"Hairy Roots, their Multiple Applications and Recent Patents"

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Abstract: In the last years, hairy root (HR) cultures are gaining attention in the biotechnology industry. This particular plant cell culture derives from explants infected with *Agrobacterium rhizogenes.* They constitute a relatively new approach to *in vitro* plant biotechnology and modern HR cultures are far away from the valuables findings performed by Philip R. White in the 1930´s, who obtained indefinite growth of excised root tips. HR cultures are characterized by genetic and biochemical stability and high growth rate without expensive exogenous hormones source. HR cultures have allowed a deep study of plant metabolic pathways and the production of valuable secondary metabolites and enzymes, with therapeutic or industrial application. Furthermore, the potential of HR cultures is increasing continuously since different biotechnological strategies such as genetic engineering, elicitation and metabolic traps are currently being explored for discovery of new metabolites and pathways, as well as for increasing metabolites biosynthesis and/or secretion. Advances in design of proper bioreactors for HR growth are being of great interest, since scale up of metabolite production will allow the integration of this technology to industrial processes. Another application of HR cultures is related to their capabilities to biotransform and to degrade different xenobiotics. In this context, removal assays using this plant model system are useful tools for phytoremediation assays, previous to the application in the field. This review highlights the more recent application of HRs and those new patents which show their multiple utilities.

Keywords: Bioreactors, biosensors, biotransformation, enzymes, phytoremediation, secondary metabolites, transgenic hairy roots.

1. INTRODUCTION

In the early 1930s, White and co-workers [1] published for the first time the continuous growth of tomato excised root tips in a proper nutrient solution. Although these roots were not obtained through *Agrobacterium rhizogenes* mediated transformation method, it was the initial idea for an indefinite plant culture system [1]. This important contribution to modern plant biotechnology, followed by the development of HR cultures and many other novel ideas and technologies promoted the evolution of techniques that are routinely used in plant regeneration and genetic transformation protocols and that constitute the actual scenario of plant biotechnology. Approximately 25% of all pharmaceuticals on the market are based on compounds originally found in plants and some are still extracted from them. In particular, plant roots produce and accumulate several secondary metabolites of pharmaceutical and industrial interest. Since plant cells have totipotent character, HRs are able to produce metabolites and/or secondary compounds in a similar way or even at higher levels than intact roots [2]. So, development of HR cultures is a remarkable alternative to avoid harvesting great amounts of plants for purification of valuable compounds.

HRs derive from the infection of wounded plant tissues by *Agrobacterium rhizogenes*, a Gram-negative soil bacterium. During infection, *A. rhizogenes* transfers the T-DNA comprising the loci with *rol* genes of Ri (*root inducing*) plasmid into the plant genome, causing neoplastic roots and root hair proliferation. A complete description about *rol* genes from *A. rhizogenes* involvement in the formation of HRs has been reported by Nilsson and Olsson [3]. The name "hairy roots" was mentioned for the first time in the literature in 1900s by Stewart and co-workers [4] and became from particular characteristics of transformed roots, such as massive adventitious growth with abundant root hairs. HR cultures have several advantages such as genotype and phenotype stability, fast and indefinite growth *in vitro* by subculture in absence of phyto-hormones under sterile conditions and high production of secondary metabolites. In Fig. (**1**), the multiple steps for the establishment of a HR culture and their main applications are shown.

Applications of HRs include several aspects as: phytochemistry, enzyme and recombinant protein production, phytoremediation, molecular breeding, rhizosphere physiology and biochemistry studies, metabolic engineering, bioreactor design and optimization, and deep studies of the system, as it was recently pointed out by Ono and Tian [5]. According to SCOPUS databases 2,025 reports are related with HRs (date of access, December 10th 2011), while considering the publications from 2008 until now, 479 reports match with "hairy root" as keyword (460 articles and 19 reviews). They are related with different subject areas with the following order of importance: Biochemistry, Genetics and Molecular Biology (37.8%); Agricultural and Biological Sciences (33.5%); Immunology and Microbiology (11.9%); Pharmacology, Toxicology and Pharmaceutics and finally, Medicine. In the same database 1,042 patents related with HRs could be

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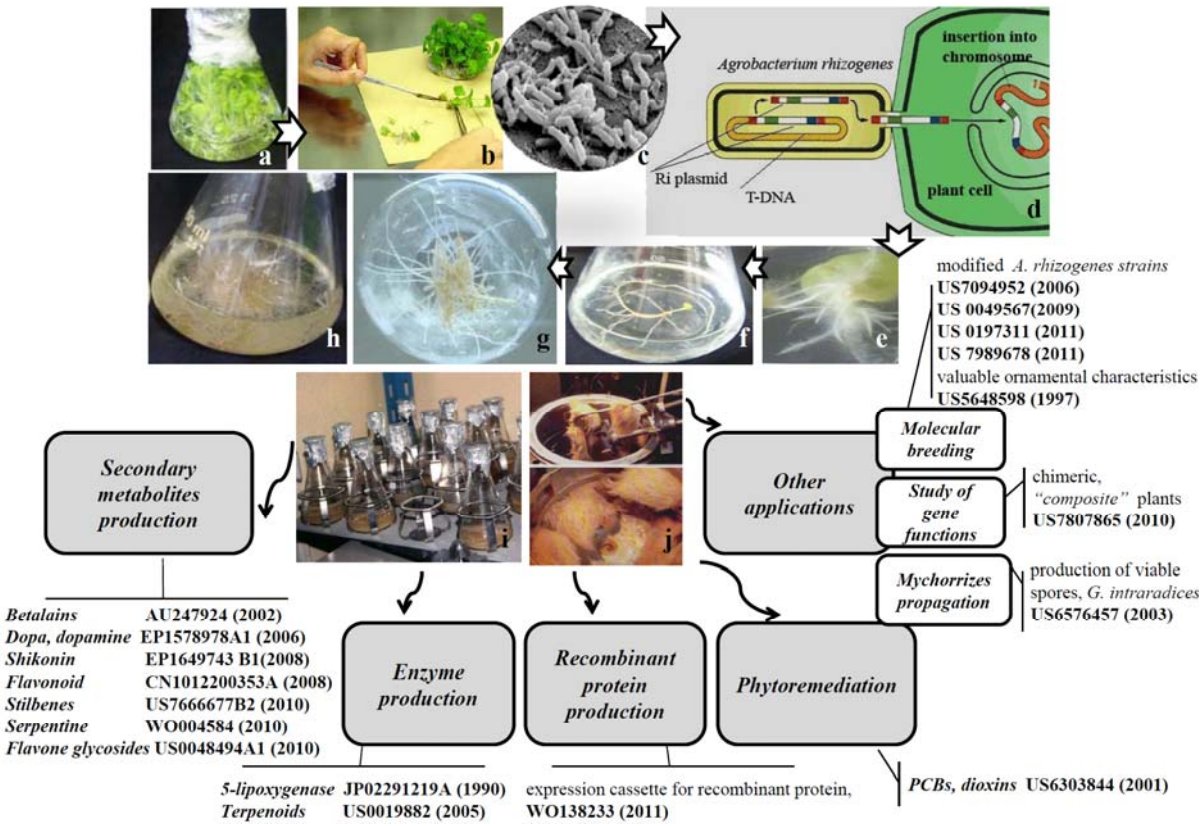

Fig. (1). Different steps for the establishment of HR cultures, multiple applications and recent patentes on the topics **a**) Sterile explants grown in hydroponic cultures, **b**, **c**) Procedure of infection, making cuts in explants and inoculation with a grown culture of *A. rhizogenes* strain, **d**) Molecular events during the transformation, transfer and integration of T-DNA of Ri plasmid in plant genome, **e**) First HR appearance on the explants on agar plates containing Murashige Skoog (MS) medium with antibiotic, **f**) First days of HRs growing in MS liquid medium, **g**, **h**) Successive stages of growth cycle until the formation of the confluent culture, **i**) Orbital shaker with HR cultures grown in liquid medium, **j**) HR bioreactor for scale up of production processes.

found, from which the majority correspond to the United State Office, followed by those from World Intellectual Property Organization (WIPO), European Patent Office, Japanese Patent Office and finally, those from UK Patent Office. Other databases were used for the present compilation of patents (Science Direct, Google Patents, PatentStorm, Patents OnLine, etc). In all of the consulted databases coincidently the first patents related with HRs are from the late 1980s and the early 1990s. They comprise the description of the obtaining of HRs as a method to produce medicinal ginseng [6] and others which described basic methods to grow HRs in large amount from horseradish [7], *Sesamum indicum* L. [8], *Beta vulgaris* [9] as well as other patents describing the production of useful substances from HR cultures [10] or enzymes such as peroxidases [11]. In the following years, there were patents related with the use of HRs as a tool to different pharmacological compounds production, which are presented here, but there are an increasing number of patents in the last years (2008-2011) comprising the use of *A. rhizogenes* technology or HRs as a basic biotechnological tool to obtain transgenic plants or chimeric plants for studying gene/protein functions.

Currently the main constraint for commercial utilization of HRs is the development and scaling up of appropriate bioreactors for HR growth. To our knowledge, the only company based on HR cultures at industrial level is a Swiss company called ROOTec Bioactives AG in Witterswil (SO) [12]. ROOTec manufactures high-value plant-derived compounds with efficient biotechnological processes, enabling its customers to generate high-quality products for the pharmaceutical and cosmetic markets, with rapid set-up of production process compared to field cultivation and chemical synthesis and competitive cost compared with conventional production. Other objectives of this company are to bring accessibility to compounds from rare and/or unexplored plants, avoid exploitation and protect rare plant species, in line with the convention of RIO (1992) [12]. For HRs, ROOTec has developed a new system, the ROOTec Mist Bioreactor (RMB), in which they can be cultured under optimal conditions and moreover the production volume can be increased by adding modular units of RMBs [12].

2. PRODUCTION OF METABOLITES, ENZYMES AND RECOMBINANT PROTEINS BY HR CULTURES

2.1. Secondary Metabolites Produced by wild Type HRs: Improvement to Increase the Production

The characteristics of HRs such as genetic stability, biosynthetic capacity comparable to native plant root and high biomass production, often make them more suitable than cell

or callus cultures for secondary metabolite production [13]. Furthermore, they often produce secondary compounds for a long period of time, unlike natural roots. Even though chemical synthesis constitutes an alternative for secondary metabolite production, it is complicated due to large number of asymmetric carbons and economic limitations. Many examples are currently in the literature about valuable secondary metabolite production from non-transgenic HRs (Table **1**). In some cases, changes on growth conditions (culture medium composition, carbon source, pH, light/dark, aeration, etc.), as well as the use of elicitors have been successfully applied to increase HR growth from different plant species and also for production or secretion of secondary metabolites [13, 14]. A recent review discusses cellular and molecular events induced by elicitors in HRs and the correlation with enhanced secondary metabolite synthesis [15]. However, new approaches are based on kinetic models for HR growth improvement [14]. In this aspect, Goel and co-workers [15] proposes the combination of elicitation with *in silico* (computer simulation) approaches to understand and identify the rate-limiting steps of biosynthetic pathways existing in HRs, in order to improve different compounds productivity, by using metabolic engineering aspects.

Here, we present selected classical and recent case studies where growth conditions, elicitation and *in silico* simulation or a combination of those have been used to improve the production of secondary metabolites.

2.1.1. Withanolide A

This is a compound with brain regenerative properties produced by HRs of *Withania somnifera*. The effect of carbon source and initial pH was studied. The authors found that biomass of HRs of *W. somnifera* and production of withanolide A was highest when sucrose was used as the carbon source; further 3% of this carbon source and pH 5.8 was optimal for biomass accumulation, while 4% sucrose and pH 6.0 favored the production of withanolide A [16].

2.1.2. Azadirachtin

Srivastava and Srivastava [17] studied the production of azadirachtin, a well-known biopesticide, during the growth cycle of *Azadirachta indica* HRs. Through an *in silico* approach, a *Plackett-Burman* experimental design protocol was used to identify key medium nutrients and concentrations to support high root biomass production and azadirachtin accumulation in HRs. When the optimal nutrients and concentrations, determined by the design protocol, were assayed in shake flask cultivation, azadirachtin accumulation increased a 68% compared with that obtained under non-optimized conditions.

Table 1. Summary of Secondary Metabolites Produced by Wild Type HR Cultures

Secondary Metabolite	Function	HR	Study/Strategy	Ref
Withanolide A	Brain regenerative properties	*Withania somnifera*	Effect of carbon source and initial pH	[16]
Azadirachtin	Biopesticide	*Azadirachta indica*	Identification of key nutrients and concentrations through *Plackett-Burman* design protocol	[17]
Stilbenoids (resveratrol, pinosylvin and derivatives)	Anti-oxidant, anti-cancer, anti-atherosclerosis neuroprotective and estrogenic activities	*Arachis hypogaea*	Elicitation with sodium acetate	[19]
Glycyrrhizin	Artificial sweetener and pharmaceutical products (peptic ulcers treatment)	*Glycyrrhiza inflate*	Optimization of growth and elicitation to improve glycyrrhizin accumulation	[24]
Camptothecin	Antitumor action, colorectal and ovarian cancers treatment	*Ophiorrhiza alata Craib*	Addition of polystyrene resin (Diaion HP-20) for an increased recovery	[26]
Dopa and dopamine	Neurotransmitters	*Beta vulgaris*	Evaluation for maximum production growth stage. Study of extraction process	[31]
Betalain	Red pigments for food industry		Improvement of extraction methods	[32]
Shikonin	Dye for silk and food industry, anti-inflammatory, anti-allergic and anti-neoplasic activities	*Arnebia*, a hispid herb mostly confined to Asia	Establishment of HRs cultures	[34]
Serpentine	Diabetes treatment	*Catharanthus roseus*	Identification, isolation and characterization of this compound	[38]
Flavone glycosides	Anti-inflammatory action (four of them)		Production of new flavone glycosides, appropriate extraction method using methanol	[39]
Sesquiterpenes	Phytoalexines	*Hyoscyamus albus*	Elicitation with copper sulphate and methyl jasmonate addition	[45]

2.1.3. Stilbenoids

Medina Bolívar and co-workers studied [18] and patented [19] the production of stilbenoids including resveratrol, pinosylvin and their respective derivatives in peanut HR cultures. These plant polyphenols are receiving considerable interest based upon a number of associated health benefits, such as antioxidant and anticancer properties and most recently their neuroprotective and estrogenic activities [20]. While stilbenes can be recovered as an extract from a selected number of plants, these products are not suitable for many applications in food/pharmaceutical sectors due to high levels of impurities, as well as the overall low concentration of resveratrol and its derivatives in the extract. Thus, many efforts have been made to develop production systems for enriched and concentrated commercial stocks of resveratrol. Among them, the expression of resveratrol pathway genes in heterologous host (yeast, bacteria) has been proved; however limitations with inefficient substrate use, high substrate cost and recombinant-based production difficulties currently limit commercialization efforts of resveratrol produced in these systems. Even though other patented strategy has considered the introduction of genes encoding resveratrol synthesis into legume plant cells [21], lengthy process steps and high costs are among the disadvantages of such systems. In addition, grapevine cell suspensions for the production of *trans*-resveratrol has been reported [22], however these plant cell cultures had low stability for secondary metabolite production and they did not respond to elicitors [23]. For these reasons, elicited peanut HRs with sodium acetate to increase the stilbene production has been proposed and patented as an efficient alternative [19].

2.1.4. Glycyrrhizin

HR cultures from *Glycyrrhiza inflata* have been developed for glycyrrhizin production, a secondary metabolite used as an artificial sweetener in some candies and pharmaceutical products and used with medical purposes, in particular, for peptic ulcers treatment and as an expectorant [24]. The optimization of growth and glycyrrhizin accumulation of *G. inflata* HRs were studied, as well as the effect of elicitors like chitosan, methyl jasmonate and yeast extract on glycyrrhizin production, being methyl jasmonate the most efficient in enhancing its production.

2.1.5. Camptothecin

This pharmaceutical valuable product has been obtained through HR cultures from different plant species like Chinese tree *Camptotheca acuminate* [25], *Ophiorrhiza alata* [26] or *Ophiorriza rugosa* [27]. Camptothecin has antitumor action along with topotecan and irinotecan, two water-soluble derivatives, which have been approved by the US Food and Drug Administration for treating colorectal and ovarian cancers [28]. The accumulation of this compound in HRs was twice that in soil-grown plants [26]. Moreover, HRs allow to improve strategies to recover the compounds of interest from the culture medium. In this sense, these authors [26] increased seven fold the recovery of camptothecin compared with controls when a polystyrene resin (which absorbs camptothecin) was added to the medium. There are some patents related with the production and extraction of camptothecin and their derivatives from different plants [29, 30], however there are no patents about the use of HRs for this alkaloid production.

2.1.6. Betalains

Beta vulgaris HR cultures have been used for the production of dopa and dopamine [31] and the proper stage of HR cultures for the maximum production was evaluated. Moreover, the extracting process with solvents to obtain them from these HRs was patented by Ravishankar and co-workers [31]. In addition, *B. vulgaris* HRs were able to produce betalains and the extraction method of these compounds have been improved and patented [32, 33].

2.1.7. Shikonin

This is a valuable red pigment produced by HRs. Since wild plant species from the *Boraginaceae* family, accumulating shikonin, fail to provide a sufficient raw material for commercial production, HR cultures become an excellent tool for overcoming these short fall in ever-increasing demand worldwide. Chaudhury [34] has patented a method for the establishment of HRs cultures of *Arnebia,* a hispid herb mostly confined to Asia, for shikonin production. Although shikonin has been known for its properties as pigment, anti-allergic, anti-pyretic and anti-neoplastic effects of shikonin and its derivatives have also been demonstrated [35]. Thus, many attempts to increase its production from different HRs cultures have been performed, as those derived from *Lithospermum canescens* [36, 37].

2.1.8. Serpentine

This is a compound used for diabetes treatment formed in *Catharanthus roseus* HR cultures. There is a patented invention that provides identification, isolation and characterization of this biologically active compound from HR extracts [38]. This herbaceous species (*C. roseus),* also known as *Vinca rosea* L. and/or *Herba catharanthi,* is widely used as ornamental and medicinal plant. It has been extensively studied because the anti-leukemia drugs vincristine and vinblastine were originally obtained from this herb. In addition, many other useful drugs have been isolated from this plant or its cell cultures. The obtaining of twelve new and useful compounds (flavone glycosides) from *C. roseus* HRs has been achieved and recently patented [39]. From them, four compounds have already been shown to have anti-inflammatory activity suggesting their use as medicaments. The patent also involves an appropriate extraction method for these compounds using methanol [39].

Other examples about elicitation to improve productivity includes the cases of the diterpenoid tanshinone in *Salvia miltiorrhiza* HRs [40], tropane alkaloids in *Anisodus acutangulus* HRs [41], spiroketal enol ether diacetylenes in *Tanacetum parthenium* HRs [42] and ginseng saponins biosynthesis in *Panax ginseng* HRs [43]. It is noteworthy that sometimes, through elicitation, HR cultures have produced compounds which were absent in the native plant [44]. For instance, phytochemical profile from *Hyoscyamus albus* HRs changed in response to copper sulphate and methyl jasmonate used as elicitors, and four new sesquiterpene phytoalexins were identified and characterized [45]. Electric current is being used as a clean elicitor and multiple electro-elicitation can be applied to enhance phytochemical produc-

tion [46]. However, a deep study is needed for that technology and currently there are no patents on this. Interestingly, there is a recent patent [47] that proposes the use of transporters, more particularly ABC-transporters, to enhance the production and/or secretion of secondary metabolites from plant systems.

All these strategies; changes in culture medium conditions and/or elicitor addition have been applied with or without success for production of valuable secondary metabolites from wild type HRs, looking for the improvement of HRs growth, metabolite production and/or secretion to the culture medium. However, biosynthesis of a valuable metabolite by HRs is sometimes limited or even it is not possible because absence of the metabolic route. Research advances on metabolomics have allowed the discovery of genes involved in several metabolic pathways. With this knowledge, new opportunities based on the expression of foreign genes involved in the synthesis of a specific compound or even the overexpression of an enzyme naturally present in HRs, are available to increase the production of a particular compound in HRs. Genes cloned between the two T-borders of the T-DNA are transferred into the plant genome by the molecular machinery of *A. rhizogenes* during the transformation process. In this sense, transgenic HR generation is a simple and well established genetic transformation process that stably integrates DNA in plant genome.

2.2. Transgenic HRs to Increase Secondary Metabolites Production or the Production of New Compounds

Since regulation, approval and public acceptance for transgenic plants grown in the field is complex, plant cell cultures containing foreign genes became a promissory tool for the production of valuable compounds. Compared with whole plants in open agriculture, the *in vitro* culture conditions of plant cultures are more readily manipulated and allow a better control over metabolite levels and quality, resulting in improved product consistency [48]. Chandra and Chandra [49] have recently reviewed the novel advances in secondary metabolites production in transgenic HR. Although metabolic engineering offers promising perspectives to improve yields in HRs, it is necessary to understand the regulation of secondary metabolite pathways, including aspects as transport and compartmentation. As it is shown in Table **2**, several strategies have been proved to be useful for increasing secondary metabolite production, such as those which avoid negative regulation or others that include overexpression or co-expression of enzymes involved in a particular metabolic pathway.

2.2.1. Co-expression of Proteins Able to Trap the Metabolite of Interest

In *Solanum khasianum* HRs, a specific antibody was synthesized that binds the anti-neoplastic agent solanoside glycoside and turns off the negative regulation mechanism and, thus leads to an increase in production of the anti-neoplastic agent [50, 51].

2.2.2. Co-expression of a Feedback-Insensitive Enzyme

The indole pathway was manipulated by introducing an *Arabidopsis* feedback-insensitive anthranilate synthase (AS) alpha subunit (*trp5*) cDNA and *C. roseus* tryptophan decarboxylase gene (TDC), under the control of a glucocorticoid-inducible promoter into *C. roseus* HRs [52, 53].

2.2.3. Expression of Antisense Gene to Increase Secondary Metabolites Production

Through transgenic HR approaches good results have been obtained for an increased secondary metabolites production with antisense strategies. In this regard, gene silencing has been useful to avoid negative regulation or even to direct or change a biosynthetic pathway and hence to improve production of specific compounds. Ginseng is a valuable metabolite since in traditional Chinese medicine, tonic properties have been attributed to this compound. Moreover, anti-aging, anti-cancer and anti-diabetes properties are related to ginsenosides compounds present in roots [54]. The increase of ginsenoside content in transgenic HR lines of *Panax ginseng* constitutes an example of antisense suppression strategy. In this case the *cs* gene, which encodes for the enzyme cycloartenol synthase (CS) was silenced in HR of *P. ginseng* and some lines showed more ginsenosides (between 50 to 100%) compared with wild type HRs. This enzyme and dammarenediol synthase are responsible for ginsenoside and phytosterol biosynthesis, respectively, derived from the precursor 2,3-oxidosqualene [55]. The authors proposed that in *P. ginseng*, the regulation of CS can control the metabolic flux from 2,3-oxidosqualene to both phytosterol and ginsenoside.

2.2.4. Overexpression or Co-expression of Enzymes Involved in the Biosynthesis Pathway of a High-Value Metabolite

Tropane alkaloids (TA) are a group of important anticholinergic drugs that acts on the parasympathetic nervous system with rapidly increasing market demand, so it is significant to improve TA production by biotechnological approaches. Among them, hyoscyamine and scopolamine have been obtained from large-scale HR cultures of several *Solanaceae* species [56, 57]. However, more recently new attempts to increase alkaloid production rates for commercial exploitation have been proposed. Moyano and co-workers [58] proposed the expression of the *pmt* gene of *Nicotiana tabacum*, which encodes for putrescine:SAM N-methyltransferase (PMT), under the regulation of the CaMV 35S promoter in *Duboisia* HRs. This enzyme catalyses the *N*-methylation of diamine putrescine to form *N*-methylputrescine, which is the first specific precursor of both tropane and pyridine-type alkaloids. For the obtainment of HRs of a *Duboisia* hybrid *(D. myoporoides* × *D. leichhardtii)*, plantlets were infected with transformed *A. tumefaciens* C58C1 carrying the rooting plasmid pRiA4 with the construction. *N*-methylputrescine levels of the resulting engineered HRs increased (2-4 fold) compared to wild-type roots, but there was not significant increase in either tropane or pyridine-type alkaloids. On the other hand, Zhang and coworkers [59] reported the simultaneous introduction and overexpression of *pmt* and *h6h* genes encoding the rate-limiting upstream enzyme putrescine *N*-methyltransferase and the downstream enzyme hyoscyamine 6-β-hydroxylase, respectively, of scopolamine biosynthesis in transgenic henbane (*Hyoscyamus niger*) HR cultures. Transgenic HR lines expressing both *pmt* and *h6h* produced significantly higher levels of scopolamine compared with the wild-type and

Table 2. Summary of Secondary Metabolites Produced by Transgenic HR Cultures

Secondary Metabolite		Function	Transgenic HR	Foreign Genes	Study/Strategy	Ref
Solanoside		Anti-neoplastic agent	*Solanum khasianum*	Gene encoding a specific antibody that binds solanoside	Binding to the antibody turns off the negative regulation mechanism and increase the production	[50] [51]
Indole		Beneficial effects on cancer, sedative and hypotensive action	*C. roseus*	Modified anthranilate synthase (AS) alpha subunit (*trp5*) and tryptophan decarboxylase gene (TDC)	Supression of feedback regulation of anthranilate synthase (AS) and stimulation of indole production by the control of a glucocorticoid-inducible promoter	[52] [53]
Ginseng		Traditional Chinese medicine, tonic, anti-aging, anti-cancer and anti-diabetes properties	*Panax ginseng*	*cs* gene for cycloartenol synthase enzyme	Silencing *cs* gene to increase ginsenosides content	[55]
	Scopolamine		*Hyoscyamus niger*	Putrescine *N*-methyltransferase (*pmt*) and hyoscyamine 6 β-hydroxylase (*h6h)* genes	Approach for large-scale commercial production of scopolamine by using HR culture systems as bioreactors	[59]
Tropane alkaloids	Catharanthine	Anticholinergic agents that acts on parasympathetic nervous system	*Catharanthus roseus*	Geraniol 10-hydroxylase (G10H) and a jasmonate-responsive transcript factor (ORCA3)	Overexpression of two genes	[60]
	Hyoscyamine, scopolamine		*Scopolia parviflora*	Putrescine *N*-methyltransferase (*pmt*) and hyoscyamine 6 β-hydroxylase (*h6h)*	Co-expression of *pmt* and *h6h* and addition of growth regulators for enhancing the production	[61]
	Anisodamine, anisodine, hyoscyamine, scopolamine		*Anisodus acutangulus*	Putrescine *N*-methyltransferase (*pmt*) and gene codifing tropinone reductase I (TRI)	Simultaneous introduction of two genes, PMT and tropinone reductase I (TRI)	[62]
	Glycyrrhizin	Medicine, health care products, food (sweetener) and cosmetics	*Glycyrrhiza uralensis*	Chalcone synthase	Overexpression of chalcone synthase	[63]
Flavones				Squalene synthase gene (*GuSQS1)*	Increased glycyrrhizin production by about 3.6 times	[64]
	baicalin, baicalein, wogonin	Diuretic, anti-inflammatory, antiseptic, antispasmodic, anticancer	*Scutellaria baicalensis*	Chalcone isomerase	Overexpression of chalcone isomerase to enhance flavones levels	[65]
Vitamin C		Antioxidant properties	*Solanum lycopersicon*	*gal*UR gene	Overexpression of *galUR* gene from *Fragaria sp.* and complemented with a precursor feeding approach	[66]
Total sterols		Hypocholesterolemic, anticarcinogenic properties	*Centella asiatica*	Farnesyl diphosphate synthase (FPS) from *Panax ginseng*	Confirmation of the regulatory function of FPS in phytosterol biosynthesis	[68]

transgenic lines harboring a single gene. This study provided an effective approach for large-scale commercial production of scopolamine by using HR culture systems as bioreactors. Overexpression of geraniol 10-hydroxylase (G10H) and a jasmonate-responsive transcript factor (ORCA3) in the HR of *Catharanthus roseus* improves catharanthine production, another valuable TA [60]. More recently, Kang and coworkers [61] using the same strategy, co-expression of *pmt* and *h6h*, obtained transgenic HR lines of *Scopolia parviflora*. Consequently to the introduction of these key enzyme genes, production of the alkaloids hyoscyamine and scopolamine was enhanced. In addition, treatment of transgenic

HRs with growth regulators further enhanced scopolamine production. Another example of two enzyme co-expression for increasing TA yields was that obtained by Kai and co-workers [62]. They obtained an increased production of TA through simultaneous introduction of PMT and tropinone reductase I (TRI) in HR of *Anisodus acutangulus*, an endemic perennial plant from China [62].

For flavonoids production, which are common active ingredients in medicinal herbs, Zhou and co-workers [63] presented a patent which describes a chalcone synthase derived from licorice (*Glycyrrhiza uralensis*). The chalcone synthase is a first key enzyme in the metabolic pathway of flavonoid compounds in plants. The experiments showed that after licorice chalcone synthase gene is overexpressed in licorice HR, total flavonoid content can be improved by about 2.5 times. The licorice chalcone synthase gene provided by the invention delivers an effective technical means for the synthesis of flavonoid compounds in the genetically engineered plants, which has great economic value and broad application prospect in medicine, health care products, food and cosmetics fields [63]. Furthermore, overexpression of a squalene synthase gene (GuSQS1) also in transgenic *G. uralensis* HR enhanced glycyrrhizin content about 3.6 times [64]. In addition, flavone levels (baicalin, baicalein and wogonin) were enhanced in HR cultures of *Scutellaria baicalensis* trough overexpression of chalcone isomerase [65].

2.2.5. Heterologous Expression of Proteins Involved in a Specific Metabolic Pathway

There are innumerable examples of this strategy. For instance, the overexpression of GalUR gene from *Fragaria sp.* involved in vitamin C biosynthesis in tomato HRs, although in this case the study was complemented with a precursor feeding approach [66]. Phytosterols have the ability to reduce low density lipoprotein (LDL) cholesterol in humans, thus foods containing this compound have therapeutic value and they have been approved for use by the US Food and Drug Administration [67]. However, free phytosterols are difficult to incorporate into food stuffs due to the low solubility while phytosterols esters can dissolve more easily. Thus, current commercial production of phytosterols-containing foods utilizes a costly fatty acid acylation procedure. In this sense, there are some patents for the production of phytosterol esters, which mostly involve the use of plants, in particular transgenic plants which overexpressed an Acyl-CoA sterol acyltransferase [67]. To our knowledge, there are not patents for the production of phytosterols in HRs despite the numerous papers in this respect. As it was reported by Kim and co-workers [68], using transgenic HR of *Centella asiatica* transformed with a construct harboring *P. ginseng* farnesyl diphosphate synthase (FPS) coupled to the cauliflower mosaic virus 35S promoter, higher total sterol content was obtained than those of the control HRs. Therefore, these results indicated that FPS performs a regulatory function in phytosterol biosynthesis.

2.3. Production of Enzymes from HR Cultures: Different Applications

One of the aspects of HR cultures that has attracted a great interest is their capacity for protein production [69]. Among the proteins that HRs can produce, important enzymes are included, such as superoxide dismutases [70], peroxidases (Px) [71, 72] and others such as phytases obtained using genetically engineered HRs [73].

In particular, Px are enzymes that have won a lot of interest for their applications in biotechnological processes. Px (EC 1.11.1.7) are oxidoreductases that catalyze the oxidation of a great variety of compounds, in presence of H_2O_2 or others peroxides as co-substrate. These enzymes are widely distributed in nature, being produced by animals, plants and microorganisms [74]. However, HR cultures have become an important source of these enzymes. Px have been applied in analytical system for H_2O_2 and organic hydroperoxide determination and coupled with enzymes which produce H_2O_2, used for the determination of many compounds such as glucose, cholesterol, uric acid, etc. in diagnostic kits. In this context, Px are probably the most suitable enzymes for the preparation of enzyme-conjugated antibodies, which are used in enzyme linked immunosorbent assay (ELISA) test [75, 76]. More recently, these enzymes have also been used in biosensor development and enzymatic remediation. In this sense, Granero and co-workers [77] proposed by the first time, an amperometric biosensor based on Px from *Brassica napus* HRs, used to determine the total polyphenolic content in wine and tea samples. The method employs carbon paste electrodes filled up with Px, ferrocene, and multi-walled carbon nanotubes embedded in a mineral oil. The biosensor exhibited a good performance, stability, reproducibility, repeatability, detection limit and linear range for the quantification of *trans*-resveratrol and caffeic acid. Furthermore, the electrochemical method had some advantages over the commonly used Folin-Ciocalteu method, such as a shorter detection time, a smaller sample volume used, higher accuracy and high simplicity. These advantages indicated that a *B. napus* Px biosensor can be used as a useful tool for rapid screening in the determination of total polyphenolic content in foods.

On the subject of enzymatic remediation, this technology has become an attractive alternative for removal of different xenobiotics, since some isolated enzymes have shown to use a wide variety of pollutants as substrate, to act in a broad range of contaminant concentrations, pH and temperatures and to have high reaction rates. Furthermore, enzymes generally do not produce toxic compounds and they are degraded *in situ* after removal treatment [78]. Next, we discuss the environmental importance of these biocatalysts, paying particular attention in the use of Px from HRs of several plant species to remediate different phenolic compounds. From the first research using the commercial horseradish peroxidase (HRP) to remove phenols from aqueous solutions [79], other sources of Px have emerged with the aim to develop more suitable and inexpensive systems. Px from HR cultures of carrot (*Daucus carota*), tomato (*Solanum lycopersicum)*, turnip *(B. napus)*, soybean *(Glycine max)* and red beet (*Ipomea batatas)* have been used for the removal of these contaminants. In this sense, interesting results have been obtained for different authors [80-82]. In our works, we established that Px HRs from several species have been very efficient in phenol and 2,4-dichlorophenol (2,4-DCP) removal [83, 84]. Moreover, we studied the involvement of particular isoenzyme Px groups from tomato and turnip HRs in removal of these phenolic compounds [82-84], which

could contribute to the selection of one enzyme that might be used as catalyst for contaminant break down.

Based on the potential applications of Px, it is very important to consider their production in large amounts with low costs. Therefore, Thimmaraju and co-workers [85] established that only some clones of red beet HRs produce high levels of Px having good thermal stability. In addition, one clone produced similar amounts of Px in both, a bubble column reactor and shake flask, indicating that clone could be exploited for scaled up production of Px. Since red beet HRs are also used for production of betalaines, red pigments for food applications, this system offer an excellent source for simultaneous production of both pigment and Px. The observation that nearly half of synthesized Px is secreted into the medium, indicates the possibility of permeabilizing the roots to enhance secretion of enzyme even in bioreactors, as reported for pigments from the same system [85]. These authors mentioned that several other unit operations could be consider to the scale up of HR biomass, and, therefore, red beet HR system appears very promising for production of this expensive enzyme.

Other enzymes produced by HRs are laccases. In this sense, it was demonstrated that not only Px are involved in removal processes, since a laccasse from HR cultures of *Brassica juncea* L. reached 92% decolorization of methyl orange. Telke and co-workers [86] purified and characterized the intracellular laccase responsible of this process and found that several textile dyes were decolorized in the presence of 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as redox mediator. These findings contribute to a better understanding of the enzymatic process involved in phytoremediation of textile dyes.

Other authors used HRs of *D. carota* to characterize *p*-hydroxybenzaldehyde dehydrogenase, which is the final enzyme for *p*-hydroxybenzoic acid biosynthesis. *p*-hydroxybenzoic acid is the precursor for the preparation of different esters, known as parabens which are used as preservatives in cosmetic industry [87].

Despite numerous research regarding different applications of HR enzymes have been presented, very few patents have been published on the subject. In 1990, Hatamoto and co-workers [88] patented the capability of potato HRs to produce 5-lipoxygenase in a large amount. This enzyme is involved in the fatty acid transformation into leukotrienes, and is a novel pharmacologic approach [88]. These compounds can be used in pharmaceutical compositions, alone or in combination with other compounds, for treating respiratory, cardiovascular, and other leukotriene-dependent diseases. Other interesting patent proposed using plant enzymes for the production of flavor, fragrance, pharmaceutical or bio-control agents from less valuable substrates. More specifically it relates with a process for terpenoid compounds production by the use of plant enzymes, which are produced by HR cultures [89].

It is noteworthy that extensive research about use of enzymes derived from HRs has been published; however, patents establishing the use of enzymes in different applications have not yet been reported.

2.4. HRs as an Important Tool for Biotransfomation of Exogenous Substrates

Biotransformation, also called bioconversion or biocatalysis, involves an enzymatic reaction catalyzed by plant cells, microorganisms and/or purified enzymes. Since chemical synthesis have sometimes several limitations, biochemical reactions occurring in plant cells appear as an interesting alternative, due to the high stereo- and regioselectivity, fewer side reactions, mild reaction conditions, simpler operation procedures, easier separation of products, lower cost and environmentally friendly properties. The reactions involved in the biotransformation of organic compounds by plant cells include oxidation, reduction, hydroxylation, esterification, methylation, isomerization, acetylation, glycosylation, etc [90].

There is a patent which establish the use of plant enzymes for bioconversion, particularly for an introduction of an oxygen group into an organic compound since this property is still a largely unresolved challenge to organic chemistry. This invention provides the conversion of sesquiterpene olefins to commercially products with high regio- and stereoselectivity using enzymes of *Asteraceae* plants [91].

Regarding biotransformations carried out by plant cell cultures there is a patent which proposed transformation of monoterpenic mixtures for the production of plant metabolites with broadly use in food and pharmaceutical industries [92].

Rational use of HRs as highly effective biotransformation systems has taken importance in the last twenty years. These *in vitro* cultures are preferred over plant cell/callus and suspension cultures as biocatalyst due to their genetic/ biochemical stability, hormoneautotrophy, multi-enzyme biosynthetic potential mimicking that of the parent plants and relatively low-cost cultural requirements. Among several useful biotransformations described for HR cultures, the oxidation-reduction like reactions of alcohols and ketones are of great interest [93]. In this context, Orden and co-workers [94] reported for the first time the stereo- and regio-selective reduction of natural prochiral diketones into the corresponding (*S*)-1-hydroxy compound catalyzed by *B. napus* HRs, which it is not feasible to be obtained by chemical transformations using usual reductive reagents. In addition, *Raphanus sativus* HRs were used as biocatalysts to afford stereoselective reduction of several prochiral alkylaryl-ketones with high yields and excellent enantioselectivities [95].

Besides, *Anethum graveolens* HRs showed biotransformation ability through different enzymes activities such as reductases, isomerases and transacetilases, among others, that readily transform geraniol and menthol into different products [96].

On the other hand, Zhou and co-workers [97] used HRs of *Polygonum multiflorum* to obtain coumarin glycosides. It is important to note that coumarins derivatives exhibit a broad range of pharmacological properties and biological activities however, their applications are limited by their low water solubility. Thus, coumarin glycosides offer a proper way of drug-administration and play an important role in the pharmaceutical utilization of coumarins [97, and references there in].

More recently, it was demonstrated for the first time the untapped potentials of a selected HR clone from *Atropa belladonna* to perform simultaneous oxidation and reduction of 3,4,5-trimethoxy benzaldehyde into 3,4,5-trimethoxy benzoic acid and 3,4,5-trimethoxy benzyl alcohol, respectively. The same HR clone also demonstrated the ability to reduce 3,4,5-trimethoxyacetophenone into the secondary alcohol, while not biotransform the carboxylic acid substrate under similar bio-reaction conditions [98]. All these examples demonstrate the great potential of HRs as model system for *in vitro* biotransformations of exogenous substrates. Furthermore, they are easy handle systems that offer the possibility of scaling up, representing an alternative to explore and use the great potential of plant enzymes.

A critical overview of the recent advances and application of HR cultures in the area of biotransformation of exogenous substrates was provided by Banerjee and coworkers [99].

2.5. Transgenic HRs as a Production System for Valuable Foreign Proteins

As previously mentioned, HRs are a fruitful source of proteins, even though for foreign proteins. It is well known that transgenic plants have been widely used for the expression of therapeutic proteins, such as vaccines, antibodies, and mammalian enzymes [100]. Moreover, heterologous proteins for pharmaceutical and industrial applications have been successfully expressed in plant cell culture-based bioreactor systems including suspended dedifferentiated plant cells, HRs, etc. Among these, HRs have enormous potential because this system combines the advantages of plant-based or "*green*" technologies such as intrinsic biosafety, scalability, low production and downstream costs, and the existence of eucariotic folding and assembling machineryto the ones of the *in vitro* technology, for example, growth under controlled and optimized conditions in confined bioreactors, continuous production, utilization of simple nutritional requirements, exclusion of transgene dissemination, reproducible product yield and easy regulatory compliance.

In 1997, a pioneering work of Wongsamuth and Doran [101] reported the first application of HRs for the synthesis of a full length murine IgG monoclonal antibody. Nowadays, 15 years later, about 15 recombinant proteins have been successfully produced in HR cultures, including other antibodies, antigens, immunomodulators, reporter proteins and enzymes, among others (Table **3**). Just in the last years, engineering HR for the production of proteins is seen as one of the exciting spin-offs of HR technology. As seen in Table **3**, tobacco (*N. tabacum*) is by far the most largely used plant species for the production of recombinant protein but many others were shown to be suitable.

Regarding patents development, progress in this field has been slow. The lack of patents dealing with protein production using HRs and other plant cultures compared to mammalian or microbial expression systems is partially related with the economic feasibility of plant cultures but also with restrictions and regulations imposed on the transgenic plant approach because of the potential for gene transfer to wild species through cross-pollination with closely related species. This is possibly the main reason that explains the scarce quantity of patents developed. Recently, Boitel-Conti and co-workers [124] patented a method comprising a vector containing an expression cassette for producing recombinant proteins from HR cultures from Brassicaceae family. The expression cassette comprises a promoter, a signal peptide, a gene encoding said recombinant protein and a polyadenylation sequence. Although scarce patents using HR cultures specifically for protein production are found, there are numerous patents that develop a transgenic plant and suggest the use of HR cultures in the future for protein production.

2.5.1. Factors Affecting Protein Production in HR

2.5.1.1. Selection of HR Line

An important factor in searching the best HR system for the production of a determined protein is exploring the properties of diverse HR lines, because of the possibility that one could be more favorable than other, in terms of by-product levels and regulatory compliance in those derived from food crops. Furthermore, other benefits of testing diverse lines include faster growth, higher expression levels, more efficient secretion and other advantages concerning process compatibility. Some researchers focus on plants with a higher protein content, for example soybean, assuming that these might more readily facilitate higher expression levels [125].

2.5.1.2. Construct Design

Regarding factors determining protein yield, the design of the construct used to express the recombinant protein is very important. Promoter choice affects the yield by determining the rate of transcription. The most commonly used promoter is the cauliflower mosaic virus (CaMV) 35S promoter or its enhanced version, but a number of alternative constitutive promoters can be used, including the hybrid *(ocs)3mas* promoter (constructed from octopine synthase (*ocs*) and mannopine synthase (*mas*) promoter sequences) [113]. In contrast to these constitutive promoters, inducible promoters could also be used. For instance, a glucocorticoid-inducible promoter controlling the expression of green fluorescent protein (GFP) was used in transgenic HR cultures of *C. roseus* [104]. Another example is the thermo-inducible expression of GUS activity in *N. tabacum* transgenic HRs transformed with the *A. thaliana* sHSP18.2 promoter fused to *E. coli gusA* gene [114].

2.5.1.3. Subcellular Localization of Proteins and Degradation

These aspects affect the economic viability of all plant based protein production. In general, proteins of less than 30 kDa tend to be secreted into the medium whereas larger proteins are quantitatively retained, although charge and/or hydrophobicity may also be important determinants [125]. In addition, foreign protein degradation can reduce levels of functional product in plant tissues after the molecules are synthesized and assembled. Moreover, being metabolically wasteful, foreign protein degradation would contaminate the product with inactive protein fragments that may be difficult to separate in large-scale recovery operations. The addition of a retention sequence that increases cytoplasmic protein and thus prevents protein degradation in the medium may enhance overall protein yields. For example, the addition of

Table 3. Proteins of Interest Produced in Transgenic HR Cultures

Protein Produced	Protein Function	Plant Specie	Scale	Ref
Murine IgG1monoclonal antibody	Murine antibody IgG1 type recognizing a protein of *Streptococcus mutans*	*N. tabacum* cv NT-1	Shake flasks Bioreactor	[101]
		N. tabacum cv NT-1	Shake flasks	[102]
		N. tabacum cv NT-1	Shake flasks	[103]
Green fluorescent protein (GFP)	Reporter protein	*Catharanthus roseus*	Shake flasks	[104]
Rabbit cytochrome P450 2E1	Mammalian liver detoxifying enzyme	*Atropa belladonna*	Shake flasks	[105]
Ricin B- GFP	Mucosal vaccine for intranasal immunization	*N. tabacum* cv *Xanthi*	Shake flasks	[106]
Human secreted alkaline phosphatase	Human enzyme	*N. tabacum*	Plastic tubes	[107]
GFP	Reporter protein	*N. tabacum L.* cv Wisconsin	Plastic tube	[108]
14D9 antibody	Murine antibody IgG1 type useful for organic synthesis	*N. tabacum*	Shake flasks	[109]
Surface protective antigen from *Erysipelothrix rhusiopathie* (swine erysipelas) fused to cholera toxin B subunit	Antigen	*N. plumbaginifolia*	Petri dishes	[110]
Human single chain antibody IgG1 and full-size IgG4	Human antibody	*N. tabacum*	Shake flasks	[111]
Hepatitis B surface antigens (HBsAg)	Hepatitis B vaccine	*Solanum tuberosum* (var. Kufri Bahar)	Shake flasks	[112]
		N. tabacum L.	Shake flasks	[113]
β-glucuronidase (GUS)	Reporter protein	*N. tabacum*	Shake flasks	[114]
GFP	Reporter protein	*N. benthamiana*	Shake flasks	[115]
Carrot ADP-ribosylation factor gene	Endoplasmic reticulum (ER) targeting signal sequence	*Atropa belladonna*	Shake flasks	[116]
Human acetylcholinesterase	Bioscavenger of organophosphate toxins	*N. benthamiana*	Shake flasks	[117]
Single chain murine interleukin-12	Immunomodulator	*N. tabacum* cv. Xanthi	Shake flasks, airlift reactor, and scalable mist reactor	[118]
Human epidermal growth factor	Growth factor	*N. tabacum*	Shake flasks	[119]
GUS	Reporter protein	*Corchorus capsularis L.*	Petri dishes	[120]
Enterotoxigenic *E. coli* B-subunit heat-labile toxin (LTB) antigen	Antigen	*N. tabacum* *Solanum lycopersicum* *Petunia parodii*	Shake flasks	[121]
Human tissue plasminogen activator (t-PA)	Thrombolytic protein that converts plasminogen into plasmin	Oriental Melon *(Cucumis melo)*	Shake flasks	[122] [123]

the endoplasmic reticulum retention sequence KDEL (Lys-Asp-Glu-Leu) to the C-terminal end of the 14D9 antibody increases accumulation levels in tobacco HR [109]. In contrast, the presence of a leader peptide, which directs the recombinant protein to the secretory pathway, may be useful in another system. For example, overexpression of a carrot ADP-ribosylation factor gene (*arf-001*) in *A. belladonna* HRs resulted in an evident enhancement of protein secretion to the medium [116].

2.5.1.4. Use of Protein-Stabilizing or Permeabilizing Agents

One of the most promising methods for retaining foreign proteins secreted into plant culture medium is the use

of protein-stabilizing agents, such as PVP and gelatin [126]. If the protein remains attached to biomass, permeabilizing agents (such as DMSO) could be used [101].

At the present, the production of a human therapeutic enzyme that has gone all the way through production and regulatory hurdles derives from carrot suspension cells, which is expected to reach soon commercial development. In the future, several other products from contained systems are expected to reach the clinical trial stage [127].

3. APPLICATION OF HRs IN PHYTOREMEDIATION

The use of the natural ability of plants to absorb and accumulate heavy metals or to transform toxic organic molecules to harmless forms enzymatically has gained increasing attention in recent years, as a cost effective and environmentally-friendly alternative, giving rise to the phytoremediation concept [128-130]. Since roots are the primary contact between plant tissues and contaminants in the soil or water, they provide a key point for assessment of the phytoremediation potential of a particular plant species. In this sense, aseptic *in vitro* cultures such as HRs, have proved to be a very useful tool and a suitable model system to study xenobiotic detoxification and the activity of central detoxification enzymes, without the interference of soil matrix and microbes. These cultures proved to be adequate systems for phytoremediation studies, including their several strategies of action, such as phytoextraction, phytostabilization, phytodegradation as well as rhyzofiltration of inorganic and organic pollutants, as it was pointed out before. HRs offer the important advantages of greater genotypic and phenotypic stability than dedifferentiated cultures, thus providing a more reliable and reproducible experimental system over time, for phytoremediation purposes [131]. It is well known that HRs are able to metabolize *per se* hazardous compounds by common metabolic pathways [132]. Furthermore, the organized nature of HR cultures provides an added advantage, making them more amenable for cultivation in bioreactors to study the process in large-scale [133].

Many investigations have demonstrated that HRs derived from different plant species could be used for the treatment of several organic contaminants, mainly polychlorinated biphenyls/dioxins (PCBs) and phenolics. In this way, Morita and co-workers [134] proposed a remediation process for a medium contaminated with PCBs, through utilization of plants' capability of absorbing, and/or decomposing PCBs or dioxins. The authors reached the inventive concept of decontaminating soil, lakes or marsh polluted with PCBs or dioxins through utilization of the abundantly growing HR or regenerated plants derived from them, thereby reducing the concentration of these xenobiotics in the contaminated soil or water. On the basis of this idea, they conducted various experiments and achieved a successful invention, because the method was not only efficient but also enables low cost treatment. As a result of their work, they found that *A. belladonna* HR were able to absorb and decompose a considerably large amount of PCBs, as well as large amounts of dioxins compared to the roots of natural *A. belladonna*. In addition, HRs derived from plants belonging to Cruciferae and Umbelliferae absorbed and decomposed large amounts of PCBs as compared to the roots of the natural counterparts of these plants. Moreover, *A. belladonna* plants regenerated from HRs were planted in the soil and ninety days later, when roots were found to have sufficiently grown inside the pot, it was observed that these plants absorbed a considerably large amount of dioxins as compared to those of natural *A. belladonna* [134]. More recently, Rezek and co-workers [135] used black nightshade (*Solanum nigrum*) HR culture SNC-9O in order to study the first step detoxification products of a wide range of PCB congeners (twelve dichlorinated, seven trichlorinated, five tetrachlorinated and one pentachlorinated). Metabolites were identified as parent PCB monohydroxylated in various positions based on their mass spectra characteristics. Free non-conjugated metabolites were detected in plant cell biomass. The number of metabolites decreases with an increasing number of chlorine atoms per molecule of PCB. The authors concluded that each plant species, even plant tissue, can have different potential for metabolising various PCB congeners and can produce different metabolites.

HRs have been also used to test the ability of plants to tolerate high levels of phenols [136]. Phenols are among the major organic contaminants found in effluents of coal conversion processes, coke ovens, petroleum refineries, manufacturing of phenolic resins, herbicides, fiberglass and petrochemicals. Phenolic contaminants can also be introduced to the environment via pesticide applications and as a result of partial degradation of aromatic organic contaminants and they pose a threat to human health [83, 84]. HRs from *B. juncea*, *B. napus*, *S. lycopersicun* and *N. tabacum* were successfully used to remove phenol and/or 2,4-DCP from aqueous solutions [81, 83, 84, 137-139]. Moreover, HRs could be re-used for the treatment of solutions containing phenolics, in several consecutive cycles, with high efficiency [84, 139]. To a greater extent, the ability of plants to metabolize contaminants will depend on the biochemical characteristics of metabolizing enzymes and other protective mechanisms that may prolong tissue survival. In this sense, HR cultures also supply valuable information about Px isoenzymes involved in the removal of phenolic compounds. In addition, HRs could be used not only to study phenolics transformation but the nature and the compartamentalization of some of the final products [139].

Nowadays, there is a vast literature describing a wide variety of organic compounds that can be removed by HR derived from different plant species, as it is shown in Table **4**.

Regarding the exploitation of plants tissues to remove inorganic contaminants, like heavy metals from polluted soils and water, hyperaccumulator species are of particular interest for phytoremediation, as they are capable of taking up and storing high concentrations of heavy metals without experiencing toxic effects. The biochemical and physiological mechanisms of hyperaccumulation and the strategies used by hyperaccumulators to tolerate high metal concentrations are not fully understood. However, it is well known that the uptake of metals and their distribution in plant tissues are both important aspects governing the capacity of plants to remove heavy metals. In this sense, HRs have demonstrated that they can be used for screening a wide variety of plant species for their capacity to extract and sequester

Table 4. Phytoremediation of Several Environmental Pollutants by Different HR Cultures

Plant Tissue Culture	Plant Species	Pollutant	Ref
HRs	*Thlaspi caerulescens*	Cadmium	[140]
HRs	*Atropa belladona*	TCE	[141]
HRs	*Brassica napus*	2,4-DCP	[83]
Transgenic HRs	*Solanum lycopersicon*	Phenol	[66]
HRs	*Solanum lycopersicon*	Phenol	[81]
HRs	*Brassica juncea*	Phenol	[137]
HRs	*Daucus carota* L. *Ipomoea batatas* L. *Solanum aviculare*	Phenol and chloroderivatives	[142]
HRs	*Solanum nigrum*	Zinc	[143]
HRs	*Solanum nigrum*	PCBs	[135]
HRs	*Helianthus annuus*	Tetracycline Oxytetracycline	[144]
HRs	*Alyssum murale*	Nickel	[145]
HRs	*Brassica napus*	Phenol	[84]
Transgenic HRs	*Nicotiana tabacum*	Phenol	[138]
HRs	*Nicotiana tabacum*	2,4-DCP	[139]
HRs	*Armoracia rusticana*	Uranium	[146]

metals and radionucleides [140, 147]. Roots of many hydroponically grown terrestrial plants, i.e. Indian mustard, sunflower and several grasses have been used to remove toxic metals such as Cu, Cr, Ni, Pb and Zn from aqueous solutions by a process known as rhizofiltration. Based on this strategy, Soudek and co-workers [148] patented a method for the removal of uranium (U) and its decay products from water by a root system of wetland plants, such as reedmace, rush, timothy grass and bulrush. This invention is related with a root purification plant [148]. Moreover, HRs, due to their highly branched nature have a large surface area in comparison with control roots and can also be used for rhizofiltration purposes. For instance, *B. juncea* and *Chenopodium amaranticolor* HRs were applied for U removal from a solution with a concentration up to 5000 μM, in a short period of incubation. In addition, Soudek and co-workers [146] described the ability of *Armoracia rusticana* HRs to accumulate U and found that the presence of phosphate has a stimulating effect on both growth of culture and accumulation of this pollutant. As a result, more than 98% of U disappeared from the medium (initial concentration 500 μM U) in presence of phosphates compared to 86% of U removal from the medium without phosphates. Besides, Straczek and co-workers [149] proposed a test to determine the threshold toxicity of U using HRs of carrot, which were grown in a gel medium. The experimental design allowed displaying the same kind of diffusion-control of U furniture in solution as in soil but in standardized and reproducible conditions. Besides it permited a sufficient time for both exposure and non-destructive growth observations and to study U distribution in roots. This *in vitro* device seems to be appropriate to study toxicity of U to plant roots in optimal conditions of both exposure and observations and is recommended to examine further physiological processes (effect on stress enzymes, on genetic material, lipid peroxidation, etc.) and the influence of microorganism interactions, because HRs play an important role of providing optimum conditions for root colonizing bacteria.

HRs have also been applied for determining the responses of plant tissues to toxic heavy metals. Some recent publications described the capacity of HR cultures to hyperaccumulate heavy metals such as Cd and Ni, allowing practical examination of the biological mechanisms responsible for high heavy metal tolerance in hyperaccumulator plant species [150]. In the last years numerous patents have been granted, by Companies and individuals, for plants showing tolerance and accumulation of inorganic contaminants, particularly As, Zn, Cd, Ni, Pb, Cu and Hg. For instance, Tamura and co-workers [151] provided a method of cleaning a contaminated soil, containing heavy metals, using a plant of Polygonaceae family, *Fagopyrum* genus, and, at the same time, proposed the addition of a biodegradable chelating agent in order to assist the absorption. Besides, Laplaze and co-workers [152] found that *Cistus libanotis* has a high ability to specifically accumulate lead. Thus, they proposed the use of *C. libanotis* for lead phytoextraction as well as for lead phytostabilization. Furthermore, this species presents the great advantage of being perennial and it has little chance to enter the food chain, since the plant is usually not ingested

by animals. However, to the best of our knowledge, despite HRs interest and importance in phytoremediation research and their potential utility, most of the studies performed with this type of *in vitro* cultures have not been addressed in patents. In spite of this fact, it is important to point out that results derived from *in vitro* HR cultures can be very useful to predict the responses of plants to environmental contaminants, and to improve the design and reduce the cost of subsequent conventional whole plant experiments.

As mentioned in section 2.2., for improving phytoremediation processes, transgenic plants can be developed by transferring genes for transport, multistep metabolic pathways, and sequestration. Genes involved in pollutant´s degradation can be isolated from bacteria, fungi, animals or plants and introduced into candidate plants. The efficacy of either increased *in planta* metabolic activity or *ex planta* enzymatic secretions may be maximized by the root-specific expression of the transgenes [153]. For instance, Wevar Oller and co-workers [66] developed a transgenic tomato (*S. lycopersicum* cv. Pera) that overexpressed *tpx1*, a native Px, resulting in higher peroxidase activity. In particular, one clone of these HR cultures removed phenol from hydroponics with higher efficiency than wild type ones. Several experiments have also demonstrated the ability of transgenic plants and HR cultures derived from them to degrade environmental contaminants that are either recalcitrant, or poorly degraded by native plant enzymatic systems. Methods for using genetically-transformed plants in the phytoremediation of Pb are described and patented [154]. These methods of phytoremediation will provide a cheap, simple and efficient alternative in the removal of contaminating Pb from oil/water/environment by ACBP-overexpressing genetically-transformed plants growing in contaminated environment. Moreover, genetically modified plants which were able to accumulate heavy metals, such as Zn, Co, Cd or Pb and to translocate them to the shoots were described and patented [155], as well as plants with resistance to metals and metalloids [156]. Recently, a method to confer formaldehyde-resistance to a plant was provided, by expressing genes in the chloroplast encoding hexulose-6-phosphate synthase and 6-phosphohexulose isomerase. Thus, a transgenic plant having a pathway to assimilate formaldehyde through the Calvin cycle was patented [157].

As was described above, phytoremediation has been the subject of many scientific publications and patents in the past decade. However, few studies deal with patents about the use of HR derived from them. With the current knowledge and a better understanding of the potentialities of HR cultures more efficient phytoremediation strategies could be designed, giving new perspectives and sustainable alternatives for environmental remediation, and therefore, new and promising patents would be expected.

4. RECENT ADVANCES IN THE SCALE UP ON HR CULTURES: DEVELOPMENT OF INNOVATIVE BIOREACTORS

Besides *in vitro* production of important metabolites in HR cultures has been reported from various plant species, bioreactors are the key step towards commercial production by plant biotechnology. Non-homogenous growth and highly branched root phenotypes present major challenges to scaled-up process of HR cultures in bioreactors. Moreover doubling time is also a very important factor that must be considered. For instance, potato *(Solanum tuberosum)* HR expressing hepatitis B surface antigen growing in shake flasks exhibited a doubling time (*dt*) of 2.32 days [158], which is interesting when compared with *N. benthamiana* and rice cells expressing alpha-1-antitrypsin in a stirred tank bioreactor have *dt* of 3 days [159] and 2 days [160], respectively.

Two important bioreactor types used for growing HR are (1) airlift bioreactors that release compressed air from the base of a liquid culture vessel, providing aeration and agitation as the air moves up through the root bed and (2) mist reactors. Most of the work examining HR growth in bioreactors has focused on phytochemical production; however, bioreactors optimized for this purpose are not directly applicable to recombinant protein production due to the susceptibility of proteins to degradation by proteases and their increased sensitivity to shear stresses. Bioreactors based on the airlift concept has been used for the scaling-up of several HR cultures, such as *B. vulgaris* HR (bubble column bioreactor) [161], *Artemisia annua* HR [162] and *Astragalus membranaceus* HR [163], among others. Similarly, airlift mesh-draught with wire-helixes bioreactor was used succesfully with *Solanum chrysotrichum* HR [164]. The mist reactor system comes from the propensity of HR to hang to a mesh support. This system offers advantages since it reduces the volume of culture medium so secreted metabolites are more concentrated. It has been used with *Tagetes patula* L. [165] and by the ROOTec Company. There is a detailed description of the system in Towler and co-workers [166]. In 2009 Shiau [167] patented a new mist type bioreactor for growing fungus, plant cell, tissue, organ, HRs and plantlet, where mists of the nutrient solution is produced by a mist generator and delivered by a mist delivery pipe which has a mist-exit port adjusted to a desired level above the culture medium so as to discharge and spray the mists over the explants.

Several studies that investigate different bioreactors have been carried out with the aim of optimizing protein production in HRs. Comparing transgenic tobacco HR grown in shake flasks and scalable airlift and mist reactors, it was found that the mist reactor worked better than the airlift reactor and produced the best yields of any mist reactor HR culture-produced protein [118].

Current progress made in the scale up of the HR cultures has paved the way for industrial exploitation of this system. In addition to bioreactor optimization for enhanced nutrient delivery, media formulations, culture conditions, and overall viability of root beds in a high-density culture environment must continue to be improved upon. Cross-disciplinary collaborations among plant biologists, bioengineers, and mathematicians (bioinformaticians) are desirable and will certainly strengthen the process of bioreactor development for HR cultures.

5. OTHERS PROLIFIC APPLICATIONS FOR HRs

As it was described, there is a multiplicity of applications for HRs related with secondary metabolites, enzymes and recombinant protein production as well as for phytoremedia-

tion purposes, aspects that have been deeply detailed in the present review along with the new patents registered in these topics. However, this plant system has been used with other purposes such as molecular breeding and other basic studies of root physiology and biosynthetic pathway elucidation, among others. Ono and Tian [5] in its review showed many interesting examples, in particular about the use of HRs as a tool for dissecting plant secondary metabolism through gene discovery, function determination and biochemical pathway elucidation. It was not our objective to point out all these applications, but it should not be omitted that most of the new patents (2008-2011) involving HRs are related with basic techniques of genetic engineering, which consider the use of *A. rhizogenes* and its possible variants as a vector for plant transformation with different applications. In this sense, Mankin and co-workers [168] patented an invention related with the proposal of "*disarmed*" strain variants of *Agrobacterium* strain K599, "*disarmed*" plasmid variants of the Ri-plasmid pRi2659, and derivatives thereof, as well as methods employing these strains and plasmids in plant transformation. In a similar way, Gilbertson and Ye [169] were interested in improving the efficiency of *Agrobacterium*-mediated plant cell transformation by the use of additional transformation enhancer sequences from a Ri plasmid of *A. rhizogenes* linked to a T-DNA border sequence. This strategy was patented by the authors. For the transformation of soybean a disarmed *A. rhizogenes* K599 strain, the methods for the incorporation of DNA into the genome and subsequent regeneration of the transformed cells into a whole plant was patented by Olhoft and co-workers [170]. Many other examples show the use of *A. rhizogenes* as mediator of transformations and hence HRs, and the reason of that became from the multiple advantages of this system. Whole viable plants can be regenerated from HRs, with the advantage that they are genetically stable, since shoot regeneration is obtained avoiding the callus phase and somaclonal variations, typical from *A. tumefaciens* mediated transformation [2]. Moreover, Ri plasmid-based gene transfer also has a high rate of transformation and regeneration of transgenic plants and they can be obtained without a selection compound thereby avoiding the use of chemicals that inhibit shoot regeneration. In addition, the plants regenerated from HRs frequently show rapid growth and leaf development as well as increased lateral bud formation, thus regenerants are useful for micropropagation of plants that are difficult to multiply or those which have long generation cycles, as trees [171]. *A. rhizogenes* mediated transformation also become as an alternative to introduce specific genes in trees since classical breeding programs are slow and tedious. In addition, this transformation strategy has also been used to improve valuable ornamental characteristics of geraniums, such as a tighter, more globular shape, unwrinkled leaves which are shorter in length and more deeply lobed, a lack of a trailing growth habit and increased fragrance [172].

Even though the following is a patent from 2006, we want to mention it here because of it curious strategy, in which *A. rhizogenes* mediated transformation appear as part of the invention [173]. This patent postulated a method for obtaining transgenic plants through *A. rhizogenes* with expression of a protein (oxalate oxidase) with H_2O_2 producing activity, which allowed selecting the transformants by a simple and rapid peroxidase-based colorimetric test. Although the possibilities of a gene transmission from transgenic plants to soil bacteria have not been demonstrated, the use of antibiotic resistance genes is very badly perceived by some authors [174]. Thus, this patented technique looks for avoiding the use of antibiotics and/or herbicides as selection markers of transgenic plants. This novel system which allows an immediate visualization on the root is particularly advantageous in the case where the gene of interest is expressed at a late stage of development, or in a vegetative organ other than that on which the selection is made or else if it is difficult to demonstrate.

In addition, *A. rhizogenes* mediated transformation has been used for the establishment of "*composite plants*", which term derives from the fact that transformed roots are induced on a whole non transformed plant [175, 176]. These methods have been shown to work efficiently for many different species of plants including several that are recalcitrant to transformation, and it has been used as a rapid tool to test gene and promoter functions in the context of a complete plant, although the transgenic trait cannot be transmitted to the progeny. Recently, composite plants have been useful for basic studies of gene function in *Prunus* [177] as well as for studies related with symbiotic associations of roots with *Frankia* [178] or rhizobacteria [179]. Even though some new application for composite plants are shown, the strategy was patented a long time ago by Strobel [180] in which case the inventor expose the beneficial growth of roots in dicotyledonous plants achieved by genetic transformation with root inducing *A. rhizogenes* ATCC 39207. More recently, Taylor and Huang [181] patented a new method of screening genetic elements of interest for functionality, more particularly that utilizing *A. rhizogenes* to transform plant tissue forming a chimeric plant expressing or containing the genetic element of interest in transgenic HR tissue.

It is well known that HRs have been used as vehicle for continuous propagation of mycorrhizes and spores [182]. In this sense, Hua and co-workers [183] patented an *in vitro* system using *Agrobacterium* transformed dicotyledonous roots (such as carrot roots) for the production of viable and aseptic spores of a vesicular arbuscular mycorrhizal fungus (*Glomus intradices*). More recently, Imanishi and co-workers [184] performed an efficient genetic transformation protocol for *Discaria trinervis* based on *A. rhizogenes* to decipher the molecular mechanisms of actinorhizal symbiosis with *Frankia* bacteria. The authors obtained composite plants with transgenic roots expressing green fluorescent protein and MtEnod11 promoter, a gene from *Medicago truncatula* widely used as a marker for early infection-related symbiotic events in model legumes, which constituted a valuable tool for studying actinorhizal symbiosis in the context of intercellular infection.

As it was shown in this review, HR cultures have multiple applications and we think that all HR potential and that of the main *"player", A. rhizogenes,* have not yet been exploited.

6. CURRENT AND FUTURE DEVELOPMENTS

Commercial exploitation of plant cell cultures and, in particular, HRs has gained importance in the last years. This

type of plant cultures constitutes an interesting system efficiently used for production of secondary metabolites, enzymes and recombinant proteins, as well as for phytoremediation purposes, as it was deeply described in the present review. Furthermore, the potential of HRs is continuously increasing since different biotechnological strategies such as genetic engineering, elicitation and application of metabolic traps are currently being explored. Although HRs have been mainly assumed as an alternative system for increasing biosynthesis and/or secretion of commercially valuable metabolites, they have also been applied to elucidation of metabolic pathways and studies related with microorganism-root interactions. Recent patents are mainly related with secondary metabolite production and purification, while few of them are related with phytoremediation, enzyme and recombinant protein production. Besides highlighting the patented advances in HR applications, our aim was to emphasize on recent research efforts through which this *green* technology might be expected to develop into a commercially competitive alternative to other methods. This is one of the biggest challenges and we think that with further exploration of inexpensive novel elicitors and bioreactor design, HRs will increase yields and reduce production costs, which will allow promptly its industrial implementation. Advances in plant transcriptomics and metabolomics joined with modeling of metabolic fluxes through *in silico* approaches and genetic engineering will allow HRs to become a powerful and sustainable phytochemical production system.

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