

Somatic embryogenesis, rhizogenesis, and morphinan alkaloids production in two species of opium poppy

My Abdelmajid Kassem^{1*} and Annie Jacquin²

¹Department of Plant, Soil, and General Agriculture, Southern Illinois University at Carbondale, Carbondale, IL 62901, USA

²Laboratoire de Pharmacognosie & Phytotechnologie, Faculte de Pharmacie, 1 rue des Louvels, 80037 Amiens, France

A study of somatic embryogenesis and rhizogenesis and their influence on production of morphinan alkaloids on two species of opium poppy is presented. We identified the ratios of auxin and cytokinin that caused somatic embryogenesis and rhizogenesis in hypocotyl and cotyledons of *Papaver somniferum album* and *Papaver orientale splendidissimum*. The hypocotyls and cotyledons both show somatic embryogenesis in *Papaver somniferum album* whereas only the cotyledons were embryogenic in *Papaver orientale splendidissimum*. For rhizogenesis, the most important response is on the cotyledons and leaves in these two species. Histology showed characteristic stages of somatic embryo: Globular, cotyledonous, and heart cotyledonary. High performance liquid chromatography analysis showed that the roots of both species synthesized codeine, thebaine, and papaverine. Morphine was only detected in aerial parts of *Papaver somniferum album*. Codeine and thebaine were detected in the rhizogenous but no embryonic callus. These results suggest that root organogenesis is causally related to alkaloid biosynthesis.

INTRODUCTION

Secondary metabolites can have many roles including pathogen defense, color, odor, and for some others the role of nitrogen source. For example, alkaloids are degraded to amino acids [1]. Several strategies have been used to enhance the production of certain metabolites, in particular alkaloids. Some authors have varied the culture conditions in order to find a satisfactory equilibrium between the growth of cells and their production of secondary metabolites. Thus, Becker [2] demonstrated that the composition of the culture medium, light, and temperature are factors that influence the production of metabolites by *Nicotiana tabacum*, *Catharanthus roseus*, and *Peganum harmala*. Increased sucrose and phytohormones concentrations can also stimulate the production of alkaloids [3, 4]. Numerous studies had shown that the production of morphinan alkaloids *via in vitro* cultures requires organogenesis of tissues in cultures [5–8].

We studied the influence of somatic embryogenesis and rhizogenesis on the accumulation of morphinan alkaloids in opium poppy. We determined the optimum culture conditions for rhizogenous callus and somatic embryos from *Papaver somniferum album* and *Papaver orientale splendidissimum*. Organogenic callus capacity to produce morphinan alkaloids was determined.

MATERIAL AND METHODS

Plant material

Seeds (of the two species) were treated using the method of Rush *et al.* [9] and germinated in darkness in magenta boxes containing Linsmaier and Skoog [10] medium. Seeds

were also germinated in distilled water with agar at 10 g/l for stock plants that provides explants; cotyledons, hypocotyls, leaves, and roots. Explants were cultured on Linsmaier and Skoog medium at different concentrations of plant growth regulators in Petri dishes. NAA/kinetin ratios were, respectively, 0.2/0.1, 0.5/0.1, 1/0.2, and 2/0.2 mg/l. The cell suspensions for callus of opium poppy were cultured in two media:

(1) S1 medium, which is the LS medium plus 2,4-D (0.2 mg/l) and kinetin (0.1 mg/l), and the sucrose at 30 g/l.

(2) S1PGRF medium, which is LS medium without plant growth regulators.

Cultures were maintained at 25°C ± 2°C, at darkness and transferred every 3 to 4 weeks.

Extraction and dosage of morphinan alkaloids

The plant material (embryogenous and rhizogenous callus, aerial parts of plants) was lyophilized for 48 hours to eliminate water. The morphinan alkaloid extraction was from 20 mg of dried material, with a mixture of chloroform and concentrated ammonium (49/1, v/v) at boiling point under reflux for two hours and repeated. After each extraction, the chloroform and ammonium extract was vaporized at 35°C under vacuum. The dried residue was dissolved by 1 ml of HPLC buffer containing acetonitrile 8% (v/v) in which the morphinan alkaloids are soluble. The mixture was agitated during one hour at 40°C and filtered for HPLC analysis. For analysis we used reverse phase chromatography, with an inverted elution gradient.

Histological study

Callus, roots, or somatic embryos tissue were suspended in one drop of gelose, and put in a fixer AAF (Alcohol Acetic/Formol) for 48 hours at room temperature [11]. Dehydration was with ethanol, at 100% (v/v), which permitted water extraction from the samples [12]. Embedded tissue was sliced with a microtome, deparaffinated, and colored with Schiff Periodic Acid (SPA) and Naphtol Blue Black (NBB). We mounted slices on slides using the EUKIT resin. The sections were dried and observed under a photonic microscope.

RESULTS AND DISCUSSION

Somatic embryogenesis

There are two common origins for somatic embryos. Direct, in which the embryo developed upon the explant, and indirect where the embryo arises from a callus [13].

We studied somatic embryogenesis in opium poppy from two different systems: A cell suspension and different explants in liquid and solid media.

Somatic embryogenesis from a cell suspension

From an embryogenic suspension of *Papaver somniferum album* cultivated on liquid media S1 and S1PGRE, small black callus were transferred to solid media containing 0.2 mg/l of NAA and 0.1 mg/l of kinetin and two different concentrations of sucrose (LS-NAA/BA-S30 and LS-NAA/BA-S60). These small black callus were also transferred to solid medium LS-S30 without plant growth regulators.

We observed that 14.5% of explants derived from callus grown in liquid medium S1HF gave somatic embryo and 18% of somatic proembryo when transferred to solid medium LS-NAA/BA-S30. When transferred to solid medium LS-NAA/BA-S60, however, callus derived from liquid medium S1 produced no somatic embryos but only 2.5% of somatic proembryos after transfer to solid media LS-NAA/BA-S30 and LS-NAA/BA-S60. We also observed that callus derived from S1HF and from S1 produced no embryonic structures when transferred to medium LS-S30 without plant growth regulators (Figure 1). The presence of some small proembryos on solid medium LS-NAA/BA-S30 after transfer of callus from the medium S1 (containing 2,4-D and kinetin) confirmed that removal from plant growth regulators improves somatic embryogenesis.

We observed the first somatic embryo after four weeks of culture in liquid suspension S1PGRE. The somatic embryos were white masses (Figure 2, photo 1) within which we observed different stages of somatic embryogenesis: Globular, cordiforme, and cotyledonous.

Increased sucrose concentration in the culture medium inhibited the development of somatic proembryos to somatic embryos. The results contrast with observations in *Panax ginseng*, embryogenous tissues cultivated on Gamborg medium with a 100 mg/l of sucrose at high temperature produced numerous somatic embryos [14].

Somatic embryogenesis and root formation occurs simultaneously in cell suspensions or cultures established on

solid media. This is remarkable because these cells were simultaneously embryogenic and organogenic. No previous data on somatic embryogenesis in opium poppy [15, 8] shows the production of roots and somatic embryo, from the same culture media. We observed similar results in *Papaver orientale splendidissimum*, however, the frequency of somatic embryogenesis was lower than in *Papaver somniferum album*.

Somatic embryogenesis from different explants

In order to study somatic embryogenesis as a function of explant type and plant growth regulator combinations, we cultivated cotyledons, hypocotyls, and roots on LS medium with different ratios NAA/kinetin.

We observed that after four months of culture, cotyledon was the explant that gave the highest percentages of somatic embryos, 16% with NAA/kinetin = 0.2/0.1 mg/l and 15% with NAA/kinetin = 0.5/0.1 mg/l. Embryogenesis from hypocotyl occurs in 5% of explants with NAA/kinetin = 0.2/0.1 mg/l and 1.5% with NAA/BA = 0.5/0.1 mg/l. Roots did not produce any somatic embryos. For both cotyledons and hypocotyls NAA/kinetin = 0.2/0.1 mg/l and 0.5/0.1 mg/l were the optimal plant growth regulator combinations (Figure 3).

On solid medium we observed somatic proembryos, somatic embryo in the heart cotyledonary stage and small plants developed from somatic embryos born directly from cotyledons (Figure 2, photos 3, 4, and 5).

The absence of somatic embryos in the presence of highest ratios of plant growth regulator combinations maybe due to the high concentration of kinetin, inhibiting embryo induction. We observed that kinetin did not promote somatic embryogenesis when the concentration passed beyond 0.2 mg/l. During some transfers, a portion of somatic embryo was lost. The mineral solution used, or inadequate transfers frequency may have caused these losses [16].

Rhizogenesis

In *Papaver somniferum album*, the plant growth regulators combinations most favorable to rhizogenesis were NAA/kinetin = 0.5/0.1 mg/l and 0.2/0.1 mg/l. The explants that were rhizogenic were cotyledons and leaves (Figure 4).

In *Papaver orientale splendidissimum*, rhizogenesis was only obtained with NAA/kinetin = 0.5/0.1 mg/l. The percentages of rhizogenic explants were lower than in *Papaver somniferum album*. As with *Papaver somniferum album*, leaves and cotyledons were the explants that produces the most rhizogenesis (Figure 5).

Root formation from *in vitro* cultures pass through two stages: Induction and initiation. The stages are inversely related to endogenous auxin concentration [17].

Root initiation, in *Papaver somniferum*, started after the passage of explants to a medium containing low concentrations of auxin. When the exogenous concentration of auxin increases, the percentage of rhizogenesis can be reduced (ratios NAA/kinetin = 1/0.2 mg/l and 2/0.2 mg/l).

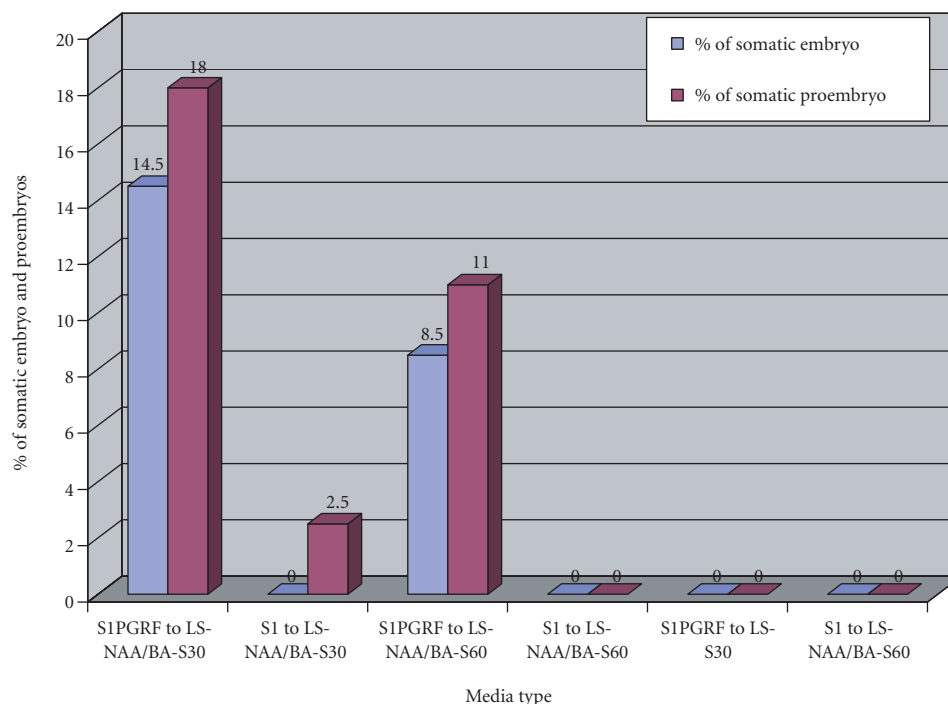


FIGURE 1: Somatic embryogenesis from a cell suspension of *Papaver somniferum album* cultivated on liquid media S1 (2,4-D 0.2 mg/l and kinetin 0.1 mg/l) and S1PGRF (plant growth regulators free). NAA, naphthaleneacetic acid; BA, benzyladenine; LS, Linsmaier and Skoog medium.

HPLC analysis of morphinane alkaloids

Roots of plants cultivated in vitro

Plants roots of *Papaver somniferum album* contained codeine (0.031%, 0.031 mg of codeine per 100 mg of dried material), thebaine (0.012%) and papaverine (0.01%) (Figure 6). The roots of plants of *Papaver orientale splendidissimum* contained the same morphinan alkaloids in lower quantities (codeine (0.014%), thebaine (0.007%), and papaverine (0.013%)) (Figure 7). The roots of both species did not contain any morphine. However, morphine is preponderant in young roots of plants of opium poppy in field [18]. The accumulation of codeine is different from the accumulation of morphine in plant organs.

Aerial parts of plants cultivated in vitro (stems and leaves)

The aerial parts of plants of *Papaver somniferum album* contained morphine (0.023%), codeine (0.013%), thebaine (0.017%), and papaverine (0.09%) (Figure 6). The aerial parts of plants of *Papaver orientale splendidissimum* did not contain morphine and papaverine but did contain codeine (0.015%), less thebaine (0.005%) (Figure 7).

With *Papaver somniferum*, a low level of thebaine was detected after the 10th day of culture whereas codeine and morphine were detected after the 15th and 20th days of culture, respectively [18]. Thebaine was the principal morphinan alkaloid synthesized by *Papaver somniferum* cultures followed

by codeine. Morphine was present in certain cases but with the lowest level, it is absent in general [19–21, 9].

Rhizogenous and nonrhizogenous callus

In nonrhizogenous callus derived from cotyledons of *Papaver somniferum album* no morphinan alkaloids were detected. However, in rhizogenous callus we detected a low amount of codeine (0.016%) and thebaine (0.004%) (Figure 6).

Hsu and Pack [20] had shown that cellular extracts from callus obtained from hypocotyls of *Papaver somniferum*, contained thebaine and a low level of codeine but morphine was not detected.

Our studies on *Papaver somniferum*, and others, shows that this species is the most able to produce morphinan alkaloids to *in vitro* culture.

Histological study

In order to confirm the presence of different stages of somatic embryos observed in opium poppy, we expected to observe laticifer cells that maybe connected to synthesis and accumulation of morphinan alkaloids in opium poppy tissues cultivated *in vitro* [19–21, 9]. We observed embryonic structures in globular and heart cotyledonary stages (Figure 8, photo 2). We observed several different characteristic stages of somatic embryo observed under binocular wen (Figure 8, photos 3 and 4). We also observed plant lets from somatic embryo that derives from cotyledons, and roots from rhizogenous callus (Figure 2, photos 5 and 6).

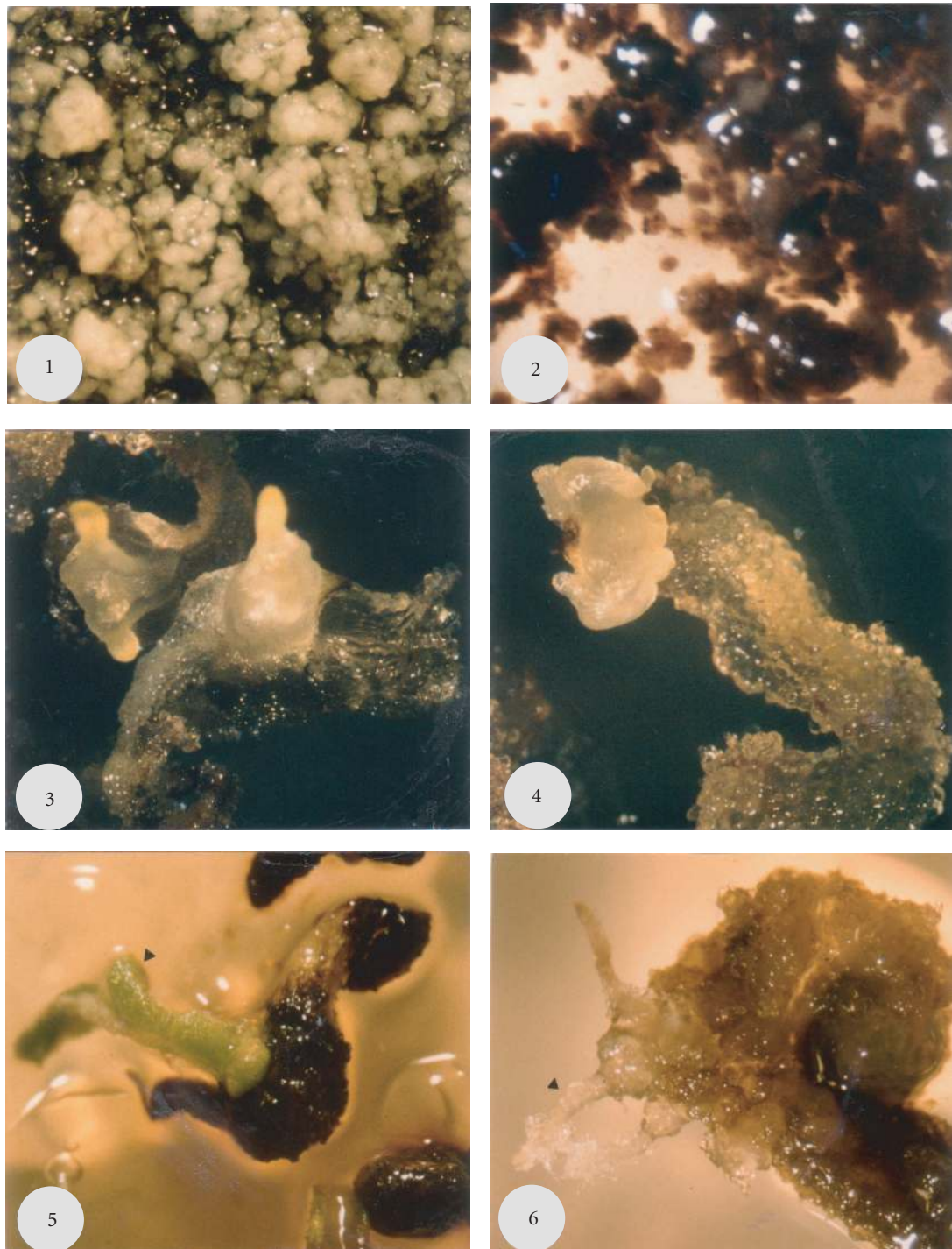


FIGURE 2: Somatic embryos of *Papaver somniferum album*. (1) Somatic proembryo and embryo in globular stage after transfer to solid medium LS-NAA/BA-S60 (NAA/BA = 0.2/0.1 mg/l) ($\times 10$), (2) Nonembryogenous suspensions after transfer to solid medium LS-S30 ($\times 10$), (3) Two somatic embryos of 20 days old, from cotyledon, on solid medium LS-NAA/kinetin = 0.2/0.1 mg/l ($\times 10$), (4) Proembryo of 20 days old, from cotyledon, on solid medium LS-NAA/kinetin = 0.2/0.1 mg/l ($\times 10$), (5) Plant of 5 weeks from a cotyledon on solid medium LS-NAA/kinetine = 0.2/0.1 mg/l ($\times 10$), (6) Rhizogenous callus from a cotyledon on solid medium LS-NAA/kinetine = 0.2/0.1 mg/l ($\times 10$). Scale: 1 cm = 1 mm.

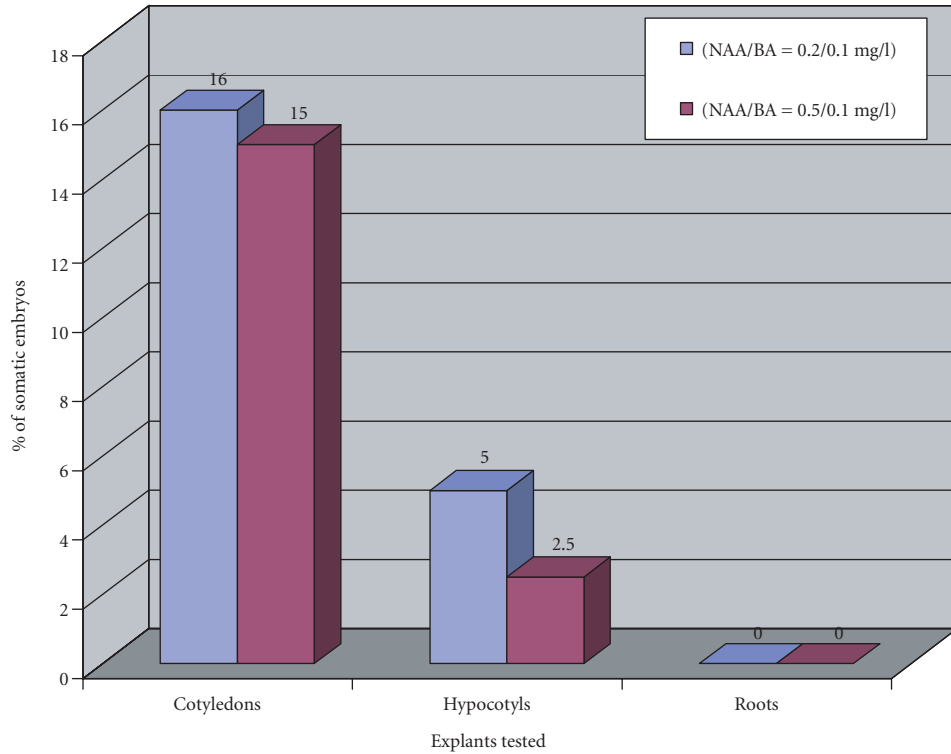


FIGURE 3: Somatic embryogenesis obtained from roots, cotyledons, and hypocotyls of *Papaver somniferum album*, with two plant growth regulator combinations (NAA/kinetin = 0.2/0.1 and 0.5/0.1 mg/l), after four months of culture.

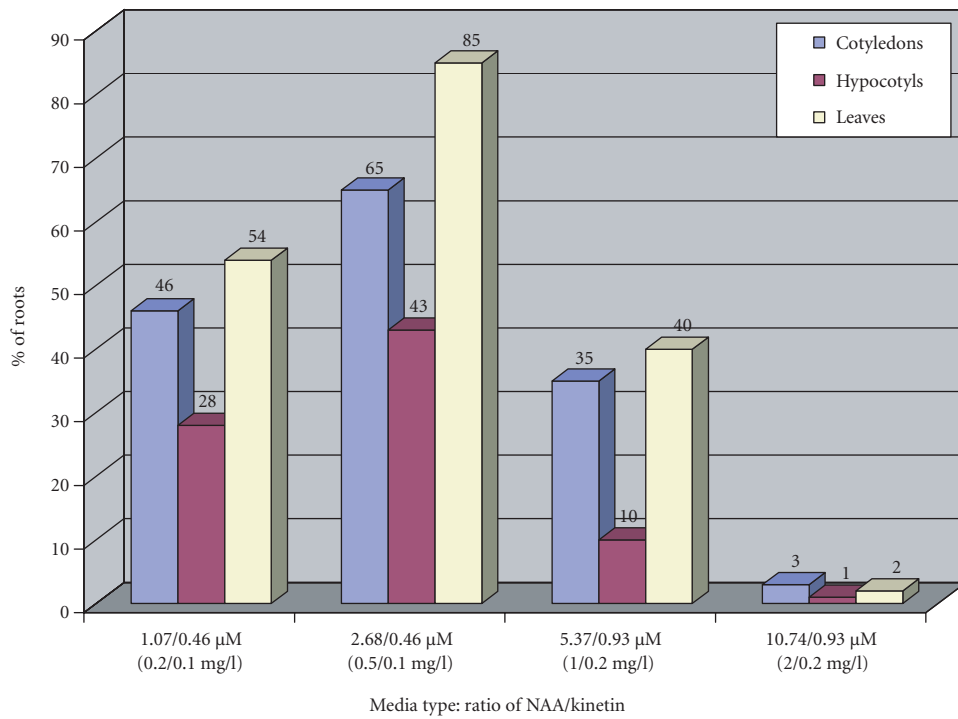


FIGURE 4: Percentages of roots obtained from callus of *Papaver somniferum album* as a function of plant growth regulator combinations, after four months of culture.

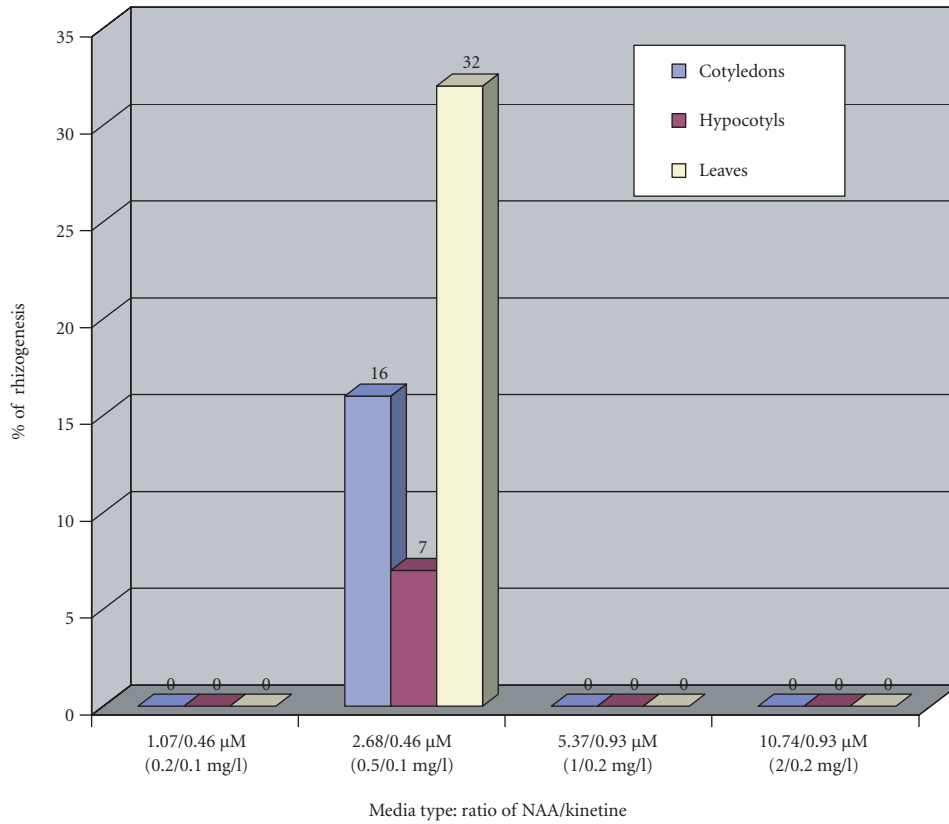


FIGURE 5: Percentages of roots obtained from callus of *Papaver orientale splendissimum* in function of plant growth regulator combinations NAA/kinetin after four months of culture.

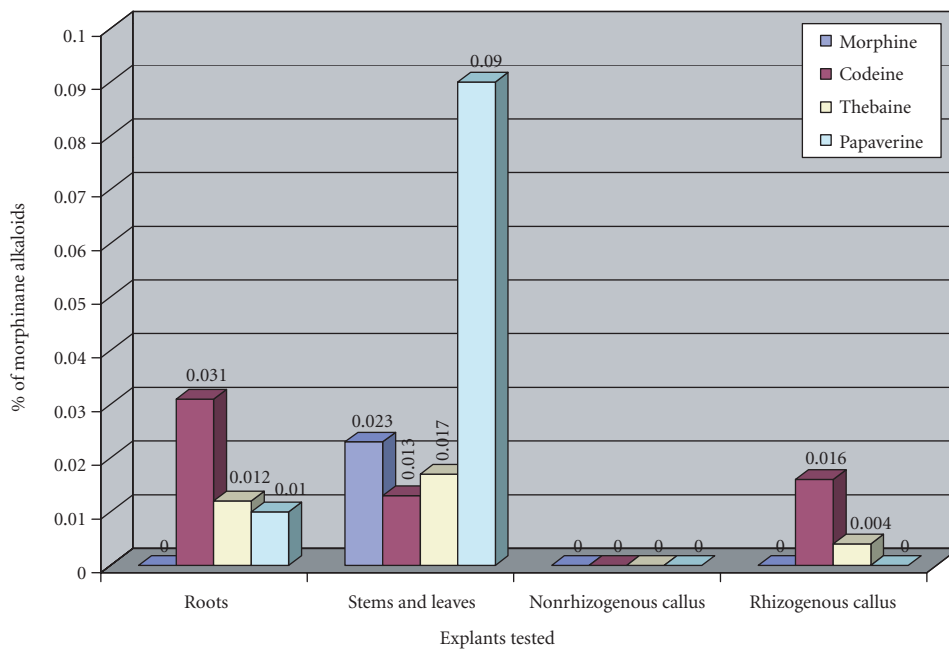


FIGURE 6: Concentrations of morphinane alkaloids (mg/100 mg of dried material) obtained in function of explants tested, in *Papaver soniferum album*.

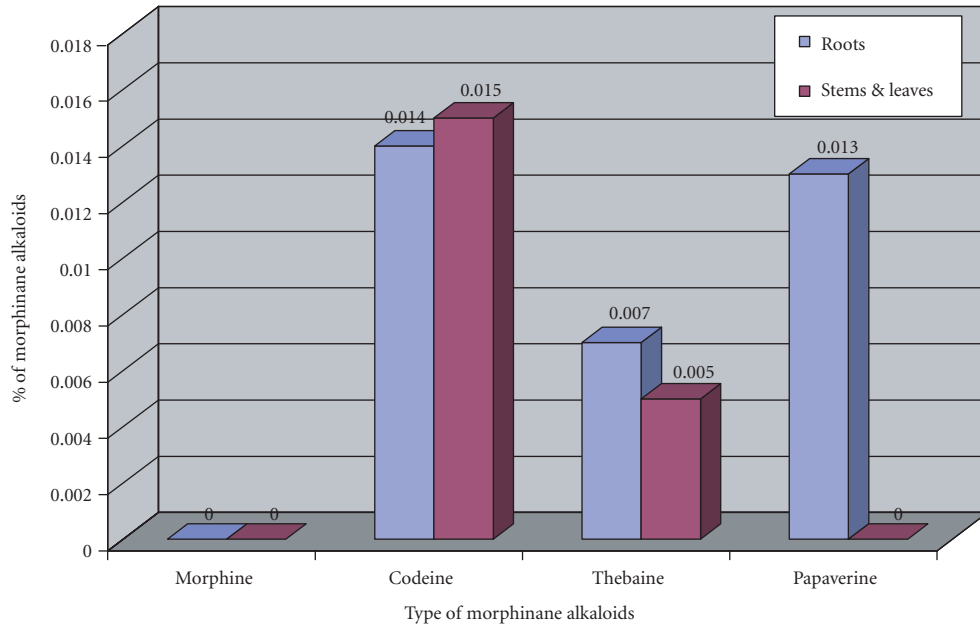


FIGURE 7: Concentrations of morphinane alkaloids (mg/100 mg of dried material) obtained in function of explants tested, in *Papaver orientale splendidissimum*.

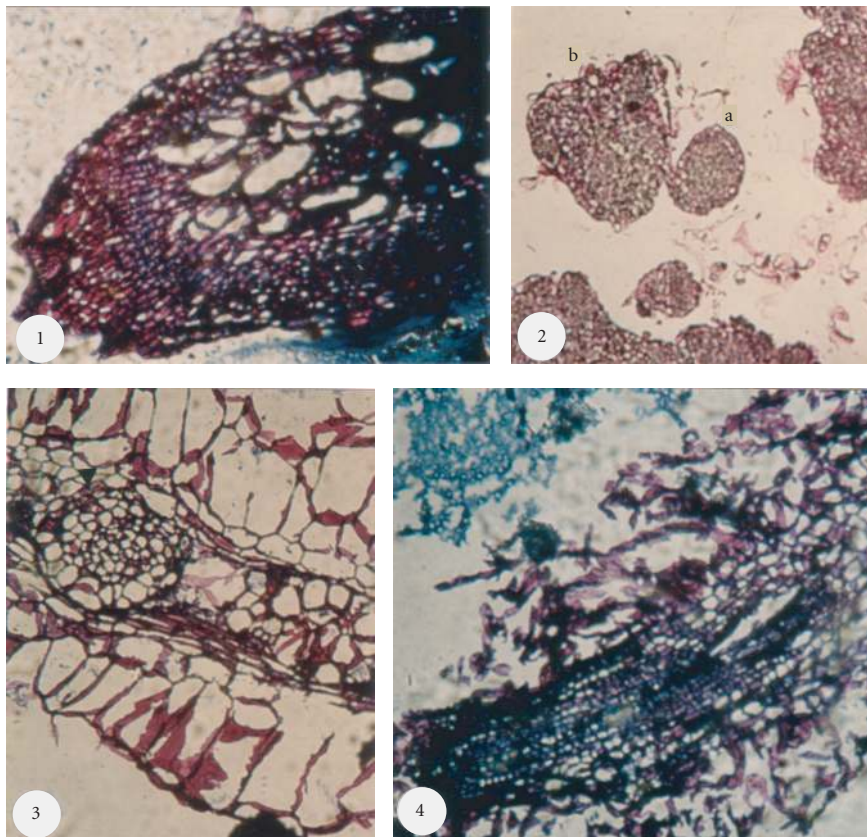


FIGURE 8: Histologic cuts of *Papaver somniferum album* cultures. (1) Roots of plants, at 3 months, cultivated in soil ($\times 10$), (2) Embryogenous callus ($\times 10$): 2a, somatic embryo in globular stage; 2b, somatic embryo in heart cotyledonary stage, (3) Rhizogenous callus ($\times 10$): Presence of a meristematic zone that shows the depart of a secondary root, (4) Rhizogenous callus ($\times 10$): Presence of vessels in helix. Scale: 1 cm = 1 mm.

Laticifer cells were not observed in the roots of plants of *Papaver somniferum album*, *Papaver orientale splendidissimum* cultivated *in vitro*, in plants of *Papaver somniferum album* obtained from the field, in embryogenous callus or in rhisogenous callus of *Papaver somniferum album*. The structures observed were the conductor vessel helixes (Figure 8, photos 1 and 4) and the meristematic zones that show secondary roots departing from the principal roots (Figure 8, photos 2 and 3).

CONCLUSION

We observed somatic embryogenesis and rhizogenesis in two species of opium poppy. We provided evidence that the ratios of NAA/kinetin = 0.2/0.1 and 0.5/0.1 mg/l were the most favorable for somatic embryogenesis and rhizogenesis (2.5% to 18% for somatic embryogenesis and 7% to 85% for rhizogenesis). Hypocotyls and cotyledons were the two explants that produced most direct somatic embryogenesis (2.5% to 18%). We also demonstrated that cotyledons and leaves gave the highest percentage of rhizogenesis.

Transfer of somatic embryos to a medium without plant growth regulators may help to optimize the survival of somatic embryos and their development in plants [17].

The HPLC analysis of morphinan alkaloids content of these cultures shows the importance of differentiation and organogenesis (roots) for the biosynthesis of these alkaloids. We observed that roots are the cardinal sites of biosynthesis of codeine but also of thebaine and papaverine. Codeine is an important molecule used in prescription in medicine, and it will be interesting to optimize its production.

ABBREVIATIONS

NAA	α -Naphthaleneacetic acid
BA	benzyladenine
LS	Linsmaier and Skoog medium
2,4-D	dichlorophenoxyacetic acid
HPLC	high performance liquid chromatography

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- * Corresponding author.
E-mail: kassem@siu.edu
Fax: +1 618 453 7457; Tel: +1 618 453 5727



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