Vikas Srivastava · Shakti Mehrotra
Sonal Mishra *Editors*

Hairy Roots

An Effective Tool of Plant Biotechnology

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This book is dedicated to
Late Dr. Arun Kumar Kukreja
(Ex-Chief Scientist, CSIR-Central Institute of Medicinal and Aromatic Plants, India)

Preface

The escalating industrial magnitude of plant-based chemicals has resulted great interest in the evolution of methods to meet their desired production. These phytochemicals are widely used in cosmetics, flavor and fragrance, dyes and pigments, food additives, and insecticide/pesticide industries as a whole or as an important ingredient in various formulations. Out of various conventional and unconventional strategies that have been applied for the production of these phytochemicals, the development of *Agrobacterium rhizogenes*-mediated hairy root cultures (HRCs) is considered as the most practically feasible approach. The HRCs are usually stable in their biosynthetic potential and, thus, offer a sustainable production system for desired metabolites. Additionally, several proof-of-principle experiments have also revealed the practical feasibility of HRCs in plant-based remediation of environment pollutants, biotransformation of important compounds, and production of therapeutic proteins. It is pertinent to mention here that perspectives in the upscaling of hairy root cultures also offer ceaseless opportunities in various objectives. Nevertheless, an easy to establish and maintain, economic, renewable and above all, the sustainability of HRCs justify the attention of the global scientific community.

At this juncture, where hairy root biotechnology is recognized as most sought-after and very dynamic research area, it is relevant to get judicious update in recent advances along with hitherto biotechnological progress of the subject. Thus, considering HRCs as a multifaceted biological tool for various applications, the present book entitled *Hairy Roots: An Effective Tool of Plant Biotechnology* has been designed. The editorial team members (**Vikas Srivastava; Shakti Mehrotra; Sonal Mishra**) have been working on various aspects of hairy root research since long time and published many articles in journals/books of international repute. The present book provides the details of conceptual as well as pragmatic information of HRCs-based research along with relevant case studies. Furthermore, an attempt has also been made to investigate the loopholes in existing methodologies and challenges and to find out possible solutions through scientific discussions from various eminent research groups working on hairy root biotechnology. The book has been

framed on the basis of three major areas and thus presents three broad parts as (i) Hairy Roots and Secondary Metabolism, (ii) Progressive Applications, and (iii) Novel Approaches and Future Prospects.

The first part (**Hairy Roots and Secondary Metabolism**) comprised of seven chapters that deals with comprehensive discussion about hitherto gradual progression of hairy root research from a simple biotechnological tool to mimic the natural phenomenon of bacterial gene transfer and occurrence of disease syndrome to a most preferred and dynamic technology for secondary metabolite production and other value-added applications. This part deals with the discovery of nature's own genetic engineer *A. rhizogenes*, its journey since then, and its successful exploitation in various fields of biotechnology and related prospects. Further discussion includes the regeneration of pRi-transformed plants, various types of HRCs-mediated secondary metabolite production, and impact of various extrinsic factors over HRCs-mediated secondary metabolite production. This part also provides an inclusive account on biotechnological interventions in HRCs of tropane and terpene alkaloid-bearing plants. The part culminates with the description of the design and development of bioreactors to achieve maximum productivity in plant cell and hairy root cultivations.

The second part (**Progressive Applications**) comprised of five chapters that provide an inclusive account on further advancement of HRCs research. This part offers vivid account on the capacity of HRCs for the biotransformation of a variety of substrates for value addition and its utility as a potential system for the production of imperative biopharmaceuticals. Further the competence of HRCs for the remediation of the environment and its utilization to study signaling pathways during nodule formation has also been incorporated. Lastly, this part also highlights the methodologies developed to generate composite plants and the applications of co-transformed hairy roots for studying gene function.

The third part (**Novel Approaches and Future Prospects**) comprised of three chapters and includes current attention on HRCs research. Here, the exploration of transcriptome sequencing in HRCs of medicinal plants and in silico perspective of HRCs growth monitoring and modeling have been presented. Finally, the utility of CRISPR/Cas9-mediated editing will offer new directions for HRCs metabolic engineering. This edited book is an attempt to ensure the research and teaching community, about the major progress in HRCs-based interventions in plant biology and applications thereof. Besides, the emerging thrust that still needs time to grow will also be considered to project the prospect trajectory of HRCs research. The book will surely provide endless opportunities in the ongoing and future research in this fascinating area.

Samba, India — Vikas Srivastava
Lucknow, India — Shakti Mehrotra
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Part I
Hairy Roots and Secondary Metabolism

Chapter 1
Progress and Prospects of Hairy Root Research

Suvi T. Häkkinen and Kirsi-Marja Oksman-Caldentey

Abstract Nature's own genetic engineer *Agrobacterium rhizogenes* was discovered more than 40 years ago, and an increasing number of publications on the use of hairy roots in biotechnology have been published since – with more than 85% of all the publications during the past 15 years. Hairy roots have been successfully exploited in various fields in biotechnology, including secondary metabolite research, recombinant protein production, and bioremediation, to mention a few. In the following chapter, we will deal with the current state of the art of hairy root research starting from evolutionary facets of hairy root generation and host-bacteria association to a range of applications where hairy roots are efficiently exploited.

Keywords Hairy root · History · Applications · Secondary metabolites · Bioreactor

1.1 Hairy Roots: Where It All Started from?

Already as early as in the late 1950s, Dr. Armin Braun from The Rockefeller University first demonstrated that tumor cells in plants are transformed, i.e., they can be freed from *Agrobacteria* – a gram-negative soil bacteria – and grown in vitro without the supplemental auxin and cytokinin required by normal plant cells in vitro (Braun 1958). Later, metabolites called octopine and nopaline were discovered from tumor cells (Petit et al. 1970). Indirect genetic evidence that *Agrobacterium* might carry a virus or plasmid with tumor-inducing genes emerged from two kinds of experiments (Hamilton and Fall 1971; Kerr 1971). It was discovered that tumor-inducing trait is recuperated in bacteria after the loss of virulence, indicating that the trait would be plasmid- or virus-borne. Simultaneously, existence of an extrachromosomal element was indicated via experiments showing transfer of virulence by

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Kerr and co-workers (1971). The exciting journey which eventually led to gene transfer of plants by *Agrobacterium* had an important step when *Ti* mega-plasmid was discovered in Ghent (Zaenen et al. 1974). Virulence of the *Agrobacterium* was located in *vir*-region (Stachel et al. 1985). The genetic engineering of plant cells was finally simultaneously accomplished by four independent research groups (Herrera-Estrella et al. 1983; Bevan et al. 1983; Fraley et al. 1983; Murai et al. 1983), which eventually made history for molecular biology and plant sciences.

The term "hairy root" dates back to 1900 when it was first associated with diseased fruit crops. Back then, hairy root syndrome, affecting mainly dicotyledonous plants, caused substantial losses in vineyards, orchards, and vegetable nurseries (Georgiev et al. 2012). Investigations revealed that causative agents for this disease were phytopathogenic *Agrobacterium rhizogenes* strains carrying an *Ri* plasmid (root-inducing plasmid) (reviewed by Sinkar et al. 1987), which displayed high resemblance to *Ti* plasmid carried by *A. tumefaciens*. While the latter causes the formation of crown gall tumor tissues in infected plants, *A. rhizogenes* induces the hairy root disease (Chilton et al. 1982). All strains of *A. rhizogenes* are known to produce opines of agrocinopine group and all or a few opines of the agropine group. The strains which produce all the agropine-type opines (agropine, mannopine, agropinic acid, and mannopinic acid) are known as the agropine-type strains, whereas the strains which produce all agropine-type opines excluding agropine are known as the mannopine-type strains (Petit et al. 1983) (Willmitzer et al. 1983). *Ri* plasmid of the mannopine strain 8196 contains only one T-DNA (Hansen et al. 1991), while two T-DNA regions have been identified in agropine *Ri* plasmids, which are separated by a 15 kb nontransferred region. The right T-DNA contains the regions similar to *Ti* plasmid, including *tms1* and *tms2*, which are responsible for the auxin biosynthesis (Inzé et al. 1984). The left T-DNA, however, does not possess close resemblance to any sequences with *Ti* plasmids (Huffman et al. 1984). Interestingly, while *virE1* and *virE2* genes are important for T-DNA transfer in *Ti* plasmids, they are not found in *Ri* plasmids (Moriguchi et al. 2001). Hairy roots induced by agropine strains frequently contain only the TL-DNA (Jouanin et al. 1987). However, in some cases, the information carried on the TL-DNA is not sufficient, and the presence of the TR-DNA greatly extends the host range of the infection. Sequence analysis has identified 18 open reading frames (ORFs) on the TL-DNA of pRiA4, and 8 of those *loci* were shown to affect the root formation, denoted as *rolA*, *rolB*, *rolC*, and *rolD* (Slightom et al. 1986). While mutants induced to *rolA*, *rolC*, and *rolD* resulted in attenuated growth or altered phenotype, mutants in *rolB* were totally avirulent confirming the very crucial role of this gene in hairy root formation (Spena et al. 1987). Furthermore, when *rolB* is introduced into the host plant genome as a single gene, it is capable of hairy root induction (Altamura 2004). Diverse and also synergistic effects of individual *rol* genes were shown by Palazón and co-workers (Palazón et al. 1997) who reported differential effects of these genes in tobacco hairy root growth and alkaloid production. Hairy roots easily regenerate into whole plants and transmit their *Ri* T-DNA into next progeny

(Oksman-Caldentey et al. 1991). Such plants display a significantly altered phenotype (reviewed in Nilsson and Olsson 1997).

Initially it was thought that monocotyledonous plants are insensitive to *Agrobacterium*-mediated gene transfer. Various molecular mechanisms for transformation resistance in monocotyledonous plants were suggested, including production of antimicrobial compounds (Sahi et al. 1990), a lack of *vir* gene inducers (Usami et al. 1987), inefficient T-DNA integration (Narasimhulu et al. 1996), and programmed cell death induced by *Agrobacterium* (Hansen 2000). A significant breakthrough occurred in 1993–1994, when highly regenerable explants of rice, immature embryos, or calli derived from mature seeds were inoculated with disarmed *Agrobacterium* harboring plant selectable marker genes resulting in fertile transgenic rice plants (Chan et al. 1993; Hiei et al. 1994). Transformation frequencies of monocotyledonous plants were improved by applying different selection markers (Negrotto et al. 2000), by modification of medium components, by optimization of co-culture and resting time periods, and by addition of *Agrobacterium* growth-inhibiting agent or bacteriocide such as silver nitrate (Zhao et al. 2001; Zhang et al. 2003). Spurred on by the success of Hiei and colleagues, there was significant interest in transforming other agronomically important crop species, such as barley and wheat. By the use of "super-virulent" *A. tumefaciens* strains and/or acetosyringone, a phenolic compound inducing expression of *vir* genes on the *Ti* plasmid, transformation via *A. tumefaciens* has become a major method also in monocots. Various factors have been identified of being important for successful transformation of monocotyledonous plants, as reviewed by Cheng et al. (2004). These include plant genotype, explant, *Agrobacterium* strain, pretreatment, and chosen selectable marker. However, there are also very few examples of successful hairy root transformation of monocotyledonous plants. Of monocotyledonous plants, onion and asparagus have been reported to be susceptible to *A. rhizogenes* transformation (Dommisse et al. 1990; Christey 1997). Maize hairy roots were recently generated offering platform for studying host-parasite interactions (Runo et al. 2012). Problems associated with difficulties of *Agrobacterium* (*tumefaciens*) transformation in monocots are reviewed by Sood and co-workers (Sood et al. 2011).

1.2 Characteristics of Hairy Roots

A. rhizogenes infects wounded plant cells because of the production of phenolic compounds that attract *A. rhizogenes*. Bacteria move to the wound site by chemotaxis. Subsequent infection at wound site followed by integration of *Agrobacterium*-derived T-DNA into the plant genome results in development of hairy root disease. Hairy root disease is characterized by high growth rate, a high degree of lateral branching, profusion of root hairs, lack of geotropism, and the tissue maintaining a highly differentiated and functional root organ (Tepfer 1984; Sevón and Oksman-Caldentey 2002). Hairy roots offer an attractive alternative for the production of a

range of high-value secondary compounds for various biotechnologically important reasons. Hairy roots are able to accumulate, e.g., the same alkaloids as the parent plant, even in higher quantities than the intact plants or undifferentiated cell cultures (Sevón and Oksman-Caldentey 2002; Ramachandra Rao and Ravishankar 2002; Akhgari et al. 2015).They gain biomass rather rapidly and have simple cultivation medium requirements, being able to grow without phytohormones. They also show high genetic stability as well as more stable metabolic production than that of undifferentiated cell cultures (Peebles et al. 2009; Häkkinen et al. 2016). This has largely been related to chromosomic stability displayed by the hairy roots (Weber et al. 2008, 2010; Dehghan et al. 2012). The chromosomic number and karyotype of hairy roots are typically the same as in the parent plant. In addition, the ability of hairy roots to grow without additional auxins increases the stability, since when exposed to growth regulators, even organized tissues modify their chromosomic numbers and display somaclonal variation (Baíza and co-workers, Baíza et al. 1999). *Catharanthus roseus* hairy roots displayed genetic and metabolic stability during a 5-year study (Peebles et al. 2009). Similarly Maldonado-Mendoza and co-workers (Maldonado-Mendoza et al. 1993) analyzed the tropane alkaloid production of hairy roots of *Datura stramonium* during 5 years and reported growth rates and alkaloid contents to be stable. In our recent study, hairy roots of *Hyoscyamus muticus* showed genetic and metabolic stability during continuous subculturing in the laboratory during 16-year follow-up (Häkkinen et al. 2016). Hitherto, this is the longest time period reported for continuous subculturing of hairy roots. Very similar results were reported by Sun and co-workers (Sun et al. 2017), with *C. roseus* hairy roots expressing anthranilate synthase. The stability was proven after 11 years of continuous subculturing. On the other hand, also contradictory findings related to high stability of hairy roots have been reported. Hairy roots of *Daucus carota* showed unstable phenotype and unstable transgene expression during a 2-year follow-up (Guivarc'h et al. 1999). Also, unstable production of tropane alkaloids in hairy roots of *Scopolia japonica* was reported, although the follow-up was rather short, 2 months (Mano et al. 1986), and usually adaptation to culture conditions requires time. Taken together, hairy roots have shown a great potential for viable industrial applications due to their high genetic and metabolic stability which surpasses that of undifferentiated cultures (Figs. 1.1 and 1.2).

1.3 Applications of Hairy Root Platform

The main applications of hairy root cultures include the biotransformation, production of high-value plant metabolites, phytoremediation, and production of artificial seeds (Georgiev et al. 2012; Guillon et al. 2006). Some of these examples are discussed further below. A number of studies related to biochemical research especially around plant secondary metabolism have been performed exploiting hairy roots. Alkaloids are compounds which are typically highly bioactive and are

Fig. 1.1 Hairy roots of *Catharanthus roseus* emerging from the wound site

Fig. 1.2 Hairy roots of *Hyoscyamus muticus* cultivated on solid and liquid medium

produced approximately in 20% of all plant species. For their interesting applications, the biosynthesis research related to alkaloids has been active, with hairy roots having an important role as research tools. Such examples are given plenty, as comprehensively listed in review by Giri and Narasu (2000). In the following section, examples of application of hairy root platform in the field of alkaloid research are described in more detail.

Tropane alkaloids are a class of alkaloids many of which are pharmaceutically interesting for their anticholinergic activities. The biosynthetic pathway of tropane alkaloids starts from amino acids arginine and ornithine and on the other hand from phenylalanine. The pathway leading to active pharmaceuticals hyoscyamine and scopolamine is rather well described. Perhaps the most significant finding related to tropane alkaloid research was reported by Hashimoto an co-workers, with isolation and characterization of an enzyme hyoscyamine-6β-hydroxylase (H6H) which converts hyoscyamine into scopolamine in a two-step process (Hashimoto and Yamada 1986) (Matsuda et al. 1991). The gene encoding for H6H has since been overexpressed in various *Solanaceae* plant species (Hashimoto et al. 1993; Parr et al. 1990; Palazón et al. 2003b; Jouhikainen et al. 1999) together with other pathway genes resulting in high accumulation of hyoscyamine and/or scopolamine

(Kang et al. 2011; Rocha et al. 2002). A remarkable yield of scopolamine (411 mg/L) was achieved in hairy root cultures of *Hyoscyamus niger*, by simultaneous overexpression of genes encoding for putrescine methyltransferase and H6H (Zhang et al. 2004). In addition to tropane alkaloid-producing species, overexpression of *h6h* was shown to catalyze the conversion of exogenously applied hyoscyamine into scopolamine in hairy root systems (Häkkinen et al. 2005; Rocha et al. 2002) and even in microbes (Kai et al. 2011; Cardillo et al. 2012). As other notable examples, Robins and co-workers (Robins et al. 1990; Hagan et al. 1999) investigated the tropane alkaloid pathway and revealed the flux regulation and littorine rearrangement pattern in *Datura* hairy roots.

Tobacco alkaloids such as nicotine, nornicotine, and anabasine are synthesized in plant roots where they are transported to plant leaves for storage and for their biological function. These tobacco alkaloids accumulate also in hairy roots, in much higher amounts than in undifferentiated cells (Hamill et al. 1986; Häkkinen et al. 2004). Similar to tropane alkaloids, also tobacco alkaloid pathway is well described except for final steps leading to nicotine and also to other nicotinic acid-derived alkaloids. Tobacco BY-2 cell culture is a widely used plant cell culture for various aspects of plant biochemistry and especially cell cycle research, due to its very high multiplication rate and easy genetic transformation (Nagata et al. 1992). Tobacco BY-2 produces alkaloids after elicitation (Goossens et al. 2003). However, it was unclear why BY-2 cell accumulates anatabine and only small amounts of nicotine after methyl jasmonate elicitation, before Shoji and Hashimoto showed the reason for this to lie in the transcriptional regulation of methyl putrescine oxidase (MPO) (Shoji and Hashimoto 2008). As BY-2 culture does not spontaneously produce alkaloids and as a result constitutive overproduction of alkaloids via genetic engineering might be detrimental to this culture, hairy roots offer an attractive alternative for tobacco pathway engineering (Häkkinen et al. 2007; Lackman et al. 2011). Recently, the biosynthetic pathway of anabasine was further revealed by using hairy root platform with ^{15}N-labelled lysine (Bunsupa et al. 2014). It was interesting to note that no significant labelling was detected in nicotine, anatabine, nor anatalline, indicating that anabasine could be synthesized via nicotinic acid-independent route.

Terpenoids are another group of important secondary compounds with a largest diversity of compound structures and are well known for their many applications in the pharmaceutical, fragrance, and cosmetics industries. Hairy root platform has mainly been exploited with *Catharanthus roseus* for pathway engineering leading to bioactive terpenoid indole alkaloids (TIAs) such as vincristine and vinblastine (Peebles et al. 2011; Hughes et al. 2004). Several TIA pathway genes have been overexpressed in hairy roots including anthranilate synthase holoenzyme (Chung et al. 2007), tryptophan decarboxylase (Hughes et al. 2004), and deacetylvindoline 4-*O*-acetyltransferase (Magnotta et al. 2007). TIA pathway genes have also been expressed in heterologous hosts. As an example, geraniol synthase gene was successfully expressed in tobacco hairy roots resulting in accumulation of geraniol and its glycosides (Vasilev et al. 2014). Engineered hairy roots were also cultivated in larger scale yielding mg amounts of geraniol. Hairy roots of *Cinchona officinalis*

expressing genes encoding for tryptophan decarboxylase and strictosidine synthase yielded high amounts of both tryptamine and strictosidine, as well as quinine and quinidine (Geerlings et al. 1999). However, many examples show that overexpression of a single gene in a specific pathway does not lead to higher accumulation of the desired metabolite, and feedback inhibition is often an encountered problem in metabolic engineering events (Palazón et al. 2008). One problem associated with homologous gene expression or expression of even heterologous genes with high sequence homology with the native genes is co-suppression. In addition, secondary metabolism in plant systems is commonly highly compartmentalized between different cellular organs, and sometimes tissue-specific expression is required, and thus the expression may not be achieved in hairy root systems. Transcription factors (TFs) are promising metabolic engineering targets due to their ability to regulate multiple biosynthetic pathway genes (Memelink and Gantet 2007). The transcription factors regulating TIA biosynthesis include the activators ORCA2, ORCA3, BIS1, BPF1, MYC1, MYC2, and WRKY1 and the repressors JAZ, ZCT1, ZCT2, ZCT3, GBF1, and GBF2 (Zhou and Memelink 2016; Rizvi et al. 2016).

1.4 Advantages and Challenges of Hairy Root Culture Systems

As a plant-based production platform, hairy roots offer several advantages over microbial- or mammalian-based systems (Häkkinen and Ritala 2010). Plant cells exhibit a potential to produce a number of small molecular weight compounds, which some are very difficult or impossible to make via chemical synthesis in an economic way. The risk of endotoxins or oncogenes in the product is nonexisting, while in microbial and mammalian systems, these risk factors should always be considered. Other advantages include the high product homogeneity and easy separation of cells and culture medium for product purification purposes. To date there are some examples of successful production of plant-based natural compound using microbial hosts (Paddon et al. 2013; Galanie et al. 2015), although sometimes the yields have remained rather low. Common problems encountered when trying to transfer the plant-based biochemical pathway to microbes are the availability of precursors; expression and activity of enzymes in prokaryotes, e.g., difficulties associated with expression of cytochrome P450s; and lack of *S*-adenosyl methionine, required in many methylation steps in plant pathways (Khosla and Keasling 2003). A notable study reported by Galanie et al. (2015) showed that the complete biosynthetic pathway of opioids could be reconstructed in yeast; however the final yields remained very low, less than 1 μg/L. While artemisinic acid, a precursor of important antimalarial compound, was successfully produced in yeast after several years of extensive research efforts with very high titers (25 g/L), the final step in the process requires a chemical conversion to reach artemisinin (Paddon et al. 2013).

When it comes to plant-based natural products, cell and tissue cultures, such as hairy roots, offer a viable option for large-scale production due to limitations posed by isolating the compounds from whole plants. Cell and tissue cultures can be cultivated in controlled and contained environment, enabling the optimization efforts for high productivities with possibility to apply GMP (good manufacturing practice). In addition, in whole plants, many plant-derived compounds accumulate in certain plant organs or in specific developmental stage making the yield optimization and production process demanding. Cell culturing enables the use of synthetic growth media, and usually the variation in yields or product quality is low (Häkkinen and Ritala 2010). When it comes to hairy roots, a specific advantage is displayed by their ability to grow relatively fast without growth hormones, reducing the costs deriving from culture medium (Georgiev et al. 2007; Häkkinen et al. 2018). Hairy roots, as other cell culture systems, offer also advantage via reduced costs deriving from product isolation and purification, since unlike whole plants, cell cultures do not possess by-products such as waxes, chlorophyll, oils, or fibers, which often are complicating these processes. However, the choice of the production host and platform should always be made by evaluating the properties of the final product against the total production costs by techno-economic feasibility assessment. It was estimated that the production of a natural product with cell and tissue culture-based host becomes economic when the price of the final product exceeds $500–1000/kg (Sajc et al. 2000). Therefore naturally this system is beneficial for high-value, complex molecules. Nielsen and Keasling estimated that engineering of microbial strains that overproduce a target compound to economically relevant levels takes 6–8 years and over US$50 million, which means much higher numbers for more complex plant cells (Nielsen and Keasling 2016).

Biotransformation has also shown to be viable option for applications with hairy root systems (Banerjee et al. 2012). Perhaps the most often hairy root-catalyzed reaction has been glycosylation, including the reactions leading to digitoxigenin glycosides (Kawaguchi et al. 1990), glycyrrhetinic acid glycosides (Asada et al. 1993), dehydroabietic acid, and phenolic acid glycosides (Fons et al. 1999; Häkkinen et al. 2012). When it comes to high-value commercial compounds, recently we showed that natural raspberry ketone, which is estimated to be the most expensive natural flavor compound after vanillin, was successfully produced in tobacco hairy roots by bioconversion strategy (Häkkinen et al. 2015). Diversity of examples shows that hairy root cultures are entering into a new era of applied research in generating pharmaceutical lead compounds by accomplishing chemical transformations aided through these unique biological systems.

1.5 Bioreactor Design for Hairy Roots

Hairy root morphology sets criteria for bioreactors suitable for cultivation of hairy roots. Tightly packed hairy roots, which are also generally considered as rather shear sensitive, typically form clumps in bioreactors causing mass transfer limitations,

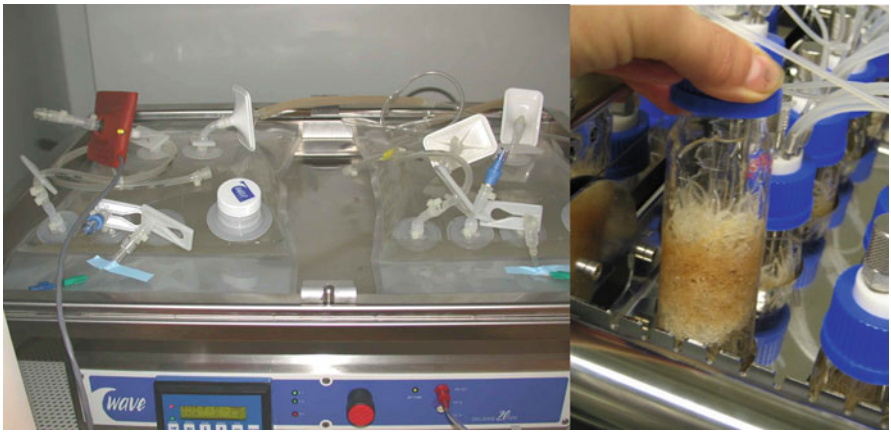

Fig. 1.3 Bioreactors for hairy root cultivation. Wave bioreactor (left) and Medicel Explorer Cultivation Unit (right)

including oxygen and nutrient availability (Georgiev et al. 2007; Eibl and Eibl 2008). Efficient ways to overcome these constraints have been shown by the use of various immobilization techniques, e.g., meshes, cages, or polyurethane foam. Thus, bioreactors with diverse configurations have been used for cultivating hairy roots, including mechanically driven reactors (e.g., stirred tank, wave, and rotating drum reactors), pneumatically driven reactors (e.g., bubble column and airlift reactors), and bed reactors (e.g., trickle bed and mist reactors) (Liu et al. 2009; Georgiev et al. 2010). Disposable bioreactors have demonstrated as promising tools for hairy root cultivations (Lehmann et al. 2014). Major advantages with the use of disposable bioreactors are the minimal cleaning and sterilization and reduced costs via reduced cleaning needs, capital investments, and maintenance (Eibl et al. 2011). Hairy roots are successfully cultivated in bioreactors with wave-induced mixing and aeration (Fig. 1.3). The performance and ginsenoside production of *Panax ginseng* hairy roots in wave bioreactors showed that both factors were significantly improved in wave cultivation compared to shake flask cultivations (Palazón et al. 2003a). Large-scale wave systems with capacities up to 600 L are now commercially available (source: Wave Biotech AG®, Tagelswangen, Switzerland). The most cited and largest hybrid bioreactor (bubble column-spray reactor) to grow hairy roots (*Datura stramonium*) so far is the 500 L Wilson Bioreactor (Wilson 1997).

1.6 Predicting the Future

Since the discovery three decades ago, hairy roots have been a tool for studying the molecular mechanism of a number of basic phenomena in plant behavior, biochemistry, and physiology. Nowadays hairy roots can be induced from practically any

plants; one of the important focuses in hairy root research should be the conservation of biodiversity and production of useful, rare, and exotic compounds from, e.g., endangered plant species. Plant kingdom has an enormous, still largely underutilized potential for the discovery of natural compounds (Newman and Cragg 2016), which may be exploited for human use. Especially for many medicinal plants, the biochemical pathways leading to interesting compounds are still much unknown, and hairy roots offer an excellent platform for pathway discovery.

The main challenge in hairy root biotechnology is still the relatively low yields of production leading to high costs for the desired product. When it comes to large-scale production of natural compounds, bioreactor technology plays a crucial role. Although hairy root cultivation technology has been studied intensively (see reviews by Mehrotra et al. 2015 and Banerjee et al. 2017), there are no flagship cases existing in hairy root-produced commercial products. However, intensive research and development work of both bioreactor design and novel computational tools applying, e.g., modelling, neural networks, and artificial intelligence, will definitely improve the understanding of processes related to hairy root technology and will lead to improved yields (Gallego et al. 2011; Mehrotra et al. 2015; Sweetlove et al. 2017).

Undoubtedly, plant metabolic engineering involving the overproduction of specialized metabolites is a technology which has resulted in great success (Farré et al. 2014). Recently, Sweetlove and co-workers showed how even primary metabolism of plant systems can be successfully engineered using computational modelling (Sweetlove et al. 2017). Another development that will clearly revolutionize plant metabolic engineering is CRISPR-Cas9-mediated genome editing. This technique is being rapidly adopted by the plant community as a robust and simple way to create targeted mutations, and it has also resulted in successful cases with application of hairy roots (Cai et al. 2015; Michno et al. 2015).

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