Application of Conventional and New Biotechnological Approaches for Improving of Morphinane Alkaloids Production

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Abstract

More than 12,000 alkaloids are known in plants, mostly used as medicine with a world market value of about 4 billion US$. Opium poppy, *Papaver somniferum*, is the most important economic source of morphinane alkaloids such as morphine, codeine, thebaine, narcotine and papaverine that are exploited by the pharmaceutical industry as analgesics, antitussives and anti-spasmodics. With regard to increasing demand for these compounds, the aim of this review is presenting an outlook of classical breeding programs that successfully applied for enhancing the alkaloid content of opium poppy. The latest biotechnological approaches also are discussed to give an outlook for future trends and possibilities.

Keywords: ***Papaver* species, Biotechnological methods, Conventional breeding, Morphinane Alkaloids, Tissue culture, Metabolic engineering**

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Introduction

Poppy seems to be one of the few species which was utilized, even as cultivated plant, in prehistoric times. There is no doubt about the evidence that the poppy was also cultivated by Sumerians, Babylonians and Assyrians about 3–6 thousand years BC [1]. The Opium poppy *Papaver somniferum* (Fig. 1) is a multipurpose crop which is used as a medicinal or ornamental plant, as well as a source for seeds and seed oil. Opium, as dried latex of unripe capsules of *P. somniferum*, contains more than 80 isoquinoline alkaloids. The main alkaloids derived in opium are morphine (4-21%), followed by codeine, thebaine, papaverine, noscapine, and narceine [2]. The Persian genius Ebne Sina, known in the West as Avicenna (980-1037), who was revered as the "prince and chief of physicians" and "the second teacher after Aristotle," recommended opium and plants of the nightshade family as analgesics and anaesthetics [3, 4]. The morphinanes (5-ring benzylisoquinoline alkaloids) include morphine, codeine, thebaine and derivatives are the most important alkaloids produced by the opium poppy. Morphine has long occupied an eminent position on the list of useful drugs. Morphine and codeine are prescribed analgesics and cough-suppressing drugs [5]. Morphine exerts its effects either by hyperpolarizing or inhibiting postsynaptic neurons, probably by increasing K^+ efflux, or by reducing Ca^{2+} influx into presynaptic nerve endings and thereby reducing transmitter release, including acetylcholine, norepinephrine, dopamine, serotonin and substance P [6]. Morphine is metabolized into morphine-3-glucuronide and morphine 6-glucuronide (M6G) in the human body; which the later one possesses analgesic activity.

Other alkaloids from poppy species have various uses: noscapine has antitussive and antitumorogenic properties; papaverine is a vasodilator and smooth muscle relaxant; sanguinarine is antimicrobial and anti-inflammatory [5, 7].



Fig. 1- Opium poppy, *P. somniferum* and different parts of its vegetative and generative organs [8]

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The opiates are industrial commodities of plant origin for which there is still considerable demand. Globally, opium poppies are legally cultivated on around 40,000 ha annually. The major supplier of opium poppies is Tasmania, supplying around 40% of the market. GlaxoSmithKline, Tasmanian Alkaloids and Johnson-Matthey operate fully integrated supply chains for opiate production. Levels of production are controlled by the UN Single Convention on Narcotic Drugs, which limits production to reasonable saleable quantities with allowance made for contingency stocks [9].

In the last few decades, the demand for poppy-derived alkaloids has raised continuously, in particular the need for thebaine, which has increased by ~ 67% in the last 6 years [10].

With five centers of chirality, the structures of morphinane alkaloids present a complexity that renders commercial synthesis uneconomic [11] and cultivation of poppy, in spite of some limitation, continues to be the most effective means to produce opiate analgesics [12]. Higher alkaloid content in the poppy crop would enhance the financial return to growers and make the industry more competitive [13]. For these purposes, different efforts by conventional and new biotechnological methods have been applied to enhance alkaloid content of *P. somniferum*. Thus in this communication we will try to present an overview of morphinanes biosynthesis in opium poppy and different aspects of genetics, breeding, tissue culture and metabolic engineering of these valuable compounds.

Benzylisoquinoline alkaloids and biosynthesis of morphinanes

The benzylisoquinoline alkaloids comprise a group of about 2,500 compounds, which can be divided into nine classes: the rhoeadines, protopines, pavines/isopavines, phthalideisoquinolines, protoberberines, the true benzylisoquinolines, the aporphines, the benzophenanthridines and the morphinanes [14]. Common to all the members of these classes are the first steps in the biosynthetic pathway that lead to the central intermediate (S)-reticuline (Fig. 2).

The presence of morphinane alkaloids (thebaine, codeine and morphine), together with the secophthalidisoquinoline alkaloids (narceine, nornarceine, narceinimide), phthalidisoquinoline alkaloids (narcotine, narcotoline), benzyltetrahydroisoquinoline alkaloids (reticuline, laudanosine, codamine, tetrahydropapaverine) and aromatic benzylisoquinoline alkaloids (papaverine, pacodine) is characteristic of *Papaver somniferum* L. The carbon skeleton of benzylisoquinoline alkaloids is derived from two molecules of tyrosine [15, 16]. The aromatic amino acids, phenylalanine, tyrosine and tryptophan are formed via the shikimate pathway. The availability of tyrosine for alkaloid biosynthetic pathways is an important determinant of the endogenous level of alkaloids.

The biosynthesis of benzylisoquinolines (BIAs) starts with the condensation of two tyrosine derivatives leading to the first tetrahydrobenzylisoquinoline norcoclaurine [17, 18]. Subsequent reactions include the methylation at position 6 of norcoclaurine by norcoclaurine 6-Omethyltransferase (6-OMT), the methylation of the nitrogen by coclaurine N-methyltransferase (CNMT), and the hydroxylation at the 3′-position by the P450 monooxygenase (S)-N-methylcoclaurine 3′-hydroxylase (Cyp80B1). One additional methylation at the 4′position by 3′-hydroxy-N-methylcoclaurine 4′-O-methyltransferase (4′-OMT) finally leads to (S)-reticuline. From this central intermediate the pathway

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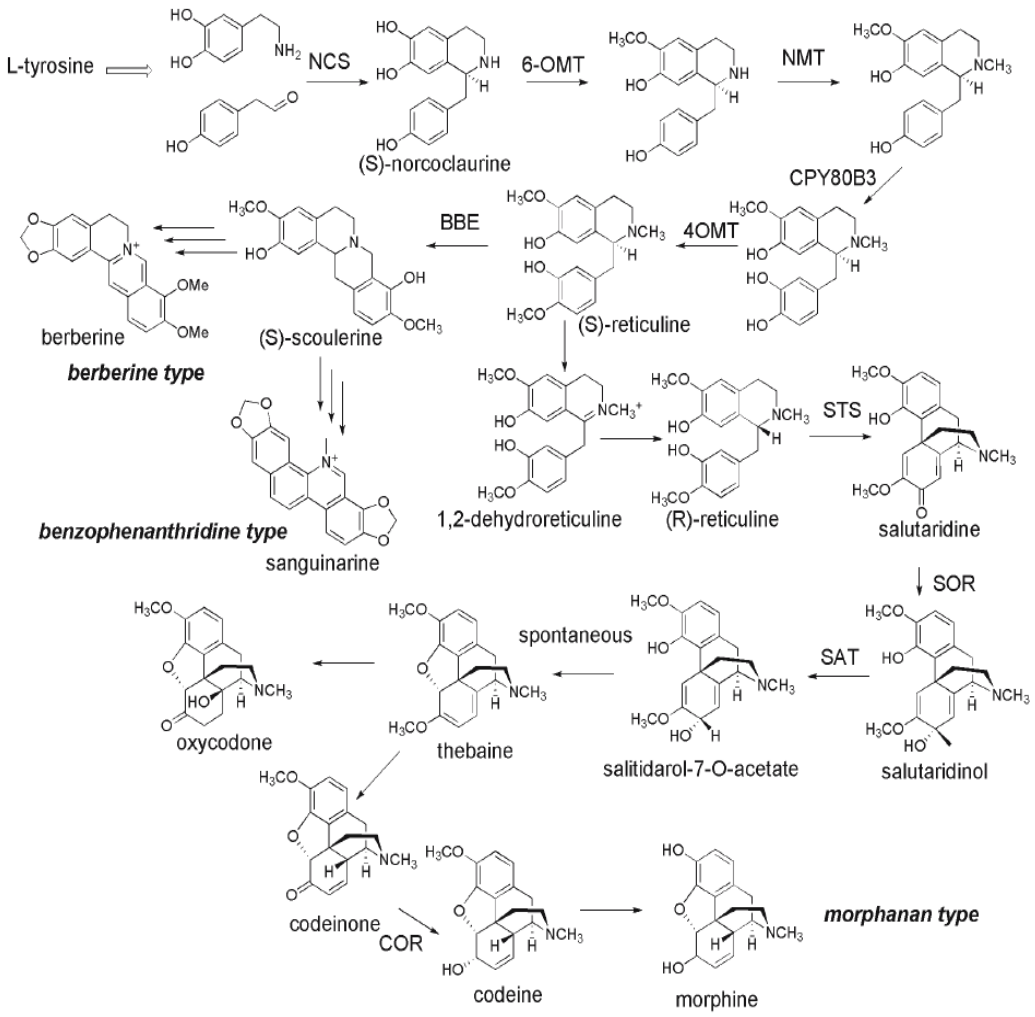


Fig. 2- Benzylisoquinoline biosynthesis pathway. NCS (norcoclaurine synthase); 6-OMT (O-methyl transferase); NMT (N-methyltransferase); CPY80B3 (cytochrome P450); 4OMT (O-methyl transferase) BBE (berberine bridge enzyme); STS (salutaridinol synthase); SAT (salutidarinol-7-O-acetyltransferase), COR (codeine reductase) [19]

bifurcates into the different benzylisoquinoline classes and a multitude of rearrangements and modifications of the benzylisoquinoline backbone takes place leading to the high structural diversity of these alkaloids. Interestingly, whereas the biosynthesis of all classes of benzylisoquinolines starts directly from (S)-reticuline, the conversion of stereochemistry to (R)-reticuline is a prerequisite for the production of morphinans.

A series of at least 17 enzymatic steps contributed in the biosynthesis of morphine. The latter steps in the pathway that lead specifically from (*S*)-reticuline, a central intermediate of isoquinoline alkaloid biosynthesis, to morphine (Fig. 2) involve three NADPH-dependent oxidoreductases [11], most probably three cytochromes P-450, and an acetyl-CoA-dependent acetyltransferase [11, 19].

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Although more than 60 species or lower taxonomic units of the genus *Papaver* have been studied, the presence of morphine has been only detected in two species from the section of *Mecones* , i.e. *P. somniferum* L. and *P. setigerum* DC. The presence of morphine has been also demonstrated in other plants, such as hay and lettuce and recently detected in mammalian tissues. Since the isolation of morphine by Serturner in 1805, a large number of isoquinoline alkaloids have been isolated from the opium poppy [21, 22]. Alkaloid biosynthesis and accumulation is constitutive, organ and cell type-specific processes in the plant. Morphine, noscapine and papaverine are generally the most abundant alkaloids in aerial organs, whereas sanguinarine typically accumulates in roots [23].

Alkaloid biosynthetic enzymes and cognate transcripts have been specifically localized to sieve elements of the phloem and associated companion cells, respectively [24, 25]. In-situ localization of alkaloid biosynthetic gene transcripts indicated that seven biosynthetic enzymes (6OMT, CNMT, CYP80B, 4′OMT and BBE in reticuline biosynthesis, and SAT and COR in a morphine pathway) are localized in sieve elements in opium poppy and corresponding gene transcripts were localized in the supporting companion cells [24, 26]. However, another immunocytochemical analysis clearly showed that 4′OMT and SAT were localized in phloem parenchyma cells, and COR, the penultimate step in morphine biosynthesis, is localized in laticifers (a vessel like series of long cells, which accompany vascular tissues throughout the plant and contain a milky sap called latex.), which is the site of morphinane alkaloid accumulation [27].

Genetics and Breeding

Papaver somniferum (2*n*=22) is a member of the genus *Papaver,* which includes some 100 species and is affiliated to the section *Mecones* comprising five species, among which *Papaver setigerum* (2*n*=44) is a close relative and probably the ancestor of the opium poppy [28]. *Papaver somniferum* is considered to be a predominantly self-pollinating species with various rates of out-crossing depending upon variety and environmental factors; large colourful flowers with numerous stamens and large amounts of pollen attract insects, especially bees; the transfer of pollen from one flower to another might also be performed by wind [29]. The divergent and long history of domestication and breeding of *P. somniferum* has resulted in the development of several different land races, chemotype varieties and cultivars adapted to various uses and climatic conditions. Cultivation of the plant therefore covers a wide geographical area from Bombay to Moscow in the Northern hemisphere and Tasmania in the Southern hemisphere [30]. Alkaloid production is controlled by the plant genotype and by environmental factors. For example cool-grown *P. somniferum* contains more morphine but has lower alkaloid content than warm-grown. Thus secondary metabolite accumulation generally is affected by water availability, exposure to soil microorganisms and variations in soil pH and nutrients [31].

Commercial cultivation of poppy have been envisaged several limitation such as availability of water for irrigation and need to fertile and free-draining soil, which is not overly acidic [13]. On the other hand several diseases like powdery mildew, root rot and leaf blight occurs in poppy. Poppy crops also suffer heavily from mildew caused by *Peronospora arborescens* and other parasitic agents. Unfortunately, at least there is no report about occurrence of completely resistance genetic stocks, in India [20]. Thus to reduce the build up of diseases in

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commercially grown poppies, crop must be grown with at least a three year rotation, before again culturing of them [12]. Regarding of these limitations, breeding strategies, not only must be enhancing morphinanes yield, but also considering the improvement of different agronomic traits.

The existence of substantial variations in the available gene pool of a species is necessary for any successful breeding program. Several independent studies on the evaluation of the genetic variation in the cultivated germplasm of *P. somniferum* reached the conclusion that only a limited variation prevails in Indian genetic stocks and European stocks for most agronomic and chemical traits. This is related to the narrow genetic base of genotypes with common ancestry. The genetic and breeding aspects of opium poppy were investigated more intensively in Europe in the early of 1960s and during the past decade in India [20].

Varietal, mass and pure line selections have been applied by several breeders of opium poppy for the development of improved cultivars [32, 33]. However, the most widely used method which has produced several commercial cultivars is the **pedigree selection** by which, through hybridization between parents with different desirable characteristics, led to development of few lines. The pedigree method has been used successfully for increasing the yield of capsules, opium and seeds, the morphine content and the lodging resistance. This method, however, markedly reduces the genetic variability and contributes to narrowing the genetic basis of the cultivated germplasm [34].

Substantial amounts of heterosis have been observed for morphine and seed yield, as well as for most of their components and a current goal is the development of hybrid species exhibiting heterosis for these characteristic. The implementation of these results into the production of commercial high-performing **hybrid cultivars** is, however, hampered by the lack of a genetic system promoting cross-pollination. Genetic-cytoplasmic male sterility is the most appropriate and widely used system in several crops for the production of hybrid cultivars. Induced male sterile mutants have been obtained in plant populations of opium poppy, either by irradiation with gamma rays [35], or by interspecific hybridization [36], but these mutants were not characterized. In the absence of male sterility, self-incompatibility can be used for the hybrid production. The use of hybrid cultivars in this crop is the most potent and rapid breeding strategy for combining several desirable characteristics from different parents and for exploiting the considerable amount of heterosis reported for morphine and seed yields [34].

There are also some reports about heterosis in *P. bractatum*. Thus heterotic effects can be used in a breeding program aimed at increasing the thebaine yield. This species is suited for the production of hybrids because of the very large quantities of seeds that are produced in each capsule due to its self incompatibility nature. A significant increase in the thebaine yield (more than three times) than that of the best parent in the cross, was found in one F1 hybrid of *P. bractatum* [37].

As an interesting method, **artificial polyploidy induction** can be useful for changing morphological characteristics [38, 39] and secondary metabolite production of plants [40]. Polyploidization is often accompanied by increased cell size and conspicuous changes in secondary metabolism. Where vegetative plant organs are the source of secondary metabolites, as is the case with most medicinal plants, ploidy

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manipulations such as direct chromosome doubling or allopolyploidization provide a rapid means to realize enhanced production of phytopharmaceuticals [40]. Triploid and tetraploid plants of *P. somniferum,* showed up to 100% increase in morphine concentration [40]. In *P. bracteatum* 3x and 4x plants had shown higher thebaine content (respectively, 4.9% and 8.8% dry weight) than diploid (2.4% dry weight) counterparts, but polyploid plants were later in flowering and their seed setting was very poor, especially in triploids [41, 42]. The polyploidy therefore seems of little use for breeding for high seed yield, but it might be considered for increasing morphinane content, which is the primary value than the plant biomass component and will facilitate the extraction process.

Spontaneous and mutagen-induced mutants have also been reported in *P. somniferum*. Such mutants can be used directly as new cultivars: the 'Soma' variety was released from a spontaneous mutant in the variety "Indra" [43]. In other cases, the mutants have been used, frequently as parents in breeding programs [44]. The occurrence of biochemical mutants induced by mutagenic treatments showed the good potential of this approach in altering the alkaloid profile of plants. By the use of mutagenic agents such as gamma ray or chemical agents, some breeder could produce opium poppy plants with different characteristics of male sterility, opium less, high morphine yield, and high number of capsules per plant [45]. A codeine chemotype in which the demethylation to morphine is blocked would be most valuable both to the pharmaceutical industry and for the prevention of the illegal use of morphine.

A world-wide effort was also invested during the 1970s to domesticate and develop *P. bracteatum* as an alternative source to the opium poppy for codeine production. The most widely used opiate in medicine, codeine, is mainly produced from the *P. somniferum*. However, the plants major alkaloid, morphine, and its highly addictive derivative heroin are also used illegally as drugs. Codeine can also be produced from thebaine, the major alkaloid of *P. bracteatum*. The species *P. bracteatum,* Persian poppy, diploid $2n$=14, is considered to be a potential alternative to opium poppy for codeine production due to its high content of thebaine and complete absence of morphine. Thebaine is a precursor of codeine and can be easily converted to codeine by the pharmaceutical industry. In some populations of this species, the capsules and roots contain almost exclusively thebaine; thus the extraction and purification of the raw material is relatively easy. The addiction potential of thebaine or its derivatives and of the minor alkaloids reported for *P. bracteatum* is negligible and no cases of abuse or illicit production of thebaine have been reported [37]. Thus whenever the climatic conditions are suitable for the growth of *P. bracteatum* it may advantageously replace the traditional *P. somniferum*.

P. bracteatum is naturally distributed in high altitudes from 1500 to 2500m. The species is found in three distinct areas: the Alborz Mountains north of Tehran, in the Iranian Kurdistan and on the Northern slope of the Caucasus [37]. A population called Arya II with a thebaine content of 3.6% of dry mater was found in Western Iran by Lalezari *et al.* [46].

However substantial progress, in the case of poppy species, has been achieved, mainly through genetic, during the last 30 years in France, where the yield of morphine has increased from 4.5 kg/ha in 1961 to 10.5 kg/ha in 1991 [34]. But unfortunately no correlations have observed between the alkaloid content and the yield of dry matter. Although

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conventional plant breeding has produced a doubling in poppy alkaloid content over the last two decades, however continued rapid improvement in morphinan yields through conventional breeding will likely be limited [13] and these methods did not give the desirable level of improvement for several reasons, including low genetic diversity, sterility, long generation time, perennial nature and complex biosynthetic pathways involved [47]. Thus today we need to use of new biotechnological methods (in combination to classic breeding programs) to efficiently manipulate morphinanes production in poppy plant.

Role of Biotechnology in morphinanes production

With regard to medicinal plants, biotechnology could be described as a method for enhancing the formation and accumulation of desirable natural products, with possible product modification in medicinal plants. Micropropagation, cell and hairy root culture as well as gene technology are all important techniques for plant propagation, but these are mostly used to improve the production and yield of desired natural products.

Plant cell and tissue cultures

Some compounds, such as shikonine and paclitaxel, can be produced with the technology of large-scale plant cell culture. However, other secondary metabolites, particularly alkaloids, are produced at low concentrations in plant cell cultures. The low or lack of productivity of these desired compounds can be explained by an insufficient level of cell differentiation to allow a production of secondary metabolites. In plants, there is a clear correlation between cellular differentiation and secondary metabolism.

Tissue cultures of different explants of the poppy plant (i.e. seedling hypocotyls, seedling roots, stalk and capsule) have been reported in the literatures [48]. Callus tissues have been obtained and the presence of alkaloids has been detected. However, other investigations have demonstrated the absence of alkaloids in *P. somniferum* tissue cultures. These conflicts reports can be due to: the use of different analytical methods with varied sensibilities, the use of different *P. somniferum* cultivars and the analysis of somatic tissue cultures at various stages of differentiation [49]. Industrial production of opiates from tissue culture is dependent on the large accumulation of alkaloids in a cell culture medium. While there has been great success in plant-cell culture in terms of cells with high yields of isoquinolines, from a commercial and pharmaceutical viewpoint, the morphinans have proved difficult to produce in plant-cell cultures [50]. As an improving strategy treatment with elicitor was not successful in all cases: codeine biosynthesis, for example, has not yet been achieved. However, compounds sharing the same precursors and intermediates, such as the antimicrobial alkaloid sanguinarine, may accumulate in quite high amounts. Using cell cultures of *P. somniferum*, the production of sanguinarine was shown to be elicited by preparations from fungal mycelia [51, 52]. Likewise, Archambault et al. [53] were able to obtain a twofold stimulation in the production of the antimicrobial compound, sanguinarine, through the use of a chitosan elicitor in *P. somniferum* cell cultures.

However numerous studies had shown that the production of morphinan alkaloids via *in vitro* cultures requires organogenesis of tissues in cultures. The induction of cell differentiation by the addition of exogenous growth regulators in the culture medium

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improves alkaloid production. However, this process is time-consuming and therefore it can be used only for the production of compounds with a high value. The transformation of medicinal plants using *Agrobacterium rhizogenes* to form hairy root cultures has the potential benefits of fast growth and rates of alkaloid production equal to or greater than that found for the intact plant. Moreover, hairy root cultures can be scaled-up for bioreactor production to allow for the large-scale recovery of alkaloids or other compounds with pharmacological activities [49].

Hairy root cultures of many other medicinal plants obtained by transformation with *A. rhizogenes* were examined as potential sources of high-value pharmaceuticals [54]. For the first time, *P. somniferum* hairy root cultures have been established after transformation of hypocotyls with the hypervirulent *A. rhizogenes* strain, LBA 9402 [55]. The total alkaloid content (morphine, codeine and sanguinarine) was higher in hairy roots (0.46 ± 0.06 % D.W.) than in untransformed roots (0.32 ± 0.05 % D.W.) and some of the alkaloids were excreted into the liquid culture medium. Rostampur et al. [56] were shown that the content of different benzylisoquinoline alkaloids produced by Persian poppy (*P. somniferum*) hairy roots was identical wild-type roots. Transformed root cultures of *P. somniferum* and California poppy, *Eschscholzia californica* had higher growth rate than wild roots and displayed benzilisoquinoline profile that were virtually identical to those of wild-type roots [57].

Tissue cultures of *P. bracteatum* have also been studied for thebaine production. Unfortunately, little success has been achieved in producing the desirable alkaloid in substantial amounts in cell or tissue culture. As for *P. somniferum,* a different alkaloid profile is obtained in culture, compared with plants. Thebaine was present in only trace amounts in cell cultures or absent. The self-incompatibility system in this species does not allow the creation of pure lines through selfing. Thus, using tissue culture as a method for mass micropropagation in *P. bracteatum* might be useful for rapid multiplication of superior individuals [37].

Production of transgenic poppy and metabolic engineering

In order to produce transgenic plants one must be able to: (1) stably integrate foreign DNA into its genome; and (2) regenerate fertile plants from transformed tissues. Unlike the transformation difficulty, it is now possible to do both of these in opium poppy. Long-term callus and suspension cultures of opium poppy have been maintained on certain media in several laboratories. Both roots and shoots have been regenerated from callus [58]; however, a much simpler method for regenerating poppy suspensions through somatic embryogenesis has been developed [59]. Hosseini [48] successfully regenerated transgenic poppy plants from meristemoid calli of hypocotyls explants (Fig. 3).

The availability of reliable transformation/ regeneration systems for opium poppy and the cloning of alkaloid pathway genes mean that it should be possible to apply metabolic engineering to alter the quantity and quality of alkaloids in this species.

A genetically modified opium poppy that produces the pharmaceutical precursor, thebaine, instead of the narcotic alkaloids morphine and codeine, has recently been described [60]. Since thebaine (in contrast to morphine) cannot be easily converted to heroin (an acetyl derivative of morphine), the genetically altered crop provides a good solution to hamper the utilization of opium poppy as a source for the illicit drug market.

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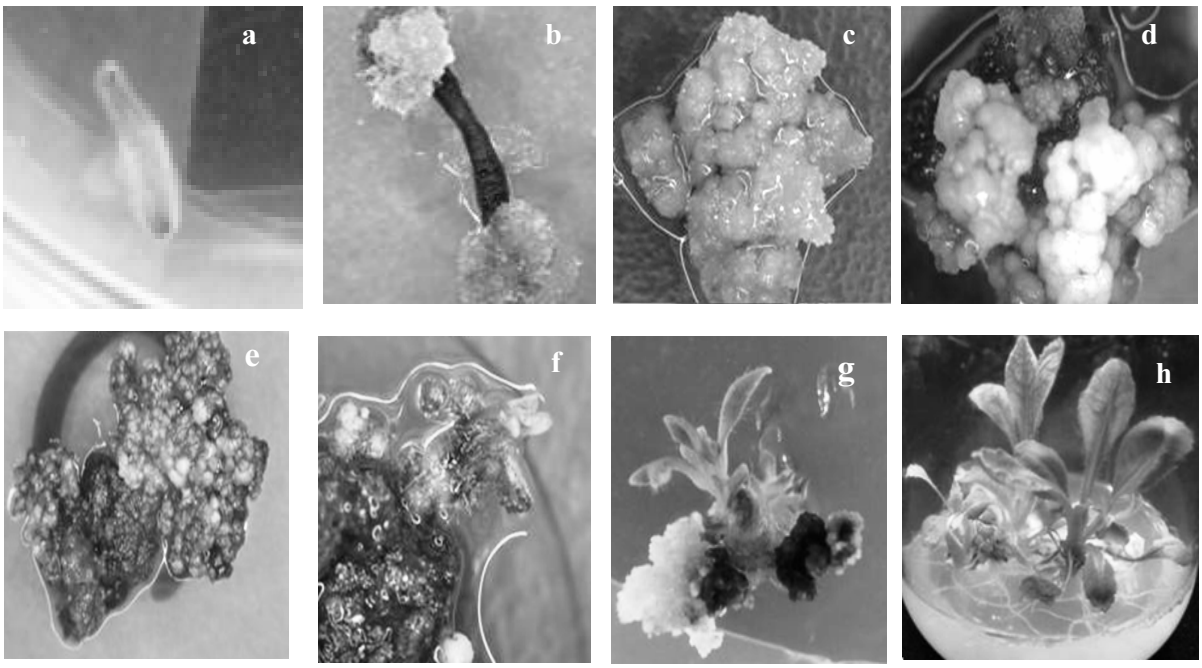


Fig. 3- *P. somniferum* transgenic plants regeneration frome hypocotyl explants. Hypocotyl explant on the B5 medium (a), callus induction (b), meristemoid calli and somatic embryogenesis (c, d and e), germination and development of a somatic embryoid (f and g), root induction and regeneration of transgenic opium poppy (h) [48]

Recently, an industrial elite line of poppy was genetically engineered to modify the alkaloid content of the plant by overexpression of the (*S*)- *N* -methylcoclaurine 3′ -hydroxylase [61]. The resulting transgenic plants contained up to 450% more alkaloids in their latex compared to untransformed plants. Overexpression of codeinone reductase in poppy led to a 30% increase of morphinan alkaloid content on a dry weight basis in transgenic plants [62]. In a different experiment, the berberine bridge enzyme (bbe), which is the first enzyme of the sanguinarine biosynthesis branch, was silenced in poppy [63]. This resulted in an increase in the concentration of several pathway intermediates from all biosynthetic branches of benzylisoquinoline alkaloids in the latex of transgenic plants. Surprisingly, the transgenic plants also exhibited an increase in concentration of (S)-scoulerine. Silencing of *BBE* in California poppy (*Eschscholzia californica)* resulted in increased levels of (S)-reticuline [64].

Silencing of salutaridinol O-acetyltransferase (SAT), an enzyme acting in the same branch caused no accumulation of its substrate salutaridinol but of salutaridine, the substrate of the previous enzyme [65, 66]. Overexpression of SAT increased the yield of morphine indicating a rate limiting function of this enzyme [65]. The data support the existence in the morphine branch of one or more metabolons, i.e. multi-enzyme complexes that allow the internal hand-over of pathway intermediates and are disrupted by the removal of one enzyme.

Codeine reductase is the penultimate step in morphine biosynthesis. Opium poppy was transformed with a chimeric cDNA hairpin

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RNA construct designed to silence codeinone reductase. However, silencing of codeinone reductase resulted in the accumulation of (S)-reticuline, but not the substrate codeinone or other compounds on the pathway from (S)-reticuline to codeine [67]. Allen and coworkers postulated that this accumulation could be due to several factors. First, accumulation of codeinone and morphinone could result in negative feedback on one of the enzymes, such as the reductase responsible for the reduction of (S)-reticuline to 1, 2-dehydroreticuline. Also, the biosynthetic intermediates and final product may regulate the transcription of pathway enzymes, through analysis of the transcript levels of a number of the morphine biosynthetic enzymes showed no change in suppressed plants. Finally, codeinone reductase could be a part of a multienzyme complex, which can't be functioned when one of the enzymes is removed. This study highlights that the complex metabolic networks found in plants are not easily or predictably redirected. The regulation of benzylisoquinoline alkaloid metabolism is complex and our understanding of opium poppy biochemistry at the molecular level can be advanced with genetic transformation and metabolic engineering biotechnology [68].

Results of over expression of two genes (*sat* and *cor*) under CaMV35s promoter showed that transgenic plants had different metabolites profiles and increased content of some metabolites. Most transgenic lines showed significant increases in capsule alkaloid content compared with non-transgenic controls the morphine alkaloid contents on a dry weight basis were 14% greater than those in control genotypes [48].

Since pure extracts of poppy-derived alkaloids that are devoid of genetic material has only industrial useable, it is expected that a transgenic source of these substances will be accepted by consumers.

Molecular markers

Molecular markers, such as restriction fragment length polymorphism (RFLP) and random-amplified polymorphic DNA (RAPD) appear to be good candidates for the identification of plant species. This methodology has become an important tool for the confirmation of somatic hybrids and, more recently, reported that RAPD markers can be utilized for genetic analysis of micropropagated plantlets regenerated from somatic embryoids. This methodology has also been used to determine the geographic variation of plants [69].

It is difficult to determine *P. bracteatum* compared with *P. orientale* L. and *P. pseudo-orientale* Medw, in Oxitona section, based on the morphological observation. Shoyama et al. [69] demonstrated that RAPD analysis can be used for a simple and rapid judgment of two parental *Papaver* species in Oxitona section (*P. bracteatum* and *P. pseudo-orientale*) and the diagnosis of their F1 hybrids. On the other hand, this method can also be used for the judgment of illegally cultivated *Papaver* species. Amplified restriction fragment length polymorphic (AFLP) analysis, also have been used to evaluate the genetic diversity of breeding populations to provide information on those lines with desired genetic heterogeneity [68].

Biotransformation/expression in other genetically modified microorganism

Although many plant cell suspension cultures fail to produce the compounds seen in the plants from which they have been established, these cells may be used in biotransformation processes where exogenous organic compounds are modified by living

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cells. Biotransformation studies in cell suspension cultures have been carried out with a view to: (i) producing new chemicals; (ii) producing known chemicals more economically; (iii) investigating the metabolic fate of xenobiotics; and (iv) elucidating metabolic pathways [70, 71].

Lenz and Zenk [20] described the biosynthetic transformation of codeine and morphine from the biological precursors: codeinone and morphinone. The biotransformation of codeinone to codeine proved to be possible in the immobilized cells. The biotransformation of thebaine via codeinone and codeine to morphine also is investigated with other strains of *P. somniferum* cell cultures. Furuya *et al.* [72] described the biotransformation of codeinone to codeine by the cell suspension culture and the cell-free system. This reduction required NADH as a co-factor in the enzyme system. In the immobilized cell system, however, this reaction proceeded without NADH. Using a column bioreactor packed with the immobilized living cells, they were also investigated the effects of various conditions, such as temperature and aeration, on the conversion of codeinone to codeine and the cell viability.

The biosynthetic pathway to morphine in the oriental poppy *P. somniferum* is currently being elucidated, and characterization will benefit the production of morphine and its semi-synthetic derivatives [73, 74]. The 17-step biosynthesis of morphine in this plant has been almost completely elucidated, with the eventual goal being the biomimetic synthesis of thebaine, codeine, and morphine. These cDNAs were all functionally expressed in insect cell culture (*S. frugiperda* Sf9 cells) or in *E. coli*. However, there are still a number of biosynthetic steps in the morphinan pathway for which the enzymes have neither been identified nor cloned. Thus, the biosynthesis of morphinan alkaloids in a microbial heterologous host is not yet fully feasible. An alternative approach is microbial biotransformations of morphine into valuable derivatives [75]. For example, a reusable efficient recombinant morphine/codeine biotransformation system was created using *Pseudomonas* enzymes [76].

Systems Biology Approaches

Recent advances in plant genomics research has generated knowledge leading to a better understanding of the complex genetics and biochemistry involved in biosynthesis of these plant secondary metabolites. This genomics research also concerned identification and isolation of genes involved in different steps of a number of metabolic pathways. Progress has also been made in the development of functional genomics resources (EST databases and micro-arrays) in several medicinal plant species, which offer new opportunities for improvement of genotypes using perfect markers or genetic transformation.

ESTs are generated by massive and random sequencing of cDNAs generated from the mRNA of the tissue of interest. ESTs are typically short (normally only partially represent the full-length clones) and are of relatively low sequencing quality. ESTs offer a quick method for cloning and examining a large number of genes known to be expressed in a particular cell population or tissue. Opium poppy (*P. somniferum*), may be the prime example for a non-model plant on the verge of becoming a model. An extensive array of genomics resources, including expressed sequence tags (ESTs) and DNA microarrays, for opium poppy plants and cell cultures has been developed, together with proteomics, allowing to the development of system biology

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approaches [77]. Decker et al. [78] and Ounaroon et al. [79] used proteomic analyses of *P. somniferum* latex (2D gel electrophoresis (microsequencing) to create proteomic maps. Beside enzymes from the primary metabolism, a codeinone reductase was identified based on homology to known isoforms [78]. In addition, *Papaver somniferum* sequences coding for reticuline 7- *O* -methyltransferase and norcoclaurine 6- *O* -methyltransferase were isolated based on peptide sequences and the respective methyl transfer enzymes of alkaloid biosynthesis were characterized [79].

Comparative macroarray analysis of opium poppy and various morphine free *Papaver* species was used to identify a *P. somniferum* *O*-methyltransferase clone [80]. In this case, *P. somniferum* seedlings were used to develop a cDNA sequence library. Of the 849 sequenced elements, three were shown on a macroarray, differentially expressed in *P. somniferum* compared to non-morphine-producing species. Whereas two of these cDNAs showed no significant homology to any known protein, one was found to encode a protein identified as *S*-adenosyl-l-methionine (R, *S*)-3′-hydroxy-*N*-methylcoclaurine 4′-OMT (4′OMT). Recently, an EST sequence database was used to obtain the *P. somniferum* clone of (*S*)-norcoclaurine synthase (NCS), which catalyzes the first committed step in benzylisoquinoline alkaloid metabolism. The use of whole genome sequences and species-specific EST collections has allowed rapid discovery of new genes involved in plant secondary metabolism. Additionally, genomic tools have provided the means necessary to understand intricate signaling and regulatory pathways, complex phylogenic relationships, and overall genetic architecture.

Metabolomics is the youngest of the so-called “omics” methods, and ultimately concerns the analysis of all metabolites in an organism. Zulak et al. [81] using Quantitative HNMR metabolomics have drawn a high-resolution map of the reprogramming primary and secondary metabolism in elicitor-treated opium poppy cell cultures. They have revealed that the response of cell cultures to elicitor treatment involves the extensive reprogramming of primary and secondary metabolism, and associated cofactor biosynthetic pathways.

In the future, potent genomics tools will be combined with metabolic profiling to identify key genes that serve for engineering secondary product pathways.

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