Response of *Arabidopsis thaliana* to Exposure to Pentachlorophenol and Influence of Boron

by

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Abstract

Pentachlorophenol (PCP) has recently been used in combination with boric acid to provide a dual treatment wood preservative for utility poles. The toxicity of chemical combinations of PCP and boric acid were examined with bioassays using the model plant *Arabidopsis thaliana* to generate both a quantifiable phenotypic and genetic response as potential biomarkers for PCP toxicity. Boric acid was not observed to have any significant effect on PCP toxicity. Fresh weight was determined to be the most consistent biomarker within this study of PCP toxicity with an EC_{50} of 5.32 mg/L. The induction of cytochrome P450 gene AT5G36220 was determined to be a suitable bioindicator of low (1-5 mg PCP/L) concentrations of PCP. These data provide a basis for the development of bioindicators of the presence of low concentrations of PCP in the environment and for understanding physiological and biochemical responses of *Arabidopsis* to exposure to PCP.

List of Abbreviations Used

BP: Biological Process

CCME: Canadian Council of Ministers of the Environment

CAT: Catalase

CC: Cellular Component

CA: Concentration Addition

CPM: Counts Per Million

DOT: Disodium Octaborate Tetrahydrate

DNA: Deoxyribonucleic acid

EDTA: Ethylenediaminetetraacetic acid

ES: Effect Summation

EPA: Environmental Protection Agency

GO: Gene Ontology

GST: Glutathione S-Transferase

H_2O_2: Hydrogen Peroxide

OH: Hydroxy

IA: Independent Action

MDA: Malondialdehyde

MF: Molecular Function

MS: Murashige and Skoog

NOEC: No Observed Effect Concentration

OCSPP: Office of Chemical Safety and Pollution Prevention

PCP: Pentachlorophenol

PMRA: Pest Management Regulatory Agency

POD: Peroxidase

PCB: Polychlorinated Biphenyls

PAH: Polycyclic Aromatic Hydrocarbon

PCR: Polymerase Chain Reaction

ROS: Reactive Oxygen Species

RPKM: Reads Per Kilobase Mapped

RNA: Ribonucleic acid

O_2: Superoxide

SOD: Superoxide Dismutase

TAIR: The Arabidopsis Information Resource

TEF: Toxicity Equivalency Factors

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Chapter 1.0 Introduction

Wood preservatives are intended to maintain the integrity of wood products by inhibiting the activity of wood degrading organisms (fungi and termites) thereby extending their service life (Lebow, 2010). Unfortunately, these compounds may also be toxic to a broad range of organisms and could be detrimental to the environment. Wood preserving chemicals can contaminate the environment in and around wood treatment facilities and can also leach, or bleed, from installed treated wood products. Leaching is when preservatives in wood are lost due to the movement of water, while bleeding is when a preservative containing oil migrates to the surface of wood (Freeman, 2010).

There are numerous water soluble and water insoluble pesticides registered for use in wood treatment, and these two groups are referred to as either water-borne, or oil-borne preservatives (Lebow, 2010). Oil-borne preservatives are more commonly used to treat utility poles due to their resistance to leaching in water. Historically, pentachlorophenol (PCP) has been the preferred oil-borne preservative (Freeman, 2010). The use of PCP in combination with boron, in the form of boric acid or borates that generate boric acid within treated wood, is presently being proposed as a better treatment system for full pole protection (Stratton, 2015). The simultaneous release of these two chemicals could affect their individual toxicity or impact on organisms in the environment.

The potential for release of environmental contaminants, such as PCP, elicits interest in detecting these compounds and their effects on organisms (Fontanetti et al., 2011). Identifying contaminants in the environment through traditional chemical analysis does not detect impacts on organisms, potential interactions (additive, antagonistic or

synergistic), or contaminant bioavailability (Fontanetti et al., 2011). Bioindicator species have the potential to provide simple, low cost, and sensitive contaminant indicators in the environment (Fontanetti et al., 2011).

Plants are exposed to the conditions in the environment in which they occur and, in the short-term, are unable to move to avoid conditions unfavorable to their growth and/or survival. To survive, they must adapt to their environment and mitigate any adverse effects caused by a stressful exposure. Plants do so by adjusting their physiology (Ramel et al., 2012). Plants are exposed to stressors, including abiotic stressors, such as industrial pollutants. When plants perceive a stress, the stress is translated into a physiological response involving hormones, secondary messengers and transcriptional regulators (Couée et al., 2013). These responses are often reproducible changes in physiology and gene regulation that can potentially serve as bioindicators for the early detection of a particular stress condition such as the exposure to toxic chemicals (Couée et al., 2013).

Bioindicators are an appealing tool to assess toxicity, as they provide an integrated assessment of the toxic affect to the organism. The term bioindicator refers to a species, or group of species, which can reflect levels of environmental contamination through alterations to their physiology (Fontanetti et al., 2011). *Arabidopsis thaliana* is considered to be a model plant system for use in various studies as it is a small plant with a short life cycle and a fully documented genome and it is easy to grow under laboratory conditions (TAIR, 2011). Therefore, it has the potential to be used as a bioindicator to contextualize effects on the genome.

Examination of the physiological, biochemical and gene expression response of *Arabidopsis thaliana* to exposure to PCP and boron, (as boric acid), will serve **i)** to identify changes in physiology and gene regulation that can be used to detect PCP toxicity **ii)** to enhance the knowledge of how physiology and gene expression changes in response to exposure to PCP and the role of boron in influencing that response and **iii)** to determine if this assay system is suitable for rapid screening of PCP toxicity associated with the wood protection industry and to ultimately produce a reliable indicator of low PCP concentrations. These goals will be achieved by addressing several specific objectives.

1.1 Objectives

To discover the phenotypic and genetic response of *Arabidopsis thaliana* to exposure to PCP several objectives will be addressed:

1. To observe the physiological responses (measured by EC_{50}) of *Arabidopsis thaliana* over a range of concentrations of PCP.

2. To determine if the duration of exposure influences the plant response endpoints.

3. To determine the influence of boric acid concentration on the toxicity of PCP.

4. To determine changes in gene expression in response to exposure to PCP and identify genes suitable as biomarkers or endpoints of the toxic response to PCP.

5. To determine how gene ontology **(**GO) is affected by exposure to PCP.

Chapter 2.0 Literature Review

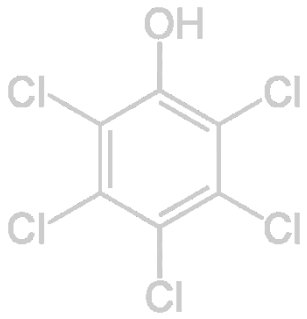
Wood preservatives are intended to inhibit wood degradation, extending the usable lifespan of wood and enhance its intended use, without posing unreasonable risks to humans or the environment (Lebow, 2010). Preservatives are designed to protect wood products from attack by organisms. Treating wood with preservatives prolongs its service life, reducing replacement costs while conserving trees that would otherwise be needed for wood products (Lebow, 2010). Wood preservatives are considered a form of pesticide and are registered and regulated by the Pest Management Regulatory Agency (PMRA) of Health Canada and the U.S. Environmental Protection Agency (EPA). The ability of preservatives and treatment processes to protect wood is evaluated by the American Wood Protection Association (AWPA) and the Canadian Standards Association (CSA) using a standardization process (CSA, 2009; Lebow, 2010; AWPA, 2014). A newly developed envelope treatment process utilizes two preservatives, boron and PCP in combination to provide full utility pole protection (Stratton, 2012).

2.1 Envelope Treatment

Dual treatment, or envelope treatment, is a newer development in wood preserving and is just beginning to be used on an industrial scale. Dual treatment is the use of two different preservatives to treat wood (Stratton, 2012). A water-soluble preservative (normally borate) is chosen to effectively treat the more difficult to penetrate heartwood, and a second oil-borne preservative (such as PCP) targets the exterior sapwood, enveloping and sealing the water-soluble borate preservative in the pole heartwood ensuring full pole treatment (Stratton, 2012).

Treatment with oil-borne PCP only permeates the outer sapwood of the pole, leaving the dense and moisture packed inner heartwood unprotected when it is later exposed through weathering. Water-borne borates are sufficiently soluble to treat the inner heartwood, but leach readily from the wood. Proprietary mixtures of boron and PCP are applied to seasoned poles, where the moisture content has been reduced to facilitate treatment. The preservative mixture is applied by way of pressure treatment after which the borate converts to boric acid and migrates throughout the heartwood of the pole while the PCP remains in the outer sapwood (Stratton, 2012).

2.2 Pentachlorophenol



Pentachlorophenol (PCP) is a fully chlorinated phenol that has a low water solubility at 20°C of 14 mg/L (pH 5), 2,000 mg/L (pH 7), or 8,000 mg/L (pH 8), with a vapor pressure of $2x10^{-6}$ kPa at 20°C, a pKa of 4.7 and an octanol/water partition coefficient of 5.01 (Freeman, 2010). Pentachlorophenol is toxic to membranes by dissipating proton transport (Steiert et al., 1988).

2.2.1 As a Preservative

The American Wood Protection Association (AWPA) is the organization responsible for setting standards for the formulation of pesticides as wood preservatives in terms of

composition and use in North America (AWPA, 2014). Methods for evaluating and measuring the levels of wood preservatives in treated materials are also proposed by the AWPA and outlined in a published book of standards each year (AWPA, 2014). It should be noted that PCP is the abbreviation for pentachlorophenol while in the wood treatment industry "penta" is commonly used to refer to commercial-grade PCP dissolved in an oil carrier (Freeman, 2010).

The widespread use of PCP as a pesticide and wood preservative has led to its release into soil environments, particularly in timber yards and treatment plants (Freeman, 2010). Since the main source of PCP in the environment is from the wood protection industry, the magnitude of production of PCP treated products can serve as an indication of the potential for environmental release. At the annual meeting of the American Wood Protection Association in April 2015, it was noted that KMG (North America's sole producer of PCP) produced about 7.5 million kg (16.5 million pounds) of PCP in 2014 for use in treating about 2 million utility poles (Stratton, 2015).

2.2.2 As a Contaminant

Pentachlorophenol contamination of soil and water is of concern both during the treatment of wood, while the treated poles are in service or storage, and following disposal (Freeman, 2010). Since PCP has been widely used for the past 60 years in a wide range of domestic, agricultural, and industrial uses, it is commonly present in the environment (Pohleven and Boh, 2007). According to the Canadian Council of Ministers of the Environment (CCME), the maximum acceptable level of PCP in soil is 7.6 mg PCP/kg soil in agricultural, residential, commercial or industrial soils (CCME, 2013).

Some countries have regulated the use of PCP or banned its use entirely (Freeman, 2010). The use of PCP is restricted in countries such as Japan, where it was once used as an herbicide in rice fields (Sato, 1987). In China, the sodium salt of PCP (Na-PCP) was used extensively in the 1930s to mitigate the spread of shistosomes, and residual PCP is commonly found in various environmental media (Cheng et al., 2015).

Volatilization of PCP from treated wood introduces it into the atmosphere, where it can be deposited back into soil and surface water (Freeman, 2010). Pentachlorophenol is also a byproduct of the metabolism of other polychlorinated aromatic pollutants, such as chlorobenzenes, hexachlorocyclohexane and pentachloronitrobenzene, which are also common pollutants in the environment (Freeman, 2010). Since 1984, indoor application of PCP has been prohibited in North America and PCP use has been restricted to industrial applications. Even though PCP use is prohibited for use outside of the wood protection industry, its presence in the environment and toxicity will persist for years (Su et al., 2007). However, since releases of PCP have been limited, the concentrations found in environmental media are decreasing (Freeman, 2010).

2.2.3 As a Toxin

The toxicity of PCP is a result of its action as an uncoupler of oxidative phosphorylation by, causing cell membranes to become permeable to protons, disrupting the proton motive force responsible for energy generation (Steiert et al., 1988). At a concentration of 0.3 mg/L PCP, 50% uncoupling was seen in the isolated mitochondria of both potato (*Solanum tubersom*) and mung bean (*Phaseolus aureus*; Ravanel and Tissut, 1986).

Cellular-level toxic affects are helpful for understanding mechanisms of toxicity, but physiologic symptoms of the whole plant are easier to observe and measure.

Growth reduction is a common functional endpoint used to assess both chronic and acute toxicity in whole plants. Growth inhibition by 50% (EC_{50}) has been calculated after PCP exposure with several plants including soybeans (*Glycine max*) at 1.1-1.4 mg/L (Pfleeger et al., 1991) and lettuce (*Lactua sativa*) at 3 mg/kg and 7 mg/kg in artificial soil, 0.03 mg/L in nutrient media, 10 mg/kg in a coarse soil and 100 mg/kg in a fine soil (Hulzebos et al., 1993; Van Gestel et al., 1995; Martí et al., 2011). Ryegrass (*Lolium multiflorum*) had an EC_{50} of 100 mg/kg (Urrutia et al., 2013), as did wheat (*Triticum aestivum*; Dams et al., 2007). Several crop species including bean (green, black, Bengal, and horse), soybean, sunflower, safflower, maize, ground nut, and wheat were screened for tolerance to PCP with concentrations between 25 mg/kg to 50 mg/kg (Marihal et al., 2009). Reductions in biomass provide an easily quantifiable way to assess the health of a complex eukaryotic system like a plant.

Pentachlorophenol is also a membrane toxin in prokaryotic cell systems, but there are a few biochemical and functional differences as compared to eukaryotic membranes that should be mentioned. Lacking membrane-limited organelles, like mitochondria and chloroplasts, the cell membrane of bacteria takes over the membrane functions of these organelles (Prosser and Killham, 2007). A susceptible target for PCP toxicity in bacterial cells is membrane fluidity, where a stress response can induce a change in the lipid composition of the membrane and thus its fluidity. Pentachlorophenol is known to have an effect on membrane fluidity (Trevors, 1983). Since PCP can influence membrane

fluidity, it can also potentially alter membrane permeability and the activities of enzymes (Trevors, 1983).

Pentachlorophenol induces oxidative stress by disturbing normal cellular conditions leading to the production of reactive oxygen species (ROS) (Michalowicz et al., 2009). Detecting the presence of ROS can be difficult due to their short half-life and low concentrations in biological systems, necessitating alternative biomarkers (Sai-Kato et al., 1995). At a concentration of 0.005 mg/L, PCP increased the point mutation rate in zebrafish p53 gene (Yin et al., 2006). Hydroxyl radical production in PCP-stressed (60 mg PCP/L) organisms has also been shown by the product of hydroxyl radicals reacting with DNA (8-hydroxy-2'-deoxyguanosine) in mouse liver cells (Sai-Kato et al., 1995). Quantities of antioxidant enzymes and their activities are frequently used as indicators of the oxidative stress induced by chlorinated phenols including PCP (Michalowicz et al., 2009; Michalowicz et al., 2010; Roy and Hännien, 1994). The effects of PCP have been reported in the leaves of wheat and reed canary grass at 0.5 mg PCP/kg soil and 5 mg PCP/kg soil resulting in the induction of antioxidant enzyme activity and lipid peroxidation (Michalowicz et al., 2009; Michalowicz et al., 2010). The aquatic plant *Eichhornia crassipes* has been used to study PCP (0.5 mg/L) effects on the antioxidant system, suggesting increases in glutathione S-transferase (GST), superoxide dismutase (SOD), and ascorbate peroxidase (APOD) as useful biomarkers of aquatic pollution (Roy and Hännien, 1994). Quantities of antioxidant enzymes and their activities have been used as indicators of stress by chemicals similar to PCP, including pesticides and polyaromatic hydrocarbons (Drążkiewicz et al., 2003; Skipsey et al., 2011; Bulgakov et al., 2012). In *Arabidopsis thaliana*, the effects of colchicine (0.2% of nutrient medium)

caused SOD activity to increase, but decreased catalase (CAT) and peroxidase (POD) activities (Drążkiewicz et al., 2003). Induction of GST was used to compare *A. thaliana* responsiveness to the rice safener (chemicals used to enhance herbicide tolerance) fenclorim (4,6-dichloro-2-phenylprimidine) and related derivatives at a concentration of 23 mg/L (Skipsey et al., 2011). Increased antioxidant enzyme gene expression was induced by 3 mg/L of paraquat in *A. thaliana* (Bulgakov et al., 2012).

2.3 Borates

Borate is the term used to refer to the oxyanionic forms of boron. Borates are highly water soluble and diffuse freely. Boric acid results from the dissolution of borate compounds and predominates at the acidic pHs found in wood, water and soil due to its high pKa (9.24) (Freeman et al., 2009). Boron is also an essential micronutrient for plant growth and is taken up in the form of boric acid (Ozturk et al., 2010).

2.3.1 As a Preservative

Borates are unrestricted use chemicals due to their low toxicity, meaning that no records of use need to be kept, and some are sold over the counter as laundry products, such as Borax™. Disodium octaborate tetrahydrate (DOT) is the main borate used in wood preservation, though it has other uses, but production and sales data are not publicly available due to there being no restrictions on the sale and use of borates (Stratton, 2015). Borates inhibit general metabolism, reducing the growth and multiplication of organisms, making them useful for biodeterioration control (Llyod, 1998). Borates are used extensively as wood preservatives, despite their ineffectiveness with wood destined for

exterior use, or on products with ground contact, as their water solubility leads to borates being readily leached from the wood (Lloyd, 1998). This solubility is not entirely detrimental as it also allows borates to be highly mobile within wood even after the treatment process, allowing them to reach the inner heartwood (Lloyd, 1998). All borates will convert to boric acid once dissolved in an acidic media (Freeman et al., 2009). This includes wood, as the moisture held within wood is around pH 4 to 5 (Sithole, 2005).

2.3.2 As a Contaminant

Wetting of any degree will cause boric acid to leach from treated wood, leaving both environmental contamination and an untreated wood product (Toussaint-Dauverge et al., 2000). Boron is generally considered not to be toxic at normal concentration and is an essential plant micronutrient. Soils vary in boron content with some containing insufficient boron to support normal plant growth. Conversely, soils containing excess boron (> 15 mg/kg soil) can cause toxicity in some plants. The concentrations at which boron is insufficient, sufficient and in excess vary by plant with <0.5 mg/kg causing deficiency and >5.0 mg/kg leading to toxicity in some (Ozturk et al., 2010).

2.3.3 As a Toxin

Boric acid is the form in which plants extract the essential micronutrient boron from the soil, due to its solubility in water and ubiquitous presence in soil (Dordas and Brown, 2000). The homeostasis of boric acid and borates in a plant is regulated using uptake and efflux transporters (Takano et al., 2008).

Even though boron is important for plants, outside of optimal concentrations deficiency or toxicity occurs (~ 50 mg/kg media) (Aquea et al., 2012). Many species, including valuable crop plants like wheat and maize, are sensitive to elevated tissue levels of boron, with severe toxicity at concentrations around 50 mg/kg (Hakki et al., 2007). Plants use over 90% of boron taken up in the formation of borate ester cross-linked rhamnogalacturonan II dimers, which are necessary for cell wall structure and function (O'Neil et al., 2001). Boron's mode of toxicity results from complexation with polyols, subsequently inhibiting enzyme systems and altering membrane function (Lloyd, 1998). When borate ions complex with compounds that are enzymatic reactants or products, it can lead to the stimulation or inhibition of specific metabolic pathways in the plant (Lloyd, 1998). This leads to alteration of metabolite pools, which can alter the growth and development of plants (Lloyd, 1998).

Transcriptome analysis of *Arabidopsis thaliana* has shown that exposure to boron at concentrations of 300 mg/kg media impacted genes involved in stress responses as well as metabolism and transport (Aquea et al., 2012). When a plant senses toxic concentrations (300 mg/kg) of boron, it elicits a molecular response to inhibit its incorporation by reducing water absorption mediated by the phytohormone abscisic acid (Aquea et al., 2012). Exposure to boron at concentrations of 300 mg/kg resulted in cellular alterations in root meristems related to a reduction of mitotic activity and modifications of the expression patterns of key core cell cycle genes (Aquea et al., 2012).

All borons' functions are fundamental to meristematic tissues, meaning boron deficiency is most damaging to organisms that are actively growing, resulting in stunting (Hänsch and Mendel, 2009). The inhibition of root meristem cell division is associated

with this stress response and results in stalled root growth and subsequent death (Aquea et al., 2012). Reactive oxygen species may also be an important signal during boron toxicity as there is evidence that exposure to boron at concentrations of 100 mg/kg and 400 mg/kg alters the antioxidant machinery and produces oxidative stress damage (Ardic et al., 2009). Since boron increases the production of reactive oxygen species (ROS), it can cause lipid peroxidation thereby damaging cell membranes (Ardic et al., 2009).

A major concern in toxicology studies is the synergistic or antagonistic effect of a combination of pollutants on toxicity. With dual treatment of utility poles with PCP and boron as the context, the potential of the known toxicity of PCP to be altered by the plant nutrient boric acid will be considered.

2.4 Pollutant Combinations

Most ecotoxicological research and regulations focus on hazard and exposure assessment of individual chemicals, with chemical mixtures in the environment receiving less attention. Chemicals usually coexist in the environment, as opposed to as single constituents, potentially enhancing risks to the environment. Several single chemicals, at concentrations below no-observed-effect-concentrations (NOECs), when in mixtures can produce significant effects or enhance responses (Backhaus et al., 2011). Chemical combinations can be additive (toxicity of compounds in combination are roughly a sum of their individual toxicities), antagonistic (toxicity is less than additive) or synergistic (toxicity is greater than additive). Assessment of combined pollutant effects is becoming a more predominant research focus due to their significance for risk assessment, particularly in situations where compounds are known to co-exist. The toxicity of

mixtures relative to the toxicity of individual components can vary over time, concentration levels or ratios (Jonker et al., 2005).

Pollutants appear as complex mixtures in air, water, and soil with their combinations potentially producing unexpected results. Combination of sublethal levels of pollutants can lead to severe injury, suggesting that relevant combinations of pollutants require better understanding of their toxicity. A major concern is whether an unpredictable toxicity from the interaction of two or more chemicals will occur, especially when no measurable effect at low concentrations is seen. The toxicity of some environmental chemicals, when in a mixture, may be underestimated when their toxicity is evaluated individually (Sexton and Hattis, 2007). Standard water quality criteria are currently derived from individual chemicals to control potential impacts to human and ecological risk and for government management of contaminants. Polycyclic aromatic hydrocarbons (PAH's) in combination have synergistic detrimental effects on mycorrihzal growth and phosphorus transport to roots (Calonne et al., 2014). The degree of toxicity of arsenic exposure relates to the presence of other pollutants and their toxic mechanism (Mecozzi and Oteri, 2011).

Four mathematical models exist to calculate the combined toxicity of mixtures from the data for individual chemicals: concentration addition (CA), toxicity equivalency factors (TEF), effect summation (ES), and independent action (IA) (Silva et al., 2002). CA assumes that the components of the mixture act in a similar way and can be replaced by an equal fraction of equal potency with no diminishing effect to the mixture's effects. TEF is the toxicity of compounds with the same mechanism of action quantified as a single standard compound based on relative toxicity. ES is based on the expectation of

the arithmetic sum of individual component effects. IA considers that dissimilar modes of action of constituents result from interactions of individual mixture components (Silva et al., 2002).

Exposing bacterial cells to combinations of PCP at concentrations of 50 mg/kg and a positively charged complex of iron or copper at non-or sub-toxic concentrations (6 mg/kg of iron, 0.2 and 8 mg/kg of copper) caused an enhanced synergistic cytotoxicity, resulting in dramatic inhibition of growth and increased cell death (Wall and Stratton, 1994a; Zhu and Chevion, 2000; Levy et al., 2007). Observed synergism is associated with the formation of non-charged and lipophilic ternary complexes containing polychlorinated anions and the iron or copper complex transport into the cells (Zhu and Chevion, 2000; Levy et al., 2007). Transition metals, especially iron and copper, significantly enhance oxidative stress, often leading to cellular injury in various biological systems (Kawanishi et al., 2002). Increases in protein carbonyl content as well as the partial protection provided by ROS scavengers and protective enzymes, indicate that ROS might be partly responsible for the synergistic cytotoxicity of these ternary complexes (Levy et al., 2007). Copper, chromium, and arsenic are the active ingredients of chromated copper arsenate, a wood preservative often found in association with PCP contamination (Wall and Stratton, 1994b; Besser et al., 2005).

Mechanistic information from studying chemical effects has contributed to the assessment of risk to humans, but has not been as successful with environmentally relevant mixtures of chemicals. When under lead stress, ethylenediaminetetraacetic acid (EDTA) had a favorable effect on brassica plants by significantly improving plant growth, biomass, gas exchange, chlorophyll content, and activities of antioxidant

enzymes (Kanwal et al., 2014). Triazine herbicides have been shown to both increase the toxicity of organophosphate insecticides and decrease the toxicity of the fungicide prochloraz to humans (Hernandez et al., 2012). Glyphosate at very low concentrations has been shown to trigger apoptosis in human cells, and the presence of its main degradation product aminomethyl-phosphonic acid dramatically enhances this particular toxic effect, even though this product is less toxic (Benachour and Séralini, 2009). Ammonium exposure has also been found to enhance multi-contaminant exposure effects (Hasenbein et al., 2014).

These examples illustrate the potential for plant growth promoting boron to have synergistic, antagonistic or additive effects on PCP toxicity. At this time, no studies have been reported which specifically examine the influence of boron on PCP toxicity. In order to assess any potential interaction of these compounds, a model organism will be used in a bioassay that has an established history of use as an indicator organism.

2.5 Bioassays and Bioindicators

Chemical and physical methods can be used for detecting the presence of various contaminants and the exact amounts of certain chemicals, but not the potential toxicity and/or induced molecular responses to the compounds (Fontanetti et al., 2011). Bioindicators show the impact of contamination by giving information about the quality of the environment through alterations of its own physiology, morphology or behavior (Fontanetti et al., 2011).

The United States Environmental Protection Agency (USEPA) has recognized the opportunity to use plant systems in environmental risk assessment (Smith, 1991). Plants

are often more sensitive to toxicants than animal models used in toxicity testing, with the added benefit of no ethical limitations (Smith, 1991). Since 2012, the USEPA has established guidelines for several higher plant phytotoxicity bioassays which can be lab based, developed by the Office of Chemical Safety and Pollution Prevention (OCSPP) for the testing of pesticides and other toxic substances. They are: seedling emergence and seedling growth (USEPA, 2012a), vegetative vigor (USEPA, 2012b), early seedling growth toxicity test (USEPA, 2012c), aquatic plant toxicity test using *Lemna* spp. (USEPA, 2012d), rhizobium-legume toxicity (USEPA, 2012e), plant uptake and translocation test (USEPA, 2012f) and terrestrial soil-core microcosm test (USEPA, 2012g).

Growth based bioassays may be good for long term (days) evaluation, however molecular and cellular changes can be of use for short term (hours) diagnostic testing (Connon et al., 2012). As molecular and cellular level changes are first to occur under environmental stress, they can be observed earlier then other physiological changes. The *Alium* (onion) test is a routinely used, simple and efficient molecular bioassay to detect genetic damage from chemical agents and their mixtures (Kwasniewska et al., 2012). It is one of the best cytotoxicity assays, as reductions in mitotic index (ratio of cells undergoing mitosis) can be observed, as well as chromosomal abnormalities (Leme and Marin-Morales, 2009). Bacterial and *Alium* tests have shown comparable results and sensitivity (Kwasniewska et al., 2012).

Both boron, at high concentrations, and PCP are known to cause oxidative stress in exposed organisms. Indicators of oxidative stress, potentially useful as biomarkers in bioassays, will be elaborated on in the next section.

2.6 Anti-oxidant System

Plants have evolved a sophisticated antioxidant system that produces antioxidative enzymes to metabolize reactive oxygen species (ROS). At normal physiological levels they serve as signaling molecules to regulate a variety of functions, however in excess they can lead to cell damage and death (Demidchik, 2015). Reactive oxygen species are formed due to the effects of chlorophenols (such as PCP). Exposure to PCP at 0.5 mg/kg and 5 mg/kg has been shown to induce an intense lipid peroxidation, oxidation of polyunsaturated fatty acids, and inhibition of the activity of antioxidant enzymes (Michalowicz et al., 2009; Michalowicz et al., 2010). In the leaves of wheat, PCP exposure at 5 mg/kg was found to have provoked a rapid release of ROS by changing antioxidant enzyme activity and increasing free phenol content (Michalowicz et al., 2009). Increased ROS production has been reported to occur in soils exposed to 100 mg/kg and 400 mg/kg of boron, inducing lipid peroxidation and hydrogen peroxide (H_2O_2) accumulation, resulting in oxidative stress and damage (Ardic et al., 2009).

The electron transport activities of the chloroplast, mitochondria, and plasma membrane organelles, as well as normal cell metabolism, also generate ROS as an unavoidable byproduct (Demidchik, 2015). Reactive oxygen species can be generated in various cell compartments in low amounts, even under normal growth conditions. At low concentrations, H_2O_2 serves a signaling role for increasing a plant's resistance to stress. However, stressful conditions can disrupt the usual cellular homeostasis to increase their production (Demidchik, 2015). During oxidative stress, ROS, including superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxy radical ($OH^{\cdot-}$) are produced in excess in plant cells (Mutlu et al., 2011).

If ROS generation exceeds antioxidant protection, as is the case of oxidative stress induced by xenobiotics, there is oxidative damage. The best measure of damage caused by increasing production of ROS is lipid peroxidation (Erdal and Demirtas, 2010). Lipid peroxidation is a key event in oxidative damage, with malondialdehyde (MDA) as one of the best biological markers (biomarkers) as it is easier to accurately quantify due to the short half-life of ROS (Erdal and Demirtas, 2010).

Plants use non-enzymatic antioxidants (i.e. glutathione, ascorbate, and carotenoids) and enzymatic ROS scavenging mechanisms, such as superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) to mitigate oxidative stress (Erdal and Demirtas, 2010). Numerous environmental stresses can lead to an increase in superoxide within plant tissues. Plants rely on the enzyme superoxide dismutase in order to detoxify superoxide, decomposing it to O_2 and H_2O_2. Further oxidation occurs to molecular oxygen and water by the enzymes catalase and peroxidase (Erdal and Demirtas, 2010).

Biochemical parameters offer an early response to visualize toxic effects. However, looking at changes in gene regulation provides another potential early response to see a mechanism-effect relationship with toxicants.

2.7 Genetics and Toxicity

The use of genetic responses presents an opportunity to understand and measure how chemicals impact the health of organisms within ecosystems. Genetic responses provide a better understanding of the mechanisms of toxicity through gene and protein expression (Snape et al., 2004). There is limited information relating to the mechanism of how plants

respond to the exposure to xenobiotics and other environmental contaminants, particularly as they influence gene regulation (Ramel et al., 2012).

A plant's response to xenobiotics often involves changes in gene expression (Ramel et al., 2012). If contaminant levels trigger different signaling pathways within a plant species, then sensing these pathways could be used to detect contaminant exposure in a particular environment (Ramel et al., 2012). Toxicogenomic approaches have demonstrated that, even with their structural variability, most xenobiotics elicit a response in plant gene expression (Couée et al., 2013). While whole organism toxicological tests with described concentration end points of lethality are useful for detecting chemicals of concern, they provide limited insight into the nature and magnitude of effects of exposure to contaminants (Snape et al., 2004).

Changes in gene regulation can provide a lot of information about the mode of action of a stressor. Through transcriptomic profiling, pollutant sources and effects can be determined (Hasenbein et al., 2014). Transcriptome analysis as a biomarker has the potential benefit of giving information about the mechanisms of a toxicity signature for chemicals of interest (Denslow et al., 2007; Connon et al., 2012). The present study assesses whether transcriptome analysis of the model organism *Arabidopsis thaliana* could be the basis for a rapid, sensitive and reliable indicator of low levels of PCP.

2.8 *Arabidopsis thaliana*

The model organism A*rabidopsis thaliana*, commonly called thale cress, is a small flowering plant belonging to the mustard (Brassicaceae) family. Although *Arabidopsis thaliana* has no agricultural significance, it is an important research tool for genetic and

molecular biology scientists. It has a small genome (114.5 Mb/125 Mb total) and all 5 of its' chromosomes have been mapped. Columbia (Col-0) is the most widely used wild type of *Arabidopsis thaliana* and the one used for sequencing the *Arabidopsis* genome (The Arabidopsis Genome Initiative, 2000). The *Arabidopsis* genome contains 25,498 genes encoding proteins from 11,000 families (The Arabidopsis Genome Initiative, 2000). It has a relatively short life cycle (about 6 weeks), produces a large amount of seeds, cultivates easily even in restricted space and has an extensive number of mutant lines and other genomic resources in addition to a broad and multinational research community (TAIR, 2011).

The plant *Arabidopsis thaliana* demonstrates commonality for use in bioassays with the toxicology standard duckweed (*Lemna*) bioassay. *Arabidopsis* can be used to study a wide range of structural complexity (metabolic, genetic, physiological). *Arabidopsis*'s small size and fast growth allows for multiple simultaneous replications that can be easily standardized and controlled (Mkandawire et al., 2014). *Arabidopsis* lends itself as a useful bioassay test organism with simple handling, sensitive responses, cheap production, ease of replication, ability to work at low concentrations of test chemicals and nutrients similar to natural environments (Mkandawire et al., 2014).

In *A. thaliana*, gene expression has been used to study oxidative stress indicators for aromatic compounds (Liu et al., 2009; Weisman et al., 2010; Skipsey et al., 2011), pesticides (Laloi et al., 2007; Ramel et al., 2007; Skipsey et al., 2011; Bulgakov et al., 2012) and other chlorinated organics (Jin et al., 2011; Zhu et al., 2012; Li et al., 2015). Exposure of *A. thaliana* to 0, 2, 10, 20, and 100 mg/L of 2,2'-3,3'-tetrachlorobiphenyl, a representative polychlorinated biphenyl, resulted in the induction of 146 and suppression

of 148 genes involved in metabolic pathways, growth and development processes using microarray sequencing (Jin et al., 2011). Trichloroethylene stress was studied in *A. thaliana* (0, 50, 100, 200, 500 mg/L) using microarray to observe transcript expression in which 1,020 transcripts were upregulated, potentially contributing to its detoxification and tolerance (Zhu et al., 2012). Microarray was also used to profile gene expression in *A. thaliana* after exposure to trichlorophenol (0, 8, 16, 24 mg/L) and identified 34 transcripts induced, while 212 were repressed. Gene ontology (GO) analysis showed the genes to be involved in many processes related to growth and development (Li et al., 2015). *A. thaliana* has also been used for toxicant specific gene discovery using microarray, with the goal of developing cheap and efficient bioindicators (Krizek et al., 2003; Bao et al., 2013; Gunning et al., 2014).

Development of bioindicators is limited primarily by the inability to identify genes specific of a toxicant. Expression profiles obtained through RNA sequencing provide candidate genes, which can be useful in the development of plants designed to serve as bioindicators. The speed and ease of this sequencing is reliant on previously developed genomic sequence data of the organism of interest. A model organism like *A. thaliana* provides a quick and cost efficient means of developing bioindicators, which are specific and sensitive to the toxicant of interest (Krizek et al., 2003).

The upregulation of specific genes in *Arabidopsis* has been used for detecting and detoxifying the explosive 2, 4, 6-trinitrotoluene (TNT) (Gunning et al., 2014). Two glutathione S-transferases (GST-U24 and GST-U25) were specifically upregulated by TNT exposure (0, 25, 50, 100 mg/kg) and found to convert it to a more biodegradable form in the environment. However, when not in the presence of TNT the increased

expression of these genes caused a reduction of biomass (Gunning et al., 2014). Gene regulation has also been used for monitoring concentrations of heavy metals and polychlorinated biphenyls (PCBs) with *Arabidopsis* (Krizek et al., 2003; Bao et al., 2013). Xenobiotic response element reporter gene system can serve as a tool to monitor PCB (at concentrations of 0.0013 mg/kg to 1.3 mg/kg) uptake and metabolism, although not all tissues showed expression (Bao et al., 2013). Nickel (5 mg/kg to 500 mg/kg) was found to be the only heavy metal to cause the induction of the gene AHB1 in *A. thaliana*, with potential for this to be further developed into a nickel specific biomonitor to report its presence and concentration (Krizek et al., 2003). Overall, however, this plant biomonitor would be less sensitive then a bacterial one, although would demonstrate bioavailability and show effects on higher organisms (Krizek et al., 2003).

To discover the phenotypic and genetic response of *Arabidopsis thaliana* to exposure to PCP an early seedling growth bioassay was used to assess the toxicity of PCP, as well as to see if boric acid will have any affect on PCP toxicity, as they are used in combination as wood preservatives. The five objectives of this research are outlined above in section 1.1. The first three objectives are addressed in chapter 3 and the last two are addressed in chapter 4.

Chapter 3: Physiological response of *Arabidopsis thaliana* to exposure to pentachlorophenol and influence of boron

3.1 Introduction

The leaching or bleeding of chemicals from treated wood products into the environment can expose organisms to toxic chemicals. This toxicity is often expressed as physiological changes in the organism. Bioassays with defined and reproducible biological endpoints are a useful, fast, accurate, and low cost method to assess this toxicity on organisms.

Arabidopsis thaliana has been used in bioassays to measure antioxidant enzymes as indicators of chemical induced oxidative stress (Drążkiewicz et al., 2003; Liu et al., 2009; Skipsey et al., 2011; Bulgakov et al., 2012; Li et al., 2015). The primary goal of this study is to test the practicality and efficacy of an early seedling growth bioassay, using *A. thaliana*, in order to detect and assess PCP toxicity. The physiological endpoints to assess toxicity will be identified and quantified by calculating EC50 concentrations.

PCP is a known toxicant, while boric acid is a plant nutrient. The objectives of this study are to observe treatment and time effects for PCP toxicity and determine whether boric acid concentration influences the toxicity effects of PCP in an additive, synergistic, or antagonistic way. The plant physiological features examined in this study, including accumulated plant biomass (fresh and dry weight), activity of the antioxidant enzymes catalase, guaiacol peroxidase, superoxide dismutase, and the oxidative stress indicator malondialdehyde, will be assessed for their suitability as early bioindicators for stress caused by PCP exposure in *Arabidopsis thaliana*.

3.2 Materials and Methods

Physiological features of seedling growth, including plant biomass (fresh and dry weight) and enzyme activity, were evaluated as early response biomarkers. PCP concentrations from 1 to 100 mg/L were initially screened for toxicity towards plant growth. Plant lethality was observed over 10 mg/L, so PCP inhibitory concentrations were selected as 1 to 5 mg/L. Concentrations of boric acid to be added were selected based on the ratio of in-pole retention during envelope treatment with PCP (0.40-0.45 pounds (0.18-0.20 kg) per cubic foot) and borates (0.17 pounds (0.08 kg) per cubic foot), which gives a ratio of borates to PCP of 0.4 of a mass basis.

3.2.1 Assay Chemicals

Technical grade (≥80% with ≤15% water) sodium pentachlorophenol (Sigma-Aldrich) was used, as it is readily soluble in water. Concentrations of sodium pentachlorophenol used for assays were 0, 1, 2, 3, 4, and 5 mg PCP/L. In addition to the 3.1 mg/L of boric acid contained in the half strength Murashige and Skoog (MS) basal medium, assay grade (≥ 99.5%) boric acid (Sigma-Aldrich) was added to establish final boric acid concentrations of 3.1, 3.5, 3.9, 4.3, 4.7, and 5.1 mg/L. This required addition of boric acid at 0.4, 0.8, 1.2, 1.6, and 2 mg/L, respectively. Data tables and figures emphasize the total boric acid levels. The interaction between PCP and boric acid was evaluated by adding both PCP and boric acid to growth media.

3.2.2 Seedling Preparation

Seeds from wild type *Arabidopsis thaliana* ecotype Columbia (Col-0) were surface-sterilized in a 1.5 mL microcentrifuge tube using sodium hypochlorite (1 mL of 100% sodium hypochlorite for 1 minute) and then rinsed thoroughly (5 times) with sterile autoclaved distilled water. The sterilized seeds were then vernalized for 48 hours in the dark at 4 °C to facilitate uniform germination.

Germination was conducted by spreading the seeds on solid growth medium (pH 5.7) containing 0.8% agar with half-strength Murashige and Skoog (1/2 MS) medium (Sigma-Aldrich) supplemented with 0.5% sucrose. Plates with germinated seeds were then transferred to room temperature under continuous light for 10 days.

3.2.3 Liquid Culture Bioassay

Wild type *Arabidopsis* seedlings (Col-0) were transferred to 24-well cell culture cluster plates containing 1 mL of liquid half-strength MS basal medium per well. After 2 days of plants acclimating in liquid MS, media was changed and treatment concentrations were added. Concentrations (listed in section 3.2.1) were chosen following a screening experiment where the physiological response was used to establish working concentration ranges. These plates were placed on an orbital shaker set at 90 rpm. Plants received light from cool fluorescent tubes with a 16:8 h photoperiod at 22 °C.

Three 24-well plates were assigned to each chemical system tested (PCP, boric acid, and PCP plus boric acid), with 4 plants on each plate assigned per chemical treatment on each plate. These 4 plants were physically combined (pooled) together before testing to provide one pooled biomass sample per plate to be weighed on an

analytical scale. Therefore, a single replicate of a chemical treatment concentration was composed of a pooled sample consisting of 4 plant seedlings. Plants were pooled to provide appropriate tissue amounts for samples and to decrease variability.

3.2.4 Biochemical Testing

All biochemical assays were conducted in 96 well plates and quantified by spectrophotometric detection methods. A single replicate of a chemical exposure treatment was composed of the material extracted from the plant biomass of the 4 pooled seedlings per plate. All assays were run in triplicate and absorbance at the applicable wavelength was measured using a BioTek Power XS2 microplate reader (VT, USA) with Gen5^{TM} software (www.biotek.com).

3.2.5 Tissue Collection and Storage

12-d-old *Arabidopsis* seedlings were taken out of liquid culture from the 24-well plates at 6 hours, 12 hours, and 24 hours post treatment. *Arabidopsis* seedlings, 4 whole 12-day-old plants pooled together as a single replicate per plate, were flash frozen in liquid nitrogen and stored at -80 ºC until use.

3.2.6 Crude Enzyme Extraction

Frozen whole seedlings were ground using a mortar and pestle and soluble proteins were extracted from 0.15 g fresh weight per sample. 1 mL of 4°C extraction buffer composed of 50 mM sodium phosphate buffer (pH 7.5), 0.5% polyvinylpyrrolidone (PVP) and 3 mM Ethylenediaminetetraactetic acid (EDTA) was added to the samples and mixed well.

The homogenate was centrifuged at 12,000 g for 20 min at 4 ºC and the supernatant was pipetted into a new 1.5 mL tube to be used for the antioxidant enzyme activity assays. This is referred to as the crude enzyme extract.

3.2.7 Total Protein Quantification

The soluble protein content of the crude enzyme extracts was estimated using the Coomassie Plus - the Better Bradford™ Assay reagent (Pierce, Rockford, IL, USA). Absorbance was read after 2 min at 595 nm in 96 well microplates with wells containing 200 µL of Bradford reagent, 35 µL of water and 5 µL of crude enzyme extract. The protein content of each sample was calculated using a bovine serum albumin (BSA) standard curve (0.1-1.0 mg/mL) made with standards reacted with Bradford™ reagent and absorbance values taken and plotted against BSA concentration (Bradford, 1976). The protein assay was done to convert enzyme activity into assay units per mg of protein.

3.2.8 Catalase (CAT) Activity Assay

Total CAT activity was estimated colorimetrically using a catalase assay kit and reagents including the chromogen catalase purpald (Cayman Chemical). One unit was defined as the amount of enzyme that will cause the formation of 1.0 nmol of formaldehyde per minute at 25°C. Absorbance was measured after 20 min at 540 nm and formaldehyde concentration of samples was calculated (µM) with CAT Activity = µM of sample/20 min x sample dilution = nmol/min-mL.

3.2.9 Guaiacol Peroxidase (GPOD) Activity Assay

Total GPOD activity was estimated using the procedure from Chance and Maehly (1955), with modifications. The 205 µL total reaction mixture contained 0.02 M guaiacol, 50 mM sodium acetate buffer (pH 5), 0.06 M H_2O_2 and 5 µL of crude enzyme extract. The formation of tetra-guaiacol product was followed every minute during a period of 5 minutes by the increase in the absorbance at 470 nm. In the calculation of change in absorbance OD470 /min, a molar extinction coefficient for tetra-guaicol of E_{A475}= 26.6 mM^{-1} cm^{-1}, were cm is length of light path, was used.

3.2.10 Superoxide Dismutase (SOD) Activity Assay

Total SOD activity was estimated by using a SOD determination kit and reagents (Sigma-Aldrich). The equation SOD activity (inhibition rate %) = {[(AbsBlank 1 – AbsBlank 3) – (AbsSample – AbsBlank 2)]/(AbsBlank 1 – AbsBlank 3)} x 100 was used.

Where:

Blank 1= WST (Dojindos water soluble tetrazolium salt)+H_2O+Enzyme working solution,

Blank 2= sample solution+WST+Dilution buffer,

Blank 3= WST+H_2O+Dilution buffer. SOD from bovine erythrocytes (S7571) from Sigma-Aldrich was used to determine SOD amounts using a standard curve. Absorbance at 450 nm was measured after 20 minutes of incubating at 37°C.

3.2.11 Lipid Peroxidation Assay

Lipid peroxidation is a key event in oxidative damage, with malondialdehyde (MDA) as one of the best biomarkers (Erdal and Demirtas, 2010). For the lipid peroxidation assay 0.15 g of fresh tissue was suspended in 5 mL of 0.1% TCA and then centrifuged at 10,000 rpm for 10 min at 4°C. 100 µL of the supernatant was taken and mixed with 100 µL of 0.5% thiobarbituric acid in 20% trichloroacetic acid. The mixture was heated at 96°C for 30 min and then cooled on ice to room temperature. The absorbance value was measured at 532 nm and 600 nm and MDA content was then calculated by subtracting the absorbance value at 600 nm from that at 532 nm and dividing by the molecular extinction coefficient, where cm relates to optical path length, of 155 mM^{-1} cm^{-1}. This procedure was taken from Dhindsa et al. (1981) with modifications for microplate.

3.2.12 Statistical Analysis

All treatments were conducted in triplicate. All data sets were tested for normality and constant variance using SAS (version 9.3, SAS Institute, Cary, C, USA). Data were transformed where required and as noted for each test. Transformed data were used for statistical tests but untransformed data are shown in figures and tables. One way ANOVA using SAS Proc GLM was employed for treatment, time, and treatment x time comparisons for these assays at p= 0.05. Significant differences between treatments were determined using Tukey's honesty significant difference (HSD) post hoc test at p= 0.05. EC_{50} was calculated by linear regression, substituting half of the control (no PCP) value for y and solving for x. The EC_{50} is the effective concentration of a chemical that causes a 50% reduction in a defined bioassay. Here it is a 50% reduction in biomass.

3.3 Results

Physiological features of seedlings were measured, including the biomass of the plant (fresh and dry weight) after growing in chemical treatments for 6 days. The activity of antioxidant enzymes was quantified, as early response biomarkers, as well as malondialdehyde (MDA) content as a biomarker of oxidative damage after 6, 12, and 24 hours. Test plants were first screened for toxic threshold level/minimum inhibitory concentration with PCP and applicable test levels chosen. After this the concentration of boric acid was based on the ratio of in-pole retention during envelope treatment with PCP and borates. Test plants were grown in 24-well plates containing 1 mL of liquid half-strength Murashige and Skoog (1/2 MS) basal medium with PCP and boric acid added. The media had a background concentration of 3.1 mg/L boric acid, so the boric acid test levels used are in addition to this.

Three 24-well plates were assigned to each chemical system tested (PCP, boric acid, and PCP plus boric acid), with 4 plants on each plate assigned per chemical treatment on each plate. These 4 plants were physically combined (pooled) together before testing to provide one pooled biomass sample per plate to be weighed on an analytical scale. Therefore, a single replicate of a chemical treatment concentration was composed of a pooled sample consisting of 4 plant seedlings.

3.3.1 Growth (biomass) Endpoint Assays

After 6 days growing in liquid culture (Figure 3.1), toxic threshold level/minimum inhibitory concentrations of PCP were screened from 1 to 100 mg/L. Lethality was observed over 10 mg/L, so concentrations were selected as 1 to 5 mg/L for experiments.

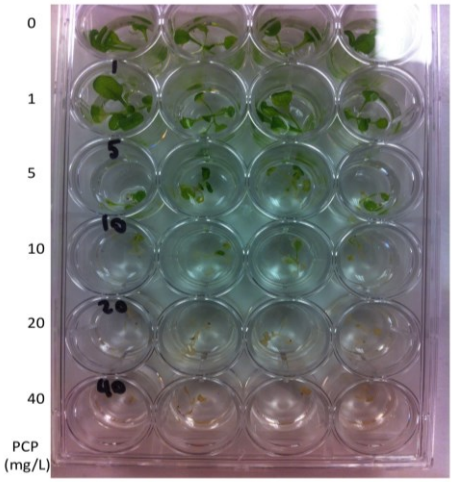


Figure 3.1: *A. thaliana* seedlings 6 days post treatment in liquid culture, exposed to PCP.

Fresh weight biomass (Table 3.1 and Figure 3.2) was significantly affected (p=0.0006) by PCP exposure, resulting in a decrease in biomass after 6 days relative to the control. Fresh weight of seedlings was found to be unaffected (p=0.4532) by additional boric acid exposure relative to seedlings grown in just MS media. There was also no significant treatment effect observed in seedlings treated with a combination of PCP and boric acid (p=0.1805) compared to the control (no PCP) seedlings. Paired T-tests showed no significant differences between PCP (with 3.1 mg/L boric acid) and PCP with added boric acid 1 mg/L against 1+3.5 mg/L boric acid (p=0.3177), 2 against 2+3.9 (p=0.5197), 3 against 3+4.3 (p=0.5163), 4 against 4+4.7 (p=0.2887) or 5 against 5+5.1 (p=0.3989). Fresh weight data required no transformation for statistical purposes.

Dry weights (Table 3.1) showed a significant (p=0.0006) decrease after 6 days of PCP exposure. In contrast to the fresh weight data, dry weights were significantly reduced by additional amounts of boric acid (p=0.0302). The combination treatments of PCP and additional boric acid were also found to have a significant negative effect

(p=0.0012) on dry weight. For all chemical treatments, the highest treatment concentration resulted in the greatest difference in dry weight. Data for boric acid dry weight was transformed ((Dry Weight)^2) for the purpose of statistical analysis. Paired T-tests showed no significant differences between PCP and PCP with a boric acid addition rate at 1 against 1+3.5 (p=0.9727), 2 against 2+3.9 (p=0.7902), 3 against 3+4.3 (p=0.5638), 4 against 4+4.7 (p=0.8086) or 5 against 5+5.1 (p=0.9156). Remaining dry weight data required no transformation.

Table 3.1: Fresh and dry weight (g) per 4 plants of *A. thaliana* seedlings exposed to PCP and boric acid addition rates alone and in combination. Table values are the mean grams of weight ± standard error. Means with the same Tukey grouping letter are not significantly different. Each chemical treatment is considered separately.

Treatment	Fresh Weight (g) Per 4 Plants	Dry Weight (g) Per 4 Plants
PCP		
Control (0 mg/L)	0.313 ± 0.022 A	$12.8 \times 10^{-3} \pm 1.33 \times 10^{-3}$ A
1 mg/L	0.253 ± 0.019 BA	$10.6 \times 10^{-3} \pm 1.03 \times 10^{-3}$ BA
2 mg/L	0.231 ± 0.018 BAC	$9.20 \times 10^{-3} \pm 7.00 \times 10^{-4}$ BAC
3 mg/L	0.196 ± 0.028 BC	$6.13 \times 10^{-3} \pm 1.16 \times 10^{-3}$ BC
4 mg/L	0.160 ± 0.018 BC	$5.20 \times 10^{-3} \pm 3.00 \times 10^{-4}$ C
5 mg/L	0.144 ± 0.009 C	$5.17 \times 10^{-3} \pm 1.13 \times 10^{-3}$ C
Boric Acid Addition Rate		
Control (3.1 mg/L)	0.268 ± 0.034 A	$12.2 \times 10^{-3} \pm 1.06 \times 10^{-3}$ A
0.4 mg/L (3.5 mg/L)	0.226 ± 0.038 A	$9.81 \times 10^{-3} \pm 3.00 \times 10^{-4}$ BA
0.8 mg/L (3.9 mg/L)	0.209 ± 0.031 A	$9.13 \times 10^{-3} \pm 7.66 \times 10^{-4}$ BA
1.2 mg/L (4.3 mg/L)	0.164 ± 0.023 A	$7.86 \times 10^{-3} \pm 1.10 \times 10^{-3}$ B
1.6 mg/L (4.7 mg/L)	0.219 ± 0.046 A	$9.07 \times 10^{-3} \pm 9.66 \times 10^{-4}$ BA
2 mg/L (5.1 mg/L)	0.213 ± 0.021 A	$7.97 \times 10^{-3} \pm 6.67 \times 10^{-5}$ B
PCP + Boric Acid Addition Rate		
Control (0+3.1 mg/L)	0.381 ± 0.096 A	$13.1 \times 10^{-3} \pm 1.63 \times 10^{-3}$ A
1+3.5 mg/L	0.301 ± 0.047 A	$10.8 \times 10^{-3} \pm 1.07 \times 10^{-3}$ BA
2+3.9 mg/L	0.241 ± 0.014 A	$8.70 \times 10^{-3} \pm 2.00 \times 10^{-4}$ BC
3+4.3 mg/L	0.216 ± 0.018 A	$8.03 \times 10^{-3} \pm 1.67 \times 10^{-4}$ BC
4+4.7 mg/L	0.239 ± 0.023 A	$7.40 \times 10^{-3} \pm 9.00 \times 10^{-4}$ BC
5+5.1 mg/L	0.205 ± 0.042 A	$5.87 \times 10^{-3} \pm 1.67 \times 10^{-4}$ C

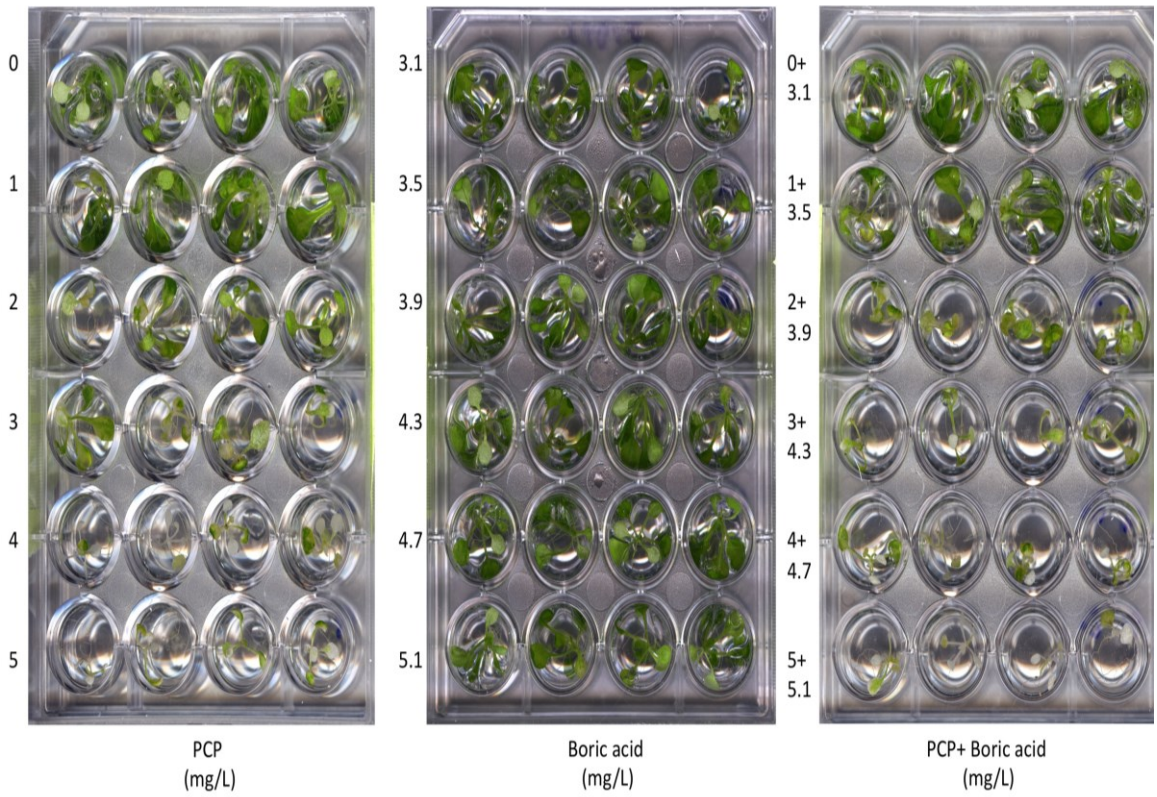


Figure 3.2: *A. thaliana* seedlings 6 days post treatment in liquid culture, exposed to PCP and boric acid addition rates.

An EC_{50} value of 5.32 mg PCP/L was calculated using linear regression of plotted fresh weight values after 6 days (Figure 3.3A) for PCP treated seedlings ($y = -0.0331x + 0.3321$, $R^2 = 0.97$). An EC_{50} value of 4.53 mg/L was also calculated for dry weights after 6 days (Figure 3.3B) for PCP treated seedlings ($y = -0.0017x + 0.0141$, $R^2 = 0.87$).

An EC_{50} for boric acid toxicity for fresh and dry weight after 6 days could not be calculated as it is well above the concentrations used of 2 mg/L additional boric acid (5.1 mg/L total).

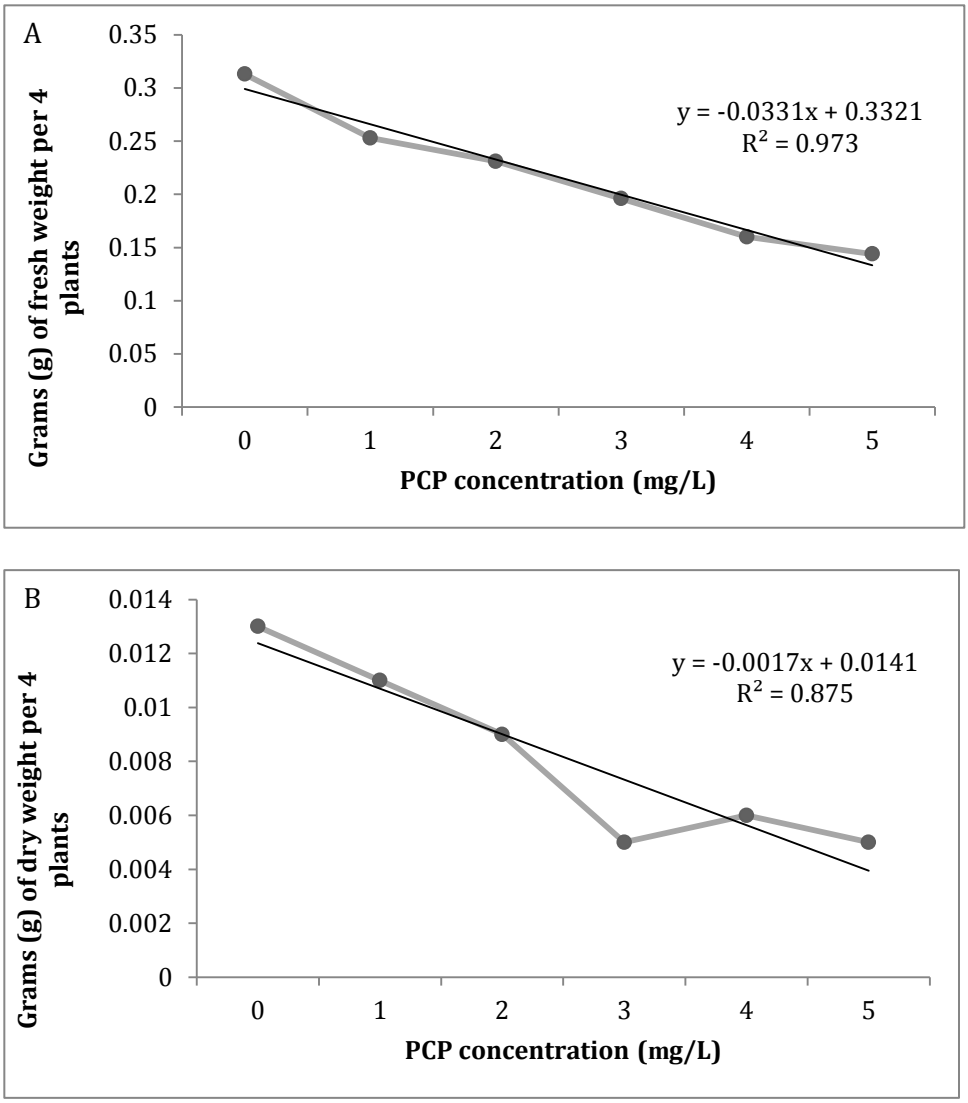


Figure 3.3: The effect of PCP on fresh (A) and dry (B) weight (g) after 6 days.

The pH of the growth medium (Figure 3.4) was found not to be affected enough by the rate of boric acid addition to result in a change of the form of PCP present in solution. The measurement of pH was done to rule out the possibility that boric acid was substantially lowering the pH, which could then have resulted in PCP becoming less water soluble, as it has a pKa of 4.7 at 25°C (Freeman, 2010).

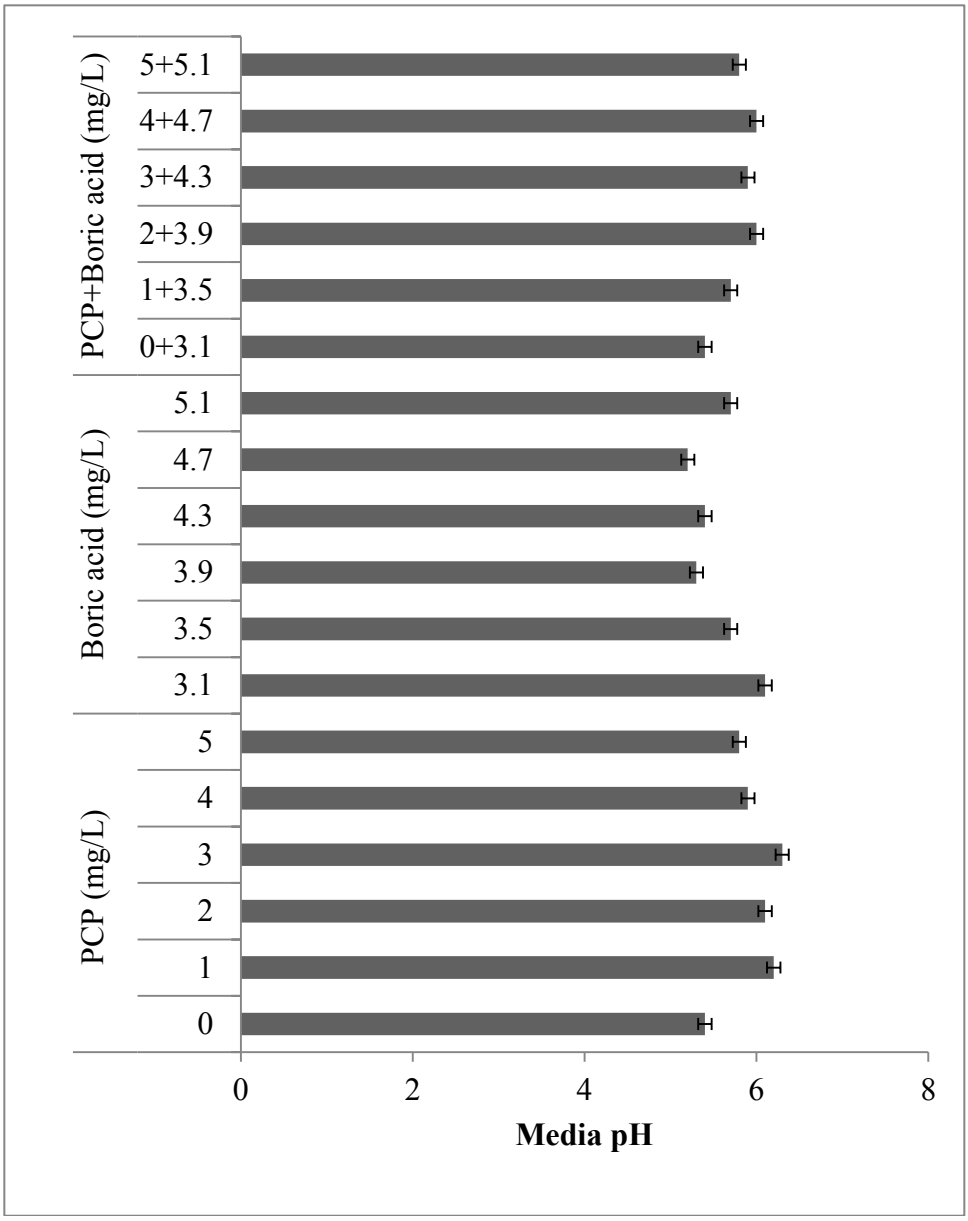


Figure 3.4: pH of liquid culture media after 6 days post treatment, with PCP and a boric acid addition rate alone and in combination. Bars are standard errors.

3.3.2 Biochemical Time Series Activity Assays

Analysis of variance (ANOVA) of the influence of PCP addition and at three time intervals was used to assess the impact on catalase activity (U CAT/mg of protein). When compared to the control (no PCP) treatment all concentrations of PCP addition showed significantly reduced activity effect of treatment (Table 3.2). The most significant decrease in activity was at 6 h were activity decreased by increasing treatment concentration. This same significant decrease in activity was observed with increasing treatments of PCP with increasing boric acid (Table 3.4). A significant increase in catalase activity was observed at 12 hours where an increase in activity was seen at the lowest treatment concentration, followed by a decrease at each further concentration. Increasing concentrations of boric acid added decreased catalase activity at 6 h, however had no significant effect on catalase activity at 12 and 24 hr (Table 3.3). Data for PCP CAT activity was transformed ($(CAT)^{0.5}$), but the remaining CAT activity data required no transformation.

A significant interaction of treatment x time required that means comparisons were performed across all treatment combinations for guaiacol peroxidase activity (U GPOD/mg of protein). No significant variation was observed with treatment concentrations of PCP (Table 3.2) or of PCP with additional boric acid (Table 3.4). The concentration of boric acid added had no significant impact on guaiacol peroxidase activity (Table 3.3). Data for PCP GPOD activity were transformed ($(GPOD)^{0.5}$) but the remaining GPOD activity data required no transformation.

Table 3.2: Antioxidant enzyme activity (U = nmol/min/mL) of *A. thaliana* seedlings exposed to PCP. Table values are the mean U/mg protein ± standard error.Means followed by the same capital letters refer to treatment differences over concentration and time period. Each chemical treatment is considered separately.

Treatment	Catalase Activity (U CAT/mg protein)			Guaiacol Peroxidase Activity (U GPOD/mg protein)			Superoxide Dismutase Activity (U SOD/mg protein)		
	6 h	12 h	24 h	6 h	12 h	24 h	6 h	12 h	24 h
PCP									
0 mg/L	804 ± 31 A	122 ± 60 B	437 ± 69 AB	131 ± 37 A	61 ± 15 BC	92 ± 15 ABC	23 ± 8 AB	30 ± 6 A	18 ± 2 BC
1 mg/L	299 ± 30 B	754 ± 314 A	443 ± 126 AB	122 ± 14 A	86 ± 28 ABC	78 ± 7 ABC	16 ± 10 BC	37 ± 16 A	16 ± 2 BC
3 mg/L	222 ± 46 B	421 ± 141 AB	703 ± 79 A	104 ± 4 AB	39 ± 11 C	129 ± 17 A	13 ± 1 C	17 ± 3 B	22 ± 1 AB
5 mg/L	170 ± 18 B	209 ± 109 B	446 ± 62 AB	97 ± 32 AB	55 ± 15 BC	95 ± 12 ABC	12 ± 6 C	17 ± 3 B	18 ± 3 BC

When compared to the control (no PCP), the addition of increasing amounts of PCP resulted in a decrease in superoxide dismutase activity (U SOD/mg protein) at 6 h (Table 3.2). At 12 h there was a more rapid decrease in U SOD activity with increasing concentration of PCP added and activity at 12 h was greater than at 6 h for the three highest concentrations. At 24 h there was no significant effect of PCP addition on SOD activity. Increasing concentrations of added boric acid resulted in a decrease SOD activity at 6 h, with an increase at the highest concentration level (Table 3.3). At 12 h there was a sharp decrease in activity as a result of the addition of 3.5 mg/L of boric acid, which continued to the further treatment concentrations. When examining the influence

of PCP and boric acid addition, at 6 h and 12 h, a significant decrease in SOD activity was observed from the control as a result of the addition of the lowest treatment concentration, but no significant effect was observed with further additions of PCP and boric acid (Table 3.4). No significant difference was observed at 24 h. Data for boric acid superoxide dismutase activity was transformed (LOG 10) for the purpose of statistics. Boric acid treatment data required no transformation.

Table 3.3: Antioxidant enzyme activity (U = nmol/min/mL) of *A. thaliana* seedlings exposed to a boric acid addition rate. Table values are the mean U/mg protein ± standard error. Means followed by the same capital letters refer to treatment differences over concentration and time period. Each chemical treatment is considered separately.

Treatment	Catalase Activity (U CAT/mg protein)			Guaiacol Peroxidase Activity (U GPOD/mg protein)			Superoxide Dismutase Activity (U SOD/mg protein)		
	6 h	12 h	24 h	6 h	12 h	24 h	6 h	12 h	24 h
Boric Acid Addition Rate									
3.1 mg/L	804 ± 31 A	122 ± 60 EF	437 ± 69 BCD	131 ± 37 AB	61 ± 15 CDE	92 ± 15 ABCDE	23 ± 8 AB	30 ± 6 A	18 ± 2 BC
3.5 mg/L	332 ± 54 CDE	276 ± 31 CDEF	459 ± 56 BC	131 ± 26 AB	39 ± 7 E	117 ± 4 ABCD	17 ± 4 BC	15 ± 1 B	20 ± 2 AB
4.3 mg/L	264 ± 47 CDEF	213 ± 76 DEF	424 ± 83 BCD	81 ± 2 BCDE	48 ± 15 DE	98 ± 6 ABCDE	11 ± 1 C	16 ± 2 AB	17 ± 3 BC
5.1 mg/L	330 ± 74 CDE	107 ± 44 F	606 ± 150 AB	129 ± 45 ABC	52 ± 11 DE	160 ± 42 A	20 ± 6 AB	15 ± 2 B	15 ± 1 C

Table 3.4: Antioxidant enzyme activity (U = nmol/min/mL) of *A. thaliana* seedlings exposed to PCP and a boric acid addition rate in combination. Table values are the mean U/mg protein ± standard error. Means followed by the same capital letters refer to treatment differences over concentration and time period. Each chemical treatment is considered separately.

Treatment	Catalase Activity (U CAT/mg protein)			Guaiacol Peroxidase Activity (U GPOD/mg protein)			Superoxide Dismutase Activity (U SOD/mg protein)		
	6 h	12 h	24 h	6 h	12 h	24 h	6 h	12 h	24 h
PCP + Boric Acid Addition Rate									
0+3.1 mg/L	804 ± 31 A	122 ± 60 E	437 ± 69 BC	131 ± 37 AB	61 ± 15 CD	92 ± 15 ABC	23 ± 8 AB	30 ± 6 A	18 ± 2 BC
1+3.5 mg/L	373 ± 90 BC	382 ± 102 BC	256 ± 21 CDE	116 ± 7 BCD	79 ± 4 ABC	89 ± 9 ABC	15 ± 1 BC	13 ± 1 C	15 ± 2 BC
3+4.3 mg/L	374 ± 37 BC	355 ± 10 BCD	426 ± 40 B	110 ± 1 ABC	80 ± 13 BCD	99 ± 12 ABC	15 ± 1 BC	15 ± 1 BC	14 ± 3 BC
5+5.1 mg/L	193 ± 35 DE	116 ± 56 E	199 ± 53 DE	127 ± 16 AB	30 ± 4 D	142 ± 43 A	18 ± 1 BC	19 ± 4 BC	18 ± 1 BC

Analysis of variance of MDA content (nm/g fresh weight) (Table 3.5) showed no significant ($p=0.6040$) affect for PCP addition. This observation was consistent over all three observation times ($p=0.0401$). Boric acid treatment showed no significant effect ($p=0.5895$), and a non-significant time effect ($p=0.0923$). ANOVA comparison of 5 mg/L PCP against 5+2 mg/L PCP+BA showed no significant ($p=0.5614$) treatment effect due to the addition rate of boric acid.

Table 3.5: MDA content (nm/g of fresh weight) for *A. thaliana* seedlings exposed to PCP and a boric acid addition rate alone and in combination. Table values are the mean MDA content ± standard error. Means with the same capital letters refer to comparisons of treatment means at a given time period. Lowercase letters refer to comparisons of means for a given treatment concentration over time.

Treatment	MDA content (nm/g Fresh Weight)		
	6 h	12 h	24 h
3.1 mg/L Boric Acid	0.99 ± 0.28 A a	1.2 ± 0.14 A a	1.54 ± 0.52 A a
5 mg/L PCP	0.86 ± 0.14 A a	0.69 ± 0.31 A a	1.81 ± 0.32 A a
2 mg/L (5.1 mg/L) Boric Acid	0.82 ± 0.27 A a	2 ± 0.17 A a	1.29 ± 0.22 A a
5 +5 .1 mg/L PCP + Boric Acid Compared to 5 mg/L PCP	1.16 ± 0.16 A	1.07 ± 0.28 A	1.59 ± 0.06 A

3.4 Discussion

3.4.1 Biomass Endpoint Assay

The purpose of plant based laboratory tests, such as the ones considered in this study, is to evaluate the potential for chemical damage to species and to assess potential environmental impacts. This is achieved by exposing organisms to toxicants for defined periods of time with a biological marker (biomarker) quantified (Mkandawire et al., 2014). Dual treatment of utility poles with PCP and boron provides the context to evaluate the potential for the known toxin PCP to have its toxicity affected by the plant nutrient boric acid.

Growth inhibition is a functional endpoint used to assess chronic and acute toxicity. Fresh weights (biomass accumulation) were significantly reduced by PCP exposure, with an EC_{50} of 5.32 mg/L, as was dry weight, with an EC_{50} of 4.53 mg/L. No significant affect was observed with additional boric acid or PCP with additional boric acid. PCP levels of 10 mg/L and higher killed the test plants. A similar range has been

reported in previous studies with short-term plant growth assays, such as 5-day whole plant growth bioassays with soybeans, which showed an EC_{50} (mg/L) of 1.1-1.4 for PCP (Pfleeger et al., 1991). A higher range was seen in lettuce (*Lactua sativa*), where Martí et al. (2011) saw 50% growth inhibition for PCP concentrations at 10 mg/kg in coarse textured soil and 100 mg/kg in fine textured soil. Significant increases in growth, however, were found at the lowest concentrations (0.001, 0.01, 0.1 and 1 mg/kg) (Martí et al., 2011). Also in *L. sativa*, Hulzebos et al. (1993) found a 50% growth reduction at a soil PCP concentration of 3.2 mg/kg at one facility, and 7 mg/kg at another. Growth reduction of 50% has also been reported at 3.4 mg/kg in artificial soil (Van Gestel et al., 1995). Growth of several plants was significantly affected at 25 mg/kg of PCP, with further inhibitory influence at 50 mg/kg (Marihal et al., 2009). Much higher concentrations then those used in this study were needed for a significant decrease of ryegrass (*Lolium multiflorum*) biomass, observed at soil concentrations of 100 mg/kg (Urrutia et al., 2013). At 100 mg/kg, the same effect in wheat (*Triticum aestivum*) was observed (Dams et al., 2007). In the present study, PCP levels ≥ 10 mg/L caused plant death.

3.4.2 Biochemical Activity Endpoint Assays

Phenotypic changes allow a better understanding of how a plant adapts to stress. Phenotypic responses include changes in enzymatic proteins and molecular level responses. In general, plants increase the enzymatic antioxidant activity in an effort to decrease damage from stress (Demidchik, 2015). If lipid peroxidation levels and H_2O_2 content decrease due to antioxidant enzyme activities, tolerance against stress can occur.

PCP and its metabolites can produce extremely reactive hydroxyl radicals and other intermediate radicals (Zhu et al., 2007; Zhu and Shan, 2009). At low concentrations, H_2O_2 serves a signaling role for increasing a plants' resistance to stress, but at high concentrations it causes oxidative stress (Demidchik, 2015).

The activities of catalase (CAT), guaiacol peroxidase (GPOD), and superoxide dismutase (SOD) were considered as toxicity endpoints in order to see if these enzyme activities mirrored trends to the biomass accumulation. The enzyme activities of the PCP treatment with basal levels of boric acid showed significance mainly at lower levels of treatment, suggesting a threshold of affect reached at that concentration. The addition of more boric acid did not change this observation. Significant effects of exposure time were observed at 6 hours for CAT activity for PCP and boric acid addition rate. The remaining exposure times showed no statistically significant effect for PCP for enzyme activities.

Michalowicz et al. (2009) showed that PCP induced oxidative stress and damage in the leaves of wheat plants. SOD activity was shown to be inhibited by PCP, and other chlorinated phenols, while CAT activity was increased by PCP at lower concentrations (0.5 mg/kg), depleted enzyme activity at higher concentrations (5 mg/kg). GPOD activity also increased through PCP exposure (Michalowicz et al., 2009). Herman et al. (1998) observed an increase of GPOD activity under the influence of aromatic herbicides (1,10-phenanthroline) in some plant species, as well as decreases in CAT activity after exposure over the treatment range (90 mg/kg to 1800 mg/kg).

The best measure of damage caused by increasing production of reactive oxygen species is lipid peroxidation (Erdal and Demirtas, 2010). PCP at 0.5 mg/kg and 5 mg/kg is known to induce lipid peroxidation, leading to an increase in MDA levels by

generating reactive oxygen species (Michalowicz et al., 2009). The products from lipid peroxidation are normally removed by antioxidants. Increases in lipid peroxidation are observed by chlorophenol exposure at 0.5 mg/kg in reed canary grass leaves, and then decreased with time, showing an ability to reduce observed toxic effects (Michalowicz et al., 2010). Peroxidase and SOD have been suggested as sensitive bioindicators of environmental pollutants including PCP at 0.5 mg/L (Roy and Hänninen, 1994).

Arabidopsis thaliana has been used in bioassays to measure antioxidant enzyme activities as indicators of oxidative stress for environmental chemicals. Including pesticides like colchicine, where decreased CAT and POD was observed with increased SOD (Drążkiewicz et al., 2003). POD, SOD activities and MDA content increased significantly after exposure to trichlorophenol (Li et al., 2015). After exposure to polycyclic aromatic hydrocarbon (PAH), which has the same toxic mechanism of chlorophenols, SOD activity was increased over the soil treatment range (45 mg/kg to 220 mg/kg) (Liu et al., 2009). PODs had peak activities at low concentrations (45 mg/kg) but declined at higher concentrations (220 mg/kg) while CAT activity was unaffected (Liu et al., 2009). H_2O_2, glutathione and MDA increased with levels of PAH (phenanthrene) chemical treatment (Liu et al., 2009). Exposure to the pesticide Paraquat was found to increase the amount of antioxidant enzyme glutathione (Bulgakov et al., 2012). Exposure to aromatic compounds, specifically 1-chloro-2,4-dinitrobenzene (CDNB), have also been shown to increase antioxidant enzymes, specifically Glutathione S-transferase (GST) on exposure (Skipsey et al., 2011). Rapid oxidative stress response is an important component of *A. thaliana* to organic environmental pollutants and pesticides such as PCP.

3.5 Conclusion

To discover the phenotypic and genetic response of *Arabidopsis thaliana* to exposure to PCP an early seedling growth bioassay was used to assess the toxicity of PCP, as well as to see if boric acid will have any affect on PCP toxicity, as they are used in combination as wood preservatives. Enzyme assays were not found to demonstrate statistically significant differences in response to treatment. No significant differences were found as a result of the presence of additional amounts of boric acid in combination with PCP. The enzyme activity assays performed at these time points do not show themselves as potential biochemical indicators of PCP chemical stress in *Arabidopsis*.

The addition of additional levels of boric acid tended to be antagonist to PCP toxicity but this trend was not statistically significant. The additional boric acid caused an increase in biomass, which may have reduced the effects of PCP toxicity. For physiology responses, fresh weight was found to be the most reliable and significant to use within this study over the range of PCP concentrations with an EC_{50} of 5.32 mg/L. However, without the specificity of the experimental conditions numerous factors could contribute to a decrease in fresh weight. From the results of this study the use of the biochemical activity biomarkers is not recommended at the time points used. This data provides a basis for the development of bioindicators and understanding physiological and biochemical responses of *Arabidopsis* to exposure to PCP, as a reliable indicator of presence of low concentrations of PCP in the environment.

Chapter 4 Genetic response of *Arabidopsis thaliana* to exposure to pentachlorophenol and influence of boron

4.1 Introduction

The use of genetics can provide information about the impacts of chemicals and could provide a better understanding of the mechanisms used by plants to deal with PCP through the expression of genes and proteins (Snape et al., 2004). The analysis of RNA expression gives information about the response of the plant to toxicity and in application can serve as a biomarker for chemicals of interest such as, PCP (Jin et al., 2011; Zhu et al., 2012; Li et al., 2015).

The primary goal of this study was to assess whether an early seedling growth bioassay with *Arabidopsis* could, practically and efficiently detect PCP toxicity. This was done through identifying and quantifying growth inhibition, antioxidant enzyme activities, and gene expression changes for toxicity biomarkers. This chapter examines changes in gene expression in response to exposure to PCP at low (5 mg/L) concentrations and the influence of the addition of boric acid on these changes.

RNA sequencing was done to generate a profile of gene expression changes after exposure to PCP for use as endpoints in toxicology (objective 4), and with an addition of boric acid, to observe any interaction effects. This profile was validated for selected genes using qRT-PCR to see how well gene expression correlated between the sequencing and PCR expression. Gene Ontology (GO) of the differentially expressed genes was examined to understand the effect of these chemicals in terms of cellular components, molecular functions, and biological processes (objective 5).

4.2 Materials and Methods

4.2.1 Assay Chemicals

Technical grade, (≥80% with ≤15% water) sodium pentachlorophenol (Sigma-Aldrich) was used, as it is readily soluble in water. The concentration of PCP used for assays was 5 mg/L. In addition to the 3.1 mg/L of boric acid contained in the half strength Murashige and Skoog (MS) basal medium, assay grade, ≥ 99.5% boric acid (Sigma-Aldrich) was added to establish a final boric acid concentrations 5.1 mg/L. The concentration of the chemical combination was 5+5.1 mg/L of PCP and boric acid, respectively. Concentrations used are representative of the highest concentrations used in previous growth and biochemical assays (see Chapter. 3). The highest concentrations were used to elicit a change in gene expression in response to PCP.

4.2.2 Seedling Preparation

Seeds from wild type *Arabidopsis thaliana* ecotype Columbia (Col-0) were surface-sterilized in a 1.5 mL microcentrifuge tube using sodium hypochlorite (1 mL of 100% sodium hypochlorite for 1 minute) and then rinsed thoroughly (5 times) with sterile autoclaved distilled water. The sterilized seeds were then vernalized for 48 hours in the dark at 4 °C to facilitate uniform germination.

Germination was conducted by spreading the seeds on solid growth medium (pH 5.7) containing 0.8% agar with half-strength Murashige and Skoog (1/2 MS) medium (Sigma-Aldrich) supplemented with 0.5% sucrose to help seedlings start growing. Plates with germinated seeds were then transferred to room temperature under continuous light for 10 days.

4.2.3 Liquid Culture Bioassay

Wild type *Arabidopsis* seedlings (Col-0) were transferred to 24-well cell culture cluster plates containing 1 mL of liquid half-strength MS basal medium per well. After 2 days of plants acclimating in liquid MS, the media was changed and treatment concentrations were added. These plates were placed on an orbital shaker set at 90 rpm. Plants received light from cool fluorescent tubes with a 16:8 h (day:night) photoperiod at a temperature of 22 °C.

Three 24-well plates were assigned to each chemical system tested (PCP, boric acid, and PCP plus boric acid), with 4 plants on each plate assigned per chemical treatment on each plate. These 4 plants were physically combined (pooled) together before testing to provide one pooled biomass sample per plate to be weighed on an analytical scale. Therefore, a single replicate of a chemical treatment concentration was composed of a pooled sample consisting of 4 plant seedlings.

4.2.4 Tissue Collection and Storage

12-d-old *Arabidopsis* seedlings were taken out of liquid culture from the 24-well plates at 12 hours post treatment. *Arabidopsis* seedlings, 4 whole 12-d-old plants pooled together as a sample, were flash frozen in liquid nitrogen and stored at -80 ºC until use.

4.2.5 RNA Extraction

Total RNA was extracted from 0.15 g ground frozen fresh tissues using the TRIzol® method according to manufacturers specifications (Sigma-Aldrich). RNase free pipette tips and microfuge tubes were used. The RNA concentration was quantified using a

NanoDrop 2000 spectrophotometer (Thermo Scientific, USA) and quality confirmed by visualizing RNA bands under UV light on a 1.3% agarose gel.

4.2.6 RNA Purification and cDNA Synthesis

Sodium acetate precipitation was performed in which 10 µL of sodium acetate was added to 100 µL of RNA and 100 µL of ethanol. Left at -20 ºC overnight, then spun at 12,000 g for 15 mins at 4 ºC, the supernatant was then discarded and the pelleted RNA was washed with 1 mL of 75% ethanol, mixed and spun again, with the supernatant discarded and the RNA left to air dry for 10 mins. RNA was treated with RQ1 DNAse kit (Promega Inc., USA) according to manufacturers specifications. Purified RNA was reverse transcribed using a high capacity cDNA reverse transcript kit (Applied Biosystems, ON, Canada) as per the instruction guide provided by the manufacturer.

4.2.7 RNA Sequencing and Bioinformatic Analysis

RNA-Seq and bioinformatics analysis was performed at the McGill University and Génome Québec Innovation Centre, Montréal, Canada. RNA sequencing generated between 106 to 161 million paired reads per library using the illumina Hiseq 2000/2500 sequencer. The protocol used for preparing libraries is the firststrand TrueSeq® mRNA protocol (Illumina, CA). Base calls (per-base estimates of error by the sequencing machines) are made using Illumina CASAVA pipeline.

Gene ontology (GO) data is the result of differential gene expression analysis generated using DESeq (Anders and Huber, 2010) and edgeR (Robinson et al., 2010) R Bioconductor package. GO analysis was performed using the goseq R Bioconductor

package, taking into account the length bias of long genes causing overrepresented categories. In order to represent the GO results graphically, the GOID list was further summarized using Revigo (Supek et al., 2011).

4.2.8 Quantitative Real-time PCR

Transcript levels of selected genes (AT5G36220, AT5G33355, AT4G26010, AT3G28550) were analyzed in order to validate the sequencing and to better understand how the genes are regulated. Primers were designed using the Roche Universal probe library design centre for each gene (Table 4.1).

A StepOneTM Real-Time qPCR System (Applied Biosystems, CA) was used to perform real time qPCR. A 10 µL reaction mixture (cDNA, gene specific primers, 5 µL of 2X SYBR green reagent and 1.5 µL nuclease free water) were used. The PCR conditions were: heat activation at 95 °C for 10 min, denaturation at 95 °C for 15 s, annealing and final extension at 60 °C for 1 min followed by 40 cycles. Relative transcript levels were analyzed using $2^{\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Transcript levels of each gene were normalized to the expression of ACT2 (Actin) gene as an endogenous control. This means kinetic PCR reactions were done for ACT2 in all samples, with values calculated for the internal control as well as this endogenous control. It is a common practice, in order to minimize sample and expression variability, to normalize expression to a reference gene. This gene is standardized across many studies and assumed to be unaffected by the experimental conditions, and is therefore expressed at a constant level among samples and replicates (Bustin, 2002). Fold changes of treated plants were relative to control plants.

4.2.9 Statistical Analysis

All experiments were replicated three times. All data sets were tested for normality and constant variance using SAS (version 9.3, SAS Institute, Cary, C, USA). Data were transformed where required and as noted for each test. Transformed data were used for statistical tests but untransformed data are shown in figures and tables. One way ANOVA using SAS Proc GLM was employed for endpoint assays and treatment comparisons. Significant differences between treatments were determined using Tukey's honesty significant difference (HSD) post hoc test at p= 0.05.

Table 4.1: Gene specific primers for validating gene expression with qRT-PCR.

Gene	Primer sequence	Gene description
ACT 2	F5'CCGCTCTTTCTTTCCAAGC 3' R5'CCGGTACCATTGTCACACAC 3'	Endogenous control
AT5G36220	F5'TTGGGAATGTGGAAGTGGAT 3' R5'AAGGAATCGCTTTGGGTACA 3'	Cytochrome p450
AT5G33355	F5'CTTAATAAACGCAAAGCATTAAAGAG 3' R5'GAAGCCACCAAAAGAACCAG 3'	DEFL (defensin like) family protein
AT4G26010	F5'ATGCTTCCCTCTTGATCGAC 3' R5'GCATTTGGTCCAGTGCTTTT 3'	Peroxidase activity
AT3G28550	F5'GCGGCATATGAGCCATACA 3' R5'CTTTGGGAAGTGGCACAGAG 3'	Proline rich Extensin like

4.3 Results

Gene expression of seedlings was quantified, through RNA sequencing and bioinformatic analysis then validated with quantitative real time PCR after growing in chemical treatment for 12 hours. Gene expression was quantified, as an early response biomarker, and Gene Ontology of the differentially expressed genes was determined to illustrate affected cellular components, molecular functions, and biological processes. Test plants were screened for toxic threshold level/minimum inhibitory concentration with PCP and applicable test levels chosen. After this the concentration of boric acid was based on the ratio of in-pole retention during envelope treatment with PCP and borates. Test plants were grown in cell culture cluster plates containing 1 mL of liquid half-strength Murashige and Skoog (1/2 MS) basal medium with PCP and boric acid added. The media had a background concentration of 3.1 mg/L boric acid, so the boric acid test levels used are in addition to this as well as the PCP concentrations.

Three 24-well plates were assigned to each chemical system tested (PCP, boric acid, and PCP plus boric acid), with 4 plants on each plate assigned per chemical treatment on each plate. These 4 plants were physically combined (pooled) together before testing to provide one pooled biomass sample per plate to be weighed on an analytical scale. Therefore, a single replicate of a chemical treatment concentration was composed of a pooled sample consisting of 4 plant seedlings.

4.3.1 RNA Sequencing and Bioinformatic Analysis

Gene read counts per million (CPM; number of reads overlapping a given gene) and transcripts reads per kilobase of exon (part of a gene that codes for a part of the RNA

product) per million fragments mapped (RPKM; exonic read density normalized to allow levels of transcripts to be compared within and between samples) values were used to detect the presence of outlier samples and explore the consistency and uniformity of the biological replicates, to reinforce the transcript analysis.

Genes with the most variable expression data (log_2 transformed CPM standard deviation), were used in visualizing the most variable genes as expressed among sequenced samples with control and boric acid treatment having 71 and 70 genes upregulated with 4 and 5 genes downregulated, respectively and the chemical treatments containing PCP had 4 genes upregulated and 71 genes downregulated. This variation is presented graphically as a Heatmap plot (Figure 4.1) indicating potential pattern of variation among genes used to discriminate between sample groups. Level of gene expression is represented as colors across comparable samples, with shades of red as upregulated (increased expression) and shades of blue as downregulated (decreased expression). Descriptions are presented to show their relationship to gene ontology (Tables 4.2, 4.3 and 4.4).

In the presence of PCP, only four genes were found to be upregulated with the majority (71) being downregulated by its exposure. It appears that at the concentrations of PCP used and the use of, additional boric acid did not have a significant effect on the gene expression when compared to control plants (Basal media boric acid concentration) or plants treated with PCP and PCP in combination with additional boric acid.

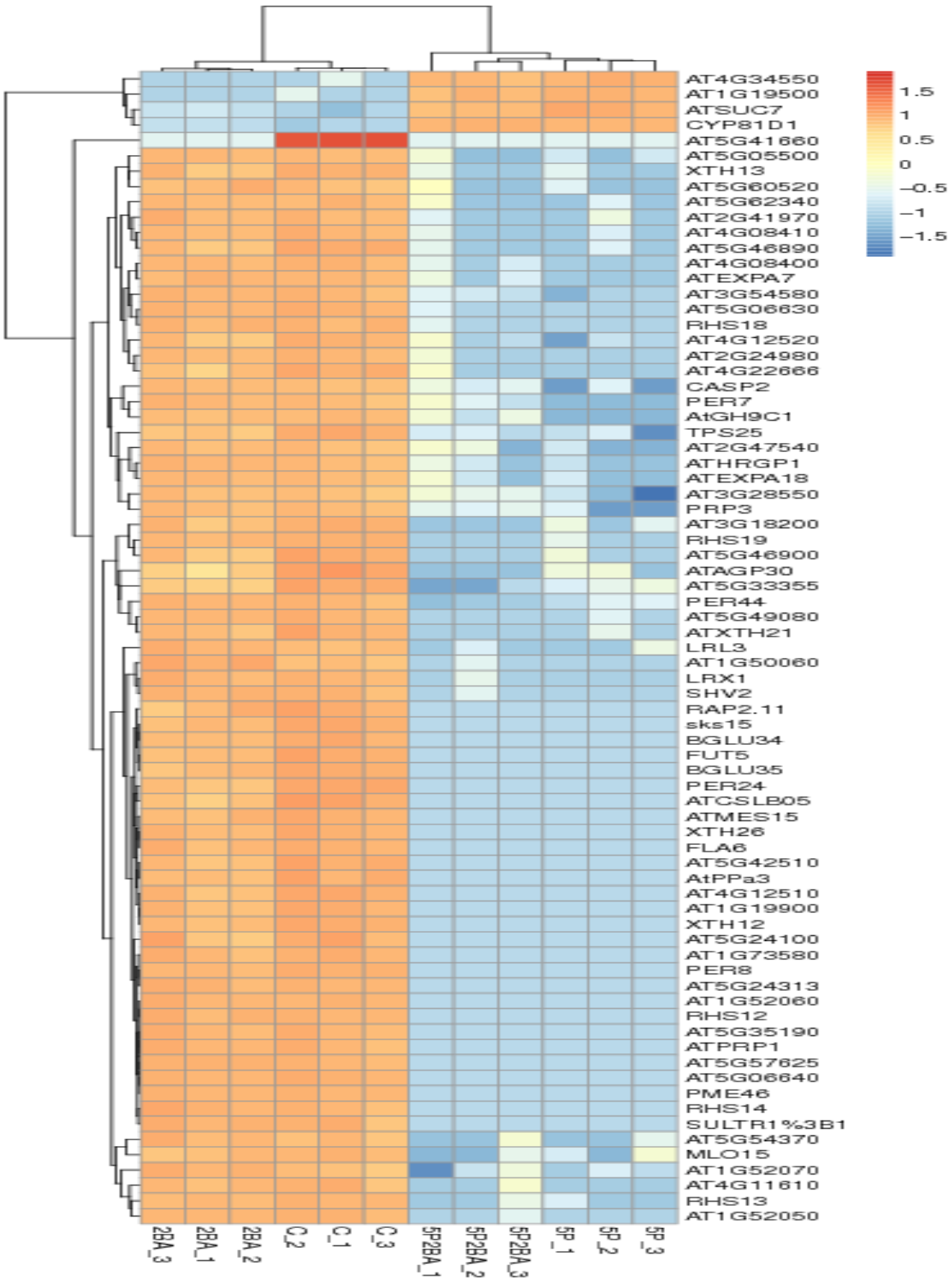


Figure 4.1: Heat map of most varying genes by $\log_2$(CPM) standard deviation. Chemical treatments C, 2BA, 5P, 5P2BA were control, 2 mg/L boric acid, 5 mg/L PCP, and 5mg/L PCP+2 mg/L boric acid respectively.

Table 4.2: Descriptions of the most up regulated genes

Gene	Description/Function
AT4G34550	F-box family protein involved in ubiquitination.
AT1G195008	Unknown protein, functions unknown.
ATSUC7	Sucrose-proton symporter
AT5G36220	Cytochrome p450s. Involved in oxidation-reduction process

Table 4.3: Descriptions of the most down regulated genes

Gene	Description/Function
LRX1	Encodes a chimeric leucine-rich repeat/extensin protein that regulates root hair morphogenesis and elongation
SHV2	Involved in successfully establishing tip growth in root hairs. Located in plasma membrane, anchored to membrane
RAP2.11	Encodes a member of the ERF (ethylene response factor) subfamily B-6 of ERF/AP2 transcription factor family
Sks15	Oxidoreductase activity, copper ion binding
BGLU34, BGLU35	Encodes a myrosinase
FUT5	Predicted fucosyltransferase
ATCSLB05	Encodes a gene similar to cellulose synthase
ATMES15	Encodes a protein predicted to act as a carboxylesterase
FLA6	Fasciclin-like arabinogalactan-protein
AT5G42510	Disease resistance-responsive
ATPPa3	Encodes a protein that might have inorganic pyrophosphatase activity
AT4G12510	Lipid binding
AT1G19900	Glyoxal oxidase-related protein
AT5G24100	Leucine-rich repeat protein kinase family protein, protein serine/threonine kinase activity, protein kinase activity, ATP binding
AT1G73580, AT4G11610	Calcium-dependent lipid-binding
AT1G52060, AT1G52070, AT1G52050	Mannose-binding lectin superfamily protein
PME46	Acts in the modification of cell walls via demethylesterification of cell wall pectin
SULTR1%3B1	Sulfate transporter in root
MLO15	Member of a large family of seven-transmembrane domain proteins specific to plants, homologs of the barley mildew resistance locus o (MLO) protein

Table 4.4: Descriptions of the most down regulated genes continued.

Gene	Description/Function
AT5G41660, AT5G24313	Function unknown
AT5G05500, PRP3, ATPRP1	Proline-rich protein. Elongation of root hairs
XTH13, ATXTH21, XTH26, XTH12	Xyloglucan endotransglucosylase/hydrolase
AT5G60520, AT5G54370	Late embryogenesis abundant protein
AT5G62340	Invertase/pectin methylesterase inhibitor
AT2G41970	Protein kinase superfamily protein
AT4G08410, AT4G08400, AT3G54580, AT5G06630, AT2G24980, AT3G28550,	Proline-rich extensin-like family protein.
AT5G49080, AT5G35190, AT5G06640	Structural constituent of cell wall
AT5G46890, AT4G12520, AT4G22666, AT5G46900	Bifunctional inhibitor/lipid-transfer protein. Functions in lipid binding
ATEXPA7, ATEXPA18	Root hair elongation and cell wall modification
RHS18, RHS19, RHS12, RHS14, RHS13	Root hair specific. Functions in peroxidase activity, heme binding. Involved in response to oxidative stress
CASP2	Cell wall modification, cell-cell junction assembly, glucuronoxylan metabolic process, xylan biosynthesic process
PER7, PER44, PER24, PER8	Removal of H202, peroxidase activity
ATGH9C1	Involved in carbohydrate metabolic process, trichoblast differentiation
TPS25	Secondary metabolite and terpenoid biosynthesis
AT2G47540, ATAGP30	Pollen Ole e 1 allergen and extensin family protein. Involved in root hair cell differentiation
ATHRGP1	Encodes a hydroxyproline- rich glycoprotein
AT3G18200	Nodulin MtN21-like transporter family protein
AT5G33355	Encodes a defensin-like family protein
LRL3	Encodes a basic helix loop-helix protein
AT1G50060, AT5G57625	Cysteine-rich secretory proteins

Genes were grouped into categories defined by common biological properties, then to terms within categories of Cellular Component (Figure 4.2), Molecular Function (Figure

4.3), and Biological Process (Figure 4.4), which are over represented amongst the differentially expressed genes.

In the Cellular Component Ontology (Figure 4.2) the terms extracellular region, cell wall, plasma membrane, anchored component of membrane, and microtubule were the terms significantly ($P<0.05$) represented with the highest frequency (according to Cuffdiff transcript quantification engine) in the genome. Kinetochore, mitochondrial respiratory chain complex IV, and proton-transporting ATP synthase complex, coupling factor F(o) were also significantly represented.

In the Molecular Function Ontology (Figure 4.3) the terms significantly represented with the highest frequency in the genome were oxidoreductase activity, and hydrolase activity. Binding of microtubule, flavin adenine dinucleotide, glutathione, iron ion, heme, protein kinase, lipid, and oxygen. As well as the activity of cinnamyl-alcohol dehydrogenase, flavin adenine dinucleotide, xyloglucan:xyloglucosyl transferase, NADH dehydrogenase, glutathione transferase, microtubule motor, cyclin-dependent protein serine/threonine kinase regulator, antiporter, terpene synthase, and electron carrier.

In the Biological Process Ontology (Figure 4.4) a large proportion of terms were found significantly ($P<0.05$) represented. The most noteworthy, relating to PCP toxicity, being metabolic process, cell communication, oxidation-reduction process, response to other organism, response to oxidative stress, response to endoplasmic reticulum stress, protein targeting to membrane, toxin catabolic process, and intracellular signal transduction.

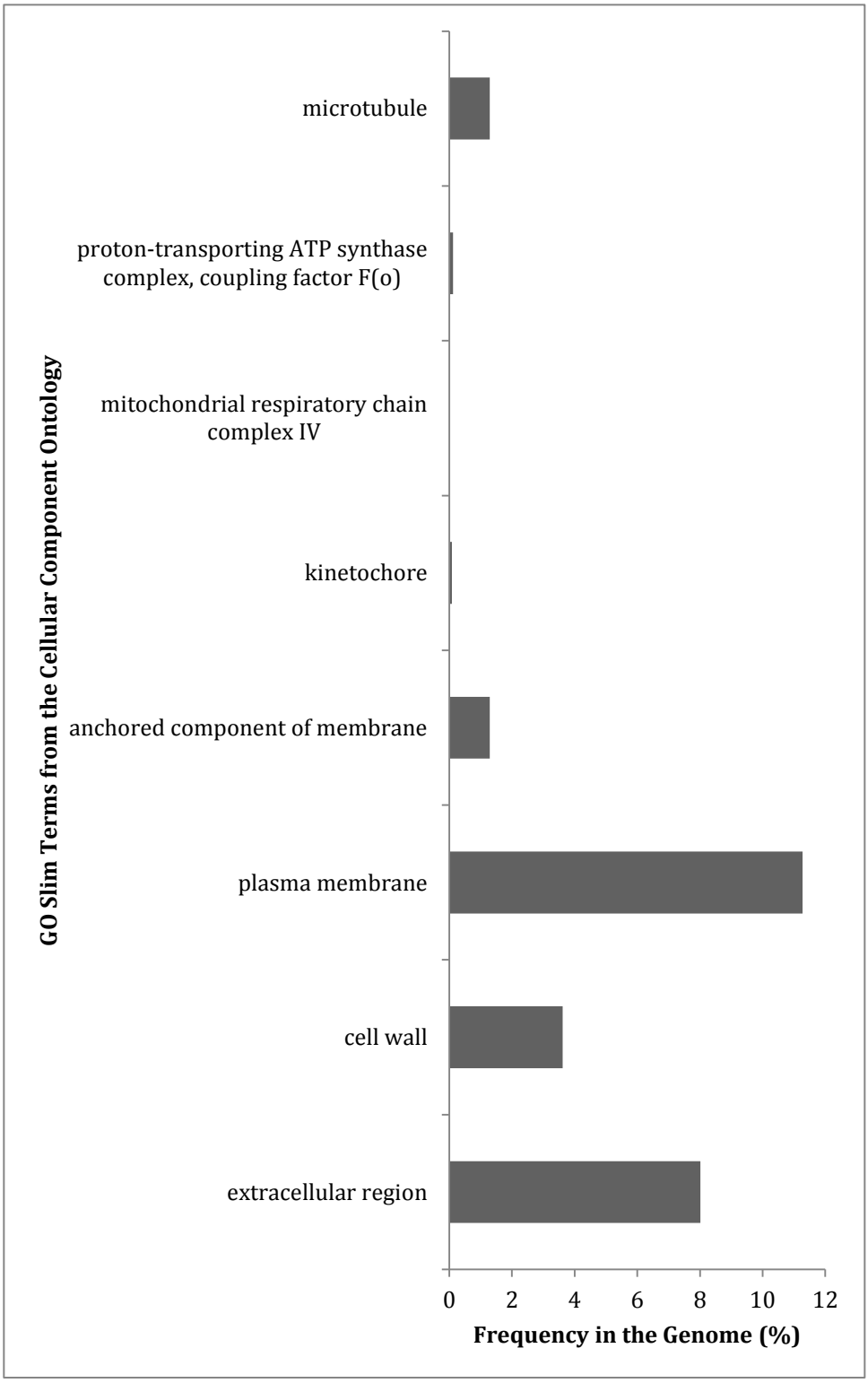


Figure 4.2: Gene Ontology (GO) terms for Cellular Component ontology by frequency in the genome (%).

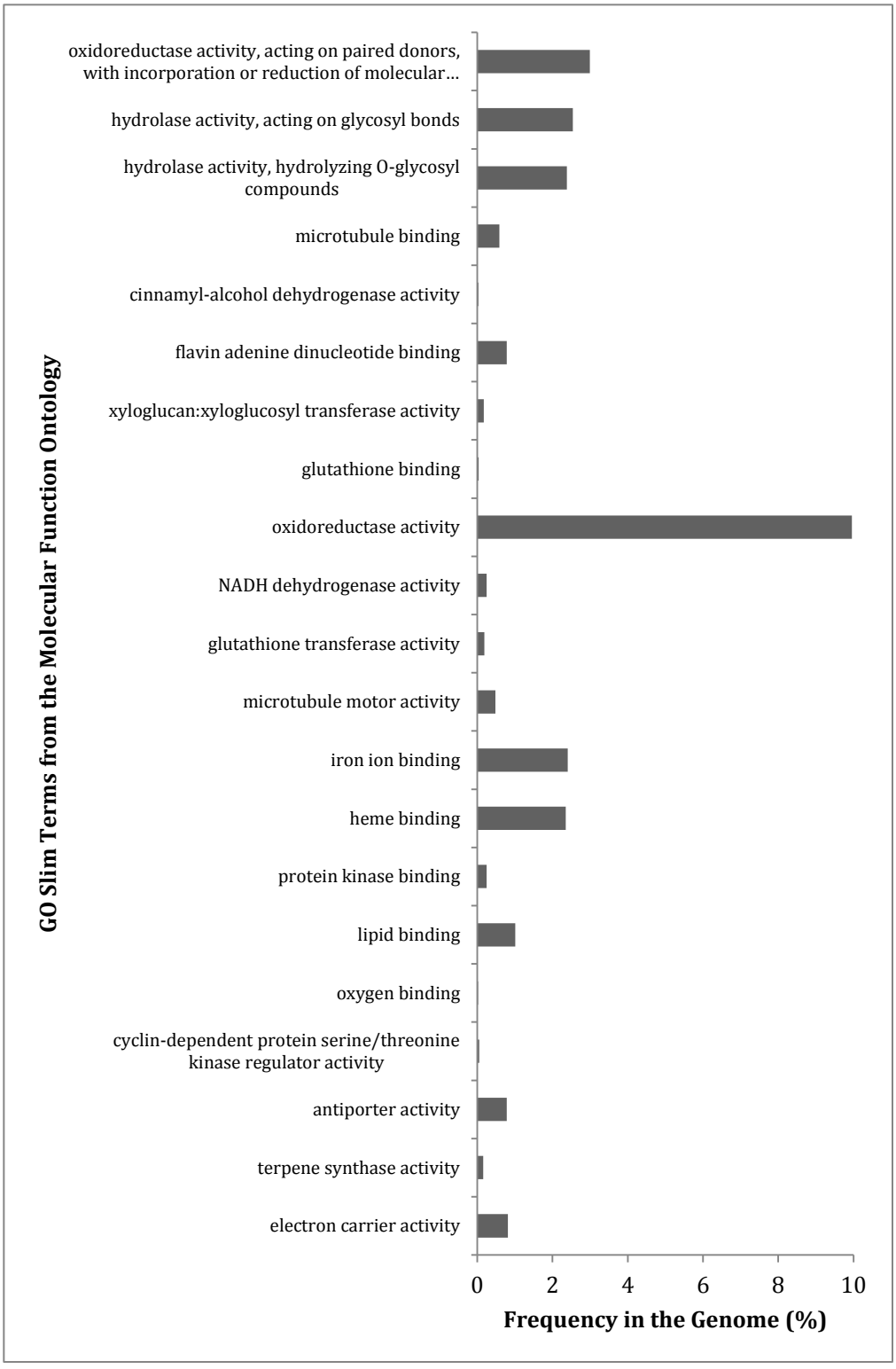


Figure 4.3: Gene Ontology (GO) terms for Molecular Function ontology by frequency in the genome (%).

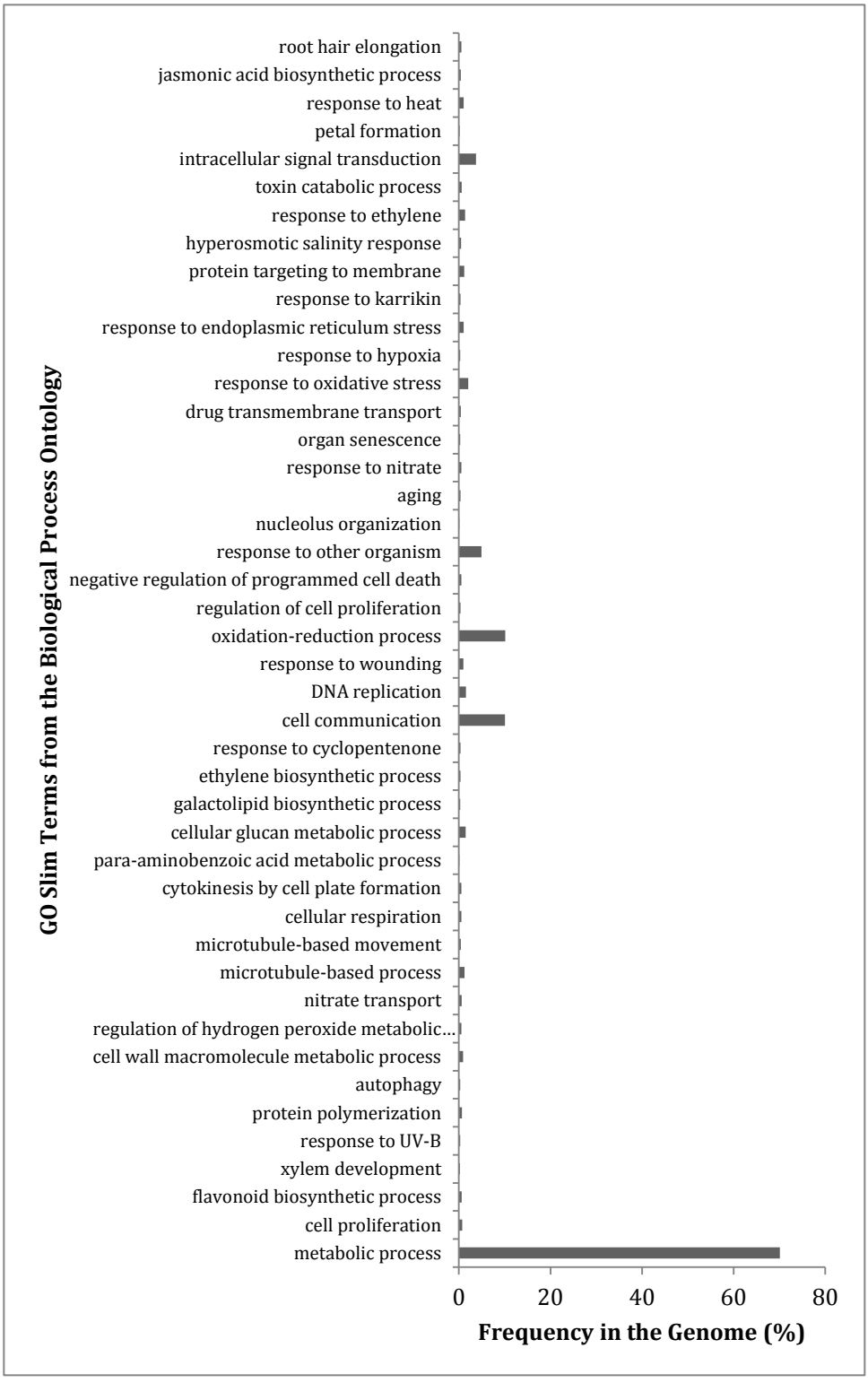


Figure 4.4: Gene Ontology (GO) terms for Biological Process ontology by frequency in the genome (%).

4.3.2 Quantitative Real-time PCR

Gene expression was quantified through qRT-PCR in order to validate the sequencing and compare expression levels determined by RNA-Seq with those from another laboratory technique. Genes were chosen based on previously found data from the TAIR database (TAIR, 2011). Primer sets were designed for a larger list of genes and the genes with the best amplification were chosen to express through real time PCR.

In order to validate the sequencing, a few genes (AT5G36220, AT5G33355, AT4G26010, and AT3G28550) which could be amplified, were expressed in real time PCR using gene specific primers with actin as an endogenous control and reference gene to compare expression levels to (Table 4.1).

The expression of AT5G36220 (Table 4.5) was found to vary significantly ($p<.0001$) across treatments. A 3-fold up regulation was observed with treatments of PCP and the combination of PCP and boric acid, consistent with the data determined by the RNA sequencing. No data transformation was necessary.

The expression of AT5G33355 (Table 4.5) was found to vary significantly ($p<.0001$) across treatments. Down regulation was observed with treatments of PCP and the combination of PCP and boric acid. Down regulation in gene expression is consistent with the data determined by the RNA sequencing. Data were transformed (LOG 10) for statistical purposes, presented are back transformed values.

The expression of AT4G26010 (Table 4.5) was found to vary significantly ($p<.0001$) across treatments. Down regulation was observed with treatments of PCP and the combination of PCP and boric acid. This down regulation in gene expression is

consistent with the data determined by the RNA sequencing. No data transformation was necessary.

The expression of AT3G28550 (Table 4.5) was found to vary significantly ($p<.0001$) across treatments. Down regulation was observed with treatments of PCP and the combination of PCP and boric acid. Down regulation in gene expression is consistent with the data determined by the RNA sequencing. Data were transformed (square root) for statistical purposes, where the presented data are back transformed values.

Table 4.5: AT5G36220, AT5G33355, AT4G26010, and AT3G28550 gene expression relative to actin in *A. thaliana* seedlings following treatment with Na-PCP and boric acid separately and in combination. Table values are the mean relative expression ± standard error. Means with the same Tukey grouping letter are not significantly different. Each gene is considered separately.

Chemical Treatment	Relative Expression	Tukey Grouping
AT5G36220		
Control	$1.00 \pm 7.89\text{x}10^{-6}$	B
5 µg/mL Na-PCP	$3.05 \pm 2.66\text{x}10^{-1}$	A
2 µg/mL Boric acid	$1.27 \pm 9.58\text{x}10^{-2}$	B
5+2 µg/mL Na-PCP+Boric acid	$3.33 \pm 2.86\text{x}10^{-2}$	A
AT5G33355		
Control	$1.00 \pm 9.89\text{x}10^{-6}$	A
5 µg/mL Na-PCP	$0.05 \pm 9.27\text{x}10^{-3}$	C
2 µg/mL Boric acid	$0.46 \pm 5.88\text{x}10^{-2}$	B
5+2 µg/mL Na-PCP+Boric acid	$0.05 \pm 1.34\text{x}10^{-2}$	C
AT4G26010		
Control	$1.00 \pm 8.72\text{x}10^{-6}$	A
5 µg/mL Na-PCP	$0.02 \pm 4.19\text{x}10^{-3}$	B
2 µg/mL Boric acid	$0.78 \pm 6.12\text{x}10^{-2}$	A
5+2 µg/mL Na-PCP+Boric acid	$0.02 \pm 5.36\text{x}10^{-4}$	B
AT3G28550		
Control	$1.00 \pm 5.22\text{x}10^{-6}$	A
5 µg/mL Na-PCP	$2.98\text{x}10^{-3} \pm 2.97\text{x}10^{-4}$	C
2 µg/mL Boric acid	$0.67 \pm 5.60\text{x}10^{-2}$	B
5+2 µg/mL Na-PCP+Boric acid	$0.01 \pm 5.36\text{x}10^{-3}$	C

4.4 Discussion

Gene expression changes in addition to the stress indicators of antioxidant enzyme activities (SOD, CAT, GPOD) and growth inhibition (see Chapter 3) were measured. In this study gene expression profiling was carried out and validated. This was done in order to identify relevant biomarkers based on gene expression changes, for the chemical treatment of PCP and to determine if there were differences with additional boric acid, and to better understand the mechanism to deal with PCP at the level of gene expression changes during chronic toxicity.

The objective of using a sequencing approach was to quantify the changing expression levels of transcripts under experimental conditions. This was done to create a list of relevant biological endpoints at the transcript level and see if there were any detectable differences in gene regulation between PCP and boric acid alone and in combination. RNA sequencing allows for capturing the relevant gene expression changes between chemical treatments. RNA is converted to a library of cDNA (complimentary DNA). They are then sequenced, using a sequencer machine, in a high throughput manner in order to obtain short sequences of DNA. The resulting DNA reads are then applied to the reference genome producing a genome scale transcription map of the level of expression for each gene (Ozsolak and Milos, 2011). For sequencing in general, it is common to see a majority of genes to be down regulated and fewer up regulated as seen in this study (Jin et al., 2011; Li et al., 2015).

In *A. thaliana*, gene expression has been used to study oxidative stress indicators for aromatic compounds (Liu et al., 2009; Weisman et al., 2010; Skipsey et al., 2011), pesticides (Laloi et al., 2007; Ramel et al., 2007; Skipsey et al., 2011; Bulgakov et al.,

2012) and other chlorinated organics (Jin et al., 2011; Zhu et al., 2012; Li et al., 2015). *A. thaliana* has also been used for toxicant specific gene discovery using microarray, with the goal of developing cheap and efficient biomonitors for heavy metal contamination (Krizek et al., 2003). All this previous gene expression work has been done using sequencing performed with microarrays, while this study employed the next generation technology of RNA-Seq.

The gene expression technology of choice is increasingly becoming RNA-Seq (Blow, 2009). RNA-Seq unlike microarrays can detect previously unknown changes as it does not require species or transcript specific probes, and offers increased specificity and sensitivity for differential expression and transcript detection (Ozsolak and Milos, 2011). RNA-Seq has been found to be very accurate and reliable for quantifying expression levels to give a large amount of data, as determined and validated using quantitative PCR (Nagalakshmi et al., 2008; Zenoni et al., 2010). This same accuracy was observed in this study.

The purpose of using Gene Ontology (GO) terms is an applicable and controlled vocabulary for genes and protein functions within eukaryotes, with transferable knowledge from one organism to another. Biological process (BP) refers to gene products that are involved in a biological objective, accomplished by several molecular functions. Molecular function (MF) refers to the biochemical activity of a gene product, and cellular component (CC) refers to where a gene product is active in the cell. The terms within these ontologies are meant to serve as tools to clarify gene expression data into more easily understandable terms (Ashburner et al., 2000). As revealed by the GO terms presented in the results section, the gene ontology obtained from the differential

expression analysis reflects what is known about the toxic mechanism of PCP. The CC, BP, and MF terms reflect that the activity of the chemical treatments is involved in oxidative stress at the membrane and in mitochondrial activities. The terms reflect damage done by the membrane toxin PCP, leading to oxidative stress by highlighting processes and functions involved with the mechanism to deal with PCP.

Cytochrome P450's biological function involves the metabolism of internal and external substances, including environmental pollutants. Cytochrome P450 gene regulation has a history of use as biomarkers for chemical and xenobiotic stress, due to their involvement in detoxification metabolism (Bucheli and Fent, 1995; Tabrez and Ahmad, 2010). Cytochrome P450 genes are also upregulated by other chlorinated organics in *A. thaliana* (Jin et al., 2011; Zhu et al., 2012). The significant 3-fold change in expression of this cytochrome P450 (AT5G36220) (Table 4.5) makes it an amenable biomarker in *Arabidopsis* for PCP exposure at the EC_{50} of roughly 5 mg/L for growth inhibition, but in hours as opposed to the days needed to see a growth reduction. Of interest is that this response was observed at a concentration below the CCME guidelines for PCP soil contamination of 7.6 µg/g in agricultural, residential, commercial or industrial soils (CCME, 2013).

The down regulation of several peroxidase genes could be partly responsible for the oxidative stress affect of PCP. The cell wall remodeling observed with several genes is also a common response to abiotic and oxidative stress (Tenhaken, 2015). Root and root hair elongation, the function of many down regulated genes, is also suppressed when peroxidase inhibitors are applied to suppress their gene expression, as well as that of other hydrogen peroxide scavengers (Causin et al., 2012; Kwon et al., 2015). Peroxidase

and cell wall remodeling are also correlated with repressed root development transcripts (Kwon et al., 2015). These trends of peroxidase, cell wall and root structure modifications are consistent with what was found with the PCP exposure at the sub lethal and low concentration used.

The molecular biomarkers appeared to be more specific to PCP than the enzyme activities presented in the previous chapter, as enzyme activities are more related to general stress response. The sensitivity of antioxidant enzyme activities and the generality of their activation on toxicity or stress make the genetic biomarkers more appealing candidates as biomarkers for PCP in this study, as well as the 50% growth inhibition observed at this concentration after several days. This sequencing work provides preliminary data and genomic resources for more in depth work, and applications in the future for studying and monitoring low concentrations of PCP contamination and its affects on plant systems.

4.5 Conclusion

To discover the phenotypic and genetic response of *Arabidopsis thaliana* to exposure to PCP an early seedling growth bioassay was used to assess the toxicity of PCP, as well as to see if boric acid will have any affect on PCP toxicity, as they are used in combination as wood preservatives. The objectives of this study were to determine any interaction effect of PCP added in combination with boric acid and to determine gene expression changes caused by PCP as observable biomarkers. As well as determining how the gene ontology (GO) is affected by exposure to PCP and the addition of increased boric acid.

All genes validated through RT-PCR in this study showed the same significant change in gene regulation in the presence of PCP as was found through the sequencing and bioinformatic analysis. This indicates the potential for these genes to be considered as biomarkers for PCP toxicity. Particular emphasis is suggested on the upregulated cytochrome P450 (AT5G36220) due to their history as gene expression biomarkers in toxicology (Bucheli and Fent, 1995; Tabrez and Ahmad, 2010). The expression fold change seen in this gene makes it an amenable biomarker in *Arabidopsis* for PCP exposure at the EC_{50} for growth reduction of roughly 5 mg/L, but in hours as opposed to the days needed to see such a growth reduction.

Additional boric acid, at the concentrations used, was not found to affect the expression of genes when compared to PCP without additional boric acid. Based on the results observed, suitable biomarkers should be used at a variety of biological levels to assess the interaction affects of pollutant combinations. The *Arabidopsis* bioassay used showed that there are several genetic biomarkers (such as AT5G36220) available for PCP detection, and that in application can be used to distinguish toxicity at the level of gene expression changes**.**

Chapter 5.0 Conclusion

To discover the phenotypic and genetic response of *Arabidopsis thaliana* to exposure to PCP an early seedling growth bioassay was used to assess the toxicity of PCP, as well as to see if boric acid will have any affect on PCP toxicity, as they are used in combination as wood preservatives. Bioassay is a practical and efficient approach for toxicant screening, using small amounts of active ingredients under controlled and standardized conditions.

For phenotypic responses, a range of concentrations was examined. Fresh weight was found to be the most consistent and statistically significant to use within this study over the range of PCP concentrations tested with an EC_{50} of 5.32 mg/L. However, without the specificity of the experimental conditions numerous factors could contribute to a decrease in fresh weight. Enzyme assays were not found to demonstrate statistically significant differences in response to treatment. No significant differences were found as a result of the presence of additional amounts of boric acid in combination with PCP. The enzyme activity assays performed at these time points do not demonstrate their potential to be biochemical indicators of PCP chemical stress in *Arabidopsis*. Fresh weight was the most suitable biomarker for chronic PCP toxicity. From the results of this study the use of the biochemical activity biomarkers is not recommended at the time points used.

Changes in gene expression to exposure of PCP were examined to identify suitable gene biomarkers. All genes validated through RT-PCR in this study showed the same significant change in gene regulation in the presence of PCP as was found through the sequencing and bioinformatic analysis. This indicates the potential for these genes to

be considered as biomarkers for PCP toxicity. Particular emphasis is suggested on the upregulated cytochrome P450 (AT5G36220) due to its history as a gene expression biomarker in toxicology (Buchell and Fent, 1995; Tabrez and Ahmad, 2010). The expression change seen in this gene makes it an amenable biomarker in *Arabidopsis* for PCP exposure at the EC_{50} for growth reduction of roughly 5 mg/L used, but in hours as opposed to the days needed to see such a growth reduction. Future research is recommended to determine the specificity of this gene biomarker in relation to other contaminants.

Gene ontology was examined to determine how it was affected by exposure to PCP. Gene ontology reflects damage done on the membrane and in mitochondrial activities, leading to oxidative stress by highlighting processes and functions involved with the mechanism to deal with PCP.

Additional boric acid, at the concentrations used, was not found to affect the expression of genes compared to PCP. The *Arabidopsis* bioassay used showed that there are several genetic biomarkers as candidates (such as AT5G36220) available for PCP detection, and that in application can be used to distinguish toxicity at the level of gene expression changes**.**

No significant differences were found as a result of the presence of additional amounts of boric acid in combination with PCP. This finding has industry significance as they are used in combination as wood preservatives. These data provide a basis for the development of bioindicators and understanding physiological and genetic responses of *Arabidopsis* to exposure to PCP, as a reliable indicator of the presence of low concentrations of PCP in the environment.

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