CALIFORNIA STATE UNIVERSITY SAN MARCOS

THESIS SIGNATURE PAGE

THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE

MASTER OF SCIENCE

IN

BIOLOGICAL SCIENCES

THESIS TITLE: DEFINING THE ROLE OF POLYPHENOL OXIDASE IN DOPMINE AND ESCULETIN BIOSYNTHESIS IN PLANTS

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DATE OF SUCCESSFUL DEFENSE: July 14th, 2016

THE THESIS HAS BEEN ACCEPTED BY THE THESIS COMMITTEE IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN BIOLOGICAL SCIENCES.

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Defining the roles of polyphenol oxidase in dopamine and esculetin biosynthesis in plants

By: Laura Walters

August 2016

Abstract:

Although the enzyme polyphenol oxidase (PPO) is well studied for its role in postharvest browning of commercial crops, its specific physiological function in plants is poorly understood. Previous research on the physiological role of PPO in walnut (*Juglans regia*) suggested that PPO is involved in secondary metabolism, namely in dopamine and esculetin biosynthesis. Dopamine is a precursor to plant-synthesized benzylisoquinoline alkaloids (BIAs) such as codeine and morphine, and esculetin is an antioxidant with promising anticancer properties. This study was performed to further define the role of PPO in the synthesis of esculetin and dopamine-derived metabolites in plants, using walnut, California poppy (*Eschscholzia californica*), and red clover (*Trifolium pratense*) as experimental systems. PPO enzyme kinetics and substrate specificity were characterized in each plant system, and PPO function was studied in plants displaying suppressed PPO gene expression (walnut, red clover) or inhibited PPO enzyme activity (poppy). Substrate specificity assays with red clover PPO revealed a lack of activity with substrates relevant to dopamine and esculetin biosynthesis. GC-MS analysis revealed the absence of both dopamine and esculetin in wild-type lines. Collectively, these results suggest that PPO is not involved in dopamine or esculetin biosynthesis in red clover. In walnut, *in vitro* enzyme kinetic assays suggest that tyramine and caffeic acid are the preferred substrates in PPO-mediated dopamine and esculetin synthesis, respectively. Poppy cell cultures in which PPO enzyme activity was chemically inhibited using the inhibitor kojic acid had significantly reduced BIA content in the culture supernatant compared to control samples. Overall, these results suggest that the previously characterized role of walnut PPO in dopamine and esculetin biosynthesis is not shared in all plant species. Instead, this PPO function may be taxonomically limited, much like previously characterized roles for specific PPO enzymes in betalain synthesis in beets (order Caryophyllales) and aurone synthesis in snapdragons (*Antirrhinum majus*).

Keywords: polyphenol oxidase, dopamine, esculetin, plant secondary metabolism, *Juglans regia*, *Trifolium pratense*, *Eschscholzia californica*

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Introduction:

The polyphenol oxidase enzyme

Polyphenol oxidase (PPO) is a type 3 copper-containing enzyme with two enzymatic activities: PPO can act as a cresolase to catalyze the *o*-hydroxylation of monophenols into diphenols (EC 1.14.18.1), and/or PPO can act as a catechol oxidase (EC 1.10.3.1) to catalyze the oxidation of *o*-diphenols to the corresponding *o*-quinone products (Figure 1). The *o*-quinones can undergo further autoxidation, resulting in the formation of complex brown polymers known as phytomelanins (Mesquita and Queiroz, 2013). PPO enzymes are found across the kingdoms of life, however the ability of specific PPO enzymes to perform cresolase and catechol oxidase activities varies (Sullivan, 2015).

In plants, the PPO enzyme is typically bound to thylakoid membranes in chloroplasts, while the phenolic substrates of PPO are primarily found in vacuoles and the cell wall (Steffens *et al.*1994). Physical damage to plant tissue causes disruption of organelle membranes, and the separation of the enzyme and substrates seen in intact cells is lost. PPO and the phenolic compounds are released into the cytosol, where they come into contact, leading to the production of phytomelanins (Steffens *et al.*1994). In cut fruit or vegetables, this process is known as postharvest browning, and it has substantial implications for the storage and transport of produce (Mesquita and Queiroz, 2013).

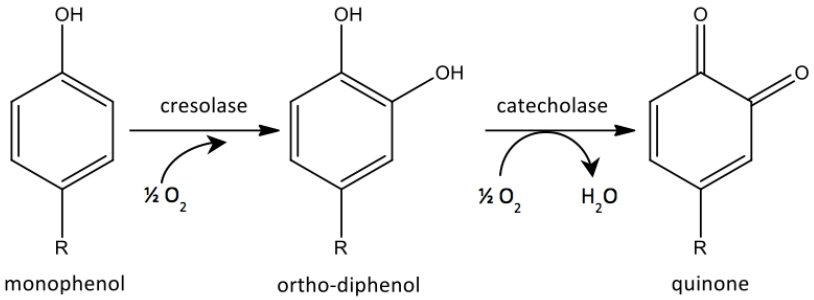

Figure 1: Enzymatic activity of polyphenol oxidase (from Gertzen and Escobar, 2014).

Several studies have suggested that PPO may play a role in plant defense against insects and pathogens. In some plant species, PPO shows increased expression and activity in response to damage caused by herbivores (Haruta *et al.,* 2001) and pathogens (Niranjan *et al.,* 2006; Taguri *et al.,* 2004). Thipyapong *et al.* (2004) found that RNAi-based silencing of PPO in tomato resulted in increased susceptibility to the bacterial pathogen *Pseudomonas syringe* pv. *tomato*. Some research suggests that products of the PPO reaction, such as quinone adducts, are responsible for these anti-pathogen and anti-herbivore responses in plants (Constable *et al.,* 1995; Melo *et al.,* 2006; Thipyapong *et al.,* 2004). Although much research has been performed on the role of PPO in pathogen response and browning, less is known about potential physiological roles of PPO in undamaged, intact plant cells.

Several studies investigating the native physiological functions of PPO have revealed specialized roles of specific PPO enzymes in plant secondary metabolism. For example, the aureusidin synthase enzyme from snapdragon flowers (*Antirrhinum majus*) was identified as a PPO (Nakayama *et al.,* 2001). This PPO converts 2,4,6,4-tetrahydroxychalcone to a quinone product through a combination of cresolase and catechol oxidase activities, and these quinone products spontaneously convert to aurones, the yellow pigment in snapdragon flowers (Nakayama *et al.,* 2001). Another specialized metabolic role of PPO was found in betalain-producing plants (Sullivan, 2015; Gandía-Herrero and García-Carmona, 2013). Betalains are yellow and violet pigments found in the order Caryophyllales (Gandía-Herrero and García-Carmona, 2013). PPO has an early role in the betalain biosynthesis pathway, catalyzing the formation of L-DOPA through the *o*-hydroxylation of tyrosine. L-DOPA is further modified by other enzymes into betalain pigments (Sullivan, 2015). Aside from involvement in pigment synthesis, PPO has also been characterized in 8-8' linked lignan synthesis in creosote bush

(*Larrea tridentata*) (Cho *et al.*, 2003). Structural characterization of the hydroxylase responsible for an intermediary step in the synthesis of the lignin intermediate nordihydroguaiaretic acid revealed that it was a PPO enzyme.

Most recently, several novel metabolic roles for PPO have been characterized using walnut as a model organism. Walnut is a good model to study the physiological functions of PPO because it has a small PPO gene family and it produces a diverse array of phenolic compounds (Escobar *et al.*, 2008; Martínez-García *et al*., 2016). The size of PPO gene families in land plants varies per species, and a recent study found from zero to thirteen PPO genes in 25 plants surveyed (Tran *et al.*, 2012). Walnut has only two PPO genes, and RNA silencing of *jrPPO1*, which encodes the PPO expressed in green tissues, caused the development of necrotic lesions on mature leaves (Araji *et al.*, 2014). Metabolite and transcript profiling analyses to investigate the cause of these lesions revealed major alterations in two metabolic pathways: tyrosine catabolism and esculetin biosynthesis.

Role of polyphenol oxidase in tyrosine and dopamine metabolism

Araji *et al.* (2014) showed that dopamine levels were significantly decreased while tocopherol and tyramine levels were significantly increased in PPO-silenced walnut lines as compared to wild-type (Figure 2). With this information, Araji *et al.* (2014) concluded that PPO probably acts in the *o*-hydroxylation of tyrosine and/or tyramine, leading to the production of dopamine. In PPO-silenced lines, tyrosine is shunted into alternative catabolic pathways, increasing the levels of tyramine and tocopherols (Figure 2).

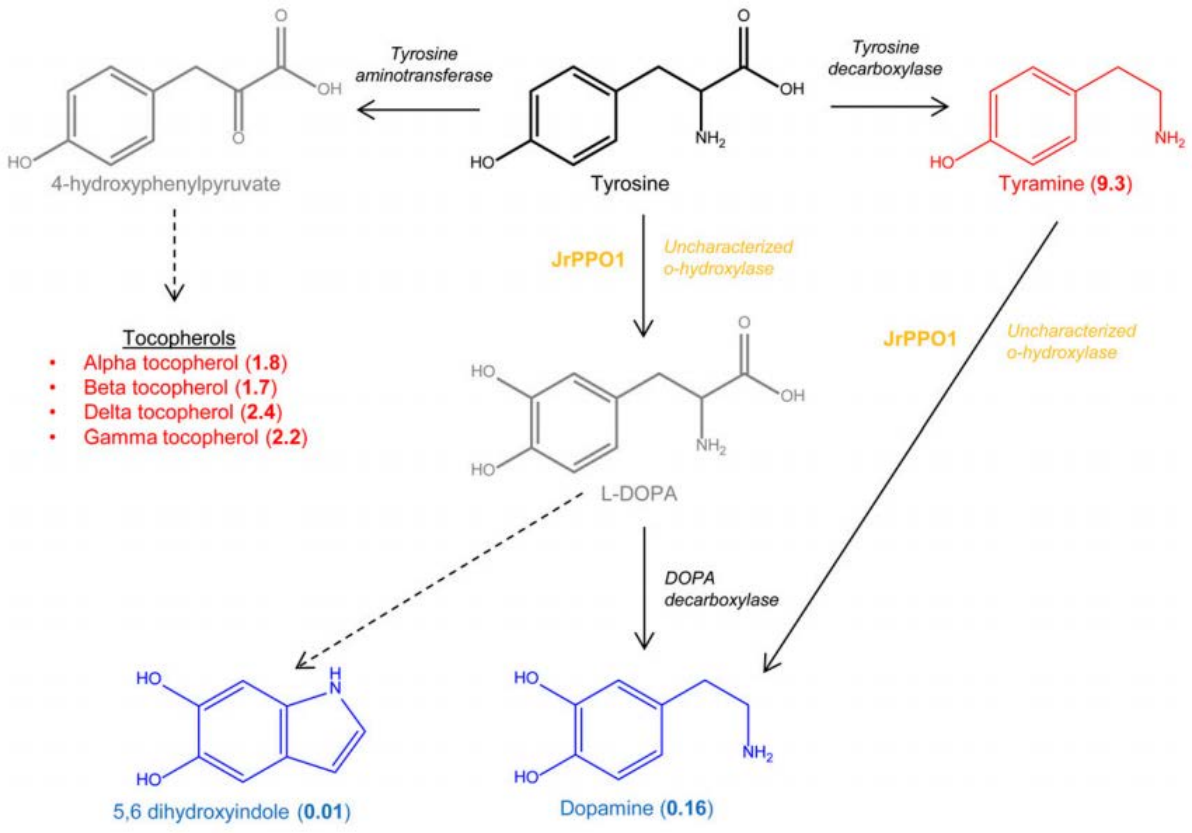

Figure 2: Alterations in tyrosine metabolism in *jrPPO1*-silenced walnut (from Araji *et al.*, 2014). Tyramine and tocopherol levels were significantly increased (red) compared to wild-type and dopamine levels were significantly reduced (blue). Values in parentheses represent the fold change (PPO-silenced lines/ wild-type). Metabolites in grey were undetected and metabolites in black had no change. Dashed arrows represent multiple enzyme-catalyzed reactions. The reactions Araji *et al.* (2014) proposed to be catalyzed by JrPPO1 are in orange.

Dopamine metabolism is a poorly studied area of plant secondary metabolism (Araji *et al.*, 2014). However, in some plant species dopamine acts as a precursor of benzylisoquinoline alkaloids (BIAs), a biomedically-important group of secondary metabolites. BIA biosynthesis has been most extensively studied in poppies (Farrow *et al.*, 2012). BIAs are formed through the linkage of 4-hydroxyphenylacetaldehyde and dopamine to form the precursor molecule to all BIAs, (S)-norcoclaurine (Figure 3) (Lee *et al.*, 2011). Among the diverse BIA compounds, biomedically-important molecules include the analgesics morphine and codeine, the

antimicrobials sanguinarine and berberine, and anti-cough agent noscapine (Hagel *et al.*, 2013; Facchini, 2001). The biosynthetic pathway for BIAs has been mostly mapped out, however, the enzyme responsible for the hydroxylation of tyrosine into L-DOPA is unknown (Hagel *et al.*, 2013). There is controversy over the identity of this enzyme. Research by Paulie *et al.* (1998) suggested that a cytochrome-P450 was responsible for this step based on enzyme kinetic studies, while Loeffler *et al.* (1990) suggested that a polyphenol oxidase (which was partially purified from poppy) was responsible. *In vivo* studies are needed to determine whether PPO is directly involved in dopamine (and BIA) synthesis in poppy, mirroring the enzyme's characterized metabolic function in walnut (Araji *et al.*, 2014).

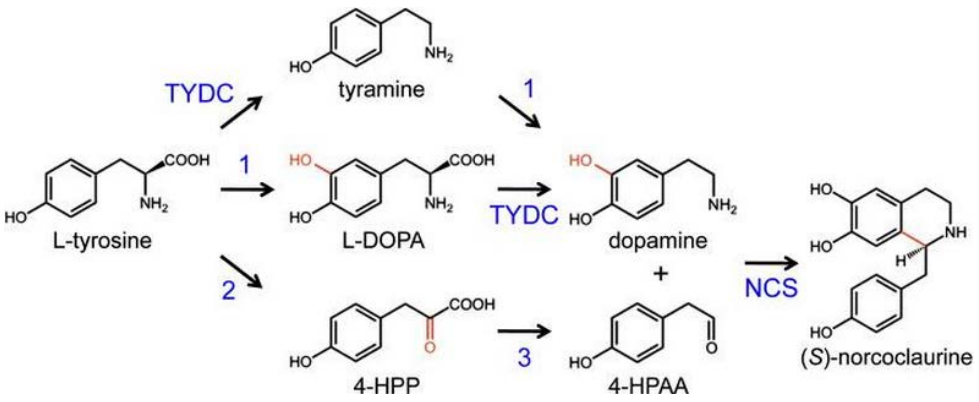

Figure 3: Initial steps in BIA synthesis in poppy (from Lee *et al.,* 2011). Enzyme #1 is a putative PPO.

Role of polyphenol oxidase in esculetin synthesis

Araji *et al.* (2014) found a highly significant reduction in the level of the hydroxycoumarin esculetin in the leaves of PPO-silenced walnut lines, compared to wild type. Interestingly, PPO involvement in esculetin synthesis has previously been investigated by Sato (1967) and Garcia-Molina *et al.* (2013). However, these two studies suggest different substrates for PPO in esculetin synthesis. Sato (1967) proposed that PPO directly hydroxylates

umbelliferone into esculetin, while Garcia-Molina *et al.* (2013) proposed that PPO oxidizes caffeic acid into an early precursor of esculetin (Figure 4). The function and abundance of esculetin in plants is not well understood, but it has been shown to have substantial antioxidant capacity *in vitro* (Vianna *et al.*, 2012). Similar to BIAs, esculetin is a biomedically-important molecule. Recently, esculetin was found to have antiproliferative effects on hepatic tumor growth in mice (Wang *et al.*, 2015).

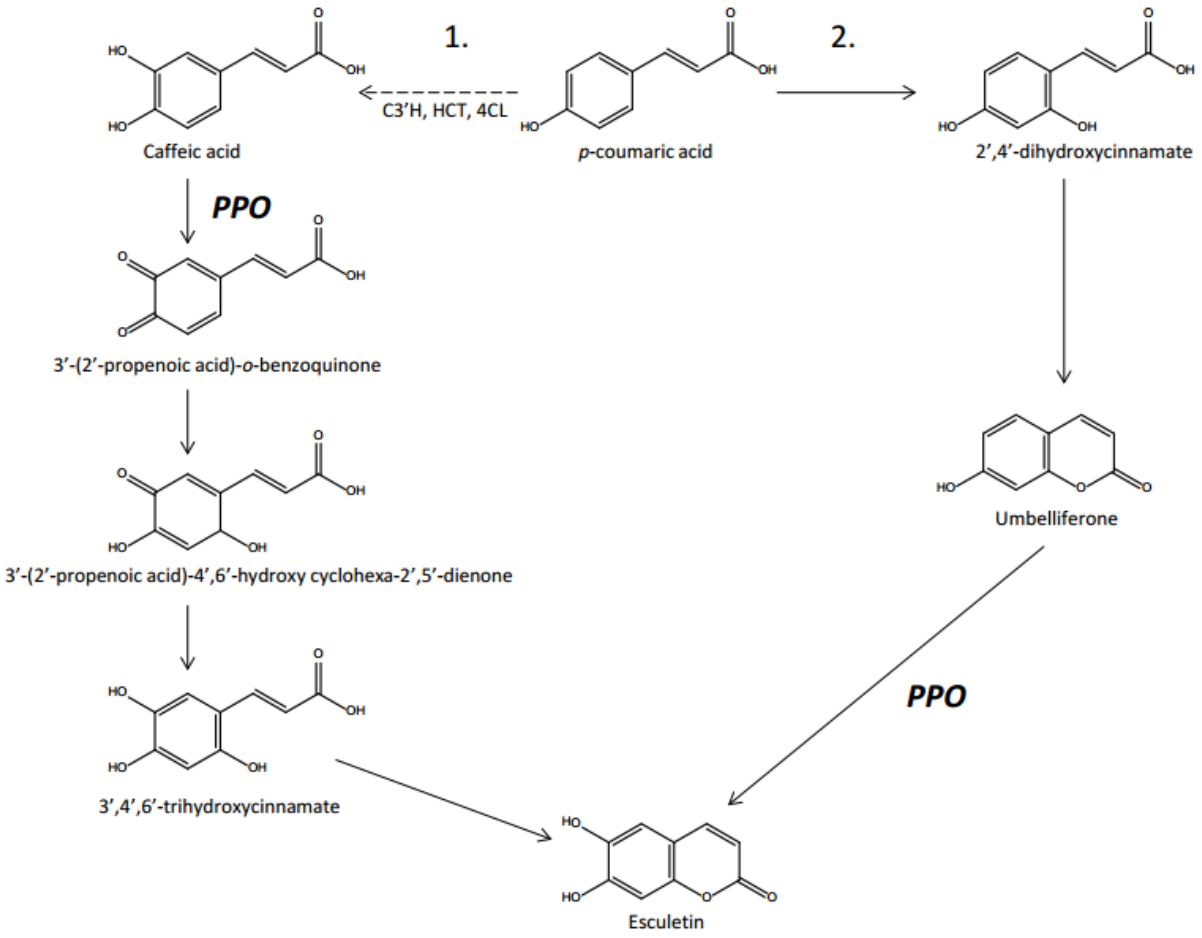

Figure 4: Possible roles for PPO in esculetin synthesis. Sato (1967) suggested that PPO catalyzes the oxidation of caffeic acid (pathway 1) and Garcia-Molina *et al.* (2013) suggested that PPO catalyzes the hydroxylation of umbelliferone directly into esculetin (pathway 2) (From Araji *et al.* (2014).

Analysis of PPO activity and function:

As outlined above, several questions remain regarding the role of PPO in plant secondary metabolism:

1) In walnut, is the native substrate of PPO tyrosine, tyramine, or both (Figure 2)?
2) In walnut, what is the specific role of PPO in the synthesis of esculetin (Figure 4)?
3) Is PPO involved in the synthesis of dopamine-derived BIAs (Figure 3)?
4) Is the role of PPO in dopamine and esculetin synthesis broadly conserved in plants other than walnut?

In order to answer these questions, PPO activity was manipulated in three plant species: walnut (*Juglans regia*), red clover (*Trifolium pratense*), and California poppy (*Eschscholzia californica*). Previous studies have utilized RNAi to essentially abolish PPO activity in the leaves of transgenic walnut and red clover plants (Araji *et al.,* 2014; Sullivan and Hatfield, 2006). In this work, we have subjected PPO-silenced walnut and red clover lines to targeted metabolite profiling to analyze the effects of PPO-silencing on dopamine, dopamine-derived metabolites, and esculetin. California poppy, which produces dopamine-derived BIAs, has a large (20 putative PPO genes, Table 3) and diverse PPO gene family. The use of a genetic strategy to analyze PPO function in this species would be very challenging. Thus, suspension cultures of poppy cells (Farrow *et al.*, 2012) were treated with kojic acid, a specific chemical inhibitor of PPO enzyme activity (Mayer, 2006; Mesquita and Queiroz, 2013; Chang, 2009). Again, effects on dopamine and BIA production were characterized by metabolite profiling. Finally, *in vitro* substrate specificity and enzyme kinetic studies were performed on PPOs from walnut, red clover, and California poppy to identify possible PPO substrates involved in secondary metabolism.

Objectives:

Objective 1: To characterize walnut, California poppy, and red clover PPO enzyme kinetics with respect to putative substrates involved in the synthesis of dopamine and esculetin.

Specific Aim 1.1: To determine the enzyme kinetics of PPO using tyrosine and tyramine (dopamine precursors) as substrates.

Hypothesis 1.1: PPOs from walnut, poppy, and red clover will use tyrosine and tyramine as substrates.

Specific Aim 1.2: To determine the enzyme kinetics of PPO using umbelliferone and caffeic acid (esculetin precursors) as substrates.

Hypothesis 1.2: PPOs from walnut, poppy, and red clover will use umbelliferone and caffeic acid as substrates.

Objective 2: To characterize the metabolic effects of silencing PPO in red clover.

Specific Aim 2.1: To perform targeted metabolite profiling of dopamine in red clover leaf extracts from wild type and PPO-silenced walnut lines

Hypothesis 2.1: PPO-silenced red clover extracts will have lower levels of dopamine than wild type extracts.

Specific Aim 2.2: To perform targeted metabolite profiling of esculetin in red clover leaf extracts from wild type and PPO-silenced lines.

Hypothesis 2.2: Esculetin levels will be lower in PPO-silenced red clover lines.

Objective 3: To characterize the metabolic effects of inhibiting PPO activity in California poppy.

Specific Aim 3.1: To perform targeted metabolite profiling of BIAs in poppy cell cultures treated with chemical inhibitors of PPO.

Hypothesis 3.1: BIAs, which are constitutively produced in *Eschscholzia californica* poppy cell cultures, will decrease in cell cultures grown in the presence of PPO inhibitors.

Materials and methods:

Protein extraction

Mature walnut leaves, mature California poppy roots, and young red clover shoots were collected and flash frozen in liquid nitrogen. Young tissue was used for red clover because PPO1 is primarily expressed in young foliage (Sullivan *et al*., 2004). Total protein was extracted as described in Escobar *et al.* (2008). Protein concentrations were determined by Bradford assay (Bradford, 1976) using bovine serum albumin as a protein standard.

Substrate specificity and enzyme kinetic assays

PPO activity assays were performed on a Clark-type oxygen electrode to determine pH and temperature optima, V_{max}, and K_m of PPO with respect each putative substrate: umbelliferone, caffeic acid, tyrosine and tyramine. Substrate reactions consisted of 1 mL of substrate buffer (100 mM sodium phosphate buffer pH 7.0, 0.15% [w/v] SDS, 8 mM substrate unless otherwise stated) and 25 µL of total protein extract. Activity was measured by calculating nmoles of oxygen consumed per milligram of total protein. Only the initial reaction rate was considered for PPO activity because quinone products can oxidize proteins, reducing the activity of the PPO enzyme (Gertzen and Escobar, 2014). Control assays were run in parallel and included: substrate reaction in the presence of the PPO inhibitor, 2 mM tropolone (Fuerst *et al.,* 2006) as a negative control, and substrate buffers in the absence of protein extract to determine background auto-oxidation levels. Auto-oxidation levels were subtracted from the PPO activity measured for each reaction. The substrate assay buffer was prepared at pH 5, 6, 7, 8, and 9 to determine pH optima. To determine temperature optima, activity assays were tested at 10° C, 25° C, 37° C, and 55° C using the optimum pH buffer. Kinetic assays were performed by testing PPO activity over a range of substrate concentrations (caffeic acid: 0.25-8.0 mM, tyramine: 0.03-

4 mM, tyrosine: 0.125-8 mM) at the optimal pH and temperature conditions for each substrate reaction, unless stated otherwise.

In addition to measuring PPO activity with the oxygen electrode, spectrophotometric assays were carried out to measure PPO activity in reactions involving monophenol substrates, which generally show lower activity (Gertzen and Escobar, 2014). The methods and reagents were the same as described above with the exception of the addition of 280 U of catalase per milliliter of assay buffer. Volumes were also scaled to perform reactions in 96-well plates (Gertzen and Escobar, 2014). Maximum product absorbance was determined for each substrate by letting the substrate reaction turn visibly brown and then running a wavelength spectrum scan. Only the initial reaction rate was considered in calculating PPO activity. One unit of enzyme activity is defined as the amount enzyme causing a change in absorbance of 0.001 per minute as described in Walker (2001). Control assays were performed as above. All kinetic assays were performed using the spectrophotometer. Lineweaver-Burke plots were used to calculate Km, Vmax and catalytic efficiency (Vmax/Km) (See Appendix A).

Sequence alignment and comparison of catechol oxidases and cresolases

An amino acid sequence alignment of polyphenol oxidase active sites was created using Clustal Omega. Walnut JrPPO1 (accession: ACN86310.1**)** and red clover PPO1 (accession: AAK13242.1) sequences were compared to the structurally well-characterized catechol oxidases sequences of *Vitis vinifera* (accession: AKG51698.1**)** and *Ipomoea batatas* (accession: AAW78869.1).

Analyzing PPO genes in California poppy

To determine the feasibility of creating a PPO-silenced transgenic poppy, the PPO gene family in California poppy was analyzed using RNA-seq data available on Phytometasyn.ca

(Facchini *et al.*, 2012; Xiao *et al.*, 2013). Phytometasyn.ca is a database of poppy tissue culture sequencing information and was used to perform a tBlastn alignment of walnut JrPPO1 protein sequence with the RNA-seq database for California poppy (E-value threshold of $1e^{-25}$). Sequencher was used to further assemble the sequences with ≥95% identity and at least 20 base pairs of overlap.

Maintenance of California poppy cell cultures

California poppy callus was donated by Dr. Peter Facchini from the University of Calgary. Protocols for growth of the callus on plates and the establishment of suspension cell cultures in liquid medium were adapted from Farrow *et al.* (2012). Callus was grown on B5 medium [3.21 g/L Gamborg salts and vitamin mix (Gamborg et. al. 1968), 20 g/L sucrose, 1 g/L casein, 1 mL/L of 2,4-dichloroacetic acid stock solution (1 mg/mL 2,4-dichloroacetic acid in 50:50 ethanol: water), 3 g/L gelzan; pH 5.8 (Facchini, 2001)]. Callus was stored at ambient temperature in the dark and was subcultured to fresh media monthly.

Suspension cell cultures were established by transferring young white callus tissue to 50 mL of liquid B5 media in a 250 mL flask. Cultures were maintained in a growth chamber (16 hr light period with ~100 µmol m^{-2} s^{-1} illumination at 22° C, 8 hr dark period at 18° C) on an orbital shaker at 125 rpm. After initial inoculation, the cultures were incubated for two weeks until cell dispersal, reduced clumping and increased cell density were achieved. Cells were subcultured to fresh media every 9-12 days.

Cell culture growth rate

Packed cell volume analysis of control and treated samples was adapted from Farjaminezhad *et al.* (2013) to monitor cell growth as a representation of suspended cell culture health. 10 mL of each cell culture was collected and transferred to a 15 mL conical centrifuge

tube. Cells were centrifuged at 900 g for 5 minutes at room temperature. The packed cell volume was marked on the side of the conical tube. The cells were removed from the tubes, replaced with water, and then the water volume was measured. For larger volumes, the water was transferred to a graduated cylinder for measurement. Smaller volumes were measured using a pipette.

Metabolite extraction from poppy cell cultures

Cell cultures were treated with either 100 µM tropolone, 1 mM 4-hexylresorcinol, or 1 mM of kojic acid 4 days after subculturing. Whole flasks were sacrificed for sample collection and packed cell volume determination at time of treatment (day 0), and 2 and 4 days after treatment. Cells and media were separated using a Buchner funnel lined with filter paper. Both cells and supernatant were flash frozen in liquid nitrogen and stored at -80° C until metabolites were extracted.

Metabolite extraction was adapted from Farrow *et al.* (2012). Cells and supernatant were lyophilized to complete dryness and dry weight was measured for normalization of data. Modified Bieleski solvent (15:1:4, MeOH: formic acid: DI water) (Hoyerovà *et al.,* 2006) was added at 20 mL per gram of dry weight. Supernatant samples were vortexed until homogenous and passively extracted for 12 hr at -20 °C. Cell samples were sonicated at a 35% duty cycle with an output control of 3.5 on a Branson Sonifier 450 (VWR scientific) for 15 seconds with a 15 second cool down on ice for a total of 2 minutes. Cell samples were then placed on an orbital shaker set to 200 rpm at 4 °C for 2 hours then moved to passive extraction for 12 hours at -20° C. All sample extracts were centrifuged at 14,000 g for 10 minutes at 4 °C in 50 mL falcon tubes to remove debris. Samples were stored at -80° C until analyzed by HPLC.

HPLC analysis of BIA content

HPLC analysis was adapted from Farrow *et al.* (2012). Metabolites were separated on a Zorbax Eclipse Plus C18 HPLC column held at 25° C (Agilent Technologies) using a binary HPLC pump (Agilent 1100, Agilent Technologies). Extracts were injected (10 µL) onto the column and compounds were eluted at a flow rate of 0.5 mL/min using a gradient of 10 mM ammonium acetate (pH 5.5) (Solvent A) and HPLC grade Methanol (Solvent B). Initial HLPC conditions were 100% solvent A: 0% solvent B changing linearly to 50% solvent A: 50% Solvent B over 10 min. Then the solvent gradient was linearly changed to 1% solvent A: 99% solvent B by 12 min. These conditions were held constant for 1 min and then returned to 100% solvent A: 0% solvent B by 13.1 min for a 4 min re-equilibration period. The total analysis time was 18.1 min per sample. The diode array detector performed a wavelength scan between 220-400 nm to provide spectra for detected compounds. Spectra and retention time were used to identify candidate BIA peaks on the HPLC chromatograms. Pure cryptopine (MP Biomedicals) and allocryptopine (gifted from Dr. Peter Facchini) were used as standards for HPLC analysis, and were quantified at their absorbance maximum of 285 nm.

Data analysis

Data was analyzed for retention times and peak integration using ChemStation software (Agilent Technologies). Abundance of the BIA allocryptopine in the samples was calculated using a standard curve with a range of allocryptopine concentrations plotted against peak integration. The concentrations of allocryptopine in the cells and in the culture medium were calculated on a per flask basis as shown below:

$$\frac{allocryptopine\ (\mu g)}{(0.5\ mg)} * Lyophilized\ mass\ (mg\ DW\ in\ flask)$$

The constant 0.5 mg is the equivalent dry weight of sample that is in a 10 µL injection.

Two tailed student's T-tests were used to test for statistical significance.

Targeted metabolite profiling of dopamine and esculetin in red clover

Lyophilized leaf tissue from wild-type and PPO-silenced red clover plants were a gift from Dr. Michael Sullivan of the U.S. Dairy Forage Research Center in Wisconsin. Red clover PPO activity assays were performed by Dr. Sullivan as previously described (Sullivan *et al.*, 2004) to confirm silencing of PPO. Metabolite extraction methods were adapted from the UC Davis Genome Center (2006). 3 mg of lyophilized tissue was ground in liquid nitrogen with a mortar and pestle. To extract metabolites, 1.5 mL of chilled extraction solution (5:2:2 methanol: chloroform: water degassed with nitrogen gas for 5 minutes) was added directly to the mortar and pestle containing the frozen tissue and was mixed until homogenous. The extracts were nutated for 6 minutes at 4° C and then were centrifuged at 14,000 g for 2 minutes at 4° C. The supernatant was removed from the pellet and completely dried down using a “medium” setting on a Savant ISS110 SpeedVac (Thermo Scientific). Comparative metabolite analysis of dopamine and esculetin in five wild type and five PPO-silenced red clover lines was performed by the UC Davis West Coast Metabolomics Center using GC-MS. Samples were run at a flow rate of 1 mL per minute, injection volume was 0.5 µL. Injection temperature was ramped from 50° C to 250° C by 12° C per second. The temperature gradient after injection was 50° C for 1 minute, and was then increased by 20° C per second until 330° C was reached. This temperature was held constant for 5 minutes. Pure esculetin and dopamine standards were analyzed using this method and expected retention times were determined.

Results:

Plant PPO enzyme kinetics

The temperature and pH optima of PPO activity were assessed in total protein extracted from walnut leaves, red clover shoots and California poppy roots for each substrate using a plate reader (absorbance) or a Clark-type oxygen electrode. Initially, the oxygen electrode was used because the rate of oxygen consumption as a direct measure of PPO activity is comparable for each substrate reaction (Figure 1). This is not the case for PPO activity assays on the spectrophotometer, which measures the rate of phytomelanin production. Different phenolic substrates produce phytomelanin products with different absorptive properties, making direct comparisons between substrates more difficult. However, the oxygen electrode method lacks sensitivity to measure the activity of most monophenolase reactions, so the indirect spectrophotometric assay was used predominantly. The specific substrates analyzed included tyrosine (monophenol) and tyramine (monophenol) for dopamine biosynthesis, and caffeic acid (diphenol), and umbelliferone (monophenol) for esculetin biosynthesis.

PPO activity assays performed on walnut leaf protein extracts showed activity with tyrosine, tyramine, and caffeic acid as substrates. For all three substrates 37° C was the optimum temperature (Figure 5). The pH optima varied per substrate, but was in the range of pH 7-8 (Figure 5). PPO activity assays performed on total protein extracts from red clover shoots showed that caffeic acid was the only substrate effectively utilized, with a pH optimum of 7.0 and a temperature optimum of 37° C (Figure 6). This result suggests that red clover PPO may lack cresolase (monophenolase) activity, as is the case for many other plant PPOs (Steffens et al., 1994). PPO activity assays performed on total protein extracted from California poppy demonstrated that tyrosine, tyramine, and esculetin could be utilized as substrates. Unlike walnut

leaves and red clover shoots, where a single PPO enzyme is predominant (Escobar *et al*., 2008; Sullivan *et al.*, 2004), California poppy has a very large number of PPO genes/enzymes (see below), which substantially complicates interpretation of the activity assays. California poppy PPOs appear to operate at very high temperatures (>50° C), when utilizing caffeic acid and tyramine as substrates (Figure 7). The poppy PPO pH optimum was 7.0 for caffeic acid and tyrosine, while a double pH optimum was observed for tyramine (Figure 7). This is not uncommon for PPO enzymes and has been documented in apples, potatoes and wheat (Reviewed in Yoruk and Marshall, 2003). This double pH optimum might also reflect the differential activities of multiple PPO enzymes in poppy. Umbelliferone did not act as a PPO substrate for any of the plant species tested. Garcia-Molina *et* al. (2013) and Gowda and Paul (2002) have shown that the presence of a reducing agent or diphenolic substrate helps to stimulate PPO activity with monophenol substrates. Even when low concentrations of catechol (a known diphenol substrate of PPO) or ascorbate (reducing agent) were added to stimulate the reaction, PPO was not able to use umbelliferone as a substrate.

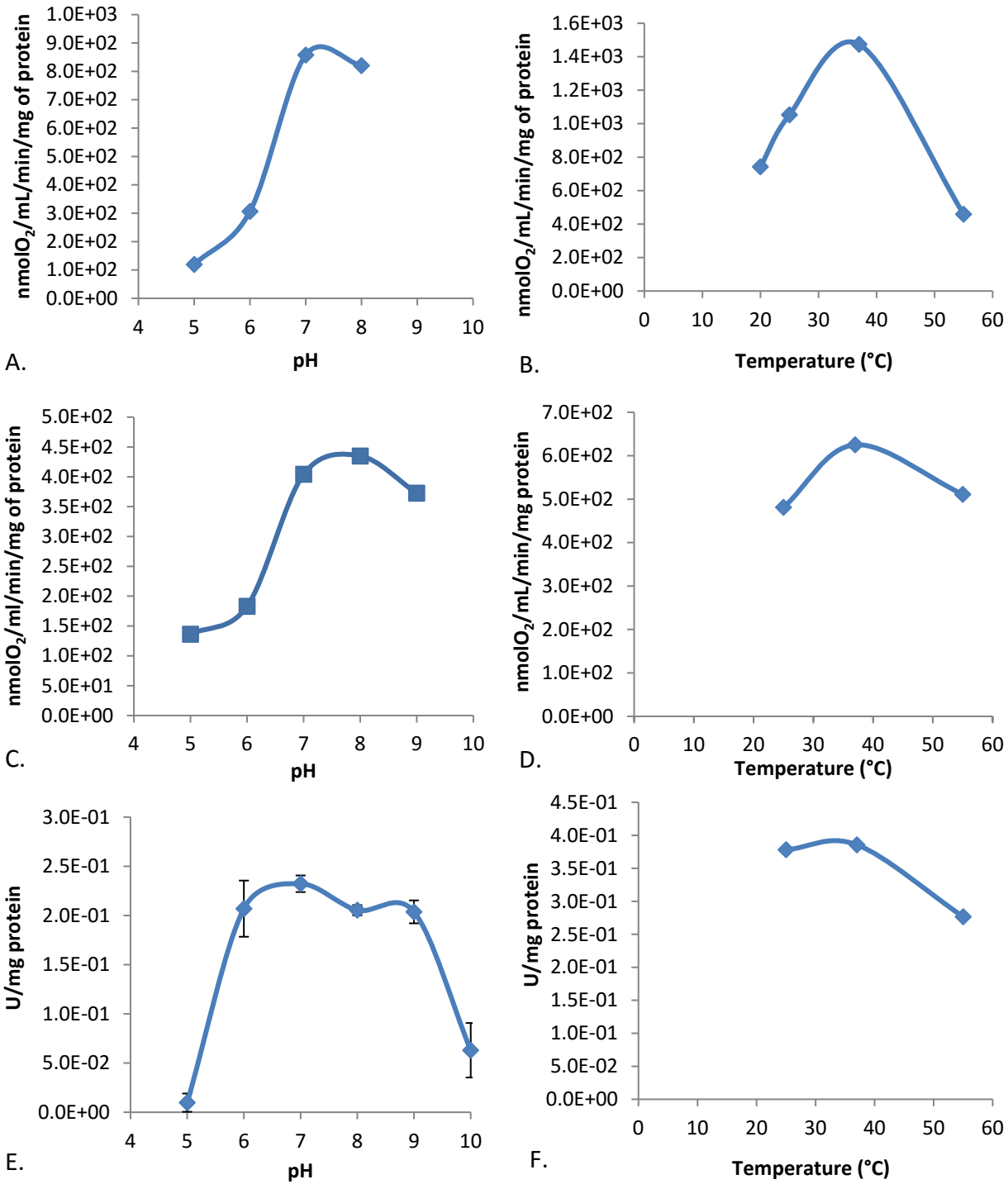

Figure 5: Temperature and pH optima of walnut PPO. Enzyme activity was measured in a leaf total protein extract using a Clark oxygen electrode (A, B, C, D) or a spectrophotometer (E, F). Substrates were 8 mM caffeic acid (A, B), 8 mM tyramine (C, D) and 8 mM tyrosine (E, F). Data points from the oxygen electrode (A-D) represent means ±SEM (n ≥ 2 technical replicates). Data points from panel E represent means ±SEM (n = 3 biological replicates). Data points from panel F represent means ±SEM (n ≥ 5 technical replicates). Note that the error bars are too small to visualize in most cases.

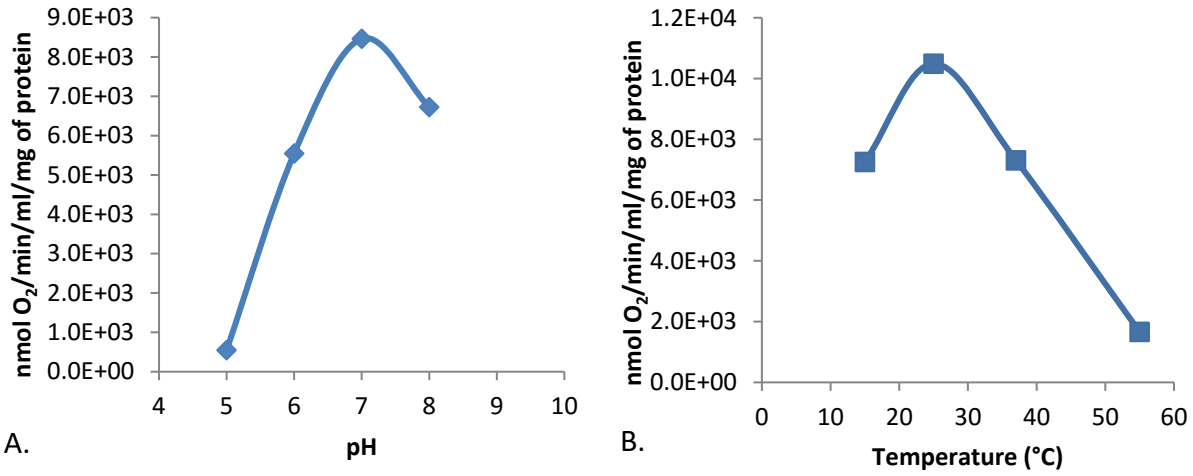

Figure 6: Temperature and pH optima of red clover PPO. Enzyme activity was measured in a shoot total protein extract using a Clark oxygen electrode with 8 mM caffeic acid as a substrate. Data points shown represent means ±SEM (n ≥ 2 technical replicates). Note that the error bars are too small to visualize in most cases.

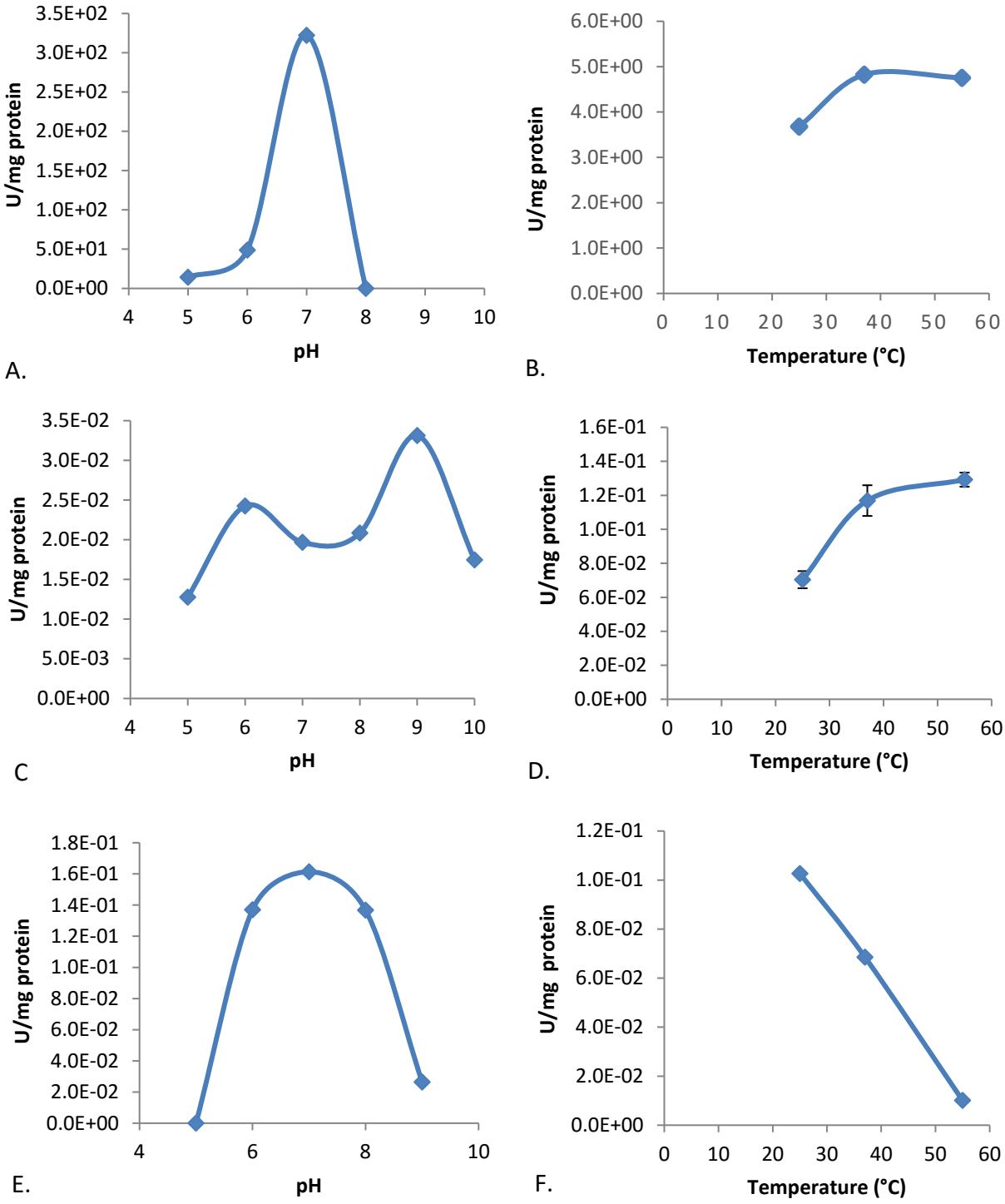

Figure 7: Temperature and pH optima of California poppy PPO. Enzyme activity was measured in a root total protein extract using a spectrophotometer at the denoted wavelengths. Substrates were 8 mM caffeic acid (A, B), 8 mM tyramine (C, D) and 8 mM tyrosine (E, F). Data points represent means ±SEM (n ≥ 2 technical replicates) except for panel D, in which n = 3 biological replicates. Note that the error bars are too small to visualize in most cases.

Enzyme kinetic assays were performed with total protein extracts from walnut leaves, California poppy roots, and red clover shoots on a plate reader. The phytomelanin products of both tyramine and tyrosine oxidation absorb maximally at 490 nm, so the enzyme kinetics for these substrates were considered comparable. The oxidation of caffeic acid produces a phytomelanin product that absorbs maximally at 350 nm, so this wavelength was used for the spectrophotometric assays. Km, Vmax and catalytic efficiency were calculated for each PPO-substrate combination based on activity tested over a range of substrate concentrations at the determined pH and temperature optima (Table 1). Kinetic assays for California poppy PPO with caffeic acid and tyramine were performed at 37° C rather than the optima because PPO was very active at this temperature and there were concerns about running the plate reader at high temperatures for several hours. Additionally, the kinetic assay for California poppy PPO with tyramine as a substrate was run at the lower pH optimum.

Walnut and California poppy PPO have greater affinity and higher catalytic efficiency for tyramine as a substrate compared to tyrosine as a substrate (Table 1). This *in vitro* result could suggest that tyramine may also be the preferred substrate for PPO-mediated dopamine synthesis in *vivo* (Figure 2). The catalytic efficiency for caffeic acid was similar between the three plant PPO enzymes, and none of the PPOs were capable of using umbelliferone as a substrate. This data supports the esculetin biosynthesis pathway suggested by Sato (1967) and is not consistent with the pathway proposed by Garcia-Molina *et al.* (2013) (Figure 4).

Table 1: PPO substrate kinetics from walnut, California poppy and red clover

Organism	Tissue	Substrate	K_m (mM)	V_{max} (U/mg total protein)	Catalytic efficiency (Vmax/Km)
Walnut	Leaf	Caffeic acid[a]	1.56	1.55	0.99
		Umbelliferone	nd	nd	nd
		Tyramine[b]	0.16	0.45	2.88
		Tyrosine[b]	7.76	0.5	0.06
Red clover	Shoots	Caffeic acid[a]	4.13	5.72	1.38
		Umbelliferone	nd	nd	nd
		Tyramine[b]	nd	nd	nd
		Tyrosine[b]	nd	nd	nd
Poppy	Roots	Caffeic acid[a,c]	23.32	29.69	1.27
		Umbelliferone	nd	nd	nd
		Tyramine[b,c]	1.49	0.2	0.13
		Tyrosine[b]	122.66	3.93	0.03

[a] The appearance of phytomelanin product from the caffeic acid reactions were measured at 350nm.[b] The appearance of phytomelanin product from the tyrosine and tyramine reactions were measured at 490 nm. [c] The enzyme kinetics for the California Poppy may be underestimated as the assay was not performed at the optimal temperature.

Some plant PPOs display cresolase and catechol oxidase activities, but many exhibit only catechol oxidase activity (Figure 1) (Steffens et al., 1994). Our data suggest that walnut and poppy PPOs are both cresolases and catechol oxidases, while red clover PPO is a catechol oxidase only. The amino acid sequences of the active sites from the walnut JrPPO1 (cresolase) (Escobar *et al.*, 2008) and red clover PPO1 (catechol oxidase) (Sullivan *et al*., 2004) were compared to other well characterized PPOs to investigate potential sequence differences associated with cresolase and catechol oxidase activities (Figure 8). Sequences for JrPPO1 in walnut and PPO1 in red clover were used in this analysis because they are the predominant PPOs expressed in leaf/shoot tissue (Escobar *et al.*, 2008; Sullivan *et al*., 2004) which was used produce the protein extract for the substrate specificity assays. California poppy PPO was not evaluated in this analysis due to the complexity of the PPO gene family in poppy (see below) as well as the lack of data on activity that can be matched to a particular gene/protein sequence. Previous studies have suggested that catechol oxidases have a restricted CuA active site, caused by an unusual thioester bond between the Cys and His residues marked with white diamonds in Figure 8 (Klaubunde *et al.*, 1998; Sendovski *et al.*, 2011; Goldfeder *et al.*, 2014). This thioester bond may stabilize the bulky Phe residue near the active site (marked with the gray diamond in Figure 8) which has previously been associated with PPOs lacking cresolase activity, and it is thought to restrict the binding of monophenol substrates at the CuA site (Klaubunde *et al.*, 1998; Sendovski *et al.*, 2011; Goldfeder *et al.*, 2014). However, as seen in Figure 8, walnut PPO possess each of these conserved residues (Cys, His, Phe) at conserved positions, but it also clearly possesses cresolase activity (Escobar *et al.*, 2008). Thus, this structural hypothesis likely needs to be reconsidered. Recently it has been suggested that amino acids in the second shell of the active site help to orient substrates into the active site and that monophenol and diphenol

substrates bind to both CuA and CuB (Bijelic *et al.*, 2015; Goldfeder *et al.*, 2014; Molitor *et al.*, 2016). The bolded and underlined residues in Figure 8 are some of the amino acids suspected to help orient the substrates and define the PPO with respect to cresolase and catechol oxidase activities, however this model is not yet fully developed (Bijelic, *et al.* 2015; Goldfeder *et al.*, 2014; Molitor *et al.*, 2016).

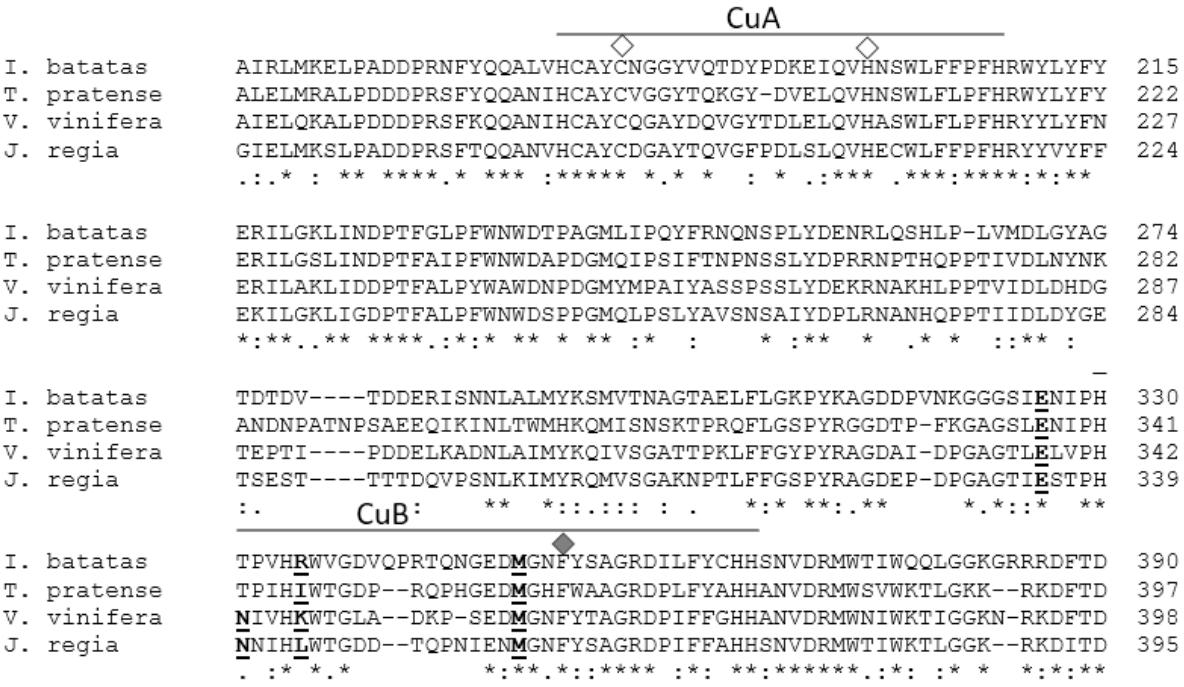

Figure 8: Comparison of polyphenol oxidase active sites from *Ipomoea batatas* (sweet potato), *Trifolium pretense* (red clover), *Vitis vinifera* (grape), and *Juglans regia* (walnut). Multiple sequence alignment was performed using Clustal Omega. White and gray diamonds signify residues suspected of causing limited monophenol substrate access to CuA site (Klaubunde *et al*., 1998; Sendovski et al., 2011; Goldfeder et al., 2014). Residues that are bolded and underlined are suspected to interact with incoming substrates and therefore add to substrate specificity of catechol oxidases or cresolases (Goldfeder et al., 2014; Bijelic et al., 2015; Molitor et al., 2016).

Targeted metabolite profiling of dopamine and esculetin in wild-type and PPO-silenced red clover

Targeted metabolite profiling of esculetin and dopamine in wild-type and PPO silenced red clover was performed by the West Coast Metabolomics Center at UC Davis. As shown in Table 2, PPO silenced red clover lines have substantially reduced PPO activity, though there is substantial variability in PPO activity amongst the wild type plants. To our knowledge, dopamine and esculetin content has never been evaluated in red clover. Surprisingly, these metabolites were not detectable in either wild-type or PPO-silenced red clover lines by highly sensitive GC-MS (Table 2). This data, combined with the inability of red clover PPO to use tyrosine and tyramine as substrates (Table 1), suggests that PPO probably does not play a role in dopamine and esculetin biosynthesis in this plant species.

Table 2: PPO activity, esculetin content, and dopamine content in wild-type and PPO-silenced red clover

Plant[a]	Silencing Construct	PPO Activity (nkat/mg protein)	Esculetin content	Dopamine content
NRC30-64-1	Yes	0	ND	ND
NRC30-64-2	Yes	4.85	ND	ND
NRC30-64-3	Yes	2.17	ND	ND
NRC30-1	No	10.43	ND	ND
NRC30-2	No	326.56	ND	ND
NRC7-64-1	Yes	2.17	ND	ND
NRC7-64-2	Yes	0.32	ND	ND
NRC7-1	No	82.43	ND	ND
NRC7-2	No	189.96	ND	ND
NRC7-3	No	603.71	ND	ND

[a] Plant: NRC30 and NRC7 are different red clover genotypes, the number 64 indicates the presence of the PPO silencing construct, and the numbers 1, 2 and 3 represent independent transformation events. ND means not detected.

Metabolic effects of inhibiting PPO in California poppy

In walnut and red clover, genes encoding PPO were silenced using RNA interference. To check the feasibility of using the same strategy in poppy, the PPO gene family was analyzed using California poppy RNA-seq data available on Phytometasyn.ca (Facchini *et al.*, 2012; Xiao *et al.*, 2013). A tBlastn search of the walnut JrPPO1 protein sequence against the RNA-seq database for California poppy was performed with an E-value threshold of 1e-25. Thirty-four sequences showed significant alignment. Contig assembly of these sequences with ≥95% identity and at least 20 base pairs of overlap was carried out using Sequencher. This analysis showed that there are approximately 20 PPO-encoding genes in California poppy (Table 3). Due the substantial number of PPO genes in California poppy, it was not reasonable to pursue RNAi as a strategy to define the role of PPO in BIA synthesis in poppy. Thus, we decided to analyze the role of PPO in this species using chemical inhibitors of PPO activity.

Table 3: The 20 putative PPO genes in California poppy.

Name	*Nucleotide sequence length (bp)*	*Identity to JrPPO1 (%)*	*Query cover (%)*	*E-value*
Contig 1	1033	34	51	8.00E-52
Contig 2	2485	41	80	4.00E-132
Contig 3	2408	48	79	2.00E-107
Contig 4	3104	38	85	9.0E-106
Contig 5	3489	53	91	0.00E+00
Contig 6	4312	41	88	1.00E-126
Contig 7	1578	49	50	1.00E-91
Contig 8	2214	58	87	0.00E+00
Contig 9	1764	45	53	1.00E-94
Singleton_rep_c16688	1856	71	55	5.00E-165
Singleton_rep_c21689	1308	34	38	2.00E-38
Singleton_rep_c2377	866	49	33	1.00E-58
Singleton_rep_c31502	575	71	28	8.00E-74
Singleton_rep_c3662	1002	42	26	7.00E-38
Singleton_rep_c5733	2011	62	65	1.00E-179
Singleton_contig25235	3804	53	93	0.00E+00
Singleton_contig28056	1784	39	45	4.00E-47
Singleton_contig3166	1069	48	51	7.00E-96
Singleton_contig6673	1810	37	95	4.00E-118
Singleton_singlet48074	1429	60	68	0.00E+00

To maximize uptake of the PPO inhibitors, California poppy cells were grown in suspension cultures, with the inhibitor added directly to the culture medium. The growth of the poppy cell cultures was characterized by measuring the packed cell volume of flasks every 3 days for 12 total days (Figure 9). The plant cell growth was exponential over this period. Cells were treated with PPO inhibitors on the fourth day and samples were collected for BIA analysis immediately thereafter and on days 6 and 8.

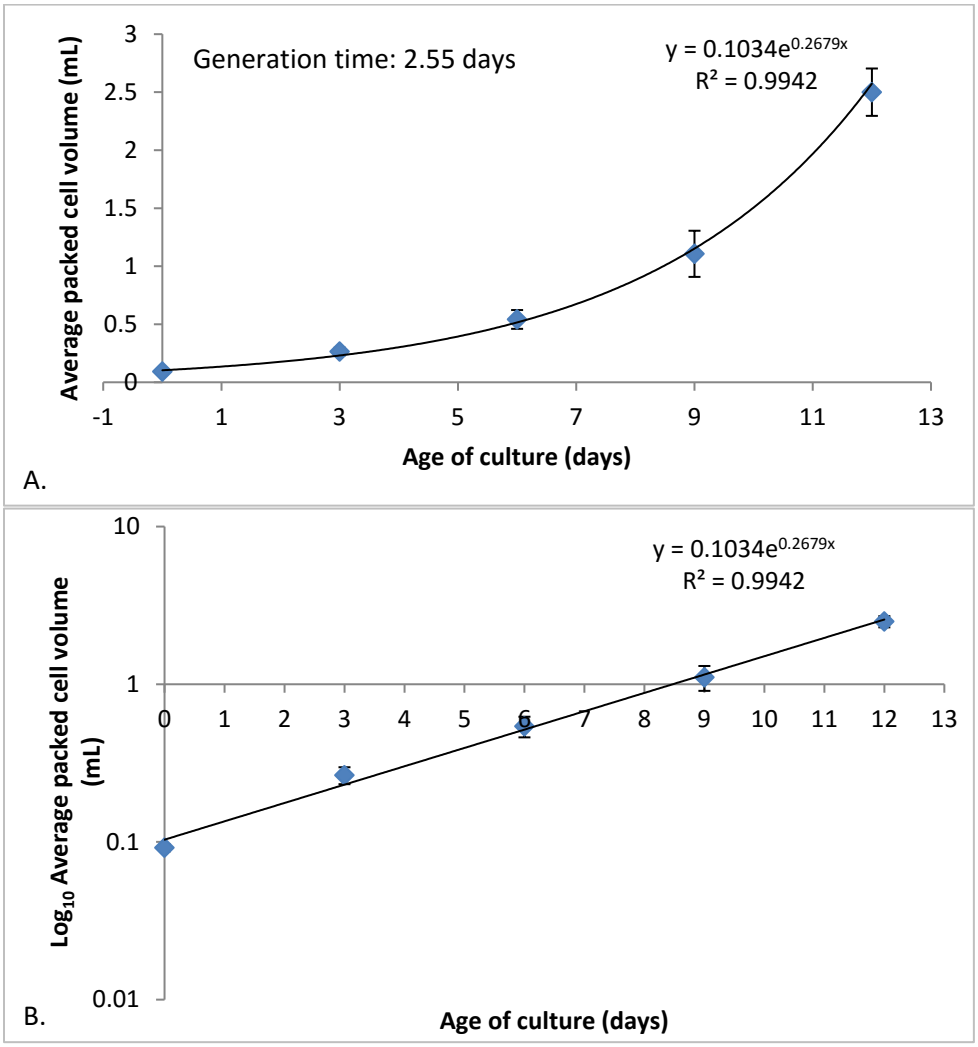

Figure 9: Characterizing California poppy cell culture growth. A) Average packed cell volume of poppy cell culture growth over time. Cells were subcultured on day 0. Generation time was calculated for growth over the 12 days. B) Log_{10} of the the average packed cell volume over time. Data points shown represent means ±SEM (n ≥ 2).

Preliminary studies of the effects of PPO chemical inhibitors on cell growth were performed on the poppy cell cultures using 100 µM tropolone, 1 mM kojic acid and 1 mM 4-hexylresorcinol. These concentrations were previously found to effectively inhibit PPO activity in wheat seeds (Fuerst *et al.*, 2006). Tropolone, kojic acid, and 4-hexylresorcinol are considered competitive inhibitors of PPO since they bind the active site (Chang, 2009). They are structural analogues of PPO substrates and all three chemicals act as slow-binding competitive inhibitors of PPO (Chang, 2009; Arias *et al*., 2007). For initial analysis of the effects of the inhibitors on the cell cultures, treatment was applied to the cell cultures 48 hr after culture inoculation. Treatment with 4-hexylresorcinol caused the cells to turn black 24 hr after treatment, and thus was not further pursued (Figure 10). Tropolone and kojic acid treatment 4 days after cell culture inoculation were evaluated for effects on cell growth (Figure 11). Tropolone had negative effects on cell growth, thus kojic acid was chosen for further testing.

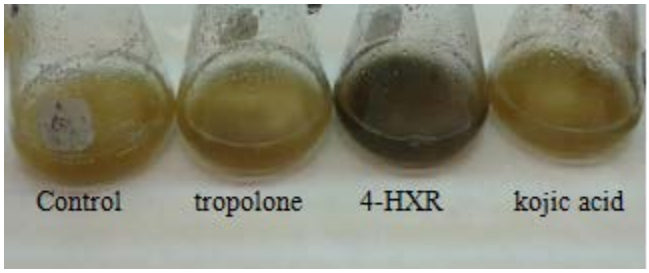

Figure 1: Characterizing the effects of various PPO inhibitors on poppy cell culture growth. Cells were treated with 100 µM tropolone, 1 mM 4-hexylresorcinol (4-HXR), or 1 mM kojic acid. Inhibitors were added 48 hr after inoculation, this photo was after 24 hr of treatment.

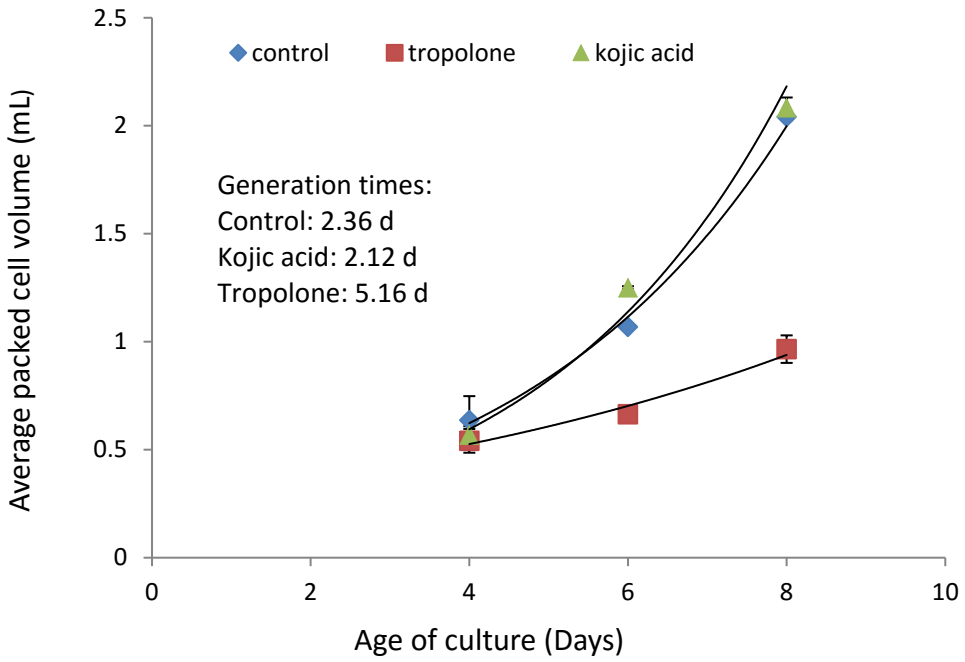

Figure 2: Characterizing the effects of tropolone and kojic acid on California poppy cell culture growth. Cells were subcultured on day 0 and treated on day 4 with 1 mM kojic acid, 100 µM tropolone, or 0.0025% (v/v) ethanol (control). Data points shown represent means ±SEM (n ≥ 2).

If PPO is involved in the synthesis of dopamine-derived BIAs in poppy (Figure 3), then chemically inhibiting PPO with kojic acid should reduce the levels of BIAs produced by poppy cell cultures. Romneine, chelirubine, scoulerine, escholidne, cryptopine, allocryptopine and several other BIAs were shown to be produced at relatively high levels in California poppy cell cultures by Farrow *et al.* (2012). Of these, we were able to purchase cryptopine (MP Biomedicals) and were gifted allocryptopine by Dr. Peter Facchini, and both were used as standards for HPLC analysis. Since dopamine is a key intermediate for the synthesis of all BIAs (Figure 2), we expect all BIA levels to be affected by the inhibition of PPO activity. HPLC retention time and UV spectra of the standards were used to identify the corresponding BIAs in cell extracts and culture supernatants (Figure 12 and Figure 13). Allocryptopine standard spiked into a control cell metabolite extract co-migrated with an abundant compound found in the

sample extract (Figure 12). The spectra of pure allocryptopine and the candidate BIA peak were similar (Figure 13). Cryptopine did not co-migrate with any distinguishable compounds and thus was considered undetectable in sample extracts.

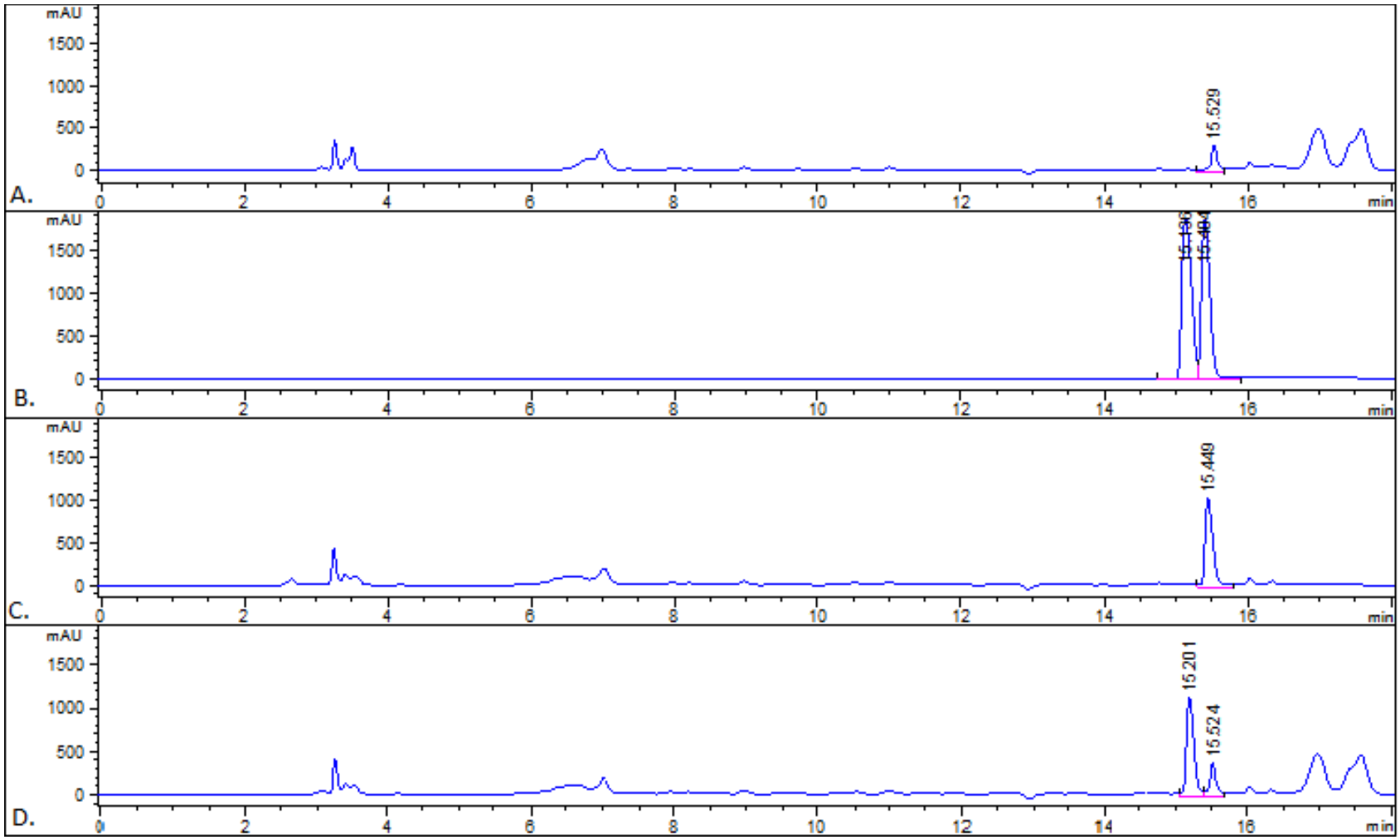

Figure 12: Identification of cryptopine and allocryptopine in standards and samples using HPLC methods adapted from Farrow *et al*. (2012). Samples are as follows: A) metabolite extract from control cell culture with suspected BIA peak at retention time (Rt): 15.529 min., B) 0.1 mM cryptopine (Rt: 15.136 min.) and 0.1 mM allocryptopine (Rt: 15.404 min.) dissolved in acetonitrile, C) 0.1 mM allocryptopine (Rt: 15.449) spiked into control cell sample extract (note co-migration of peaks), D) 0.1 mM cryptopine (Rt: 15.201) spiked into control cell sample day 4 with suspected BIA peak at Rt 15.524 min.

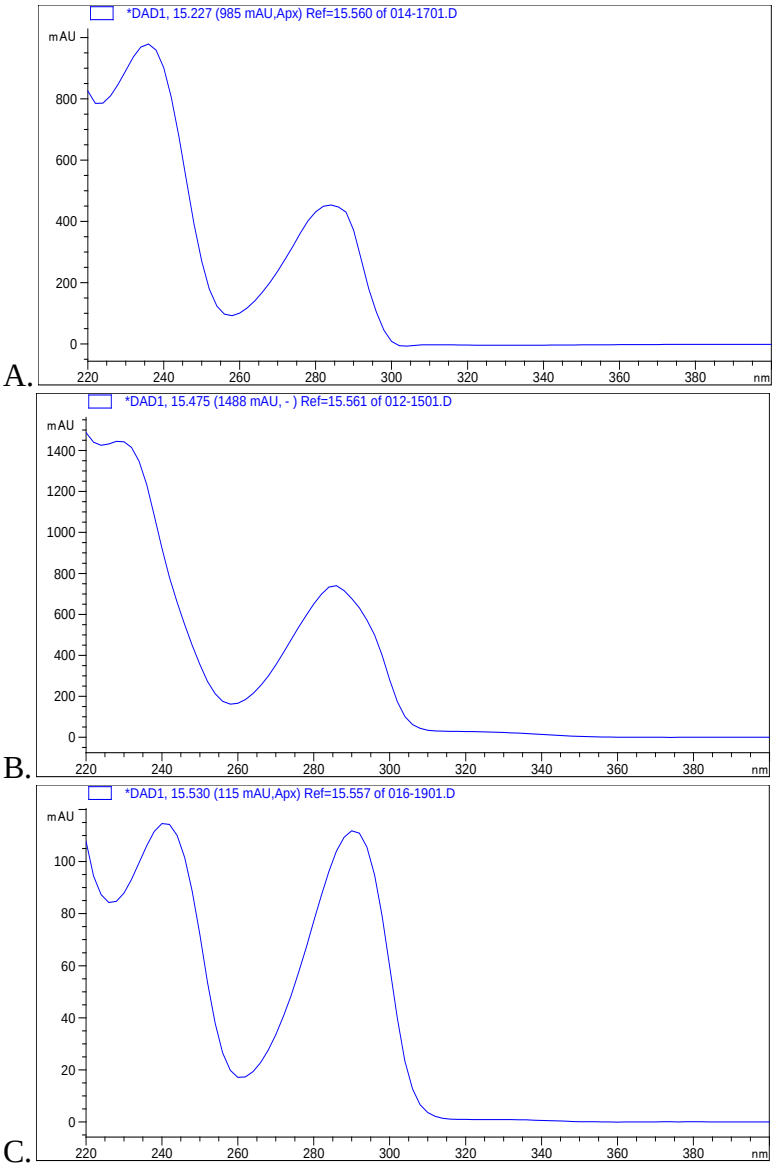

Figure 13: Absorbance spectra of cryptopine (A), allocryptopine (B) and candidate BIA peak (Rt: 15.5 min) in samples (C) as detected by the HPLC-diode array detector (Agilent Technologies).

The inhibitor treatment, metabolite extraction, and HPLC analysis of the California poppy cell cultures was performed during three separate experiments (total $n \geq 10$ flasks per treatment and time). Each experimental run had slightly different trends in allocryptopine content in the cell extracts, and similar trends in allocryptopine content of the culture medium. Thus to see a clearer trend, data from each individual trial was normalized to the fourth day control

sample data, and then data sets were merged (Figure 14). The growth of poppy cells treated with kojic acid was not affected in any of the experimental runs (Figure 15). While allocryptopine content increased over time in the cells, there were no differences between the controls and kojic acid treated cells (Figure 15A). Suspension cell cultures from poppy species have been observed to excrete various BIAs into the culture medium with or without the help of elicitors (Nakagawa *et al.*, 1984; Hussain *et al.*, 2012). Thus, we also quantified BIA content in the culture medium. In contrast to cell extracts, the culture medium of kojic-acid treated samples had significantly lower allocryptopine levels than untreated samples (Figure 15B). Thus it appears that inhibition of PPO may have an effect on BIA production, however it is unclear why BIA content in the cells and in the culture supernatant are differentially affected.

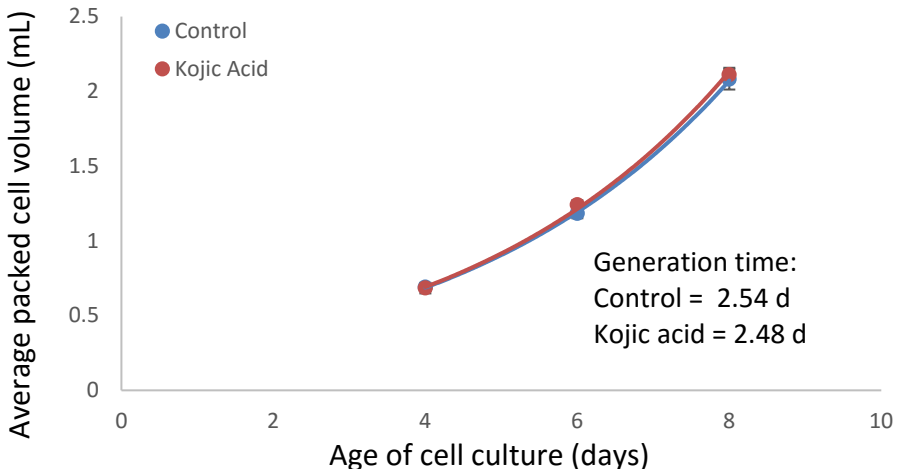

Figure 14: Growth of California poppy cell cultures used in HPLC analysis. Cells were subcultured on day 0 and treated on day 4 with 1 mM kojic acid or 0.0025% (v/v) ethanol (control). Data points shown represent means ±SEM (n ≥ 10).

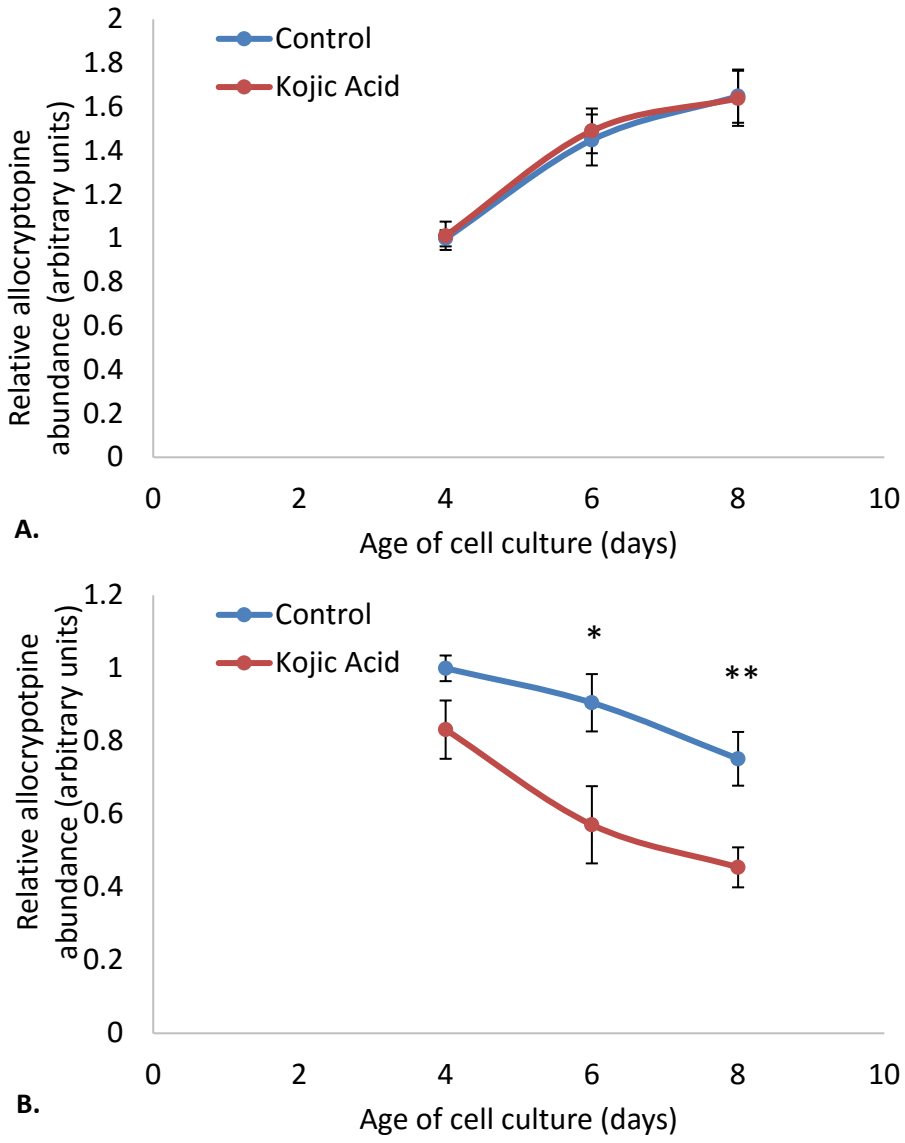

Figure 15: Effects of treating California poppy cell cultures with kojic acid on relative allocryptopine abundance in cell metabolite extracts (A) and culture medium metabolite extracts (B). California poppy cell cultures were treated with 1 mM kojic acid or 0.0025% (v/v) ethanol (control) on day 4 after subculture. Flasks were sacrificed for collection on day 4, 6 and 8. Data points shown represent the means ±SEM (n ≥ 10). Allocryptopine abundance data for day 4 control samples was arbitrailiy assigned a value of 1. * represents significance of P≤0.05 (Student's t-test, two tailed). ** represents significance of P≤0.01 (Student's t-test, two tailed)

Discussion:

I*n vitro* substrate specificity and enzyme kinetic assays were performed to further define the role of PPO in esculetin and dopamine synthesis in plants. The enzymatic properties of the three plant PPOs characterized in these studies were within typical ranges of PPOs characterized from other species. Previous studies have shown that PPOs typically have relatively low affinity for their phenolic substrates (i.e. Km values in the low millimolar range) (Mayer and Harel, 1979). The Km values for walnut, California poppy, and red clover PPOs using the diphenol caffeic acid as substrate (Table 1) were comparable to corresponding Km values previously characterized for apple (0.14 mM) (Janovitz-Klapp *et al.,* 1990), spearmint (0.83 mM) (Neves *et al.*, 2009), and pear (16 mM) (Ziyan and Pekyardimci, 2004) PPOs. Likewise, the calculated Km values for walnut and California poppy PPOs using the monophenol tyramine as a substrate (0.16 mM and 1.49 mM, respectively) were similar to those previously reported for PPO from beet leaves (0.25 mM) (Escribano *et al.*, 1997) and apple (1.7 mM) (Espín *et al.*, 1998). The Km of walnut PPO with tyrosine as a substrate (7.76 mM) falls within the range of Km values seen in moss rose (0.5 mM) (Yamamoto *et al*., 2001), *Amorphophallus campanulatus* (1.33 mM) (Paranjpe *et* al., 2003), elephant yam (3.6 mM) (Balakrishnan and Kalirajan, 2015), jackfruit (20.7 mM) (Geetha Rani *et al.*, 2014), and pear (32.67 mM) (Ziyan and Pekyardimci, 2004). In contrast, California poppy PPO showed extremely low affinity for tyrosine (Km = 122 mM), suggesting that tyrosine oxidation by PPO probably does not occur at a significant rate in California poppy plants.

Previous studies have implicated PPO in the biosynthesis of esculetin, but the phenolic substrate involved is controversial. Sato (1967) described a chloroplastic phenolase (PPO) as responsible for the converting caffeic acid into a precursor of esculetin. More recently, PPO was

described to be directly responsible for converting the substrate umbelliferone to esculetin (Garcia-Molina *et al.,* 2013). From our *in vitro* study, we found that all tested plant PPOs were capable of using caffeic acid, but not umbelliferone, as a substrate (Table 1). This suggests that the esculetin biosynthesis pathway described by Garcia-Molina *et al.* (2013), which was developed using a mushroom PPO as a model, may not operate in plants.

As described by Araji *et al.* (2014), PPO appears to be involved in the biosynthesis of dopamine in walnut. Two dopamine synthesis pathways are possible: 1. PPO-mediated hydroxylation of tyrosine into L-DOPA with subsequent decarboxylation of L-DOPA into dopamine, or 2. PPO-mediated hydroxylation of tyramine directly into dopamine (Figure 2). Based on Km and catalytic efficiency values of the walnut PPO enzyme, it is apparent that tyramine is the preferred PPO substrate *in vitro* (Table 1). However, enzymes involved in the synthesis of tyrosine are localized in the chloroplast (Tzin and Galili, 2010), as is PPO (Steffens *et al.*, 1994), while the enzyme responsible for tyramine synthesis from tyrosine has been localized in the cytosol of *Arabidopsis thaliana* (Lehmann and Pollman, 2009). Thus, increased catalytic efficiency of the tyramine reaction *in vitro* may not represent what is happening *in vivo*. Radioactive labelling studies of tyrosine and tyramine metabolism may provide a better alternative to answer this question.

Dopamine is a key intermediate in the synthesis of biomedically-important BIAs. To determine if PPO plays a role in the synthesis of dopamine-derived BIAs, we analyzed the effect of chemically inhibiting PPO in California poppy cell cultures. We quantified allocryptopine, a BIA known to be abundant in these cell cultures (Farrow *et al*., 2012), as a representative BIA. Metabolite extracts from poppy cells treated with kojic acid had nearly identical allocryptopine levels compared to the control cells over the treatment period (Figure 15A). This does not

support our hypothesis that PPO is involved in the synthesis of dopamine (and dopamine-derived BIAs) in poppy. In contrast, the culture medium from cell cultures treated with kojic acid had a significantly lower concentration of allocryptopine over the treatment period compared to control samples (Figure 15B). However, allocryptopine content unexpectedly decreased in the medium during the growth of the cell culture.

One possible explanation for the observed decrease in allocryptopine may be that the California poppy cells are reabsorbing the allocryptopine to protect themselves from the toxic effects of this metabolite (Weiss *et al*., 2006). Allocryptopine is in a sub-group of alkaloids called benzophenanthridies, which are derived from S-scoulerine and S-reticuline (Weiss *et al*., 2006, Farrow *et al.*, 2013). Benzophenanthridies are often produced in the presence of stressors and elicitors, and they can be excreted into cell walls and externally into the medium where they act as defense compounds (Weiss *et al.*, 2006). As these compounds can be toxic to the poppy cells, they are reabsorbed and then are recycled or converted to less harmful intermediates (Weiss *et al*., 2006). Thus, the decreasing allocryptopine content in the supernatant could possibly be due to poppy cell defense behavior. Despite these confusing results, California poppy PPO was found to use both tyramine and tyrosine as substrates *in vitro* (Table 1), meaning that PPO could be responsible for the synthesis of dopamine in poppy. This would be reliant the whether the substrates come into contact with PPO *in vivo*. Unfortunately, a firm conclusion as to whether PPO is involved in dopamine-derived BIA synthesis in poppies cannot be reached from this data.

In contrast, data from our analyses of red clover PPO clearly suggest that the enzyme is not involved in dopamine or esculetin synthesis in this species. GC-MS analysis of dopamine and esculetin content in wild-type red clover leaves revealed the absence of both compounds

(Table 2). Complementing this result, red clover PPO could not utilize tyramine or tyrosine as substrates (Figure 2). Thus, PPO is clearly not involved in dopamine synthesis. Although caffeic acid was a substrate for red clover PPO, the lack of esculetin in the foliage indicates that PPO1 is not involved in esculetin synthesis. It is possible that other enzymes required to synthesize esculetin may not be present in red clover (Figure 4).

A recent study which examined the metabolic effects of silencing PPO in potato tubers (Shepherd *et al*., 2015) provides an interesting contrast to the previously described effects of silencing *JrPPO1* in walnut (Araji *et al.,* 2014). Shepherd *et al.* (2015) did not observe a large increase in tyramine or a decrease in dopamine and esculetin in PPO-silenced potato. Instead, they observed less robust alterations in metabolites involved in wound response. Similarly, in our study, we did not see an effect of PPO silencing in red clover on dopamine or esculetin content, as these compounds were absent in both the wild-type and PPO-silenced lines. Additionally, there did not seem to be a consistent effect of PPO inhibition on the content of dopamine-derived BIAs in poppy. Thus, it appears that the role of PPO in dopamine and esculetin biosynthesis may be specific to walnut, much like the taxonomically-limited roles for specific PPOs in betalain synthesis in beets (order Caryophyllales) and aurone synthesis in snapdragons (*Antirrhinum majus*).

Future directions:

To further clarify our understanding of the role of PPO in the synthesis of dopamine and dopamine-derived BIAs in California poppy, additional BIA standards should be used beyond allocryptopine. It may also be appropriate to use more sensitive BIA detection methods, such as HPLC-ESI-MS/MS, to detect and monitor alternative BIAs, which may be less abundant than allocryptopine. If these studies generate clear evidence that chemical inhibitors of PPO suppress BIA synthesis in poppy cell cultures, then a genetic approach to verify this effect would be appropriate. Silencing one or more target PPO genes in California poppy by virus-induced gene silencing (VIGS), as described in Lee *et al*., (2011), could provide the most direct test of the roles of specific PPOs in BIA biosynthesis.

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Appendix A:

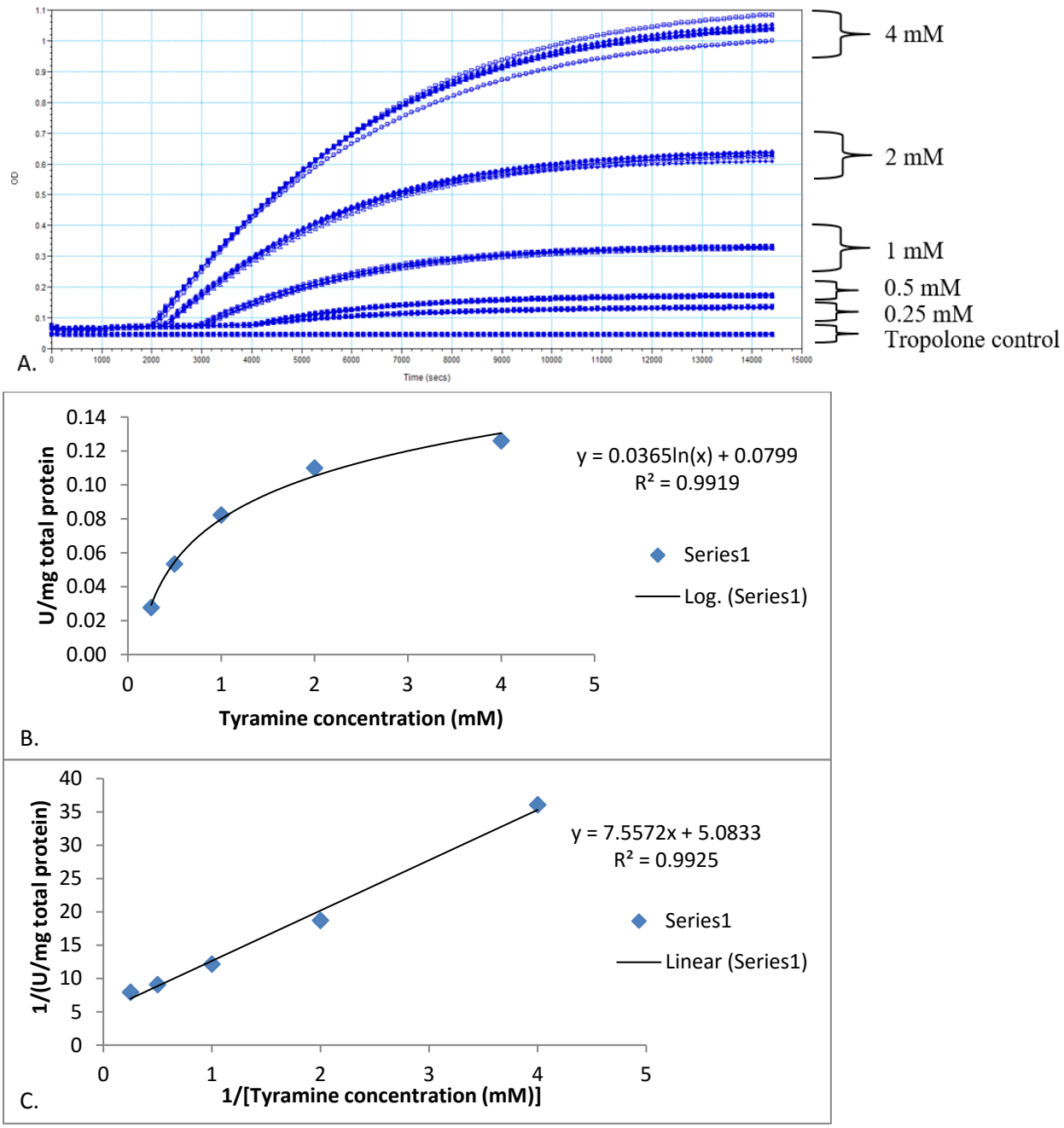

Figure A1: Characterizing enzyme kinetics for California poppy PPO using tyramine as a substrate. A) Example spectrophotometric data (Abs 490 nm vs. time) from PPO enzyme activity assays using varying concentrations of tyramine (replicates of 6). B) Calculated PPO activity at varying concentrations of tyramine (0.25 – 4 mM). C) Lineweaver-Burk plot calculated using the inverse of initial reaction rates and substrate concentrations from B.