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Disposable Bioreactors for Plant Micropropagation and Mass Plant Cell Culture

Jean-Paul Ducos, Bénédicte Terrier, and Didier Courtois

Abstract Different types of bioreactors are used at Nestlé R&D Centre – Tours for mass propagation of selected plant varieties by somatic embryogenesis and for large scale culture of plants cells to produce metabolites or recombinant proteins. Recent studies have been directed to cut down the production costs of these two processes by developing disposable cell culture systems. Vegetative propagation of elite plant varieties is achieved through somatic embryogenesis in liquid medium. A pilot scale process has recently been set up for the industrial propagation of *Coffea canephora* (Robusta coffee). The current production capacity is 3.0 million embryos per year. The pre-germination of the embryos was previously conducted by temporary immersion in liquid medium in 10-L glass bioreactors. An improved process has been developed using a 10-L disposable bioreactor consisting of a bag containing a rigid plastic box ('Box-in-Bag' bioreactor), insuring, amongst other advantages, a higher light transmittance to the biomass due to its horizontal design. For large scale cell culture, two novel flexible plastic-based disposable bioreactors have been developed from 10 to 100 L working volumes, validated with several plant species ('Wave and Undertow' and 'Slug Bubble' bioreactors). The advantages and the limits of these new types of bioreactor are discussed, based mainly on our own experience on coffee somatic embryogenesis and mass cell culture of soya and tobacco.

Keywords Box-in-bag, Coffee, Scaling-up, Slug bubble, Somatic embryogenesis, Somatic seedling, Temporary immersion, Wave and undertow

J.P. Ducos, B. Terrier, and D. Courtois
Nestlé R&D Centre Tours, 101 Avenue Gustave Eiffel, Notre Dame D'Oé, BP 49716,
37097 Tours Cedex 2, France

Contents

Abbreviations

FW Fresh weight
SB Slug bubble
TIB Temporary immersion bioreactor
WU Wave and undertow

1 Introduction

For more than 20 years, Nestlé R&D Centre – Tours has been involved in in vitro cultivation of plant material, either for the production of metabolites by cell cultures [1–8] or for mass propagation by somatic embryogenesis [9–19]. Despite biochemical and physiological studies allowing improved experimental protocols at laboratory level, these improvements have seldom been matched by large scale or mass propagation of these plant materials. The gap between lab and production scales arises from the type and cost of the facilities necessary for scaling-up.

Plant micropropagation refers to the production of true-to-type plants from organogenic cultures (stem cuttings, axillary buds, meristem clusters, protocorm-like bodies) under aseptic and controlled environment. Somatic embryogenesis, which is the process for the development of embryos from somatic cells, offers a tremendous potential for mass propagation [20]. Current commercial applications are still restricted to a limited number of plant species because these methods are based on manual aseptic division of the plant tissues and their transfer on numerous small containers containing semi-solid media. In the 1985s to 1990s, studies described micropropagation scaling-up into two types of bioreactor: those in which the cultures are continuously submerged and those in which the cultures are temporarily immersed in the medium (temporary immersion bioreactor). The first approach consists in growing plants tissues in fermentation vessels either mechanically agitated (stirred tank bioreactor, rotating drum bioreactor) or pneumatically agitated (bubble column bioreactor, air-lift bioreactor) [21, 22]. The second involves placement of plant

tissues on solid supports which are periodically perfused with nutrients solutions [23–26]. Reports on micropropagation using disposable bioreactors remain rare as confirmed by two books reviewing the recent advances in plant tissue culture engineering in liquid cultures [27, 28]. Mainly based on our own experience on coffee somatic embryogenesis, the present chapter will describe the status of this technique and discuss its advantages.

The culture of undifferentiated plant cells for the production of metabolites has been achieved at an industrial scale by various groups since the late 1970s, in conventional stainless stirred bioreactors, up to 75 m^3. In spite of the interest in this technology, and numerous scientific and technical advances, there are very few examples of economical production of metabolites [29, 30], such as the red dye shikonin [31], ginseng cells [32], berberine [33] and more recently taxus-derived drugs [34].

Recently, interest in mass plant cell cultures increased again, using plant cell as a host cell for the production of recombinant proteins [35–40] and as an alternative to genetically modified plants in field ('biopharming'). In January 2006, the USDA Centre for veterinary biologics approved Dow AgroSciences vaccine (virus in poultry) produced in tobacco plant cell cultures in bioreactor. Also in 2007, and for the first time, the FDA gave approval to Protalix (Israel) to begin clinical trials for a drug produced in carrot cell cultures [41], which demonstrates a renewed interest in plant cell culture systems for the production of biopharmaceuticals. Also food flavourings have been recently produced by hairy-root of *Catharanthus roseus* cells [42], as well as indigo precursor indican in genetically modified tobacco plants and cells cultures [43] and therefore considered as an alternative to genetically modified plants in field.

Nevertheless, the limited development of the technology is mainly due to plant cell low growth rate (doubling time currently between 24 and 48 h) and often low productivity: one cannot expect more than 10–15 batches per year per bioreactor and, even with continuous systems, the cost of the produced biomass remains high, limiting this use to very high value products. Usual equipment and support facilities associated with aseptic bioprocess are extremely expensive, partly because large-scale production is based on stainless steel vessels, sterilized in situ. For plant cells, some estimation has been made concluding that more than 60% of the production costs are due to the fixed costs: high capital costs of fermentation equipment, depreciation, interest and capital expenditure [44]. Running costs are also high due to low yields and the need to clean and sterilize the bioreactor after each culturing cycle.

Economical viable solutions for large scale vegetative propagation of various plant species or in vitro mass cell cultures demand improved selection of the highest producing strains or the high propagation ability, determination of the right physiological conditions for growth and production, and cheaper facilities for the cultivation itself.

This is why we have recently developed new, cheap, disposable equipment for the cultivation of undifferentiated plant cells of various species and the vegetative propagation via somatic embryogenesis.

2 Micropropagation and Somatic Embryo Cultures

2.1 Bioreactors for Plant Propagation

2.1.1 Usual Bioreactors

Production of about 10,000 shoots per batch in 10- to 20-L stainless steel or glass bioreactor has been reported [21, 22]. This type of culture has often been criticized because of the limitation of oxygen exchange, problems of excessive foaming and high shear stress. Moreover, a well known and major issue is anomalous morphogenesis when green propagules are grown in liquid media, due to hyper-hydricity of leaves and stems, greatly affecting the plant survival after transplanting. Particularly, for dicotyledonous plant species, most shoots are etiolated, succulent and easily damaged by handling and environmental stress when they are transplanted to the soil [22]. The submerged type bioreactor is usually used for high-density multiplication of cultures where submersion does not result in abnormal plant development, such as the proliferation of storage organs (bulbs, corms, microtubers), meristematic clusters, embryogenic callus and small size somatic embryos [45].

In the past, we have used stirred-tank bioreactors to investigate critical parameters for the success of torpedo stage embryo production of carrot and coffee somatic embryos [11, 12]. For both species, we observed that constant submergence in liquid media completely inhibited leaf development from somatic embryos, even if the biomass was diluted. To extend the development of the embryos beyond the torpedo stage, it was necessary to subculture them onto gelose medium for their development into plantlets suitable for acclimatization. Another drawback of these bioreactors is their low performance regarding light transmittance through the biomass. When the purpose is to produce micro-plants ready for transplantation in the soil, high illumination is required in the bioreactor. However, among the different technologies involved in bioreactor engineering for plant propagation, the most difficult is the introduction of light into the biomass [22, 45].

2.1.2 Temporary Immersion Bioreactor

To avoid the problems associated with submerged cultures, a new type of bioreactor appeared in the late 1980s. These pieces of equipment were constructed to allow cycling of the culture medium, thus exposing the plant tissues to the liquid media intermittently rather than continuously. They have been used for shoot cultures of cow tree [23], *Pinus radiata* [24] and serviceberry [25, 26]. These temporary immersion bioreactors (TIB), also termed temporary immersion systems (TIS) or ‘Ebb and Flow methods’, offer the advantages of cultures in liquid medium, therefore reducing labour cost without the disadvantages of a liquid environment.

Thereafter, different versions of TIB have been developed: nutrient mist bioreactors [46], tilting and rocking vessels [47, 48] or single containers with two compartments, the

upper one containing the tissues, such as the so-called Recipient for Automated Temporary Immersion (RITA®, Vitropic, France) [49]. This small bioreactor has been used to produce torpedo-shaped embryos of *Coffea arabica* F1 hybrids [50, 51], tea [52] and sugarcane [53]. However, a system consisting of a pair of bottles connected by a silicone tube, known as the twin flask system [54], is generally preferred because construction and operation are very simple. Easy to scale up to 10–20 L, it represents a very attractive low-cost alternative. This typical design consisting of two vessels (plastic or glass), one holding the liquid medium and the other the cultures, becomes more and more popular for large-scale propagation. Air pressure is applied to push the medium from one container to the other to immerse the explants or to withdraw the medium. This process is repeated at preset intervals, and can be easily automated. It has been used for shoot multiplication of pineapple and various other tropical crops [54, 55], *Phalaenopsis species* [56, 57], and *Prunus* and *Malus species* [58]. These simple pieces of apparatus were also used for the optimization of secondary metabolite production from shoots of diverse species, such as *Ruta graveolens* or *Hypericum* [59, 60].

In our laboratory, we have implemented the Temporary Immersion Bioreactor for the scaling-up of coffee embryo conversion from torpedo to cotyledonary stages. This step, pre-germination, is mainly characterized by the greening and the acquisition of photo-autotrorophic characters. Our TIB version is similar to the twin flask systems. It consists of two glass jars (Fig. 1a) [17, 18]: a 10-L jar con-

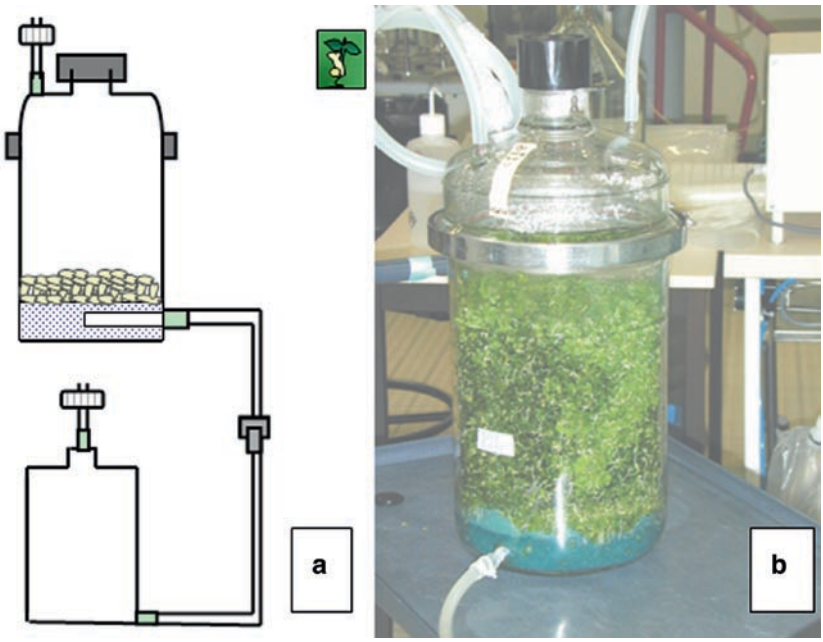

Fig. 1a,b Glass jar temporary immersion bioreactor. **a** Diagram. **b** View of a 10-L bioreactor at the end of the pregermination phase

taining the somatic embryos (20 cm diameter × 30 cm height) and a 5-L bottle containing the medium and placed below the 10-L jar and connected to an air pump. When the pump is turned on, the pressure pushes the liquid medium to the upper part. When the pump is switched off, the medium flows back down due to gravity. The main characteristic of this TIB is a polyurethane foam disk laid on the bottom of the 10-L jar. Between the immersion periods this disk isolates the embryos from the thin liquid medium layer which remains in the vessel. It retains about 1 L of liquid medium inside the vessel, and therefore maintains a sufficient relative humidity (85–90%). During the immersion, this disk has the function of an air sparger and facilitates the good ventilation of the headspace.

Temporary immersion culture brings several advantages [61]. It insures adequate oxygen transfer because the tissues are not permanently immersed in liquid media in which oxygen is poorly soluble. Shear stresses are almost suppressed due to the lack of mechanical agitation or permanent aeration. The hyper-hydricity is limited and can be controlled by manipulating the frequency and duration of immersions.

Nevertheless, targeting commercial production, the current TIB systems have to be improved, due to some limitations, such as the size of the vessels and their disposability. We encountered issues with insufficient mixing which led to the accumulation of coffee embryos forming compact aggregates. Furthermore, for some Robusta clones, the top of the biomass reaches the cover of the vessel (Fig. 1b). In this case, about 20,000 transplantable embryos can be collected from such cultures. The embryos present a large heterogeneity in size, from precocious (1 mm) to the fully expanded cotyledon stage (20 mm). Approximately, only half of the embryos have a hypocotyl larger than 5 mm which is the main criterion to select the embryo at the sowing time in the greenhouse. Most likely a non-uniform light distribution inside the TIB may be responsible for differences in growth and quality among embryos. When shoots are grown at a high density in a bioreactor and are illuminated externally, light becomes a rate-limiting factor as it can only penetrate a few centimetres through the compact biomass [45].

Consequently, we looked after large polycarbonate containers offering a greater surface-to-volume ratio to overcome light limitation. Unfortunately, these trials were not successful due to frequent contaminations at the level of the cover ring and also because of the deformation of the polycarbonate with repeated autoclaving. Therefore, the use of glass or rigid plastic TIBs at a commercial scale is possible but with limited size and performances.

2.1.3 Disposable Bioreactors

Disposable containers, up to 0.5-L, are commonly used for industrial micropropagation on semi-solid media. They are made of rigid polyethylene and bulk sterilized by gamma radiation but they are not adapted for culture in liquid medium.

In India, a commercial facility was adapted for the production of shoots of sugarcane to a new system which uses 20× 30 cm polypropylene bags, without any outlet and inlet ports [62]. After pouring 30 mL of sterile liquid medium under the

laminar flow, the bags are inoculated with single plants which grow into a bunch of plants within 1 month. The bags are closed with a heat-sealing machine and hung on ropes in a greenhouse under natural light illumination. In a 50 m^2 greenhouse, 100,000 bags can be accommodated. According to the authors, once closed, the polypropylene bags contain enough oxygen for the cultures. To avoid oxygen limitation in non-ventilated bags, disposable vessels have been developed using fluorocarbon polymer films (Neoflon® films, Daikin Industries, Ltd) [63]. This device, referred to as the 'Culture Pack', consists of a 3-L box-shaped culture vessel with an external stainless steel frame. Due to the fluorocarbon polymer gas permeability, the culture pack system has no adverse effect on shoot development of *Cymbidium* and *Spathiphyllum.* Each vessel can contain 16 shoots growing on agar medium or on rock wool plugs soaked with liquid medium.

The first disposable apparatus suitable for bulk-cultivation of propagules cited in the literature is an airlift bioreactor: it is made of non-autoclavable clear flexible plastic film, sterilized by gamma radiation, and has a vertical and conical shape [64, 65]. This so-called LifeReactor® (Osmotek LTD, Rehovot, Israel) has a working volume of 1 or 5 L and contains a sparger for bubble production. Meristematic clusters were cultured in this disposable bioreactor to grow propagules of potato, fern, banana and gladiolus [64]. The clusters' biomass increased five- to eightfold within 1 month. At the end of the culture, the propagules must be subcultured onto agar medium for their development into plants which can be transplanted to the greenhouse. This bioreactor has been adapted for temporary immersion culture just by coupling two unit devices (Ebb and Flow Bioreactor®, Osmotek LTD, Rehovot, Israel). For laboratories with limited resources, as in developing countries, a procedure to make a simple version of 1 L or less of this temporary immersion bioreactor was described [66].

Recently, commercial implementation based on 5- to 10-L autoclavable plastic bags was mentioned for two major propagation laboratories in North America but no details were given [67]. To produce metabolites, embryogenic calli of *Allium sativum* were grown in 2-L plastic bags by wave-induced agitation [68].

As a conclusion, reports on micropropagation using disposable bioreactors made of flexible plastic still remain much less frequent than those describing polycarbonate devices, such as RITA® or twin flask systems.

2.2 Box-In-Bag Bioreactors

By providing a larger surface-to-volume ratio, a horizontal design is more convenient than a vertical one to produce micro-plants that can be directly transplanted from the bioreactor to the greenhouse. However there is a serious concern about how to maintain a headspace between the immersion periods in a large and horizontal TIB made of flexible plastic. This point can be solved by developing three types of containers: (1) 3D structures such as cubes, (2) 2D bags with an external frame, and (3) 2D bags with an internal frame. Cubic structures were tested but their fabrication was found

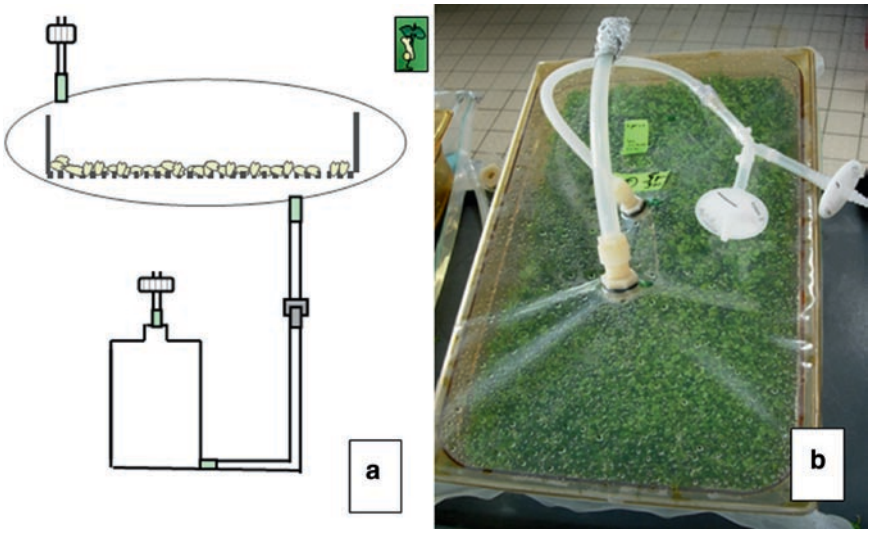

Fig. 2a,b Box-in-bag temporary immersion bioreactor. **a** Diagram. **b** View of a 10-L bioreactor at the end of the pregermination phase

to be too expensive for micropropagation purposes. In 2006, at the 27th International Horticultural Congress (Seoul), we presented a very simple solution corresponding to the third option and consisted of placing a rigid box inside a plastic bag (Fig. 2) [16]. This so-called 'box-in-bag' bioreactor is easier to handle than an empty bag having an external frame. We describe a detailed account of how to prepare an example of this bioreactor for the pre-germination of coffee somatic embryos.

Bags are made from a transparent plastic film composed of polyethylene and nylon (CPL613, Charter Medical, Lydall Group, NC, USA) and are supplied closed on three sides. They are 750× 420 mm in size and have two polyethylene ports moulded into the film. The port A (12 mm diameter) is positioned above the bag; it is used for the inoculation step and then for air outlet (Fig. 3a). The port B (7 mm diameter) is located below the device and used for air inlet and medium entrance and exit.

A 50 × 30× 10 cm rigid box made of a transparent and ionisable plastic, for instance polycarbonate (Gastronorm 1/2, Cambro, Huntington Beach, USA), is introduced without its cover into the bag. The bottom of the box is perforated with 1–1.5 mm diameter holes, or better, with two 50 mm diameter holes in which 90 × 30 mm polyurethane foam disks are fixed (Tramopen 45 ppI, Javaux, Maintenon, France) (Fig. 3b). One of the foam disks is located just above port B, which functions as both the medium inlet and air entrance inlet. A funnel made of silicone tubing is fixed through this disk to permit the rapid introduction of the medium inside the box at the beginning of the immersion periods. Silicone tubing is fixed to each port and female polycarbonate connectors and plugs (Cole Parmer, Minneapolis, USA) are placed at their extremities (Fig. 3c). The fourth side is heat sealed and the system gamma-sterilized (Ionisos, Sablé, France).

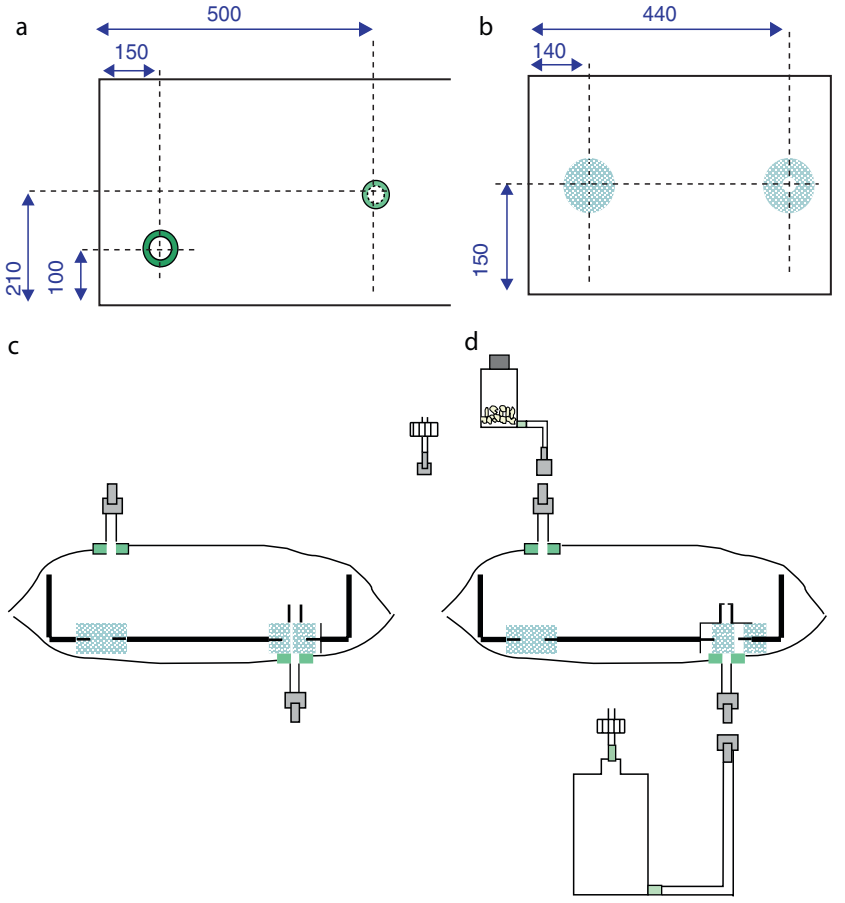

Fig. 3a–d Details for the preparation of a 10-L box-in-bag temporary immersion bioreactor. **a** Bag. **b** Box. **c** Closing the bioreactor and preparation for sterilization. **d** Inoculation and connection to the medium vessel

The torpedo-shaped embryos are aseptically introduced into the bioreactor by using an inoculator bottle connected to port A (Fig. 3d). A 0.2-µm sterilizing air filter (Midisart or Sartofluor, Sartorius, Germany) is then connected to this port. The glass bottle containing 5 L of autoclaved medium is connected to port B. The whole system is placed in the culture room, the medium tank beneath. The later is connected to a compressed air source. During the culture, overpressure at 0.5 bar is applied through the 0.2-mm vent filter of the reservoir bottle at repeated intervals, generally twice a day for 6 min, forcing the medium into the bag. In these conditions, 5 L of medium can be transferred in 1 min from the reservoir to the bioreactor. Fresh air is then injected inside the bag which is inflated over a period of 5 min. Using CO_2 as a gas tracer, it has been checked that this period of time is sufficient to refresh fully the atmosphere.

2.3 Case Study: Coffee Somatic Embryogenesis

During the 1990s, three major progress steps led to the scaling up of somatic embryogenesis of the allogamous species *Coffea canephora* (var. Robusta) by reducing the labour cost input: (1) culture of embryogenic cells and torpedo stage embryos in liquid media [9, 10, 12], (2) pre-germination from the torpedo to the cotyledonary stage by temporary immersion in liquid media [50], and (3) ex vitro germination by directly sowing cotyledonary stage embryos, without true leaves, in the greenhouse [14, 69]. From 1996 to 2000, large-scale Robusta field trials were set up representing a total of 12,000 somatic seedlings from ten clones. The trees did not show major undesired somaclonal variation and no significant differences were seen between the somatic seedlings and the microcutting-derived trees for the observed morphological traits and the yield characteristics [15, 17].

Based on the progress mentioned above, a pilot process for large-scale production of pre-germinated Robusta somatic embryos was implemented in our centre [18]. Three operators can produce a total of 3.0 million pre-germinated embryos per year using 10-L glass TIBs. A production cycle is started every month and requires a total of 4–5 months to produce somatic embryos ready to be sown in the greenhouse. A cycle can be summarized as follows:

- Each run starts from 60 g FW of embryogenic cells multiplied in liquid medium.
- For the production of torpedo stage embryos (Fig. 4a), the cells are transferred into submerged cultures, stirred-tank bioreactor or Erlenmeyer flasks, inoculated at a density of 1.0 g L−1.
- For pre-germination up to cotyledonary stage (Fig. 4b), the torpedo stage embryos are transferred into 35–40 10-L glass TIBs. Each bioreactor is inoculated with 30–60 FW g of embryos. When most of the embryos turn green, generally within 2–4 weeks, the medium is replenished by fresh medium.
- Within 2–3 months, a total biomass of about 7–9 kg FW is collected from the bioreactors, corresponding to 2–250,000 cotyledonary embryos.

A significant part of the labour is devoted to the handling and the cleaning of the glass jars. However, this material is heavy and breakable. In 2005, we started trials to develop bioreactors made of flexible plastic film. By a step-by-step approach, these trials led us to the box-in-bag design (Fig. 4c). At the end of the pregermination phase, the box-in-bag bioreactor can be easily cut to harvest the embryos (Fig. 4e). We compared its performance to the 10-L glass jar TIB by inoculating the bioreactors with the same quantity of embryos issued from the same cell lines. The biomass and the number of embryos having a hypocotyl longer than 5 mm are significantly higher than in the 10-L glass vessel (Table 1), probably because the area is 1,260 cm^2, i.e., four times the area of the 10-L glass jars for a similar volume. The embryos look very green and their ability to develop a plant is similar to those grown in the glass bottles. This experiment confirms the importance of light intensity on embryo quality, as they are greener and taller under higher light intensity conditions. In 2008, we are planning to replace all the glass vessels by such disposable containers. A significant increase in term of embryos produced per operator, about 50%, is expected.

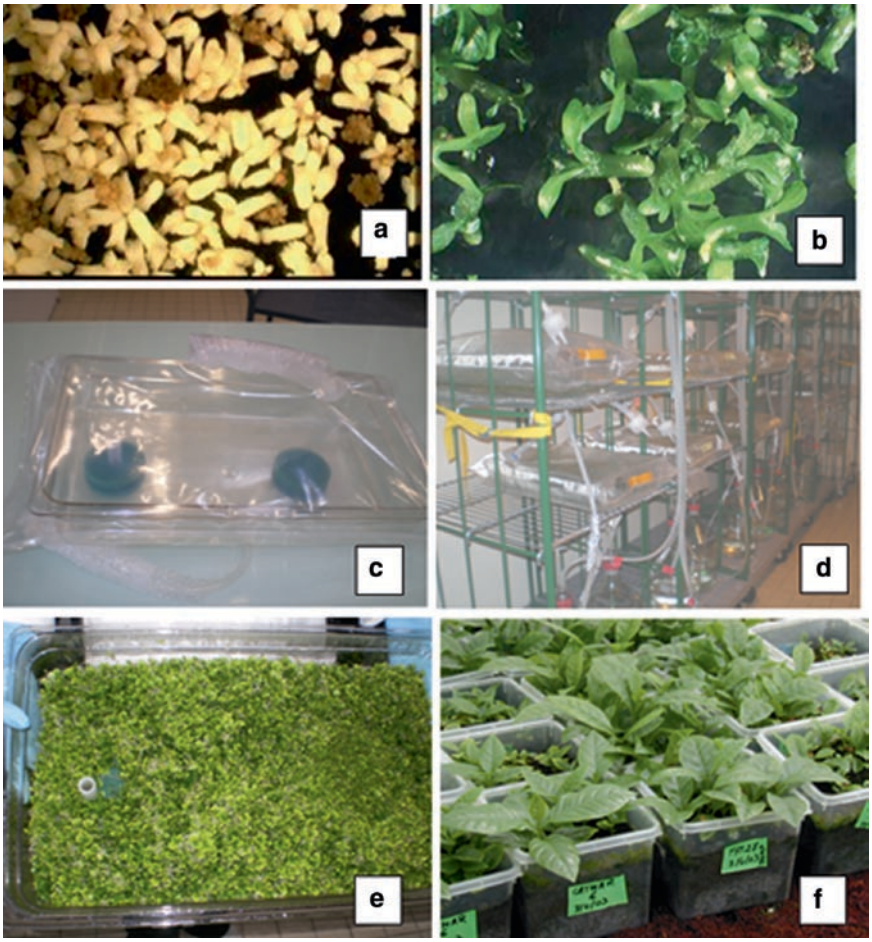

Fig. 4a–f Pilot process for the production of pre-germinated Robusta embryos. **a** Torpedo-stage embryos. **b** Cotyledonary-stage embryos. **c** A 10-L box-in-bag disposable bioreactor before inoculation. **d** Overview of a culture room with disposable bioreactors. **e** View of cotyledonary embryos produced in a disposable bioreactor. **f** Ex vitro germination for the conversion to fully developed plantlets

Table 1 Comparison of two types of 10-L temporary immersion bioreactors for the pre-germination of Robusta somatic embryos (clone FRT23) (reproduced from [16])

	Pregermination		Ex vitro germination
TIB type	FW[a]	Pregerminated embryos[a]	Embryo-to-plantlet conversion rate[b]
	g/TIB	nbr/TIB	%
Glass jars	519	18,576	42
Box-in-bag	943	26,794	57
	S[c]	S[c]	NS

[a]Means of three experiments
[b]Means of five replicates of 25 embryos
[c]S: significant ($P < 0.01$)

Considering germination tests conducted under a plastic tunnel in our greenhouse (Fig. 4f), our current production capacity is enough to regenerate potentially about 1.0 million plantlets. This potential can be increased up to 1.5–2.0 million if the ex vitro germination is conducted under a microenvironment, achieved by placing a transparent cover 2–3 cm above the embryos which would benefit from the positive effect of the CO_2 released by horticultural media as peat or coconut fibres [19]. The embryos are sent to coffee producing countries where they are sown in ex vitro conditions under a tunnel plastic to develop plantlets bearing true pairs of leaves within 4–6 months. The plantlets are then grown in polyethylene bags in the nurseries during 6–12 months before their transplantation to the field [18, 70].

2.4 Advantages and Limitations

The box-in-bag disposable TIB combines the advantages provided by the two types of plastics, rigid and flexible. The rigid plastic box facilitates the manipulations, maintains a culture headspace between the immersion periods and allows a horizontal distribution of the biomass, allowing better oxygenation and illumination. Moreover, the possibility of stacking several boxes one top of another makes this system easy for transportation: it is possible to send in vitro plants keeping them inside the bioreactor in which they have grown. The international exchanges of sterile plant material are therefore greatly facilitated. The flexible plastic is a disposable device (low cost, simple to operate) and offers a high process security and a great versatility by allowing a large diversity in sizes and designs. Both plastics can be used together as a mini-greenhouse for storage, shipment, hardening, and probably even for ex vitro germination under microenvironment conditions.

The box-in-bag TIB is very easily scalable because its size can be increased without the cost impact of custom-made moulders; a lot of rigid and translucent plastic boxes of different sizes are commercially available, for instance from gathering retailers. In order to illustrate the versatility it offers in design, we present a very simple TIB consisting of a bag containing both embryos and medium (Fig. 5). The immersion is simply achieved by manually moving the box into the medium.

The security of disposable devices allows the implementation of the cultures in greenhouses instead of expensive culture rooms. Moreover, growth under natural illumination is a relevant strategy to insure an excellent survival rates when the in vitro plants are transferred to the soil [62]. The box-in-bag system also offers the possibility for bulk-cultivation of coffee somatic embryos in photoautotrophic conditions, i.e., without sugar and with CO_2 enrichment. This culture method improves the quality of the vitroplants when they are grown individualized on gelose media or plugs [71–74]. Obviously, cultures conducted under photoautotrophic conditions will only be efficient if the embryos are enough illuminated.

Unsatisfactory mixing sometimes remains a sticking point in this large TIB. Nevertheless, if it is necessary to disperse the immersed embryos, the operator can easily move the bag when it is inflated due to its light weight. It is not totally

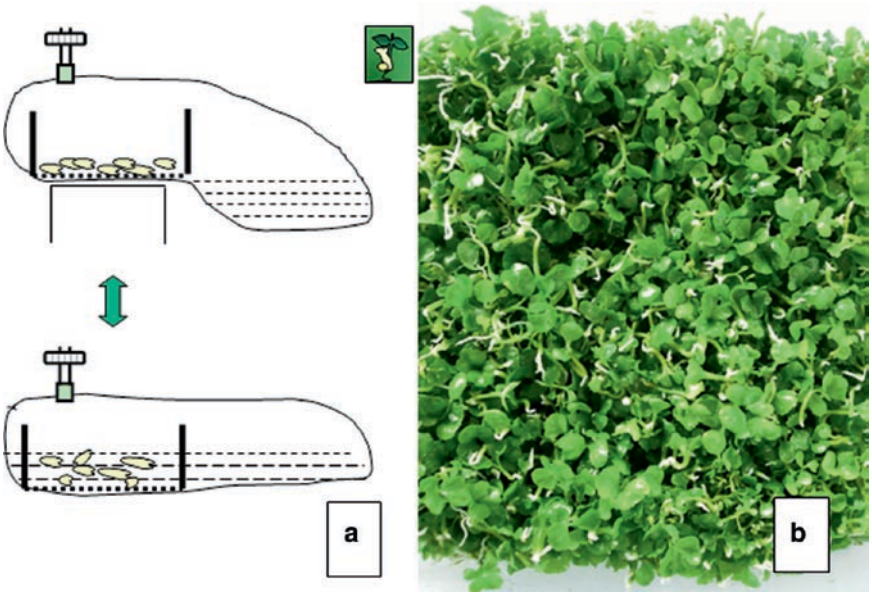

Fig. 5a,b Very simple temporary immersion bioreactor. **a** Diagram. **b** View at the end of the pregermination phase

disposable because some components are too expensive, as the connector systems. Moreover, it is not available 'ready-to-be-used' yet. We are investigating different sizes and designs of ready-to-be- used bioreactor manufactured by Hegewald Medizinprodukte (Lichtenberg, Germany).

3 Mass Plant Cell Culture and Metabolite Production

3.1 Disposable Bioreactors for Plant Cells

In order to minimise production costs, a few alternatives to traditional stainless steel bioreactors have been developed [75–77]. Singh [75] developed a disposable bioreactor with an original agitation apparatus, using an inflated bag placed on a rocking mechanism that induces a wave-like motion to the liquid contained therein. This system is mainly used for animal cell cultures. Few papers have been published in plant cell domain, and only with small working volumes [68, 78, 79].

We are developing two new flexible, scalable, plastic disposable bioreactors [8]. The first is based on the principle of a wave/undertow mechanism providing convenient mixing and aeration to the plant cell culture ('WU bioreactor'). The second is a new bubble column bioreactor that allows an easy increase of working volumes (up to several hundred liters) with the use of multiple units ('SB' bioreactor). Both

systems are pre-sterilized and have been designed to allow for medium introduction, inoculation and sampling.

To assess the performances of these two new systems, they will be compared in terms of biomass accumulation (tobacco and soya) and isoflavone production (soya) to two so-called 'traditional' systems: Erlenmeyer flasks and a 14-L stirred tank bioreactor, which are widely used in the laboratory.

3.2 Wave and Undertow and Slug Bubble Bioreactors

3.2.1 Description

WU

The Wave and Undertow (WU) bioreactor consists of a large flexible plastic container partly filled with medium and inflated with air (Fig. 6). The system is located on a horizontal table equipped on one side with a moveable platform. The intermittent rising movement of the platform to the rest point, and down/descending movement back to initial position enable continuous mixing and aeration through the wave/undertow

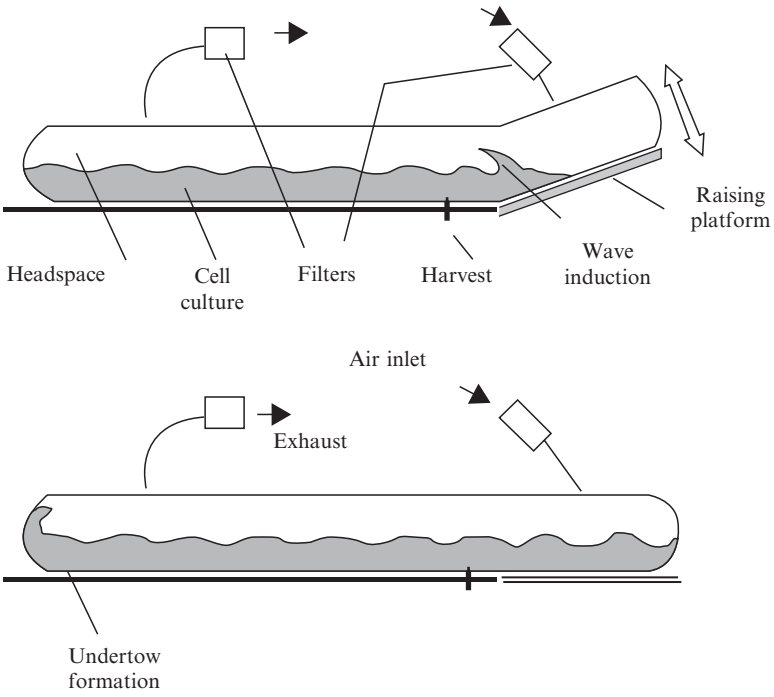

Fig. 6 Schematic diagram of the WU bioreactor (reproduced from [8])

motion. The platform ascension leads to the formation of a wave that propagates through the bag and bounces off the extremity creating an undertow which returns to the initial point. This action is repeated, creating a new impulse to ensure persistence of flow within the WU bioreactor. Sterile air is continuously fed in the headspace. Wave/undertow induction provides liquid culture mixing and bubble-free aeration. Oxygen transfer is accomplished by transport from the headspace air to the liquid culture.

SB

The Slug Bubble (SB) bioreactor consists of a vertical flexible plastic cylinder filled with medium up to circa 80% of its height (Fig. 7). Agitation and aeration are achieved through the intermittent generation of large cylindrical single bubbles at

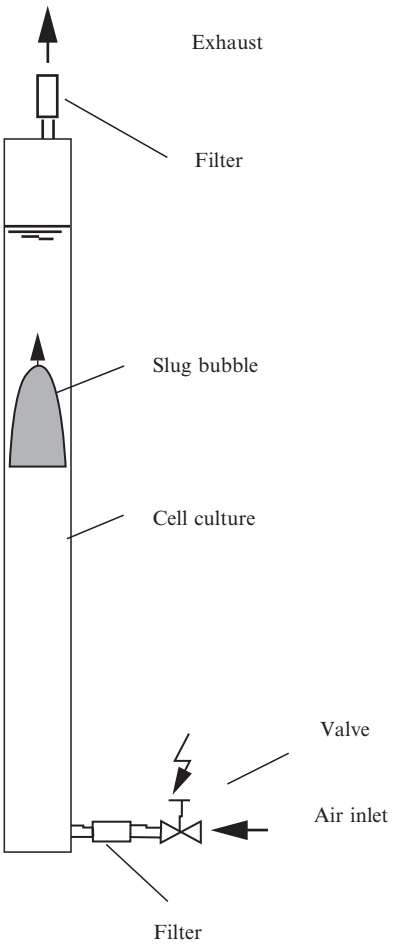

Fig. 7 Schematic diagram of the SB bioreactor (reproduced from [8])

the bottom of the system that rise to the top of the cylinder. These bubbles are comparable to 'Taylor bubbles', or 'slug bubbles' [80–82]. These bubbles can be described as long bullet-shaped bubbles, which nearly occupy the entire cross-section of a pipe. The rear of the slug bubble is a region characterized by strong mixing, where all transfer processes are enhanced. Mixing and oxygen transfer are therefore achieved at the same time.

3.2.2 Engineering Aspects (Manufacturing/Working)

Both systems are entirely made of plastic components. They are either 'home-made' or contract-manufactured and they are designed to allow air inlet, medium introduction, inoculation, sampling and air outlet. They are maintained in an air-conditioned room for temperature control.

WU

With regard to manufacturing, as the wave generation (which is the basis of the agitation) leads to the bioreactor to be regularly bent (always at the same spot, from 200,000 to more than 1 million times in a batch), the plastic film used to manufacture the WU needs to be flexible and capable of enduring this repeated movement. Two types of plastic films have been found that can satisfy that demand: PVC (polyvinylchloride) and PU (polyurethane).

Most systems have been built in-house from biopharmaceutical grade PVC (Achilles, WA, USA); however this film displayed two main disadvantages. First, it was so pliable that it loosened during system manufacture and during pressure test leading to a non- symmetrical and extensible systems (that is of variable volume for given lengths). Second, it was not flexible enough to avoid perforations, followed by leaks, at the weakest points. Two PU films (a polyurethane ester and a polyurethane ether, manufactured by Epurex Films, a Bayer MaterialScience company) have also been used. However, whilst being flexible, they do not show the extreme extensibility displayed by the Achilles PVC, which permits bags to be manufactured more easily and of constant volume. Finally, another PVC film, 'Transfufol', provided by the company Lider, to whom the making of WU bags was contracted, has also been used. For sterilisation, PVC films are autoclaved (Fedegari, Italy) for 40 min at 121 °C. PU films are sterilized by gamma radiation (12–25 kGy).

With regard to working, the pre-sterilized system is set up on the table and undergoes a pressure test (air filling) to check for potential leaks. If the bag is intact, medium can be added and the system prepared before inoculation. Platform movements are simply achieved by pneumatic jacks located under the platform. The time needed to allow for the platform to rise and stay up (T1), and the time necessary for the platform to descend and stay down (T2) can be adjusted easily. Other parameters are adjustable: the percentage of culture volume located on and lifted by the platform(s) (v), the platform raising angle (α), and the air inlet flow rate (Q). The agitation

Table 2 WU bioreactor volumes and dimensions

Total volume (L)	Length (*L*) (cm)	Width (*W*) (cm)	*L/W*	Working volume (L)	Filling level (%)
60	175	35	5	20	33
				30	50
200	280	55	5	70	33
				100	50
750	390	75	5	250	33

Table 3 SB bioreactor volumes and dimensions

Total volume (L)	Working volume (L)	Diameter (*D*) (cm)	Floor surface (cm^2)	Height (cm)	Unaerated suspension height (cm)	Aspect ratio (*H/D*)
14	10	8.5	60	250	175	21
24	20	11.0	100	250	210	19
64	50	18.0	250	250	200	10
90	70	18.0	250	350	280	15
135	100	20.2	320	350	310	21
175	125–150	22.5	400	420	315–380	14–17

intensity is adjusted depending on the batch volume and on the oxygen demand of the cell strain (Table 2).

SB

With regard to manufacturing, the system is made from biopharmaceutical grade polyethylene (CPL613; Charter Medical, Lydall Group, NC, USA) and gamma-sterilized (Ionisos, France) or produced and pre-sterilized by Charter Medical using the same flexible plastic film.

With regard to working, the pre-sterilized system is set up in a rigid plastic tube and undergoes a pressure test (air filling) to check for leaks. As previously, if the bag is intact, medium can be added and the system prepared before inoculation. The slug flow regime is artificially produced by intermittent gas supply, using a solenoid valve and compressed air. The valve relieves a predetermined quantity of air at the given frequency. The quantity of air can be adjusted by changing the inlet pressure (P), the valve opening duration (T1), or the bubble frequency (f). The usual inlet pressure is from 0.03 to 0.05 MPa for 10–70 L (working volume) reactor. The corresponding averaged flow rates vary between 0.1 and 0.5 vvm, which is consistent with values usually encountered with plant cell culture [29]. The aeration intensity is defined according to the batch volume thanks to the programming device. The rigid plastic tube (PVC) maintains the reactor vertical. A horizontal slot (3–7 cm wide) is cut up lengthwise for the crossing of different inlets and outlets and the observation of the culture. Table 3 presents different sizes of SB bioreactors.

For both systems, aeration is achieved with compressed air sterilized trough membrane air filter (Sartofluor, Sartorius AG, Germany). Culture medium is either sterilized by autoclave and aseptically transferred to the systems, or sterilized by membrane filtration (Sartobran, Sartorius AG, Germany).

3.2.3 Characteristics (k_La/O_2 Transfer)

The volumetric oxygen mass transfer coefficient (k_La) of the Erlenmeyer flask, stirred-tank bioreactor, WU and SB systems were measured in duplicate by the dynamic gassing-out (air) method using a polarographic, temperature-compensated, dissolved oxygen probe. The liquid (water) in the system is deoxygenated by gassing nitrogen through the inlet filter. When the dissolved oxygen (DO) probe (Ingold) reached 0% saturation, aeration and agitation were started and the rising DO is recorded as a function of time. The value of k_La is defined as:

$$\frac{dC}{(C^*-C)} = k_L a.dt$$

where C: oxygen concentration in the liquid at time t (mg L^{-1})
C*: oxygen solubility in the liquid (mg L^{-1})

For the Erlenmeyer flasks, the same procedure was used, the only difference being that the O_2 probe is a Clark oxygen electrode immerged in the chamber body of an oxygen system, through which the liquid from the Erlenmeyer flask circulates along a closed circuit by a peristaltic pump. This system avoids the perturbations provoked by the direct immersion of the probe in the Erlenmeyer flask.

Oxygen mass transfer coefficients measured in the WU bioreactor (Table 4) were lower than those observed in a traditional stirred tank bioreactor, but comparable to or higher than those encountered in Erlenmeyer flasks or other known flexible disposable cell culture systems such as the Wave Bioreactor [68, 75].

Table 4 Volumetric mass transfer coefficient (k_La)

Culture system	Working volume	Agitation/aeration				k_La (h^{-1})
250-mL Erlenmeyer flasks	100 mL	100 rpm				5
14-L stirred-tank bioreactor	10 L	0.04 vvm; 150 rpm				3
		0.25 vvm; 200 rpm				15
		0.5 vvm; 100 rpm				29
					vvm	
60-L WU	20 L	At given conditions			0.12	10
60-L WU	30 L	At given conditions			0.18	9
		P	T1	T2	vvm	
24-L SB	20 L	0.04	0.5	7.0	0.31	7
			0.5	4.0	0.49	16
64-L SB	50 L	0.05	0.3	7.0	0.17	10
			0.5	7.0	0.21	17

vvm: air flow rate (vvm); P: air inlet pressure (MPa); T1: valve opening duration (s); T2: time interval between two successive valve openings (s)

The Slug Bubble bioreactor showed good oxygen transfer capacities. Oxygen transfer coefficients (Table 4) were comparable to coefficients for traditional culture systems such as Erlenmeyer flasks and traditional bioreactors in the range of agitation and flow rates compatible with the oxygen demand and low shear stress required for plant cell cultures. As it might have been expected, these results also show that increasing the valve opening time (bubble size) or the opening frequency (bubble frequency), both leading to the increase of the average gas flow rate, resulted in higher $k_L a$ values.

3.3 Case Studies: Tobacco and Soya Cell Cultures

3.3.1 Plant Material, Methods

The tobacco cell strain and the isoflavone-producing soya strain are grown in 250-mL Erlenmeyer flasks containing 100 mL medium on a gyratory shaker (New Brunswick Scientific, USA) at 100 rpm (shaking diameter 20 mm), at 25 °C in darkness. The *Nicotiana tabacum L* BY2 cell strain [83] is grown in MS components [84] with KH_2PO_4 (270 mg L^{-1} instead of 170 mg L^{-1}), 0.2 mg L^{-1} of 2,4-dichlorophenoxyacetic acid and 30 g L^{-1} of sucrose, at pH 5.8 and subcultured every week. The *Glycine max* (L.) Merr cell strain is cultivated in Gamborg medium [85] supplemented with 30 g L^{-1} sucrose and 1 mg L^{-1} 2,4-dichlorophenoxyacetic acid at pH is 6.0 and subcultured every 2 weeks [4]. For both strains, initial density is close to 30 g L^{-1} and medium is sterilized by autoclave (30 min at 115 °C).

The stirred-tank bioreactor is a 14-L vessel (New Brunswick Scientific, USA) equipped with a pitched blade impeller (10 L working volume). The bioreactor containing 9 L of fresh medium is autoclaved for 40 min at 121 °C. Dissolved oxygen is maintained at 30% by increasing or decreasing airflow rate. The bioreactor is equipped with a sterilisable oxygen probe (lnPro 6110, Ingold Mettler Toledo GmbH, Switzerland), and a mass flowmeter. The stirrer speed is adjusted at 100 rpm. For cultures in WU bioreactors (10, 20, 30 and 100 L working volumes) and SB bioreactors (10, 20, 50 and 70 L working volumes), medium addition, agitation and aeration have been described earlier.

In all systems, inoculation is performed as follows: 14-day-old soya cells or 7-day-old tobacco cells are aseptically transferred from Erlenmeyer flasks or from the stirred-tank bioreactor (for the WU and SB bioreactors) to the bioreactor via a sterile container. The inoculum is prepared in order to reach circa 30 g L^{-1} fresh weight in the inoculated bioreactor.

Cell doubling time (td) is defined by the expression: $td = \ln 2/\mu$, where (μ), the apparent growth rate, is calculated as:

$$\mu = \frac{\ln (final\ DW\ /\ initial\ DW)}{\Delta t} \quad \text{during exponential growth phase.}$$

Extraction and analysis of isoflavones are described in [8].

3.3.2 Results

Tobacco Cell Culture

Figures 8 and 9 show an example of the growth kinetics currently obtained in the different culture systems. Tobacco cells were cultivated in four different volumes in the WU bioreactor and in the SB bioreactor. The results obtained were similar to

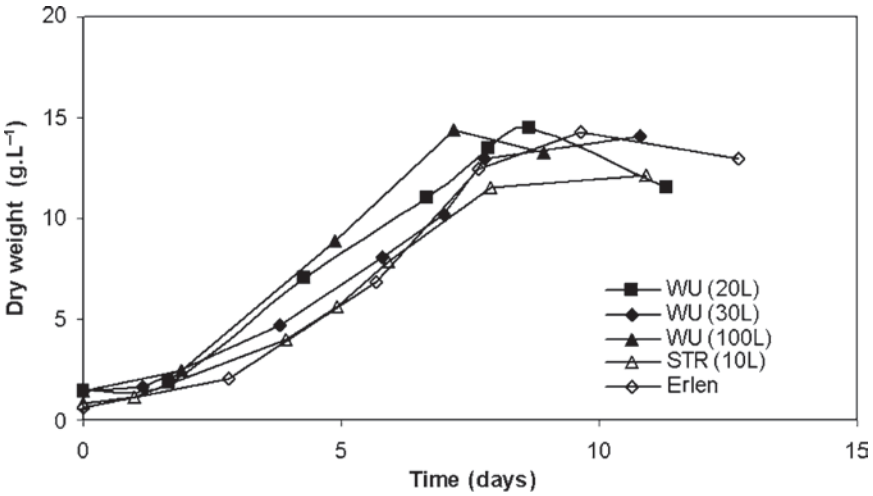

Fig. 8 Growth of tobacco cell cultures in Erlenmeyer flasks, stirred-tank bioreactor and WU bioreactor (reproduced from [8])

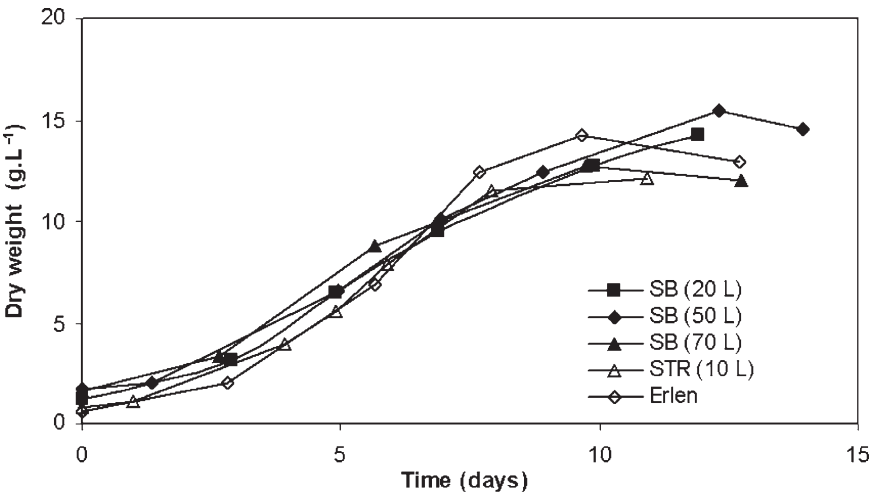

Fig. 9 Growth of tobacco cell cultures in Erlenmeyer flasks, stirred-tank bioreactor and SB bioreactor (reproduced from [8])

Table 5 Growth parameters of tobacco and soya cell cultures in Erlenmeyer flasks, 10-L stirred-tank, *WU* and SB bioreactors

	Tobacco cells			Soya cells		
Type of system (working volume)	Number of independent experiments	Max dry weight ($g\ L^{-1}$)	Doubling time (days)	Number of independent experiments	Max dry weight ($g\ L^{-1}$)	Doubling time (days)
Erlenmeyer flask[a]	3	13.9 ± 0.5	2.1 ± 0.4	3	14.6 ± 0.4	2.8 ± 0.6
STR (10 L)[b]	3	14.4 ± 2.1	1.9 ± 0.3	3	12.9 ± 4.7	3.5 ± 1.5
WU (10 L)	2	13.6 ± 0.0	2.3 ± 0.1	1	14.3	2.5
WU (20 L)	4	12.8 ± 1.9	2.3 ± 0.4	5	13.8 ± 1.8	3.2 ± 0.7
WU (30 L)	3	12.6 ± 1.3	2.0 ± 0.2	2	16.5 ± 0.5	2.4 ± 0.2
WU (100 L)	5	13.0 ± 1.1	2.3 ± 0.3	2	15.5 ± 0.1	2.2 ± 0
SB (10 L)	2	17.2 ± 0.6	2.0 ± 0.2	–	–	–
SB (20 L)	5	13.7 ± 0.6	2.1 ± 0.2	6	13.9 ± 0.9	2.8 ± 0.3
SB (50 L)	3	14.2 ± 1.2	2.0 ± 0.4	3	14.7 ± 2.0	2.7 ± 0.5
SB (70 L)	2	12.9 ± 0.3	2.4 ± 0.1	–	–	–

[a]Erlenmeyer flask: 250 mL with 100 mL medium, 100 rpm on a gyratory shaker. 26 °C
[b]10-L stirred-tank bioreactor: New Brunswick bioreactor, 100 rpm, 0.25–0.5 VVM, 26 °C

Table 6 Isoflavone production

Type of system (working volume)	Number of experiments	Max. Isoflavone concentration ($mg\ g^{-1}$ DW)
Erlenmeyer flask[a]	6	61 ± 35
Stirred-tank bioreactor (10 L)[b]	3	28^{NS} ± 20
WU (20 L)	5	39^{NS} ± 39
SB (20 L)	6	23* ± 11
SB (50 L)	3	48^{NS} ± 34

[a]Erlenmeyer flask: 250 mL with 100 mL medium, 100 rpm on a gyratory shaker. 26 °C
[b]10-L stirred-tank bioreactor: New Brunswick bioreactor, 100 rpm, 0.25–0.5 VVM, 26 °C
ANOVA: *NS*: nonsignificantly different from Erlenmeyer flasks*:significantly different from Erlenmeyer flasks; $p < 0.05$

those observed in traditional systems (Erlenmeyer flasks and bioreactor). This was confirmed using data collected from several independent experiments (Table 5). These results establish that cultivation in WU bioreactor with a 100 L working volume can be used instead of a traditional stainless steel stirred tank bioreactor. For the SB bioreactor, cultivation up to 70 L working volume has been demonstrated.

Soya Cell Culture and Isoflavone Production

The results are different from those obtained with tobacco cells (see Table 4): the stirred tank bioreactor is the least efficient culture system. Soya cell suspensions are much more aggregated (clump formation) than tobacco suspension. This result could be due to a higher shear stress sensitivity of the cells due to mechanical impeller agitation. The cultivation in WU and SB bioreactors is similar to cultivation in Erlenmeyer flasks.

Isoflavone production has been measured in three systems (20-L WU and SB bioreactors, and 50-L SB bioreactors), in comparison with Erlenmeyer flasks and stirred-tank bioreactor (Table 6). A large variability is observed between experiments, whatever the culture system, showing that the optimal control of the culture conditions for isoflavone production is not reached in any culture system. Statistical analysis (ANOVA) confirms that the culture systems are not significantly different from the Erlenmeyer flasks, except the SB bioreactor (20-L scale) which appears to give lower concentrations. New culture systems are not detrimental to the production of isoflavones, but further investigation is required to identify the key parameters linked to the biosynthesis and accumulation of isoflavones.

3.4 Scale-Up; Advantages and Limitations

3.4.1 Scale-Up and Volume Increase

Studies are still on-going to scale-up the systems, that is increase the working volumes while maintaining similar growth conditions.

The WU systems are geometrically similar (constant bag length over width ratio) with a filling level ranging from 33 to 50%; for a given size, increasing the filling level favours the system compactness but also impacts the wave formation or quality and therefore the growth conditions. For the SB bioreactor, the most crucial parameter is the column diameter; a small increase deeply impacts the total volume but is also, at the same time, truly detrimental to bubble formation and therefore to mixing and oxygenation.

Besides traditional scale-up as described above, both systems also present another form of scaling-up. For large culture volumes, the WU bioreactor can be adapted to grow the inoculum and the batch in the same bag (Fig. 10). The bioreactor

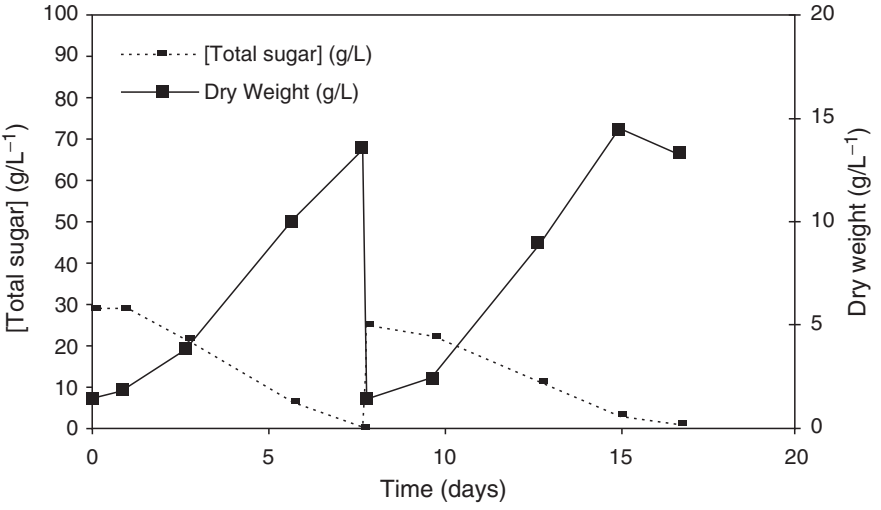

Fig. 10 Growth of tobacco cell culture in two successive steps (10 and 100 L working volumes) in a 200-L WU bioreactor (reproduced from [8])

is partitioned during the first part of the culture (smaller surface area) before going back to its initial shape when medium is added to grow the entire batch. This technique can reduce the risks of contamination through successive inoculations and decrease scale up time. Nevertheless, the difference of several parameters (headspace volume, filling level, k_La, etc.) from one phase to the other has to be studied to validate this improvement. For the SB bioreactor, running multiple experiments in parallel or increasing production volumes can be rapidly achieved having several culture systems close together (or even connected) in a small area.

3.4.2 Advantages and Limitations

The disposable systems described here offer many benefits and practical advantages in comparison with traditional systems, especially lightness and versatility and they also permit new designs (that would have been impossible in glass or stainless steel, for example the WU system). The new design (and the novel agitation mode) also implies new engineering studies to prove at least the innocuity, at best the added value of these novel systems in comparison with well known and trustable ancient systems, especially if these bioreactors are to be used in the biopharmaceutical industry. Improved or simplified designs finally mean less or no maintenance and minimal needs for cleaning. Scale up is simplified and faster, up to a certain limit, since flexible containers will not be able to hold large volumes without any support. Working with disposable bioreactors instead of re-usable ones also implies to trust and validate the manufacturer and/or the manufacturing process (whether the systems are home-made or contract-manufactured) since each bioreactor is a novel process unit.

4 Conclusion

There is a consensus according to which temporary immersion cultures will play a dominant role on the future of plant micropropagation but ideal commercial equipments have yet to be invented [86]. Particularly, it is a promising way to easily optimize light illumination inside bulk cultures of plant tissues. So far, the various illuminated bioreactors designed to introduce light to the cultures through optical fibre are not yet efficient [45]. Nevertheless, one of the key points is the quality of ports and welds: for a commercial implementation, this material must be manufactured by plastic specialists. Scale-up of micropropagation may probably be facilitated by the commercialization of large and adapted plastic bags. The box-in-bag bioreactor can be an example of such innovations and a promising technology even if it is too early to say if such a disposable device could be applied for other plant species or for the micropropagation from shoots or other organogenic tissues.

The use of disposables is slowly but steadily increasing in the field of cell culture where their advantages for the manufacturing of biopharmaceuticals are well-known and largely agreed on [87]:

– Simplified facility design
– Greater flexibility for small to medium scale operations
– Modular manufacturing done in a ‘rapid factory’ based on disposable, pre-validated units that can be deployed very quickly
– Producing otherwise uneconomical drug candidates
– Possible multi-product facilities: multi-product manufacturing in one suite using disposables will allow high capacity utilization
– Disposables minimize cross contamination (cell therapy procedures)

The present chapter underlines the interest of developing disposable plastic-based systems with two different applications in the field of plant biotechnology: small to medium scale plant cell cultures can be easily obtained for biomass, metabolites or recombinant proteins production; for plant propagation, the system we have developed is, to our knowledge, the first one allowing the routine production of millions of coffee plantlets each year.

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