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Alkaloids in Plant Cell Cultures

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Abstract

Alkaloids are natural substances used largely for human medication. Destruction of natural habitat of various alkaloid-producing plants has led to cultivation. But large areas of cultivation are required due to very low content of the substances, which presents a consequence of high production cost. Cell cultures, undifferentiated or differentiated, have exhibited the capacity to synthesize alkaloids *in vitro*, which may be developed as alternative source of phytochemicals. The discoveries of biosynthetic pathways, sites of stepwise synthesis, and accumulation of alkaloids in plant and cultured cells, also factors involving in or influencing those mechanisms, are all mosaics that build an increasingly obvious picture of the case. Despite enhancement in alkaloids production in *in vitro* culture through many kinds of manipulation, variability in the yield still occurs. Improvements on the basic knowledge of cell mechanisms, including metabolomics, along with genetic engineering are expectable to solve the problems.

Keywords: alkaloids, cell culture, *in vitro* production, accumulating structures

1. Introduction

Many alkaloids are pharmacologically active and have been used since decades as valuable medicines. They are produced by a large variety of organisms such as bacteria, fungi, plants, and animals. People in many parts of the globe used to extract natural substances from plants that actually contain alkaloids to cure them against certain diseases or to maintain their health. Alkaloids are known as having antimicrobial and pharmacological activities such as analgesic, antipyretic, antihypertensive, cardiac antiarrhythmic, antimalarial, and anticancer [1–5]. Nevertheless, only few of those natural alkaloids have been investigated through a proper pharmacological test, while we are being run after by massive deforestation and physical

development in many countries. Moreover, natural production of alkaloids is quite vulnerable vis-à-vis the climatic condition and produced in very low level. Therefore, one of the efforts to save any invaluable plants' substances from extinction and at the same time to increase and stabilize the production is by culturing plant cells and tissue *in vitro*. A suitable term for this is cell farming.

Secondary metabolites biosynthesis and accumulation are associated with a variety of cell types in different plants, including epidermis, endodermis, pericycle, phloem parenchyma, phloem sieve elements and companion cells, specialized mesophyll, and laticifers. There have been many evidences that plant cells cultured *in vitro* are capable to synthesize secondary metabolites, then people tried to culture tissues or organs of the plant from which a desirable phytochemical used to be highly accumulated, for instances roots for ginsenoside, seeds of *Trigonella* spp. for diosgenine, or leaves of *Catharanthus roseus* for vinblastine. However, the case is not always true, since some alkaloids are synthesized in one plant organ and then translocated to another one for accumulation [6]. Contrarily, plant tissues isolated from different parts of the naturally producing plant have been reported as being capable to synthesize the substance in *in vitro* cell culture; the cells are either undifferentiated or differentiated. Due to this fact, choosing the type of plant tissue is of critical importance for the success. Once a phytochemical production is established *in vitro*, it is subject to upscale the production, particularly of substances which have high commercial value. Through various ways, that is, chemically, technologically, and genetically, some of them have reached industrial cost-benefit level. Metabolic along with genetic engineering work together or in parallel to improve the cells' yields.

This chapter describes *in vitro* production of alkaloids and factors that may influence the synthesis and storage in cultured cells which were found in some cases different from those discovered in plant. It also reports the existence of specialized structures in *in vitro* cell cultures compared to natural secretory cells that occur in intact plant.

2. Alkaloids in *in vitro* cell cultures

Secondary metabolites are generally synthesized in very low level in plants (only up to 1% of total carbon) as they have specific functions. Mostly, they serve in plant protection against biotic and/or abiotic stresses. The use of biotechnology strengthens the efforts to improve the yield of a desired compound. Cultured plant cells can be considered as factory cells that produce secondary metabolites due to their capability for bio-transformations. Thus, some processes such as hydrogenation, dehydrogenation, isomerization, glycosylation, hydroxylation, and addition of carbon atoms might be performed in plant cell cultures.

The biosynthesis pathways of indole alkaloids and the others have been investigated for many years, but the whole process is not completed yet. Good knowledge on the mechanism of alkaloids biosynthesis in cell cultures and cultural condition will considerably enhance the production of natural alkaloids for medication. In plant, the tissue of synthesis is not always the same as the tissue of accumulation. Certain substance is derived from a precursor that

presents in one organ, and then the whole pathway from one intermediate to subsequent ones is performed in different organs/sites, each with its specific enzyme. When this is the case, the substance must be synthesized in differentiated plant where translocation takes place from one plant part to another. That substance might not be found in *in vitro* cells culture. Another case is that alkaloid biosynthesis and storage are in a particular organ or tissue; then performing *in vitro* cultures of a specific organ or tissue is likely to get the desired substance. When biosynthesis and accumulation occur in any cells of a plant, the alkaloid of interest can be produced in the culture [6].

It was suggested that the synthesis and storage of quinoline alkaloids in Cinchona plant is determined by the extent of differentiation. Experiments with hairy roots culture of Cinchona [7, 8] resulted in an increased yield of the alkaloids. In mature plant of *C. roseus*, genes expression for tryptophan decarboxylase (TDC) and strictosidine synthase (STR), both are among the key enzymes for terpenoid indole alkaloids (TIAs) biosynthesis, were detected much higher in roots than in the aerial part of the plant [9]. The research results led to the integration of *Cinchona officinalis* hairy roots with *tdc* and *str* genes originated from *C. roseus*, in an attempt to increase the alkaloid production [10]. Indeed, the transformed hairy roots culture produced higher level of quinoline alkaloids, suggesting that in *Cinchona* spp., roots may be the main site for those two enzymes. Many other researchers reported that undifferentiated cultured cells of Cinchona exhibited quinine alkaloid in control medium [11, 12] as well as in elicitors treated medium [12]. This means that all Cinchona cells can synthesize the alkaloids in some ways.

Similar stories also applied to reserpine biosynthesis in *Rauwolfia serpentina*. Reserpine was found in cells or callus culture of the plant [13, 14] as well as in its shoots culture [15], while naturally people collect reserpine from its roots. Dimeric TIAs, vinblastine and vincristine, have been detected in higher level in new-formed roots and callus-roots cultures of *C. roseus*, compared to that existed in its petiole [16]. Those examples suggest that *Rauvolfia* spp. and *Catharanthus* spp. are capable to synthesize alkaloids regardless of the tissue type as biosynthetic site.

Regenerated shoots of *Papaver bracteatum* contain much higher thebaine/morphine alkaloids than meristemoids or callus. The shoots have laticifer-like cells and tracheids, whereas meristemoids and callus occasionally do not show the presence of laticifer [17]. It was reported also that only greenish callus with tracheary elements produced thebaine and codeine in *P. somniferum* [18]. Ginsenoside is specifically produced in ginseng roots; therefore, *in vitro* root culture is required. Similarly, herbal plants such as *Hypericum perforatum* (St. John's wort), which accumulates hypericins and hyperforins in foliar glands, have not demonstrated the ability to accumulate the phytochemicals in undifferentiated cells [19]. Those cases indicate that cytodifferentiation has correlation with alkaloids synthesis, and most presumably cells culture of those certain plant species cannot produce the corresponding alkaloids. **Table 1** summarizes some alkaloids that have been successfully synthesized in *in vitro* culture.

Alkaloids	Plant source	*In vitro* culture	Yield (%)	References
Indole alkaloids				
Serpentine	*Rauvolfia serpentina*	Cells suspension	0.03-0.06	[13]
		Plantlets	4.02-5.76	[15]
	Catharanthus roseus	Regenerated roots	0.06	[16]
Vinblastine	*Catharanthus roseus*	Regenerated roots	0.0005	[16]
Vincristine		Regenerated roots	0.01	[16]
Ajmalicine	*Catharanthus roseus*	Compact callus	0.003-0.005	[32]
		Cells suspension	0.16-0.7	[31, 36, 40, 57]
Reserpine	*Rauvolfia tetraphylla*	Callus	0.09-0.21	[14]
	Rauvolfia serpentina	Callus	0.1	[53]
Quinoline alkaloids				
Quinine	*Cinchona ledgeriana*	Hairy roots	0.058-0.23	[7, 8]
		Cells suspension	0.12-10.90	[12, 26]
	Cinchona officinalis	Hairy roots	0.05	[10]
Isoquinoline alkaloids				
Thebaine/codeine	*Papaver bracteatum*	Callus	0.01/0.0033	[17]
	Papaver somniferum	Embryogenic callus	0.0025/0.012	[84]
Berberine	*Coscinium fenestratum*	Cells suspension	0.004-0.02	[85]
	Thalictrum rugosum	Cells suspension	0.0015	[86]
Tropane alkaloids				
Atropine	*Datura metel*	Hairy roots	0.13-0.26	[60]
	Anisodus acutangulus	Hairy roots	0.0004-0.0007	[44]
Scopolamine/hyosciamine	*Brugmansia candida*	Hairy roots	0.24/0.06	[45]
Hyosciamine	*Hyosciamus muticus*	Callus	3.72-4.25	[61]
Terpenoid alkaloids				
Aconitine	*Aconitum napellus*	Cells suspension	0.035-0.043	[62]
Amaryllidaceae alkaloids				
Galanthamine	*Leucojum aestivum*	Plantlets	0.068–0.05	[41, 42, 58]
		Shoots	2.0–9.7	[37, 43]

Table 1. Report on some alkaloids produced in *in vitro* culture.

2.1. Biosynthesis pathway of alkaloids: different facts in cell cultures

TIAs are derived from amino acid tryptophan. **Figure 1** shows the branching pathways of many TIAs biosynthesis. Tryptophan combines with secologanin to form strictosidin. It seems that strictosidin is the intermediate base for many TIAs. From this stage, reserpine is synthesized. Strictosidin which is de-glycosylated to strictosidine aglycoside will convert to dehydrogeissoschizine. This compound in separate way via corynantheal will become cin-chonidinone that by a reductive reaction forms cinchonidine and then quinine. Two

NADP-dependent enzymes specific to cinchona quinoline biosynthesis have been identified [20]. On the other hand, Alahmad *et al*. [21] reported that quinine is detected in some plants experiencing biotic stresses where quinine is presumably derived from phenolic compounds through the activity of polyphenol oxidase and/or phenol oxidase.
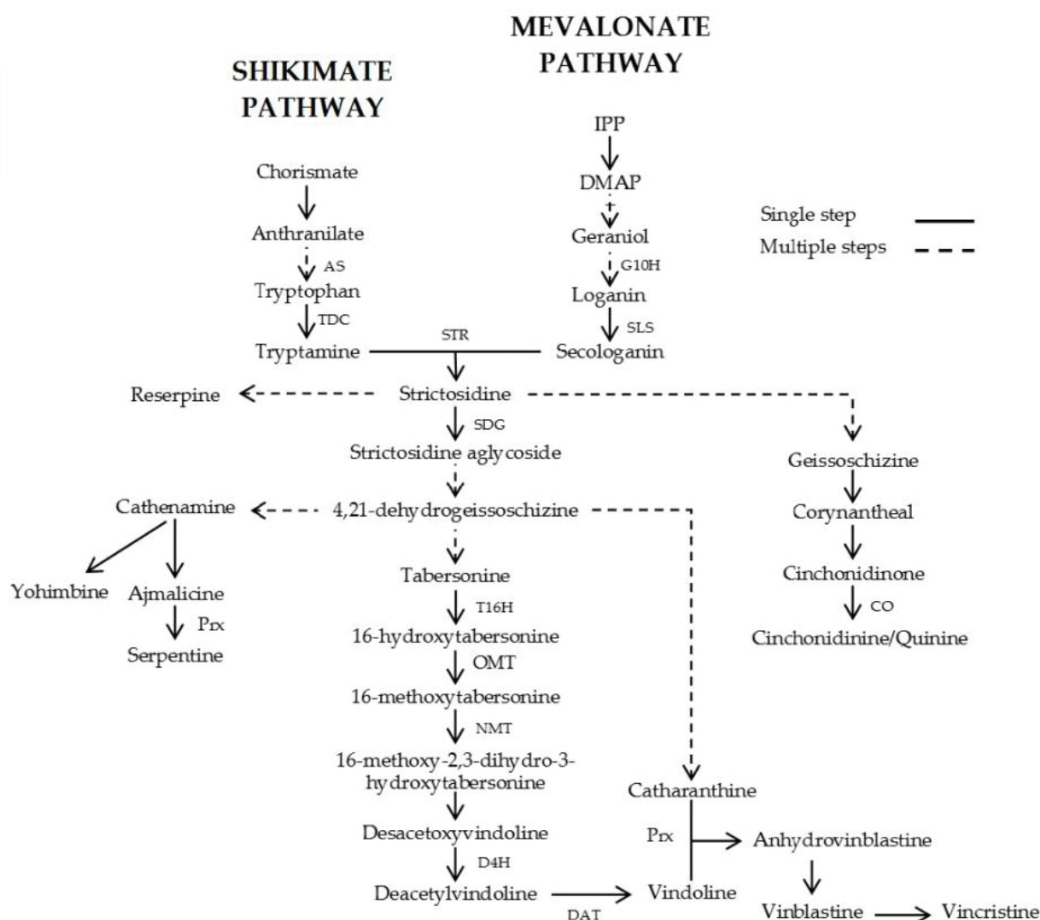
Figure 1. Biosynthesis pathways of terpenoid indole alkaloids (TIAs).

Dehydrogeissoschizine through six further steps via tabersonine pathway would arrive to form vindoline, with which catharanthine will be converted to bisindole alkaloids vinblastine and then vincristine. Dehydrogeissoschizine via cathenamine pathway produces yohimbine and ajmalicine. By oxidation, the latter compound becomes serpentine [20, 22, 23]. **Figure 2** demonstrates the basic molecular structures of strictosidine and its essential alkaloid derivatives.

The initial source for berberine biosynthesis is ʟ-phenylalanine. From phenylalanine, through reticuline, coulerine, and canadine consecutively, berberine is produced. It belongs to the group benzyl isoquinoline alkaloids. The biosynthesis of atropine starting from ʟ-phenylalanine too first undergoes a transamination forming phenylpyruvic acid which is then reduced to phenyl-lactic acid. The latter compound combines with co-A to become tropine. Following many further steps, it converts to hyoscyamine which is then racemized to atropine [24].
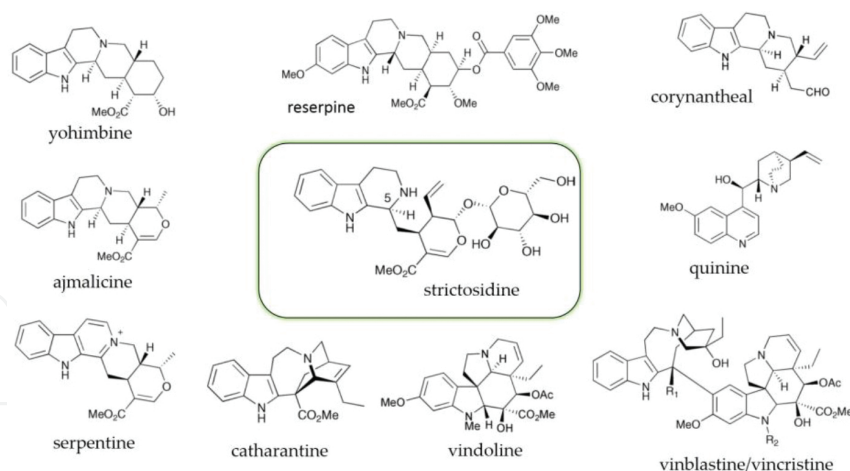
Figure 2. Strictosidine as central basic molecule of TIAs and its derivative molecules.

The biosynthesis of galanthamine also starts with enzymatic conversion of L-phenylalanine. The enzyme phenylalanine ammonialyase (PAL) converts it to protocatechuic aldehyde, and L-tyrosine, which includes the enzyme tyrosine decarboxylase producing tyramine. The junction of the tyramine and the protocatechuic aldehyde results in Schiff's base, which is converted to norbelladine. Norbelladine or related compounds undergo an oxidative coupling in Amaryllidaceae plants. 4-O-methylnorbelladine is considered as a key intermediate. The biosynthesis of galanthamine involves the phenol oxidative coupling of O-methylnorbelladine, and then following further steps, galanthamine is produced [25].

By identifying the pathway, the application of a precursor in culture media is a common practice. In *C. pubescens* cells, tryptophan as a precursor, fed in the culture media, did not improve quinoline alkaloids level [11, 12], but *C. ledgeriana* cells successfully increased the synthesis of cinchonidine by tryptophan feeding [26]. Culture media, with or without Cinchona cells in it, treated with tryptophan accumulated β-carboline alkaloids and indole-3-aldehyde [11, 27]. It indicates that tryptophan does not go along the pathway to quinoline alkaloids biosynthesis in Cinchona-cultured cells. Moreover, it was reported by [28] that simple β-carboline alkaloids such as tetrahydro-β-carboline-3-carboxylic acid and 1-methyl-tetrahydro-β-carboline-3-carboxylic acid are readily formed from tryptophan as well as from tryptamine by Pictet-Spengler reaction in food and beverages.

Feeding geraniol, 10-hydroxygeraniol, or loganin to a *C. roseus* hairy root culture resulted in significant increases in the accumulation of tabersonine [29], but the addition of tryptophan or tryptamine separately had no effect. It is presumed that only the early parts of the pathway are present in the hairy roots cultures where certain enzymes involved considerably in alkaloid production. An elicitor, jasmonic acid, combined with feeding either loganin or tryptamine did not further enhance the accumulation of indole alkaloids [30]. Precursors loganin, tryptamine, or their combination were fed to noninduced and methyl jasmonic acid (MeJA)-induced cultures. TIA production was not significantly enhanced in either noninduced or MeJA-induced cultures with precursor feeding. It seems that in noninduced cells, steps downstream

of loganin and tryptamine were disturbed because of the accumulation of loganin or trypta-mine in the cells with precursor feeding.

An increase in reserpine content was observed in 50 mg L^{-1} tryptophan fed callus culture than in other concentrations in *R. tetraphylla* callus [14]. Addition of secologanin increased the alkaloid ajmaline production 10-folds in *C. roseus* [31]. More results were reported [32] that succinic acid, tryptamine, and tryptophan feedings also significantly increased ajmalicine and catharanthine production by compact callus cultures of *C. roseus*, while geraniol feeding inhibited biomass and alkaloid accumulation. Treatment on the whole plant culture of *R. serpentina* with tryptamine (1.0 mg L^{-1}) alone resulted in enhanced reserpine production while the highest reserpine yield was obtained after 36 days of elicitation in cultures treated with 0.1 mmol salicylic acid and enriched with 1.0 mg L^{-1} tryptamine [15]. Research on the utilization of precursor to upscale *in vitro* production of other alkaloids is still limited, probably due to limited revelation of the biosynthesis mechanism.

2.2. Factors affecting alkaloids *in vitro* production

2.2.1. Propagules as the initial plant material

Theoretically all plant parts are capable of producing callus which then grows undifferentiated or differentiated. We have discussed above that some alkaloids are synthesized in particular organelles or organs, meaning that cytodifferentiation is required. An identification process for the most productive cell clones or propagules is important to be done, as the initial cultured plant material.

Cell type-specific localization applies also for berberine biosynthesis and accumulation which are temporally and spatially separated. In a berberine-producing plant, *Thalictrum flavum*, it was reported that the transcripts of its nine biosynthetic genes were confined to the pericycle and adjacent cortical cells of roots, and protoderm of leaf primordia, whereas berberine accumulation occurred in the root's endodermal cells, also in pith and cortex of rhizomes [33]. Based on this report, the use of root tissues or transformed hairy roots can be considered.

2.2.2. Plant growth regulators

Increasing or lowering the growth regulators in culture media gives impacts in alkaloids production. Growth regulators determine secondary metabolites biosynthesis as well as biomass production. It is well known that growth promoters such as auxins and cytokinins have important roles in plant growth and differentiation. However, it is also recognized that secondary metabolites are synthesized when the plant growth rate is decreasing, meaning when the plant starts to senesce or becomes stressed due to biotic or abiotic environment, secondary metabolites, including alkaloids, will appear. Based on this contradiction, a balanced treatment between growth promoters and stress-creating substances (growth inhibitors) has to be taken into account.

It was reported that auxins negatively influence alkaloid biosynthesis and accumulation at all levels in *C. roseus* cell culture [34]. Subculturing cells on an auxin-free medium results in

increased *Tdc* and *Str* mRNA levels, while the addition of auxins rapidly decreases the *Tdc* mRNA. During the growth phase, 2,4-D strongly inhibits alkaloid production, but it recovers during the stationary phase. This is the reason that auxins are commonly added to the medium for callus induction, but they are added at a low concentration or omitted for the production of secondary metabolites. By contrast, the addition of cytokinin zeatin to an auxin-free *C. roseus* cell cultures resulted in an increase in ajmalicine alkaloid accumulation [35]. It was reported [36] that cytokinins and ethylene increased alkaloid accumulation in periwinkle callus or cell suspension cultures. It was mentioned that either exogenously applied cytokinins or ethylene (supplied by ethephon) greatly enhanced ajmalicine and serpentine accumulation in cells subcultured in a 2,4-D-free medium. Ethylene precursor, 1-aminocyclopropane-1-carboxylic acid, amplified galanthamine and lycorine content to sixfold in *Leucojum aestivum* while ethylene was reducing [37].

Abscisic acid (ABA) regulates various aspects of plant growth and development including seed maturation and dormancy, as well as adaptation to abiotic environmental stresses [38]. A report [39] stated that ABA stimulated accumulation of catharanthine and vindoline in *C. roseus*. Treatment of precursors fed *C. roseus* cells with ABA did not induce the accumulation of alkaloids but it delayed the catabolism of strictosidine [40]. In *C. ledgeriana* cell culture, ABA (1–3 mg L^{-1}) exhibited a growth promotion compared to the control medium containing picloram as an auxin source and BA, while the quinine content is comparable to that of ABA-free medium [12, 26], whereas paclobutrazol (PBZ) reduced the cell growth but induced significantly the quinine production [12].

There were variations in the accumulation of galanthamine in *L. aestivum in vitro* cultures grown in media with various combinations of growth regulators. In the absence of growth regulators, the amount of galanthamine was 0.0011% (DW). When cultivated in media with 10 μM α-naphthyl acetate and 0.5 μM BA, the highest yield was obtained, 0.0068% (DW) [41]. Cytokinin thidiazuron demonstrated better effect in *L. aestivum* shoot culture in which galanthamine biosynthesis achieved maximum yield of 0.05% (DW) [42].

2.2.3. Culture conditions

2.2.3.1. Nutrient and pH of culture media

Mineral and organic substances that are commonly incorporated in culture media are subject to modification. For galanthamine, the optimization of nitrate, ammonium, phosphate ions, and sucrose concentration increased the production in *L. aestivum* shoot culture [43]. The nitrogen concentration and NH_4^+/NO_3^- molar ratio of the culture medium often influence the synthesis of alkaloid. In *Anisodus acutangulus* hairy roots culture, cell growth and alkaloid yield were inhibited at low or high total nitrogen concentrations and the most favorable concentration of nitrogen for the maximum biomass and the highest tropane alkaloid yield was found to be 90 mM (NH_4^+/NO_3^- = 4:1). Regarding carbon source, medium containing sucrose, glucose, fructose, and galactose was compared; 3% sucrose gave the best yield in terms of cell growth and metabolism resulting in alkaloid productivity among different carbon sources used [44].

Medium component is not only the ingredients, but also pH. At pH 5.5 and at certain concentrations of acetic acid, the content of scopolamine and hyoscyamine increased in root cultures of *Brugmansia candida* and also promoted the release of both alkaloids into the medium. Lowering the pH to 3.5 and 4.5 reduced the accumulation of both alkaloids, but at a pH of 4.5, their release increased significantly. Acetic and citric acid stimulated the release of scopolamine and hyoscyamine, presumably due to permeability change of cell membrane [45]. A hairy root line, which was induced from leaves of *A. acutangulus*, was subjected to pH variation in the culture medium [44]. The biomass yield of hairy roots grown at pH 6.5 was two times higher than that at pH 4.5. However, the maximum production yield of tropane alkaloid was reached at pH 4.5.

2.2.3.2. Light

Light is an environmental factor most frequently reported having significant effect in secondary metabolite biosynthesis. Light can affect plant differentiation, morphology, and metabolic activities. Cultures that need certain stage of shoot differentiation to produce a secondary metabolite require light, indicating that some enzymes for the biosynthesis will be activated by light exposure. It was reconfirmed [46] that in lupine (*Lupinus* spp.), enzymes of quinolizidine alkaloids (QAs) and the precursor lysine are localized in the chloroplast. Consequently, the alkaloid formation requires cell differentiation to greening tissue and is governed by light. The conversion of ajmalicine to serpentine involves the role of peroxidase. Light-grown cell cultures of *C. roseus* had a 20-fold higher vacuolar peroxidase activity compared to those of dark-grown cells and the accumulation of serpentine in light-grown cells was higher [47]. Vindoline biosynthetic pathway is also regulated by light as proven with callus culture of *C. roseus* [32, 48]. Maximum galanthamine production was achieved in cultures grown under light conditions. Galanthamine in the cultures under light is more than twice (73.8 μg g^{-1} DW) than those cultivated under dark conditions (38.5 μg g^{-1} DW) [25].

It seems that alkaloids biosynthesis in Cinchona, at least partially, is light regulated. In contrast to the abovementioned cases, transformed cells of *C. ledgeriana* grown in the dark produced a remarkable enhancement of alkaloid accumulation, 50 times greater than those cultured under the light [49]. Blue light was found detrimental to alkaloid accumulation, but red or green light has the same effect with the darkness. Furthermore, alternating the dark and light periods for every 28 days resulted in alternate high and low alkaloid productivity, suggesting that this was not simply an adaptation effect.

2.2.3.3. Temperature

Cultivation of *C. roseus* hairy roots at different temperatures was found to have an effect on growth rate and indole alkaloid content. When lowering the temperature, the roots responded by increasing the degree of unsaturation of cellular lipids, which was mainly due to an increased proportion of linolenic acid. The modifications in lipid composition might be necessary for the roots to retain the proper cell membrane fluidity at each temperature. Although changes in membrane lipids might happen, the distribution of indole alkaloids

between the roots and the medium was undetectable. Instead, the level of alkaloid accumulation in the roots increased significantly with lowering temperature [50].

2.3. Improvements to enhance *in vitro* alkaloids production

Plant alkaloids are usually produced in very low level, both in intact plant and in cell cultures. It depends greatly on the physiological and developmental stages of the plant or the plant cells. There are an increasing number of reports that plants and endophytic fungi produce secondary metabolites through mutualistic symbiosis. This issue is attracting, but the aseptic method of *in vitro* culture has to consider some modifications to benefice the involvement of endophytic microbes. Fungal endophytic *Curvularia* spp. and *Choanephora infundibulifera* have been found in *C roseus* enhancing vindoline content by 229–403% [51]. An example of producing a non-alkaloid substance, paclitaxel, has been reported [52]. Suspension cells of *Taxus chinensis* var. *mairei* was co-cultured with its endophytic fungi, *Fusarium mairei*, in a 20-L co-bioreactor for paclitaxel production. By using co-bioreactor that consists of two-unit tanks (10 L each) separated by a membrane, and then culturing *Taxus* suspension cells in one tank and the fungi in another, a desirable yield of paclitaxel was obtained in *Taxus* cell cultures. The co-cultured *Taxus* cell cultures produced 25.63 mg L^{-1} of paclitaxel within 15 days or equivalent to 1.71 mg L^{-1} per day and 38-fold higher than that by uncoupled culture (0.68 mg L^{-1} within 15 days).

Many other metabolic manipulations can be applied along with genetic engineering in attempting a higher production of desirable alkaloid compounds. To achieve an industrial scale of production, one has to obtain a stable, high-producing cell line of the plant of interest. At least two approaches of metabolic manipulation are being considered: (a) metabolic improvements such as screening and selection for high-producing cell lines and the stimulation of biosynthetic activities through various methods, and (b) optimization of growth and production medium.

2.3.1. Metabolic improvements

2.3.1.1. Cells screening

Screening of germplasm and selection methods to obtain highly productive cell clones is suggested. *R. serpentina* callus culture was divided into two cell strains of different fluorescence under 365-nm UV light [53]. The results indicated that the yellow-green fluorescent cell strain produced much more reserpine than the blue-white strain. Screening on the slow-growing cells of 13-year-old cultures of *R. serpentina* through four successive subcultures into a liquid medium has revived the cells. After three further generations, the cells recovered their growth rate and enhanced reserpine production [13].

Transformed hairy root cultures of *C. roseus* established approximately 150 transformants from four different cultivars. They were screened for desirable traits in growth and indole alkaloid production. Five hairy root clones grew well in liquid culture. The levels of alkaloids ajmalicine, serpentine, and catharanthine in these five clones were higher compared with cell suspensions reported elsewhere; the experiment also indicated the presence of vindoline in two clones at

levels over three orders of magnitude, greater than the minute amounts reported in cell culture [54]. It was observed [55] from 11 cell lines of *C. roseus* derived from protoplast, 2 cell shapes, spherical and cylindrical. The production of ajmalicine and catharanthine was significantly greater when the cell aspect ratio (cell length/width) was more than 2.8.

2.3.1.2. Elicitors

In nature, a wide range of environmental stresses are threatening the plants. Secondary metabolites are frequently increased when the plant encounters to environmental stresses, biotic or abiotic. The phenomena found led us to believe that specific secondary metabolite functions as protective agents against the stresses. Based on those reasons, the use of artificial stresses or elicitors is common in *in vitro* cultures to enhance a desirable compound production. The most frequently used elicitors are high or low temperature, drought, medium salinity, growth retardants, microbial toxin, fungal carbohydrates, yeast extract, MeJA, and chitosan.

Polyethylene glycol (PEG) as a drought-creating agent incorporated in the callus culture media of *C. roseus* did not affect the production of vinblastine nor vincristine [56]. In contrast with ajmalicine, *C. roseus* cell suspension cultures treated with cadmium [57] resulted in the enhancement of ajmalicine content as well as TDC enzyme activity. Treatment with 250 mM mannitol and 4 g L^{-1} KCl as growth-stressing agents in compact callus culture of *C. roseus* yielded ajmalicine about four-fold higher than the control [32]. For quinine, paclobutrazol combined with mannitol in the media of *C. ledgeriana* suspension cultures significantly improved quinine content [12]. Galanthamine and lycorine were 1.36- and 1.67-fold higher compared to the control, respectively, achieved after elicitation with jasmonic acid in *L aestivum* cell culture [58]. The addition of MeJA at the start of the cultivation resulted in a two-fold increase in the concentration of galanthamine. Among the given elicitors, copper sulfate, silver nitrate, salicylic acid, and MeJA in *L. aestivum* shoot cultures [59], it was found that MeJA increased galanthamine biosynthesis the most.

Biotic (*Bacillus cereus* and *Staphylococcus aureus*) and abiotic (AgNO$_3$ and nanosilver) elicitors were added to the hairy root cultures of *Datura metel* [60]. All the elicitors influenced biomass accumulation and atropine production. Among the tested elicitors, nanosilver was the most effective in enhancing the hairy roots' atropine content. Chitosan increased the content of scopolamine and hyoscyamine in transformed roots of *B. candida* [45].

2.3.1.3. Optimization of culture conditions

Generally after having basic conditions required by cell cultures in regard to obtaining an acceptable cell growth rate containing certain level of alkaloids, it is necessary to optimize the culture media as well as the culture conditions to reach the maximum and consistent production of the desired substance. Some examples are given below.

Full-strength MS medium was the best for nourishing the growth of *Hyoscyamus muticus* cell cultures. The equal combination ratios of BA and NAA at 1.0 mg L^{-1} gave the highest biomass accumulation and alkaloid production of hyoscyamine. Culture grown under light was higher in their growth rate than that grown in the dark, but the alkaloid content was

relatively higher under dark condition. Furthermore, low inoculum density of cell suspension cultures (25 g L^{-1} (FW) presented a shortest doubling time (2.93 days), while those obtained with a high inoculum (100 g L^{-1} (FW) reached 13.47 days. At inoculum density of 50 g L^{-1} (FW), the highest dry cell weight and alkaloid content were attained [61].

To increase aconitine alkaloid production in cell culture of *Aconitum napellus*, liquid MS medium supplemented with 1 mg L^{-1} and kinetin 0.1 mg L^{-1} gave the best growth rate and aconitine yield. Cell density of 3 g (FW) per flask resulted in the highest amount of cell biomass, and the addition of salicylic acid and yeast extract combined with 5% sucrose improved the alkaloid level 2.5–3-fold higher compared to that from the control media [62].

By modification of a standard MS culture medium and further optimization with the inclusion of lactose as the carbohydrate source, NAA and kinetin as growth regulators, a system has been developed for *C. roseus* cell suspension cultures to elevate both the growth and catharanthine alkaloid accumulation in a single-stage culture of 14–21 days [39].

Basic practices related to metabolic production process of any substance is pre-requisite for scaling-up by using of bioreactor. This means that certain level of alkaloids productivity has been achieved in general with particular method of cells/tissue cultures. A laboratory scale of bioreactor or biofermenter is a large vessel, 10 L of maximum capacity. Culturing cells suspension in bioreactor for a substance production needs a balanced combination of technical design and internal culture conditions that may be created. Control of medium pH, dissolved oxygen, flow rate of gas exchange, nutrient supply, cells density, agitation speed, as well as temperature are among the factors of custodian importance [63, 64].

2.3.2. Genetic engineering

Many achievements have been made in modifying various metabolic pathways by using specific genes encoding biosynthetic enzymes or regulatory proteins. Full-length cDNAs namely *CrRR2* and *CrRR3* originated from a *C. roseus* cDNA library have been introduced into cell suspension culture *of C roseus*. *CrRR2* gene was expressed at a very low level, if any, that treatment by cytokinins did not trigger its transcription. The *CrRR3* gene expression is root-specific and the transcripts are transiently up-regulated. In cells suspension, transcript amounts of *CrRR3* remained unchanged even by the treatment with NaCl, abscisic acid, or jasmonic acid, but was increased with 10^{-6} M trans-zeatin [65].

In *C. officinalis* hairy roots [10], a binary plant vector construct whose T-DNA region contained constitutive-expression versions (CaMV35S promoter with double enhancer and *nos* terminator) of *tdc* and *str* cDNA clones was used from *C. roseus*. It was then combined with an intron possessing the β-glucuronidase (*gus*-int) reporter gene and a hygromycin phospho-transferase (*hpt*) selection gene. The products of TDC and STR, tryptamine and strictosidine, were found in high amounts, 1200 and 1950 mg g^{-1} (DW), respectively. Quinine and quinidine levels were found to rise up to 500 and 1000 mg g^{-1} (DW), respectively. However, the genes were not expressed anymore one year after. They had completely lost their capacity to accumulate alkaloids.

Based on the knowledge of biosynthetic pathways, a logic strategy can be made. Some efforts to induce up-regulation of the transcript levels of key pathway genes are also commonly performed, but the successes are not universal. In cultures of *C. roseus* elicited with MeJA (250 μM) on day 21, *g10h, tdc, str*, and *sgd* expression increased by three- to nine-fold after 24 h. Up-regulation of the gene expression was followed by a 160, 440, and 420% increase in strictosidine, ajmalicine, and tabersonine levels, respectively, after 5 days [30].

Genetic engineering is still promising for elaboration since the real problems encountered in stabilizing the alkaloids production after transformation or induction rest to be elucidated.

3. Alkaloids accumulation in cultured cells

The fact is that alkaloids are synthesized and accumulated in plants to cope with unfavorable environment. They hold adaptive significance to survive in diverse ecological situation. But a quite number of these compounds are toxic and can be dangerous to the producing plant itself [66]. Consequently, to maintain homeostasis, feedback inhibition of certain enzymes in the biosynthesis pathway can occur in particular cell compartment that leads to cease further alkaloids production. As a prerequisite for the defense function, however, the sequestration of a respective alkaloid to a critical amount is necessary, then the compound is frequently confined in vacuoles of specialized cells, as a means of detoxification.

3.1. Secretory structures

Plants have various specialized cells where a wide variety of products are often synthesized and/or stored, namely secretory cells. External secretory structures include trichome, gland, and nectary, but alkaloids are found mostly in internal structures such as idioblast, laticifer, or secretory cavity and canal [67]. They are isolated cells that differ from the surrounding cells.
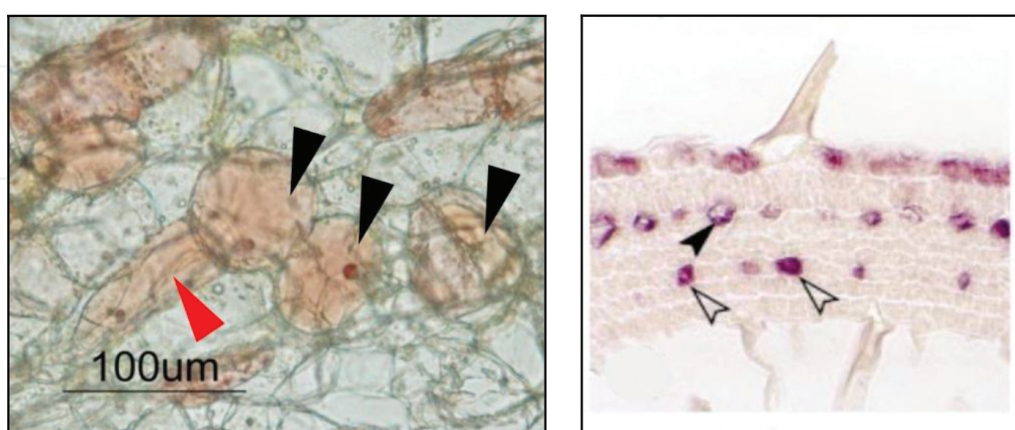
Figure 3. Spheric and elongated laticifers containing alkaloids among the cell aggregates cultures (left); idioblasts (open arrowheads) and laticifer (solid arrowhead) in an intact leaf (right) of *Catharanthus roseus* (left: courtesy of Iskandar and Iriawati [56]; right: with permission of the American Society of Plant Physiologists from [48]).

It was reported [48, 68] that vinblastine and vincristine are confined in idioblast and laticifer cells located among the palisade and spons cells, whereas catharanthine is found in leaf cuticle, and specialized cells of cortex [69]. According to [70], the capacity of alkaloid synthesis in *C. roseus* depends on the number of cells accumulating the alkaloid compounds. The genes for the enzymes involved in the final stages of those two bisindole alkaloids synthesis, D4H and DAT, are only expressed and activated within the idioblast and laticifer. Those cells are also the site of the secondary metabolite accumulation [48, 71, 72]. **Figure 3** demonstrates the existence of secretory cells of *C. roseus* in callus culture and in intact leaf, suggesting that in *in vitro* cultures, such a structure can be regenerated.

In *Papaver somniferum*, three different sites have been identified; benzylisoquinoline alkaloids were stored in laticifers, the biosynthetic gene transcripts were restricted to companion cells, and the enzymes were detected in sieve elements [73].

Cinchona bark does not exhibit any specialized structure that may be the site of biosynthesis and/or storage of quinoline alkaloids. It was suggested [74] that quinoline alkaloid is located most probably in intercellular spaces of the bark, which are schizogenous, lysigenous, or schizo-lyzigeneous secretory ducts or canals. These are tube-like structures containing and producing secretions. No report on secretory structures in cultured Cinchona cells is found so far.

3.2. Alkaloids accumulation and harvest

Three factors affecting the yield of secondary metabolite should be considered, for example, biosynthesis of the product, accumulation of the product, and breakdown of the product. The actual yield will be the result of biosynthesis minus breakdown of the product, which provides storage in a certain cellular compartment as a mechanism of avoiding autointoxication of the producing cells [75].

Isoquinoline alkaloids, for example, berberine, are also synthesized in small vesicles which later fuse with tonoplast to release the alkaloids content into the vacuole. Both *Coptis japonica* and *Thalictrum flavum* cells, which have an ability to synthesize berberine, took up exogenous berberine from the culture medium to accumulate it exclusively in vacuoles. By contrast, *T. minus* cells, which excrete indigenous berberine mostly into the medium, did not take up exogenously supplied berberine [76]. Vacuole seems to serve as particular compartment to store high concentration of secondary metabolites, since some of them might be toxic for the producing plant itself when they exist in higher amount than needed for particular function, for instance in the defense or signaling [6]. A study on *C. ledgeriana* reconfirmed this [77]. The toxicity of cinchona alkaloids to cell cultures was related to alkaloid uptake from the medium with the purpose for selecting high-yielding cell lines. Quinine, the strongest, was completely toxic at 5.5 mM. Both quinine and quinidine were more toxic than their unmethoxylated precursors, cinchonidine and cinchonine.

Elimination of feedback inhibition of metabolic enzymes and inhibition of membrane transport can be made by certain products newly synthesized in a second phase introduced into liquid culture medium. Organ culture often demonstrates separate sites of synthesis and storage of

secondary metabolites [78] which facilitates to avoid a saturated condition of a substance. Some *in vitro*–cultured cells have also different subcellular compartments for biosynthesis and storage of secondary metabolites. Increasing the permeabilization of vacuolar and cell membranes would let the metabolites excretion to occur into the medium or into the apoplast. Some chemicals have been used to alter membrane permeabilization,. Cd ranging from 0.05 to 0.4 mM for 24–48 h increased ajmalicine production and excretion into culture medium, particularly for cells at the mid-exponential growth phase [57], Tween 20 added for 24 h to the medium, after 2–4 weeks, the hairy roots line of D. *metel* culture excreted scopolamine into the culture medium, from 8.7 to 70% [79], or Tween 80 in roots culture of *D. inoxia* elevated hyoscyamine release in the medium [80].

3.3. Industrial-scale production of alkaloids by cell cultures

The high production cost to extract a secondary metabolite that creates the consequence of high price of the product has been described. The small taxol yield of *Taxus* species requires 10,000 kg of bark to produce 1 kg of taxol [81]. In 1993, it was reported that quinine and quinidine were produced at 300–500 metric tons yearly, which were extracted from 5000 to 10,000 metric tons of Cinchona bark [75]. Concern was raised about the environmental impact of the sourcing, leading to search for alternative sources of the alkaloid. In the end, an alternative environment-sustainable approach using plant cell culture has been developed.

Large-scale production of plant secondary metabolites through plant cells culture in bioreactors is technically feasible [64, 75]. However, the cost-benefit of such a production is still the major barrier. For some pricey products, it is still feasible. Another obstacle is that unfortunately some of the most interesting products exist only in very small amounts or are not at all produced in plant cell cultures. Hence, for industrial production, two crucial questions were raised [75], whether plant cells can be grown in large fermenters and whether the price of a product from such a large-scale plant cell culture will be competitive with conventional production methods. The questions are still relevant nowadays for some extent. The answers remain case-dependent, as during these 30 years several industrial-scale productions of secondary substances indeed have been developed [64].

In another group of secondary metabolite paclitaxel, Phyton Biotech [81] and Samyang Genex Corporation [82], both are the largest paclitaxel producer, have successfully increased the productivity of taxanes by using plant tissue culture in large scale fermenters and through the application of several strategies. By selecting high-yielding cell lines, Mitsui group produced berberine on a large scale with a productivity of 1.4 g L^{-1} over 2 weeks [83].

4. Conclusion

The facts that plant alkaloids can be produced by cultured cells have proven that cell culture system is feasible to produce enhanced yield of desirable substances as an alternative for natural sources. Plant cells have plasticity in some extent and are subject to manipulations.

Therefore, substantial advanced and continuous researches still need to be established, particularly in the biosynthetic pathway, the enzymes involved and their properties, the sites of each reaction step, the factors affecting those steps of biosynthesis and accumulation, and the consistency, as well as factors affecting the respective genes' expression in cultured cells. Specific cell type of a plant species necessitates a detailed exploration for its requirements to produce certain alkaloids.

Collective reports from all over the world will be likely to improve the basic knowledge as well as the technical approaches that will promote the development of a stable cells system serving as technological matter. Industrial production of secondary metabolites in bioreactors is not an impossible matter in the near future. It has been developing with steady progress, particularly for certain alkaloids.

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