The First Step of Biotechnological Approaches for Alkaloid Biosynthesis in Papaveraceae: *In vitro* Plant Regenerations

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ABSTRACT : Alkaloid producing species of plants have long been a major component of the medicinal, social and magico-religious aspects of human culture. A diverse array of biological activities has been attributed to different alkaloids including numerous members of benzylisoquinoline family of alkaloids. For biotechnological approaches of alkaloid biosynthesis in poppy family, plant regeneration protocol through somatic embryogenesis or shoot organogenesis is a first step. This paper describes the methods and applications of plant regeneration of poppy family.

Key words : Papaveraceae, alkaloid biosynthesis

INTRODUCTION

Alkaloid producing species of plants have long been a major component of the medical, social and magico-religious aspects of human culture. The alkaloids are nitrogenous natural products incorporating structures from diverse primary metabolites. As secondary metabolites, these compounds are known to accumulate in numerous higher plant species and to a limited extent in other organisms including mammals and fungi.

A diverse array of biological activities has been attributed to different alkaloids including numerous members of the benzylisoquinoline family of alkaloids (BIAs). Derived from two units of tyrosine through coupling of dopamine and 4−hydroxyphenacetaldehyde, norcoclaurine represents the basal skeleton for all BIAs. The level of accumulation of alkaloids of interest in plants is often too low for medicinal use as in the classic example of paclitaxal, the anti−cancer compound derived in miniscule quantities from *Taxus* species. In other cases it may be desirable to modify

the composition of the alkaloids such as the desire for high thebaine or high codeine varieties of the opium poppy, *P. somniferum* L. The alternative to isolation from natural sources, synthesis, although useful in some cases, it is limited due to the common structurally complexity of many alkaloids. Biotechnological approaches are a feasible alternative to these two approaches, but requires tissue culture methods and an understanding of the molecular biology of the plant to manipulate these pathways for altered substrate accumulation and composition.

Numerous BIAS accumulating species are present in the Papaveraceae family including a couple species for which numerous alkaloid analyses are available including opium poppy (*Papaver somniferum* L.) and greater celandine (*Chelidonium majus* L.). The latex of *P. somniferum* (crude opium) is commonly collected for the antinociceptives morphine and codeine (Calixto *et al.*, 2000), although papaverine and narceine are other medicinally important high abundance alkaloids in the latex. *C. majus* accumulates antifungal benzophenathridines such

as chelidonine, dihydrochelerythrune and dihydrosanguinarine as well as a number of protoberberines including berberine itself, which has demonstrated cytotoxic properties (Iwasa et al., 2001a).

Regeneration protocols have also been described for a number of plant species for which chemical compositions are lacking or minimal including California, Japanese and Himalayan blue poppies. The Californica poppy, E. californica Cham. lacks protoberberines but does accumulate benzo[c] phenanthridines such as sanguinarine as a major product along with a number of related compounds such as macarpine and chelerubine. Similarly, the spring celandine or Japanese poppy (Hylomecon vernalis=H. vernale) and the Himalayan blue poppies (Meconopsis spp.) are known to accumulate various sanguinarine and isopavine derivatives (Kang et al., 2003; Xie et al., 2001).

The medicinal activity of many BIAs have been determined through various means from human cell proliferation to reverse transcriptase activity assays. Papaverine derivatives have been shown to have anti-malarial activity (Iwasa et al., 2001b), berberine and sanguinarine have been suggested as leads in development of anticancer drugs (Ahmad et al., 2000, Iwasa et al., 2001a). While berberine also appears to have antiplasmodial activities (Wright et al., 2000), sanguinarine has displayed protein kinase inhibitory action (Wang et al., 1997). Finally, numerous BIAs have displayed antifungal activities including dicentrine, glaucine, protopine, -allocryptopine (Morteza-Semnani et al., 2003) as well as dihydrosanguinarine, dihydrochelerythrine, papavoriendine and chelidonine among others BIAs (Ma et al., 2000). The bioactivity of these BIAs suggests they may confer selective advantages in terms of direct defense or antifeedant activities for these plants.

Initial attempts to alter metabolite accumulation or product composition in these plants through genetic engineering have met with mixed results (Sato et al., 2001). A greater understanging of the regulation of these pathways in response to developmental and environmental queues is a key to increasing our ability to manipulate these pathways. The opium poppy (P. somniferum) and California poppy (E. californica)

have been chracterized extensively at the chemical and biochemical levels and are beginning to be used as models to understand these higher levels of regulation. Use of regulators such as transcription factors or promoter elements show promise as a way to manipulate these compounds using the existant molecular regulatory networks of these plants in combination with variations on culture methods..

Biotechnologically approached studies such as these and attempts to engineer plant natural product composition initially require plant regeneration systems such as somatic embryogenesis or shoot organogenesis. These sorts of regeneration systems are often a key to plant transformation as well as for studies into environmental and developmental cues in alkaloid accumulation. For biotechnological approaches of benzylisoquinoline alkaloid biosynthesis in poppy family, plant regeneration protocol through somatic embryogenesis or shoot organogenesis is a first step. In this paper we describe the methods and applications of plant regeneration of poppy family.

Plant regeneration

Plant cell and tissue culture plays an important role in plant biotechnology. Plant regeneration protocols are an essential part of plant genetic transformation leading to plant improvement. Early last century, Haberlandt (1902) suggested that individual nucleated plant are totipotent, that they have the genetic capacity to be converted to complete plants. More recently, the term 'regeneration' has been broadly used in the context of tissue culture as the production of whole plants from cells, tissues, organs, meristems or zygotic embryos cultivated in vitro.

Almost all current practical transformation systems require a plant regeneration system to allow selection for and regeneration of transgenic plant tissues or cells. Many of these regenerations systems may be adapted from the plant regeneration systems that have long been developed commercially for the micropropagation of ornamentals and disease-free stock. There are two major systems of plant regeneration, organogenesis and somatic embryogenesis. These systems are defined based on the developmental stages through which a whole plant is regenerated.

Organogenesis

Organogenesis is a developmental pathway in which shoots or roots (i.e., organs) are induced to differentiate from a cell or group of cells. Plant regeneration through organogenesis generally involves induction and development of a shoot from explant tissue, followed by transfer to a different medium for the induction of roots, although less commonly shoots may be regenerated from root cultures. In 1957, Skoog and Miller performed classic experiments demonstrating that shoot and root initiation in callus cultures of tobacco could be regulated by manipulation of the ratio of auxin and cytokinin present in the growth medium. Generally in organogenesis protocols, high cytokinin to auxin ratios induce shoots, high auxin to cytokinin ratios produce roots, and more equal concentrations of theses phytohormones are found to cause callus proliferation (Pierik, 1987). Since Skoog & Miller's (1957) original experiments, research has demonstrated that successful organogenesis in many plant species, beyond hormone manipulations requires establishment of medium components, selection of a suitable explant, and control of the physical environment (Brown & Thorpe, 1986; Thorpe, 1990).

Currently, organogenesis is the most widely used method of *in vitro* plant regeneration in transformation systems. Most Agrobacterium-mediated transformation systems use the leaf disk procedure, or a modified form or plant regeneration based on direct or indirect organogenesis (Ritchie & Hodges, 1993).

Somatic Embryogenesis

Steward *et al.* (1958) originally observed plant regeneration by somatic embryogenesis in cultured carrot (Daucus carota) cells. In somatic embryogenesis, somatic cells develop by division to form complete embryos, with a bipolar structure consisting of shoot and root meristems. These embryos are then induced to develop, progressing through the distinct structural steps of the globular, heart, torpedo, cotyledonary, and mature stages, analogous to development of a zygotic embryo. Somatic embryogenesis can occur directly from cells of the explant tissue without an intervening callus phase. However, the indirect embryogenesis pathway, where somatic embryos are induced and develop from a proliferated callus, is generally more common (Pierik, 1987; Rashid, 1988). During the initiation of embryogenic cultures, a high concentration of auxin (usually 2,4-dichlorophenoxyacetic acid (2,4-D)) induces cellular proliferation (callus induction) along with the embryogenic pathway of development. It is generally thought that the embryogenic pathway is induced and becomes determined very early in embryogenic cultures, and this clearly seems to be the case in carrot, a model species in this system. The high concentration of auxin used for induction, however, is usually inhibitory to the development of somatic embryos into advanced stages. A hormone-free medium is often used for the development of globular-staged somatic embryos into plantlets, however low concentrations of hormones in the development medium may be beneficial or necessary, depending on the species, to promote normal development of the embryos. Cytokinin for example is required for somatic embryogenesis in some monocot species.

Induction of somatic embryogenesis can generally be considered to require a single hormonal signal to induce a bipolar structure capable of forming a complete plant. In contrast, organogenesis requires two different hormonal signals and thus two different culture media. The first media is designed for shoot development the second for initiation of a root organ. Somatic embryogenesis often does not require a different medium after promotion of embyrogenic cells and uses lower concentrations of hormones, if any, to promote the development of embryogenic cells into plantlets (Phillips *et al.*, 1995). Somatic embryogenesis has the potential for rapid and efficient regeneration, making it a valuable tool in transformation of plants.

Plant regeneration in poppy family
Chelidonium majus

Chelidonium majus L., a member of the Papaveraceae, is widely distributed in Asia and Europe. This plant contains a yellow, unpleasant smelling, and poisonous latex in all parts of the plant, resulting in the common name baby excrement plant in Korea. The whole plant produces benzylisoquinoline alkaloids, including chelidonine,

chelerythine and sanguinarine. Traditionally it has been employed as an ophthalmic to treat eye infections while in modern herbal medicine it is used as a mild sedative, antispasmodic and detoxifying herb and has value in relaxing the muscles of the bronchial tubes, intestines and other organs. Finally, the latex is commonly used externally to treat warts. Caution should be employed, especially when the plant is used internally however, as many of the alkaloids may be toxic dependant on dosage. The alkaloids chelidonine and protopine for example have anticancer properties (Colombo & Bosisio, 1996), likely through similar mechanisms which make them toxic.

Somatic embryogenesis and plant regeneration protocols have been developed for *Chelidonium majus* L. using various explants and culture conditions. These have included cultures derived from pedicels (Woo *et al.*, 1996), immature ovule-derived embryogenic cell suspension cultures (Kim *et al.*, 1999) as well as use of epicotyl explants (Vinterhalter *et al.*, 2002) of seedlings after prolonged cultivation on Murashige and Skoog (MS) medium with or without plant growth regulators.

Eschscholtzia californica Cham. (California poppy)

Eschscholzia californica Cham. is a common ornamental and traditional medicinal plant of many North America natives (Cheney, 1964). Sanguinarine is one of the principal benzophenanthridine alkaloids produced in *E. californica* roots and is used commercially as an antiplaque agent in oral hygiene products due to its potent antimicrobial activity (Dzink & Socransky, 1985). The regeneration of *E. californica* plants via somatic embryogenesis has been previously reported (Kavathekar, 1974; Kavathekar & Ganapathy, 1973; Kavathekar *et al.*, 1977). However, the embryogenesis and regeneration efficiencies were low, the embryos developed into plantlets only after exposure to low temperature, and few embryos displayed normal developmental morphology (Kavathekar *et al.*, 1977).

We developed a rapid protocol for high efficiency somatic embryogenesis and plant regeneration from seed-derived embryogenic callus cultures of California poppy (Park & Facchini, 1999). The optimized procedure

required less than 13 weeks from the initiation of seed cultures to the recovery of plantlets, and involved the sequential transfer of cultures onto solid MS basal medium containing three different combinations of growth regulators. Callus was induced from seeds of *E. californica* cultured on medium supplemented with 1.0 mg ℓ^{-1} 2,4-D. This primary callus was then transferred to medium containing 1.0 mg ℓ^{-1} 1-napthalene acetic acid (NAA) and 0.5 mg ℓ^{-1} 6-benzylamino purine (BAP) to establish embryogenic callus and promote somatic embryogenesis. These regenerated plantlets, which displayed normal development, were recovered after germination of somatic embryos on medium containing 0.05 mg ℓ^{-1} BAP.

A protocol for mass production of somatic embryos was developed for *E. californica* using embryogenic cell suspensions in optimized media conditions (Park & Facchini, 2001). Somatic embryo production was substantially reduced at shaker speeds above 40 rpm. These data are in agreement with those reported for the production of somatic embryos from embryogenic cell cultures of an *Agrobacterium rhizogenes*-transformed cell line of California poppy grown in an 11-1 helical-ribbon-impeller bioreactor containing B5 liquid media and 30 g ℓ^{-1} sucrose (Archambault *et al.*, 1994). Glucose and sucrose were the most effective carbon sources, whereas fructose, galactose, and maltose resulted in a reduced yield and growth of somatic embryos. The development of somatic embryos was promoted by $AgNO_3$ at concentrations below 10 mg ℓ^{-1}. A semi-solid medium containing 1.5 g ℓ^{-1} Gel-rite produced the highest frequency of somatic embryo conversion, and promoted the efficient growth of plantlets. Using the reported protocol, over 500 viable somatic embryos were produced per 25 mℓ of embryogenic cell suspension culture

Hylomecon vernalis

Hylomecon vernalis Max. is a traditional medicinal plant in Asia. The root of this plant has been considered as herbal medicine for the treatment of arthritis, neuralgia, and eczema. Recently somatic embryogenesis and plant regeneration of *H. vernalis* Max. using embryogenic calluses from petiole and leaf explants was reported (Kim *et al.*, 2003).

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Meconopsis spp. (Himalayan poppy)

This genus of plants, often grown as ornamentals belong to the poppy family, Papaveraceae. Himalayan blue poppy, *Meconopsis simplicifolia* (D.Don) Walp. Has been regenerated by induction of adventitious shoots from callus produced from hypocotyl, cotyledon and rosette leaf explants of 4-month-old seedlings (Sulaiman & Babu, 1993) and by somatic embryogenesis from embryogenic callus cultures (Sulaiman *et al.*, 1991). Plant regeneration via shoot organogenesis in callus cultures of Himalayan yellow poppy, *Meconopsis paniculata* D.Don (Prain) has also been reported (Sulaiman *et al.*, 1994).

Papaver somniferum L. (opium poppy)

The opium poppy (*Papaver somniferum* L.) is one of the oldest and historically most significant of all cultivated plants. Archaeological records suggest that the domestication of opium poppy predates recorded history (Brownstein, 1993). The plant remains an important source of several benzylisoquinoline alkaloids including the analgesic and antitussive drugs morphine and codeine, the muscle relaxant papaverine, and the anti-tumorigenic agent noscapine (Ye *et al.*, 1998).

The production of callus and suspension cultures of opium poppy has been widely reported, however the regeneration of intact plants has proven to be considerably more difficult (Facchini *et al.*, 2000). Somatic embryogenesis in opium poppy is generally inducible by transferring embryogenic cultures to medium containing low levels of exogenous hormones (Kamo *et al.*, 1982; Nessler & Mahlberg, 1979; Nessler, 1982; Yoshikawa & Furuya, 1983; Galewsky & Nessler, 1986; Ovecka *et al.*, 1996; Laurain *et al.*, 1999; Kassem *et al.*, 2001). However, somatic embryogenesis in opium poppy results in a relatively inefficient production of somatic embryos, and requires approximately nine months to regenerate intact plants from explant or primary callus tissues (Nessler, 1982). Shoot organogenesis in opium poppy is generally initiated from embryogenic callus cultures using auxins and cytokinins. (Ovecka *et al.*, 2000) Some reports described *in vitro* plant regeneration of opium poppy through two different pathways, somatic embryogenesis and organogenesis (Ovecka *et al.*,

1997, 1999; Alkhimova *et al.*, 2001).

Although the direct organogenesis of opium poppy roots has been described (Staba *et al.*, 1982), a reliable protocol for the regeneration of intact plants via shoot organogenesis has not been reported. In our efforts to develop a simple and effective plant regeneration protocol that can be used for transformation purposes, we investigated the effect of cytokinins, auxins, gelling agents, and ethylene inhibitors on the efficiency of shoot organogenesis in opium poppy. Shoot development from wounded callus tissue that formed on excised cotyledons did not occur in the absence of exogenous cytokinin. The addition of 2 mg ℓ^{-1} BAP was optimal for the development and growth of opium poppy shoots. BAP was superior to kinetin in all aspects of shoot organogenesis. Although these initial experiments were performed on medium solidified with Phytagar, shoot organogenesis was found to be more efficient when Gelrite was used as the gelling agent. The number of shoots produced per cotyledonary explant was 35% higher, and the growth of shoots was 25% greater, on 3 g ℓ^{-1} Gelrite compared to 6 g ℓ^{-1} Phytagar. The addition of the auxin NAA and to a lesser extent indole-3-butyric acid (IBA), reduced the regeneration frequency and growth of shoots. The number of shoots produced per explant was marginally higher when 0.1 mg ℓ^{-1} indole-3-acetic acid (IAA) was added to the basal medium containing 2 mg ℓ^{-1} BAP and 3 g ℓ^{-1} Gelrite, but the growth of shoots was reduced by about 20%; thus, auxins were not included in the optimized shoot regeneration medium. Addition of the ethylene inhibitor $AgNO_3$ improved the shoot regeneration frequency by about 20%, and shoot growth by 30%, at an optimal concentration of 5 mg ℓ^{-1}. Based on these results, our optimized shoot regeneration medium consisted of B5 salts and vitamins, 30 g ℓ^{-1} sucrose, 2 mg ℓ^{-1} BAP, 5 g ℓ^{-1} $AgNO_3$, and 3 g ℓ^{-1} Gelrite (Park & Facchini, 2000).

Other Papaver spp.

The blood or Iranian poppy, *Papaver bracteatum* and the oriental poppy, Papaver orientale are generally used as ornamental plants, but have also been used traditionally as medicinals.

The first plant regeneration of *P. bracteatum* through

somatic embryogenesis was developed by Day *et al.*, (1986). Iiahi & Ghauri (1997) reported plant regeneration in embryogenic callus cultures of *P. bracteatum* using growth hormones (1 mg/ℓ NAA and 0.5 mg/ℓ BAP) and low temperature (15℃). Recently the improved protocol for callus induction, somatic embryogenesis and organogenesis of *P. bracteatum* was established (Alkhimova *et al.*, 2001).

Papaver orientale has been regenerated via somatic embryogenesis through embryogenic callus. Alkaloid, lipid, starch and triacylglycerol metabolism related to somatic embryogenesis in *Papaver orientale* tissue cultures has also been studied (Schuchmann & Wellmann, 1983; Hara *et al.*, 1985; Kassem & Jacquin, 2001)

CONCLUSIONS

Plant regeneration systems are an essential part of molecular approaches leading to plant improvement in the poppy family. These methods are an intermediary step whereby advances made by molecular biologists in gene isolation and modification may be transferred to plant cells. These transgenic plants that are regenerated in culture can then be full evaluated either as a tool to study the transgene in question or to determine success of a plant improvement attempt. Cells and tissues of poppy plant species have proven to be difficult to culture and establishing optimal growing conditions *in vitro* is not a trivial matter. Therefore there continues to be an urgent need for extensive work in the field of basic tissue culture protocols for poppy plants to further plant improvement through molecular biology approaches as well as to improve the use of these plants as tools in further studies.

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