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Production of sanguinarine by elicited plant cell culture II. Further nutritional aspects

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Abstract

Papaver somniferum cell suspension cultures carried out in shake flasks were elicited with chitin under batch and nutrient feeding conditions to increase sanguinarine production. Batch cultures yielded maximum production of 270 mg l^{-1} upon elicitation during exponential growth phase at extracellular phosphate depletion. The addition of concentrated medium solutions prior to elicitation of stationary phase cultures allowed a modest increase in production to 350 mg l^{-1}. Further optimization of the nutrient solution yielded production of 575 mg l^{-1}. Examination of the composition of this nutrient solution showed an absolute requirement of elicited cultures for the presence of carbohydrates, phosphate and sulfate during fedbatch additions for maximum sanguinarine production.

Keywords: Sanguinarine; *Papaver somniferum*; Plant cell culture; Elicitation; Nutrient

1. Introduction

In the first paper of this series, the market potential of sanguinarine for dental hygiene products was highlighted (Archambault et al., 1996). Current methods of production of this valuable phytochemical involve agricultural and plant biomass extraction practices which may not afford consistent and sufficient supply to meet market demand. Intact plants of *Papaver somniferum* accumulate high quantities of alkaloids, mostly of the morphinan type (Williams and Ellis, 1989). Cell lines derived from these plants, however, generally do not accumulate these alkaloids (Rueffer, 1985), but usually produce benzophenanthridine alkaloids of which sanguinarine is the main component (Rueffer, 1985; Williams et al., 1992; Williams and Ellis, 1993).

Sanguinarine production by *Papaver* cultures can occur endogenously or by elicitation. The first approach is generally not attractive due to the low yields (~ 1% of the biomass dry weight (dw)) and productivities (< 10 mg l^{-1} d^{-1}) as well as the instability of this process (Rueffer, 1985; Hook et al., 1988; Williams and Ellis, 1993; Chauret et al., 1990; Archambault, 1991; Archambault et al., 1996). Alternatively, higher production levels have been achieved upon treating *P. somniferum* cell cultures with the biotic elicitors *Botrytis* (Eilert et al., 1985)

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or chitin (Williams et al., 1992; Archambault et al., 1996). This production can also be enhanced by the addition of an alkaloid sink (Williams et al., 1992) such as the polymeric adsorbent resin Amberlite XAD-7 which afforded easier harvesting of most (> 85%) sanguinarine produced for eventual culture reuse (Eilert et al., 1985; Tyler et al., 1988).

A great deal of studies have been carried out on elicitation. However, specific knowledge about the production aspects of this process remains sketchy at best. Most research on the induction of secondary metabolites by elicitation has been empirical and culture conditions have rarely been investigated. Previously, we have shown that the growth conditions and stage and the nutritional regime prior, at, and during elicitation of *P. somniferum* cell cultures were critical to high and reproducible production of sanguinarine (Archambault et al., 1996). Thus, extracellular phosphate concentration near depletion was found to be a reliable culture marker for best elicitation induction while carbohydrates and ammonium were essential for high production and culture survival for reuse. According to these findings, modification of the growth medium formulation and first nutrient additions at elicitation resulted in increase of sanguinarine concentration and productivity from 200 to 385 mg l^{-1} and from 12 to 26 mg l^{-1} d^{-1}, respectively.

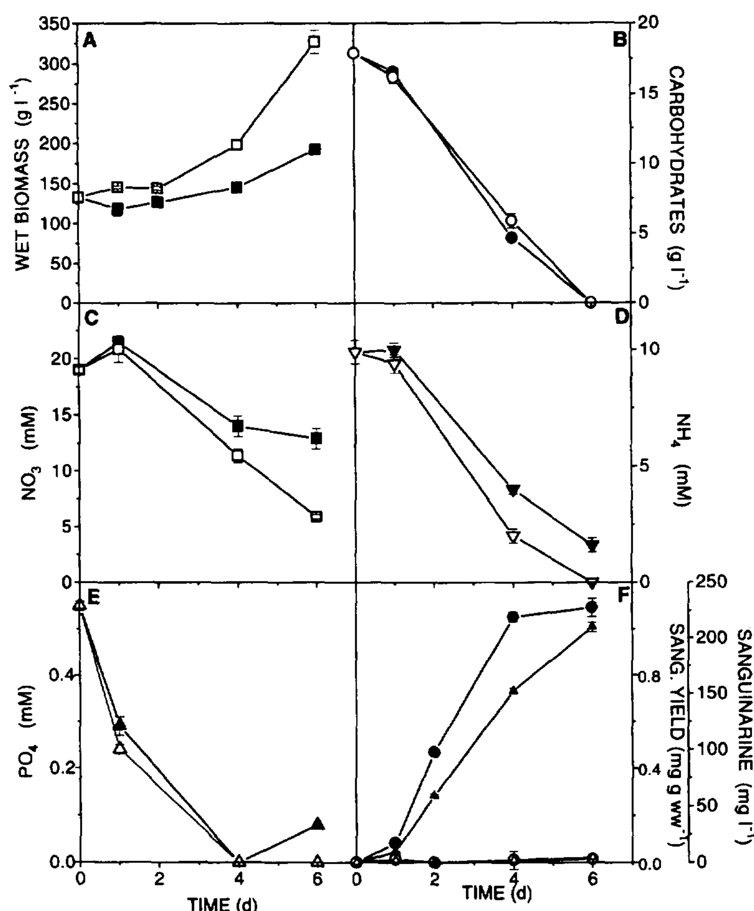

Fig. 1. Growth, nutrients uptake and sanguinarine production curves of elicited *P. somniferum* cell suspension batch cultures. Solid symbols are for elicited cultures. Error bars indicate one standard deviation from the average value. (F) ○ and ●, sanguinarine yield (mg g ww^{-1}); △ and ▲, sanguinarine concentration (mg l^{-1}).

These productivity levels, however, were achieved at low dry biomass concentration (< 9 g l^{-1}) and remain too low for economical production (≥ 600 mg l^{-1} d^{-1}; Archambault et al., 1996). Consequently, the objectives of this second study were to further investigate and characterize the nutrient requirements during elicitation of higher density *Papaver somniferum* cell cultures carried out in shake flasks to increase sanguinarine production.

2. Materials and methods

2.1.1. Plant cell cultures

The *Papaver somniferum* cell line PS-SBR1 used in this study was initiated, grown in MS (Murashige and Skoog, 1962) medium supplemented with 0.1 mg l^{-1} 2,4-dichlorophenoxyacetic acid, 0.5 mg l^{-1} benzyladenine and 30 g l^{-1} sucrose, and maintained as previously described (Archambault et al., 1996).

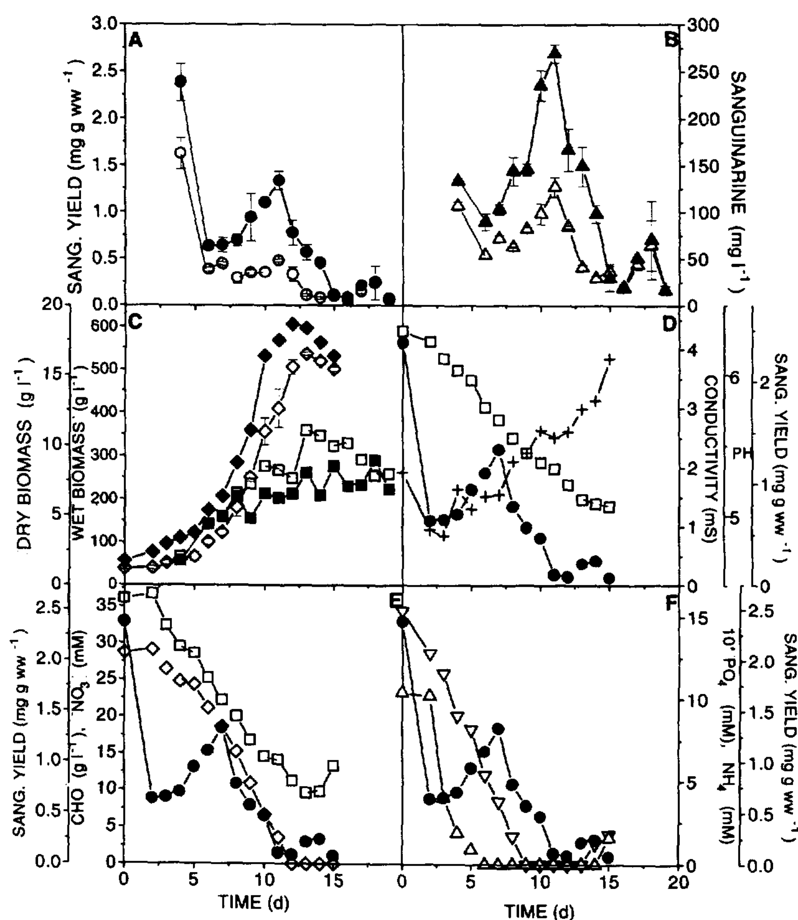

Fig. 2. Growth, nutrients uptake and sanguinarine production curves of batch cultures elicited along the growth curve. (A and B) Solid symbols are for elicited cultures. Results are for total culture time (growth and production). (C) ◆, dry biomass concentration of untreated cultures (g l^{-1}); ◇, wet biomass concentration of untreated cultures (g l^{-1}); ■, dry biomass concentration of elicited cultures (g l^{-1}); □, wet biomass concentration of control cultures (g l^{-1}). (D) ●, sanguinarine yield of elicited cultures (mg g ww^{-1}); □, conductivity of untreated cultures (mS); +, pH of untreated cultures. (E) ●, sanguinarine yield of elicited cultures (mg g ww^{-1}); ◇, carbohydrates concentration of untreated cultures (g l^{-1}); □, nitrate concentration of untreated cultures (mM). (F) ●, sanguinarine yield of elicited cultures (mg g ww^{-1}); △, phosphate concentration ($\times 10$) of untreated cultures (mM); ▽, ammonium concentration (mM).

2.1.2. Growth and elicitation experiments

For the production kinetics experiment (Fig. 1), a culture was grown in a 1-l flask containing 300 ml fresh medium and inoculated with 100 ml of a 14-day-old suspension which resulted in an initial dry biomass concentration of 2 g l^{-1}. After 7 d, this culture was distributed in 10-ml volumes in 50-ml flasks containing 0.5 g wet XAD-7 resin (Williams et al., 1992). Four series of triplicate flasks were then elicited with 300 μl chitin slurry, while others were used as controls. Triplicate elicited and control flasks were harvested as indicated for sanguinarine determination.

In the case of the elicitor concentration experiment, a culture was grown in a 1-l flask for 7 d, distributed in 50-ml flasks containing resin as above and elicited with varying volumes of the chitin slurry as indicated. Triplicate elicited flasks were harvested after 4 d for sanguinarine content measurement.

For the growth curve experiment (Fig. 2), cultures were grown in 125-ml flasks containing 30 ml fresh medium and inoculated with 10 ml of a 14-day-old suspension (initial dry biomass of 2 g l^{-1}). At the indicated times, triplicate flasks were removed and part of these cultures were distributed in 50-ml flasks containing resin and elicited with 300 μl chitin slurry or grown as controls as above. The remaining suspension was harvested and analyzed for biomass and extracellular main nutrients content. Duplicate elicited and control 50-ml flasks were harvested after 4 d for sanguinarine determination.

For all nutrient addition experiments (Figs. 3, 6–8), cultures were grown in 1-l flasks for 14 d as above. Thereafter, they were distributed in 80-ml volumes into 250-ml flasks and the indicated solutions, which pH were adjusted to 5.5 prior to steam sterilization (20 min), were added at a ratio of 20% (v/v). These cultures were incubated for 0 to 5 days, as indicated, and subsequently distributed into 50-ml flasks containing resin and elicited with 300 μl chitin slurry as above. Duplicate elicited flasks were harvested after 4 d for sanguinarine determination.

In the case of the first phosphate experiment (Figs. 4 and 5), 80-ml cultures were initiated and grown in 250-ml flask as above using regular MS medium, except for the phosphate concentration which was varied from 0 to 200% of the original MS concentration of 1.2 mM KH_2PO_4. At the indicated times, two 250-ml flasks were harvested and part of these cultures was distributed in 50-ml flasks containing resin which were elicited as above. The remaining suspension was analyzed for biomass con-

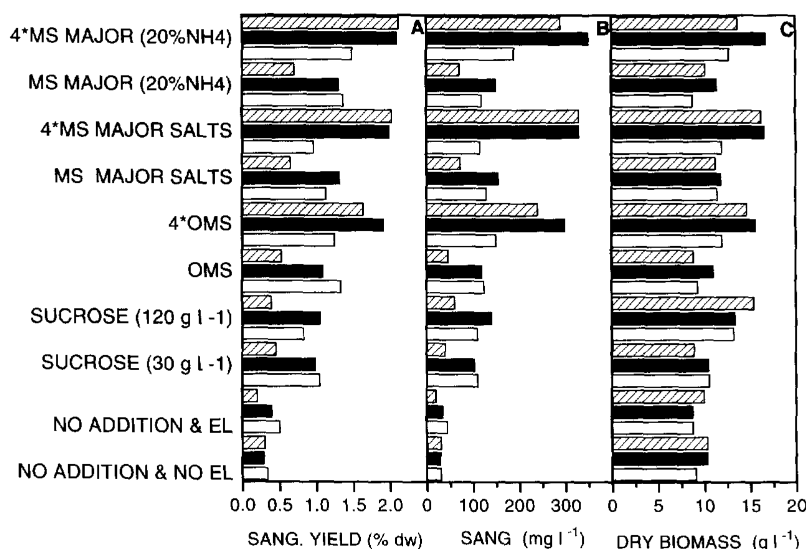

Fig. 3. Sanguinarine production and final biomass concentration of elicited fedbatch stationary phase cultures. Open bars, results following nutrients addition with no incubation prior to elicitation. Solid bars, results following nutrients addition and 2 d of incubation prior to elicitation. Striped bars, Results following nutrients addition and 5 d of incubation prior to elicitation.

tent. Triplicate elicited flasks were harvested after 4 d for sanguinarine determination.

For the experiment on the effect of the culture volume and oxygen transfer rate on the elicitation process (Fig. 10), a culture was grown in a 1-l flask for 7 d as above. At that time, this culture was distributed in 25-, 50- and 100-ml volumes in duplicate 250-ml flasks containing proportional amounts (5% (w/v); Williams et al., 1992) of wet XAD-7 resin as above. These cultures were elicited with 25 $\mu l\ ml^{-1}$ culture of chitin slurry, maintained as above and harvested after 4 d for sanguinarine content measurement. The oxygen transfer coefficient ($k_L a$) of this culture system containing 25, 50 and 100 ml water was measured at 26°C and 120 rpm using an Inglod polarographic dissolved oxygen probe by the conventional gassing–degassing method. The resulting $k_L a$ values (average of two measurements for each liquid volume) were $32.9 \pm 1.5\ h^{-1}$, $18.4 \pm 0.8\ h^{-1}$ and $15.9 \pm 0.1\ h^{-1}$.

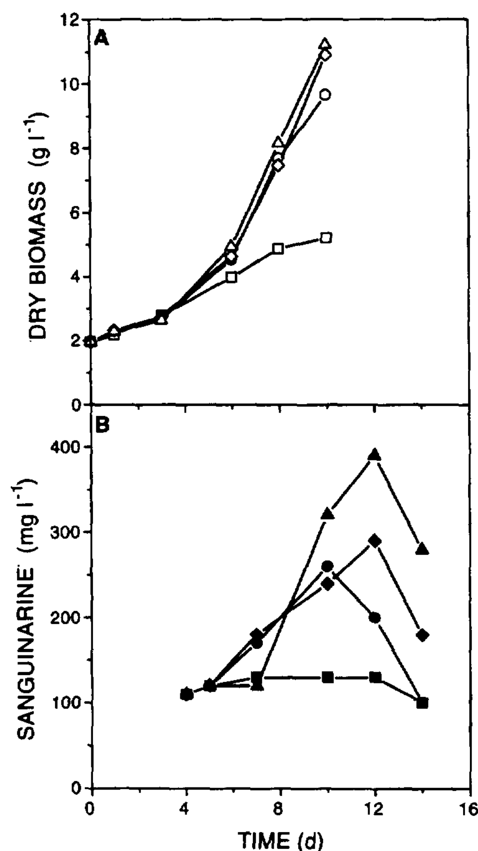

Fig. 4. Growth and sanguinarine production of phosphate supplemented and elicited batch cultures. □ and ■, cultures without phosphate; ○ and ●, cultures with 50% phosphate supplementation; ◇ and ◆, cultures with normal (100%) phosphate supplementation; ▲ and △, cultures with 200% phosphate supplementation.

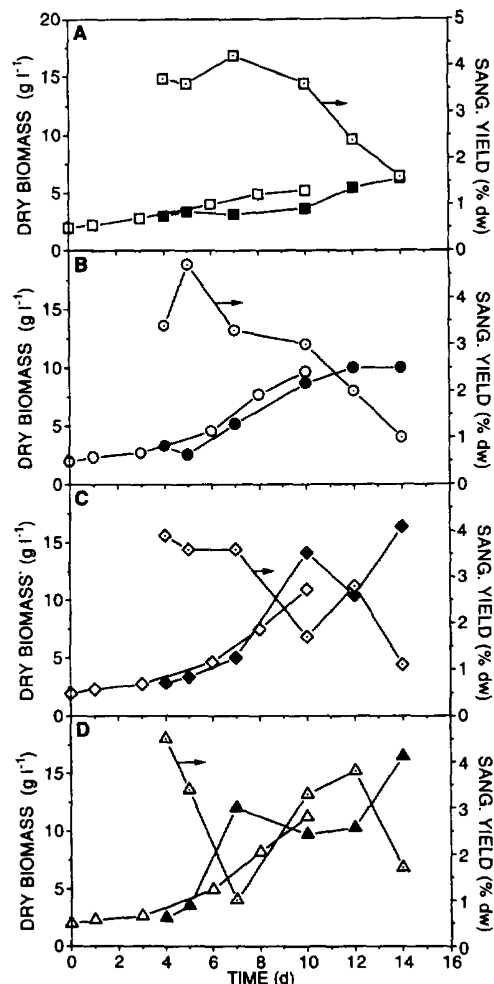

Fig. 5. Growth and sanguinarine yield of the phosphate supplemented and elicited batch cultures of Fig. 4. (A) Cultures without phosphate; (B) cultures with 50% phosphate supplementation; (C) cultures with normal (100%) phosphate supplementation; (D) cultures with 200% phosphate supplementation. Open symbols, dry biomass concentration prior to elicitation (g l^{-1}); solid symbols, dry biomass concentration after elicitation (g l^{-1}); dotted symbols, sanguinarine yield (% dw).

2.1.3. Elicitors preparation

All biotic elicitors tested (xantham gum, karaya gum, pectin and chitin) were obtained from Sigma (St. Louis, MO). They were prepared as a slurry in deionized water (100 mg per 10 ml) and steam sterilized for 20 min prior to use.

2.1.4. Analytical

Suspension culture samples were assayed for pH, conductivity, wet and dried (80°C, 24 h) biomass, main extracellular nutrients and sanguinarine concentration as previously described (Archambault et al., 1996).

3. Results

3.1. Batch cultures

3.1.1. Growth and production of elicited batch cultures

The growth, major nutrients uptake and sanguinarine production curves of *P. somniferum* cell cultures elicited 7 d post inoculation are illustrated in Fig. 1. These results represent typical behaviors of elicited cultures. They experienced lower growth, lower nitrate and similar carbohydrate and ammonium uptakes as compared to control cultures. Elicitation did not lead to culture death as indicated by the absence of phosphate or ammonium release.

The maximum sanguinarine yield (1.2% dw) occurred 4 d after elicitation, as expected (Eilert et al., 1985), followed by a plateau. Consequently, all subsequent cultures were tested for sanguinarine production only 4 d post elicitation. This levelling of production coincided with a slight increase in growth of elicited cultures from days 4 to 6. This may correspond with the removal (uptake or degradation) of the elicitor from the medium.

During this time, control cultures displayed minimal production (0.01%). There were, however, a few occasions when non-elicited cultures showed endogenous production of up to 0.4%.

3.1.2. Elicitor selection and quantity

When the level of elicited plant cell biomass is increased, the concentration of the elicitor may need to be adjusted accordingly for optimal production. Consequently, the concentration of the chitin slurry required for proper elicitation of *P. somniferum* cell cultures was investigated. Results indicate a typical dose–response curve (not shown) where there was low response at low concentration (< 0.3 μl ml^{-1} culture) and exponential increase in sanguinarine production thereafter up to 10–20 μl ml^{-1}. At very high level of elicitor (100 μl ml^{-1}) a 14% decrease in yield was observed. Similar results were obtained for both crude and purified chitin as well as for *Botrytis* (Eilert et al., 1985). Consequently, a wide range of elicitor concentrations, from at least 10 to 30 μl ml^{-1}, can be used for proper elicitation of *P. somniferum* cell cultures without negatively affecting production.

In similar experiments, a variety of putative elicitors were tested for higher production of sanguinarine. Xantham gum, karaya gum and pectin were examined over a similar range of concentrations under identical conditions. The only elicitor that showed sanguinarine production of the same magnitude as chitin was xantham gum (not shown). Maximum production, however, never reached more than 60% of that obtained with chitin. Not surprisingly, elicitors of plant origin (karaya gum and pectin) showed no increase in alkaloid content when they were prepared by steam sterilization. Other methods of preparation, however, can lead to suitable elicitors (Cline and Coscia, 1988). Consequently, chitin was used as the elicitor for all subsequent experiments at a concentration of 20–30 μl ml^{-1} of culture, due to its efficiency, ease of preparation and uniformity.

3.1.3. Elicitation along the growth curve

It was reported that best production required elicitation of cultures around the middle of their exponential phase of growth (Kombrink and Hahlbrock, 1985; Eilert et al., 1985; Eilert, 1987; Williams et al., 1992). This was better defined in the first paper of this series (Archambault et al., 1996) whereby best elicitation time was found to coincide with extracellular phosphate depletion of *P. somniferum* cell cultures. In order to better characterize this critical parameter of this bioprocess, the production of sanguinarine by elicitation was closely examined over the complete growth cycle of a typical *P. somniferum* cell line in relation to major nutrients uptake (Fig. 2). Sanguinarine was obtained by elici-

tation at the indicated times followed by 4 d of production (Fig. 2A and B) in the presence of XAD-7 resin.

The untreated cultures showed slow initial growth up to ~ day 4 (Fig. 2C), which resulted from sucrose hydrolysis (≤ 2 d) and high phosphate and ammonium uptake (Fig. 2F), the latter yielding a sharp decrease in culture pH to 4.9 at day 2–3 (Fig. 2D) likely from high H^+/NH_4^+ exchange (Smith and Raven, 1976). Thereafter, growth proceeded until extracellular carbohydrates depletion (Fig. 2E). Elicited and control cultures displayed similar wet biomass increase as untreated cultures until day 8 (Fig. 2C). Thereafter, elicited cultures experienced limited wet biomass increase during high sanguinarine production. Control cultures, on the other hand, displayed growth in between that of untreated and elicited cultures as a result of their content in XAD-7 resin which was shown to adsorb growth regulators and limit growth (Williams et al., 1992).

Sanguinarine production, in terms of yield (Fig. 2A) and concentration (Fig. 2B), for both elicited and control cultures clearly varied with the growth phase of the stimulated cultures. Remarkably, elicited and control cultures displayed similar production patterns and three peaks of production. Best yields and concentrations obtained by elicitation after 4 and 10–11 d of total culture time, including the 4-d production phase, were 47% and 180–210%, and 25% and 110–140%, respectively, higher than those observed for control cultures. Production of the latter cultures, however, was not negligible as compared to elicited cultures, especially at inoculation (4.3% dw vs. 5.1% for elicited cultures).

The three peaks of production (Fig. 2A and B) were observed upon product induction at inoculation and after 7 and 14 d of growth. Surprisingly, the highest yield of sanguinarine with respect to biomass was found when the cells were first transferred into fresh medium. It appears that the inclusion of XAD-7 resin, which removes growth regulators and acts as a sanguinarine sink (Williams et al., 1992), and the chemical shock or the presence of large quantities of nutrients at inoculation stimulate significantly elicited and endogenous production. In fact, this may be an amplification of the limited increase in production obtained upon treating 14-day-old stationary phase cultures devoid of extracellular nutrients.

The maximum sanguinarine concentration (270 mg l^{-1}) was obtained upon eliciting 7-day-old cultures of low dry biomass content (6.4 g l^{-1}). As seen previously (Archambault et al., 1996), this maximum elicitability of growing *P. somniferum* cell cultures coincided with extracellular phosphate depletion (Fig. 2F), pH increase (Fig. 2D) and the presence of carbohydrates and ammonium. Subsequent decrease in production resulted at least from extracellular exhaustion of the later two nutrients during elicitation. Similar growth and production patterns were observed for other *P. somniferum* cell lines.

Consequently, *P. somniferum* cell cultures can be elicited successfully and reproducibly at extracellular phosphate depletion and at stationary phase, upon addition of fresh medium (and XAD-7 resin) in this last case. However, maximum production was always at low biomass concentration. Obviously, productivity could be increased if the same cultures can be grown and elicited at high dry biomass concentrations (~ 20–35 g l^{-1}). In this context, the elicibility of stationary phase cultures represents the basis for developing a two phase process to increase sanguinarine production towards economical levels. However, the nutrient requirements of high density elicited cultures need to be defined to maximize this production. This was the focus of the second part of this study.

3.2. Fedbatch elicited cultures

3.2.1. Effect of nutrient additions

In order to see if high density suspensions can be induced into the correct physiological state for successful elicitation and sanguinarine production, stationary phase cultures (Fig. 2C: ~ 19 g l^{-1}) were fed with various nutrients, elicited with chitin in the presence of XAD-7 resin as above and examined for sanguinarine production. Initial addition experiments involved a variety of nutrient solutions based on normal MS medium without growth regulators (OMS medium) since high sanguinarine yields were obtained upon eliciting stationary phase cells transferred into fresh medium (Fig. 2A). These solutions included sucrose only (30 and 120 g l^{-1}), OMS medium, MS major salts and MS major salts with 20% of the original content in NH_4NO_3 (20.6 mM) and 4 × concentrates of these solutions. All MS

based solutions and 4× concentrates were supplemented with 30 and 120 g l^{-1} sucrose, respectively. These solutions were added to the cultures at a 20% (v/v) ratio. The resulting suspension were incubated for 0, 2 and 5 d prior to elicitation.

Cultures without addition (Fig. 3) showed little increase in sanguinarine production over non-elicited control cultures indicating lack of nutrients. The addition of carbohydrates alone resulted in limited increase in production (~ 1% dw or 110–140 mg l^{-1}) which was less than those obtained following the addition of other nutrients. Two other definite trends are noticeable. Firstly, fed cultures required an incubation time of at least 2 d before elicitation for highest production. Consequently, all subsequent cultures fed similarly with nutrients were incubated 2 d prior to elicitation. Secondly, addition of concentrated solutions resulted in highest productions from increases of both yield and biomass concentrations as compared to control cultures.

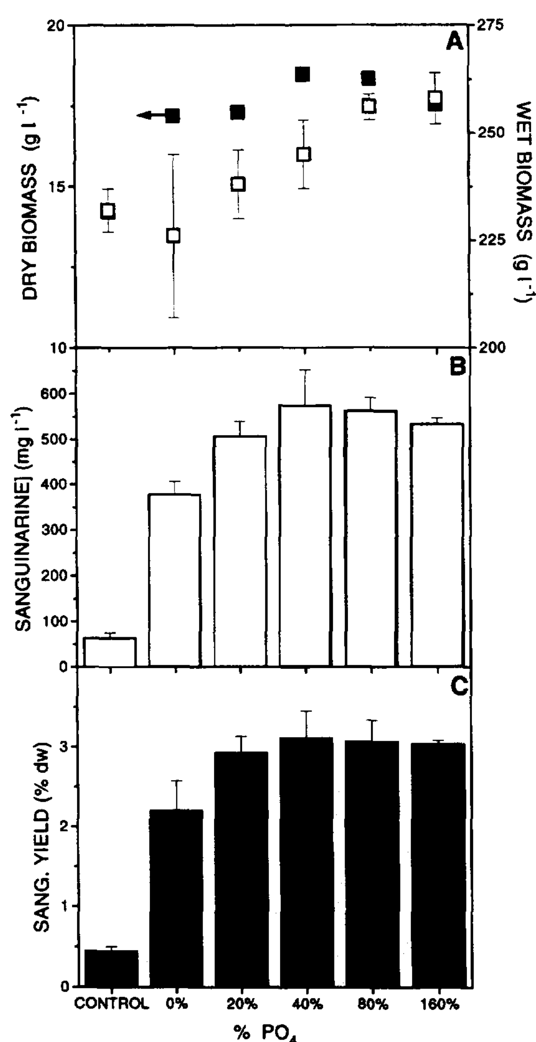

Fig. 6. Sanguinarine production and final biomass concentration of elicited stationary phase cultures fed with concentrated solutions of MS major salts and sucrose supplemented with increasing concentrations of phosphate. (A) ■, dry biomass concentration (g l^{-1}); □, wet biomass concentration (g l^{-1}).

Sanguinarine concentrations reached 350 mg l^{-1}, which is only slightly greater than the production obtained from eliciting growing cultures at phosphate depletion (Fig. 2: 270 mg l^{-1}). This increase in production resulted from much higher dry biomass levels (16.7 g l^{-1} vs. 6.4 g l^{-1} (Fig. 2C)) but lower yields ($\leq$ 2.1% dw) as compared to less mature cultures (Fig. 2: 7-day old, 4.2%). Obviously, nutrient requirements, including dissolved oxygen, of high density elicited cultures need to be better defined. However, there was clearly an absolute requirement for a carbon source and one or more components of MS medium.

The addition of normal MS medium used for culture maintenance to elicited high density cultures yielded very low levels of sanguinarine (not shown). This resulted likely from the presence of growth regulators in this medium. Removal and subsequent culturing of plant cells in growth regulator free medium has been found to stimulate secondary metabolite production in a number of cases (Cline and Coscia, 1988) and, as seen for low density control cultures containing XAD-7 resin (Fig. 2: Williams et al., 1992).

3.2.2. *Effect of phosphate*

To further investigate the action of phosphate on this process, the effect of altered levels of this nutrient on growth and elicitation was examined over the course of normal culture growth. The concentration of phosphate in nutrient solutions added to stationary phase cultures prior to elicitation was also examined in relation to sanguinarine production.

P. somniferum cell cultures showed little difference in growth under different phosphate regimes

(0.6 to 2.4 mM), except for those grown without phosphate (Fig. 4A). Lack of phosphate clearly limited biomass production ($\mu \sim 0.10\ d^{-1}$) while all other levels resulted in identical growth curves ($\mu \sim 0.17\ d^{-1}$) indicating that the phosphate concentration of normal MS medium is sufficient for proper growth of *P. somniferum* cell cultures.

The sanguinarine production curves of these cultures elicited without nutrient addition, however, were very different (Fig. 4B). Cultures grown in MS without phosphate showed lowest production (100–130 mg l^{-1}) but high yields (Fig. 5A: 3.6–4.2%) when elicited within 6 d from inoculation. Thereafter, limited growth occurred and yield declined. Cultures carried out with higher levels of phosphate displayed delayed and higher productions (Fig. 4B and Fig. 5).

Consequently, it appears that low phosphate availability favor high initial sanguinarine yields over an extended culture duration while high phosphate uptake induces low early yields but significant increases following extracellular depletion of this nutrient (Fig. 5). These results support the hypothesis that extracellular phosphate is a suitable marker for elicitation of growing *P. somniferum* cell cultures. Furthermore, they suggest that its effect during the growth phase of batch flask cultures on subsequent sanguinarine production is mainly related to biomass formation rather than yield increase, assuming that all other nutrients were not limiting (Fig. 2).

The effect of phosphate addition on sanguinarine production during the incubation and production phases of elicited fedbatch stationary phase cultures was also investigated (Fig. 6). In this case, the nutrient solution used was a 4 × concentrate of MS major salts supplemented with 120 g l^{-1} sucrose and increasing quantities of phosphate to give initial concentrations in the incubated and elicited cultures of 0 to 160% that found in normal MS medium. The previous addition of nutrient solution without phosphate resulted in increases in sanguinarine yield and production to 2.2% and 380 mg l^{-1} (Fig. 3). The addition of phosphate to the nutrient solution led to further yield and production increases. Maximum production was obtained at a phosphate level of 40% that normally found in MS and reached 3.1% dw and 575 mg l^{-1}. This concentration, which is much higher than found during normal growth and elicitation (Fig. 2: 270 mg l^{-1}) and previous nutrient additions (Fig. 3: 350 mg l^{-1}), resulted from limited increases in yield and biomass concentration (Fig. 6A). Therefore, it appears that phosphate addition is not essential for the production of sanguinarine by elicited fedbatch cultures but that some enhancement of sanguinarine levels can be obtained with such additions.

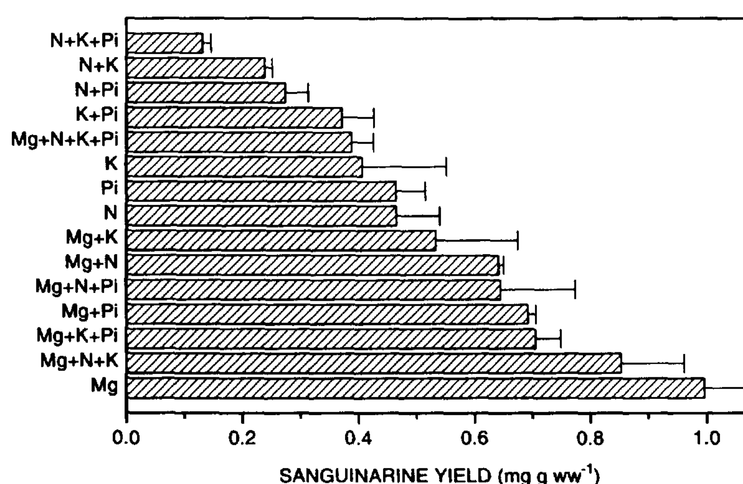

Fig. 7. Effect of specific nutrient additions on sanguinarine yield of elicited stationary phase cultures. N, NH_4NO_3; K, KNO_3; P_i, KH_2PO_4; Mg, $MgSO_4$.

3.2.3. Requirements for specific major salts

There is definitely an absolute need for carbohydrates, ammonium (Archambault et al., 1996) and phosphate during the incubation and elicitation phases of high density *P. somniferum* cell suspension cultures for high sanguinarine production. The next series of experiments were performed to elucidate which other nutrients in the major salts solution were essential for high sanguinarine production during elicitation of fedbatch cultures.

The major salts present in MS (NH_4NO_3 (N), KNO_3 (K), $MgSO_4$ (Mg) and KH_2PO_4 (P_i)) were added, singly or in combinations, with sucrose (30 g l^{-1}), to stationary phase cultures which were incubated and elicited as above. The addition of $CaCl_2$ alone was not evaluated since preliminary experiments suggested a possible inhibitory action of this nutrient on sanguinarine production during elicitation. When the resulting alkaloid yields were plotted from the lowest to highest, a clear pattern emerged (Fig. 7). All combinations containing magnesium sulfate, except for the addition of sucrose and all major salts, showed highest levels of production. Surprisingly, the highest alkaloid yield obtained (1.7%) resulted from the addition of $MgSO_4$ alone (plus sucrose).

The actual effect of $MgSO_4$ was further examined to see if the stimulatory effect was due to the magnesium ion or to sulfate (Fig. 8). It appears that the stimulation of sanguinarine production was due to the presence of sulfate. The addition of $(NH_4)_2SO_4$ gave identical yields as the addition of $MgSO_4$, while the addition of $MgCl_2$ showed only marginal increase in production over control levels. The addition of concentrated (4 ×) MS medium showed an increase in sanguinarine yield (2.7%) over that found for the addition of $MgSO_4$ or $(NH_4)_2SO_4$ alone, indicating further stimulation of alkaloid production presumably from a synergistic effect of all nutrients present when their concentrations are higher than that originally found in MS (Fig. 8 vs. Fig. 7). Highest overall production of these experiments reached ~ 500 mg l^{-1}, which compares to the best results presented in Fig. 6. These results were confirmed for other *P. somniferum* cell lines.

3.2.4. Effect of oxygen transfer rate

In reexamining the best production results presented in Fig. 2, Fig. 3, Fig. 5, Figs. 6 and 8 upon proper elicitation of *P. somniferum* cell cultures, an inverse relationship can be observed between sanguinarine yield and biomass concentration at harvesting (Fig. 9). Furthermore, these yield results (1.0–5.1%) remain low as compared to highest yields reported (Songstad et al., 1990) for this particular bioprocess (up to 12% dw). Obviously, this suggests

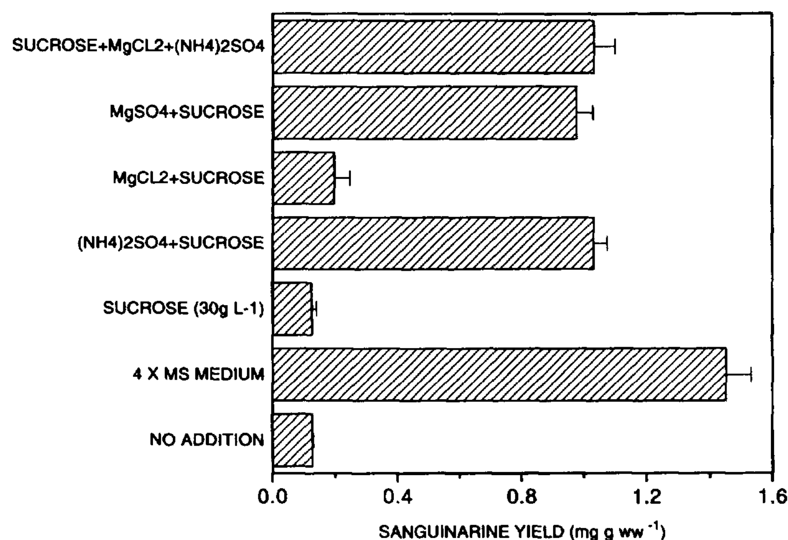

Fig. 8. Effect of nutrient additions on sanguinarine yield of elicited stationary phase cultures.

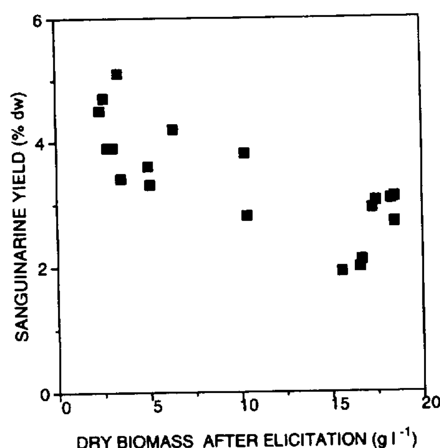

Fig. 9. Correlation between sanguinarine yield and dry biomass concentration after elicitation of *P. somniferum* cell cultures.

that higher nutrient feeding during elicitation may be required to increase sanguinarine production, especially for high density cultures.

In this context, dissolved oxygen may also become limiting in flask cultures as a result of growth, increase in biomass concentration (Tyler et al., 1988; Williams et al., 1992) and expected higher oxygen demand during elicitation (Apostol et al., 1989; Cline et al., 1990). In order to verify this, at least in first approximation, 25-, 50- and 100-ml 7-day-old cultures were elicited in 250-ml flasks with XAD-7 resin and crude chitin as above. This increase in cell

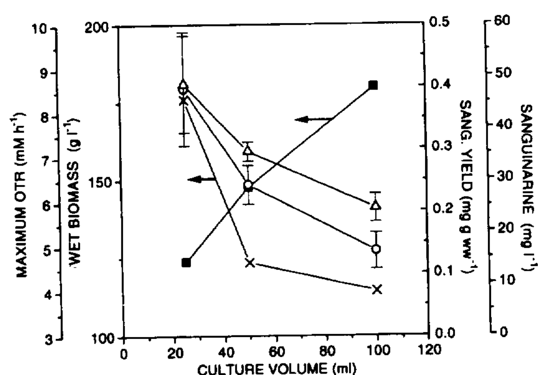

Fig. 10. Effect of estimated maximum oxygen transfer rate on sanguinarine production by elicited batch cultures. ■, final wet biomass concentration (g l^{-1}); ×, estimated maximum oxygen transfer rate (mM h^{-1}); ○, sanguinarine yield (mg g ww^{-1}); △, sanguinarine concentration (mg l^{-1}).

suspension volume resulted in $k_L a$ reductions, mostly through decrease of the specific surface area for oxygen transfer ('a' in $k_L a$), of 44% and 52% for the 50- and 100-ml cultures, respectively, as compared to that of the lowest volume used. Although, in this case, elicitation may not have been initiated at the best time (Fig. 2: at PO_4 depletion) as indicated by the low yields obtained, the results presented in Fig. 10 show a clear promoting effect of higher initial estimated maximum oxygen transfer rate (OTR_{max}) on sanguinarine production. In fact, sanguinarine yield and concentration decreased in the same trend as OTR_{max} with increasing culture volume and seemed to experience an inverse relationship with biomass concentration increase during elicitation. Consequently, further development of this bioprocess will also need a closer examination of this culture parameter.

4. Discussion

The first two papers of the series illustrate clearly the important, but often neglected, close relationship existing between the actual, ever changing chemical environment and nutritional regime of cultured plant cells, and their growth and production behavior. A close examination of this aspect of *Papaver somniferum* cell cultures is providing for valuable insights into this process as well as means to improve their performance.

4.1.1. *Elicited batch cultures*

Elicited culture must be in a correct physiological state for maximum production and survival (Archambault et al., 1996), which is essentially governed by their chemical environment and nutritional status at, and during elicitation. Growing cell suspensions are best elicited at extracellular phosphate depletion (Fig. 2), which represents an efficient and reproducible culture marker for proper induction of this particular production. Furthermore, stationary phase cultures were also found elicitable and high producing upon proper nutrition. Evidently, this opens the door to the development of two phase process strategies for sanguinarine production by elicitation at high biomass concentration and high yield to attain economical productivity levels.

Surprisingly, high sanguinarine yields were obtained from control cultures containing resin at inoculation (Fig. 2). This indicates that diluted stationary phase cultures responded to the chemical shock of inoculation, in the presence of growth regulators and sanguinarine sink (XAD-7 resin), by rapid (≤ 4 d) endogenous production at levels of $\sim 80\%$ of that obtained following the elicitation of similar cultures. How this remarkable result could be reproduced, using similar culture protocols, for the endogenous production of valuable secondary metabolites by cultured plant cells of other species remains an interesting opportunity to explore.

4.1.2. Elicited fedbatch cultures

The two phase process is clearly feasible (Fig. 3, Fig. 6, Figs. 7 and 8). These results, however, need to be reassessed in a more integrated perspective in view of the multiplicity of possible physico-chemical limiting and interacting factors involved in these cultures. First, high density flask cultures with resin (≥ 200–300 g ww l^{-1}) experienced mixing difficulties and, likely, reduced gas transfer efficiency, as did similar cultures performed in bioreactors (Spieler et al., 1985; Fowler et al., 1987; Panda et al., 1989; Smith and Skidmore, 1990; Jolicoeur et al., 1992; Ballica and Ryu, 1993). Consequently, all these cultures were basically mass transfer (gases, nutrients availability and sanguinarine adsorption) limited to some degree. Furthermore, best sanguinarine productions obtained reached ~ 500–600 mg l^{-1}. Considering the capacity of XAD-7 resin for sanguinarine adsorption (10 mg g wet^{-1} resin; Williams et al., 1992) and the quantity of resin (50 g l^{-1}) present in these cultures, it is likely that their product sink was close to saturation. This may have resulted in low production of high density cultures.

In this context, production results for high density cultures, although still valid, take on a somewhat different meaning. Thus, the effect of extracellular phosphate and its uptake on the overall process appear to be mainly related to the formation of biomass (Fig. 4A) and its conditioning in part or in full into the correct physiological state for high responsiveness to elicitation. As suggested previously (Archambault et al., 1996), this could be associated with the culture's transition from cellular division to expansion (Pépin et al., 1995). This role of phosphate is supported by the results of batch cultures (Fig. 2 (at day 7), 4 and 5) and the preincubation requirement of fedbatch cultures (Figs. 3 and 6) prior elicitation for maximum production, likely for rapid phosphate uptake by the high quantity of biomass present. Higher phosphate concentrations yielded more biomass into the correct physiological state for elicitation to allow for increased yields. However, one significant result remains puzzling: the high yields obtained from eliciting the inoculated, low density and nutrient richest, including phosphate and dissolved oxygen, cultures of Fig. 2. The synergistic effect of these conditions and/or the combined chemical shock of inoculation and elicitation may have played critical roles in stimulating production as found for similar control cultures which also showed high endogenous production.

Other nutrients, like carbohydrates, ammonium (Archambault et al., 1996), sulfate and dissolved oxygen, at high concentrations also stimulate sanguinarine production during elicitation. They appeared to act synergistically on this process together, possibly, with other major ions of MS medium. Their actual individual and combined effect at high biomass concentration, however, need to be further examined under unrestricted physical and (monitored) supply conditions to assess their exact roles, some of which are obvious (carbohydrates) or have been investigated to some extent (oxygen; Apostol et al., 1989), as well as their full stimulating potential. This work will also lead to reevaluating other aspects of this process, in particular the elicitor concentration required, its production kinetics as well as improved nutrient feeding and product harvesting strategies for maximum performance.

4.1.3. Towards economical production

Although a great deal of valuable results were presented and discussed in this study, progress in terms of increased productivity were limited. Best productions obtained (Fig. 2, days 0 and 7: 134 and 270 mg l^{-1}; Fig. 6: 575 mg l^{-1}) resulted in productivities of 33.5, 24.5 and 28.7 mg l^{-1} d^{-1} for the cultures' duration. These levels compare to our previous results (Archambault et al., 1996) of 385 mg l^{-1} and 26 mg l^{-1} d^{-1}, but remain far from our objectives of 300–600 mg l^{-1} d^{-1} for the culture's duration. However, clear directions are now outlined

to improve this production. Further yield increases should result from the development of unrestricted elicited cultures at high biomass concentration. Furthermore, it can be seen that 80% of the total production time of high density cultures was essentially for biomass formation and conditioning. Further examination of this less glamorous phase of the process is also required to maximize its performance. Results from this work as well as reelicitation of same cultures should allow significant increases in productivity. The next two paper of this series will look at the scale-up of this process in immoblization and suspension culture bioreactors.

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