During the 20th Century, the average human lifespan increased by approximately 30 years, mainly by virtue of vaccination. In spite of the great economic value of healthcare, the number of vaccine manufacturers has decreased in recent years, probably due to regulatory hurdles and modest financial returns. However, new vaccines, produced with novel technologies, have recently been licensed for human use, such as the anti-human papillomavirus (HPV) recombinant subunit vaccines based on virus-like particles (VLPs), and obtained by the expression of viral L1 capsid protein in yeast and insect cell lines. This has led to the expectation that the vaccine business will grow at a greater rate than the pharmaceutical drug business over the next decade [1]. Therefore, new biotechnology strategies are facing some major challenges, such as increasing the chances of success for important new vaccines and making low-cost innovations available for developing countries, where medical infrastructure and coordinated healthcare policies are lacking and preventative measures are also poor.

Plants have been used for therapeutic purposes since ancient times. Nowadays, plants, together with other approaches (i.e., DNA vaccines, VLPs, novel adjuvants, delivery systems and 'reverse vaccinology') represent a novel platform for the development and production of safer and more potent vaccines. Research over the past 20 years has significantly increased our knowledge of gene regulation and protein synthesis in plants, many recombinant proteins have been produced in a range of different species [2–4] and the first plant-derived biopharmaceutical proteins have approached commercial approval [5].

In addition to using whole plants, contained systems are gaining importance for the production of recombinant therapeutic molecules. These systems include plant cell suspensions, hairy root cultures, novel plants grown in contained conditions and microalgae. These systems show intrinsic advantages, such as control over growth conditions, production in compliance with good manufacturing practice and avoidance of political resistance to the release of genetically modified field crops. At present, one of the two plant-produced vaccine-related products that have gone all the way through production and regulatory hurdles derives from tobacco cell suspensions, and the second is a human therapeutic enzyme, which is expected to reach commercial development soon and derives from carrot suspension cells. In the future, several other products from contained systems are expected to reach the clinical trial stage.

Keywords: cell suspensions • GMP • microalgae • plant-derived vaccine • root cultures
significantly higher than for agriculture, contained systems provide more benefits compared with whole plants in the field: culture conditions are more readily manipulated and controlled in vitro (growth is independent on the climate, soil quality, season, day length and weather, resulting in improved product consistency), production times are shorter, problems related to pathogens (i.e., mycotoxin), herbicides or pesticides are abolished, and the potential to secrete the product into the culture medium might imply simpler and cheaper downstream processing and product purification [7]. Moreover, contained plant systems can be cultivated in bioreactors, making these systems amenable to good manufacturing process (GMP) procedures, with no worries that genetically modified cells or foreign genes might be released into the environment, allowing regulatory and environmental requirements to be met with less difficulty [8].

Two main types of products have been produced by in vitro plant cultures: secondary metabolites and heterologous proteins [6]. Historically, the first attempts at large-scale plant tissue cultures aimed at the production of pharmaceutical secondary metabolites date back to the 1950s, but in spite of different efforts to enhance yields, up till now the most significant examples of commercially viable production of a secondary metabolite in vitro are represented by ginseng saponins, shikonin, paclitaxel (Taxol®) and berberine [6,7].

Regardless of the tissues or cells used, the first requirement for obtaining high levels of products by the plant tissue/cell factory is the generation of high amounts of biomass that might then be stimulated for the production of high amounts of product. Once high economical productivity of both biomass (g/l per day) and product (mg/g dry weight per plant tissue per liter per day) is achieved, then the process has the potential to be commercialized [7].

The selection of suitable bioreactors depends on the system used (e.g., plant cells in suspension, hairy roots or microalgae). Stirred airlift tanks and bubble column reactors are most widely used for plant cells [8]. Other types of bioreactors (e.g., liquid dispersed, mist or spray reactors, or liquid or gas phase) are more suitable for culture of hairy roots [9,10]. One recent development in cell tissue and organ culture is the use of low-cost, disposable and scalable reactors, usually plastic bags [11,12] that, in spite of the limited scale-up size, the multiple units employed reduce the risk of product loss due to contamination.

The two main approaches developed for recombinant protein production in whole plants, nuclear/chloroplast transformation and transient expression (by vacuum infiltration or by using engineered ‘first’- or ‘second’-generation viral vectors) can both be applied in in vitro system, but with some differences and limitation. In fact, while plant tissue culture of stably nuclear-transformed plant material has been widely used, the potential of plastid transgenics for foreign protein expression in plant tissue cultures is unclear since most plant tissue cultures are heterotrophic and do not possess functional chloroplasts. An important exception is represented by chloroplast transformation of microalgae, which is evolving as a very promising tool in the biopharmaceutical field. Also, in vitro foreign-protein production using transient expression has been limited and requires further development and testing. Although viral infection of plant tissue cultures was reported as early as the 1950s, progress in this field has been slow, mainly because virus accumulation was generally very low [13]. However, in recent works, hairy roots were found to support moderately high levels of plant virus replication [14], indicating that hairy root culture is a potential candidate for in vitro production of foreign proteins using plant viral vectors [15,16]. An interesting tool for algal transient expression might be represented by the use of dsDNA viruses that infect Chlorella-like green algae as genetic vectors [17].

In Figure 1 the principal contained systems (mammal, yeast, and insect cells, plant tissue/cells and microalgae) used for the production of recombinant therapeutic molecules and vaccines are compared. In this article, we shall focus on the state-of-the-art and advantages of contained plant systems (represented by cell suspensions, hairy roots and other contained plant production systems such as microalgae and alternative plants) for the production of vaccine antigens and other therapeutic molecules (Table 1).

### Plant suspension cell cultures

Plant suspension cell cultures are composed of plant cells and small aggregates growing in liquid medium in a fermenter. They are usually derived by shaking friable callus cultures in shaker flasks or fermenters to form single cells and small aggregates [6]. While a lot of different plant species have been used for basic studies and secondary metabolites production, the application of plant cell culture for recombinant protein production has focused only on a small number of well-characterized plant cell lines, the most popular of which are derived from the tobacco cultivars bright yellow 2 (BY-2; considered as the ‘HeLa’ cell in the cell biology of higher plants) and Nicotiana tabacum 1 (NT-1), preferred because of easy transformation, propagation and growth characteristics. However, other diverse cell lines (rice, soybean, tomato, alfalfa and carrot) have been explored for better performance in terms of faster growth, higher expression levels, higher protein content, more efficient secretion, regulatory compliance, byproducts level and higher process compatibility.

Although plant cell densities and growth rates are lower than those of microbial cells, principles developed for the culture of microbial and mammalian cells also apply to plant cells [8,18], and many challenges associated with cell suspensions (i.e., formation of aggregates, tendency to adhere to the walls of the fermenter vessel, somaclonal variation and gene silencing) have been addressed by improving fermenter design (i.e., better agitation or aeration conditions), optimization of the nutrient supply or by the careful selection of the starting callus cell lines.

After the first demonstration that transgenic plants were able to produce functional antibodies [19], the human serum albumin (HSA) was the first recombinant human protein produced in suspension cultures approximately 20 years ago [20]. Since this initial demonstration, several other recombinant proteins have also been produced in whole plants and plant cultures [4,21].

Recombinant antigen production is achieved by using transgenic explants to derive the cultures, or transforming cells in suspension, usually by co-cultivation with Agrobacterium tumefaciens or particle bombardment [18]. Co-culture of suspended plant cells with transformed A. tumefaciens has also been described for transient expression of foreign protein in bioreactors [22].
The most critical challenge of the plant suspension technology is represented by the relatively low protein yields (0.01–0.2 g/l) with respect to mammalian (1–3 g/l) or other eukaryotic systems (0.5–5 g/l). This is partly due to the undifferentiated state of the suspension cells and the lack of promoters that are very active under such conditions. To optimize protein yields, instead of the

Figure 1. Comparison of plant-based production systems with other platforms. The benefits and limitations of each system for the production of vaccines and related molecules are shown with respect to product manufacturing costs (cost), system scalability (scale-up), product manufacturing time (time), similarity to the original human product for the presence of PTMs in the product, amenability of the system to production according to GMP, safety of the system in terms of presence of pathogens or toxic compounds in the final product (safety) and ability to reach (or of having already reached) the market (market).

GMP: Good manufacturing practice; PTM: Post-translational modification.
generally used cauliflower mosaic virus (CaMV) 35S constitutive promoter, a sucrose-inducible rice α-amylase gene (RAmy3D) promoter has been successfully used in rice cell cultures to express recombinant proteins such as the human granulocyte–macrophage colony-stimulating factor (hGM-CSF) [23]. In addition, an inducible virus infection system, based on tomato mosaic virus (ToMV) and controlled by an estrogen-inducible promoter, was developed in tobacco BY-2 suspension-cultured cells [24]. More recently, it has been demonstrated that a chemical inducible viral (cucumber mosaic virus) amplicon expression system can significantly improve the productivity of the recombinant α1-antitrypsin, compared with that using the CaMV35S promoter expression system in transgenic tobacco cell bioreactor cultures [25,26].

**Table 1. Examples of recombinant vaccines and therapeutics expressed in contained plant systems†.**

<table>
<thead>
<tr>
<th>Culture type and species</th>
<th>Proteins</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Suspension cells</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Daucus carota</td>
<td>Human enzyme GCD (for treatment of Gaucher’s disease)</td>
<td>[33]</td>
</tr>
<tr>
<td>Nicotiana tabacum cv BY-2</td>
<td>IFN-α</td>
<td>[27]</td>
</tr>
<tr>
<td>N. tabacum cv Havana</td>
<td>Human IL-12</td>
<td>[40]</td>
</tr>
<tr>
<td>N. tabacum cv NT-1; Glycine max</td>
<td>Heat-labile toxin from Escherichia coli</td>
<td>[201]</td>
</tr>
<tr>
<td></td>
<td>Hemagglutinin neuraminidase from Newcastle disease virus</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hemagglutinin from avian influenza</td>
<td></td>
</tr>
<tr>
<td></td>
<td>VP2 from infectious bursal disease</td>
<td></td>
</tr>
<tr>
<td>N. tabacum cv Xanthi</td>
<td>Human antirabies monoclonal antibody</td>
<td>[39]</td>
</tr>
<tr>
<td>N. tabacum cv BY-2</td>
<td>Active dust mite allergens</td>
<td>[46]</td>
</tr>
<tr>
<td>Nicotiana rustica cv</td>
<td>Human GM-CSF</td>
<td>[23]</td>
</tr>
<tr>
<td>Lycopersicon esculentum</td>
<td>Rotavirus VP6 protein</td>
<td>[42]</td>
</tr>
<tr>
<td><strong>Hairy roots</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transgenic N. tabacum cv Xanthi</td>
<td>Single-chain murine IL-12</td>
<td>[54]</td>
</tr>
<tr>
<td>Transgenic potato (var. Kufri Bahar)</td>
<td>HBsAg</td>
<td>[55]</td>
</tr>
<tr>
<td>Transgenic N. tabacum cv L.</td>
<td>HBsAg</td>
<td>[56]</td>
</tr>
<tr>
<td>Transgenic N. tabacum cv NT-1</td>
<td>Ricin B</td>
<td>[53]</td>
</tr>
<tr>
<td>Transgenic N. tabacum (also in cell suspensions)</td>
<td>Murine IgG, recognizing a 185-kDa cell-surface protein of Streptococcus mutans</td>
<td>[37,38]</td>
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<tr>
<td>N. tabacum</td>
<td>Human growth hormone</td>
<td>[16]</td>
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<tr>
<td>Nicotiana plumbaginifolia</td>
<td>Surface protective antigen from Erysipelothrix rhusiopathie (swine erysipelas) fused to cholera toxin B subunit</td>
<td>[57]</td>
</tr>
<tr>
<td><strong>Green microalgae</strong></td>
<td></td>
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<tr>
<td>Chlamydomonas reinhardtii</td>
<td>Aquaculture vaccines and related adjuvants</td>
<td>[205]</td>
</tr>
<tr>
<td></td>
<td>Footh-and-mouth disease virus VP1 protein fused with cholera toxin B subunit</td>
<td>[91]</td>
</tr>
<tr>
<td></td>
<td>Staphylococcus aureus fibronectin-binding domain D2 fused to cholera toxin B subunit</td>
<td>[90]</td>
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<tr>
<td></td>
<td>Bovine mammary-associated serum amyloid sequence</td>
<td>[93]</td>
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<tr>
<td></td>
<td>Antiglycoprotein D (herpes simplex virus) antibody (human IgA)</td>
<td>[70,88]</td>
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<td></td>
<td>Monoclonal antibody against protective antigen 83 from Bacillus anthracis</td>
<td>[89]</td>
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<tr>
<td><strong>Duckweed</strong></td>
<td></td>
<td></td>
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<tr>
<td>Lemna minor</td>
<td>Monoclonal antibody anti-CD30 (non-Hodgkin’s B-cell lymphoma)</td>
<td>[100]</td>
</tr>
<tr>
<td></td>
<td>Monoclonal antibodies anti-CD20 (non-Hodgkin’s B-cell lymphoma) and anti-IFN-α2b</td>
<td>[207]</td>
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<tr>
<td><strong>Moss</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Physcomitrella patens</td>
<td>Monoclonal antibody for the prevention of deep vein thrombosis</td>
<td>[288]</td>
</tr>
<tr>
<td></td>
<td>Antitumor antibody</td>
<td>[112,113]</td>
</tr>
</tbody>
</table>

†Many products in development remain undisclosed.

GCD: Glucocerebrosidase; GM-CSF: Granulocyte–macrophage colony-stimulating factor; HBsAg: Hepatitis B surface antigen; VP: Viral particle.
Leader peptides, from plant and nonplant proteins, can be used to target foreign proteins for accumulation in specific areas of plant cells or organs. The secretory pathway is a more suitable compartment for folding and assembly than the cytosol. The endoplasmic reticulum (ER) provides an oxidizing environment and an abundance of molecular chaperones (i.e., binding protein [BiP]), and few proteases. In the absence of further targeting information, the expressed protein is secreted to the apoplast. Depending on its size as well as other physicochemical properties (i.e., charge and/or hydrophobility), the protein can be retained therein or might leach from the cell.

Plant cells retaining the protein of interest can be easily separated from the fermentation supernatant, often accomplished by filtration steps. Expression levels can be increased even further if the protein is retrieved to the ER lumen using a His/Lys–Asp–Glu–Leu C-terminal tetrapeptide tag. When targeted to the ER using a retention signal, many proteins that are unstable in plant cell culture media accumulate to levels much higher than identical proteins targeted for secretion [21]. Molecules retrieved in this way are not modified in the Golgi apparatus and will have mannose glycans but not plant-specific xylose and fucose residues. This is an important issue since there are differences in post-translational modifications between mammals and plants; while early glycosylation steps are similar in plants and in mammals, later steps are different. Plant-derived recombinant human proteins tend to have the carbohydrate groups β1,2-xylose and α1,3-fucose, which are absent in mammals, but lack the terminal galactose and sialic acid residues that are found on many native human glycoproteins [3].

Fusion with a hydroxyproline-rich glycoprotein (HRGP) tag in tobacco and Arabidopsis has been used as a strategy to optimize yields of a recombinant protein [27,28]. Glycosylation of the HRGP tag greatly extended the serum half-life of the secreted therapeutic proteins (interferon and growth hormone). For most applications, however, the HRGP tag must be removed from the fusion protein to generate the native protein and this increases production costs.

It has been demonstrated that plant N-glycans might be immunogenic in humans [29]. Strategies are under investigation to engineer glycosylation in all eukaryotic expression systems, including plants, so that the expression host will be suitable for the production of recombinant therapeutic glycoproteins harboring humanized nonimmunogenic N-glycans. It has been shown that it is possible to manipulate specific glycosyl transferases in plant cells to yield human-like glycosylation patterns on recombinant proteins [30]. Recently, transgenic Nicotiana tabacum BY-2 cells expressing rat β1,4-N-acetylgalactosaminyltransferase III (GnTIII; that introduces a bisecting N-acetylgalactosamine [GlcNAc] residue into N-glycans) localized within the Golgi were obtained. In this way, plant-specific fucosylation and xylosylation could be greatly reduced, obtaining up to 59% of human-compatible N-glycans on endogenous secreted protein lacking the plant-specific modifications [31].

For the formulation of more potent vaccines, as well as for cancer immunotherapy, plant-derived glycans might represent an advantage rather than a disadvantage by increasing the immune ‘visibility’ of the antigen of interest, targeting antigen-presenting cells, most particularly through lectins or mannose/fucose receptors expressed on the cell surface of dendritic cells [32]. Also, therapeutic molecules with plant-derived glycosylation could represent superior biosimilar (or ‘biobetters’). This seems the case of the human enzyme glucocerebrosidase (GCD) for therapy of Gaucher’s disease, a chronic congenital disorder of lipid metabolism characterized by an enlarged liver and spleen, increased skin pigmentation and painful bone lesions. At first, GCD purified from human placenta was used to treat this disease, but this required protein purification from 50,000 placenta for each patient per year. The current replacement therapy is represented by intravenous administration of recombinant GCD expressed in Chinese hamster ovary (CHO) cells. As complex glycans in mammalian cells do not terminate in mannose residues (essential for the macrophage uptake of GCD via mannose receptors in human patients with Gaucher’s disease), an in vitro glycan modification is required. Human GCD was produced in recombinant form in a contained disposable bioreactor system with suspension-cultured tobacco cells (Protalix Biotherapeutics, Carmiel, Israel). The plant-derived recombinant protein (prGCD), targeted to the storage vacuoles, presents uniform glycosylation with exposed mannose residues on its complex glycans, probably as a result of the activity of a special vacuolar enzyme that modifies complex glycans [33].

The safety and pharmacokinetics of this novel plant cell culture-derived human recombinant enzyme was also evaluated after administration to primates and human subjects. Neither clinical drug-related adverse effects nor neutralizing antibodies were detected in the animals. In a Phase I clinical trial, six healthy volunteers were treated with prGCD and no anti-prGCD antibodies were detected. The pharmacokinetic profile of the prGCD revealed a prolonged half-life compared with the commercial enzyme, manufactured at high prices in mammalian cell systems. A pivotal Phase III clinical trial for prGCD was US FDA approved and is currently ongoing [34]. Hence, plant glycosylation in some cases might even represent an advantage and prGCD will probably be the first plant-made product approved for parental human use.

When a recombinant protein produced by in vitro cultures is secreted into the culture medium, its recovery is expected to be much simpler and less expensive than recovery from homogenized biomass since the plant cell culture media are very simple and do not contain exogenous protein compared with the supernatants of microbial cultures. However, the cost advantages associated with foreign protein secretion would depend on whether protein stability and protection from proteases and nonbiotic losses can be maintained within the extracellular environment of plant cell cultures. In fact, protein yields are often too low due to degradation by proteases that are produced during cell culture, aggregation or protein adsorption to culture system walls [35,36]. Another drawback might be the loss of biological activity of secreted (extracellular) products as a result of alteration in protein conformation.

The recovery of recombinant proteins from plant cell cultures can be enhanced by modifying the culture conditions or adding various agents to the growth medium (e.g., simple inorganic...
compounds, amino acids or stabilizing agents such as dimethyl sulfoxide [DMSO], polyethylene glycol [PEG], polyvinylpyrrolidone, gelatin, bovine serum albumin [BSA] and protease inhibitors). Product yields can be improved not only by medium optimization but also through process development. In a recent study, it has been demonstrated that a pH-shifting culture strategy applied during the protein production phase reduced protease activity derived from cell cultures and improved stability of a human blood protein expressed in transgenic tobacco cell cultures [25,26]. To further maximize the recombinant protein production, an optimized semicontinuous bioreactor process was adopted. This process periodically allows for harvesting and purification of active recombinant proteins, simultaneously removing cell growth-limiting and protein production-limiting factors in the culture broth such as proteases (derived from cell cultures) as well as nonprotein components, such as phenolics that react with the recombinant protein, causing changes in protein properties and complicating the purification process [7].

**Vaccine antigens from plant suspension cell cultures**

Research to date has demonstrated that plant cell cultures (mainly tobacco BY-2 and NT-1) are capable of expressing multiple classes of biologically active proteins including immunoglobulins (e.g., IgG) [37–39], IL-12 [40], hGM-CSF [23], immunotoxins [41], human IFN-α [27], and some of these (i.e., human GCD, Protalix Biotherapeutics [33]) are near the level of commercial developments.

Albeit the recombinant rotavirus VP6 antigen was one of the first antigens expressed in transgenic tomato cells (with a yield of 0.15–0.33 mg/l in the intracellular fraction) [42], most studies on vaccine antigens produced *in vitro* by plant cell cultures have focused on the hepatitis B surface antigen (HBsAg), a complex, extensively disulfide cross-linked, lipoprotein entity. It was first, expressed in soybean and tobacco NT-1 cell suspension cultures [43]. Stable, high-level expression was achieved in the soybean cell line with antigen titers reaching 65 mg/g fresh weight (20–22 mg/l), while titers in the most productive tobacco cell line was approximately tenfold lower. This difference was probably due to the higher transgene copy number in soybean, 4–6 versus one for the tobacco line and/or to a strong downregulation of antigen expression that occurred with successive subculture in tobacco cultures. HBsAg processing and assembly *in planta* was characterized and, similar to yeast, the plant-expressed HBsAg was retained intracellularly. Similarly, Sojikul et al. expressed HBsAg protein attached with eukaryotic ER signal peptide from soybean vegetative storage protein (VSPαS) or ER retention signal (Ser–Glu–Lys–Asp–Glu–Leu) at the N-terminal and C-terminal ends, respectively, in the tobacco NT-1 cell line culture [44]. The signal peptide directly targeted the protein to the ER and remained uncleaved, enhancing VSPαS–HBsAg fusion accumulation and stability in tobacco plant cells. Later, Kumar et al. reported HBsAg expression in NT-1 tobacco cell line suspension cultures [45]. Preclinical studies demonstrated the ability of this plant-derived vaccine to elicit an antigen-specific immune response in mice.

The potential of suspension-cultured BY-2 tobacco cells as bioreactors for the production of recombinant allergens from the house dust mite under controlled and environmentally safe conditions has also been investigated. The plant-made allergens showed similar proteolytic maturation and folding, as well as comparable immunoreactivity to their natural counterparts, demonstrating that suspension-cultured BY-2 tobacco cells represent a low-cost and environmentally safe expression system suitable to produce recombinant allergens from *Dermatophagoides pteronyssinus* in an appropriate form for therapeutic purposes [46].

A number of vaccine antigens for veterinary use have been expressed in a biologically active form (Table 1). In particular, Dow AgroSciences (Indianapolis, IN, USA) developed a plant cell line-based production system of veterinary vaccines able to induce immune responses in poultry and swine. In particular, in 2006 this company produced a Newcastle disease virus (NDV) vaccine for poultry by using a less-defined suspension-cultured tobacco cell line, and successfully tested it as a purified injectable product in chickens [201], This has been registered and approved by the US Department of Agriculture (USDA) – the final authority for veterinary vaccines — and, although it is (still) not for sale, it represented the world’s first regulatory approval for a plant-made vaccine.

**Root cultures**

Hairy roots develop as the consequence of the interaction between *Agrobacterium rhizogenes*, a Gram-negative soil bacterium, and the host plant. To generate hairy roots, wounded plant tissues are inoculated with *A. rhizogenes*, which transfers the T-DNA comprising the loci between the T₅ and T₄ regions of the Ri plasmid into the plant genome. The T-DNA carries the *rol* and *aux* genes, the former being responsible for the hairy root differentiation under the influence of endogenous auxin synthesis directed by *aux* genes [47].

To induce hairy roots, the proper explant material (e.g., hypocotyl, leaf, stem, stalk, petiole, shoot tip, cotyledon, protoplast, storage root or tube) for a certain plant species is wounded, and co-cultivated or inoculated with *A. rhizogenes*. A few days later, the explant can be transferred into solid media with antibiotics, such as cefotaxime, to eliminate redundant bacteria. The hairy roots will emerge at the wounding site within a short period of time, which varies from 1 week to over 1 month, depending on the plant species. Next, the decontaminated hairy roots can be sub-cultured individually on phytohormone-free medium [48]. Thus, hairy roots are organs characterized by extensive secondary branching and by an increase in the number of root hairs that can be excised and cultivated indefinitely under sterile conditions. In contrast to ordinary root cultures (nontransformed roots), which often require a balanced supplement of auxin and cytokinin to maintain growth and phenotype, transformed roots can be maintained on hormone-free media, thus reducing production costs [49]. Furthermore, the media used for hairy root growth do not contain materials of animal origin or selective agents such as kanamycin.

Transformed root cultures have been established from numerous species of dicotyledonous plants and, thanks to their properties, hairy root use in plant biotechnology has been promoted for a wide
range of research and industrial purposes, such as plant improvement, plant interaction with the environment, secondary metabolites, phytochemicals, pharmaceuticals and cosmetics study/overproduction by metabolic engineering [49]. In addition, hairy roots opportunely engineered by introduction of heterologous genes can be used as an excellent, continuous starting material for the production of valuable therapeutic proteins, vaccines and monoclonal antibodies, holding immense potential for the biopharmaceutical field [49].

Although aseptic bioprocessing is not very cost competitive, in vitro root cultures provides undoubted benefits compared with production in whole plants and other contained systems. They are genetically stable over time, and they have normal mitoses and normal cell divisions. Besides avoidance of political resistance to genetically modified field crops, root cultures can provide economic and fast-growing organ-specific culture, allow uniform expression at high levels of a target gene product in a time-efficient manner (60 days to obtain a master culture) with a relevant biomass increase (120-fold over 4–5 weeks), and show consistency of target protein expression during subcultures (batches) over a long period of time. Hairy root cultures also hold the potential to secrete product into the culture medium, possibly resulting in a reduction in purification costs. The extraction and purification of proteins from plant tissues is, in effect, a laborious and expensive process, and a major obstacle to large-scale protein manufacturing in plants (the cost of recombinant protein isolation and purification can be as high as 90% of the total production costs [37,50]). Secretory signal sequences directing the protein of interest to the secretory pathway of hairy roots aseptically cultivated in vitro, besides promoting proper folding and disulfide bond formation (and increasing the functional protein expression level), can be exploited in secretion-based systems where isolating recombinant proteins from a simple hydroponic medium rather than from complex plant extracts represents a simplified and cost-effective method. As an example, targeting an antibody in single-chain variable fragment (scFv) format to the root secretory pathway was successfully obtained by means of the polygalacturonase-inhibiting protein leader peptide, obtaining the scFv secretion in culture hydroponic medium in a stable and continuous manner [51]. Borrowing from many well-developed and tested methods of commercial hydroponic plant cultivation, rhizosecretion would be safe and could have some potential to be scaled-up. On the other hand, the economic feasibility of rhizosecretion-based systems also depends on preventing the degradation or loss of product after its secretion into the culture medium [35]. The stability of the rhizosecreted protein can be increased by using protein stabilizers such as polyvinylpyrrolidone and gelatin [18,52]. Nevertheless, further attempts are required in this direction since the influence of additives, substances increasing root permeability and stabilizers must be studied and optimized for each expressed protein in order to establish a competitive production system.

Hairy roots represent a very well-regulated and controlled system for which cultivation in bioreactors supporting efficient cell nutrition, activity, differentiation and function is an important step towards the development of high-technological products [7,49]. Indeed, bioreactor culture is the key step towards commercial production of bioactive therapeutics by plant biotechnology.

The examples reported in the following sections demonstrate that hairy root cultures offer potential for biotechnological purposes and for the production of vaccine antigens and therapeutic
proteins in containment. Nevertheless, no industrial applications in this field will be described since they have not been developed so far. Many attempts have been made to develop economical bioreactors containing airlifting, bubble-column, mist, dual and wave reactors [7,49]. To resolve the bottleneck of the application of hairy root culture systems, future research should focus on the establishment of effective and economical scaled-up culture systems that can reduce consumption but obtain the biggest benefits. If such a breakthrough is achieved, a promising application of hairy root culture systems will be more likely.

**Vaccine antigens from hairy root cultures**

Most of the work with hairy roots in reactors has focused on secondary metabolites. Only very recently, hairy roots are being exploited to produce functional and pharmaceutical proteins in view of combining the advantages of recombinant protein-based vaccines with the potential benefits provided by contained plant production (Table 1 & Figure 1).

With the purpose of producing valuable heterologous proteins, hairy root cultures can be obtained from transgenic plants by co-cultivation or inoculation with *A. rhizogenes* (Figure 2).

Hairy roots from transgenic tobacco plants have been successfully used to produce the immune modulator ricin B (the nontoxic galactose/N-acetylgalactosamine-binding subunit of ricin) fused to the green fluorescent protein (GFP) as a model antigen, and tested in mucosal vaccine delivery/adjuvancy [53]. Data from this work present the first evidence that a recombinant ricin B genetically fused to an antigen and expressed in roots, is able to deliver the antigen to the mucosa surface and to enhance the immunogenicity of the fused antigen upon intranasal immunization in mice with superior adjuvancy to cholera toxin in eliciting a Th2-type systemic and mucosal response. As a consequence, the amount of ricin B—GFP necessary to induce equivalent antibody responses was tenfold lower than cholera toxin.

Tobacco lines yielding high levels of murine IL-12 with equivalent bioactivity to the animal cell-derived line, were further transformed with *A. rhizogenes* to obtain hairy roots that also produced IL-12 at high levels, demonstrating the successful production of a therapeutic protein in a mit bioreactor with potential for large-scale applications in root cultures [54].

In 2006, Sunil Kumar et al. reported HBsAg expression for the first time in roots induced from transgenic potatoes. Higher levels of HBsAg were obtained when using a C-terminal ER retention signal [55]. In 2007, Rukavtsova et al. obtained hairy root cultures also carrying the HBsAg as a result of retransformation of *N. tabacum* plants carrying the same antigen with *A. rhizogenes* under the control of a hybrid Agrobacterium-derived promoter [56].

Transgenic hairy roots for a surface protective antigen (SpA) from *Erysipelothrix rhusiopathiae* (swine erysipelas) fusion to cholera toxin B subunit (CTB), provided with an ER retention signal, were obtained from untransformed tobacco leaf disks by using *A. rhizogenes* harboring the CTB–SpA fusion [57]. Unfortunately, low expression levels were found; multiple transfer of T-DNA copies or ‘position effects’ were the hypothesized reasons.

Besides improving vaccine expression through the development of better promoters or ER targeting, the avoidance of foreign protein degradation, which can reduce levels of functional protein in plant tissues and which can be wasteful in large-scale recovery operations, is of importance. A murine IgG recognizing a 185-kDa cell-surface protein of *Streptococcus mutans* to be used as an anticaries vaccine (Guy’s 13 monoclonal antibody [mAb] [58]) was produced in hairy root cultures derived from transgenic tobacco. The expressed antibody was partially lost due to degradation. Inhibition of protein secretion and retention of antibody in the ER and/or Golgi reduced fragmentation and increased antibody accumulation levels, probably by reducing exposure to protease activity both in the secretory pathway and in the apoplast [37,38].

**Perspectives in vaccine antigen production by hairy root cultures**

Unlike the well-known transgenic hairy root system, for which the available examples of therapeutic protein production have been given previously, the works that will be described in the following lines are based on episomal replication of viral RNA in root cultures. This last section has only the goal of describing the potential of transient expression technologies in root cultures with pharmaceutical biotechnology as a possible application.

Plant viral vectors for foreign protein and epitope vaccine production rely on technologies developed by several research and commercial groups. As primary examples, researchers from Icon Genetics (Halle, Germany) [202], Kentucky Bioprocessing [203] or PBL Biotechnology [204] might be cited. These technologies are based on the plant viruses’ property to accumulate to very high levels in plants; if a foreign protein is coexpressed with the virus, either as free protein or as a fusion to the viral coat protein, large amounts of product can be formed [59].

Because most plant pathogens, including viruses, are not infectious in animals, plant-virus-based vaccines are expected to be relatively safe for humans. However, environmental concerns regarding the containment of recombinant viruses, and the desirability from a regulatory point of view of exercising close control over environmental conditions during foreign protein synthesis, make transient viral infection of plant tissue/organ cultures in large-scale bioreactors a possible alternative.

Only very recently, technologies for transient expression of recombinant proteins in hairy roots, based on viral infection, have been developed. In contrast to the unfortunate early attempts to develop in vitro systems for plant virus infection in plant cell suspensions (mostly for studying viral pathogenesis and plant viral resistance, see previous sections), recent reports demonstrate that root cultures infected with recombinant viruses have the potential to synthesize foreign proteins [16,36].

Initially, it was demonstrated that plant viruses can transiently infect, replicate within and accumulate to reasonably high levels in hairy root cultures. Tobacco hairy roots were breached and infected with the particles purified from plants infected with an in vitro transcript from a tobacco mosaic virus (TMV)-based viral vector expressing GFP (TMV–GFP). Infection and virus
accumulation did not affect growth and hairy roots accumulated the recombinant virus (with the hypersusceptible *N. benthamiana* roots being more productive than *N. tabacum*) better than suspended plant cells (1–2 mg/g dry weight or 20–28% of the total soluble protein [TSP]) infected with the same initial virus:biomass ratio [14]. The authors speculate that this probably happens thanks to the presence of fixed connections (plasmodesmata) between root cells, facilitating cell-to-cell transmission of the virus in root tissue compared with cell suspensions where direct cell-to-cell movement of the virus by plasmodesmata relies on the formation of cell aggregates [14]. On the other hand, a lower average concentration of virus was observed in hairy roots (1.6 ± 0.25 mg/g) compared with leaves of infected whole plants (10–29 mg/g), probably due to the different dynamics of long-distance transport in roots compared to leaves. In fact, although cultured roots contain vascular tissue (with the exception of the active root meristems, where the virus cannot replicate), transpiration-driven convection does not occur in submerged cultures and this would be expected to reduce the rate of viral spread compared with intact plants [36]. Moreover, GFP expression and infectivity of virus in the biomass declined during the culture, probably due to genetic instability of the vector and loss of the GFP gene with passaging of the viral vector from leaves.

The potential of 'deconstructed' viral vector technology was also investigated for hairy root culture application. A viral vector based on the yellow dwarf bean virus containing the GUS reporter gene was delivered to *Nicotiana glutinosa* cell suspension or hairy root cultures via *A. tumefaciens*. This vector made the cell culture generate a greater yield of reporter gene expression than the root culture, especially when the viral vector containing the GUS reporter gene and the *cis*-acting viral regulatory elements was codelivered with the viral replication-associated proteins provided on a secondary vector. Reporter protein was generated 3 days after the initiation of *A. tumefaciens* co-culture, providing a faster generation of the protein in cell culture than in hairy root culture [60].

In another study, the infection approach was different: *N. benthamiana* clonal root cultures were generated from the leaves of plants that had previously been infected with the *in vitro* transcript from a TMV-based viral vector for the expression of GFP or human growth hormone (Figure 2) [16]. The hairy roots stably maintained the virus expression vector and expressed GFP at concentrations of 50–120 µg/g fresh weight (a level that is comparable with other plant production systems using transgenic hairy roots [55]) and human growth hormone in the range of 3–6 µg/g fresh weight. This variability could be due to different reasons, including the sequence of the gene, stability of the RNA, biochemical properties of the protein or sensitivity to proteases. Also in this study, a large variation in virus levels was observed between hairy root clones, but clonal roots maintained their ability to produce the foreign proteins over a 3-year period through multiple subcultures and in the absence of antibiotic selection.

The persistence of the viral vector in the clonal root lines suggests that the virus is not subjected to the same selective pressures as in aerial plant tissues.

Exploiting a TMV-derived vector, the E7 oncogene from high-risk HPV-16 (causative agent of cervical cancer and many head and neck tumors) has been transiently expressed in tobacco whole plants as a fusion to a bacterial glucanase (LicKM) in view of a therapeutic vaccine against HPV-related malignancies [61,62]. The target antigen was purified and evaluated in a mouse model for its potential as a therapeutic vaccine. The vaccine induced E7-specific IgG and cytotoxic T-cell responses inhibiting tumor development following challenge with an E7-expressing tumor cell line. Very recently, hairy root cultures have been established from

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**Figure 3. Microalgae as bioreactors for the production of biopharmaceuticals.** Example of *Chlamydomonas reinhardtii* chloroplast-based platform: *C. reinhardtii* chloroplast transformation can be performed by different protocols (the glass beads method is shown in the picture: cells are mixed with exogenous DNA and glass beads). After several selection cycles to obtain homeoplasmy, transformants are grown and analyzed for the presence of the recombinant protein.
Microalgae

Microalgae comprise a highly heterogeneous group of photosynthetic, heterotrophic microscopic organisms that are found in both marine and freshwater environments. They are unicellular species that exist individually, in chains or groups. They include two divisions of prokaryotic and nine divisions of eukaryotic organisms. Some species are currently used for the commercial production of high-added-value compounds for use in health and medical applications [64]; furthermore, microalgae are being investigated for the production of different biofuels [65] and for bioremediation [66]. Photosynthetic microalgae (‘green microalgae’, Chlorophyceae class), such as Chlamydomonas, Chlorella, Haematococcus and Dunaliella, have been proposed as an attractive system for the expression of heterologous proteins, including vaccines, antibodies and other therapeutic proteins [64,67–70]. Unlike viruses and prions, these microalgae do not contain toxins and human pathogens, and are defined as ‘generally regarded as safe’ (GRAS), offering a safe platform for vaccine production. In addition, microalgae require simple media and present fast vegetative growth; for example, Chlamydomonas reinhardtii doubles in approximately 8 h under a 12-h light–12-h dark regime [71]. Generally, green microalgae are more efficient, compared with land plants, at converting solar energy into biomass because they have a simple cellular structure and live submerged in aqueous environment where they have easy access to water, CO₂ and other nutrients. In addition, transformation methods are simple and the time required from initial transformation to protein production is significantly less. Since the cellular population of microalgae is uniform in size and type, there is no gradient of recombinant protein distribution, simplifying the purification procedure.

Many microalgae species can grow heterotrophically or photoautotrophically. Nevertheless, the success of the commercial production of recombinant proteins from microalgae depends on the development of cost-effective, large-scale, closed-culture systems, in compliance with GMP [68]. Heterotrophic growth (based on the addition of acetate or glucose as a carbon source in the culture medium) presents the advantages of using well-established dark fermenters allowing high cell densities (20–100 g/l) [72]. Photoautotrophic, large-scale cultivation in contained system (‘photobioreactors’) only requires water, salts and CO₂. However, the distribution and utilization of light (natural or artificial, passing through the transparent container walls), represents the rate-limiting factor for cell growth. In the past few years, progress has been made in the design of photobioreactors, in order to overcome the problems related to light intensity/penetration in dense cultures, allowing a better conversion of energy to biomass. Principally, horizontal and vertical tubes or plates made of either glass or transparent plastic membranes have been developed [73–75]. Mutagenesis approaches have been used to increase the photosynthetic rate and productivity of dense cultures in photobioreactors [76,77]. This underlines how the availability of genomic information is another important factor/variable in the effective use of microalgae as biofactories. Many microalgal nuclear and/or organelle genomes are completely sequenced or soon to be sequenced [78]. To date, C. reinhardtii is the best characterized with its three genomes (nuclear, mitochondrial and plastid) completely sequenced [79]. For this species, different nuclear and chloroplast transformation methods (e.g., glass beads, carbide whisker agitation, electroporation, particle bombardment and Agrobacterium tumefaciens infection) have been established, as well as several resistance markers, reporter genes, promoters and mutant strains [64]. In particular, chloroplast transformation was first reported by Boynton and co-workers by particle gun-mediated bombardment [80] and, until now, microprojectile bombardment represents the method of choice for chloroplast transformation of C. reinhardtii; however, Kindle and colleagues demonstrated the possibility of using the simpler glass bead method for strains lacking a cell wall, although with lower efficiency (Figure 3) [81].

Principal factors affecting the protein yield have been identified [69,82,83]. In particular, codon-optimization plays a significant role in protein accumulation in C. reinhardtii. In fact, in this species, highly expressed nuclear and chloroplastic genes show a strong bias for specific codons. In particular, nuclear expression can be optimized by using synthetic genes with a high GC content (~61%) [84], whereas for optimized chloroplast expression, codon usage should be based on highly expressed Chlamydomonas chloroplast genes. Moreover, using only the most frequently used codon for each amino acid will result in low expression of the gene due to tRNA depletion. Therefore, a codon adaptation index (CAI) is adopted as a quantitative method to predict the expression level of the codon-adapted gene of interest. The CAI should be approximately 0.8, since high CAI values (i.e., only using the codon for each amino acid that is most frequently used, CAI = 1.0) will result in low expression of the gene [82].

However, in spite of all these findings, the protein yield obtained by nuclear transformation is still very low. Bock and colleagues suggest that nonconventional epigenetic suppression mechanisms and/or the structure of extremely compact chromatin could be involved [85]. Nuclear expression of transgenes was described for other green microalgae such as Chlorella, Haematococcus spp. [86] and Dunaliella [67].

To date, the unique way to produce consistent amounts of recombinant protein is to express them in C. reinhardtii chloroplasts. Unlike higher plants, counting approximately 100 chloroplasts per cell (each one with approximately 100 genome copies), Chlamydomonas has a single chloroplast per cell with approximately 80 genomes that occupy approximately 40% of the total cell volume: hence, the transformation of this organelle is relatively easy and allows the generation of transformant lines within 4–6 weeks [70]. Recombinant protein accumulation is higher than that obtained by nuclear transformation, due to the lack of positional effects (the transgene is inserted by homologous recombination), to lower proteases content and to the presence of molecular chaperones. Although lacking the machinery to perform some post-translational modifications, Chlamydomonas chloroplast permit
the formation of disulfide bonds, making it a suitable host for the production of complex molecules, such as nonglycosylated proteins and antibodies that turned out to have a similar affinity to their mammalian counterpart in spite of the lack of glycosylation [87–89].

Vaccine antigens from microalgae
The potential of using *Chlamydomonas* chloroplast transformation has been exploited by PhycoBiologics Inc., to produce oral aquaculture vaccines (freeze-dried or live algae) able to induce antigen-specific immune response in both serum and mucus [205].

Lyophilized microalgae, expressing the *Staphylococcus aureus* fibronectin-binding domain D2, fused to the CTB mucosal adjuvant have been used for oral immunization in preclinical studies [90]. Immunized mice developed both systemic and mucosal immune responses; the pathogen load was reduced in the spleen and in the intestine, and 80% of them were protected against lethal doses of *S. aureus*. This vaccine was stable for more than 1.5 years at room temperature.

The potential of this system has also been exploited by scientists from the Scripps Research Institute (La Jolla, CA, USA) to produce human therapeutic proteins [206]. The CTB was used as a carrier for the expression of foot-and-mouth disease virus VP1 protein. The fusion protein was successfully expressed in *Chlamydomonas* chloroplast with a yield of 3–4% of the TSP [91]. In another study, glutamic acid decarboxylase-65, an insulin-dependent autoantigen, was expressed at 0.3% of the TSP, purified and recognized by diabetic serum samples collected from NOD mice, demonstrating the validity of using *Chlamydomonas* chloroplast as a vaccine production platform [92]. To date, the highest accumulation of recombinant antigen has been obtained by replacing the chloroplast *psbA* coding region with the bovine mammary-associated serum amyloid sequence (encoding a protein of mammalcolostrum that protects newborns against intestinal bacterial and viral infections) that lead to accumulation of the recombinant protein above 5% of the TSP [93].

In addition, a single-chain antibody against herpes simplex virus glycoprotein D [87,88] and a full-length mAb against the protective antigen 83 of *Bacillus anthracis* were expressed [89].

Novel plant-contained systems
Novel platforms currently used for the production of pharmaceuticals in contained systems include *Lemnaceae*, higher aquatic plants and the moss *Physcomitrella patens*.

*Lemnaceae* are fresh-water, free floating, edible plants, currently used for human and animal nutrition. They present a high protein content (up to 45% dry weight) and can be grown with low investments in full containment in aseptic modules without aeration [94–96]. Owing to a fast clonal proliferation (doubling time as short as 1.5–2 days) and to a peculiar plant architecture (they require little mechanical support or vascular tissue), this system can be easily scaled-up, with high biomass accumulation [96]. Moreover, Lemnana, pectic polysaccharide from *Lemna minor*, demonstrated adjuvant properties following oral administration with protein antigens via induction of both Th1- and Th2-type responses [97], suggesting an added value for the development of oral vaccines.

Stable nuclear transformation of many species of *Lemnaceae* has been accomplished using *Agrobacterium*-mediated gene transfer or biolistic method [98], and expression levels vary depending on the protein and the species chosen. At present, *Spirodela oligorhiza*, with 25% of the TSP accumulation for the GFP, represents the best example of foreign protein production by nuclear transformation in a higher plant [99].

Many therapeutic proteins have been successfully expressed in this system and Biolex Therapeutics Inc. [207], in 2005 acquired the French company Lemnagene, currently uses the two genera *Lemna* and *Spirodela* as a platform for the production of pharmaceuticals for human and animal uses, such as IFN-α2b (that is entering Phase III of clinical trial), plasmin and the mAb anti-CD20 for the treatment of non-Hodgkin’s B-cell lymphoma. Also, species of the genus *Wolfia* are under development for molecular farming purposes [99].

Glycosylation of a mAb against human CD30 was optimized by coexpressing the heavy and light chains of the mAb with RNA interference construct targeting expression of the endogenous α,1,3-fucosyltransferase and β1,2-xyllosyltransferase genes. The resultant mAb showed undetectable plant-specific N-glycans and had even better functionality (cell-mediated cytotoxicity and effector cell receptor binding) than the mAb expressed in cultured CHO cells [100].

The moss *P. patens* can be grown under contained conditions during its complete lifecycle, offering unique advantages for the production of biopharmaceuticals [101–103]. While sexual reproduction occurs predominantly when growing moss on agar plates, vegetative growth is favored when cultured in liquid media (flasks and bioreactors). At this stage (‘protonema’), *Physcomitrella* can be cultured as tissue suspension cultures in simple media (e.g., inorganic salts and airborne CO2, as a carbon source) using stirred glass tanks or modular glass tubular photobioreactor. Several photobioreactors have been established for moss cultivation and, currently, a 100-L modular photobioreactor is employed for the production of biopharmaceuticals according to GMP standards [208]. Since *Physcomitrella* tolerates a broad pH range, this parameter can be adapted to any protein that has to be secreted into the culture medium [104]. The *Physcomitrella* genome has been fully sequenced [105] and a second, ‘more user friendly’ version of this genome is expected by the end of this year [209]. The analysis of expressed genes disclosed that, unlike higher plants, there is no significant bias for codon usage, so transgenes can be expressed without codon optimization [106]. In addition, *Physcomitrella* presents the possibility of gene targeting by homologous recombination (HR) in its nuclear DNA, and by this method, precise genetic modifications including base-specific targeted gene knockouts can be easily accomplished at frequencies of up to 100% [107], whereas in flowering plants, HR occurs at a much lower frequency (104–105) [108].

In this context, double-knockout strains of *P. patens* for β1,2-xylosyltransferase and α1,3-fucosyltransferase have been developed [109] as well as other engineering strategies in view to humanize the moss glycosylation pattern [110,111]. Scientists at Greenovation (Freiburg, Germany, [208]) have successfully
expressed a humanized mAb for the prevention of deep vein thrombosis, which was secreted in the culture medium. This antibody was shown to be correctly assembled and functional, and it is currently in preclinical development. Another humanized antibody developed by Greenovation is an antitumor antibody presenting an enhanced antibody-dependent cellular cytotoxicity, fundamental for therapeutic efficacy [112,113].

Expert commentary
Many important advantages (i.e., cost–effectiveness, speed and safety) are associated with plant cell cultures for production of commercially valuable foreign proteins, compared with mammalian cell cultures. However, before this platform becomes generally acceptable and commercially feasible, many improvements are required. In particular, it is essential to enhance productivity, working both at the basic research level (genotype selection, appropriate promoter and enhancer elements, codon optimization and control of glycosylation) as well as in adequate bioreactor design, overcoming the same bottleneck that has previously been encountered with animal cell cultures. Moreover, attention is also required to address problems of post-synthesis degradation and nonbiotic product losses within plant tissues and in the medium of plant cell cultures.

If the challenge can be met to obtain human proteins in plant cells that are structurally and functionally equivalent to their native counterparts (and are produced at high yields), plant cells will definitely be a competitor. In this respect, the Protalix Biotherapeutics recombinant glucocerebrosidase (prGCD), obtained in carrot cells, behaving as a superior biosimilar, represents an encouraging example. In addition, another important example is represented by the first plant-derived veterinary vaccine developed by Dow AgroSciences in tobacco cell culture that obtained USDA approval in 2006 [114].

Hence, plant cell suspensions represent a realistic resource for the production of high-grade pharmaceuticals: where good production settings are lacking, where existing vaccines are expensive, where efficacy/half-life of the product has to be improved or where there are no vaccines at all.

These features are maintained even in cases where the expression level is low but the value of the product is high, as in the case of GCD, for which costs are estimated to be US$200,000 a year per patient.

Nevertheless, in general, high product yields are required, with the main challenge being in translating the laboratory product into a large-scale production in a reproducible and economically competitive manner. In this respect, hairy roots represent a promising system. The great genetic stability of hairy roots, together with their feature of being a clear example of plant viral vector–based transient expression in containment, make them an interesting pharmaceutical production system, even though no advanced-stage products are available to date.

Other contained systems (e.g., aquatic plants, moss and microalgae) are also very promising. Most of these systems are used by companies: Greenovation has developed a proprietary system for protein production in the moss P. patens, as a custom manufacturer of several undisclosed products that are in preclinical development for undisclosed partners [208]. Biolex uses proprietary technology to produce proteins in duckweed in a contained process such as Locteran (an IFN-α fused to a novel agent for controlled release activity, currently in Phase II clinical trials) [207] with positive results in patients with chronic hepatitis C [3]. From this scenario, it appears that these contained systems are exclusive properties of few companies, suggesting that research should focus on other related species/models. As a consequence, a large gap is recorded between the few products that are being commercialized and the many products that have been expressed but whose efficacy has not been tested even in preclinical trials.

Five-year view
At present, besides the veterinary vaccine for poultry by Dow AgroSciences, a second plant-produced vaccine-related product has overcome all production and regulatory hurdles, and is in the market. It is a plant-made antibody produced in transgenic tobacco and used as a reagent for the purification of a recombinant anti-hepatitis B vaccine in Cuba [115].

Plant-based technology for human vaccines is not currently supported by ‘big pharma’, with its own plants and technologies. Moreover, globally, there are not many GMP facilities that can process bulk plant material to an acceptable degree of purity and at sufficient quantities for Phase IIb/III human trials, necessary to establish collaborations between public/nonprofit organizations and small–medium pharma companies.

Besides the well-known example of Bayer AG (Leverkusen, Germany; which in 2006 acquired ICON Genetics) that opened production facilities based on the use of tobacco to manufacture biopharmaceuticals (such as a patient-specific antibody vaccine for non-Hodgkin’s lymphoma therapy), it is also very encouraging that recently, Medicago Inc. of Canada was invited to the sixth WHO’s meeting on evaluation of pandemic influenza prototype vaccines in clinical trials [116,210]. One of the purposes of the WHO’s meeting was to make recommendations on research activities that will contribute to the development of effective pandemic vaccines. Medicago recently reported positive interim results from a Phase I human clinical trial with its H5N1 avian influenza vaccine candidate (a VLP-based vaccine produced with a transient expression system). The vaccine was found to be safe, well tolerated and also induced a solid immune response. Based on these results, Medicago will proceed with a Phase II clinical trial with the first plant-made influenza vaccine.

If these novel products win approval, the field should really take off. Other potential first-line plant-derived vaccines may be new versions of blockbuster products such as the new HPV or possibly rotavirus vaccines, given their current very high pricing [117]. Hopefully, the development will also be driven by small companies, independent/philanthropic organizations (i.e., the Bill & Melinda Gates Foundation) and governments able to support innovative thinking, larger investments and new regulatory issues specific to new cell substrates that, therefore, for contained system-derived vaccines should be more easily achievable.
Financial & competing interests disclosure
The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.
No writing assistance was utilized in the production of this manuscript.

Key issues
- Contained production systems (i.e., plant cell suspension or hairy root cultures, novel plants and microalgae) are gaining greater recognition for the production of recombinant therapeutic molecules.
- While maintaining the benefits of whole-plant systems, further advantages of contained production systems are represented by controlled growth in bioreactors, easier downstream processing and purification, compliance with good manufacturing practice procedures, and less environmental, regulatory, society and political concerns.
- Most of these systems have an established track record for the production of secondary metabolites that now, together with advances in knowledge and technology, can be exploited for the production of useful proteins.
- Plant cell cultures still need optimized and stable expression levels of the product in long-term cultures. In spite of these limitations, at present, the only two recombinant plant products close to commercialization derive from cell suspensions (recombinant human glucocerebrosidase and a poultry vaccine).
- One interesting system is represented by hairy roots, which now can also be generated without the need of transgenic plants, by plant viral vector-mediated expression.
- Other contained systems (e.g., aquatic plants, moss and microalgae) are also very promising.
- In the future, several other products (at the moment undisclosed) from all these contained systems are expected to reach the clinical trial stage and the market.

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Papers of special note have been highlighted as:
- of interest
- interesting
- essential
- highly relevant

8 Comprehensive review on plant cell/tissue systems, cultivation and potentials. Provides references to recent relevant reviews along with a critical analysis of the latest improvements in plant cell culture, co-cultures and disposable reactors for production of small secondary product molecules, transgenic proteins and other products. Some case studies for specific products or production systems are used to illustrate principles.
17 Breakthrough in the application of transient expression technology in root cultures. Describes a fully contained system for expressing recombinant proteins that is based on clonal root cultures and episomal expression vectors, showing promise for commercial production of vaccine antigens and therapeutic proteins in contained facilities.


Karg SR, Frey AD, Kallio PT. Reduction of N-linked xylose and fucose by expression of rat 1,4-N-acetylgalcosaminyltransferase III in tobacco BY-2 cells depends on Golgi enzyme localization domain and genetic elements used for expression. J. Biotechnol. 146(1–2), 54–65 (2010).


Critical review on plant cell/tissue systems, cultivation and potentials/limitation. Transient expression of foreign proteins using plant viral vectors is also described as a practical approach for producing foreign proteins in plants with adaptation required to allow infection and propagation of engineered viruses in plant tissue cultures for transient protein expression in vitro.


Critical review on bioreactor cultivation of hairy roots.


- Provided the first evidence that complex mammalian proteins could accumulate as soluble correctly folded proteins in algal chloroplasts. Also the first demonstration of a therapeutic protein expressed and shown to have biological activity, in this case antibody binding to its antigen.

• Production of an antitumor antibody in a plant-based system (Lemna minor) with engineered glycan structures (via RNAi) provided enhanced antibody-dependent cell-mediated cytotoxicity compared with the Chinese hamster ovary-produced antibody.


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