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Advances and challenges on the *in vitro* production of secondary metabolites from medicinal plants

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ABSTRACT

The production of secondary metabolites from medicinal plants, also called Plant-Derived Medicinal Compounds (PDMC), is gaining ground in the last decade. Concomitant to the increase in the knowledge about pharmacological properties of these compounds, horticultural plants are becoming the most important, sustainable and low-cost biomass source to obtain high-complex PDMCs to be used as medicaments. Biotechnological tools, including plant cell and tissue culture and plant genetic transformation, are increasingly being employed to produce high quality and rare PDMC under *in vitro* conditions. The proper use of these technologies requires studies in organogenesis to allow for better control of *in vitro* plant development and, thus, to the production of specific tissues and activation of biochemical routes that result in the biosynthesis of the target PDMCs. Either biotic or abiotic factors, called elicitors, are responsible for triggering the PDMC synthesis. *In vitro* techniques, when compared to the conventional cultivation of medicinal plants in greenhouse or in the field, have the advantages of (1) producing PDMCs in sterile and controlled environmental conditions, allowing better control of the developmental processes, such as organogenesis, and (2) producing tissues with high PDMC contents, due to the efficient use of different biotic and abiotic elicitors. Nevertheless, the process has many challenges, e.g., the establishment of step-by-step protocols for *in vitro* biomass and PDMC production, both involving and being affected by many factors. Other limitations are the high costs in opposition to the relatively cheaper alternative of growing medicinal plants conventionally. This paper aims to quickly review the general origin of plant secondary metabolites, the leading techniques and recent advances for PDMC *in vitro* production, and the challenges around the use of this promising technology.

Keywords: plant-derived medicinal compounds, *in vitro* cell and tissue culture, techniques, biotic and abiotic factors, biofactories.

RESUMO

Avanços e desafios na produção *in vitro* de metabólitos secundários de plantas medicinais

A produção de metabólitos secundários de plantas medicinais, também chamados de Compostos Medicinais Derivados de Plantas (PDMC), vem ganhando importância na última década. Concomitante ao aumento do conhecimento sobre as propriedades farmacológicas destes compostos, as plantas da horticultura tornam-se a fonte de biomassa mais importante, sustentável e de baixo custo para a obtenção de PDMCs de alta complexidade a serem utilizados como medicamentos. Ferramentas biotecnológicas, incluindo cultura de células e tecidos e transformação genética, estão sendo cada vez mais empregadas para produzir PDMCs raros e de alta qualidade sob condições *in vitro*. O uso adequado dessas tecnologias requer estudos em organogênese para permitir melhor controle do desenvolvimento *in vitro* das plantas e, assim, da produção de tecidos específicos e ativação das rotas bioquímicas que resultam na biossíntese dos PDMCs alvo. Elicitores bióticos ou abióticos são responsáveis por desencadear a síntese desses PDMCs. As técnicas de produção *in vitro*, quando comparadas ao cultivo convencional de plantas medicinais em casa de vegetação ou no campo, têm as vantagens de (1) produzir PDMCs em condições ambientais estéreis e controladas, permitindo um melhor controle de processos de desenvolvimento, como a organogênese, e (2) produzir tecidos com alto teor de PDMCs, devido à aplicação eficiente de diferentes elicitores. No entanto, o processo tem muitos desafios como, por exemplo, o estabelecimento de protocolos para produção *in vitro* de biomassa e PDMC, ambos envolvendo e sendo afetados por muitos fatores. Outras limitações são os altos custos em oposição à alternativa relativamente mais barata de cultivar plantas medicinais convencionalmente. O objetivo deste artigo é revisar brevemente a origem dos metabólitos secundários das plantas, as principais técnicas e avanços recentes para a produção *in vitro* de PDMCs e os desafios em torno do uso dessa tecnologia promissora.

Palavras-chave: compostos medicinais derivados de plantas, cultura de células e tecidos *in vitro*, técnicas, fatores bióticos e abióticos, biofábricas.

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Medicinal plants as a source of metabolites used as medicaments

Humans have been using plants for thousands of years as a means for maintaining their health. Medicinal plants continue to be the primary type of medicament used in the world currently, especially in tropical and in economically underdeveloped countries. In the last century, society, in its turn, has advanced in knowledge on chemistry and biology, which allowed the isolation of new molecules from plants, animals, and microorganisms, as well as the development of synthetic molecules, giving rise to the present generation of conventional medicines. However, despite the significant progresses observed in the pharmaceutical field, problems related to many of the conventional medicaments, such as high costs and collateral effects, are present complications of medication overuse.

Antibiotics illustrate well this

context: they are efficient against infectious diseases and avoid the death of millions in the world every year if used correctly and safely. However, their inappropriate use may lead to different types of bacterial resistance, which is one of the reasons for the current antibiotic crisis, along with the limited development of new molecules (Martens & Demains, 2017).

Based on these constraints, the use of medicinal plants is becoming increasingly important, especially in the last decade. Part of this is due to a return to the principles of dealing with some diseases, while, in another direction, the current capacity of science to prove both the efficacy and the safety of plants used as a medication, also plays a role (Hoareau & Silva, 1999; Petrovska, 2012). Among their advantages over conventional drugs, medicinal plants make highly complex organic biosynthesized molecules available naturally, reducing the costs of using them as medicaments, with fewer collateral effects.

It is worth considering also that megadiverse countries, such as Brazil, have important issues to address. For example, the need to prospect plants, animals, and microorganisms' natural molecules and their uses, including their medicinal properties, and how to deal with the vast and unexplored biodiversity in the face of the need to expand other economics activities that may threaten the environment. Part of the strategy for biodiversity management in megadiverse countries might be the use of biodiversity E-infrastructures to meet public demands and to open access to data about species (Canhos *et al*., 2015).

What is the origin of the Plant-Derived Medicinal Compounds (PDMC)?

In this paper, we concentrate only in the secondary metabolites used as medicaments, and we employ the term Plant-Derived Medicinal Compounds (PDMC) to distinguish these from other types of secondary metabolites used for other purposes.

Most PDMC come from the so-called secondary metabolism of plants, whose primary ecological role for the plant is to biosynthesize molecules to increase the plant capacity to adapt to the wild environment, especially in terms of defense and signaling (Wink, 2003). Plant primary metabolism includes essential life mechanisms, such as photosynthesis, respiration, water, and nutrient uptake and assimilation. Plant secondary metabolism, in its turn, uses energy and other molecules generated in the primary metabolism to produce a range of thousands of types of other compounds involved in increasing plant adaptability to different environmental conditions, especially molecules to respond to the biotic and abiotic stresses characteristic from wild environments. Besides, the secondary metabolism allows plants to communicate, at least from a chemical point of view, with animals and microorganisms, opening room to synergistic and antagonistic relationships, with many ecological functions.

Therefore, it is expected that many of the secondary metabolites a plant uses as their defense mechanism against pathogens can also serve as antimicrobial compounds, since they have the capacity to partial or completely inhibit the proliferation of some pathogens. The so-called PDMC arises from the correlation between the plant defense mechanism and the type of action of these secondary metabolites since this type of action can be extended and used as a product to fight diseases also in animals and even humans. It is even very likely that humans had instinctively realized the medicinal potential of plants, by observing other animals' habits of feeding in some specific plants when they were sick or with some disease or pain (Stojanoski, 1999).

It is difficult to say when humans began to use plants as medicines, but it must be associated with the emergence of humans themselves, once plants are the oldest type of medication recorded (Halberstein, 2005). The use of medicinal plants by civilizations is biblical and already reported thousands of years before Christ. Medicinal plants are often related to religious cults and beliefs or even to the popular tradition, which employs many plants to fight infectious diseases, in addition to other uses. Records from 6000 to 3000 BC, in Egypt, China, and India showed one of the earliest collections of plant species with pharmacological principles (Ang-Lee *et al*., 2001). Nevertheless, some authors claim that, much before, Neanderthals already used plants for medicinal purposes, 60 thousand years ago (Shanidar, 1975).

This previous awareness, coupled with up-to-date scientific evidence of the bioactive chemicals present and produced in the plant biomass, along with the safety of using some medicinal plant species, lead to the rediscovery of PDMC as medicaments. Such a revival implied in significant economic growth for the sector, reaching and moving an industry of billions of dollars worldwide, with relevant consequences especially to developing countries, where the access to conventional treatments is limited (Bukar *et al*., 2016).

It is important to note that medicinal plants may be used in their entirety, fresh or dried and ground, and contain several PDMC at once. Such characteristics are founding principles of the phytotherapy. On the other hand, most conventional medicinal products, including those containing molecules derived from medicinal plants, in this case, isolated from the whole, contain a single PDMC as a chemical marker of reference. Cinnamon is an illustrative example of these two treatments: while the use of the cinnamon bark as infusion to treat infectious diseases characterizes the use of the medicinal plant; its main secondary metabolite, cinnamaldehyde, which is isolated from the bark, proved to be an efficient antimicrobial agent (Zhang *et al*., 2015) and can be used as a conventional medicament, similar to other types of synthetic antimicrobials.

Many of the conventional drugs produced by the pharmaceutical industry come from molecules isolated from plants or other living organisms. Approximately 67% of anti-cancer drugs have molecules isolated or primarily synthesized by plants or animals in their composition (NCI-NHI, 2007). Many of them are references in the treatment of cancer and specially used as chemotherapeutics. That is the case of Taxol (Paclitaxel), a PDMC

obtained from the *Taxus brevifolia* bark and currently on the FDA-approved list of cancer treatments. Unfortunately, this use placed the species in danger due to the drastic reduction of its natural populations in the wild: the need for the bark to obtain Taxol led to a drastic reduction in *T. brevifolia* populations (Thomas, 2013, Weaver, 2014) since the bark removal results in the death of the trees. Other important issues to consider when using medicinal plants are those related to health risks (Smet, 2004) that can be caused, for example, by species misidentification, mistakes in dosage, intoxication, and interaction between plants and other drugs. Finally, we should not overlook environmental matters. Unsustainable exploitation can dangerously reduce natural populations and narrow the genetic basis of the species used as the PDMC source (Cardoso & Silva, 2013).

In this sense, horticulture can contribute in many ways, from the domestication of wild medicinal plants, increasing plant biomass and PDMC productivity as a way of stimulating its production in a commercial basis, to the use of plants as natural bioreactors, thus indirectly mitigating the unsustainable exploitation of natural resources. The horticultural science can provide methods to produce native and exotic PDMC-source plants in agroecosystems, using some techniques proved efficient in regular horticulture, i.e., for food productions, such as, for example, physical and chemical soil preparation, fertilization and other specific treatments.

In Brazil, different programs to improve the production of medicinal plants have been stimulated by different agents of the government or industry, in which the farmer-company-government partnership has been successful in increasing the production of some medicinal plants by both extending the growing area and using adequate technology to each specific crop. As far as the government is concerned, it is worth mentioning three initiatives: (i) the National Policy for Medicinal Plants and Phytotherapy (Brazil, 2006), also launched to improve the research involving medicinal plants and to value and appraise the extensive and diverse traditional knowledge related to their uses; (ii) the presence of some medicinal plants in the Brazilian National Registry of Medicaments (RENAME) and; (iii) the list of 71 species published by the Brazilian Ministry of Health with the objective of improving the knowledge and the bulk of technical-scientific information related to these species, aiming at their registration and further use as medication.

Despite the contribution medicinal plants can give to individuals and health systems, their use as drugs, without recommended dosages and professional care, can result in unsuccessful treatment and undesirable collateral effects. All medicinal plants have one or more PDMCs that act in human metabolism. Thus, it is necessary to control the medicinal compound (drug) dosages and frequency for achieving positive and safety results in the treatment. The Brazilian Policy of Medicinal Plants and Phytotherapy also recommends capacity strengthening for health professionals aiming at improving the accuracy of prescriptions and the quality of patient orientation when using medicinal plants. Thus, it is expected that medicinal plants could be employed more often in public health, with easier and cheaper access than conventional drugs. The low number of studies investigating the actual action mechanism of medicinal plants on human disorders currently limits their broader use in public health systems. More evidence warranting the safety use of medicinal plants as drugs, with robust scientific background, would contribute to improving their use in health systems.

From a business point of view, we highlight three main examples of improved medicinal plant chains in Brazil: Itaipu Binacional and its encouragement to the regional community towards sustainable production, commercialization and use of medicinal plants; the Centro Pluridisciplinar de Pesquisas Químicas, Biológicas e Agrícola (Multidisciplinary Center for Chemical, Biological and Agricultural Research, University of Campinas) (CPQBA/UNICAMP), which preserves collections of different medicinal plants and carries researches on breeding, physiology, phytochemistry, propagation, and crop technology involving medicinal plants, and; the company Centroflora that has partnerships with local growers aiming at stimulating the growth and production of some species containing PDMCs the industry is interest in, for isolating and stabilizing these components which are later sold to the pharmaceutical industries. The comprehensive structuration of these chains is crucial to allow farmers to invest resources in producing crops with an exceptional economic value, assuring sales to the industry, which, in its turn, needs a stable flow of high-quality plant raw matter to extract PDMCs.

Pests and diseases are one of the significant challenges currently faced by those growing medicinal plants in Brazil. There is a general lack of pesticides registered for medicinal plants and the need of using unregistered ones often results in undesired contamination of extracted or isolated PDMC (Zuin & Vilegas, 2000, Gerth *et al*., 2007, Tripathi *et al*., 2015). The methods of PDMC preparation, purification, and isolation also concentrate some pesticides and other toxic undesirable molecules. The sensitivity of most PDMC to environmental factors and growing techniques (Gobbo-Neto & Lopes, 2007) also challenges the chain, once it makes it very difficult to standardize lots with a minimum concentration of the target PDMC to warranty the efficiency of the treatment. Other factors that influence PDMC production are the genotype and the plant organ used and the life cycle: in some tree species, the PDMC is available only in the adult stage, after a long growth period.

These factors influence significantly the biosynthesis of the secondary metabolites that result in the PDMCs of interest, which can belong to three main classes, basically: the terpenes, the phenolic compounds, and the alkaloids. Each class of secondary metabolites is a result of a complex network of precursors, enzymes and co-factors, some of them leading to specific PDMCs.

The concept of PDMC *in vitro* production

In vitro culture involves the production of new cells, tissues, and organs derived exclusively from the mitotic cell division, thus generating cloned cells, tissues, and individuals, namely, with the same genetics of the mother plant. The use of *in vitro* techniques to produce secondary metabolites, especially PDMCs, has as main advantages: less environmental interference due to the controlled conditions in *in vitro* growing rooms; possibility of more control over the PDMC production by developing step-by-step protocols to accelerate fresh biomass accumulation and increase PDMC concentration in the tissues; the season-independent staggered production of PDMCs; PDMC production under sterile conditions and with very few risks of contamination by undesired toxic compounds.

PDMC *in vitro* production consists of growing explants from the target medicinal plant employing plant cell and tissue culture techniques. Explants are surface sterilized and inoculated on *in vitro* conditions, using a previously formulated culture medium, and cultivated for different phases under controlled environmental conditions. Culture media contain organo-mineral formulated solutions to nurture the tissues; sugars, such as sucrose, to act as the energy source to a system (*in vitro* conditions) in which light conditions and low CO_2 concentrations severely limit photosynthesis (photomyxotrophyc conditions); plant growth regulators, used to control tissue development and to stimulate the increase of plant biomass and PDMC contents; as well as other additional products such as amino acids, vitamins, activated charcoal, antioxidants, and specific substances used for each individualized protocol.

In this context, explants such as shoot tips containing the apical stem meristem, leaf segments, and others, are surface sterilized (asepsis) to reduce the occurrence of microorganisms in plant tissues using alcohol 70% or sodium hypochlorite. Following, these explants are inoculated into a specific culture medium for the establishment of the *in vitro* culture, i.e., the development of shoot meristems into callus (undifferentiated tissue), plantlets (aerial part + roots), shoots (aerial part) or only roots *in vitro*. Some explants, such as leaf segments, have an additional *in vitro* phase called organogenesis, in which specialized cells resume their capacity of cell division and develop callus (indirect organogenesis) or buds and shoots directly from the original tissue (direct organogenesis).

After the successful establishment of the *in vitro* culture without microorganism contamination, next phases consist of multiplication of cells, tissues, shoots, and roots. These phases are critical to the economic viability of the process, mainly because the more cells and tissues are produced, the higher the yield of the target PDMC.

The remaining stages of micropropagation are rooting and acclimatization of the *in vitro* plantlets under greenhouse conditions, but these are performed only to produce large-scale clonal plantlets, including some medicinal plants. The whole process, except for acclimatization, is carried out under *in vitro* cultivated conditions, in glass or plastic bottles sealed to prevent contamination, keeping control of temperature, light (quality and intensity) and photoperiod.

The *in vitro* cultivation of PDMC plant species can help the production of medicinal plants in several ways. Here, we highlight the following techniques: 1) large scale propagation of clonal plantlets with high genetic and sanitary quality and high PDMC concentration and the propagation of species which are difficult to multiply by conventional methods, such as those with long growth cycle or systemic phytosanitary problems, for example, the occurrence of viruses and bacteria that are typically potentiated by conventional vegetative propagation; 2) the use of elicitors (biotic or abiotic treatments applied to the biomass to stimulate the production of secondary metabolites) to stimulate tissues usually obtained under *in vitro* conditions to produce target PDMCs, followed by the multiplication of these tissues, and; 3) genetic transformation of species used as bioreactors to induce PDMC development in specific tissues, aiming at improving the production efficiency of biomass, cells and, thus, PDMCs from rare species or species with difficult cultivation or very long cycle, or even of PDMCs of interest, but produced at low concentration in the original species.

The 1st technique can contribute to the production of herbal medicines and conventional products that use PDMCs extracted from medicinal plants. The *in vitro* cloning techniques accelerate the production of clonal plantlets from those genotypes performing better on both agronomic aspects and aspects related to the medicinal characteristics of interest. Although the 2nd and 3rd techniques may also result in the production of herbal medicines, their most significant application is the *in vitro* production of PDMCs of higher commercial value, also due to the higher costs involved in the use of these techniques (Cardoso *et al*., 2018). In the case of technique 3, the inherent contained conditions of *in vitro* systems circumvent the issues related to the release of transgenic medicinal plants for *ex vitro* cultivation, which involves complex regulatory processes and could also result in several inquiries from consumers, mainly for some food species (Cardoso, 2018). In this case, *in vitro* transgenic live tissues will be meant only to produce *in vitro* biomass (transgenic), aiming at the extraction of the purified chemical PDMC (with non-transgenic residues).

An example of the later would be the development of transgenic tobacco lines with the biosynthetic capacity to produce rare PDMCs, such as the alkaloids Vincristine and Vinblastine. These alkaloids are extracted originally from *Catharantus roseus* and used for producing vincristine and vinblastine sulfates, two chemotherapeutic agents widely used to treat some types of cancer. In this case, callus cells from transgenic tobacco would serve as a bioreactor for the *in vitro* production of the chemicals. These fast-multiplication cells would allow increasing the PMDC production exponentially. Tobacco is the choice because it is an excellent model for genetic transformation and allow controlling the development of *in vitro*

tissues. Transgenic tobacco could also be used to produce, in addition to PDMCs, different biopharmaceuticals, such as vaccines, hormones, and antibodies (Goldstein & Thomas, 2004).

The 2nd and the 3rd techniques have the most significant bulk of studies involving PDMC production from tissues, performed in several species that biosynthesize target metabolites. This momentum comes from the advantages of producing PDMCs using these techniques in comparison to producing plants using technique 1, which requires a long multiphase protocol, including genotype choice, asepsis, *in vitro* inoculation and regeneration, multiplication of callus, cells, roots and/or shoots, followed by elicitation, extraction, and quantification of the PDMC of interest.

Main factors affecting PDMC production

The genotype has unquestionable direct effects over the production of plant biomass and PDMCs. The prospection of genotypes with high PDMC contents and the establishment of breeding programs are both excellent strategies to increase the productivity of these compounds in domesticated and cultivated medicinal plants. As an example of these possibilities, in jaborandi (*Pilocarpus microphyllus*) the pilocarpine content ranges from 16.3 to 235.9 μg g^{-1} in dry weight (Sandhu *et al.*, 2006), a difference of 14.5 times in the native pilocarpine content. Nevertheless, research prospecting genotypes of medicinal plants with high PDMC contents is limited and has been carried out mainly by private companies or growers, limiting public access to more promising genetic material from the economic point of view.

The use of genotypes with high natural PDMC contents is an essential condition to the success of the *in vitro* production due to the higher costs of the system compared to other conventional techniques. Besides, as *in vitro* cultivation is based on mitoses (clonal origin), more efficient genotypes will result in significantly higher PDMC concentrations in the plant or tissues grown *in vitro*. However, growing conditions, especially in the field, can induce significant variations in the production of plant mass and PDMC contents, even in high-yielding genotypes (Vaz *et al.*, 2006). Therefore, it is essential that the conditions that provide the best genotype-environment relation are known, even for *in vitro* systems. In field conditions, both endogenous factors, to mention genotype, plant organ and age, circadian clock; and exogenous factors, such as photoperiod, light intensity and wavelength, temperature, water availability, cropping system, soil type, and atmospheric composition, can individually or jointly affect both the composition and the concentration of a single or different PDMCs (Gobbo-Neto & Lopes, 2007; Cui *et al.*, 2011).

These same factors can also influence the PDMC *in vitro* production. For example, Kapoor *et al.* (2018) reported the influence of light quality on the production of accumulated biomass, growth rate, and concentration of the phenolic compound Salidroside in *Rhodiola imbricata*. Kapoor *et al.* (2018) found the highest callus growth index (2.97) in red-light and the highest concentration of the total phenolic compound and Salidroside using blue light, in 21-day callus. As they observed, *in vitro* systems are established using protocols based mainly in culture medium and environmental conditions adjusted to the different phases of the *in vitro* growth. If the highest plant *in vitro* growth was obtained using white light, but the PDMC biosynthesis required blue light, a protocol should comprise at least two stages, one for plant tissue growth and another for increasing the PDMC concentration in those tissues. In other words, if the protocol uses callus as the primary source of cells for the PDMC *in vitro* production, callus proliferation should be carried out under white light, particularly in the exponential phase of cell or tissue growth, to accelerate and increase the biomass. Under the stationary growth phase, these tissues should be placed under blue light to increase the production of the target PDMC in each of the cells obtained in the previous phase (Figure 1). Other strategies can be designed to improve the efficiency, such as combining blue/white light in adequate rates for cell proliferation and PDMC stimulation. Similar tactics should be used for other factors, combining the ideal requirements for plant tissues and PDMC production on *in vitro* conditions.

In vitro environments offer the possibility of controlling the conditions

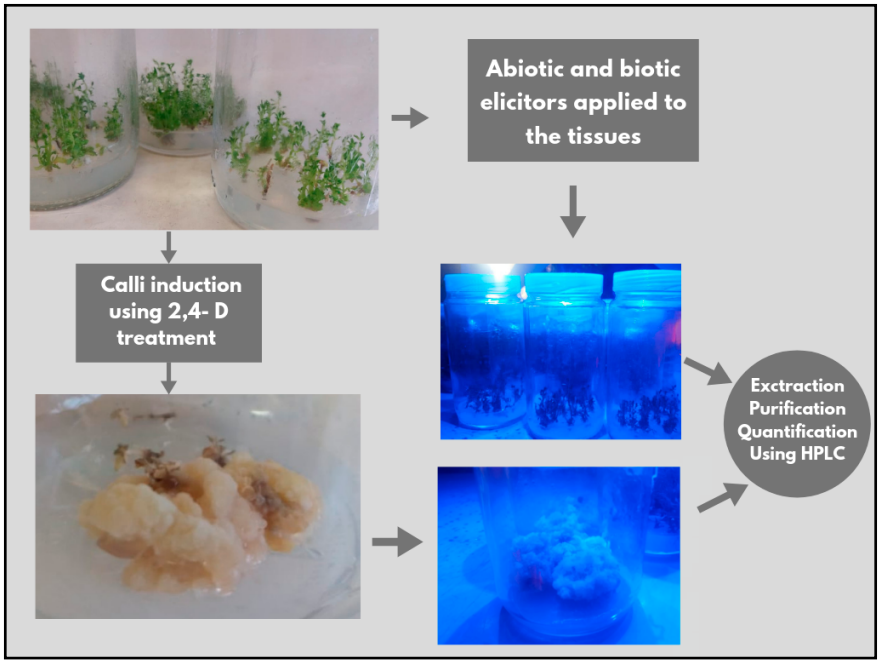

Figure 1. *In vitro* culture of *Phyllanthus amarus* established to produce the PDMCs phyllanthin and hypophyllanthin through shoot and callus. Araras, UFSCar, 2019.

that optimize the production of metabolites, independent of the time of the year and under aseptic conditions, which are both significant advantages of the system. On the other hand, the main disadvantage lies in the high production cost, which should be compensated by using the area and time provided by the system as intensely as possible. The main factors used for calculating the PDMC yield on *in vitro* environments should be fresh or dry mass and the PDMC concentration on them, the area used by the cultivation bottle or bioreactor (liquid media), and the time spent to obtain a PDMC production cycle. Therefore, we can summarize that the PDMC productivity can be calculated by different points of view and under different systems of cultivation. Other factors, such as the volume of culture medium, could also be used in this formula. However, culture media usually represents less than 5% of the total cost. Thus, considering the *in vitro* culture system, all factors influencing the fresh/dry biomass production, the PDMC biosynthesis, the area used by the flasks, and the culture cycle is crucial to determine the efficiency and productivity of PDMC under *in vitro* conditions.

The area used by the culture bottles varies according to the type and size of bottles, the efficiency of biomass accumulation in the protocols, and the system used. Conventional systems use semi-solid medium, containing agar, or liquid medium, under intermittent agitation, or even in culture under temporary immersion bioreactors (TIB). The use of the TIB has resulted in increases in the accumulation of plant biomass with good PDMC concentration compared to other conventional techniques using agar-based protocols (Gerth *et al*., 2007; Thakore *et al*., 2017).

Abiotic factors, such as temperature, light (intensity, photoperiod, and wavelength), relative humidity, and the atmospheric composition inside the bottle strongly influence the fresh and dry biomass accumulation, the PDMC concentration in the biomass and the cultivation period, which are, in their turn, also biological-dependent characteristics. The culture medium and its components, such as water availability and water potential of the culture medium, macro and micronutrients, carbohydrates, vitamins and amino acids, and phytoregulators of different classes, such as auxins, cytokinins (Grzegorczyk-Karolak *et al*., 2015) gibberellins, jasmonates, and salicylates (Ali *et al*., 2015) also influence these characteristics.

Other factors of influence are the type, intensity, and period of exposition of tissues to elicitors, which are used to stimulate the biosynthesis and increase in the PDMC concentration at the end of the growth cycle, or the accumulation of plant biomass (Siddiqui *et al*., 2013). Elicitors are chemical substances applied to the culture medium, ionizing radiation or other physical factors applied to the cells and tissues (Naik & Al-Khairy, 2016), or even co-culture of plant tissues with specific microorganisms (Tonk *et al*., 2016) or application of environmental stresses, such as high or low temperatures for a given period. Among the chemical substances applied to the culture medium, there are some phytoregulators, such as the salicylic and jasmonic acids (Sharma *et al*., 2015), chitosan (Ferri & Tassoni, 2011), microorganisms extracts (Maqsood & Abdul, 2017), in addition to other new generation molecules (Ramirez-Estrada *et al*. 2016). Among physical factors, ultraviolet radiation has been used frequently and has effects over different active principles produced *in vitro* (Klein *et al*., 2018).

The co-cultivation of plant cells with microorganisms has been used to elicitate some PDMCs. In *Catharanthus roseus*, the use of *Aspergillus flavus* in co-cultivation with plant tissues resulted in increases of 7.88 and 15.5% in the vinblastine and vincristine concentration, respectively, compared to non-elicited control tissues (Tonk *et al*., 2016). It is very probable that the activation of the synthesis of some secondary metabolites in response to microorganisms is associated with the biological function of these PDMCs in pathogen control. Ludwig-Müller (2015) reported the induction of secondary metabolism in plants also by endophyte microorganisms. However, the co-cultivation of plant tissues with microorganisms could also result in increases in the PDMC production costs because their use involves two types of organisms and, consequently, two different protocols, followed by the co-cultivation period. Also, the microorganism used for co-cultivation may produce toxic components and increase risks of contamination of plant cells.

Therefore, the development of a protocol for the *in vitro* culture of cells, tissues, and the medicinal plant itself to produce the target PDMC requires an extensive search for the existing knowledge on the species and the PDMC. Based on all previous factors presented by the literature and tested both *ex*- and *in-vitro*, including the use of elicitors to increase the biochemical routes to the PDMC, one is more likely to develop successful high-yielding *in vitro* systems customized for specific plants and target PDMCs.

The main systems used for the *in vitro* production of secondary metabolites

In vitro micropropagation, followed by plantlet acclimatization and growth in greenhouse or in the field, is a promising strategy to produce plant secondary metabolites, especially in rare or endangered species, in those difficult to propagate (Kapoor *et al*., 2018) or of slow growth (Cardoso & Silva, 2013), and in species vulnerable to recurrent phytosanitary problems, such as those of vegetative propagation. In the later, the techniques used to produce virus-free plants may be helpful also to increase both plant biomass productivity and PDMC contents, as observed in *Allium sativum*. *In vitro* techniques also allow for and accelerate the large-scale propagation of selected genotypes with high PDMC contents, either native or developed in breeding programs. In addition to conventional micropropagation, PDMC can be produced *in vitro* by callus induction and growth, cell culture, shoot proliferation, and the protocol of inducing hairy roots using transgenic techniques.

Shoot culture and proliferation

Shoot proliferation is similar to

conventional micropropagation. It involves the inoculation of surface-sterilized tissues in culture medium for shoot regeneration, followed by inducing shoot proliferation, usually by adding cytokinin as the main phytoregulator to the culture medium. After some cycles of shoot proliferation and *in vitro* mass accumulation, an elicitor is applied to increase the PDMC concentration in the tissues. Plantlets are then used to extract, characterize, and quantify PDMCs, instead of being directed to rooting and acclimatization, as it is in conventional micropropagation.

To determine and quantify the main secondary metabolites produced by shoot culture in *Scutellaria alpine*, Grzegorczyk-Karolak *et al*. (2015) cultivated 0.5 cm-long shoot tips in different cytokinin types and concentrations. Shoot tips came from surfaced sterilized seeds, germinated *in vitro* on MS medium. These authors found that, while 6-Benzylaminopurine (2-4 µM) resulted in the highest shoot proliferation and biomass accumulation, cytokinin type and concentration, as well cytokinin combination with the auxin Indole Acetic Acid, influenced both the composition and concentration of secondary metabolites in shoots. Regarding the use of elicitors, Sharma *et al*. (2015) reported that maximum bacoside contents on *in vitro* shoot cultures (8.73 mg g^{-1} dry weight) of *Bacopa monnieri* resulted from using 45 mg L^{-1} of $CuSO_4$ in the culture medium, combined with a shoot elicitor incubation period of 6 to 9 days. They also found that both jasmonic acid at 1.0 mg L^{-1} and salicylic acid at 50 µM, with 6- to 9-day incubation, resulted in increased bacoside concentration, 8.46 and 8.14 mg g^{-1} dry weight, respectively, compared to control conditions without elicitor (6.41 mg g^{-1} dry weight).

Callus and cell suspension culture

Callus induction and multiplication have been extensively used in PDMC *in vitro* production. It is an efficient approach to produce PDMCs in large scale when compared to other techniques, mainly because the *in vitro* callus induction is a straightforward and rapid system of cell multiplication (Kapoor *et al*., 2018). Besides, the factors that induce callogenesis are well studied (Ahmad *et al*., 2016), highly consistent, and used commercially already for some decades in tissue culture for other applications.

The use of callus culture is also associated with the competence of many plant cells and tissues, from different plant organs, to produce calli in response to a simple stimulus, generally with the application of auxins, especially 2,4-Dichlorophenolic Acid, and/or cytokinin phytoregulators in the culture medium (Ahmad *et al*., 2016). This great advantage comes from the fact that most callus cells acquire a continuous growth capacity if the conditions of the culture medium remain favorable. Therefore, cells maintain cycles of constant division, extending significantly a period known as the growth exponential phase, which is followed by a stationary phase with reduced growth. The ability to maintain these cells in a non-differentiating condition makes the process more manageable from the biological point of view and results in higher mass production per unit of time and area when compared to other regeneration processes of tissues, organs or individuals, generally more complex and more expensive.

In *Stevia rebaudiana*, leaf-piece explants were surface-sterilized and inoculated in MS culture medium, containing 2.0 mg L^{-1} of 6-Benzyladenine (BA) and 2.0 mg L^{-1} 2,4-D to induce callus formation (Ahmad *et al*., 2016). These authors tested the influence of the quality of the light on callus production.

The genus *Phyllanthus* has important PDMCs, especially the alkaloids phyllantine and hypophyllanthin (Lee *et al*., 2016). Seeds of a genotype of *Phyllanthus amarus* with high concentrations of these alkaloids, selected by the CPQBA/UNICAMP (Vaz *et al*., 2006), were previously germinated *in vitro* using the MS culture medium at half-strength. Derived 1-cm long micro-cuttings were transferred for callus induction into similar medium, adding 0.5 mg L^{-1} 2,4-D. The induced calli were kept in the same medium for proliferation, in the dark, with cycles of transference of 20-30 days after inoculation (unpublished data, Figure 1). Various elicitors will be used to test the capacity of the callus cells to produce phyllantine and hypophyllanthin. The callus obtained *in vitro* also can be used also for establishing new suspensions of cell cultures. Thus, new cell cultures become a regular additional phase of the callus culture, in which the friable type of callus starts a new cell culture in liquid media.

In *Hypericum perforatum*, cell cultures were induced in liquid MS medium containing 1.0 mg L^{-1} 2,4-D and 0.2 mg L^{-1} BA, obtained from a previous induced friable callus, using the same culture medium, but with agar. The friable callus was essential for successfully establishing the cell suspension. Only stem explants resulted in this type of callus, while leaf and calyx tissues originated hard and green non-friable calli. After transferring the friable calli to liquid media, the flasks were placed on a rotary shaker and transferred to a new culture medium every 20 days. These authors reported that maximum fresh (≡200 g L^{-1} culture medium) and dry (≡7-8 g L^{-1} culture medium) weight were obtained after 20-25 days, when also the highest contents of flavonoid accumulation (≡15-16 mg g^{-1} dry weight) were found in tissues. Wang *et al.* (2015), in their turn, reported that the use of methyl jasmonate and salicylic acid as elicitors resulted in flavonoid increases in cell suspension cultures, 2.1 and 1.5 times higher than the control treatment.

Hairy root cultures are those in which the genetic transformation mediated by the bacteria *Agrobacterium* rhizogenes is used to induce and obtain hairy-root lines (Chen *et al*., 2018) in tissues already established *in vitro*. Hairy-root lines are important sources of stable cells specialized in the production of useful PDMCs in amounts higher than those obtained from cell cultures (Sujatha *et al*., 2013), in several species.

Sujatha *et al*. (2013) used four strains of *Agrobacterium* rhizogenes to transform shoot tips, leaves, and nodes of *Artemisia vulgaris*, and observed that the A4GUS strain combined with leaf explants resulted in the highest transformation response, with 92.6% of transformation success. From all

transgenic hairy-root strains obtained from leaf explants, Sujatha *et al*. (2013) selected AV1 and AV2 because they grew better, with higher root elongation, ramification, and biomass accumulation. The cultivation of these transgenic hairy-root strains resulted in higher growth rate (9x), types (87), and concentration (0.51%) of essential oils, compared to non-transformed roots (7x, 77, and 0.43%, respectively). The hairy-root induction, combined with the temporary bioreactor immersion system, can also improve the PDMC productivity of hairy-roots. Aiming the production of Ajmalicine from *Catharanthus roseus*, Thakore *et al*. (2017) observed the effect of days of cultivation, sucrose concentration in the culture medium, inoculum size, photoperiod, type of the bioreactor system, and aeration volume on the growth of hairy roots and concentration of Ajmalicine, and concluded that the bubble gum bioreactor combined with polyurethane support improved significantly the hairy-root dry biomass (15.4 times in 30-day cultivation) and the concentration of Ajmalicine (34 mg L^{-1}), when compared to conventional rotating drum system of *in vitro* cultivation (3.4 times, and 4.6 mg L^{-1}).

Most of the authors who compared hairy root cultures with cultures of cell, callus, and shoots to PDMC production observed many advantages for hairy roots and concluded that, overall, the most important are the genetic stability of hairy-root cultures, their long-lasting capacity to keep cell viability, and their good capacity to produce different PDMCs in high concentration (Sujatha *et al*., 2013; Thiruvengadam *et al*., 2016; Thakore *et al*., 2017). Therefore, the hairy-root technique is definitely an appropriate and viable choice to the large-scale PDMC *in vitro* production.

Challenges and conclusions about PDMC *in vitro* production

Among the limitations for the *in vitro* production of secondary metabolites, the low biomass yield, resulting in reduced PDMC production on these conditions, stands out. Besides, the higher production costs, due to the controlled environmental conditions and need of specialized labor (Cardoso *et al*., 2018), when compared to field grown medicinal plants, also contributes to the low number of large-scale, profitable systems to produce PDMC *in vitro* we currently observe. Most of the *in vitro* techniques are still more appropriate to research and to understand the factors affecting the production of different PDMCs in several medicinal plant species. Like other plant tissue culture applications, large-scale PDMC *in vitro* production can be useful first for high-value and rare target PDMCs. The increase in the knowledge about the biochemical routes and genes that control the complex routes resulting in PDMCs, associated with advances in plant synthetic biology, specially related to metabolic engineering, will result in significant improvements in genetic transformation or organism reconstruction techniques, similar to some already experienced in the production of secondary metabolites from microorganisms (Wu & Hong, 2013).

Another limitation is the high cost for extraction, purification, and analysis of PDMCs obtained *in vitro*. Most of the methods involve the use of High-Performance Liquid Chromatography (HPLC) or ultra HPLCs for analysis, i.e., a few hundred dollars per analysis. Nevertheless, these costs may be diluted in large-scale systems and worth in case of high-value PDMCs.

Natural products are powerfully re-emerging in the genomic era, and metabolomics and metagenomics are increasingly efficient in identifying new ones (Harvey *et al*., 2015). In this context, the generation of transgenic plants with high capacity to produce PDMCs opens a new age for the discovery and re-discovery of natural molecules, derived from either plants or plant-microorganism interactions, valuable for medical ends and other purposes. Despite some technical limitations of the current systems for PDMC *in vitro* production, the highly controlled environment in plant/cell/callus biofactories, associated with the large capacity of *in vitro* systems to obtain high-concentrated and contamination-free compounds will constitute a realistic part of the production of different PDMCs. Such systems are among this century's cutting-edge technologies for drug production and development.

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