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Improvement of plastic-based disposable bioreactors for plant science needs

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Abstract The present article describes two new applications of plastic-based cell culture systems in the plant biotechnology domain. Different types of bioreactors are used at Nestlé R&D Center-Tours for large scale culture of plants cells to produce metabolites or recombinant proteins and for mass propagation of selected plant varieties by somatic embryogenesis. Particularly, recent studies are directed to cut down the production costs of these two processes by developing disposable cell culture systems. For large scale 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). Vegetative propagation of elite plant varieties is achieved through somatic embryogenesis in liquid medium. A pilot scale process has been recently set up for the industrial propagation of *Coffea canephora* (Robusta coffee). The current production capacity is 2.5–3.0 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 in 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.

Keywords Coffee · Disposable · Plant cell culture · Somatic embryogenesis · Temporary immersion

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Introduction

Plastic-based culture systems have become a useful tool in research and biotech processes and are now widely spread, at small and medium scales, especially in mammalian cell-based manufacturing units. This technology is a great potential for various applications, notably for plant biotechnology, where such culture systems can be used for two applications, as described below.

(a) Mass plant cell culture for the production of metabolites has been studied for many years. In spite of the interest of this technology, there are very few examples of economical production (Takahasi and Fujita 1991; Hibino and Ushiyama 1999; Matsubara and Fujita 1991; Venkat 1998). The limited development of the technology at an industrial scale is due to well-known drawbacks, such as low growth rate, low productivity and/or instability of the productive

cell strains and high cost of the traditional bioreactor technology (stainless steel). However, recent publications dealing with the production of recombinant proteins by plant cells indicate a renewed interest in this technology (Hellwig et al. 2004; Girard et al. 2004, 2006; Soderquist and Lee 2005; McDonald et al. 2005). Furthermore, Dow AgroSciences received in January 2006 from USDA the world's first regulatory approval for a plant cell-made vaccine. Finally, the Israeli biotech company Protalix has recently reported the production of glucocerebrosidase by plant cells in disposable bioreactors (Shaaltiel et al. 2007).

(b) Somatic embryogenesis in liquid medium is a powerful alternative to other vegetative propagation techniques for mass propagation of selected varieties of plants. Our aim is to propagate plants with added genetic value, such as coffee genotypes selected from a Core Collection established in the field in Ecuador and Thailand (Pétiard et al. 2004). With the collaboration of National Institutes of different coffee producing countries, the genotypes are selected according to different characters (yield, quality...), multiplied by somatic embryogenesis and then distributed to the farmers. The first somatic embryos in coffee species were reported by Staritsky (1970) and the first indirect somatic embryogenesis by Söndhal and Sharp (1977). Then, three major advancements have led to the scaling up of coffee somatic embryogenesis: (1) liquid media cultures for the multiplication of embryogenic cells and for the production of torpedo embryos, both described, for the first time, in our laboratory (Zamarripa et al. 1991a, b; Ducos et al. 1993), (2) temporary immersion method for the culture of green cotyledonary embryos (Berthouly et al. 1995), and (3) ex vitro germination allowing the suppression of embryo individual manipulations in the laboratory (Ducos et al. 1999). Based on these improvements, we have recently set up a pilot process for the propagation of elite clones for the self-incompatible species Robusta (*Coffea canephora*) coffee (Ducos et al. 2007a, b). The current capacity allows the production of about 2.5–3.0 million cotyledonary embryos per year.

The present article describes the set up and use of new plastic-based culture systems in order to cut down the production costs of processes developed in our research centre.

Disposable bioreactors for mass plant cell cultures

In order to decrease the production costs, a few alternatives to traditional stainless-steel bioreactors have recently been developed (Singh 1999; Hsiao et al. 1999; Curtis 1999), but these systems, when used for plant cell cultures, concern only small working volumes (Palazon et al. 2003; Bentebibel et al. 2005; Eibl and Eibl 2006). We are developing two new flexible, scalable, plastic-made disposable bioreactors (Terrier et al. 2007). The first one 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 ("SB bioreactor") that allows an easy increase of working volumes (up to several 100 l) with the use of multiple units. Both systems are pre-sterilized and have been designed to allow for medium introduction, inoculation and sampling.

Plant material

An isoflavone producing *Glycine max* (L.) Merr. soya cell strain (Federici et al. 2003) and a *Nicotiana tabacum* L. BY2 cell strain (Nagata 2004) were used as models to assess the performances of the disposable bioreactors in comparison with two traditional systems: Erlenmeyer flasks and a 14 l stirred-tank bioreactor (New Brunswick Scientific, USA). Conditions for cell cultures are detailed in Terrier et al. (2007).

WU bioreactor

The Wave and Undertow (WU) bioreactor consists of a large flexible plastic container partly filled with medium and inflated with air. It is made of biopharmaceutical grade flexible PVC (Achilles, WA, USA) and sterilized by autoclave (40 min at 121°C).The system is located on a horizontal table equipped on one side with a platform. The intermittent rising movement of the platform to the rest point, and

descending movement back to initial position, enable continuous mixing and aeration through the wave/undertow motion, which, in turn, provides liquid culture mixing and bubble-free aeration (Fig. 1). Platform movements are simply achieved by pneumatic jacks located under the platform; the times needed to allow for the platform to raise and to descend can be adjusted easily. Other parameters are adjustable. Growth parameters for cell cultures in WU bioreactors are very close to those obtained with current vessels, or even better in the case of soya cell culture (Terrier et al. 2007); tobacco growth pattern in WU bioreactors at different volumes is comparable to growth observed with traditional cell culture systems (Fig. 2).

SB bioreactor

The Slug Bubble (SB) bioreactor consists of a vertical flexible plastic cylinder filled with medium up to circa 80% of its height (Fig. 3). It is made from biopharmaceutical grade polyethylene (CPL613; Charter Medical, Lydall Group, NC, USA) and gamma-sterilized. Agitation and aeration are achieved through the intermittent generation of large cylindrical single bubbles at the bottom of the system that rise to the top of the cylinder. These bubbles are comparable to "slug bubbles" (Davies and Taylor 1950; Sousa et al. 2005) which are known to provide

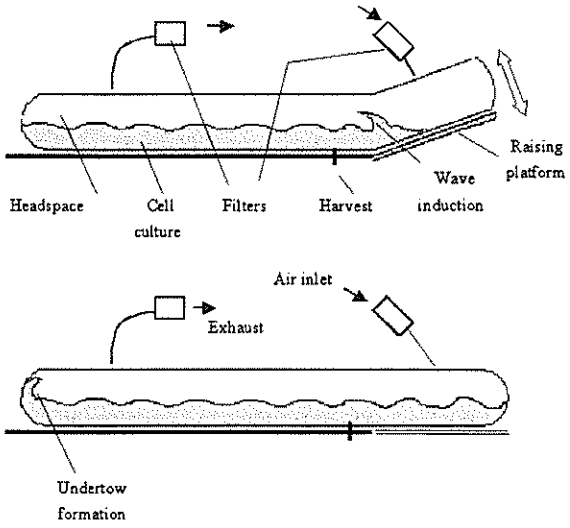

Fig. 1 Diagram of wave and undertow bioreactor (reproduced from Terrier et al. 2007)

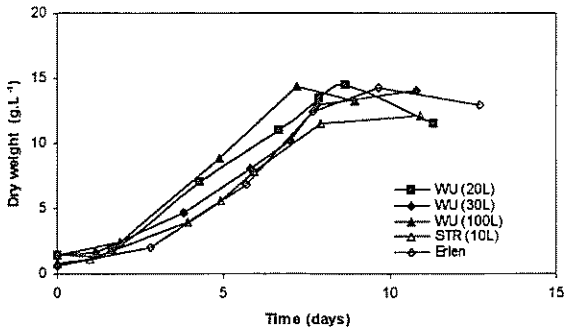

Fig. 2 Growth of tobacco cells in Erlenmeyer flasks ("Erlen"), stirred-tank bioreactor ("STR") and WU bioreactors at 3 different scales (10, 20 and 100 l working volumes)

strong mixing in their wake. Air bubbles are produced by intermittent gas supply, using a solenoid valve and compressed air: the valve relieves a predetermined quantity of air at the given frequency. Average air flow rate and mixing intensity can be adjusted by changing the inlet pressure, the valve opening duration or the bubble frequency. The results establish that cultivation in SB bioreactors at 20, 50 or 70 l working volumes can be used instead of Erlenmeyer flasks or a traditional stainless steel stirred-tank bioreactor (Fig. 4).

Several plant cell species have been successfully cultivated in these two new flexible plastic-based disposable bioreactors: *Nicotiana tabacum, Glycine max, Pilocarpus heterophyllus;* isoflavone production by soya cells has also been shown (Terrier et al. 2007). Both systems rely on very different mechanisms to provide oxygen and ensure proper mixing and culture homogeneity. They are easy to operate and do not require time for cleaning, sterilization or maintenance. Due to their simplicity, these culture systems lead to the development of efficient, low cost cell culture systems applicable to small and medium scales and complementary to traditional stainless-steel bioreactors.

Bioreactors for the production of somatic embryos

The process consists in two main steps. The production of coffee torpedo embryos, starting from embryogenic callus, is achieved in Erlenmeyer flasks. The torpedo stage embryos are not autotrophic and require a maturation step to reach the cotyledonary stage which is the earliest embryo stage having

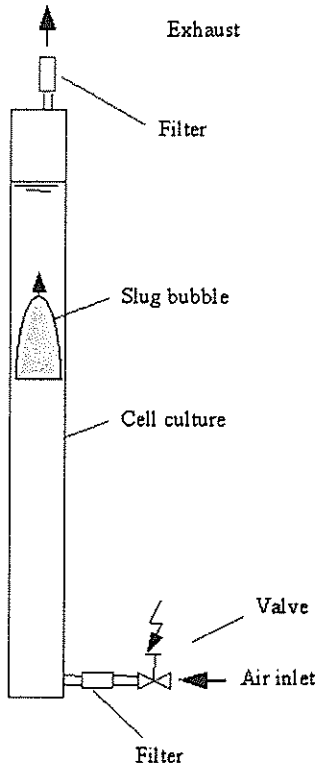

Fig. 3 Diagram of a slug bubble bioreactor (reproduced from Terrier et al. (2007))

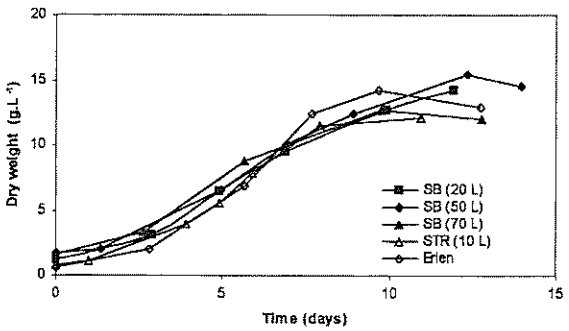

Fig. 4 Growth of tobacco cells in Erlenmeyer flasks ("Erlen"), stirred-tank bioreactor ("STR") and SB bioreactors at 3 different scales (20, 50 and 70 l working volumes)

functional photosynthesis. They are then transferred into a 10-l temporary immersion bioreactor (TIB) for their development as green cotyledonary-stage embryos (pre-germination phase).

Temporary immersion method involves placement of plant tissues on solid supports which are periodically perfused with nutrients solutions. Etienne and Berthouly (2002) described the various TIBs which have been used for plant micropropagation since the 1980s by some pioneers such as Tisserat and Vandercook (1985) and Aitken-Christie et al. (1985). TIBs offer the advantages of culture in liquid medium, with reduced labour cost, without the disadvantages of a liquid environment. The benefits compared with conventional bioreactors are:

- limitation of hyperhydricity due to avoidance of continuous immersion
- limitation of shear stress due to the lack of mechanical agitation or permanent aeration
- optimization of the provision of adequate oxygen transfer (because the tissues are not permanently immersed in liquid media in which oxygen is poorly soluble)

A small 0.2-l working volume bioreactor was commercialized in 1995, so-called Recipient for Automated Temporary Immersion (RITA®, Vitropic, France). According to Etienne and Berthouly (2002), new simplifications are possible and highly desirable to reduce the price of the TIBs or to increase their efficiency. In fact, different authors described several modifications, the most frequently reported being to increase the device volume for commercial scale-up. The twin flask system, consisting of a pair of bottles connected by a silicone tube, is generally the preferred system, because it allows larger volumes, up to 10 l. It was first reported for the culture of pineapple shoots (Escalona et al. 1999).

Our own temporary immersion bioreactor consists of a 10-l glass jar containing the embryos and a 5-l jar containing the medium (Fig. 5). Overpressure is applied twice a day during 5 min through the vent filter of the medium bottle to immerse the embryos. When the overpressure stops, the medium goes down by gravitation. From some vessels, up to 25,000 pre-germinated coffee embryos can be collected. Nevertheless, all the embryos do not have a normal development and there is a need to improve the quality of the embryos i.e. their ability to develop a plantlet in the nursery. Particularly, the lighting becomes a rate-limiting factor during the culture, as light can only penetrate the first few centimetres into the biomass. A non-uniform light distribution inside the TIB may be responsible for differences in growth and quality among the embryos.

There is therefore a need for a new culture system allowing an improved development of the embryos

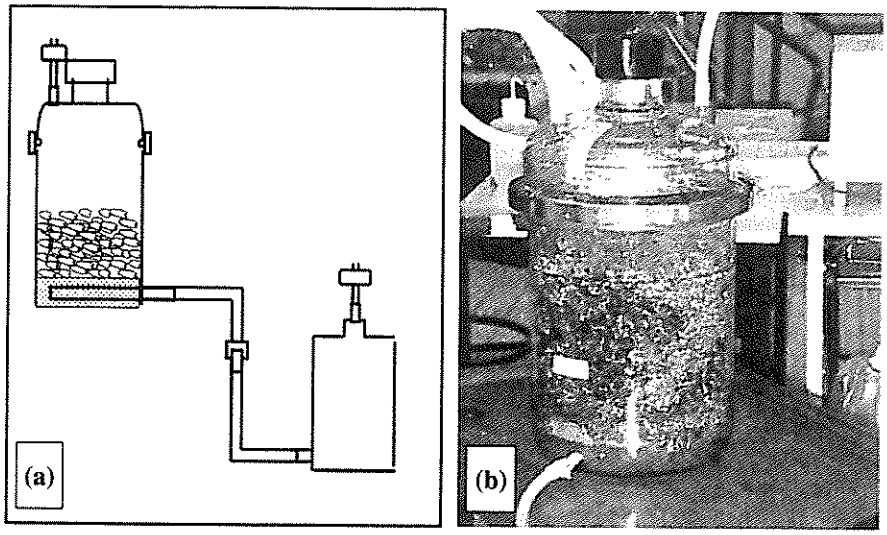

Fig. 5 Glass bottle temporary immersion bioreactor: (**a**) Diagram, (**b**) View of a culture of pre-germinated Robusta somatic embryos after 2 months

and possibly at least partially disposable. One improvement would be to increase the surface-to-volume ratio; one possibility is to place a rigid box inside a plastic bag. We presented this new TIB, so-called "Box-in-Bag" in the 27th International Horticultural Congress (Séoul, August 2006) (Ducos et al. 2007c). This experimental culture system is sterilized by gamma radiation and connected to the usual 5-l medium bottle (Fig. 6). The embryo growth is significantly improved comparing to the one in glass jars: in the case of the first clone assessed, the fresh weigh of the biomass collected at the end of the culture was 943 g instead of 519 g (Ducos et al. 2007b, c). The "Box-in-Bag" bioreactor combines the advantages provided by both types of plastics. The flexible plastic bag gives the advantages of a disposable device (low cost, simple to operate), high process security, and versatility by allowing diversity in designs and sizes. The rigid plastic box maintains a culture headspace between the immersion periods. Moreover, the possibility to stack boxes up makes this system easy to transport: it will be possible to send the embryos inside the bioreactor in which they have grown. Nestlé's devices were provided by Hegewald–Medical (Lichtenberg, Germany) to assess different designs for optimised mixing and aeration.

The scaling up of the ex vitro germination phase was managed for the first time in this country, up to 100 to 200,000 plants per year, in the Chumphon Horticultural Research Center (Thailand) (Sanpote et al. 2006). Up to now, a total of 600,000 somatic seedlings have been produced.

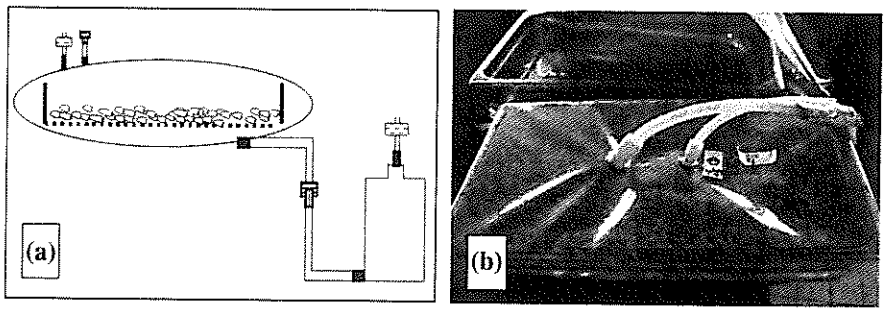

Fig. 6 Horizontal disposable temporary immersion bioreactor ("Box-in-Bag"): (**a**) Diagram, (**b**) View of a culture of pre-germinated Robusta somatic embryos after 2 months

Conclusion

The disposable systems described here offer many benefits and practical advantages in comparison with traditional systems: lightness and handiness (in the case of the TIB), versatility; they also enable new designs (that would have been impossible in glass or stainless steel, for example the WU system), improved or simplified designs, with less or no maintenance; they exhibit 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/manufacturing process (whether the systems are "home-made" or contract-manufactured) since each bioreactor is a novel process unit.

This articles 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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