reviewed in (Berthouly and Etienne 2005; Georgiev <i>et al.</i> 2014; González 2005; Watt 2012). Also see Table 1.5.
	Modified BCR or BTBR for temporary immersion	<b>Bulblets</b> of <i>Lilium spp</i> <b>Protocorm-like-bodies</b> of <i>Phalaenopsis</i> <b>Shoots/plantlets</b> <i>Solanum tuberosum</i> <i>Cymbidium sinense</i>	(Lian <i>et al.</i> 2003) (Young <i>et al.</i> 2000)  (Piao, <i>et al.</i> 2003) (Gao, <i>et al.</i> 2014)
	TRI, Box-in-bag	Embryo-plantlet conversion of <i>Coffea spp</i>  Photoautotrophic shoots/plantlets of a variety of species	(Afreen 2006; Ducos, <i>et al.</i> 2007b) (Kubota, <i>et al.</i> 2005; Xiao, <i>et al.</i> 2011)
	BIB®	<b>Shoots/plantlets</b> <i>Ananas comosus</i> <i>Melaleuca alternifolia</i>	(Scheidt <i>et al.</i> 2009) (Scheidt <i>et al.</i> 2011)
Gas-phase	Mist reactor	<b>Somatic embryos</b> of <i>Dacus carrota</i> <b>Shoots/Plantlets</b> of <i>Dianthus caryophyllus</i>	(Fei and Weathers 2014) (Correll and Weathers 2001a)

Due to the growth conditions in submerged cultures, plants best cultivated in liquid-phase bioreactors are leafless structures (bulblets, microtubers, protocorms, and protocorm-like bodies) and somatic embryos (Table 1.4 and 1.5). Nevertheless, some successful examples of species grown in liquid-phase reactors were *Stevia rebaudiana* (Takayama and Akita 2006), *Chrysanthemum* (Sivakumar, *et al.* 2005), *Alocasia amazonica* (Jo, *et al.* 2008) and *Anoectochilus formosanus* Hayata (Yoon, *et al.* 2007).

### **1.7.2 Gas/liquid-phase reactors**

Since liquid phase culture is suboptimal for shoot cultivation, the tissues from which most species are micropropagated (Alvard, *et al.* 1993; Hahn and Paek 2005; Kim *et al.* 2011), a wide variety of bioreactors have been developed with designs and configurations offering periodic and/or partial immersion in liquid medium. These reactors integrate features that provide a better gaseous environment for plant materials and also simplify operation compared to the standard stirred tank reactor. For example, temporary immersion reactor systems (TIS; Figure 1.3 M,N,O and P) that allow for periodic wetting of the inoculum with nutrient medium yield better somatic embryo development, microtuber quality with high sprouting rates, and growth of shoots and roots than in gelled medium or liquid phase reactors (Berthouly and Etienne 2005; Etienne and Berthouly 2002; Georgiev, *et al.* 2014; Nhut *et al.* 2006; Watt 2012; Yan *et al.* 2013). The headspace of some gas/liquid-phase reactors can be further integrated with forced ventilation to improve the headspace environment for plantlet growth (Wang, *et al.* 2013; Zhao, *et al.* 2012).

### 1.7.2.1 Continuous partial immersion reactor (CPI)

Reactors in this category can simply be a container installed with a support (raft, plugs) inside (Figure 1.3F). Explants are positioned on the culture support and fed with liquid medium continuously at the root zone, akin to *in vitro* hydroponic culture. A CPI reactor can be easily built in-house using a glass jar capped with a ventilation port and a porous raft (Hahn and Paek 2005). Compared to liquid-phase reactors, CPI reactors showed better microtuber production (Piao, *et al.* 2003) and more successful in shoot cultures of ornamental plants (Table 1.4).

**Table 1.5** Some examples of somatic embryos cultivated in bioreactors

Bioreactor Type	Species	Volume (L)	Ref.
BTBR	<i>Eleutherococcus senticosus</i>	500	(Shohael, <i>et al.</i> 2014)
	<i>Eleutherococcus koreanum</i>	18	(Park <i>et al.</i> 2005)
	Transgenic <i>E. senticosus</i> SEs	130	(Kang, <i>et al.</i> 2006)
	<i>Panax notoginseng</i>	3	(You, <i>et al.</i> 2012)
	<i>Santalum album</i>	10	(Misra and Dey 2013)
TIS	<i>Coffea arabica</i>	1 1-10	(Etienne-Barry, <i>et al.</i> 1999) (Ducos, <i>et al.</i> 2007b)
	<i>Saccharum spp.</i> cv Q165	≤ 1	(Mordocco <i>et al.</i> 2009)
	<i>Hevea brasiliensis</i>	1	(Etienne <i>et al.</i> 1997)
	<i>Theobroma cacao</i>	1	(Niemenak <i>et al.</i> 2008)
	<i>Bactris gasipaes</i> Kunth	1	(Heringer <i>et al.</i> 2014)
BCR	<i>Castanea dentate x mollissima</i>	0.1-1.0	(Kong <i>et al.</i> 2014)
	<i>Eleutherococcus senticosus</i>	10	(Yang <i>et al.</i> 2012)
	<i>Lilium x formolongi</i> (5 cvs)	2	(Ho, <i>et al.</i> 2006)
	<i>Picea sitchensis</i>	2	(Ingram and Mavituna 2000)
Mist	<i>Dacus carota</i>	4	(Fei and Weathers 2014)
Shake flasks	<i>Quercus suber</i> L.	0.1-0.25	(Jiménez <i>et al.</i> 2011)

The Lifeguard (Figure 1.3F) is one example of a CPI; there is a floating raft placed inside a plastic box (Ziv 1999). However, there is no medium replenishment or ventilation. Similar to the Lifeguard, the Growtek reactor (Figure 1.3G) included a side tube for medium exchange

(Kar *et al.* 1999). The side tube could be modified to act as a ventilation port to provide diffusive or forced ventilation to the headspace environment (Sharma *et al.* 2011).

The BCR and BTBR can be modified to be the CPI reactor (Figure 1.3H), by installing a raft/net inside the reactor to avoid plantlet submersion. Compared to complete submersion, the CPI yielded better bulblet formation in garlic (Kim, *et al.* 2004a), better shoot proliferation in *Spathiphyllum cannifolium* (Dewir, *et al.* 2006a) and greater rooting percentage in *Alocasia amazonica* (Jo, *et al.* 2008). Growth of these plants using a CPI reactor was also better than temporary immersion, suggesting these species may prefer continuous contact with nutrient medium. However, dicotyledonous species (e.g. *Gypsophila paniculata*) suffered from hyperhydricity in a modified BCR reactor (Zhang *et al.* 2007). Hyperhydricity in this reactor, was significantly reduced when the headspace was ventilated (Wang, *et al.* 2013).

Akita *et al.* (1998) developed a simple rotary drum bioreactor (Figure 1.3D) composed of a cylinder shaped bottle equipped with an air-permeable membrane on the cap, a layer of polyurethane at the bottom of the container and a stainless steel mesh column to immobilize the plant materials; these bottles were slowly rotated on a bottle roller. Compared to static submersion, microtuber formation of potato and yam was better in the rotary drum reactor (Akita and Ohta 1998; Akita and Ohta 2002).

Fari *et al.* (2010) invented an “artificial plant ovary” (APO, Figure 1.3I) consisting of 1) a horizontal placed column as a growth chamber, 2) a medium-filled artificial “umbilical cord”

going through the longitudinal axis of the growth chamber, 3) several medium reservoirs connected to the ends of the medium supply cord, 4) ventilation ports at both ends of the column and 5) a controller to adjust the medium level inside the chamber by controlling medium supply. This APO provides flexible control on medium, switching between media and ventilation, and thus plant materials placed inside the column progressed through shooting, rooting and pre-acclimatization without any manual transfers between developmental steps (Adelberg and Fári 2010). The reactor was tested on some economically import plants including *Hosta*, *Gerbra*, *Arundo*, *Eucalyptus*, and is currently used for commercial production of *Arundo donax* (Adelberg and Fári 2010).

#### ***1.7.2.2 Temporary immersion system (TIS)***

In a TIS, plant materials are cultured in a humid gas phase and periodically immersed in liquid medium. Compared to liquid-phase reactors, TIS reactors are more suitable for shoot cultures and therefore have a longer list of plants that have been successfully cultivated (Table 1.4). Immersion and drainage can be achieved by mechanical driven tilting, rotating or lifting, by pneumatically driven ebb and flow or by gravity driven ebb and flow. The RITA<sup>®</sup> and twin flask reactor are most popular among TIS reactors and have been used to propagate a large variety of plants via shoot cultures (Berthouly and Etienne 2005; Georgiev, *et al.* 2014; Watt 2012).

***System with tilting, rotary or lifting machines:***

A thin-film liquid rocker system (Figure 1.3J) composed of individual rectangular culture boxes and a rocker platform was designed by Adelberg (2005). Due to the high cost and contamination problems of the initial version of the culture box, a more recent version (2.7 L) made of polycarbonate with diffusive ventilation was developed (Kämäräinen-Karppinen, *et al.* 2010). The thin-film rocker box has been used to propagate *Alocasia amazonica* (Adelberg and Toler 2004), *Hosta* (Adelberg 2005), *Hemerocallis* (Adelberg, *et al.* 2007), minirhizomes of *Curcuma longa* (Adelberg and Cousins 2007), and microtubers of *Solanum tuberosum* (Kämäräinen-Karppinen, *et al.* 2010). One drawback of this system is the depletion of nutrients; there is only a thin layer of medium at the bottom of culture boxes. To overcome this problem, vessels are being redesigned to provide nutrient replenishment (Adelberg and Fári 2010), but to my knowledge, there is not yet any application.

The BioMINT bioreactor (Figure 1.3K) also rides on a rocker platform to achieve temporary immersion (Robert *et al.* 2006). It is built of two polypropylene vessels, one for the plant materials and the other for the liquid culture medium. The vessels are coupled together through a perforated adaptor allowing the flow of the liquid media from one vessel to the other. Ventilation ports on the vessels enable diffusive or forced ventilation. Spanish red cedar (Peña-Ramírez, *et al.* 2010; Peña-Ramírez *et al.* 2012) and Habanero pepper (Bello-Bello, *et al.* 2010) cultured in BioMINT showed better shoot growth, root development and *ex vitro* survival than in gelled medium.

A rotary driven system 3R (Figure 1.3L) has a segment across the center to separate plant materials from media (Mészáros *et al.* 2004). The vessel rolls 180 degrees back and forth to provide periodic immersion (Fari *et al.* 2006; Mészáros, *et al.* 2004). This system was used to propagate pineapple and transformed tobacco shoots in large scale (Adelberg and Fári 2010). Unfortunately the capital cost of building and running this system is high (Adelberg and Fári 2010).

***Pneumatically driven TIS:***

Transfer of medium in pneumatically driven TIS is achieved by applying air pressure onto the medium. Many TIS belong to this type; the twin flask system and the RITA<sup>®</sup> are representatives of this group.

In a twin flask system (BIT<sup>®</sup>, Figure 1.3M), liquid is passed horizontally between two flasks (Escalona *et al.* 1999). Air pressure alternatively applied to the medium reservoir and the growth chamber periodically transfers medium between the two bottles; this system is easy to build in-house. Transfer of medium can also be achieved by alternatively changing the interrelated vertical position of the two vessels (FÁRI *et al.* 2010). BIT<sup>®</sup> can be easily scaled up by using larger containers (Hempfling and Preil 2005; Wilken *et al.* 2014). Forced ventilation can be readily integrated into the growth chamber to improve the headspace environment (Wang, *et al.* 2013; Zhao, *et al.* 2012). Twin flask reactors are used for shoot proliferation, elongation and rooting in a variety of species (Berthouly and Etienne 2005; Georgiev, *et al.* 2014; Watt 2012).

The RITA<sup>®</sup> system (Figure 1.3N) is a 1 L vessel comprising two compartments, an upper plant growth chamber and a lower medium reservoir (Teisson and Alvard 1995). Liquid is pressure fed from the medium reservoir into the top growth chamber to the level of the plant materials. Gas vents with sterile filters are used to equalize pressure. The liquid is held in the top chamber for a short period of time and then drained back to the bottom chamber until the next filling. This can occur at any regularly set interval, which is often species specific. This system was mainly intended for mass propagation of somatic embryos but has also been used for proliferating shoots, bud clusters and microtubers (Akdemir *et al.* 2014; Berthouly and Etienne 2005; Georgiev, *et al.* 2014; Polzin *et al.* 2014; Ramos-Castellá *et al.* 2014). The original small RITA<sup>®</sup> system was the basis for larger scaled systems based on the same principles.

The Plantima vessel, a RITA<sup>®</sup> variant (Figure 1.3N), uses a larger two-compartmented rectangular container (Yan *et al.* 2010). It was reported to improve plantlet growth and reduce hyperhydricity in *Siraitia grosvenorii* and *Dioscorea* (Yan, *et al.* 2010; Yan, *et al.* 2013).

A Plantform bioreactor (Figure 1.3N) is another variation of RITA<sup>®</sup> using a plastic container (4L) fitted with a perforated plastic raft as the culture vessel (Sayegh and Welander 2012; Welander *et al.* 2014). This system has been used to propagate apple, blueberry, raspberry, blackberry, rhubarb, potato, several types of grasses and banana ([www.plantform.se/pub/](http://www.plantform.se/pub/)).

Soccol *et al.* (2008) developed a bubble immersion bioreactor (BIB<sup>®</sup>, similar to Figure 1.3L) with a 90 cm high growth chamber fitted with multiple tiers to accommodate plant materials. Instead of immersion in liquid, plant materials were periodically immersed in bubbles of nutrient medium. This reactor reportedly yielded better shoot proliferation than RITA<sup>®</sup> for pineapple and tee tree (Scheidt, *et al.* 2009; Scheidt, *et al.* 2011; Soccol *et al.* 2008).

The BCR and BTBR have also been modified to be vertical twin-flask systems (Figure 1.3O). To provide temporary immersion, gas pressure is periodically applied to the medium reservoir connected to the bottom of the growth chamber by tubing. Similar to RITA<sup>®</sup>, liquid is drained back into the reservoir by gravity after the air pressure is released. The growth chamber in the modified BCR and BTBR is fitted with a supportive net to prevent plant materials from submersion. A vertical twin-flask system also allows the two bottles to be stacked. For example, the SETIS<sup>™</sup> reactor (similar to Figure 1.3O <http://www.setis-systems.be/>) is comprised of two stacked flat containers with the growth chamber on top of the medium reservoir. Horizontal placement of stacked vessels saves shelf space.

Afreen *et al.* (2002) developed a temporary root zone immersion reactor system (similar to Figure 1.3O) for photoautotrophic micropropagation. In this system, plant materials fixed in porous plugs are periodically immersed in their root zone (plug part); forced ventilation with CO<sub>2</sub> enriched air and high light intensity are also provided to stimulate autotrophic growth (Afreen *et al.* 2002b). The system is also available at a commercial scale (Kozai *et al.* 2006). Compared to the TIS that submerses explants during immersion time (e.g. RITA<sup>®</sup> and twin

flask system), the TRI reactor showed better embryo-to-plantlet development as well as shoot and root growth (Afreen 2006).

The growth chamber of a TIS can also be made of transparent disposable plastic as shown in the box-in-bag TIS (Figure 1.3P). To provide a reasonable height of headspace for explant growth, the plastic bag is fitted outside a box with a lateral screen on which plant materials reside and grow. This reactor provides uniform light transmittance, and was used to produce pre-germinated embryos using torpedo stage embryos as inoculum (Ducos, *et al.* 2007c; Ducos *et al.* 2008). Despite the high light transmittance, this bioreactor has a large foot print; it also has a problem with medium mixing and the sterile vent connector parts on the bag were too costly to be disposable (Ducos *et al.* 2007a), so the design was abandoned.

### ***Application strategies of TIS***

Many studies compared TIS reactors with conventional gelled medium, and the results were consistent in favoring TIS; they yielded greater biomass with better quality (normal leaf anatomy, higher chlorophyll content, less hyperhydricity) (Georgiev, *et al.* 2014; Watt 2012; Yan, *et al.* 2010; Yan, *et al.* 2013; Yang and Yeh 2008). When used in production of somatic embryos, the TIS also yielded more embryos with better quality compared to semi-solid medium (Heringer, *et al.* 2014; Mallón *et al.* 2012; Niemenak, *et al.* 2008). On the other hand, protocorm-like-bodies proliferated better in the BTBR than in the TIS (Gao, *et al.* 2014; Yang, *et al.* 2010).

Compared to continuous partial immersion reactors, the TIS is a better option for dicotyledonous and tree species in terms of shoot proliferation, root development and embryo-to-plantlet conversion (Georgiev, *et al.* 2014; Kim, *et al.* 2011). In contrast monocotyledonous species generally prefer continuous partial immersion to temporary full immersion (Dewir, *et al.* 2006a; Jo, *et al.* 2008; Kim, *et al.* 2004a; Nesi, *et al.* 2014).

Comparisons between different temporary immersion systems are still limited and there are no clear cut suggestions on which bioreactor to choose. Somatic embryogenesis seems better in a RITA<sup>®</sup> than a twin flask system (Heringer, *et al.* 2014; Sankar-Thomas and Lieberei 2011). As embryos mature and become chlorophyllous in the cotyledonary stage, light transmittance becomes important for germination of somatic embryos and their subsequent plantlet development (Afreen *et al.* 2002a). However, the cylindrically shaped vessels in most TIS reactors e.g. the twin flask system, restricted light penetration into their center and thus, also restricted somatic embryo development in the center of the culture vessel (Ducos, *et al.* 2007b). Compared to twin flask systems, the RITA<sup>®</sup> was more effective for embryo-to-plantlet conversion and microshoot production, which was probably because there was more uniform light transmittance in the RITA<sup>®</sup> (Heringer, *et al.* 2014; Scherer *et al.* 2013). Likewise, the box-in-a-bag TIS yielded more synchronized somatic embryo maturation after the torpedo stage than did the cylindrically shaped twin flask systems probably because the former provided more uniform light transmittance (Ducos, *et al.* 2007b; Ducos, *et al.* 2007c; Ducos,

*et al.* 2008). Scheidt *et al.* (2009, 2011) found BIB<sup>®</sup> to be superior to RITA<sup>®</sup> for the propagation of pineapple and tea tree seedlings.

Immersion cycle in a TIS affects somatic embryo development, microtuber formation, shoot proliferation, and root development, with cycle length dependent on species and development step (Berthouly and Etienne 2005; Georgiev, *et al.* 2014; Ramos-Castellá, *et al.* 2014). While microtuber and bulblets develop well under long or frequent immersions (e.g. 10 min h<sup>-1</sup>) (Ashraf *et al.* 2013; Balogun *et al.* 2014; Kämäräinen-Karppinen, *et al.* 2010; Ramos-Castellá, *et al.* 2014), shoot tissues require short, infrequent immersion cycles (e.g. 5 min 12 h<sup>-1</sup>) (Georgiev, *et al.* 2014; Valdez-Tapia *et al.* 2014; Watt 2012). Somatic embryos grow well under short, frequent cycles (several minutes every few hours) (Albarrán *et al.* 2005; Mallón, *et al.* 2012; Niemenak, *et al.* 2008; Oliva *et al.* 2014; Pérez *et al.* 2013; Teisson and Alvard 1995).

### **1.7.3 Gas-phase reactors**

The mist reactor is, to our knowledge, the only truly gas-phase reactor. It uses aeroponics and provides nutrient medium to cells, explant tissues or organs via an ultrasonic nozzle that yields a fine mist that coalesces and drips back into the medium reservoir. With the elimination of immersion in liquid phase, plant materials grown in nutrient mist bioreactors have unlimited gas exchange, and thus showed better *in vitro* growth than gelled medium or liquid-phase reactors in terms of shoot proliferation, *in vitro* rooting, biomass yield, hyperhydricity and secondary metabolite contents (Towler *et al.* 2006).

The original mist reactor had gas-driven spray nozzles to generate nutrient mist, and was used to culture a variety of plant tissues and species with improved shooting and estimated reduced production cost (Weathers and Giles 1988). The spray nozzles, however, were easily clogged. A submerged ultrasonic transducer was then used for mist generation (Liu *et al.* 1998; Tisserat *et al.* 1993), and was used to propagate shoot tissues and microtubers (Cheetham *et al.* 1992; Hao *et al.* 1998; Liu *et al.* 2003; Weathers *et al.* 1988; Woo and Park 1993). However, deterioration of the ultrasonic element occurred as a result of direct contact with medium (Tisserat, *et al.* 1993). The acoustic window mist bioreactor was then developed and simplified later with the design to avoid direct contact of medium to the transducer (Buer *et al.* 1996; Chatterjee *et al.* 1997). By altering the misting cycle, plant leaves developed functional stomata and epicuticular wax, and hyperhydricity was greatly reduced; *in vitro* rooting and acclimatization was also merged into one step yielding high *ex vitro* survival in the acoustic window mist bioreactor (Correll, *et al.* 2001; Correll and Weathers 2001a; Correll and Weathers 2001b).

The earlier versions of the mist reactor were difficult to scale up, and the ventilation was completely controlled by the misting cycle. So an improved version of the mist reactor (Figure 1.3Q) was then developed using a liquid-driven ultrasonic spray nozzle for nutrient dispersion and a disposable plastic bag as a growth chamber (Liu *et al.* 2009a).

## **1.8 Summary**

Micropropagation is a powerful tool in rapid clonal propagation of plants and offers great potential in germplasm preservation. However, current methods used in commercial production are time consuming and labor intensive, which limits economic expansion. Use of bioreactors to automate plant tissue growth and improve plant quality has shown great potential for reducing plantlet production cost.

## Chapter 2 Hypothesis and research objectives

### 2.0 Hypothesis

The potential of the mist reactor to maximize plant growth, reduce hyperhydricity and increase *ex vitro* survival is tremendous because of its flexible control on headspace environment and nutrient feeding. The medium exchanges, CO<sub>2</sub> levels, and nutrient feeding cycles can be manipulated with ease to provide desired nutrient supply and culture environment at different development steps including shooting, rooting and acclimatization. Plant material transfers associated with these *in vitro* culture steps can therefore be eliminated in the improved mist reactor. Thus, I hypothesize that one-step micropropagation from cell or leaf inoculum through shooting, rooting and acclimatization to soil-ready plants can be achieved using this reactor. The challenge was to scale vertically in a gas phase environment.

### 2.1 Research objectives

To achieve the goal of one-step micropropagation, there are several technical objectives:

- 1) To investigate the effects of misting cycle, ventilation, and CO<sub>2</sub> levels on somatic embryo development using carrot as the model species;
- 2) To study how growth and key aspects of plant physiology respond to misting cycle, ventilation, CO<sub>2</sub> level and light intensity during shooting, rooting and acclimatization in these propagation steps compared to gelled medium and use *Artemisia annua* as the model species;
- 3) To explore the potential of integrating poly-cation amino acid, poly-L-lysine (PLL), coating technology to provide a hanging style vertical growth akin to a “hanging garden” through

surface attachment of plant materials to PLL-coated substrates hanging inside the mist reactor with the aim of decreasing labor and increasing quality and yield capacity while minimizing the cultivation footprint.

## **2.2 Thesis organization**

The work on one-step micropropagation through somatic embryogenesis and integration of surface adhesion technology for somatic embryo development in a “hanging garden” style is described in Chapter 3. In Chapter 4, comparisons of plant growth in mist reactor and traditional gelled medium are presented for different development steps including shooting, rooting, and acclimatization. Chapter 5 contains an investigation on the attachment of small pieces of shoot tissue to substrates with PLL coating, and one-step micropropagation of *A. annua* by PLL attachment. Chapter 6 presents conclusions and suggests future directions. Chapters 1 and 3 have already been published in part or in their entirety. Chapters 4 and 5 are written for publication submission.

## **Chapter 3 From cells to embryos to rooted plantlets in a mist bioreactor**

Published as:

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### 3.1 Abstract

A mist bioreactor using a disposable bag as culture chamber was used to propagate carrot embryogenic cells into rooted plantlets. The best operating configuration was akin to a vertical hanging garden using 50–90  $\mu\text{m}$  nylon mesh for explant attachment. Cells spray inoculated into the reactor were 51.2 % viable. Misting cycle and aeration conditions were studied and showed that under the same hourly volumetric nutrient feed and 0 VVM, embryo development in the reactor was best using a 0.3 min on/2.7 min off misting cycle, yielding about 23 % post heart stage embryos. Compared to 0 VVM, 3 %  $\text{CO}_2$  enrichment improved embryo development in reactor culture. Spray inoculated cells also attached to several vertically hung poly-L-lysine coated strips and then developed in situ into embryos. Cell attachment was significantly improved when they were suspended in salt-free sucrose solution during spray inoculation. Almost 90 % of the originally attached cells remained on the nylon mesh 24 h later after spraying with B5 medium in the mist reactor. Strip grown embryos had the same post heart stage ratio but shorter overall length compared to those developed on a horizontal platform. Young plantlets developed uniformly up and down the hanging strips and did not detach after 3 weeks of culture suggesting this technology may prove useful for improving micropropagation.

**Key words:** Mist bioreactor, Somatic embryogenesis, Poly-L-lysine, Adhesion, Micropropagation, Plant tissue culture

**Abbreviations:**

N50, N70, N90: nylon screens with openings of 50, 70 and 90  $\mu\text{m}$ , respectively;

P74, P105: polypropylene screen with opening of 74 and 105  $\mu\text{m}$ , respectively;

## 3.2 Introduction

Millions of plants are commercially propagated annually via micropropagation (Loberant and Altman 2010), which is labor intensive and associated with developmental abnormalities (Hazarika 2006; Pospisilova, *et al.* 1999; Towler, *et al.* 2006). There have been many efforts to develop cost-effective, and simple bioreactors with the aim of automating micropropagation, but designing reactors for plant tissue culture must reconcile environmental factors (shear stress, aeration, RH, nutrient supply) with healthy plant development, cost and simplicity of use. These factors determine plant quality, *ex vitro* survival, and overall production costs (Lowe *et al.* 2003; Ziv 1991a). Indeed, bioreactors offer possibility of automation, decreased labor costs and potential for mass propagation scale-up (Paek *et al.* 2001; Paek, *et al.* 2005).

Liquid-phase reactors developed for micropropagation include the stirred tank reactor, bubble column reactor, balloon type bubble reactor, and air lift reactor, but they often produce hyperhydric shoots due to submergence in liquid (Aitken-Christie, *et al.* 1995a; Ziv 2010).

Attempts to reduce hyperhydricity used temporary immersion systems (TIS) that either completely or partially submerge plant tissues in liquid medium (Afreen 2006; Etienne and Berthouly 2002; Weathers *et al.* 2010) using various mechanisms (Adelberg and Fári 2010; Robert, *et al.* 2006); (Fari, *et al.* 2006); (Afreen 2006; Ducos, *et al.* 2009). In TIS, the immersion cycle, volume of nutrient medium and the container are critical for efficient shoot proliferation (Snyman *et al.* 2011). Compared to continuously submerged cultures, growth

generally improved, but hyperhydricity was still a problem and was greater than 50% for some species (Shaik *et al.* 2010; Snyman, *et al.* 2011).

Nutrient mist reactors are gas-phase reactors that provide *in vitro* plants with small droplets of culture medium fully infused with whatever gas or gas mixture is generated as an aerosol into the growth chamber. Mist bioreactors of various configurations promote better plant growth with increased shooting, somatic embryo formation and regeneration rate (Towler, *et al.* 2006). Using a disposable plastic bag as growth chamber also offers an inexpensive means for cultivating *in vitro* plantlets with adjustable control of nutrient feeding and ventilation (Liu, *et al.* 2009a). Plantlets are exposed to improved gas exchange, controlled relative humidity and medium feeding, all resulting in healthy growth with high *ex vitro* survival (Correll and Weathers 2001a).

In earlier studies using an older version of the mist reactor, control of the headspace environment, including relative humidity, CO<sub>2</sub> and nutrient supply, produced high quality plantlets (Correll, *et al.* 2001; Correll and Weathers 2001a; Correll and Weathers 2001b). Mist-grown *Dianthus* developed a better quality cuticle and functional stomata than plantlets grown in Magenta boxes (Correll and Weathers 2001a). Mist reactor-grown plantlets also rooted better and showed higher *ex vitro* survival than controls (Correll and Weathers 2001a). Together these studies suggested the potential for automation of *in vitro* plant propagation using the mist bioreactor by connecting the otherwise discrete stages of micropropagation into a one-step process within a single culture system. This study used carrot as a model to

investigate the possibility of developing cells through embryogenesis to fully rooted plantlets in a mist reactor. We chose carrot as model because of its well-established protocol to initiate embryogenic cells and subsequent conversion to embryos. Embryo developmental response to nutrient supply and aeration was studied using two configurations of the reactor. In particular, we were interested in the following questions: Would the cells survive the inoculation process? Would they develop into embryos? Would they immobilize via adhesion to PLL-coated substrates after spray inoculation, and would they develop *in situ* into rooted plantlets?

### **3.3 Material and Methods**

#### **3.3.1 Plant material and maintenance**

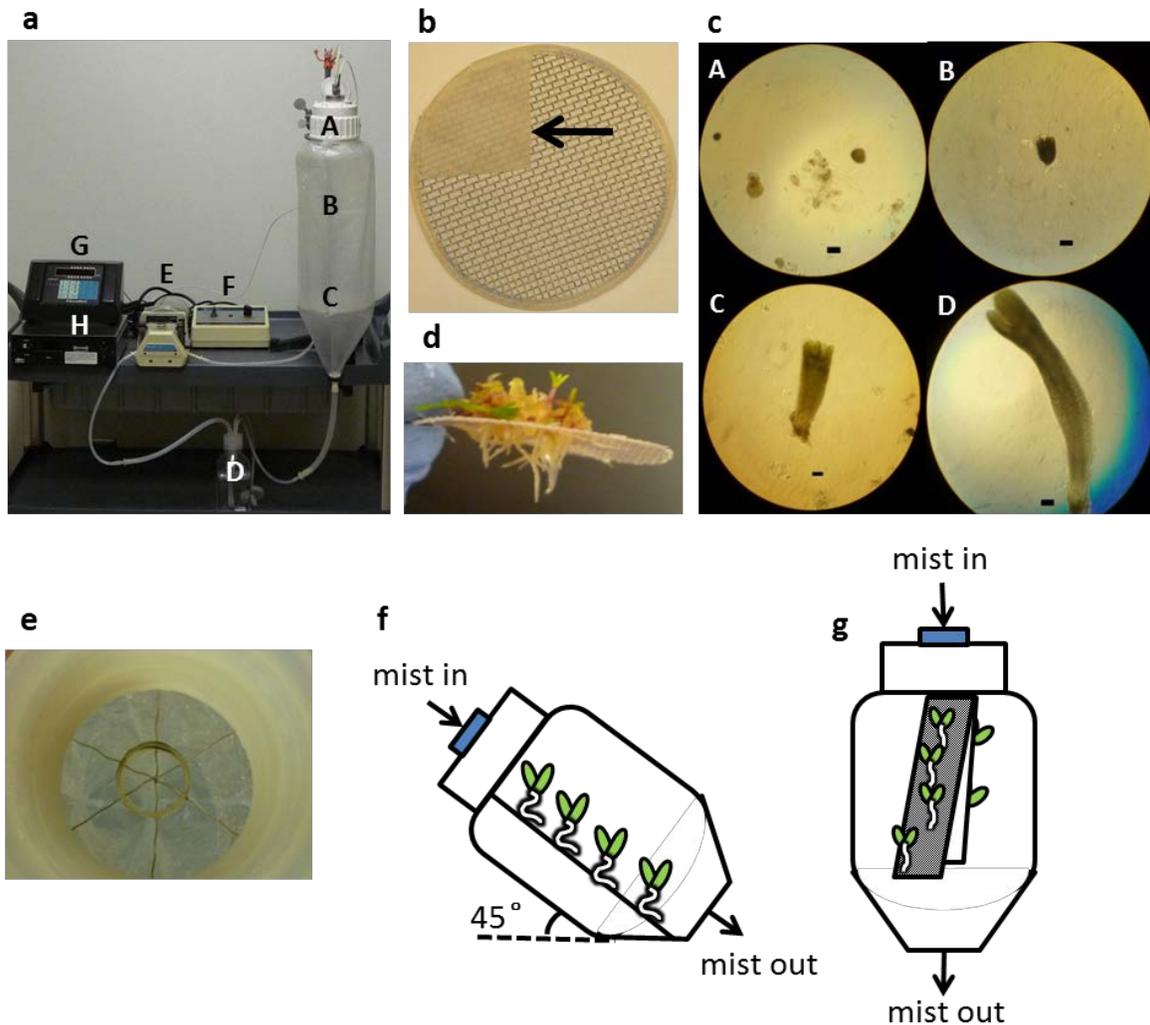
Carrot (*Daucus carota*) suspension cultures were initiated from callus developed from explants of commercially purchased carrots grown on semi solid Gamborg's B5 medium with 30 g L<sup>-1</sup> sucrose (B5; (Gamborg *et al.* 1976) and 1 mg L<sup>-1</sup>, 4-dichlorophenoxyacetic acid (2,4-D; B5+2,4-D). Cells were grown in liquid B5+2,4-D in 250ml Erlenmeyer flasks at 120 RPM and 25°C and sub-cultured every 2 weeks. To induce somatic embryos, 7-day-old undifferentiated cells were successively sieved through 500 and 350 µm sterile stainless steel screens, washed with B5 3 times by centrifugation at 200 × g for 2 min, and resuspended in B5 to a density of 10<sup>5</sup> cells mL<sup>-1</sup> for further experiments. Embryogenic cells spontaneously develop into somatic embryos between 14-21 days during growth on B5 (without 2,4-D).

### **3.3.2 Mist reactor**

Details of mist reactor (Figure 3.1a) construction, assembly of components and operation were previously reported (Liu, *et al.* 2009a). Operational parameters related to this study are provided in descriptions for each experiment. All parts were autoclaved except the mister located in the screw cap, which was sterilized as follows: tap water washed to remove large debris; soaked in 200 ppm fresh ClO<sub>2</sub> for 5-6 hr; then wrapped with 4 layers of Al foil, heated at 100°C for 20 hr, cooled to room temperature in a sterile hood before being unwrapped and aseptically screwed onto the shoulder of the reactor that also supports the culture bag.

### **3.3.3 Viability of cells after spray inoculation**

Inoculation was carried out by spraying an 8-day-old cell suspension into the culture bag. To test viability, 30 mL of cells (10<sup>5</sup> mL<sup>-1</sup>) in B5 were sprayed through the ultrasonic nozzle using various ultrasonic power inputs ranging from 4.5-6 watts. Cells emerging from the nozzle tip were collected for 30 sec and stained with 0.01% (w/v) fluorescein diacetate (FDA, Sigma 31545) for 20 min (Widholm 1972). Viable cells were counted before and after spraying through the ultrasonic nozzle.



**Figure 3.1** Mist reactor system. **a** Photo of reactor system layout: **A** mist cap, **B** growth chamber, **C** support platform, **D** medium reservoir, **E** peristaltic pump, **F** liquid flow controller, **G** time controller, **H** ultrasonic power supply. **b** Photo of stainless steel mesh support platform inside reactor culture bag with example of mesh (arrow). **c** Carrot embryos at different developmental stages under 100 $\times$ : **A** globular, **B** heart, **C** torpedo, **D** cotyledonary. **d** Rooted embryos developed on P105 mesh after 4 weeks. **e** Top view of 6 spoke shaped scaffold inside reactor for strips to hang. **f** Growth chamber tilted at 45 $^{\circ}$  with plantlets growing on a platform inside the bag. **g** Growth chamber vertically placed with plantlets growing on vertically hung +PLL strips inside the bag. Bars **c** 100  $\mu$ m

### **3.3.4 Somatic embryogenesis in the mist bioreactor with various misting cycles**

For initial embryogenesis experiments, a 16 cm diameter platform, composed of nylon mesh (Small Parts, various opening sizes stated later, obtained from Amazon.com supply) covering a stainless steel mesh screen (3 mm opening) supported by an outer ring of silicon tubing was placed inside the reactor bag to provide a supportive surface for embryo development (Figure 3.1aC, b). The bag was first aerated with sterile filtered humidified air to its full 3D inflated shape and then kept at 0 VVM over the entire culture period of 2 weeks. Aeration rate VVM is defined as gas volume flow per unit of culture space volume per minute. During inoculation, the inflated bag was flushed with 38-60 mL min<sup>-1</sup> of B5 using 4.5-6.0 watts of ultrasonic power for 15 min before spraying with carrot cells at 50 mL min<sup>-1</sup> through the ultrasonic nozzle. For each reactor run, 150 mL of 10<sup>5</sup> mL<sup>-1</sup> cells were used. The medium reservoir was filled with 350 mL B5, and after optimization testing (Figure 3.2), ultrasonic power was set at 4.5 watts for all subsequent runs. Embryo development was quantified at total volumetric medium feed rates of 150 or 300 mL hr<sup>-1</sup>, and misting cycles of either 4 or 20 mistings hr<sup>-1</sup> (15 vs. 3 min cycles). Embryos formed on nylon screens were harvested after 2 weeks axenic culture under continuous light. Embryos at the heart, torpedo, and cotyledonary stages of development were counted (Li and Kurata 2005). For controls, deep petri-dishes filled with 15 ml B5 onto which were placed 9, 1.9 cm diameter nylon mesh discs of either 50 (N50, Small Parts CMN-0050-C) or 90 μm (N90, Small Parts CMN-0090-C) nylon mesh, or 105 μm polypropylene mesh (P105; Small Parts CMP-0105-D). A 20 μL aliquot of cells was

pipetted onto each disc. For all experiments dishes and reactors were placed under continuous cool white fluorescent light of  $12 \mu\text{mol s}^{-1}\text{m}^{-2}$  and  $25^\circ\text{C}$ . After 2 weeks of incubation embryos at the heart (H), torpedo (T), and cotyledonary (C) stages (Figure 1c) were counted on each disc and the ratio (R) of post-heart embryos that developed was defined as:  $R=(T+C)/(H+T+C)$ .

### **3.3.5 Somatic embryogenesis in the mist bioreactor with various aeration conditions**

Reactor set up and initiation were the same as those in the misting cycle study: 2 weeks growth,  $300 \text{ mL hr}^{-1}$  total volumetric feed, misting cycle of 0.3 min on, 2.7 min off ( $30 \text{ mistings hr}^{-1}$ ). Aeration conditions included 0 VVM, 0.05 VVM of ambient air and 0.05 VVM of 3%  $\text{CO}_2$  enriched air. VVM was based on the 7 L total volume of the plastic culture bag. Controls were the same cells inoculated on B5 Phytigel in vented 1 L screw cap polycarbonate boxes (DiIorio *et al.* 1992) with the same aeration. Overall length and developmental stage of embryos were measured.

### **3.3.6 Cell adhesion to Poly-L-lysine (PLL) coated substrates**

Strips of  $2 \text{ cm} \times 4 \text{ cm}$  N50 and N70 screen or polypropylene sheet were submerged in 0.01% of PLL (Sigma Aldrich P1274) solution for 30 min and then air dried for 30-120 min (Towler and Weathers 2003). To measure cell attachment the difference in blotted cell fresh weight (FW) was measured after application to the nylon or polypropylene strips  $\pm$  PLL and then weighed again after being held vertically and washed 10 times from top to bottom each time

with 0.3 mL dH<sub>2</sub>O. Using this procedure, attachment incubation times of 15-240 min at 0.0025 - 0.05% (W/V) PLL were investigated. Retention ratio was determined as: post wash FW/ initial FW cells.

Cell adhesion in spray inoculation was measured by using cells washed twice with dH<sub>2</sub>O, resuspended in 15 g L<sup>-1</sup> sucrose solution (10<sup>5</sup> mL<sup>-1</sup>), pH 5.8, ± B5 medium salts and then sprayed into a reactor bag hung with strips (2 × 4 cm) of N50, N70 or P74 polypropylene (Small Parts CMP-0074-A, now discontinued) mesh ± PLL. The flow rate during inoculation was 10-15 mL min<sup>-1</sup>, at 5.0 watts, and 30 min duration. Strips were removed from the reactor bag and attached cells were counted at 15 locations with 15 grids of 0.01 cm<sup>2</sup> averaged to represent the adherent cells for that strip. Cell retention after spray inoculation was measured on hanging strips that were put back into the reactor bag and sprayed with B5 for 24 hours at feed rate of 50 mL min<sup>-1</sup> (300 mL hr<sup>-1</sup>) and a misting cycle of 0.3 min on, 2.7 min off. Cells remaining on the strip after 24 hr were considered attached.

### **3.3.7 Somatic embryogenesis on PLL coated N70 mesh hanging inside the mist reactor**

Cells were also sprayed into the mist reactor in which strips of PLL coated N70 (Small Parts PN CMN-0070-C) were hung and a N70 covered platform was placed (Figure 3.1aC) to maintain the 3D shape of the bag. Cells at 10<sup>5</sup> cells mL<sup>-1</sup> were spray inoculated at 10-15 mL min<sup>-1</sup> onto PLL coated strips already hung within the reactor (Figure 3.1e). Cells were sprayed as a single passage of the entire suspension volume with no recirculation through the

peristaltic pump. After inoculation, cells were fed B5 with a misting cycle of 0.3 min on, 2.7 min off, 300 mL hr<sup>-1</sup> feed rate, at 0 VVM for 2 weeks to encourage embryogenesis and rooting. Controls were the same cells inoculated on B5 Phytigel medium. After harvest, developing embryos on N70 strips and platform were counted and measured as previously described. To test the concept of one-step growth from cells to plantlets, manually attached cells on N70 strips were also grown in the reactor using the same cultivation conditions for three weeks. For manual cell application, cells were concentrated by centrifugation (200 × g for 2 min), washed three times with 15 g L<sup>-1</sup> sucrose, and then sterile PLL coated strips were dipped into the slurry of cells and incubated for 5 min prior to hanging in the reactor.

### **3.3.8 Statistical Analyses**

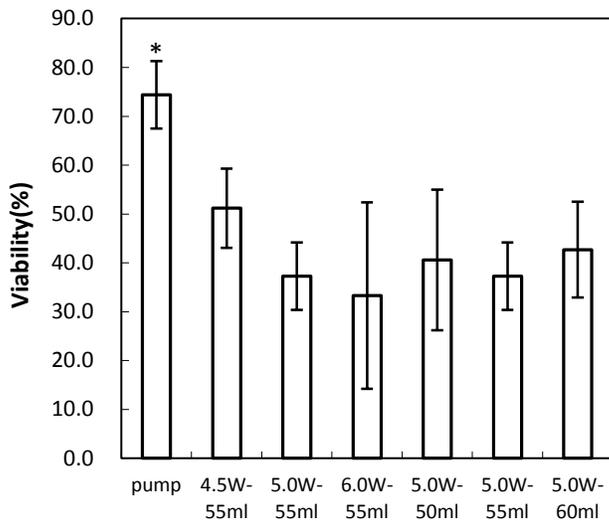
All experiments had  $\geq 3$  replicates and One-way ANOVA and Duncan's range analysis were used to measure statistical significance. Reactors in the misting cycle study were run in duplicate and results analyzed using nested ANOVA (Grafen and Hails 2002).

## **3.4 Results and Discussion**

### **3.4.1 Cell viability after spray inoculation**

After passage from the reservoir through the peristaltic pump, cell viability decreased about 25%. Spraying through the ultrasonic nozzle further decreased viable cells to 30-50% (Figure 3.2). Though not statistically different, viability appeared to decrease slightly with increasing sonic power at constant flow rate, but when flow rate was varied with constant sonic power input, viability was not affected. At 4.5 watts, 51.2% of all cells sprayed remained viable using

a 55 mL min<sup>-1</sup> flow rate (Figure 3.2). These results showed that cells could be inoculated via spraying into the reactor without excessive loss of viability.



**Figure 3.2** Viability of carrot cells at various combinations of ultrasonic power and flow rate. Cells were pumped through ultrasonic nozzle and then cell viability was measured using fluorescein diacetate. N=5;  $\pm$ SE; \* $p \leq 0.05$

### 3.4.2 Embryogenesis on horizontal platforms in the mist reactor

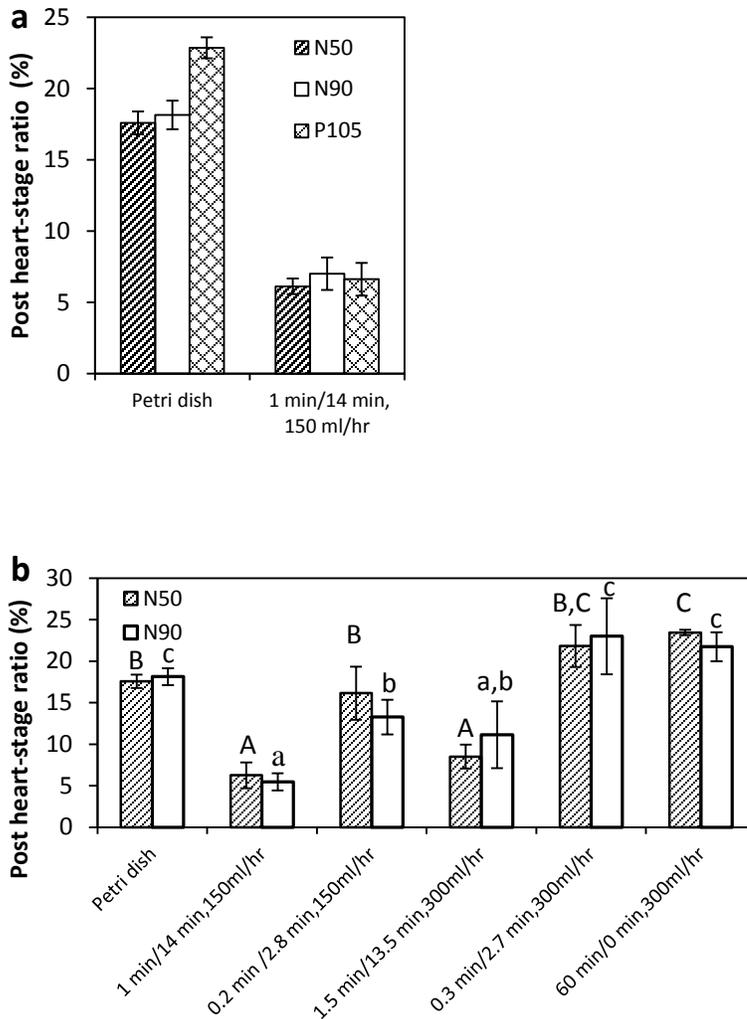
Embryo development was studied in two reactor configurations: on a horizontal platform, or on vertical hanging strips inserted into the culture bag (Figure 3.1 b, e). Average single cell size is about 50  $\mu$ m, so N50, N90 and polypropylene (P105) meshes were aseptically laid on the platform and then spray inoculated with cells. After 2 weeks only heart, torpedo and cotyledonary stage embryos were counted because globular stage embryos were difficult to differentiate from cell aggregates (Figure 3.1c). About 18% of control embryos developed to either torpedo or cotyledon stage for both N50 and N90, and about 23% for P105; all were about three times their mist reactor counterparts (Figure 3.3a). Although the more open meshes, N90 and P105, seemed to provide better substrates for embryo development than the

less open mesh, N50, in the Petri dish controls, roots grew through the openings of P105 (Figure 3.1d). Because this would result in root shear damage during harvest, P105 was rejected for further study. There was no significant difference between either N50 or N90, so both were further studied.

To improve embryo development in the mist reactor, various feeding strategies were tested including changing the frequency and volume of medium fed to cells and the VVM. When total volumetric delivery was  $150 \text{ mL hr}^{-1}$ , increasing the feeding frequency from 1 min on/14 min off (4 mistings  $\text{hr}^{-1}$ ), to 0.2 min on/2.8 min off (20 mistings  $\text{hr}^{-1}$ ) doubled the formation of post heart stage embryos (Figure 3.3b). When the delivery feed was doubled from 150 to  $300 \text{ mL hr}^{-1}$ , it appeared that embryo development increased, but results were not statistically significant ( $p = 0.09$ ) at 4 mistings  $\text{hr}^{-1}$  (1.5 min on, 13.5 min off) unless the frequency of misting was increased again to 20 mistings  $\text{hr}^{-1}$  (0.3 min on, 2.7 min off) (Figure 3.3b). Then developed embryos exceeded that of the controls. Mesh size of the two tested substrates, N50 and N90, had no effect. Petri dish controls showed about 18% embryo development to the post heart stages, better than most of the mist reactor experiments. However, once the total volumetric feed was  $300 \text{ mL hr}^{-1}$  and at a frequency of 20 mistings  $\text{hr}^{-1}$ , then the number of developed embryos in the mist reactor exceeded 20% (Figure 3.3b). Continuous misting at  $300 \text{ mL hr}^{-1}$  did not further increase embryogenesis and embryo development remained at about 21- 23% (Figure 3.3b). Taken together, increasing the total volumetric feed promoted better embryo development, but was less effective than increasing misting frequency.

Somatic embryos lack chlorophyll and stomata resulting in a low rate of photosynthesis (Afreen, *et al.* 2002a). Embryos live solely on their exogenous nutrient supply and growth improves with better nutrient contact. In this study, short but frequent misting produced more developed embryos compared to less frequent cycles, and this seemed to indicate that the cells were using the delivered nutrients more efficiently when medium contact was short, but frequent. It is possible that nutrient absorption during frequent misting cycles is saturated. The more frequently cells are fed small amounts of nutrients, the more they are likely to absorb those nutrients resulting in better growth. In the case of 300ml hr<sup>-1</sup>, cells had 20 feedings hr<sup>-1</sup> with a 0.3 min/2.7 min misting cycle, whereas they only had 4 feedings hr<sup>-1</sup> with the 15 min misting cycle. Therefore, frequent short mistings may result in a higher percentage of nutrient absorbance and thus better embryo development compared to less frequent but longer exposure to mist. Similar results were observed for coffee somatic embryos in TIBs (Albarrán, *et al.* 2005).

Headspace gas was also investigated as an important factor affecting embryo development. Compared to 0 VVM, 3% CO<sub>2</sub> yielded a significantly greater post heart stage ratio in reactor culture, but root length was not significantly different (Table 3.1).



**Figure 3.3** Embryo development in mist reactor with different substrates (a) and nutrient supply cycles (b). Misting cycles were indicated as on time/off time per cycle. N=3 for petri dish control; N=2 for each reactor condition; all reactor data are shown in min/max and Petri dish data are shown in  $\pm$ SE, in b, A,B,C compares embryo development on N50 in all conditions and a,b,c compares embryo development on N90 in all conditions,  $p \leq 0.05$

In Phytigel, the post heart stage ratio was greatest when 0.05 VVM ambient air was continuously flushed through the system (Table 3.1). In all conditions, reactor-grown embryos had significantly greater post heart ratio and longer overall rooted embryos than those grown in Phytigel (Table 3.1). The only exception was at 0.05 VVM air; there was no significant difference between embryos grown in the reactor and Phytigel.

**Table 3.1** Effect of aeration on carrot somatic embryogenesis after 14 d culture.

Aeration condition	Post heart stage ratio (%)		Rooted embryo length (cm)	
	Phytigel	Reactor	Phytigel	Reactor
0 vvm	19.5 b	24.9 b*	0.25 a	0.46 a*
0.05 vvm air	31.2 a	36.8 ab	0.29 a	0.48 a*
0.05 vvm 3% CO <sub>2</sub>	26.3 ab	40.1 a*	0.36 a	0.60 a*

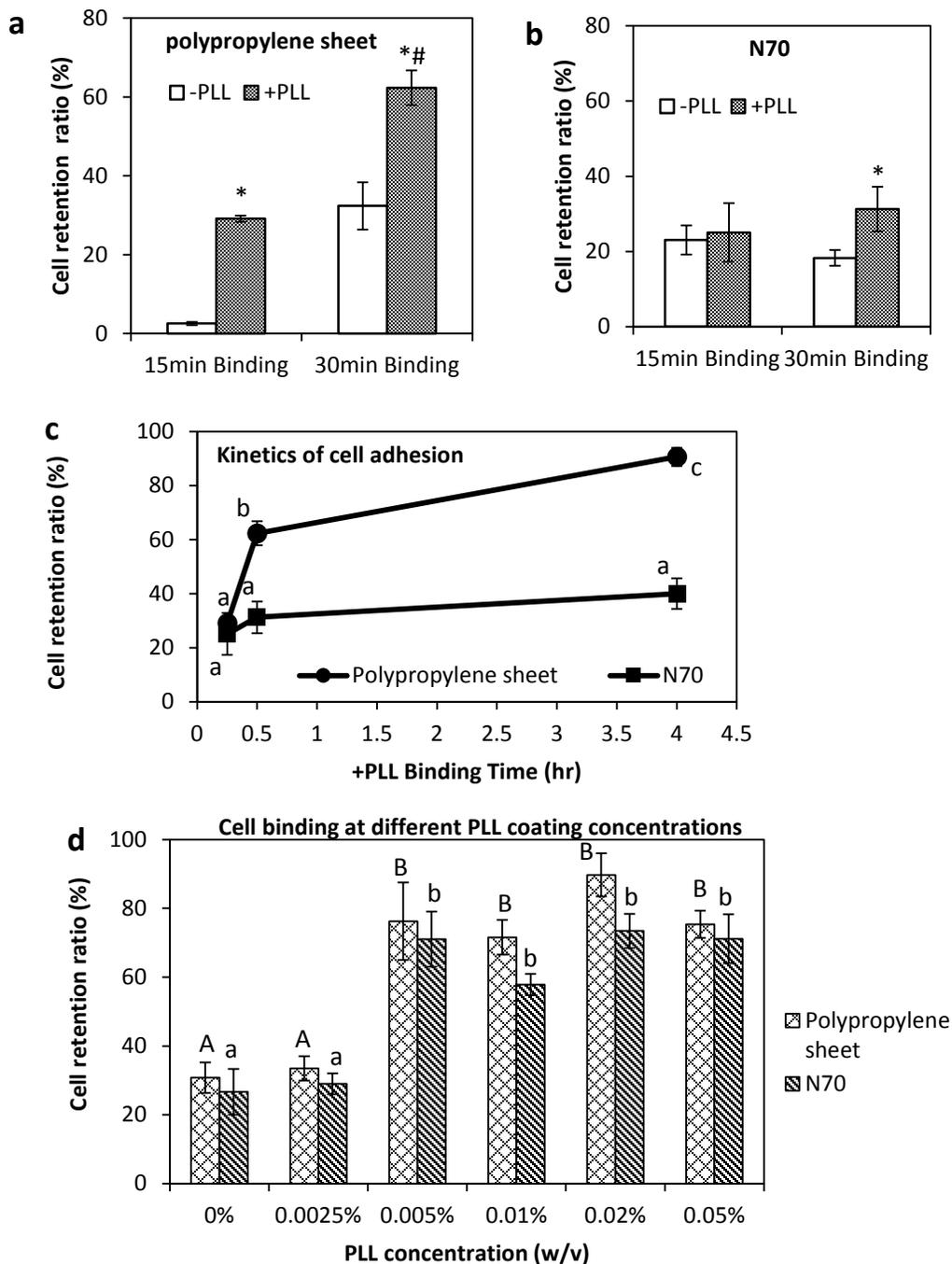
Post heart stage ratio %: torpedo + cotyledon stage embryos over sum of all embryo stages. Average of N50 and N90 were presented in “Reactor” columns since no significant difference was observed between these two substrates. Comparisons within columns with the same letter are not significantly different at  $p \leq 0.05$  by Duncan's multiple range test; \*  $p \leq 0.05$  for comparisons between columns, i.e. between reactor and Phytigel control; N= 3.

Gases, i.e. O<sub>2</sub>, CO<sub>2</sub> and C<sub>2</sub>H<sub>4</sub>, are important for embryo development. O<sub>2</sub> consumption usually increases as embryos mature (Jay, *et al.* 1992; Shigeta, *et al.* 1996; Shimazu and Kurata 1999). Similar to observations by Ducos *et al.* (2009), embryo development in shake flasks was inhibited (data not shown), suggesting O<sub>2</sub> limitation. CO<sub>2</sub> is produced during embryogenesis and increasing concentrations often promote embryo development (Barbón, *et al.* 2008a; Buddendorfjoosten and Woltering 1994; Chung and Bae 2000; Huang, *et al.* 2006; Rosnow, *et al.* 2011; Takamura, *et al.* 2010), so excessive aeration, while improving O<sub>2</sub> availability, may actually inhibit embryogenesis by stripping away CO<sub>2</sub>. Since carrot cell regeneration did not respond to CO<sub>2</sub> enrichment up to 2% (Tate and Payne 1991), we chose 3% (v/v) to investigate its effect on carrot embryo development. Compared to the other two conditions, 3% CO<sub>2</sub> enrichment seemed to expedite embryo development (Table 3.1). Although CO<sub>2</sub> stimulation of root growth is well known (DiIorio, *et al.* 1992; Fisichella and Morini 2003; Jeong, *et al.* 2006; Weathers and Zobel 1992; Wyslouzil *et al.* 2000), this was not observed in our study. C<sub>2</sub>H<sub>4</sub>, also produced by embryos, inhibits development at high concentrations in tightly-sealed vessels with a limited headspace (Roustan, *et al.* 1994; Roustan, *et al.* 1989;

Roustan, *et al.* 1990; Wang, *et al.* 2011). Possible inhibition by C<sub>2</sub>H<sub>4</sub> was only observed in 0 VVM Phytigel, but not in the 0 VVM reactor where cells developed into healthy embryos suggesting that in this large bag (7L) at this low density of cells (< 1 g FW) , there were adequate nutrients including gases. Further investigation of the role of headspace gases is warranted when larger amounts of biomass are involved, e.g. during scale-up.

### **3.4.3 Cell adhesion to PLL coated surfaces**

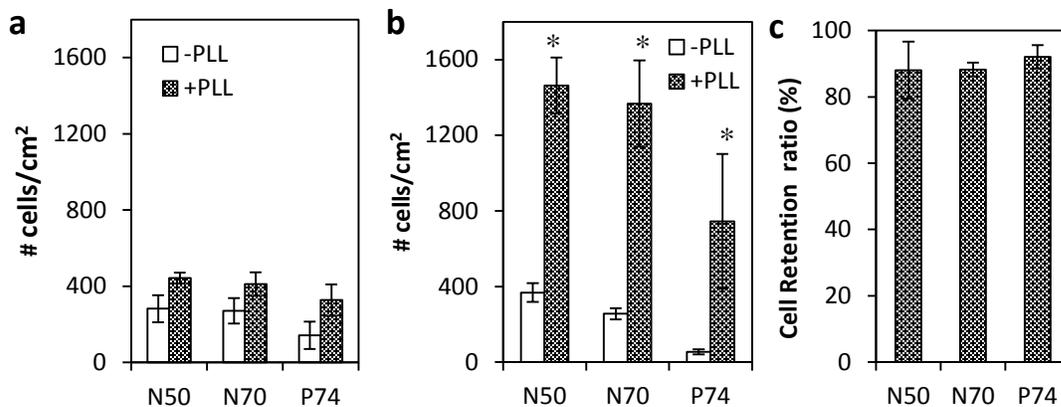
To move from a horizontal to a vertical reactor configuration, cells must attach to a substrate; a hanging garden of carrot plantlets was envisioned. PLL coated sheeting or mesh were vertically hung inside the culture bag and cell attachment measured. PLL enhanced cell binding on polypropylene sheeting as did longer binding times (Figure 3.4a). PLL also enhanced binding on N70 mesh, but only when subjected to longer binding times, e.g. 30 min vs. 15 for sheeting (Figure 3.4b). Cell binding kinetics showed that the process was swift and nearly complete in ~1 hr; more than twice as many cells attached to sheeting than to the mesh (Figure 3.4c). The minimum effective PLL concentration for either sheeting or mesh was 0.005% (Figure 3.4d). Although sheeting showed better cell binding, in the reactor bag cells did not remain bound during vertical spray inoculation tests (data not shown), so we continued our study with only mesh.



**Figure 3.4** Cell binding when manually applied to PLL coated materials. Cell retention ratio (%) measured as cells remaining after vertically flushing ten times of 0.3 mL water down each strip,  $\pm$ SE. **a**  $\pm$  PLL coated polypropylene sheet. **b**  $\pm$ PLL coated nylon screen (N70). **c** Kinetics of cell adhesion on polypropylene sheet and N70. **d** Cell binding at different PLL coating concentrations. SE bars shown, in **a**, \* +PLL vs. -PLL,  $p \leq 0.05$ ; #, 30 min binding +PLL vs. 15 min binding +PLL,  $p \leq 0.05$ ; in **b**, \* +PLL vs. -PLL,  $p \leq 0.05$ ; in **c**, a, b, c, compares binding on each material,  $p \leq 0.05$ ; in **d**, A, B indicate statistical difference between polypropylene sheet and a, b indicate statistical difference between N70 for PLL concentrations,  $p \leq 0.05$

### 3.4.4 Effect of sucrose and salts on binding

To improve cell binding on the mesh we tested different binding solutions. We initially tried to spray cells in deionized water, but they failed to develop into embryos, likely the result of an imbalance in osmotic pressure and subsequent changes in cell physiology (Guo *et al.* 2005; Shoji *et al.* 2006). When different meshes were compared, there was no significant difference in binding  $\pm$  PLL using  $\frac{1}{2}$  strength B5 with  $15 \text{ g L}^{-1}$  sucrose (Figure 3.5a).



**Figure 3.5** Cell binding when sprayed into mist reactor hung with various meshes and sprayed with different suspension media. **a** Cells were suspended in half strength B5 with  $15 \text{ g L}^{-1}$  sucrose. **b** Cells were suspended in  $15 \text{ g L}^{-1}$  sucrose. **c** Cell retention on +PLL meshes after binding in  $15 \text{ g L}^{-1}$  sucrose after spraying B5 for 24 hr,  $\pm$ SE, N=3 for all tests, \*  $p \leq 0.05$

When the B5 salts were eliminated, binding increased substantially on all 3 meshes (Figure 3.5b), and of those bound cells,  $>85\%$  remained attached 24 hr after being sprayed with B5 (Figure 3.5c). The increased cell attachment to PLL coated substrates resulting from removal of B5 was similar to that observed for *Catharanthus roseus* cells (Facchini *et al.* 1989). There are a variety of free cations in B5, and it is postulated that they may interfere with cell adhesion to surfaces by affecting the electrical potential of the plant cell wall (Shomer *et al.* 2003).

These results showed that cells can bind to PLL coated mesh and remain attached potentially for long duration.

### **3.4.5 Somatic embryogenesis and subsequent growth on PLL coated mesh hanging inside the mist reactor**

Spray inoculated cells attached irregularly, but developed into small rooted embryos (Figure 3.6a); development was statistically the same as those on a N70 covered platform and the petri dish controls. Although overall length of rooted embryos on the hanging strips (0.34 cm) was significantly greater than those on the petri dish controls (0.25cm), it was less than embryos on the N70 covered platform (0.45 cm). It is possible that nutrient availability to embryos on the horizontal platform was greater than for the embryos on the vertically hanging strips.

To determine if cells attached to PLL coated surfaces could develop into rooted plantlets, we first set up a reactor hung with PLL coated N70 strips to which cells had been manually applied. After 3 weeks of culture using a misting cycle of 0.3 min on, 2.7 min off at 50 mL min<sup>-1</sup> medium flow rate, fully rooted healthy plantlets formed on the vertically hanging strip (Figure 3.6b). Both sides of the strip had attached plantlets and there was no observed difference in growth or quality from top to bottom of the hanging strip suggesting a reasonably uniform distribution of the nutrient mist was provided to the embryos (Figure 3.6b).



**Figure 3.6** *In vitro* propagation on PLL coated surfaces in a mist bioreactor. **a** Embryos developed on PLL coated surface after 14 days of reactor culture by spray inoculation. **b** Fully rooted plantlets developed on PLL coated surface after 20 days of reactor culture by manual inoculation

### 3.4.6 Considerations between vertical and horizontal culture

Using a vertical hanging culture system offers a series of advantages over horizontal culture (Table 3.2), but it also requires that plantlets attach to the hanging strips so they do not succumb to gravity and detach. To estimate possible productivity, we harvested 0.013 g FW biomass  $\text{cm}^{-2}$  of N70 strip, which was 65% of the yield obtained per  $\text{cm}^2$  on the N70 platform. This seeming disadvantage can be compensated by the larger potential culture surface area for the vertical configuration (Table 3.2). Vertical scaling also occupies less floor space, which is an advantage in reducing production cost. Advantages and disadvantages of each design orientation are summarized in Table 3.2.

**Table 3.2** Comparison of two designs of reactor orientation options.

<b>Horizontal (45° angle) (Figure 1f)</b>	<b>Vertical (Figure 1g)</b>
Easier to inoculate	Need additional binding step
One flat useable surface for cultures: total usable culture surface ~450 cm <sup>2</sup> (based on current bag size)	Multiple surfaces available via 6 × 30 cm strips hung; each strip has two sides: total usable culture surface ~2,880 cm <sup>2</sup> (based on current bag size)
Light intensity may vary down slope depending on source location.	Light intensity may vary top down strip depending on source location.
Tangled, twisted roots	Linearly elongated roots
Floor space occupied for a single reactor: 668 cm <sup>2</sup>	Floor space occupied for a single reactor: 191 cm <sup>2</sup>
Less uniform nutrient mist distribution on platform	Uniform distribution of nutrient mist along the strip

### 3.5 Conclusions

We showed here that a mist reactor using a disposable bag offers the potential for one step micropropagation from cells via embryogenesis to fully rooted plantlets. Using hanging mesh strips, three important results emerged from this study: embryos developed well within the mist environment of the reactor, development was uniform up and down the strip, and despite producing some considerable biomass, the young plantlets did not fall off the hanging strips. Vertical culture using the described attachment technology provides process advantages including use of less floor space and greater plantlet productivity per unit area. By controlling nutrient supply and aeration, embryo development in the mist reactor was improved and

equaled or exceeded that of controls, mainly by providing a more frequent feeding cycle. Together both the mist reactor and attachment technology may offer opportunities for at least partial automation of micropropagation. The ability to control all aspects of the culture environment in the mist reactor offers a good potential for combining the otherwise discrete process steps common to micropropagation resulting in a one-step process for producing high quality plants ready for field conditions. Together these results suggested this may be a reasonable propagation method.

### **Acknowledgments**

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# Chapter 4 Comparing the mist bioreactor with traditional micropropagation of *Artemisia annua*

## 4.1 Introduction

Plant tissue culture and its commercial application in micropropagation are of great importance in plant propagation. Currently, the most widely used micropropagation method is proliferation via axillary bud explants. Therefore, an *in vitro* culture procedure generally includes culture initiation, shoot proliferation, root development and finally acclimatization. Culture medium for each of these steps is different and thus explants need to be transferred from one medium to the next at each step. Traditional micropropagation relies on gelled medium and small containers requiring intensive labor during subculture and medium transfer.

Automation of the *in vitro* propagation process in a bioreactor that uses liquid medium is considered labor and time efficient (Takayama and Akita 2006). Design of bioreactors for micropropagation, however, is challenging because plant tissues have diverse morphologies and at different development stages their demand on culture conditions varies. For example, production of protocorm-like bodies was better in submersion culture vs. temporary immersion culture (Gao, *et al.* 2014; Yang, *et al.* 2010). In contrast shoot proliferation from tissues was better in temporary immersion than in submersion culture (Gao, *et al.* 2014).

Submersion culture can be problematic for shoot tissues and severe hyperhydricity often occurs as a result of continuous contact with liquid medium (Ziv 1991a), so ventilation is

another condition that needs dynamic control at different developmental stages. Shoot initiation requires little ventilation (Majada, *et al.* 1997; Mills *et al.* 2004; Nour and Thorpe 1994). On the other hand, shoot proliferation is better with high ventilation ( $\geq 0.1$  vvm) to minimize accumulation of volatiles like  $C_2H_4$  (Heo, *et al.* 2001; Majada *et al.* 2000; Majada, *et al.* 2002; Ogasawara 2003; Santamaria *et al.* 2000a; Zobayed 2005). Thus, the mode of medium contact, irradiance, and headspace gas composition and ventilation are all key factors to be controlled for *in vitro* propagation to ensure high yield and quality of plantlets.

A mist reactor provides independent and flexible control of nutrient mist feeding cycle and ventilation, and has the potential to be used for shooting, rooting, acclimatization, possibly combining all these steps together into a single step. Previously, plantlets of carnation, a plant prone to hyperhydricity, showed less hyperhydricity and greater *ex vitro* survival when acclimatized in an acoustic window mist reactor than in gelled medium (Correll, *et al.* 2001; Correll and Weathers 2001a). Using a mist reactor made of a disposable plastic bag with independent ventilation (Liu, *et al.* 2009a), healthy rooted somatic embryos were produced from carrot cells (Fei and Weathers 2014). These carrot plantlets grew on hanging strips of mesh inside the reactor. However, it was not known if plantlets could be similarly grown from leaf explant inoculum.

The medicinally important plant *Artemisia annua*, which produces the current antimalarial drug, artemisinin, was used as an experimental plant. Clonal propagation of high yield plants through micropropagation is an attractive alternative for producing plants with uniform

chemical constituents. Studies on *in vitro* propagation of *A. annua* have mostly focused on growth hormone and medium composition in relation to artemisinin content (Davies *et al.* 2009; Ferreira 2007; Liu *et al.* 2006; Liu, *et al.* 2003). Here *A. annua* was used as a model plant to study the effect of mist reactor conditions (misting cycle, ventilation, CO<sub>2</sub> concentration, irradiance, and relative humidity) on shooting, rooting and *in vitro* acclimatization.

## **4.2 Materials and Methods**

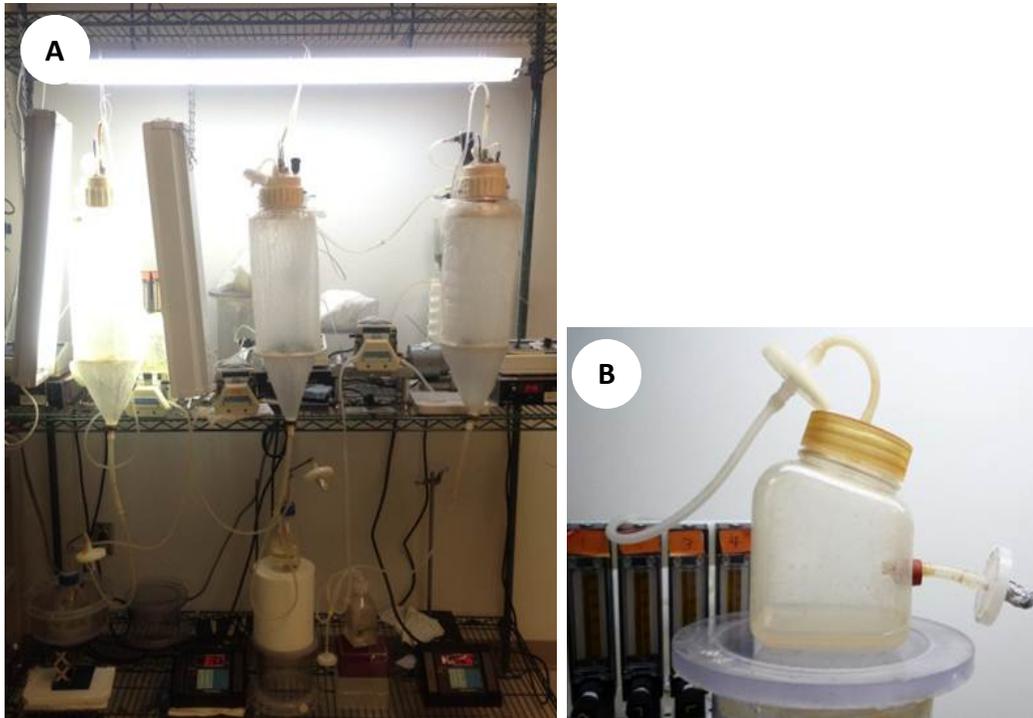
### **4.2.1 Plant materials and maintenance of culture**

The clonal SAM cultivar of *A. annua* L. (Nguyen *et al.* 2013) was maintained on gelled rooting medium: 20 g L<sup>-1</sup> sucrose, 2.22 g L<sup>-1</sup> Murashige & Skoog (MS) salts with vitamins (Murashige and Skoog 1962), and 5 g L<sup>-1</sup> Agargellan<sup>TM</sup>, pH 5.8, and subcultured by nodal cuttings every month.

### **4.2.2 Shoot induction in the mist reactor**

Using a scalpel scissors, shoots of 4-week-old *in vitro* *A. annua* were manually chopped into four types of inoculum: leaves (L), leaves + petiole (LP), nodes (N) and internodes (IN). Each nodal inoculum had one node. Reactor preparation and sterilization was as described in detail in Fei and Weathers (2014; Chapter 3, Figure 3.1a). Each reactor (5 L headspace) was inoculated with about 1g fresh weight containing at least 30 pieces of each type of inoculum and then cultured for 2 weeks with shooting medium: 30 g L<sup>-1</sup> sucrose 4.43 g L<sup>-1</sup> MS salts with vitamins, 0.25 μmol L<sup>-1</sup> α-naphthaleneacetic acid (NAA), and 2.5 μmol L<sup>-1</sup> N-6-

benzyladenine (BA), pH 5.8 (Nguyen, *et al.* 2013). During shoot induction, different ventilations (V), CO<sub>2</sub> concentrations (C), light intensities (I) misting cycles (M) and sucrose levels (S) were investigated (Table 4.1). The forced ventilation rate was described as vvm, which is defined as the number of volumetric exchanges of headspace gas per unit time (e.g., per minute) within the culture vessel. For example, at 0.1 vvm ventilation, 10 % of the headspace gas is renewed per minute. Regardless of misting cycle, the hourly volumetric nutrient medium delivery was maintained at 30 mL h<sup>-1</sup>. Prior to entry into reactors, ambient or CO<sub>2</sub> enriched air was humidified by a Nafion tube (Perma Pure, MH-110-48F-4) filled with dH<sub>2</sub>O. The 1I light condition was set up using four overhead full spectrum fluorescent light bulbs (GE brand F40T12-SR), and the 3I (50 μmol m<sup>-2</sup>·s<sup>-1</sup>) light included four additional hanging fluorescent light bulbs vertically flanking both sides of the reactor (Figure 4.1A). To query osmotic potential, medium equivalent to the 3S medium contained 10.75 g L<sup>-1</sup> mannitol (MNT) along with 10 g L<sup>-1</sup> sucrose (1S). Controls on gelled medium (liquid medium supplemented with 5 g L<sup>-1</sup> Agargellan<sup>™</sup>, pH 5.8) were treated with the same ventilation, CO<sub>2</sub> level, and light intensity as their corresponding reactors (Figure 4.1B)



**Figure 4.1** Mist reactor system and gelled medium controls. **A**, mist reactors with different light intensities. The two reactors on right were treated with 1I light and the one on the left was treated with 3I light. **B**, culture vessel used for gelled medium controls.

**Table 4.1** Reactor conditions for shooting study

<b>Condition</b>	<b>Misting cycle (M) (min on/min off)</b>	<b>Ventilation (V) (vvm)</b>	<b>CO<sub>2</sub> (C) (%)</b>	<b>Light (I) (<math>\mu\text{mol m}^{-2}\cdot\text{s}^{-1}</math>)</b>	<b>Sucrose (S) (<math>\text{g L}^{-1}</math>)</b>
<b>0V1C1I3S2M</b>	0.5/29.5	0	0.04	15	30
<b>1V1C1I3S2M</b>	0.5/29.5	0.1	0.04	15	30
<b>1V1C1I3S1M</b>	0.75/59.25	0.1	0.04	15	30
<b>1V1C3I3S2M</b>	0.5/29.5	0.1	0.04	15	30
<b>1V4C1I3S2M</b>	0.5/29.5	0.1	0.16	15	30
<b>1V4C3I3S2M</b>	0.5/29.5	0.1	0.16	50	30
<b>1V4C3I1S2M</b>	0.5/29.5	0.1	0.16	50	10
<b>1V4C3I1S2M+MNT</b>	0.5/29.5	0.1	0.16	50	10

Ventilation (V) was 0 and 0.1 vvm for 0V and 1V, respectively. CO<sub>2</sub> concentration (C) was 0.04% (v/v, ambient level) and 0.16% (v/v) for 1C and 4C, respectively. Light intensity (I) was 15 and 50  $\mu\text{mol m}^{-2}\cdot\text{s}^{-1}$  for 1I and 3I, respectively. Sucrose level (S) was 3S and 1S for 30  $\text{g L}^{-1}$  and 10  $\text{g L}^{-1}$ , respectively. Misting cycle (M) was 0.75 min on/59.25 min off and 0.5 min on/29.5 min off for 1M and 2M, respectively. MNT: mannitol (10.75  $\text{g L}^{-1}$ ).

### 4.2.3 Analysis of shoot growth, flavonoids and artemisinin

New shoot growth was measured in terms of gain in % shoot FW, shooting percentage, number of shoot apical meristems (ShAM), number of new leaves and length of new shoots.

$$\% \text{ shoot FW gain} = \frac{(FW \text{ of shoots post harvest} - FW \text{ of initial inoculum}) \times 100}{FW \text{ of initial inoculum}} \quad \text{Eq (4.1)}$$

Shooting percentage was determined as

$$\% \text{ new shoots} = \frac{\# \text{ inoculum pieces with new shoots} \times 100}{\# \text{ inoculated pieces}} \quad \text{Eq (4.2)}$$

Explants with new shoot development were also examined for hyperhydricity and the percent of shoots that were hyperhydric was arbitrarily scored for 6 features including swollen and glassy appearance, brittle texture, dark color, narrow and elongated shape, and curling edges.

Hyperhydricity percentage was measured as:

$$\% \text{ hyperhydricity} = \frac{\left[ \frac{1}{6}(a+2b+3c+4d+5e+6f) \right] \times 100}{(a+b+c+d+e+f)} \quad \text{Eq (4.3)}$$

In Eq (4.3), a, b, c, d, e, f are the number of explants developing new shoots that showed 1, 2, 3, 4, 5, and all 6 of the hyperhydric features, respectively. Hyperhydric leaves were also compared to normal leaves for their water content and number of glandular trichomes (see section 4.2.4). Artemisinin was measured using GC/MS according to (Weathers and Towler 2012). Total flavonoids were measured using the AlCl<sub>3</sub> method of (Arvouet-Grand *et al.* 1994) with quercetin as standard and flavonoid amount expressed as quercetin units. To measure water content, new shoots were first weighed for FW right after harvest, and then incubated at 60 °C to constant weight (DW). Water content was then determined as

$$\% \text{ water} = \frac{(FW \text{ of shoot tissue} - DW \text{ of shoot tissue}) \times 100}{FW \text{ of shoot tissue}} \quad \text{Eq (4.4)}$$

#### 4.2.4 Glandular trichome counts

The 4<sup>th</sup> fully expanded leaf from the shoot apical meristem (ShAM) of each new shoot was used for counting glandular trichomes. Each leaf sample was sandwiched in water between two microscope slides. The adaxial and abaxial side of the sample was then counted for autofluorescent glandular trichomes under a 120× magnification and GFP 470 filtered UV light using a Zeiss brand, SteREO Discovery V12 microscope. The number of trichomes on each leaf sample was averaged from 6 different locations per leaf.

#### 4.2.5 Rooting of shoots in the mist reactor

**Table 4.2** Reactor conditions for rooting study

Condition	Misting cycle (M) (min on/ min off)	# Mistings/h	Ventilation (V) (vvm)	CO <sub>2</sub> (C) (%)	Light intensity (I) (μmol m <sup>-2</sup> ·s <sup>-1</sup> )
<b>0V1C1I2M</b>	0.5/29.5	2	0	0.04	15
<b>1V1C1I2M</b>	0.5/29.5	2	0.1	0.04	15
<b>1V4C1I2M</b>	0.5/29.5	2	0.1	0.16	15
<b>1V4C3I2M</b>	0.5/29.5	2	0.1	0.16	50
<b>1V1C1I1M</b>	0.75/59.25	1	0.1	0.04	15
<b>1V1C1I4M</b>	0.25/14.75	4	0.1	0.04	15

Ventilation (V) was 0 and 0.1 vvm for 0V and 1V, respectively. CO<sub>2</sub> concentration (C) was 0.04% (v/v, ambient level) and 0.16% (v/v) for 1C and 4C, respectively. Light intensity (I) was 15 and 50 μmol m<sup>-2</sup>·s<sup>-1</sup> for 1I and 3I, respectively. Misting cycle (M) was 0.75 min on/59.25 min off, 0.5 min on/29.5 min off and 0.25 min on/14.75 min off for 1M, 2M and 4M, respectively.

Single node cuttings from four-week-old *A. annua* were used as inoculum. Explants were cultured in rooting medium (20 g L<sup>-1</sup> sucrose, 2.22 g L<sup>-1</sup> MS salts with vitamins, pH 5.8) under different conditions for 12 days (Table 4.2). Regardless of misting cycle, the hourly volumetric nutrient delivery was maintained at 30 mL h<sup>-1</sup>. Ambient or CO<sub>2</sub> enriched air was again

humidified by Nafion tube. Controls on gelled medium (liquid medium supplemented with 5 g L<sup>-1</sup> Agargellan<sup>TM</sup>, pH 5.8) were treated with the same ventilation, CO<sub>2</sub> level, and light intensity as their corresponding reactors.

After 12 days of culture in rooting medium, rooted plantlets were harvested and measured for % shoot FW gain (Eq 4.1), rooting percentage, total number and total length of primary roots per plant, total number and total length of branches per plant, and the maximum diameter of each primary root per plant. Rooting percentage was determined as

$$\% \text{ root} = \frac{\# \text{ inoculum cuttings with new roots} \times 100}{\# \text{ inoculated cuttings}} \quad \text{Eq (4.5)}$$

#### **4.2.6 *In vitro* acclimatization in mist bioreactor**

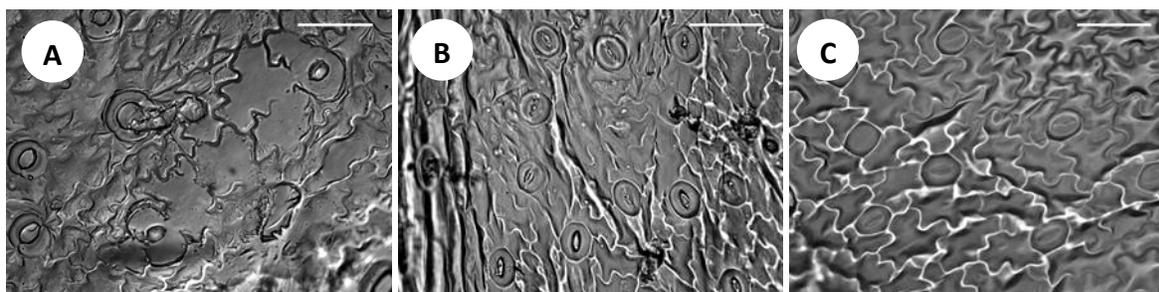
Four-week-old rooted plantlets of *A. annua* grown in Magenta boxes were used in the acclimatization study and grown in rooting medium for 10 days under different misting cycles (M), ventilation (V) and relative humidity (RH) conditions (Table 4.3). Regardless of misting cycle, the hourly volumetric nutrient delivery was maintained at 30 mL h<sup>-1</sup> for all reactors unless otherwise indicated (see Table 4.3). To study the effect of ventilation and RH, ambient air with different RH was passed into the bioreactor and gelled medium controls at 0.1 vvm. Fully humidified (100% RH) air was generated by passing ambient air through a dH<sub>2</sub>O filled Nafion tube (Perma Pure, MH-110-48F-4). Air with 52% RH was achieved by directly passing ambient air into the reactor. Low humidity air (11% RH) was generated by passing ambient air through a column (4.5 cm × 22 cm) filled with Driarite<sup>TM</sup>.

**Table 4.3** Reactor conditions for *in vitro* acclimatization

<b>Condition</b>	<b>Misting cycle (min on/ min off)</b>	<b>Volumetric feed (mL h<sup>-1</sup>)</b>	<b>Ventilation (vvm)</b>	<b>Relative humidity (RH)</b>
<b>0 vvm</b>	0.5/29.5	30	0	>96 %
<b>100RH</b>	0.5/29.5	30	0.1	100 %
<b>52RH</b>	0.5/29.5	30	0.1	52 %
<b>52/11RH</b>	0.5/29.5	30	0.1	52 % first 5 days and then 11%
<b>Stepped down feed</b>	0.5/29.5 (D 0-1) 0.75/59.25 (D 2-4) 0.75/119.25 (D 5-7) 0.75/239.25 (D 8-10)	30 (D 0-1) 22.5 (D 2-4) 11.3 (D 5-7) 5.6 (D 8-10)	0.1	100 %
<b>Stepped cycle</b>	0.5/29.5 (D 0-1) 1/59 (D 2-4) 2/118 (D 5-7) 4/236 (D 8-10)	30	0.1	100 % first 5 days and then 11%

D, day. All reactors and controls were grown under 11 light.

Controls were treated with the same condition as their corresponding reactors. Each condition was done in triplicate with 10 plantlets per replicate. Imprints of the detached 3-6<sup>th</sup> fully expanded leaf were made using clear nail polish and stomatal function was measured as described by Correll and Weathers (2001a): open, partially open, closed (Figure 4.2). Closed stomata were deemed functional.



**Figure 4.2** Stomata status at open (A), partially open (B) and closed (C). Bar: 50  $\mu$ m.

Counts were made at 400 $\times$  magnification at 5 positions on each sampled leaf, and 4 leaves were sampled from each plantlet. The average of these 20 counts was then taken to represent

each plantlet, and the average of 10 plantlets was taken to represent each reactor. Closed stomata were deemed functional. Plantlets harvested from mist reactors and gelled medium controls were transplanted to soil:perlite (2:1 V/V) pots and *ex vitro* survival was measured after one week.

#### **4.2.7 Stem lignin assay**

Stems were stained for lignin using phloroglucinol (Speer 1987; Yeung 1998). Free-hand cross sectioned stem slices between 9<sup>th</sup> and 10<sup>th</sup> node were incubated in saturated (2 mol L<sup>-1</sup>) solution of phloroglucinol dissolved in 2 mol L<sup>-1</sup> hydrochloric acid and then observed under a visible light microscope. Phloroglucinol-stained sections were photographed within 30 min; lignified cell walls stained red.

#### **4.2.8 Statistics**

All reactor experiments had three replicates. Data from all experiments was subjected to one-way ANOVA, and Duncan's multiple range analysis by SPSS.

### **4.3 Results**

#### **4.3.1 Shooting in mist bioreactor and gelled medium**

##### ***4.3.1.1 Comparison between the mist reactor and gelled medium controls***

Regardless of condition, reactors yielded greater fresh biomass than gelled medium controls (Table 4.4, Figure 4.3A). The mist reactor also yielded more shoot apical meristems, new leaves and longer shoots than controls (Table 4.5, Figure 4.3B-D). There was no significant difference in shooting percentage between plantlets grown in the mist reactor and controls in

most conditions. However, when misting frequency was once per hour (1M) and when there was CO<sub>2</sub> enrichment (4C1I), reactor-grown explants had about 50% more new shoot emergence than controls (Figure 4.3E, Table 4.4). Hyperhydricity generally increased with more frequent misting (Figure 4.3F) unless CO<sub>2</sub> and light intensity were increased (Table 4.4). Under the latter conditions plantlets in the mist reactor were hyperhydric similar to gelled medium controls (Table 4.4).

#### ***4.3.1.2 Comparison among conditions***

Compared to the unventilated condition, 0 vvm (0V), 0.1 vvm ventilation significantly reduced hyperhydricity in reactors (Table 4.4). Biomass, new shoots and shoot length also increased in reactors that were ventilated (Table 4.4). When misting frequency was increased from once (1M) to twice an hour (2M), meristems, leaves and shooting percentage all decreased (Figure 4.3B, C and E), and plantlets were more hyperhydric (Figure 4.3F). There was no overall change in biomass due to alteration in misting frequency (Figure 4.3A).

Light but not CO<sub>2</sub> was the limiting factor for enhanced shoot proliferation (% new shoots). Increasing CO<sub>2</sub> (4C) alone did not yield significant differences compared to conditions of low light and low CO<sub>2</sub> (1C1I) (Table 4.4). Shooting percentage in the mist reactor increased significantly only after tripling the light intensity (3I) without CO<sub>2</sub> enrichment (Table 4.4). Increasing both light (3I) and CO<sub>2</sub> (4C) did not further increase shoot production (Table 4.4).

**Table 4.4** Overall growth of new shoots during shooting in reactor and control

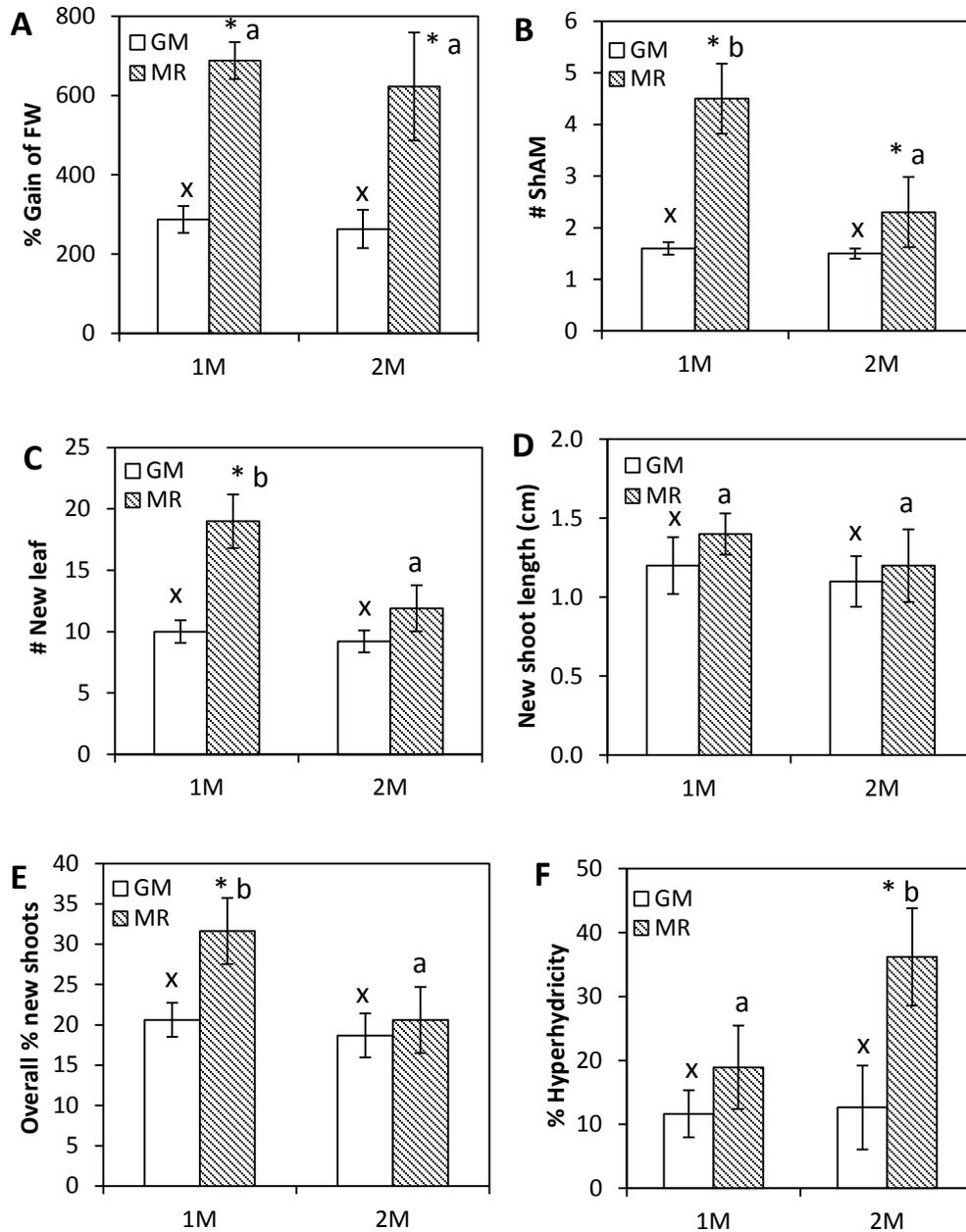
Condition code	Reactor			Gelled medium		
	% shoot FW gain	% new shoots	% hyper-hydricity	% shoot FW gain	% new shoots	% hyper-hydricity
0V1C1I3S	493.3 * a	18.8 a	49.2 * d	172.2 a	19.7 a	13.0 a,b
1V1C1I3S	622.8 * a,b	20.6 a,b	36.2 * c	263.4 a,b	18.7 a	12.6 a,b
1V1C3I3S	758.9 * b,c	32.6 c	29.2 * c	237.1 a,b	29.8 c	9.9 a,b
1V4C1I3S	771.6 * b,c	24.9 * b	27.8 * b,c	273.6 a,b	16.5 a	8.3 a
1V4C3I3S	1257.7 * d	31.7 c	14.8 a	376.1 b,c	26.9 b,c	7.8 a,b
1V4C3I1S	1611.1 * e	40.7 d	32.3 * c	520.8 c	36.9 d	14.3 b
1V4C3I1S+M	965.5 * c,d	27.2 b,c	21.9 * a,b	224.6 a,b	24.1 b	11.5 a,b
NT						

Ventilation (V) was 0 and 0.1 vvm for 0V and 1V, respectively. CO<sub>2</sub> concentration (C) was 0.04% (v/v, ambient level) and 0.16% (v/v) for 1C and 4C, respectively. Light intensity (I) was 15 and 50  $\mu\text{mol m}^{-2}\cdot\text{s}^{-1}$  for 1I and 3I, respectively. Sucrose level (S) was 3S and 1S for 30 g L<sup>-1</sup> and 10 g L<sup>-1</sup>, respectively. Misting cycle was 0.5 min on/29.5 min off (2M) for light intensity, CO<sub>2</sub> level and sucrose concentration studies. MNT: mannitol (10.75 g L<sup>-1</sup>). Letters compare measured factors among different conditions; \* compares between reactor and the gelled medium control;  $p \leq 0.05$ .

**Table 4.5** Growth of normal new shoots from single nodes during shooting

Condition	Reactor			Gelled medium		
	# ShAM	# New Leaves	Shoot length (cm)	# ShAM	# New Leaves	Shoot length (cm)
0V1C1I3S	2.2 * a	12.5 * a	1.0 a	1.4 a	9.0 a	1.0 b
1V1C1I3S	2.3 * a	11.9 * a	1.2 a,b	1.5 a	9.2 a	1.1 b
1V1C3I3S	3.1 * b	14.9 * a,b	1.6 * c	1.8 a,b	8.3 a	1.0 a,b
1V4C1I3S	2.3 * a	15.2 * a,b	1.5 * c	1.6 a	9.6 a	1.1 b
1V4C3I3S	7.5 * d	32.4 * d	1.4 * b,c	3.4 c	16.9 b	1.0 b
1V4C3I1S	4.1 * c	22.0 * c	1.3 b,c	3.5 c	20.2 b	1.0 a,b
1V4C3I1S+MNT	3.5 * b,c	18.9 * b,c	1.2 a,b	2.2 b	9.6 a	0.8 a

Ventilation (V) was 0 and 0.1 vvm for 0V and 1V, respectively. CO<sub>2</sub> concentration (C) was 0.04% (v/v, ambient level) and 0.16% (v/v) for 1C and 4C, respectively. Light intensity (I) was 15 and 50  $\mu\text{mol m}^{-2}\cdot\text{s}^{-1}$  for 1I and 3I, respectively. Sucrose level (S) was 3S and 1S for 30 g L<sup>-1</sup> and 10 g L<sup>-1</sup>, respectively. Misting cycle was 0.5 min on/29.5 min off (2M) for light intensity, CO<sub>2</sub> level and sucrose concentration studies. MNT: mannitol (10.75 g L<sup>-1</sup>). Letters compare measured factors among different conditions; \* compares between reactor and the gelled medium control;  $p \leq 0.05$ .



**Figure 4.3** The effect of misting cycle on new shoot development. GM: gelled medium; MR: mist reactor. Two misting cycles: 2 mistings  $\text{h}^{-1}$  (2M, 0.5 min on/29.5 min off), 1 misting  $\text{h}^{-1}$  (1M, 0.75 min on/59.25 min off). **A**, % gain of FW; **B**, number of shoot apical meristems (ShAMs) on new shoots; **C**, number of new leaves; **D**, length of new shoots; **E**, over all shooting percentage; **F**, % hyperhydricity. Letters a and b compare between two reactor misting cycles; \* compares between gelled medium and mist reactor,  $p \leq 0.05$ . Other conditions for both reactor and gelled medium controls were: 0.1 vvm (1V), ambient air (1C), at  $15 \mu\text{mol m}^{-2}\cdot\text{s}^{-1}$  (1I),  $30 \text{ g L}^{-1}$  sucrose (3S).

Although shoot proliferation (% new shoots) did not increase with extra CO<sub>2</sub>, emergence of apical meristems (# ShAM) and new leaves (# new leaves) increased significantly when both CO<sub>2</sub> and light were increased (Table 4.5). There was also significantly less hyperhydricity in reactor-grown plantlets but only when both extra CO<sub>2</sub> and light were added (Table 4.4). Controls showed similar trends in response to light and CO<sub>2</sub> (Table 4.4).

When light and CO<sub>2</sub> were increased, sucrose concentration also had significant effects on new shoot development in reactors. When sucrose was reduced from 30 g L<sup>-1</sup> (3S) to 10 g L<sup>-1</sup> (1S) in the medium, shoots grown in reactors had greater biomass and shooting percentage, but fewer shoot meristems (# ShAM) and leaves (# new leaves) (Table 4.4 and 4.5). There was also an increase in hyperhydricity from 14.8% to 32.2% (Table 4.4). Changes in shoot parameters seemed more pronounced in reactors than in gelled medium controls. The only significant difference for sucrose reduction in gelled medium was the increase in shoot proliferation (% new shoots) (Table 4.4). Shoot length was not affected by sucrose concentration either in reactors or gelled medium controls (Table 4.5).

Since a major difference between medium with 30 g L<sup>-1</sup> and 10 g L<sup>-1</sup> sucrose is osmotic potential, it was hypothesized that the increase in hyperhydricity at the low sucrose concentration (10 g L<sup>-1</sup>, 1S) was the result of altered osmotic potential and could be reversed by using mannitol to restore osmotic potential. The addition of mannitol to 10 g L<sup>-1</sup> sucrose medium significantly reduced hyperhydricity in the mist reactor to the same level as the comparable higher sucrose (30 g L<sup>-1</sup>, 3S) (Table 4.4). Although hyperhydricity of controls

with mannitol also decreased slightly, the change was not significant (Table 4.4). Biomass and emergence of new shoots (% new shoots), however, decreased significantly in both reactors and controls after addition of mannitol (Table 4.4), and explant type was particularly sensitive to mannitol (Table 4.6). Mannitol also seemed to affect other shoot parameters including proliferation of shoot meristems (# ShAM), new leaves (# new leaves) and shoot length in both reactors and controls (Table 4.5).

#### **4.3.1.3 Comparison between inoculum types on shoot proliferation**

Among all 4 types of inoculum explants, nodes (N) showed greatest shooting percentage (>80 %) regardless of culture conditions for both reactor and gelled controls (Table 4.6). Compared to nodal explants, those from leaves (L) and leaves with their petiole (LP) not only showed much lower % of new shoots under all conditions but also had different morphology from the axillary shoots emerging from nodal explants displaying compact clusters of adventitious micro-shoots (Figure 4.4).



**Figure 4.4** Morphology of an axillary shoot (**A**) and a cluster of adventitious micro-shoots (**B**) from *A. annua*. The unit of numbers on the ruler was cm in both A and B.

Light seemed to be the limiting factor for new shoot development from nodal and internodal explants (Table 4.6). The shooting percentage of leaf + petiole explants increased to the same level as that of internodal explants under the CO<sub>2</sub> and light enriched condition with 30 g L<sup>-1</sup> of sucrose (Table 4.6, 1V4C3I3S). When sucrose was reduced to 10 g L<sup>-1</sup> (1S), shoot regeneration from leaf explants increased (Table 4.6, 1V4C3I1S). Positing an osmotic effect, the addition of mannitol to the medium containing lower sucrose did not affect shoot regeneration from nodal, internodal or leaf + petiole explants, but significantly reduced shoot organogenesis of leaf explants (Table 4.6, 1V4C3I1S+MNT).

**Table 4.6** Shooting percentage of various inoculum explants during shoot production

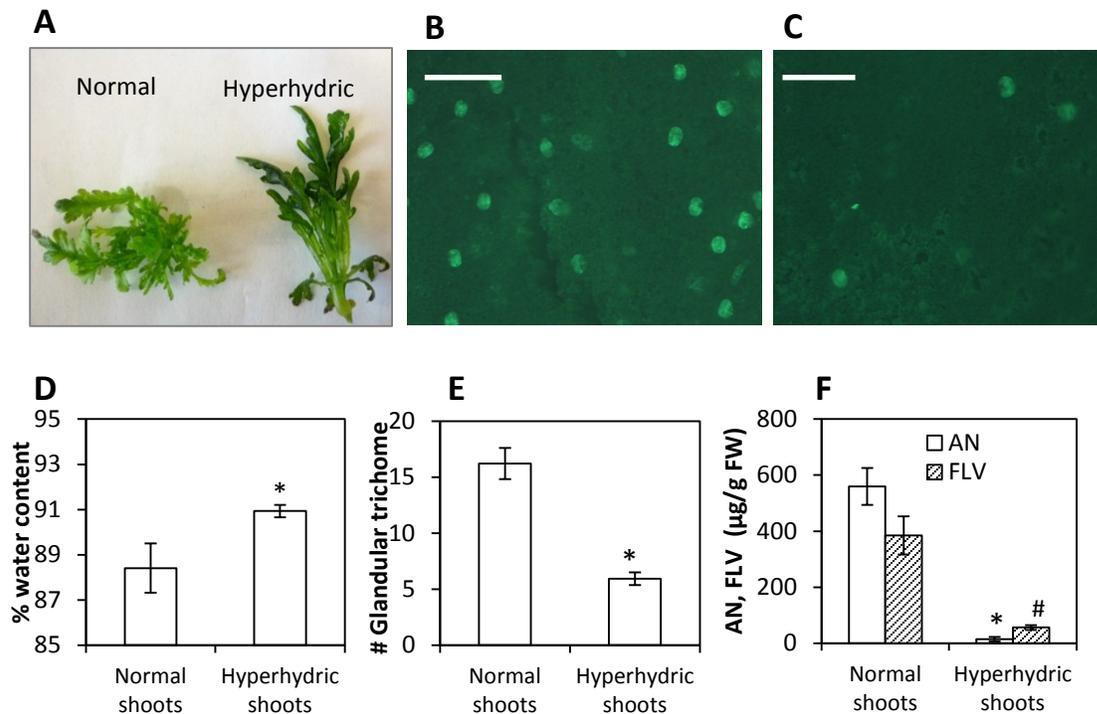
Condition	% new shoots in reactor				% new shoots in gelled medium			
	N	IN	L	LP	N	IN	L	LP
0V1C1I3S	81.7 U a	21.1 T a	1.8 R a	9.6* S a	86.4 T a	16.4 S a	0.9 R a	0.8 R a
1V1C1I3S	88.3 U a	21.7 T a	2.6 R a,b	8.5* S a	88.6 T a	20.6 S a	1.5 R a	1.9 R a
1V1C3I3S	90.6 U b	38.5* T b	4.2 R b,c	10.1 S a,b	88.6 T a	21.6 S a	2.2 R a,b	4.2 R a,b
1V4C1I3S	83.7 T a	29.4 S a,b	2.4 R a,b	13.8* S a,b	86.5 T a	17.7 S a	1.2 R a	1.8 R a
1V4C3I3S	92.6 T b	36.7* S b	9.9 R c	27.4 R,S b	89.1 T a	20.0 S a	5.1 R b	10.5 R b
1V4C3I1S	91.8 T b	38.9* S b	14.1 R c	16.7 R a,b	89.7 T a	26.8 S a	16.4 R c	20.3 R,S b
1V4C3I1S +MNT	94.5 T b	30.4 S a,b	2.2 R a,b	18.5 S a,b	86.8 T a	26.7 S a	3.5 R b	20.7 S b

Ventilation was 0 and 0.1 vvm for 0V and 1V, respectively. CO<sub>2</sub> concentration was 0.04% (v/v, ambient level) and 0.16% (v/v) for 1C and 4C, respectively. Light intensity was 15 and 50 μmol m<sup>-2</sup>•s<sup>-1</sup> for 1I and 3I, respectively. Sucrose level was 3S and 1S for 30 g L<sup>-1</sup> and 10 g L<sup>-1</sup>, respectively. Mist cycle was 0.5 min on/29.5 min off (2M) for light intensity, CO<sub>2</sub> level and sucrose concentration studies. MNT: mannitol (10.75 g L<sup>-1</sup>). N: node, IN: internode, L: leaves, LP: leaves with petiole. Upper case letters (R,S,T,U) compare among different inocula, lower case letters (a,b,c,) compare among different conditions, \* compares between reactor and control,  $p \leq 0.05$ .

#### 4.3.1.4 Comparison between hyperhydric and normal shoots

Compared to normal shoots, hyperhydric shoots appeared to be larger in size with elongated swollen leaves that were dark green, thick, glassy, and brittle (Figure 4.5A). Besides the

morphological differences, hyperhydric shoots also had more water, fewer glandular trichomes, and a reduced content of artemisinin and total flavonoids (Figure 4.5B-F).



**Figure 4.5** Comparison of normal and hyperhydric shoots grown in reactors that were air ventilated and with enhanced light (1V1C3I3S). **A**, picture of a typical normal shoot and a hyperhydric shoot; **B**, fluorescing glandular trichomes on normal leaf (adaxial side), bar:250  $\mu\text{m}$ ; **C**, fluorescing glandular trichome (bright spots) from hyperhydric leaf (adaxial side), bar:250  $\mu\text{m}$ ; **D**, water content of normal and hyperhydric shoots,  $N=4$ ,  $\pm$  SE,  $p \leq 0.05$ ; **E**, number of glandular trichomes on normal and hyperhydric leaves in a view of  $1 \text{ mm}^2$ ,  $N=6$ ,  $\pm$  SE,  $p \leq 0.05$ ; **F**, Content of artemisinin (AN) and total flavonoids (FLV) from normal and hyperhydric shoots; \* compares normal shoot AN vs. hyperhydric shoot AN; # compares FLV in normal shoots vs. hyperhydric shoots,  $N=4$ ,  $\pm$  SE,  $p \leq 0.05$

### 4.3.2 Rooting in mist bioreactor and gelled medium

#### 4.3.2.1 Comparison between reactors and gelled medium controls

Bioreactor-grown plantlets increased in shoot fresh weight more than controls (Table 4.7). The hyperhydricity of plantlets was about 10% or less in both reactor and controls except in the

unventilated (0 vvm) reactor which was about 16% (Table 4.7). When explants were misted once (1M) or twice (2M) per hour without addition of CO<sub>2</sub> or light, emergence of new roots and primary root growth in reactor cultures was less than controls (Table 4.7).

When extra light and CO<sub>2</sub> were present, the primary root development in the reactor appeared to be better than that in controls (Table 4.7). When the misting frequency in the reactor increased to four times per hour (4M), primary root initiation and growth increased and seemed better than controls (Figure 4.6A-C). Under this misting cycle, root branch growth in the reactor was also better than controls (Figure 4.6D, E). Regardless of condition, primary roots that developed in reactors were thicker than in controls (Table 4.7).

#### ***4.3.2.2 Comparison among conditions***

It appeared that shoot growth was not affected by various conditions during the rooting stage (Table 4.7). All ventilated reactors yielded less hyperhydricity than unventilated (0 vvm) and there was no difference in hyperhydricity among ventilated conditions (Table 4.7). Also, in both bioreactors and controls under the same ventilation and light conditions, hyperhydricity during rooting was less than during the shooting stage (Table 4.4 and 4.7).

**Table 4.7** Growth of roots and shoots during the rooting stage

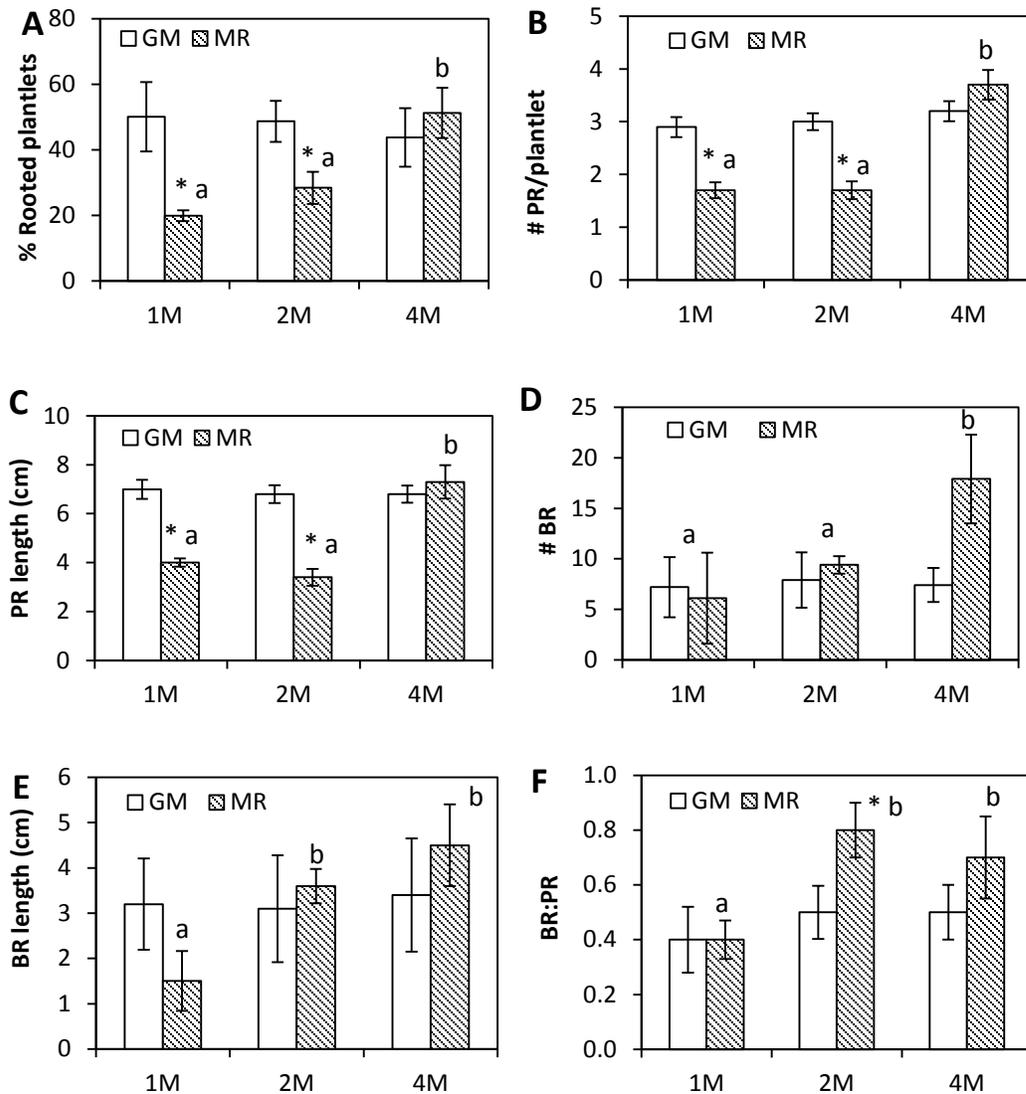
Condition	Reactor										Gelled medium controls									
	% SH FW	% HYP	% RT	Ø PR (µm)	# PR	PR (cm)	# BR	BR (cm)	BR: PR	% SH FW	% HYP	% RT	Ø PR (µm)	# PR	PR (cm)	# BR	BR (cm)	BR: PR		
0V1C1I	331.9 * a	16.3 * b	39.8 a,b	412.0 *a	1.6 a	2.4 a	3.1 a	0.8 a	0.3 a	105.1 a	8.1 b	53.9 a,b	348.0 a	3.3 *a	6.9 *a	5.0 a	1.9 *a	0.3a, b		
1V1C1I	279.2 * a	11.3 a	28.4 a	450.0 *a	1.7 a	3.4 a,b	9.4 b	3.6 b	0.8 * b	133.1 a,b	8.2 b	42.9 a,b	364.7 a	2.8 * a	6.6 *a	7.8 a	3.3 a	0.5b		
1V4C1I	264.4 * a	10.6 a	35.3 a,b	611.4 *b	2.4 b	4.7 b,c	22.1 * c	10.3 *d	1.8 * c	157.5 b	7.0 b	49.0 * b	385.7 a	3.1 a	7.5 *a	7.5 a	3.4 a	0.3 a		
1V4C3I	333.2 * a	7.1 a	43.5 b	656.4 *c	3.1 b	6.2 c	22.1 c	12.1 d	1.5 c	193.1 b	4.3 a	37.2 a	449.3 a	3.3 a	8.5 b	25. 8b	11.7 b	1.5c		

Ventilation (V) was 0 and 0.1 vvm for 0V and 1V, respectively. CO<sub>2</sub> concentration (C) was 0.04% (v/v, ambient level) and 0.16% (v/v) for 1C and 4C, respectively. Light intensity (I) was 15 and 50 µmol m<sup>-2</sup>·s<sup>-1</sup> for 1I and 3I, respectively. Mist cycle (M) was 0.5 min on/29.5 min off (2M). SH, shoot; PR, primary roots per plantlet; BR, root branches; BR:PR, branch length:primary root length per plantlet; HYP, hyperhydricity; Ø, diameter. Both numbers and length of root and branches were measured for each rooted plantlet. Letters compare among all conditions, \* compares between reactor and gelled medium control,  $p \leq 0.05$ .

When forced ventilation was introduced into the reactor, not only did hyperhydricity decrease, but the number and length of root branches also increased (Table 4.7). CO<sub>2</sub> enrichment significantly increased all parameters on root development in the reactor but not in the control (Table 4.7). When both CO<sub>2</sub> and light were increased, there was significant increase in root development and root branching in both reactor and gelled medium compared to low light and CO<sub>2</sub> conditions (Table 4.7).

In contrast to its effects on shoots, increasing the misting cycle significantly increased the number of primary roots, primary root length, number of root branches and branch length, and the ratio of branch length to primary root length (Figure 4.6). Misting cycle had no effect on primary root thickness (Appendix S3).

Compared to primary roots, root branching seemed more sensitive and responsive to environmental changes. Both number and length of branches increased more than that of the primary root after added ventilation, increased CO<sub>2</sub>, and increased misting frequency (Table 4.7). Greater branch growth was reflected in the increase in the branch: primary root ratio (Table 4.7, BR:PR). The total length of branches exceeded that of the primary roots with CO<sub>2</sub> and light enriched (4C3L) conditions in both reactor and gelled medium (Table 4.7).



**Figure 4.6** The effect of misting cycle on root development. GM, gelled medium; MR, mist reactor; PR, primary roots; BR, branches; BR: PR, branch length: primary root length per plantlet. Three misting cycles: 1 misting h<sup>-1</sup> (1M, 0.75 min on/59.25 min off), 2 mistings h<sup>-1</sup> (2M, 0.5 min on/29.5 min off), 4 mistings h<sup>-1</sup> (4M, 0.25 min on/14.75 min off). **A**, rooting percentage; **B**, number of primary roots per plantlet; **C**, length of all primary roots per plantlet; **D**, number of branches per primary root; **E**, length of all branches per plantlet; **F**, ratio of all branches over all primary roots. Letters a and b compares between three misting cycles of reactor; \* compares between gelled medium and mist reactor,  $p \leq 0.05$ . Other conditions for both reactor and gelled medium controls were: 0.1 vvm (1V), ambient air (1C), at 15  $\mu\text{mol m}^{-2}\cdot\text{s}^{-1}$  (1I), 20 g L<sup>-1</sup> sucrose (2S).

### **4.3.3 Acclimatization in mist bioreactor and gelled medium**

During the acclimatization stage, the major goal is to stimulate development of functional stomata and well developed epicuticular wax so leaves do not lose water when transplanted to soil. Stomatal function is tightly regulated by ambient gases and water status, so ventilation, relative humidity (RH) and misting cycle were studied here for their effects on stomatal development and function.

Immature stomata are unable to close in response to changes like low RH. When plantlets were ventilated, stomatal closure improved especially for cultures on gelled medium (Table 4.8, Appendix S4). When RH was reduced, stomatal closure improved on both sides of the leaf for all plantlets in both reactors and gelled medium cultures and especially for the adaxial side of leaves in the reactor (Table 4.8). There was no significant difference in stomatal closure between a stepped or non-stepped reduction in hourly nutrient volume feed, suggesting misting cycle did not affect stomatal development (Table 4.8). *Ex vitro* survival was 100% for all conditions unless cultures were not ventilated (Table 4.8, Appendix S5).

### **4.3.4 Stem fragility**

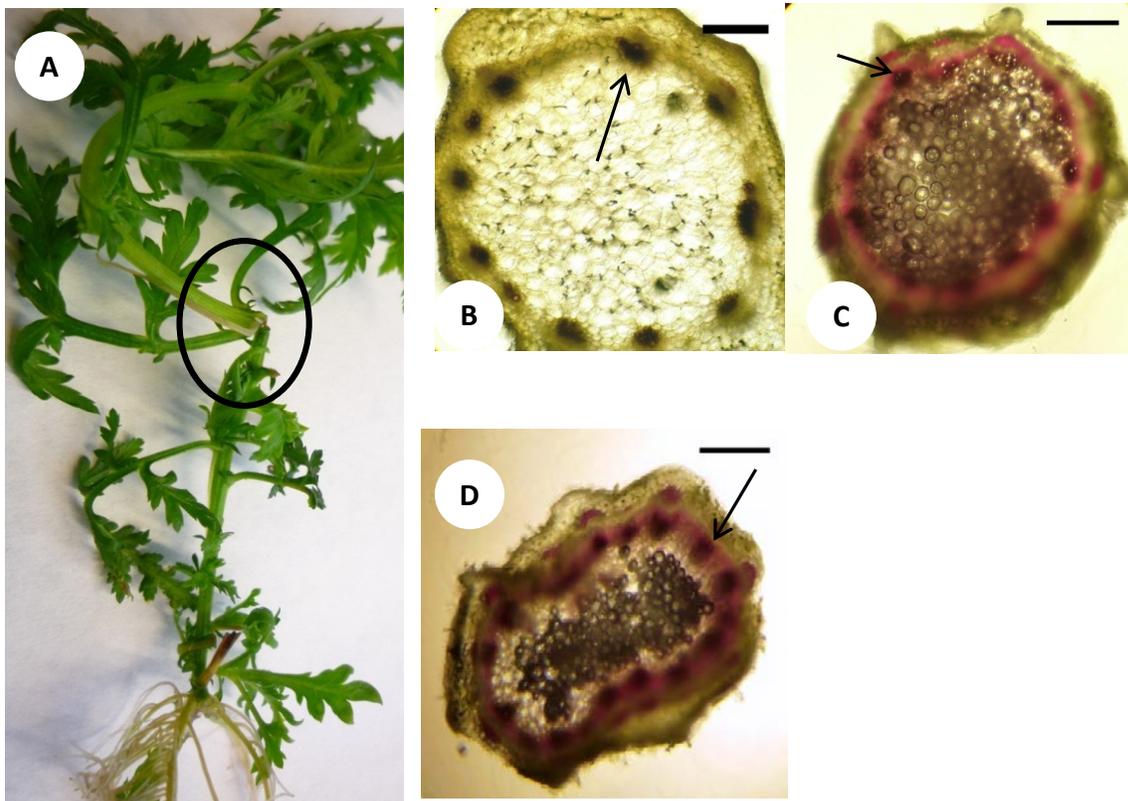
A high percentage (>90%) of plantlets that were grown in unventilated (0 vvm) reactors had fragile or broken stems (Figure 4.7A). This fragile stem response was not seen in plantlets grown in other reactor conditions. Since lignin is the main component of secondary walls of plant cells that provides the mechanical and structural strength for stems, cross sections of

**Table 4.8** Growth, stomatal function and *ex vitro* survival of *A. annua* under different acclimatization conditions

Condition	Reactor				Gelled medium			
	FW gain per plantlet	% Closed stomata		<i>Ex vitro</i> survival (%)	FW gain per plantlet	% Closed stomata		<i>Ex vitro</i> survival (%)
		Adaxial	Abaxial			Adaxial	Abaxial	
0 VVM	0.22 * a	87.1 * # a	71.5 * a	90 * a	0.13 a	7.9 a	5.6 a	84 a
100 RH	0.29 a	94.9 * # a,b	80.3 a	100 a	0.21 b	72.2 b	71.8 b	100 a
52 RH	0.25 a	98.7 * b	95.8 * b	100 a	0.23 b	94.5 c	89.8 c	100 a
52/11 RH	0.22 a	97.8 * b	94.2 b	100 a	0.20 b	93.4 c	90.1c	100 a
Stepped down feed	0.22 a	93.7 * # a,b	78.2 a	100 a	0.21 b	74.8 b	73.1 b	100 a
Stepped cycle	0.27 a	99.1 * b	94.8 b	100 a	0.20 b	92.8 c	90.8 c	100 a

Ventilation was 0.1 vvm for all conditions except with 0 vvm, whereby there was no forced ventilation. Relative humidity (RH) was >96%, 100% and 52% for 0 vvm, 100RH and 52RH, respectively. The RH condition was maintained at 52% for the first 5 days and then at 11% for the remaining 5 days for 52/11RH. The misting cycle for 0 vvm, 100RH, 52RH and 52/11RH was 0.5 min on/29.5 min off. In the stepped down feed culture, the RH was maintained at 100%, and plantlets were misted twice every hour with 30 mL h<sup>-1</sup> medium delivery for day 0-1, once every hour with 22.5 mL h<sup>-1</sup> medium delivery for day 2-4, once every two hours with 11.3 mL h<sup>-1</sup> medium delivery for day 5-7 and once every four hours with 5.6 mL h<sup>-1</sup> medium delivery for day 8-10, respectively. In stepped cycle culture, the RH was 100% for the first 5 days and then 11% for the remaining 5 days. The misting cycle regimen in stepped cycle was 0.5 min on/29.5 min off for day 0-1, 1 min on/59 min off for day 2-4, 2 min on/118 min off for day 5-7, and 4 min on/236 min off for day 8-10, respectively. The hourly volumetric medium delivery for these cycles was maintained at 30 mL h<sup>-1</sup>. Light intensity for all conditions was 11. Letters compare all conditions; \* compares between reactor and gelled medium controls; # compares % closed stomata between adaxial and abaxial side of leaf for the same condition; ; p ≤ 0.05.

stems from unventilated reactors, normal stems from reactors as well as from gelled medium controls were stained with phloroglucinol-HCl to assess lignin deposition.



**Figure 4.7** Fragile stems from unventilated (0 vvm) bioreactors and lignin stained with phloroglucinol-HCl. **A**, fragile stem with roots developed from the broken site (circle) from a 0 vvm bioreactor; **B**, lignin stain of free-hand cross section of stems from 0 vvm bioreactor, arrow indicates vascular bundles; **C**, lignin stain of free-hand cross section of stems from 52RH bioreactor, arrow indicates vascular bundles; **D**, lignin stain of free-hand cross section of stems from 0 vvm gelled medium, arrow indicates vascular bundles; B,C, and D, bar: 250  $\mu\text{m}$ .

Red stained tissues indicated lignin, typically around the vascular bundles arranged in a circle near the periphery of a dicot stem (arrows in Figure 4.7). Stems from the unventilated reactor lacked any apparent stain (Figure 4.7B) in the peripheral ring otherwise outside of the stained xylem (Figure 4.7C). In addition, the fragile stems appeared to have larger cortical cells with

less stain in their walls than in normal stems (Figure 4.7B-D). Together these results suggested stems from plantlets grown in unventilated reactors were poorly lignified.

## **4.4 Discussion**

### **4.4.1 Comparison between mist bioreactor and gelled medium**

Bioreactors are proposed to reduce labor cost and promote biomass yield in plant micropropagation (Adelberg and Fári 2010; Takayama and Akita 2006). However, liquid medium reactor cultures have a high rate of hyperhydricity in shoots (Ziv 1995b). To avoid continuous submersion in reactors, temporary immersion systems (TIS) were developed for periodic nutrient immersion and headspace gas exchange. Application of TIS for micropropagation has been reported for many species with better yields and plantlet quality compared to gelled medium (Berthouly and Etienne 2005; Hahn and Paek 2005; Watt 2012; Welander, *et al.* 2014; Yan, *et al.* 2010; Yan, *et al.* 2013). Compared to gelled medium controls, the gas-phase mist reactor also showed better biomass yield, shoot proliferation, and stomatal function in this study. Better shoot proliferation rate in reactor cultures has generally been ascribed to increased availability of cytokinins and other nutrients (Debergh 1983).

Cytokinins, however, were also identified to induce hyperhydricity in both gelled and liquid medium (Debnath 2011; Tsay, *et al.* 2006; Ziv 1991a). This stimulating effect was concentration-dependent with hyperhydricity increasing with increasing cytokinin concentration (Ivanova and van Staden 2008; Ivanova and Van Staden 2011). In correlation

with these reports, there was a significant reduction in hyperhydricity when explants during the rooting stage were instead cultured in hormone-free medium under the same reactor conditions as those in the shooting stage. In addition, shoots in reactor cultures were more sensitive to cytokinin than in gelled medium and became more hyperhydric in this study. Similar results were also observed with *Aloe polyphylla* in stagnant liquid culture (Ivanova and Van Staden 2011) and berry crops in TIS culture (Debnath 2011).

Rooting percentage measured the success of root induction. Similar to the results in this study, root initiation in plantain (*Musa AAB*) in a TIS was not superior to that in gelled medium (Roels *et al.* 2005). On the other hand, root induction of rose in a hydroponic culture vessel was better than in gelled medium, showing greater rooting percentage, root length and root numbers (Pati *et al.* 2005). Although this suggested that root induction may require continuous misting, an earlier hairy root study showed that hairy roots of *A. annua* barely grew in continuous mist (Towler *et al.* 2007). Nevertheless, root branching in the mist reactor with frequent misting (4M) was greater than in gelled medium.

#### **4.4.2 Demand on misting cycle was diverse during different propagation stages**

An appropriate nutrient feeding cycle is a major factor to ensure both growth and quality of explants in reactors. In the mist reactor, shoot proliferation was also better with less frequent misting. A number of studies of different species in TIS also recommended a short and sparse feeding frequency of 2-3 times a day with several minutes of immersion each time (Georgiev,

*et al.* 2014; Valdez-Tapia, *et al.* 2014; Watt 2012). Short and sparse feeding cycles not only maintained good shoot proliferation but also reduced hyperhydricity by providing a growth condition with less water stress (Valdez-Tapia, *et al.* 2014; Zhao, *et al.* 2012). Residual nutrients remaining on tissue surfaces after immersion were probably sufficient for shoot proliferation, and thus, reduced immersion frequency helped to decrease liquid availability of plant tissue in TIS without limiting nutrient availability or reducing relative humidity (Scherer, *et al.* 2013). Similarly, residual nutrients after each misting may also be adequate for shoots to grow well in a less frequent cycle.

On the other hand, root development increased with more mistings per hour. Similar results were also observed with *Dianthus* (carnation) (Correll, *et al.* 2001) and *Pseudostillaria* (elephant ear) (Wang and Qi 2010). Compared to periodic feeding, root initiation also improved with continuous nutrient feeding at the root zone using a liquid-based medium (Afreen, *et al.* 2005; Jo, *et al.* 2008; Pati, *et al.* 2005; Wang, *et al.* 2013). High demand for nutrients during rooting was probably because cell division of the root meristem is carbohydrate-dependent (Van't Hof 1985). Root growth also needs O<sub>2</sub> for production of ATP to provide energy during cell division (Lambers *et al.* 1996). Since the gas phase is continuous within the mist reactor, O<sub>2</sub> transport to roots was not limited as in a liquid-based reactor (Curtis and Tuerk 2006).

Misting frequency did not affect hyperhydricity during the rooting stage. This was probably because rooting medium contained no phytohormones. Misting cycle also did not influence stomatal function or *ex vitro* survival of *A. annua*, suggesting this plant seems relatively resistant to hyperhydricity and easy to acclimatize. Carnation, on the other hand, is a hyperhydric sensitive species and in earlier mist reactor studies reduced misting frequency alleviated hyperhydricity during rooting and acclimatization (Correll, *et al.* 2001; Correll and Weathers 2001a). Thus repeating this study perhaps with a more challenging species may provide more information on the impact of reactor conditions on acclimatization.

#### **4.4.3 Forced ventilation is necessary in mist reactor**

Increasing ventilation benefits growth by increasing CO<sub>2</sub> level and reducing RH as well as potentially toxic volatiles (i.e. C<sub>2</sub>H<sub>4</sub>) in the headspace of a culture container (Thongbai, *et al.* 2010; Xiao, *et al.* 2003; Zobayed, *et al.* 2001a). As a result of these environmental changes, plantlets *in vitro* should have less hyperhydricity, better photosynthesis, better root development, improved stomatal function and less water loss during *in vitro* acclimatization (Chen *et al.* 2006a; Chen, *et al.* 2006b; Ivanova and Van Staden 2010; Majada, *et al.* 2001; Mills 2009; Tsay, *et al.* 2006; Zhao, *et al.* 2012).

In small culture containers, improved *in vitro* growth can be sustained by using porous closures or gas permeable membranes on the closure (Chen, *et al.* 2006b; Fujiwara and Kozai 1995; Mohamed and Alsadon 2010; Tsay, *et al.* 2006). By using these strategies, the gas exchange rate can be elevated from 0.04 times h<sup>-1</sup> (0.00066 vvm) under non-ventilated

conditions to around 5 times  $\text{h}^{-1}$  (0.083 vvm) (Chen and Chen 2002; Cui, *et al.* 2000; Thongbai, *et al.* 2010). For reactors with several L of volume, however, these strategies are not sufficient to maintain an appropriate gas exchange rate and gas composition for *in vitro* growth (Xiao, *et al.* 2003). Indeed in a TIS, intermittent gas exchange between immersion cycles was not sufficient to control hyperhydricity, and continuous forced ventilation had to be implemented (Zhao, *et al.* 2012). In this study, ventilation was essential to three major *in vitro* stages, shooting, rooting and acclimatization, and 0.1 vvm (1V) was required to achieve an air flow of  $600 \text{ mL min}^{-1}$ .

While excess accumulation of  $\text{C}_2\text{H}_4$  in the headspace from inadequate ventilation can be toxic to cultures (Jin Goh, *et al.* 1997; Kevers, *et al.* 1992; Neto, *et al.* 2009; Reis, *et al.* 2003), too little  $\text{C}_2\text{H}_4$  as a result of excessive ventilation can also inhibit shoot development (Chatfield and Raizada 2008; Kevers, *et al.* 1992; Kumar *et al.* 1987; Majada, *et al.* 1997; Mills, *et al.* 2004; Nour and Thorpe 1994; Tsay, *et al.* 2006; Zhao, *et al.* 2012). Moreover, increasing passive ventilation in gelled medium through use of membrane filters can lead to medium desiccation and growth therefore may become limited due to alteration of osmotic potential (Shim, *et al.* 2003; Yann, *et al.* 2010). Indeed, Majada *et al.* (1997) showed that shoot proliferation in liquid medium was better than in gelled medium even though both cultures were ventilated. An optimum ventilation rate for shoot proliferation also varies with species and cultivar, and thus has to be studied case by case (Majada, *et al.* 1997; Mills, *et al.* 2004; Zhao, *et al.* 2012). In this study, neither shooting percentage nor shoot proliferation rate was

reduced after ventilation with ambient air, suggesting the gas exchange rate was appropriate for *A. annua*.

#### **4.4.4 Added CO<sub>2</sub> and light stimulated shoot and root growth**

CO<sub>2</sub> and light are critical for photosynthesis and thus *in vitro* growth of plantlets. Growth of *A. annua* shoots and roots was stimulated with added CO<sub>2</sub> and light, and similar results were also reported in previous studies on a variety of plants (Emam and Esfahan 2014; Fan *et al.* 2013; Saldanha *et al.* 2013; Suthar *et al.* 2009; Vyas and Purohit 2003; Vyas and Purohit 2006).

Increased shoot multiplication was probably due to elevated photosynthesis under added CO<sub>2</sub> and light (Kozai, *et al.* 1990; Mosaleeyanon, *et al.* 2004; Thongbai, *et al.* 2011). At the lower light intensity (1I, 15  $\mu\text{mol m}^{-2}\cdot\text{s}^{-1}$ ) used in this study, increasing CO<sub>2</sub> alone was not sufficient to stimulate photosynthesis and shoot proliferation until the light intensity was elevated to 50  $\mu\text{mol m}^{-2}\cdot\text{s}^{-1}$  (3I).

Although light *per se* cannot stimulate root growth (Correll and Weathers 2001b; Jo, *et al.* 2008; Mills 2009), increased photosynthesis under added CO<sub>2</sub> and light may result in better root growth. Increased root growth may also occur by CO<sub>2</sub> fixation via phosphoenolpyruvate carboxylase in roots especially under low light intensity (Bihzad and El-Shora 1996; Jeong, *et al.* 2006; Shin *et al.* 2013). CO<sub>2</sub> enrichment in this study also increased root thickness and similar results were observed in *Macadamia tetraphylla* (Cha-um, *et al.* 2011), *Cistus incanus* (Mills 2009) and *Pfaffia glomerata* (Spreng.) (Saldanha, *et al.* 2014). The positive effect of

CO<sub>2</sub> on root development may help explain the slight increase in rooting in unventilated reactors compared to ventilated reactors both at low light intensity because CO<sub>2</sub> level under unventilated conditions may well have accumulated in the headspace. Interestingly, the stimulating effect of CO<sub>2</sub> on root growth was only observed in plantlets grown in the mist reactor but not in the gelled controls. This was possibly due to restricted solubility of CO<sub>2</sub> into the root zone submerged in gelled medium. Indeed root development has been shown to improve after switching plants from gelled to porous medium (Cha-um, *et al.* 2011; Saldanha, *et al.* 2014). Limited CO<sub>2</sub> solubility in gelled medium may also explain reduced root thickness of plants grown therein compared to those grown in the mist reactor.

#### **4.4.5 Low sucrose reduced shoot multiplication and increased hyperhydricity**

The 3I light intensity of 50  $\mu\text{mol m}^{-2}\cdot\text{s}^{-1}$  in this study falls into a common effective range for photomixtrophic propagation (Fujiwara and Kozai 1995). It was known that plants *in vitro* can shift from photomixtrophic to photoautotrophic growth when grown under gradually decreased exogenous sugar together with an increase in the headspace CO<sub>2</sub> (Kozai 1991; Kozai 2010; Van Huylenbroeck and Debergh 1996). So the growth of *A. annua* shoots in the mist reactor was tested under low sucrose concentration with added CO<sub>2</sub>.

Although low sucrose increased emergence of new shoots (% new shoots) overall and those from leaf explants, there was higher hyperhydricity rate in the mist reactor than the 3S condition (30 g L<sup>-1</sup>). Both results were probably from alteration in osmotic potential (Debergh

1983). When the osmotic potential was restored by mannitol, the emergence of new shoots overall decreased to the same level as for plants in 30 g L<sup>-1</sup> sucrose; hyperhydricity was also reduced. Reduction in hyperhydricity with alteration in osmotic potential was also observed in other species (Kadota, *et al.* 2001; Mills 2009; Yadav, *et al.* 2003).

Low sucrose also reduced the number of new shoots grown in the mist reactor. The sub-optimal shoot multiplication under low sucrose was probably from insufficient nutrients from the medium and photosynthesis, suggesting the shoots needed more nutrients either from more exogenous sucrose or a higher rate of photosynthesis. On the other hand, shoot multiplication in gelled medium controls was not reduced in low sucrose. It is possible the low sucrose concentration was adequate for shoots in gelled medium but not sufficient for shoots in the mist reactor. Shoots in the mist reactor consumed more sucrose to develop greater number of new shoots and biomass than in the gelled medium controls. Indeed, elimination of sucrose in the medium around the light intensity of 50  $\mu\text{mol m}^{-2}\cdot\text{s}^{-1}$  resulted in decreased shoot multiplication in a variety of plants including *Wrightia tomentosa* (Vyas and Purohit 2003), *Feronia limonia* (L.) Swingle (Vyas and Purohit 2006), *Terminalia bellerica* Roxb (Suthar, *et al.* 2009) and *Chlorophytum borivillianum* Sant. et Fernand (Joshi *et al.* 2009).

#### **4.4.6 Physio-morphological abnormalities of *A. annua* during *in vitro* culture**

The major physio-morphological abnormality observed in this study was hyperhydricity. Explants of *A. annua* with hyperhydric signs displayed the same characteristics observed in

other plant species (Dewir, *et al.* 2006b; Ziv 1991c). The onset of hyperhydricity is known to be triggered by excessive water accumulation in the apoplast (Gribble, *et al.* 1998; Kevers and Gaspar 1986; Rojas-Martinez *et al.* 2010). Water lodging then leads to hypoxia and oxidative stress (Rojas-Martinez, *et al.* 2010; Saher, *et al.* 2005c; van den Dries, *et al.* 2013), which induces abnormal physiological processes and ultimately hyperhydricity (Dewir, *et al.* 2006b; Fernandez-García, *et al.* 2008; Saher, *et al.* 2004; Sreedhar, *et al.* 2009; Wu, *et al.* 2009). Fragile stems observed in the unventilated reactors are also considered a sign of mild hyperhydricity. Stems from unventilated conditions have been shown to have excess water accumulation, characteristics of reduced lignification and mechanical support in the vascular tissue (Donnelly *et al.* 1985; Iarema *et al.* 2012; Jausoro *et al.* 2010; Majada, *et al.* 2000). As observed in this study and other reports, ventilation, water availability, CO<sub>2</sub> and light conditions are important environmental factors that should be controlled during *in vitro* culture to minimize hyperhydricity (Casanova, *et al.* 2008; Ivanova and Van Staden 2010; Jausoro, *et al.* 2010; Majada, *et al.* 1997; Valdez-Tapia, *et al.* 2014; Zobayed, *et al.* 2000).

Besides abnormal morphology, hyperhydric *A. annua* shoots also contained fewer glandular trichomes and less artemisinin and flavonoids than normal shoots. Artemisinin, an important antimalarial drug, is synthesized and stored in the glandular trichomes of *A. annua* (Duke *et al.* 1994). A decrease in trichome number is associated with reduced artemisinin content (Kapoor *et al.* 2007; Lommen *et al.* 2006; Nguyen *et al.* 2011). In this study, artemisinin decreased by 97%, while trichome numbers decreased 62.5%. Previously it was reported that

increased ventilation led to elevated trichome numbers in *A. annua* (Yann 2010) and *Wigandia urens* (Pérez-Estrada et al. 2000), suggesting the biosynthesis of secondary metabolites may be affected by culture conditions. Similar to the results in this study, flavonoid biosynthesis was also reduced in hyperhydric shoots of *Scutellaria* spp. (Tascan, et al. 2010). These findings suggested that trichomes as well as the biosynthesis of secondary metabolites were sensitive to environmental change, and maintaining an *in vitro* environment comparable to *ex vitro* conditions would benefit both the anatomy and secondary metabolite production of this species.

## 4.5 Conclusion

The mist reactor was used to measure how culture environment affected shoot proliferation, rooting and acclimatization of *A. annua* with the aim towards using the mist reactor to simplify the labor intensive steps of micropropagation. Maintenance of an appropriate condition for each stage was important to *in vitro* propagation and secondary metabolite production. All of these different culture stages required adequate ventilation, CO<sub>2</sub> enrichment (0.16%) and light ( $50 \mu\text{mol m}^{-2}\cdot\text{s}^{-1}$ ) for high quality growth. Aside from these common requirements, each stage had different demands on nutrient delivery and misting cycle. The best mist frequency for shoot proliferation and root development was once per hour (1M) and four times per hour (4M), respectively. While misting cycles during acclimatization did not affect stomatal function and *ex vitro* survival of *A. annua*, these parameters appeared better when ventilating with air at 52% RH. Plantlets grown under these conditions in the reactor had the least

hyperhydricity and produced more biomass yield than those in gelled medium controls. This study has shown the potential of the mist reactor to combine otherwise discrete steps in micropropagation into one step.

# Chapter 5      Attachment of leaf tissue to poly-L-lysine (PLL) coated substrates for use in micropropagation

## 5.1 Introduction

Using plant cell, tissue and organ culture techniques, billions of commercially important plants are clonally produced annually through micropropagation (Singh and Shetty 2011). The traditional micropropagation process, which is based on non-scalable gelled medium, is labor intensive, requiring manual handling of a large number of single containers (Takayama and Akita 2006). To reduce labor, bioreactors are being developed to provide control of the *in vitro* microenvironment to secure the growth and physiological integrity of the plantlets (Steingroewer *et al.* 2013).

Existing bioreactors for micropropagation can be classified into liquid-phase bioreactors (e.g. airlift and balloon type bubble bioreactors), temporary immersion systems (TIS; e.g. RITA<sup>®</sup> and Twin flask) and gas-phase bioreactors (e.g. nutrient mist bioreactor) (Steingroewer, *et al.* 2013; Weathers, *et al.* 2010). In liquid-phase bioreactors, plant materials remain suspended in the culture medium. Although liquid suspension is ideal for culturing protocorm-like bodies and storage organs, shoots do not grow as well often developing physio- morphological abnormalities (i.e. hyperhydricity) caused by low oxygen and osmotic potential of the liquid medium (Afreen 2006; Dewir *et al.* 2014; Georgiev, *et al.* 2014; Yang, *et al.* 2010). TIS, on

the other hand, provide flexible nutrient feeding regimes with periodically altered gaseous growth environment. Thus, TISs have been widely used for micropropagation of economically important species (Berthouly and Etienne 2005; González 2005; Watt 2012).

Traditionally, plant materials in TISs are horizontally placed on a supportive raft or net, creating a large footprint for each bioreactor. To save costly floor space, multi-tiered shelves have to be used in commercial production (Adelberg 2006; Adelberg and Fári 2010; Wilken, *et al.* 2014). In mist reactors, plant materials have thus far been horizontally placed to receive nutrient mist fed top down, which limits yield per footprint area. To maximize batch yield and also minimize footprint, a vertical “hanging garden” style of culture could prove useful as suggested by Fei and Weathers (2014). This method requires rapid and predictable attachment of plant materials onto vertically hanging substrates within a bioreactor.

Surface attachment of plant materials has been primarily studied using plant cell cultures for production of secondary metabolites, bioconversion and biomanufacturing (Huang and McDonald 2012; Ruffoni *et al.* 2010; Tyler *et al.* 1995). Adhesion of plant cells to a surface is the result of interfacial tensions and electrostatics (Dicosmo *et al.* 1989), and is affected by hydrophobicity of substrates as well as pH and ionic strength of the liquid medium (Facchini *et al.* 1988b; Facchini, *et al.* 1989). Since plant cells have a net negative surface charge (Dicosmo, *et al.* 1989; Facchini *et al.* 1988a) and many substrates used for immobilization are also negatively charged, poly-cation coating of substrates has the potential for reducing repulsion and enhancing attachment between plant tissues and substrates. Using poly-L-lysine

(PLL) coated polypropylene and nylon as substrates, Fei and Weathers (2014) showed that pre-embryogenic carrot cells attached to vertically hung substrate strips and developed *in situ* into fully rooted embryos in a mist bioreactor. That study suggested a similar approach may work for culturing differentiated tissues (shoots and roots) via micropropagation. Although root tissues were shown to adhere to PLL-coated substrates via root hairs (Towler and Weathers 2003), it is unclear if shoot tissues can similarly bind. Using *A. annua* as a test species, attachment ability of leaf tissues to PLL-coated substrates was investigated along with the potential of using blenderized shoot tissues as inoculum for micropropagation. Also investigated was the feasibility of one step micropropagation that included shooting, rooting and acclimatization with tissue attaching to PLL-coated substrates hanging inside the mist reactor.

## **5.2 Materials and methods**

### **5.2.1 Plant materials for attachment experiments**

Rooted *Artemisia annua* L. (clone SAM) shoots were grown in Magenta boxes containing 50 mL semi-solid hormone-free rooting medium (RTM): 20 g L<sup>-1</sup> sucrose, 2.22 g L<sup>-1</sup> Murashige & Skoog (MS) salts with vitamins, and 5 g L<sup>-1</sup> Agargellan<sup>TM</sup>, pH 5.8 (Nguyen, *et al.* 2013). All cultures were grown at 25 °C and continuous irradiation at 50 μmol m<sup>-2</sup>·s<sup>-1</sup> with cool white fluorescent bulbs (GE brand, F15T8-CW) unless otherwise specified. Subculture was every 4 weeks by nodal cuttings. Leaf tissues of the 4-week-old cultures were used in attachment experiments.

## **5.2.2 Mist reactor**

Mist reactor preparation and set up was already detailed in Fei and Weathers (2014). The reactor briefly had a 7L disposable bag fitted with silicone tubing wrapped around the perimeter of a stainless steel mesh plate to 3D shape. The disposable bag also fitted around a Nalgene bottle neck to be screw capped with an Osmotek<sup>TM</sup> mister head. Tubing linked an air pump via a Nafion tube to the reactor chamber and is shown in Figure 3.1a. Separate tubing connected the culture medium reservoir to the mister head and another was between the culture bag and the reservoir.

## **5.2.3 Preparation of substrates and PLL coating**

Pieces of polypropylene (PP) sheet and nylon mesh (70  $\mu\text{m}$ ; N70) were cut into 2×4 cm strips for binding experiments. PLL coating was done by soaking the PP sheet and N70 mesh in 0.01 % (w/v) PLL (Sigma, P1274) solution for 0.5 h and then air dried (Fei and Weathers 2014; Towler and Weathers 2003). Binding experiments were done under non-sterile conditions for 1-24 h. Initially bound tissues within 12 h were considered “retained” since it was uncertain if they would remain bound for longer time. Initially retained tissues were considered truly “attached” after they remained on substrates for at least 24 h.

## **5.2.4 Retention of chopped leaf tissue by manual application**

Leaf tissue of 4-week-old *in vitro* cultivated *A. annua* (section 5.2.1) was randomly chopped into pieces about 5 mm length and then incubated  $\pm$  PLL onto PP sheeting or N70 mesh in 30 g L<sup>-1</sup> sucrose to maintain osmotic potential and avoid tissue desiccation. After incubation for 1-6 h, the substrates were held vertically and washed 10 times from top to bottom each time with 0.3 mL dH<sub>2</sub>O. Percent tissues retained on substrates were determined as:

$$\% \textit{retention} = \frac{\textit{post wash \# leaf pieces} \times 100}{\textit{initial \# applied leaf pieces}} \quad \text{Eq (5.1)}$$

### **5.2.5 Quick dip and retention of manually chopped vs. blenderized leaf tissue**

Small pieces of leaf tissue were produced either from manually chopped leaves as described in section 5.2.4 or blenderized by using a 4-blade blender (Hamilton Beach Commercial). The blender was filled with about 4 g of fresh leaf tissues and 200 mL of 30 g L<sup>-1</sup> sucrose, and tissues blended for 30, one-second pulses, under low input power. The small pieces of tissue from manually chopped or blenderized leaves were then suspended in 30 g L<sup>-1</sup> sucrose at various concentrations (25, 33, 50, 100 g L<sup>-1</sup>) in Magenta boxes; each suspension was incubated with PP sheet and N70 mesh  $\pm$  PLL for 5-10 sec (quick dip). Then substrates were moved out of the tissue suspension, blotted dry, and measured for fresh weight (FW) of retained tissues.

### **5.2.6 Attachment of manually chopped vs. blenderized leaf tissue after quick dip**

Initially retained tissues (section 5.2.5) were hung inside the mist bioreactor and sprayed with hormone-free rooting medium (RTM, 20 g L<sup>-1</sup> sucrose and 2.22 g L<sup>-1</sup> MS salts with vitamins,

pH 5.8) for 24 h at a misting cycle of 0.3 min on/2.7 min off (300 mL h<sup>-1</sup>). The % attachment was measured and defined as:

$$\% \textit{ attachment} = \frac{\textit{post misting FW of leaf pieces} \times 100}{\textit{FW of initially retained leaf pieces}} \quad \text{Eq (5.2)}$$

### 5.2.7 Quick dip and attachment of various sizes of blenderized leaf tissue

Blenderized leaf tissue produced as described in section 5.2.5 was successively sieved through stainless steel screens of 1, 0.5, 0.35 and 0.15 mm to obtain four groups of tissues: 0.15-0.35 mm, 0.35-0.5 mm, 0.5-1 mm and >1 mm. At a biomass concentration of 1g FW in 20 mL 30 g L<sup>-1</sup> sucrose, each size group was measured for instant retention on ± PLL N70 following the quick dip method (section 5.2.5). Initially retained tissues were then misted with rooting medium as described in section 5.2.6 for 24 h, and % attachment was measured (Eq 5.2).

### 5.2.8 Shoot regeneration of blenderized leaf tissue

Shoot tissues of different sizes generated from 30 pulses of blending (section 5.2.5) were cultured for 6 weeks on gelled shooting medium (SHM): 30 g L<sup>-1</sup> sucrose, 4.43 g L<sup>-1</sup> MS salts with vitamins, 0.25 μmol L<sup>-1</sup> α-naphthaleneacetic acid (NAA), 2.5 μmol L<sup>-1</sup> N-6-benzyladenine (BA), and 5 g L<sup>-1</sup> Agargellan<sup>TM</sup>, pH 5.8 (Nguyen, *et al.* 2013). All cultures were grown under 25 °C and continuous light at 50 μmol m<sup>-2</sup> s<sup>-1</sup>. The shooting percentage of leaf tissues in each size group was then measured as follows:

$$\% \textit{ new shoots} = \frac{\# \textit{ inoculum pieces with new shoots} \times 100}{\# \textit{ inoculated leaf pieces}} \quad \text{Eq (5.3)}$$

Each of the four sizes of tissue was loaded in 3 wells in a 12-well plate with four replicate plates.

### **5.2.9 Optimization of shooting medium to reduce callus formation and enable root development**

To reduce callus formation and thus enable root development, shooting medium was optimized by altering plant growth regulators (PGRs) and MS salts. BA and NAA were proportionally reduced to 10%, 20%, 40%, 60% and 80% of the current concentration with either full or ½ strength MS salts (section 5.2.8, Table 5.1). Single nodal cuttings were inoculated into Magenta boxes filled with the different shooting media to induce shoots, and then to induce roots explants with new shoots were transferred onto hormone-free rooting medium (RTM, section 5.2.1) at day 7 and 14, respectively. New shoots that developed on explants from each tested shooting medium were recorded at the time of transfer and after 3 weeks cultivation rooting percentage was calculated (Eq 5.4). Each tested shooting medium had 6 explants in each of three Magenta boxes. Rooting percentage was defined as:

$$\% \textit{ root} = \frac{\# \textit{ explants having roots} \times 100}{\textit{initial \# inoculated explants}} \quad \text{Eq (5.4)}$$

### **5.2.10 Addition of auxin into rooting medium to enhance root initiation after shoot proliferation**

To further improve rooting percentage, hormone-free RTM was optimized by testing two types of auxin: NAA and indole-3-butyric acid (IBA). Single nodal cuttings were first cultured for 7 days on ½ strength shooting medium (SHM50: 30 g L<sup>-1</sup> sucrose, 2.22 g L<sup>-1</sup> MS salts with vitamins, 0.125 μmol L<sup>-1</sup> NAA, 1.25 μmol L<sup>-1</sup> BA, pH 5.8), and then transferred to Magenta boxes each filled with a different rooting medium (Table 5.1). Rooting percentage was measured after 3 weeks. Each rooting medium had 12 explants and the experiment was repeated three times.

**Table 5.1** Various medium tested in optimization of shooting and rooting medium

Tested medium	Sucrose (g L <sup>-1</sup> )	MS salts with vitamin (g L <sup>-1</sup> )	BA (μmol L <sup>-1</sup> )	NAA (μmol L <sup>-1</sup> )	IBA (μmol L <sup>-1</sup> )
SHM	30	4.43	2.5	0.25	0
SHM10a	30	4.43	0.25	0.025	0
SHM10b	30	2.22	0.25	0.025	0
SHM20a	30	4.43	0.5	0.05	0
SHM20b	30	2.22	0.5	0.05	0
SHM40a	30	4.43	1.0	0.1	0
SHM40b	30	2.22	1.0	0.1	0
SHM60a	30	4.43	1.5	0.15	0
SHM60b	30	2.22	1.5	0.15	0
SHM80a	30	4.43	2.0	0.2	0
SHM80b	30	2.22	2.0	0.2	0
RTM	20	2.22	0	0	0
RTM1x	20	2.22	0	0.125	0
RTM1y	20	2.22	0	0.25	0
RTM2x	20	2.22	0	0	2.5
RTM2y	20	2.22	0	0	5

SHM is the original shooting medium and served as the control for all tested shooting medium SHM10a,b-SHM80a,b. SHM10-80 have 10%, 20%, 40%, 60%, and 80% of the BA and NAA levels in SHM, respectively. Lowercase letters a and b within each level of SHM has either full (a) or ½ (b) strength MS salts with vitamins. RTM is the original hormone-free rooting medium and served as the control for all tested rooting medium RTM1x,y-RTM2x,y. RTM1x,y was supplemented with NAA at two concentrations, and RTM2x,y was supplemented with IBA at two concentrations.

### 5.2.11 One-step micropropagation of *A. annua* by attachment to PLL-coated substrates

Shoot tissues from 4-week-old cultures in hormone-free rooting medium (RTM, section 5.2.1) were manually chopped into single nodes and then suspended in 30 g L<sup>-1</sup> sucrose solution at the ratio of 1 g FW per 20 mL. N70 and PP sheet were cut into 4×20 cm strips, and every two strips were connected by fish line (Tom Mann brand, CS-65). The strips were hot seamed using an impulse sealer every 3 cm lengthwise to create a zig-zag shape to facilitate tissue retention longitudinally along the hanging strips. The strips were then autoclaved and coated

with PLL as described in Fei and Weathers (2014). Air dried PLL-coated strips were subsequently soaked in a suspension of shoot tissues for 5 min prior to inoculation into the mist reactor and misted for 7 days with SHM50 (section 5.2.10) followed by misting for another 21 days with RTM1y (Table 5.1) at  $50 \mu\text{mol m}^{-2}\cdot\text{s}^{-1}$  light intensity and  $25 \text{ }^\circ\text{C}$ . Reactor ventilation was 0.1 vvm of 0.16% (v/v)  $\text{CO}_2$  enriched air. Unless otherwise specified,  $\text{CO}_2$  enriched air was humidified using a Nafion tube (Perma Pure, MH-110-48F-4) connected with a  $0.2 \mu\text{m}$  sterile filter prior to entry into the mist reactor. Gelled medium controls had the same ventilation and light conditions as the mist reactor.

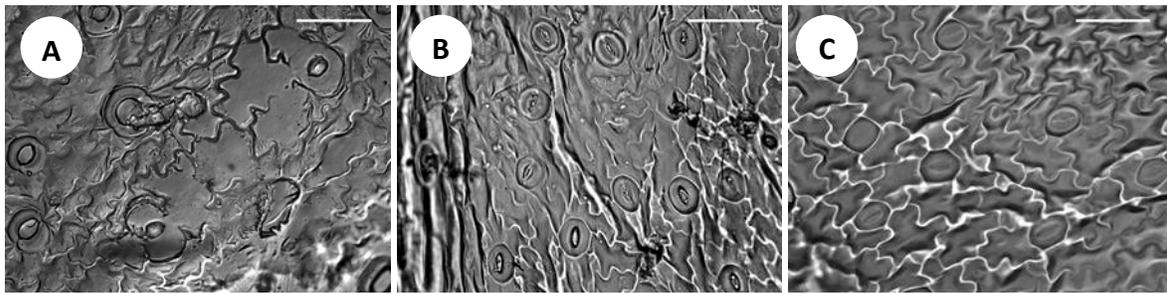
During the 7-day shooting stage, the mist cycle was 0.75 min on/59.25 min off with medium flow rate of  $40 \text{ mL min}^{-1}$  to deliver  $30 \text{ mL h}^{-1}$ . After 7 days of shoot proliferation, RTM1y (Table 5.1) was switched into the feeding line to initiate root development. The misting cycle was 0.25 min on/14.75 min off during the first 12 days for root induction, and then switched to 0.5 min on/29.5 min off during the remaining 9 days of culture for acclimatization. Volumetric medium delivery was maintained at  $30 \text{ mL h}^{-1}$  during the 21 days of culture in RTM1y. Ventilation during acclimatization was 0.1 vvm of 0.16% (v/v)  $\text{CO}_2$  enriched air with about 52% relative humidity.

Plantlets from mist reactor and gelled medium controls were measured for their shooting percentage (Eq 4.2), number of new shoots per plantlet (# shoot), rooting percentage (Eq 5.4), number of primary roots per plantlet (# roots), and hyperhydricity percentage (Eq 4.3). Detached 3-6<sup>th</sup> fully expanded leaves were sampled and imprints of their abaxial side were

made to measure stomatal function as described by (Correll and Weathers 2001b). Stomatal function was scored as open, partially open, closed (Figure 5.1). Closed stomata were deemed functional. Counts were made at 400× magnification at 5 positions on each sampled leaf, and 2 leaves were sampled from each plantlet. The average of these 10 counts was then taken to represent each plantlet, and the average of 10 plantlets was taken to represent each reactor plantlet harvested from mist reactors and gelled medium controls were transplanted to soil:perlite (2:1 V/V) pots and *ex vitro* survival was measured after one week.

### **5.2.12 Statistics**

All attachment experiments had four replicates. Medium adjustment and one-step micropropagation experiments had three replicates. Data from all experiments was subjected to Students T test between  $\pm$  PLL substrates, one-way ANOVA, and Duncan's multiple range analysis by SPSS.



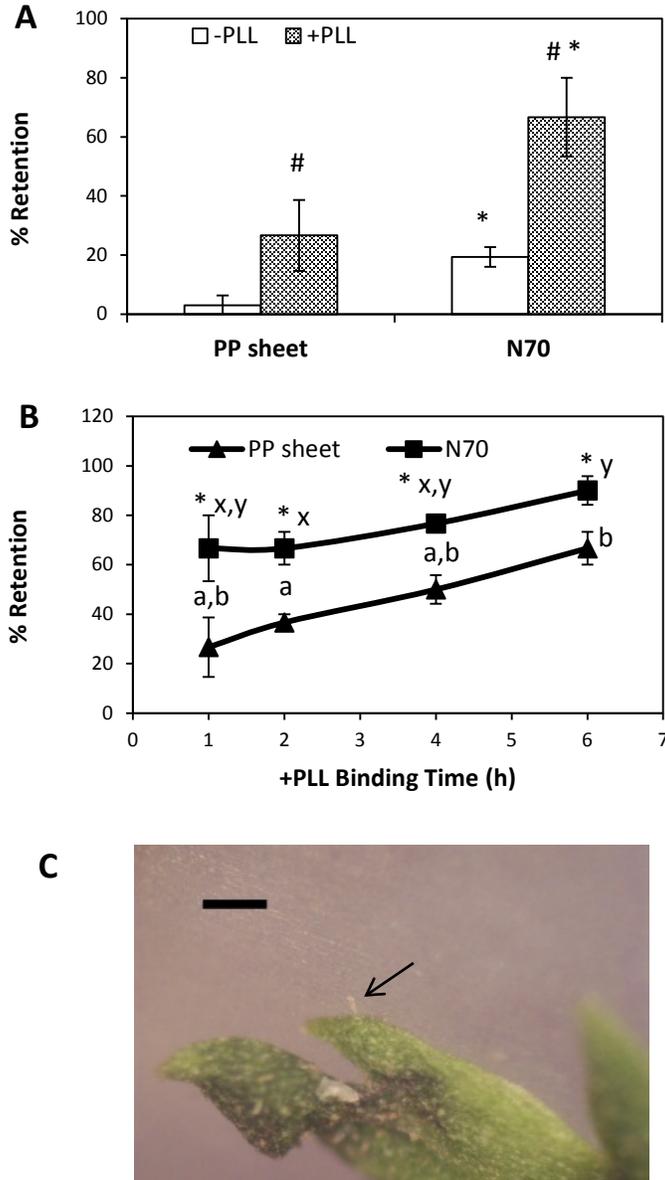
**Figure 5.1** Stomata status at open (A), partially open (B) and closed (C). Bar: 50  $\mu\text{m}$ .

## 5.3 Results and discussion

### 5.3.1 Retention of manually chopped and applied leaf tissue

It was initially uncertain if small pieces of leaf tissue could attach to PLL-coated strips. Using manually chopped and applied leaf pieces, there was a significant increase in retention percentage with PLL-coated PP sheets and N70 mesh compared to controls lacking PLL (Figure 5.2A). Regardless of PLL coating, leaf tissue retention rate was higher on N70 than on PP sheet (Figure 5.2A). Tissue binding kinetics showed a nearly linear increase in retention on both substrates as incubation time increased from 1 to 6 h (Figure 5.2B). This was different from the kinetics of cell binding, which showed a rapid interaction between cells and PLL (Davis *et al.* 2004; Fei and Weathers 2014).

After 1 h of binding incubation, more than twice as many leaf tissues were retained on N70 mesh than on PP sheets (Figure 5.2A). This was in contrast to the results using manually applied carrot cells (Fei and Weathers 2014).



**Figure 5.2** Retention of leaf tissue to substrates  $\pm$  PLL. **A**, retention percentage of manually applied leaf tissue with 1 h incubation after 10 successive vertical washes; # compares +PLL vs. -PLL; \* compares PP sheet vs. N70 mesh,  $N = 4$ ,  $\pm$  SE,  $p \leq 0.05$ ; **B**, kinetics of leaf piece retention Letters compare across different incubation times PP sheet (a, b, c) and N70 mesh (x, y, z), \* compares PP sheet vs. N70 mesh,  $N = 4$ ,  $\pm$  SE,  $p \leq 0.05$ ; **C**, leaf tissue attached to +PLL substrate *via* filamentous trichome (arrow), bar: 250  $\mu$ m.

Unlike spherical cells, differentiated tissues like leaves consist of complicated structures, so the retention of a piece of leaf tissue is a function of binding force of the structures interacting with the PLL-coated surface and the weight of the tissue piece. Microscopic observation showed that the *A. annua* leaf pieces were at least in part also attached to substrates via their filamentous trichomes (Figure 5.2C), which was similar to root attachment by root hairs to PLL-coated substrates (Towler and Weathers 2003). *A. annua* leaves have two types of trichomes: T-shaped filamentous and glandular (Duke et al., 1994). The T-shaped filamentous trichomes are long outgrowths on the leaf surface and give rise to a hairy appearance of leaves (Figure 5.2C). The higher tissue retention on N70 mesh than on PP sheet was thus probably because fine openings in the mesh structure further facilitated trichome anchorage.

### **5.3.2 Quick dip retention vs. attachment: manually chopped vs. blenderized leaf tissue**

Since adherence of leaf tissue on PLL-coated substrates was deemed feasible, a “hanging garden” style of culture through quick dip inoculation was envisioned. Using manually chopped or blenderized leaf pieces, there was a significant increase of initially retained leaf FW with PLL-coated PP sheet and N70 mesh compared to controls lacking PLL (Table 5.2). Generally there was no difference in initially retained leaf FW between PP sheet and N70 mesh for both chopping methods (Table 5.2). However, there was more initial retention of tissue pieces at higher tissue concentrations (100 and 50 g L<sup>-1</sup>) than at lower tissue concentrations (33 and 25 g L<sup>-1</sup>) regardless of chopping method or substrate type (Table 5.2). Quick dip tissue concentration, however, did not affect initial retention of manually chopped

tissues on PLL-coated N70 mesh (Table 5.2). Regardless of substrate type, PLL coating or tissue concentration, blenderized tissue showed significantly fewer initially retained tissues than manually chopped tissue (Table 5.2).

**Table 5.2** Quick dip retention of leaf tissue after 5-10 sec incubation

Tissue Concentration (g L <sup>-1</sup> )	Manually chopped (mg FW)				Blenderized (mg FW)			
	PP sheet		N70		PP sheet		N70	
	-PL L	+PLL	-PL L	+ PLL	-PL L	+ PLL	-PLL	+ PLL
100	Nd	316 b	nd	197 a	nd	111 #* c	nd	102 b c
50	45 a	400 # b	58 a	330 # a	21* a	103 #* c	25 *	126 #* c
33	54 a	213 #a,b	46 a	316# a	28* a	51 #* b	32 a	74 #* b
25	58 a	133 #a	57 a	196 # a	20* a	26 * a	19 *	36 * a

Letters compare within columns for different biomass suspensions; # compares +PLL vs. -PLL; \* compares manual chop vs. blender, N=4,  $p \leq 0.05$ .

Compared to controls lacking PLL, there was significantly more attachment of the initially retained tissues on PLL-coated substrates (Table 5.3). Most retained tissues on PLL-coated substrates remained attached after 24 h of misting regardless of substrate type or chopping method, suggesting the retained tissues were likely to remain on PLL-coated hanging strips during cultivation in a bioreactor (Table 5.3). For controls lacking PLL, attachment was greater on N70 mesh than on PP sheet, and tissues that were manually chopped also showed greater attachment than those from the blender (Table 5.3). The main difference between manually chopped and blenderized leaves was size, so variation in attachment was likely due to tissue size.

**Table 5.3** Attachment of retained leaf tissue after 24 h in the mist reactor

	Manually chopped (%)		Blenderized (%)	
	-PLL	+ PLL	-PLL	+ PLL
PP sheet	37 a	87 # a	32 a	80 # a
N70 mesh	85* b	97 # a	72 b	89 # a

Letters compare between PP sheet and N70 mesh; # compares +PLL vs. -PLL; \* compares manually chopped vs. blenderized, N=4,  $p \leq 0.05$ .

### 5.3.3 Quick dip, attachment and shoot regeneration of various sized shoot tissues

To determine if the smaller size of blenderized leaf tissue correlated with its lower retention compared to manually chopped leaves, quick dip retention and attachment experiments were conducted using the four leaf sizes shown in Table 5.4. The tissue concentration for quick dip was 50 g FW L<sup>-1</sup>, and N70 was used as the binding substrate. As shown in Table 5.4, the retention FW on N70 + PLL was greatest in the >1 mm group, but was significantly reduced once the size decreased to < 0.5 mm. The finest pieces (0.15- 0.35 mm) showed lowest % attachment to N70 + PLL (Table 5.4).

The blenderized leaf tissue was actually a mixture of the four sizes of tissues, so the initially retained FW of blenderized tissue was estimated by integrating the fraction of each size group. Using the numbers in Table 5.4, the calculated overall retained FW on PLL-coated N70 mesh was about 160 mg, which was very close to the experimental result (126 mg) under the same tissue concentration of 50 g FW L<sup>-1</sup> during the quick dip (Table 5.2). It was posited that this reduced retention was likely the result of the smaller pieces having fewer intact filamentous

trichomes. The regeneration ability of leaf explants also progressively decreased as explant size decreased (Table 5.4), possibly due to mechanical damage during blending.

**Table 5.4** Adherence and shoot regeneration of various sizes of blenderized tissue

Leaf tissue size (mm)	% in 30 - pulse mixture	Initially retained tissue FW (mg) on N70		% Attached, 24 hr post misting		% new shoots
		-PLL	+ PLL	-PLL	+ PLL	
0.15-0.35	4 a	24 a	51 a	68 a	66 a	0.7 a
0.35-0.5	4 a	30 a	50 a	82 a	89 b	2.8 b
0.5-1	36 b	30 a	108 b	70 a	93 # b	11.3 c
>1	56 c	47 b	216 c	83 a	96 # b	23.3 d

To determine initially retained tissue, the tissue concentration for quick dip was 50 g FW L<sup>-1</sup>. Letters compare across various sizes; # compares +PLL vs. -PLL, N=4,  $p \leq 0.05$ .

### 5.3.4 Optimization of shooting and rooting medium for zero manual transfer between shoot proliferation and rooting stage

The goal of one-step micropropagation in the mist reactor was to accomplish shoot proliferation, root development and *in vitro* acclimatization in one batch culture by shifting the nutrient medium and culture conditions. There would be no manual transfers. Unfortunately, the initial trials of one-step micropropagation in the mist reactor yielded callusing of shoot stems during the shoot proliferation stage and roots did not form unless the callus was excised prior to switching to the hormone-free rooting medium (RTM). Compact callus at the bottom of stems also inhibited root induction in *Artemisia absinthium* (Zia *et al.* 2007). Others have shown that cytokinins (e.g. BA) in the medium generally inhibited root induction (Jarvis 1986) and having both BA and NAA in the medium induced callus formation on shoots (Jamaledine *et al.* 2013; Sudha *et al.* 2012; Zia, *et al.* 2007). Root initials originate in stem but not callus tissue (Lane 1979), so root induction may be improved by reducing

callus formation during shoot proliferation. To reduce callus formation and improve root induction, the phytohormone and salt composition of shooting medium was investigated. In addition, the exposure time to different shooting media was investigated.

As shown in Table 5.5, neither the strength of MS salts nor the incubation time for shoot proliferation affected the production of new shoots. Shoot proliferation remained constant as long as the phytohormones were  $\geq 40\%$  of the original concentration (Table 5.5).

**Table 5.5** Number of new shoots of *A. annua* upon direct transfer from shooting medium

% of original PGR concentration	Full MS (SHM10-100a)		½ MS (SHM10-100b)	
	1 wk transfer	2 wk transfer	1 wk transfer	2 wk transfer
10 (SHM10)	1.3 x	1.4 x	1.2 x	1.2 x
20 (SHM20)	1.8 x,y	1.7 x,y	1.9 y	1.7 x,y
40 (SHM40)	2.2 y	2.0 y	2.1 y	2.1 y
60 (SHM60)	2.2 y	2.2 y	2.3 y	2.1 y
80 (SHM80)	2.3 y	2.3 y	2.4 y	2.2 y
100 (SHM)	2.1 y	2.2 y	2.0 y	2.0 y

PGR: plant growth regulator; SHM: shooting medium. SHM is the original shooting medium and served as the control for all tested shooting medium SHM10a,b-SHM80a,b. SHM10-80 have 10%, 20%, 40%, 60%, and 80% of the BA and NAA levels in SHM, respectively. Lowercase letters a and b within each level of SHM has either full (a) or ½ (b) strength MS salts with vitamins. Letters x and y compare across different hormone concentrations, N=3,  $p \leq 0.05$ .

On the other hand, none of the new shoots from full MS shooting medium had roots after 3 weeks regardless of time of transfer (Table 5.6). Root development only happened with those explants previously cultured on ½ MS shooting medium containing phytohormones up to 60% of SHM when transferred on day 7 (Table 5.6). The majority of new shoots transferred on day 14 had formed callus at the time of transfer and few roots were observed later on rooting

medium, suggesting the duration of exposure to BA and NAA during shoot proliferation also played a role in subsequent root induction. Taken together the results from Table 5.5 and 5.6, shooting time was adjusted to 7 days on ½ strength SHM in order to enhance root development without reducing shoot proliferation. The sucrose concentration of the adjusted shooting medium remained at 30 g L<sup>-1</sup>.

**Table 5.6** Rooting percentage of *A. annua* upon direct transfer from shooting medium

% of original PGR concentration	Full MS (SHM10-100a, %)		½ MS (SHM10-100b, %)	
	1 wk transfer	2 wk transfer	1 wk transfer	2 wk transfer
10 (SHM10)	0 x	0 x	22.2 # * y	0 x
20 (SHM20)	0 x	0 x	27.8 # * y	0 x
40 (SHM40)	0 x	0 x	44.4 # * y	11.1 # y
60 (SHM60)	0 x	0 x	33.4 # * y	16.7 # y
80 (SHM80)	0 x	0 x	0 x	0 x
100 (SHM100)	0 x	0 x	0 x	0 x

PGR: plant growth regulator; SHM: shooting medium. SHM100 was the original shooting medium and served as the control for all tested shooting medium SHM10a,b-SHM80a,b. SHM10-80 have 10%, 20%, 40%, 60%, and 80% of the BA and NAA levels in SHM100, respectively. Lowercase letters a and b within each level of SHM has either full (a) or ½ (b) strength MS salts with vitamins. Letters x and y compare across different hormone concentrations, # compares between full and ½ MS and \* compares between the time of transfer, N=3,  $p \leq 0.05$ .

To further improve rooting, NAA and IBA were tested in RTM. Both auxins were found to effectively induce root development in various cultivars of *A. annua*; NAA and IBA concentrations were selected based on prior studies (Alam and Abdin 2011; Gopinath *et al.* 2014; Hailu *et al.* 2014; Han *et al.* 2005; Hong *et al.* 2009; Janarthanam *et al.* 2012; Lin *et al.* 2011a; Sharma and Agrawal 2013). As indicated by Table 5.7, 0.25 µmol L<sup>-1</sup> of NAA and both concentrations of IBA improved root initiation. There was no difference in root induction efficiency between the two IBA concentrations (Table 5.7).

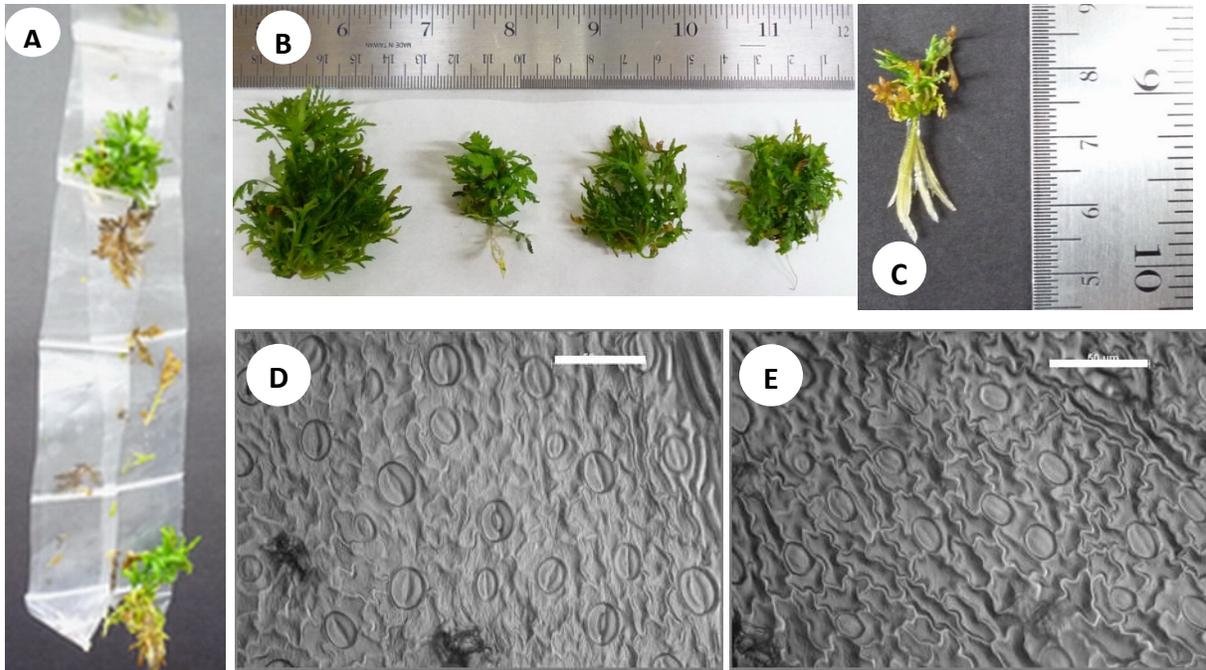
**Table 5.7** Rooting percentage of *A. annua* on different rooting medium

Tested medium	Auxin concentration ( $\mu\text{mol L}^{-1}$ )		Rooting percentage (%)
RTM0	None	0	30.7 a
RTM1x	NAA	0.125	27.2 a
RTM1y	NAA	0.25	60.8 b
RTM2x	IBA	2.5	55.6 b
RTM2y	IBA	5	56.7 b

Letters in column compare across different auxin concentrations,  $N=3$ ,  $p \leq 0.05$ .

### 5.3.5 One-step micropropagation of *A. annua* by attachment to PLL-coated substrates

Propagation by attachment to PLL-coated strips was successfully achieved through somatic embryogenesis of carrot (Fei and Weathers 2014). When this concept was tested using nodal cutting inoculum followed by rooting, the majority of plantlets grew large and unfortunately fell off the strips when harvested 28 days after inoculation (Figure 5.3A). The adherent force between these large plantlets (Figure 5.3B) and PLL-coated strips was not adequate to retain their increased weight. The zig-zag design of strips marginally helped plantlet retention. In contrast, when inoculated with embryogenic cells, the binding force was strong enough and resulting plantlet weight was low enough to enable retention of rooted plantlets (Fei and Weathers 2014).



**Figure 5.3** One-step micropropagation of *A. annua* in mist reactor by attachment to PLL-coated strips. **A**: rooted plantlets harvested on PLL-coated polypropylene strip with zig-zag seam; **B**: rooted plantlets harvested from the mist reactor after one-step culture; **C**: rooted plantlets harvested from gelled medium control; **D**: representative photo of stomata on abaxial side of leaf harvested in mist reactor; **E**: representative photo of stomata on abaxial side of leaf harvested in gelled medium. Bars in D and E: 50 µm.

Although the process still requires optimization, one-step culture in the mist reactor is feasible and plantlets showed better growth than in gelled medium controls (Figure 5.3B and C, Table 5.8). Compared to gelled medium controls, better growth in the mist reactor was probably due to increased availability of nutrients in liquid medium as suggested by other reactor systems (Adelberg and Fári 2010). Regardless of similar stomatal developmental status (Figure 5.3D and E, Table 5.8), *ex vitro* survival from the mist reactor also appeared greater than from gelled medium controls ( $p=0.08$ ). This was probably because plantlets from gelled medium were too small to survive transplant into soil, suggesting that *in vitro* culture duration on gelled medium needs to be longer than in the mist reactor.

When considering bioreactors for shoot cultures, a TIS is frequently used because they yield high amounts of biomass with high quality (Adelberg 2006; Hahn and Paek 2005; Roels, *et al.* 2005; Roels *et al.* 2006; Welander, *et al.* 2014; Yan, *et al.* 2010; Yan, *et al.* 2013; Yang and Yeh 2008). Similar to a TIS, the mist reactor offers an alternate option for micropropagation as shown in this and prior studies (Correll, *et al.* 2001).

**Table 5.8** *A. annua* plantlets from one-step micropropagation in the mist reactor

	Mist reactor	Gelled medium
% new shoot	98 *	83.3
# Shoots	6.8 *	2.9
Shoot length (cm)	3.7 *	1.8
% root	55.8	43.3
# primary roots	3.7 *	2
% hyperhydricity	16.3	16.7
% closed stomata	93.7	96.4
% <i>ex vitro</i> survival	89	65
Estimated time in days from inoculum to soil-ready plants	28	45

\* indicates significant difference between the mist reactor and gelled medium controls, N=3,  $p \leq 0.05$

Bioreactors usually save labor by increased culture scale and also by circumventing otherwise piecemeal handling in gelled medium (Alister, *et al.* 2005; Lorenzo, *et al.* 1998; Takayama and Akita 2006). In an estimate of labor time, one-step micropropagation in the mist reactor yielded nearly three times the plantlets with half the labor time compared to traditional methods (Appendix S6). By using more vertical space in the reactor, greater productivity should also be possible.

## 5.4 Conclusions

*A. annua* leaf tissues attached to PLL-coated polypropylene sheeting and nylon mesh via filamentous trichomes. Most of the initially retained tissue remained attached to the substrate after a 24 hr misting treatment in the mist reactor, suggesting the feasibility of a “hanging garden” style of culture. Although automation of leaf tissue preparation by blending is possible, the timing has to be carefully controlled to prevent over production of very fine tissue pieces <0.5 mm, which have a reduced capacity for both attachment capacity and shoot regeneration. These results show that if large enough, leaf explants will bind to PLL-coated materials and produce shoots and roots, thereby enabling development of alternative cultivation technologies for possible use in micropropagation. In a final test of one-step micropropagation through attachment to PLL-coated strips, single nodal tissues attached to PLL-coated strips and developed into acclimatized rooted plantlets ready for direct transplant into soil. This proof of concept study should aid automation of this labor intensive industrial process.

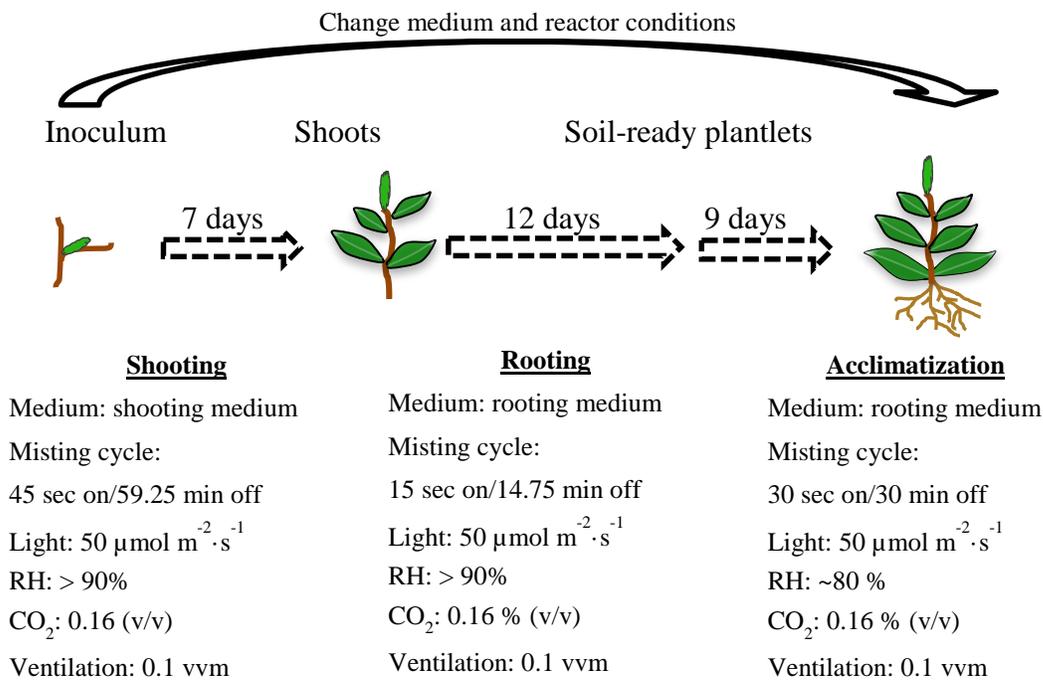
## Chapter 6 Conclusions and future work

### 6.1 Conclusions

Traditional micropropagation is extremely labor intensive. Automation of *in vitro* cultures in bioreactors offers great potential to save labor and meanwhile increase production efficiency. The artificial environment used for *in vitro* culture (light, relative humidity, CO<sub>2</sub> etc) is critical for *in vitro* plantlet quality and *ex vitro* survival, and thus needs to be controlled. A mist reactor was used to study plant growth and development under various environmental conditions towards the production of healthy plantlets ready for soil transplant in one step from inoculation. In addition, a 3D type of cultivation via surface attachment of explants to vertically hanging strips inside the mist reactor was also investigated to maximize productivity with a minimal footprint. The major conclusions are:

- The mist reactor using a disposable bag offered the potential for one-step micropropagation either from cells via embryogenesis or from nodal explants via shoot multiplication to fully rooted plantlets. The plantlets developed from nodal explants were also demonstrated ready for direct soil transplant when harvested from the mist reactor.
- Using hanging strips, inoculum attached to poly-L-lysine (PLL) coated strips and developed *in situ* into rooted plantlets:
  - Embryogenic cells attached efficiently and the attachment was improved by eliminating ions in the incubation medium.
  - Embryo development was more or less uniform up and down the strip.

- Despite producing some considerable biomass, young plantlets developed from embryos did not fall off the hanging strips.
- Manually chopped or blenderized shoot tissues attached to PLL-coated polypropylene or nylon via non glandular trichomes after substrates were immersed into an explant suspension.
- Although nodal explants developed into fully rooted plantlets, the majority fell off the hanging strips by harvest due to their large size.



**Figure 6.1** One step micropropagation of *A. annua* from nodal cuttings in the mist reactor

- Compared to gelled medium controls, plant development was better in the mist reactor in terms of somatic embryo development, shoot proliferation, stomatal function and biomass yield.

- Hyperhydric shoots of *A. annua* not only appeared swollen, brittle and dark, they also had fewer glandular trichomes, artemisinin and flavonoids than normal shoots.
- The headspace environment was critical for development of *in vitro* plants:
  - Increasing irradiance, ventilation and CO<sub>2</sub> in the headspace improved somatic embryogenesis, shoot proliferation, root development, stomatal function and *ex vitro* survival.
  - With the above improvements in headspace conditions, hyperhydricity in the mist reactor was also reduced.
- Mist feed rate had a significant effect on plant development:
  - Increasing misting frequency stimulated embryo development and root induction.
  - Decreasing misting frequency improved shoot multiplication and reduced hyperhydricity in the mist reactor.
- To achieve one-step micropropagation from nodal explants to whole plants, culture medium and process were optimized by:
  - reducing by 50% the phytohormones and MS salts in the shooting medium,
  - reducing the culture time on shooting medium from 14 to 7 days, and
  - adding auxins to the rooting medium to improve root initiation.

The ability to control all aspects of the culture environment in the mist reactor enabled combining the otherwise discrete process steps common to micropropagation resulting in a

one-step process for producing high quality plants ready for field conditions. Together with the surface attachment technology, the mist reactor offers an alternative labor-saving approach to traditional micropropagation.

## **6.2 Future work**

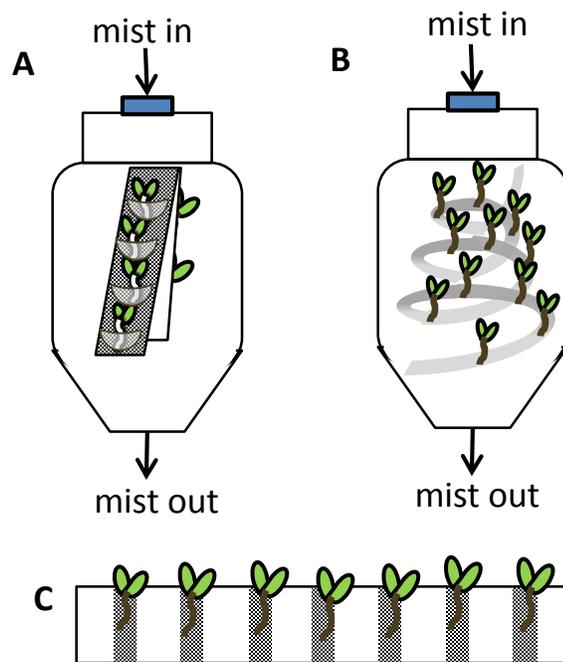
There are several aspects that need to be further investigated regarding implementing the one-step micropropagation with 3D growth into a production option.

### **6.2.1 Improving tissue immobilization in mist bioreactor**

Although somatic embryos remained attached to PLL-coated substrates and developed into fully rooted plantlets, nodal explants developed quite large shoots that did not remain attached, succumbing to gravity. To address this, either smaller rooted plantlets should be grown by spending less time in the reactor, or current surface adhesion technology should be improved to provide a stronger adhesive force between plants and substrates.

Current attachment technology mainly relies on electrical attraction between negatively charged plant tissues and positively charged substrates through the PLL-coating. When plant shoots grow well and large, the adhesive force may not be strong enough to counteract gravity. To increase adhesion, alternative attachment technology bearing different adhesion chemistry could be used. For example, large algae, e.g. *Fucus serratus*, produce a strong adhesive to anchor them to a variety of surfaces under the water. Shellfish like mussels, barnacles and oysters, also exude highly sticky proteins together with other inorganic compounds to help

them stay attached to rocks, boats and other objects in the water (Burkett *et al.* 2010; Sever *et al.* 2004; Silverman and Roberto 2007; Wilker 2010). Bio-adhesives that mimic the adhesive ability of large algae (Bitton and Bianco-Peled 2008) or shellfish (Meredith *et al.* 2014; Wilker 2014; Zhong *et al.* 2014) could potentially be used to coat substrates possibly by improving plant tissue adhesion.



**Figure 6.2** Proposed immobilization methods for micropropagation in the mist reactor. **A**, plantlets inside pockets on hanging strips; **B**, plantlets on a hanging spiral substrate; **C**, plantlets on designated positions where adhesives are loaded prior to inoculation.

Physical entrapment can also be used to enhance plant tissue retention. For example, the polypropylene strips can be hot-seamed with pockets or small slashes to “catch” explants during inoculation by tissue immersion and then hold the inoculum until they develop into plants (Figure 6.2A). Plants can also grow on a spiral support hanging inside the mist reactor

(Figure 6.2B). Use of a spiral support provides each plant with a slight horizontal plane with a small angle, and thus plants may be better retained.

### **6.2.2 Automation of other steps in micropropagation**

To warrant producing single nodes during explant preparation, shoot tissues were manually chopped. Although a 4-blade blender was also used to chop shoot tissues, the chopping was random and thus yielded a mix of a various sized inocula. A different mechanical chopping apparatus is thus needed to produce more uniform explants. For example, a food processor with multiple blades along the vertical axis should be tested to chop shoot tissue.

Soil transplant can also be automated if plants grow at designated positions on adhesive surfaces and/or physical entrapments (Figure 6.2A, C).

### **6.2.3 Exploring the use of the mist reactor for production of leafless structures and facilitating the screening of genetic modified plants**

In addition to somatic embryos and shoot tissues, the mist reactor is also a good candidate for production of leafless structures like bulblets, protocorm-like-bodies and microtubers possibly by inoculating into hanging mesh bags. Investigation of *in vitro* growth of these structures in the mist reactor could provide a device for controlling environments for mass production of healthy leafless structures. One-step protocols can then be established for such species.

Because of its flexible control on growth conditions, the mist reactor can also be used to expedite the screening process after genetic modification. Genetically transformed explants

could be positioned on adhesive surfaces or pockets and then developed into plants subsequently subjected to screening analysis.

The mist reactor has been shown to have the potential for one-step micropropagation from inoculum to fully acclimatized plants ready for soil transplant. The labor associated with shooting, rooting and acclimatization transfer steps can therefore be eliminated. The one-step culture method shown in this study should also be tested with a variety of other plant species.

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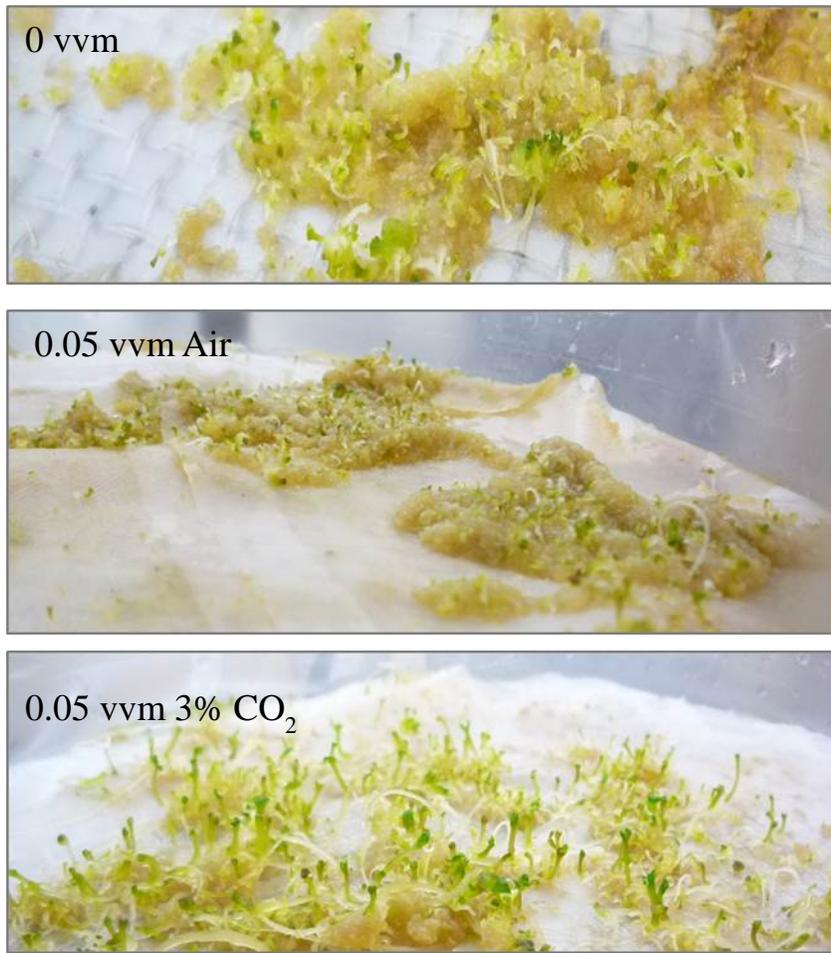
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## **Appendix: Supplemental Figures and Tables**



**Figure S1** Rooted somatic embryos of carrot grown under different ventilation and CO<sub>2</sub>

**Table S2** Effect of misting cycle on shooting percentage of various inocula

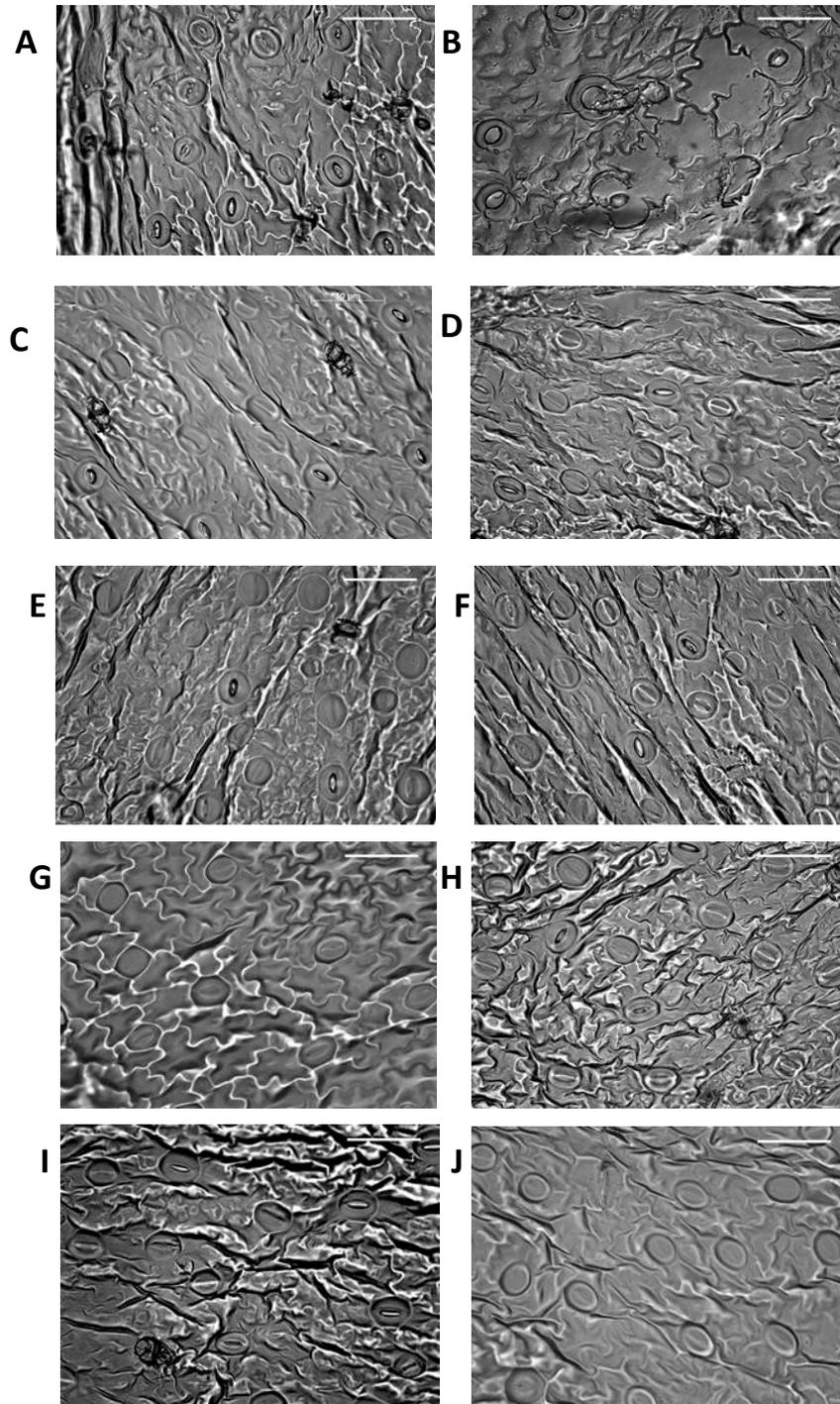
Condition	% new shoots in reactor				% new shoots in gelled medium			
	N	IN	L	LP	N	IN	L	LP
0V1C1I3S2M	81.7 U a	21.1 T a	1.8 R a	9.6* S a	86.4 T a	16.4 S a	0.9 R a	0.8 R a
1V1C1I3S2M	88.3 U a	21.7 T a	2.6 R a,b	8.5* S a	88.6 T a	20.6 S a	1.5 R a	1.9 R a
1V1C1I3S1M	86.6 T a	28.2 S a,b	5.4* R b,c	6.1* R a	88.2 T a	21.1 S a	1.2 R a	1.7 R a

Ventilation (V) was 0 and 0.1 vvm for 0V and 1V, respectively. Misting cycle (M) was 0.75 min on/59.25 min off and 0.5 min on/29.5 min off for 1M and 2M, respectively. Light intensity (I) was 15  $\mu\text{mol m}^{-2}\cdot\text{s}^{-1}$  (1I) and sucrose level (S) was 30 g L<sup>-1</sup> (3S). Upper case letters (R,S,T,U) compare among different explants, lower case letters (a,b,c,) compare across different conditions, \* compares between reactor and control,  $p \leq 0.05$ .

**Table S3** Effect of misting cycle on roots and shoots during rooting stage

Condi- -tion	Reactor										Gelled medium controls											
	% SH FW	% HYP	% RT	Ø PR (µm)	# PR (cm)	# BR (cm)	BR: PR	% SH FW	% HYP	% RT	Ø PR (µm)	# PR (cm)	# BR (cm)	BR: PR	% SH FW	% HYP	% RT	Ø PR (µm)	# PR (cm)	# BR (cm)	BR: PR	
1M	252.2 *a	9.5 a	19.9 a	467.4 * a	1.7 a	4.0 a,b	6.1 b	1.5 b	0.4 a	140.1 a,b	8.3b	50.1 * b	371.7 a	2.9 *a	7.0 *a	7.2 a	3.2 a	0.4 a,b				
2M	279.2 *a	11.3 a	28.4 a,b	450.0 * a	1.7 a	3.4 a,b	9.4 b	3.6 c	0.8 * b	136.5 a,b	7.8 b	48.7 b	382.3 a	3.0 * a	6.8 *a	7.9 a	3.1a	0.5 b				
4M	302.9 *a	10.9 a	51.3 c	428.6 * a	3.7 b	7.3 c	17.9 *c	4.5 c	0.7 b	138.3 a,b	7.9 b	43.8 a,b	374.4 a	3.2 a	6.8 a	7.4 a	3.4 a	0.5 b				

Three misting cycles: 1 misting h<sup>-1</sup> (1M, 0.75 min on/59.25 min off), 2 mistings h<sup>-1</sup> (2M, 0.5 min on/29.5 min off), 4 mistings h<sup>-1</sup> (4M, 0.25 min on/14.75 min off). Other conditions for both reactor and gelled medium controls were: 0.1 vvm (1V), ambient air (1C), at 15 µmol m<sup>-2</sup>.s<sup>-1</sup> (1D), 20 g L<sup>-1</sup> sucrose (2S). SH, shoot; PR, primary roots per plantlet; BR, root branches; BR:PR, branch length:primary root length per plantlet; HYP, hyperhydricity; Ø, diameter. Both numbers and length of root and branches were measured for each rooted plantlet. Letters compare among all conditions, \* compares between reactor and gelled medium control, p ≤ 0.05.



**Figure S4** Picture of abaxial side leaf stomata acclimatized under different conditions. **A**, 0 vvm reactor; **B**, 0 vvm gelled medium; **C**, RH100 reactor; **D**, RH 100 gelled medium; **E**, RH52 reactor; **F**, RH52 gelled medium; **G**, RH52/11 reactor; **H**, RH 52/11 gelled medium; **I**, stepped down feed reactor (RH100); **J**, stepped cycle reactor (RH100 for the first 5 days and then RH11). Bars in A-J: 50  $\mu$ m.



**Figure S5** Picture of one-week-old *ex vitro* plantlets after acclimatization under different conditions. **A**, 0 vvm reactor; **B**, 0 vvm gelled medium; **C**, RH100 reactor; **D**, RH 100 gelled medium; **E**, RH52 reactor; **F**, RH52 gelled medium; **G**, RH52/11 reactor; **H**, RH 52/11 gelled medium; **I**, stepped down feed reactor (RH100); **J**, stepped cycle reactor (RH100 for the first 5 days and then RH11).

**Table S6** Comparison of operational time between the mist reactor and gelled medium

	Mist reactor (min)	Gelled medium (min)
Medium preparation	30	50 *
Inoculation	20	30
Shift from SHM to RTM	5 #	30 @
Removing cultures	5	20
Vessel washing	5	5
Total	65	135
# harvested plantlets	43	15

SHM: adjusted shooting medium (1/2 strength of PGRs and MS salt); RTM: rooting medium supplemented with NAA. \*Gelled medium need aliquot and solidified in culture containers prior to inoculation. # Only the media supply line was switched and cultures remained in mist reactor. @ Cultures in SHM were transferred to RTM one by one in sterile hood.