

Application of bioreactor technology in plant propagation and secondary metabolite production

Приложение на биореакторната технология в размножаването на растенията и производството на вторични метаболити

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Abstract

Plant tissue cultures have been widely used in both fundamental and applied types of research on various biological species, and the scientific interest to transfer that technology in industrial scale has been rapidly growing. The use of *in vitro* technology for commercial propagation of different plant species and the production of bioactive compounds from them has become profitable industry worldwide. In the past decades, the progress in plant tissue culture technology was directed towards the introduction of the liquid medium for cultivation under submerged conditions in different bioreactor types, and automation of the entire process. Some applications of modified bioreactor systems and their importance for the advancement of plant biotechnology in the fields of agriculture, medicine, and pharmacy are discussed in this review.

Keywords: bioreactor systems, plant biotechnology, propagation *in vitro*, secondary metabolites, TIS RITA[®]

Абстракт

Растителните тъканни култури се използват широко във фундаментални и приложни изследвания на различни биологични видове, а научният интерес за трансфер на тази технология в промишлен мащаб бързо нараства.

Използването на *in vitro* технологии за комерсиално размножаване на различни растителни видове и производството на биологично активни съединения от тях се превърнаха в печеливша индустрия в световен мащаб. През последните десетилетия напредъкът в технологията на растителното тъканно култивиране се насочи към въвеждането и използването на течна хранителна среда за култивиране при различни условия, осигурени от технически различни типове биореактори и към автоматизация на целия процес. В този обзор са разгледани някои приложения на модифицирани биореакторни системи и тяхното значение за развитието на растителната биотехнология в областта на земеделието, медицината и фармацията.

Ключови думи: биореакторни системи, растителна биотехнология, размножаване *in vitro*, вторични метаболити, TIS RITA®

Introduction

Plant cell and organ cultures have emerged as potential sources of secondary metabolites, which are used as pharmaceuticals, agrochemicals, flavors, fragrances, coloring agents, biopesticides, and food additives (Murthy et al., 2014). *In vitro* micropropagation of plant species is a traditional technique which is labor-intensive enough and economic assessment indicates a higher cost of resulting plants. This fact is limiting in expanding the use of *in vitro* technology for the propagation of various plant species. The value of labor is between 40 and 60% of the total price of the obtained plants (Afreeen, 2006; Hanhineva and Kärenlampi, 2007). Because of this reason, many researchers are directing their efforts to maximize the mechanization of the propagation process of different plant species (Berthouly and Etienne, 2005). One of the most effective ways for increasing of micropropagation effectiveness and reducing the labor costs was to move the cultivation of *in vitro* plants from gelled to liquid medium (Hvoslef-Eide and Preil, 2005). Various conventional bioreactor systems have been successfully modified and adapted for the specific needs of plant micropropagation over the past decade (Steingroewer et al., 2013). However, the complete submersion of the plantlets into the culture medium has been found to cause another issue, mainly associated with changes in plants morphology, appearance of physiological malformations as hyperhydricity (vitrification), dwarfism, abnormal growth and organs development, etc., and finally causing problems with acclimatization and survival of the resulting plants (Etienne and Berthouly, 2002). A new type of bioreactors, known as temporary immersion systems (TIS) has been developed and adapted specially for the needs of plant micropropagation (Georgiev et al., 2014) and sustainable production of biopharmaceuticals (Georgiev, 2015). Present review highlights the progress in the application of bioreactors for micropropagation of economically important plants, with the accent on temporary immersion RITA® type system.

Plant propagation aspects

Benefits and problems of bioreactor cultivation

Different strategies exist for in vitro multiplication of plants. However, their effectiveness in achieving one of the primary goals of the propagation process, to obtain genetically identical and stable individuals, could significantly vary depending on the used method. Scientific reports confirmed with success the possibility to use both liquid and gelled media for plant propagation (Hvoslef-Eide and Preil, 2005). During the last years, the improvement of the efficiency of plant tissue method was towards commercialization of micropropagation by robotization or automation of the process and application of liquid medium in different bioreactor types. Recently, diverse bioreactor systems, as well as various temporary immersion systems have been developed in attempts to scale-up the micropropagation process to industrial level by improving its productivity and economic effectiveness (Steingroewer et al., 2013; Georgiev et al., 2014).

The use of bioreactors for micropropagation was reported first for *Begonia*, cultured in bubble column bioreactor (Takayama and Misawa, 1981). Gradually, cultivation in submerged bioreactor systems became possible for many explant types such as shoots, bulbs, microtubers, corms and somatic embryos (Paek et al., 2001).

The main benefits of bioreactor system could be summarized as fast growth of the cultures, better mass transfer of nutrients and gases, easy scalability of the process, and reduced labor costs resulting in the price of the propagated plants. Moreover, the bioreactor systems can ensure constant micro-environmental conditions and allow a high degree of automation of the cultivation process, using a liquid medium.

Different culture conditions could affect plant morphogenesis and biomass accumulation in the cultures, grown in bioreactors. The lack of gaseous atmosphere requires improved mass transfers of dissolved oxygen and nutrients, which could be realized only by precise control on oxygen supply and CO₂ exchange, pH, as well as by optimization of minerals, carbohydrates and growth regulators in the liquid medium (Heyerdahl et al., 1995; Georgiev, 2014; Gatti et al., 2017).

The various propagation aspects of several plant species and some of the problems associated with the operation of bioreactors were reviewed (Takayama and Akita, 1998; Steingroewer et al., 2013; Paek et al., 2014). In the beginning, the authors defined microbial contamination as one of the main problems, affecting both the introduced plant material and the operation procedures of large-scale bioreactors. Appearing of such an issue on a large scale could result in massive losses of money and time. However, over the past decades, a significant advance in bioreactor technology was realized (Table 1a, 1b, 1c), and now there are many bioreactor designs, including single-use and disposable vessels, reducing the risk of contamination (Weathers et al., 2010).

Genetic stability

True-to-type propagules and genetic stability are prerequisites for the application of micropropagation. The occurrence of somaclonal variation could be affected by different factors including genotype, the presence of chimeral tissue, the explant

type, size, age and origin, the basal media composition (inorganic and organic constituents), type and concentrations of the growth regulators and their timing of application, cultural environment and duration of the culture (Yancheva et al., 2003; Graham 2005; Nas et al., 2010; Ikeuchi et al., 2016). Plant cell totipotency and the realization of the regeneration program are additionally strongly dependent on physical and chemical influences controlling the occurrence of somaclonal variation during the propagation in liquid culture. Since one of the primary physiological problems in liquid-cultured plants is the malformation of the shoots, caused by hyperhydricity, the induction of meristematic or bud clusters with arrested leaf growth could help to overcome this problem (Ziv, 1991; Ziv and Shemesh, 1996). The choice of regeneration program that does not allow induction of variations is essential for ensuring the genetic stability of *in vitro* plants in conservation and micropropagation programs (Bose et al., 2017). Further studies are needed to optimize culture conditions for the specific genotypes. Clonal fidelity of micropropagated plants has to be monitored by their morphological, biochemical, physiological and genetic characteristics using molecular markers as powerful tools for genetic identification (Ziv, 1991; Debnath, 2011; Bose et al., 2017).

Mechanization and automation of the micropropagation process can significantly contribute to overcoming the limitation imposed by existing conventional labor-intensive methods, based on solid medium growth. The progress of the automated systems required expanding the range of knowledge and ability to decipher correctly the biochemical and physiological signals of plants propagated in a specific microenvironment. Another critical point is to develop and apply advanced mathematical models for optimization of nutrient medium composition and cultivation conditions for each plant species, which could help to predict and control the morphogenesis of plants growing under submerged conditions (Ziv, 2005; Debnath, 2011).

Table 1a. Summary of the plant species propagated by different bioreactor systems (based on Ziv, 2010; supplemented)

Species	Type of bioreactor	References
<i>Abies nordmanniana</i>	Temporary immersion bioreactors	Businge et al. (2017)
<i>Aloe barbadensis</i>	Continuous immersion bioreactor	Cardarelli et al. (2014)
<i>Amaryllis hyppeastrum</i>	Aerobic-bioreactor techniques	Takayama and Akita (1998)
<i>Ananas comosus</i>	Temporary immersion bioreactors	Escalona et al. (1999)
<i>Apium graveolens</i>	Mechanical gas-sparget system	Nadel et al. (1990)
<i>Araceae species</i>	Aerobic-bioreactor techniques	Takayama and Akita (1998)
<i>Begonia x hiemalis</i>	Aerobic-bioreactor techniques	Takayama et al. (1981)
<i>Betula pendula</i> , <i>Betula pubescens</i>	Temporary immersion bioreactors	Businge et al. (2017)
<i>Brodiaea complex</i>	Gas-bubble column bioreactor	Ilan et al. (1995)
<i>Coffea Arabica</i>	Temporary immersion bioreactors	Teisson and Alvard (1995)
<i>Coffea canephora</i>	Temporary immersion bioreactors	Etienne and Berthouly (2002)
<i>Colocasia esculenta (Taro)</i>	Rotary drum bioreactor	Takayama and Akita (1998)
<i>Cyclamen persicum</i>	Temporary immersion bioreactors	Hvoslef-Eide and Munster (2005)

Table 1b. Summary of the plant species propagated by different bioreactor systems (based on Ziv, 2010; supplemented)

Species	Type of bioreactor	References
<i>Daucus carota</i>	Temporary immersion bioreactors	Archambault et al. (1995)
<i>Dianthus caryophyllus</i>	Temporary immersion bioreactors	Chatterjee et al. (1997)
<i>Eschcholtzia californica</i>	Helical ribbon impeller bioreactor	Archambault et al. (1994)
<i>Eucalyptus grandis</i> x <i>E. urophylla</i>	Temporary immersion bioreactors	Businge et al. (2017)
<i>Euphorbia pulcherrima</i>	Balloon-type bubble bioreactor	Preil (1991)
<i>Fragaria ananassa</i> Duch.	Aerobic-bioreactor techniques	Takayama and Akita (1998); Debnath (2008, 2009b)
<i>Gerbera jamesonii</i>	Temporary immersion bioreactors	Mosqueda Frómata et al. (2017)
<i>Gladiolus grandiflorum</i>	Bioreactors with gas-sparged	Ziv (1990); Ziv et al. (1998)
<i>Hevea brasiliensis</i>	Temporary immersion bioreactors	Alvard et al. (1993); Teisson and Alvard (1995)
<i>Hypericum perforatum</i> L.	Shake flask culture	Cui et al. (2010)
<i>Hyacinthus orientalis</i>	Aerobic-bioreactor techniques	Takayama and Akita (1998)
<i>Lilium</i> spp.	Continuous immersion bioreactors	Takayama (1991)
<i>Lessertia frutescens</i> L.	Glass ballon-type bubble bioreactors	Shaik et al. (2011)

Table 1c. Summary of the plant species propagated by different bioreactor systems (based on Ziv, 2010; supplemented)

Species	Type of bioreactor	References
<i>Lowbush blueberry</i>	Temporary immersion bioreactors	Debnath (2011)
<i>Medicago sativa</i>	Multi-specimen perfusion bioreactor	Stuart et al. (1985, 1987); Chen et al. (1987); McDonald and Jackman (1989)
<i>Musa spp.</i>	Temporary immersion bioreactors	Alvard et al. (1993); Teisson and Alvard (1995); Ziv et al. (1998)
<i>Nephrolepis exaltata</i>	Glass spargers in the airlift bioreactors	Ziv and Hadar (1991); Ziv et al. (1998)
<i>Nerine sarniensis</i>	Temporary immersion bioreactors	Lilien-Kipnis et al. (1994); Ziv et al. (1994)
<i>Ornithogalum dubium</i>	Temporary immersion bioreactors	Ziv and Lilien-Kipnis (1997)
<i>Populus tremula</i>	Glass bubble - column bioreactor	McCown et al. 1988; Carmi and Altman (1997)
<i>Picea glauca</i>	Bioreactor with large chamber, supported by flat absorbent bed	Attree et al. (1994)
<i>Picea glauca-engelmannii</i> and <i>Picea marianna</i>	Mechanically stirred bioreactors	Taurus et al. (1994)
<i>Solanum tuberosum</i>	Aerobic-bioreactor	Akita and Takayama (1994); Ziv and Shemesh (1996); Takayama and Akita (1998); Ziv et al. (1998)
<i>Stevia rebaudiana</i>	Temporary immersion bioreactor	Sreedhar et al. (2008)
<i>Xanthosoma sagittifolium</i>	Temporary immersion bioreactor	Niemenaket et al. (2013)

Bioreactors application for plant propagation

Bioreactors used for plant propagation demonstrate continual improvement of their technical performance (Table 1a, 1b, 1c). Initially, they have been classified into two types: with constantly and with partially immersed cultures in the medium (Takayama and Akita, 1994). Evolution in the development of this technology has allowed bioreactors nowadays to be classified mainly into the following categories: mechanically agitated, pneumatically agitated, non-agitated and temporary immersion systems (TIS).

Bioreactor systems with liquid media have been introduced for mass propagation of horticultural plants as a cost reduction approach (Levin and Vasil, 1989). The application of large-scale liquid cultures and automation has the potential to solve the problem with the expensive manual handling used in various stages of micropropagation. The cultures growing in a bioreactor vessel containing liquid medium exhibit the following conditions: a) floating just beneath the medium surface (Strawberry plants); b) freely moving in the medium as in case of Begonia and Gloxinia; c) sinking or submerged to the bottom of the vessel (Lilies, Gladiolus, potatoes) (Takayama and Misawa, 1981; Takayama, 1991).

Liquid cultures systems have significant effects on the multiplication rates and morphology of shoots, somatic embryos, micro-tubers or bulblets produced in vitro (Preil, 2005). The main challenge with using the classical submerged bioreactor systems like a stirred tank and bubble column is the hyperhydricity (vitrification) of cultured plantlets, therefore for overcoming this problem, temporary immersion systems (Figure 1) have been developed and applied for the differentiated plant in vitro cultures (Georgiev et al., 2014).

Described by Harris and Mason (1983), TISs with different frequencies of immersion were reported as an effective system for improving the plant quality and multiplication rates of various plant species as banana, coffee, small and wild fruits (Alvard et al., 1993; Teisson and Alvard, 1995; Debnath, 2009a, 2009b, 2009c; Dzhambazova et al., 2015; Georgieva et al., 2016) (Table 1a, 1b, 1c). Such bioreactor cultivation systems, providing lower levels of hydrodynamic stress can resolve the problems associated with the mass-exchange constraints accompanying the cultivation of plant tissues on the agar medium. Some small fruits as *Fragaria*, *Rubus*, and *Vaccinium* were the plant species subjected to bioreactor propagation resulting in few times increased proliferation in comparison with micropropagation on gelled medium (Debnath, 2009a, 2009d, 2011; Georgieva et al., 2016). Hvoslef-Eide and Preil (2005) described lower cost and less labour-intensive clonal propagation of shoots, bud-clusters and somatic embryos of *Anoectochilus*, apple, *Chrysanthemum*, garlic, ginseng, grape, *Lilium*, *Phalaenopsis* and potato through the use of modified air-lift, bubble column, bioreactors, together with temporary immersion systems.

The critical factor in TIS is the immersion time since it governs nutrient uptake and expression of hyperhydricity. Hence, the optimization of shoot proliferation and reducing hyperhydricity, it is essential to find the balance between immersion frequencies and immersion duration (Steingroewer et al., 2013; Georgiev et al., 2014; Regueira et al., 2018; Zhang et al., 2018).

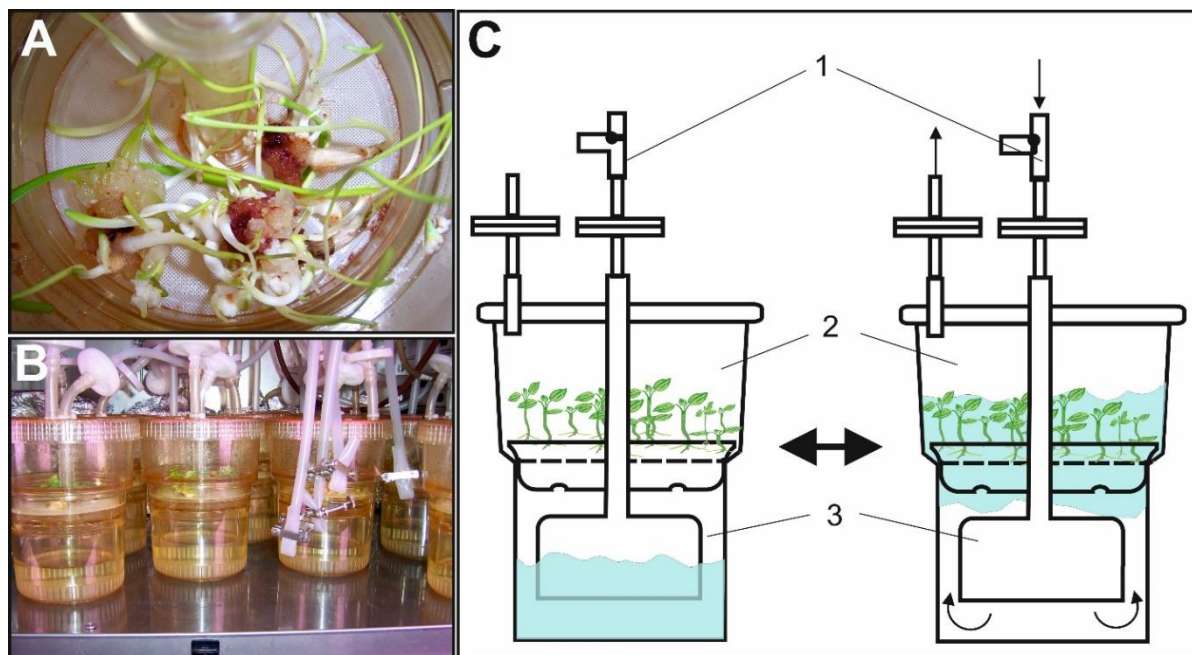


Figure 1. A and B - propagation of *Pancretium maritimum* L. species in TIS RITA[®], C - schematic diagram of a temporary immersion RITA[®] system, 1- three-way solenoid valve, 2 - culture chamber, 3 - medium compartment

Bioreactor propagation become increasingly preferred due to additional advantages as precise control of temperature, light, dissolved oxygen and carbon dioxide, improved mass transfer of nutrients and waste metabolites, reduced risk of contamination, providing an easier for the handle and scalable technology (Takayama and Akita, 1994; Mamun et al., 2015). However, some disadvantages as the high sensitivity of *in vitro* plantlets to mechanical and shear stress, slowing down the mass implementation of that technology for commercial micropropagation. The appearance of plant malformations and somaclonal variation, the oxidative stress occurring in some tissues as changes in the antioxidative enzyme activity can influence the anatomy and physiology of the plants and their survival. A lot of such problems could be overcome by selecting the most optimal bioreactor design for each propagated genotype (Steingroewer et al., 2013).

The successful exploitation of bioreactors as a commercial micropropagation systems was demonstrated by examples as *Anoectochilus*, apple, chrysanthemum, garlic, ginseng, grape, *Lilium*, *Phalaenopsis* and potato (Paek, 2005). However, the large-scale plant production in bioreactors depends on careful studies of genotype specifics, plant morphogenesis in liquid media and the understanding of the complex control mechanisms of organ and embryo development from meristematic or bud clusters (Ziv, 2010). The effects of aeration, mixing, consumption, and depletion of the various components present in the medium could provide relevant information for establishing semi-continuous or continuous culture system as a prerequisite for optimal biomass growth, differentiation and future production of quality plants. The fact that organogenic and embryogenic cultures of lots of economically important species have the potential for continuous proliferation and virtually unlimited production of propagules has stimulated the use of bioreactor culture for mass

propagation. Hence, the further development and use of bioreactors for the propagation of plant species and their transformation into conventional technology require profound multidisciplinary knowledge.

Production of valuable secondary metabolites

Plant cell culture technology, based on bioreactor cultivation of elite species has gradually become an efficient system for propagation of a wide range agricultural, forest, wild and medicinal plants species (Ziv, 2005, 2010; Businge et al., 2017). Plant biotechnology has also been recognized as an alternative source for the production of valuable secondary metabolites (Table 2).

Due to the specific morphology of the plants developing in vitro, the accessibility of nutrient during their cultivation in bioreactors is one of the primary factors, influencing both their development and their secondary metabolism (Steingroewer et al., 2013). Biomass accumulation and metabolite biosynthesis are often two-stage events, which require the development of a two-step cultivation strategy. In that cultivation scenario, the factors that control the growth and multiplication of cultured cells/organs and biomass accumulation differs from the stimuli that assist the biosynthesis of secondary metabolites. Under these circumstances, the typical two-steps cultivation process requires the control and monitoring of different parameters on each stage. In the first stage – selection of high-producing cells or organ clones, the optimization of the suitable medium (salts, sugar, nitrogen and phosphate sources), plant growth regulator levels, and physical factors such as temperature, illumination, light quality, pH, agitation, aeration, and environmental gas (oxygen, carbon dioxide, and ethylene) play critical role. Elicitation, replenishment of nutrient and precursor feeding, permeabilization and product recovery strategies assist with the accumulation of secondary metabolites and have an impact in the second stage of the culture process. Following the two-steps specific strategy, it is possible to produce large amounts of biomass with an increase in the accumulation of secondary compounds (Murthy et al., 2014).

Agrobacterium rhizogenes mediated transformation of plants has application mainly for the production of secondary metabolites. Genetically transformed root cultures are recognized as the most effective expression systems for valuable phytochemicals (Hu and Du, 2006; Georgiev et al., 2010, 2012; Steingroewer et al., 2013; Georgiev et al., 2014). Furthermore, an essential characteristic of hairy roots is their genetic stability. The correlation between secondary metabolite and morphological differentiation give more stimuli for the production of phytochemicals. It is proved that the synthesis of two different secondary metabolites is possible simultaneously by adventitious root co-cultures (Wu et al., 2006). Hairy root cultures continue to maintain interest as a way to produce valuable plant components using bioreactors with special hangers inside the vessel (Furuya et al., 1984; Kieran et al., 1997; Georgiev et al., 2012).

Table 2. Groups of natural products isolated from tissue and suspension cultures of higher plants (adapted from Ramachandra Rao and Ravishankarb, 2002)

Phenylpropanoids	Alkaloids	Terpenoids	Quinones	Steroids
Anthocyanins	Acridines	Carotenes	Anthroquinones	Cardiac glycosides
Coumarins	Betalains	Monoterpenes	Benzoquinones	Pregnenolone
Flavonoids	Quinolizidines	Sesquiterpenes	Naphthoquinones derivatives	
Hydroxycinnamoyl	Furonoquinones	Diterpenes derivatives	Harringtonines	
Isoflavonoids	Isoquinolines	Triterpenes		
Lignans	Indoles			
Phenolenones	Purines			
Proanthocyanidins	Pyridines			
Stilbenes	Tropane alkaloids			
Tanins				

Bioreactors used for hairy roots are classified commonly into three types - liquid-phase, gas-phase and mixed, which is a combination of both. Recently, the temporary immersion systems have been demonstrated to be very appropriate for cultivation of hairy roots, and production of valuable plant-derived secondary metabolites (Georgiev et al., 2012; Steingroewer et al., 2013; Georgiev et al., 2014). An important conclusion is that in bioreactor cultivation of plant cell suspensions, usually specialized cells in distinct developmental stages produce secondary metabolites (Balandrin et al., 1985). When the cells are undifferentiated, they wholly or partially lose their biosynthetic ability to accumulate secondary products (Rokem et al., 1985). Contrary, the hairy roots have attractive properties for secondary metabolite production because of their differentiation, which is the same as the root system of intact plants, and faster growth in lack of hormonal treatment than plant cell cultures. Another advantage of hairy roots is that they often exhibit similar or greater biosynthetic capacity for secondary metabolite production compared to their mother plants (Banerjee et al., 1998; Kim et al., 2002).

The quality and quantity of active substances production from wild or field grown plants varied depending on the specific environmental conditions. With the in vitro cultivated plants in bioreactors, such problems could be avoided by the precisely controlled microenvironment. Studies of selected plant species prove that the bioreactor technologies allow obtaining of new pharmaceutically active compounds not existed in the field growing plants (Pavlov et al., 2005; Georgiev et al., 2010; Georgiev et al., 2014).

Recent advances towards development and commercialization of plant cell culture processes for synthesis of biomolecules, some products produced commercially via plant cell culture and examples of secondary metabolites commercialized through natural harvest have been reviewed by Wilson and Roberts (2012). Nowadays, scientific efforts are concentrating on plant genomics and the synthesis of natural

products participating in the development of modern biopharmaceuticals as recombinant proteins and vaccines derived from transgenic plants (Gerth et al., 2007), known as “molecular farming” (Georgiev, 2015) and giving strategies for the production of natural products from either plant cell suspensions or hairy root culture for the pharmaceutical, cosmetic or food industries (Ochoa-Villarreal et al., 2016) (Figure 2).

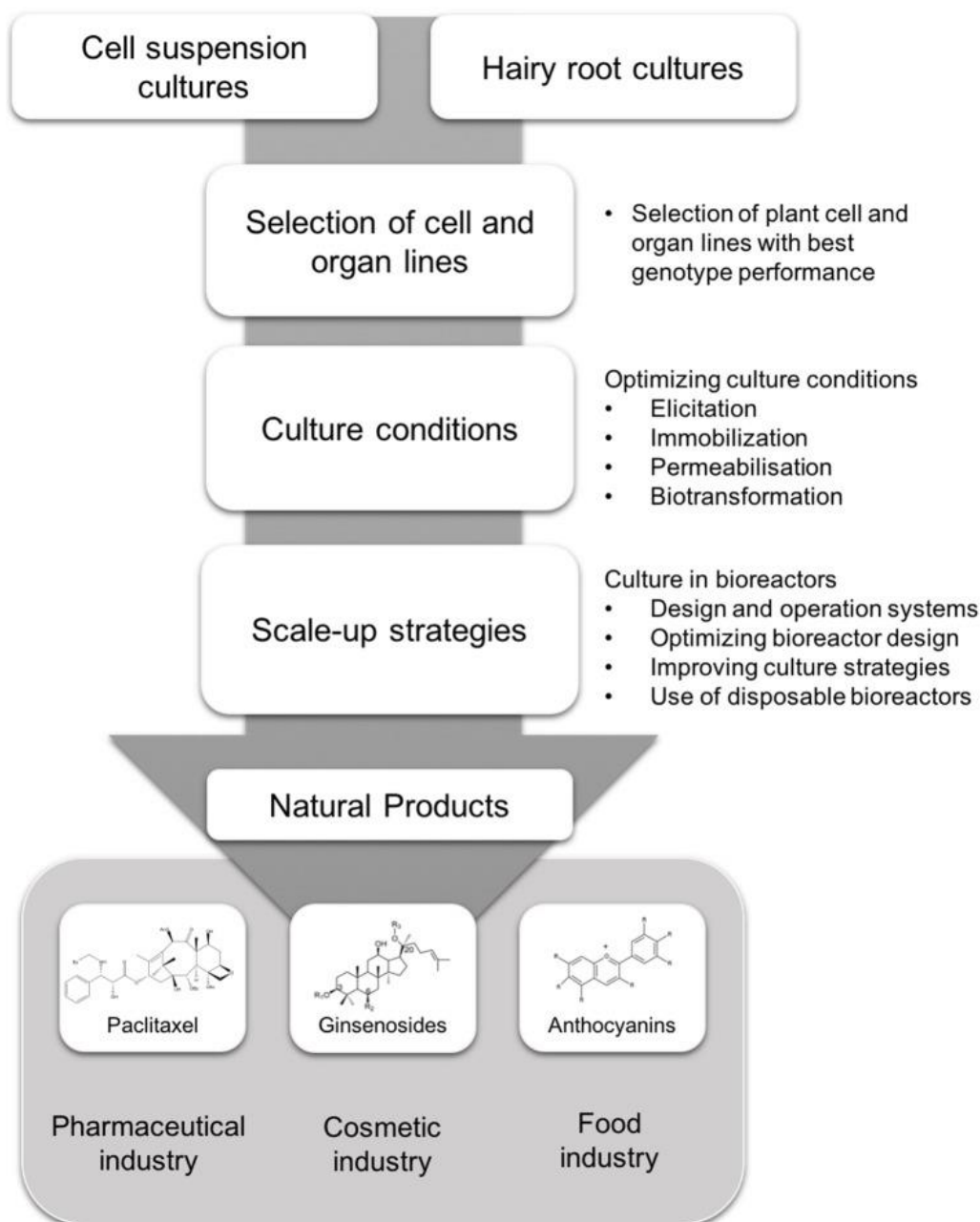


Figure 2. Strategies for the production of natural products from either plant cell suspensions or hairy root culture for the pharmaceutical, cosmetic or food industries (Ochoa-Villarreal et al., 2016)

The evaluation of different types of bioreactors applicable to plant micropropagation determined Temporary Immersion RITA® Systems as one of the most effective. What makes them attractive are their simple design, low cost, and considerable advantages. Cultivation conditions of TIS RITA® have minimal effect on plantlets morphology, which is essential for the quality of the obtained plants and their subsequent development. Another advantage of the system is the ability of propagated plants to biosynthesize high amounts of valuable plant-derived secondary metabolites. TIS can be used for the production of secondary metabolites with economic importance in rare and endangered medicinal plants without any risk for their natural habitats. Moreover, changing the immersion program of RITA® TIS could be used to manipulate the metabolic processes of the in vitro plants, depending on the primary goal - micropropagation or accumulation of target secondary metabolites.

Plant cell and tissue cultures are worldwide used techniques, and their focal directions of applying are the commercial micropropagation of different plant species, obtaining of pathogen-free plants, production of haploid plants, inducing genetic variation. Their further development is related to the facilitation of the production through automation of the processes and computerization of the information. The rapid evolution of "Omics" technology in recent years, combined with the benefits of plant tissue culture allows understanding and overcoming challenges to fundamental biological processes.

Conclusion

In vitro technology development has top priorities including the conservation of plant genetic resources; restoring the balance between the studies related to plant genetic transformation, with the aim of providing sufficient, quality and safety foods for the world population, and the research designed to determining the risk of growing and consuming them; creating transgenic plants with a sufficient protein content; and the use of plant resources possessing valuable biologically active substances for the pharmaceutical industry. The successful use of bioreactors as a commercial system generally depends on a deep understanding of the specific physicochemical parameters that influence the morphogenesis of the plants propagated in liquid cultures. The continuous upgrading of the bioreactor systems for micropropagation and, or secondary metabolite production, the optimization of the nutrient media and cultivation conditions, and the results obtained in various plant species are encouraging for the future development of this technology.

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