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A Novel Approach for Production of Colchicine as a Plant Secondary Metabolite by *in Vitro* Plant Cell and Tissue Cultures

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Abstract: The secondary metabolites synthesized by plants are economically important chemical compounds in the agricultural and industrial areas such as food, perfumery and pharmaceutical sectors. In recent years, attempts for their production by *in vitro* plant cell and tissue cultures have been accelerated considerably. Colchicine, the principle secondary metabolite of *Colchicum autumnale* L. and *Gloriosa superba* L., is an important alkaloid that has poison effect used for treatment of various diseases and plant breeding studies. Presently, colchicine has been produced by using the seeds of *C. autumnale* L. and the tubers of *G. superba* L. through different chemical extraction methods. Applying *in vitro* plant cell and tissue cultures together with metabolic and genetic engineering techniques, large-scale production of colchicine can be achieved from the above two plant species.

Key words: Colchicine, plant secondary metabolite, *Colchicum autumnale*, *Gloriosa superba.*

1. Introduction

Plant secondary metabolites play major role for survival of plants in their ecosystems such as being chemical defender against microorganisms, insects, predators, etc. and attractant of pollinators [1]. They include alkaloids, terpenoids, phenolic compounds, tannins and glucosides which have been used for commercial applications as drug, fragrance, flavor, dye, insecticide, etc. [2]. Alkaloids are a group of organic nitrogenous compounds with complex chemical structures isolated from some plants and are found in approximately 20% of plant species. Benzylisoquinoline alkaloids (BIAs) are a very large and diverse class of alkaloids and contain varied physiologically active members such as colchicine, emetine (an antiamoebic), morphine and codeine [3].

Colchicine is the principle alkaloid of *Colchicum autumnale* L. and *Gloriosa superba* L. but also found in some other genera belonging to the Colchicaceae family, namely *Androcymbium*, *Merendera*, *Sandersonia* and *Littonia* [4]. The main commercial source for colchicine is obtained from two members of the Colchicaceae family, *Colchicum* species and *G. superba* L., as they have higher colchicine content than the other species. *C. autumnale* L. seeds contain the highest concentration in the plant, which is 0.5%-1.2% of dry weight, while the corm contains 0.6% colchicine [5]. On the other hand, it was reported that *G. superba* L. seeds gave a yield of 0.61% of dry weight and tubers from the plants from which the seeds were obtained yielded 0.9% of dry weight [6].

Despite the fact that is poisonous, colchicine has many medicinal uses, including treatments of diseases especially gout, cirrhosis of the liver, Familial Mediterranean fever (FMF), Behcet's disease [7]. On the other hand, colchicine has been used for artificially inducing polyploidy in plants. Due to its antimitotic properties, colchicine prevents spindle formation at metaphase of dividing cells and leads to doubling in chromosome number. The advantages of

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increased chromosome number include production of large flowers or fruit, increased hardiness, increase in size of various organs or change in season of maturity, novel flower coloration, improved drought tolerance and increased photosynthetic capacity, etc. [8].

There are currently two major approaches for the manufacture of plant secondary metabolites: firstly through the harvesting and extraction of whole plant parts, and secondly through synthetic organic chemistry. The production by plant is not always satisfactory because it is often restricted to a genus or species and might be activated only during a particular growth or developmental stage, stress or nutrient availability conditions [1]. Therefore, a lot of effort in the past years has been put into plant cell cultures as a possible production method for plant secondary metabolites of commercial interest. The use of plant cell and tissue cultures for production of secondary metabolites under controlled conditions has many advantages rather than the whole plant [9]. However, the production of many of the pharmaceuticals is too low in the cultured cells, even in the large-scale bioreactors, because the yield is economically insufficient and costly in many cases. Therefore, in recent years genetic engineering has been incorporated into plant cell cultures to improve the production of desired compounds by manipulating the plant secondary metabolism [10].

The production of colchicine by chemical synthesis is not economically feasible compared to extraction from the plant due to considerably low product yield. Recently, attempts have been made by a few researchers for tissue and cell cultures on *C. autumnale* L. [11, 12] and *G. superba* L. [13, 14]. It was reported that callus and root cultures of *C. autumnale* L. and *G. superba* L. were capable of producing colchicine and the accumulation was stimulated by precursor feeding [15]. Consequently, *in vitro* plant cell and tissue cultures combined with genetic and metabolic engineering techniques can be a new promising approach to produce colchicine as a secondary metabolite from *C. autumnale* L. and *G. superba* L.

2. Plant Secondary Metabolites

The secondary metabolites are an extremely various group of natural products synthesized by bacteria, fungi, algae, plants and animals. Plant secondary metabolites do not affect plant life as much as primary metabolites, which have active functions in the photosynthesis and respiration, but may play a crucial role in the adaptation of plants to their environments. They are essential for plants in point of defense against pest and diseases, attraction of pollinators, etc. to ensure survival of plants in their ecosystems [2]. It has been proposed that the genes involved in secondary metabolism procure a "genetic playing field" which allows mutation and natural selection to fix beneficial traits through evolution [16].

Besides about 25% of all drugs used in western medicine are derived from plant natural compounds, almost 80% of the world population depends on traditional medicinal plants for their primary health care. Morphine, codeine, atropine, scopolamine, vinblastine, vincristine, digoxin and paclitaxel are some examples of pure compounds from plants used for manufacturing of drugs in the pharmaceutical sector [17]. Moreover, some of plant secondary metabolites have been used economically in the food sector as flavors and natural additives for taste (e.g., vanillin from *Vanilla plenifolia*, monellin from *Dioscorephyllum cumminsii*, humulone from *Humulus lupulus*, etc.) and color of foods (e.g., betalain from *Beta vulgaris* and lycopene from *Lycopersicum esculentum*, etc.), in the perfumery and cosmetic sectors as fragrances (e.g., essential oils obtained from *Lavandula officinalis*, *Jasminum* spp., *Rosa damascena*, etc.) and in the agricultural sector as biopesticides (e.g., pyrethrin from *Chrysanthemum cinerariafolium*, nicotine from *Nicotiana tabacum*, etc.) [18].

Plant secondary metabolites can be classified in

different ways according to their biosynthetic origins, plant origins or chemical characteristics. They are simply classified into three main groups based on their biosynthetic origins: terpenoids (such as plant volatiles, cardiac glycosides, carotenoids and sterols), phenolics (such as phenolic acids, coumarins, lignans, stilbenes, flavonoids, tannins and lignin) and nitrogen containing compounds (such as alkaloids and glucosinolates). Examples of their classification in point of their plant origin are the opium alkaloids, *Strychnos* alkaloids and *Digitalis* cardenolides. Based on their chemical characteristics, they can be classified into a number of groups, such as alkaloids, characterized by a basic nitrogen function, or phenolics, which are characterized by aromatic ring systems having a phenolic hydroxyl group, or other groups with a certain type of basic skeleton, e.g., anthracene, coumarine, quinone, indole, isoquinoline, etc. [19].

Alkaloids, which are defined as pharmacologically active basic compounds synthesized by living organisms, containing one or more heterocyclic nitrogen atoms and derived mostly from amino acids, compose a very large group of secondary metabolites. Plants are estimated to produce more than 21,000 different alkaloids coming from different biosynthetic pathways [3]. Many of alkaloids in plants are toxic and can cause death even in small quantities providing defense against herbivores and pathogens. BIAs represent approximately 2,500 elucidated structures possessing potent pharmacological properties and include the narcotic analgesics morphine and codeine, the muscle relaxant papaverine, the antimicrobial agents sanguinarine and berberine, and the anti-inflammatory & anti-mitotic agent colchicine [20].

3. Colchicine

Colchicine ($C_{22}H_{25}NO_6$), a well-known toxic compound and named after *C. autumnale* L., commonly known as autumn crocus, meadow saffron or naked lady, is the largest group of alkaloids within the family Colchicaceae. It is soluble in water, freely soluble in alcohol and in chloroform, and slightly soluble in ether. Being a microtubule-depolymerizing agent, colchicine interferes with the microtubular cytoskeleton, which plays a crucial role in the cell cycle and in mitosis, by inducing microtubule depolymerization preventing the formation of the mitotic spindle. As a result, chromatids fail to move to the poles at metaphase of mitosis and consequently become enclosed in a new nuclear membrane and proceed to interphase as a doubled number of chromosomes. Because colchicine binds to tubulin to interrupt microtubule dynamics, it has been used in tubulin binding assays as a positive control [21].

3.1 Usage of Colchicine

The autumn crocus has been used as a medicinal plant for more than 3,000 years. The corm of autumn crocus was mentioned in the London Pharmacopoeia between 1618 and 1639, after which it was removed until the 1788 edition [22]. Treatment with *C. autumnale* L. was reintroduced by the French army officer Nicolas Husson in 1780 due to its particular efficacy on gout. Colchicine-based medicines have been continuously employed as a remedy for especially acute attacks of gout since the very early 19th century [23]. In 2009, the U.S. Food and Drug Administration approved the use of colchicine for treatment of acute gout flares in adult patients and the prophylaxis of gout flares in patients aged > 16 years, based on clinical experiments defining dosage and efficacy [24]. Today colchicine has been used for also treatments of FMF, cirrhosis of the liver, and Behcet's disease. Although its use has been limited because of its toxicity, colchicine can still be used as a lead compound for the generation of potent anti-cancer drugs.

Due to its anti-mitotic activity, colchicine was determined as a "mitotic poison" in the early 1930s. Since 1937, when its use for production of polyploid

plants was described, colchicine has been used extensively as a "genome doubling agent" in the plant breeding. However, *in vitro* colchicine-induced polyploidy is characterized by low induction rates and a high frequency of chimeras depending on the concentration of colchicine, duration of exposure, explant type and tissue penetrability [25].

3.2 Sources of Colchicine

Investigation of crude alkaloid extracts with mass spectrometry has confirmed that colchicine is present in, and restricted to, the family Colchicaceae [26] consisting of 15 genera and about 280 species, of which more than half belong to genus *Colchicum* [27]. Plants of both genera *Colchicum* and *Gloriosa* contain colchicine in toxic amounts. By applying an approach of taking phylogenetic classification into account for the evaluation, Larsson and Ronsted (2014) assorted Colchicaceae alkaloids under eight different structural types, such as phenethylisoquinolines, homoproaporphines, homoaporphines, androcymbines, colchicines, allocolchicines, lumicolchicines and homoerythrinans [28].

The genus *Colchicum* includes 159 species, most of which are endemic in Southern Europe, Northern Africa and the Middle East [27]. Within these species, *C. autumnale* is the major source of tropolone alkaloids and the overall concentration fluctuates from 0.1% to 2% in different plant parts. The alkaloid content ranks from 0.15% to 0.4% in fresh leaves, 0.1% to 0.6% in corms, 0.5% to 1.2% in seeds and 1.2% to 2% in fresh flowers. Although the highest colchicine level is occurred in flowers and seeds, all parts of plant are toxic. Colchicine contributes 50%-70% of the total alkaloid content along with minor amounts of demecolcine, colchicoside, and various related tropolone derivatives [5].

G. superba L., belongs to genus *Gloriosa*, is an ornamental climbing herb native of tropical Asia and Africa and its roots and tubers have been used in traditional Indian medicine. It has been reported that *G. superba* tubers contain about 0.9% colchicine and its levels are the highest during the initial growth of plant and decline during maturation [6]. Therefore *G. superba* has been utilized as alternative source for commercially production of colchicine.

4. Production of Secondary Metabolites by *in Vitro* Plant Cell and Tissue Cultures

Currently, many plant secondary metabolites have been commercially produced by extraction and purification from the plant materials which either field cultivated or collected from natural habitats. However, their production by plants via field cultivation has several disadvantages, such as low yields, fluctuations in concentrations due to environmental and seasonal variations, restriction to a species or genus and to a particular plant growth or developmental stage, activation of their biosynthesis under stress conditions, etc. On the other hand, using wild plant material collected from natural habitats has considerable risk related to extinction of many valuable and even endemic species. Therefore, plant cell and tissue culture techniques have been investigated extensively as an alternative method for production of secondary metabolites of commercial interest since the end of the 1950s [29].

Plant secondary metabolites can be produced by two major groups of *in vitro* cultures: organized cultures of differentiated tissues (i.e., organ cultures as root, shoot and embryo cultures) and unorganized cultures of undifferentiated cells (i.e., callus and cell suspension cultures). Investigations have showed that differentiated plant tissues produce the same products as the plant itself and they were relatively more stable in the production of secondary metabolites than the undifferentiated cells. Shoot cultures have been used for many medicinal plants, which have been showed to accumulate secondary metabolites much greater than that of natural plants. Besides, many of the valuable secondary compounds, such as tropane alkaloids, hyoscyamine and scopolamine, have been

produced quite well in the root cultures [30]. However, plant roots in cultures generally grow slower than undifferentiated plant cells and their harvest is difficult. Therefore, plant hairy root cultures have been applied as an alternative method for the production of compounds synthesized in the plant roots. Hairy roots obtained by *Agrobacterium rhizogenes*-mediated transformation exhibit higher growth rates than cell suspension cultures and produce secondary metabolites over successive generations without losing genetic or biosynthesis stability [31]. Furthermore, production of two different secondary metabolites is possible simultaneously by co-culturing of adventitious roots. Natural adventitious roots have been induced in many medicinal plants via flask scale to large-scale bioreactor cultivation for the production of several bioactive compounds [32].

Non-embryogenic plant callus cultures, consisting of more or less homogeneous clumps of dedifferentiated cells, have been used for production of secondary metabolites. During the past decades, plant cell suspension cultures initiated from callus cultures have been extensively studied and have emerged as attractive alternatives for production of the range of valuable compounds found in the whole plant. However, production of many of the pharmaceuticals is too low or even zero in cultured cells due to controlling their production by a tissue-specific manner and loss of production capacity resulting from dedifferentiation. Therefore in recent years, various strategies have been developed for use in biomass accumulation and regulation of biosynthesis of secondary metabolites, such as selection of cell lines, optimization of medium and culture conditions, elicitation, immobilization, nutrient and precursor feeding, permeabilization and biotransformation techniques [1, 30].

Because secondary metabolite accumulation in plants is genotype and tissue specific, explants should be selected from particular parent plants and tissues, which have higher contents of desired compound, to initiate cell and organ cultures. Levels and types of various chemical components, such as carbohydrates, nitrate, phosphate and growth regulators which could affect biomass accumulation and biosynthesis of secondary metabolites in plant cell and organ cultures, have been taken into consideration to optimize the medium. Environmental conditions of cultures, such as temperature, light, etc., may affect formation of secondary metabolites. Agitation and aeration are also important factors that should be controlled in flask-scale to large-scale bioreactor cultures for optimization of biomass growth and secondary metabolite production [33].

Secondary metabolites have synthesized in plant cells to respond various abiotic (e.g., temperature, salinity, water, heavy metal, etc.) and biotic (e.g., pathogen or insects) stresses. Therefore these stress factors have been designated as “elicitors” to induce biosynthesis of secondary metabolites. Unfortunately elicitation does not always lead to product of interest because it activates certain plant species in the specific pathway [1]. By utilizing preexisting enzyme systems, many plant cell cultures have also been used to convert precursors into products. Biotransformation is another technique that can be utilized for the over production of high-value metabolites using plant cell and organ cultures [31]. Recently, a wide types of bioreactors, such as stirred tank reactor, bubble column reactor, airlift reactor, ebb and flood reactor, have been designed and used for cultivation of plant cells to produce secondary metabolites [32]. However, the yield of secondary metabolites is still economically insufficient and costly in many cases. Therefore, metabolic and genetic engineering techniques have been incorporated into plant cell cultures to improve the production of secondary metabolites via regulation of their biosynthesis [10].

5. A Novel Approach for Production of Colchicine

Today colchicine has been isolated commercially

from the related ornamental species autumn crocus (*C. autumnale* L.) and flame lily (*G. superb* L.). The production of colchicine by chemical synthesis is not economically feasible compared to extraction from the plant due to considerably low product yield. Therefore several techniques have been developed for extraction of colchicine from different members of the Colchicaceae family, among which the Soxhlet and solid-liquid techniques are the most commonly used [34].

Recently, attempts have been made by a few researchers for initiating callus & cell suspension cultures on *C. autumnale* L. [11, 12] and root & callus cultures on *G. superba* L. [13, 14]. Aroud (2005) reported that callus tissues of *C. autumnale* L. and callus and root tissues of *G. superba* L. were capable of producing colchicine and the accumulation was stimulated by precursor feeding with coumaric acid [15]. Sivakumar *et al*. (2004) used phenylalanine and tyrosine for precursor feeding on *G. superba* L. callus tissues to enhance colchicine accumulation [14]. Ghosh *et al*. (2015) studied elicitation strategies and found that the accumulation of colchicine in non-transformed root cultures of *G. superba* was significantly increased by supplementing the media with mannitol, serine and phenylalanine [35].

Metabolic engineering offers the opportunity to overcome issues related to restricted availability, diversification and productivity of plant alkaloids. Engineered plant, plant cells and microbial cell cultures can act as "biofactories" by providing their metabolic machinery for the aim of optimizing the conditions and increasing the productivity of a specific alkaloid. However, efforts to improve yields of plant alkaloids are often prevented from limitations in metabolic engineering due to lack of genetic tools, long development cycles of plants and the complex interaction between primary and specialized metabolic pathway [36].

RNAi technology has potential applications in metabolic modifications. It has been reported that knockdown of berberine bridge enzyme (BBE) by RNA interference in California poppy (*Eschscholzia californica*) cells caused suppression of both BBE mRNA accumulation and enzyme activity in transgenic cells. Thereupon, end-products of isoquinoline alkaloid biosynthesis, such as sanguinarine, were considerably reduced while reticuline, a key compound in the biosynthetic pathway for isoquinoline alkaloids such as morphine, codeine and berberine, was accumulated at a maximum level of 310 μg/g-fresh weight [37]. On the other hand, it has been stated that transformation of opium poppy (*Papaver somniferum* L.) with antisense berberine bridge enzyme gene (*anti-bbe*) via somatic embryogenesis altered the ratio of morphinan and tetrahydrobenzylisoquinoline alkaloids in latex but not the benzophenanthridine alkaloids in roots [38].

However, very few reports are available on biotechnological approaches toward the production of colchicine [39, 40]. Clearly, the increasing commercial importance of colchicine in recent years will accelerate studies by plant biotechnologist leading to exciting opportunities to engineer colchicine metabolism in plants.

6. Conclusions

Even though a few investigations so far has showed that the levels of colchicine produced by *in vitro* cell and tissues are still relatively low in comparison with the intact plant, it is clear that callus and root cultures of *C. autumnale* L. and *G. superba* L. have the capacity to produce colchicine. Therefore, the production of colchicine by *in vitro* cultures of these two species can be enhanced by applying biotransformation (i.e., hairy root cultures by *Agrobacterium rhizogenes*-mediated transformation), using bioreactors and other techniques of genetic and metabolic engineering to regulate colchicine biosynthesis in cells and tissues. In this way, industrial-scale biomanufacturing of colchicine can be established.

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