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REVIEW

Exudation: an expanding technique for continuous production and release of secondary metabolites from plant cell suspension and hairy root cultures

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Abstract This review addresses methods of obtaining secondary metabolites from plant cell suspension and hairy root cultures and their exudates, particularly the physiological mechanisms of secondary metabolites release and trafficking. The efficiency for product recovery of metabolites can be increased by various methods, based on the principle of continuous product release into the cultivation medium. The most common methods for metabolite recovery are elicitation, influencing membrane permeability, and in situ product removal. The biosynthetic pathways can be influenced by cultivation conditions, transformation, or application of elicitors. The membrane permeability can be altered through the application of chemical or physical treatments. Product removal can be greatly increased through a two-phase system and the introduction of absorbents into the cultivation medium. In this review, we describe some improved approaches that have proven useful in these efforts.

Keywords Exudation · Cell culture · Hairy root · Secondary metabolite · Medium · Elicitor · Membrane permeabilization · Product recovery

Abbreviations

DW	Dry weight
FW	Fresh weight
MS	Murashige and Skoog
SA	Salicylic acid
JA	Jasmonic acid
MJ	Methyl jasmonate
CWE	Cell wall elicitor
PEF	Pulsed electric fields
HHP	High hydrostatic pressure
US	Ultrasound
ISPR	In situ product removal
DMSO	Dimethylsulfoxide

Introduction

Plant in vitro culture has received a lot of attention as an effective technology for the production of valuable secondary metabolites. Some metabolites in plant cell and hairy root cultures can be accumulated with a higher yield than those in parent plants, i.e. product yield of rosmarinic acid from *Coleus blumei* cell culture was ninefold of that from intact plant (Petersen and Simmonds 2003), suggesting that the production of plant-specific secondary metabolites by plant in vitro cultures instead of whole plants cultivation possesses great potential. Recently, a review concerning advances on plant cell cultures was given by Weathers et al. (2010), in which recent

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developments on metabolic engineering of useful products were summarized.

However, most of the useful metabolites biosynthesized by plant cells are stored within the cells, thus making their efficient and continuous production very difficult. Moreover, the cells must be disrupted in order to extract and purify the desired product, adding to both the complexity of the process and the production cost. The amounts produced and the rates of production of these metabolites in plant cell cultures are still very low, and only a few plant cell culture produced secondary metabolites are commercialized. To overcome these problems, some methods for inducing the release of useful products into the culture medium have been developed. Perhaps, the most important advantage of plant cells over whole plants is the ease of product isolation and purification, especially when the product is secreted into the medium.

Plant secondary metabolites are of keen interest, because they have shown potential as food additives, nutraceuticals, pharmaceuticals, and cosmetic ingredients. Much effort has been made and different culture systems developed for the production of secondary metabolites (Bourgaud et al. 2001; Rao and Ravishankar 2002; Srivastava and Srivastava 2007). A relatively new technology for the production of plants' secondary compounds utilizes extraction via exudates. Exudates are relatively simple mixtures in comparison to plant tissues, allowing for reduced costs in the production of secondary metabolites. Moreover, one of the advantages of exudation is its continuity. Exudation offers a novel and efficient way of "phytomanufacturing" secondary metabolites.

One of the most fruitful areas of research for the production of secondary metabolites is the study of methods to induce product release or exudation from cells into the surrounding growth medium, where they can be more easily recovered. A study of the intracellular compartments in which the synthesis of chemicals occurs is also necessary, because the substances are often transported to the vacuole for accumulation, e.g. phenolic acids in the cell cultures of *Vitis vinifera* (Cai et al. 2011b, c). Plants often have sites of synthesis and storage of the secondary metabolites in separate cells or organs. Inhibition of metabolic enzymes as well as inhibition of membrane transport can be eliminated by the accumulation of synthesized products in a second phase introduced into the aqueous medium or two-phase system. There are several strategies to increase exudation. Special treatments include application of elicitors, membrane permeabilization, cell immobilization and in situ product removal (ISPR).

Exploiting the secretion process of plant secondary metabolites would allow them to be accumulated in a higher content and be more easily isolated. However, only limited information is available about how to enhance the production of secondary metabolites in exudates. The present review highlights the recent advances in production of secondary metabolites via plant cell suspension and hairy root cultures especially in their exudation, new developments in plant cell and exudation bioprocess, and emerging research on phytochemical recovery.

Principles of exudation

Definition of exudates

The chemicals secreted by plant hairy roots, suspension cells and callus into the surrounding growth medium are broadly referred to as exudates (Walker et al. 2003). Exudates, containing a wide range of bioactive compounds, play an important role in the adaptation of plants to their environment. The ability to secrete a vast array of compounds into the medium is one of the most remarkable metabolic features of plant cells, in fact 5–21% of all photosynthetically fixed carbon being transferred out of the root cells occurs through root exudates (Walker et al. 2003). The exudation process includes the secretion of ions, free oxygen and water, enzymes, mucilage, and a diverse array of carbon-containing primary and secondary metabolites.

Exudates include low-molecular weight compounds such as amino acids, organic acids, sugars, phenolics; and high-molecular weight compounds such as polysaccharides and proteins, which are less diverse but often compose a larger proportion of the exudates by mass. Since several review articles have been published on this topic recently (Badri and Vivanco 2009; Bais et al. 2004, 2006), the fascinating role of plant root exudates will not be reviewed here extensively.

A compositionally diverse array of more than 100,000 different low-molecular weight compounds are produced by plants (Bais et al. 2004). From the mechanisms for improved defense against a broad array of microbes, insects, and physico-chemical stress, the rich diversity of phytochemicals arises (Merbach et al. 1999). In plant root cells the functional importance of exuded chemicals includes: activity and turnover of microbes (Bais et al. 2004), influence on nutrient availability (Garcia et al. 2001), and inhibition of growth of competing plant species (Walker et al. 2003).

Mechanisms of exudation and metabolite trafficking

The study of the mechanisms and regulatory processes of controlling exudation just began in the last decade. One coping mechanism for plants under attack from pathogens is the production of a "front line" of detached, living cells

named border cells (Bais et al. 2004). These cells exude a wide array of biological compounds which influence the fungi and bacteria behavior. Border cells, together with their associated products, constitute up to 98% of the carbon-rich material exuded by plants. Exudates can be released from the cells via at least two mechanisms, passively (diffusates) and actively (secretions). Currently, little is known about the molecular mechanisms for the trafficking of these chemicals. Channels are likely to be involved in the secretion of organic acids normally present at high levels in the cytoplasm in at least some plants (Walker et al. 2003). Release of major low-molecular weight organic compounds from plant exudates is a passive process along their own steep concentration-gradient that usually exists between the cytoplasm of intact cells and the external solution (Neumann and Roemheld 2002). Direct passive diffusion through the lipid bilayer of the plasmalemma is determined by membrane permeability, which depends on the physiological state of the cell and the polarity of the exuded compounds, facilitating the permeation of lipophilic exudates.

Storage vacuoles, which often occupy 40–90% of the inner volume of plant cells, play a pivotal role in the accumulation of secondary metabolites in plants. Two major mechanisms are proposed for the vacuolar transport of secondary metabolites: H^+-gradient-dependent secondary transport via H^+-antiport and directly energized primary transport by ATP-binding cassette (ABC) transporters (Martinoia et al. 2002). ABC transporters use ATP hydrolysis to actively transport chemically and structurally unrelated compounds from cells. Membrane transport is fairly specific and highly regulated for each secondary metabolite. Not only genes that are involved in the biosynthesis of secondary metabolites but also genes that are involved in their transport will be important for systematic metabolic engineering aimed at increasing the productivity of valuable secondary metabolites in plant cell cultures (Yazaki 2005).

Here, we discuss the transport of resveratrol in plant cell cultures as a representative example. Resveratrol (3,4′,5-trihydroxystilbene) is a naturally occurring phytoalexin produced by some spermatophytes, such as *V. vinifera* and other members of Vitaceae as a response to infection, injury, fungal attack or exposure to ultraviolet light (Langcake and Pryce 1976). Resveratrol has been reported to exhibit a wide range of important biological and pharmacological properties. In plant cell suspension cultures, this metabolite is much more localized in the medium than within the cell (Zamboni et al. 2009). The secretion of resveratrol in growth medium of *V. vinifera* cell suspension cultures could be related to active transport mechanisms involving ABC transporters, or H^+-gradient-dependent mechanisms (Donnez et al. 2011; Santamaria et al. 2011), as described also for other secondary metabolites such as alkaloids (Hashimoto and Yamada 2003; Yazaki 2005, 2006). Moreover, it has been suggested that the localization of the stilbene synthase enzyme (STS) close to the cell wall in grape berries of *Vitis* sp. is linked to an excretion mechanism of resveratrol (Fornara et al. 2008). This is of practical importance since excretion of most of the resveratrol produced in the culture medium could facilitate its extraction.

Biochemical analysis of exudation

The extraction and purification of phytochemicals from the biochemically complex plant in vitro cells or tissues is a laborious and expensive process that presents a major obstacle to large scale manufacturing. The non-destructive exudation process may provide high yields of phytochemicals over the lifetime and facilitate their downstream purification. Exudates are relatively simple mixtures, in comparison to solvent extracts of plant tissue, which makes the isolation of chemicals an easier task. Exudation can be operated continuously without destroying the plant cells in vitro, thus producing a higher total yield of the phytochemicals over the lifetime of the culture. Glucosinolates, for example, can be separated from plant tissues, based on bio-refining of cruciferous oilseed crops or aqueous enzyme-aided extractions, utilizing a "Green chemistry" technique (Bagger et al. 1998). "Green chemistry" is an improvement over previous techniques, because it uses enzymes instead of chemical solvents for degradation of plant cell walls. The separation occurs in aqueous emulsion without the use of organic solvents, and oilseeds are allowed to be transformed into such products as lipids, oil, protein products, carbohydrates, special fibers, and various types of low-molecular weight compounds, including glucosinolates and derived substances.

Exudates can be removed from the circulating nutrient solution in open flowing culture system. Collection of exudates in solution generally yields a very dilute solution (Engels et al. 2000). The problem can be solved by decreasing the volume of trap solution, e.g. by freeze-drying or rotoevaporation (depending on the stability of the compounds). The volume restriction may lead to high salt concentrations resulting in precipitation of exudates, but interfering salts can be removed by ion exchange resins prior to volume reduction. Low- and high-molecular weight compounds can be separated by ultrafiltration. These techniques were applied to concentrate the exudates from hydroponically grown *Brassica rapa* (Schreiner et al. 2011). Solid phase extraction technique and more sensitive fluorescent detection parameters were used to quantify catechin in the picomolar range from the growing medium of hydroponically grown *Centaurea stoebe* (Tharayil and Triebwasser 2010).

Plant cell suspension and hairy root culture as efficient production system of exudates

There are two main types of plant in vitro culture that are of potential use for the production of useful secondary metabolites from exudation: cell suspension culture and hairy root culture. The majority of secondary metabolites are hydrophilic and, therefore, the main storage compartment associated with the cell is the aqueous environment of the vacuole (Martinoia et al. 2007). However, hydrophobic secondary metabolites typically accumulate in membranes, vesicles, dead cells or extracellular sites such as the cell wall (Roberts et al. 2003). Figure 1 shows the storage compartments for different secondary metabolites.

To reduce the costs for product recovery, it is useful to enhance transfer of compounds from the vacuole to the culture medium. To obtain valuable metabolites from the surrounding medium, the easiest and most efficient way is that this medium is liquid. Plant in vitro cultures such as cell suspension and hairy root cultures, therefore, are culture systems of increasing interest, because they grow in liquid medium and can secrete some metabolites into the medium. Below, the two possible production systems are characterized and their applications named.

Cell suspension culture

Medium is an essential part of a cell suspension culture, functioning as a nutrient source on one hand and as a storage and lytic compartment on the other hand and thus complements the activities of the vacuole (Wink 1994). It can be assumed that the medium functions as a big, extracellular vacuole, and most importantly it is big compared to vacuole, thus large amounts of metabolites can be sequestered there. Therefore, obtaining extracellular secondary metabolites from growth medium shows potential and remains relatively understudied.

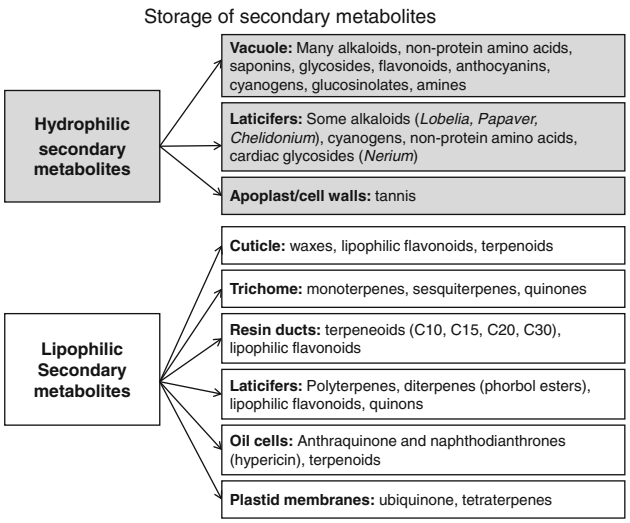

Fig. 1 Storage compartment for hydrophilic and lipophilic secondary metabolites (Wink 2010)

Cell suspension cultures can exude valuable secondary metabolites into growth medium. Here, we discuss resveratrol again as a representative example. In plant cell suspension cultures, this metabolite is much more localized in the medium than within the cell (Zamboni et al. 2009). In *V. vinifera* cv. Barbera cell suspension cultures in 100 ml flasks, the release of *trans*-resveratrol into the culture medium was about 60% (Tassoni et al. 2005); in 175 ml flasks, up to 67% of the total stilbene amounts were secreted to medium (Ferri et al. 2011b); in a 1 l stirred bioreactor, the vast majority of the produced resveratrol (up to 95%) was released into the culture medium (Ferri et al. 2011a). In *V. vinifera* cv. Chasselas × *Vitis berlandieri* cell suspension cultures in a 2 l stirred bioreactor, 90% of the total resveratrol can be secreted into the liquid medium (Donnez et al. 2011). According to Lijavetzky et al. (2008), endogenous stilbenoids represent less than 1% of the total stilbenoids in *V. vinifera* L. cv. Monastrell cell suspension cultures; therefore, the extracellular *trans*-resveratrol correlates with the actual biosynthetic activity of the cells. Furthermore, resveratrol can also be released by hairy root cultures. Abbott et al. (2010) and Condori et al. (2010) reported that majority of resveratrol produced in hairy root cultures of peanut (*Arachis hypogaea*) can be released and recovered from the growth medium.

Many other secondary metabolites can also be released and recovered in growth medium. Kajani et al. (2010) found that up to 74.9% of total taxanes was secreted to growth medium (5.584 mg/l) in *Taxus baccata* L. cell culture. In *Taxus canadensis* cell suspension culture, more than 90% of the total paclitaxel was recovered in the extracellular medium following treatment with cell wall enzymes (Roberts et al. 2003). In *Taxus chinensis* cell cultures, 90% of paclitaxel was found in the culture medium of the chitosan-adapted cell cultures (Zhang et al. 2007). Ye et al. (2004) reported that up to 5.3% taxuyunnanine C and 25.1% phenolics in *T. chinensis* cell culture were released into the liquid medium. More than 70% of the plumbagin produced by *Plumbago rosea* cell cultures was released into the medium (Komaraiah et al. 2003). In *L. erythrorhizon* cell culture, 60–70% shikonin could be excreted (Lin and Wu 2002). In *Catharanthus roseus* cell suspension culture, majority of ajmalicine production was found in growth medium (Almagro et al. 2011). Many extracellular antioxidants (mainly phenolics) and antioxidant enzymes can be continuously produced from medium of cell suspension cultures. For example, the supernatant from suspensions of *Solanacearum tuberosum*

and *Nicotiana tabacum* that had been incubated for 5 h had nearly 95% of the activity of the cell suspension, while the cell residue retained less than 5% antioxidant capacity, the antioxidant activity paralleling the content of phenolics (Baker et al. 2002). Antioxidant enzymes, superoxide dismutase (SOD), guaiacol-type peroxidase (POD), and glutathione peroxidase (GPX) were secreted at high levels into cultured medium of *Ipomoea batatas*. The extracellular activities of the three enzymes in culture medium were much higher than were the intracellular activities (Kim et al. 2004).

Hairy root culture

Since cell suspension cultures are dedifferentiated, there is lack of storage tissue and the product released in the culture media is prone to degradation by the enzymes released in the culture media (Wink 1994). The development of a certain level of differentiation is considered to be important in the successful production of phytochemicals by cell cultures. Organized cultures such as hairy root cultures can make significant contribution in the production of secondary metabolites.

Due to their fast growth rates and biochemical stability, hairy root cultures remain unsurpassed as a choice for model root systems and have shown promise as a bioprocess system. The applications are wide-ranging, from the production of natural products and foreign proteins, to a model system for the phytoremediation of organic and metal contaminants (Shanks and Morgan 1999). Secondary metabolites are accumulated in both hairy roots and culture medium, e.g. glucosinolates accumulated in *Brassica rapa* (Kastell et al. 2011), alkaloids in *Catharanthus roseus* (Li et al. 2011), terpenoids in *Salvia sclarea* (Kuzma et al. 2009) and flavonoids in *Glycyrrhiza uralensis* (Zhang et al. 2011). In some hairy root cultures, some metabolites are accumulated more in the medium than in the cells. For instance, in *G. uralensis* hairy root culture, up to 98 and 94% of the total licochalcone A and total flavonoids were secreted into the culture medium, respectively (Zhang et al. 2011). In *Arachis hypogaea* hairy root culture, over 90% of the total resveratrol, arachidin-1 and arachidin-3 were accumulated in the medium (Condori et al. 2010).

A. rhizogenes-transformed hairy roots are characterized by high growth rate, genetic stability, and growth in hormone-free media. These genetically transformed root cultures can produce a level of secondary metabolites comparable or higher to that of intact plants. Banerjee et al. (1998) reported the total valepotriate content was highest in the 20-week-old *A. rhizogenes* LBA 9402 line-induced *Valeriana wallichii* DC hairy root cultures (8.52% DW), followed by that of the A4 line of the same age (5.10% DW), which were 3.3 and 2 times higher, respectively, than that of the untransformed roots (2.58% DW).

However, many valuable secondary metabolites are synthesized by roots in vivo, and the synthesis is often connected to root differentiation (Flores et al. 1999). Even in cases where a particular secondary metabolite accumulates only in the aerial part of an intact plant, hairy root cultures have been shown to accumulate the same metabolite (Kim et al. 2002). For example, lawsone normally accumulates only in the aerial part of the plant, but hairy roots of *Lawsonia inermis* grown in a half- or full-strength Murashige and Skoog (MS) medium can produce lawsone under dark conditions (Bakkali et al. 1997). Although artemisinin was thought to accumulate only in the aerial part of *Artemisia annua* plant (Wallaart et al. 1999), several research efforts have shown that hairy roots can produce artemisinin (Liu et al. 2002; Wang and Tan 2002; Wang et al. 2001).

Hairy root cultures can produce a spectrum of secondary metabolites that are not present in the parent plant. Furthermore, a transgenic root system offers tremendous potential for introducing additional genes along with the Ri plasmid, especially modified genes, into plant cells with *A. rhizogenes* vector systems. Hairy root cultures have become a useful tool to study the biochemical properties and gene expression profile of metabolic pathways. They can also be used to elucidate the intermediates and key enzymes involved in the biosynthesis of secondary metabolites (Srivastava and Srivastava 2007). Hairy root cultures from more than 200 species of higher plants, mostly dicots, representing at least 30 plant families, have been studied, and represent a truly remarkable range of biosynthetic capabilities (Table 1).

Hairy root culture is well established as an experimental system and, most importantly, it provides many insights into root-specific metabolism and regulation. A summary of hairy root as secondary metabolites producing system, hairy root metabolic engineering, phytoremediation and scale-up process in recent years was given by Guillon et al. (2006) and Chandra and Chandra (2011).

Strategies for increasing exudation

There are several strategies to improve secondary metabolites production in plant in vitro culture: elicitation, membrane permeabilization, cell immobilization and ISPR. The approaches to improve secondary metabolite production have a vast literature base and have been reviewed recently either in general or by specific compound or chemical class, or by individual cultivation system. In

Table 1 Recently studied secondary metabolites from hairy root cultures

Plant species	Secondary metabolite	Volume	Content	Reference
Arachis hypogaea	Resveratrol	250 ml F	4.3 nmol/g DW in root, 420.7 nmol/g DW in medium	Condori et al. (2010)
			1.2% DW in medium extract	Abbott et al. (2010)
Artemisia annua	Drimartol A	250 ml F	383.2 mg/l	Zhai and Zhong (2010)
Beta vulgaris	Betalains	500 ml F	47.1 mg/g DW	Georgiev et al. (2010b)
Brassica rapa	Glucosinolates	250 ml F	80 μmol/g DW	Kastell (2009)
Brugmansia candida	Anisodamine	1.5 l B	10.1 mg/g DW	Cardillo et al. (2010)
Centella asiatica	Triterpenoids	100 ml F	0.55% DW	Kim et al. (2010a)
Catharanthus roseus	Catharanthine	250 ml F	1.96 mg/g DW	Wang et al. (2010)
	Alkaloid	250 ml F	4 mg/g DW	Li et al. (2011)
Chinese cabbage	Indole glucosinolates	100 ml F	1.6 μmol/g FW	Zang et al. (2009)
Coleus blumei	Rosmarinic acid	100 ml F	78 mg/g DW	Bauer et al. (2009)
Datura stramonium L.	Hyoscyamine	250 ml F	110.3 mg/l	Amdoun et al. (2010)
Fagopyrum esculentum M.	Rutin	100 ml F	1.3 mg/g DW	Kim et al. (2010b)
Gentiana macrophylla	Gentiopicroside	150 ml F	0.11 mg/g DW	Zhang et al. (2010)
Glycyrrhiza uralensis	Flavonoid	100 ml F	28.38 mg/g DW	Zhang et al. (2009)
Gossypium hirsutum L.	Gossypol	250 ml F	2.43 mg/g DW	Verma et al. (2009)
Panax quinquefolium L.	Ginsenoside	250 ml F	200 mg/g DW	Mathur et al. (2010)
Plumbago indica	Plumbagin	250 ml F	11.96 mg/g DW	Gangopadhyay et al. (2011)
Psoralea corylifolia	Daidzein	250 ml F	2.06% DW	Shinde et al. (2010)
	Genistein	250 ml F	0.37% DW	
	Psoralen	250 ml F	3 mg/g DW	Baskaran and Jayabalan (2009)
Salvia miltiorrhiza	Tanshinone	200 ml F	6.9 mg/l	Yan et al. (2011)
		250 ml F	2.727 mg/g DW	Kai et al. (2011)
		125 ml F	1.59 mg/g DW	Zhao et al. (2010)
Salvia sclarea	Diterpenoid	10 l B	67.5 mg/g DW	Kuzma et al. (2009)
Taxus x media var. Hicksii	Paclitaxel	250 ml F	568.2 μg/l	Syklowska-Baranek et al. (2009)

F flask, *B* bioreactor, *DW* dry weight

order to design an optimal production process for secondary metabolites, it is essential that the majority of metabolites be released into the extracellular medium. Although some key examples of novel approaches to metabolic engineering of plant secondary metabolites are briefly mentioned below, the main focus will be on secondary metabolites release in exudation by in vitro systems.

Elicitation

Exudation is affected by multiple biotic and abiotic factors such as nutritional status, light intensity, temperature, activity of retrieval mechanisms, and sorption characteristics of the growth medium (Engels et al. 2000). To increase the yield of exuded compounds, multiple techniques have been developed, as an example to increase the area of absorptive root surface, and to increase the content of the compounds in plants, leading to enhancement of the content in exudates, which is possible by elicitation (Kiddle et al. 1994).

Elicitor is defined as a substance which, when introduced in small concentration to a living cell system, initiates or improves the biosynthesis of specific compounds (Chandra and Chandra 2011). Depending on their origin, they are classified as biotic or abiotic (Smetanska 2008). Biotic elicitor includes: (1) enzymes, cell wall fragments of microorganisms, polysaccharides derived from microorganisms (chitin or glucans) and glycoproteins; (2) phytochemicals produced by plants in response to physical damage, fungi or bacteria attack, polysaccharides derived from plant cell walls (pectin or cellulose), fragments of pectin, formed by action of microorganisms on plant cell wall; (3) chitosan, glucans, salicylic acid (SA), methyl jasmonate (MJ, formed by the action of plant on microbial cell walls). Abiotic elicitors are the substances of non-biological origin. The causes of the abiotic stress can be of chemical or physical nature; among them are chemicals such as inorganic salts, heavy metals and some chemicals that disturb membrane integrity, physical factors such as mechanical wounding, ultraviolet irradiation, high salinity,

high or low osmolarity, extreme temperature (freezing, thawing) and high pressure. Elicitors mimic stresses on plants, activating biochemical defense systems and resulting in quantitative and qualitative changes in the composition of the exudates (Gaume et al. 2003). Results can be quite dramatic; useful summaries were given by Rao and Ravishankar (2002).

Elicitation at right stage of culture, concentration of elicitors, and appropriate combination of medium and elicitor are also the regulating factors responsible for enhancement of secondary metabolites. Elicitors stimulate the defense mechanisms in plant cells causing secondary metabolite production as well as their exudation. Elicitation greatly increases the exuded chemical diversity as well as the quantity of many compounds. In *T. canadensis* cell suspension culture, extracellular paclitaxel levels increased from 2.0 to 6.5 mg/l in the control cell cultures and from 22.9 to 52.7 mg/l in MJ-elicited cell culture (Roberts et al. 2003). Yeast elicitor increased tanshinones production in *Salvia miltiorrhiza* cell suspension cultures, both in the cells and in the culture medium (Chen and Chen 2000). Yeast elicitor was also found to increase oleanolic acid (OA) content in culture medium of *Calendula officinalis* L., and the addition of jasmonic acid also stimulated secretion of OA. Furthermore, it was noted that the increase in levels of OA in the culture medium did not result in a decrease in the intracellular accumulation of this compound (Wiktorowska et al. 2010). Interestingly, some elicitors only increase the extracellular accumulation of metabolites, without impact on intracellular amount. Recently, it was observed that 80 mM sucrose increased the release of *trans*-resveratrol and of piceid in the grape Gamay Fréaux cell suspension cultures, without inducing any intracellular stilbene accumulation (Belhadj et al. 2008).

Gleba et al. (1999) saw evidence that elicitors stimulate various plant roots to exude phytochemicals in much higher quantities than non-elicited plants. Moreover, elicited plant in vitro culture may exude compounds which are not detected in the exudates of non-elicited culture, e.g. exudation of rosmarinic acid from *Ocimum basilicum* hairy root culture was induced by elicitation with fungal cell wall elicitors (CWE) from *Phytophthora cinnamon* (Bais et al. 2002).

Elicitors do not function equally in all species. They are most effective at optimum concentrations and at right stage of culture. Several previous works have demonstrated that the developmental stage is crucial for the response to elicitation. For instance, the recent work of Condori et al. (2010) shows that production of resveratrol and the prenylated resveratrol analogues arachidin-1 and arachidin-3 in growth medium of peanut hairy root cultures upon sodium acetate-mediated elicitation is highly dependent on the developmental stage. Highest yields were found during the exponential growth. Similarly, hypericin production in *Hypericum perforatum* cell suspension cultures treated with ozone at exponential phase was higher than that of lag and stationary phase (Xu et al. 2011b). But in contrast, the highest peruvoside production of 8.93 mg/l in growth medium of *Thevetia peruviana* cell suspension cultures was obtained when methyl jasmonate was applied at the lag phase (Zabala et al. 2010). It is possible that at certain developmental stages, the plant cells are more prone to activate metabolic pathways associated with the production and secretion of these metabolites. Elicitors can dramatically increase the quantities of certain compounds in the exudates. The release of glucosinolates from in vitro grown *A. thaliana* was studied, at the same time the effect of signaling molecules such as SA, MJ and nitric oxide on the gene expression was also studied (Badri et al. 2008). A partial summary of recent elicitors used in plant cell suspension and hairy root cultures to improve production of secondary metabolites is shown in Table 2.

Membrane permeabilization

Cultivated plant cells often accumulate secondary metabolites intracellularly in vacuoles; therefore, efforts have been made to develop procedures for induced release of such products into medium. In order to release metabolites from vacuoles, two membrane barriers, plasma membrane and tonoplast, should be penetrated. Cell permeabilization depends on the pore formation in one or more of the membrane systems, enabling the passage of molecules into and out of plant cell (Brodelius and Pedersen 1993). Many attempts have been made to permeabilize the plant cells transiently, to maintain the cell viability and to have short time periods of increased mass transfer of substrate and metabolites to and from the cell (Smetanska 2008).

A wide variety of methods and agents have been used to increase membrane permeability, including chemical treatments, e.g. solutions of high ionic strength, external pH change, dimethylsulfoxide (DMSO), Tween 20 (polyoxyethylene sorbitan monolaurate) and chitosan addition, and physical treatments, e.g. pulsed electric fields, ultrasound and high hydrostatic pressure. Below, the chemical and physical methods of permeabilization will be discussed.

Chemical permeabilization

The chemical permeabilization includes the change of nutritious status, addition of chemical agents, etc. For instance, serpentine was released from *Catharantus roseus* cells when the cells were filtered and re-suspended in fresh

Table 2 Elicitors for production of secondary metabolites in plant cell suspension and hairy root cultures

Elicitor	Plant species	Product	Culture system and volume	Content	Increase (fold of control)	Reference
Biotic						
Fungi	*Abrus precatorius* Linn.	Glycyrrhizin	CS, 250 ml F	53.62 mg/l	5.22	Karwasara et al. (2010)
	Artemisia annua	Artemisinin	HR, 250 ml F	2.1 mg/g DW	2.6	Wang et al. (2009)
	Calophyllum inophyllum L.	Inophyllum	CS, 250 ml F	6.84 mg/100 g elicited biomass	751	Pawar et al. (2011)
Yeast extract	*Glycyrrhiza uralensis*	Flavonoid	HR, 100 ml F	28.38 mg/g DW	1.6	Zhang et al. (2009)
	Pueraria candollei	Isoflavonoid	HR, 125 ml F	60 mg/g DW	4.5	Udomsuk et al. (2011)
Salicylic acid	*Fagopyrum sculentum*	D-chiro-inositol	CS, 100 ml F	5.521 mg/g DW	2.7	Hu et al. (2011)
	Psoralea corylifolia L.	Daidzein	HR, F	2.2% DW	1.4	Shinde et al. 2009)
	Salvia miltiorrhiza	Phenolic acid	CS, 100 ml F	na	6–10	Dong et al. (2010)
Jasmonic acid	*Cannabis sativa* L.	Tyrosol	CS, 250 ml F	27 µmol/g DW	2	Peč et al. (2010)
	Datura stramonium L.	Hyoscyamine	HR, 250 ml F	110.3 mg/l	1.74	Amdoun et al. (2010)
	Vitis vinifera L. cv Italia	Resveratrol	CS, 250 ml F	1.6 mg/l	4	Santamaria et al. (2011)
Methyl jasmonate	*Artemisia annua*	Arteminisin	CS, 100 ml F	14.4 µg/g DW	2.4	Caretto et al. (2011)
	Centella asiatica	Centellosides	CS, 500 ml F	1.11 mg/g DW	6.9	Bonfill et al. (2011)
		Asiaticoside	HR, 100 ml F	7.12 mg/g DW	nd in control	Kim et al. (2007)
	Glycine max	Isoflavonoid	CS, 200 ml F	800 mg/g DW	5–6	Gueven and Knorr (2011)
	Salvia sclarea	Aethiopinone	HR, 10 l B	40 mg/g DW	9	Kuzma et al. (2009)
Sodium acetate	*Arachis hypogaea*	Resveratrol	HR, 250 ml F	420.7 nmol/g DW	30	Condori et al. (2010)
				1.2% DW in medium extract	na	Abbott et al. (2010)
Cyclodextrin	*Catharanthus roseus*	Ajmalicine	CS, 250 ml F	220 mg/l	3	Almagro et al. (2011)
	Vitis vinifera L. cv. Monastrell	Resveratrol	CS, 250 ml F	220 µmol/g DW in medium	nd in control	Lijavetzky et al. (2008)
	Vitis riparia	Resveratrol	CS, 250 ml F	911.3 mg/l in medium, 622.90 µg/g in cells	2,462 in medium, 227 in cells	Zamboni et al. (2006)
Ethephon	*Vitis vinifera*	Anthocyanins	CS, 200 ml F	2 mg/g DW	2.2	Cai et al. (2011b)
Chitosan	*Pueraria* var. *mirifica*	Isoflavonoids	HR, 125 ml F	32.33 mg/g DW	2.1	Korsangruang et al. (2010)
Pectin	*Vitis vinifera*	Anthocyanins	CS, 100 ml F	3.4 mg/g DW	2.5	Cai et al. (2011a)
Abiotic						
Electric current	*Cicer arietinum* L.	(+)-Pisatin	HR, 250 ml F	34.5 µg/g FW	13	Kaimoyo et al. (2008)
High hydrostatic pressure	*Glycine max*	Isoflavonoids	CS, 200 ml F	800 mg/g DW	1.3	Gueven and Knorr (2011)
	Vitis vinifera	Resveratrol	CS, 200 ml F	0.95 µmol/l	3.4	Cai et al. (2011c)

Table 2 continued

Elicitor	Plant species	Product	Culture system and volume	Content	Increase (fold of control)	Reference
Ultrasound	*Morinda citrifolia*	Anthraquinones	CS, 250 ml F	16.74 mg/g DW	2.5	Komaraiah et al. (2005)
	Taxus yunnanensis	Taxol	CS, 125 ml F	5.46 mg/l	15	Wang et al. (2006)
Pulsed electric fields	*Glycine max*	Isoflavonoids	CS, 200 ml F	800 mg/g DW	1.9	Gueven and Knorr (2011)
	Vitis vinifera	Resveratrol	CS, 200 ml F	0.5 µmol/l	3.6	Cai et al. (2011b)
Hyperosmotic stress	*Salvia miltiorrhiza*	Tanshinone	HR, 125 ml F	0.72 mg/g DW	4.5	Shi et al. (2007)
	Vitis vinifera	Anthocaynins	CS, 175 ml F	24 △Abs/g DW	2.6	Ferri et al. (2011b)
		Stilbenes	CS, 175 ml F	0.4 µmol/g DW	3.5	
Heat shock	*Taxus yunnanensis*	Taxol	CS, 100 ml F	6.8 mg/l	6	Zhang and Fevereiro (2007)
Cold shock	*Glycine max*	Isoflavonoid	CS, 200 ml F	800 mg/g DW	1.4	Gueven and Knorr (2011)
Metal ions: Cu^{2+}, Cd^{2+}, Al^{3+}, Zn^{2+}, Cu^{2+}, Va^{2+}, Ag^{+}, Hg^{2+}	*Pueraria candollei* var. *candollei*	Isoflavonoids	HR, 125 ml F	27.99 mg/g DW	1.4 by Cu^{2+}	Korsangruang et al. (2010)
	Taxus chinensis	Paclitaxel	CS, 125 ml F	39 mg/l	4.6 by Ag^{+}	Zhang et al. (2007)
Light irradiation	*Catharanthus roseus*	Lochnericine	HR, 250 ml F	3 mg/g DW	2	Binder et al. (2009)
	Echinacea purpurea	Caffeic acid derivatives	HR, 250 ml F	35 mg/g DW	1.6	Abbasi et al. (2007)
Ozone exposure	*Hypericum perforatum*	Hypericin	CS, 250 ml F	28 mg/l	4	Xu et al. (2011b)
	Taxus chinensis	Taxol	CS, 250 ml F	20 mg/l	2.7	Xu et al. (2011a)

HR hairy root culture, *CS* cell suspension culture, *F* flask, *B* bioreactor, *DW* dry weight, *na* not available, *nd* not detected

or conditioned medium possibly induced by the temporary membrane uncoupling (Moreno et al. 1995). Permeabilizing agents are those chemical compounds which are not inhibitory to the cell growth and at the same time have the ability to reversibly increase the pore size of the cell wall (Prakash and Srivastava 2011). The normal pore size of the cell should be restored upon removal of the ideal permeabilizing agent. Dornenburg and Knorr (1997) reported *Chenopodium rubrum* cells could be permeabilized by chitosan treatment. Long-term permeabilization with chitosan showed a time-dependent amaranthin release from *C. rubrum* cells into the culture medium. Zhang et al. (2007) characterized the growth and paclitaxel production and extracellular release of *T. chinensis* cell suspension cultures adapted to chitosan by comparing with the unadapted cells. The paclitaxel yield of the chitosan-adapted cells was about fourfold that of the unadapted cells, while the paclitaxel release ratio of the chitosan-adapted cells was about threefold that of the unadapted cells.

Brodelius and Pedersen (1993) investigated five permeabilization agents on three different species, and although product release was achieved, cell viability dropped in most cases. Permeabilization of plant membranes for the release of secondary metabolites is often connected with the loss of viability of the plant cells, but there are exceptions, for example DMSO and Triton X-100 applied to *C. roseus* cells (Moreno et al. 1995), Triton X-100 applied to *Beta vulgaris* cells (Trejo-Tapia et al. 2007), *n*-hexadecane applied to *Azadirachta indica* cells (Prakash and Srivastava 2011) and Tween 80 applied to *G. uralensis* hairy root culture (Zhang et al. 2011). According to Trejo-Tapia et al. (2007), *B. vulgaris* cell culture treated with 0.7 mM Triton X-100 for 15 min induced a release of 30% betacyanines without loss of cell viability (≥70%). After this permeabilization treatment, *B. vulgaris* cultures actually re-grew faster, reaching a maximum biomass concentration of 48% higher than non-permeabilized cultures after 14 days. Tween 20 was used as permeabilizing agent on tropane alkaloids from *Datura innoxia* Mill. hairy root culture (Boitel-Conti et al. 1995, 1996). For various Tween 20 concentrations both hyoscyamine and scopolamine accumulated in the culture medium, meanwhile plant material viability could be preserved after a 24-h 2% Tween 20 concentration treatment (Boitel-Conti et al. 1995). In cell suspension culture of *Tessaria absinthioides*, the total production of the sesquiterpene tessaric acid (TA) at day 25 of the culture period reached 0.086 mg/g DW, with extracellular accumulation accounting for 0.027 mg/g DW. DMSO-induced permeabilization of the cells effected both total production and extracellular accumulation of the sesquiterpene to reach levels of 148 and 271%, respectively (Sanz et al. 2000). *n*-hexadecane led to higher azadirachtin concentration in *A. indica* cell culture and up to 13% release in the culture medium without affecting the cell viability (Prakash and Srivastava 2011). Tween 80 significantly elevated licochalcone A (ninefold higher) and total flavonoid (elevenfold higher) production from hairy root cultures of *G. uralensis* Fisch, and stimulated the secretion of licochalcone A (98%) and total flavonoid (94%) into the culture medium, without loss of cell viability (Zhang et al. 2011).

Physical permeabilization

Physical treatments such as pulsed electric fields (PEF), high hydrostatic pressure (HHP) and ultrasound (US) can cause membrane permeabilization. The principle of PEF is based on the development of membrane pores under external electric fields. PEF treatment on cell material leads to pore formation in cell membrane and thus modifies diffusion of intra- and extracellular media. PEF enhances release of intracellular molecules from permeabilized tissue as well as improves uptake of low molecular substances into the cells (Janositz et al. 2011). The pore formation can be reversible or irreversible, depending on electric field strength and pulse number. Application of PEF caused high levels of cell permeabilization in *C. rubrum* cell culture, but with ten pulses at field strengths beyond 0.75 kV/cm the cells lost their viability (Knorr et al. 1993). PEF application between 1.6 and 2.0 kV/cm (5 Hz, 36 s) induced reversible membrane permeability and increased isoflavonoid concentrations in *Glycine max* cell culture, but further increase in voltage resulted in decrease in isoflavonoid concentration (Gueven and Knorr 2011). A significant increase in intracellular and extracellular accumulation of taxuyunnanine C was observed by exposing the *T. chinensis* cells in the early exponential growth phase to PEF (50 Hz, 10 V/m; Ye et al. 2004). Application of PEF increased the phenolic acids accumulation in *V. vinifera* suspension culture medium, the total extracellular phenolic acids was 11% higher than that of the control (Cai et al. 2011b). It was also reported that PEF led to the permeabilization of plant membrane and breakage of cells and tissues of food material (Ersus and Barrett 2010; Fincan and Dejmek 2003).

HHP is also an efficient method to increase membrane permeability. In their research, Knorr et al. (1993) have demonstrated that HHP of 50 MPa increased the production of amarantin and antraquinones in *C. ruburum* and *Morinda citrifolia* cell cultures. But pressures greater than 250 MPa caused loss of cell viability, most likely due to permeabilization of the tonoplast. A recent research has found that HHP of 50 Mpa resulted in a 10–30% increase in isoflavonoid biosynthesis in *G. max* cell culture, but higher pressures did not cause further increase (Gueven and

Knorr 2011). It was also reported that HHP of 40 MPa increased the phenolic acids production up to ninefold in exudates from *V. vinifera* cell culture (Cai et al. 2011c). It is assumed that the pressure-dependent destruction of the tonoplast, the loss of compartmentation, and subsequent release of the content of the vacuoles causes the pH change in the medium and the resultant cell death. HHP at high levels may also cause denaturation of enzymes which are necessary for metabolites biosynthesis, influence membrane integrity causing irreversible permeability (Corrales et al. 2008; Gueven and Knorr 2011). Although cell permeabilization was coupled with concurrent loss of cell viability, recent researches have demonstrated that application of both PEF and HHP could become useful tools for product recovery from plant cells and tissues with minimum effect on cell viability and product composition. For the industrial use of PEF and HP, possible mechanism of membrane permeabilization and the induced changes in cell structures, see Olsen et al. (2010) and Soliva-Fortuny et al. (2009).

Another efficient permeabilization method is ultrasound (US). Studies have shown that exposure of plant cells to low-energy US enhances the biosynthesis of some secondary metabolites, e.g. shikonin from *Lithospermum erythrorhizon* (Lin and Wu 2002), anthraquinone from *Morinda citrifolia* (Komaraiah et al. 2005), taxol from *T. chinensis* (Wu and Ge 2004) and *Taxus yunnanensis* (Wang et al. 2006). Exposure to US can also enhance exudation of metabolites into medium. For instance, Lin and Wu (2002) demonstrated the use of US to enhance shikonin production in *L. erythrorhizon* cell cultures. Suspension cells were exposed to US (power density $\leq$ 113.9 mW/cm^3) for short periods (1–8 min). The US exposure significantly stimulated the shikonin biosynthesis of the cells, and under certain US treatment conditions, increased the volumetric shikonin yield by 60–70%. Meanwhile, the shikonin excreted from the cells was increased from 20 to 65–70%, due partially to an increase in the cell membrane permeability by sonication. The enhanced secondary metabolite biosynthesis of plant cells by US has been proved to be a physiological activity of the cells stimulated by US rather than the mass transfer effects proposed for other biological systems (Wang et al. 2006). The unique effect of US on secondary metabolite production in plant cell cultures is of both fundamental and applied significance.

Utilizing cell wall digesting enzymes

Hydrophobic secondary metabolites typically accumulate in membranes, vesicles, dead cells or extracellular sites such as the cell wall. Paclitaxel is hydrophobic and essentially insoluble in aqueous solutions (including cellular cytoplasm) and is suggested to be stored in the cell wall. The application of cell wall digesting enzymes cellulase (1%) and pectolyase (0.1%) to *Taxus canadensis* suspension cultures induced a significant increase in the paclitaxel present in the extracellular medium while maintaining membrane integrity, and more than 90% of the total paclitaxel was recovered in the extracellular medium following treatment with the enzymes (Roberts et al. 2003). The addition of cell wall digesting enzymes to a cell culture bioprocess may be an effective way of enhancing paclitaxel release to the extracellular medium and hence simplify product recovery. This simple method may be applicable to the localization of other hydrophobic secondary metabolites in suspension cultures. The addition of cell wall digesting enzymes to cell cultures where secondary metabolites are stored in the cell wall is additionally a simple and effective way of enhancing release into the extracellular medium, and studies are currently underway to optimize this protocol for secondary metabolites production via plant cell suspension culture.

In situ product removal

The synthesis and storage of secondary metabolites in plant cells often occurs in separate compartments. Synthesis of desired compounds can be enhanced through the accumulation of synthesized products in a second phase introduced into the aqueous medium. ISPR is a process that quickly removes product from the producing cells thereby preventing the subsequent interference with cellular or medium components. It is established that ISPR greatly benefited the production of metabolites limited by inhibitory or toxic products, as well as unstable products or reactions which are thermodynamically unfavorable (Woodley et al. 2008).

Low accumulation of secondary metabolites in cell cultures may not be due to lack of key biosynthetic enzymes but rather feedback inhibition, enzymatic or non-enzymatic degradation of the product in the medium, or volatility of substances produced (Smetanska 2006). In such cases, it is possible to increase the net production by addition of an artificial site for product accumulation, e.g. introduction of second solid or liquid phase into the aqueous medium (Fig. 2). These "two-phase systems" have the ability to accumulate traces of secondary compounds from the culture medium. For instance, Amberlite XAD-7 increased the yields of ajmalicine and serpentine produced by *Catharantus roseus*, and altered the ratio between the alkaloids produced (Lee and Shuler 2000). It is of high interest that production of these alkaloids, which accumulate intracellularly, was affected by the presence of Amberlite XAD-7 resin.

To date, a number of different ISPR methods have been investigated (Table 3). The removal and sequestering of the product in a non-biological compartment often increases its

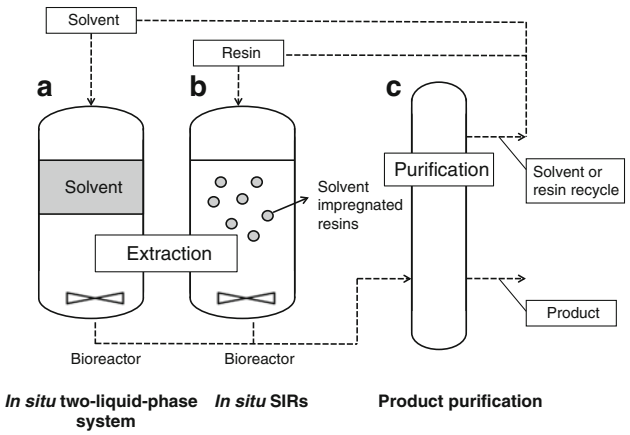

Fig. 2 Schematic diagram of the in situ product removal (ISPR) experimental system: in situ two-liquid-phase extraction (*a*), in situ extraction using solvent impregnated resins (SIRs) (*b*) and downstream product purification (*c*)

total production. For example, the addition of Amberlite XAD-4 to *M. elliptica* cell suspension culture medium on day 18 for 6-day contact period achieved comparable cell growth to control (41 g/l), but with 1.3-fold higher intracellular anthraquinones (AQ, 124 mg/g DW) and twofold increase in extracellular AQ (14.3 mg/l). With 5–8.3 g XAD-4 adsorbent per liter *M. elliptica* culture in production medium, 60–90% AQ was recovered from extracellular AQ after 24–26 days of culture period (Chiang and Abdullah 2007).

The addition of a second site for the accumulation of secondary compounds can be a useful tool for increasing biosynthetic pathways in plant in vitro cultures. When the product is subject to feedback inhibition or intracellular degradation, the removal and sequestering of the product in an artificial site may increase total metabolite yield. Downstream purification can be reduced if ISPR from the culture medium and cells is selective. Consequently, recovery and purification are generally simplified, thus reducing production costs.

The advantages of using ISPR include the stimulation of the secondary metabolite biosynthesis and the easy separation of products. Among the adsorbents used, polymeric adsorbents are the most attractive due to their biocompatibility, large surface per unit mass leading to high accumulation capacity, regeneration characteristics, low toxicity, effective separation from very dilute aqueous solutions, and special selectivity (Barkakati et al. 2010). The capturing of low concentration plant metabolites directly from the particulate-laden culture medium simplifies downstream product recovery units. The net production and volumetric yields are increased, when the products are localized in a reduced volume compared to the

Table 3 Adsorbents used for two-phase plant cell cultivation systems

Adsorbent	In vitro culture	Culture system and volume	Product	Yield	Recovery (%)	Reference
Activated charcoal	*Taxus baccata* L.	CS, 250 ml F	Taxane	5.584 mg/l	52.7	Kajani et al. (2010)
Liquid paraffin	*Echium italicum* L.	CS, 250 ml F	Shikonin acetate	16 mg/l	na	Zare et al. (2010)
Miglyol	*Pimpinella anisum*	na	Ethanol	na	na	Mulder-Krieger et al. (1988)
XAD-4	*Morinda elliptica*	CS, 250 ml F	Anthraquinones	124 mg/g DW in cells, 14.3 mg/l in medium	60–90	Chiang and Abdullah (2007)
	Lavandula vera	CS, 500 ml F	Fatty acids	8.5% from the total volatiles	50.1	Georgiev et al. (2010a)
	Rosa damascena Mill 1803	CS, 500 ml F and 3 l B	Acids and esters	7.2% from the total volatiles	99	Pavlov et al. (2005)
XAD-7	*Catharanthus roseus*	CS, 125 ml F	Ajmalicine	130 mg/l	100	Lee and Shuler (2000)
			Catharanthine	80 mg/l	100	
	Plumbago rosea	CS, 250 ml F	Plumbagin	92.1 mg/g DW	70	Komaraiah et al. (2003)
X-5	*Salvia miltiorrhiza*	HR, 200 ml F	Diterpenoid tanshinones	87.5 mg/l	76.5	Yan et al. (2005)
RP-8	*Mentha piperita*	CS, 250 ml F	Menthol	148 mg/l	na	Chakraborty and Chattopadhyay (2008)
HP2MGL	*Vitis vinifera*	CS, 500 ml F	Resveratrol	2666.7 mg/l	100	Yue et al. (2011)
Diaion HP 20	*Tinospora cordifolia*	CS, 250 ml F	Arabinogalactan	0.49% DW	na	Roja et al. (2005)
	Arachis hypogaea	HR, 250 ml F	Resveratrol	1.2% DW	91.7	Abbott et al. (2010)

HR hairy root culture, *CS* cell suspension culture, *F* flask, *B* bioreactor, *DW* dry weight, *na* not available

bigger volume medium. The most commonly used absorbents are the non-ionic Amberlite XAD resins. Available as white translucent beads, the resins have high specific surface area, and mechanical strength, and maintain effectiveness in typical pH ranges (Chiang and Abdullah 2007). Adsorption-based separation is one of the most promising methods for separation since they are non-denaturing, highly selective, energy efficient, and relatively inexpensive. In addition, the resin-based extraction permitted to use less amount of organic solvent during the extraction. As shown by Komaraiah et al. (2003), more than 70% of the plumbagin produced by *Plumbago rosea* cells was released into the growth medium, leading to easy recovery of the product, at the same time the sucrose utilization rate of the cells was higher when subjected to ISPR using Amberlite XAD-7. Interesting results were also observed on tanshinone production, while the addition of X-5 resin to the *Salvia miltiorrhiza* hairy root culture only increased the tanshinone yield slightly, but recovered more than 80% of tanshinones from the roots (Yan et al. 2005).

Challenges and outlook

The limited success of industrial utilization of plant cell cultures for the production of secondary metabolites is due to various reasons ranging from low yields of the desired compounds, incomplete understanding of the biosynthetic pathways and the technological processes involved in extraction and purification of these compounds. The biggest challenge of obtaining secondary metabolites from exudates is, however, the secretion of products into the extracellularly medium is imperative. The most common metabolites are stored intracellularly in plant cells. Several secondary metabolites are hydrophobic in nature and are minimally secreted in the growth medium. The study of exudates just began in the last decade, and many questions remain open issues.

In cell suspension cultures, undifferentiated cells are far less efficient than cultured organs in terms of metabolite production and biosynthetic pathways are often not complete. In cell suspension cultures, there is lack of storage tissue and the product released in the culture media is prone to degradation by the enzymes released in the culture media (Wink 1994). Thus, where secretion occurs, there may be problems of extracellular degradation of the products.

Repressed secondary metabolite production in pant cell cultures might also be due to the specific location of some of the key enzymes involved in the biosynthetic pathways. Subcellular localization of various enzymes and transport of intermediates to the respective compartments for further activity of enzymes limits the target of sustainable accumulation of product in the culture medium. This is probably one reason for the low production or release of metabolites in the culture medium for sustainable harvest and downstream processing at the commercial scale (Chandra and Chandra 2011). Proper understanding of biosynthetic pathways, exploration of respective enzymes and investigations regarding their developmental regulation are further needed. Furthermore, strategies to increase metabolite accumulation/secretion in culture medium are often lack of success or accompanied with loss of cell viability. An approach for preserving viability of biomass and its reuse needs to be developed. Optimization of these methods could be explored by utilizing different kinds of bioreactors, combining elicitors and adsorbents, and simultaneous extraction of metabolites from the exhausted medium. The introduction of newer techniques of extraction and purification of metabolites from liquid medium is also likely to be a significant step towards making exudates more generally applicable to the commercial production of secondary metabolites.

In conclusion, exudates of plants are a fascinating and largely unexplored biological frontier. Exudates have remarkable diversity of secondary metabolites, and a striking ability to adjust their metabolic activities in response to biotic and abiotic stresses. In the past, research on plant exudation has been hampered by the underground growth habit of roots and by the lack of a suitable experimental system. However, recent progress in growing roots and cells in isolation has greatly facilitated the study of exudation metabolism and contributed to our understanding of this remarkable plant behavior. Plant in vitro cultures offer promise for high production and collection of valuable secondary metabolites, through the plants and cells, as well as exudates. The yield of secondary metabolites from exudates can be increased by: elicitation, membrane permeabilization, cell immobilization and ISPR, etc. Exudation is increasingly recognized as a potential source of valuable phytochemicals, but still much work is needed to turn exudation into a stable and productive source of the secondary metabolites. The combined efforts of scientists from the fields of plant physiology, food technology, biochemistry, molecular biology, and agriculture can further our understanding in this field, helping to exploit the potential of exudates for the production of secondary metabolites.

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