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REVIEW ARTICLE

Bioreactor-based advances in plant tissue and cell culture: challenges and prospects

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ABSTRACT

Bioreactors are engineered systems capable of supporting a biologically active situation for conducting aerobic or anaerobic biochemical processes. Stability, operational ease, improved nutrient uptake capacity, time- and cost-effectiveness, and large quantities of biomass production, make bioreactors suitable alternatives to conventional plant tissue and cell culture (PTCC) methods. Bioreactors are employed in a wide range of plant research, and have evolved over time. Such technological progress, has led to remarkable achievements in the field of PTCC. Since the classification of bioreactors has been extensively reviewed in numerous reviews, the current article avoids repeating the same material. Alternatively, it aims to highlight the principal advances in the bioreactor hardware s used in PTCC rather than classical categorization. Furthermore, our review summarizes the most significant steps as well as current state-of-the-art of PTCC carried out in various types of bioreactor.

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Introduction

Classical *in vitro* morphogenesis, including direct and indirect pathways has been developed for different plant species, where plants can be generated from the explant via organogenesis or embryogenesis [1]. Plant micropropagation via somatic embryogenesis (SE) may offer numerous advantages over organogenesis, including the bipolar structure of the embryo, feasibility of single cell regeneration that provides no vascular connection with maternal callus tissue or the cultured explant, and single hormonal induction [2]. Regardless of the above-mentioned advantages, automating large-scale production of embryos is only achievable using bioreactor technology [3]. In addition, bioreactors possess other privileges, including constant regulation of conditions during operation, enhanced nutrient uptake, and easy handling of large quantities of a culture [4]. Considering these facts, bioreactors have become suitable systems for the modern industrial plant tissue and cell culture (PTCC). Bioreactors, however, have been developed, since their first use in 1981 for *Begonia hiemalis* propagation [5]. Accordingly, investigating bioreactor technological progress s over time creates a clear picture of the current status as well as future developments. To this end, the present review aims to highlight trends and significant advances of bioreactor systems in PTCC as well as related challenges and prospects.

Principles of bioreactor designs for PTCC

The concept of bioreactors for PTCC was based on a transition from solid (agar) medium and shakes culture (flasks) to the use of liquid medium for enhancing the productivity and efficiency of plant propagation. In line with such concepts, an ideal bioreactor design should basically consider several factors, including the production of large number of plantlets in a single batch,

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scalability, low number of culture vessels, simple processes of inoculation and harvest, complete and continuous contact between the surface of cultures and the medium to enhance the uptake of nutrients and growth rate, aeration capability for oxygen supply to increase the growth rate and final biomass. Finally, development of an efficient circulating system (free-flow) for moving the cultures to suppress the apical dominance in favor of improving the growth of shoot buds [6].

Considering the above-mentioned characteristics, glass and stainless steel bioreactors integrating impellers, designed for plant micropropagation, are mainly categorized as: (a) mechanically agitated (e.g. aeration-agitated bioreactors, rotating drum bioreactors, and spin filter bioreactors), (b) pneumatically driven (unstirred bubble bioreactors, bubble column bioreactors [BCBs], air-lift bioreactors) and (c) non-agitated systems (e.g. gaseous phase bioreactors, oxygen permeable membrane aerator bioreactors, overlay aeration bioreactors) [7,8]. Furthermore, modern designs of bioreactors also tend to solve the foaming, shear- and stress-related problems.

Principal types of bioreactors for PTCC (advances, applications and solutions)

Airlift bioreactors and BCBs

Airlift and bubble column systems were basically designed to improve the biological oxygen demand of the culture. Airlift bioreactors (ALBs) are commonly used bioreactors for the plant cell suspension culture [9]. Technically, an ALB differs from BCB due to the pattern of fluid flow caused by the presence of an internal or external draft-tube, which results in vigorous axial flow circulation, more oxygen mass transfer, and reducing bubble coalescence and foaming. Despite dynamic recirculation, ALBs provide often lower oxygen transfer rates (OTRs) than BCBs, with a similar airflow. These groups of bioreactors represent simple structures, since no mechanical force for propeller stirring or shaking are required [10]. Indeed, the first bioreactor-based plant micropropagation was conducted in 1981 for *Begonia* propagation using a BCB [5]. Since then, ALBs have been employed to grow embryogenic cultures of various plant species, such as alfalfa (*Medicago sativa*) [11], carrot (*Daucus carota*) [12], grape (*Vitis vinifera*) [13] and cork oak (*Quercus suber*) [14]. These bioreactors have higher energy efficiency than stirred tanks. Moreover, ALBs have no moving parts, which can reduce maintenance time and costs and, in general, they provide low shear. However, foaming and bubble aggregation can be a major problem [9,15]. In a recent study, the application of ALBs could accelerate genetic transformation in the American chestnut (*Castanea dentata*) [10].

Stirred tank reactors

Stirred tank reactors (STRs) are currently the most functional bioreactors in PTCC [16]. STRs are scalable devices with efficient fluid mixing systems and high OTR that can be integrated with various types of impeller. Additionally, STRs comply with the current Good Manufacture Practices [17]. The greater microturbulence and higher shear rates are the major problems of STRs. Shear stresses are generated by the impeller and bursting gas bubbles in STRs [18,19]. However, these forces occasionally lead to beneficial elicitor effect, and therefore, boost the plant secondary metabolism. For instance, cysteine protease production by a cell suspension of the species of papaya (*Jacaratia mexicana*) as well as its proteolytic activity in STRs were found to be higher compared with the ALBs and BCBs due to the greater microturbulence force in STRs [20].

Advances in solving the technical glitches, such as foaming, shear and oxidative stresses

Different cultures are exposed to the problems, such as bubble coalescence, foaming, shear and oxidative stresses in bioreactors, especially in conventional STRs. Plant cells with rigid cell walls made of cellulose, are larger than microbial cells, and more sensitive to destructive shear forces [16]. Modified impeller types, silicone-based and polypropylene glycol antifoam agents, mechanical foam disruption [21], and bubbling systems [22], are used to overcome these problems. In some cases, the employed technologies, to combat these problems, have led to the creation of a new generation of bioreactors, such as membrane bioreactors (MRBs) [23]. Some of the related achievements are discussed below.

Development of different impeller designs in STRs

Different types of impellers, such as rushton and rushton-like impellers, pitched-blade, marine-blade, spin filter, helical and double helical ribbon, cell lift impellers, basket and anchor impellers have been described in STRs with different objectives [24]. Various types of impellers and baffles can affect the mixing rate, OTR and turbulent flow as well as shear stress in a bioreactor [25]. The effect of different impellers and baffles on the performance of cultures in STRs can be calculated using computational fluid dynamics (CFDs). Recently, MATLAB programs were employed for the

mathematical modeling of CFD, while ANSYS Fluent (ANSYS, Inc., USA), and Autodesk simulation CFD software (Autodesk, Inc., USA) helped to generate the related simulations [26]. Sometimes, the type (shape) of impeller inspires the names of bioreactors.

Helical ribbon impeller bioreactors

Helical [27], and double helical ribbon impellers (HRIs) [28] were embedded in the bioreactors to be well adapted for shear sensitive suspension cultures, by the early 1990s. Despite the occurrence of surface baffles that affect the power input, HRI bioreactors reduced shear damage, and improved the power dissipation, mixing rate and OTR, especially in viscous plant cell suspensions by generating, re-dispersing and entraining bubbles from the upper gas interface [28]. Helical agitators appeared to be efficient for the suspension culture of high-density plants ($\sim$27 g dw L^{-1}) [29]. In another attempt, SE of California poppy (*Eschscholzia californica*) was successfully conducted in HRI bioreactors [27].

Rotating wall vessel (RWV) bioreactors

Rotating wall vessel (RWV) bioreactors were originally designed to be used in mammalian cells under microgravity, and are still employed for human cell studies [30]. As a unique attempt, the cell lines of the Japanese yew plant (*Taxus cuspidata*) were subjected to a RWV bioreactor. The results showed that a secondary metabolite produced by the plant called Taxol could be optimized with respect to shear stress. As such, 2 mg L^{-1} Taxol was produced on day 14 in the culture at 2.3×10^{-3} Nm^{-2} shear stress [18]. Additionally, production of the component was suppressed under high shear conditions, at which cells might have been extensively injured in the later stages of the culture period. Under zero shear circumstances, Taxol productivity was as great as at moderate shear stress conditions. The mechanism by which plant cells detects shear stress forces and convert them to electrophysiological and biochemical reactions remains uncertain [18].

Membrane bioreactors MBRs and hollow-fiber systems

Technically, MBRs have a convenient in-situ separation capability that is lacking in other types of bioreactor [31]. Moreover, MBRs provide complete cell retention and selective removal of products as well as harmful metabolites and by-products [32]. Membranes are categorized into diverse geometries, including ceramic capillaries, plate-and-sheet (flat), tubular, spiral-wound and polymeric hollow-fiber modules. Polymeric hollow-fiber membranes are the most commonly used type due to their low price and wide range of application. Scalability of the hollow-fiber-based systems, such as multiple membrane plate bioreactors, is limited by the cartridge diameter, and the possibility of the axial length extension of fiber, without incoming O_2 transfer restrictions. Moreover, the mechanical flexibility of the membrane and the pump capacity can be a barrier to maximum operable flow rates [32,33]. The main features of MBRs application in plant cell cultures are high cell density, high protein volumetric productivity that preserve the secreted foreign protein in the cell compartment, and concentrating the product before harvest [33]. Furthermore, the foaming and bubbling problems as well as the cell damage caused by the shear stress found in STRs are diminished in MBRs, while the cultured cells are aggregated in a relatively static zone, in which the cells are protected from mechanical damage and are not in direct contact with the gas bubbles [33,34]. The membrane technology can also be integrated with STRs to enhance the performance of culture. According to Sorvari et al. [35], the integration of silicone or track-etch membranes with a specific type of STR with a cell lift impeller, resulting in a reduced adherence of somatic embryos of carrot var. Duke to the cell lift impeller.

Tubular membrane bioreactor (TMBR)

Tubular MRBs (TMBRs) with different membrane systems, such as hollow-fibers or ceramic capillaries have been designed with high surface-to-volume ratios for high dense plant cell cultures under low shear stress. Poor OTR, complex operation, and scalability are the drawbacks of these bioreactors. The TMBR concept imitates tissue-like conditions similar to the network of arteries, veins, and lymphatics in a living tissue. TMBRs can be perfectly integrated with cell immobilization systems, as the membrane performs like an immobilizer, to retain the cell culture in the reactor compartment. It also acts as an attachment surface for adherent cell types [32]. TMBR have been successfully employed to enhance the extraction of betanin from beetroot (*Beta vulgaris*), as well as ajmalicine and yohimbine from Madagascar periwinkle (*Catharanthus roseus*) cells [36].

Silicone-tubing aerated bioreactors

Silicon-made materials are replaced with polypropylene membranes in various types of bioreactor. Bioreactors

equipped with silicone tubing provide a bubble free oxygen supply, which are suitable for somatic embryo production [37,38]. Interestingly, the use of silicon tubing in STRs, prevents foaming when stirrer operates at low speed [6]. Furthermore, silicon membrane tubing led to doubling the gas mass transfer, under the same shear stress in membrane-aerated and mechanically agitated bioreactors [39]. The efficiency of silicon in bioreactor systems, for reducing the induction of hyperhydric shoots in *Ornithogalum dubium*, recently been realized [40,41]. Probably, the silicon membrane tubing could play the same role in the reduction of hyperhydricity problems under temporary immersion systems (TISs).

Slug bubble bioreactors and microbubbles

Oxygen transfer rate and suitable mixing with a low shear force are critical issues for plant micropropagation in bioreactor systems. Microbubbling is an approach for oxygen supplementation, so that purified oxygen is separately sparged in the form of very small bubbles into the culture medium. However, the first generation of spargers used in ALBs, did not receive sufficient attention due to high energy consumption [15,42], but the new systems, combined with multichannel ceramic membranes and fluidic oscillators, resulted in a larger gas holdup, microbubble distribution, interfacial area, mixing rate, and OTR [43,44]. The formation of slug bubbles (also known as Taylor-like bubbles) in the cell culture of devil's claw (*Harpagophytum procumbens*), reduced shear stress, increased the OTR, and harmonized the culture medium within a glass-column bioreactor under pulsed aeration mode [22]. The slug bubble bioreactors (SBBs) have been developed based on slug bubble flow models in flat sheet MRBs [45]. These systems have been used in tobacco (*Nicotiana tabacum*) and soybean (*Glycine max*) cell suspension cultures, in order to produce isoflavones [46].

Bed bioreactor

Bed bioreactors use cell immobilization technology. These bioreactors mainly consist of: (a) a fixed or packed bed (PBB), and (b) fluidized bed bioreactors (FBB). In an FBB, a cellular or enzymatic biocatalyst is immobilized in or onto a solid support while a fluid (gas or liquid) is passed through a solid granular material (usually a catalyst possibly shaped as small spheres) at high velocities to suspend the solid phase, making it function as a fluid [47]. In contrast, the PBBs consist of high-density packet microcarrier material (usually sphere particles, from 100 to 300 μm in size), which forms a fixed bed. Circulating liquid nutrients across beds provides nutrients and oxygen for cultured cells/tissues, and organs [48]. Immobilized cell particles remain in the bed by gravity, while small separate cells are washed out. Therefore, setting velocity is a crucial parameter to increase the cell culture performance [49]. Tobacco cells fixed on the cell-attachment net in a PBB shows growth rates similar to flask cultures [50]. Bed bioreactors were recently used for PTTC to produce therapeutic proteins [48]. However, the main disadvantages of PBBs are the low mass transfer coefficient and heat transfer. Contrary to PBBs, the main advantage of FBBs is their efficiency in high heat transfer between the high velocity fluid and the catalyst surface [51]. As an advantage, the shear stress at the particle surface is much lower in FBBs compared with the STRs using immobilized cell systems [49].

Disposable bioreactors

Disposable bioreactors (DBRs), also known as single-use bioreactors are made of single-use plastic bags, suitable for a plant cell suspension culture. DBRs are time- and cost-effective, capable of eliminating cross-contamination and reducing turnover time between each run [33]. Considering the tremendous impact of genoproteomics-assisted strategies in the field of medicinal plant breeding and molecular farming [52], DBRs can provide novel features to produce biopharmaceuticals and recombinant proteins [51]. DBRs can be incorporated with various bioreactor systems; however, scalability poses a major challenge to their development [53].

Disposable wave and orbitally shaken bioreactors

Wave and orbitally shaken bioreactors are categorized as DBRs. These single-use systems were designed, based on a non-invasive rocking motion that provides excellent mixing and gas transfer for cell growth [54]. These bioreactors are simple, cost-efficient, and capable of scaling up to the cubic meter. The wave and orbitally shaken single-use reactors are merely suitable for certain uses, such as cultivating animal or plant cells with low oxygen demand [55]. The cultivation of human IgG-secreting BY-2 cells was recently scaled-up in a 200 L disposable wave and orbitally shaken bioreactor. Tobacco BY-2 cells are an attractive platform for manufacturing a variety of biopharmaceutical proteins such as antibodies. Cell growth and recombinant protein yields were comparable with those obtained from

cultivations in 500-mL shake flasks. Furthermore, an efficient downstream process has been developed for antibody recovery from the viscous spent medium using expanded bed adsorption chromatography [56].

Mist/spray bioreactors

The initial attempts to develop hairy root cultures using classic impeller-based systems, such as STRs, caused damage to sensitive plant tissues, extra callus formation and poor biomass production [57]. Probably, the discussed problems ignited ideas towards extension of mist/spray reactors considering the nutrient mist/spray technology or aeroponics systems to assure efficient gas exchange and low shear conditions. Mist bioreactors are gas-phase instruments with the highest potential for the cultivation of hairy roots. Unlike the growth conditions in liquid bioreactors, roots grown in mist/spray reactors are not oxygen limited even at high bed densities; and, the production of secondary metabolites is often greater in mist bioreactors than in liquid phase reactors [58]. Liquid dispersal can be achieved using a spinning disk, a compressed gas atomizer, or an ultrasonic droplet generator. In addition, droplet size is a critical issue in mist reactors as smaller droplets have a larger surface to volume ratio that facilitates gas transport into liquids. The minimum applicable droplet size is about 1 μm for roots. Nozzles with small orifice generate small droplets; however, as a negative point, they clog easily [58]. Hairy root culture of the neem tree (*Azadirachta indica*) by using mist bioreactors has led to production of 13.3 g L^{-1} dry weight of Azadirachtin [59]. Recently, the extract of St John's wort (*Hypericum perforatum*) roots, grown in a mist bioreactor, has shown great performance against planktonic cells and biofilm of a skin fungus (*Malassezia furfur*) [60]. In the latest generation of mist bioreactors, the cell culture is carried out within a mist-chamber full of aerosols. These aerosols are indeed the vaporized medium generated by a piezoelectric ultrasonic vaporizer [61]. These systems have successfully been employed for the propagation of *Saccharomyces cerevisiae* (yeast), *Aspergillus niger* (a pathogenic fungus), and *Flammulina velupites* (also known as Enokitake or Enoki; a long-thin white mushroom) [61]. Seemingly, the mist intervals and aeration rate are the key factors in the performance of mist/spray bioreactors [62].

Photobioreactors

Photobioreactor (PBR) systems utilize a light source for cultivating phototrophic microorganisms, such as plant, microalgae, and cyanobacteria to generate biomass. To this end, the culture is constrained by transparent material in a PBR [63]. The new generation of PBRs, called compact PBR or compact tubular PBR, are efficient systems for supplying biofuel/electricity, and synthesis of novel materials [64]. Microalgae are suitable cultures for PBRs due to their high photosynthetic efficiency and salt tolerance compared with the field crops. Moreover, compact PBRs succeed greater photosynthetic capacity by increasing the chlorophyl area due to the vertical growth position of the culture [65]. Hybrid PBRs become more profitable by being integrated into other systems such as column, tank, spiral, bubble and ALBs. The panel and tubular horizontal PBRs are efficient systems under the PBR category due to their high level of lighting and photosynthetic area. Nevertheless, scaling up, algae-glass wall adhesion and biofilm formation are the disadvantages of these systems [63].

Super-spinner bioreactors

The super-spinner bioreactors, designed with a central membrane stirrer at low speed, are suitable systems for plant cell suspension culture, since they are capable of reducing shear forces [66]. However, the performance of super-spinner bioreactors differs in different plant species. For instance, Ho et al. [67], achieved excellent results with cell culture of Maire's yew (*Taxus mairei*), while the same bioreactor did not perform successfully when it was used for the SE of *Lilium* × *formolongi* Hort. hybrid cultivars [68].

Membrane-based strategies in PTCC

Cell immobilization

Immobilization systems were originally developed to stabilize enzymes used in the industrial production of sugars, amino acids and pharmaceutical products [69]. These systems were subsequently utilized to immobilize plant cells in bioreactors [34]. Cell immobilization is a method in which the cells are deposited on the surface of a synthetic fibrous membrane that prevents cell entry into a mobile phase carrying the substrate and the product [70]. The membrane also provides strong binding of the growing plant tissue biomass in a submerged culture. The immobilized plant cells remain highly viable with catalytic activity [71]. Surface immobilization of plant cells decrease the hydrodynamic shear stress caused by agitation or sparging, especially in mechanically agitated bioreactors, and prevents inadvertent cell removal with the effluent during continuous culture. The method also provides convenient cell

recovery from the culture broth for repeated use without any necessity for inoculation [70]. The system also promotes the natural tendency of plant cells to aggregate, which may improve the synthesis and accumulation of secondary metabolites [72]. The most common immobilization agent is calcium alginate [73], vanillin, ajmalicine and capsaicin [74]. Reportedly, calcium alginate has been used in the production of paclitaxel [73]. Bodeutsch et al. [75], stated that cell immobilization in alginate, carrageenan and/or agar could enhance the production of pharmaceutical proteins, such as the human granulocyte-macrophage colony-stimulating factor in tobacco cells. In another experiment, cell immobilization led to increasing the production of ajmalicine by Madagascar periwinkle from 2 mg L^{-1} to 90 mg L^{-1} [76].

TIS/Temporary immersion bioreactor

The initial concept of TIS dates back to 1983, when scientists designed an apparatus called "auxophyton" capable of merging aeration and liquid medium culture together [77]. Auxophyton turned the culture containers on a wheel, exposing the experimental plants alternately to air, or immersing them in liquid. After 20 days, the carrot tissue weighed 2.6 times more than the tissue cultured on an agar medium [78]. Previous attempts with carrot tissue culture, under complete immersion mode, failed, possibly due to lack of oxygen [79]. Since then, TIS-based bioreactors have been exposed to many changes. However, all devices meet the requirements described by Teisson et al. [80], including: (a) no continuous immersion, (b) sufficient mixing and OTR, (c) consecutive medium changes and automation, (d) low shear stress, contamination and costs. The positive effects of TIS on: shoot proliferation [81], shoot vigor [82], SE [83], plant material quality [84], as well as microcuttings and microtuberization [85,86] have been reported for different plant species. Hyperhydricity and optimizing the immersion time are the most crucial parameter affecting the efficiency of TIS [87,88].

Specific achievements in bioreactor-based SE

Secondary embryogenesis and embryogenic cell suspension for enhancing genetic stability

Embryogenic suspensions (ESPs), including extensive cell division, are risky in respect to both genome and epigenome instability. Consequently, elevated frequencies of somaclonal variation (SV) in ESP-derived plants have been reported in numerous species, such as coffee (*Coffea arabica*) [89], tea (*Camellia sinensis*), oak (*Quercus robur*) [90], and rubber (*Hevea brasiliensis*) [91]. Culture conditions that allow moderate cell proliferation, such as low 2,4-D concentrations, short proliferation periods, and limited number of subcultures are suitable strategies to overcome this problem. Accordingly, the temporary immersion system (TIB)-based secondary embryogenesis (SCE) and ESP protocols have been developed with an emphasis on very low concentrations of auxin (1.36 μM 2,4-D), low concentrations of cytokinin (5.6 μM 6-BA), and short proliferation periods (6 months), to decrease SV, and increase genetic/epigenetic stability. These strategies ensured very low SV and high genetic conformity in SE-derived plants (emblings) of coffee [89].

SV and molecular approaches as conservation and breeding tools

As mentioned, scaling up ESP often leads to the production of large numbers of plantlets, and high rates of SV, which is not desired for genetic stability. Regardless of the desirability of SV, its occurrence in bioreactor-based ESPs, can be detected through molecular markers. For example, methylation-sensitive amplified polymorphism, and amplified fragment length polymorphism markers have been used to confirm the low SV, and high genetic stability (high similarity) in coffee emblings regenerated within a TIB [89]. Clonal fidelity analysis of St John's wort plants regenerated within a simple bioreactor, using Random Amplified Polymorphic DNA markers exhibited 84–99% similarity. These markers detected low polymorphism amongst the mother plants and emblings, i.e. ranges of 0.07–0.18 and 0–0.003%, respectively [92]. In contrast, Inter-simple Sequence Repeat markers have revealed high SV (low similarity) for sugarcane (*Saccharum* spp. hybrids) plants micropropagated in a TIB. The subcultures 1°, 9° and 10° showed the highest percentage of polymorphism with 10.1, 15.6 and 10.1%, respectively [93]. Since SV generates variation, it is regarded as a suitable source for plant breeding, especially in the species with low genetic diversity [94]. For the same reason, SV is useful for the conservation of endangered species [95]. Recently, the salt tolerance of pineapple (*Ananas comosus*) somaclonal variants using different sodium chloride (NaCl) concentrations were screened within TIBs for a breeding program [96].

Efficient SE methods have recently been developed using bioreactors, and coupled with molecular techniques, such as *Agrobacterium*-mediated transformation. Golden pothos (*Epipremnum aureum*) [97], tobacco [98],

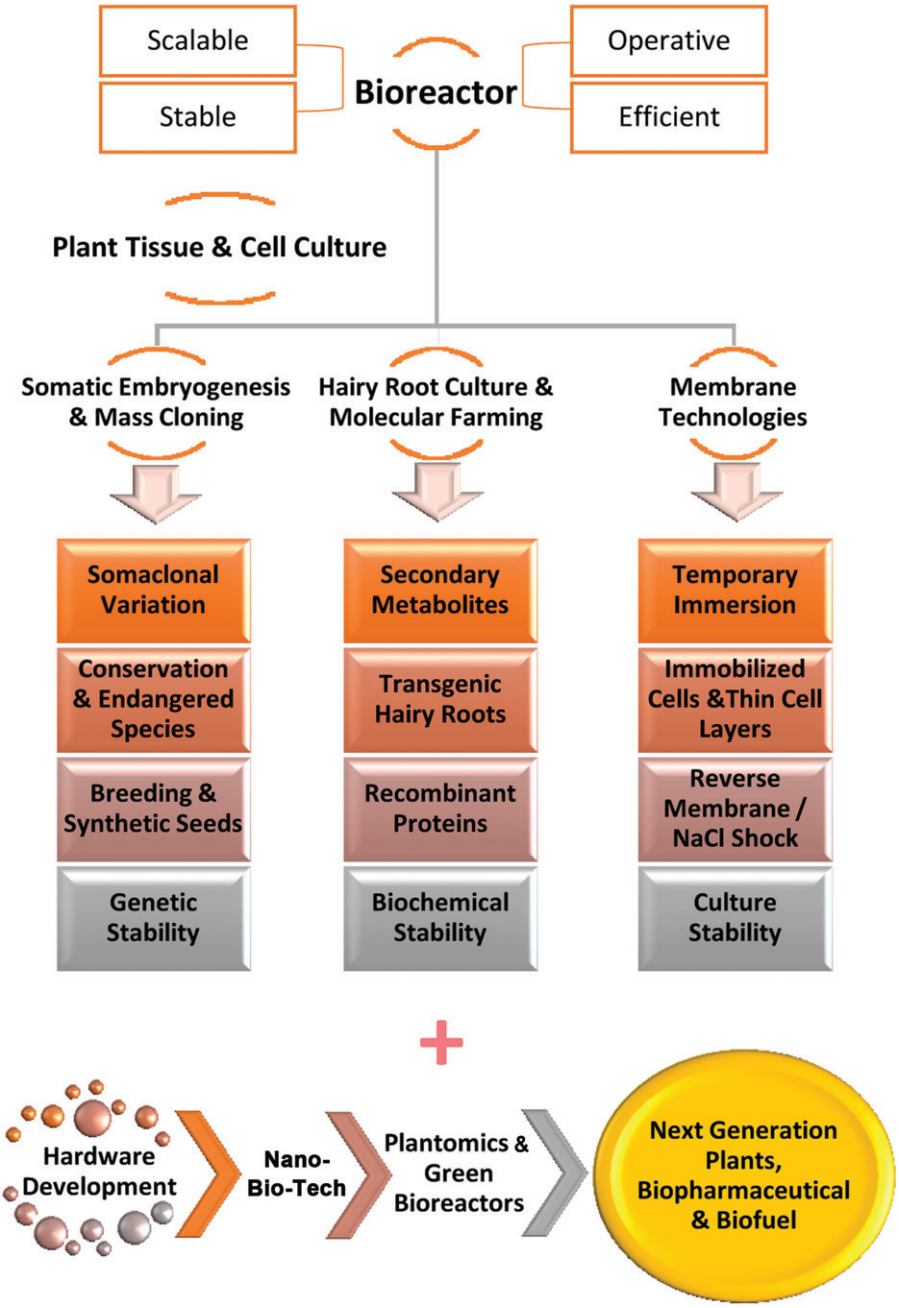

Figure 1. Schematic representation of the main achievements and prospects of bioreactor-based studies in the field of plant tissue and cell culture.

and cassava (*Manihot esculenta* Crantz) [99] are good examples for employing a joint process, including efficient SE protocol and an effective *Agrobacterium*-mediated transformation.

Abscisic acid-based prevention of polar auxin transport

SE exhibits great potential for large-scale plant propagation in various automated bioreactor systems. Despite this, somatic embryos sometimes suffer deformities, such as cleavage polyembryony, and lack suspensor. Moreover, unlike the zygotic embryos, somatic embryos often show SCE, pluricotyledony (producing more than two cotyledons), precocious germination (viz. in immature somatic embryos), asynchronous development, and double-triple vascular systems caused by polar transport of auxin in undeveloped shoots [100]. Some of these abnormalities can be corrected by the exogenous application of low concentrations of abscisic acid, for instance, in cocoa [101], and some by directional auxin transport mechanisms in

land plants, such as moss (*Physcomitrella patens*) [102]. Bioreactor technology facilitates the application of plant growth regulators (PGRs) for different aims [103]. MBRs possess such capacity for the treatment of 2,4-D, mecoprop and dicamba [104].

Challenges and prospects

Bioreactor-based studies in the field of PTCC are accompanied with challenges and prospects. The main achievements and prospects of this field have been represented in Figure 1.

Green bioreactors (transgenic plants as bioreactors)

Currently, the concept of PTCC (e.g. transgenic hairy roots) and microalgae (e.g. *Chlamydomonas reinhardtii*) are utilized as green bioreactors to produce valuable secondary metabolites, edible vaccines [105,106] and recombinant proteins [107,108]. The cultivation of transgenic hairy roots has recently attracted a great deal of attention for the production of secondary metabolites. Hairy roots are fast growing, genetically stable, and simple to maintain in PGR-free media [109]. Initial studies on the cultivation of hairy roots were carried out using the hairy roots of bindweed (*Calystegia sepium*) and belladonna or deadly nightshade (*Atropa belladonna*) to produce tropane alkaloids in STRs, during the late 1980s [109]. The liquid-phase (submerged), gas-phase (mist/spray), disposable, and hybrid bioreactors, which are a combination of liquid and gas-phase bioreactors, are suitable for hairy root culture [110]. The hairy roots of black henbane (*Hyoscyamus niger*) grown in a hybrid bubble-column/spray bioreactor have resulted in substantial production of alkaloids [111]. In addition, the hairy roots of various plant species, including cucumber (*Cucumis sativus*) [112], soybean [113], tomato (*Solanum lycopersicum*) [114], tobacco, and a vine species, called *Tetrastigma hemsleyanum* [115], have become, in the service of biotechnology, living bioreactors. The transgenic hairy roots of *T. hemsleyanum* were recently used as bioreactors for producing recombinant proteins. Since the natural slow growth rate barely meets market demand, the rapid and large-scale production of this species was achieved through the induction of its hairy roots by using *Agrobacterium rhizogenes* [115]. Despite the fact that the hairy root cultures are mainly employed under aerobic conditions, transgenic methods in low oxygen conditions are alternatively optimized for secondary metabolite production. Accordingly, transgenic *Arabidopsis thaliana* hairy roots, containing alcohol dehydrogenase and pyruvate decarboxylase, showed a similar growth rate under low oxygen conditions, similar to those with full aeration [116].

Bioreactor-derived synthetic seeds

Synthetic seeds are basically produced by encapsulating any *in vitro* or *in vivo* generated explant with an artificial endosperm and a seed coat. With this definition, the center of synthetic seeds can be filled with somatic embryos, shoot buds, axillary buds, cell aggregates, protocorms or other meristematic tissues [117]. The technology responds to the concerns related to the preservation of germplasms [118], as well as the conservation of rare and endangered plants which are suffering from a low seed set during conventional methods [119]. A conifer, namely white spruce (*Picea glauca* [Moench.] Voss.), was amongst the first plant species subject to production of synthetic seeds using somatic embryos and bioreactor systems [120]. Modern bioreactors integrated with TIS have been used for the synthetic seed production of *Cannabis sativa* [121].

Protoplast culture in bioreactors

Despite a tangible need to develop and optimize the bioreactor systems for the mass production of plant protoplasts, there is a large research gap since Hohe and Reski [122], tried to optimize a bioreactor culture of the moss for mass production of protoplasts. Considering numerous advantages of bioreactor systems compared with conventional methods, any progress in the development of bioreactor-based protocols for protoplast culture, could potentially lead to advances in related techniques, such as somatic hybridization or protoplast fusion—a non-GMO method used in plant breeding.

Hyperhydricity, gas exchange and gas-based decontamination

Continuous contact of plant tissues with the liquid medium is a source of hyperhydricity [123]. Hyperhydricity can cause accumulation of high concentrations of C_2H_4 (ethylene) and CO_2. Technically, the presence of O_2, progressive ventilation, and sufficient gas exchange during culture can prevent the negative effects of hyperhydricity [124]. Alternatively, application of $CaCl_2.2H_2O$ in the culture medium increases the calcium content of tissues and decreases hyperhydricity [125]. Regardless of contrary reports on the positive

and negative impacts of O_2- CO_2-enriched atmospheres of bioreactors [126,127], a combination of O_2/CO_2 is used to control the culture media pH and decrease the risk of contamination. The CO_2 gas decreases the pH, while NaOH increases the pH [128]. Theoretically, gaseous CO_2 reacts with the media H_2O and forms H_2CO_3 (carbonic acid), which immediately decomposes into HCO_3^- (bicarbonate) as well as H^+ ions, and results in the pH reduction. Low CO_2 will produce fewer $H+$ ions. This process consequently leads to a rise in pH value. Despite the occurrence of pH reduction (due to the consumption of ammonium and H^+ diffusion) in the beginning of the course of any culture [6], when stepwise CO_2 gassing (8.5–2%) is applied to batch cultures with pH ranging 6.8–7.2, perhaps to decrease the risk of cross-contamination [129]. Furthermore, concerns regarding the side effects and inhibitory impact of antibiotics in plant micropropagation, resulted in the development of gas-based decontaminators. Recently, ozone (O_3) treatment for 5 min day^{-1} with a flow rate of 1.0–2.0 L min^{-1} on *in vitro* propagation of *Lilium*, under continuous immersion bioreactors, has reduced the risk of contamination [130].

Non-invasive oxygen monitoring in 3D plant scaffolds

Previously, scientific reports relied on written descriptions and 2D illustrations, but recently 3D virtual modeling has been introduced. Unlike 2D environments such as petri dishes, bioreactors permit cells *in vitro* to grow in all directions (3D) [131]. Monitoring of intracellular oxygen content in 3D scaffolds pose a challenge for tissue engineering applications [132]. Currently, non-invasive real-life oxygen monitoring methods in green plants are mostly based on optical micro- and nano-sensors [133–135]. These approaches could potentially be replaced with methods measuring the dissolved oxygen content of the media in a bioreactor system.

Bioprinting, phenotyping and X-ray tomography

Computed tomography remains underutilized in plant studies despite its potential to deliver high-resolution 3D phenotypic data. The available methods suffer from the low X-ray absorption of most plant tissues. However, X-ray computed tomography has become an efficient method for the quantification of cancer cell proliferation within perfusion bioreactor [136]. Seemingly, recent achievements in plant tissue X-ray tomography by developing efficient contrasting agents [137], as well as studying other influencing factors [138], make it conceivable to experience quantification, phenotyping, and preparing high-resolution images of 3D plant scaffolds, such as somatic embryos in different growth stages within bioreactor systems. Additionally, tomography based on CFDs, is a potent tool for determining the shear stresses within perfusion bioreactors [139].

Thin cell layers and protocorm-like body

Thin cell layer (TCLs) are small and versatile explants for the *in vitro* culture of plants [140]. TCLs have been applied to *in vitro* culture of many field crops, vegetables, ornamental and medicinal plants as well as protocorm-like bodies (PLBs). Aranda or flax lily (*Dianella caerulea*), and orchid species, such as *Coelogyne cristata*, *Cymbidium* spp., *Dendrobium* spp., *Doritaenopsis, Paphiopedilum, Renanthera, Rhynchostylis, Spathoglottis* and *Xenikophyton* have successfully been subjected to TCL-based propagation [141]. Considering the similarities of PLBs and somatic embryos, the technique seems to be useful for largescale mass propagation in bioreactors or for long-term storage as synthetic seeds. In line with this, steps towards incorporating the TCLs in bioreactors have been taken recently through the mathematical modeling of cell layer growth in MBRs with hollow fiber membranes [142].

Plant-microbial exopolysaccharide production

Exopolysaccharides (EPSs) are natural extracellular polymeric substances (EPSs) with high molecular weights secreted by microorganisms into their environment. Bioreactor systems have already been operated for microbial EPS production [143]. In line with current achievements, bioreactors can be exploited for further investigations of plant-bacterial interactions as well as future EPS production [144].

Reverse MBR

The latest generation of MBRs developed for biofuel production is based on a novel idea called reverse MBR (rMBR). The rMBR applies high native cell density and the membrane separation of cell/feed to the conventional immersed MBR (iMBR) set up [145]. Considering the interdisciplinary approaches in bioreactor technologies, the employment of the rMBR concept for plant micropropagation aims is predicted.

Bioreactors and plant metabolomics

Significant fluctuations in the content of active components (e.g. melatonin and serotonin) within the same products of the pharmaceutical companies has become a serious issue [146]. Melatonin and serotonin are not only deemed as medicinal compounds but also as potential regulators of plant growth and development [147]. As already discussed, establishing bioreactor-based hairy root cultures is an alternative to obtain homogeneous plant-derived products in large quantities. More specifically, a high content of plant-derived secondary metabolites can be achieved through either over-expression of the relevant enzymes of the metabolic pathways in specific gene [148], or elicitors [149].

Salinity and application of transient NaCl shock

High salinity imposes a challenge to agriculture. Despite this, *in vivo* studies showed that NaCl can be used as an elicitor for increasing the biosynthesis of secondary metabolites (e.g. diterpenoids) in a medicinal plant, called *Andrographis paniculata* [150]. *In vitro* studies on the response of pineapples to salinity within a TIB, revealed similar outcomes, whereas 200 mM NaCl increased the levels of other aldehydes by 2.4 times, and the soluble phenolics in shoots by 1.4 times [96]. Recently, developmental transition of chloroplasts of Bienertia sinuspersici [151], and SE of rice (Oryza sativa) have been investigated *in vitro* with increasing concentrations of NaCl [152]. Furthermore, salinity is known to have toxic effects on bacterial strains, and is capable of altering the microbial population. This property of NaCl salt has recently been used for wastewater treatment using MBRs. Fortunately, the robustness of MBRs against transient NaCl shocks in the wastewater treatment process has already been approved [153,154]. According to these achievements, it seems that bioreactors are going to be increasingly employed in plant metabolomics and abiotic stress resistance on both the research and industrial scales.

Nanodevices and nanoparticles for membranes and immobilization

Similar to cell immobilization, that was initially used during enzyme research [69], nanotechnology based advances were also used for enzyme investigations as well. However, as mentioned previously, due to the interdisciplinary nature of the current science, nanodevices and nanoparticles can be employed for bioreactor-based plant micropropagation in the future [155].

Woodchip bioreactors or future bioreactors on chips?

Woodchip bioreactors are defined as subsurface trenches filled with a carbon source, mainly wood chips, through which water is permitted to flow before leaving the drain to enter a surface water body [156]. These systems are modern agricultural tools for reusing carbon and nitrate wastes in favor of crop production. Considering the trend of technological progress, bioreactor development would not be confined to concerns on shear stress, gas and medium exchange, sterility and synchronization, and also on developing bioreactors on a chip.

Conclusions

We have reviewed the trend of bioreactor-based researches in the field of PTCC. However, the classification of bioreactors, as well as their hardware designs and differences, were not in the center of our attention, but the application of different types of bioreactors in PTCC studies was considered in this review. As mentioned, bioreactors are increasingly involved in PTCC studies due to their numerous advantages over conventional methods. Bioreactor-related technologies rapidly transfer from one field to another, meaning that a specific type of bioreactor that is currently used in a different industry, such as wastewater treatment, can be employed in PTCC researches in future. SE and SV play important roles in breeding new plants and the conservation of endangered species. The current trend shows that alongside technological progress in bioreactor hardware, PTCC develop mostly towards metabolomics, recombinant proteins production, molecular farming, transgenic hairy root culture, microalgae research and biofuel production, and membrane-based strategies. All these aspects are going to be integrated with plantomics and nano-bio-technologies. Despite positive horizons, the costs and scale-up engineering developments are major challenges.

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