Identifying and Investigating Metabolic Pathways Activated in High-Producing California Poppy Suspension Cultures

A Thesis Presented

By

John Thornton Oldham

to

The Department of Chemical Engineering

In partial fulfillment of the requirements
For the degree of

Doctor of Philosophy

In the field of

Chemical Engineering

Northeastern University
Boston, Massachusetts

April 23, 2010
ACKNOWLEDGEMENT

I thank my advisor, Dr. Carolyn Lee-Parsons, for the opportunity to work in her laboratory. I am thankful for the opportunity to grow and work in an exciting field. Under her supervision and guidance I have grown as a researcher and as a person. Her patience and wisdom have been a blessing and will strengthen me in my future endeavors.

I would also like to thank Dr. Marina Hincapie for teaching me a wide range of protein extraction, separation, and analysis techniques. Her patience, wisdom, encouragement, and generous time investment have been invaluable. I also thank Dr. Tomas Rejtar for guidance, support, and help with experimental techniques and for teaching me the principles of protein identification and also for many thoughtful discussions. I thank Dr. Hincapie and Dr. Rejtar for their support and guidance with the proteomics experiments in Chapter 5. Also, I am thankful to Dr. Rejtar, Dr. Thomas Plasterer, and Dr. Rebecca Carrier for their valuable comments and insight and for serving on my thesis committee.

I am also thankful to my fellow laboratory members Sheba Goklany and Lütfiye Kurt for their assistance with experiments and thoughtful discussions. Also, I thank Majilinda Kullolli, Agnes Rafalko, Zhi Zeng, and Dipak Thakur from the Barnett Institute for their advice, support, and time.

I thank my family for their constant support throughout the past six years. Without their support none of this research would have been possible. I am thankful to NSF, Northeastern University, Giner Inc., the department of Chemical Engineering, and the Graduate School of Engineering for financial support.
ABSTRACT

Plant cell culture offers an alternative production system capable of providing consistent, high yields of pharmaceutical compounds. Currently, 9 pharmaceutical compounds have been produced through plant cell culture [1]. However, further increases in productivity for other compounds from plant cell cultures will require a greater understanding of metabolic pathways which limit production. Proteins and pathways with changes in abundance in high-producing cell lines represent potential targets for further increases in production. In this thesis, *Eschscholzia californica* cultures which produce benzophenanthridine alkaloids, including the anti-microbial and anticancer drug sanguinarine, were used as a model system to study metabolic bottlenecks using proteomics.

Enhancements in BPA production was explored using elicitation, *in situ* product extraction, and medium composition. Cultures treated with a purified yeast elicitor (PYE) enhanced BPA production to greater than a 20-fold. *In situ* extraction combined with PYE-treatment increased production 63-fold, *i.e.* up to 85 mg BPAs / g dry weight. Medium optimization (hormones, sugar, nitrogen, and phosphate) did not increase production in elicited cultures.

A proteomic comparison of unelicited and PYE-elicited cultures was performed to assess differences in global metabolism associated with enhanced BPA production. Approximately 650 proteins were identified using a liquid chromatography / mass spectrometric method. Three proteins related to (S)-adenosylmethionine (SAM) biosynthesis and metabolism were significantly induced in elicited cultures. SAM is a universal methyl donor in biological reactions including 6 steps in sanguinarine
biosynthesis. The results suggest a coordination between primary metabolic pathways and increased BPA production.

The role of the SAM biosynthetic pathway in supporting enhanced BPA synthesis was investigated further by adding methionine, ethylene, and the ethylene biosynthesis inhibitor cobalt chloride to *E. californica* suspension cultures. Methionine did not affect production in elicited cultures suggesting that methionine is not limiting BPA production. Amino acid analysis of culture media from elicited cultures reflected increased methionine biosynthesis with elicitation. Both ethephon (soluble ethylene) and cobalt chloride did not affect production suggesting that ethylene is not required for elicitor-induced BPA production.

Recommendations include enhancing production using cyclodextrin for *in situ* extraction, improved protein identification using fractionation by differential centrifugation, and investigation of the role of DNA methylation in supporting increased production. These proposed experiments will provide insight into pathways which can be manipulated by genetic engineering to enhance production.
TABLE OF CONTENTS

LIST OF FIGURES ........................................................................................................ viii
LIST OF TABLES ......................................................................................................... ix

1.0 INTRODUCTION .....................................................................................................1

2.0 CRITICAL LITERATURE REVIEW ........................................................................8

2.1 Plant Cell Culture Technology ............................................................................... 9
2.2 Benzophenanthridine Alkaloids from *Eschscholzia californica* .............................. 10
2.3 Benzophenanthridine Alkaloid Biosynthetic Pathway ............................................ 10
2.4 Factors Affecting Benzophenanthridine Alkaloid Production ................................. 16
    2.4.1 Elicitation of BPA Production ...................................................................... 16
    2.4.2 *In situ* Extraction ...................................................................................... 18
    2.4.3 Alginate Immobilization ............................................................................. 19
    2.4.4 Media Optimization .................................................................................... 19
    2.4.5 Genetic Engineering of the BPA Pathway .................................................... 21
2.5 Genomic and Proteomic Investigation of Secondary Metabolism ........................... 22
    2.5.1 Applications of Proteomic Methods ............................................................. 23
    2.5.2 Proteomic Analysis Applied to Recombinant Protein Production ................. 24
    2.5.3 Proteomic Studies Applied to Plants ............................................................. 27
2.6 Proteomic Methods for Investigating Global Metabolism ....................................... 29
    2.6.1 Protein Extraction ......................................................................................... 29
    2.6.2 Protein Digestion .......................................................................................... 30
    2.6.3 Protein and Peptide Separation ................................................................... 31
    2.6.4 Protein Identification .................................................................................... 34
    2.6.5 Mass Spectrometry ....................................................................................... 35
    2.6.6 Protein Quantification .................................................................................. 37

3.0 MATERIALS AND METHODS .................................................................................44

3.1 Maintenance of *Eschscholzia californica* Suspension Cultures and Calli .............. 44
3.2 Elicitation Experiments ........................................................................................... 46
    3.2.1 Preparation of Purified Yeast Extract ........................................................... 46
    3.2.2 Preparation of Methyl Jasmonate Stock Solution ......................................... 47
    3.2.3 Preparation of Ethephon Stock Solution ....................................................... 47
    3.2.4 Preparation of Salicylic Acid Stock Solution ............................................... 47
3.3 Alkaloid Analysis .................................................................................................... 48
    3.3.1 Alkaloid Extraction ....................................................................................... 48
    3.3.2 Validation of Alkaloid Extraction Method .................................................... 48
    3.3.3 High Performance Liquid Chromatography Separation & Quantification of Alkaloids ................................................................. 51
    3.3.4 HPLC Validation .......................................................................................... 57
3.4 Protein Quantification & Validation of Protein Extraction Method ........................ 59
    3.4.1 Protein Concentration Assays ...................................................................... 59
    3.4.2 Validation & Comparison Between Bradford & BCA Assay ....................... 60
3.5 Protein and Peptide Separation ............................................................................ 64
6.0 INVESTIGATING THE ROLE OF (S)-ADENOSYL METHIONINE BIOSYNTHESIS IN SUPPORTING OR MEDIATING ENHANCED BENZOPHENANTHRIDINE ALKALOID PRODUCTION IN CALIFORNIA POPPY (*ESCHSCHOLZIA CALIFORNICA*) SUSPENSION CULTURES ..........112

6.1. Introduction ............................................................................................................ 112
6.2. Materials and Methods ........................................................................................... 115
  6.2.1. Maintenance of Cell Cultures .....................................................................115
  6.2.2. Preparation of Purified Yeast Extract .........................................................116
  6.2.3. Methionine Feeding ....................................................................................116
  6.2.4. Elicitation with Ethylene .............................................................................117
  6.2.5. Addition of Cobalt Chloride .......................................................................117
  6.2.6. Alkaloid Analysis ........................................................................................117
  6.2.7. Amino Acid Analysis ..................................................................................118
6.3. Results and Discussion ........................................................................................... 119
  6.3.1 Effect of Methionine Feeding on BPA Production .....................................119
  6.3.2. Effect of the Plant Hormone Ethylene on BPA Production .....................124
6.4 Conclusions ............................................................................................................ 127

7.0 CONCLUSIONS .......................................................................................................129
8.0 RECOMMENDATIONS ............................................................................................133
9.0 REFERENCES .........................................................................................................140
10.0 APPENDICES ........................................................................................................155
  Appendix A ..............................................................................................................156
  Appendix B ..............................................................................................................158
  Appendix C ..............................................................................................................163
LIST OF FIGURES

Figure 1: Chemical structures of sanguinarine (left) and chelerythrine (right) .......... 3
Figure 2: BPA biosynthetic pathway from tyrosine to macarpine .................................................. 12
Figure 3: De novo peptide sequencing ........................................................................... 35
Figure 4: Schematic representation of MALDI spotting system used by Chen et al [130-131] ................................................................. 36
Figure 5: Isotope-coded affinity tag (ICAT) reagent structure (A) and experimental workflow (B) [135] ........................................................................ 39
Figure 6: Workflow for measuring relative protein abundance using the ICAT reagent ........................................................................ 39
Figure 7: Chemical structure of the iTRAQ reagent and how the iTRAQ reagent is linked to the peptide ................................................................. 40
Figure 8: *E. californica* suspension and calli cultures .................................................. 45
Figure 9: Separation of BPAs by HPLC ........................................................................ 52
Figure 10: UV spectra of the BPA compounds identified in Figure 9 ........................................ 53
Figure 11: Sanguinarine calibration curve ....................................................................... 54
Figure 12: Estimation of chromatographic peak area ........................................................... 55
Figure 13: Bradford assay calibration curve .................................................................. 61
Figure 14: Separation of peptides by SCX chromatography ........................................... 69
Figure 15: Peptide purification by HPLC .......................................................................... 70
Figure 16: BPA production is affected by timing and dosage of PYE ............................... 73
Figure 17: BPA production profile with time in elicited *E. californica* suspensions ....... 75
Figure 18: Effect of XAD-7 on BPA production ............................................................. 77
Figure 19: Effect of XAD-7 dosage and harvest time on BPA production ..................... 79
Figure 20: Elicitation of BPA production in elicited *E. californica* suspensions and cells immobilized in alginate beads ......................................................... 80
Figure 21: Effect of 2,4-D on BPA production ................................................................... 82
Figure 22: Effect of NAA concentration on alkaloid production ...................................... 84
Figure 23: Effect of methyl jasmonate on BPA production ............................................. 85
Figure 24: Effect of sucrose on BPA production .............................................................. 86
Figure 25: Elicitation of BPA production in *E. californica* suspensions with varying ammonium and nitrate concentrations ................................................ 88
Figure 26: Elicitation of BPA production in *E. californica* suspensions with varying ammonium and nitrate concentrations ................................................ 89
Figure 27: Elicitation of alkaloid production using purified yeast extract (PYE) .......... 104
Figure 28: Metabolic pathways leading to (S)-adenosyl methionine (SAM) production from glycolysis ........................................................................ 107
Figure 29: SAM biosynthesis and metabolism of related amino acids adapted from Ravanel et al. [195] .................................................................. 114
Figure 30: Effect of methionine feeding on BPA production in *E. californica* suspension cultures ........................................................................ 120
Figure 31: Accumulation of amino acids in the media of cultures fed with methionine .... 122
Figure 32: Treatment of *E. californica* cultures with ethephon ...................................... 125
Figure 33: Treatment of *E. californica* cultures with cobalt chloride ............................. 126
LIST OF TABLES

Table 1: Summary of studies on enzymes in the BPA biosynthetic pathway .......... 13
Table 2: Comparison of sanguinarine extracted (mg/g DW) by sonication and cell beater.......................................................................................................................... 50
Table 3: Extinction coefficients (expressed as log ε) and corresponding wavelengths for the 6 BPAs obtained from the literature [150].................. 57
Table 4: Reproducibility and stability of sanguinarine standard solution ............ 58
Table 5: Variability of sanguinarine calibration curves........................................ 58
Table 6: Reproducibility of protein extraction ..................................................... 62
Table 7: Comparison of protein extraction buffers.............................................. 64
Table 8: Summary of biological and technical replicates used in proteomic analysis .................................................................................................................. 99
Table 9: Quantification of selected differentially abundant proteins............... 106
Table 10: Comparison of HPLC peak area, base peak intensity, total peptides, and total proteins for each replicated included in the proteomic study......... 159
1.0 INTRODUCTION

Plants are a valuable source of pharmaceutical compounds. Approximately 25% of pharmaceutical compounds are derived from plants [2]. Currently many plant-derived pharmaceutical compounds are produced by extraction from whole plants. Commercially available plant secondary metabolites include analgesics such as morphine and codeine from *Papaver somniferum*, stimulants such as caffeine from *Coffea arabica* and nicotine from *Nicotiana tabacum*, and anti-cancer compounds such as paclitaxel from *Taxus chinensis*, vincristine and vinblastine from *Catharanthus roseus*. The pharmaceutical compounds extracted from plants occur in very small concentrations and are difficult to extract in bulk, leading to high market values. For example, vincristine is produced at 0.0005 wt % [3] in *C. roseus* plants and has a market value of $4 MM/kg [4]. Traditional organic synthesis is not an efficient and economical method for producing plant secondary metabolites because of their complex chemical structures which frequently include multiple chiral centers [5].

In recent years, plant cell culture technology has developed as another means of producing secondary metabolites. Instead of growing the plants in their natural habitats subject to environmental conditions, plant cell cultures produce valuable secondary metabolites under controlled conditions. Plant cell cultures consist of cells grown in simple, defined media (sucrose, inorganic salts, plant hormones, and vitamins) free from external influences such as weather or pathogen invasion which may otherwise affect the yield of secondary metabolites. Currently fourteen compounds have been produced through plant cell culture including nine pharmaceutical compounds [1]. Plant cell culture has been demonstrated to provide a steady source of pharmaceutical compounds.
with high yields. However, in some cases, the volumetric productivity (g/L-day) may be low leading to a high bulk price [6-7]. The lack of a viable large-scale production method for many high-value plant-based products provides a need for research into improving the yield of pharmaceutical compounds from plant cell culture.

The increase in production required to make a process commercially viable and competitive with extraction from whole plants is dependent on the volumetric productivity and the bulk price from whole plants. For example, Drapeau et al. [6] estimated that a 40-fold increase in ajmalicine production from *C. roseus* is necessary to enable production competitiveness with extraction from whole plants. Shuler reported a revenue of $0.02/(L*day) is expected for a commercially viable cell culture pharmaceutical process [7]. Based on Shuler’s calculations, the highest sanguinarine production reported in this thesis in Chapter 4 (50 mg sanguinarine / g dry weight grown to approximately 10 g/L and harvested after 11 days) would require a bulk price of $4.40/g to be commercially viable. Comparatively, 100 mg sanguinarine can be purchased from 3B scientific (http://www.3Bscientific.com) for $625 ($6250/g) suggesting that the *E. californica* cell line and elicitation method used in this thesis can be used for economical production. Therefore this cell culture system provides an opportunity to understand the pathways which have enabled economical production. This thesis investigates global changes in metabolism associated with increased secondary metabolite production in plant cell cultures to identify pathways which may play a critical role in enhancing production.

In this thesis, California poppy (*Eschscholzia californica*) cell suspensions were used as a model system for investigating the global changes in metabolism associated
with significant increases in secondary metabolite production in a plant cell culture system. *E. californica* produces a class of secondary metabolites known as benzophenanthridine alkaloids (BPAs); examples of BPAs include sanguinarine and chelerythrine (Figure 1), which possess anticancer [8-13] and antimicrobial activity [14]. Sanguinarine has been detected in the rhizomes of *Sanguinaria canadensis* plants up to 4% by dry weight [15]. Cell cultures of *E. californica* can produce up to 8.5% alkaloids by dry weight in cell culture (See Chapter 4) and was used as a model system for investigating secondary metabolite production from plant cell culture in this thesis.

![Chemical structures of sanguinarine and chelerythrine](image)

**Figure 1: Chemical structures of sanguinarine (left) and chelerythrine (right)**

The production of secondary metabolites from plant cell cultures requires energy (e.g., ATP, NADPH) and precursors from primary metabolism (e.g. amino acids such as tyrosine). While production has successfully been enhanced in many plant cell cultures, less is known about which biochemical pathways are critical for supporting increased production. Our hypothesis is that primary metabolic pathways (e.g. glycolysis, citric acid cycle, pentose phosphate pathway) provide precursors and energy resources to support secondary metabolism. Thus, our hypothesis is that primary and secondary metabolic pathways are coordinated to support increased secondary metabolite production. Investigating the proteome could provide a more global view of cellular metabolism and its coordination in response to external stimuli. The activities of cellular metabolic pathways are coordinated by proteins. Changes in abundance of proteins
suggest the pathways and cellular functions that are activated or repressed to enable enhanced secondary metabolite production.

Proteomic methods have not been widely applied to investigate the metabolic processes involved in secondary metabolite production. In Chapter 2 of this thesis, the application of proteomics to investigating protein and secondary metabolite production is reviewed. Traditional plant proteomics experiments have utilized two-dimensional gel electrophoresis for protein separation. In this thesis we applied a gel-free chromatographic method for peptide separation to enhance the number of proteins identified.

The overall goal of the thesis was to identify and investigate pathways associated with increased secondary metabolite production in *E. californica*. The first objective of this thesis was to screen *E. californica* culture conditions yielding enhanced production of BPAs (>20-fold increase compared to untreated cultures; Chapter 4). The second objective was to compare the protein profile of high- and low-producing culture conditions using proteomics and identify potential metabolic pathways that are important in supporting elevated secondary metabolite production (Chapter 5). The final objective was to validate the findings from the proteomics experiment and demonstrate the ability of proteomics to identify critical metabolic pathways in high-producing plant cell cultures (Chapter 6).

To accomplish the first objective, production from *E. californica* cultures was increased using established methods including elicitation, *in situ* product removal, immobilization, and medium optimization (Chapter 4). Elicitors are molecules which activate plant natural defense responses leading to increased secondary metabolite
production. Elicitation using purified yeast extract [16-17] and fungal elicitors [18] has increased BPA production in *E. californica* suspension cultures. Elicitation is believed to increase BPA production by inducing expression of BPA biosynthetic genes [19-22] leading to increased levels of their corresponding enzymes [16, 23]. In *in situ* extraction utilizes either immiscible solvents or polymeric resins to absorb products as they are secreted by the cell. *In situ* extraction using Amberlite XAD-7 increased alkaloid production 20-fold in yeast-elicited *E. californica* cultures [24]. Immobilization consists of entrapping cells in polymeric beads (e.g. alginate). Immobilizing *E. californica* suspensions in alginate beads increased production 800-fold to 8 mg/g dry wt (DW) [25]. With medium optimization, total alkaloid production increased 38-fold up to 3163 mg/g DW when the nitrate concentration was reduced from 25.0 to 12.5 mM in elicited *E. californica* cultures [26].

As part of the first objective, elicitation, *in situ* extraction, immobilization, and medium optimization were investigated (Chapter 4) but elicitation and *in situ* extraction resulted in the most significant increase in BPA production. Elicitation increased production approximately 20-fold (20 mg BPAs / g dry weight) compared to the uninduced cultures. Addition of XAD-7 combined with elicitation increased production 63-fold compared to untreated cultures (up to 88 mg BPAs / g dry weight).

To accomplish the second objective, the metabolism of untreated and treated (elicited) cultures was compared using a proteomics approach (Chapter 5). Methods for protein extraction, solubilization, separation, digestion, and identification were developed (Chapter 3). Proteins were extracted from unelicited and elicited cultures, digested into peptides, and analyzed by mass spectrometry. A total of 646 proteins were identified.
Proteomic studies on other medicinal plants using the traditional gel electrophoresis method [27-29] have identified less than 120 proteins. Increasing the number of proteins identified improves the capability of proteomics to extract biologically relevant information from the experiment. Three proteins from the (S)-adenosylmethionine (SAM) biosynthetic pathway were induced by elicitation demonstrating the ability of proteomics to identify pathways correlated with enhanced production.

In the third objective, the role of SAM and methionine in supporting or activating enhanced BPA production was investigated using external methionine feeding (SAM precursor) and the addition of ethylene, a plant hormone produced from SAM (Chapter 6). Methionine (0.5 and 1.0 mM) did not enhance production in unelicited cultures or elicited cultures. The extracellular concentrations of amino acids related to the methionine biosynthetic pathway increased with elicitation, suggesting that the activity of the SAM and methionine biosynthetic pathways was increased. Ethylene addition in unelicited *E. californica* cultures did not affect production suggesting that ethylene is a byproduct rather than a mediator of elicitor-induced production. Addition of the ethylene biosynthetic inhibitor cobalt chloride did not affect BPA production at lower concentrations, also suggesting that ethylene is not necessary for increasing production.

In summary, this thesis demonstrates one of the first studies on understanding global metabolism in cell cultures of an unsequenced medicinal plant. First, high producing cell culture conditions were developed using established methods that resulted in up to a 63-fold increase in total BPA production (89 mg / g dry weight) compared to untreated cells. Second, the protein profiles of high- and low-producing conditions were compared to determine proteins and associated metabolic pathways correlated with
increased BPA levels. Finally, the increased activity of the SAM biosynthetic pathway was confirmed. Together, these results demonstrate the potential for proteomics in identifying pathways correlated with increased secondary metabolite production in plant cell cultures and the role of primary metabolic pathways such as SAM in supporting secondary metabolite production. Investigating the mechanisms leading to production in high-producing *E. californica* cultures will provide targets for increasing production of high-value pharmaceutical compounds from other plant cell culture systems.
2.0 CRITICAL LITERATURE REVIEW

Plant cell culture provides an alternative technology for the production of valuable plant-derived pharmaceutical compounds. The overall goal of this thesis is to advance plant cell culture technology towards the goal of developing economically feasible, large-scale processes for the production of pharmaceutical compounds. Specifically, this thesis investigates the global metabolism of California poppy cell cultures producing benzophenantheridine alkaloids (BPAs, reviewed in Section 2.2) to determine potential bottlenecks to its production. The BPA biosynthetic pathway involves twenty enzyme-catalyzed reactions starting from the amino acid tyrosine (reviewed in Section 2.3). In addition, multiple metabolic pathways providing precursors and energy must be coordinated to support enhanced production.

The first specific aim of this thesis is to determine and select cell culture conditions where increased BPA production occurs. In Section 2.4, a literature review of the methods for increasing production will be covered, including elicitation (Section 2.4.1), in situ extraction (Section 2.4.2), alginate immobilization (Section 2.4.3), media optimization (Section 2.4.4), and genetic engineering (Section 2.4.5).

The second specific aim of this thesis is to investigate global metabolism in California poppy cultures using proteomics. The application of proteomic methods involves monitoring differences in protein abundance between multiple cell culture conditions. The application of proteomic methods to the production of therapeutic proteins from mammalian cell culture and small molecules from plant cell culture in the literature are discussed in Sectio 2.5. The proteomic workflow including extraction from
cell culture samples, protein separation, digestion, peptide separation, and mass spectrometry are covered in Section 2.6.

2.1 Plant Cell Culture Technology

Plants produce a vast array of pharmaceutical compounds. At least 25% of the pharmaceutical compounds sold in the United States are produced from plants or synthetic derivatives of plant-produced compounds [2]. Many of these compounds are obtained commercially through extraction from the whole plant. However, the production rates are often low and are subject to variations in environmental conditions. As an alternative, plant cell culture provides a stable environment for production of plant pharmaceutical compounds. Several pharmaceutical compounds are currently produced from plant cell culture including the anti-cancer treatment paclitaxel [30].

Plant cell cultures are originated by transferring growing tissue from the whole plant to a solid medium containing sugar, organic salts, and plant growth hormones. The resulting callus culture can be propagated on solid media or transferred to liquid media and grown as a suspension culture. Suspension cultures are maintained by transferring cells and spent media to fresh media after the cells have reached the stationary phase (usually every 2 – 4 weeks). Suspension cultures can also be induced using chemical cues to form differentiated cultures including shoot and root cultures. One of the first steps in developing a plant cell culture process is cell line selection. In this thesis, California poppy (Eschsholzia californica) suspension cultures were provided by Dr. Hwa-Young Cho and Dr. Sung-Yong Yoon of POSTECH (Pohang, South Korea) and used in all experiments.
2.2 Benzophenanthridine Alkaloids from *Eschscholzia californica*

Benzophenanthridine alkaloids (BPAs) are a class of secondary metabolites derived from the amino acid tyrosine and produced by several plants including *E. californica*, *Papaver somniferum* (opium poppy), *Macleaya cordata*, *Thalictrum bulgaricum* and *Sanguinaria canadensis* (Bloodroot). Examples of BPAs include sanguinarine and chelerythrine, shown in Figure 1, which have potential medicinal properties. For example, sanguinarine was once included as an antimicrobial supplement in toothpaste. Sanguinarine and chelerythrine are under investigation for the treatment of cancer [9-10], atherosclerosis [31], and other diseases. For instance, sanguinarine induced apoptosis in epidermoid carcinoma [9] and prostate cancer cell lines [9-10]. Sanguinarine and chelerythrine have been shown to bind to and inhibit the activity of the anti-apoptotic proteins Bcl-XL [13] and MKP-1.

2.3 Benzophenanthridine Alkaloid Biosynthetic Pathway

The benzophenanthridine alkaloid (BPA) pathway is the longest plant secondary metabolic biosynthetic pathway for which all of the enzymatic steps are known. The BPA biosynthetic pathway consists of 20 enzyme-catalyzed steps and 1 spontaneous rearrangement [32]. Many of the enzymes have been sequenced and nearly all enzymes have been isolated and characterized by activity assays (Table 1). Unlike the BPA biosynthetic pathway, the biosynthetic pathways for other commonly studied plant medicinal compounds such as taxanes from *Taxus* and terpenoid indole alkaloids from *Catharanthus roseus* [33] are not completely mapped. For example, production of the anticancer compound paclitaxel from *Taxus* plants requires at least twelve steps downstream of primary metabolism but many are not known [34-35]. Production of the
antimalarial compound artemisinic acid from the mevalonate precursor is completely known and involves at least 10 steps, which is much shorter than the BPA pathway [36]. Precursors for artemisinic acid have been produced by genetic engineering of two enzymes in *Saccharomyces cerevisiae* [37] and converted to artemisinic acid by chemical synthesis. However, the BPA pathway is longer than the artemisinic acid pathway and would thus be significantly more difficult to produce through genetic engineering in yeast.

Genetic engineering of BPA pathway enzymes into yeast [38-39] and *Escherichia coli* [39] has led to production of the intermediate reticuline (See Figure 2). However, sanguinarine was also not produced because several of the BPA biosynthetic enzymes converting reticuline to sanguinarine have not been sequenced and thus could not be engineered into yeast. Thus, production from cell culture remains the most attractive option for large scale production of BPAs. Viable large scale BPA production still requires a greater understanding of mechanisms leading to enhanced production.

Formation of BPAs involves twenty experimentally verified enzymatic steps and one spontaneous rearrangement. A summary of the enzymes, required co-factors, subcellular localization, and whether the gene has been sequenced is presented in Table 1. The first step in the formation of BPAs is the conversion of tyrosine to norcoclaurine by two separate routes. Tyrosine is converted to tyramine by the enzyme tyrosine decarboxylase (TYDC). TYDC was isolated from *E. californica* suspension cultures and increased approximately 6-fold with a yeast extract elicitor [20-21]. Tyramine is then converted to dopamine by phenolase or to 4-hydroxyphenylacetaldehyde by tyrosine transaminase [40]. Dopamine and 4-hydroxyphenylacetaldehyde are then condensed by
norcoclaurine synthase (NCS) to form (S)-norcoclaurine as shown in Figure 2. NCS represents the first committed step in benzylisoquinoline alkaloid (BIA) biosynthesis in plants.

Figure 2: BPA biosynthetic pathway from tyrosine to macarpine. Dashed arrows represent multiple reactions. See Table 1 for enzyme abbreviations.
Table 1: Summary of studies on enzymes in the BPA biosynthetic pathway. Unless noted otherwise, the cofactors required for enzyme activity were determined based on sequence homology by ExPaSy (www.expasy.org).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Cofactors</th>
<th>Cellular location</th>
<th>Organism and sequence or characterization status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyrosine decarboxylase (TYDC)</td>
<td>Pyridoxal phosphate [40]</td>
<td>Unknown</td>
<td>P. somniferum [20], [40]</td>
</tr>
<tr>
<td>Norcoclaurine synthase (NCS)</td>
<td>Endoplasmic reticulum (ER) [41]</td>
<td></td>
<td>P. somniferum [42-43]</td>
</tr>
<tr>
<td>(S)-norcoclaureine-6-O-methyltransferase (6OMT)</td>
<td>SAM</td>
<td>Unknown</td>
<td>C. japonica [44]</td>
</tr>
<tr>
<td>Coclaurine N-methyltransferase (CNMT)</td>
<td>SAM</td>
<td>Unknown</td>
<td>C. japonica [45]</td>
</tr>
<tr>
<td>(S)-methylcoclaurine 3’-hydroxylase (CYP80B1)</td>
<td>NADPH, Heme</td>
<td>Unknown</td>
<td>E. californica [46]</td>
</tr>
<tr>
<td>3’-hydroxy-(S)-N-methylcoclaurine-4’-O-methyltransferase (4’OMT)</td>
<td>SAM</td>
<td>Unknown</td>
<td>C. japonica [47-48]</td>
</tr>
<tr>
<td>Berberine bridge enzyme (BBE)</td>
<td>O₂, FAD, metal ion [49]</td>
<td>ER [50]</td>
<td>E. californica [51]</td>
</tr>
<tr>
<td>Cheilanthifoline synthase (CFS, CYP719A5)</td>
<td>NADPH, O₂, [52]</td>
<td>ER [50]</td>
<td>E. californica Pubmed</td>
</tr>
<tr>
<td>Stylopine synthase (SPS)</td>
<td>NADPH, O₂, [52]</td>
<td>Unknown</td>
<td>E. californica n/a</td>
</tr>
<tr>
<td>Tetrahydroprotoberberine cis-N-methyltransferase (TPMT)</td>
<td>SAM [53]</td>
<td>Unknown</td>
<td>E. californica n/a</td>
</tr>
<tr>
<td>N-methylstylopine-14-hydroxylase (MSH)</td>
<td>NADPH, O₂, [54]</td>
<td>ER [50]</td>
<td>Unsequenced n/a</td>
</tr>
<tr>
<td>Protopine 6-hydroxylase (P6H)</td>
<td>NADPH, O₂, [22]</td>
<td>ER [50]</td>
<td>Unsequenced [22]</td>
</tr>
<tr>
<td>Dihydrobenzophenanthridine oxidase (DHBO)</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Unsequenced n/a</td>
</tr>
<tr>
<td>Dihydrosanguinarine 10-hydroxylase (DS-10H)</td>
<td>NADPH, O₂ [55]</td>
<td>Unknown</td>
<td>Unsequenced n/a</td>
</tr>
<tr>
<td>10-hydroxydihydrosanguinarine 12-hydroxylase (10DS-10OMT)</td>
<td>SAM [55]</td>
<td>Unknown</td>
<td>Unsequenced n/a</td>
</tr>
<tr>
<td>Dihydrochelirubine 12-hydroxylase (DHCHR-12H)</td>
<td>NADPH, O₂, [56]</td>
<td>Unknown</td>
<td>Unsequenced n/a</td>
</tr>
<tr>
<td>12-hydroxydihydrochelirubine 12-hydroxylase (12DHCHR-12OMT)</td>
<td>SAM, [56]</td>
<td>Unknown</td>
<td>Unsequenced n/a</td>
</tr>
</tbody>
</table>
(S)-norcoclaurine is converted to (S)-coclaurine by the enzyme S-adenosyl-L-methionine:norcoclaurine 6-O-methyltransferase (6OMT). Enzyme activity was inhibited by berberine (seven steps downstream of coclaurine in the berberine biosynthetic pathway) in enzyme activity assays which indicates possible feedback inhibition of 6OMT [44]. The conversion of (S)-norcoclaurine to (S)-coclaurine is one of six methylation reactions in the BPA pathway requiring S-adenosyl-L-methionine (SAM) (See Table 1). (S)-coclaurine is converted to (S)-N-methylcoclaurine by coclaurine-N-methyltransferase (CNMT). (S)-N-methylcoclaurine is a branchpoint between the biosynthesis of bisbenzylisoquinoline alkaloids and BIAs. In the BIA pathway, (S)-N-methylcoclaurine is converted to (S)-3'-hydroxy-N-methylcoclaurine by the cytochrome P450 reductase (S)-N-methylcoclaurine 3'-hydroxylase (CYP80B1) in the presence of NADPH and O₂ [46]. The conversion of (S)-3'-hydroxy-N-methylcoclaurine to (S)-reticuline is catalyzed by SAM: 3'-hydroxy-N-methylcoclaurine 4'-O-methyltransferase (4’OMT) [47].

(S)-Reticuline is a branchpoint to a number of BIA biosynthetic pathways including morphinan alkaloids such as morphine and codeine as well as BPAs. The first committed step in the conversion of (S)-reticuline to BPAs is catalyzed by berberine bridge enzyme (BBE). BBE converts (S)-reticuline to (S)-scoulerine in the presence of molecular oxygen [51, 57]. BBE was found to contain a FAD cofactor [49]. BBE and CYP80B1 are localized to the ER [58] and BBE is directed to the ER after translation by a 22 amino acid N-terminal peptide sequence which is subsequently cleaved [51, 57]. Scoulerine is also the branch point for chelerythrine biosynthesis. Currently, the chelerythrine biosynthetic pathway has not been elucidated [59].
A cytochrome P450 oxidase, (S)-cheilanthifoline synthase (CFS), converts (S)-scoulerine to (S)-cheilanthifoline which is then converted to (S)-stylopine by another cytochrome P450 oxidase, (S)-stylopine synthase (SPS) [52, 60]. CFS and SPS are cytochrome P450 enzymes requiring NADPH and O₂ for activity. (S)-stylopine is converted to (S)-cis-N-methylstylopine by (S)-tetrahydroprotoberberine-(cis)-N-methyltransferase (TPMT) [61]. (S)-cis-N-methylstylopine 14-hydroxylase (MSH) then converts (S)-cis-N-methylstylopine to protopine [54]. Another cytochrome P450 enzyme (Protopine 6-hydroxylase, P6H) was identified from a microsomal membrane *E. californica* extract and converts protopine to 6-hydroxyprotopine in the presence of NADPH and O₂ [22]. Protopine then spontaneously converts to dihydrosanguinarine. Dihydrosanguinarine is converted to dihydrochelirubine by cytochrome P450 dependent enzyme dihydrosanguinarine-10-hydroxylase (DS-10H) and SAM-dependent methyltransferase 10-hydroxy-dihydrosanguinarine 10-O-methyltransferse (10DS-10OMT) [55]. Dihydrochelirubine is converted to dihydromacarpine by cytochrome P450 dependent oxidation (dihydrochelirubine 12-hydroxylase, DHCHR-12H) and methylation (12DHCHR-12OMT) [56]. Dihydrosanguinarine and the other intermediates leading to dihydromacarpine are all oxidized by dihydrobenzophenanthridine oxidase (DHBO) [62-63].

Several BPA biosynthetic enzymes are predicted to be localized to the endoplasmic reticulum based on sequence homology (BBE, NCS, [41, 50]) or function (CYP80B1, CFS, SPS, MSH, P6H) [50]. BBE and NCS were both found to contain peptide sequences consistent with integral endoplasmic reticulum proteins. However, it has been suggested that alkaloid biosynthesis is localized to a specialized vesicle separate
from the endoplasmic reticulum [64] to minimize cytotoxic effects. CYP80B1, CFS, SPS, MSH, and P6H are all cytochrome P450 enzymes and thus are predicted to be possible membrane proteins. It is unclear where the methyltransferases are localized. However, CNMT was predicted to be a hydrophilic protein based on primary structure analysis [45] suggesting a possible cytoplasmic localization.

2.4 Factors Affecting Benzophenanthridine Alkaloid Production

Several strategies have been employed for increasing BPA production. Specifically, elicitation, \textit{in situ} extraction, alginate immobilization, medium composition, and genetic engineering have been used to increase BPA production in \textit{E. californica}.

2.4.1 Elicitation of BPA Production

BPA production has been increased using both biotic and abiotic elicitors. Biotic elicitors are derived from living organisms and are used to simulate a pathogen attack. Examples of biotic elicitors include yeast and fungal extracts. Abiotic elicitors are natural plant hormones such as methyl jasmonate, salicylic acid, and ethylene. Methyl jasmonate is a natural plant hormone which is produced in response to yeast or fungal elicitation [65]. Examples of enhancements in BPA production, gene expression, and enzyme activity by yeast elicitation, methyl jasmonate, and salicylic acid are presented.

Farber et al. [17] reported enhanced BPA production upon addition of either yeast extract or methyl jasmonate (MeJA). Yeast elicitation (0.2 g/l) enhanced sanguinarine up to 400\% (146 mg/L) but did not affect dihydrosanguinarine production significantly in \textit{E. californica} cultures [16]. In the same study, 100 \(\mu\)M MeJA enhanced dihydrosanguinarine production 150\% (509 mg/L) [16]. However, MeJA did not enhance sanguinarine production [23]. Gundlach et al. [66] observed an increase in BPA
production (25 mg/g DW total alkaloids) with MeJa elicitation up to 100 μM. Addition of MeJA to *E. californica* cultures increased BPA production 15-fold to 32 mg/L [19]. Dihydrosanguinarine production increased approximately 100% to 300 mg/L in *E. californica* cultures 48 hours after the addition of 1.5 mg/L salicylic acid while sanguinarine production was not affected at the same conditions [67].

In elicitation studies with in *E. californica*, Farber et al. [17] demonstrated that the yeast elicitor activated a pH-dependent signal transduction pathway separate from the octadecanoid pathway activated by MeJa. Farber et al. [17] reported that elicitation with either MeJa or yeast elicitor followed by a second addition of the same elicitor yielded no further increases in alkaloid production while elicitation with one elicitor followed by the other one after 24 hours enhanced alkaloid production. Interestingly, the alkaloid enhancement was observed regardless of the order of elicitation.

Facchini and Park found increased expression of the TYDC, 6OMT, CNMT, CYP80B1, 4’OMT, and BBE genes following elicitation of opium poppy suspension cultures with a fungal elicitor [18]. Induction of TYDC began 1 hour after elicitation while induction of 6OMT, CNMT, CYP80B1, 4’OMT, and BBE did not begin until 2 hours after elicitation. Further, expression of 4’OMT and 6OMT peaked 5 – 10 hours after elicitation. Expression of CYP80B1 peaked 10 – 20 hours after elicitation while expression of TYDC, CNMT, and BBE reached their maximum expression 2 hours after induction and remained constant up to 80 hours after elicitation. The differences in the time for maximum expression for individual genes suggests a complex mechanism for regulating flux through the BPA biosynthetic pathway during enhanced production.
The level or activities of enzymes in the BPA biosynthetic pathway are inducible by MeJA and yeast extract elicitor. Elicitation of *E. californica* cultures with MeJA upregulated expression of 6 BPA biosynthetic enzymes (6OMT, CNMT, CYP80B1, 4’OMT, BBE, and DHBO) [16, 23]. Other studies showed that CYP80B1, BBE, CFS, SPS, MSH, and P6H activities were elicited by MeJa [19, 46]. TYDC [20-21], BBE [51], and P6H [22] activities are inducible by yeast extract elicitors. The yeast elicitor and MeJa studies did not measure the effect of elicitation on BPA production so the relationship between enzyme activity and BPA production were not be studied.

Blechert et al. [19] examined the effect of yeast elicitor and MeJa on seven enzymes in the BPA pathway and found the activity of several membrane-associated enzymes (BBE, CFS, SPS, MSH and P6H) increased at least 6-fold to 16-fold while the cytosolic enzymes (*i.e.* TNMT, DHBO) were unaffected. The increase in activity was similar for treatment with MeJa and the yeast elicitor. The elicited enzymes are also cytochrome P450-dependent enzymes indicating the possible presence of a transcriptional regulator which activates cytochrome P450s. The enzymes with the greatest change after elicitation were five of the six enzymes between reticuline and dihydrosanguinarine (see Figure 2). However, DHBO was not affected by the elicitor so any subsequent BPA increase would be in the dihydro forms. Total alkaloid production was not reported by Blechert et al.

### 2.4.2 *In situ* Extraction

*In situ* extraction utilizes either immiscible solvents or polymeric resins to absorb products as they are secreted by the cell. Production increases potentially due to reduced feedback inhibition and increased driving force for product secretion [68-69].
products are secreted and absorbed onto the resin, both the intracellular and medium concentrations decrease.

BPAs are believed to inhibit cell growth by several mechanisms including DNA-binding and microtubule disruption [70] and thus should be sequestered or removed from the cell to prevent cytotoxic activity. BPAs are localized to the endoplasmic reticulum [58] suggesting an effort by the cell to sequester BPAs and reduce their cytotoxic effect. Thus adsorption onto the resin decreases the bulk concentration in the media which enhances the driving force for transport into the media. For example, in situ extraction with Amberlite XAD-7 was used to enhance alkaloid production in Catharanthus roseus cultures [71-73]. In situ extraction using Amberlite XAD-7 resin to yeast-elicited E. californica suspension cultures increased alkaloid production 20-fold to 10 μmol/g DW [24]. The effect of extraction using tricaprylin (glyceryl tri-n-octanoate) increased production 4-fold in E. californica cultures [74].

2.4.3 Alginate Immobilization

Alginate immobilization consists of trapping cells in alginate beads. BPA production was enhanced 800-fold (up to 8 mg/g DW) by immobilizing unelicited E. californica suspensions in alginate beads [25]. Immobilization of Taxus brevifolia cultures followed by methyl jasmonate elicitation produced 8 mg/L paclitaxel compared to 4 mg/L with methyl jasmonate alone [75].

2.4.4 Media Optimization

Media that has been optimized for growth may not be optimal for secondary metabolite production. Concentrations of hormones [76], sugar [77], phosphates [26],
and nitrates [26] have been shown to affect secondary metabolite production in *E. californica* cultures.

The plant hormones 2,4-dichlorophenoxyacetic acid (2,4-D) and \(\alpha\)-naphthalene acetic acid (NAA) are common components of plant cell culture media. While 2,4-D and NAA promote growth, alkaloid production decreased as hormone concentration increased in *E. californica* suspension cultures [78]. Currently, the mechanism by which 2,4-D acts on plant cell cultures is unknown.

The effect of the plant ripening hormone ethylene on secondary metabolite production has also been studied. Treatment of *E. californica* cell cultures with ethylene has resulted in both increased production [78] and no change in production [79]. The increase in production with ethylene addition suggests ethylene is part of a signal transduction pathway that is required for BPA production. However, Songstad et al. [79] showed that ethylene may be a byproduct of elicitation rather than required for BPA production.

The sugar (*e.g.* sucrose) concentration in the medium may also affect BPA production. Elevated sucrose concentrations in the media (up through 8% w/v) enhanced alkaloid production in *E. californica* from 13 mg/L in 2% w/v sucrose to 120 mg/L in 8% w/v sucrose [77].

Lamboursain and Jolicoeur studied the effect of nitrate and phosphate concentration on alkaloid production in *E. californica* cultures treated with chitin and Amberlite XAD-7 resin. Lamboursain and Jolicoeur [26] observed a 3-fold increase in alkaloid production to 345 mg/g DW in elicited *E. californica* when the total phosphate concentration was reduced from 1.1 mM to 0.8 mM. When the nitrate concentration was
reduced from 25.0 to 12.5 mM, total alkaloid production increased 38-fold up to 3163 mg/g DW. The intracellular glucose concentration increased 16-fold and total starch content increased 4.7-fold in low nitrate media suggesting a change in metabolic pathways in high alkaloid producing cultures.

### 2.4.5 Genetic Engineering of the BPA Pathway

BBE and 6OMT have been overexpressed in *E. californica* and related cultures. Overexpression of BBE increased BPA production by 5-fold in *E. californica* cultures [59]. Inui et al. [80] overexpressed 6OMT in *E. californica* resulting in a 7.5-fold increase in BPA production. Also, CYP80B3 was overexpressed in *P. somniferum* plants leading to a 450% increase in total alkaloid production [81]. In addition to single enzymes, a protein with a W-R-K-Y N-terminal amino acid sequence (WRKY) has been identified as a possible transcriptional regulator of alkaloid biosynthesis in *Coptis japonica* [82]. Silencing of the WRKY gene reduced transcripts of several BPA biosynthetic genes (TYDC, NCS, 6OMT, CNMT, CYP80B2, 4’OMT, and BBE) to between 40 and 60% of the control. The expression of primary metabolic enzyme transcripts (glyceraldehyde-3-phosphate dehydrogenase, 3-deoxy-D-arabino heputulosonate 7-phosphate synthase, dehydroquinate shikimate dehydrogenase, and chorismate mutase) was not significantly changed by silencing of the WRKY gene. Similarly, ectopic expression of WRKY induced expression of the BPA biosynthetic genes but not the primary metabolic genes. However, the effect of overexpression and RNAi of WRKY on berberine accumulation was not monitored. Further, WKRY transcript accumulation was similar in high and low berberine accumulating cultures.
The ability to manipulate multiple genes at the same time would be a valuable tool for developing economically feasible plant cell culture processes.

2.5 Genomic and Proteomic Investigation of Secondary Metabolism

The combined use of genomic, transcriptomic, proteomic, and metabolomic methods can potentially contribute greater understanding about the regulation of metabolic pathways leading to alkaloid production.

For example, Zulak et al. [83] analyzed the transcript profile of fungal-elicited *P. somniferum* cell cultures using expressed sequence tags (ESTs) and characterized the profile of primary metabolites and alkaloids using mass spectrometry. An increase in transcripts of both sanguinarine biosynthetic enzymes and enzymes in primary metabolic pathways including sugar metabolism, the shikimate pathway, and amino acid metabolism was observed. Specifically, all of the steps between sucrose and sanguinarine were upregulated. The systems biology approach by Zulak et al. [29, 83] revealed a coordination of primary and secondary metabolism during increased BPA biosynthesis in *P. somniferum* cell cultures induced with a homogenate from the fungus *Botrytis cinerea*.

For example, 8 genes from the BPA biosynthetic pathway were upregulated in fungal-elicited cultures (TYDC, NCS, 6OMT, CNMT, CYP80B1 4′OMT, BBE, and TNMT). Genes from primary metabolic pathways which were upregulated by elicitation include glycolysis and several genes from the methionine/SAM biosynthetic pathway. The genes encoding for enzymes related to S-adenosyl-L-methionine (SAM) biosynthesis which were upregulated included methionine synthase, adenosylhomocysteinase, and SAM synthetase (SAMS); methionine is converted to (S)-adenosylmethionine (SAM) by
the enzyme SAM synthase (SAMS). SAM is a universal methyl donor in a variety of biosynthetic reactions including 6 steps in the BPA pathway. Increased SAM production is believed to enhance BPA production by promoting the methylation reactions in the BPA pathway.

Zulak et al. also analyzed elicited *P. somniferum* cultures using a proteomic methodology [29]. Protein extracts from elicited and unelicited *P. somniferum* cultures were separated by two-dimensional gel electrophoresis. Spots showing intensity differences between the two cultures were excised and analyzed by LC-MS/MS for identification. Using a combination of a *P. somniferum* expressed sequence tag (EST) database and existing plant protein databases, 219 of 340 selected spots were identified. Proteins identified included heat shock proteins, chaperones, primary and secondary metabolic enzymes, and defense-related proteins. Proteins induced by elicitation included primary metabolic enzymes (SAMS, glutamate dehydrogenase, malic enzyme, and phenylalanine ammonia lyase), secondary metabolism (6OMT), and heat shock proteins. The results agree with the gene transcript data and suggest both primary and secondary metabolic pathways are affected by elicitation in *P. somniferum*.

### 2.5.1 Applications of Proteomic Methods

Proteomic methods can detect hundreds of proteins from a single sample and identify quantitative differences in individual proteins. Proteomic methods are a useful tool for the identification of disease biomarkers. Researchers have applied proteomic techniques to understand disease metabolism and identify targets for novel treatments. Quantitative differences in protein abundance have been observed between normal and
cancer cells [84]. Increased abundance of a particular protein in diseased cells or tissue compared to healthy cells or tissue can result in a protein marker for disease.

Identifying differences in protein abundance when cells are exposed to an external stimulus can yield clues to the cellular processes. For example, identification of differentially abundant proteins is useful in the biotechnology industry for optimizing production of proteins or small molecules from recombinant bacterial and mammalian cell cultures (Section 2.4.2). Recently, proteomics has also been used to analyze developmental processes in plants and to a lesser extent, the production of small molecules from plant cell culture (Section 2.4.3).

### 2.5.2 Proteomic Analysis Applied to Recombinant Protein Production

The biotechnology industry applies recombinant cell cultures to produce therapeutic proteins or small molecules in host cultures such as bacterial (*Escherichia coli*) or mammalian (Chinese hamster ovary, CHO) cells. A gene encoding for the protein product or for the biosynthetic enzymes involved in the biosynthesis of a small molecule is genetically engineered into the cells. The increased production of the desired protein/enzyme causes a burden on the cell as additional metabolic and energy resources are diverted to new or amplified pathways. This metabolic burden leads to slower production rates.

The metabolic burden of *E. coli* grown under high cell density conditions was demonstrated by proteomic methodologies. For example, Yoon et al. [85] observed increased abundance of several genes coding for enzymes of the tricarboxylic acid (TCA) cycle during high density *E. coli* fermentation. Similarly, in another study using shotgun proteomics, several TCA cycle enzymes and transport proteins were identified as being
expressed at high levels in *E. coli* cultures genetically engineered to overproduce 6-phospho-gluconolactonase [86]. Enzymes of the TCA cycle and glycolysis (*i.e.* malate dehydrogenase, pyruvate dehydrogenase, and phosphofructokinase-2) were also significantly increased in *E. coli* mutant cultures producing elevated threonine levels [87]. Malate dehydrogenase (which converts malate to oxaloacetate, the precursor for threonine), citrate synthase, and threonine dehydrogenase (enzymes that divert intermediates from threonine synthesis), were decreased in abundance in threonine overproducing cultures. Citrate synthase converts oxaloacetate to citrate and threonine dehydrogenase converts threonine to aminoacetone. Thus, the pathways of primary metabolism were altered to favor accumulation of threonine.

The Chinese hamster ovary (CHO) genome is not sequenced which presents an additional challenge in identification of proteins. Several proteomic studies have analyzed recombinant protein production in CHO [88-90] and the murine myeloma cell line NS0, another industrially-relevant cell line [91]. Most of these studies [89-91] utilized two-dimensional gel electrophoresis (SDS-PAGE) technology for protein separation prior to protein identification. One study used a gel-free approach utilizing strong cation exchange and reversed phase chromatography for peptide separation, resulting in the identification of 864 proteins including 392 identified by more than 1 peptide [88]. During high production, proteins increased in abundance included those involved in protein synthesis, transcriptional regulators, and glycolytic enzymes (pyruvate kinase, malate dehydrogenase). Six enzymes involved in protein folding and trafficking were more also abundant in the high-producer than the low-producer. The
results suggest that activity of primary metabolic pathways and the ability to provide precursors (e.g. amino acids, energy) for protein synthesis is limiting production.

Once a key protein (i.e. its abundance correlates with productivity) is identified, production of the desired product can be further enhanced by adding an inducer that results in overexpression of the target protein. Hayduk and Lee performed a proteomic comparison of four CHO cell lines producing 0.2 – 3.7 pg/cell/day of secreted alkaline phosphatase (SEAP) [92]. Twenty one proteins were found to be correlated with SEAP production, including CapZ which is involved in actin capping and cytoskeleton formation. The authors then simulated CapZ activity by adding the small molecule cytochalasin, resulting in a 3-fold increase in SEAP production.

Production can also be enhanced by overexpression of a target protein. For example, Ku et al. observed up to a 2.5-fold increase in erythropoietin productivity with XBP-1S overexpression of XBP [93]. The endoplasmic reticulum folding protein immunoglobulin binding protein (BIP) and the chaperone protein disulfide isomerase (PDI) increased in abundance in high monoclonal antibody producing NS0 cultures [94]. Also, BIP was overexpressed at the transcript level in human embryonic kidney cells overproducing recombinant human transferrin [95]. XBP-1S is a key regulator of the protein folding pathway. Ku et al. [93] also discovered that the overexpression of XBP-1S had a greater effect in high-producing cell lines but less effect on low-producing cell lines. In high-producing cell lines, the protein secretion pathway is likely limiting and thus more susceptible to enhancement by genetic engineering.
2.5.3 Proteomic Studies Applied to Plants

Proteomic studies in plants have primarily aimed to understand developmental processes in whole plants. *Arabidopsis thaliana* is the most commonly used plant in proteomics experiments as its genome has been sequenced and the protein database has been well-annotated. A large, well-annotated protein database increases the number of protein identifications and increases the chance of identifying important proteins. Currently, there are seven plants defined as completely sequenced by the NCBI (*Arabidopsis thaliana, Glycine max, Medicago truncatula, Oryza sativa, Populus tricocharpa, Sorgum bicolor*, and *Zea mays*; [http://www.ncbi.nlm.nih.gov/genomes/PLANTS/PlantList.html](http://www.ncbi.nlm.nih.gov/genomes/PLANTS/PlantList.html)). Protein identification is presented in more detail in section 2.5.1.4. Due to the lack of plant protein sequence databases, other methods including *de novo* sequencing of peptides and expressed sequence tags (ESTs) have been used in proteomics of non-model plants. *De novo* sequencing has been used to identify proteins from the insect *Triatoma infestans*, Brazilian pine *Araucaria angustifolia* [96], spinach, and bell pepper [97]. However, *de novo* sequencing is time-intensive and thus not ideal for high-throughput proteomic analysis. Expressed sequenced tags have been used for proteomic studies in *Glycine max* [98] and banana [99].

Proteomic experiments in *Arabidopsis* have included studies of seed germination [100], response to external treatment including fungal elicitation [101-102], nutrient conditions [103], and light stress [104]. Comparison of elicited and non-elicited *Arabidopsis* cells by two-dimensional gel electrophoresis resulted in visualization of approximately 1500 spots with 154 changing by over 20% [101]. However, of the 45 differentially abundant proteins actually listed in the paper, only seventeen changed by
more than two-fold which is a more common cutoff for proteomic studies. In the same study the most significant differences in abundance were the antioxidative enzyme glutathione-S-transferase (GST) and tryptophan synthase. GST upregulation is consistent with pathogen defense in plants and tryptophan is the precursor for a number of glucosinolates which provide protection against pathogen attack [105-106].

Proteomic methods has been applied to a number of medicinal plants including *Catharanthus roseus* [28], *P. somniferum* [27, 29], *Taxus cuspidata* [107], *Cannabis sativa* [108-109], ginseng [110-111], and *Chelidonium majus* [112]. The majority of proteomic research on medicinal plants has concentrated on maximizing the number of proteins identified. However, only a few of these papers investigated how primary and secondary metabolic pathways are altered in plant cell cultures producing high levels of secondary metabolites. Jacobs et al. [28] studied the protein profile at different time points in *C. roseus* but did not correlate protein abundance to alkaloid production. They observed approximately 1000 spots per gel. Database searching using the non-redundant plant database (NCBI) yielded 56 identifications from the 88 spots selected for analysis. Identified proteins included enzymes from glycolysis, the TCA cycle, alkaloid biosynthesis, and structural proteins. Zulak et al. [29] studied the protein profile with elicitation in *P. somniferum* and observed 46 differentially abundant proteins (excluding unidentified differentially abundant spots and multiple isoforms of the same protein). To date, the above proteomic studies in plants have identified differences in abundance of metabolically relevant proteins. However, the results have not been used to develop methods for further enhancement in secondary metabolite production.
2.6 Proteomic Methods for Investigating Global Metabolism

Proteomics refers to the study and profiling of the full complement of proteins produced by the cell at a given point in time. Proteomic methodologies have been applied for discovery of disease markers and of bottlenecks to the production of therapeutic proteins from mammalian cell culture (Section 2.4.2). Transcriptomics, the large-scale monitoring of gene expression, has also been used for biomarker discovery and analysis of production of therapeutic proteins. However, gene expression is not always correlated with protein abundance.

Each step in the proteomic method must be performed carefully so as not to alter the protein profile or affect the downstream steps. The workflow for a proteomics experiment includes protein extraction, digestion, protein and peptide separation and mass spectrometric analysis.

2.6.1 Protein Extraction

Proteins are isolated by first lysing the cell and releasing the intracellular content. Several commonly used lysis methods include grinding with a mortar and pestle in liquid nitrogen, mechanical disruption (i.e. homogenizer, bead beater, French press) and sonication. Lysis with a mortar and pestle or a homogenizer utilizes mechanical force to break open the cell. Sonication uses high energy sound waves for cell lysis.

Proteins extracted during lysis are released and solubilized by the extraction buffer. The extraction buffer is designed to maximize protein solubility without altering the protein profile. Common extraction buffer components include denaturants (e.g. urea and sodium dodecyl sulfate) and buffers to maintain pH (Tris, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, |HEPES|). Cells contain a number of natural proteases
which cleave proteins in an uncontrolled manner in this setting. Thus, inhibitors for a range of proteases (serine, cysteine, metallo-, and aspartic proteases) are added to the extraction buffer to preserve the protein profile. Extraction of proteins from plant tissue is further complicated by the presence of secondary metabolites including phenol-derived compounds (e.g. phenols, tannins, lignins, coumarins, and flavonoids) [113]. These compounds can interfere with protein assays and bind covalently to proteins leading to protein precipitation. Polymeric resins including polyvinylpolypyrrolidone (PVPP) and polystyrene (Amberlite XAD-4) have been used to adsorb interfering compounds [114-115]. The resins are insoluble in water and can thus be removed from the protein extract by centrifugation. PVPP has been included in several plant proteomics experiments [116-119].

### 2.6.2 Protein Digestion

The purpose of protein digestion is to break the protein into smaller fragments (peptides) which are more amenable to mass spectrometric analysis. Sample preparation prior to digestion involves denaturation, reduction, and alkylation. Denaturation unfolds the protein to expose the entire amino acid sequence to the protease. Commonly used denaturing agents include urea and guanidine hydrochloride. Upon denaturation, the protein secondary structure is still intact due to disulfide linkages on cysteine residues. The disulfide linkages are broken by reduction, usually with dithiothreitol or TCEP. After reduction, the proteins are alkylated with iodoacetamide or iodoacetic acid. The alkylation step places an alkyl group on the exposed cysteine residue thus blocking reformation of the disulfide bonds. Digestion is performed with a protease that cleaves proteins at specific locations. Prior to digestion, the denaturant must be diluted or
removed in order for the protease to maintain its activity. The most commonly used protease, trypsin, cleaves at the C-terminal end of lysine and arginine residues.

2.6.3 Protein and Peptide Separation

A protein extract from a whole cell lysate is a highly complex mixture of proteins with variations in abundance, size, and hydrophobicity. Without fractionation, a proteomic analysis of a complex mixture will only identify the most abundant proteins. Separation of a complex mixture into less complex fractions enhances the number of proteins identified. Crude separation methods such as ultracentrifugation can be used for protein fractionation. Commonly used methods for separating proteins include gel electrophoresis and high performance liquid chromatography (HPLC). While several methods exist for protein separation, chromatographic separation is the most common method for peptide separation in proteomics.

Ultracentrifugation can be included in proteomic methods to produce fractions enriched in proteins from specific organelles (e.g. mitochondria, ribosome) or to purify membrane proteins. Density gradients using sucrose or percoll are commonly used for organelle fractionation. For example, sucrose density centrifugation followed by LC-MS/MS proteomics was used to identify photosynthetic proteins from barley, spinach, and Arabidopsis leaves [120]. Liver protein extracts have also been separated into mitochondrial, cytosol, and microsomal fractions using differential centrifugation [121]. Nuclear proteins were precipitated by centrifugation at 1,000 g. The supernatant was saved and mitochondrial proteins were sedimented by centrifugation at 15,000 g. The supernatant from the 15,000 g centrifugation was centrifuged at 100,000 g. The resulting supernatant was enriched in cytosolic proteins and the pellet was enriched in
mitochondrial proteins. Differential centrifugation led to approximately twice as many protein identifications compared to analysis of the whole cell lysate [121].

Gel electrophoresis utilizes a polyacrylamide gel to separate proteins based on molecular weight. When an electric field is applied, proteins migrate through the gel. Smaller proteins migrate faster than larger proteins as they diffuse through the gel matrix easier. One dimensional sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) allows for qualitative analysis of a protein sample. Many proteomic analyses, particularly the earlier studies, have utilized two-dimensional SDS-PAGE for protein separation. Two-dimensional SDS-PAGE utilizes isoelectric focusing (IEF) as the first dimension and SDS-PAGE as the second dimension for separation. Isoelectric focusing takes advantage of the protein’s isoelectric point (pI) for separation; pI is the pH where the given protein has a zero net charge. Samples are loaded into a strip containing a medium with a pH gradient. Under an electric field proteins will move through the medium until they reach the point where the pH is equal to the pI. IEF is followed by SDS-PAGE where a group of proteins with the same pI are then separated by molecular weight. Proteins are then visualized as spots after staining the gel with a stain (e.g. Coomassie blue, silver, Sypro red). In proteomic studies, spots of interest (i.e. those changing in intensity between samples) are cut out, digested with trypsin, and analyzed individually by LC-MS.

Recently, chromatographic methods have been used in place of gel electrophoresis for protein and peptide separation. Proteomics using SDS-PAGE has a number of well known limitations including a bias against very hydrophobic proteins, as well extremes in pI and molecular weight. Also, chromatographic columns with high
loading capacities allow for a greater amount of protein to be analyzed compared to SDS-PAGE leading to improved detection of low-abundance proteins. In general, chromatography separates a sample mixture based on a given property of the individual analytes. For protein separation, the most common forms of chromatography are reversed-phase liquid chromatography (RPLC) and ion exchange chromatography (IEC). Other forms of chromatography used in protein separation or purification include gel filtration chromatography and affinity chromatography.

RPLC separates based on hydrophobicity. Samples are loaded onto a column containing a nonpolar stationary phase (e.g. C_{18}, C_{8} or C_{4}) and eluted with a gradient from polar solvents such as water to organic solvents such as acetonitrile, methanol or isopropanol. Individual peaks or groups of peaks can be collected and digested separately potentially resulting in a greater number of protein identifications.

IEC uses charge as the mechanism of separation. There are two major types of IEC: anion-exchange chromatography and cation-exchange chromatography. Anion-exchange chromatography utilizes a positively charged matrix covalently attached to the stationary phase. Anions then bind to the positively charged matrix. Increasing salt concentration in the mobile phase disrupts the binding of the sample to the stationary phase causing it to elute. Anion exchange chromatography has been used to fractionate Arabidopsis proteins prior to digestion [122]. Anion exchange chromatographic separation of proteins combined with 2D-LC peptide separation resulted in identification of 1032 unique proteins compared to 297 using 2D-LC peptide separation alone. Depending on the strength of the stationary phase, anion exchangers can be categorized as strong or weak exchangers.
Strong cation exchange chromatography (SCX) is frequently used in multi-dimensional peptide fractionation. SCX takes advantage of the fact that nearly all peptides are positively charged at a pH below 3. The net charge on the protein then depends on the number of positively charged groups (i.e. peptide N-terminus, lysine, arginine, and histidine). SCX has been used for fractionation of peptides from yeast [123], *Arabidopsis* [122], *Medicago trunculata* [124], and rice [125-126]. SCX can lead to an increased number of identifications. For example, Natera et al. [126] identified 687 proteins using 2D-LC-MS/MS compared to 486 proteins using Gel-LC-MS/MS. Frequently, SCX fractionation is followed by reversed phase peptide separation. The reversed phase separation removes the elution buffer salt and enables further peptide fractionation.

### 2.6.4 Protein Identification

After digestion, peptides are analyzed by mass spectrometry. Fragmentation of peptides in the mass spectrometer allows the peptide sequence to be determined. Comparison of the peptide sequence with the theoretical sequences generated from a database allows determination of the protein from which the experimental peptide originated. Peptide sequences are primarily determined by database searching but could be determined also by for example *de novo* sequencing. Database searching compares the masses of peaks in the MS/MS spectrum with the masses of theoretically determined peptide fragments derived from the database of all potential proteins that could be in the sample. *De novo* sequencing uses the mass spectrum to directly infer the amino acid sequence and subsequently search for proteins with similar sequences as shown in Figure
3. *De novo* sequencing is more time-consuming than database searching and thus reserved for spectra which cannot be identified by database searching.

![Figure 3: De novo peptide sequencing. De novo sequencing is used to determine the amino acid sequence of a peptide. The difference in mass between successive peaks indicates an amino acid from the sequence [127].](image)

2.6.5 Mass Spectrometry

Developments in mass spectrometry have greatly enhanced the field of proteomics. The main components of a mass spectrometer are the ionization method, mass analyzer, and detector.

The two most common ionization methods used in proteomics are electrospray ionization (ESI) and matrix assisted laser desorption/ionization (MALDI). ESI was originally developed to allow mass spectrometry of large biomolecules and polymers [128]. In ESI, the analyte is ionized by the application of an electric field. ESI instruments can be coupled on-line with an HPLC for peptide separation prior to mass spectrometry.

MALDI requires the peptide to be mixed with a low molecular weight matrix solution. Common matrix solutions include α-cyano-hydrocinnaminic acid (CHCA), nicotinic acid, 2,5-dihydroxybenzoic acid, sinapinic acid and succinic acid [129]. A dilute sample/matrix mixture is allowed to crystallize on a plate with the matrix in a large
excess. The crystallized matrix is then bombarded with a laser beam. The energy of the laser is sufficient to ionize the matrix. Energy is transferred from the matrix to the analyte, which is then ionized in the vapor phase. A MALDI instrument cannot be directly coupled to a HPLC; therefore in proteomics experiments, peptides must be first separated by HPLC then mixed with matrix and spotted on a plate offline prior to MS analysis. An automated spotting device depicted in Figure 4 was developed by Chen et al. to couple HPLC peptide separation to MALDI analysis [130-131].

![Diagram of MALDI spotting system](image)

**Figure 4:** Schematic representation of MALDI spotting system. The eluent from the HPLC is mixed with the MALDI matrix and spotted onto a plate positioned on a platform which moves in the X and Y directions [130-131].

Briefly, the device consists of a HPLC for peptide separation where the effluent stream is mixed with a matrix solution flowing from a syringe pump. The mixed peptide and matrix solution flows through a capillary and onto a plate which is moved at defined time intervals to collect peptide fractions. After the matrix crystallizes the plate is analyzed by MALDI-MS.
2.6.6 Protein Quantification

The ability to accurately detect quantitative differences in protein abundance between samples is critical to a proteomics experiment. Current available methods for protein quantification include spot intensity from SDS-PAGE, isotope labeling, and spectral counts.

The ability to accurately detect quantitative differences in protein abundance between samples is critical to a proteomics experiment. Current available methods for protein quantification include spot intensity from SDS-PAGE, isotope labeling, or for example spectral counts.

In SDS-PAGE, the spot intensity is visualized by staining and is roughly proportional to the amount of protein present. The gel is scanned and a computer program is trained to detect stain intensity and look for differences between the same spot on different gels. Typically in a 2D gel experiment only the spots showing at least a two-fold intensity difference are analyzed. The drawback of intensity-based quantitation is a lack of accuracy as one spot may contain several proteins. Also, in order to identify proteins, spots of interest have to be cut from the gel and analyzed individually which requires additional reagents and instrument time. Currently, SDS-PAGE is the most common choice for plant proteomic experiments [27-28, 132].

Labeling of proteins or peptides with stable isotopes provides the greatest accuracy and sensitivity. Isotope labeling methods are performed metabolically or chemically. Metabolic labeling includes growth in labeled media components \( \text{i.e. } ^{15}\text{NO}_3^- \) and stable isotope labeling with amino acids in cell culture (SILAC) [133]. Utilization of labeled media results in proteins synthesized with \(^{15}\text{N}\) instead of \(^{14}\text{N}\).
leading to a mass shift of a particular peptide in the MS spectrum. The ratio of the MS peaks from the light and heavy peptide is then used for quantitation. SILAC has successfully been applied to identify proteins associated with prostate cancer [134]. Though very powerful, SILAC method is applicable only to organisms with at least some essential amino acids. However, plant cell cultures have the ability to synthesize all amino acids, and thus SILAC cannot be used. On the other hand, plans cells can be effectively labeled with ammonium and nitrate as the exclusive source of $^{15}$N. Importantly, since the samples can be mixed directly after cell lysis and protein extraction, the effect of variation in downstream sample handling steps is reduced.

Chemical labeling methods incorporate isotopes through reaction with for example amino acid side chains and peptide N-terminal amine groups. For instance, isotope-coded affinity tags (ICAT) label cysteine residues with a light or a heavy tag containing eight deuterated hydrogens as shown in Figure 5. In MS mode there is a mass difference of eight for a given peptide labeled with the heavy tag (Figure 6). The large mass difference between heavy and light chain tags allows the MS peaks to be easily distinguished from each other. Figure 6 shows the workflow for labeling and quantifying peptides using the ICAT reagent. However, ICAT reagents are expensive and only peptides with cysteine residues can be quantified.
Figure 5: Isotope-coded affinity tag (ICAT) reagent structure (A) and experimental workflow (B) [135]

Figure 6: Workflow for measuring relative protein abundance using the ICAT reagent.
The same peptide will differ in mass between the light and heavy sample [136].

Alternatively, proteins can be labeled after digestion. The most common peptide labeling reagent is isobaric tag for relative and absolute quantitation (iTRAQ) [137]. Unlike ICAT which only labels cysteines prior to digestion, iTRAQ labels the N-terminus of every tryptic peptide by a reaction of a N-hydroxy succinimide (NHS) with the N-terminal peptide. Several other tags utilizing succinimide groups have also been employed including 3-sulfobenzoic acid succinimidyl ester labeling [138-140]. The iTRAQ reagent incorporates NHS group (Figure 7, top) to react with primary amines such as the N-terminus of a peptide (Figure 7, bottom) and includes a reporter group and a balancer group. Up to eight samples are labeled with tags of varying reporter group masses. The balancer group incorporates isotope labels such that the sum of the reporter and balancer masses is the same for each label. Thus in the MS and MS/MS spectra the each peptide has the same mass for each tag used.

Figure 7: Chemical structure of the iTRAQ reagent and how the iTRAQ reagent is linked to the peptide.
Then in the MS/MS fragmentation process the reporter group is cleaved and the balancer group is lost as a neutral species. The ratio of the peak areas for the reporter ions is used for quantitation allowing up to eight samples to be analyzed at the same time. All peptide fractionation is done with the samples mixed together after the labeling step which reduces the effect of sample loss due to multiple processing steps. The effect of run-to-run variation is also minimized by running all samples at the same time. Also, spectral quality is enhanced as the intensity of each labeled sample contributes to the total peak intensity. Despite these advantages, iTRAQ reagents are expensive and require a mass spectrometer with the sensitivity to distinguish between the masses of the reporter ions (114, 115, 116 and 117). Commonly used mass spectrometers in proteomics including the LTQ instruments lack the sensitivity at the low-mass range to enable use with iTRAQ.

Alternative label-free quantitation methods, such as the ion chromatogram peak area calculation and spectra counting, do not require labeling, can provide improved accuracy compared to SDS-PAGE, and require fewer reagents. However, samples must be analyzed separately increasing the total processing time for the experiment. Also, labeling proteins and peptides or proteins may cause a shift in chromatographic retention time making sample comparison more challenging.

Peak area measurement involves extraction of ion intensity data for a specific peptide mass and plotting as a function of retention time. The result is similar to an absorbance-based chromatogram except that only the contribution of the specified mass is included. Co-eluting chromatographic peaks with different masses are not included in the extracted ion chromatogram. However, as with absorbance chromatograms, the peak
area for the precursor ion is proportional to its abundance. Mass spectrometric peak area and protein abundance have been correlated with less than 16% error [141-142]. Label free approaches have been applied to both simple proteins and complex mixtures. However, for some mass spectrometers, calculation of peak area must be done manually and is a time consuming procedure particularly if a large number of peptides from a complex sample are to be quantitated.

For experiments where it is sufficient only to observe a large, statistically significant abundance difference between two samples the number of spectra counts can be used for relative quantitation. Spectra count refers to the number of times a peptide from a particular protein was observed. Spectra counts has been established as an effective technique for quantifying protein abundance differences [143-146].

Spectra counting and peak integration methods were compared using dilutions of a 6-protein standard mixture and it was shown that both methods were linear over a 40-fold dilution range [147]. Using spectra counts for quantitation provides a rough approximation and should be used with caution. Important differentially abundant proteins, particularly those with low spectra counts, should still be quantified by peak area. Also, the error in spectral counting was found to be higher for low molecular weight peptides which was expected as low molecular weight proteins don’t produce as many unique peptides leading to a reduced chance of identifying a peptide [147].

In summary, there are advantages and disadvantages of each quantitation method. Chemical labeling methods at the protein and peptide levels provide more accurate quantitation and require less sample handling as multiple samples can be analyzed.
simultaneously. However, chemical labeling methods are more expensive. Label-free methods do not require expensive reagents but require additional sample processing time.
3.0 MATERIALS AND METHODS

3.1 Maintenance of *Eschscholzia californica* Suspension Cultures and Calli

The *E. californica* (California poppy) suspension cultures (cell line ELDN01) were a gift from Dr. Song-Yong Yoon of Pohang Institute of Science and Technology (POSTECH, Pohang, South Korea). Suspension cultures of *E. californica* were maintained in 250 and 1000 ml Erlenmeyer flasks. Subculturing was performed every fourteen days by pipetting ten ml of culture (containing three ml of packed cells) to 40 ml of fresh media.

To obtain 3 ml of packed cell volume, approximately 7 – 8 ml of culture is drawn into a 10 ml pipette and then media is expelled while the pipette tip is pressed against the bottom of the flask. After 3 ml of cells have accumulated in the pipette, 7 ml of media is drawn into the pipette. The cells and media are then transferred to a new flask containing fresh media. For 250 ml flasks, 20 ml of culture were added to 80 ml of autoclaved fresh media. For 1000 ml flasks, 80 ml of culture were added to 320 ml of autoclaved fresh media. The cultures were then agitated in a rotary incubator at 120 rpm, 22°C with a 16 hr light photoperiod. The cells grown in flasks, 6-well plates, and on solid agar medium are shown in Figure 8.
Figure 8: *E. californica* suspension and calli cultures. *E. californica* cells are grown in flasks (left), 6-well culture plates (center), and as calli on agar plates (right).

In preparation for subculturing, empty 250 and 1000 ml flasks were covered with foam closures (Bellco, Vineland, NJ) and autoclaved for 30 min at 121°C. Fresh media was then added to each flask (*i.e.* 80 and 320 ml of media in 250 and 1000 ml flasks, respectively) and autoclaved for 30 min at 121°C. Fresh media consisted of 4.43 g/L Linsmaier-Skoog macro- and micronutrients (Caisson Laboratories, Rexburg, ID, #LSPC0130), 30 g/L sucrose (Sigma, #S-5390), 0.37 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D, Sigma #D-8407), and 0.11 mg/L α-napthaleneacetic acid (NAA, Sigma, #N-0640) and adjusted to pH 5.5 with 1 N NaOH.

As back-up to suspension cultures, *E. californica* calli were also maintained on agar petri dishes. The media for agar plates consisted of the suspension culture media described above plus 8 g/L agar (Type E, Sigma #A4675). The agar-containing media was autoclaved for 30 min at 121°C then poured into sterile petri dishes, solidified, and sealed with parafilm prior to storing at 4°C. Cells from suspension cultures (day 14 of the growth curve) were collected by vacuum filtration and then spread evenly over the surface of an agar-containing petri dish. The cultures on petri dishes were then sealed with parafilm and stored at room temperature under fluorescent light.
3.2 Elicitation Experiments

Elicitation experiments were performed to determine the effect of purified yeast extract (PYE), methyl jasmonate (MJ), ethephon, and salicylic acid (SA) on alkaloid production in \textit{E. californica} cell suspensions. For elicitation experiments, cells were cultured in growth media lacking 2,4-D to maximize alkaloid production as 2,4-D has been shown to inhibit alkaloid production in \textit{E. californica} and \textit{Catharanthus roseus} [78-79, 148]. After 7 days in 2,4-D free media (exponential phase), 5 ml of culture were pipetted into 6-well cell culture clusters (Corning Incorporated, Corning, NY, #3516) and then the elicitor or hormone was added. The wells were inoculated in triplicate for each elicitor dosage: 1) 10, 20, or 40 mg of PYE/g fresh cell weight (FCW), 2) 50, 100, or 500 μM MJ, 3) 50, 250, or 1000 μM ethephon, and 4) 1, 5, or 10 mM SA. Plates were wrapped in parafilm and placed in a rotary incubator at 120 rpm, 22°C with a 16 hr light photoperiod.

Cells were harvested by removing the liquid using a 10-ml glass pipette pressed against the bottom of the well and the cells were subsequently scraped into petri dishes (Fisher Scientific, #08-757-100A) after 24, 48, 72, and 96 hours; cells were lyophilized for 72 hours using a Flexi-Dry MP Freeze Dryer (Kinetics Thermal Systems, FD-3-85A-MP, Stone Ridge, NY).

3.2.1 Preparation of Purified Yeast Extract

Purified extract was prepared based on Hahn and Albersheim [149] and modified by Cho et al. [16, 23]. Yeast extract (50 g, Becton-Dickinson #212750, Sparks, MD) was solubilized in 200 ml of water in a 1 L Erlenmeyer flask; ethanol (Fisher-Scientific, A406P) was added to a final concentration of 80% v/v and then the mixture was sealed

46
with aluminum and parafilm and then stored at 4 °C for 4 days. The precipitate settled to
the bottom and supernatant was discarded without filtering. The gummy precipitate was
redissolved in 200 ml of water and precipitated again with ethanol (80% v/v) at 4 °C for 4
days. The final precipitate was then resuspended in 200 ml of deionized water,
lyophilized for 72 hours, and stored at -20 °C. The PYE stock solution was prepared by
dissolving 1 g of lyophilized powder in 4 ml of water followed by autoclaving for 30
minutes at 121 °C.

3.2.2 Preparation of Methyl Jasmonate Stock Solution

A 62.5 mM methyl jasmonate (MeJa) stock solution was prepared by transferring
21 μl 95% MeJa (Aldrich, #W34100-2) and 1479 μl of ethanol to a 1.5 ml
microcentrifuge tube. The solution was sterilized in the laminar flow hood using a 0.20
μm syringe filter (Corning Incorporated #431221, Corning NY).

3.2.3 Preparation of Ethephon Stock Solution

A 8 mg/ml ethephon stock was prepared by dissolving 12 mg of ethephon (Sigma
# C0143) in 1.5 ml of water. The solution was sterilized in the laminar flow hood using a
0.20 μm syringe filter (Corning Incorporated #431221, Corning NY). Ethephon was
added to a final concentration of 50, 250, and 1000 μM.

3.2.4 Preparation of Salicylic Acid Stock Solution

A 1.81 M stock solution was prepared by adding 2.5 g of salicylic acid (Acros,
#14-770-26) to 10 ml of ethanol. The solution was sterilized in the laminar flow hood
using a 0.20 μm syringe filter (Corning Incorporated #431221, Corning NY).
3.3 Alkaloid Analysis

Benzophenanthridine alkaloid (BPA) levels in the *E. californica* cells were determined by extracting and isolating the metabolites from freeze-dried cells, then separating and quantifying the metabolites by high performance liquid chromatography (HPLC).

3.3.1 Alkaloid Extraction

Freeze-dried cells (10 mg) were added to Eppendorf microcentrifuge tubes and then 1 ml of HPLC grade methanol (Fisher Scientific A452-4) containing 0.2% (v/v) HCl was added to each tube. The tubes were then briefly vortexed and then sonicated for 1 hour in an ultrasonic cleaner (Fisher Scientific, FS14H). Then the tubes were vortexed for an additional 30 minutes and centrifuged for 20 minutes at 13,200 g and 4°C (Eppendorf tabletop centrifuge #5415; Eppendorf, Westbury, NY). The resultant supernatants were filtered through 0.45 μm syringe filters (Millipore, Billerica, MA, #SLHN 013 NL) and stored in 1 ml glass vials (Waters #186000384c, Milford MA) at -20°C in preparation for HPLC analysis.

3.3.2 Validation of Alkaloid Extraction Method

The effectiveness and reproducibility of alkaloid extraction from California poppy cell cultures using the following variations on the general extraction method (from Section 3.3.1) was tested to maximize the alkaloids extracted from freeze-dried cell: 1) a sonicator versus a bead beater for tissue destruction and 2) a second extraction step. The validation experiment is described in section 3.3.4.

*E. californica* cell line ELDN01-2 was maintained as described in Section 3.1. After 14 days of growth, cells were transferred to fresh medium lacking 2,4-D to
maximize BPA production. After 7 days of growth in 2,4-D free medium, cells were elicited with 40 mg PYE per FCW and harvested after 96 hours. For each extraction method, four samples were taken from the same 250 mL flask.

Alkaloids were extracted by adding 10 mg dry weight either to 1) an Eppendorf microcentrifuge tube for sonication or 2) a centrifuge tube (VWR Scientific, #20170-217) half-filled with 0.5 mm glass beads (BioSpec, Bartlesville, OK #11079105) for extraction with the cell beater (BioSpec, Bartlesville, OK, #3110BX). In the sonication method, cells (10 mg dry wt) were extracted with 1 ml methanol (+ 0.2% HCl), sonicated for 1 hour, vortexed for 30 min, and then centrifuged for 20 min at 13,200g and 4 °C, as described in Section 3.3.1. Alternatively, cells (10 mg dry wt) in bead beater vials were extracted with 1 ml of methanol (+ 0.2% HCl), shaken in the bead beater for 30 seconds at 4800 rpm, and then placed on ice. This shaking and cooling process was repeated two more times and then the vial was centrifuged for 20 min at 13,200g and 4 °C.

Alkaloids extracted from freeze-dried cells using either a sonicator or a bead beater were then analyzed by HPLC. In particular, sanguinarine was used as a marker for alkaloid extraction efficiency and was identified by comparison of retention time and UV spectra with a standard (Sigma #84480). The eight extracts (four using the sonicator and four using the cell beater) were injected consecutively into the HPLC (30 μl injections each; see Section 3.3.3 for HPLC method); the injection sequence was repeated two more times so that each sample was injected every 7.2 hours.

As shown in Table 2, the amount of sanguinarine extracted using both methods were comparable. In the first extraction step, using the sonicator yielded an average of 3.76 mg sanguinarine / g dry wt (DW) compared to 4.02 mg sanguinarine / g DW using
the cell beater; accounting for the standard deviation the difference between the methods was not significant. Both methods yielded less than 10% variation between the four extracted samples. In addition, low variations in quantifying the sanguinarine concentration from a single vial by HPLC were observed when each sample was injected 3 times. For example, subsequent injections from the same vial resulted in 0.8 - 2.3% variation (data not shown in Table 2). Also the sanguinarine in a cell extract does not break down during the first 24 hours after extraction (data not shown in Table 2).

Table 2: Comparison of sanguinarine extracted (mg/g DW) by sonication and cell beater. Sanguinarine concentrations for each sample are the average of three injections per sample. The total average represents the average of 4 aliquots from the same flask. The average and standard deviation are from triplicate measurements.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sonicator 1st extraction</th>
<th>Sonicator 2nd extraction</th>
<th>Cell Beater 1st extraction</th>
<th>Cell Beater 2nd extraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.45 ± 0.08</td>
<td>0.61 ± 0.01</td>
<td>3.99 ± 0.06</td>
<td>0.77 ± 0.07</td>
</tr>
<tr>
<td>2</td>
<td>4.16 ± 0.04</td>
<td>0.90 ± 0.01</td>
<td>4.27 ± 0.05</td>
<td>0.79 ± 0.07</td>
</tr>
<tr>
<td>3</td>
<td>3.76 ± 0.04</td>
<td>0.75 ± 0.01</td>
<td>3.46 ± 0.05</td>
<td>0.62 ± 0.08</td>
</tr>
<tr>
<td>4</td>
<td>3.68 ± 0.03</td>
<td>0.78 ± 0.00</td>
<td>4.38 ± 0.05</td>
<td>0.75 ± 0.07</td>
</tr>
<tr>
<td>Average</td>
<td>3.76</td>
<td>0.76</td>
<td>4.02</td>
<td>0.73</td>
</tr>
<tr>
<td>Std Dev.</td>
<td>0.27</td>
<td>0.11</td>
<td>0.37</td>
<td>0.09</td>
</tr>
<tr>
<td>% Std Dev.</td>
<td>7.3</td>
<td>14.1</td>
<td>9.2</td>
<td>12.7</td>
</tr>
</tbody>
</table>

Alkaloids extracted must be maximized to represent the true level of production; any unextracted alkaloids must be small and consistent. Thus extraction from the same pellet was performed twice by adding a fresh aliquot of methanol to the cells after the first extraction. Extraction for a second time with a fresh aliquot of methanol yielded 0.76 mg sanguinarine /g DW using the sonicator and 0.73 mg sanguinarine /g DW using the cell beater (Table 2). The sonicator extracted a total of 4.52 mg/g DW compared to 4.75 for the cell beater. The percentage of total sanguinarine found in the first extract was similar for both methods (83.2% for sonicator vs. 84.7% for bead beater).
In conclusion, the extraction method using the sonicator versus the cell beater does not significantly affect the amount of sanguinarine obtained. Therefore, the sonicator was used since it required less sample handling than the cell beater and was capable of processing up to 30 samples at a time while the bead beater could only process one sample at a time. The second extraction step will not be included in the future protocol since sanguinarine content is increased by only 15% with the second extraction step, which is similar to the variability of the extraction method between replicates (i.e. 10%).

3.3.3 High Performance Liquid Chromatography Separation & Quantification of Alkaloids

Separation of alkaloids was performed using a high performance liquid chromatography system (HPLC, Waters Corp., Milford, MA, #2695) with a photodiode array UV detector (PDA, Waters Corp., Milford, MA, #996). Separation was achieved using a Phenomenex C18 Luna column (150 x 4.60 mm, 5 μm, Phenomenex, Torrence, CA, 00F-4252-E0). The solvent system for HPLC analysis consisted of 1) solvent A: Prepared by adding 0.8 of 40% (w/v) t-butyl ammonium phosphate (Sigma, #178780) to 1 L of water then adjusted to pH 3.0 with phosphoric acid (Fisher, #A242), 2) solvent B: acetonitrile (HPLC grade, Fisher Scientific, part #A998-4), 3) solvent C: methanol (HPLC grade, Fisher Scientific, part #A452-4), 4) solvent D: 95% reverse osmosis water / 5% acetonitrile. Aqueous solvents were suction-filtered through 0.45 μm nylon filter membranes (Phenomenex, Torrance, CA, AF0-0504) and organic solvents were suction-filtered through PTFE filter membranes (Phenomenex, Torrance, CA, AF0-0514) prior to HPLC.
The column conditions included five linear segments at 1.0 ml/min and were adapted from Cho et al. [16, 23, 67] as follows: 1) 80% A, 10% B, 10% C to 75% A, 10% B, 15% C in 5 minutes; 2) to 60% A, 15% B, 25% C in 5 minutes; 3) to 40% A, 25% B, 35% C in 10 minutes; 4) to 1% A, 10% B, 89% C in 15 minutes; 5) to 80% A, 10% B, 10% C in 10 minutes; and 6) isocratically at 80% A, 10% B, and 10% C for 15 minutes. The column was then washed with solvent D overnight at 0.1 ml/min after all of the samples were run. The column was then washed with 20 column volumes (50 ml) of 95% acetonitrile / 5% water then washed with 20 column volumes (50 ml) of 30% water and 70% acetonitrile for storage. An example chromatogram from the separation of BPAs by HPLC is shown in Figure 9.

Identification of sanguinarine and chelerythrine was based on comparison of retention time and UV spectra with standards (Sigma; sanguinarine #84480; chelerythrine, #C292; see Figure 10). Reference UV spectra were provided by Dr. Hwa-Young Cho. Confirmation of alkaloid identities by LC-MS/NMR is in progress.

**Figure 9:** Separation of BPAs by HPLC. The HPLC chromatogram of a *E. californica* methanol extract injected on a Phenomenex C18 column and separated using the LC gradient described in Section 3.3.3 is shown with the identified BPAs.
Figure 10: UV spectra of the BPA compounds identified in Figure 9.

The calibration curve for sanguinarine (Figure 11) was generated by injecting 30, 20, and 10 µl of a 20 mg/l standard solution dissolved in methanol + 0.2% HCl.
Figure 11:  Sanguinarine calibration curve. The sanguinarine standard solution (0.02 mg/ml) was injected in 3 different volumes (10, 20, and 30 ul).

Only sanguinarine and chelerythrine are commercially available as standards. Thus, concentrations of other alkaloids were determined using extinction coefficients found in organic electronic spectral data [150]. The absorbance of a solution is governed by the Beer-Lambert law (Equation 1) where \( a \) is the absorbance in absorbance units, \( \varepsilon \) is the extinction coefficient (M\(^{-1}\)cm\(^{-1}\)), \( c \) is the concentration (M), and \( l \) is the path length (cm).

\[
a = \varepsilon cl
\]

(1)

The eluent from the HPLC column passes through a photodiode array (PDA) detector and the absorbance is measured at an array of wavelengths from 210 to 600 nm. As a compound elutes from the column, a plot of the absorbance versus time yields a chromatographic peak as shown below in Figure 12.
The area under the curve can be approximated using the trapezoid rule where each point on the curve corresponds to the absorbance of a plug of solvent passing through the detector. Thus the area can be calculated using the trapezoid rule as shown in Equation 2 where \( t_0 \) is the time where the peak begins to elute and \( t_n \) is the end point of the peak. \( N \) is the number of segments used to calculate the peak area.

\[
\text{Area} = \left( \frac{t_n - t_0}{2n} \right) \left( a_0 + 2a_1 + 2a_2 + ... + 2a_{n-1} + a_n \right) \tag{2}
\]

Substitution of Equation 1 into Equation 2 yields the area in terms of concentration (Equation 3). After factoring out \( \varepsilon l \), the concentrations can be summed as in Equation 4; \( c_0 \) and \( c_n \) (concentrations) are defined as the endpoints of the peak and are assumed to be zero.

\[
\text{Area} = \left( \frac{t_n - t_0}{2n} \right) \left( \varepsilon l c_0 + 2\varepsilon l c_1 + 2\varepsilon l c_2 + ... + 2\varepsilon l c_{n-1} + \varepsilon l c_n \right) \tag{3}
\]
\[
\text{Area} = \frac{\epsilon l(t_n - t_0)}{n} (c_1 + c_2 + \ldots + c_{n-1} + c_n) = \frac{\epsilon l(\Delta t)}{n} \sum_{i=1}^{n} c_i
\]  

(4)

From a mass balance, the sum of the individual concentrations becomes the total concentration as shown in Equation 5 where \( \Delta t \) is the peak width time. Area can also be related to concentration using a calibration curve for the compound of interest as shown in Equation 6 where \( m \) is the slope of the calibration curve determined from a standard. Equations 5 and 6 are equal to each other as shown in Equation 7.

\[
\text{Area} = \frac{\epsilon l(\Delta t)}{n} c_i
\]  

(5)

\[
\text{Area} = mc_i
\]  

(6)

\[
\frac{\epsilon l(\Delta t)}{n} c_i = mc_i
\]  

(7)

Dividing Equation 7 for a compound for which a calibration curve is available (denoted by subscript 1) by Equation 7 for a compound with no standard available (denoted by subscript 2) is used to estimate the unknown calibration curve according to Equation 8.

\[
\frac{\frac{\epsilon_2 l(\Delta t)}{n} c_2}{\frac{\epsilon_1 l(\Delta t)}{n} c_1} = \frac{m_2 c_2}{m_1 c_1}
\]  

(8)

Assuming the peak width is similar for both compounds, the \( l(dt)/n \) and concentration terms cancel, and Equation 8 is rearranged to solve for the slope for compound 2 as shown in Equation 9. The table of extinction coefficients used with Equation 9 is shown in Table 3. In equations 8 and 9, \( \epsilon_2 \) and \( \epsilon_1 \) are determined from the literature while \( m_1 \) is determined experimentally. Then, \( m_2 \) is determined experimentally from \( \epsilon_2, \epsilon_1, \) and \( m_1 \). The concentration of compound 2 is then calculated by substituting
m_2 into equation 6 and solving for the unknown concentration using the peak area of compound 2.

\[ m_2 = m_1 \left( \frac{\varepsilon_2}{\varepsilon_1} \right) \]  

(9)

**Table 3: Extinction coefficients (expressed as log ε) and corresponding wavelengths for the 6 BPAs obtained from the literature [150].**

<table>
<thead>
<tr>
<th></th>
<th>λ</th>
<th>log ε</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sanguinarine</td>
<td>285</td>
<td>4.53</td>
</tr>
<tr>
<td>Chelirubine</td>
<td>281</td>
<td>4.46</td>
</tr>
<tr>
<td>Chelerythrine</td>
<td>283</td>
<td>4.47</td>
</tr>
<tr>
<td>Dihydrosanguinarine</td>
<td>282</td>
<td>4.56</td>
</tr>
<tr>
<td>Dihydrochelirubine</td>
<td>280</td>
<td>4.52</td>
</tr>
<tr>
<td>Dihydrochelerythrine</td>
<td>282</td>
<td>4.65</td>
</tr>
</tbody>
</table>

### 3.3.4 HPLC Validation

The purpose of this section is to determine the variability due to the HPLC method. Calibration curves were prepared from a single standard solution and injected multiple times on the HPLC.

Stock solutions of sanguinarine and chelerythrine were prepared using the following method. Sanguinarine hydrochloride powder (10 mg, Sigma, #84480) was dissolved in 10 ml of methanol (+ 0.2% HCl to stabilize the sanguinarine). The sanguinarine solution (1 mg/ml) was split into 1 mg aliquots and evaporated using a Savant SpeedVac (Thermoquest; model SC210A; Holbrook, NY). The sanguinarine powder was then stored at -80 °C until future use. Stock solutions for HPLC calibration curve were prepared by diluting the sanguinarine from 1 mg/ml to 0.02 mg/ml solution using 0.2% HCl in HPLC grade methanol. The 0.02 mg/ml solutions are stored at -80°C. Calibration curves were prepared by injecting 10, 20, and 30 μl of the 0.02 mg/ml
sanguinarine solution into the HPLC and plotting of the equivalent sanguinarine concentration versus peak area.

First, the reproducibility between HPLC injections and the stability of the alkaloid standard was tested by injecting 30, 20, and 10 μl of the 0.02 mg/ml sanguinarine solution. The sequence of injection volumes was performed three times so that each volume was analyzed approximately every 3 hours since the run time was 54 minutes per sample. Table 4 shows that the peak area for each injection volume is consistent with less than 0.24% variation; these results also indicate that the alkaloids are stable for the length of time required to generate the calibration curve.

**Table 4: Reproducibility and stability of sanguinarine standard solution**

<table>
<thead>
<tr>
<th>Injection vol. μL</th>
<th>Area Replicate 1</th>
<th>Area Replicate 2</th>
<th>Area Replicate 3</th>
<th>Avg</th>
<th>St Dev</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>2.3E+06</td>
<td>2.3E+06</td>
<td>2.3E+06</td>
<td>2.3E+06</td>
<td>5.4E+03</td>
<td>0.24</td>
</tr>
<tr>
<td>20</td>
<td>1.6E+06</td>
<td>1.6E+06</td>
<td>1.5E+06</td>
<td>1.6E+06</td>
<td>3.7E+03</td>
<td>0.22</td>
</tr>
<tr>
<td>10</td>
<td>7.5E+05</td>
<td>7.5E+05</td>
<td>7.5E+05</td>
<td>7.5E+05</td>
<td>4.6E+02</td>
<td>0.06</td>
</tr>
</tbody>
</table>

Second, three calibration curves were prepared using standards prepared from three different aliquots of lyophilized sanguinarine powder as shown in Table 5. There was approximately 11% variation in the slope between the three standards. Table 5 suggests that the preparing of the standards contributes more to the variability than the HPLC injections.

**Table 5: Variability of sanguinarine calibration curves. Calibration curves were prepared from three different sanguinarine standard solutions.**

<table>
<thead>
<tr>
<th>Preparation date</th>
<th>Slope</th>
<th>Intercept</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vial 1, 12/4/06</td>
<td>8.0E-09</td>
<td>-1.8E-04</td>
<td>0.998</td>
</tr>
<tr>
<td>Vial 2, 12/14/06</td>
<td>6.9E-09</td>
<td>1.3E-04</td>
<td>0.999</td>
</tr>
<tr>
<td>Vial 3, 12/14/06</td>
<td>8.6E-09</td>
<td>2.0E-04</td>
<td>0.999</td>
</tr>
<tr>
<td>Average</td>
<td>7.9E-09</td>
<td>4.7E-05</td>
<td></td>
</tr>
<tr>
<td>Stdev</td>
<td>8.3E-10</td>
<td>2.0E-04</td>
<td></td>
</tr>
<tr>
<td>% Variation</td>
<td>10.62</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.4 Protein Quantification & Validation of Protein Extraction Method

3.4.1 Protein Concentration Assays

Protein concentrations in the extracts were determined quantitatively using the Bradford assay [151] or the bicinchoninic acid (BCA) assay [152]. The Bradford assay was developed by Marion Bradford [151] to quantitate microgram amounts of protein. The Bradford assay utilizes a reaction of the protein with Coomassie Blue reagent which forms a complex which changes the reagent from brown to blue and the absorbance can then be measured at 595 nm. In the Bradford assay, protein quantitation is performed by a chemical reaction with a chemical reagent known to react with arginine, histidine, lysine, and aromatic amino acid side chains (tyrosine, phenylalanine, and tryptophan) [153], causing a color change which can be measured spectrophotometrically. The BCA assay utilizes the reduction of copper from $\text{Cu}^{2+}$ to $\text{Cu}^{1+}$ by protein. The $\text{Cu}^{1+}$ complexes with BCA resulting in a purple color which can be measured spectrophotometrically at 562 nm.

Both assays were performed in a 96-well plate (Corning Inc., Corning NY; #3364) using bovine serum albumin (BSA, Pierce #23209, Rockford, IL) as a standard. BSA (10 μl) of varying concentrations from 125 to 1500 μg/ml were added in quadruplicate to the 96-well plate. Each sample being tested was added in quadruplicate to the 96-well plate.

For the Bradford assay, 200 μl of Coomassie blue reagent (Pierce # 23236, Rockford, IL) is added to each well (containing 10 μl of sample or BSA standard). After 10 minutes, the absorbance at 595 nm is measured and a calibration curve is generated for the BSA standards and used to calculate the concentration of the samples. The Bradford
assay is only linear over a small range of concentrations (from 125 to 1000 μg/ml). The analyte protein concentration must therefore be in the linear range of the BSA calibration curve. Samples with a protein concentration above the upper limit of the assay (1000 μg/ml [151]) are diluted with water to less than 1000 μg/ml.

For the BCA assay, the BCA reagent is prepared by first adding reagent A to reagent B from the BCA Protein Assay Kit (Pierce 23227) in a 50:1 ratio. Standards (125 to 1000 μg/ml of BSA) and samples are added to each well in either 3-4 replicates (10 uL per well). The reagent mixture (200 μl) is then added to each well. The 96-well plate is placed in a plate-reader (Molecular Devices SpectraMax 340 PC) preheated to 37° C and incubated for 30 minutes before the absorbance at 562 nm is measured. A calibration curve is generated in the same manner as the Bradford assay and used to determine protein concentration. The BCA assay was shown to be linear from 100 to 1200 μg/ml [152].

3.4.2 Validation & Comparison Between Bradford & BCA Assay

The purpose of this section is to determine the reproducibility of the Bradford assay versus the BCA assay and to evaluate the protein extraction buffer applied using the BCA assay. The BCA assay has several advantages over the Bradford assay. The BCA assay is reported to be more sensitive than the Bradford assay. Further, extraction buffer components such as sodium dodecyl sulfate (SDS) are incompatible with the Bradford assay.

The reproducibility of the Bradford assay for quantifying BSA standards (Fig. 13) and poppy protein extracts (Table 6) are presented. Standards (125, 250, 500, 750, and 1000 μg/ml of bovine serum albumin) were added in triplicate (10 μl) to a 96-well plate.
A calibration curve was prepared using the 5 concentrations of BSA. The CV of the 125 μg/ml solution was approximately 10% compared to less than 5% for the other standards. Since the analysis was done in triplicate and there were still 4 BSA concentrations for the calibration curve, the 125 μg/ml data point was discarded. The calibration curve is shown in Figure 13.

![Calibration Curve](image)

**Figure 13:** Bradford assay calibration curve. The standard concentration (μg/ml) of the BSA standard is plotted versus the raw optical density (OD) data using a linear fit. Concentrations are averages of triplicate measurements and error bars represent standard deviations.

Next, the reproducibility of the Bradford assay for quantifying poppy protein extracts is presented. Protein extracts from freeze-dried poppy cells were prepared as described in Section 3.5.1 using three aliquots of cells from the same culture flask. The cells were extracted using the urea extraction buffer consisting of 7 M urea (Fisher, #U15-3), 2 M thiourea (Fisher, #AC42454-2500), 0.1 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Fisher #BP299-1), ROCHE protease inhibitor
cocktail (Roche Diagnostics, Mannheim, Germany, #04 693 159 001), and benzonuclease (Novagen, Madison, WI, 70664-3; dithiothreitol, DTT, was omitted in this case). The protein concentrations from 3 cell aliquots were compared to evaluate the extraction reproducibility while 3 replicates of each protein extract were compared to evaluate the protein assay reproducibility. Protein extracts were diluted 1:5 with 0.05 M HEPES (pH 8.0) to lower the urea concentration to 1.4 μM (less than 3.0 μM is required for the Bradford assay); based on previous experience, at least a 1:5 dilution was necessary to lower the protein concentration to within the linear range of the assay.

The concentrations of each sample based on the BSA calibration curve are shown below in Table 6. The combined values reflect the average, standard deviation, and CV for all 9 samples (3 replicates for each of the 3 samples).

<table>
<thead>
<tr>
<th>Aliquot</th>
<th>Replicate Measurements Conc. in μg/ml</th>
<th>Average and Variation of Triplicate Measurements from Given Aliquot</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>1</td>
<td>1953</td>
<td>1660</td>
</tr>
<tr>
<td>2</td>
<td>2365</td>
<td>2010</td>
</tr>
<tr>
<td>3</td>
<td>2178</td>
<td>2473</td>
</tr>
<tr>
<td>Combined</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The reproducibility of the protein quantitation between triplicate measurements of each aliquot using the Bradford Assay was less than 18%. BSA is only a model protein while a complex sample such as a cell lysate has a wide variety of proteins which interact with the Coomassie dye differently. The manufacturer’s manual provides data for the ratio of the absorbance of a given protein to the absorbance of a BSA solution of the
same concentration showing a CV of 28.8%. For comparison in the manufacturer’s manual, the same experiment was performed with the BCA assay with a CV of 14.7% indicating the Bradford assay is less precise than the BCA assay.

In proteomics experiments it is critical to start with the same amount of protein for each sample. The CV between triplicate extractions was 12.7% and was shown to be within the inherent variability of the Bradford assay; this means that unless there is a significant difference in concentration between two samples (e.g. control vs. elicited), it is safer to assume both samples have the same concentration and start with the same volume of each sample.

Table 7 shows the results for the reproducibility of the BCA assay for quantifying proteins extracted from freeze-dried poppy cells using two different extraction buffers. The SDS buffer consisted of 0.1% SDS (Fisher #02674-25) in 100 mM HEPES pH 8.0 (DTT was omitted in this buffer). The urea buffer consisted of 7 M urea (the 2 M thiourea commonly used in urea extraction buffers was omitted as it interferes strongly with the BCA assay) in 100 mM HEPES pH 8.0. Both buffers were supplemented with ROCHE protease inhibitor cocktail and benzonase nuclease. Less than 4.3% standard deviation in protein concentration was observed in the protein extracts of three cell aliquots from the same flask using the BCA assay. Thus the BCA assay provides a more reproducible measurement of protein concentration than the Bradford assay.
Table 7: Comparison of protein extraction buffers. Protein was extracted using SDS and urea buffers and the concentration (mg/ml) was measured from 3 aliquots from the same flask. The protein content was evaluated using the BCA assay. Each aliquot represents extraction of cells from the same flask. The average for each vial is from 3 replicates of a single extract.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Aliquot 1</th>
<th>Aliquot 2</th>
<th>Aliquot 3</th>
<th>Avg</th>
<th>Stdev</th>
<th>CV(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDS</td>
<td>3.6 ± 0.04</td>
<td>3.8 ± 0.03</td>
<td>3.7 ± 0.05</td>
<td>3.70</td>
<td>0.1</td>
<td>2.7</td>
</tr>
<tr>
<td>Urea</td>
<td>3.5 ± 0.03</td>
<td>3.7 ± 0.04</td>
<td>3.4 ± 0.03</td>
<td>3.53</td>
<td>0.2</td>
<td>4.3</td>
</tr>
</tbody>
</table>

3.5 Protein and Peptide Separation

Protein and peptide separation methods were used to simplify complex samples prior to mass spectrometric analysis. Ultracentrifugation (Section 3.5.1) and gel electrophoresis (Section 3.5.2) was used as a crude protein separation technique. Proteins were then digested (Section 3.5.3) and peptides were separated by strong cation exchange (SCX, Section 3.5.4) or reversed phase liquid chromatography (Section 3.5.5 and 3.5.6).

3.5.1 Ultracentrifugation for Fractionating Proteins Based on Subcellular Localization

The purpose of ultracentrifugation in protein separation is to prepare less complex fractions based on subcellular location (e.g. cytosol, membrane). An overview of the ultracentrifugation method consists of the following steps: 1) destruct cells in the bead beater in the presence of a urea extraction buffer, 2) centrifuge the supernatant at 5,000 g to remove cellular debris, 3) centrifuge at 16,000 g to sediment mitochondrial proteins, and then 4) centrifuge at 100,000 g to sediment microsomal proteins. Differential centrifugation was used to generate subcellular protein fractions from liver extracts [121]. Each of these steps will be described in greater detail below.

*E. californica* cells were harvested by suction-filtration and frozen in liquid nitrogen. Proteins were extracted using the following extraction buffer: 7 M urea.
(Fisher, #U15-3), 2 M thiourea (Fisher, #AC42454-2500), 5 mM dithiothreitol (DTT, Sigma, #150460), 50 mM Tris-HCl pH 8.0 (Fisher, #BP1758), ROCHE complete protease inhibitor cocktail (Roche diagnostics, Mannheim, Germany, #04 693 159 001), and benzonase nuclease (Novagen, Madison, WI, 70664-3). The urea, thiourea and Tris are prepared separately and stored at 4 °C until use. The DTT and ROCHE protease inhibitor cocktail are prepared as concentrated stock solutions, stored at -20 °C, and added right before extraction along with the nuclease.

Extraction was performed using a bead beater (BioSpec Products #3110BX) using the following steps: 1) 2.0 ml vials (VWR Scientific, #20170-217) were filled approximately one-fourth full with glass beads (BioSpec Products, #11079105), 2) 500 mg fresh weight of frozen cells was added to the top of the vials and followed by 0.5 ml of protein extraction buffer, and 3) the cells were disrupted in the bead beater in which each cycle consists of 30 seconds of agitation at 5000 rpm followed by cooling on ice and repeated two more times. After 3 beater cycles, the vials were centrifuged for 15 minutes at 5,000 g and 4°C (Eppendorf, Westbury, NY, #5415) and the resulting supernatant was saved and replaced with an equal volume of fresh protein extraction buffer. The process was repeated two additional times (a total of 3 aliquots of protein extraction buffer was added per 500 mg of frozen cells) and the resulting supernatants were combined. The combined supernatants were centrifuged for 30 minutes at 16,000 g and 4°C (Eppendorf, Westbury, NY, #5415).

The supernatant resulting from 16,000 g centrifugation was transferred to 3.5 ml thick-walled polycarbonate ultracentrifuge tubes (Beckman Coulter #349622) and loaded in a Beckman Coulter Ultracentrifuge (TI100.3 rotor). The centrifuge was operated at
100,000 g (55,000 rpm) for 40 minutes. The supernatant was transferred to an Eppendorf microcentrifuge tube using a plastic transfer pipette; care was taken to avoid disturbing the pellet. The supernatant was analyzed according to the methods describe in Sections 3.5.3 and 3.5.5. The pellet was then washed 3 times in 3 ml of phosphate buffered saline (PBS, ATCC, Manassas, VA, #SCRR-2201). The wash buffer was discarded and the pellet was resuspended in 7 M urea, 2 M thiourea in 50 mM Tris pH 8.0.

3.5.2 Gel Electrophoresis for Separating Proteins Based on Molecular Weight

Gel electrophoresis was performed using Invitrogen NuPAGE 4 – 12% Bis-Tris gels (Invitrogen, NP0321BOX). The gels were placed inside an XCell Sure Lock Novex-Mini-Cell; the Mini-Cell was filled with 2-(N-morpholino)ethanesulfonic acid loading buffer (MES, Invitrogen #NP0002).

Samples were prepared by adding 30 μg of total protein (10 – 15 μl, adjusted to 15 μl with 50 mM Tris) to a 1.5 ml Eppendorf microcentrifuge tube. To each tube, 3 volumes of sample (15 μl) was mixed with 1 volume of 4X SDS loading buffer (5 μl, Invitrogen #NP0007); the samples were reduced with 1 μl of 1M DTT and heated for 10 min at 70°C in a heating block (Fisher Isotemp 125D).

Individual samples are loaded into separate wells on the same gel using gel loading tips (Fisher #02-707-138). To estimate the range of molecular weights in the sample, a mixture of molecular weight protein markers (Invitrogen SeeBlue Prestained Standard #LC5925) was also added to separate wells on the gel. Once the samples were loaded in the wells, separation was started by application of an electric field with 200 V, 400 mA, and 100 W as the starting conditions. By default, the electrophoresis apparatus sets the voltage to 150 V with the current and power varied accordingly. After the
electric field was applied for 1 hour (or until the prestained standards have been clearly 
resolved on the gel), the gel was removed from the gel housing and washed 3 times for 5 
minutes each with Milli-Q water to remove residual MES running buffer. The gel is then 
stained with Simply Blue Safe Stain (Invitrogen, 46-504) for 1 hour with gentle shaking 
then destained with water for at least one hour. Longer destaining times can reduce the 
background resulting in clearer images.

3.5.3 Protein Digestion

Protein digestion is more effective on unfolded proteins as the protease has easier 
access to the cleavage sites. Proteins are maintained in an unfolded state using a 
denaturant such as urea. Reduction of disulfide bonds further unfolds the protein. 
Alkylation of cysteine sulfhydryl groups protects the protein against refolding due to 
reforming of the disulfide linkage.

Protein extracts (100 ug) were reduced in by adding 0.25 M tris (2-carboxyethyl) 
phosphine hydrochloride (TCEP, Pierce, #77720) to a final concentration of 5 mM and 
incubating for 20 minutes at room temperature. Proteins were alkylated by adding 0.5 M 
ioodoacetamide (IAA, Sigma, #A3221) to a final concentration of 15 mM and incubating 
in the dark for 30 minutes at room temperature. The alkylation reaction was quenched by 
adding 0.25 M dithiothreitol (DTT, Sigma, #150460) to a final concentration of 5 mM 
and incubating for 5 minutes at room temperature. Digestion was performed by adding 
trypsin (Promega, Madison, WI, #V511A, 1:40 w/w ratio) and incubating overnight at 37 
C. The digestion reaction was stopped by adjusting the pH to less than 4 using 3% 
formic acid. The peptide digest was purified for mass spectrometry as described in 
Section 3.5.7.
3.5.4 Strong Cation Exchange: Fractionation of Peptide Digests Based on Charge

Complex digests from poppy protein extracts were fractionated by strong cation exchange chromatography (SCX). The entire peptide digest (<100 μl) was diluted 1:10 with buffer A (see below for buffer composition) and adjusted to pH 3.0 using 1 M phosphoric acid. The entire sample (100 μl) was then loaded on a polysulfoethyl A column (Cat #054SE0303, 50 x 4.6 mm, 3 μm particle size, 300 A, PolyLC, Columbia, MD) and separated using a Shimadzu HPLC (Shimadzu Scientific Instruments).

The mobile phases used were A) 10 mM KH₂PO₄ and 10% acetonitrile, adjusted to pH 3.0 with phosphoric acid and B) 1 M KCl, 10 mM KH₂PO₄, and 10% acetonitrile, adjusted to pH 3.0 with phosphoric acid; the solvent flow rate was 1.0 ml/min. The gradient consisted of several isocratic steps: 0% B for 15 minutes, 5% B for 8 minutes, 10% B for 8 minutes, 15% B for 8 minutes, 30% B for 8 minutes, 100% B for 15 minutes, and 0% B for 15 minutes. The eluate from the column at 5, 10, 15, and 30% B was collected as 4 separate fractions which were then concentrated to less than 1 ml in a Speed Vac (Labconco, Kansas City, MO). An example chromatogram is shown in Figure 14. The peptide fractions were then cleaned up by RPLC as described in Section 3.5.5.
3.5.5 Cleanup, Isolation, and Concentration of Peptides by Reversed Phase HPLC

Peptide fractions from SCX (Section 3.5.4) or in solution digestion (Section 3.5.3) were loaded on a reversed phase column packed with POROS R1 resin (4.6 mm x 50 mm, 20 μm particles, 4000 A pore size, Applied Biosystems, Framingham, MA, #1-1029-26). The mobile phases used were A) 0.1% v/v trifluoroacetic acid (TFA, Pierce, Part #28904) in water and B) 0.085% v/v TFA in acetonitrile; the solvent flow rate was 3.0 ml/min. The HPLC method consisted of several isocratic steps: 2% B for 4 minutes, 30% B for 4 minutes, 90% B for 4 minutes, and 2% B for 4 minutes. Peptides were eluted in 30% B and monitored at 214 nm, concentrated in a SpeedVac (Labconco, Kansas City, MO), and then adjusted to 20 μl with 0.1% TFA in water. An example chromatogram is shown in Figure 15.
Figure 15: Peptide purification by HPLC. Digested samples were loaded on a reversed-phase column. The absorbances at 214 and 280 nm are shown in pink and blue, respectively. Peptides elute in 30% acetonitrile (4 – 5 min) while undigested proteins elute in 98% acetonitrile (7-7.5 min).

3.5.6 Fractionation of Peptides by Nano-HPLC

Peptides were separated by HPLC using a LC-Packings UltiMate system (Dionex, Sunnyvale, CA, UltiMate #160534) and a 100 μm I.D. x 150 mm C_{18} column packed using C_{18} resin in empty 300 μm I.D. fused silica capillaries (Upchurch Scientific, #FS-110). The mobile phases used were A) 0.1% trifluoroacetic acid (TFA, Pierce) in water and B) 0.1% TFA in acetonitrile. An injection volume of 2 μl and a solvent flow rate of 0.5 μl/min was used. Absorbance was monitored at 214 and 280 nm. Peptides eluted in 15 – 50% solvent B.

3.5.7 MS Analysis of Peptides

Using an Eksigent HPLC system (Eksigent Technologies, Dublin, CA), 2.5 μl of each peptide sample was injected onto a Magic C_{18} reversed phase column (75 μm i.d. X
175 mm, 5 μm, Michrom Biosciences, Auburn, CA). The mobile phases used for peptide separation were A) 0.1% formic acid in water and B) 0.1% formic acid in acetonitrile. The method consisted of an isocratic segment at 5% B for 5 minutes, gradient to 35% B in 115 minutes, gradient to 80% B in 20 minutes, isocratic at 80% B for 10 minutes, gradient to 5% B in 1 minute, and then isocratic at 5% B for 4 minutes; the flow rate was set to 0.25 μl/min. The LTQ mass spectrometer (Thermo Fisher, San Jose, CA) was operated with the ion transfer tube at 245 °C, a spray voltage of 2.0 kV, and normalized collision energy of 35%. The mass spectrometer was operated in data-dependent mode with 1 MS scan from m/z 400 to 1600 followed by MS/MS scans of up to the ten most abundant peaks. Precursor ions were excluded for 1 min.

Proteins were identified by processing the MS/MS data using the computational proteomics analysis system (CPAS) [154] using the Sequest search algorithm and a previously published *E. californica* EST database [155] translated into protein sequences (4700 protein sequences) combined with 15 protein sequences from 10 BPA pathway enzymes (http://www.expasy.org). The database also include a random protein database created by reversing the protein sequences in order to facilitate estimation of the false positive rate [156]. The false discovery rate represents the probability that the MS/MS spectrum is matched to an incorrect peptide sequence. A list of identified proteins was generated by filtering the data using PeptideProphet probability >0.9 and Sequest Xcorr values greater than 1.9 for 1+, 2.2 for 2+, and 3.8 for 3+ ions.
4.0 ENHANCEMENT OF ALKALOID PRODUCTION

This chapter describes the development of culture conditions for enhancing the production of benzophenanthridine alkaloids (BPAs) from California poppy cultures. The optimum culture conditions for production determined in this chapter will be selected for proteomic analysis in Chapter 5. Factors including elicitation, *in situ* extraction, alginate immobilization, hormone treatment, and medium optimization were studied. Elicitation and *in situ* extraction were found to have the greatest effect on alkaloid production.

4.1 Optimization of BPA Production with Purified Yeast Extract Elicitation

Elicitors are compounds which activate signal transduction pathways in plants, leading to increased gene expression and secondary metabolite production. Elicitors include biotic (*e.g.* fungal or yeast extracts) and abiotic elicitors (methyl jasmonate, salicylic acid, and ethylene). The usage of elicitation for increasing secondary metabolite production is presented in Chapter 2, Section 2.4.1.

In this thesis, a purified yeast extract (PYE) was used to enhance BPA production from *E. californica* suspension cultures. In the literature, PYE increased sanguinarine production up to 400% (146 mg/L) in *E. californica* cultures [16]. Yeast elicitors have been shown to activate a signal transduction pathway leading to the expression of genes involved in BPA biosynthesis [20-22, 51] and the increase of BPA production levels [16, 23, 67].

In this thesis, purified yeast elicitor dosage, addition timing, and harvesting timing were optimized. The effect of dosage and timing of elicitor addition were investigated by
our research group previously for *Catharanthus roseus* [157] and Cho et al. for *E. californica* [67].

### 4.1.1 Effect of Purified Yeast Elicitor Dosage and Addition Timing on BPA Production

To investigate optimum elicitor dosage and timing, cultures were elicited with PYE dosages of 0, 10, 20, and 40 mg/g FW, added on either day 4 or 7, and harvested after 48 hours.

![Figure 16: BPA production is affected by timing and dosage of PYE. Cultures were grown with 30 g/L sucrose, Linsmaier Skoog salts, 0.37 mg/L 2,4-D, and 0.11 g/L NAA; the cultures were elicited on day 4 or 7 with 10, 20, or 40 mg of PYE/g fresh wt of tissue and harvested after 48 hours. Error bars represent standard deviation from triplicate measurements. Abbreviations: DHCHY: Dihydrochelerythrine; CHY: Chelerythrine; DHSA: Dihydrosanguinarine; SA: Sanguinarine; DHCHR: Dihydrochelirubine; CHR: Chelirubine. Total alkaloid production increased dramatically after elicitation on either day 4 or 7 with all PYE dosages as shown in Figure 16. Elicitation on day 4 (early exponential growth) was more effective than day 7.
phase) produced 7 – 10 mg of BPAs /g FW and was not significantly affected by elicitor concentration (Figure 16). Elicitation on day 7 (mid-exponential phase) increased total alkaloid content to 16 – 18 mg of BPAs / g FW (Figure 16); for example, elicitation with 40 mg of PYE /g FW increased total alkaloid production by 67% compared to the same PYE dosage on day 4.

The ratio of BPAs to dihydro-BPAs increased when PYE was added on day 7 versus day 4. In addition, when cultures are elicited on day 7, increasing elicitor concentration from 10 to 40 mg/g FW increased the ratio of BPAs to dihydro-BPAs. This suggests that the enzyme dihydrobenzophenanthridine oxidase (DHBO), which converts dihydro-BPAs to their oxidized forms, is activated by timing of elicitation and higher elicitor dosages.

In summary, BPA production was dramatically and similarly enhanced by PYE dosages of 10 – 40 mg/g FW and optimized with PYE addition on day 7 compared to day 4.

### 4.1.2 Effect of Harvesting Time on BPA Production

To investigate the effect of harvesting time on BPA production, cells were elicited on day 7 with 40 mg/g FW PYE (based on optimal results from Section 4.1.1) and harvested after 24, 48, 72, and 96 hours.
Alkaloids accumulated approximately linearly from 7 mg/g DW at 24 hours after elicitation to 20 mg/g DW at 72 hours after elicitation (Figure 17). Dihydrosanguinarine and dihydrochelerythrine remained relatively constant after elicitation while sanguinarine, chelerythrine, and chelirubine increased 11, 27 and 27-fold, respectively between 24 and 96 hours after elicitation. The increase in the ratio of BPAs to dihydro-BPAs suggests that a higher percentage of the flux is being directed to BPAs by the enzyme dihydrobenzophenanthridine oxidase (DHBO) with harvest time and therefore longer harvest times are preferred for accumulation of the desired products chelerythrine, sanguinarine, and chelirubine. Chelirubine production is still increasing between 72 and
96 hours after elicitation. In summary, alkaloid production increases linearly between 24 and 72 hours after elicitation and dihydroBPAs are converted to BPAs between 48 and 96 hours after elicitation. After 96 hours, production levels off.

4.2 Optimization of BPA Production with in situ Extraction and PYE Elicitation

In situ extraction utilizes either immiscible solvents or polymeric resins to absorb products secreted by the cell. In situ extraction has been shown to enhance production in C. roseus [71-73] and E. californica [24]. In this thesis, the combined effect of in situ extraction using Amberlite XAD-7 and PYE elicitation on BPA production was studied in E. californica suspension cultures.

4.2.1 Effect of XAD on BPA Production

To investigate the effect of Amberlite XAD-7 on BPA production, PYE was added to E. californica cultures at 10 and 40 mg/g FW with and without addition of Amberlite XAD resin (0.24 mg/5 ml culture). Cultures were harvested 48 and 96 hours after elicitation and BPAs were recovered from the XAD-7 resin by extraction in methanol.
Figure 18: Effect of XAD-7 on BPA production. Addition of XAD-7 enhances BPA production in elicited and nonelicited E. californica suspension cultures grown with 30 g/L sucrose, Linsmaier Skoog salts and 0.11 g/L NAA. Cultures were transferred to 6-well plates on day 7 and elicited with PYE (10 or 40 mg/g FW), supplemented with XAD-7 resin (0.24 g/5 ml culture) and harvested after 48 (top) or 96 (bottom) hours. Error bars represent standard deviation from triplicate measurements. Abbreviations: DHCHY: Dihydrochelerythrine; CHY: Chelerythrine; DHSA: Dihydrosanguinarine; SA: Sanguinarine; DHCHR: Dihydrochelirubine; CHR: Chelirubine.
The effect of XAD-7 resin addition on BPA production was investigated in the presence of PYE elicitation. BPA production was similarly enhanced in cells treated with 10 or 40 mg/g FW PYE at both 48 (approximately 20 mg/g DW total BPAs, Figure 18) and 96 hours (approximately 25 mg/g DW) as previously observed in Section 4.1. In the presence of XAD, elicited cultures produced approximately 20 - 30 mg/g DW after 48 hours and 86 mg/g DW after 96 hours; a greater than 3-fold increase in BPA production was observed between elicited cultures with and without resin after 96 hours. Even unelicited cultures produced 17 mg/g DW BPAs in the presence of resin after 48 hrs and 28 mg/g DW after 96 hours. While total BPA production leveled off between 48 and 96 hours in the absence of XAD, total BPA production continued to increase in elicited cultures with XAD between 48 and 96 hours, suggesting that BPA production may be limited by feedback inhibition by BPA intermediates.

XAD is believed to enhance production by reducing feedback inhibition caused by high product accumulation [68-69]. Also, some secondary metabolites, particularly BPAs, may be cytotoxic and thus their removal is beneficial for cell survival and metabolite production [70]. Our results suggest that BPA production may be limited by feedback inhibition of secondary metabolic enzymes in elicited cultures or cytotoxicity of BPAs at production levels greater than 20 mg/g DW. The feedback inhibition or cytotoxicity of the BPAs appears to be alleviated by the addition of XAD resin.

At 96 hours after elicitation, production with elicitation and XAD addition levels off with PYE dosage, suggesting another factor is limiting once higher production is achieved. One explanation is that higher elicitor dosages increase production but the
resin becomes saturated leading to higher medium and intracellular concentrations and potential feedback inhibition

4.2.2 Effect of XAD Dosage and Timing on BPA Production

To test if the XAD had become saturated in elicited cultures at 96 hours, the amount of XAD added to each well was increased and the harvest time was increased.

![Figure 19: Effect of XAD-7 dosage and harvest time on BPA production.](image)

Addition of XAD-7 enhances BPA production in elicited and nonelicited *E. californica* suspension cultures grown with 30 g/L sucrose, Linsmaier Skoog salts and 0.11 g/L NAA. Cultures were transferred to 6-well plates on day 7 and elicited with PYE (10 or 40 mg/g FW), supplemented with XAD-7 resin (0.24 or 0.36 g/ 5 ml culture) and harvested after 96 or 144 hours. Error bars represent standard deviation from triplicate measurements. Abbreviations: DHCHY: Dihydrochelerythrine; CHY: Chelerythrine; DHSA: Dihydrosanguinarine; SA: Sanguinarine; DHCHR: Dihydrochelirubine; CHR: Chelirubine.

After 96 hrs, increasing the amount of resin added from 0.24 to 0.36 g/well increased production from 90 to 108 mg/g DW in elicited cultures but the increase was not statistically significant due to the large error bars (Figure 19). After 144 hours,
production in elicited cultures with 0.24 g XAD/well increased to 108 mg/g DW but decreased in elicited cultures with 0.36 g XAD/well.

4.3 Effect of Alginate Immobilization on BPA Production

Alginate immobilization consists of entrapping cells in polymeric beads. The mechanism leading to increased production after immobilization is currently unknown. The usage of immobilization for increasing production in plant cell cultures is described in Section 2.4.2.

In the literature, BPA production was enhanced 800-fold (up to 8 mg/g DW) by immobilizing unelicited *E. californica* suspensions in alginate beads. In this thesis, *E. californica* suspension cultures were immobilized in alginate beads, elicited with 40 mg/g FW PYE on day 7, and then harvested after 48 and 96 hours.

**Figure 20:** Elicitation of BPA production in elicited *E. californica* suspensions and cells immobilized in alginate beads. Cells were cultured in 30 g/L sucrose, LS media and 0.11 mg/L NAA for 7 days then elicited with PYE (40 mg/g FW) and harvested after 48 or 96 hours. Cells were immobilized then elicited (Immobilize 1st) or elicited then immobilized after 30 minutes (Immobilize 2nd). Error bars represent standard deviation from triplicate measurements. Abbreviations: DHCHY: Dihydrochelerythrine; CHY: Chelerythrine; DHSA: Dihydrosanguinarine; SA: Sanguinarine; DHCHR: Dihydrochelirubine; CHR: Chelirubine; Susp.: Suspensions.
Total alkaloid production in suspensions approximately doubled from 13 mg/g DW to 26 mg/g DW from 48 to 96 hours (Figure 20). Consistent with previous results, the ratio of sanguinarine to dihydrosanguinarine production increased significantly (from 0.2 after 48 hours to 1.2 after 96 hours). However, the total alkaloid production in elicited and immobilized cells was 40% of the production in suspensions after 48 hours and 31% after 96 hours. The lower production in immobilized cells may have resulted from the reduced diffusion of elicitors to immobilized cells. Hence, cells were also elicited for 30 minutes first prior to immobilization. However, the order of elicitation of immobilized cultures did not improve alkaloid production.

Farber et al. showed that transferring elicited cells into fresh media 30 minutes after elicitation did not affect production compared to continuous presence of the elicitor [17]. It was expected that waiting 30 minutes after elicitation prior to immobilization would enhance production because the signal transduction pathway would be fully activated 30 minutes after elicitation. As shown in Figure 20, there was no significant difference with the order of elicitation and immobilization. While 30 minutes may be sufficient time for the elicitor to trigger the signal transduction pathway, disturbing the cells by immobilization may have another effect on the cell that inhibits production.

4.4 Effect of Plant Growth Hormones (Auxins) on BPA Production

Plant cell culture usually contains small molecule hormones such as auxins and cytokinins. In the whole plant, auxins and cytokinins regulate physiological processes including root development, cell growth and differentiation, and organ development. In cell cultures, these hormones promote cell growth and division. Commonly used
hormones include the synthetic auxins 2,4-dichlorophenoxyacetic acid (2,4-D) and α-naphthalene acetic acid (NAA).

In this thesis, the effect of 2,4-D and NAA on BPA production was studied. Cells were cultured in the absence of 2,4-D and various NAA concentrations. In the literature, these hormones have been shown to promote growth but reduce productivity [78].

![Figure 21: Effect of 2,4-D on BPA production. BPA production in E. californica suspension cultures grown with and without 2,4-D and transferred to 6-well plates and elicited on day 7 with PYE (40 mg/g FW 40) and harvested after 48 hours and 96 hours. Error bars represent standard deviation from triplicate measurements. Abbreviations: DHCHY: Dihydrochelerythrine; CHY: Chelerythrine; DHSA: Dihydrosanguinarine; SA: Sanguinarine; DHCHR: Dihydrochelirubine; CHR: Chelirubine.](image)

BPA production in elicited cultures (40 mg/g FW PYE) with and without 2,4-D was approximately 8 mg/g DW after 48 hours. After 96 hours, BPA production in elicited cultures was 50% higher in cells cultured without 2,4-D (15 mg/g DW) than with 2,4-D (Figure 21). However, this difference may not be statistically significant due to the
nearly overlapping error bars. Higher production in 2,4-D free media was observed with a previous study [78]. Also, at 96 hours, the ratio of sanguinarine to dihydrosanguinarine production increased from 0.3 to 2.1 when the cells are cultured without 2,4-D. The mechanism leading to increased production in cells cultured without 2,4-D is unknown.

Cells were grown in 3 different NAA concentrations and elicited with PYE (Figure 22). Total alkaloid production (22 – 24 mg/g DW) was not significantly different between the 3 NAA concentrations. Similarly, after 96 hours the production was not different at 0.1 and 0.6 μM NAA but the production was approximately 26% higher at 2.0 μM NAA (p = 0.04 using the Student’s t-test in Excel); the ratio of BPAs to dihydro-BPAs were different between the different NAA concentrations. NAA may not have as much effect compared to 2,4-D because the NAA concentration was lower in the maintenance culture. Since the NAA concentration was lower it may have had less of an effect on cell growth and alkaloid production in maintenance cultures.
4.5 Effect of Methyl Jasmonate Compared to PYE on BPA Production

Methyl jasmonate (MeJa) is a plant signal transduction hormone. Methyl jasmonate is produced through the octadecanoic acid pathway. In *C. roseus* MeJa activates the octadecanoid-responsive *Catharanthus* AP-2 domain protein (ORCA)
transcription factor leading to increased expression of genes in the terpenoid indole alkaloid biosynthetic pathway [158].

In this thesis, methyl jasmonate was applied to enhance BPA production from *E. californica* cell cultures. In the literature, methyl jasmonate was applied to *E. californica* suspension cultures to increase BPA production. Addition of 100 μM methyl jasmonate to *E. californica* suspension cultures enhanced dihydrosanguinarine production 150% (509 mg/L) [16]. Methyl jasmonate also increased expression of several enzymes in the BPA biosynthetic pathway (BBE, CFS, SPS, MSH and P6H) [19].

![Figure 23](image-url)

**Figure 23:** Effect of methyl jasmonate on BPA production. BPA production in *E. californica* suspension cultures grown without 2,4-D and transferred to 6-well plates and elicited on day 7 with methyl jasmonate (100 μM) or PYE (10 mg/g FW) and harvested after 24, 48, and 96 hours. Error bars represent standard deviation from triplicate measurements.

BPA production was not increased by MeJa addition while elicitation with PYE increased production up to 28 mg/g FW (Figure 23) after 96 hours consistent with
Section 4.1. The results suggest the genes required for increasing the BPAs monitored in our study are not activated by MeJa in the *E. californica* cell line studied in this thesis.

4.6 Effect of Media Components on BPA Production

Plant cell culture contains sugar (*e.g.* sucrose or glucose), nitrogen (*e.g.* ammonium and nitrate salts), phosphate, potassium and other essential nutrients. Media concentrations are often optimized to maximize secondary metabolite production. The effect of sucrose, nitrate, and phosphate on elicited *E. californica* cultures is presented in Section 2.4.4.

4.6.1. Effect of Media Sucrose Concentration on BPA Production

Cells were cultured in maintenance media containing 30 g/L or 60 g/L sucrose. In the literature, increased sucrose concentrations have increased secondary metabolite production in *E. californica* suspension cultures [77].

![Figure 24: Effect of sucrose on BPA production. BPA production in *E. californica* suspension cultures grown in either 30 g/L or 60 g/L sucrose and elicited on day 7 with PYE (40 mg/g FW) and harvested after 96 hours. Error bars represent the standard deviation from triplicate measurements.](image-url)
Elitation with PYE (40 mg/g FW) in cultures grown in 60 g/L sucrose produced approximately 50% alkaloid content (13 mg/g DW) compared to elicited cultures grown in 30 g/L sucrose (Figure 24). No increase in production was observed for nonelicited cells (Figure 24) suggesting that elevated sucrose alone does not enhance production. However, Berlin et al. [77] observed a 7-fold increase (up to 15 mg/g dry weight) in alkaloid production from *E. californica* cultures in 80 g/L sucrose compared to 20 g/L sucrose. In our study, the dry weight concentration was lower in 60 g/L sucrose than in 30 g/L sucrose which indicates the volumetric productivity is also lower in the reduced sucrose media.

4.6.2. Effect of Media Ammonium and Nitrate Concentrations on BPA Production

In the literature, ammonium and nitrate have been manipulated to increase production from plant cell cultures. Lamboursain and Jolicoeur observed a 39-fold increase in alkaloid production from *E. californica* suspensions when the nitrate concentration was reduced from 25 to 12.5 mM at a constant ammonium concentration [26]. In this thesis, *E. californica* suspension cultures were grown in media containing low, medium, and high nitrate and ammonium concentrations.
Figure 25: Elicitation of BPA production in E. californica suspensions with varying ammonium and nitrate concentrations. Cells were cultured in LS media with varying levels of ammonium and nitrate, 30 g/L sucrose, and 0.11 mg/L NAA for 7 days, then elicited with PYE (40 mg/g FW) and harvested 96 hours after elicitation. Error bars represent standard deviation from triplicate measurements. Abbreviations: DHCHY: Dihydrochelerythrine; CHY: Chelerythrine; DHSA: Dihydrosanguinarine; SA: Sanguinarine; DHCHR: Dihydrochelirubine; CHR: Chelirubine.

Cells were cultured in media containing combinations of ammonium and nitrate at low, medium (i.e. maintenance media), and high concentrations. The maximum total BPA content was observed with low and medium nitrate concentrations. Increasing the nitrate concentration from 39.4 mM to 56.4 mM decreased production by at least 50% at each of the ammonium concentrations tested (Figure 25). This experiment was repeated six times (see Appendix A) and no trends were consistently observed in repeated experiments. In the literature, artemisinin production decreased with increasing nitrate concentration [159].
Figure 26: Elicitation of BPA production in E. californica suspensions with varying ammonium and nitrate concentrations. Cells were cultured in LS media with varying levels of ammonium (10.3 and 20.6 mM) and nitrate (18.8 and 39.4 mM), 30 g/L sucrose, and 0.11 mg/L NAA for 7 days, then elicited with PYE (40 mg/g FW) and harvested 96 hours after elicitation. Error bars represent standard deviation from triplicate measurements. Abbreviations: DHCHY: Dihydrochelerythrine; CHY: Chelerythrine; DHSA: Dihydrosanguinarine; SA: Sanguinarine; DHCHR: Dihydrochelirubine; CHR: Chelirubine.

Thus, the three highest conditions from the previous experiment were tested along with the normal media grown using the regular media supply instead of the ammonium nitrate-free media adjusted to the same composition. Without XAD, low NH₄/low NO₃ and normal media concentration produced the highest total BPA levels (Figure 26). The normal media concentration produced different results depending on whether the media was prepared from NH₄NO₃-free media and adjusted to the usual composition or prepared using the standard media. Using the ammonium nitrate-free media may affect production because myo-inositol and thymine were added separately to nitrate media.
compared to the media used for maintenance cultures which is supplied with myo-inositol and thymine.

4.7. Conclusions

The highest BPA production was achieved using a combination of elicitation with purified yeast extract and in situ extraction using Amberlite XAD-7 resin. The optimum conditions for elicitation were elicitation on day 7 with 40 mg/g FW PYE and harvested after 96 hours. The optimum conditions for in situ product adsorption were 0.24 g of XAD-7 added to elicited cultures on day 7 and harvested after 96 hours. A proteomic comparison of unelicited, elicited (40 mg/g FW PYE), and elicited cells with XAD-7 added is presented in Chapter 5. A qRT-PCR analysis of cells grown at two PYE dosages (10 and 40 mg/g FW) with and without XAD-7 will be performed.
5.0 SHOTGUN PROTEOMIC ANALYSIS OF YEAST-ELICITED CALIFORNIA POPPY (ESCHSCHOLZIA CALIFORNICA) SUSPENSION CULTURES PRODUCING ENHANCED LEVELS OF BENZOPHENANTHRIDINE ALKALOIDS

The research described in this chapter was done in collaboration with Dr. Marina Hincapie (expertise in protein separation and mass spectrometry) and Dr. Tomas Rejtar (expertise in protein identification and bioinformatics) of the Barnett Institute (Northeastern University), and Dr. John Carlson and Dr. P. Kerr Wall (originator of the EST database with expertise in bioinformatics) (Pennsylvania State University). This chapter was submitted as a manuscript to the Journal of Proteome Research.

5.1 Introduction

The California poppy, *i.e. Eschscholzia californica*, produces benzophenanthridine alkaloids (BPs) such as the biologically active compounds sanguinarine and chelerythrine [8-14, 31, 160]. Sanguinarine and chelerythrine (Figure 1) show activity against multiple targets associated with viral replication [14], inflammation [31], cell cycle regulation, and apoptosis of cancer cells [8-13]. In addition, sanguinarine was previously incorporated as an anti-plaque agent in Viadent toothpastes and mouthwashes [160]. Due to BPA’s important biological activities, cell cultures of *E. californica* are being investigated as an alternative and scalable method for producing these valuable compounds. The advantage of the cell culture system is that
environmental conditions can be controlled and easily manipulated for improving production.

For example, BPA production is significantly enhanced in cell cultures of *E. californica* and *Papaver somniferum* with elicitation [16, 19, 23, 66, 83, 161]. Elicitors activate plant natural defense responses, including increased secondary metabolite production [162-164]. Examples of elicitors include hormones (e.g. jasmonates or salicylic acid), crude biological extracts (e.g. fungal homogenate), and purified biological extracts (e.g. polysaccharides derived from the yeast cell wall) [149, 165]. In *E. californica* cell cultures, yeast elicitor is believed to increase BPA production by activating a pH-dependent signal transduction pathway [166-167] and by activating the production of the hormone, jasmonic acid [65]. Activation of signal transduction pathways triggers gene transcription leading to increased enzyme abundance and secondary metabolite production. For instance, gene transcripts of several BPA biosynthetic enzymes were elevated by elicitation with yeast extract [20, 22, 51], fungal homogenate [18, 161, 168], and methyl jasmonate [19, 46]. Also, the levels of 6 BPA enzymes were increased by elicitation with methyl jasmonate, salicylic acid, and yeast extract [16, 23].

However, less is known about how primary and secondary metabolic pathways are coordinated to support increased secondary metabolite production. Investigating the proteome could provide a more global view of cellular metabolism and its coordination in response to external stimuli. In this paper, changes in the metabolism of *E. californica* cell cultures with enhanced BPA production were explored using a proteomic...
methodology. The proteomic methodology has been employed for this purpose in only a few medicinal plants [28-29, 107].

Proteomic studies in plant systems have primarily been performed in sequenced model systems such as *Arabidopsis thaliana*, *Oryza sativa* (rice), *Populus trichocarpa* (black cottonwood), and *Vitis vinifera* (grape vine) since mass spectrometry (MS)-based proteomics requires the availability of a protein database. However, the proteomes of a few medicinal plants with unsequenced genomes have also been explored, including *Catharanthus roseus* [28], *P. Somniferum* [27, 29], *Taxus cuspidata* [107], *Cannabis sativa* [108-109], ginseng [110-111], and *Chelidonium majus* [112]. For proteomic analysis of unsequenced organisms, one approach utilizes sequence homology to proteins already in a database (*e.g.* *A. thaliana*). This approach allows identification of highly conserved proteins such as primary metabolic pathways proteins but not necessarily enzymes involved in alkaloid biosynthesis which are specific to individual plant species. Alternatively, an EST database for a given organism has been used to identify proteins from tomato [169] and ginseng [110-111].

For the unsequenced *E. californica*, an EST database has also been constructed [155]. In addition, unlike other alkaloid biosynthetic pathways, the biosynthesis of BPAs such as macarpine (the most oxidized BPA, shown in Figure 1) is completely known and involves twenty enzyme-catalyzed steps and one spontaneous step starting from two tyrosine molecules [32]. Furthermore, ten enzymes in the BPA biosynthetic pathway (seven steps leading up to scoulerine in Figure 2 and three steps after scoulerine, *i.e.* chelianthifoline synthase, stylopine synthase, and (S)-tetrahydroprotoberberine-(cis)-N-
methyltransferase) have been sequenced (http://www.expasy.org), enabling the profiling of a number of genes and enzymes involved in BPA biosynthesis.

Typical plant proteomic experiments utilize two-dimensional gel electrophoresis (2-DE) for protein separation prior to identification [27-28, 132]. Though very powerful and offering a number of advantages, 2-DE yields relatively low number of identified proteins due to inherent limitations of this approach [170-171]. In order to increase the coverage of the proteome, we implemented an alternative but complimentary gel-free method. Recently, shotgun proteomic technologies have been applied to plants such as the A. thaliana [122], O. sativa [126], and Medicago truncatula [124]. In this paper, we implemented the shotgun proteomic method based on nano-LC-MS/MS. Proteins were identified using a database generated by translation of a published E. californica uniGene library constructed using ESTs [155]; 15 enzyme sequences from 10 BPA pathway enzymes were also incorporated into this protein database. Shotgun proteomics with nano-LC-MS/MS analysis and identification utilizing a well-annotated, translated unigene database is an approach that has been applied to only a few plant systems with unsequenced genomes.

Using the combined approach described above, we performed a proteomic investigation of E. californica cell cultures elicited with purified yeast extract (PYE). Elicited cultures produced up to 23 mg/g dry weight (DW) of BPAs corresponding to a 20-fold increase over untreated cultures. Nearly 646 proteins from primary and secondary metabolism and other functional protein classes were identified. Furthermore, differential abundance of proteins from several pathways was observed. Hence, in this paper, we demonstrate the application of the shotgun proteomics method utilizing a well-
annotated, translated unigene database to 1) identify proteins from an unsequenced plant 
(*E. californica*) and 2) explore changes in global metabolism associated with enhanced 
alkaloid production.

5.2 Material and Methods

5.2.1 Maintenance of Cell Cultures

*E. californica* suspension cultures were a gift from Dr. Sung-Yong Yoon (formerly at Pohang Institute of Science and Technology; currently at Exelixis Plant Sciences). The suspension cultures were initiated from leaf sections of seedlings germinated on agar-containing medium, as described by Park et al (2006) [172]. Maintenance conditions were provided by Drs. Hwa-Young Cho (Pohang Institute of Science and Technology) and Sung-Yong Yoon and were modified from previously published reports [16, 23, 67]. Cultures were maintained on Linsmaier and Skoog’s medium (Caisson Laboratories, North Logan, UT) [173], supplemented with 30 g/L sucrose (Sigma), 0.37 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D, Sigma), 0.11 mg/L α-naphthaleneacetic acid (Sigma), and adjusted to pH of 5.5 with 1 N NaOH. Cells were subcultured every 14 days by transferring 20 ml of culture (containing 6 ml of packed cell volume) to 80 ml of fresh medium in 250 ml Erlenmeyer flasks. Cultures were maintained at 22 °C with 16 hours light per day, at 120 rpm in a Forma Scientific incubator shaker (Marietta, OH). Sterile water was added weekly to compensate for evaporation.

5.2.2 Elicitation of Cell Cultures

Purified yeast extract (PYE), containing polysaccharides associated with the yeast cell wall, was prepared based on the method of Hahn and Albersheim [149]. Hahn and
Albersheim isolated the active component of the yeast cell wall (i.e. a glycan) which elicited the production of the phytoalexin, glyceollin, from soybeans.

Yeast extract (50 g, Becton-Dickinson, Sparks, MD) was solubilized in 200 ml of water; ethanol (Fisher-Scientific) was added to a final concentration of 80% v/v and then the mixture was stored at 4 °C for 4 days. The supernatant was discarded and the precipitate was redissolved in 200 ml of water and precipitated again with ethanol (80% v/v) at 4 °C for 4 days. The final precipitate was then resuspended in 200 ml of water, lyophilized, and stored at -20 °C.

For proteomic analysis, cells were transferred from growth medium on day 14 to fresh medium without 2,4-D in 250 ml Erlenmeyer flasks (50 ml of culture); these cells were cultured in 2,4-D free medium for 7 days and then elicited with 40 mg/g fresh cell weight (FW) PYE on day 7 (mid-exponential phase). Cells were harvested for alkaloid analysis after 48 and 96 hours by vacuum filtration, frozen, lyophilized, and stored at -20 °C. For proteomic analysis, cells were harvested after 48 hours by vacuum filtration, flash frozen in liquid nitrogen, and stored at -80 °C.

5.2.3 Alkaloid Analysis

The alkaloid extraction and HPLC protocols were provided by Drs. Hwa-Young Cho and Sung-Yong Yoon and were modified from previously published reports [16, 23, 67]. Freeze-dried cells (10 mg) were extracted in 1 ml of 0.2% HCl in methanol. Extracts were sonicated for 1 hr, vortexed for 30 minutes, and then centrifuged for 20 minutes at 13,200g and 4 °C. The supernatant was filtered through a Millex-FH 0.45 μm syringe filter (Millipore, Billerica, MA) prior to HPLC analysis.
Alkaloids were separated and quantified by HPLC using a Waters Alliance 2695 separations module, 996 photodiode array detector (Waters, Milford, MA), and a Phenomenex C\textsubscript{18} reversed phase column (4.60 mm i.d. x 150 mm, 5 μm) with a C\textsubscript{18} precolumn (Phenomenex, Torrance, CA). The mobile phases used for separation were A) 0.032% tetrabutylammonium hydroxide (Sigma) in water, pH 3.0, B) acetonitrile (Fisher, HPLC grade), and C) methanol (Fisher, HPLC grade). The HPLC protocol was provided by Drs. Hwa-Young Cho and Sung-Yong Yoon and was adapted from Cho et al. 2008 [23]. The protocol consisted of gradients at 1.0 ml/min from 1) 80% A, 10% B, 10% C to 75% A, 10% B, 15% C in 5 minutes, 2) to 60% A, 15% B, 25% C in 5 minutes, 3) to 40% A, 25% B, 35% C in 10 minutes, 4) to 1% A, 10% B, 89% C in 15 minutes, 5) to 80% A, 10% B, 10% C in 10 minutes, and 6) isocratically at 80% A, 10% B, and 10% C for 15 minutes. 30 μL of the cell extract was injected into the HPLC. Alkaloids were detected by UV absorbance at 283 nm and quantified by peak areas using sanguinarine as a standard (Sigma). Calibration curves for chelerythrine, chelirubine, dihydrochelerythrine, dihydrochelirubine, and dihydrosanguinarine were estimated using the calibration curve for sanguinarine and published extinction coefficients [150].

5.2.4 Protein Extraction and Fractionation by Differential Centrifugation

Freeze-dried cells were lysed in in a BioSpec bead beater (BioSpec, Bartlesville, OK) with protein extraction buffer as modified from Cho et al. [16]. The protein extraction buffer consisted of 7 M urea (Fisher), 2 M thiourea (Sigma), 50 mM tris-HCl (Fisher) pH 8.0, 5 mM dithiothreitol (DTT, Sigma), ROCHE protease inhibitor cocktail, and benzonase nuclease (Novagen, Madison, WI) Vials containing cells (500 mg FW), extraction buffer (500 μl), and glass beads (BioSpec) were shaken for 30 seconds at
5,000 rpm, cooled on ice, and then the cycle was repeated 3 more times. Vials were centrifuged at 5,000 g for 15 minutes at 4 °C. The supernatant was saved while the pellet was then resuspended with fresh protein extraction buffer. The extraction procedure was repeated 2 more times.

The supernatant fractions from the 3 extraction procedures were combined and centrifuged for 20 minutes at 16,000 g at 4 °C. The resulting supernatant was centrifuged for 45 minutes at 100,000 g at 4 °C in a Beckman-Coulter TI-100 ultracentrifuge. The supernatant enriched in soluble proteins were transferred to Eppendorf tubes. The remaining pellet was thoroughly washed with phosphate-buffered saline (PBS) to obtain maximum recovery of soluble proteins and then the remaining pellet was saved and stored at -80 C. The protein concentration of the resulting fractions was then determined by the Bradford assay [151] using BSA (Pierce, Rockford, IL) as a standard. Two biological replicates for each cell culture condition (e.g. 2 unelicited cultures and 2 elicited cultures) were analyzed. In addition, 2 technical replicates were obtained from each biological replicate by separately digesting 2 aliquots from the 100,000 g supernatant fraction. Table 8 summarizes the preparation of biological and technical replicates.
Table 8: Summary of biological and technical replicates used in proteomic analysis. Under the “Label” column, C and E represent cultures which were untreated and elicited with purified yeast extract, respectively. Biological replicates refer to samples from cultures treated similarly (e.g. untreated or elicited) but grown in separate flasks; biological replicates are denoted as 1 and 2. Technical replicates were obtained by separately digesting two separate aliquots from the 100,000 g supernatant protein fraction of one biological replicate; technical replicates are referred to as A and B. The technical replicate from only a single biological replicate (sample labeled as C1A) was also injected twice on the mass spectrometer and is denoted as C1A-a and C1A-b.

<table>
<thead>
<tr>
<th>Culture condition</th>
<th>Biological replicate (flask)</th>
<th>Technical replicate (digestion)</th>
<th>Technical replicate (mass spec)</th>
<th>Label</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unelicited</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>C1A-1</td>
</tr>
<tr>
<td>Unelicited</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>C1A-2</td>
</tr>
<tr>
<td>Unelicited</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>C1B</td>
</tr>
<tr>
<td>Unelicited</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>C2A</td>
</tr>
<tr>
<td>Unelicited</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>C2B</td>
</tr>
<tr>
<td>Elicited</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>E1A</td>
</tr>
<tr>
<td>Elicited</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>E1B</td>
</tr>
<tr>
<td>Elicited</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>E2A</td>
</tr>
<tr>
<td>Elicited</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>E2B</td>
</tr>
</tbody>
</table>

5.2.5 In-Solution Trypsin Digestion of Protein Fractions

Only the supernatant from the 100,000 g ultracentrifugation step was analyzed. Briefly, for each sample, 100 μg of protein was reduced in 5 mM tris (2-carboxyethyl) phosphine hydrochloride (TECEP) (Pierce, Rockford, IL), alkylated with 15 mM iodoacetamide (Sigma), and quenched with 5 mM DTT. Samples were digested overnight with trypsin (Promega, Madison, WI, 1:40 w/w ratio) and the digestion reaction was stopped by adjusting the pH to less than 4.0 with 3% formic acid. Peptides resulting from protein digestion were desalted and separated from any undigested or partially digested proteins by reversed phase liquid chromatography using a Discovery
BIO C18 cartridge (3 µm, 4.6 mm X 30 mm; Sigma-Aldrich, St Louis, MO) and a Shimadzu HPLC system (Shimadzu Scientific Instruments, Columbia, MD). The mobile phases used were A) 0.1% trifluoroacetic acid (TFA, Pierce, Rockford, IL) in water and B) 0.1% TFA in acetonitrile. The HPLC method consisted of three isocratic steps: 2% B for 3 minutes at 2.0 ml/min to remove salts; 30% B for 6 minutes at 0.6 ml/min to elute tryptic digested peptides and 90% B for 1.5 minutes at 2.0 ml/min to elute very hydrophobic peptides, large protein fragments or non-digested protein. The cartridge was re-equilibrated to initial conditions (2% B) for 1.5 minutes at 2.0 ml/min. Peptides eluted with 30% organic solvent were concentrated under vacuum using a CentriVap concentrator (Labconco, Kansas City, MO) to a volume of approximately 10-15 µl and then adjusted to 20 µL with 0.1% formic acid (Pierce, Rockford, IL) in water.

5.2.6 Nano-LC-ESI-MS/MS Analysis

The complex peptide mixture was separated using a 2D nano Eksigent HPLC system (Dublin, CA) on a reversed phase C18 capillary (175 mm x 0.075 mm i.d.) column in-house packed with Magic C18 media, particle size 5 µm, pore size 300 Å (Michrom Bioresources, Auburn, CA) and directly connected to a linear ion trap (LTQ) instrument (Thermo Fisher, Waltham, MA). The electrospray conditions were: temperature of the transfer tube, 245°C; spray voltage, 2.0 kV; normalized collision energy, 35%. The mass spectrometer was operated in the data dependant mode and switched automatically between MS and MS/MS using MS acquisition software (Xcalibur 2.0, Thermo Fisher, Waltham, MA). Each MS full scan (mass range of m/z 400 to m/z 1600) was followed by MS/MS scans of the 7 most intense peaks.
Peptides (5 µL injection) were loaded at a higher flow rate (1 µL/min) and concentrated on a peptide Captrap cartridge (Michrom Bioresources, Auburn, CA). The peptides were then eluted onto the capillary column and chromatographed at 250 nL/min. The mobile phases used for peptide separation were A) 0.1% formic acid in water and B) 0.1% formic acid in acetonitrile. The method consisted of an isocratic segment at 5% B for 5 minutes, followed by a linear gradient to 35% B in 115 minutes, gradient to 80% B in 20 minutes, isocratic at 80% B for 10 minutes, gradient to 5% B in 1 minute, and then isocratic at 5% B for 4 minutes. The capillary column was equilibrated for 30 min at 250 nL/min before injection of the next sample.

5.2.7 Protein Identification and Quantitation

The *E. californica* unigene sequences (build 5) were downloaded from http://pgn.cornell.edu and run through several iterations of SeqClean (http://compbio.dfci.harvard.edu/tgi/software/) to trim contaminants, including vector, low quality, and low-complexity sequences. Each cleaned unigene was run through the program ESTScan [174] to predict coding sequence, using the *Arabidopsis* hidden Markov model. To annotate each unigene sequence, each of the four sequenced angiosperm genomes was downloaded, which included 31,921 gene models from *A. thaliana* [175], 45,555 from *P. trichocarpa* [176], 66,710 from *O. sativa* [177], and 30,434 from *V. vinifera* [178]. The predicted proteins for all four species were compared in an all-against-all BLASTP (evalue < e-10) using the NCBI BLAST package [179]. The program orthoMCL [180] was then used to identify all ortholog/co-ortholog gene sets. Co-orthologs are defined as two or more genes within a species that share orthology with one or more genes in another species due to duplication events after the two species
diverged. Each orthoMCL group was annotated with a representative *Arabidopsis* gene. The poppy unigene sequences were searched against the entire protein dataset using BLASTX and best hits were recorded.

MS/MS spectra were processed by the CPAS system [154] using the Sequest search against the *E. californica* EST database translated into protein sequences (4700 protein sequences) combined with 15 protein sequences from 10 BPA pathway enzymes ([http://www.expasy.org](http://www.expasy.org)). This protein database was appended with a random protein database created by reversing the protein sequences in order to facilitate estimation of the false discovery rate [156]. The database search was performed with trypsin specified as the digestion enzyme, up to 2 missed cleavages and carbamidomethylation as a fixed modification of cysteines. Mass tolerance was set to 1.6 Da for the precursor ion while 1 Da was used for MS/MS fragment ions. The peptide matches were filtered using PeptideProphet probability >0.9 and Sequest Xcorr values greater than 1.9 for 1+, 2.2 for 2+, and 3.8 for 3+ ions. Then, the protein list was generated by the Protein-Prophet tool with the protein probability cutoff set to 0.9 for protein groups. These filtering criteria led to approximately 3% false positive rate at the protein level. Special care was taken to remove protein redundancy and only protein groups with at least one peptide that is not shared by any other group were retained for further evaluation.

Semi-quantitative analysis was performed using spectral counting; the relative protein abundance was expressed as the total number of peptides identified for a particular protein in elicited or control sample [181-182]. In addition, spectral index [183] and permutation analysis were used to determine the level of significance for differentially abundant proteins. Based on the permutation analysis, proteins with
spectral index values greater than 0.55 or less than -0.60 were considered differentially abundant with 95% confidence. Finally, all differentially abundant proteins, 95% confidence and at least 3-fold change in the spectral counts were also manually validated. The reproducibility of the spectral counts method among biological replicates is shown in Appendix B.

5.3 Results

5.3.1 Elicitation of Alkaloid Production

Benzophenanthridine alkaloid (BPA) production was elicited in *E. californica* suspension cultures using purified yeast extract (PYE). Elicitation conditions (*i.e.* 40 mg of PYE/g FW added on day 7, mid-exponential phase) were chosen to optimize total BPA production [16, 23, 67]. After 48 hours, elicited cultures accumulated 23.1 mg of BPAs/g DW, a 20-fold increase over unelicited cultures (Figure 27). After 96 hours, the total alkaloid production did not increase in the elicited cultures (*i.e.* 16.7 mg/g DW) but the profile of alkaloids changed. For example, sanguinarine and chelerythrine accounted for 13.4% and 2.5%, respectively, of the total BPA production in elicited cultures after 48 hours compared to 72.5% and 11.3% after 96 hours. The protein profile of the cultures under low (*i.e.* untreated) and high BPA-producing (*i.e.* PYE-elicited) conditions were then compared.
Figure 27: Elicitation of alkaloid production using purified yeast extract (PYE). PYE (40 mg/g FW) was added on day 7 to E. californica cultures and cells were harvested after 48 and 96 hours. Error bars represent the standard deviation of triplicate cultures. Abbreviations: DHCHY, dihydrochelerythrine; CHY, chelerythrine; DHSA, dihydrosanguinarine; SA, sanguinarine; DHCHR, dihydrochelirubine; CHR, chelirubine.

5.3.2 Summary of Protein Identification

Soluble protein extracts from untreated and PYE-elicited cells were prepared by ultracentrifugation (see Section 5.2.4). Extracts enriched in cytosolic proteins were then digested with trypsin; the resulting peptides were separated by capillary reversed phase chromatography and analyzed by MS/MS. MS/MS spectra were searched using the Sequest algorithm against the E. californica protein database. For untreated and PYE-elicited cultures at 48 hours, two biological replicates with two technical replicates (samples digested in parallel) each were analyzed.

Using the constructed E. californica protein database (see Protein Identification and Quantitation Section of the Materials and Methods), approximately 646 proteins were
identified with a false positive rate of 3% at the protein level. The classes of identified proteins included structural proteins (e.g. ribosomal proteins, actin, tubulin), protein synthesis (e.g. elongation factor 1A), chaperone proteins (e.g. heat shock proteins, peptidyl cis-trans isomerase), calcium binding proteins (e.g. calreticulin), ubiquitin-associated proteins, defense response proteins (glutathione-S-transferase), and enzymes involved in nucleic acid biosynthesis and binding, amino acid biosynthesis, energy metabolism, and secondary metabolism.

5.3.3 Summary of Differentially Abundant Proteins

Protein abundance was evaluated using a semi-quantitative approach called spectral counting. This method relies on counting the number of MS/MS spectra that are matched to peptides associated with a particular protein. Since the selection of the precursor ion for MS/MS analysis is a stochastic process, it is expected that a higher number of MS/MS spectra associated with a particular protein is a result of the higher abundance of this protein in the sample. It has been shown that the spectral count is proportional to the amount of the protein present in the sample [156, 184]. However, the dependence is not linear and as a result, the fold change in spectral counts does not directly correspond to the change in protein abundances. In addition, the spectral index [183] was calculated to determine the level of significance for the differentially abundant proteins. Proteins with spectral index values greater than 0.55 or less than -0.60 were considered differentially abundant with 95% confidence. The differentially abundant proteins are summarized in Table 9.
Table 9: Quantification of selected differentially abundant proteins. Selected differentially abundant proteins identified with spectral index values greater than 0.55 or less than -0.6. Relative protein abundance is expressed by the average spectral counts from elicited samples (from 2 technical replicates for each of the 2 biological replicates – 4 samples total) and the average spectral counts from unelicited samples (from 2 technical replicates for each of the 2 biological replicates including duplicate injections of a single technical replicate – 5 samples total). The spectral index was calculated according to Fu et al. [183]. aThree isoforms for methionine synthase were detected using unique peptides from each isoform. The high sequence homology prevented exact determination of spectral counts for each isoform.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Name</th>
<th>Average Spectral Counts</th>
<th>Spectral Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS</td>
<td>Methionine synthase</td>
<td>Elicited 17.50</td>
<td>Unelicited 3.80</td>
</tr>
<tr>
<td>SAMS</td>
<td>S-adenosyl methionine synthase</td>
<td>9.00</td>
<td>1.20</td>
</tr>
<tr>
<td>SAHH</td>
<td>S-adenosylhomocysteine hydrolase</td>
<td>3.00</td>
<td>1.00</td>
</tr>
<tr>
<td>4’OMT</td>
<td>3’-hydroxy-N-methylcoclaurine 4’-O-methyltransferase</td>
<td>14.50</td>
<td>0.60</td>
</tr>
<tr>
<td>PFK</td>
<td>Phosphofructokinase</td>
<td>2.00</td>
<td>0.60</td>
</tr>
<tr>
<td>Aldolase</td>
<td>Fructose bisphosphate aldolase</td>
<td>2.50</td>
<td>0.80</td>
</tr>
<tr>
<td>PEPC</td>
<td>Phosphoenolpyruvate carboxylase</td>
<td>0.00</td>
<td>2.20</td>
</tr>
<tr>
<td>GS</td>
<td>Glutamate synthase</td>
<td>3.25</td>
<td>0.00</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione-S-transferase</td>
<td>5.00</td>
<td>1.40</td>
</tr>
</tbody>
</table>

The increased abundance of several enzymes within a specific pathway suggests a potential effort by the cell to increase flux through this pathway. For instance, the levels of three enzymes involved in the biosynthesis of methionine and (S)-adenosylmethionine SAM were increased in the high alkaloid-producing cultures (Figure 28, Table 9): cobalamin-independent methionine synthase (MS), SAM synthase (SAMS), and (S)-adenosyl homocysteine hydrolase (SAHH). In addition to primary metabolic enzymes, one enzyme from the BPA biosynthetic pathway (4’OMT) was identified in the high-producing cultures. Importantly, increased flux in the BPA pathway is consistent with a higher abundance of 4’OMT. As shown in Figure 2, the biosynthesis of BPAs begins
with the conversion of two molecules of tyrosine in several steps to form (S)-norcoclaurine. Then, (S)-norcoclaurine is converted to (S)-reticuline by three methylation reactions, one of which is catalyzed by 4’OMT, and finally one hydroxylation reaction (\textit{i.e.} CYP80B1).

**Figure 28:** Metabolic pathways leading to (S)-adenosyl methionine (SAM) production from glycolysis. See Table 9 for abbreviations.

Other proteins that exhibited a significant difference based on spectral index values above the 95% cutoff included glycolytic enzymes (\textit{e.g.} phosphofructokinase, fructose bisphosphate aldose, phosphoenolpyruvate carboxylase, see Figure 28), stress response proteins (\textit{e.g.} glutathione-S-transferase), and structural proteins (\textit{e.g.} ribosomal subunits and histones).

### 5.3.4 Discussion

Few studies have applied proteomics for investigating secondary metabolism of medicinal plants [28-29, 107] and in particular have focused on applying proteomics for
discovering new enzymes involved in secondary metabolism [185-186]. In Ounaroon et al. [186], a proteomic analysis of latex from the opium poppy using 2-D gel electrophoresis led to the isolation and identification of two methyltransferase enzymes involved in benzylisoquinoline alkaloid biosynthesis. In Jacobs et al. [28], a proteomic analysis of *C. roseus* cell cultures at different production stages using 2-D gels was pursued to identify novel proteins associated with terpenoid indole alkaloid (TIA) biosynthesis. While unique sequences were discovered, the analysis also demonstrated that a variety of proteins could be identified for an unsequenced plant, including enzymes associated with primary and secondary metabolism, photosynthesis, oxidative phosphorylation, and protein synthesis as well as chaperone and structural proteins.

The absence of a sequenced genome presents a significant challenge in the proteomic analysis. Several approaches have been employed to overcome this problem, for example 1) sequence homology to *A. thaliana* and other model plant systems was used to identify highly conserved proteins from medicinal plants such as those in *C. roseus* [28], *P. Somniferum* [27], and *T. Cuspidata* [107], and *C. Sativa* [108-109], and 2) expressed sequence tags (EST) databases were used for identification of proteins in *P. Somniferum* [29], tomato [169], and ginseng [110-111]. The ability to perform proteomic analyses of medicinal plants without a fully sequenced and annotated genome will provide a useful platform for exploring and investigating the global metabolism of these medicinal plants.

In this paper, we demonstrate the application of a gel-free shotgun proteomic method and a well-annotated, translated unigene database for identifying proteins in the unsequenced species, *E. californica*. Specifically, we identified nearly 650 proteins from
E. californica cell cultures with minimal protein fractionation (i.e. differential centrifugation) followed by peptide separation and analysis using nano-LC-MS/MS. The number of proteins identified compared favorably to reports in the literature investigating other unsequenced organisms, including the industrially important Chinese Hamster Ovary (CHO) cell lines for producing recombinant therapeutic proteins [88, 90-91, 187], C. roseus [28], and P. somniferum cultures [29]. For instance, Nissom et al. [88] used a gel-free approach based on two-dimensional liquid chromatography and used a combined mouse, rat, and human database to identify 864 proteins from CHO protein extracts. In Jacobs et al. [28], a 2-D gel-based proteomic study of C. roseus cell cultures resulted in a maximum number of 988 spots; by comparing the protein profiles from different culture days, 88 spots were selected, leading to 58 identified proteins using a homology-based search. In Zulak et al. [29], a 2-D gel proteomic study of P. somniferum identified 219 proteins from 340 spots using an EST-database for searching.

Comparing the protein profile of the low and high alkaloid-producing cultures of E. californica resulted in the identification of several proteins with significant changes in abundance based on the spectral index method. In particular, we identified several differentially abundant proteins ranging from the glycolytic pathway to the methionine and SAM biosynthetic pathways (i.e. phosphofructokinase, aldose, MS, SAMS, and SAHH) and one enzyme from the BPA biosynthetic pathway (4’OMT). The same proteins were identified to be differentially abundant in a duplicate proteomics experiment with our E. californica cell line performed using a different subculture, demonstrating the reproducibility of these results. The increased abundance of enzymes associated with the methionine, SAM, and BPA biosynthetic pathways was also observed
in a recent transcript profiling [83] and a proteomic study [29] with fungal-elicited *P. somniferum* suspension cultures, as discussed below.

SAM is a universal methyl donor in biological reactions and is required in 6 steps of the BPA biosynthetic pathway (*e.g.* 6OMT, CNMT, and 4’OMT shown in Figure 2). In our study, several enzymes involved in the biosynthesis of methionine and SAM (*e.g.* MS, SAMS, SAHH) increased in abundance. Interestingly, using transcript profiling and proteomic studies, Zulak et al.[29, 83] observed increased levels of either the transcript or protein of several SAM biosynthetic enzymes (*e.g.* MS, SAMS, and SAHH) in fungal-elicited opium poppy cultures, further supporting our findings. SAMS levels were also increased in tobacco cultures treated with methyl jasmonate [188-189], suggesting that the increased levels of methionine and SAM biosynthesis may be common in several plant species with elicitation and may be important for secondary metabolite production.

In our study, the levels of one enzyme catalyzing early steps in the BPA biosynthetic pathway (4’OMT) were also increased. Similarly, Zulak et al. [29] observed the increased abundance of enzymes in the BPA biosynthetic pathway, including 6OMT using 2-D gel proteomics and increased abundance of 6OMT, CNMT, CYP80B3, 4’OMT, and BBE through Western blotting. The differential induction of at least six genes coding for enzymes from the BPA pathway (*e.g.* TYDC, NCS, 6OMT, CNMT, CYP80B1, 4’OMT, and BBE shown in Figure 2) were also observed in fungal-elicited *P. somniferum* cultures [18, 83].

In addition to proteins from the methionine, SAM, and BPA pathways, we observed the following differentially abundant proteins in elicited poppy cultures: stress-related proteins (*e.g.* glutathione-S-transferase), proteasome components, glycolytic
enzymes (e.g. fructose-bisphosphate aldolase, phosphofructokinase), and amino acid metabolism (glutamate synthase), which is in agreement with results of Zulak et al. [29, 83]. Since the literature results were based on a related poppy species treated with similar elicitors, it supports the validity of our proteomic approach and the application of the unigene-derived protein sequences for protein identification. Our proteomic study also observed the differential abundance of several enzymes in both primary and secondary metabolism, suggesting the potential of proteomics to explore and investigate the global metabolism of cultures under different environmental conditions.
6.0 Investigating the Role of (S)-Adenosyl Methionine Biosynthesis in Supporting or Mediating Enhanced Benzophenanthridine Alkaloid Production in California Poppy (*Eschscholzia californica*) Suspension Cultures

The research described in this chapter was performed in collaboration with Maria-Louisa Izamis (who performed the amino acid analysis) of the Center for Engineering in Medicine of Massachusetts General Hospital and will be submitted as a manuscript to a biotechnology journal.

6.1. Introduction

The California poppy (*Eschscholzia californica*) produces a class of biologically active secondary metabolites known as benzophenanthridine alkaloids (BPAs). BPAs produced by *E. californica* include sanguinarine and chelerythrine (shown in Figure 1), compounds shown to have anti-cancer [8-13, 190], anti-inflammatory [14], and antimicrobial properties [191]. The production of these valuable BPAs was significantly enhanced in *E. californica* cultures with the addition of yeast extract or methyl jasmonate [16, 23, 67].

Previously, we performed a shotgun proteomic analysis of *E. californica* cell cultures elicited with purified yeast extract (PYE) to explore changes in metabolism associated with elicitor-induced BPA production [192]. In particular, several enzymes in the (S)-adenosyl methionine (SAM) biosynthetic pathway were significantly increased in abundance in elicited cultures, including methionine synthase (MS), SAM synthetase (SAMS), and (S)-adenosylhomocysteine hydrolase (SAHH) (Figure 2). For instance, the levels of MS, SAMS, and SAHH were increased by 6, 2.4, and 3.7 fold (based on spectral counts) in elicited cultures compared to the controls [192]. Further, the levels of a methyl
transferase involved in BPA production, 3'-hydroxy-N-methylcoclaurine 4'-O-
methyltransferase (4’OMT, Figure 1), was increased by 24-fold in the yeast-elicited
cultures [192].

The coordinated increase in SAM biosynthetic enzymes and BPA production is
interesting since the production of the most oxidized BPA, macarpine, from tyrosine
requires 6 methyltransferases (Figure 2) with SAM serving as the methyl donor in these 6
reactions. This suggests that SAM biosynthesis may be required and coordinately
induced to support increased BPA production. The increased abundance of SAMS
(SAHH and MS were not detected) was also observed in fungal-elicited Papaver
somniferum suspension cultures by Zulak et al. (2009) [29]. Increased SAM biosynthesis
associated with elicitation was also observed in other plant secondary metabolic systems.
For instance, increased expression of MS, SAMS, and SAHH genes was also observed in
unelicited, high-alkaloid producing Nicotiana tabacum cell lines [193]. Alkaloid
production from Nicotiana involves one methylation step (putrescine methyltransferase)
which is consistent with increased SAMS expression in high-producing cultures. SAMS,
SAHH, and MS transcripts were also induced by methyl jasmonate in N. tabacum
cultures [189] and by fungal elicitation in Petroselinum crispum cultures and leaves
[194].
Figure 29: SAM biosynthesis and metabolism of related amino acids adapted from Ravanel et al. [195]. SAM is synthesized from 3-phosphoglycerate and oxaloacetate precursors. SAM is used as a general methyl donor and as a precursor for ethylene production in plants. Enzymes with increased abundance in elicited cultures are shown in red. Amino acids produced from precursors in the methionine pathway are shown in blue. SAMS: (S)-adenosyl-methionine synthetase, MS: Methionine synthase, SAHH: (S)-adenosyl-homocysteine hydrolase, ACC, 1-aminocyclopropane-1-carboxylate.

SAM biosynthesis may potentially be required for mediating increased BPA production through the production of ethylene. Ethylene is a well-known plant hormone produced through the SAM biosynthetic pathway (Figure 29). Songstad et al. reported that ethylene generation reached a maximum increase of 3-fold 7 hours after elicitation with a fungal elicitor in *P. somniferum* cell cultures [79]. Some studies have suggested that ethylene participates in the signal transduction pathways that activate secondary metabolism and that adding ethylene to plant cell cultures can enhance secondary metabolite production [78, 196]. Ju et al. [78] observed a 63% increase in total alkaloid production when ethephon was added up to 1000 μM. For example, Kobayashi et al. observed a 5-fold decrease in berberine production when ethylene biosynthesis was inhibited using silver thiosulfate [197].
In this paper, the role of the SAM biosynthetic pathway in supporting or mediating enhanced BPA production is explored. First, the effect of SAM supply on BPA production is investigated in elicited *E. californica* cultures using methionine feeding. Methionine is the precursor to SAM [195] suggesting that externally supplied methionine would be converted to SAM and then used in methylation reactions to support BPA production. The amino acid profiles of PYE-elicited cultures were measured and validated the increased flux to SAM biosynthesis. Second, the role of increased SAM biosynthesis in mediating or inducing BPA production through the plant hormone, ethylene, is also studied. The results in which *E. californica* suspension cultures are treated with methionine, ethylene (added as soluble ethephon), and cobalt chloride (ethylene biosynthesis inhibitor) are presented.

6.2. Materials and Methods

6.2.1. Maintenance of Cell Cultures

*E. californica* suspension cultures were a gift from Dr. Sung-Yong Yoon (formerly at Pohang Institute of Science and Technology; currently at Exelixis Plant Sciences). The suspension cultures were initiated from leaf sections of seedlings germinated on agar-containing medium, as described by Park et al (2006) [172]. Maintenance conditions were provided by Drs. Hwa-Young Cho (Pohang Institute of Science and Technology) and Sung-Yong Yoon and were modified from previously published reports [16, 23, 67].

Cultures were maintained on Linsmaier and Skoog’s medium [173] (Caisson Laboratories, North Logan, UT), supplemented with 30 g/L sucrose (Sigma), 0.37 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D, Sigma), 0.11 mg/L α-napthaleneacetic acid
(Sigma), and adjusted to pH of 5.5 with 1 N NaOH. Cells were subcultured every 14 days by transferring 20 ml of culture (containing 6 ml of packed cell volume) to 80 ml of fresh medium in 250 ml Erlenmeyer flasks. Cultures were maintained at 22 °C with 16 hours light per day, at 120 rpm in a Forma Scientific incubator shaker (Marietta, OH). Sterile water was added weekly to compensate for evaporation.

6.2.2 Preparation of Purified Yeast Extract

Purified yeast extract (PYE), containing polysaccharides associated with the yeast cell wall, was prepared based on the method of Hahn and Albersheim [149]. Hahn and Albersheim [149] isolated the active component of the yeast cell wall (i.e. a glycan) which elicited the production of the phytoalexin, glyceollin, from soybeans.

Yeast extract (50 g, Becton-Dickinson #212750, Sparks, MD) was solubilized in 200 ml of water; ethanol (Fisher-Scientific) was added to a final concentration of 80% v/v and then the mixture was stored at 4 °C for 4 days. The supernatant was discarded and the precipitate was redissolved in 200 ml of water and precipitated again with ethanol (80% v/v) at 4 °C for 4 days. The final precipitate was then resuspended in 200 ml of water, lyophilized, and stored at -20 °C.

6.2.3 Methionine Feeding

Cells were cultured in growth media lacking 2,4-D for seven days to maximize production. On day 7, suspensions were transferred to 6-well plates (Corning Incorporated, Corning, NY, #3516, 5 ml culture/well) and elicited with PYE (40 mg/g FW). Methionine (Sigma, 200 mM stock solution in water) was filter-sterilized and then added to the 6-well plates to a final concentration of 0.5 and 1.0 mM on day 7 or added twice to a final concentration of 0.25 and 0.5 mM on both day 7 and day 9. Cells were
harvested on day 11 and then lyophilized for 72 hours using a Flexi-Dry MP Freeze Dryer (Kinetics Thermal Systems, FD-3-85A-MP, Stone Ridge, NY) in preparation for alkaloid analysis.

6.2.4. Elicitation with Ethylene

Ethylene was added as soluble ethephon as ethylene is poorly soluble in solution. Upon dissolution, ethephon releases ethylene gas. Suspensions were transferred to 6-well plates (5 ml culture/well) on day 7 and elicited with ethephon (Sigma, 50, 250 or 1000 μM) or PYE (40 mg/g FW). Cells were harvested either after 48 or 96 hours and then lyophilized for 72 hours in preparation for alkaloid analysis.

6.2.5. Addition of Cobalt Chloride

Cobalt chloride is an ethylene biosynthesis inhibitor [198-199] Suspensions were transferred to 6-well plates (5 ml culture/well) on day 7 and elicited with PYE (40 mg/g FW). A 500 mM cobalt (II) chloride hexahydrate (Sigma, C8661) was prepared in deionized water, filter-sterilized, and added to unelicited and elicited cultures at final concentrations of 70, 700, or 7000 μM based on Lau et al. [198]. Cells were harvested either after 48 or 96 hours and then lyophilized for 72 hours in preparation for alkaloid analysis.

6.2.6. Alkaloid Analysis

The alkaloid extraction and HPLC protocols were provided by Drs. Hwa-Young Cho and Sung-Yong Yoon and were modified from previously published reports [16, 23, 67]. Freeze-dried cells (10 mg) were extracted in 1 ml of 0.2% HCl in methanol. Extracts were sonicated for 1 hr, vortexed for 30 minutes, and then centrifuged for 20
minutes at 13,200g and 4 °C. The supernatant was filtered through a Millex-FH 0.45 μm syringe filter (Millipore, Billerica, MA) prior to HPLC analysis.

Alkaloids were separated and quantified by HPLC using a Waters Alliance 2695 separations module, 996 photodiode array detector (Waters, Billerica, MA), and a Phenomenex C\textsubscript{18} reversed phase column (4.60 mm i.d. x 150 mm, 5 μm) with a C\textsubscript{18} precolumn (Phenomenex, Torrance, CA). The mobile phases used for separation were A) 0.032% tetrabutylammonium hydroxide in water, pH 3.0, B) acetonitrile, and C) methanol. The HPLC protocol was provided by Drs. Hwa-Young Cho and Sung-Yong Yoon and was adapted from Cho et al. 2008 [23]; the protocol consisted of gradients at 1.0 ml/min from 1) 80% A, 10% B, 10% C to 75% A, 10% B, 15% C in 5 minutes, 2) to 60% A, 15% B, 25% C in 5 minutes, 3) to 40% A, 25% B, 35% C in 10 minutes, 4) to 1% A, 10% B, 89% C in 15 minutes, 5) to 80% A, 10% B, 10% C in 10 minutes, and 6) isocratically at 80% A, 10% B, and 10% C for 15 minutes; 30 μL of the cell extract was injected into the HPLC. Alkaloids were detected by UV absorbance at 283 nm and quantified by peak areas using sanguinarine as a standard (Sigma). Calibration curves for chelerythrine, chelirubine, dihydrochelerythrine, dihydrochelirubine, and dihydrosanguinarine were estimated using the calibration curve for sanguinarine and published extinction coefficients [150].

6.2.7. Amino Acid Analysis

Media from methionine-treated and elicited samples was saved and stored at -20 C in 15 ml centrifuge tubes. Aliquots of media were centrifuged to remove cell debris and derivatized using the Waters AccQ-Fluor Reagent Kit (#WAT052880) according to the manufacturer’s protocol. Derivatized samples were separated and quantified using a
Waters 2695 separations module and Waters 474 scanning fluorescence detector. Eluents A and B were prepared by dissolving 14.8 g of sodium acetate trihydrate, 607 mg of triethylamine, and 0.5 g of sodium azide in 1 L water; the pH was adjusted to 5.70 (eluent A) or 6.80 (eluent B) with 50% phosphoric acid. Eluent C was 100% acetonitrile. The HPLC protocol consisted of gradients from 1), 90% A, 0% B, 10% C to 89 % A, 1% B, 10% C in 0.5 minutes at 1.0 ml/min, 2) to 88% A, 2% B, 10% C in 16.5 minutes at 1.0 ml/min, 3) to 86% A, 5% B, 9% C in 7 minutes at 1.0 ml/min, 4) to 63% A, 12% B, 25% C in 8 minutes at 1.0 ml/min, 5) to 12.5% B, 87.5% C in 1.5 min at 1.0 ml/min, 6) to 22% A, 12.5% B, 65.5% C in 0.3 min at 1.3 ml/min, 7) to 22% A, 13% B, 65% C in 3.2 minutes at 1.3 ml/min, 8) to 22% A, 15% B, 63% C in 11 minutes at 1.3 ml/min. Derivatized amino acids were detected using a Waters 474 Scanning Fluorescence Detector set at an excitation of 250 nm and an emission of 395 nm. Amino acids were identified by comparison of retention time to those from an amino acid standard mixture consisting of Amino Acid Standard H (Pierce, Rockford, IL), to which asparagine, glutamine, and ornithine were added.

6.3. Results and Discussion

6.3.1 Effect of Methionine Feeding on BPA Production

Due to the increased flux in SAM biosynthesis in elicited cultures, the effect of methionine feeding on benzophenanthridine alkaloid (BPA) production was examined in untreated and elicited *E. californica* cultures (Figure 30). In this study, PYE was added on day 7 (40 mg/g FW) to maximize BPA production (see Chapter 4; Section 1) followed by methionine addition (0.5 and 1.0 mM) to elicited and unelicited cultures also on day 7. To avert potential feedback inhibition of methionine biosynthetic enzymes, methionine
was added to a final concentration of 0.5 and 1 mM either as a single dose on day 7 or as two lower doses (0.25 and 0.5 mM) on days 7 and 9 (Figure 30). All cultures were harvested on day 11, 96 hours after the initial elicitation or methionine feeding.

Figure 30: Effect of methionine feeding on BPA production in *E. californica* suspension cultures. Methionine (0.5 and 1.0 mM) was added on day 7 to unelicited (left panel) and elicited cultures (middle and right panels). In the middle panel, aliquots of 0.5 and 1.0 mM methionine were added on day 7 to elicited cultures (40 mg/g FW PYE also added on day 7). In the right panel, aliquots of 0.25 and 0.5 mM methionine were added on day 7 and again on day 9 (0.5 and 1.0 mM total methionine added) to elicited cultures (40 mg/g FW PYE added on day 7). Cells were harvested 96 hours after the initial elicitation. Error bars represent standard deviation from triplicate measurements.

Methionine feeding did not significantly affect the alkaloid profile or the total alkaloid production in either untreated or yeast-elicited cultures (Figure 30). There was also no significant difference between single and dual feeding of methionine on alkaloid production (Figure 30).
The biosynthesis of methionine and SAM from primary metabolites is shown in Figure 29. SAM is synthesized from oxaloacetate and 3-phosphoglycerate produced from the TCA and glycolytic cycle, respectively (Figure 29). Literature suggests that methionine can be taken up into the cell. For instance, the SAM biosynthetic enzymes, SAMS and SAHH (see Figure 29), have been identified as cytosolic enzymes [195], indicating that externally added methionine does not have to cross intracellular membranes to be converted to SAM. Also, addition of SAM to the media of murine monocyte cultures led to an increase in intracellular SAM concentration [200]. To confirm that methionine was transported into the cells, the culture medium from the methionine feeding experiment was analyzed for amino acid content by HPLC.

As shown in Figure 31, less than 20 μM of methionine was observed in the media of cultures fed with 500 – 1000 μM methionine, suggesting that most of the methionine had either been taken up by the cell or degraded in the media. Conditioned media was centrifuged to remove cells on day 7 and then spiked with 500 μM methionine. After incubating for 96 hours, the methionine concentration had decreased to only 420 μM, suggesting that methionine did not break down in conditioned media and that low methionine levels observed in elicited and unelicited cultures were due to conversion to other metabolites (e.g. SAM, isoleucine, ethylene as shown in Figure 29). Interestingly, no extracellular methionine was observed in unelicited cultures (with no methionine fed) whereas less than 20 μM of methionine was detected in elicited cultures with no methionine fed, consistent with the proteomic results that methionine and SAM biosynthesis is increased in elicited cultures.
Figure 31: Accumulation of amino acids in the media of cultures fed with methionine. *E. californica* cells were treated on day 7 with purified yeast extract (40 mg/g FW) and methionine (0, 500, 1000 μM) and harvested after 96 hours. The concentrations of several amino acids related to methionine biosynthesis (Met, Ile, Thr, Lys, and Asp,) in unelicited (A) and elicited (B) cultures were monitored by HPLC. Two amino acids unrelated to methionine biosynthesis (Ala and Tyr) in unelicited (C) and elicited (D) cultures were monitored by HPLC. Abbreviations: Met, methionine; Thr, Threonine; Ile, Isoleucine; Lys, Lysine; Asp, Aspartic acid; Ala, Alanine; Tyr, Tyrosine. Error bars represent standard deviation from duplicate experiments.

Furthermore, the profile of extracellular amino acids associated with the biosynthesis of methionine from oxaloacetate and 3-phosphoglycerate was distinctly different in untreated versus elicited cultures. In particular, Figure 31 profiles aspartate, threonine, lysine, isoleucine, alanine, and tyrosine in the medium for the following reasons. Aspartate and cysteine are directly required in methionine biosynthesis but cysteine was not quantified because it could not be separated by HPLC. Other amino
acids such as threonine, lysine, and isoleucine are generated from pathways branching off from the primary methionine biosynthetic pathway. Isoleucine can be generated either through methionine breakdown or using threonine as a precursor [201]. Thus, changes in flux through the metabolic pathway are expected to affect accumulation of both methionine and related amino acids.

As shown in Figure 31, elevated levels of aspartate was observed in the medium of untreated cultures whereas elevated levels of threonine, isoleucine, and lysine were observed in the medium of elicited cultures. An increased diversion of aspartate towards threonine, lysine, and isoleucine production is expected if methionine biosynthesis is saturated; this observation is consistent with the proteomic results that methionine and SAM biosynthesis is increased in elicited cultures [192]. In addition, higher isoleucine levels are expected to result from the degradation of methionine. Interestingly, amino acids (alanine and tyrosine) not associated or involved in methionine biosynthesis were affected less than 50% between elicited and unelicited) than the amino acids in the methionine biosynthetic pathway.

These results suggest that methionine was not limiting the biosynthesis of SAM, despite the increased levels of SAM biosynthetic enzymes in the yeast-elicited cultures. Hence, biosynthetic and recycling pathways are sufficient to provide new SAM required for BPAs or other processes associated with BPA biosynthesis. After methylation, SAM forms SAH which is then recycled to SAM in three steps, all of which were upregulated in the proteomic study [192]. If these enzymes are sufficient to meet the SAM demands of the cell, then additional methionine is either unnecessary or potentially being broken down into isoleucine. Cycloleucine was also tested as a SAMS inhibitor [202] but did
not enter the cell as evidenced by HPLC analysis of spent medium from cycloleucine-treated cultures.

6.3.2. Effect of the Plant Hormone Ethylene on BPA Production

Ethylene is a natural plant hormone produced in response to elicitation [79, 203] or involved in inducing secondary metabolite production in certain plant cell cultures [196, 204-205]. For instance, a fungal elicitor increased ethylene production in *P. somniferum* cultures [79]. In *E. californica* cell cultures, ethylene treatment increased alkaloid production in some cases [78, 196] and resulted in no change in other cases [79]. Ethylene also increased alkaloid production in *Coffea arabica* and *Thalictrum rugosum* cell cultures [205]. Addition of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) increased ethylene production in soybean cotyledons but secondary metabolite production was not affected [203]. Overall, there is evidence that ethylene is either a byproduct of elicitation or inducer of secondary metabolite production.

Ethylene biosynthesis in plants begins with the conversion of SAM to ACC which is then converted to ethylene (see Figure 29). Increased SAM biosynthesis in our yeast-elicited *E. californica* cultures [192] presumably also promotes increased ethylene production. In this study, the role of ethylene in mediating increased BPA production with yeast elicitation was investigated by adding ethephon (soluble ethylene) and cobalt chloride (ethylene biosynthesis inhibitor).
Figure 32: Treatment of E. californica cultures with ethephon. Ethephon was added to E. californica cultures up to 1000 μM with no observed increase in alkaloid production. Total alkaloid production and individual alkaloid profiles are shown at 48 after addition of ethephon (0, 50, 250 or 1000 μM) or elicitation with PYE (40 mg/g FW). Error bars represent standard deviation from triplicate measurements.

In this study, ethephon was added to E. californica cultures at 0, 50, 250, or 1000 μM on day 7 and harvested after 48 and 96 hours (only 48 hrs data shown). For comparison, cells were also elicited with PYE (40 mg/g FW). As shown in Figure 32, addition of ethephon did not enhance alkaloid production and elicitation with PYE alone produced significant amounts of alkaloids (27 mg/g DW) after 48 hours (Figure 32). The presence of ethylene in ethephon-treated cultures was confirmed by gas chromatography (See Appendix C). Similarly, Songstad et al. [79] did not observe an increase in sanguinarine production when ethephon (up to 2.7 mM) was added to P. somniferum cultures.

The role of ethylene in BPA production was also investigated using the ethylene biosynthesis inhibitor CoCl₂. If PYE increases production by inducing ethylene production, then inhibition of ethylene production in elicited cultures would decrease BPA production. CoCl₂ inhibits the conversion of ACC to ethylene by the enzyme
aminocyclopropanecarboxylate oxidase. CoCl$_2$ inhibited ethylene production in *Phaseolus aureus* [198] and *Vigna radiate* seedlings [199]. In this experiment, CoCl$_2$ (70, and 700 μM) was added to unelicited and elicited *E. californica* cultures on day 7 and harvested after 48 and 96 hours.

![Figure 33: Treatment of *E. californica* cultures with cobalt chloride. Cobalt chloride was added to unelicited and PYE-elicited (40 mg/g FW) *E. californica* cultures up to 7000 μM with no observed increase in alkaloid production. Total alkaloid production and individual alkaloid profiles are shown at 48 and 96 hours (bottom) after the addition of CoCl$_2$ (70 or 700 μM). Top left: Unelicited cells after 48 hrs; Top right: Elicited cells after 48 hours; Bottom left: Unelicited cells after 96 hrs; Bottom right: Elicited cells after 96 hours. Error bars represent standard deviation from triplicate experiments.](image)

After 48 hours, cobalt chloride (70 and 700 μM) did not significantly affect production compared to untreated cultures (2.3 mg/g DW of BPAs, Figure 33). After 48
hours, PYE-elicited cultures (40 mg/g FW) produced 23.9 mg/g DW of BPAs; cobalt chloride (70 and 700 μM) did not significantly affect total BPA production suggesting that ethylene is not required for PYE-induced BPA production.

After 96 hours, production in untreated cultures did not change significantly compared to after 48 hours. BPA production was not affected by CoCl$_2$ treatment (70 and 700 μM) in unelicited cultures. After 96 hours, PYE-elicited cultures (40 mg/g FW) produced 29.6 mg/g DW of BPAs. Cobalt chloride treatment (70 μM) of PYE-elicited cultures did not significantly ($p = 0.47$) affect total BPA production. Treatment with 700 μM CoCl$_2$ increased total BPA production by 56% ($p=0.05$) compared to elicitation alone. CoCl$_2$ treatment (20, 50, and 100 μM) increased paclitaxel production in *Taxus chinensis* cultures [206] and induced anthocyanin production in *Vaccinium pahalae* cultures [207].

The results in Figure 33 show that yeast elicitation increases alkaloid production but that ethylene is not responsible for mediating and inducing this effect in *E. californica* cell cultures. Cobalt chloride did not decrease BPA production in PYE-treated cultures suggesting ethylene is not required for enhanced BPA production.

### 6.4 Conclusions

The goal of this study was to investigate the role of the SAM biosynthetic pathway in supporting or inducing BPA production. The effect of the SAM biosynthetic pathway was explored by methionine feeding, ethephon treatment, and cobalt chloride treatment of elicited *E. californica* cultures.

Addition of methionine to either unelicited or PYE-elicited cultures did not enhance BPA production, suggesting that methionine and SAM were not limiting the
production of BPAs. While methionine was taken up into the cells, levels of amino acids produced from precursors in common with methionine biosynthesis (i.e. threonine, isoleucine, and lysine) were significantly increased in elicited cultures, suggesting that the supply of methionine towards SAM biosynthesis was saturated and being degraded or diverted towards the biosynthesis of other amino acids. Elicitation leads to increased SAM levels and presumably increased ethylene production but neither addition of ethylene nor the inhibition of ethylene biosynthesis affected BPA production. These results suggest that ethylene generated from increased SAM biosynthesis is not a mediator or inducer of BPA production.
7.0 CONCLUSIONS

The overall goal of this thesis was to investigate and identify the metabolic pathways that lead to increased secondary metabolite production in high alkaloid-producing cultures of the *E. californica*. Determining the metabolic pathways that are correlated to increased production suggests a potential mechanism for enhancing production in low-producing cultures. The specific objectives of this thesis were to 1) screen and identify *Eschscholzia californica* culture conditions yielding high production of benzophenanthridine alkaloids (>20-fold increase compared to untreated cultures); 2) identify proteins and metabolic pathways associated with increased production by applying global proteomics to compare high and low-producing cultures; and 3) further validate and elucidate the importance of key pathways identified by proteomic methods using cell culture experiments.

Production from *E. californica* cultures was increased using established methods including elicitation [16, 23, 67], *in situ* product removal [24], immobilization [25], and medium optimization [26]. These strategies have been used previously in *E. californica* cultures to increase benzophenanthridine alkaloid (BPA) production. However, few studies have attempted to use proteomics to understand changes in global metabolism in response to induction of alkaloid production in plant cell cultures [29]. In this thesis, elicitation, *in situ* extraction, immobilization, and medium optimization were investigated (Chapter 4). Elicitation using purified yeast extract (PYE) increased production by approximately 20-fold (over 20 mg of BPA/g dry weight) compared to unelicited cultures (Chapter 4; Section 1). BPA production increased with PYE dosages from 10 to 40 mg of PYE/g fresh weight (FW) and then leveled off, suggesting that production may be
limited by feedback inhibition of the BPAs at higher PYE dosages. Thus, cultures were treated with Amberlite XAD-7 resin to adsorb BPAs in situ and reduce feedback inhibition. Addition of XAD-7 combined with PYE elicitation increased production up to 63-fold (i.e. 85 mg/g DW) compared to untreated cultures (Chapter 4; Section 2). Production in elicited, resin-treated cultures also leveled off at higher dosages. The leveling off may have been due to saturation of the resin or another factor limiting production. Neither immobilization or medium composition improved BPA production significantly.

Next, a shotgun proteomic analysis of unelicited and PYE-elicited *E. californica* cultures (40 mg PYE/g FW) was performed to explore changes in metabolism associated with enhanced BPA production (Chapter 5). The *E. californica* genome has not been sequenced and annotated, leading to an additional challenge in the proteomic analysis. However, a published expressed sequence tag (EST) database [155] was available and was translated to protein sequences used for identifying proteins in our samples. Approximately 646 proteins (3% false positive rate at the protein level) were identified. Multiple enzymes involved in (S)-adenosyl methionine (SAM) biosynthesis were significantly more abundant in elicited cultures. SAM provides methyl groups for a range of biochemical reactions including DNA methylation, protein methylation, and alkaloid biosynthesis. Also, the enzyme 4OMT, which is involved in BPA biosynthesis, was also significantly more abundant in elicited cultures. The results demonstrate the utility of proteomics in identifying pathways associated with enhanced BPA production and also suggest an important role for methylation reactions in supporting enhanced BPA production.
The role of methylation reactions in supporting increased production was further explored using methionine feeding, inhibition of SAM biosynthesis, and treatment with ethylene (a plant hormone derived from SAM; Chapter 6). If SAM is limiting production in elicited cultures, then methionine feeding would be expected to increase SAM production since SAM synthetase was upregulated in the proteomic analysis. However, methionine feeding did not significantly affect the alkaloid profile or the total alkaloid production in either untreated or PYE-treated cultures. Analyzing the extracellular amino acid profile of elicited cultures fed with methionine confirmed that SAM and methionine biosynthesis was increased since the extracellular concentrations of amino acids related to the methionine biosynthetic pathway were increased in these cultures. The results suggest that the additional methionine was not limiting further increases in BPA production. SAM is also a precursor to the plant hormone ethylene. Cultures were also treated with the SAMS inhibitor cycloleucine which was unable to enter the cell. Ethylene production was induced by elicitation in *Papaver somniferum* cultures [79]. However, ethylene addition did not increase alkaloid production in *P. somniferum*, suggesting that increased ethylene production results from increased SAM production rather than required as an activator of BPA production [79]. To test this hypothesis, ethylene was added to unelicited *E. californica* cultures. Ethylene addition in our *E. californica* cultures did not affect production. Also, the ethylene biosynthesis inhibitor cobalt chloride did not affect production suggesting that ethylene is a byproduct rather than a mediator of elicitor-induced production.

In this thesis, the ability to increase BPA production from *E. californica* cultures was demonstrated and a proteomic analysis was performed to assess the global changes
in metabolism associated with increased BPA production. The best producing conditions were elicitation with 40 mg/g FW PYE in combination with \textit{in situ} extraction using Amberlite XAD-7 leading to a 63-fold increase in total BPA production compared to untreated cultures. A proteomic analysis of low and high producing cultures was then performed. Approximately 650 total proteins were identified which is significantly more than other proteomic studies with unsequenced medicinal plants [28-29]. Several proteins associated with SAM biosynthesis and a BPA biosynthetic enzyme (4OMT) were significantly more abundant in elicited cultures. The increased activity of the SAM pathway was confirmed using methionine feeding. The results demonstrated the ability of the proteomic platform to identify a large number of proteins from an unsequenced plant and also the ability to detect proteins and pathways associated with secondary metabolite production in plant cell culture. The role of SAM in supporting increased production will be further explored by investigating additional SAM-related pathways (\textit{e.g.} DNA methylation) as described in Chapter 8 (Recommendations).
8.0 RECOMMENDATIONS

This thesis presents the development of high-producing conditions in *Eschsholzia. californica* cell cultures, the application of proteomics for the identification of differentially abundant proteins in high-producing cell cultures, and the validation of the proteomics results using cell culture experiments. This thesis presents the first step in the application of proteomics to investigating global metabolism of high-alkaloid producing plant cell cultures. The recommendations for future research include:

1. Improve benzophenanthridine alkaloid (BPA) production by *in situ* extraction using cyclodextrin.

2. Improve the power of proteomics for detecting changes in important metabolic pathways by applying a more detailed protein fractionation method. Improved protein fractionation will increase the number of proteins identified and strengthen the identification of pathways associated with increased production in other high-producing conditions.

3. Validate the importance and the role of the methyl donor S-adenosylmethionine (SAM) using DNA methyltransferase inhibitor.

**Recommendation 1: Enhancement of BPA production by *in situ* extraction using cyclodextrin.** The methods investigated in Chapter 4 for increasing BPAs from *E. californica* cell cultures resulted in production levels of up to 85 mg BPAs / g dry weight (*i.e.* 8.5% by dry wt) using a combination of purified yeast extract elicitation and *in situ* extraction with Amberlite XAD-7. While elicitation combined with XAD-7 resin yielded the highest BPA production, the cells may be damaged by shearing as evidenced by lower cell dry weights. An alternative approach is to utilize a compound which binds to
BPAs produced by *E. californica* cells but which is soluble in aqueous media. For instance, cyclodextrins are cyclical oligosaccharides with a hydrophilic exterior and a hydrophobic interior shell. They have been used in the pharmaceutical industry to enhance drug bioavailability [208-209] and have also been used to increase both the productivity and the recovery of secreted plant secondary metabolites [210]. Cyclodextrins may enhance production by binding hydrophobic alkaloids to the inner shell as they are secreted by the cell and reducing feedback inhibition of the soluble alkaloids.

Several modified β-cyclodextrins (βCDs) have been used to enhance resveratrol production from grape cultures [211-212]. The combination of methyl jasmonate (MJ) and (2,6-di-O-methyl)-βCD (DIMEB) increased resveratrol production by over 5-fold, i.e. up to 1600 μmol / g DW compared to 300 μmol / g DW with MJ alone. Untreated cultures produced no significant resveratrol levels [212]. The most effective βCDs were (2,6-di-O-methyl)-βCD (DIMEB; 66.5 g/L or 50 mM) and 2-hydroxypropyl-βCD (HYPROB; 69 g/L or 50 mM) resulting in up to 14.8 and 13.3 mM of resveratrol, respectively compared to untreated cultures which did not produce resveratrol. Similarly, the combination of 100 mg/L βCD and fungal elicitor increased production of the sesquiterpene rishitin 3.3- and 2.7-fold (up to 200 μg/g DW) compared to βCD and fungal elicitation alone, respectively [213]. Lee et al. observed a 2.5-fold increase in digoxin production from *Digitalis lanata* suspension cultures when treated with 3.33 g/L βCD [214]. Cho and Pedersen [215] used a dansyl-glycine-β-cyclodextrin system to bind sanguinarine in extracts from *E. californica* cultures. The system demonstrated the binding of sanguinarine to the hydrophobic interior of the cyclodextrin molecules with
the resulting florescence signal used for alkaloid quantification. Bru et al. [211]
measured trans-resveratrol production by diluting spent medium with fresh medium,
filtering through a 0.2 \( \mu \text{m} \) filter and directly injecting into an HPLC suggesting that
cyclodextrins are compatible with downstream processing steps.

A future recommendation for this research is adding cyclodextrins (\( \beta \text{CD}, \text{DIMEB}, \text{and HYPROB} \)) at final concentrations of 6.25, 12.5 and 25 mM. Komariah et
al. [213] used a 100 mg/L cyclodextrin solution and observed production equivalent to a
6:1 cyclodextrin to metabolite ratio. Bru et al. [211] used a 5 – 50 mM cyclodextrin
solutions and observed up to a 3.5:1 cyclodextrin to metabolite ratio. The highest
production observed in Chapter 4 was 85 mg total BPAs / g DW (approximately 2.5
mM). Thus CDs will be added in 2.5, 5, and 10:1 mole ratios to PYE-treated cultures to
increase production without damaging the cells. If increased production is observed
using the cyclodextrin and elicitor-treated cells, proteomic methods can be applied to this
enhanced condition and the analysis extended to multiple culture conditions and time
points. The goal is to identify pathways which are induced when feedback inhibition is
removed and production is enhanced beyond the 30 mg/g DW observed with elicitation
alone.

**Recommendation 2: Increase Protein Identification using Differential
Centrifugation as a Fractionation Method.** Increased protein identification strengthens
the conclusions drawn from proteomic studies. The use of additional protein and peptide
separation techniques has led to increased protein identification. For instance, in plants,
protein fractionation by anion exchange chromatography followed by two-dimensional
liquid chromatographic peptide separation increased the number of proteins identified
from *Arabidopsis thaliana* by 3.5-fold compared to two-dimensional liquid chromatographic peptide separation alone [122]. Chromatographic separation methods produce fractions enriched in proteins with particular physical properties (e.g. hydrophobicity, size, glycosylation).

As an alternative, differential centrifugation can be used to produce fractions enriched based on function due to subcellular localization. In this thesis, a crude separation utilizing differential centrifugation was utilized. Samples were separated into a cytosol-enriched fraction and a membrane-enriched fraction and only the cytosol-enriched fraction was analyzed. Potentially key enzymes including NADPH-producing enzymes from the pentose phosphate pathway (observed in both the cytosol and plastid fractions in plants [216]) and cytochrome P450 enzymes from the BPA biosynthetic pathway (associated with the endoplasmic reticulum [41, 50]) are membrane-associated proteins and would not have been identified in our proteomic study. Other potentially key proteins not identified in our proteomic study such as transcription factors and DNA methyltransferases may be localized to the nucleus.

Arnold et al. [121] used differential centrifugation to prepare nuclear, mitochondrial, cytosolic, and microsomal fractions leading to 50% more protein identifications. In their differential centrifugation method, the protein is extracted and centrifuged at a low speed (1,000 g). The pellet is saved as a nuclear-protein enriched fraction and the supernatant is then centrifuged at 15,000 g to obtain a mitochondrial protein-enriched fraction. At 100,000 g, the resulting supernatant is enriched in cytosolic proteins and the pellet is enriched in microsomal proteins. Differential centrifugation was also used to identify seven of the ten glycolytic enzymes in a mitochondrial fraction.
from *Arabidopsis* cells [217]. Millar et al. used differential centrifugation to isolate mitochondrial proteins from *Arabidopsis* cell cultures leading to identification of all of the enzymes from the TCA cycle [218].

Differential centrifugation was used by several studies to identify nuclear proteins from plants. Repetto et al. [219] identified transcription factors, chromatin-modifying enzymes, and DNA-methylating enzymes from *Medicago truncatula* seed protein extracts centrifuged at 1,200 g. Aki et al. [220] used differential centrifugation (1,000 g) and DNA-affinity chromatography to produce nuclear fractions enriched in histones. These results suggest subcellular fractionation is an effective method for targeting nuclear proteins (e.g. transcription factors and DNA methyltransferases). Identifying transcription factors and DNA methyltransferases will strengthen the power of the proteomics experiments to detect changes in pathways which correlate with changes in BPA production.

Thus, a differential centrifugation method for protein separation is proposed to potentially yield fractions enriched in proteins of interest (e.g. pentose phosphate, cytochrome P450, transcription factors) localized to specific locations (mitochondria, membrane-bound organelles, and nucleus, respectively) while minimizing variability and protein losses that occur using chromatographic methods for peptide or protein separation. The proposed method will generate four fractions: Pellets from the 1,000 g (nuclear proteins, transcription factors), 15,000 g (mitochondria), and 100,000 g (cytochrome P450 enzymes) centrifugations and the supernatant from the 100,000 g centrifugation.
Recommendation 3: Validate the importance and the role of the methyl donor S-adenosylmethionine (SAM) using DNA methyltransferase inhibitors. The proteomics experiment and subsequent methionine feeding experiment indicated increased activity through the SAM biosynthetic pathway in high-producing cultures of *E. californica*. Methionine feeding to these high-producing elicited cultures did not enhance further production, indicating additional SAM was not required. More studies should be performed to determine the fate of the increased SAM produced by elicitation.

SAM is produced in response to several factors (nutrient stress and induction using biological extracts and hormones). For instance, SAM synthetase (SAMS) increased in abundance in response to aluminum stress in rice roots [221] or was induced by methyl jasmonate treatment in tobacco cell cultures [222]. Thus determining how SAM is used by the cell is important for investigating pathways and mechanisms leading to increased secondary metabolite production.

In addition to methylation of BPA pathway intermediates, SAM is also used in the biosynthesis of several other compounds including spermine, ethylene, and in other methylation reactions such as DNA methylation. In spermine biosynthesis, SAM is decarboxylated and then converted by spermine synthase. Spermine has been shown to enhance DNA synthesis [223] and also protect DNA from reactive oxygen species [224]. Inhibition of spermine biosynthesis decreased DNA synthesis in *Catharanthus roseus* suspension cultures [225]. The role of ethylene in supporting enhanced BPA production was investigated in Chapter 6. In summary, ethylene addition did not affect BPA production in unelicited cultures. Ethylene inhibition using cobalt chloride did not affect
BPA production in elicited cultures suggesting that ethylene is not necessary for elicitor-induced BPA production.

DNA methylation also utilizes SAM as a methyl donor. DNA methylation is believed to regulate transcription [226-227] by methylating the promoter regions [228]. DNA methylation is believed to inhibit transcription by interfering with transcription factor binding [229]. Also, methylated DNA attracts proteins which bind to methylated DNA [230-231] and activate histone deacetylases leading to chromatin remodeling [232-233]. In mammalian culture systems, sodium butyrate is believed to enhance recombinant therapeutic protein production by inhibiting histone deacetylation [234-236], suggesting that the acetylation state can affect global metabolism.

The effect of DNA methylation and the inhibitory effect on secondary metabolite production has not been studied extensively in plant cell culture. The DNA methylation inhibitors 5-azacytidine (AzaC, 10 and 20 μM) and 5-aza-2'-deoxycytidine (AzadC, 1 and 5 μM) inhibited shoot bud regeneration in Petunia leaf disks [237]. Although metabolite production was not measured, a decrease in cytosine methylation was observed confirming the de-methylating ability of AzaC and AzadC. AzaC and AzadC are cytosine analogs which are incorporated into DNA but cannot be methylated [238]. To follow-up on results from Chapter 6, the addition of DNA methylation inhibitors AzaC (5, 10, 20, and 50 μM) and AzadC (0.1, 1.0, 5.0, and 10.0 μM) to unelicited and PYE-elicited (40 mg/g FW) cultures is proposed for investigating the role of DNA methylation on secondary metabolite production; the concentrations proposed were used by Prakash and Kumar [237] in the initial experiment. The effect of AzaC or AzadC on BPA production in E. californica cultures has not yet been studied.
9.0 REFERENCES


46. Pauli, H.H. and T.M. Kutchan, *Molecular Cloning and Functional Heterologous Expression of Two Alleles Encoding (S)-N-Methylcoclaurine 3’-Hydroxylase (Cyp80b1), a New Methyl Jasmonate-Inducible Cytochrome P-450-Dependent...*


10.0 APPENDICES
Appendix A

Reproducibility of Elicitation in Reduced Nitrate Media
Appendix A

Reproducibility of Elicitation in Reduced Nitrate Media

In Chapter 4, *E. californica* cultures grown in reduced nitrate media were treated with PYE. Reduced nitrate did not enhance BPA production despite reports in the literature where reduced nitrate increased BPA production [26]. This experiment was repeated several times and reduced nitrate consistently did not increase production in elicited cultures.

Elicitation of BPA production in *E. californica* in reduced nitrate media. Cells were cultured in LS media with reduced (18.8 mM) and normal (39.4 mM) nitrate concentrations. Cultures were grown for 7 days, then elicited with PYE (40 mg/g FW) and harvested 96 hours after elicitation. Error bars represent standard deviation from triplicate measurements. Abbreviations: DHCHY: Dihydrochelerythrine; CHY: Chelerythrine; DHSAl: Dihydrosanguinarine; SA: Sanguinarine; DHCHR: Dihydrochelirubine; CHR: Chelirubine.
Appendix B

Reproducibility of Proteomic Methods
Appendix B

Reproducibility of Proteomic Methods

The proteomics experiments (Chapter 5) involve several steps thereby propagating the error. First, two biological replicates for each experimental condition were used. Each biological replicate was processed separately from protein extraction up through ultracentrifugation (See Chapter 3 for proteomic workflow). From each biological replicate, two aliquots were digested, cleaned up by HPLC, and analyzed by mass spectrometry separately. The peak areas from HPLC cleanup, base peak intensity (measure of abundance in mass spectrometry), and the total number of proteins and peptides identified were used as markers of reproducibility in proteomics experiment.

<table>
<thead>
<tr>
<th></th>
<th>Peak area</th>
<th>Base peak intensity</th>
<th>Total peptides</th>
<th>Total proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1A</td>
<td>8.2E+06</td>
<td>1.6E+05</td>
<td>507</td>
<td>227</td>
</tr>
<tr>
<td>C1B</td>
<td>3.4E+07</td>
<td>2.4E+06</td>
<td>1132</td>
<td>353</td>
</tr>
<tr>
<td>C2A</td>
<td>4.0E+07</td>
<td>2.9E+06</td>
<td>1110</td>
<td>342</td>
</tr>
<tr>
<td>C2B</td>
<td>9.0E+07</td>
<td>6.8E+05</td>
<td>1162</td>
<td>375</td>
</tr>
<tr>
<td>E1A</td>
<td>6.5E+07</td>
<td>2.2E+06</td>
<td>1332</td>
<td>370</td>
</tr>
<tr>
<td>E1B</td>
<td>4.1E+07</td>
<td>1.7E+06</td>
<td>1174</td>
<td>368</td>
</tr>
<tr>
<td>E2A</td>
<td>7.9E+07</td>
<td>1.9E+06</td>
<td>1253</td>
<td>373</td>
</tr>
<tr>
<td>E2B</td>
<td>3.0E+07</td>
<td>1.2E+06</td>
<td>1141</td>
<td>365</td>
</tr>
<tr>
<td>r²</td>
<td>0.49</td>
<td>0.26</td>
<td>0.94</td>
<td></td>
</tr>
</tbody>
</table>

As shown in table 1, neither peptide cleanup HPLC peak areas nor base peak intensity were correlated with total proteins. Excluding sample C1A due to operator error, there was approximately 3% variation in total proteins. The low variability in total proteins identified despite the large variation in HPLC peak areas is possibly due to overloading of the trap column. The samples were prepared from 100 μg of protein extract by digestion, reversed-phase HPLC cleanup, and concentration of the peptide fraction to 20
μl. Then, 5 μl of the 20 μl sample (less than 20 μg) were injected but only a small amount which was the same for each sample was eluted from the trap column. Therefore, the data spectral counts data reported in Chapter 5 is based on the same amount of peptides entering the mass spectrometer.

Variation in Identified Proteins between Biological Replicates

The spectral count method was used as a measure of relative protein abundance. The variability between biological replicates (e.g. C2A vs C2B) is shown below. The number of spectral counts for each protein in one replicate is plotted versus the number of spectral counts for the same protein in the biological replicate.

Reproducibility of the spectral counts method. The number of spectral counts for each protein in one replicate is plotted versus the number of spectral counts for the same protein in the biological replicate.
$y = 0.8814x + 0.0582$

$R^2 = 0.8576$
Appendix C

Effect of cycloleucine and ethephon on BPA production
Appendix C

Effect of cycloleucine and ethephon on BPA production

In chapter 5, three enzymes from the SAM biosynthetic pathway were more abundant in elicited cultures. Specifically, SAM synthetase (SAMS) which converts methionine to SAM was induced in elicited cultures. To test the role of SAMS, cycloleucine was added to elicited cultures. Cycloleucine is a synthetic amino acid which has been used to inhibit SAMS enzyme. For instance, 1 mM cycloleucine inhibited SAMS enzyme activity in *C. roseus* extracts up to 57% [239]. Cycloleucine (5 - 20 μM) also inhibited SAM production in rat hepatocytes without any effect on cell viability [202].

The importance of SAM in supporting BPA production was investigated by adding cycloleucine to the cultures. Inhibiting SAMS would reduce the pool of available SAM for methylation reactions. Cycloleucine was added to elicited cultures (40 mg/g FW PYE) on day 7 with the addition of elicitors or postponed until day 9 (2 days after elicitation) when the rate of BPA production is highest.
Effect of cycloleucine on BPA production in elicited E. californica suspension cultures grown with 30 g/L sucrose, Linsmaier Skoog salts and 0.11 g/L NAA. Cycloleucine (0.1, 0.5, and 1.0 mM) was added on day 7 (left) or day 9 (right). Cells were harvested on day 11. Error bars represent standard deviation from triplicate measurements. Cycloleucine treated samples which are significantly different (p<0.05 using Student’s t-test in Excel) from elicited samples are designated by *.

Cycloleucine was added at dosages of 0, 0.1, 0.5, and 1 mM to E. californica cultures to inhibit the enhanced levels of SAM synthase observed in yeast-elicited cultures (SAMS; pathway shown in Fig. 2). As shown in Fig. 5, cycloleucine treatments on day 7 appeared to increase BPA production in elicited cultures by 23 – 37%; only treatment with 0.1 mM cycloleucine significantly increased BPA production compared to yeast elicitation alone. An explanation for the lack of effect on BPA production is that cycloleucine was not able to diffuse into the cell and thus had no effect on the cell. Cycloleucine addition was also postponed until day 9 to allow cycloleucine to specifically act on SAM synthase when BPAs accumulation was most rapid. If SAM synthase was critical for BPA production, then the addition of cycloleucine would be expected to also decrease BPA production. Note the result when cycloleucine was
postponed until day 9 & then summarize what this means. Addition of 0.5 or 1.0 mM cycloleucine on day 9 did not affect production in elicited cultures suggesting that sufficient methionine for BPA biosynthesis was already produced by elicitation prior to day 9.

To test if cycloleucine was able to enter the cell, spent media from elicited, cultures treated with 500 μM cycloleucine was derivatized using the amino-acid labeling method described in Chapter 6. Also, the cycloleucine concentration in cell-free media immediately after addition and after 96 hours of incubation was measured.

**Cycloleucine concentration in cell-free media spiked with 0.5 μM cycloleucine and incubated for 0 or 96 hours, and conditioned media from cultures elicited on day 7 with 40 mg/g FW of PYE and treated with 0.5 μM cycloleucine for 96 hours. Values are the average of duplicate measurements.**

<table>
<thead>
<tr>
<th>Sample</th>
<th>cLEU concentration, μM</th>
<th>% of initial cycloleucine remaining</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell free media, no incubation</td>
<td>389</td>
<td>78</td>
</tr>
<tr>
<td>Cell free media, 96 hour incubation</td>
<td>325</td>
<td>65</td>
</tr>
<tr>
<td>Conditioned media, 96 hours</td>
<td>215</td>
<td>43</td>
</tr>
</tbody>
</table>

In cell free media, cycloleucine concentration was reduced by 16% after 96 hours of incubation possibly due to cycloleucine breakdown. In conditioned media, cycloleucine levels were reduced by 45% compared to cell free media with no incubation. The results suggest that because the cycloleucine only partially entered the cell, the intracellular cycloleucine concentration was still low and SAMS was not inhibited.

The effect of ethylene addition on BPA production was examined by adding ethephon which produces ethylene gas as it dissolves. In chapter 6, adding ethephon had no effect on BPA production in untreated cultures. Ethylene production from ethephon-treated cultures, ethephon stock solutions, and elicited cultures was monitored by gas chromatography.
Ethylene production was measured from unelicited flasks, elicited flasks (40 mg/g FW PYE added on day 7), elicited flasks treated with 0.7 mM CoCl₂, flasks treated with 1000 μM ethephon, and a 1000 μM ethephon solution. A representative chromatogram from each condition is shown above.

As shown in the figure above, the ethephon standard solution and ethephon-treated flask produced a single peak corresponding to ethylene gas. Neither unelicited nor elicited flasks produced detectable amounts of ethylene. The lack of ethylene production in elicited cultures is consistent with ethylene not being required for elicitor-induced BPA production as suggested in Chapter 6.