PHYTOREMEDIATION FOR DYE DECOLORIZATION

by

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B.Sc., University of Mumbai, Mumbai, India, 2006
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AN ABSTRACT OF A DISSERTATION

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Abstract

Synthetic dyes are capable of producing the whole color spectrum on account of their structural diversity but this diversity poses challenges in the degradation of dyeing wastes. Laccases and peroxidases from bacterial or fungal sources and parts of plants in the presence of hydrogen peroxide (H_2O_2) plus a mediator have been exploited in the bioremediation of synthetic dyes. However, intact plants have not found much favor despite their phytoremediation potential. The goal of this research was to further clarify ways by which whole plants bring about decolorization of different types of synthetic dyes. Hydroponically cultivated plants from two dicot families namely *Arabidopsis thaliana* and sunflowers (*Helianthus annuus*) were exposed to representative dyes from several classes: monoazo (Methyl Red and Methyl Orange), disazo (Trypan Blue, Evans Blue and Chicago Blue 6B), and arylmethane (Brilliant Blue G, Bromocresol Green, Malachite Green and Phenol Red). Tests were done in presence or absence of externally added H_2O_2, with or without a free radical mediator, 1-hydroxybenzotriazole, using UV-Visible spectrophotometry. The initial rate of decolorization and the overall percentage decolorization was calculated for each dye in the different treatments. Decolorization of the dyes from different classes varied between plant species and depending on the treatment. Except for Methyl Red, all dyes required added H_2O_2 as well as mediator to achieve rapid decolorization. Added H_2O_2 was found to be the limiting factor since it was degraded by plants within a few hours. Both species were able to slowly decolorize dyes upon daily addition of fresh dye even in the absence of added H_2O_2 and mediator, provided that nutrients were supplied to the plants with the dye. *A. thaliana* was found to be more effective in dye decolorization per gram tissue than sunflower when treated under similar conditions. Analysis of the residual dye solution by ESI/MS did not reveal any potential by-products following the decolorization treatment with plants, suggesting that the plant roots might be trapping the by-products of dye decolorization and preventing their release into the solution. All these findings support the potential application of whole plants for larger scale remediation.

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Synthetic dyes are capable of producing the whole color spectrum on account of their structural diversity but this diversity poses challenges in the degradation of dyeing wastes. Laccases and peroxidases from bacterial or fungal sources and parts of plants in the presence of hydrogen peroxide (H_2O_2) plus a mediator have been exploited in the bioremediation of synthetic dyes. However, intact plants have not found much favor despite their phytoremediation potential. The goal of this research was to further clarify ways by which whole plants bring about decolorization of different types of synthetic dyes. Hydroponically cultivated plants from two dicot families namely *Arabidopsis thaliana* and sunflowers (*Helianthus annuus*) were exposed to representative dyes from several classes: monoazo (Methyl Red and Methyl Orange), disazo (Trypan Blue, Evans Blue and Chicago Blue 6B), and arylmethane (Brilliant Blue G, Bromocresol Green, Malachite Green and Phenol Red). Tests were done in presence or absence of externally added H_2O_2, with or without a free radical mediator, 1-hydroxybenzotriazole, using UV-Visible spectrophotometry. The initial rate of decolorization and the overall percentage decolorization was calculated for each dye in the different treatments. Decolorization of the dyes from different classes varied between plant species and depending on the treatment. Except for Methyl Red, all dyes required added H_2O_2 as well as mediator to achieve rapid decolorization. Added H_2O_2 was found to be the limiting factor since it was degraded by plants within a few hours. Both species were able to slowly decolorize dyes upon daily addition of fresh dye even in the absence of added H_2O_2 and mediator, provided that nutrients were supplied to the plants with the dye. *A. thaliana* was found to be more effective in dye decolorization per gram tissue than sunflower when treated under similar conditions. Analysis of the residual dye solution by ESI/MS did not reveal any potential by-products following the decolorization treatment with plants, suggesting that the plant roots might be trapping the by-products of dye decolorization and preventing their release into the solution. All these findings support the potential application of whole plants for larger scale remediation.

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Dedication

This research is dedicated to my parents Babli Rajaram Kamat and Sushila Babli Kamat for teaching me the importance of plants in everyday life.

Chapter 1 - Introduction and Review of literature

This chapter will discuss synthetic dyes, their classification, their applications and the health hazards associated with their usage. Current research of conventional dye remediation methods with their possible drawbacks and bioremediation methods with special focus on phytoremediation of dyes involving whole plants and their enzymes will be reviewed. Lastly the objective of the research will be discussed.

1.1 Synthetic dyes

Synthetic dyes are artificial, colored compounds that have diverse industrial and biotechnological applications (Robinson *et al.*, 2001; Zollinger, 2003; Hunger, 2007; Gupta and Suhas, 2009). These dyes are different from natural dyes which are generally sourced from live organisms and have been in use since 32-11 kyr B.P. (thousand years before present) (Balter, 2009; Kvavadze *et al.*, 2009; Gupta and Suhas, 2009). The very first synthetic dye prepared in 1856 by the chemist William Henry Perkin using coal-tar residues was Mauveine also known as aniline purple or Perkins mauve (Zollinger, 2003; Hunger, 2007; Gupta and Suhas, 2009). The discovery of mauveine led to the production of other artificial synthetic dyes derived from benzene, phenol, aniline and other compounds having cyclic ring structures mixed in proper stoichiometric proportions (Zollinger, 2003; Hunger, 2007; Gupta and Suhas, 2009). Today there are over 100,000 distinct synthetic dyes used in commercial applications with an annual production exceeding 7×10^5 metric tons, replacing the classical natural dyes due to their low cost of production, easy availability of raw material and efficient production, formation of more diverse range of colors and improved properties of adhering to and staining various fabrics (Robinson *et al.*, 2001; Zollinger, 2003; Hunger, 2007; Gupta and Suhas, 2009; Carmen and Daniela, 2012).

These synthetic dyes are heavily used not only in the textile, denim, wool, fur, leather, tanning, paper-pulp and ink manufacturing industries, but also have applications as food colors, analytical pH indicators and in pharmaceuticals (Zollinger, 2003; Hunger, 2007; Husain, 2010).

For a molecule to be classified as a synthetic dye it needs to possess two groups, namely the chromophore and the auxochrome (Gupta and Suhas, 2009). The chromophore group is the molecule that gives the dye its specific hue while the function of the auxochrome is to ensure that it not only makes the dye more water soluble, thus increasing its binding capacity to fibers, but also can supplement the main chromophore group (Gupta and Suhas, 2009). Synthetic dyes are classified in many different ways based on their structural organization, their relative solubilities or their intended applications (Zollinger, 2003; Hunger, 2007; Gupta and Suhas, 2009). Based on their chemistry, dyes are classified into many major groups according to their characteristic central linkage group such as azo linkage (strong, cost-effective, reasonable all round characteristics); anthraquinone linkage (weak, expensive); nitro; carotenoid; diphenylmethane and triphenylmethane; xanthene; acridine; quinolone; indamine; sulfur; amino- and hydroxy ketone; indigoid; phthalocyanine; inorganic pigment (Zollinger, 2003; Hunger, 2007; Gupta and Suhas, 2009; Carmen and Daniela, 2012). The second method of classification, based on the dye solubility classifies the dyes into either water soluble class which includes acid, mordant, metal complex, direct, basic and reactive dyes or water insoluble class which includes azoic, sulfur, vat and disperse dye (Zollinger, 2003; Hunger, 2007; Gupta and Suhas, 2009). The most preferred and logical classification is classifying the dyes according to their application in staining natural fibers such as cotton, silk, wool, etc. or artificial fibers such as polyester, rayon, nylon, etc. (Zollinger, 2003; Hunger, 2007; Gupta and Suhas, 2009). This method of classification not only forms the basis of identifying the dye using color index (C.I) system but

also simplifies the classification for individuals who might not be aware of the molecular nature of the dyes (Zollinger, 2003; Hunger, 2007; Gupta and Suhas, 2009). Thus, based on usage synthetic dyes are classified into the following classes: acid (anionic) dyes; basic (cationic) dyes; reactive dyes; direct dyes; solvent dyes; disperse dyes; vat dyes; sulfur dyes and other dye classes (Zollinger, 2003; Hunger, 2007; Gupta and Suhas, 2009).

Based on applications monoazo dyes, disazo dyes and arylmethane dyes have the most diverse industrial applications. So it makes sense to select representative members from the above classes for further studies and investigate these dyes in terms of their structure and ultimately their function.

1.1.1 Azo dyes

Dyes bearing azo linkages are the most diverse, not only in their number and structure, but also their commercial applications in textiles, food colors, pharmaceuticals, petroleum products, and tanning industries (Zollinger, 2003; Hunger, 2007; Gupta and Suhas, 2009; Carmen and Daniela, 2012). These dyes account for 65-80% of the total amount of synthetic dyes produced and are the most researched in terms of their structure, applications, ecological impacts and toxicological studies (Hunger, 2007; Gupta and Suhas, 2009; Carmen and Daniela, 2012). Azo dyes are characterized by the presence of at least one azo linkage or group where the core nucleus is composed of double bonded nitrogen (-N=N-), although they may contain more than one azo group (Wallace, 2001; Zollinger, 2003; Hunger, 2007; Gupta and Suhas, 2009; Carmen and Daniela, 2012). Based on the number of azo groups these dyes are classified into monoazo dyes having single azo group or polyazo dyes bearing multiple azo groups which includes disazo, trisazo, tetrakisazo or polyazo bearing two, three, four or more than four azo groups respectively (Zollinger, 2003; Hunger, 2007).

Monoazo dyes are represented as R-N=N-R' where R and R' are the side groups (Wallace, 2001; Zollinger, 2003; Hunger, 2007; Gupta and Suhas, 2009; Carmen and Daniela, 2012). Usually in case of monoazo dyes, R is the electron accepting side group while R' is the electron donating side group (Hunger, 2007). Various permutations and combinations of these side groups make it possible to have a diverse range of structures, each with distinct color characteristics and absorption pattern (Hunger, 2007). Depending on their application, these side groups are comprised of conjugated phenyl rings, benzene rings, naphthalene rings, heterocyclic aromatic rings or an enolizable aliphatic group bearing functional groups such as hydroxyl, sulfonic acid, phosphoric acid, carboxylic acid or amino moieties (Wallace, 2001; Zollinger, 2003; Hunger, 2007; Carmen and Daniela, 2012). These combined side groups act as the chromophores due to the easy movement of the pi-electrons around the double bond and their close proximity to the sp^2 hybridized carbons of the aromatic or phenyl rings, resulting in the resonance stabilized structure that is responsible for the vibrant range of colors (Brown and De Vito, 1993; Wallace, 2001; Zollinger, 2003; Hunger, 2007). Monoazo dyes are usually moderately soluble in water but adding cations such as sodium or potassium to the acidic dyes or anions such as chloride to the basic dyes increases their water solubility.

Disazo dyes contain two azo groups and are structurally more complex than monoazo dyes (Zollinger, 2003; Hunger, 2007). In fact, they are basically two monoazo dyes linked together. Like monoazo dyes, even these are moderately soluble in water and their solubility can be increased by addition of cations although they do produce darker color intensities than monoazo dyes (Zollinger, 2003; Hunger, 2007).

1.1.2 Arylmethane dyes

These dyes are derivatives of methane (CH_4) where the hydrogen atoms are substituted by complex ring structures such as phenolic or benzene linked to hydroxyl, sulfonic acid, phosphoric acid, carboxylic acid or nitro functional groups (Gorman, Hepworth and Mason, 2000; Zollinger, 2003; Hunger, 2007). Based on the number of hydrogen being substituted, these dyes can be further divided into diaryl or diphenylmethane where two hydrogens are replaced by two phenyl rings and triaryl or triphenylmethane where three phenyl rings substitute three hydrogens (Zollinger, 2003; Hunger, 2007). Their production and synthesis might be limited and not as diverse as compared to azo dyes, but arylmethane dyes, especially triarylmethane derivatives, still have applications in textiles, printing, clinical studies, biological stains, pharmaceuticals as well as food colors (Zollinger, 2003; Hunger, 2007).

1.1.3 Issues associated with synthetic dyes

Being completely artificial there are potential issues and health hazards associated with the release of these synthetic dyes (Robinson *et al.*, 2001; Zollinger, 2003; Hunger, 2007; Husain and Husain, 2008; Gupta and Suhas, 2009; Carmen and Daniela, 2012; Ramachandran *et al.*, 2013). The textile industry, one of the largest users of synthetic dyes consumes nearly 95% of the available water as processing water. This is amongst the highest usage of potable water for non-human consumption (Carmen and Daniela, 2012). Wastewater from the textile and other industries using synthetic dyes mainly contains residual dyes and other potentially harmful contaminants, that if untreated gets easily released into water bodies contamination and causes high levels of water pollution (Robinson *et al.*, 2001; Zollinger, 2003; Hunger, 2007; Husain and Husain, 2008; Gupta and Suhas, 2009; Kaushik and Malik 2009; Carmen and Daniela, 2012; Ramachandran *et al.*, 2013). Nearly 20% of the synthetic dyes get mixed in the processing water

and nearly 15% of the dyes directly enter and interact with the environment (Robinson *et al.*, 2001; Husain and Husain, 2008; Husain, 2010; Carmen and Daniela, 2012). This is a more pressing issue in developing nations since the textile industry is more prevalent and thriving in these nations (Kaushik and Malik 2009). Recently in articles published in the Ecotextile news, there were concerns raised on the rising pollution levels due to release of textile dyes in India (Article: 2013120512341, 5 December 2013) as well as flouting of rules in terms of treatment of the effluents released from the textile industry in certain towns of China (Article: 2014010312375, 3 January 2014; Article: 2014022112460, February 2014). Use and sale of these synthetic dyes, especially azo dyes and their by-products, is banned in most of the developed economies (Directive 2002/61/EC).

Presence of synthetic dyes in natural water systems is characterized by vibrant colors which makes the water not only unpalatable but also makes it aesthetically undesirable due to their high levels of biological oxygen demand as well as chemical oxidation demand and increased content of total dissolved solids (Robinson *et al.*, 2001; Gupta and Suhas, 2009; Kaushik and Malik 2009; Van der Zee and Cervantes, 2009; Srinivasan and Viraraghavan, 2010; Carmen and Daniela, 2012; Ramachandran *et al.*, 2013). The inherently complex structural organization of the dyes make them quite rigid, stable and resistant to break down by a variety of physical and chemical methods including certain techniques employing biological sources (Robinson *et al.*, 2001; Husain and Husain, 2008; Gupta and Suhas, 2009; Kaushik and Malik 2009; Husain, 2010; Srinivasan and Viraraghavan, 2010; Carmen and Daniela, 2012). The complicated structures makes these dyes recalcitrant while also increasing their half-lives which in turn makes them a potential health hazard (Robinson *et al.*, 2001; Husain and Husain, 2008; Gupta and Suhas, 2009; Kaushik and Malik 2009; Van der Zee and Cervantes, 2009; Husain, 2010;

Srinivasan and Viraraghavan, 2010; Carmen and Daniela, 2012). Being completely non-natural, release of these dyes into water is directly and indirectly linked to a number of drastic effects on the abiotic world and the biotic population of the environment (Robinson *et al.*, 2001; Wallace, 2001; Srivastava, Sinha and Roy, 2004; Husain and Husain, 2008; Gupta and Suhas, 2009; Kaushik and Malik 2009; Van der Zee and Cervantes, 2009; Husain, 2010; Srinivasan and Viraraghavan, 2010; Carmen and Daniela, 2012). Exposure to high concentrations of synthetic dyes has been shown to not only be toxic but also potentially mutagenic, genotoxic and/or carcinogenic to the flora and fauna of the environment including fishes, animals and humans (Bonin, Farquharson and Baker, 1981; Brown and De Vito, 1993; Robinson *et al.*, 2001; Wallace, 2001; Hunger, 2007; Mathur and Bhatnagar, 2007; Gupta and Suhas, 2009; Kaushik and Malik 2009; Van der Zee and Cervantes, 2009; Husain, 2010; Srinivasan and Viraraghavan, 2010; Carmen and Daniela, 2012; Ramachandran *et al.*, 2013). These potential health hazards were found to be true for both azo dyes and arylmethane dyes with increased cases of sensitivity and irritation of eyes and skin being reported (Bonin, Farquharson and Baker, 1981; Brown and De Vito, 1993; Wallace, 2001; Mathur and Bhatnagar, 2007; Husain and Husain, 2008; Gupta and Suhas, 2009; Husain, 2010; Carmen and Daniela, 2012; Ramachandran *et al.*, 2013). Knowing that synthetic dyes are the causative agents of such potential health hazards, it is imperative to explore feasible and sustainable techniques that can completely remove the recalcitrant dyes from the industrial effluents without causing damage to the ecosystem.

1.2 Conventional methods of dye removal

Various methods have been explored for remediation of recalcitrant xenobiotics found in the wastewaters and effluents released from various industries (Robinson *et al.*, 2001; Wallace, 2001; Husain and Husain, 2008; Gupta and Suhas, 2009; Kaushik and Malik 2009; Van der Zee

and Cervantes, 2009; Husain, 2010; Srinivasan and Viraraghavan, 2010; Carmen and Daniela, 2012). The techniques employed for remediation of recalcitrant synthetic dyes are generally grouped as physical methods, chemical methods, or physicochemical methods, based on the chemistry of the dye with different physical substances or polymers, and methods involving biotic sources (Robinson *et al.*, 2001; Wallace, 2001; Husain and Husain, 2008; Gupta and Suhas, 2009; Kaushik and Malik 2009; Van der Zee and Cervantes, 2009; Husain, 2010; Srinivasan and Viraraghavan, 2010; Carmen and Daniela, 2012; Ramachandran *et al.*, 2013).

1.2.1 Physical methods of dye removal

Physical methods employ the fluid mechanics principles and/or membrane based systems to clear the water system of dye residues (Robinson *et al.*, 2001; Gupta and Suhas, 2009; Srinivasan and Viraraghavan, 2010; Carmen and Daniela, 2012). Some of the current methods that have been employed to reduce the levels of the recalcitrant compounds especially synthetic dyes present in industrial and municipal sewage treatments include sedimentation, centrifugation, flotation, filtration using synthetic membranes, microfiltration, ultra- and nanofiltration, reverse osmosis, and desalting (Robinson *et al.*, 2001; Gupta and Suhas, 2009; Srinivasan and Viraraghavan, 2010; Carmen and Daniela, 2012).

1.2.2 Chemical methods of dye removal

Chemical methods of dye removal are based on the chemistry of the dye molecule and the behavior of dye with other chemicals under variable conditions of pH and temperature (Robinson *et al.*, 2001; Gupta and Suhas, 2009; Srinivasan and Viraraghavan, 2010; Carmen and Daniela, 2012). Oxidation of the synthetic dyes with strong oxidizing agents such as bleaching treatments with chlorine, hypochlorites and chlorate salts as well as interactions with hydrogen peroxide and cations such as iron (Fenton's reaction), ozonation, light-dependent photochemical oxidation,

electrochemical oxidation using conductive polymers, electricity-dependent dye decolorization, and advanced oxidation processes which combines the different oxidation techniques, while involving their simultaneous applications to increase the efficiency of dye decolorization are some of the well researched chemical methods employed in removal of dyes (Robinson *et al.*, 2001; Gupta and Suhas, 2009; Srinivasan and Viraraghavan, 2010; Carmen and Daniela, 2012; Ramachandran *et al.*, 2013).

1.2.3 Physico-chemical methods of dye removal

Physico-chemical methods of treating effluents rich with synthetic dyes are usually techniques based on the chemical interaction of the synthetic dye with various solid surfaces or gel based matrices under variable conditions of pH, temperature, surface area available for reaction and contact time of reaction (Robinson *et al.*, 2001; Gupta and Suhas, 2009; Srinivasan and Viraraghavan, 2010; Carmen and Daniela, 2012). Adsorption using substances like activated charcoal, alumina, alum, clay, wood chips, silica gels, peat, coal, fly-ash, zeolites as well as low-cost adsorbents such as saw dust, polysaccharides, chitin, chitosan, lignin, sunflower seed shells and other natural polymers, flocculation, electrokinetic coagulation, ion exchange, sonication, and irradiation are the most prominent techniques which have shown great potential in removing the recalcitrant dyes from industrial wastewater (Robinson *et al.*, 2001; Gupta and Suhas, 2009; Srinivasan and Viraraghavan, 2010; Carmen and Daniela, 2012; Ramachandran *et al.*, 2013).

1.2.4 Disadvantages

Each method mentioned in the preceding sections has distinct advantages over the other methods but they all also have equal number of disadvantages, some of them more concerning than others, making them quite unviable for long-term and large scale usage (Robinson *et al.*, 2001; Gupta and Suhas, 2009; Srinivasan and Viraraghavan, 2010; Carmen and Daniela, 2012;

Ramachandran *et al.*, 2013). The major factor is the high cost and expenditure involved in employing these techniques in industry, along with the infrastructure required to ensure the continuous operation with minimal loss in efficiency (Robinson *et al.*, 2001; Gupta and Suhas, 2009; Srinivasan and Viraraghavan, 2010; Carmen and Daniela, 2012). Also barring a few, most of these techniques have not been entirely successful in completely removing the dye and producing reasonable quality of water to be recycled and reused (Robinson *et al.*, 2001; Gupta and Suhas, 2009; Srinivasan and Viraraghavan, 2010; Carmen and Daniela, 2012; Ramachandran *et al.*, 2013). Combining some of the dye removal techniques could potentially solve the problem but the costs involved as well as the long-term sustainability is still unknown (Robinson *et al.*, 2001; Gupta and Suhas, 2009; Srinivasan and Viraraghavan, 2010; Carmen and Daniela, 2012; Ramachandran *et al.*, 2013). Other disadvantages include formation of sludge, extreme high levels of both biological oxygen demand and chemical oxygen demand and the formation of toxic by-products due to the of the parent dye (Robinson *et al.*, 2001; Gupta and Suhas, 2009; Srinivasan and Viraraghavan, 2010; Carmen and Daniela, 2012). These potential hazards are quite commonly observed for nearly all of these techniques raising questions about their environmental friendliness and their safety to human health (Robinson *et al.*, 2001; Gupta and Suhas, 2009; Srinivasan and Viraraghavan, 2010; Carmen and Daniela, 2012). Moreover, this would mean additional treatments of the wastewater before its release which adds to the total expense, making these methods unfeasible to use on a large scale (Robinson *et al.*, 2001; Gupta and Suhas, 2009; Srinivasan and Viraraghavan, 2010; Carmen and Daniela, 2012; Ramachandran *et al.*, 2013).

Thus, employing physico-chemical methods on their own in remediation of dye in wastewater is not very feasible in terms of the expenses involved (Robinson *et al.*, 2001; Gupta and Suhas,

2009; Husain, 2010; Srinivasan and Viraraghavan, 2010; Carmen and Daniela, 2012). The criteria for a dye remediation method to be feasible yet sustainable on a large scale would be that it needs to be cost-effective, efficient yet eco-friendly in its ability to remove synthetic dye from textile effluents and wastewater while ensuring low level of damage to the flora and fauna in the environment. One potential solution would be to involve biotic sources in the remediation of synthetic dyes.

1.3 Remediation of textile dyes using biological sources

Using biotic organisms, entirely or in parts for remediation of xenobiotic as well as other toxic contaminants from abiotic sources such as soil and water has gained momentum for the last few decades as these provide cost-effective and environmentally friendly alternatives to the conventional techniques. The United States Environmental Protection Agency (EPA) defines the term 'bioremediation' as "the use of microbes and natural processes to clean up harmful chemicals in contaminated soil and ground water of the environment" (US EPA 2001; US EPA, 2012) while the Office of Technology Assessment (OTA) defines 'bioremediation' as "the act of adding materials to contaminated environments to cause an acceleration of the natural biodegradation processes" (OTA, 1991). The EPA also defines bioremediation agents as "microbiological cultures, enzyme additives, or nutrient additives that significantly increase the rate of biodegradation to mitigate the effects of the discharge" (Venosa, 2004). The use of biotic sources such as bacteria, fungi as well as plants to clear up effluents and wastewater containing recalcitrant and toxic xenobiotic compounds especially synthetic dyes from the natural environment has been well explored and will be discussed in the following sections.

1.3.1 Bioremediation using bacteria

Remediation of synthetic dyes and other recalcitrant components using bacteria forms the basis for the treatment of effluents carried out in the municipal treatment plants (Robinson *et al.*, 2001; Gupta and Suhas, 2009; Srinivasan and Viraraghavan, 2010; Carmen and Daniela, 2012; Ramachandran *et al.*, 2013). Aerobic, anaerobic or a combination of both bacterial systems with their respective action on all classes of synthetic dyes has been well documented (Robinson *et al.*, 2001; Wallace, 2001; Husain and Husain, 2008; Gupta and Suhas, 2009; Kaushik and Malik 2009; Van der Zee and Cervantes, 2009; Husain, 2010; Srinivasan and Viraraghavan, 2010; Carmen and Daniela, 2012). Aerobic bacteria depend on oxygen for their survival and various strains have been shown to bring about complete break down and subsequent mineralization of azo, anthraquinone as well as arylmethane dyes (Robinson *et al.*, 2001; Wallace, 2001; Husain and Husain, 2008; Gupta and Suhas, 2009; Kaushik and Malik 2009; Van der Zee and Cervantes, 2009; Husain, 2010; Srinivasan and Viraraghavan, 2010; Carmen and Daniela, 2012). Anaerobic bacteria on the other hand only survive in oxygen-free environments but have been shown to bring about degradation of synthetic dyes from the azo as well as arylmethane class through the reduction process since they cannot tolerate oxygen (Robinson *et al.*, 2001; Wallace, 2001; Husain and Husain, 2008; Gupta and Suhas, 2009; Kaushik and Malik 2009; Van der Zee and Cervantes, 2009; Husain, 2010; Srinivasan and Viraraghavan, 2010; Carmen and Daniela, 2012). Both continuous feeding activated sludge bioreactor and stage flow batch feeding systems have been employed using different strains of bacterial cultures (Robinson *et al.*, 2001; Gupta and Suhas, 2009; Van der Zee and Cervantes, 2009). Also there are reports of enhancing the process of dye decolorization and mineralization process if small molecules such as mediators were added to the reaction system (Van der Zee and Cervantes, 2009; Husain, 2010; Carmen and Daniela, 2012). Combining both aerobic and anaerobic bacterial systems can get rid of a diverse

variety of synthetic dyes from wastewater without much damage to the environment and it is more cost-effective than the conventional methods (Robinson *et al.*, 2001; Husain and Husain, 2008; Gupta and Suhas, 2009; Van der Zee and Cervantes, 2009; Husain, 2010; Srinivasan and Viraraghavan, 2010; Carmen and Daniela, 2012). However, there are disadvantages associated with employing live bacterial cells for dye decolorization (Robinson *et al.*, 2001; Husain and Husain, 2008; Gupta and Suhas, 2009; Van der Zee and Cervantes, 2009; Husain, 2010; Srinivasan and Viraraghavan, 2010; Carmen and Daniela, 2012). The synthetic dye mineralization process is not very common and not all of the different bacterial systems that were employed proved capable of carrying out this process (Robinson *et al.*, 2001; Husain and Husain, 2008; Gupta and Suhas, 2009; Van der Zee and Cervantes, 2009; Husain, 2010; Srinivasan and Viraraghavan, 2010; Carmen and Daniela, 2012). Maintaining optimal pH and temperature and removal of potential inhibitors in order to reduce the inactivation in continuous systems are some of the things to monitor on a daily basis (Robinson *et al.*, 2001; Husain and Husain, 2008; Gupta and Suhas, 2009; Van der Zee and Cervantes, 2009; Husain, 2010; Srinivasan and Viraraghavan, 2010; Carmen and Daniela, 2012). The most concerning issues are the degradation products formed from the parent dye compound and the time required for the decolorization which is generally slow and requires longer reaction periods when compared to the physical and chemical methods (Robinson *et al.*, 2001; Husain and Husain, 2008; Gupta and Suhas, 2009; Van der Zee and Cervantes, 2009; Husain, 2010; Srinivasan and Viraraghavan, 2010; Carmen and Daniela, 2012). The dye break down products in case of the azo dyes where the azo linkage is susceptible to break down through reduction forming toxic aromatic amines, are shown to be potentially carcinogenic and mutagenic (Robinson *et al.*, 2001; Husain and Husain, 2008; Gupta and Suhas, 2009; Van der Zee and Cervantes, 2009; Husain, 2010;

Srinivasan and Viraraghavan, 2010; Carmen and Daniela, 2012). Most bacterial systems do not completely internalize or mineralize the dyes, thus releasing toxic by-products in the water system thereby increasing the health risks (Robinson *et al.*, 2001; Husain and Husain, 2008; Gupta and Suhas, 2009; Van der Zee and Cervantes, 2009; Husain, 2010; Srinivasan and Viraraghavan, 2010; Carmen and Daniela, 2012; Ramachandran *et al.*, 2013). These azo break down products are a major health concern and the reason why usage of azo dyes has been banned in the EU and USA (Directive 2002/61/EC). Also the effect of the possible mutations on using these dyes in bacterial cells, the potential formation of bacterial biofilms which creates a new added problem, formation of sludge, side reactions made by the bacterial cells due to exposure to dye, inactivation of the bacterial system due to inhibitors thereby reducing the dye decolorization efficiency, and the huge amount of biomass generated, are some of the potential issues that have not been explored but they need to be addressed before employing these systems on a large scale in treatment plants (Robinson *et al.*, 2001; Husain and Husain, 2008; Gupta and Suhas, 2009; Van der Zee and Cervantes, 2009; Husain, 2010; Srinivasan and Viraraghavan, 2010; Carmen and Daniela, 2012).

1.3.2 Bioremediation using fungi

Whole fungal organisms or parts of fungal filaments have also shown potential in bioremediation of synthetic dyes (Robinson *et al.*, 2001; Baldrian, 2006; Couto and Herrera, 2006; Husain and Husain, 2008; Gupta and Suhas, 2009; Kaushik and Malik 2009; Husain, 2010; Srinivasan and Viraraghavan, 2010; Harms, Schlosser and Wick, 2011; Strong and Claus, 2011; Ramachandran *et al.*, 2013). Yeast cells (*Saccharomyces cerevisiae*); various fungal genera from the phyla Ascomycota and Basidiomycota namely *Aspergillus*, *Candida*, *Coriolus*, *Funalia*, *Ganoderma*, *Hirschioporus*, *Ionotus*, *Irpex*, *Neurospora*, *Phlebia*, *Pluerotus*, *Rhizopus*, *Thelepora*, and others

have been exhaustively explored for their ability to grow on as well as break down a large variety of textile dyes (Robinson *et al.*, 2001; Baldrian, 2006; Couto and Herrera, 2006; Husain and Husain, 2008; Gupta and Suhas, 2009; Kaushik and Malik 2009; Husain, 2010; Srinivasan and Viraraghavan, 2010; Harms, Schlosser and Wick, 2011; Strong and Claus, 2011). Two of the most prominently studied in bioremediation of a variety of xenobiotics include the white-rot fungi *Phanerochaete chrysosporium* and *Tramates versicolor*, both from the phylum Basidiomycota. They have shown great potential on their own or in combination with other systems to bring about biodegradation of the structurally complex synthetic dyes (Robinson *et al.*, 2001; Baldrian, 2006; Couto and Herrera, 2006; Husain and Husain, 2008; Gupta and Suhas, 2009; Kaushik and Malik 2009; Husain, 2010; Srinivasan and Viraraghavan, 2010; Harms, Schlosser and Wick, 2011; Strong and Claus, 2011; Ramachandran *et al.*, 2013). The fungal systems provide an ecofriendly and less expensive alternative to the conventional remediation of dyes, as do bacterial cells but the disadvantages include the long-term sustainability of these systems in decolorizing synthetic dyes as well as in general less reliability in terms of production and secretion of enough quantity of enzymes that are responsible for the decolorization and mineralization of the dye. There is high variance seen in the biological samples as well as the need for strict regulation of pH, temperature, fermentation conditions (Robinson *et al.*, 2001; Baldrian, 2006; Couto and Herrera, 2006; Husain and Husain, 2008; Gupta and Suhas, 2009; Kaushik and Malik 2009; Van der Zee and Cervantes, 2009; Husain, 2010; Srinivasan and Viraraghavan, 2010; Harms, Schlosser and Wick, 2011; Strong and Claus, 2011; Ramachandran *et al.*, 2013). Moreover, it is expensive to culture fungal systems since the growth media require sources of carbon and nitrogen which adds to the cost. This often makes it unfeasible for large scale operations (Robinson *et al.*, 2001; Baldrian, 2006; Couto and Herrera, 2006; Husain and

Husain, 2008; Gupta and Suhas, 2009; Kaushik and Malik 2009; Van der Zee and Cervantes, 2009; Husain, 2010; Srinivasan and Viraraghavan, 2010; Harms, Schlosser and Wick, 2011; Strong and Claus, 2011). However, one important disadvantage is the huge biomass of fungi that is left behind after the dye treatment.

1.3.3 Phytoremediation

Phytoremediation processes employ plants as whole or in parts to remove the contaminants present in soil and water. This has now gained importance as researchers around the world try to figure out innovative, sustainable yet ecofriendly as well as cost-effective ways of reducing levels of inorganic and organic contaminants (Pivetz, 2001; McCutcheon and Schnoor, 2004; Marmiroli, Marmiroli and Maestri, 2006; Agostini *et al.*, 2013). The EPA defines the term 'Phytoremediation' as "the direct use of green plants and their associated microorganisms to stabilize or reduce contamination in soils, sludges, sediments, surface water, or ground water" (US EPA, 2012). Phytoremediation technologies are divided into several subtypes based on the characteristics of the contaminant, the environment in which the plants will be exposed to the contaminant and the processes involved in neutralizing the contaminant (Pivetz, 2001; McCutcheon and Schnoor, 2004; Marmiroli, Marmiroli and Maestri, 2006; Agostini *et al.*, 2013; Kang, 2014). Broadly, the different phytoremediation technologies are grouped into the following types (Pivetz, 2001; McCutcheon and Schnoor, 2004; Marmiroli, Marmiroli and Maestri, 2006; Kang, 2014):

- Degradation or alteration of the contaminant. Included in this are Rhizodegradation or Phytostimulation and Phytotransformation processes.

- Accumulation and interaction but not break down of the contaminant. Two broad applications processes namely Rhizofiltration and Phytoextraction or Phytoaccumulation are included in this group.
- Phytovolatilization processes involving dissipation or release of the contaminant back into the environment in less toxic and relatively harmless form using plants.
- Immobilization of the contaminants either on a long-term basis or short-term basis and involves Hydraulic control and Phytostabilization processes.

Thus, phytoremediation technologies have proven to be advantageous since the processes involved are not only diverse and non-selective in their ability to capture and remove contaminants from the environment, but also these processes do not require much technical knowledge about start-up and infrastructure, thereby reducing the cost as compared to other bioremediation methods. Phytoremediation works independent of the origin or characteristics of the contaminant (Pivetz, 2001; McCutcheon and Schnoor, 2004; Marmiroli, Marmiroli and Maestri, 2006; Kang, 2014). In additions, the aesthetics of the environment are preserved due to additional benefits of oxygen which makes using phytoremediation processes quite feasible on a large scale (Pivetz, 2001; McCutcheon and Schnoor, 2004; Marmiroli, Marmiroli and Maestri, 2006; Kang, 2014). Also plants usually sequester and trap the xenobiotic contaminant and may even utilize the transformed contaminants for their own growth thereby reducing the toxicity of the contaminant which would otherwise be a potential health hazard (Pivetz, 2001; McCutcheon and Schnoor, 2004; Marmiroli, Marmiroli and Maestri, 2006; Agostini *et al.*, 2013; Kang, 2014). However, phytoremediation like all other biological methods of remediation has some minor drawbacks which need to be addressed to improve outlook for remediation of metals, inorganic and organic contaminants (Pivetz, 2001; McCutcheon and Schnoor, 2004; Marmiroli, Marmiroli

and Maestri, 2006; Kang, 2014). The most important considerations are that phytoremediation processes are generally very slow and are very hard to control as compared to the other methods (Pivetz, 2001; McCutcheon and Schnoor, 2004; Marmiroli, Marmiroli and Maestri, 2006; Kang, 2014). Some plants may not completely remove the contaminant if it is very toxic or if its effect is detrimental to the plant, thereby allowing it to not only remain in the ecosystem but also affect the flora and fauna of the newer environment (Pivetz, 2001; McCutcheon and Schnoor, 2004; Marmiroli, Marmiroli and Maestri, 2006; Kang, 2014). The long-term effect of the contaminant on the health, adaptability as well as survivability of the plant is still unclear (Pivetz, 2001; McCutcheon and Schnoor, 2004; Marmiroli, Marmiroli and Maestri, 2006; Kang, 2014). Transformation of the contaminant by plants is based on its characteristics and accumulation of the by-products in the consumable organs such as leaves or fruits is possible (Pivetz, 2001; McCutcheon and Schnoor, 2004; Marmiroli, Marmiroli and Maestri, 2006; Kang, 2014). This increases the potential of the transformed contaminant entering the food chain since plants are consumed by nearly every living organism. This may cause secondary toxicity and become another potential health hazard (Pivetz, 2001; McCutcheon and Schnoor, 2004; Marmiroli, Marmiroli and Maestri, 2006; Kang, 2014). Also phytoremediation processes are occasionally plant-specific and no one plant can carry out many different types of phytoremediation processes on its own without being stressed (Pivetz, 2001; McCutcheon and Schnoor, 2004; Marmiroli, Marmiroli and Maestri, 2006; Kang, 2014). Another concern is the huge biological variability not only between plants from different families but also between different species for the contaminant (Pivetz, 2001; McCutcheon and Schnoor, 2004; Marmiroli, Marmiroli and Maestri, 2006; Kang, 2014). Despite these drawbacks plant-dependent remediation of xenobiotic compounds is now becoming a feasible alternative to the commercial and conventional methods

of neutralizing contaminants (Pivetz, 2001; McCutcheon and Schnoor, 2004; Marmiroli, Marmiroli and Maestri, 2006; Kang, 2014).

1.3.3.1 Phytoremediation of synthetic dyes

Although whole plants, their parts or even their secretory enzymes have been exhaustively explored and have shown promising potential in the remediation of all sorts of metals, diverse inorganic and organic contaminants, by-products from the petroleum industry, solvent industry, mining industry, ammunition as well as explosive industries, pesticides as well as herbicide industries and even pharmaceutical drugs (Pivetz, 2001; McCutcheon and Schnoor, 2004; Marmiroli, Marmiroli and Maestri, 2006; Kang, 2014). It was only in the last decade that the phytoremediation of textile dyes and other by-products from the textile industry using different types of plants gained a lot of interest (Pivetz, 2001; Marmiroli, Marmiroli and Maestri, 2006; Agostini *et al.*, 2013; Kang, 2014). Indeed interaction of both land and aquatic plants including algae with synthetic dyes from different classes has been studied. Some of the plants that are reported to show dye decolorization potential have been listed in Table 1.1. Hairy root cultures of *Armoracia rusticana* (Horseradish), *Helianthus annuus* (sunflower), *Tagetes patula (Marigold)* and some other plants have been successful in remediation of diverse organic contaminants with complex structures (Patil *et al.*, 2009; Agostini *et al.*, 2013; Jha *et al.*, 2013). Recently transgenic *Arabidopsis* plants overexpressing the gene encoding for triphenylmethane reductase which originated from the bacterium *Citrobacter* has been shown to be successful in decolorizing triphenyl or triarylmethane dyes (Fu *et al.*, 2013). Even exotic, ornamental plants and their secretory enzymes are being explored for synthetic dye break down (Agostini *et al.*, 2013; Vafaei *et al.*, 2013a, 2013b; Watharkar *et al.*, 2013; Watharkar and Jadhav, 2014).

Table 1.1 Some of the plants employed in dye decolorization

Name of the plant	Reference
Armoracia rusticana	Veitch, 2004
Aster amellus	Kabra *et al.*, 2011
Azolla filiculoides	Khataee *et al.*, 2013a
Blumea malcolmii	Kagalkar *et al.*, 2009
Brassica juncea	Ghodake *et al.*, 2009
Brassica rapa	Kulshrestha and Husain, 2007
Chara vulgaris	Mahajan and Kaushal, 2013
Eichhornia crassipes	Shah *et al.*, 2010; Muthunarayanan *et al.*, 2011
Gaillardia grandiflora	Watharkar and Jadhav, 2014
Glandularia pulchella	Kabra *et al.*, 2012
Helianthus annuus	Ibbini *et al.*, 2009; Xie *et al.*, 2011a, 2011b
Hydrocotyle vulgaris	Vafaei *et al.*, 2013a, 2013b
Lemna minor	Movafeghi *et al.*, 2013
Medicago sativa	Zhou and Xiang, 2013; Gallego-Giraldo *et al.*, 2014
Momordica charantia	Husain, 2010
Nopalea cochenillifera	Adki, Jadhav and Bapat, 2012
Petunia grandiflora	Watharkar *et al.*, 2013; Watharkar and Jadhav, 2014
Phragmites australis	Davies *et al.*, 2005
Portulaca grandiflora	Khandare *et al.*, 2013
Rheum rabarbarum	Aubert and Schwitzguébel, 2004
Rosmarinus officinalis	Zheng and Shetty, 2000
Rumex hydroplantarum	Aubert and Schwitzguébel, 2004
Sesbania cannabina	Zhou and Xiang, 2013
Sesuvium portulacastrum	Patil *et al.*, 2012
Spirogyra	Khataee *et al.*, 2013b
Tagetes patula	Patil *et al.*, 2009
Thymus vulgaris	Zheng and Shetty, 2000
Typhonium flagelliforme	Kagalkar *et al.*, 2010

1.3.4 Enzymes involved in bioremediation of synthetic dyes

In majority of plants that were successful in phytoremediation of the different synthetic dyes, enzymes from the oxidoreductase class namely laccases, peroxidases, oxidoreductase, reductases, tyrosinases, superoxide dismutase, and catalase were found to be upregulated (Khataee *et al.*, 2013a, 2013b; Mahajan and Kaushal, 2013; Movafeghi *et al.*, 2013; Vafaei *et al.*, 2013a, 2013b; Watharkar *et al.*, 2013; Watharkar and Jadhav, 2014). Of these, laccases and peroxidases have been well researched for their ability to break down a wide-variety of xenobiotic contaminants with complex structures.

1.3.4.1 Laccases

Laccases (benzenediol:oxygen oxidoreductases EC 1.10.3.2) are oxidoreductase enzymes belonging to the blue multicopper oxidase (MCO) group (Giardina *et al.*, 2010; Strong and Claus, 2011; Jeon and Chang, 2013). These enzymes usually get activated in presence of oxygen and use it as the electron acceptor to bring about oxidation of a wide variety of aromatic and phenolic compounds (Baldrian, 2006; Couto and Herrera, 2006; Solomon *et al.*, 2008; Giardina *et al.*, 2010; Jeon *et al.*, 2012). Since laccases can act on a diverse range of substrates, they show promise as potential candidates for carrying out bioremediation of complex organic molecules. Expression of laccase enzymes have been observed across the biotic world including bacteria (Claus, 2003; Strong and Claus, 2011), fungi (Baldrian, 2006; Strong and Claus, 2011; Jeon *et al.*, 2012), insects (Dittmer and Kanost, 2010; Jeon *et al.*, 2012) and plants (Ranocha *et al.*, 1999, 2002; Gavnholt, Larsen and Rasmussen, 2002; McCaig, Meagher and Dean, 2005; Strong and Claus, 2011; Jeon *et al.*, 2012). Laccases characterized from the fungal species belonging to either Basidiomycetes (Kunamneni *et al.*, 2007; Strong and Claus, 2011; Jeon *et al.*, 2012) which comprises of white-rot and brown-rot fungi or Ascomycetes (Baldrian, 2006; Kunamneni *et al.*,

2008) have been extensively researched and reviewed. Nearly all the fungal laccases are secreted as extracellular glycoproteins (Baldrian, 2006; Strong and Claus, 2011; Jeon *et al.*, 2012) and mostly exist as isoenzymes (Baldrian, 2006; Strong and Claus, 2011). However, the levels of glycosylation as well as the actual structural arrangements differ not only for laccases from different fungal sources but also for different laccases within the same fungal species (Baldrian, 2006; Dwivedi *et al.*, 2011; Strong and Claus, 2011). The alignment of gene sequences as well as amino acid sequences for laccases from different fungal sources showed very low levels of similarities except for conservation of the copper binding domains in the active site of the enzyme (Suresh Kumar *et al.*, 2003; Hoegger *et al.*, 2006; Dwivedi *et al.*, 2011). Fungal laccases appear to be evolutionarily different from those obtained from plants and bacteria (Suresh Kumar *et al.*, 2003; Hoegger *et al.*, 2006; Dwivedi *et al.*, 2011). This makes sense since fungal laccases in terms of certain substrates have contradictory roles than those observed for plant laccases (Baldrian, 2006; Dwivedi *et al.*, 2011; Strong and Claus, 2011). Fungal laccases usually break down the complex polymer of lignin into smaller components (Baldrian, 2006; Dwivedi *et al.*, 2011) while plant laccases play a role in building up lignin from its precursors (Gavnholt and Larsen, 2002; Dwivedi *et al.*, 2011). Despite these obvious antagonistic functions as well as evolutionary distinct placements, fungal laccases exhibit certain similarities with laccases from bacterial as well as plant sources (Suresh Kumar *et al.*, 2003; Hoegger *et al.*, 2006; Dwivedi *et al.*, 2011). The arrangement of the copper binding motifs in the reaction pocket is highly conserved in laccases from different kingdoms despite differences in actual functions (Dwivedi *et al.*, 2011; Jeon *et al.*, 2012). Fungal laccases and plant laccases are both glycoproteins but the level of glycosylation and the nature of glycosylation differs between them (Dwivedi *et al.*, 2011; Strong and Claus, 2011). High rates of gene duplication events in both plants and fungi as

they adapt and evolve to environmental changes have led to specific functions of these laccase genes despite certain redundancies in their expression profiles (McCaig, Meagher and Dean, 2005; Giardina *et al.*, 2010; Dwivedi *et al.*, 2011).

Laccases, being from the MCO group, have characteristic multi-copper domains (Baldrian, 2006; Kunamneni *et al.*, 2007; Giardina *et al.*, 2010; Dwivedi *et al.*, 2011; Jeon and Chang, 2013). Using single electron transfer and oxygen as the electron acceptor, these enzymes are able to oxidize a diverse range of phenolic ring bearing compounds with low redox potentials (Baldrian, 2006; Kunamneni *et al.*, 2007; Giardina *et al.*, 2010; Strong and Claus, 2011; Jeon *et al.*, 2012). The reaction mechanics in the copper-rich active site are quite complicated but involve both enzymatic as well as non-enzymatic steps (Baldrian, 2006; Kunamneni *et al.*, 2007; Giardina *et al.*, 2010). It involves electron transfer from the phenolic substrate to oxygen generating a free radical of the substrate which due to its instability losses more electrons and is thus oxidized while reducing oxygen to water (Baldrian, 2006; Kunamneni *et al.*, 2007; Giardina *et al.*, 2010; Jeon *et al.*, 2012). Overall, since oxygen is divalent and requires four electrons to be reduced to water, the substrate undergoes oxidation by loss of four electrons (Baldrian, 2006; Kunamneni *et al.*, 2007; Giardina *et al.*, 2010). Laccases, however, can only bring about oxidation of compounds with low redox potentials. So with few exceptions of fungal laccases (Nagai *et al.*, 2002; Husain, 2006; Cherkashin *et al.*, 2007; Jeon *et al.*, 2012) they are not able alone to oxidize substrates with complex aromatic structures and high redox potentials (Baldrian, 2006; Kunamneni *et al.*, 2007; Giardina *et al.*, 2010; Strong and Claus, 2011; Jeon *et al.*, 2012). Characterization of laccases in terms of their *in vivo* functions and diverse substrate specificities has been quite successful in case of fungal laccases (Giardina *et al.*, 2010; Strong and Claus, 2011; Jeon *et al.*, 2012); insects (Dittmer and Kanost, 2010) and only recently in plants (Cai *et*

al, 2006; Berthet *et al.*, 2011; Zhao *et al.*, 2013). Some of the *in vivo* functions of fungal laccases include break down of complex polymeric compounds such as lignin (Baldrian, 2006; Strong and Claus, 2011; Jeon *et al.*, 2012), humic acids (Strong and Claus, 2011) and lignocellulose (Jeon *et al.*, 2012); oxidizing and interconverting a wide variety of compounds having aromatic or phenolic ring structure (Baldrian, 2006; Strong and Claus, 2011; Jeon *et al.*, 2012); playing a role in defense against reactive oxygen species (Jeon *et al.*, 2012), melanization (Jeon *et al.*, 2012) as well as during the growth and developmental stages of the fungi (Strong and Claus, 2011; Jeon and Chang, 2013). In insects the enzyme has been associated with insect-cuticle sclerotization (Arakane *et al.*, 2005) and also immune functions and melanization (Dittmer and Kanost, 2010). In plants, laccases are involved in lignin polymerization (Gavnholt, Larsen and Rasmussen, 2002; Gavnholt and Larsen, 2002; Jeon *et al.*, 2012; Zhao *et al.*, 2013; Gallego-Giraldo *et al.*, 2014); synthesis of polyflavanoids (Dardick *et al.*, 2010; Jeon *et al.*, 2012); cell wall regulation (Ranocha *et al.*, 2002; Pourcel *et al.*, 2005; Jeon *et al.*, 2012).

In *Arabidopsis thaliana*, a plant from the Brassicaceae family, 17 annotated laccase genes have been identified (McCaig, Meagher and Dean, 2005; Cai *et al.*, 2006). All of these except one have been shown to be expressed in roots (Cai *et al.*, 2006). Specific functions of certain laccases in *Arabidopsis* have been explored. *Lac4*, *Lac7* and *Lac11* have been implicated in lignin polymerization (Berthet *et al.*, 2011; Turlapati *et al.*, 2011; Zhao *et al.*, 2012) while *Lac15* has been shown to play a role in flavonoid production in seeds thereby affecting the color of the seed coat (Pourcel *et al.*, 2005; Turlapati *et al.*, 2011). *Lac2* plays a role in root elongation as well as during growth and developmental stages of the plant while *Lac8* seems to be responsible for the onset of flowering (Cai *et al.*, 2006; Turlapati *et al.*, 2011; Zhang, Zhang and Wang, 2012). A high rate of gene duplication events in plants as they constantly evolve has led to a redundancy

in the expression as well as difficulties in assigning specific functions to these laccase genes (McCaig, Meagher and Dean, 2005; Cai *et al.*, 2006; Dwivedi *et al.*, 2011).

Due to the ability of laccases to accept and act on a wide range of substrates, these enzymes serve as excellent candidates for bioremediation of xenobiotic compounds (Couto and Herrera, 2006; Kunamneni *et al.*, 2008; Canas and Camarero, 2010; Strong and Claus, 2011). Indeed a number of laccases identified from bacterial and fungal sources have been exploited on a large scale to bring about conversion of various organic compounds which normally would be very difficult to degrade (Strong and Claus, 2011; Jeon *et al.*, 2012). Exhaustive research has been conducted for commercially employing laccases on their own or in conjugation with other enzymes in the food industry, paper-pulp industry, soil bioremediation, cosmetics, diagnostic indicators, pharmaceutics, biosensors, and nanotechnology as well as remediation of compounds from the wastewater released by the textile industry and effluents released from other industrial sources (Nagai *et al.*, 2002; Couto and Herrera, 2006; Husain, 2006; Riva, 2006; Kunamneni *et al.*, 2007; 2008; Giardina *et al.*, 2010; Strong and Claus, 2011; Jeon *et al.*, 2012). However, not much is described about using laccases from plant sources for the above mentioned applications despite their relative abundance in many higher plants and the economic benefits in terms of cost and infrastructure (Ranocha *et al.*, 1999; Gavnholt, Larsen and Rasmussen, 2002; McCaig, Meagher and Dean, 2005; Husain 2010; Strong and Claus, 2011).

1.3.4.2 Peroxidases

Peroxidases are one of the several groups of enzymes that get activated in the presence of hydrogen peroxide and use it as the electron acceptor to bring about transformation of a wide variety of structurally diverse substrates (Passardi *et al.*, 2005; Cosio and Duand, 2010; Mathe *et al.*, 2010). Peroxidases are ubiquitously expressed across the different biotic kingdoms and are

essential in every stage of life, from growth, development and maintaining homeostasis to defense and responses to stress conditions (Zámocký and Obinger, 2010). Numerous gene duplications during evolution have led to an extensive redundancy in the functional expression of many of these peroxidases across kingdoms (Passardi *et al.*, 2007a; Loughran *et al.*, 2008; Zámocký and Obinger, 2010). Peroxidases are grouped into two major classes namely heme containing peroxidase and non-heme containing peroxidases based mainly on the presence of heme as the prosthetic group (Hiraga *et al.*, 2001; Loughran *et al.*, 2008; Mathe *et al.*, 2010). Non-heme peroxidases usually do not possess a heme group and are phylogenetically distinct from heme peroxidases (Zámocký and Obinger, 2010). Heme-containing peroxidases are further divided into three large superfamilies, established primarily on their origin in animals and/or plant and/or other biotic sources such as protists, bacteria and fungi, although there are certain exceptions in each group (Welinder, 1992; Hiraga *et al.*, 2001; Mathe *et al.*, 2010; Zámocký and Obinger, 2010). The first superfamily is called peroxidase-cyclooxygenase superfamily. This is further divided into five distinct subfamilies consisting of bacterial peroxicins, peroxidockerins and primordial peroxidases subfamily; Ecdysozoan and Echninozoan peroxinectins subfamily; Ecdysozoan and Deuterostomian peroxidasins subfamily; subfamily of bacterial, fungal and animal cyclooxygenases; and subfamily of Chordata peroxidases (Zámocký and Obinger, 2010). The cyclooxygenases and the Chordata peroxidases are discussed below since members from these two subfamilies are the most reviewed. The most prominent member from the cyclooxygenases subfamily is prostaglandin endoperoxide synthase (E.C. 1.14.99.1) (Welinder, 1992; Hiraga *et al.*, 2001; Zámocký and Obinger, 2010). Prostaglandin endoperoxide synthase, also called cyclooxygenase, has two reported isozymes which play an important role in prostanoid production and arachidonate pathway in autocrine as well as paracrine functions

(Smith and Marnett, 1994). The Chordata peroxidases subfamily is further characterized based on function, into eosinophill peroxidase (E.C. 1.11.1.7); myeloperoxidase (E.C. 1.11.1.7); lactoperoxidase (E.C. 1.11.1.7); thyroid peroxidase (E.C. 1.11.1.8) (Welinder, 1992; Hiraga *et al.*, 2001; Loughran *et al.*, 2008; Mathe *et al.*, 2010; Zámocký and Obinger, 2010). The second superfamily consists of catalases (E.C. 1.11.1.6) which are ubiquitously expressed across the animal, plant, fungi and yeast kingdoms but are not seen in bacteria or protists (Welinder, 1992; Hiraga *et al.*, 2001). These enzymes usually are expressed to destroy hydrogen peroxide and other free radicals that are generated during oxygen burst (Chelikani, Fita and Loewen, 2004; Chelikani, Ramana and Radhakrishnan, 2005). Thus, catalases protect the cells from oxidative damage caused by reactive oxygen species (Chelikani, Fita and Loewen, 2004; Chelikani, Ramana and Radhakrishnan, 2005). They are expressed as homotetramers and bring about break down of hydrogen peroxide to water and oxygen (Chelikani, Fita and Loewen, 2004; Chelikani, Ramana and Radhakrishnan, 2005). The third superfamily of heme peroxidases was earlier named plant peroxidases based on their expression mostly in plants, but since this superfamily also contains enzyme groups which are expressed in bacteria, yeast and fungi, this superfamily is now called peroxidase-catalase superfamily which also takes its functional role in consideration (Welinder, 1992; Hiraga *et al.*, 2001; Cosio and Duand, 2010; Mathe *et al.*, 2010; Zámocký and Obinger, 2010). This is one of the most researched and diverse group of peroxidases (Passardi *et al.*, 2005; Cosio and Duand, 2010; Mathe *et al.*, 2010). Peroxidases classified in this superfamily of peroxidase-catalase have certain common structural and functional characteristics despite poor agreement in the primary amino acid sequence alignment, catalytic mechanisms, cellular expression patterns as well as sub-cellular localization and major functional roles (Hiraga *et al.*, 2001; Passardi *et al.*, 2005, 2007a; Cosio and Duand, 2010; Mathe *et al.*, 2010; Zámocký and

Obinger, 2010). Besides the fact that these enzymes require hydrogen peroxide for their activation, all the members from this superfamily also have heme moiety in the form of ferriprotoporphyrin (consisting of protoporphyrin IX and Fe(III) in resting state), conserved histidine as well as arginine residues in their active site and similarities in their three-dimensional structures (Welinder, 1992; Edwards *et al.*, 1993; Veitch, 2004; Passardi *et al.*, 2007a; Cosio and Duand, 2010; Zámocký and Obinger, 2010). This peroxidase-catalase superfamily further branches into three subfamilies named class I, class II and class III peroxidases, classified based on their primary amino acid sequences as well as functional roles and localizations (Welinder, 1992; Hiraga *et al.*, 2001; Passardi *et al.*, 2005, 2007a; Cosio and Duand, 2010; Mathe *et al.*, 2010; Zámocký and Obinger, 2010).

Class I peroxidases consist of the cytochrome c peroxidase (CcPs; E.C. 1.11.1.5); the ascorbate peroxidase (APxs; E.C. 1.11.1.11) and the catalase-peroxidase (CPs or KatGs; E.C. 1.11.1.6) subfamilies (Welinder, 1992; Hiraga *et al.*, 2001; Passardi *et al.*, 2005, 2007a; Cosio and Duand, 2010; Mathe *et al.*, 2010; Zámocký and Obinger, 2010). These are expressed in all biotic sources but animals (Hiraga *et al.*, 2001; Passardi *et al.*, 2005, 2007a; Cosio and Duand, 2010; Mathe *et al.*, 2010; Zámocký and Obinger, 2010). Structurally they are quite unrelated to peroxidases from the other two classes since they are not glycosylated, lack a secretory signal peptide, and do not show any interaction with calcium ions or possess any disulfide linkages in their structures (Passardi *et al.*, 2007a; Cosio and Duand, 2010; Mathe *et al.*, 2010; Zámocký and Obinger, 2010). Also they are mostly intracellular, unlike the other two classes which are extracellular (Passardi *et al.*, 2007a; Cosio and Duand, 2010; Mathe *et al.*, 2010; Zámocký and Obinger, 2010). Despite differences in subcellular localizations as well as diversities in specific functions, the rudimentary role of class I peroxidases is protecting the cell from harmful effects of reactive

oxygen species generated during oxygen burst, especially break down of excess hydrogen peroxide inside the cell (Passardi *et al.*, 2007a; Cosio and Duand, 2010; Mathe *et al.*, 2010; Zámocký and Obinger, 2010). *In vivo*, Ccps are expressed in the mitochondrial intermembrane space and play an important role in destruction and neutralization of hydrogen peroxide generated during aerobic respiration (Passardi *et al.*, 2007a; Cosio and Duand, 2010; Mathe *et al.*, 2010; Zámocký and Obinger, 2010). APxs also play an important role in reducing levels of hydrogen peroxide inside the cell using ascorbate as the electron donor (Passardi *et al.*, 2007a; Cosio and Duand, 2010; Mathe *et al.*, 2010; Zámocký and Obinger, 2010). They have subcellular localization and are further classified into cytosolic, peroxisomal and chloroplastic based on their expression in the specific cell compartment (Teixeira *et al.*, 2004, 2005; Passardi *et al.*, 2007a; Cosio and Duand, 2010). Cps play dual roles since they can behave as catalase (mainly) as well as peroxidase despite having a single catalytically active heme domain and are evolutionarily different from catalases with no sequence similarities (Yamada *et al.*, 2002; Klotz and Loewen, 2003; Khersonsky, Roodveldt and Tawfik, 2006; Passardi *et al.*, 2007a, 2007b; Zámocký and Obinger, 2010). However, their application in biotechnology and bioremediation is still unexplored.

Class II peroxidases are comprised of manganese peroxidases (MnPs; E.C. 1.11.1.13), lignin peroxidases (LiPs; E.C. 1.11.1.14) and versatile peroxidases (VPs; E.C. 1.11.1.6) subfamilies (Welinder, 1992; Hiraga *et al.*, 2001; Passardi *et al.*, 2005, 2007a; Cosio and Duand, 2010; Mathe *et al.*, 2010; Zámocký and Obinger, 2010). Unlike class I and class III peroxidases these are expressed solely in fungi with no indication of orthologs or paralogs in other biotic kingdoms (Passardi *et al.*, 2005, 2007a; Morgenstern, Klopman and Hibbett, 2008; Cosio and Duand, 2010; Mathe *et al.*, 2010; Zámocký and Obinger, 2010). All of them possess a secretory signal peptide

sequence which makes them extracellular (Passardi *et al.*, 2007a; Cosio and Duand, 2010; Mathe *et al.*, 2010; Zámocký and Obinger, 2010). Unlike class I, class II peroxidases are synthesized as glycoproteins, have disulfide linkages and show interaction with calcium ions (Welinder, 1992; Passardi *et al.*, 2007a; Cosio and Duand, 2010; Mathe *et al.*, 2010; Zámocký and Obinger, 2010). All of them require metal ions as cofactors and all of them play a major role in soil bioremediation as well as soil recycling since they can break down complex organic molecules such as lignin (Conesa, Punt and van den Hondel. 2002; Passardi *et al.*, 2007a; Cosio and Duand, 2010; Mathe *et al.*, 2010; Zámocký and Obinger, 2010). In fact this ability of class II peroxidases to degrade complex molecules with high redox potential makes them unique and different from not only from class I and class III peroxidases but also could be exploited in bioremediation and biotechnological applications (Piontek, Smith and Blodig, 2001; Gianfreda and Rao, 2004; Martinez *et al.*, 2005; Passardi *et al.*, 2007a; Ruiz-Duenas *et al.*, 2009; Marques *et al.*, 2010; Fernandez-Fueyo *et al.*, 2014).

Class III peroxidases are members of a large multigene family expressed in all higher land plants (Welinder, 1992; Tognolli *et al.*, 2002; Duroux and Welinder, 2003; Passardi *et al.*, 2005, 2007a; Cosio and Duand, 2010; Mathe *et al.*, 2010; Zámocký and Obinger, 2010). The peroxidases from this class are quite diverse in their functions, despite nearly all of them having conserved structural motifs and very similar amino acid sequences (Welinder, 1992; Duroux and Welinder, 2003; Veitch, 2004; Passardi *et al.*, 2005, 2007a; Cosio and Duand, 2010; Zámocký and Obinger, 2010). They are only expressed in plants with higher levels of organization which have evolved and adapted to grow on land but some members are also expressed in plants of lower complexity such as algae (Passardi *et al.*, 2004, 2007a; Cosio and Duand, 2010; Mathe *et al.*, 2010; Zámocký and Obinger, 2010). Numerous gene duplication events during evolution have led to extensive

structural and functional redundancy between members of this class of peroxidase (Welinder *et al.*, 2002; Passardi *et al.*, 2004, 2005, 2007a; Cosio and Duand, 2010; Mathe *et al.*, 2010; Zámocký and Obinger, 2010). Moreover, different post-transcriptional modifications have resulted in various isoenzymes being synthesized from the same mRNA (Hiraga *et al.*, 2001; Duroux and Welinder, 2003; Cosio and Duand, 2010; Mathe *et al.*, 2010; Zámocký and Obinger, 2010). The number of genes encoding for distinct functional class III peroxidase has been identified for some of the Angiosperms (Mathe *et al.*, 2010; Zámocký and Obinger, 2010). The speculated numbers for the model plant *Arabidopsis thaliana* is 73 while it is 15 for *Armoracia rusticana* (horseradish), 138 for *Oryza sativa* (rice) and 151 for *Zea mays* (Maize) respectively (Tognolli *et al.*, 2002; Welinder *et al.*, 2002; Duroux and Welinder, 2003; Veitch, 2004; Passardi *et al.*, 2004, 2007a; Cosio and Duand, 2010; Mathe *et al.*, 2010; Zámocký and Obinger, 2010). Like class II peroxidases, class III peroxidases possess a signal peptide which targets them to the endoplasmic reticulum for secretion across a membrane (Welinder *et al.*, 2002; Duroux and Welinder, 2003; Veitch, 2004; Passardi *et al.*, 2004, 2007a; Mathe *et al.*, 2010; Zámocký and Obinger, 2010). Nearly all of them also possess signal peptides which directs them to vacuoles or the cell wall (Matsui *et al.*, 2003; Veitch, 2004; Passardi *et al.*, 2004, 2005, 2007a; Cosio and Duand, 2010; Mathe *et al.*, 2010). Class III peroxidases are glycosylated polypeptides containing two different metal centers, one with ferriprotoporphyrin heme (protoporphyrin IX and Fe(III) in resting state) and the other center containing two calcium atoms (Veitch, 2004) with conserved disulfide bridges, α-helices and β-strands (Gajhede, 1997; Veitch, 2004; Ros Barcelo, Gomez Ros and Esteban Carrasco, 2007; Almagro *et al.*, 2009).

Another discovery was that cyanobacteria, bacteria and fungi are capable of expressing a distinct type of heme peroxidase that can decolorize synthetic dyes yet they share no substrate specificity

and very low sequence similarities with the other classes of heme peroxidases (Sugano *et al.*, 2007; Sugano, 2009; Sugano *et al.*, 2009; Hofrichter *et al.*, 2010 Liers *et al.*, 2010; Zámocký and Obinger, 2010; Liers *et al.*, 2013; Sezer *et al.*, 2013; Strittmatter *et al.*, 2013). These were named as 'Dye Decolorizing Peroxidases (DyPs)' and classified into Dyp-type heme peroxidase (E.C. 1.11.1.13) also called Reactive-Blue-5:hydrogen peroxide oxidoreductase (Sugano *et al.*, 2007; Sugano, 2009; Hofrichter *et al.*, 2010 Liers *et al.*, 2010; Liers *et al.*, 2013 Strittmatter *et al.*, 2013). Although their natural substrates and physiological roles in bacteria and fungi are still not clear, they have been shown to transform a variety of structurally different synthetic dispersive dyes such as anthraquinone dyes by using them as electron donors (Sugano *et al.*, 2007; Sugano, 2009; Sugano *et al.*, 2009; Hofrichter *et al.*, 2010 Liers *et al.*, 2010; Zámocký and Obinger, 2010; Liers *et al.*, 2013; Sezer *et al.*, 2013; Strittmatter *et al.*, 2013). These Dyp-type peroxidase have a promising bioremediation potential and it appears that they have been explored with that role in mind (Sugano *et al.*, 2007; Sugano, 2009; Sugano *et al.*, 2009; Hofrichter *et al.*, 2010 Liers *et al.*, 2010; Zámocký and Obinger, 2010; Liers *et al.*, 2013; Sezer *et al.*, 2013; Strittmatter *et al.*, 2013).

Class II and class III heme peroxidases generally catalyze hydrogen peroxide-dependent oxidoreduction of structurally simple as well as relatively complex molecules, which means they can act on and break down a wide variety of substrates (Veitch, 2004; Harms, Schlosser and Wick, 2011). Like laccases, peroxidases are also quite promiscuous in their choice of substrates and by single electron transfer mechanism using hydrogen peroxide as the electron acceptor bring about oxidation of substrates with complex structures (Passardi *et al.*, 2004; Veitch, 2004; Harms, Schlosser and Wick, 2011; Jeon *et al.*, 2012). Both the Fe(III) of the heme and calcium atoms participate in the catalytic process as well as stability of the peroxidase enzyme (Veitch,

2004). Peroxidases can accept and transform molecules with high redox potentials much more readily than laccases (Passardi *et al.*, 2004; Veitch, 2004; Harms, Schlosser and Wick, 2011; Jeon *et al.*, 2012). However, unlike catalases and even possibly class I peroxidases which primarily are hydrogen peroxide scavengers by breaking down excess of hydrogen peroxide to water and releasing oxygen, class III peroxidases *in vivo* can participate in two different types of reaction processes (Passardi *et al.*, 2004; Veitch, 2004; Cosio and Duand, 2010; Mathe *et al.*, 2010). Class III peroxidases in their primary peroxidative-oxidoreductive cycle transfers electrons from various natural substrates such as lignin precursors, secondary metabolites, phenolic compounds or auxins to hydrogen peroxide thereby reducing hydrogen peroxide to water and oxygen while bringing about oxidation of the above mentioned natural substrates (Hiraga *et al.*, 2001; Passardi *et al.*, 2007a; Cosio and Duand, 2010; Mathe *et al.*, 2010). The kinetics and reaction mechanism for this single electron transfer has been well studied using horseradish peroxidase (HRP) which is a classic class III peroxidase, in addition to class III peroxidases from *A. thaliana* (Passardi *et al.*, 2004; Veitch, 2004; Almagro *et al.*, 2009; Mathe *et al.*, 2010). Class III peroxidases can also conduct the hydroxylic cycle which produces reactive oxygen species plus producing more hydrogen peroxide which can act as a signal molecule in various signal transduction pathways during stress and oxygen burst (Liszkay, Kenk and Schopfer, 2003; Passardi *et al.*, 2004, 2005, 2007a; Mathe *et al.*, 2010). Both cycles generate very reactive and highly unstable free radicals (Passardi *et al.*, 2004; Veitch, 2004; Cosio and Duand, 2010; Mathe *et al.*, 2010).

Class III peroxidases are not only diverse in terms of the substrates they can transform but also their levels of expression varies strongly during the different stages of growth and development of the plant (Valerio *et al.*, 2004; Passardi *et al.*, 2004, 2005; Veitch, 2004; Cosio and Duand,

2010; Mathe *et al.*, 2010). On account of the large number of the duplication events that resulted in increased redundancy in the amino acid sequences of the members of class III peroxidases, elucidating the functions of each peroxidase has become very difficult (Hiraga *et al.*, 2001; Passardi *et al.*, 2005, 2007a; Zámocký and Obinger, 2010). This is true especially if these peroxidases are grouped in the same clade or family where no visible effect is seen on silencing the particular gene since it gets replaced by another functional peroxidase which can compensate for the loss of the particular peroxidase (Passardi *et al.*, 2004, 2005; Veitch, 2004; Cosio and Duand, 2010; Mathe *et al.*, 2010). In general, class III peroxidases have been implicated to play important role in every stage of the plant's life cycle from germination to senescence (Hiraga *et al.*, 2001; Welinder *et al.*, 2002; Passardi *et al.*, 2004, 2005; Veitch, 2004; Cosio and Duand, 2010; Mathe *et al.*, 2010). These include roles in lignification, suberization, germination, cell wall cross-linking, loosening and metabolism, cell elongation, auxin catabolism, secondary metabolite oxidation, defense against pathogens and pests, wound healing, abiotic as well as biotic stress responses, tolerance to different environmental conditions, roots, leaf, hypocotyl and stem elongation, oxidative burst responses, signal transduction pathways, homeostasis, senescence, organogenesis, symbiotic associations including nodulation and mycorrhization, flowering, fruit growth and ripening, nutrient deficiency, pollen-pistil interactions, seed protection, chlorophyll bleaching (Hiraga *et al.*, 2001; Welinder *et al.*, 2002; Valerio *et al.*, 2004; Passardi *et al.*, 2005; Veitch, 2004; Cosio and Duand, 2010; Mathe *et al.*, 2010).

In *A. thaliana* 73 gene loci are predicted to code for functional class III peroxidase enzymes and around 36 isoforms have been identified using microarrays, sequencing and functional analysis (Tognolli *et al.*, 2002; Welinder *et al.*, 2002; Duroux and Welinder, 2003; Valerio *et al.*, 2004; Passardi *et al.*, 2004, 2007a; Cosio and Duand, 2010; Mathe *et al.*, 2010; Zámocký and Obinger,

2010). Fully 79% of the genes encoding for functional class III peroxidases are expressed in the roots of *A. thaliana* with AtPrx12, AtPrx14, AtPrx17 and AtPrx65 expressed exclusively in roots (Valerio *et al.*, 2004). Putative functions for 44 of these peroxidases have been identified (Cosio and Duand, 2010).

The prospect of using peroxidases as biotechnological and bioremediation tools is immense based on their ability to act on and break down a wide variety of complex xenobiotic compounds (Veitch, 2004; Husain, 2006; Husain, 2010; Harms, Schlosser and Wick, 2011; Strong and Claus, 2011). Dyp-type peroxidases from bacteria and fungi on account of their potential to carry out decolorization of structurally distinct dyes have recently gained momentum since these peroxidases are quite versatile in showing transformation by either regular redox conversion while even showing hydrolase and oxygenase activities (Sugano *et al.*, 2007; Sugano, 2009; Sugano *et al.*, 2009; Hofrichter *et al.*, 2010 Liers *et al.*, 2010; Liers *et al.*, 2013; Sezer *et al.*, 2013; Strittmatter *et al.*, 2013). Members of secretory class II and class III peroxidases from various fungal and plant sources as well as bacterial peroxidases have been identified and purified for their efficiency in getting rid of compounds with high oxidation potential especially synthetic dyes (Gianfreda and Rao, 2004; Veitch, 2004; Husain, 2006; Husain, 2010; Harms, Schlosser and Wick, 2011; Strong and Claus, 2011). Class II peroxidases namely MnPs, LiPs as well as Vps whose primary role is in lignin break down have shown immense potential in degrading synthetic dyes from all classes.(Gianfreda and Rao, 2004; Husain, 2006; Husain, 2010; Harms, Schlosser and Wick, 2011). Plant peroxidases or class III peroxidases which have shown great versatility in terms of the structurally complicated substrates like textile dyes have been identified, isolated and purified from various plant families (Shaffiqu *et al.*, 2002; Gianfreda and Rao, 2004; Husain, 2006; Husain, 2010; Harms, Schlosser and Wick, 2011). HRP

is the most researched and effective class III peroxidase with ability to transform a wide variety of compounds having high redox potential including synthetic dyes and other xenobiotics (Veitch, 2004; Husain, 2006; Husain, 2010). Like laccases, peroxidases are also capable of oxidizing small molecules such as mediators in order to enhance as well as increase the efficiency of degrading and decolorizing complex dyes (Husain, 2006; Kulshrestha and Husain, 2007; Tinoco, Verdin and Vazquez-Duhalt, 2007; Husain, 2010). Thus, class II and class III peroxidases make ideal candidate for various biotechnological and bioremediation applications (Gianfreda and Rao, 2004; Veitch, 2004; Husain, 2006; Husain, 2010; Harms, Schlosser and Wick, 2011; Strong and Claus, 2011).

There are certain disadvantages of using isolated enzymes in synthetic dye decolorization (Robinson *et al.*, 2001; Strong and Claus, 2011). The biggest factor is the stability of enzyme outside cell once it is isolated and this can depend on variables such as pH conditions, temperature, nature of solution and substrate availability (Robinson *et al.*, 2001; Strong and Claus, 2011). Unless the enzymes are immobilized these enzymes only carry out a fixed number of decolorization cycles and also can undergo irreversible inhibition due to the presence of certain compounds (Robinson *et al.*, 2001; Strong and Claus, 2011). Also the cost-expenditure as well as infrastructure involved in isolating, purifying and stabilizing the enzyme is not trivial (Robinson *et al.*, 2001; Strong and Claus, 2011). Like with most remediation methods using biological sources, the dye decolorization process catalyzed by the enzymes produces by-products that may be hazardous to health with uncertain long-term effects, making the enzyme-based decolorization infeasible in field trials on a large scale (Robinson *et al.*, 2001; Strong and Claus, 2011).

1.4 Mediators

Mediators are low molecular weight substances that behave as co-substrates or primary substrates for various oxidoreductase enzyme-dependent transformations of xenobiotic compounds (Bourbonnais and Paice, 1990; Kunamneni *et al.*, 2007, 2008; Husain and Husain, 2008; Giardina *et al.*, 2010; Strong and Claus, 2011). They are usually compounds that have high redox potential and hence are called redox mediators (Kunamneni *et al.*, 2007, 2008; Van der Zee and Cervantes, 2009; Canas and Camarero, 2010). These compounds on account of their relatively small size can easily enter the active site of the enzymes and then undergo oxidation (Kunamneni *et al.*, 2007; Canas and Camarero, 2010). Mediators are also known as electron shuttles, since on oxidation these compounds diffuse away from the enzyme's active site before carrying about further conversion of molecules with complex aromatic structures (Kunamneni *et al.*, 2007, 2008). Combining these molecules with enzymes such as laccases and peroxidases opens up new avenues towards remediation of structurally complex molecules which otherwise would otherwise be recalcitrant and difficult to degrade (Couto and Herrera, 2006; Van der Zee and Cervantes, 2009; Canas and Camarero, 2010; Strong and Claus, 2011; Jeon *et al.*, 2012). Presence of mediator in the vicinity of the two enzymes has been shown to not only initiate break down of complex compounds but also to increase the transformation efficiency of these xenobiotic compounds compared to treatments which lack mediators (Bourbonnais and Paice, 1990; Kunamneni *et al.*, 2007, 2008; Husain and Husain, 2008; Giardina *et al.*, 2010; Strong and Claus, 2011). Both synthetic as well as natural compounds that could serve as stable yet economically sustainable mediators have been explored towards remediation of xenobiotic compounds such as synthetic dyes (Couto and Herrera, 2006; Canas and Camarero, 2010; Strong and Claus, 2011; Jeon *et al.*, 2012). Two of the most notable mediators that are widely used and

have shown promise in bioremediation of organic pollutants are 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) [ABTS] and 1-hydroxybenzotraizole (HOBt).

1.4.1 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) [ABTS]

ABTS has displayed great potential as a mediator, when employed in laccase dependent degradation of lignin polymer (Bourbonnais and Paice, 1990; Bourbonnais, Leech and Paice, 1998; Canas and Camarero, 2010; Giardina *et al.*, 2010). ABTS has a higher redox potential than laccases themselves and therefore can undergo rapid reversible transitions between its oxidized and reduced state (Bourbonnais *et al.*, 1995; Bourbonnais, Leech and Paice, 1998; Morozova *et al.*, 2007). Not only are these two forms relatively stable but the reaction carried out by ABTS is cyclic which means that a large conversion of the recalcitrant compounds with high redox potentials to their oxidized form can occur for many cycles unless the oxidized ABTS in its free radical form is removed or the enzyme loses its function (Fabbrini, Galli and Gentili, 2002; Riva, 2006; Kunamneni *et al.*, 2007; Morozova *et al.*, 2007; Strong and Claus, 2011). ABTS being relatively water soluble can enter the active site of the enzyme, undergo oxidation, diffuse out and bring about oxidation of the secondary xenobiotic compounds (Kunamneni *et al.*, 2007; Morozova *et al.*, 2007; Canas and Camarero, 2010). The redox reaction carried out by ABTS on undergoing enzymatic oxidation involves two steps (Fabbrini, Galli and Gentili, 2002; Morozova *et al.*, 2007). The first step is the generation of free radical intermediates of ABTS in the form of single cation radical ($ABTS^{+\cdot}$) and dication radical ($ABTS^{2+}$) (Fabbrini, Galli and Gentili, 2002; Kunamneni *et al.*, 2007, 2008; Morozova *et al.*, 2007; Canas and Camarero, 2010). The second step involves oxidation of the high redox potential secondary aromatic compounds by these free radical intermediates via electron transfer (ET) mechanism (Fabbrini, Galli and Gentili, 2002; Morozova *et al.*, 2007). The ET mechanism proceeds by transfer of electrons from the secondary

substrate to the mediator resulting in the formation of oxidized by-products of the target substrate while the mediator undergoes reduction and can be recycled for further break down of the secondary compounds (Fabbrini, Galli and Gentili, 2002; Morozova *et al.*, 2007). This mechanism usually generates by-products bearing aldehyde functional groups due to the oxidation of C-C bonds (Fabbrini, Galli and Gentili, 2002; Morozova *et al.*, 2007). The oxidation of these secondary xenobiotic compounds by enzymatic catalysis of ABTS is much dependent not only on the nature of the secondary substrate that the enzyme encounters, but also on the treatment conditions (Bourbonnais, Leech and Paice, 1998; Branchi, Galli and Gentili, 2005; Morozova *et al.*, 2007). Interaction of ABTS with the enzyme and its subsequent oxidation has shown immense potential not only in the biotechnology and bioremediation of aromatic compounds including textile dyes (Canas and Camarero, 2010; Giardina *et al.*, 2010; Strong and Claus, 2011; Jeon *et al.*, 2012), but also as an useful assay to detect the activity of laccase and peroxidase in the medium using colorimetric tools (Childs and Bardsley, 1975). Unoxidized ABTS is colorless with absorption maximum around 340 nm, while on oxidation to its free radical ($ABTS^{+\cdot}$) turns greenish-blue in color with the absorption maximum shifting to 414 nm (Childs and Bardsley, 1975; Kunamneni *et al.*, 2007). The intensity of the blue-green coloration serves as the indicator of laccase (in presence of oxygen) or peroxidase (in presence of hydrogen peroxide) activities (Childs and Bardsley, 1975). Since the overall reaction involves cycling of the ABTS between its oxidized and relatively reduced forms, the molar absorptivity of 36 mM^{-1} cm^{-1} at either wavelength remains the same (Childs and Bardsley, 1975).

1.4.2 1-hydroxybenzotriazole (HOBt)

Another artificial compound that shows potential as a mediator in enzymatic degradation of complex aromatic xenobiotic molecules is HOBt (Bourbonnais, Leech and Paice, 1998; Xu *et*

al., 2000; Fabbrini, Galli and Gentili, 2002; Canas and Camarero, 2010; Strong and Claus, 2011; Jeon *et al.*, 2012). Based on its structure, HOBt belongs to the mediator class of compounds with a characteristic heterocyclic ring structure bearing N-OH functional groups represented as R-N-OH (Call, 1994). Like ABTS, HOBt is small enough to enter the active site of the enzymes such as laccases and peroxidases where it is oxidized (Bourbonnais, Leech and Paice, 1998; Fabbrini, Galli and Gentili, 2002; Kunamneni *et al.*, 2007; Morozova *et al.*, 2007; Canas and Camarero, 2010). However, the reaction mechanism followed by enzymatic oxidation of HOBt is different from ABTS despite the formation of a free radical intermediate (Xu *et al.*, 2000; Fabbrini, Galli and Gentili, 2002; Riva, 2006; Morozova *et al.*, 2007). The redox reaction carried out by HOBt also follows two steps (Xu *et al.*, 2000; Fabbrini, Galli and Gentili, 2002; Morozova *et al.*, 2007). In the first step HOBt, on enzymatic oxidation, is converted from its reduced form (N-OH) to its highly reactive nitroxyl free radical ($N\text{-}O^{\cdot}$) due to sequential loss of electrons followed by removal of protons (Xu *et al.*, 2000; Fabbrini, Galli and Gentili, 2002; Cantarella, Galli and Gentili, 2003; Morozova *et al.*, 2007). In the next step the $N\text{-}O^{\cdot}$ free radical attacks the secondary aromatic substrates with higher oxidation potential (Xu *et al.*, 2000; Fabbrini, Galli and Gentili, 2002; Morozova *et al.*, 2007). Aromatic substrates bearing labile C-H bonds are oxidized by the HOBt free radical via radical hydrogen atom transfer (HAT) mechanism (Xu *et al.*, 2000; Fabbrini, Galli and Gentili, 2002; Morozova *et al.*, 2007; Canas and Camarero, 2010). The HAT mechanism proceeds by the free radical-mediated dissociation of the C-H bond from the secondary aromatic compound resulting in the formation of its oxidized by-product while the free radical of the mediator gets reduced on taking up the abstracted hydrogen from the aromatic compound and then, like ABTS, can be recycled for further break down of the aromatic compounds (Xu *et al.*, 2000; Fabbrini, Galli and Gentili, 2002; Morozova *et al.*, 2007). This

mechanism of oxidation usually produces by-products which are ketone derivatives (Xu *et al.*, 2000; Fabbrini, Galli and Gentili, 2002; Morozova *et al.*, 2007; Canas and Camarero, 2010). As with ABTS, the oxidized by-products generated by enzyme-dependent oxidation of HOBt are also dependent on the nature of the secondary substrate that the enzyme encounters as well as treatment conditions (Xu *et al.*, 2000; Fabbrini, Galli and Gentili, 2002; Morozova *et al.*, 2007; Canas and Camarero, 2010).

As discussed above, laccases on their own are quite restrictive in their ability to oxidize synthetic dyes with complex aromatic structures since the active site is not modular enough to accommodate such large and steric-hindrance causing compounds (Kunamneni *et al.*, 2007; Giardina *et al.*, 2010). Although laccases isolated and purified from certain fungal sources have been successful in degrading and decolorizing the different classes of synthetic dyes (Nagai *et al.*, 2002; Nyanhongo *et al.*, 2002; Palmieri, Giardina and Sannia, 2005; Husain and Husain, 2008), these enzymes were less successful in decolorizing textile dyes with larger aromatic rings and relatively high redox potential in the absence of any mediators (Zilly *et al.*, 2002; Trupkin *et al.*, 2003; Couto and Herrera, 2006; Husain, 2006). When laccases are coupled to mediators, an increase in the efficiency of remediation of synthetic dyes as well as other organic compounds with higher redox potentials was observed. (Kunamneni *et al.*, 2007; Husain and Husain, 2008; Canas and Camarero, 2010; Giardina *et al.*, 2010). This arrangement of laccase in which the mediator serves as the co-substrate is known as laccase mediator system or LMS (Bourbonnais and Paice, 1990; Couto and Herrera, 2006; Kunamneni *et al.*, 2008; Canas and Camarero, 2010). LMS was initially used for its success in the break down of lignin (Bourbonnais and Paice, 1990; Canas and Camarero, 2010) but has now been exploited for remediation of various, structurally unrelated xenobiotics (Riva, 2006; Canas and Camarero, 2010; Jeon *et al.*, 2012) especially dyes

(Husain, 2006; Camarero *et al.*, 2007; Canas and Camarero, 2010). However, laccases are not the only enzymes that are capable of breaking down structurally complex compounds in presence of mediators (Xu *et al.*, 2000; Fabbrini, Galli and Gentili, 2002; Husain, 2006; Morozova *et al.*, 2007; Canas and Camarero, 2010). Peroxidases from different sources have also shown promise as enzymes capable of acting on wide variety of compounds bearing complex aromatic structures and high redox potential with mediator (Husain, 2006; Husain and Husain 2010).

However, there are some disadvantages associated with using mediator-dependent enzyme based transformation processes (Riva, 2006; Canas and Camarero, 2010; Strong and Claus, 2011; Jeon *et al.*, 2012). Unless the mediators are from natural sources, the use of synthetic mediators in LMS has been shown to be toxic, even irreversibly inhibiting laccase activity if used at higher concentrations which not only increase the cost of running LMS but also decreases its transformation efficiency as well as the number of transformation cycles (Kunamneni *et al.*, 2007; Canas and Camarero, 2010; Strong and Claus, 2011; Jeon *et al.*, 2012). Ensuring stability of the enzyme outside its host without inactivating the enzyme is a major challenge, but newer and better techniques of immobilization mitigate these issues to certain extent (Kunamneni *et al.*, 2007; Canas and Camarero, 2010; Strong and Claus, 2011; Jeon *et al.*, 2012). Further isolating as well as purifying these enzymes from their sources along with manufacturing the mediators, both synthetic as well as those derived from natural sources, are expensive to use on large scale in field-based bioremediation and biotechnological applications (Kunamneni *et al.*, 2007; Canas and Camarero, 2010; Strong and Claus, 2011; Jeon *et al.*, 2012). There is also concern about the nature of the transformed by-product being more toxic and dangerous as compared to the parent compound as well as the LMS process not being very environmental friendly unless paired with commercial inorganic cleaning techniques (Jeon *et al.*, 2012). Still these are minor issues

compared to the huge advantages and benefits for the potential of using biotic sources for remediation of complex xenobiotic compounds.

1.5 Research Objectives and Significance

This study has three major objectives:

I. To establish and compare the processes of decolorization of representative monoazo dyes MR and MO; disazo dyes TB, EB and CB6B as well as arylmethane dyes BBG, BG, MG and PR, between plants from two different families *Arabidopsis thaliana* (thale cress) and *Helianthus annuus* (sunflower) under conditions of added hydrogen peroxide and mediator. The major drawback of phytoremediation process is the very long time required for transformation when the plants are exposed to the synthetic dye (Pivetz, 2001). One solution to enhance or accelerate the dye decolorization process in presence of plants would be to externally add mediators along with the dye and monitor the decolorization process (Riva, 2006; Canas and Camarero, 2010).

II. To determine if laccases and peroxidases are the major oxidoreductase enzymes that are expressed in *A. thaliana* and sunflowers when appropriate conditions of enzyme activation are applied to the dye treatments, since these enzymes been implicated for their ability to decolorize synthetic dyes (Husain, 2010; Jeon *et al.*, 2012; Agostini *et al.*, 2013; Watharkar *et al.*, 2013)

III. To characterize the dye decolorization pathway followed by the plants in the above described systems, based on the by-products formed under specific dye decolorization conditions.

Chapter 2 - Materials and Methods

This chapter will discuss the techniques and methodologies employed for the research.

2.1 Plants considered

Plants chosen for the comparison were *Arabidopsis thaliana* and *Helianthus annuus* (sunflower). *Arabidopsis thaliana* belongs to the mustard family *Brassicaceae* (http://www.arabidopsis.org). Despite having no commercial usage, *Arabidopsis* is a model plant with a relatively short life cycle whose genome is completely sequenced. Furthermore, gene knockouts using T-DNA insertions leading to mutant plants lacking a particular gene of interest are available for majority of the genes. The other plant used for this study was *Helianthus annuus*, commonly known as sunflower. Sunflower is a commercial crop for oil and seed production. Sunflower is a member of the family *Asteraceae* and is also the state flower of Kansas. These two plants were chosen since the similarities and differences in the dye decolorization pattern between a model plant and a commercial plant under similar treatments would not only give better understanding of the decolorization process but also could be extrapolated to field studies in real world situations.

2.1.1 Hydroponics

Hydroponics was selected as the method of cultivation for both plants because the objective was to expose the plants directly to the synthetic dye in absence of soil which is known to bind organic dyes and prevent them from reaching the plants (Harms, Schlosser and Wick, 2011). Hoagland's solution was chosen as the nutrient solution because it provides all the nutrients essential to support the growth of plants (Norén, Svensson and Andersson, 2004). Hoagland's nutrient formulation is comprised of four stock solutions labeled 'macronutrients stock' comprised of 1 M KNO_3, 0.2 M KH_2PO_4, and 0.4 M $MgSO_4.7H_2O$; 'calcium nitrate stock'

containing 1 M $Ca(NO_3)_2$; 'micronutrients stock' comprised of 0.01164 M H_3BO_3, 0.00227 M $MnCl_2.4H_2O$, 0.000191 M $ZnSO_4.7H_2O$, 0.00022 M $CuSO_4.5H_2O$ and 0.00031 M $H_2MoO_4.H_2O$; and 'Iron-EDTA stock' containing 0.005 M $FeSO_4.7H_2O$ and 0.0057 M EDTA-Na_2. The salts for making the stock solutions were certified ACS reagent grade obtained from Fisher Scientific Inc. (Pittsburgh, PA). Stock salt solutions were autoclaved at 121^0C for 20 minutes. To make a working nutrient solution of half-strength Hoagland's solution 2 mL of the macronutrients, 2.5 mL calcium nitrate, 2 mL micronutrients and 2 mL Iron-EDTA salt solutions were mixed together in ~500 mL (no less than 500 mL) sterile distilled water (D/W) and the volume made to 1 liter using sterile D/W. This gives a final concentrations of 2 mM KNO_3, 0.4 mM KH_2PO_4, 0.8 mM $MgSO_4$; 31.5mM $Ca(NO_3)_2$; 23.28 µM H_3BO_3, 4.54 µM $MnCl_2$, 0.382 µM $ZnSO_4$, 0.44 µM $CuSO_4$ and 0.62 µM H_2MoO_4; and 10µM $FeSO_4$, 11.4 µM EDTA for the different salts in half-strength Hoagland's solution.

For preparing 1% agar plugs, 1 g of granulated agar powder (Becton Dickinson Microbiology Systems, Cockeysville, MD) was dissolved in 100 mL of half-strength Hoagland's solution and the mixture was autoclaved. The autoclaved agar was then poured into 1.7 mL polypropylene, research grade microcentrifuge tubes (GeneMate, Kaysville, UT). The lids of the tubes were removed. The agar was allowed to solidify in the tubes at room temperature for 3-4 hours. Once the agar was set as plugs the tubes were cut near the 0.5 mL mark using scissors so that the tubes were open at both ends (Figure 2.1A). The agar plugs were then inserted into the lids of Folgers coffee containers (~2.3 L) which were punched with holes to fit the agar plugs. Coffee containers were autoclaved and then filled to the brim with sterilized half-strength Hoagland's solution (Figure 2.1A). The reason for making the agar in half-strength Hoagland's solution instead of water was to prevent the agar plugs from cracking on the surface after dipping the plugs in

excess of Hoagland's solution. Matching salt concentrations in the agar and the solution maintains an osmotic balance and ensures the constant supply of the nutrients from the Hoagland's solution in the container travels through the agar plugs by capillary action. Agar plugs made in water have a tendency to crack at the surface when placed in salt solutions. This also leads to poor availability of nutrients to the germinating plant.

2.1.1.1 *Arabidopsis thaliana* Columbia ecotype

Seeds of wild-type (WT) *Arabidopsis thaliana* Columbia ecotype were surface-sterilized using 70% ethanol for two minutes followed by 10 minutes exposure to 2.5% hypochlorite followed by washing 3-4 times with sterile distilled water (D/W). The treated seeds were placed on the microcentrifuge tubes containing 1% agar plugs (Figure 2.1A). To assure a good stand, 4-5 seeds were placed per agar plug. The lids with the tubes containing agar with seeds were then placed on the coffee container containing half-strength Hoagland's solution and was latched shut. The whole set up was covered with plastic food-wrap to maintain constant humidity and temperature and was placed under continuous fluorescent white light (Philips CW Supreme Plus). The plastic wrap was removed after 2-3 weeks when the plants reached the flowering stage. Plants used for the assays were 5-6 weeks old from the day of planting on agar (Figure 2.1A).

2.1.1.2 *Helianthus annuus* cv HySun 521

Seeds of *Helianthus annuus* cv HySun 521 were germinated on vermiculite (Figure 2.1B). This formulation contains a mixture of mica particles that has very strong water holding capacity. It is however devoid of any sort of nutrients that can support plant growth and hence was moistened with half-strength Hoagland's nutrient solution. The wet vermiculite was then layered on trays and sunflower seeds were placed on the surface of the vermiculite under continuous fluorescent light. After one week the young seedlings were removed, their roots washed to remove any

excess vermiculite that may have adhered to it and then transferred to bottles containing sterile half-strength Hoagland's solution (Figure 2.1B). The seedlings were placed in the nutrient solution for 2-3 days so that the plants could continue to grow and acclimatize to the liquid nutrient solution in order to reduce the stress if the plants were directly exposed to the dyes without any acclimatization to the liquid solution. The sunflower plants used for the assay were 2 weeks old from the day of planting (Figure 2.1B).

2.1.2 Crushed turnip root

Turnip has been shown to be a rich source of peroxidase enzymes (Husain and Husain, 2008; Husain, 2010). Like horseradish, some of the peroxidases from turnip have been identified, isolated, characterized and purified to homogeneity. They have shown strong potential in synthetic dye decolorization (Veitch, 2004; Kulshrestha and Husain, 2007; Husain, 2010). Crushed turnip root extract which was not further purified was used as the positive control for all the dye decolorization experiments. Freshly harvested turnip root was provided by Lawrence Davis from his garden. Around 1 g of the turnip root was weighed and crushed in 10 mL of distilled water in a mortar using a pestle. The crushed extracts were centrifuged in Marathon Micro A microcentrifuge (Fisher Scientific, Pittsburgh, PA) at 12,000 rpm for 10-15 minutes. The supernatants from the tubes were pooled together while the pellets containing the cellular debris and crushed roots were discarded. The peroxidase activity of the crude extract was measured using 1 mM hydrogen peroxide and 50 μM ABTS in order to confirm the potency of the crushed turnip root extract.

2.2 Synthetic dyes and other chemicals

The representative synthetic dyes that were selected were Methyl Red (MR) and Methyl Orange (MO) from the monoazo class; Trypan Blue (TB), Evans Blue (EB) and Chicago Blue 6B

(CB6B) from the disazo class; and Brilliant Blue G (BBG), Bromocresol Green (BG), Malachite Green (MG) and Phenol Red (PR) from the arylmethane class (Table 2.1).

2.2.1 Monoazo dyes

2.2.1.1 Methyl Red (MR)

Methyl Red (MR) also known as C.I. Acid Red 2 is a monoazo dye which functions as a pH indicator in various physiological, analytical and biochemical techniques (Lillie and Conn, 1969). Structurally, MR has a carboxylic acid at the ortho-position of the azo benzene ring (Table 2.1). Although MR is not used as a textile dye it has applications in microbiology where it is employed as a part of IMViC tests to determine and differentiate the bacteria from the colon aerogenes family which can cause mixed acid fermentation. It is also used in vital staining of protozoa (Clark and Lubs, 1915; Carter, 1933; Parr, 1936; Lillie and Conn, 1969). MR is also used in the textile printing industry (Ebrahimi and Modrek, 2013). However, the dye is not stable in solution for longer periods of time as it can easily undergo loss of color due to reduction (Lillie and Conn, 1969). MR is classified by the International Agency for Research on Cancer as a class 3 category agent in terms of its carcinogenicity to humans (http://www-dep.iarc.fr/; http://www.inchem.org/documents/iarc/monoeval/eval.html) and there is evidence of its mutagenicity, carcinogenicity and oral toxicity potentials (Toxicity Profile for Methyl Red, 1993). MR has a pKa around 5.1 (Lillie and Conn, 1969). Thus, at relatively acidic pH values of 4.4 and below where the protonated form dominates, the dye appears reddish-pink in color with a wavelength maximum around 430 nm while the deprotonated form at pH of 6.0 and above appears yellow with the wavelength maximum shifting to 520 nm (Lillie and Conn, 1969). The dye has an isosbestic point wavelength at 465 nm (Korzun and Miller, 1986). The isosbestic point is the wavelength seen for pH sensitive dyes where the molar absorptivity values of the

different dye forms are no longer affected by the pH and is the preferred wavelength to monitor the changes in dye properties such as decolorization since it removes any biases due to preferential selection of one pH dependent form over the other leading to error in the trends observed at the wavelength maxima (Korzun and Miller, 1986; Hoxter, 1979). The molar absorptivity coefficient determined for MR at 430 nm is around 23.36 mM^{-1} cm^{-1} (Chen, Hopper and Cerniglia, 2005; Daher 2012). Due to its distinct pH dependent color changes and relatively high pKa value the dye serves as a good model to study decolorization of pH indicators from the azo dye class especially for plants. MR for the dye decolorization assay was obtained from General Chemical Company, NY with the starting concentration of 40 mg/L.

2.2.1.2 Methyl Orange (MO)

Methyl Orange (MO) like MR is a monoazo dye which serves as a pH indicator (Lillie and Conn, 1969). MO is structurally similar to MR except for the replacement of the carboxylic acid group at the ortho position in MR by the sulfonic acid group at the para position on the benzene ring (Table 2.1). Apart from being a pH indicator this dye is also used in dyeing and printing textiles (Gattermann, 1961; Pourbabaee *et al.*, 2005). Exposure to MO shows both mutagenic and carcinogenic potential in bacterial cells as well as mammalian cells but only at very high concentrations of dyes (Toxicity Profile for Methyl Orange, 1992). The pKa of MO is much more in the acidic range ~3.47 which makes studies involving the yellow color of the dye in the relatively less acidic conditions more preferable (Lillie and Conn, 1969). The protonated form of the dye which exists at pH of 3.1 and below appears red in color with a wavelength maximum around 520 nm while the yellow-orange color representing the deprotonated form of the dye has a wavelength maximum of 460 nm which is very close to its isosbestic point ~470 nm (Lillie and Conn, 1969; Hoxter, 1979; Agilent Technologies, 2000). The molar absorptivity coefficient for

MO around 460 nm was found to be 24.6 mM^{-1} cm^{-1} (Daher, 2012). Recrystallized MO was obtained from Kenneth Burkhard. The starting concentration of the dye was 40 mg/L.

2.2.2 Disazo dyes

The selected disazo dyes are from the Direct Blue dye family with none being pH indicators (Lillie and Conn, 1969).

2.2.2.1 Trypan Blue (TB)

Trypan Blue (TB) is a disazo dye which has several clinical applications as a vital stain (Lillie and Conn, 1969) besides being a textile dye (Forgacs, Cserháti and Oros, 2004). Structurally TB is very similar to Evans Blue (EB) with the only difference being the placement of the sulphonic acid groups around the napthol ring at the corners of the dye structure at positions 3 and 6 from the amino group of the 1-naphthylamine moieties (Table 2.1). TB is taken up by the endothelial system. It is used in the dye exclusion method for determining cell viability since it helps to distinguish live cells (do not uptake TB) from dead cells (stains blue due to uptake of TB). It was used for killing of the causative agent of sleeping sickness namely trypanosomes (Tennant, 1964; Lillie and Conn, 1969; Phillips and Hayman, 1970: Feron *et al.*, 2002; Harrington *et al.*, 2010). However, TB was also shown to have teratogenic potential, cause ocular toxicity, necrosis of the kidney and congenital anomalies such as spina bifida and hydrocephalus (Gillman, Gilbert and Gillman, 1948; Good and Thomas, 1952; Beck, Lloyd and Griffiths, 1967; Veckeneer *et al.*, 2001). The molar absorption coefficient for TB is 51,700 M^{-1} cm^{-1} with a peak wavelength around 580-600 nm (Pease, 2000).

2.2.2.2 Evans Blue (EB)

Evans Blue (EB) also called Direct Blue 53 is a disazo textile dye (Forgacs, Cserháti and Oros, 2004) which is structurally similar to TB except for the sulphonic acid groups at positions 2 and

4 from the amino group of 1-napthylamine moieties at either side in EB instead of positions 3 and 6 in TB (Table 2.1). These dyes serve different functions due to these visible differences despite the same molecular weight (Table 2.1). EB has been known to tightly bind to cellulose in the presence of calcium ions. This binding is reversed by washing with organic solvents such as ethanol Tris-EDTA solutions when EDTA chelates calcium (Davis LC, unpublished). Some of the clinical applications of EB includes it being used as a vital stain similar to TB, monitoring endothelial cell turnover and vascular permeability, monitoring cell-death and apoptosis and quantifying blood-retinal barrier break down (Lillie and Conn, 1969; Saria and Lundberg, 1983; Saria and Lundberg, 1983; Baker and Mock, 1994; Matsuda, Nishikawa and Tanaka, 1995; Xu, Qaum and Adamis, 2001). As seen with TB, exposure to high levels of EB is toxic and is found to be detrimental towards the growth of mice (Balzarini *et al.*, 1989; Israël *et al.*, 2001). EB has a wavelength maximum around 600-610 nm with a molar absorption coefficient of 78,100 M^{-1} cm^{-1} (Roberts and Palade, 1995).

2.2.2.3 Chicago Blue 6B (CB6B)

Another member of the Direct Blue family of dyes used as a textile dye is Direct Blue 1, also called as Chicago Blue 6B (CB6B) (Husain, 2006). CB6B is almost identical in structure to EB except for the replacement of the methyl groups on the central benzidine moiety in EB by methoxy groups in the CB6B (Table 2.1). However, unlike EB, CB6B does not appear to bind cellulose in presence of calcium. Although not as widely used as TB or EB, CB6B has few clinical applications in studies involving modeling of dye-surface interactions, measuring permeability of lymphatic capillaries and behaving as an anti-coagulant of whole blood, besides functioning as a vital stain (Rous, Gilding and Smith, 1930; Hudack and McMaster, 1932; Lillie and Conn, 1969; Anacker, 1994; Kútvölgyi, Stefler and Kovács, 2006). Not much is known

about toxicity due to CB6B though like EB and TB it may display similar levels of toxicity (Huggett and Rowe, 1933). The molar absorption coefficient obtained for CB6B is 73,000 M^{-1} cm^{-1} with the peak wavelength around 610-620 nm.

EB and CB6B were obtained from Sigma-Aldrich Co. (St. Louis, MO) while TB was ordered from JT Baker Chemical Co. (Phillipsburg, NJ). The starting concentrations of EB, CB6B and TB used were 20 mg/L.

2.2.3 Arylmethane dyes

2.2.3.1 Brilliant Blue G (BBG)

Brilliant Blue G (BBG) also known as Coomassie Brilliant Blue G is a well-known stain that helps in the identification as well as quantification of proteins by binding to them (Bradford, 1976; Neuhoff *et al.*, 1988). BBG should not be confused with Coomassie Brilliant Blue R (BBR) which is structurally similar to BBG except for the absence of two methyl group in BBR (Wong *et al.*, 2011). Structure of BBG is displayed in Table 2.1. The dye is a disulfonated triarylmethane (Zollinger, 2003; Hunger, 2007). The ability of BBG to develop different pH sensitive hues is well explored (Bradford, 1976; Neuhoff *et al.*, 1988; Chial and Splittgerber, 1993; Congdon, Muth and Splittgerber, 1993). The dye has affinity for various proteins and binding of protein to BBG in acidic solution can be detected at 620 nm which happens to be the middle wavelength between the blue form (590 nm) and the green form of the dye (650 nm) (Chial and Splittgerber, 1993; Congdon, Muth and Splittgerber, 1993). This tight binding property makes BBG suitable to detect proteins quite accurately. Apart from protein quantification BBG is also used in treatment of spinal injury and in retinal surgery (Remy *et al.*, 2008; Peng *et al.*, 2009; Shimada *et al.*, 2009). BBG also has very low level of toxicity as compared to the blue azo dyes and was not harmful to mice when ingested, although it does

behave antagonistically towards certain ATP-dependent receptors (Jiang *et al.*, 2000; Remy *et al.*, 2008; Peng *et al.*, 2009; Shimada *et al.*, 2009). BBG appears reddish in color at really acidic pH of 0 with a peak wavelength at 470 nm while at pH of 1 the peak wavelength shifts to 650 nm and the dye appears greenish in color (Chial and Splittgerber, 1993; Congdon, Muth and Splittgerber, 1993). The dye shows blue coloration at pH of 2 with a wavelength maximum around 590 nm and BBG continues to appear blue from pH 2 till pH 11 (Chial and Splittgerber, 1993; Congdon, Muth and Splittgerber, 1993). BBG does not have an isosbestic point indicating these three forms of the dye to be distinct species with no fixed overlapping wavelength (Chial and Splittgerber, 1993). A fourth form of the dye which appears at highly alkaline pH of 12 which appears pink has also been reported (Chial and Splittgerber, 1993). BBG at pH 7.0 has a molar absorption coefficient of 43,000 M^{-1} cm^{-1} at 620 nm (Chial and Splittgerber, 1993). BBG for the assays was purchased from Sigma-Aldrich Co. (St. Louis, MO) with the starting concentration of 40 mg/L.

2.2.3.2 Bromocresol Green

Bromocresol Green (BG) is a triarylmethane dye from the phenolphthaleins and phenolsulfonphthaleins group (Lillie and Conn, 1969). BG is also known as tetrabromo-m-cresolsulfonphthalein because it has both a core phthalein group as well as four bromine atoms at 3, 3', 5 and 5' positions of the two phenol rings (Lillie and Conn, 1969; Table 2.1). Some applications of BG include estimation of serum albumin levels since BG interacts with the albumin. It is also used as pH sensitive sensors in various electrical apparatus and biological indicators (Hoxter, 1979; King and Kester, 1989; Duly *et al.*, 2003; Pacquit *et al.*, 2006; Vinay *et al.*, 2012). Toxicity due to exposure to BG is still unclear. BG behaves as a pH indicator with pKa ~4.7 (Lillie and Conn, 1969). At relatively acidic pH of 3.8 and below where the protonated

species of BG is in excess, the dye appears yellow in color with a wavelength maximum ~440 nm while the deprotonated form of the dye which appears at pH 5.4 and above shows blue coloration with the wavelength maximum shifting to ~610 nm (Lillie and Conn, 1969; Pacquit *et al.*, 2006). The dye appears greenish in color in solutions with pH close to its pKa. BG has an isosbestic point wavelength ~510 nm which like with other pH indicators is the preferred wavelength to study actual decolorization or effects on dye independent of the pH of the solution (Hoxter, 1979; King and Kester, 1989). The molar absorptivity for BG is found to be 18,000 M^{-1} cm^{-1} at 480 nm (Jansen, 2008). BG for the dye decolorization experiments was purchased from Fisher Scientific (Pittsburgh, PA) with the starting concentration of 40 mg/L.

2.2.3.3 Malachite Green (MG)

Malachite Green (MG) is a textile dye which belongs to the diaminotriphenylmethane group of arylmethane dyes (Lillie and Conn, 1969). MG exists in two forms, the more oxidized cationic form which appears green in color and called MG cation, or its more reduced form which is known as leucomalachite green (LMG) and which has no visible coloration (Lillie and Conn, 1969; Alderman, 1985). Table 2.1 shows the MG cation structure. The difference between MG and LMG is the addition of hydrogen at the core methyl carbon in LMG which is not present in MG. Only the MG cation shows green coloration with a wavelength maximum ~610-620 nm and a molar extinction coefficient of 100,000 M^{-1} cm^{-1} while LMG shows no spectra in the visible region (Alderman, 1985). MG has various applications in histology as vital stain, staining bacterial endospore, aquaculture, parasiticide, antibacterial agent, detection of hemoglobin in blood and protein phosphatase activity (Lillie and Conn, 1969; Alderman, 1985; Geladopoulos *et al.*, 1991; Srivastava, Sinha and Roy, 2004). MG toxicity has been explored in various contaminated fresh and sea water species of fish. Higher than tolerable limits of both MG and

LMG have been detected, raising concerns over the release of this dye into water bodies and its deposition in the flora and fauna in the contaminated environment (Alderman, 1985; Srivastava, Sinha and Roy, 2004). MG has also shown to have both mutagenic and carcinogenic potential (Culp and Beland, 1996) and thus its usage as a textile dye is now being monitored (Srivastava, Sinha and Roy, 2004).

MG oxalate salt was obtained from Allied Chemical and Dye Co., NY with the starting concentrations of 40 mg/L.

2.2.3.4 Phenol Red (PR)

Phenol Red (PR) like BG is a triarylmethane dye that belongs to the phenolphthaleins and phenolsulfonphthaleins group (Lillie and Conn, 1969). Like BG, PR is also a pH indicator with a pKa value around 7.9 (Lillie and Conn, 1969). At pH 6.4 and below, the protonated form of the dye is yellow in color with a wavelength maximum ~430 nm (Tamura and Maeda, 1997; Yao and Byrne, 2001). Around neutral pH the dye appears yellowish-orange which consists of both the protonated and deprotonated forms of the dye. The partially deprotonated form of the dye is reddish in color and is visualized around pH 7.6-7.8. At relatively alkaline pH of 8.2 and above PR turns to dark pink (fuchsia) color with a wavelength maximum ~550 nm (Tamura and Maeda, 1997; Yao and Byrne, 2001). The isosbestic point wavelength for PR is 480 nm (Lisman and Strong, 1979; Baylor, Chandler and Marshall, 1982). PR has several applications in the textile and printing industry (Gomaa, 2005; Abdullah et al., 2012), quantification of hydrogen peroxide from cells (Pick and Keisari, 1980; Pick and Mizel, 1981), used in cell culture as indicator of acidification (Moreno-Cuevas and Sirbasku, 2000), behaves as an estrogen mimic for estrogen receptors (Berthois, Katzenellenbogen and Katzenellenbogen, 1986; Welshons *et al.*, 1988), induces oogenesis in surface ovarian cells (Bukovsky, Svetlikova and Caudle, 2005), reduces

cell apoptosis and cell cycle arrest (Wesierska-Gadek *et al.*, 2006). It is also a component of coolants used in motor engines (Richard and Claude, 1960). Phenol Red WS dye was purchased from Nutritional Biochemical Co. (Cleveland, OH). The changes in absorbance value at the isosbestic point wavelength of 480 nm was monitored rather than the peak wavelength of 550 nm seen for the dye in alkaline solution. Change of absorbance at the isosbestic point removes any biases that favors one species of the dye over the other due to changes in pH. The molar absorptivity at 480 nm for the dye is 11 mM^{-1} cm^{-1} (Lisman and Strong, 1979; Baylor, Chandler and Marshall, 1982) while it is 31.62 mM^{-1} cm^{-1} at 550 nm (Green, 1990). At 610 nm, that represents the oxidized product of the dye the molar absorptivity is 22 mM^{-1} cm^{-1} (Rajan, Kurup and Abraham, 2010).

2.2.4 Mediators

ABTS and HOBt were free radical mediators employed for the assays. Both mediators were purchased from Sigma-Aldrich Co. (St. Louis, MO). ABTS in its unoxidized form is colorless with an absorbance maximum ~340 nm and on oxidation to its free radical cationic form turns green in color which can be monitored at 414 nm (Childs and Bardsley, 1975). The molar extinction coefficient obtained for ABTS is 36 mM^{-1} cm^{-1}. There is only cyclic inter-conversion between the unoxidized and the oxidized ABTS. The extinction coefficient does not change at either wavelength making it a good indicator of laccase and peroxidase activity using spectrophotometric techniques (Childs and Bardsley, 1975). The stock ABTS solution was prepared in D/W and had a concentration of 50 mM while the working concentration was established at 50 µM for each assay. However, ABTS is expensive in terms of its cost and is not feasible to use on commercial scale. Hence, the replacement mediator used for the dye decolorization experiments was HOBt. HOBt is relatively inexpensive in its cost and shows

similar mediator potential as seen for ABTS. HOBt does not produce a color and has a wavelength maximum around 300 nm which ensures no overlap of its spectrum with the dye spectra. The stock concentration of HOBt was 33 mM while the working concentration used for the dye decolorization assays was 50 μM, similar to the final concentration of ABTS.

2.2.5 Hydrogen peroxide (H_2O_2)

Since peroxidases are the enzymes that have shown potential in dye decolorization and these enzymes get activated or induced in plants in the presence of hydrogen peroxide, external source of hydrogen peroxide was added to the dye treatments in order to compare the decolorization pattern of the dyes in presence or absence of hydrogen peroxide when plants are exposed to the dye. Hydrogen peroxide used for all the experiments was obtained from the Kroger Co. (Cincinnati, OH). As per specifications, the bottle contained 3% hydrogen peroxide as active ingredient which would give a stock concentration of 1 M. This was confirmed by reading its absorbance in the UV-region at 230 nm and using the determined molar absorptivity at 230 nm for hydrogen peroxide which is 81 M^{-1} cm^{-1} (Homan-Muller, Weening and Roos, 1975). A thousand-fold dilution of the stock was made in appropriate solutions so that the starting hydrogen peroxide concentration used for the experiments was 1 mM.

2.2.6 Phosphate buffer

Certified ACS Reagent grade monobasic potassium phosphate (KH_2PO_4) and dibasic potassium phosphate (K_2HPO_4) were obtained from Fisher Scientific (Pittsburgh, PA). Separate stock 1 M KH_2PO_4 and 1 M K_2HPO_4 were prepared. Using these, 10 mM pH 4.6 phosphate and pH 6.3 phosphate buffer were prepared.

The 10 mM pH 4.6 potassium phosphate buffer was prepared by adding 1 mL of 1 M KH_2PO_4 per 100 mL of D/W giving a 1:100 dilution. The pH of the buffer solution was confirmed using a pH meter (Fisher Scientific, Pittsburgh, PA).

The 10 mM pH 6.3 potassium phosphate buffer was prepared by adding 0.8 mL of 1 M KH_2PO_4 and 0.2 mL of 1 M K_2HPO_4 per 100 mL of D/W. Using the pH meter pH of the buffer solution was confirmed.

Synthetic dyes especially the pH indictors were prepared in both the buffers since the pKa of nearly all the selected pH indicator dyes is in between these two pH values and hence the preference of the plants in terms of decolorization of one form of the dye over other based on pH can be studied.

2.2.7 Ethanol Tris-EDTA extraction solution

Alcohol USP Specs 190 proof that contains 95% pure, undenatured ethanol was from Decon Labs Inc. (King of Prussia, PA) while disodium salt of ethylenediaminetetraacetic acid (Na_2EDTA) and Tris were obtained from Sigma-Aldrich Co. (St. Louis, MO). For preparing the ethanol Tris-EDTA extraction solution 9.7 g of Na_2EDTA and 12.1 g of Tris were dissolved in 190 mL of D/W which gives final concentrations of 0.5 M for Tris and 0.167 M for EDTA. This is diluted 1+10 in 80% ethanol.

Dissolving Tris-EDTA in ethanol makes a solution which is capable of extracting any of the loosely bound or unutilized dye from the roots after the decolorization assay as well as extraction of the dye products from the roots after the assay (Scott and Kyffin, 1978). Ethanol is relatively non-polar as compared to water and is capable of extracting any of the loosely-bound synthetic dyes from the roots. Tris acts as a buffer while EDTA chelates or sequesters a diverse variety

metal ions especially calcium and magnesium which may play a role in the binding of the dye to plant roots (Scott and Kyffin, 1978).

2.3 Ultraviolet-Visible spectrophotometry

The Ultraviolet-Visible (UV-Vis) spectra for the dye solutions treated with plants and their controls were generated with a Hitachi U-2900 double beam UV-Visible spectrophotometer purchased from Hitachi High Technologies America Inc. (Chicago, IL). For all the experiments and data formulation, the pathlength of light was set at 10 mm or 1.0 cm. Quartz cuvettes (3.0 mL) with pathlength of 10.00 mm (Fisher Scientific, Pittsburgh, PA) were used for the analysis and are preferred over glass since glass absorbs UV light and plastic cuvettes are prone to scratching. The reference solution used for all the experiments was D/W.

2.3.1 ABTS activity and Km determination

ABTS oxidation rate is a measure of the laccase and the peroxidase (in presence of hydrogen peroxide) activity of the plants. *A. thaliana* and sunflower plants each in triplicates (n=3) were placed in either pH 4.6 phosphate buffer containing 50 μM ABTS with no added peroxide for determining the laccase activity, or in pH 4.6 phosphate buffer containing 50 μM ABTS and 1 mM hydrogen peroxide for estimating the peroxidase activity (Figure 2.2). The absorbance readings were taken at 414 nm, the wavelength maximum for the green form (Figure 2.2) representing the oxidized ABTS free radical. Samples of ~3 mL were withdrawn from the total reaction mixture at intervals of 60 seconds for a total of 300 seconds, read in the spectrophotometer and then returned to the reaction mixture. At the end of the reaction, the plants were removed from the solution and the gram fresh weights of the roots were measured.

The ABTS oxidation rate was calculated using the following formula and expressed in nanomoles of ABTS oxidized per hour per gram fresh root weight of the plants.

$$\text{ABTS oxidation in mM per hour} = \frac{\text{Absorbance per hour at 414 nm}}{\text{Molar extinction coefficient of ABTS at 414 nm}}$$

$$\text{ABTS oxidation rate} = \frac{[\text{ABTS oxidation in mM per hour} * \text{Volume of solution}]}{\text{Gram fresh weight of roots}}$$

Similarly, the ABTS oxidation rate for both the plants were estimated in pH 6.3 phosphate buffer and compared to the rates obtained in pH 4.6 phosphate buffer.

For determination of the apparent Km value for hydrogen peroxide, the plants were exposed to pH 6.3 phosphate buffer solution containing 50 µM ABTS with either 1 mM hydrogen peroxide or 0.03 mM hydrogen peroxide. The peroxide values were chosen arbitrarily, one from the lower range of concentrations and the other from relatively higher range of concentrations. The ABTS oxidation rate for the plants under the above conditions was estimated and the apparent Km value obtained using the following equation (Davis, 1980).

$$Km = \frac{V_1 - V_2}{(V_2/[S_2]) - (V_1/[S_1])}$$

where V_1= Rate of ABTS oxidation obtained in presence of 1 mM hydrogen peroxide

V_2 = Rate of ABTS oxidation obtained in presence of 0.03 mM hydrogen peroxide

S_1 = Concentration of hydrogen peroxide i.e. 1 mM

S_2 = Concentration of hydrogen peroxide i.e. 0.03 mM

2.3.2 Dye decolorization assay

A. thaliana and sunflower plants each in triplicates (n=3) were exposed to the following treatments: dye alone; dye with 1 mM hydrogen peroxide; dye with 50 µM HOBt and dye with 1

mM hydrogen peroxide plus 50 μM HOBt. All the dyes were prepared in pH 6.3 potassium phosphate buffer. To determine the preference of the plant towards one particular species based on pH, the pH indicator dyes namely MR, MO and BG were also prepared in pH 4.6 potassium phosphate buffer and tested under the above mentioned dye treatments. The starting concentrations tested was 40 mg/L of MR, MO, BBG, BG and MG while for EB, CB6B and TB the starting concentration tested was 20 mg/L since concentrations higher than 20 mg/L in phosphate buffer gave absorbance readings which were out of the sensitive range of the spectrophotometer and made the readings difficult. The plants exposed to the different dye treatments were then placed on Orbit water bath shaker (Lab-line Instruments Inc., Melrose Park, IL) set at 25 rpm under continuous illumination of fluorescent white light at room temperature for 72 hours. At intervals of 6 hours, 12 hours, 24 hours, 48 hours and 72 hours aliquots of the different dye treatments were read against the water blank in the Hitachi U-2900 double beam UV-Visible spectrophotometer. The residual dye solutions were brought back to the starting volumes using distilled water to account for the loss of water while also preventing the dyes from getting concentrated. Wavelength scans of average absorbance values from the three plants from 200-700 nm range were generated for each time interval and for each treatment (See Results for averaged spectra). At the end of the treatment after 72 hours the gram fresh root weights of the plants were measured and the roots were washed twice initially with water followed by two washes with ethanol Tris-EDTA extraction solution to remove the loosely bound dye which might stick to the roots. The extracted solutions from the roots were pooled together and the ethanol were evaporated overnight in the fume hood. The left over residues were re-dissolved in an equivalent amount of distilled water and the wavelength scans of the extracts were generated. This was done since the spectra of the dyes in organic solvents were slightly altered as compared

to the spectra of the dyes in distilled water. For instance in the case of MO, the dye peak shifted to 430 nm in ethanol Tris-EDTA extraction solution (Figure 3.9C) from the 460 nm (Figure 3.9A) seen in distilled water due to the differences in polarities of the solution. This in turn alters the molar absorptivity coefficient of the dye in the two solvents.

Crushed turnip root extract served as the positive control. An 80 mg/L of the stock monoazo and arylmethane dyes, and 40 mg/L of the stock disazo dyes prepared in phosphate buffer were mixed with the crushed turnip root extract to give final concentrations of 40 mg/L for the monoazo dyes as well as the arylmethane dyes, and 20 mg/L for the disazo dyes. Thus, the dye concentrations were comparable to those used for the whole plants. The dye treatments were conducted in similar manner as seen for the plants and the crushed root extract with the dye were placed under similar conditions of shaker and light conditions as those employed for the plants. The 40 mg/L monoazo as well as arylmethane dye solutions and 20 mg/L disazo dye solutions with or without added hydrogen peroxide and with or without mediator which were never exposed to the crushed root extract were used as negative controls. The negative dye control solutions were also placed under similar conditions as those employed for the positive control. The absorbance changes over 6-10 hour time intervals were used to estimate the initial rate of decolorization carried out by the plants for each dye and expressed in nanomoles of dye decolorized per hour per gram fresh root weight of plants.

$$\text{Decolorization in mM per hour} = \frac{\text{Absorbance per hour at peak or isosbestic point}}{\text{Molar extinction coefficient of dye at peak or isosbestic point}}$$

$$\text{Initial rate of dye decolorization} = \frac{[\text{Decolorization in mM per hour} * \text{Volume of dye}]}{\text{Gram fresh weight of treated roots}}$$

Similarly, the percentage (%) dye decolorization values in 24 hours were estimated for both the plants under the different treatments.

$$\% \text{ dye decolorization} = \frac{[\text{Absorbance of negative control} - \text{Absorbance of plant treatment}] * 100}{\text{Absorbance of negative control}}$$

2.3.3 Repeated dye addition

The time duration of exposure of the dye to *A. thaliana* as well as sunflowers plants was restricted in the dye decolorization experiments described in section 2.3.2, so that the ability of the plants to decolorize synthetic dyes when exposed to fresh synthetic dyes for longer time durations was still unclear. To carry out this experiment MO and BBG were selected as the representative azo and arylmethane dyes. The dyes were dissolved in half-strength Hoagland's nutrient solution instead of phosphate buffer so that the plants were not stressed for the lack of nutrients. The starting dye concentrations tested were 40 mg/L of MO and BBG. Since the objective was to know about the interaction of dye on its own with the plants external hydrogen peroxide and mediator were left out through the entire time duration of the experiment. Again *A. thaliana* and sunflowers in triplicates (n=3) were selected. The experimental conditions were similar to those described in section 2.3.2 with one change. Instead of replenishing the daily consumption of the dye by the plants with D/W, fresh dye in half-strength Hoagland's solution was added. The experiment was carried out until the equivalent volume of the original dye from the start of the experiment was completely replaced with a like volume of fresh dye. At the end of the experiment the wavelength scans of the dye without plant (negative control) were measured along with the scans of the completely replenished dye treatments which on the last day were made to the final volume using D/W. Also the gram fresh weight of the roots were measured. The roots were then given washes with the extraction mixture of ethanol Tris-EDTA

to remove the loosely bound dye as described in sections 2.3.2. The total amount of dye consumed by the plants, at the end of the experiment was calculated.

2.3.4 Binding of dyes to dried roots and cellulose

The aim of the experiment was to determine the extent of adsorption and absorption of the synthetic dyes from each class by dead roots in order to differentiate the actual dye decolorization process from simple binding of dye in the absence of active roots. MO, BBG and EB were selected as the representative dyes from each class. The representative dyes were prepared in pH 6.3 potassium phosphate buffer and in half-strength Hoagland's nutrient solution in order to study the interaction of the excess salts in the nutrient solution in the presence or absence of added hydrogen peroxide, with or without HOBt, on the binding of dyes to dried roots. Dried sunflower roots in triplicates (n=3), weighing ~50-100 mg were placed in 10 mL of the dye solutions with starting concentrations of 40 mg/ L in presence or absence of 1 mM hydrogen peroxide and/or 50 μM HOBt. This is a much higher ratio of root mass per dye volume than with living plants (approximately 15-fold higher). The dye solutions with the roots and their respective negative controls without any roots were placed on the shaker moving at 25 rpm under constant source of fluorescent white light for 48 hours. At the end of 48 hours, spectra of the dye solutions following removal of the roots were generated. The roots were then given one wash with 10 mL of extraction mixture of ethanol Tris-EDTA to remove the loosely bound dye and the spectra of the supernatant were generated. The negative control solution contained 40 mg/L of the BBG and EB dye in 80% ethanol Tris-EDTA while for MO the concentration was 20 mg/L. The differences in the shape of the spectra and the shift in the absorbance values of the dye in the water-based solutions versus the extracted dye in the alcohol-based extraction solution was due to the differences in the polarities of the two solutions. Molar absorptivity of the dyes in the two

solvents also varies. The total amount of dye recovered from the roots was estimated from these spectra.

Another objective was to check whether the presence of externally added hydrogen peroxide enhanced permanent binding of EB to pure cellulose in presence of salts. EB is known to bind complex polysaccharides such as cellulose and the binding is enhanced in presence of divalent ions such as calcium (Davis LC, unpublished). Hence, the dye decolorization experiment as described above was repeated using Whatman Chromatography Paper # 1 which represents pure cellulose since it is composed of dehydrated cellulose fibers. As per specifications, the basis weight is 87 g/m^2 with a thickness of 0.16 mm and width of 1 inch. The paper roll was cut into rectangles of 25 X 35 mm. The rectangles weighed between 75-80 mg. The rectangles were then placed in 10 mL of 40 mg/L EB (~35 μM) prepared in either pH 6.3 Evans Blue or in half-strength Hoagland's nutrient solution, under the different treatments on the shaker moving at 25 rpm for 48 hours. The negative control solutions that were not exposed to the Whatman Chromatography Paper # 1 under the different treatments were also similarly handled. At the end of 48 hours, wavelength spectra of the dye solutions after removal of the papers were generated. The papers were then given two washes with 10 mL of extraction mixture of ethanol Tris-EDTA to remove the loosely bound dye and the spectra of the pooled supernatant were generated. The negative control solution contained 40 mg/L of the dye in 80% ethanol Tris-EDTA and was used for the comparison. The differences in the shape of the spectra and the shift in the absorbance values of the dye in the water-based solutions versus the extracted dye in the alcohol-based extraction solution was due to the differences in the polarities of the two solutions. Molar absorptivity of the dyes in the two solvents also varies. The total amount of dye recovered from the papers was estimated from these spectra.

2.3.5 Estimation of hydrogen peroxide using Phenol Red

Phenol Red is used in the quantitative estimation of hydrogen peroxide (Pick and Keisari, 1980; Pick and Mizel, 1981). The method is based on horseradish peroxidase (HRP)-dependent oxidation of PR dye (Pick and Keisari, 1980; Pick and Mizel, 1981). When hydrogen peroxide is added to the dye in presence of suitable enzymes which can utilize peroxide such as peroxidase enzymes, a colored compound is generated (Pick and Keisari, 1980). The formation of this compound whose characteristics are not yet defined is completely dependent on the amount/concentration of peroxide available to react with the dye if excess PR is present. The compound formed is not only stable under both acidic and alkaline conditions for long time periods, but also has an absorbance maximum quite distinct from unreacted PR dye (Pick and Keisari, 1980). In presence of strong alkali, the unreacted (unoxidized) PR has an absorbance maximum in the 550-560 nm region and is distinctly dark pink in color (Figure 2.3A) with very little absorbance around 600-620 nm (Pick and Keisari, 1980; Pick and Mizel, 1981). On the other hand the compound formed due to the oxidation of dye under similar alkaline conditions has relatively lower absorbance at the 550-560 nm wavelengths while the absorbance in the region 600-620 nm increases (Figure 3.36). The compound under alkaline conditions appears maroon in color (Figure 2.3B) and the intensity of the color is proportional to the amount of oxidation i.e. the concentration of peroxide present in the solution (Pick and Keisari, 1980). In fact, the color change observed ranges from dark pink to dark maroon to dark purple-violet (Figure 2.3A and 2.3B) in order of increasing concentration of peroxide. The previous method used for detecting peroxide, based on the oxidation of a fluorescent compound scopoletin (Root *et al.*, 1975; Nathan and Root, 1977) had a number of drawbacks. Since the method was based on detection of loss of fluorescence of scopoletin on oxidation with peroxidase a lot of calibration and standardization in terms of background as well as interfering fluorescence needed to be

done. Not only was a spectrofluorometer essential but the samples also had to be maintained at a constant temperature which was tedious. Also at one time only single samples could be analyzed and the linear relationship between loss of fluorescence of the scopoletin and peroxide concentration could only be sustained for brief time intervals due to complete loss of substrate. This PR method of peroxide detection overcomes these drawbacks since it is based on UV-Visible spectrophotometric detection of spectral changes due to oxidation of Phenol Red (Pick and Keisari, 1980). While this method was originally developed to detect hydrogen peroxide from macrophages and monolayer of cells (Root *et al.*, 1975; Nathan and Root, 1977) it has been modified to monitor the effects of externally added hydrogen peroxide in plants with different solution conditions of distilled water, pH 6.3 potassium phosphate buffer or half-strength Hoagland's nutrient solution.

Healthy *A. thaliana* plants (n=3) matched for similar weights and size were placed in one of the four solutions: distilled water, pH 6.3 potassium phosphate buffer, half-strength Hoagland's solution and half-strength Hoagland's solution with 50 µM HOBt. All of these contained 1 mM added hydrogen peroxide. Plants placed only in half-strength Hoagland's solution with no added hydrogen peroxide served as biotic control, while non-biotic controls were composed of all the above solutions with similar treatments but with no exposure to plants. All the plant treatments as well as the biotic and the non-biotic control solutions were placed under constant source of fluorescent white light under shaker conditions as described in Section 2.3.2.

The peroxidase enzyme used for the assay is horseradish peroxidase. Horseradish roots are rich in number of enzymes from the oxidoreductase family especially peroxidases which are potent enough to carry out bioremediation of various compounds (Veitch, 2004; Husain, 2010). This peroxidase is replaceable with peroxidases from other roots such as turnip. For the purpose of

this experiment, freshly crushed horseradish root extract was the source of peroxidase instead of purified peroxidase enzyme.

For the assay, ~1.0-1.5 grams of freshly chopped horseradish root was weighed. The roots were cleaned and washed with distilled before being crushed using a mortar and pestle. For each gram of root 10 mL of deionized distilled water was used. The crushed extract was then centrifuged in a benchtop centrifuge for 25 minutes at ~10,000 rpm. The supernatants were collected and diluted 1+4 final dilution using distilled water. The oxidation rate of the mediator, ABTS for each of the diluted crude extracts was carried out before using it for further experiments. The extracts were found to be stable for over two weeks with little loss in activity, at 4^0C.

To test the stability of peroxide solutions, from the starting hydrogen peroxide solution with 1 mM concentration, aliquots representing concentrations of 20, 40, 60 and 100 µM were each removed at time intervals of 8 hours, 24 hours, 48 hours, 72 hours and 120 hours. Appropriate aliquots at the stated time intervals were removed only from half-strength Hoagland's solution containing 1 mM hydrogen peroxide placed under source of constant light. A negative control of half-strength Hoagland's solution containing no added hydrogen peroxide was also tested under similar conditions. The stock PR dye solution had a concentration of 1 mM. For the standardization of the method, starting concentration of the dye was selected as 100 µM since it gave absorbance readings which were within the sensitive range of the UV-Visible spectrophotometer. Thus, not only will the interaction of the dye with increasing concentration of hydrogen peroxide in the assay be studied, but also the stability of the hydrogen peroxide over time. At the stated time intervals appropriate aliquots representing increasing concentration of hydrogen peroxide from the half-strength Hoagland's solution as well as the negative control solutions were added to tubes containing 100 µM Phenol Red and five-fold diluted crushed

horseradish roots extract. The remaining volume was made up to 3.0 mL using half-strength Hoagland's solution. The reaction in the tubes was allowed to take place at room temperature for 30 minutes. At the end of 30 minutes, one-tenth volume of 1 M NaOH was added to solution to raise the pH to visualize the compound formed from the interaction of hydrogen peroxide with the dye. The final concentration of NaOH was 0.1 M. The samples were run against distilled water in quartz cuvettes of 10.00 mm pathlength in the Hitachi 2900 UV-Visible Spectrophotometer where the spectra from 400-700 nm and the absorbance values at 610 nm were obtained.

2.3.5.1 Direct exposure of Phenol Red with plants

For this series of experiments the aim was to study the direct interaction of *A. thaliana* plants with Phenol Red dye in presence or absence or externally added hydrogen peroxide and or added mediator. Stock 1.0 mM Phenol Red dye prepared in half-strength Hoagland's solution was chosen with the concentration of added hydrogen peroxide fixed at 1.0 mM and 50 μM HOBt when added. These concentrations not only ensured a stoichiometric 1:1 interaction between the dye and hydrogen peroxide but also reflected the hydrogen peroxide and mediator concentrations employed in the decolorization studies of dyes from other classes so that the trends could become comparable. The plants were divided into three sets, with each set containing three plants. The first set of plants was exposed to only dye solution with no other additions, the second set to dye with added hydrogen peroxide, while the third set was placed in solutions of dye containing both added hydrogen peroxide and HOBt. All the above treatments including the controls were placed on the shaker moving at 25 rpm under constant light with few exceptions. While the treatments containing added hydrogen peroxide with or without HOBt were monitored for only 72 hours, the treatments involving only PR dye without any additions were monitored

for 7 days. Each day the volume of water lost was brought back to volume using half-strength Hoagland's solution. Also aliquots representing 50 μM dye from the plant-treated as well as control solutions were removed every 24 hours for 72 hours and tested for the treatments involving added hydrogen peroxide while it was 100 μM dye tested after 72 hours for the treatment not involving added hydrogen peroxide but containing only dye. The dye solution was made alkaline to 0.1 M NaOH and was read against D/W in quartz cuvette in Hitachi U-2900 UV-Visible spectrophotometer. At the end of the treatments, the roots of the plants were cut, washed under distilled water and the gram fresh weight of the roots were measured. The roots were then placed in equivalent amount of the extraction solution containing 80% ethanol Tris-EDTA extraction solution for 4 hours. The washings were repeated 2 to 3 more times with the extraction solution and all the washings were pooled together. The alcohol was then allowed to evaporate overnight at room temperature and the residue left behind was re-dissolved in equivalent amount of distilled water. The spectra of the re-dissolved residual dye were determined. Untreated roots that were never exposed to the dyes served as negative controls and were treated similarly. The roots after extraction were washed under distilled water and placed in either distilled water with 0.1 M NaOH or distilled water with 0.1 M acetic acid. The color of the roots was noted and the spectra of the solution in which the roots were placed were generated.

2.4 Phylogenetic relationships

A. thaliana which belongs to the mustard family that also includes horseradish and turnip, has 17 putative laccase genes (McCaig, Meagher and Dean, 2005; Cai *et al.*, 2006) and 73 putative peroxidase genes (Welinder *et al.*, 2002; Cosio and Duand, 2010).

2.4.1 Phylogenetic tree construction

Predicted amino acid sequences of the 17 putative laccase and 73 putative peroxidase encoding functional proteins were obtained from GenBank (Benson *et al.*, 2013; http://www.ncbi.nlm.nih.gov). These sequences were also pasted in Kalign, an online tool that is freely accessible (Lassmann and Sonnhammer, 2005; http://www.ebi.ac.uk/Tools/msa/kalign) and multiple sequence alignments of the protein sequences were carried out. Kalign also generated the percent identity matrix that indicates the level of similarities between the different sequences. Pairwise comparisons indicative of probable percentage similarities in the sequences within the same clade and the neighbouring branch, down the branches of the phylogenetic tree were generated and compared. The percentage similarities were calculated based on the level of similarities in members from the same clade with those from the closest neighbouring branch. The mean of the pairwise comparisons was calculated in order to know the probable similarities of members in the two enzyme families. The phylogenetic tree for the laccases and peroxidases was constructed using the 'One Click' mode in Phylogeny.fr (Dereeper *et al.*, 2008; http://www.phylogeny.fr/), a freely accessible online tool that generates phylogenetic tree from predicted protein sequences. Separate phylogenetic trees were constructed for laccases and peroxidases. Sequences with high percentage similarity values usually are grouped in the same clade and have smallest number of substitutions in them while dissimilar sequences with low levels of similarities are grouped into different branches. The length of the branch is directly proportional to the most probable number of changes seen in the members of the particular subfamily or even the whole family. The scale bar at the bottom of each tree indicates the probable number of amino acid substitution per site or position and is a prediction of the phylogenetic divergence. Both the above softwares are claimed to be robust in their alignment methods (Lassmann and Sonnhammer, 2005; Dereeper *et al.*, 2008).

2.4.2 T-DNA insertion lines of *Arabidopsis*

Since laccases and peroxidases from a variety of bacteria as well as fungi have shown great potential in decolorization and break down of synthetic dyes (Canas and Camarero, 2010; Husain, 2010; Strong and Claus, 2011), laccases and peroxidases sourced from plants are expected to show similar potentials. Peroxidases and laccases isolated from a variety of plants including horseradish, turnip and other plant species have been shown to decolorize a wide variety of dyes (Gianfreda and Rao, 2004; Veitch, 2004; Husain, 2006; Husain, 2010; Harms, Schlosser and Wick, 2011; Strong and Claus, 2011; Agostini *et al.*, 2013; Watharkar and Jadhav, 2014). Information on T-DNA insertions for most of these genes is available through The Arabidopsis Information Resource (TAIR, http://www.arabidopsis.org) and The Salk Institute Genome Analysis Laboratory (SIGnAL, http://signal.salk.edu). T-DNA insertion typically leads to disruption of the gene of interest in the host plant *Arabidopsis*, by *Agrobacterium* transferred DNA. This method has been successful in generating mutant *Arabidopsis* plants with an insertion in the gene of interest that can be studied for its effect on the plants phenotype and genotype (Alonso *et al.*, 2003; http://signal.salk.edu). Seeds for the available T-DNA insertion lines corresponding to predicted laccases and peroxidases in *A. thaliana* were ordered from The Arabidopsis Biological Resource Center (ABRC) at the Ohio State University though TAIR. Each T-DNA insertion line is identified by a unique name which can be used to look up information about the source of for the T-DNA insertion, the gene it targets, the region of the gene where the T-DNA is inserted. Nearly all of them are made in *A. thaliana* Columbia-O (CS60000) ecotype background (Alonso *et al.*, 2003; http://www.arabidopsis.org; http://signal.salk.edu). For instance the gene locus AT1G14540 which encodes for *AtPrx4* peroxidase enzyme has two available T-DNA insertion line identified by SALK_044730C where the insertion is in the exon region of *AtPrx4* gene and SALK_110617C where the insertion is in

the promoter region of *AtPrx4* gene (Alonso *et al.*, 2003; http://www.arabidopsis.org; http://signal.salk.edu). The letter 'C' at the end means the line is homozygous (Alonso *et al.*, 2003; http://www.arabidopsis.org; http://signal.salk.edu). Seeds for some of the T-DNA insertion lines were however, not available either at TAIR or SIGnAL and hence were not covered in this research. The seeds were grown hydroponically on agar plugs like WT *A. thaliana* Col ecotype (Figure 2.1A) as described in section 2.1.1.1 of Materials and Methods.

These T-DNA insertion mutant *Arabidopsis* plants were tested for their dye decolorization activity. For the single laccase mutant *Arabidopsis* plant which are predicted to have T-DNA insertion in laccase genes, the ability of these plants to bring about decolorization of MR was observed and the % dye decolorization in 24 hours was estimated. For the single peroxide mutant *Arabidopsis* plants which are predicted to have T-DNA insertion in peroxidase genes, the % dye decolorization of MO in 24 hours in the presence of added hydrogen peroxide and HOBt was carried out. These dye decolorization activities were compared to WT *A. thaliana* subjected to similar conditions. The objective was to determine whether particular laccase or peroxidase genes play a role in dye decolorization compared to the WT.

2.5 Reverse phase HPLC-ESI/MS

Reverse Phase HPLC is a chromatographic separation technique that preferentially retains and separates molecules based on hydrophobicity. The more hydrophobic the molecule, the more its interaction with a C18 column and thus its retention times in the column. This ensures the enrichment of the compound of interest as well as removal of other molecules and impurities which have different solubility profiles compared to the parent compound of interest. Hydrophilic compounds on the other hand will not interact much with column and will elute out much quicker than relatively hydrophobic compounds. Thus, separation of the fragments

generated from dye decolorization is based on polarity. If the dye fragments generated are more hydrophobic, they will have longer retention times than the parent dye. These fragments will thus be eluted after the parent dye which will result in them missing detection in the time that the cycle is run. Moderately polar fragments on the other hand will have lower retention times than the relatively non-polar fragments but can be missed if their elution times is much quicker than that employed in the instrument and will fail to be detected. Oxidation of the parent dye is expected to increase fragment polarity for most portions of the dye.

Coupling reverse phase HPLC with ESI/MS (Electron Spray Ionization-Mass Spectrometry) ensures not only enrichment and separation of the molecule of interest but also aids in the identification of the compound. ES/MS is based on the mass/charge (m/z) ratio which is characteristic for every compound. Each compound has a distinct m/z signature based on the charge acquired by the molecule as well as its intact molecular weight. ESI/MS thus sorts ions based on charge as well as polarity and thus the analysis is based on ion guidance. This technique also helps in identifying the salts of the compounds such as compounds enriched with cations of sodium or potassium and even anions like chloride or bromide. Based on the relative abundance of the ions generated as well as their natural isotopic abundance each molecule has unique, identifiable peaks that can be detected using ESI/MS.

The instrument employed to conduct the mass spectrometry of dye was Agilent Esquire 3000 ion trap Mass Spectrometer. It is capable of conducting reverse phase HPLC with tandem MS/MS capability at the same time. After enrichment of the sample using reverse phase HPLC the compound is injected at high pressure with a fixed flow rate into the ionization chamber where it undergoes nebulization after being hit with a laser wave of high radiofrequency. The laser cleaves the parent intact molecule into various components. Based on the charge they carry and

their relative abundance the ions travel towards the oppositely charged electrode where they are collected by the detector. Thus, relative intensity of the molecule of interest is detected. Since we are employing the positive ion mode, the charged molecule being positively charged will travel to the negatively charged electrode which is the cathode. The instrument employs octopole which ensures that the ions move in a straight direction to the detector quite rapidly. This ensures that all the ions are accounted for after the nebulization. One drawback of using ESI/MS is that compounds which can undergo polymerization and precipitate out of the solution will escape detection. So if the fragments formed are free radicals which have a high tendency to polymerize it becomes difficult to identify the polymerized by-products using ESI/MS.

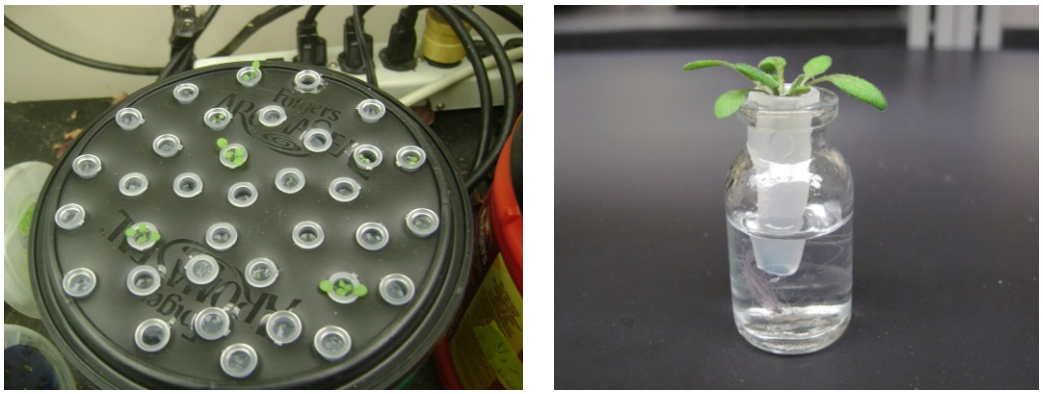
All the reagents were HPLC grade from Fisher Scientific (Pittsburgh, PA). Solution A was 0.01% Formic acid in water while solution B was 90% acetonitrile with 10% solution A. These were run isocratically at 50:50 Solvent A:Solvent B so as to ensure that the conditions employed throughout the run time are kept uniform. These solvent conditions ensures that more hydrophobic molecules are retained for longer times on the C18 Synergi 2.5 μm 20 X 2.00 mm Hydro-Rp mercury column (Phenomenex, Torrence, CA) than comparatively more polar compounds. Flow rate of sample injection was maintained at 0.05 mL per minute. Trifluoroacetic acid (TFA) which is added to all the samples serves two functions: one is to acidify the compound so that the sulfonic acid groups become protonated, thereby allowing the compound to interact with C18 2.5 microcolumn much longer and the other is to act as a counterion in the positive ion mode of ESI/MS so that the dye takes flight in the machine while undergoing fragmentation.

The synthetic dye is dissolved in solvents which are easily gasified or are volatile when they are exposed to the laser. This ensures that there is no interference from any of the buffer ions as the

signal due to the buffer ions being large in concentration usually overshadows that of the dye molecule fragments which are relatively lower in concentration. Phosphate ions in the phosphate buffers are known to build up in sample and interfere with the dye spectra while masking the peak. Hence, it is preferred to dissolve the synthetic dyes in water or in buffers such as ammonium carbonate which can easily volatilize thereby reducing any interference from the other ions. The counterion solution used to dissolve the dye solution contained 0.01%TFA-70% ethanol. The solutions tested were the representative dye treatments involving added hydrogen peroxide and HOBt which were exposed to *A. thaliana* plants for 48 hours after removing the plants. Also included was the positive dye control solutions of crushed turnip root extract exposed to comparable treatments of hydrogen peroxide and HOBt. The negative dye control solution under similar treatments acted as the standard for comparison.

The ESI/MS analysis was restricted to continuous MS1 mode since the objective was to detect and identify the molecule of interest based on its mass. There was uncertainty in terms of the enrichment and analysis of particular dye fragments and hence the MS2 mode of the analysis was not used.

A



B

Figure 2.1 A. Images of *Arabidopsis thaliana* grown under hydroponic conditions and **B.** Sunflowers grown in vermiculite.

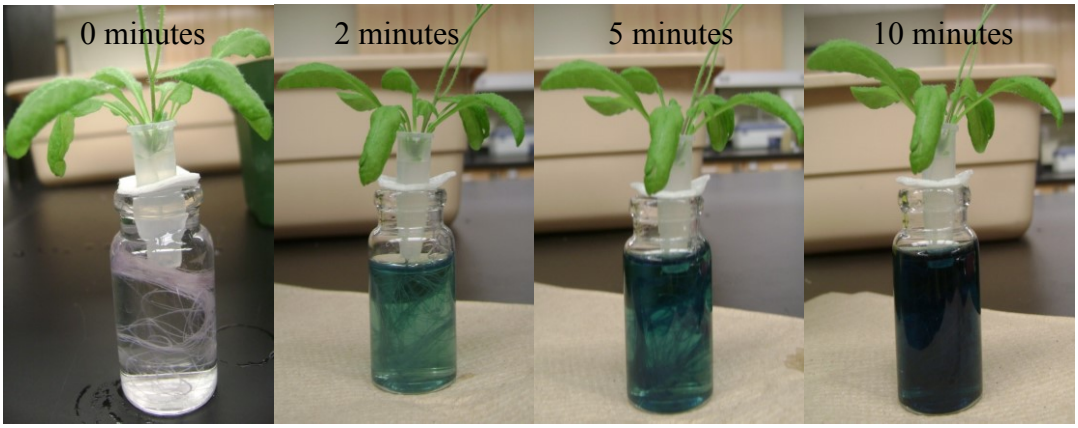


Figure 2.2 Images showing progression of ABTS oxidation activity of *A. thaliana* plants.

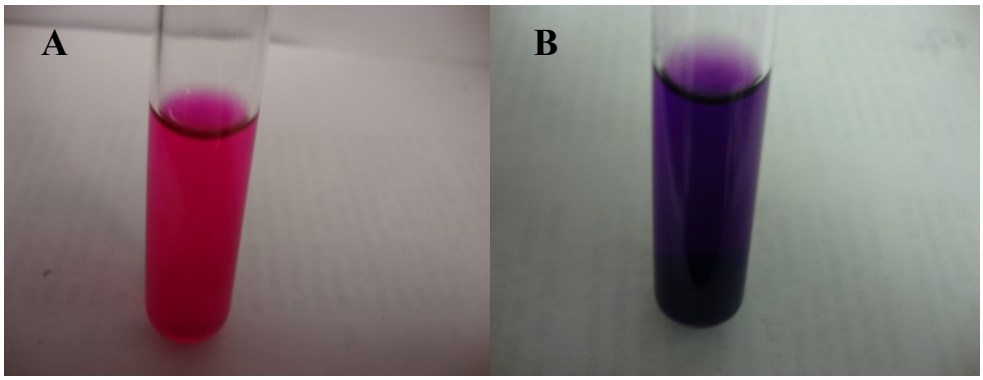
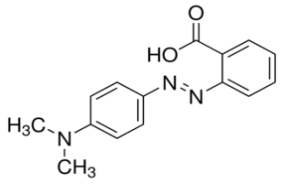
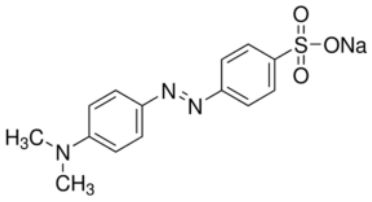
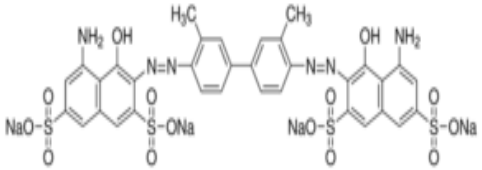
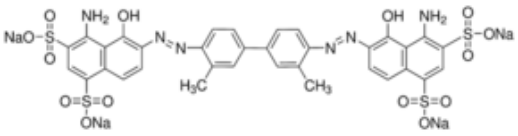
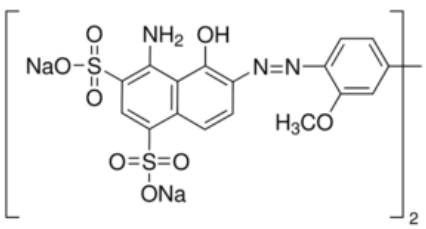
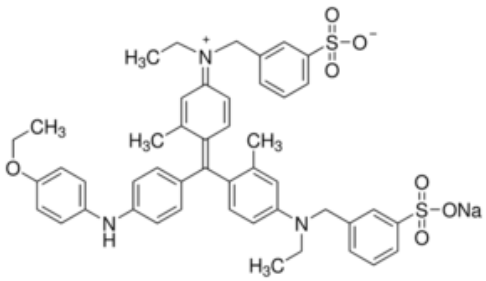
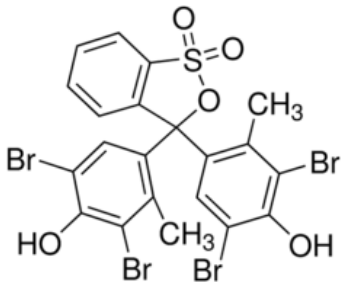
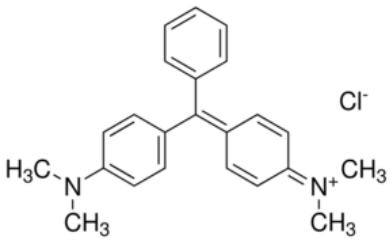
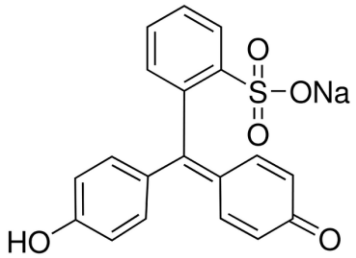
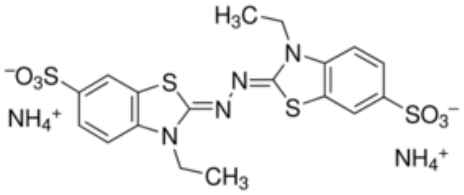
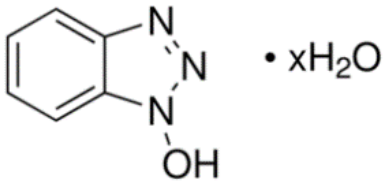


Figure 2.3 Images of Phenol Red with HRP in 0.1 M NaOH in **A.** absence of hydrogen peroxide and **B.** presence of hydrogen peroxide.

Table 2.1 Characteristics of the selected synthetic dyes and mediators. Structures were obtained from Sigma-Aldrich website: http://www.sigmaaldrich.com/catalog

Common name and Alternate	Structure and Formula weight	C.I. numbers and CAS numbers and Molecular weight of free dye
Methyl Red C.I. Acid Red 2	 $C_{15}H_5N_3O_2$	C.I. 13020 CAS 493-52-7 M.W. 269.30
Methyl Orange C.I. Acid Orange 52	 $C_{14}H_{14}N_3NaO_3S$	C.I. 13025 CAS 547-58-0 M.W. 305
Trypan Blue C.I. Direct Blue 14	 $C_{34}H_{24}N_6Na_4O_{14}S_4$	C.I. 23850 CAS 72-57-1 M.W. 872
Evans Blue C.I. Direct Blue 53	 $C_{34}H_{24}N_6Na_4O_{14}S_4$	C.I. 23860 CAS 314-13-6 M.W. 872

Chicago Blue 6B C.I. Direct Blue 1	 $C_{34}H_{24}N_6Na_4O_{16}S_4$	C.I. 24410 CAS 2610-05-1 M.W. 905
Brilliant Blue G Coomassie Brilliant Blue G	 $C_{47}H_{48}N_3NaO_7S_2$	C.I. 42655 CAS 6104-58-1 M.W. 832
Bromocresol Green 3,3',5,5'-Tetrabromo-*m*-cresolsulfonphthalein	 $C_{21}H_{14}Br_{43}O_5S$	No C.I. Number CAS 76-60-8 M.W. 698.01
Malachite Green Basic Green 4	 $C_{23}H_{25}ClN_2$	C.I. 42000 CAS 569-64-2 M.W. 330

Phenol Red Phenolsulfonphthalein	 $C_{19}H_{13}NaO_5S$	No C.I. Number CAS 34487-61-1 M.W. 354.38
2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt ABTS	 $C_{18}H_{24}N_6O_6S_4$	No C.I. Number CAS 30931-67-0 M.W. 548.68
1-hydroxybenzotriazole HOBt	 $C_6H_5N_3O$	No C.I. Number CAS 123333-53-9 M.W. 135.12 (anhydrous)

Chapter 3 - Results

3.1 Plants showed ABTS oxidation in presence of external hydrogen peroxide

The ABTS oxidation rate for *A. thaliana* plants in pH 4.6 phosphate buffer with added 1 mM hydrogen peroxide was found 84,000 $\pm$ 4500 nanomoles per hour per gram fresh weight of roots (Figure 3.1A) while the rate was 73,300 $\pm$ 3400 nanomoles per hour per gram fresh weight of roots (Figure 3.1B) in pH 6.3 phosphate buffer. Similarly for sunflowers under identical conditions the rates at pH 4.6 and pH 6.3 were estimated to be 50,700 $\pm$ 2100 (Figure 3.1A) and 46,600 $\pm$ 1700 nanomoles per hour per gram fresh weight of roots (Figure 3.1B). However, in absence of added hydrogen peroxide the ABTS oxidation rate obtained for *A. thaliana* in pH 4.6 phosphate buffer was 145 $\pm$ 30 nanomoles per hour per gram fresh weight of roots (Figure 3.1A) which was close to the rate determined in pH 6.3 buffer which was 110 $\pm$ 25 nanomoles per hour per gram fresh weight of roots (Figure 3.1B). The rates determined for the sunflowers were relatively higher at 510 $\pm$ 80 nanomoles per hour per gram fresh weight of roots for pH 4.6 phosphate buffer (Figure 3.1A) and 380 $\pm$ 60 nanomoles per hour per gram fresh weight of roots in pH 6.3 phosphate buffer (Figure 3.1B). In absence of added hydrogen peroxide, the rates of ABTS oxidation observed were at least 100 times lower than those containing externally added hydrogen peroxide. The pH of the solution did not affect the plant's ability to oxidize ABTS. Also sunflowers had some ABTS utilization in the absence of hydrogen peroxide. The apparent Km values obtained in pH 6.3 buffer for *A. thaliana* plants was 0.065 mM for hydrogen peroxide while for sunflower plants it was lower at 0.018 mM.

3.2 Batch decolorization of MR by *A. thaliana* and sunflower

Spectra with live plants and dead roots in Figures 3.2 to 3.34 are averages of three replicates. The negative control (without plants) and the positive control with turnip root extract are done in

single replicate. Both *A. thaliana* and sunflower plants were able to decolorize the monoazo dye MR without mediator or external peroxide (Figures 3.2; 3.3; 3.4 and 3.5). For both plants, presence of external hydrogen peroxide and/or mediator did not enhance the % dye decolorization (Figures 3.2; 3.3; 3.4 and 3.5). Even the initial rate of dye decolorization obtained for both the plants was quite comparable (Table 3.1). Also pH of the solution did not affect the ability of the plants to decolorize MR (Figures 3.2; 3.3; 3.4 and 3.5). At pH 4.6 the protonated form of MR predominates (Figures 3.2 and 3.3) while at pH 6.3 where the deprotonated form of the dye predominates (Figures 3.4 and 3.5). The amount of decolorization for MR at pH 4.6 was ~85% (Figures 3.2 and 3.3) while at pH 6.3 it was ~79% in 24 hours (Figures 3.4 and 3.5). This points towards the non-specificity of the plants towards either species of MR independent of their pH. Both plants are able to decolorize MR without the need for any added hydrogen peroxide and mediator.

The positive control MR solutions with the crushed turnip root extract under similar treatments behaved similar to the plants (Figure 3.6B) while the negative control MR dye solutions retained their color and showed negligible decolorization (Figure 3.6A) when treated under similar conditions as those used for the plants.

3.3 Batch decolorization of MO by *A. thaliana* and sunflower

MO is structurally similar to MR with the only difference being the replacement of the carboxylic acid group at the ortho position in MR by a sulphonic acid group at the para position in MO (Table 2.1). So it was expected that the decolorization trend and behavior of both plants towards MO should be similar to MR. However, it was found that MO is more difficult to decolorize than MR unless suitable additions were added along with the dye. MO like MR also behaves as a pH indicator with pKa ~ 3.47. Since the more protonated form (reddish-pink) will

require the dye to be prepared in buffer with pH 3.0 or below which is too acidic to support the growth and development of whole plants, only the orange-yellow color species of MO (more deprotonated form of the dye) in pH 6.3 phosphate buffer was used for the decolorization studies with both plants (Figure 3.7). On exposing both *A. thaliana* and sunflower to MO in pH 6.3 buffer without any additions of hydrogen peroxide or mediator, around 10-15% of the dye was decolorized in 24 hours (Figure 3.7A and Figure 3.8A) with very similar results obtained when the mediator alone was added to the dye (Figure 3.7C and Figure 3.8C). When external hydrogen peroxide was added along with MO both the plants achieved nearly 30% dye decolorization in 24 hours (Figure 3.7B and Figure 3.8B). However, when both hydrogen peroxide and mediator were added together to the dye and then exposed to the plants ~73% of MO was decolorized in 24 hours, nearly five times the decolorization achieved with only external hydrogen peroxide was added (Figure 3.7D and Figure 3.8D). Washes of the roots of plants with ethanol Tris-EDTA extraction solution, after exposure to the various dye treatments did not result in the removal of any dye (Figure 3.9C). The roots which showed yellowish-brown coloration before the extraction continued to appear yellowish-brown in color even after the extraction, while the roots of the plants which were never exposed to MO dye remained white in color when treated with the extraction solution. This indicates that the not all of the bound dye was extractable using the extraction solution. Even the initial rate of decolorization obtained for the different dye treatments with both the plants reflected this trend where the rates of MO decolorization in presence of added hydrogen peroxide and mediator were higher than those obtained for the other treatments including the treatment with just hydrogen peroxide but no mediator (Table 3.1). This was found to be true for both the plants.

Negative controls of the dye without any plants showed negligible decolorization (Figure 3.9A). The decolorization trends obtained for the positive control solution of crushed turnip root extract were comparable to the plants exposed to MO under similar conditions (Figure 3.9B).

3.3.1 Decolorization of MO on repeated addition of fresh dye

The next objective was to determine the effect of repeated addition of MO on the plants and their ability to decolorize the dye in absence of added hydrogen peroxide and mediator for more than 72 hours. A 40 mg/L starting concentration of MO was prepared in half-strength Hoagland's nutrient solution to ensure that the plants are adequately supplied with nutrients and are not stressed during the duration of the experiment. This would not be possible with phosphate buffer and D/W. This will help in understanding the interaction of the salts other than phosphate present in the Hoagland's nutrient solution in the ability of the plants to decolorize MO.

An additional comparison was made in the decolorization pattern of the synthetic dyes carried out by sunflowers when placed under shaker conditions was compared with those placed on the static table in order to understand the effects of diffusion of the dye to the active roots and the total availability of the dye. When placed under shaker conditions, it was observed that for the sunflower plants, the total amount of daily MO dye added during 10 days as replenishment was 38.36 ± 3.1 mg (Figure 3.10A). The roots of the plants developed yellow-brown coloration at the end of the experiment, while the control roots which were never exposed to the dye continued to retain their white appearance. At the end of 10 days the residual dye solution after removing the plants showed yellow-orange in color which was of lower color intensity than the color of the comparable control which was never exposed to any plant. After subjecting the treated roots to the extraction solution, amount of the dye removed from the roots was found to be 10.44 ± 1.3 mg (Figure 3.10B). The final amount of MO dye decolorized by sunflower plants over 10 days

MO was 21.3 ± 3.6 mg. The overall decolorization of MO at the end of 10 days was found to be 55%.

Under static conditions on the bench, the sunflower plants showed trends which were indistinguishable to those placed under shaker conditions (Figure 3.10A). The total amount of daily dye added as replenishment during 10 days was a very similar 32.73 ± 0.40 mg (Figure 3.10A). After subjecting the treated roots to the extraction solution, amount of the dye removed from the roots was found to be 8.92 ± 1.33 mg (Figure 3.10B). The final amount of dye decolorized by sunflower plants in 10 days when exposed constantly to MO was 17.28 ± 1.84 mg with an overall decolorization of around 53%. All of the above values were found to be very similar to those observed for sunflowers under shaker conditions when treated in similar manner. This indicates that there were no differences in the MO decolorization trends observed for sunflowers when placed in shaker conditions or under static conditions (Figure 3.10). The above results also indicate that aeration may not play a contributing role towards the decolorization process.

Similarly, *A. thaliana* plants were capable of decolorizing MO under daily replenishment conditions with addition of fresh MO dye. Since the presence of static or shaker conditions did not affect the ability of sunflowers to bring about MO decolorization, the studies with *A. thaliana* were only conducted under shaker conditions (Figure 3.10A). *A. thaliana* took 7 days to complete the entire MO dye replenishment volume (Figure 3.10A). The total amount of daily dye added as replenishment in 7 days was 3.05 ± 0.37 mg (Figure 3.10A). The roots of the *A. thaliana* plants appeared yellow-brown in color at the end of the experiment similar to sunflower roots when treated in similar manner, while the control roots which were never exposed to the dye continued to retain their white appearance. At the end of 7 days the residual dye solution

after removing the plants appeared yellow-orange in color which was of lower color intensity than the color of the comparable control which was never exposed to any plant. After subjecting the treated roots to the extraction solution, the amount of the dye removed from the roots was found to be 0.32 $\pm$ 0.07 mg (Figure 3.10B). So the final amount of dye decolorized by sunflower plants when exposed constantly 40 mg/L MO in 7 days was 1.43 $\pm$ 0.5 mg with an overall decolorization of around 47% which was found to be close to the value obtained for sunflower. This signifies that both the plants were capable of utilizing MO even after daily addition of fresh dye and constant exposure to MO. Also it appears that decolorization of MO is not a single cycle process and as long as the nutrients are provided to plants they will continue to decolorize freshly added dye (Figure 3.10A).

3.4 Batch decolorization of BBG by *A. thaliana* and sunflower

On placing *A. thaliana* in BBG dissolved in pH 6.3 phosphate buffer without any mediator or external peroxide, the plants were capable of decolorizing ~15% of BBG in 24 hours (Figure 3.11A). A very similar value of ~20% was observed when the mediator, HOBt was added to the dye (Figure 3.11C). However, when external hydrogen peroxide was present along with the dye *A. thaliana* achieved nearly 68% dye decolorization (Figure 3.11B) which was found to be higher than the treatments without added hydrogen peroxide. Similarly in presence of both peroxide and HOBt the plants brought about ~80% decolorization (Figure 3.11D). It was observed that BBG has a tendency to stick and adsorb to the active plant roots. Surprisingly, addition of external hydrogen peroxide with or without added HOBt (Figures 3.11B and 3.11D; Table 3.2) triggered much higher usage of the BBG dye by plant roots resulting in higher dye decolorization values in 24 hours. This was in contrast to the treatments in which *A. thaliana* were only exposed to dye with or without HOBt but not containing external hydrogen peroxide,

where binding of BBG to the roots was more prominently seen (Figures 3.11A and 3.11C; Table 3.2). Also it was observed that before the ethanol Tris- EDTA extractions, the *A. thaliana* roots irrespective of the treatment appeared blue in color but only the dye treated roots exposed to added hydrogen peroxide retained their bluish appearance while the ones without any exposure to hydrogen peroxide lost their blue coloration after the extraction and appeared white in color comparable to the control plant roots which were never exposed to BBG (Figure 3.13C). The initial rates of dye decolorization were also found to reflect the above trend where the plants exposed to BBG with added hydrogen peroxide with or without HOBt were relatively higher by at least 3-4 times than the decolorization rates obtained for the treatments without any added hydrogen peroxide (Table 3.2).

However, the behavior of sunflower plants towards BBG in pH 6.3 phosphate buffer was much different than that seen for *A. thaliana* when subjected to similar treatments. On exposing sunflowers to BBG in pH 6.3 phosphate buffer without any added hydrogen peroxide or HOBt around 15% dye decolorization was observed in 24 hours (Figure 3.12A). Addition of HOBt to the dye treatment resulted in identical 15% decolorization (Figure 3.12C). Both the above values were comparable to the values obtained when *A. thaliana* was subjected to similar dye treatments. However, unlike *A. thaliana*, when external hydrogen peroxide was added to the dye, sunflowers accomplished only 16% dye disappearance (Figure 3.12B) which is surprisingly not much higher than the above mentioned treatments not containing added hydrogen peroxide. Also the dye decolorization did not change much even when external hydrogen peroxide and HOBt were added together where again only 18% of BBG disappeared in 24 hours which was not much higher than the values obtained for the other three treatments (Figure 3.12D). Like *A. thaliana* roots treated with BBG the sunflower roots at the end of the experiment appeared dark blue in

color. However, unlike *A. thaliana* all of the roots irrespective of their pretreatment lost their blue coloration after washing with the extraction solution of ethanol Tris-EDTA (Figure 3.13C). The rate of dye decolorization reflected the above trend for sunflower plants where exposing them to BBG results in binding as well as adsorbing of the dye to the sunflower roots rather than being utilized by the plant (Table 3.2). The presence of external hydrogen peroxide does not alter that mechanism (Table 3.2). This means that in the case of sunflower plants, BBG was mostly bound to the active plant roots rather than getting utilized by the plants.

The trends and the decolorization pattern shown by the positive control involving exposing the crushed turnip root extract to BBG under similar treatments as those used for the plants were quite comparable those seen for *A. thaliana* plants but not sunflower plants (Figure 3.13B). The negative control dye solution which were never exposed to any plants under similar conditions were stable and did not get decolorized during the reaction time (Figure 3.13A). The above results indicate that the plant roots are able to decolorize only a relatively small fraction of the BBG in absence of external hydrogen peroxide and that *A. thaliana* plants were relatively better than sunflowers in terms of decolorizing BBG in presence of peroxide. The BBG dye mostly bound to sunflower roots and was completely extractable using the extraction solution containing ethanol Tris-EDTA.

3.4.1 Decolorization of BBG on repeated addition of fresh dye

As conducted for MO the goal of this study was to determine the effect of repeated addition of BBG on the plants and their interactions with the dye in absence of added hydrogen peroxide and mediator for more than 48 hours so as to compare and contrast the behavior of plants towards representative monoazo dyes and arylmethane dyes. BBG of 40 mg/L starting concentrations was prepared in half-strength Hoagland's nutrient solution since this ensures that the plants are

adequately supplied with nutrients and are not stressed over the experimental time which would not be possible if the dye were prepared in phosphate buffer and D/W. Also this will help in understanding the effect of the salts in the nutrient solution in the ability of the plants to decolorize BBG.

On subjecting *A. thaliana* to 40 mg/L BBG under shaker conditions the plants were capable of completing their entire dye replenishment volume in 10 days (Figure 3.14A). The total amount of dye added in 10 days as replenishment was 3.57 $\pm$ 0.06 mg (Figure 3.14A). The roots of the plants which appeared nearly white before the start of the experiment showed dark blue color intensity at the end of the experiment, while the control roots that were never exposed to the dye retained their white appearance. The residual dye solution after the plants were removed after 10 days was nearly colorless signifying that large fractions of the dye was no longer present in the solution. After subjecting the dye treated roots to the extraction solution of ethanol Tris-EDTA the amount of bound dye removed from the roots was found to be 1.94 $\pm$ 0.37 mg (Figure 3.14B). The extracted solution had a bluish appearance but was of lighter intensity than the comparable control of BBG in the extraction solution. The final amount of 40 mg/L Brilliant Blue G dye utilized by *A. thaliana* plants was 1.59 $\pm$ 0.31 mg. In 10 days, this represents ~45% of the freshly added 40 mg/L BBG dye being removed by *A. thaliana* plants which surprisingly was observed to be very close to the values obtained for *A. thaliana* plants when exposed to the monoazo dye MO under similar conditions.

The above experiment was similarly repeated with sunflower plants, despite the low decolorization and high binding of BBG to sunflower roots since it was thought that BBG in presence of the salts in the nutrient solution might alter the interaction of the active roots of the plants with the BBG dye. On exposing sunflower to 40 mg/L BBG under shaker conditions the

plants were capable of completing their entire dye replenishment cycle in a relatively shorter 6 days (Figure 3.14A). The sum total amount of dye added in 6 days as replenishment was 9.2 $\pm$ 0.68 mg (Figure 3.14A). As seen with *A. thaliana* roots exposed to BBG, the roots of the sunflower plants showed dark blue color intensity at the end of the experiment while the control roots which were never exposed to the dye retained their white appearance. The residual dye solution after the plants were removed was found to be light blue of low color intensity, much darker than those observed for *A. thaliana* plants. After subjecting the dye treated roots to the extraction solution of ethanol Tris-EDTA the amount of bound dye removed from the roots was found to be 5.78 $\pm$ 0.23 mg (Figure 3.14B). The extracted solution had bluish appearance comparable to the control of BBG in the extraction solution which again was different from the trend seen for *A. thaliana* plants. The final amount of 40 mg/L Brilliant Blue G dye utilized by *A. thaliana* plants was 3.15 $\pm$ 0.45 mg. In 6 days, this represents ~34% of the freshly added 40 mg/L BBG dye being removed by sunflower plants which was observed to be not only lower than the values obtained for *A. thaliana* plants exposed to BBG under similar conditions but also lower than the values obtained for sunflowers when exposed to monoazo dye MO under similar conditions.

Though both plants are capable of removing BBG on repeated addition the decolorization of dye was found to be higher in *A. thaliana* plants than in sunflowers when treated in similar manners. Binding of BBG to sunflower roots was more commonly seen (Figure 3.14B). Also as observed for MO, the plants continued to decolorize BBG after the initial exposure to the dye (Figures 3.10A and 3.14A). Thus, the decolorization of BBG using plants in absence of added hydrogen peroxide and mediator is not a one cycle process and as long as the nutrients are provided to plants they will continue to decolorize freshly added dye if the concentrations are not high

enough to be toxic or detrimental to the plants (Figure 3.14A). The plants appeared healthy and continued to grow without any signs of stress, comparable to the control plants through the experiment. The negative control solutions of the dye which were never exposed to plants but were subjected to in similar conditions showed negligible decolorization.

3.5 Batch decolorization of BG by *A. thaliana* and sunflower

BG like MR and MO behaves as a pH indicator. When *A. thaliana* was placed in BG dissolved in pH 4.6 phosphate buffer without any mediator or external hydrogen peroxide the decolorization value calculated was around 25% in 24 hours (Figure 3.15A). A very similar value of 26% was observed when the plants were exposed to the dye with added HOBt (Figure 3.15C). When external hydrogen peroxide was added along with the dye *A. thaliana* achieved 64% dye decolorization (Figure 3.15B) which was found to be not much different from the values obtained when of both external hydrogen peroxide and HOBt were added to the dye and exposed to the plant (Figure 3.15D). Under such condition *A. thaliana* could bring about 65% decolorization of BG (Figure 3.15D). Thus addition of external hydrogen peroxide to the dye results in nearly 2-3 times higher dye decolorization over the dye treatments with plants which were never exposed to the added hydrogen peroxide. When *A. thaliana* plants were placed in BG dissolved in pH 6.3 phosphate buffer without any added HOBt or hydrogen peroxide, the plants achieved nearly 15% decolorization in 24 hours (Figure 3.17A). A value of 10% was observed when HOBt was added to the dye (Figure 3.17C). When external hydrogen peroxide was added along with the dye *A. thaliana* achieved 28% dye decolorization (Figure 3.17B) while it was 45% in presence of both added hydrogen peroxide and HOBt (Figure 3.17D). Thus as observed for BG at lower pH the decolorization values obtained for BG at relatively higher pH in presence of external peroxide was found to be nearly 2-3 times higher than the treatments in which

external hydrogen peroxide was not added. Washing of the roots of the plants which were exposed to the various dye treatments with the ethanol Tris-EDTA extraction solution did not result in the removal of the dye (Figure 3.19C). The roots which showed greenish-yellow coloration before the extraction appeared yellowish-brown in color after the extraction, while the roots of the plants which were never exposed to BG dye remained white in color when treated with the extraction solution. This indicates that the bound dye was not extractable. The initial rate of decolorization obtained for BG at both pH values for *A. thaliana* plant treatments were comparable and similar to the above mentioned trends where the rates in presence of added hydrogen peroxide and/or mediator were relatively higher than those obtained for the other treatments (Table 3.2).

Placing sunflower plants in pH 4.6 BG without any added mediator and external hydrogen peroxide resulted in around 16% of BG getting decolorized in 24 hours (Figure 3.16A). A very similar value of 17% was observed when HOBt was added to the dye (Figure 3.16C). When external hydrogen peroxide was added along with the dye sunflowers accomplished 64% dye decolorization (Figure 3.16B) while in presence of added hydrogen peroxide and HOBt, the BG decolorization achieved was 68% (Figure 3.16D). These dye treatments involving added hydrogen peroxide for the sunflowers were surprisingly similar to those obtained from *A. thaliana* when placed under similar dye treatments. On exposing sunflower plants to BG dissolved in pH 6.3 phosphate buffer in the absence of HOBt or external hydrogen peroxide, in 24 hours the plants were capable of achieving around 13% decolorization (Figure 3.18A). However, plants were only capable of decolorizing ~9% of BG when HOBt was also added to the dye (Figure 3.18C). When external hydrogen peroxide was added along with the dye around 27% dye decolorization (Figure 3.18B) was obtained which became 35% in presence of added

hydrogen peroxide plus HOBt (Figure 3.18D). This was ~3 times higher than the dye treatments with plants in which external hydrogen peroxide was not added. These trends were quite similar to those obtained for *A. thaliana* plants when treated in similar manner (Figures 3.16 and 3.18). The initial rates of BG decolorization obtained for sunflowers at either pH were in agreement with the above noticed trends (Table 3.2). The sunflower roots appeared yellow-green in color before washing the roots with the extraction solution, compared to the white colored control roots which were never exposed to BG. The roots hardly lost their coloration after the extraction and the extracted solution had no coloration indicating that the decolorized dye was not extractable (Figure 3.19C).

Negative controls of BG dye which were not exposed to plants under similar conditions showed negligible decolorization (Figure 3.19A). The decolorization trends obtained for the positive control solution of crushed turnip root extract with BG were similar to the plants under similar conditions (Figure 3.19B).

Thus, pH did not influence the decolorization or the rate of decolorization of BG with both *A. thaliana* and sunflower plants. The presence of added hydrogen peroxide worked the best in achieving rapid BG decolorization.

3.6 Batch decolorization of MG by *A. thaliana* and sunflower

On treating *A. thaliana* plants with 40 mg/L Malachite Green dissolved in pH 6.3 phosphate buffer, in the absence of added HOBt and external hydrogen peroxide, ~40% of the dye was decolorized in 24 hours (Figure 3.20A). When HOBt was added along with the dye the value obtained was ~25% (Figure 3.20C). When external hydrogen peroxide was added to MG *A. thaliana* plants achieved ~65% dye decolorization (Figure 3.20B) while in presence of both added hydrogen peroxide and HOBt, the value obtained was ~83% (Figure 3.20D). These values

with added hydrogen peroxide, as observed with MO and BG, were found to be nearly 3 times higher than those obtained for dye treatments in which plants were not exposed to any added hydrogen peroxide. The active plant roots appeared green in color irrespective of their pretreatment before being washed with the extraction solution of ethanol Tris-EDTA. The treated roots exposed to added hydrogen peroxide appeared brownish-black in color after the extraction while the roots without any exposure to added hydrogen peroxide lost nearly all of their greenish coloration and appeared nearly white-brown, similar in appearance to the control roots which were never exposed to the dye (Figure 3.22D). The initial rate of dye decolorization obtained for the *A. thaliana* plants under comparable dye treatments were higher in the presence of externally added hydrogen peroxide than the dye treatments in which the plants were never exposed to the added hydrogen peroxide (Table 3.2).

On exposing sunflowers to MG dissolved in pH 6.3 phosphate buffer without any added mediator or added hydrogen peroxide, nearly 57% of the dye was decolorized in 24 hours (Figure 3.21A). Addition of HOBt to the treatment resulted in a very similar value of 58% (Figure 3.21C). When external hydrogen peroxide was supplied to the MG sunflowers accomplished 78% (Figure 3.21B) while the value was 89% when external hydrogen peroxide plus HOBt were given together to the dye solution with the plants (Figure 3.21D). Here again it was observed that adding external hydrogen peroxide improved the amount of dye decolorized by the plants. The dye treated sunflower roots before washing with the extraction solution appeared dark green. On washing the dye treated roots with the extraction solution it was seen that the green color of the roots was not extractable, indicating that the decolorization process was not completely reversible and that the dye did not merely bind to or adsorb on the roots. Also there was no coloration seen in the extraction solution (Figure 3.22D). Again the initial rate

of dye decolorization showed that addition of external hydrogen peroxide improved the decolorization of MG (Table 3.2).

The negative control dye samples without any plants under similar treatments were quite stable and did not get decolorized during the reaction time (Figure 3.22A). The control dye solutions in pH 6.3 phosphate buffer appeared dark blue-green in color. The positive control solution in which the crushed turnip root extract was exposed to MG under similar conditions showed trends of decolorization which were quite comparable to the plants exposed to MG (Figure 3.22C). Sunflowers were found to be better in MG decolorization than *A. thaliana* under similar treatments. Addition of external hydrogen peroxide with or without mediator improved that dye decolorization abilities of both plants over the dye decolorization treatments not involving added hydrogen peroxide.

3.6.1 Bleaching of MG in absence of plants

The control dye samples without any live plants under similar conditions were stable and did not undergo any decolorization on their own during the reaction time only when external hydrogen peroxide was not added to the dye. The various control dye samples in pH 6.3 phosphate buffer appeared dark blue-green in color. While the control dye solutions which either had only HOBt added to it or had no external additions continued to appear dark blue-green in coloration, the control solutions which had external hydrogen peroxide added to it with/without HOBt started to lose their dark blue-green coloration over the reaction time (Figure 3.22B). At the end of 72 hours these control solutions containing external peroxide appeared very pale shade of green, almost colorless as if they underwent ‘auto’ bleaching (Figure 3.22B) while the similarly plant treated dye solutions appeared pale green much more rapidly and in shorter time (Figures 3.20B and 3.21B). Comparing the UV-Visible spectra of the control dye solutions containing external

peroxide with/without HOBt to the plants treated under similar conditions, it was observed that over time while the major peak in the region of 600-620 nm representing MG started decreasing in case of all the treatments, a new peak in the region 350-370 nm began to rise (Figures 3.20B; 3.21B and 3.22B). This was observed only in for the control solutions, while the plant treated dye solution showed no development of new peak (Figures 3.20B; 3.21B and 3.22B). Occurrence of this new peak was not seen in case of the control MG dye solutions which contained no external hydrogen peroxide (Figures 3.20A; 3.21A and 3.22B). This was surprising since the 'green' form of MG happens to be the more oxidized form of the dye, with leucomalachite green (LMG) being the more reduced form (Alderman, 1985). This points towards interaction of the external hydrogen peroxide with MG and triggering its decolorization even in the absence of the plant. However, this possibly represents only inter-conversion between the different dye forms while there was an actual decolorization of dye on exposure to plants due to no new peak development (Figures 3.20B; 3.21B and 3.22B).

3.7 Batch decolorization of disazo dyes by *A. thaliana* and sunflower

Since the three disazo dyes are closely related to each other in their structures the hypothesis was that the pattern of the dye decolorization on exposure to both plants will be very comparable to each other under similar conditions.

3.7.1 Decolorization of TB

On placing *A. thaliana* in TB dissolved in pH 6.3 phosphate buffer without any additions, ~17% of the dye was decolorized in 24 hours (Figure 3.23A). This was also observed when HOBt was added along with the dye where again the value was nearly 17% (Figure 3.23C). However, when external hydrogen peroxide was added to the dye, *A. thaliana* achieved ~30% TB dye decolorization which was nearly double than the treatments without added hydrogen peroxide

(Figure 3.23B). The decolorization amount was found to be more enhanced in presence of added hydrogen peroxide plus HOBt where ~60% of TB was decolorized (Figure 3.23D). Thus, in presence of external hydrogen peroxide and added mediator the decolorization carried out by *A. thaliana* plants was found to be nearly two times the value obtained for the dye treatments without added hydrogen peroxide and/or mediator while also being double the value observed for the plants exposed to TB in presence of just added hydrogen peroxide. Even the initial rates of TB decolorization reflected these trends where the presence of external hydrogen peroxide and HOBt showed relatively higher decolorization than the ones without added hydrogen peroxide including the treatments involving only added hydrogen peroxide (Table 3.3). The dye solutions exposed to plant with or without HOBt appeared blue but with less intensity than their respective negative controls. Addition of hydrogen peroxide to TB with plants resulted in reduced intensity of blue color. However, the most prominent color change was when external hydrogen peroxide and HOBt were present together which resulted in maroon-purple coloration of the solution while the negative control solution of the same treatment remained blue comparable in intensities with the other TB negative control solutions. This was found to be true only for *A. thaliana* plants exposed to added hydrogen peroxide and HOBt. The active roots of the plants exposed to TB before washing them with the extraction solution containing ethanol Tris-EDTA appeared blue in color except for the plants exposed to TB with added hydrogen peroxide plus HOBt where the roots appeared purple. This was in contrast to the roots of control plants, never exposed to TB which remained white-brown. This coloration of the roots was retained even after washing with the extraction solution, with very low color being seen in the extraction solution (Figure 3.25C).

On exposing sunflowers to TB dissolved in pH 6.3 phosphate buffer without any mediator or added hydrogen peroxide around 24% of the dye was decolorized in 24 hours (Figure 3.24A). The treatments involving addition of HOBt alone to the dye or external hydrogen peroxide alone to the dye achieved a similar ~24% dye decolorization (Figures 3.24B and 3.24C). This was different than with *A. thaliana* placed under similar treatments, where addition of hydrogen peroxide seemed to slightly increase TB dye decolorization. Also surprising was that when hydrogen peroxide and HOBt were present together ~35% of TB was decolorized in 24 hours, which was comparable to the decolorization values observed for sunflower plants exposed to the other TB treatments (Figure 3.24D). The initial rate of TB decolorization also showed similar trends where presence of added hydrogen peroxide and HOBt did not seem to enhance the TB decolorization by sunflower plants over the other treatments of TB with plants (Table 3.3). The plant treated dye solutions as well as the roots of the plants irrespective of the presence of external hydrogen peroxide and/or HOBt, appeared blue with lower intensity than the comparable negative control solution which were never exposed to plants. Also there was not much difference observed in the color intensity of the roots after subjecting them to extraction solution of ethanol Tris-EDTA between the different dye treatments. This was in complete contrast to the results observed when *A. thaliana* was exposed to the TB dye under similar conditions (Figure 3.25C). This points towards the ability of *A. thaliana* plants to decolorize higher amounts of TB dye in presence of added hydrogen peroxide and HOBt as compared to sunflowers under similar conditions (Figures 3.23 and 3.24).

The negative control dye samples without any exposure to live plants when placed under similar treatments were quite stable and did not undergo decolorization due to absence of any ions or salts in the buffer (Figure 3.25A). The positive control solution involving the crushed turnip root

extract with TB showed decolorization trends that were very similar to the *A. thaliana* plants when placed under similar conditions (Figure 3.25B).

3.7.2 Decolorization of CB6B

On subjecting *A. thaliana* to CB6B dissolved in pH 6.3 phosphate buffer without any additions of hydrogen peroxide and HOBt nearly 17% of the dye was decolorized in 24 hours (Figure 3.26A). When HOBt was added along with the dye the CB6B decolorization value was found to be nearly 27% (Figure 3.26C). When external hydrogen peroxide was added to the dye *A. thaliana* achieved ~35% CB6B dye decolorization which was found to be nearly double the value obtained for plants exposed to dye treatments without any additions (Figure 3.26B). The decolorization of CB6B jumped to ~90% in presence of added hydrogen peroxide plus HOBt which happens to be nearly five times the value obtained for the dye treatments without added hydrogen peroxide and/or mediator and nearly three times the value observed for the plants exposed to the dye in presence of added hydrogen peroxide (Figure 3.26D). The above trends were also seen in the initial rates of CB6B decolorization where the presence of external hydrogen peroxide and HOBt showed comparatively higher decolorization than the ones without added hydrogen peroxide including the treatments involving only added hydrogen peroxide (Table 3.3). The dye solutions exposed to plant with or without added HOBt appeared blue but with lower intensity than their comparable negative controls. Addition of hydrogen peroxide to CB6B resulted in reduced intensity of blue color. However, as seen for TB the most prominent color change was when external hydrogen peroxide plus HOBt were present together which resulted in maroon-pink coloration of the solution. This was in contrast to the other CB6B treatments. The, negative control solution of the same treatment remained blue comparable in intensities with the other CB6B negative control solutions. This decolorization pattern was found

to be true only for *A. thaliana* plants exposed to the dye solution with added hydrogen peroxide and HOBt, as observed for TB treatments under similar conditions.

On exposing sunflowers in pH 6.3 CB6B without any mediator or added hydrogen peroxide ~21% of the dye was decolorized in 24 hours (Figure 3.27A) which was very similar to the value obtained on placing plants in the dye in presence of externally added hydrogen peroxide (Figure 3.27B). This was observed only for sunflowers. On addition of HOBt to the dye the plants achieved a slightly higher value of ~36% dye decolorization (Figure 3.27C) which happens to be different than that observed with *A. thaliana* under similar treatments, where addition of hydrogen peroxide seemed to slightly enhance CB6B dye decolorization (Figure 3.26B) . However, when hydrogen peroxide plus HOBt were added together, ~43% of CB6B was decolorized in 24 hours (Figure 3.27D) which was different from the *A. thaliana* plants under similar conditions (Figure 3.26D). The initial rates of CB6B decolorization also showed trends which were similar to the above observations where addition of external hydrogen peroxide and HOBt did not seem to enhance the CB6B decolorization in presence of sunflower plants over the other treatments of CB6B (Table 3.3). The plant treated dye solutions as well as the roots of the plants irrespective of the presence of external hydrogen peroxide and/or HOBt appeared blue with lower intensity than the comparable negative control solution which were never exposed to plants. However, the decolorization values achieved by both plants for CB6B dye were comparatively better than those obtained for TB under similar conditions. The negative control dye solutions without any exposure to live plants when placed under similar treatments were quite stable and did not undergo decolorization due to absence of any ions or salts in the buffer (Figure 3.28A). The positive control solution involving the crushed turnip root extract with

CB6B showed decolorization trends that were very similar to the *A. thaliana* plants when placed under similar conditions (Figure 3.28B).

3.7.3 Decolorization of EB

The decolorization trends observed for the plants exposed to EB were comparable and similar to those observed for CB6B under similar treatment conditions. Minor differences are observed in their structures where methoxy groups in CB6B are replaced by methyl group in EB (Table 2.1). On exposing *A. thaliana* to EB dissolved in pH 6.3 phosphate buffer without any additions of hydrogen peroxide and HOBt ~12% of the dye was decolorized in 24 hours (Figure 3.29A). When HOBt was added along with the dye the plants brought about ~16% dye decolorization value (Figure 3.29C). This was comparable to TB (Figure 3.23C) and CB6B (Figure 3.26C) decolorization under similar treatments with *A. thaliana*. When external hydrogen peroxide was added to the dye the plants achieved ~35% EB decolorization (Figure 3.29B) which was nearly double the value obtained for plants exposed to dye treatments without any additions and comparable to the value obtained when the plants were exposed to TB and CB6B under similar conditions (Table 3.3). As observed for both TB (Figure 3.23D) and CB6B (Figure 3.26D) the decolorization of EB was also enhanced to ~85% in presence of added hydrogen peroxide plus HOBt (Figure 3.29D). This is similar to CB6B decolorization achieved by *A. thaliana* under similar dye treatments (Figures 3.26 and 3.29). The initial rates of EB decolorization were quite similar to those estimated for TB and CB6B in the presence of external hydrogen peroxide and/or HOBt with *A. thaliana* plants (Table 3.3). As seen for TB and CB6B the EB solutions exposed to plant with or without added HOBt appeared blue but with lower intensity than their comparable negative controls. Addition of hydrogen peroxide to EB with plants resulted in reduced intensity of blue color as seen with CB6B. The most prominent color change was when

external hydrogen peroxide and HOBt were present together which resulted in maroon-pink coloration of the solution which was different to the other EB treatments. The negative control solution of the same treatment remained blue comparable in intensities with the other EB negative control solutions. As observed for the other disazo dyes this decolorization pattern was found to be true only for *A. thaliana* plants exposed to the dye solution with added hydrogen peroxide plus HOBt.

As seen for CB6B, the roots of the plants exposed to EB before washing them with the extraction solution containing ethanol Tris-EDTA appeared blue in color except for the treatments in which the plants exposed to EB with added hydrogen peroxide plus HOBt where the roots appeared purple-pink which was different from the nearly white colored roots of control plants which were never exposed to CB6B. This coloration of the roots was retained even after washing with the extraction solution with very low color being seen in the extraction solution indicating that the decolorized dye was not completely extractable, comparable to the trend seen for CB6B (Figure 3.31C).

Placing sunflowers in pH 6.3 EB without any mediator or added hydrogen peroxide resulted in ~15% of the dye being decolorized in 24 hours (Figure 3.30A) while on addition of HOBt to the dye the plants achieved very similar dye decolorization value of ~10% dye (Figure 3.30C). This trend was comparable to the trends observed for TB (Figure 3.24A and 3.24C) and CB6B under similar dye treatments (Figures 3.27A and 3.27C) with sunflowers. Adding external hydrogen peroxide to EB and exposing the dye to sunflower plants resulted in the dye decolorization of ~28% (Figure 3.30B). Adding hydrogen peroxide plus HOBt resulted ~45% EB decolorization in 24 hours (Figure 3.30D), comparable to the decolorization of the other two disazo dyes when exposed to sunflower plants under similar conditions (Figures 3.24D and 3.27D). The initial rates

of EB decolorization with sunflower also showed trends which were similar to the above decolorization pattern, where addition of external hydrogen peroxide and HOBt did not seem to enhance the decolorization of EB in the presence of sunflower plants over the other dye treatments involving interaction of EB with sunflower plants (Table 3.3). The plant treated dye solutions as well as the roots of the plants irrespective of the presence of external hydrogen peroxide and/or HOBt, appeared blue with lower intensity than the comparable negative control solution which were never exposed to plants. Also there was not much difference observed in the color intensity of the roots after subjecting them to extraction solution of ethanol Tris-EDTA between the different dye treatments (Figure 3.31C). This was different to the results observed when *A. thaliana* was exposed to the EB dye under similar conditions but was comparable to the decolorization pattern of TB and CB6B when exposed to sunflower plants under similar treatment conditions. However, the decolorization values obtained for both plants under the different dye treatments with EB (Figures 3.29 and 3.30) were comparable to CB6B (Figures 3.26 and 3.27) and were relatively better than those obtained for TB (Figures 3.23 and 3.24) under similar conditions.

The negative control dye samples without any exposure to live plants, when placed under similar treatments were quite stable and did not undergo decolorization due to absence of any ions or salts in the buffer (Figure 3.31A). The positive control solution involving the crushed turnip root extract with EB showed decolorization trends that were very similar to the *A. thaliana* plants when placed under similar conditions (Figure 3.31B) as well as being comparable to CB6B decolorization (Figure 3.28B).

The above observations points towards the preference of *A. thaliana* plants to decolorize EB, CB6B and TB when external hydrogen peroxide plus HOBt were added together with the dyes.

Sunflowers, on the other hand, did not show the same dye decolorization trend when exposed to similar dye treatments and conditions.

3.7.3.1 Decolorization of EB in half-strength Hoagland's solution

The decolorization trends obtained for EB as mentioned in section 3.7.3 was conducted in pH 6.3 phosphate buffer and it was still unclear if the decolorization trends would be replicable if EB was prepared in Hoagland's nutrient solution which have been shown to contribute to its tight binding to roots, in presence of calcium (Davis, unpublished). Very similar results were generated when both *A. thaliana* as well as sunflower were exposed to EB dissolved in half-strength Hoagland's nutrient solution (Data not shown). It should be noted that though the negative control samples of EB in nutrient solution containing externally added hydrogen peroxide and/or HOBt resulted in EB getting decolorized, the decolorization process was quicker with the dye exposed to the plants as compared to the comparable negative control solution. The dye in nutrient solution also appeared less intense in its blue color when compared with the intensity of the dye dissolved in pH 6.3 phosphate buffer.

3.8 Binding of dyes to dried roots and cellulose

The objective of this study was to understand the interaction of the salts on dye adsorption and absorption by dried roots. Hence, the comparison was carried out for each representative dye from the monoazo (MO), arylmethane (BBG) and disazo (EB) class of dyes for their ability to adsorb and/or absorb on dried roots in presence of salts (Hoagland's nutrient solution) and relatively less salts (pH 6.3 phosphate buffer).

The starting concentration of MO when prepared either in pH 6.3 phosphate buffer or in half-strength Hoagland's solution was around 91 μM (Figures 3.32A and 3.32C). After the dried sunflower roots were placed in MO prepared in either pH 6.3 or in half-strength Hoagland's

solution for 48 hours, visible differences in the dye binding to the roots were observed (Figures 3.32A and 3.32C). The roots appeared yellowish-brown in color while the dye solution appeared light orange in color (Figure 3.32A and 3.32C). After 48 hours, the amount of dye in pH 6.3 phosphate buffer stuck to the roots in the absence of external hydrogen peroxide and HOBt was ~39 μM while it was ~32 μM with HOBt but no added peroxide. In presence of both added hydrogen peroxide and HOBt ~10 μM of the dye stuck to the roots while ~40 μM of the dye was stuck to the roots exposed only to added hydrogen peroxide (Figure 3.32A). The amount of MO stuck to roots when prepared in half-strength Hoagland's solution was ~59 μM in the absence of both hydrogen peroxide and HOBt, ~16 μM in the presence of HOBt but no added peroxide, ~28 μM in the presence of externally added hydrogen peroxide and ~18 μM in the presence of both added hydrogen peroxide and HOBt (Figure 3.32C). Addition of hydrogen peroxide with or without HOBt did not alter the binding of the dye to the roots since even after 48 hours the peak at 460 nm was observed for the different treatments, indicating the absence of dye decolorization (Figures 3.32A and 3.32C). On washing the dye treated roots with the extraction solution of ethanol Tris-EDTA, nearly all of the dye bound to the dried roots came out in the extraction solution (Figures 3.32B and 3.32D). This was observed for all the roots exposed to the dye and presence of excess salt did not affect the extraction levels (Figures 3.32B and 3.32D). The ethanol Tris-EDTA extracts appeared yellowish-orange in color while the roots after the extraction appeared light brown in color similar in appearance to the unstained roots. The negative control solutions without any roots under the different treatments remained stable and were negligibly decolorized.

Similar results were obtained for treatments involving interactions of BBG with the dried sunflower roots (Figure 3.33). However, interactions of EB (Figure 3.34) with the dried roots in

the treatments involving external hydrogen peroxide were different from MO (Figure 3.32) and BBG (Figure 3.33). The starting concentration of Evans Blue when prepared in pH 6.3 phosphate buffer as well as in half-strength Hoagland's solution was ~36 μM (Figures 3.34A and 3.34C). When the roots were placed in EB prepared in pH 6.3 or in half-strength Hoagland's solution under the different treatments, a large proportion of the EB dye was found to be bound to the roots (Figures 3.34A and 3.34C). The roots treated with the dye appeared dark blue in color. However, the dye solutions showed variability in their colorations. The EB solutions in pH 6.3 buffer which were exposed to added hydrogen peroxide with or without HOBt appeared dark blue in color than those observed for the treatments without the added hydrogen peroxide which were nearly colorless (Figure 3.34A). The dye from the above treated was found to be completely extractable and recoverable (Figure 3.34B). The extracted solution appeared dark blue while the roots after the extraction appeared light brown in color comparable to the control roots not exposed to EB (Figure 3.34B). However, the trends observed for interactions of EB prepared in pH 6.3 phosphate buffer with the dried sunflower roots (Figures 3.34A and 3.34B) were quite different from the interactions of the dried sunflower roots with EB prepared in half-strength Hoagland's nutrient solution (Figures 3.34C and 3.34D). After 48 hours, the color of the EB solutions prepared in half-strength Hoagland's nutrient solution with added hydrogen peroxide appeared light purple-pink in color while the dye treatments without any added hydrogen peroxide appeared light blue in color despite the roots in all the treatments appearing dark blue in color (Figure 3.34C). On washing the treated roots with the extraction solution, the roots which were not exposed to the added hydrogen peroxide with or without HOBt, lost their color and the dye was found to be completely extractable (Figure 3.34D). However, there was tight binding of EB to the roots which were exposed to external hydrogen peroxide with or

without added HOBt and hardly any of the color was extractable from the roots (Figure 3.34D). All the above observations indicate towards some interaction of the salts from Hoagland's solution with the added hydrogen peroxide affecting the binding of EB to the roots (Figures 3.34C and 3.34D). Calcium ions are known to enhance the binding of EB to cellulose which is a major component of plant roots. The recovery of EB dye from the roots was extremely poor for the roots exposed to EB in presence of added hydrogen peroxide with or without HOBt, even after extraction with ethanol Tris-EDTA (Figures 3.34C and 3.34D). In the presence of some Hoagland's salts some dyes such as EB were found to be tightly and irreversibly bound to the roots and were not easily extractable after interacting with added hydrogen peroxide (Figures 3.34C and 3.34D).

The experiment was repeated to check for binding of EB with pure cellulose in the form of Whatman Chromatography Paper # 1 (Figure 3.35). The results showed that in the absence of salts, binding of EB to cellulose was reversible and the dye was recovered with the extraction solution, even after exposure to hydrogen peroxide (Figures 3.35A and 3.35B). However, in the presence of salts and added hydrogen peroxide with or without HOBt, the binding of EB to cellulose was tight and was not completely recovered using the extraction solution of ethanol Tris-EDTA (Figures 3.35C and 3.35D). These results were obtained without replication.

3.9 Studies involving Phenol Red

3.9.1 Estimation of externally added hydrogen peroxide

Phenol Red exposed to hydrogen peroxide and crushed horseradish root extract shows a spectral difference compared to the same solution without any added hydrogen peroxide. This appears as a distinct shoulder in the 590-620 nm region, tapering out beyond 640 nm (Figure 3.36). The difference is maximal near 610 nm, as suggested by Pick *et al.* (1980). See Figure 3.36. Plotting

absorbance observed at 610 nm against increasing concentration of hydrogen peroxide revealed a linear relationship obeying Beer-Lamberts Law (Figure 3.37A). The absorbance value at 610 nm represented the amount of product formed due to oxidation of the dye by hydrogen peroxide and was in direct proportion to the concentration of hydrogen peroxide. This trend was true only for hydrogen peroxide concentrations of 20 μM, 40 μM and 60 μM. At concentrations of 100 μM the linear relationship was no longer followed with the line starting to plateau (Figure 3.37A). This was found to be one of the drawbacks of the method where accurately estimating higher concentrations of hydrogen peroxide based on absorbance at 610 nm was no longer reliable (Figures 3.37A). Interestingly, this linear relationship held true over time till 72 hours since it was observed that the absorbance readings at each time-intervals for the same set of peroxide concentrations was not altered (Figure 3.37A). However, by 120 hours the absorbance values for all the tested hydrogen peroxide concentrations dropped but these values never reached the baseline absorbance observed due to PR dye with no interaction with added hydrogen peroxide (Figure 3.37A). Externally added hydrogen peroxide was found to be relatively stable in the different tested solutions when not exposed to plants (Figures 3.37A). When *A. thaliana* plants were treated in a similar manner it was seen that within 8 hours, the plants were capable of reducing the levels of externally added hydrogen peroxide (Figure 3.37A). The plants reduced the absorbance at 610 nm to baseline absorbance. The absorbance values at 610 nm for the dye solution treated with plants and the control dye solution which contained no added hydrogen peroxide were found to overlap with each other at all the other time intervals of 24, 48, 72 and 120 hours as well (Figure 3.37A). This indicates that any traces of hydrogen peroxide in the solution in which plants were placed were no longer detectable using this method (Figure 3.37A). All the plants appeared healthy and alive at the end of 120 hours meaning that exposure

to 1.0 mM hydrogen peroxide did not alter its normal growth pattern and the plants were not visibly stressed as long as nutrients were provided for its growth.

Since it was observed that plants reduced the amount of externally added hydrogen peroxide within eight hours (Figure 3.37A), the next goal was to estimate and compare the levels of external peroxide within the initial time of eight hours for the plants treated solution with those observed for the control solution containing similar amount of hydrogen peroxide. The experiment was repeated with modifications. The time interval for detecting the added hydrogen peroxide was reduced to initial 8 hours from 0-8 hours with aliquots being removed every 60 minutes after the first 30 minutes from exposing the plants to the externally added hydrogen peroxide (Figure 3.37B). Since the absorbance at 610 nm representing the oxidized dye product was only linear for lower concentrations of hydrogen peroxide, an arbitrarily selected concentration within that range was decided. The selected hydrogen peroxide concentration to be tested at each time point was fixed at 60 μM. Thus, at each time interval the aliquots equivalent to 60 μM hydrogen peroxide concentrations were removed from the solution and tested. 1.0 mM hydrogen peroxide was added to solutions of distilled water, pH 6.3 phosphate buffer or half-strength Hoagland's solution. Positive control solutions containing 1.0 mM hydrogen peroxide as well as negative control solutions having no added hydrogen peroxide, neither of which contained plants, were also treated in similar manner. One other thing that was tested was the influence of the mediator on hydrogen peroxide levels since HOBt was always added along with the external hydrogen peroxide to achieve rapid dye decolorization. It was observed that nature of solution did not alter the reduction of added hydrogen peroxide by plants (Figure 3.37B). The trends observed when comparing the reduction in absorbance value at 610 nm obtained for all the plants placed in different solution conditions of distilled water, pH 6.3 potassium phosphate

buffer and half-strength Hoagland's nutrient solution were very similar to each other (Figure 3.37B). The plants were capable of reducing the levels of externally added hydrogen peroxide to the baseline negative control solution levels for all the solutions independent of their salt concentrations (Figures 3.37B and 3.38A). The constituents of the solution did not appear to affect the behavior of plants towards reducing levels of hydrogen peroxide (Figure 3.37B). For all the solutions exposed to plants, the absorbance at 610 nm decreased very rapidly in the first 2.5 hours (Figure 3.38A). By 4.5 hours, the absorbance value at 610 nm for all the solutions in which plants were placed were comparable with the baseline absorbance spectra of PR, comparable to respective negative control solutions containing no hydrogen peroxide (Figure 3.38A).

There were no changes observed in the levels of the added hydrogen peroxide over 6 hours in the positive control treatments of distilled water, pH 6.3 phosphate buffer and half-strength Hoagland's solution when assayed under similar conditions (Figures 3.37B and 3.38B). Added hydrogen peroxide was detectable when assayed at every time point for 6 hours (Figures 3.37B and 3.38B). Also the levels of hydrogen peroxide estimated at each time interval when observing the absorbance at 610 nm were comparable to each other, signifying no loss in the concentration of the added hydrogen peroxide over time in absence of plants (Figures 3.37B and 3.38B). This shows that the added hydrogen peroxide was quite stable and not destroyed over time in any of the three solutions without any exposure to plants (Figures 3.37B).

Presence of HOBt has no effect on removal of hydrogen peroxide by plants (Figures 3.37B). The concentration of HOBt added to half-strength Hoagland's solution was 50 μM which was similar to the amounts employed in the decolorization of other dyes (Figure 3.37B). It was observed that adding the mediator did not enhance or slow the removal of hydrogen peroxide by *A. thaliana*

plants. Presence of HOBt did not affect the removal of hydrogen peroxide by plants or the hydrogen peroxide levels in the control solution (Figure 3.37B). The absorbance values at 610 nm obtained for the solutions containing added HOBt compared really well with the reduction in absorbance value at 610 nm obtained for all the different solution treatments without the mediator when treated in similar manners (Figure 3.37B). Whether HOBt was present or not, all the plants decreased the levels of externally added hydrogen peroxide to the baseline negative control solution levels within a similar time of 4.5 hours (Figures 3.37B and 3.38B). As seen before there were no changes observed in the levels of the added hydrogen peroxide over 6 hours in the control treatments of half-strength Hoagland's solution in presence or absence of HOBt when assayed under similar conditions. The added hydrogen peroxide was detectable in comparable amounts at each time interval from 0 to 6 hours (Figure 3.37B). This suggests that the oxidation of HOBt caused by the enzymes of the plant shows no influence on the elimination of the hydrogen peroxide when plants are involved. Also there was no evidence found to suggest that the oxidation of HOBt was carried out by hydrogen peroxide and due to low availability of hydrogen peroxide the mediator was no longer oxidized over time. This makes sense and was found to be in agreement with the decolorization trends seen for all the dyes exposed to externally added hydrogen peroxide where there is a sharp drop in the absorbance values of the dye in the initial time period between 0-10 hours followed by gradual absorbance decrease over the remaining time, unless more external hydrogen peroxide is provided to the plants.

It was also observed that the half-times calculated for reduction of added hydrogen peroxide in the different solutions by plants were similar to each other. Since the plants were capable of getting rid of the added hydrogen peroxide in six hours the next thing to understand was the mechanism by which plants diminished the added hydrogen peroxide. When logarithmic

function to base 10 of the absorbance values obtained at each time interval was plotted against the time interval for all the plant treatments in different solution conditions a sharp decrease in the absorbance value from 0-2.5 hours was observed (Figure 3.39). The values seem to stabilize and plateau after the 2.5 hours indicating that the levels of detectable hydrogen peroxide dropped considerably in the initial 2.5 hours. This indicates that not much hydrogen peroxide was detectable beyond 2.5 hours (Figures 3.37B and 3.38B) and the absorbance values at 610 nm for time intervals following 2.5 hours reflect the values obtained for the PR dye which had no interaction with hydrogen peroxide (Figure 3.39). Hence, the slope of the best-fit trend lines used for calculating half-times for disappearance of the added hydrogen peroxide under the different solution conditions was obtained from considering the trend followed in the initial 2.5 hours. However, it appears rather difficult to determine the reaction mechanism for this process (Figure 3.39). The best guess is that the lines fitting to the curve in the initial time appears to follow the first-order elimination kinetics where the concentration of hydrogen peroxide drops considerably in the initial 2.5 hours from exposing plants to the solutions containing externally added hydrogen peroxide. The half-time for detection of the added hydrogen peroxide was found to be 39 minutes for the treatments of half-strength Hoagland's solution with added HOBt and 37.5 minutes for the treatments of half-strength Hoagland's solution containing no added HOBt (Figure 3.39). These times are very close to each other which means that in less than an hour ~50% of the added hydrogen peroxide was removed by the plants. Presence of HOBt plays no role in the reduction of the added hydrogen peroxide in presence of plants (Figure 3.39). Similarly the half-time obtained for pH 6.3 buffer was 48 minutes and for distilled water it was 68 minutes (Figure 3.39). These times were relatively longer than those obtained for half-strength Hoagland's solution. The differences in times can be attributed to the biological

variability between the plants since the gram fresh weight of the roots for all the tested plants independent of the solution treatments were very similar to each other and thus these half-times for reduction in concentration of added hydrogen peroxide were based on amount of roots exposed per equivalent amount of solution. As expected the control solutions without plants but with added hydrogen peroxide showed no decrease in the hydrogen peroxide concentration over the same time which was also seen for the control solutions containing combination of added hydrogen peroxide and HOBt.

3.9.2 Decolorization of PR on direct exposure with *A. thaliana*

It was found out that nearly 50% of the PR dye was decolorized by *A. thaliana* plants in presence of added hydrogen peroxide. Within 24 hours, *A. thaliana* plants were capable of decolorizing ~43% of PR dye on direct exposure of the plant roots with the dye in presence of added hydrogen peroxide with or without HOBt (Figures 3.40A and 3.40C). These values were comparable to the decolorization values obtained for BG in pH 6.3 phosphate buffer when exposed to the *A. thaliana* plants under similar treatment conditions after 24 hours (Figures 3.15B and 3.15D). At the end of 72 hours, the decolorization values estimated were ~75% for the dye treatments in which plants were exposed to added hydrogen peroxide with added HOBt (Figure 3.40C) while the decolorization rate was ~66% for the dye treatments in which plants were exposed to externally added hydrogen peroxide without any HOBt (Figure 3.40A). After washing the roots with the extraction solution nearly 10% of the color from the roots was recovered (Figures 3.40A and 3.40C). Over the same time of 72 hours ~34% of the PR dye was decolorized when exposed to plants without any added hydrogen peroxide and HOBt. This was found to be lower than the values obtained for plants in the above two treatments (Figure 3.41A). At the end of 168 hours, nearly 70% of the dye was decolorized and ~20% of the PR dye was

extracted from the roots using the extraction solution (Figure 3.41A). Thus, added hydrogen peroxide triggers rapid decolorization of the dyes. As long as nutrients are provided to the plants and if the dye is not toxic to the plants, they will continue to decolorize synthetic dye though at comparably slower pace requiring no additions of added peroxide and mediator the plants.

The spectra for the negative control half-strength Hoagland's solution containing PR dye with added hydrogen peroxide (Figure 3.40B); dye with the combination of added hydrogen peroxide and HOBt (Figure 3.40D) or just the dye with no additions (Figure 3.41B) changed negligibly in their absorbances over the experimental time which showed that they were stable in absence of plants. Relatively lower absorbances of the PR dye in the treatments involving added hydrogen peroxide could be attributed to hydrogen peroxide bringing about some level of dye decolorization on its own since no plant was added to the negative control which should keep the levels of added hydrogen peroxide unchanged. However, this decrease in the absorbance values was relatively small as compared to the dye solution exposed to the plants (Figures 3.40B and 3.40D). The effect was most prominent for the control dye solution containing added hydrogen peroxide and HOBt together (Figure 3.40D).

Oxidation of PR dye in presence of hydrogen peroxide and HRP results in a purple colored compound in 0.1 M NaOH. This compound can be identified by monitoring the absorbance at 610 nm, different from the absorbance of the parent dye. Untreated PR in 0.1 M NaOH appears light fuchsia (pink) and has an absorbance maximum around 550-560 nm with very low baseline absorbance at 610 nm (Figure 3.36). When the dye is oxidized absorbance at 610 nm in alkali solution increases over the baseline dye absorbance (Figure 3.36). However, the characteristics of this oxidized dye by-product are still unclear. On carrying out extraction of the roots of *A. thaliana* plants which were treated with PR, nearly 15% of the color from the roots was

extractable (Figures 3.40A; 3.40C and 3.41A). When monitoring the ratio of the absorbance at 610 nm versus absorbance at the isosbestic point at 480 nm for the root extracts, it was observed that ~50% of the extracted root solution showed purple appearance (Figures 3.40A; 3.40C and 3.41A). Since the amount of dye which was extractable using ethanol Tris-EDTA was low to begin with the relative amount of oxidized dye to the non-oxidized parent dye was even lower (Figures 3.40A; 3.40C and 3.41A). Thus, these values only represent the fractions which were extractable using the extraction solution of ethanol Tris-EDTA and do not take into account the non-extractable portion which still presumably resides within the roots. This means that a large amount of the by-product was not extractable using organic solvents such as ethanol Tris-EDTA and remains unaccountable. Roots of the dye treated plants for all the treatments appeared reddish-brown before any extractions were carried out with them. They showed dark blackish-brown coloration after the extraction, losing most of their reddish coloration in the extraction solution. This reddish coloration turned light maroon-purple on addition of 0.1 M NaOH which indicates that both the loosely bound dye as well as some of the by-product was extractable (Figure 3.42). On adding 0.1 M NaOH directly on these roots which have undergone extraction they became dark purple-violet in coloration. Reading the spectra of this purple colored solution showed that the ratio of unconverted PR dye (A480) to the oxidized form of the dye (A610) was nearly 1:1 (Figure 3.42). The spectrum of this root solution either in acid or alkali was much different than the equivalent dye spectra when treated in similar manner (Figure 3.42). This hints towards the purple colored solution being different from the original PR dye and shows promise of being the oxidized dye by-product (Figure 3.42).

3.10 Phylogenetic relationships

Predicted protein sequences of the 17 putative laccases of *Arabidopsis* were clustered into a single family (Figure 3.43). Pairwise comparisons in terms of percentage identity in the amino acid sequence for the laccases revealed that the highest identity in the sequences predicted was ~89% while the lowest predicted similarity was ~44% (Figure 3.43). The mean value predicted by the program was ~60% similarities in identity of the members of the laccase family in *Arabidopsis*. Similarly, the clustering of predicted protein sequences of the 73 putative peroxidases of *Arabidopsis* showed that the class III peroxidases superfamily was grouped into three subfamilies (Data not shown). For the convenience of displaying the phylogenetic relationships, the subfamilies of peroxidases were arbitrarily named as subfamily A (Figure 3.44), subfamily B (Figure 3.45) and subfamily C (Figure 3.46). Individual clustering of the members within the same subfamily after being segregated from the main branching affected the apparent phylogenetic relationships, but not by a huge margin. Pairwise comparisons in terms of percentage identity in the amino acid sequence for the members within the peroxidase subfamily A revealed that the highest predicted sequence identity predicted was ~89% while the lowest predicted identity was ~31% (Figure 3.44). For the peroxidases subfamily B, the highest predicted sequence identity was ~100% with the lowest predicted identity being ~36% (Figure 3.45) and for the peroxidases subfamily C, the highest predicted sequence identity predicted was ~93% with the lowest predicted identity being ~42% (Figure 3.46). The mean values predicted by the program for the percentage identity for peroxidase subfamily A was ~56%, for peroxidase subfamily B was ~56% and for peroxidase subfamily C was ~64%. The amino acid sequences of the members of the laccase family and those predicted for the peroxidase family in *Arabidopsis* possibly takes into account the redundancy in functions of the different members from the independent families as these sequences are predicted have conserved residues highlighting their

common evolutionary ancestor. It is also possible that at gene level, the substitutions in nucleotide might be higher than at protein level, leading to the diversity in the number of laccases and peroxidases which perform similar functions while accounting for their highly evolved nature. Note that the pairwise comparison values displayed for the pairs were predicted the algorithms of the programs. These were not global matches or pairing. The algorithms employed by the two programs are certainly different (Lassmann and Sonnhammer, 2005; Dereeper *et al.*, 2008). The above results were considered to be the best based on the parameters that were chosen for studying the phylogenetic relationships between the different laccases and peroxidases of *Arabidopsis* but the results may be refined by tweaking the parameters and stringency of the prediction programs.

3.11 Characterization of T-DNA insertion mutants of *Arabidopsis*

T-DNA insertions in single laccase and peroxidase genes in *Arabidopsis* did not hinder the plant's capacity to decolorize synthetic dyes (Tables 3.4 and 3.5). No additional growth requirements were observed for the T-DNA insertion lines. The various T-DNA insertion single mutant lines corresponding to laccases showed about the same amount of MR decolorization in absence of added hydrogen peroxide and mediator as the WT *Arabidopsis* plants (Table 3.4). The mutant plants decolorized MR in pH 4.6 and pH 6.3 phosphate buffers and the decolorization trends were comparable to the WT plant (Table 3.4). Some of the T-DNA insertion lines for laccase were heterozygous and hence the gene would be expected to be expressed at 50% of the WT level. A certain level of redundancy among laccases in *Arabidopsis* plants is indicated by the observation that the T-DNA mutants maintain the dye decolorization potential presumably through compensation by other laccases (Figure 3.43). There are 17 laccase genes predicted in

Arabidopsis (McCaig, Meagher and Dean, 2005; Cai *et al.*, 2006) and 16 of them are shown to be expressed in roots (Cai *et al.*, 2006).

Similarly the single T-DNA insertion mutant plants corresponding to peroxidases showed MO decolorization in presence of added hydrogen peroxide and mediator with comparable trends as seen for the WT *Arabidopsis* plants (Table 3.5). This provides evidence for the functional redundancy of the peroxidases of *Arabidopsis*. This redundancy along with gene duplication results in other peroxidases being possibly expressed since there are 73 peroxidase genes predicted *Arabidopsis* (Welinder *et al.*, 2002; Cosio and Duand, 2010) grouped into several families and subfamilies (Figures 3.44; 3.45 and 3.46).

3.12 Reverse phase HPLC-ESI/MS studies

The hypothesis was that the process of decolorization of synthetic dyes in presence of added hydrogen peroxide and the mediator by active plants occurs within the roots and the resulting break down products of the parent dye stay trapped within the roots or undergo polymerization and precipitate out of the solution thereby escaping detection using this technique. The fragmentation pattern of the control dye, that was never exposed to the plants, but includes added hydrogen peroxide plus HOBt should be comparable and resemble the pattern seen for the synthetic dye solution exposed to plants under similar conditions.

3.12.1 Reverse phase HPLC-ESI/MS analysis of MO

In reverse phase HPLC it was noticed that MO did not appear to interact much with the C18 microcolumn and was eluted around 2.0-2.6 minutes (Figure 3.47A). The negative control untreated dye solution showed a single high resolution peak which indicated that the dye was relatively free of salts. Similarly for the plant treated dye solution, the elution peak around 2.0-2.5 minutes was seen while the elution peak for the positive control using crushed turnip roots

was around 1.5-2.3 minutes (Figure 3.47B). However, the intensities of the plant treated as well as the positive control dye peaks were found to be relatively lower than that observed for the negative control dye solution and contained noise in the form of smaller peak. The relative peak intensity for the negative control dye solution was ~1.6 X 10^7 (Figure 3.47A) while it was ~1.5 X 10^6 for the plants treated dye solution and ~2.0 X 10^5 for the crushed turnip root extract treated dye solution (Figure 3.47B)

On integrating the whole area under the peak obtained in reverse phase HPLC, the distinct m/z peaks representative of MO dye were observed for the negative control dye solution as well as the dye solution exposed to plants (Figures 3.48A and 3.48B). Three high intensity peaks with m/z 170.76 Da, 305.42 Da and 419.72 Da were prominently seen (Figures 3.48A and 3.48B). The 170.76 Da represents sodium salt of phthalic anhydride while the 419.72 Da peaks represents sodium salts of either diisooctylphthalate or triton. These two peaks represent the contaminant peaks which are commonly observed when the positive ion mode of ESI/MS machine is operated. Their origin can be traced back to plastic tubing, lining of the walls of plastic tubes or contaminated solvent. These peaks were difficult to eliminate and appear in all the ESI/MS scans. Of more interest was the 305.42 Da peak which represents MO dye without its sodium salt (Figures 3.48 and 3.49). Two other peaks of 306.40 Da and 307.37 Da which were of relatively lower intensities were also seen (Figures 3.49A and 3.49B). These two peaks denote both the protonation of the dye as well as the naturally occurring carbon isotope (C13) which has a ratio 98.90:1.10 on % atom basis for C12:C13 (Figures 3.49A and 3.49B).

The above characteristic peaks of MO dye were also observed for the dye solutions exposed to plants. However, the intensities of the plant-treated dye peaks were much lower than those compared with the untreated control dye solution. The peak intensities for 305.4 Da, 306.3 Da

and 307.3 Da representative of C14H15N3O3S, C14H16N3O3S and C14H17N3O3S forms were ~1,527,929; ~274,012 and ~82,611 with combined sum of ~1,884,552 for the negative untreated dye control (Figure 3.49A) while these intensities reduced to ~92,624; ~17,068 and ~6,982 with combined sum of ~116,674 for the solution of the dye left behind after exposure with *A. thaliana* plants in presence of added hydrogen peroxide and HOBt (Figure 3.49B). Based on the intensities this represented ~95% of the MO dye being decolorized by the *A. thaliana* plants in presence of external peroxide and HOBt after 48 hours which was comparable to the ~90% dye decolorization obtained over 48 hours when calculated using UV-Visible spectrophotometry which confirmed the dye decolorization values between the two detection methods.

However, no new fragments which would represent the possible break down products on decolorization of MO were detected in the negative dye control as well as the dye solution exposed to plants (Figure 3.50A). The positive control solution on the other hand showed no distinct peaks representative of the intact MO dye or its salts in the m/z 300-340 region (Figure 3.50B). This showed that MO was completely decolorized and broken down by the crushed turnip root extract and thus diminished the intensity of the parent dye (Figure 3.50B). The data confirmed that intensity of the eluted peak for the positive control dye solution was much lower than that observed for the negative dye control solution which meant that the parent dye was indeed broken down and was no longer detectable. However, the identification of particular dye fragments representative of break down became really difficult due to the overlapping of the masses of HOBt and its break down products along with the other contaminants and no dye break down product could be detected using ESI/MS (Figure 3.50).

3.12.2 Reverse phase HPLC-ESI/MS analysis of BG

The observed retention time for BG in reverse phase HPLC was around 3.5-4.0 minutes, which was slightly later than the retention time seen for MO (Figure 3.51A). This made sense since BG happens to be relatively more hydrophobic than MO thereby interacting for longer with the C18 microcolumn. However compared to MO many smaller peaks were coupled with the high intensity peak in case of BG (Figure 3.51A). The observed retention time seen for the positive control dye solutions containing crushed turnip root extract was around 1.5-2.8 minutes and like the negative control dye solution noise in the form of smaller peaks was also detectable (Figure 3.51B). Also the intensity of the peak for the dye solutions exposed to plants as well as the positive control dye solutions were relatively lower than those detected for negative control dye solution (Figure 3.51). The relative peak intensity for the negative control dye solution was ~3.8 X 10^6 (Figure 3.51A) while it was ~0.7 X 10^6 for the BG dye solution exposed to plants and ~6.2 X 10^5 for the positive control dye solution (Figure 3.51B).

On integrating the entire area under the peak obtained in reverse phase HPLC as observed for MO distinct m/z peaks characteristic of BG were noted in MS analysis. The molecular weight of BG as stated in literature is ~698.01 Da (Figure 3.52). The dye has four bromine (Br) atoms in its structure and these needs to be taken into account in terms of the relative abundance of naturally occurring isotopes of Br. Br has two known naturally occurring isotopes: Br (79) and Br (81) with abundance of 50.69 % and 49.31 % respectively. In the m/z 690-710 region the peaks with m/z 694.73 Da, 695.82 Da, 696.74 Da, 697.76 Da, 698.75 Da, 699.71 Da, 700.71 Da, 701.65 Da and 702.66 Da were detected (Figure 3.53A). Being halogenated the halogen-skip isotope technique was followed where every alternate peak was added together starting from 696.74 Da till 700.71 Da. So the intensity values of the peaks 696.74 Da, 698.75 Da and 700.71 Da were added together to represent the final intensity value for Bromocresol Green (Figure 3.53). In

terms of Br isotopes, peak 696.74 Da represents Br (79) abundance while 700.71 Da represents Br (81) abundance since both are nearly 50:50 in terms of their intensities which corresponds perfectly to their relative natural abundance. The m/z 698.75 Da peaks takes both the Br isotopes into consideration (Figure 3.52) and therefore was relatively higher in intensity than the other two peaks representing individual isotopes (Figure 3.53). Similarly for the BG dye solutions exposed to plants these very same peaks but of relatively lower intensities were seen (Figures 3.52B and 3.53B). On summation of the three peaks 696.74 Da, 698.75 Da and 700.71 Da the overall intensity calculated for the negative control dye solution was ~402,559 (Figures 3.52A and 3.53A) while it was ~142,408 for the BG dye solution exposed to *A. thaliana* plants (Figures 3.52B and 3.53B). This represents ~35% of the BG dye being left in the solution after treatment with plants exposed to added hydrogen peroxide and HOBt. This trend as seen with MO showed that the detection of relative amounts of synthetic dyes following their decolorization using UV-Visible spectrophotometry and ESI/MS methods were comparable to each other.

As seen for MO, no new fragments representative of the break down products of BG dye were detected in the negative dye control as well as the dye solution exposed to plants (Figures 3.52A; 3.52B and 3.54A). In case of the positive control dye solution exposed to BG dye, the characteristic m/z peaks of 696.74 Da, 698.75 Da and 700.71 Da representing intact BG were missing and were not detectable using ESI/MS (Figure 3.54B). The only other high intensity m/z peaks that were detected were 170.7 Da, 390.65 Da and 419.75 Da which represents the contaminants present in the machine (Figures 3.52 and 3.54). As observed with MO the identification of particular dye fragments representative of break down became really difficult due to the overlapping of the masses of HOBt and its break down products along with the other

contaminants and no break down products of the parent arylmethane BG dye could be easily detected using ESI/MS (Figure 3.54).

3.12.3 Reverse phase HPLC-ESI/MS analysis of BBG

In reverse phase HPLC, intact BBG from the negative control dye solution showed a retention time around 3.0-4.0 minutes (Figure 3.55A). This retention time was very similar to the times obtained for BG since both these dyes are relatively hydrophobic as compared to MO. As observed for MO, the formation of single high intensity peak indicated that the dye was free of salts and other contaminants (Figure 3.55A). However, the elution times were found to be around 1.8-2.4 minutes for the dye solutions which were exposed to plants as well as for the BBG dye exposed to crushed turnip root extract in the positive control dye solution and were composed of noise in the form of numerous smaller peaks (Figure 3.55B). The relative intensity of the peaks for the untreated dye solution in the negative control was ~2.0 X 10^7 (Figure 3.55A) while it was ~2.3 X 10^6 for the plant treated dye solutions and ~~2.0 X 10^6 for the positive control (Figure 3.55B).

Integration of the area under the single high intensity peaks obtained in reverse phase HPLC, distinct m/z peaks characteristic of BBG dye were detected in MS1 analysis (Figure 3.56A). The molecular weight of BBG when synthesized as sodium salt as reported in literature is 856 Da (Table 2.1). On removing sodium the compound left behind gives a mass ~833 Da (Figure 3.56A). A distinct peak corresponding to 832.92 Da representing BBG dye without sodium was obtained for the negative control dye solution (Figure 3.56A). Along with 832.92 Da peak other peaks corresponding to 833.61 Da, 834.63 Da and 835.72 Da were also seen (Figure 3.57A). These peaks account for the protonation of the dye molecule as well as the relative abundance of

the naturally occurring C13 carbon isotope which is found to be in the ratio 98.90:1.10 on % atom basis for C12:C13 (Figure 3.57A).

However, unlike MO and BG the above characteristic peaks of BBG were not found in either of the treated dye solutions (Figures 3.56B; 3.56B and 3.57). No peaks were seen in the m/z 830-860 region (Figures 3.56B and 3.57B). The only similarities between the control untreated dye and the plant treated dye solution were the contaminant peaks of 170.7 Da and 419.76 Da (Figure 3.56 and 3.58). The peak 135.7 Da which corresponds to intact HOBt was also seen (Figure 3.58A and 3.58B). The relative peak intensities for 832.92 Da; 833.61 Da; 834.63 Da and 835.72 Da were ~218,318; ~84,499; ~38,867 and ~14,933 which gave a combined intensity of ~356,617 for the negative control of the dye (Figure 3.57A). However, when it came to *A. thaliana* plants being exposed to BBG combined intensities reduced to ~18,258 while it was ~ 18,038 of the positive control dye solution. These were comparable to each other (Figure 3.57B). This indicated ~95% of the BBG dye disappeared from the solution when exposed to either whole *A. thaliana* plants in presence of externally added hydrogen peroxide and HOBt as the mediator or crushed turnip root extract under similar conditions. It's possible that a large fraction of the BBG dye was removed from the solution either by binding to the roots or by interaction with the crushed turnip root extract and hence was no longer detected in the residual solution. As observed with MO and BG possible by-products from the dye decolorization released into the solution were not detectable using ESI/MS (Figures 3.56 and 3.58). The new peaks that were observed in the BBG dye solutions of the positive control appeared to be contaminants similar to that observed for the other two dyes (Figures 3.56 and 3.58).

Unfortunately, the heavier disazo dyes namely EB, TB and CB6B did not fly through ESI/MS despite adding a counter ion in either positive or negative mode. Even MG refused to show a

decent MS1 spectra. It was not possible to detect any proper fragmentation pattern for these dyes under any conditions so far tested.

Thus, ESI/MS analysis could only detect the relative abundance of the intact parent dye compound but failed to detect any possible dye break down product in the leftover solution treated with *A. thaliana* plants in presence of added hydrogen peroxide and HOBt as well as crushed turnip root extract when subjected to similar treatments.

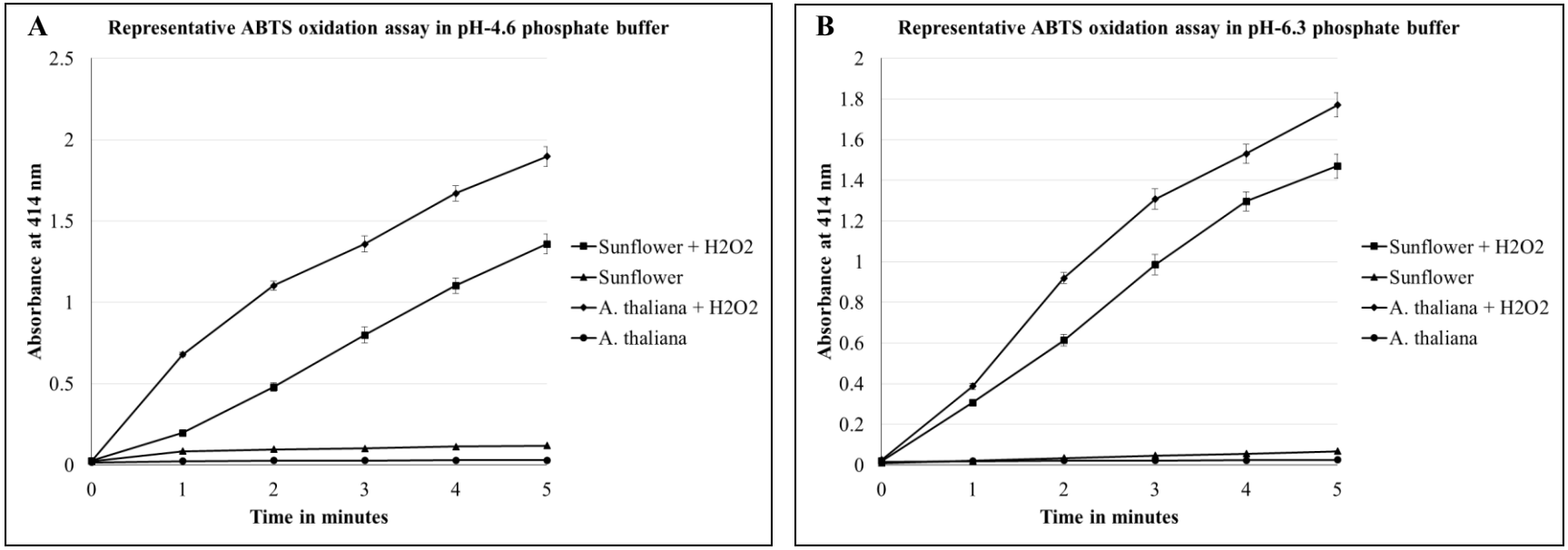


Figure 3.1 Oxidation of ABTS by *A. thaliana* and sunflower in presence or absence of added hydrogen peroxide in **A.** pH 4.6 phosphate buffer and **B.** pH 6.3 phosphate buffer.

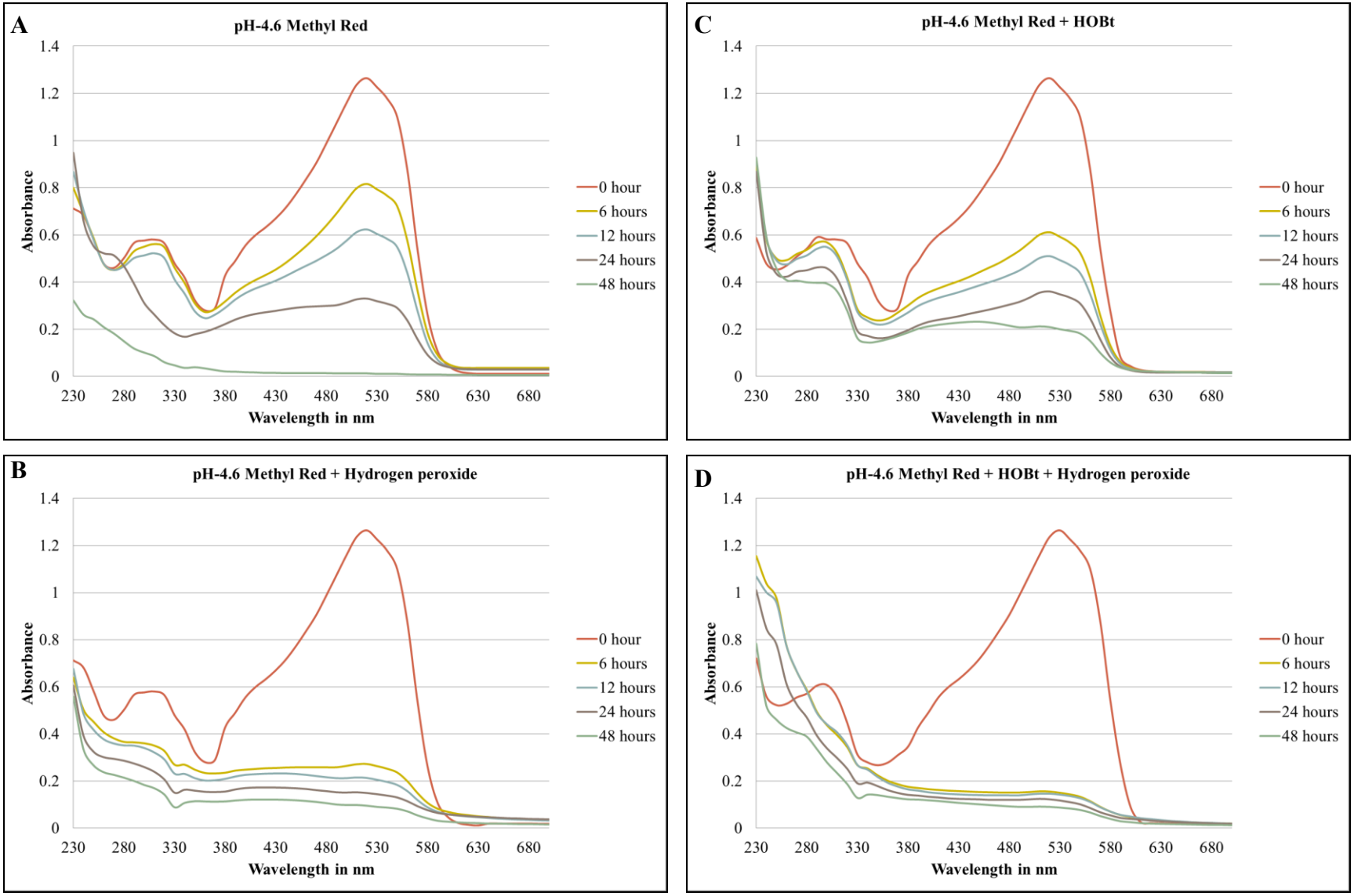


Figure 3.2 Spectra of Methyl Red exposed to *A. thaliana* plants in pH 4.6 phosphate buffer containing **A.** no additions; **B.** 1 mM hydrogen peroxide; **C.** 50 µM HOBt and **D.** combination of 1 mM hydrogen peroxide with 50 µM HOBt.

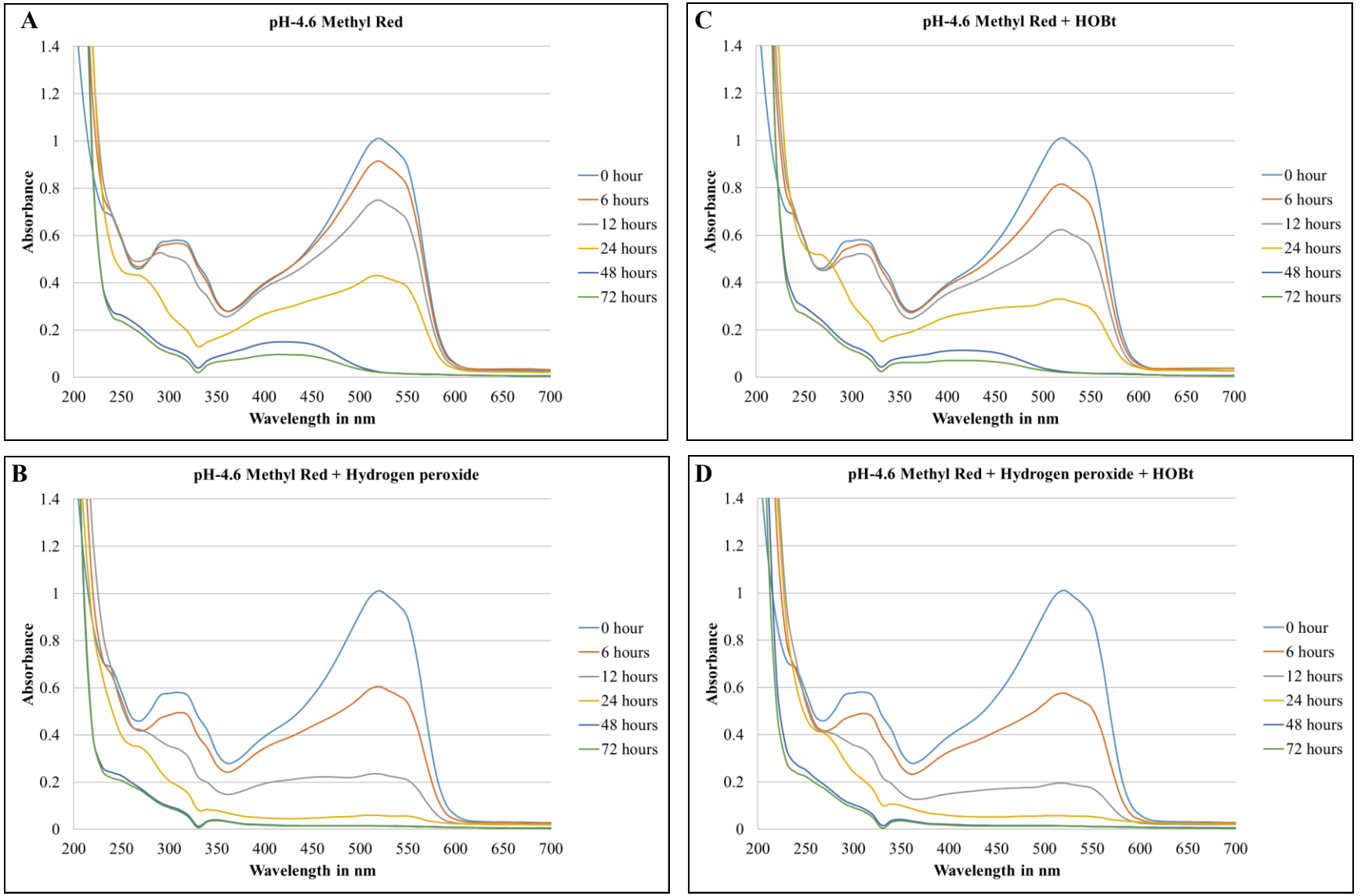


Figure 3.3 Spectra of Methyl Red exposed to sunflowers in pH 4.6 phosphate buffer containing **A.** no additions; **B.** 1 mM hydrogen peroxide; **C.** 50 µM HOBt and **D.** combination of 1 mM hydrogen peroxide with 50 µM HOBt.

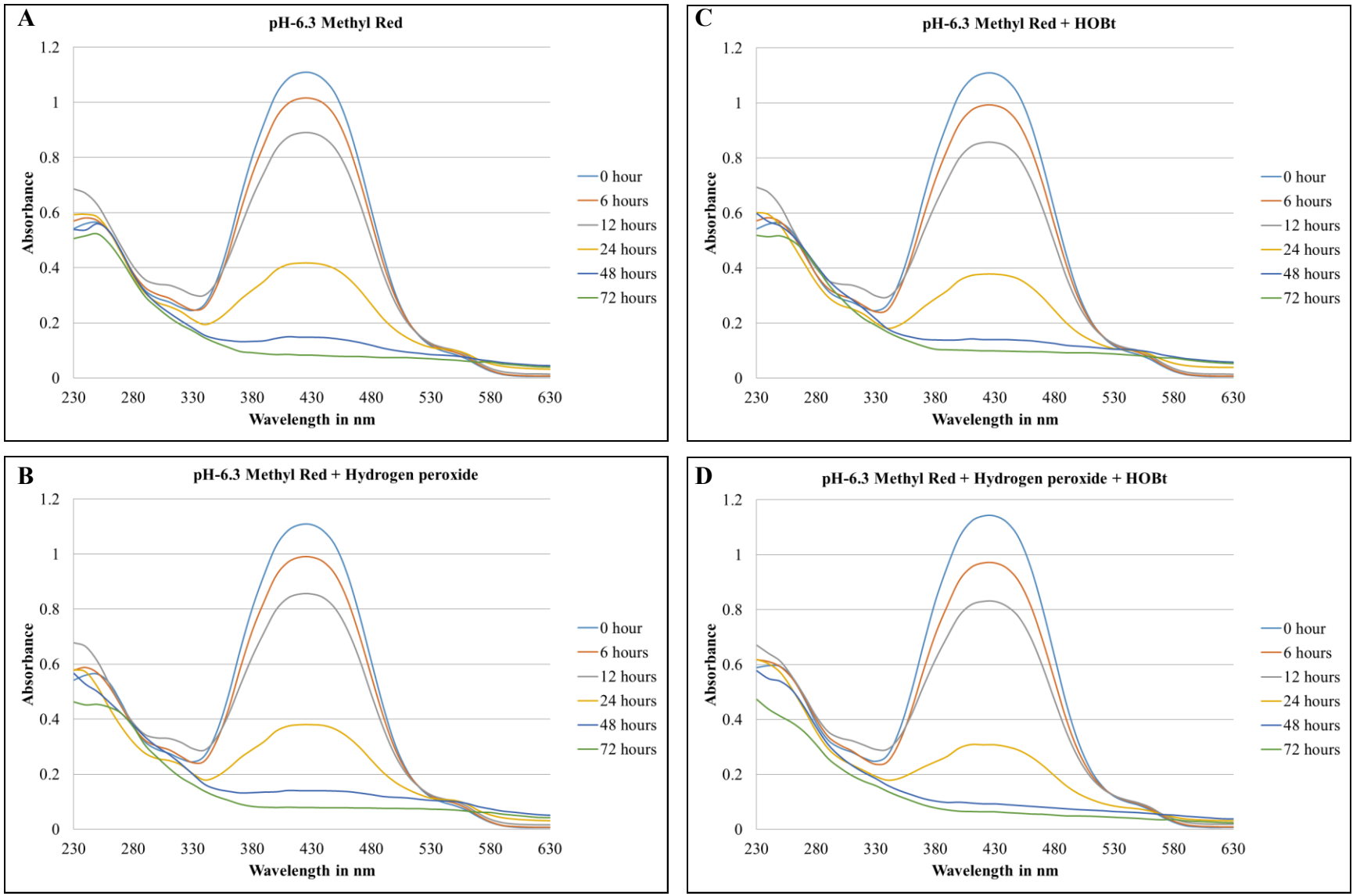


Figure 3.4 Spectra of Methyl Red exposed to *A. thaliana* plants in pH 6.3 phosphate buffer containing **A.** no additions; **B.** 1 mM hydrogen peroxide; **C.** 50 µM HOBt and **D.** combination of 1 mM hydrogen peroxide with 50 µM HOBt.

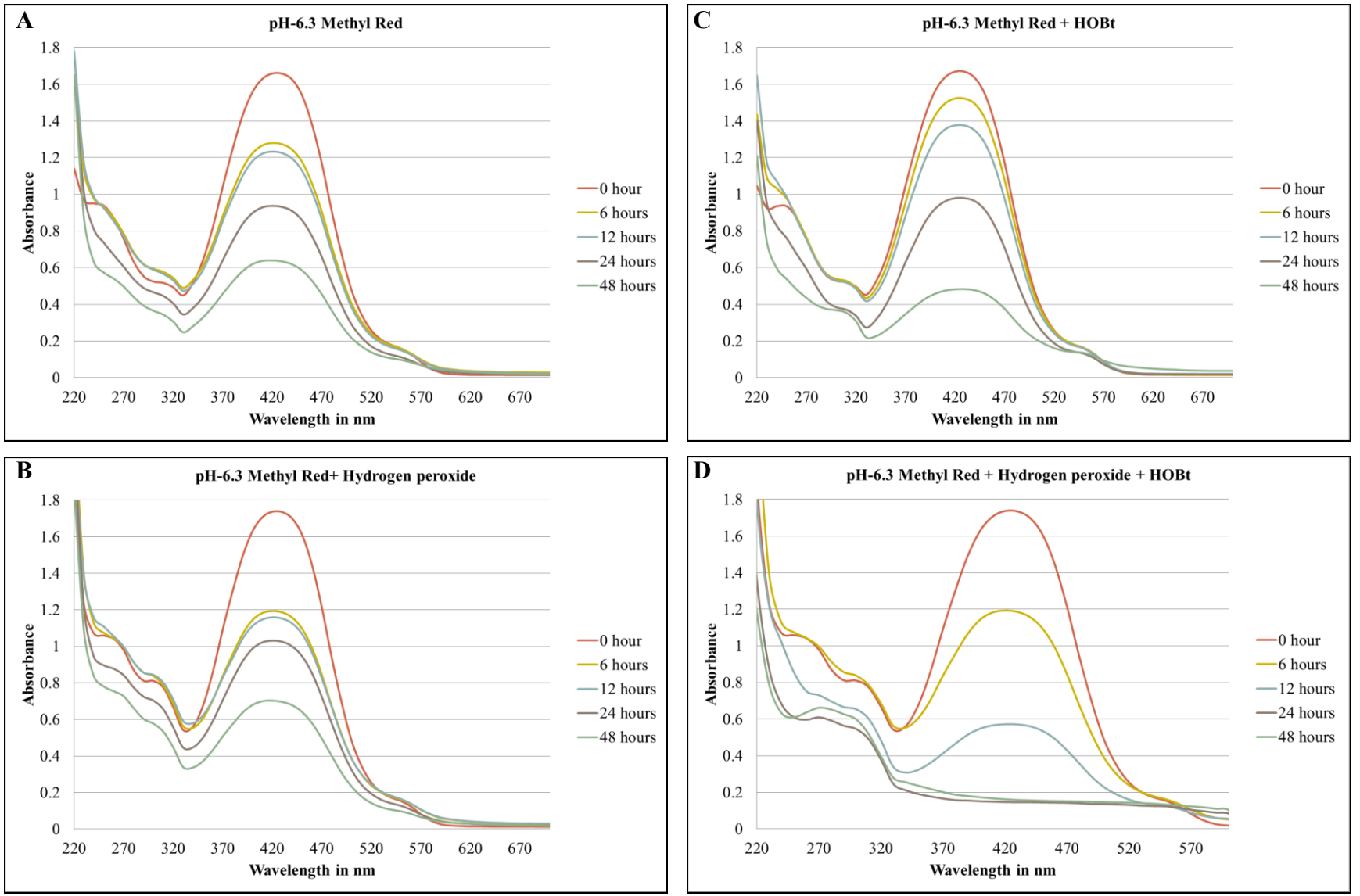


Figure 3.5 Spectra of Methyl Red exposed to sunflowers in pH 6.3 phosphate buffer containing **A.** no additions; **B.** 1 mM hydrogen peroxide; **C.** 50 µM HOBt and **D.** combination of 1 mM hydrogen peroxide with 50 µM HOBt.

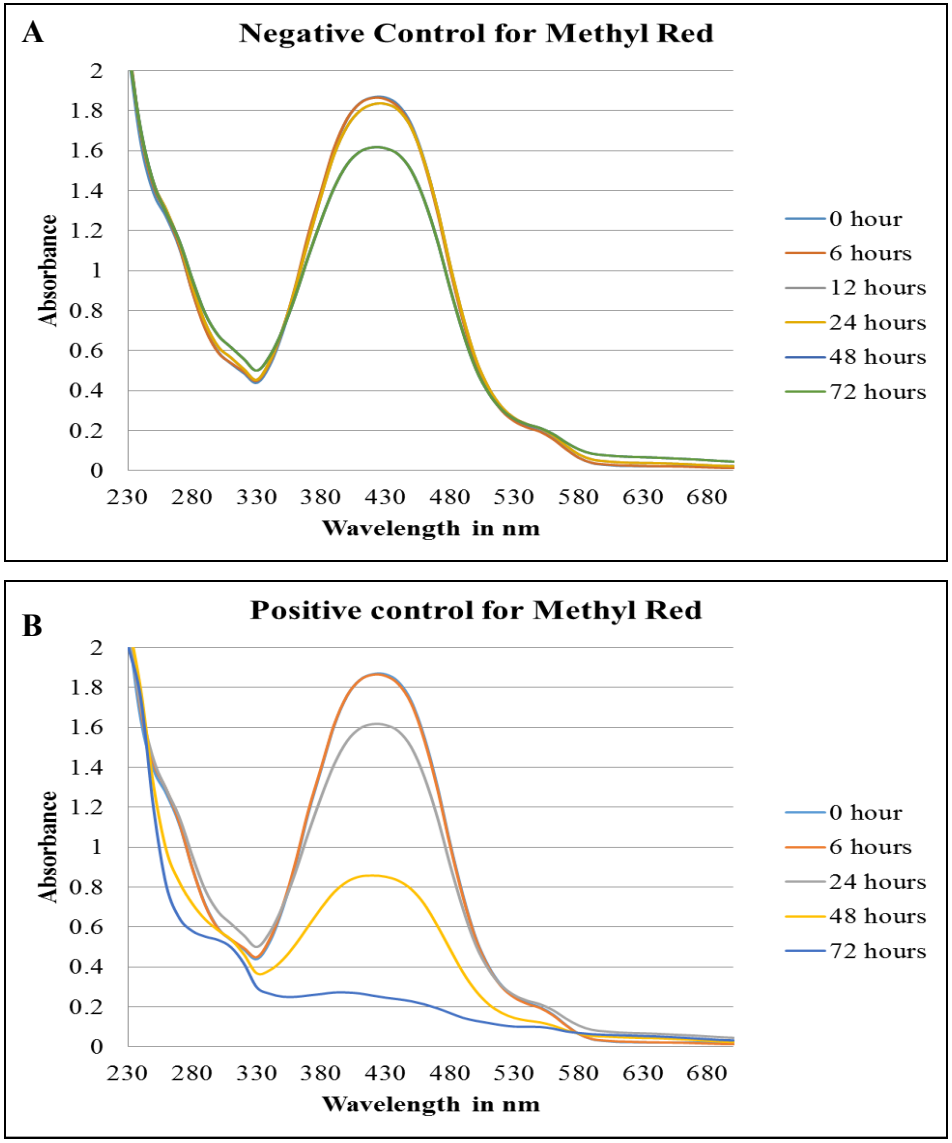


Figure 3.6 Spectra of **A.** Methyl Red not exposed to plants (Negative control) and **B.** Methyl Red with crushed turnip root extract and 1 mM hydrogen peroxide plus 50 µM HOBt (Positive control).

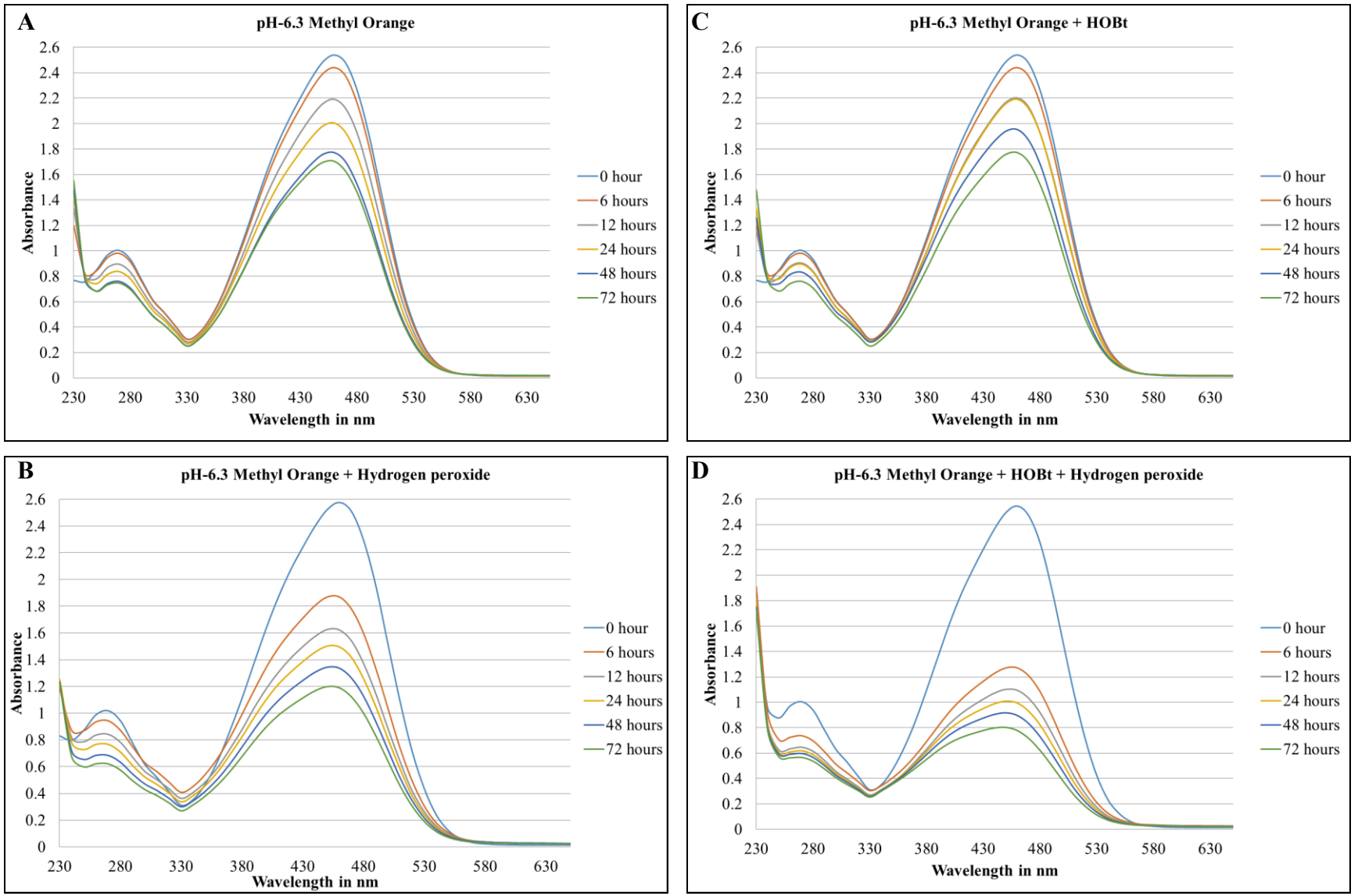


Figure 3.7 Spectra of Methyl Orange exposed to *A. thaliana* plants in pH 6.3 phosphate buffer containing **A.** no additions; **B.** 1 mM hydrogen peroxide; **C.** 50 µM HOBt and **D.** combination of 1 mM hydrogen peroxide with 50 µM HOBt.

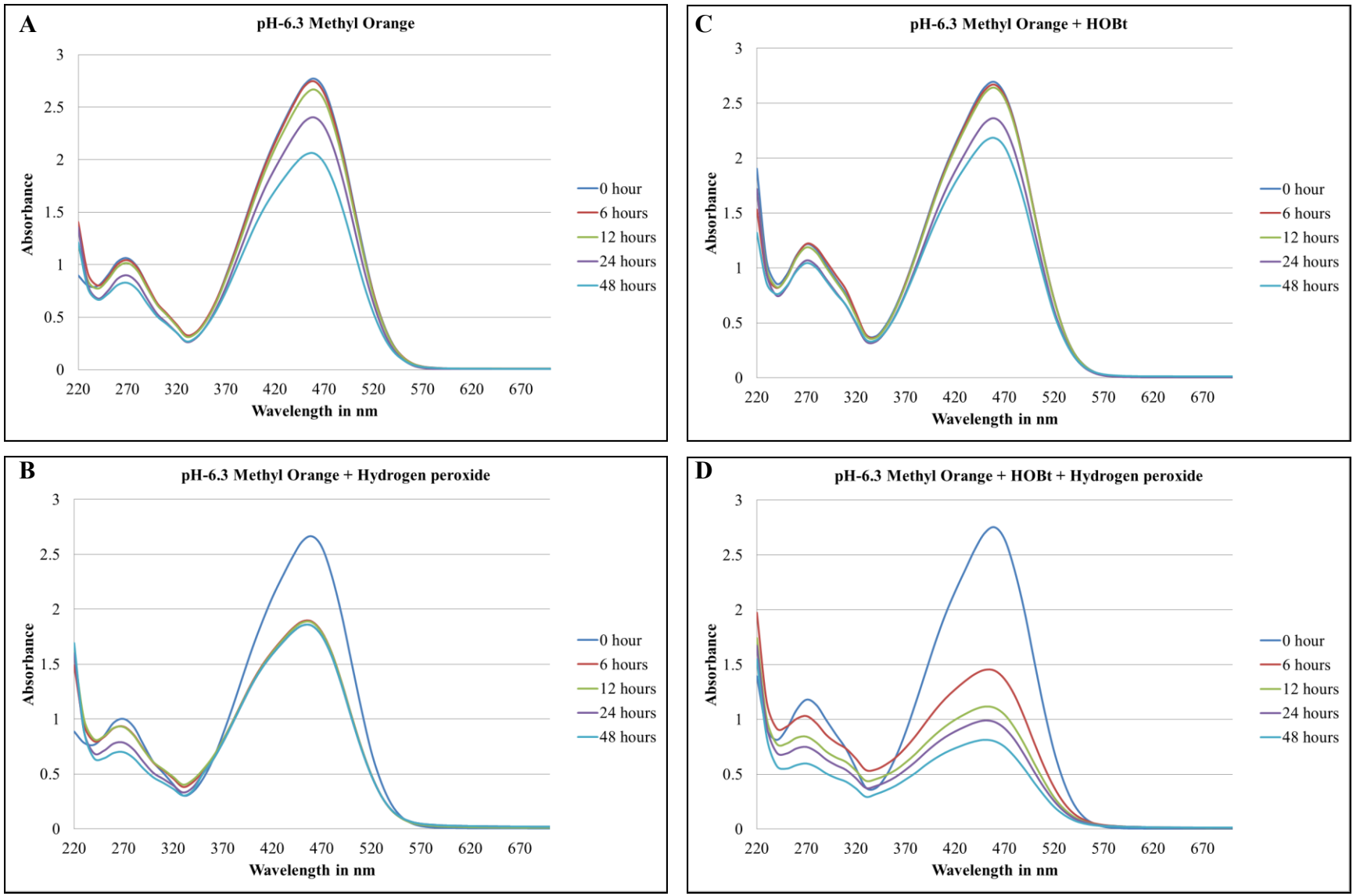
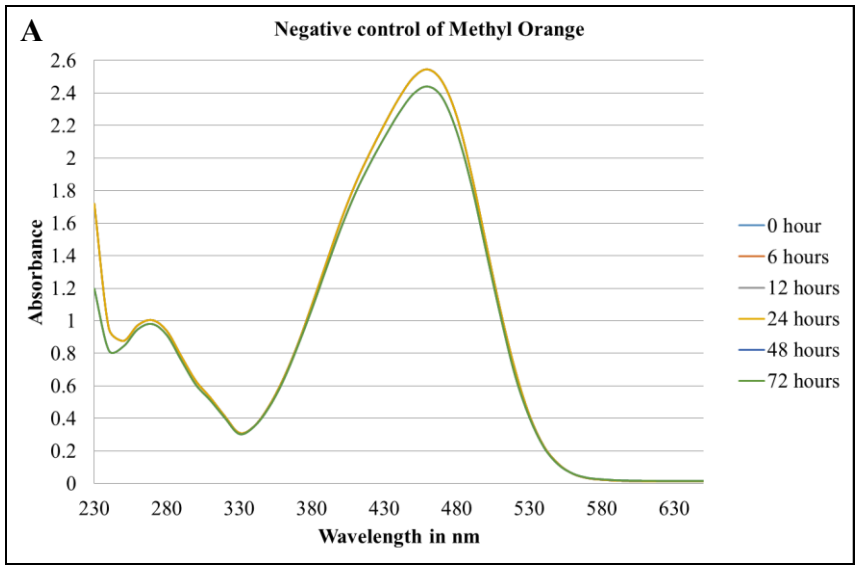
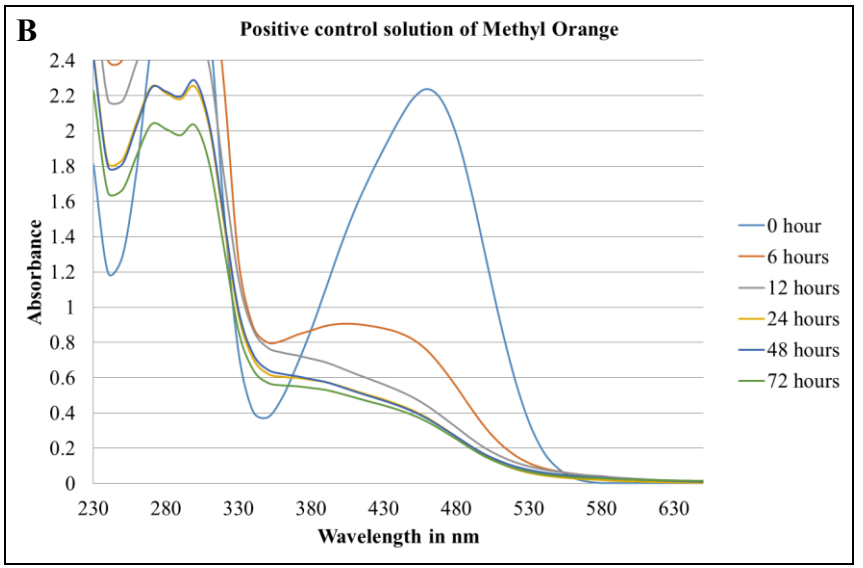


Figure 3.8 Spectra of Methyl Orange exposed to sunflowers in pH 6.3 phosphate buffer containing **A.** no additions; **B.** 1 mM hydrogen peroxide; **C.** 50 µM HOBt and **D.** combination of 1 mM hydrogen peroxide with 50 µM HOBt.





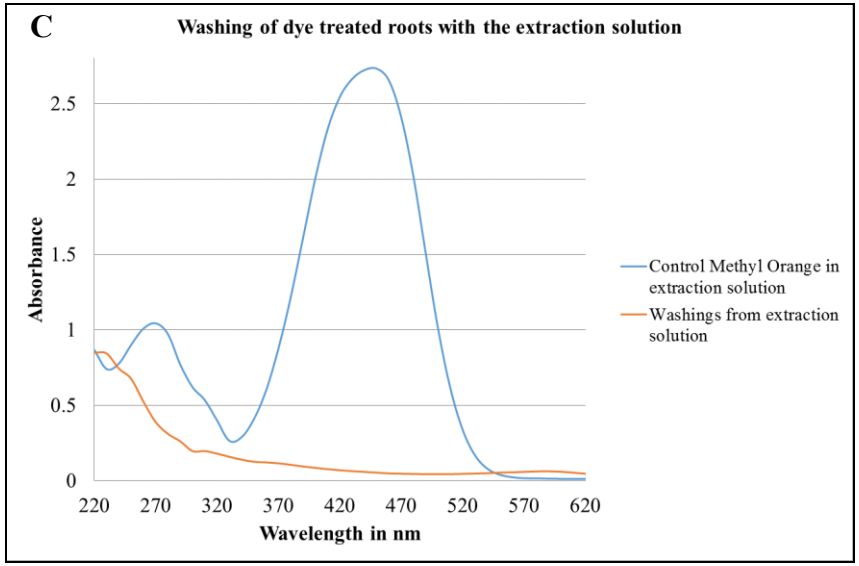


Figure 3.9 Spectra of **A.** Methyl Orange not exposed to plants (Negative control); **B.** Methyl Orange with crushed turnip root extract and 1 mM hydrogen peroxide plus 50 µM HOBt (Positive control) and **C.** Extraction solution of treated roots.

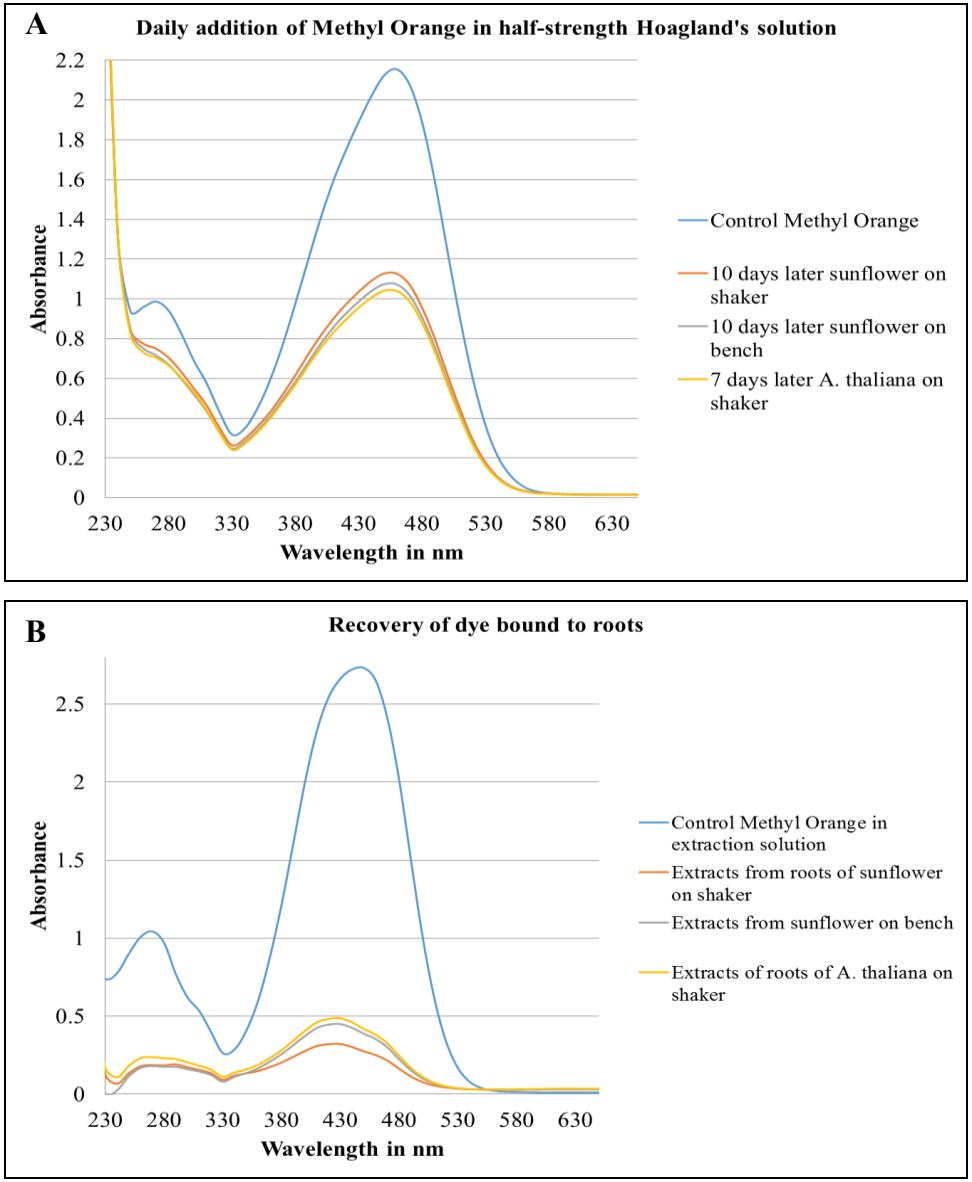


Figure 3.10 A. Daily addition of 40 mg/L Methyl Orange and **B.** Recovery of the dye bound to roots using extraction solution.

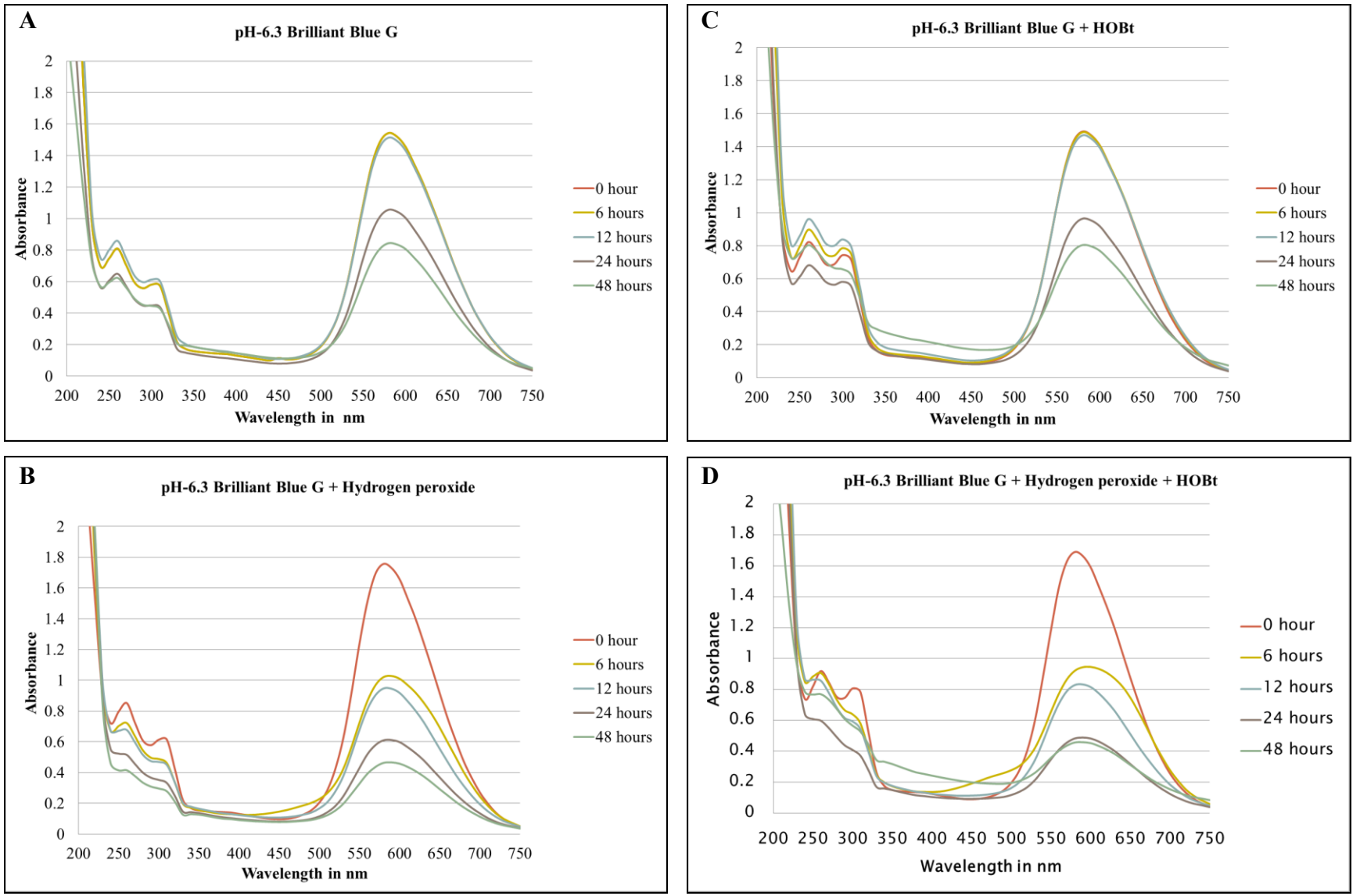


Figure 3.11 Spectra of Brilliant Blue G exposed to *A. thaliana* plants in pH 6.3 phosphate buffer containing **A.** no additions; **B.** 1 mM hydrogen peroxide; **C.** 50 µM HOBt and **D.** combination of 1 mM hydrogen peroxide with 50 µM HOBt.

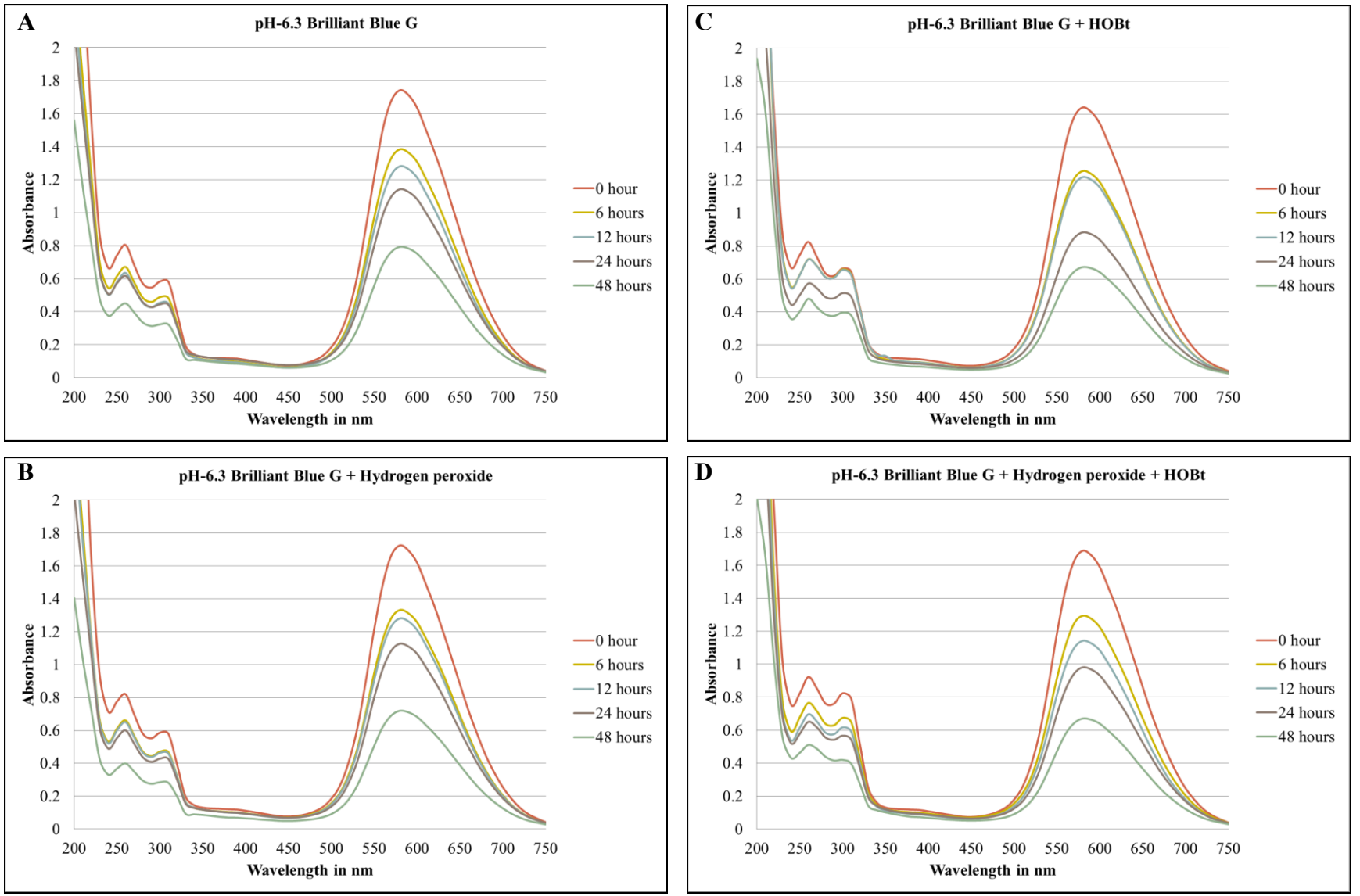
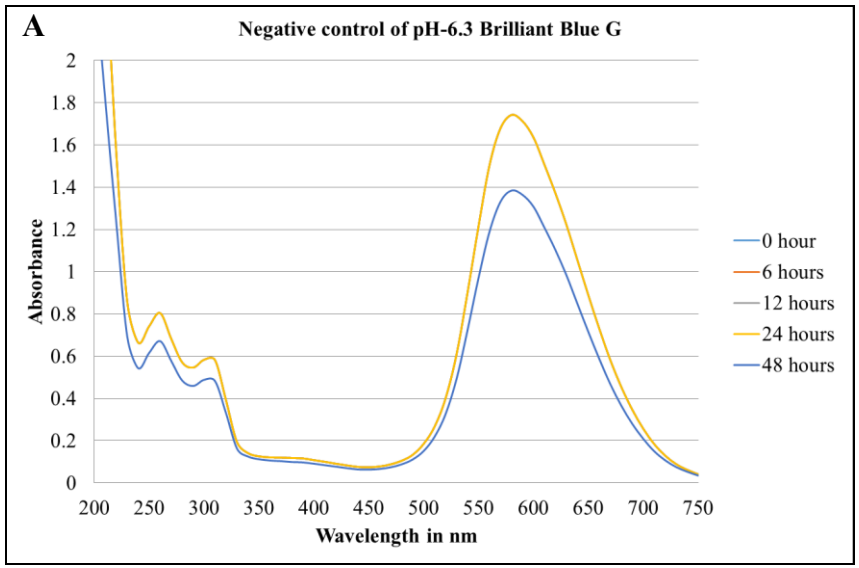
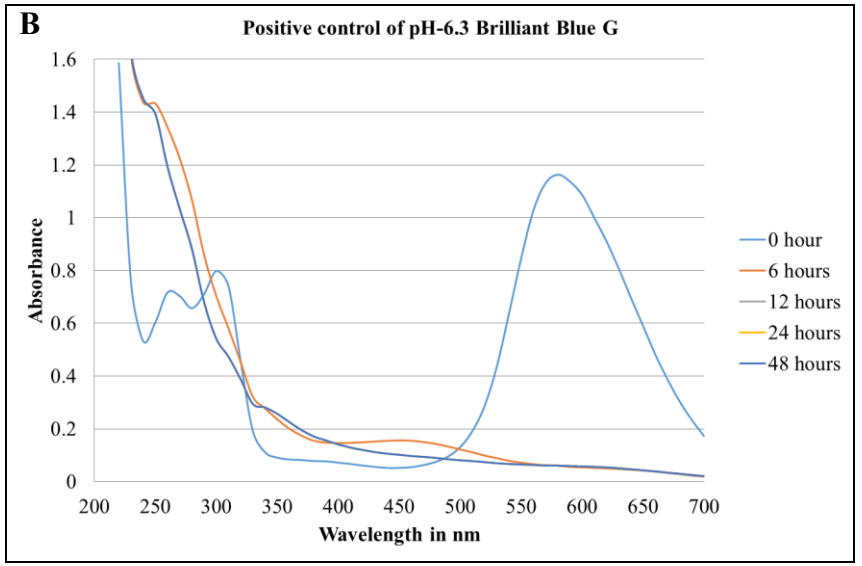


Figure 3.12 Spectra of Brilliant Blue G exposed to sunflowers in pH 6.3 phosphate buffer containing **A.** no additions; **B.** 1 mM hydrogen peroxide; **C.** 50 µM HOBt and **D.** combination of 1 mM hydrogen peroxide with 50 µM HOBt.





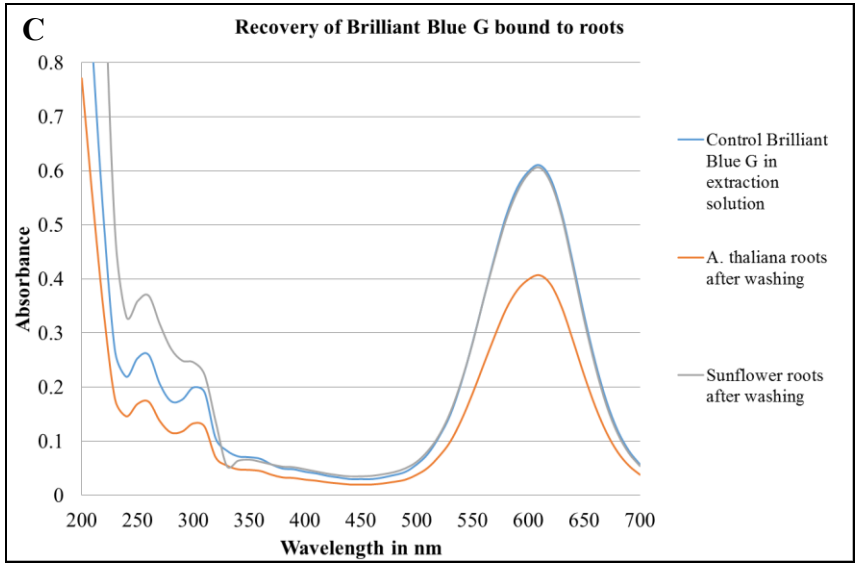


Figure 3.13 Spectra of **A.** Brilliant Blue G not exposed to plants (Negative control); **B.** Brilliant Blue G with crushed turnip root extract and 1 mM hydrogen peroxide plus 50 µM HOBt (Positive control) and **C.** Extraction solution of treated roots.

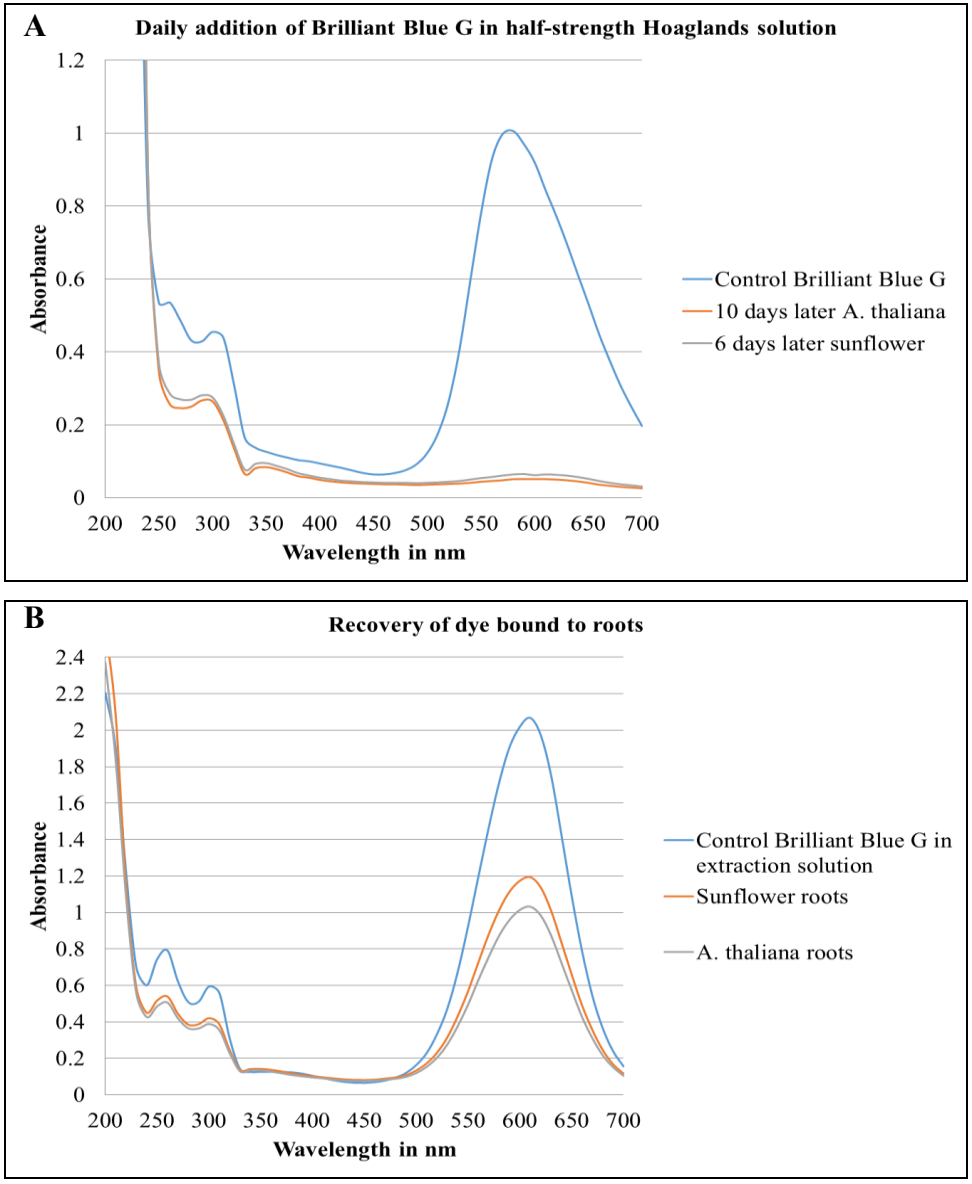


Figure 3.14 A. Daily addition of 40 mg/L Brilliant Blue G and **B.** Recovery of the dye bound to roots using extraction solution.

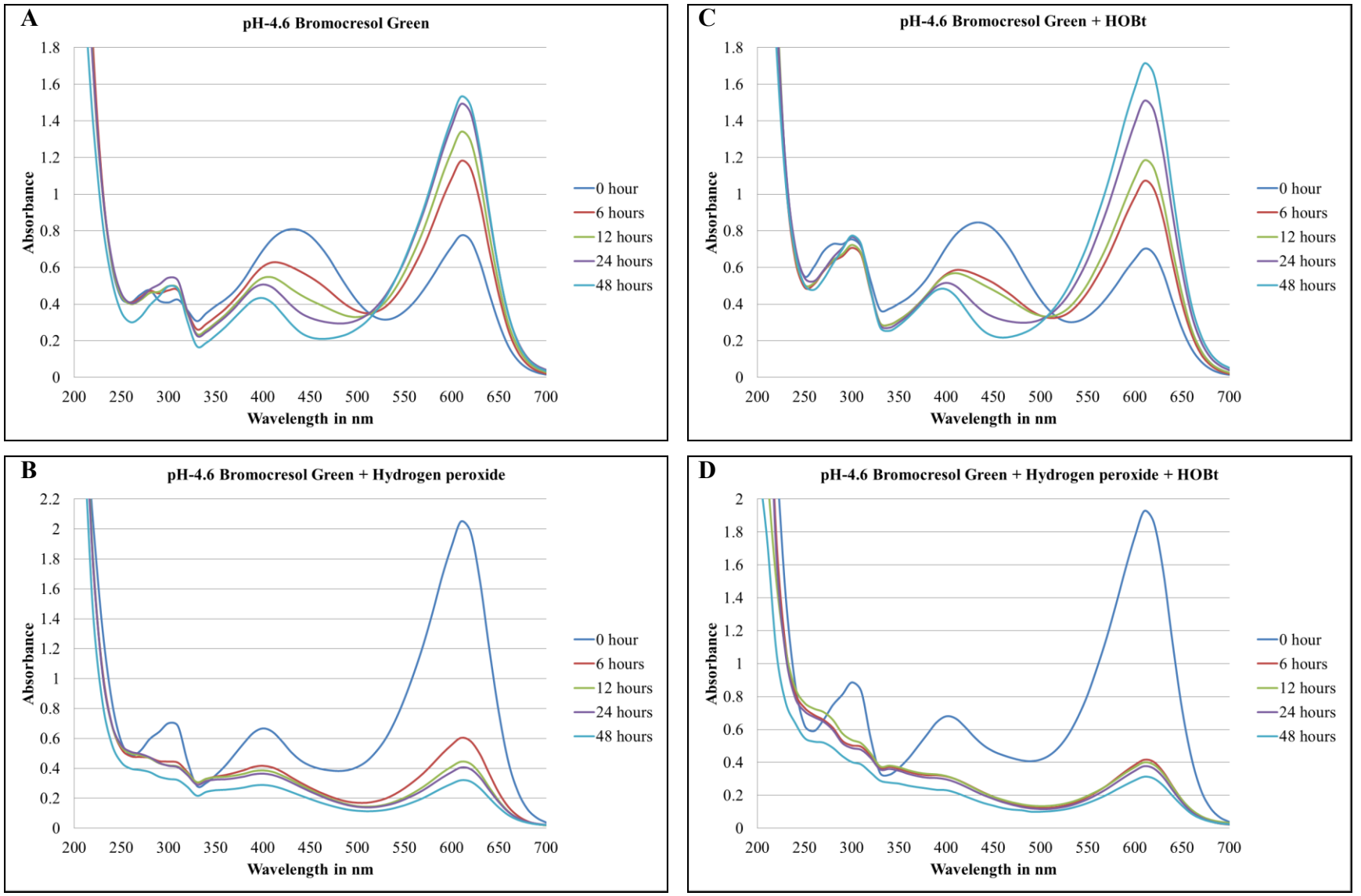


Figure 3.15 Spectra of Bromocresol Green exposed to *A. thaliana* plants in pH 4.6 phosphate buffer containing **A.** no additions; **B.** 1 mM hydrogen peroxide; **C.** 50 µM HOBt and **D.** combination of 1 mM hydrogen peroxide with 50 µM HOBt.

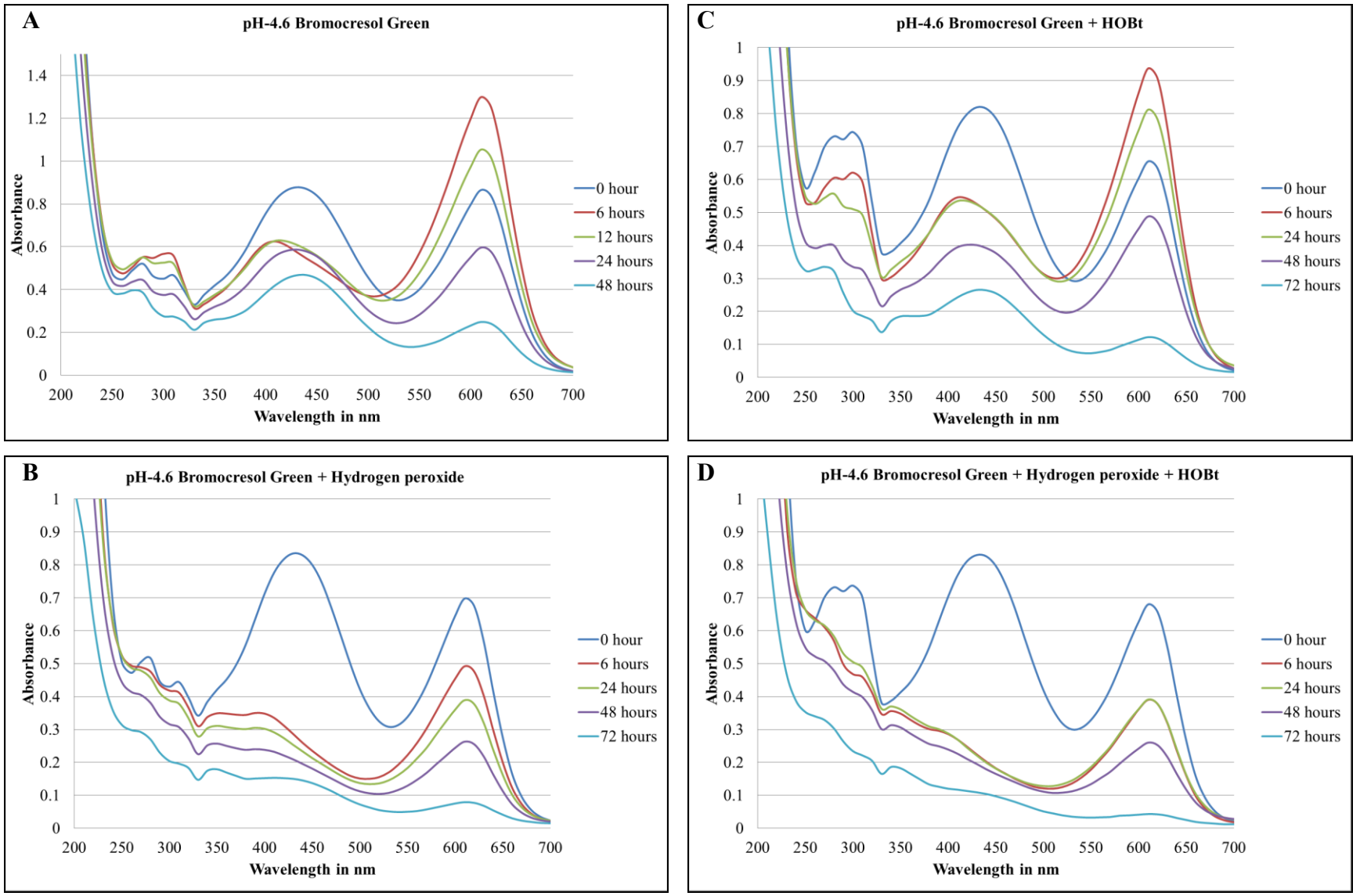


Figure 3.16 Spectra of Bromocresol Green exposed to sunflowers in pH 4.6 phosphate buffer containing **A.** no additions; **B.** 1 mM hydrogen peroxide; **C.** 50 µM HOBt and **D.** combination of 1 mM hydrogen peroxide with 50 µM HOBt.

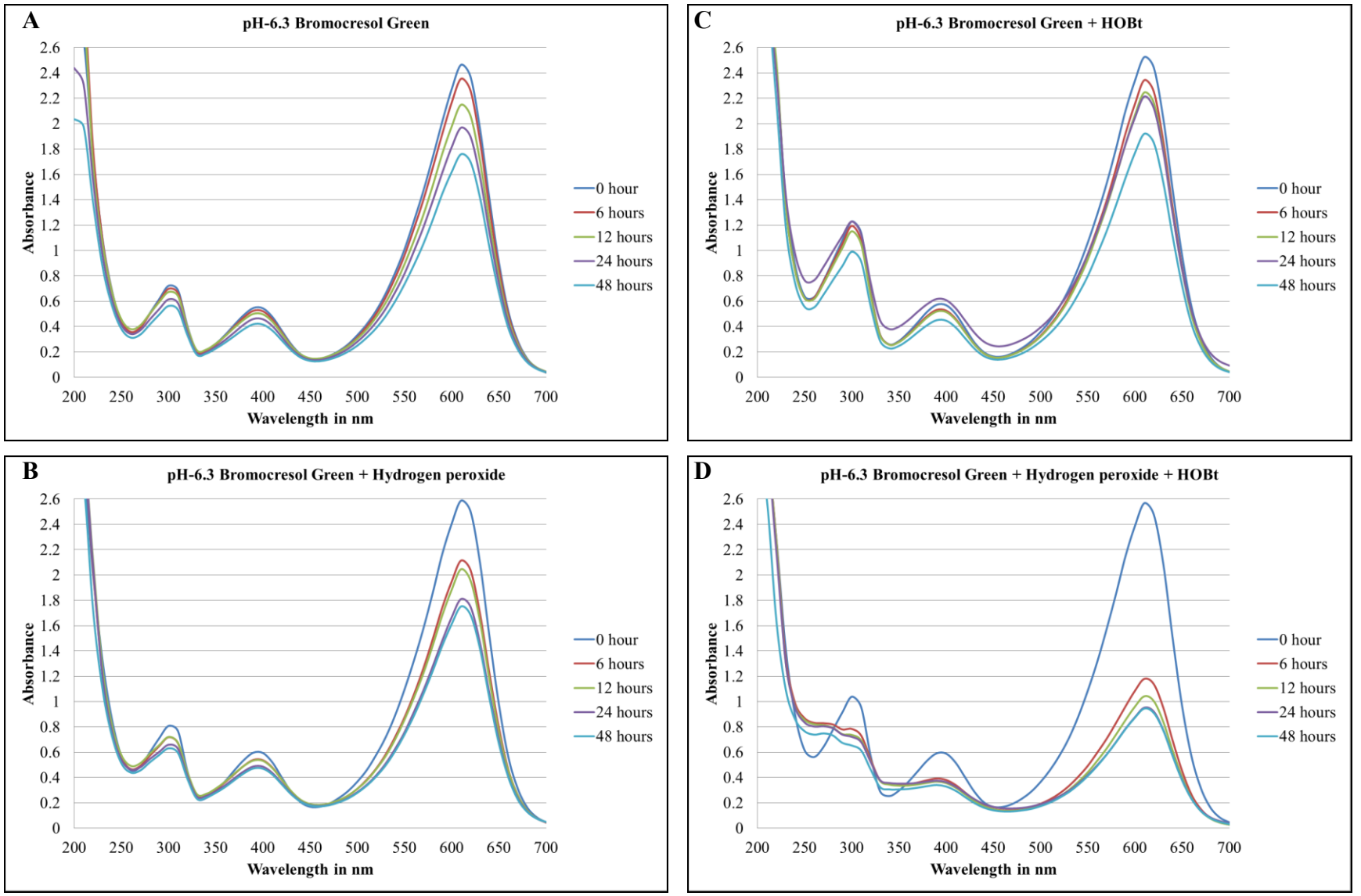


Figure 3.17 Spectra of Bromocresol Green exposed to *A. thaliana* plants in pH 6.3 phosphate buffer containing **A.** no additions; **B.** 1 mM hydrogen peroxide; **C.** 50 µM HOBt and **D.** combination of 1 mM hydrogen peroxide with 50 µM HOBt.

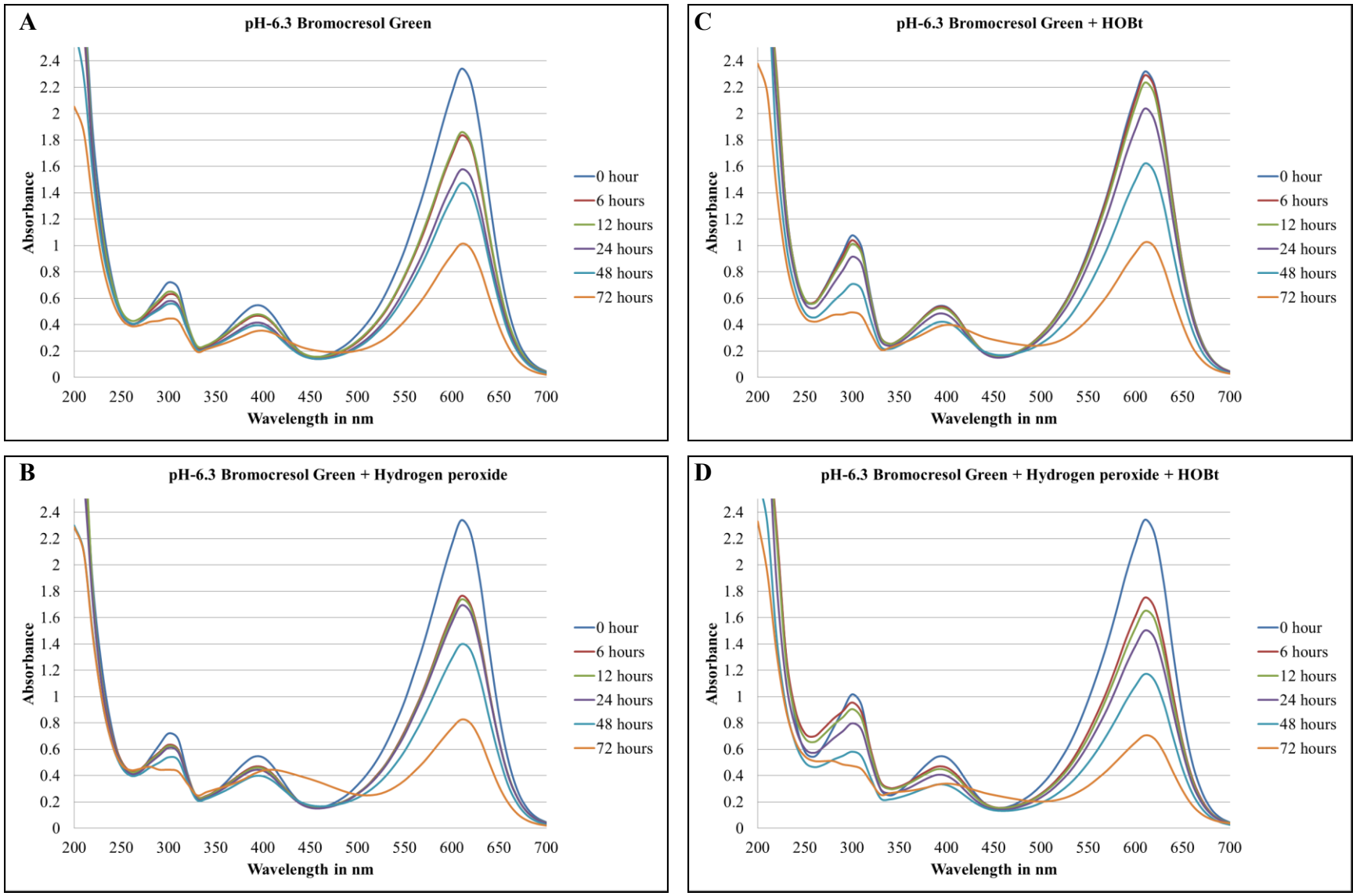


Figure 3.18 Spectra of Bromocresol Green exposed to sunflowers in pH 6.3 phosphate buffer containing **A.** no additions; **B.** 1 mM hydrogen peroxide; **C.** 50 µM HOBt and **D.** combination of 1 mM hydrogen peroxide with 50 µM HOBt.

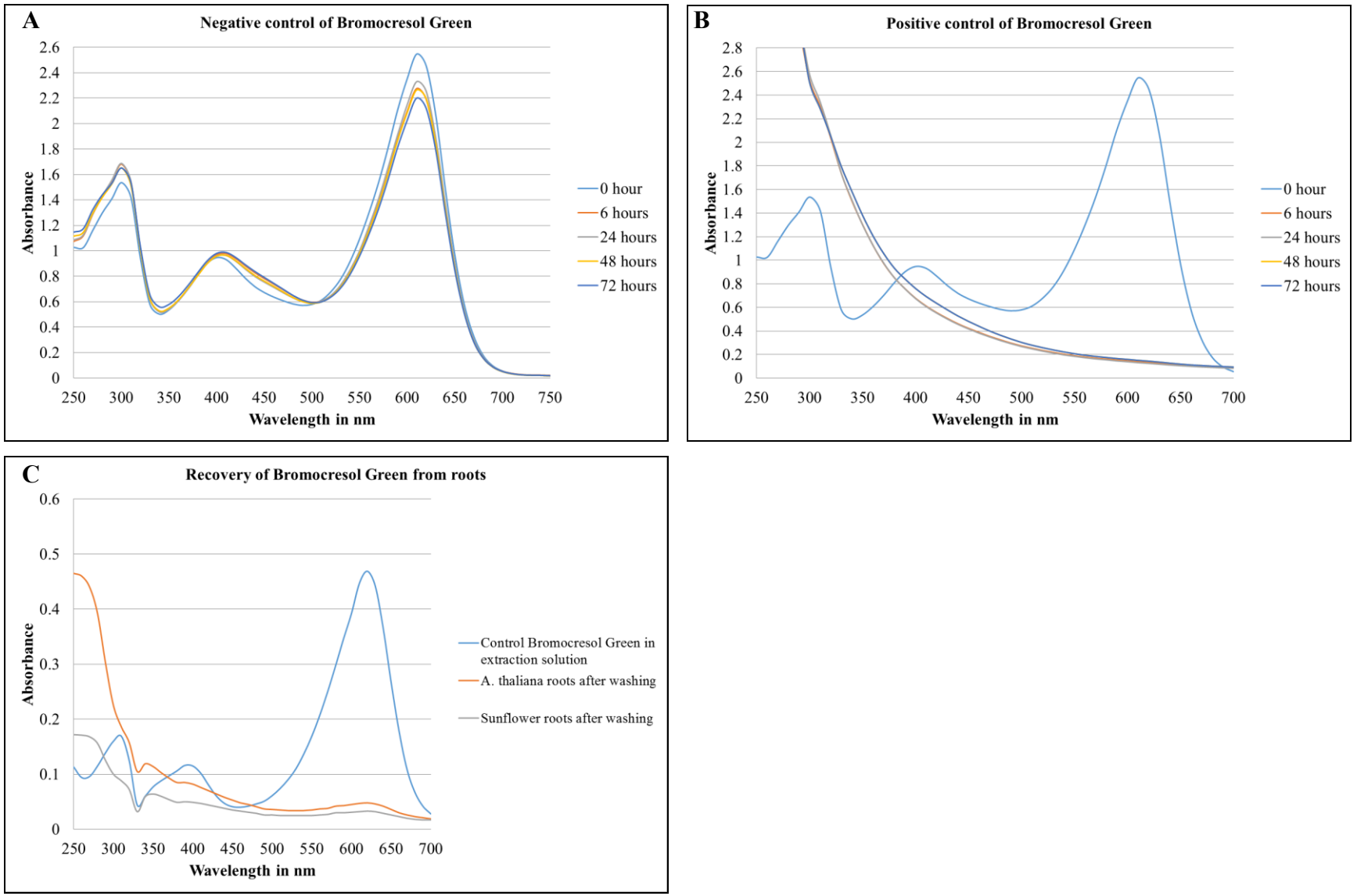


Figure 3.19 Spectra of **A.** Bromocresol Green not exposed to plants (Negative control); **B.** Bromocresol Green with crushed turnip root extract and 1 mM hydrogen peroxide plus 50 µM HOBt (Positive control) and **C.** Extraction solution of treated roots.

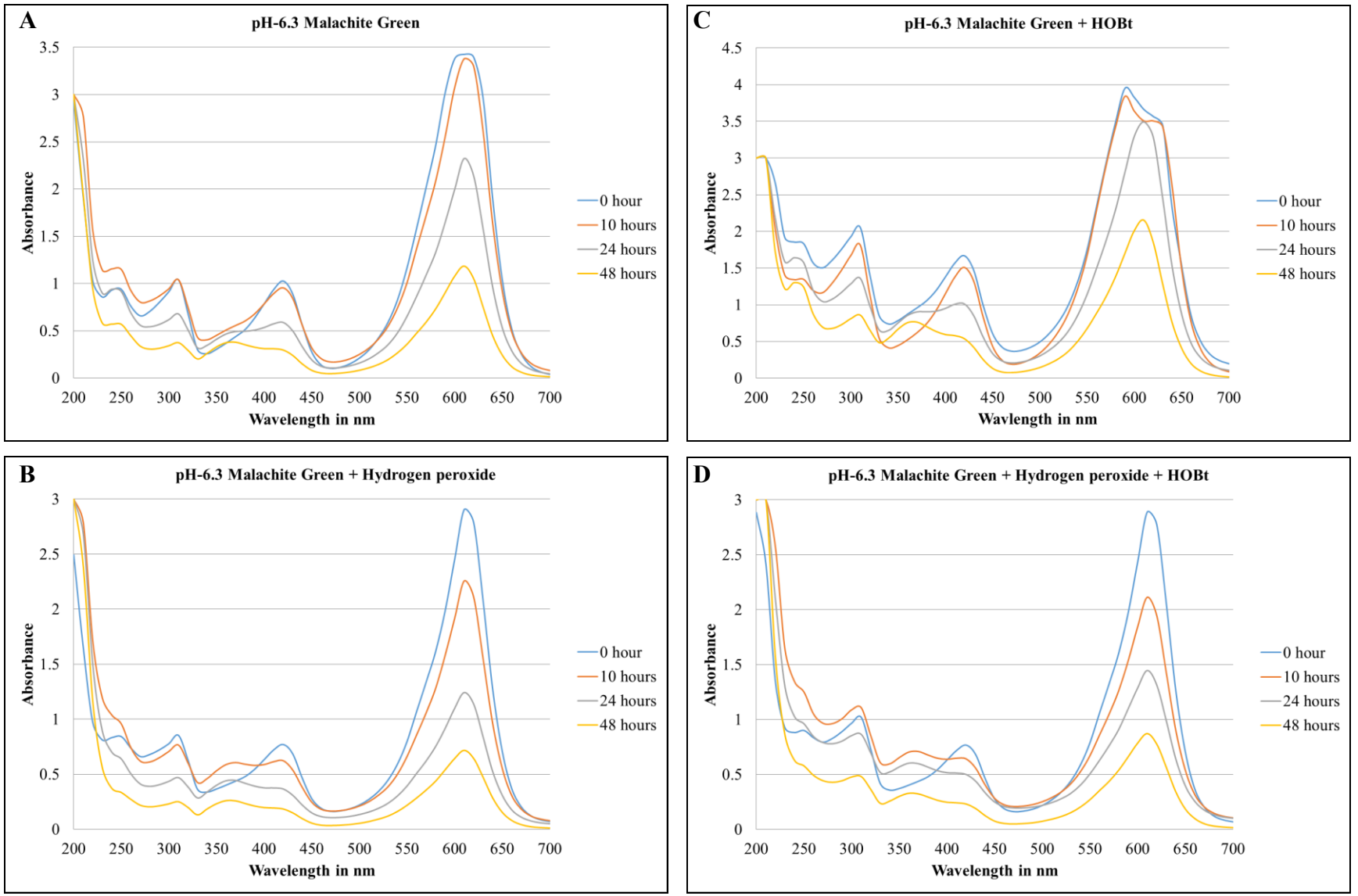


Figure 3.20 Spectra of Malachite Green exposed to *A. thaliana* plants in pH 6.3 phosphate buffer containing **A.** no additions; **B.** 1 mM hydrogen peroxide; **C.** 50 µM HOBt and **D.** combination of 1 mM hydrogen peroxide with 50 µM HOBt.

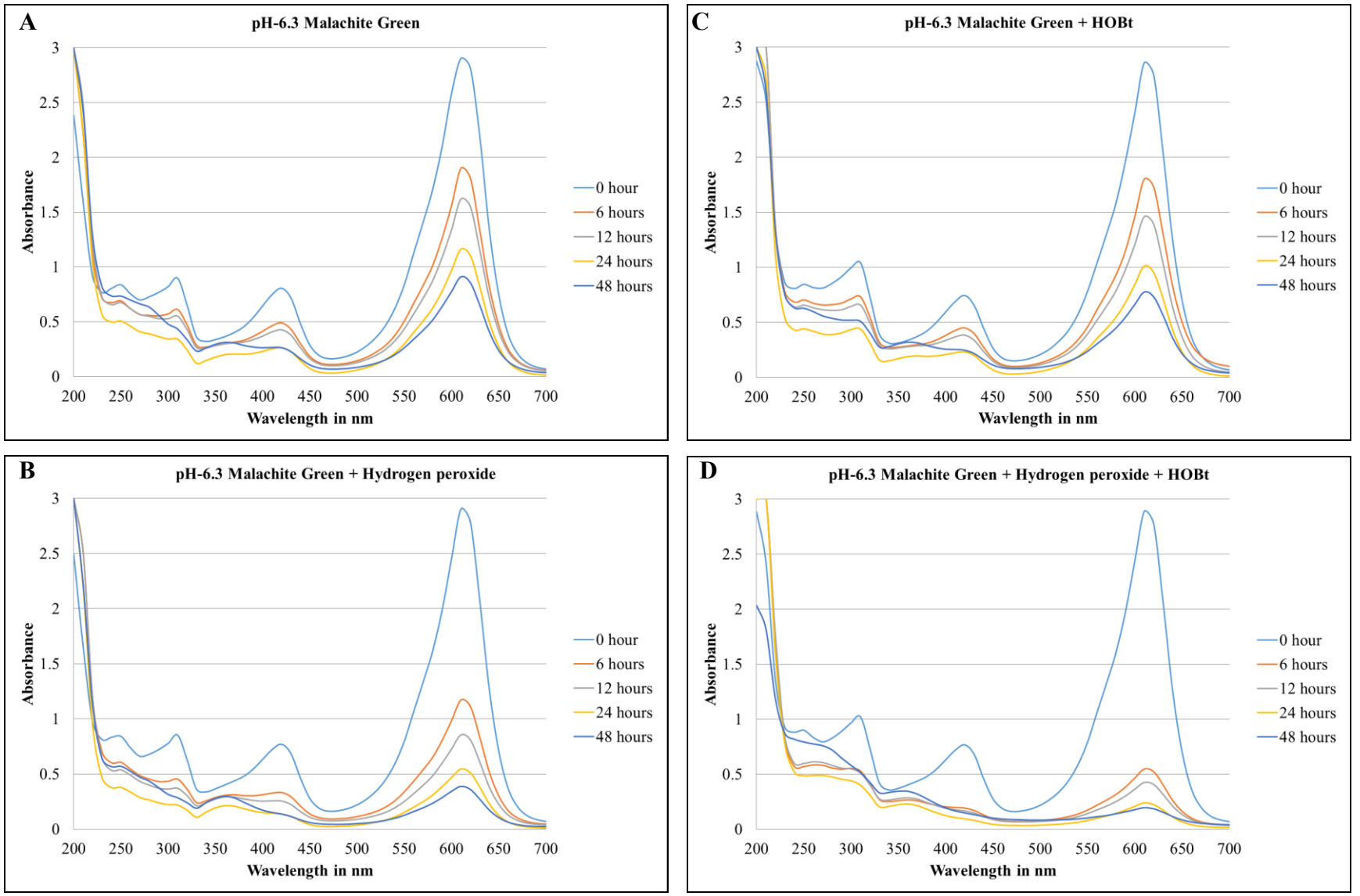


Figure 3.21 Spectra of Malachite Green exposed to sunflowers in pH 6.3 phosphate buffer containing **A.** no additions; **B.** 1 mM hydrogen peroxide; **C.** 50 µM HOBt and **D.** combination of 1 mM hydrogen peroxide with 50 µM HOBt.

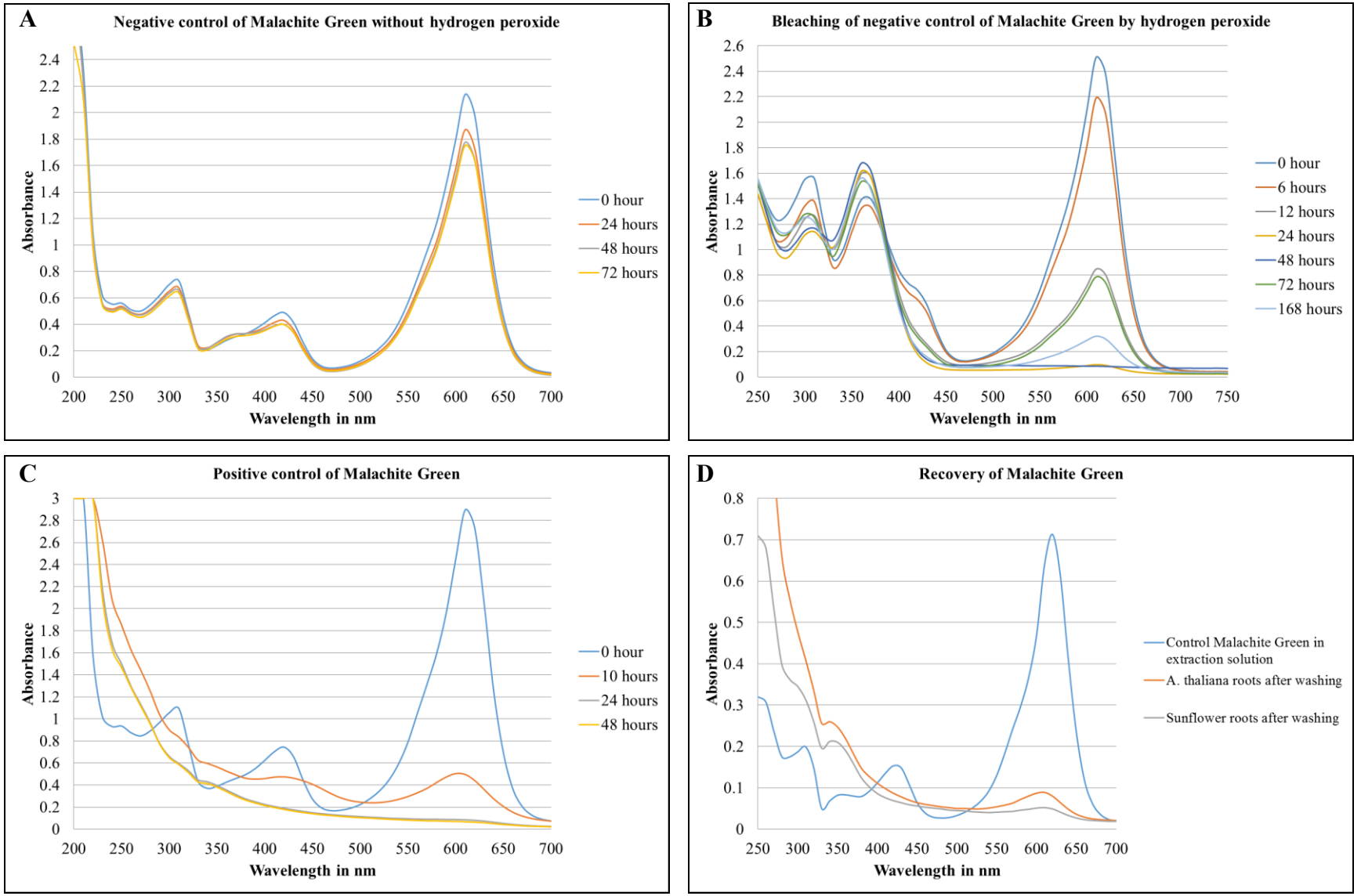


Figure 3.22 Spectra of Negative control of Malachite Green **A.** without hydrogen peroxide **B.** with hydrogen peroxide; **C.** Positive control of Malachite Green and **D.** Washing of the roots with the extraction solution after exposure to Malachite Green.

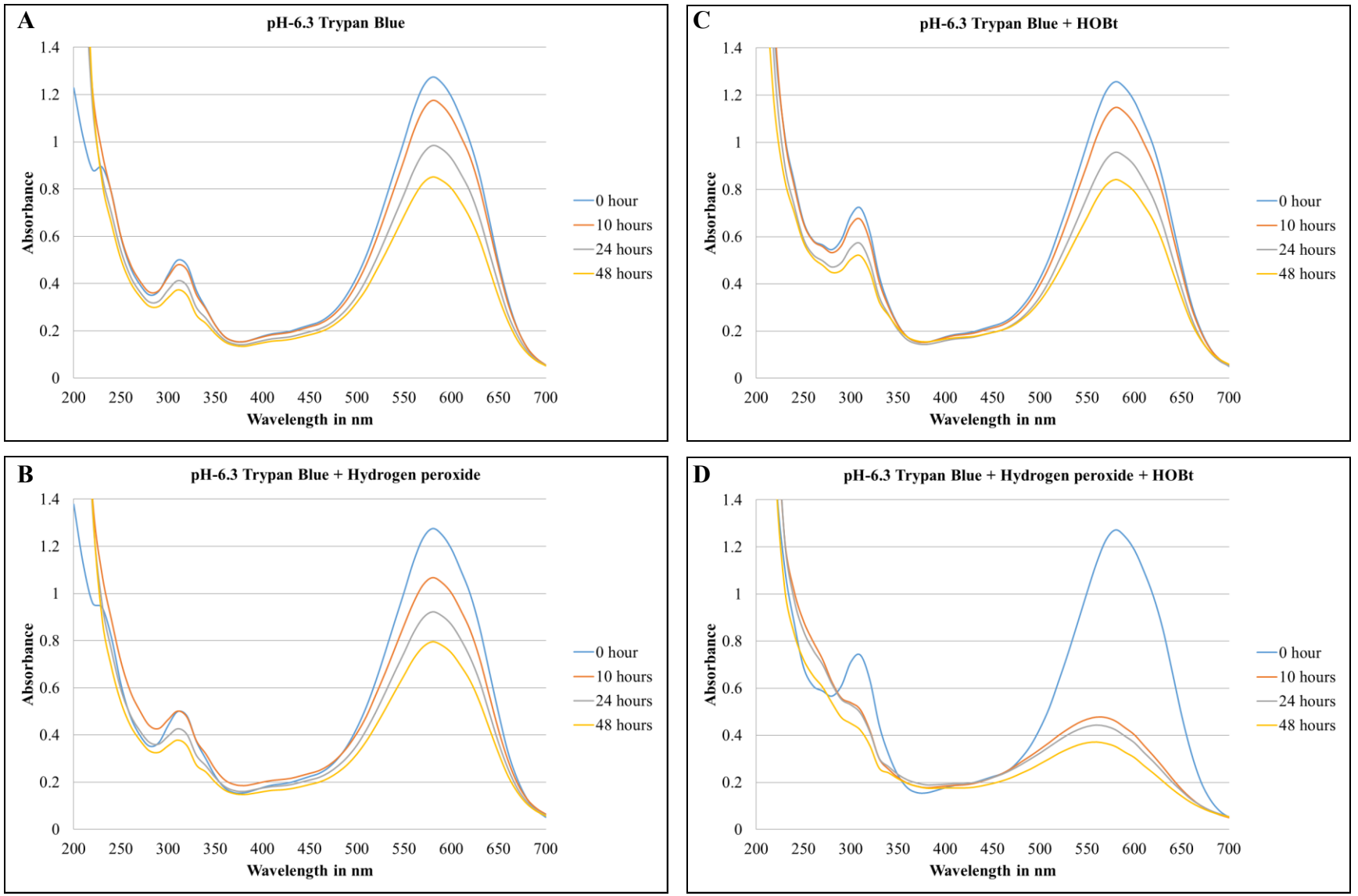


Figure 3.23 Spectra of Trypan Blue exposed to *A. thaliana* plants in pH 6.3 phosphate buffer containing **A.** no additions; **B.** 1 mM hydrogen peroxide; **C.** 50 µM HOBt and **D.** combination of 1 mM hydrogen peroxide with 50 µM HOBt.

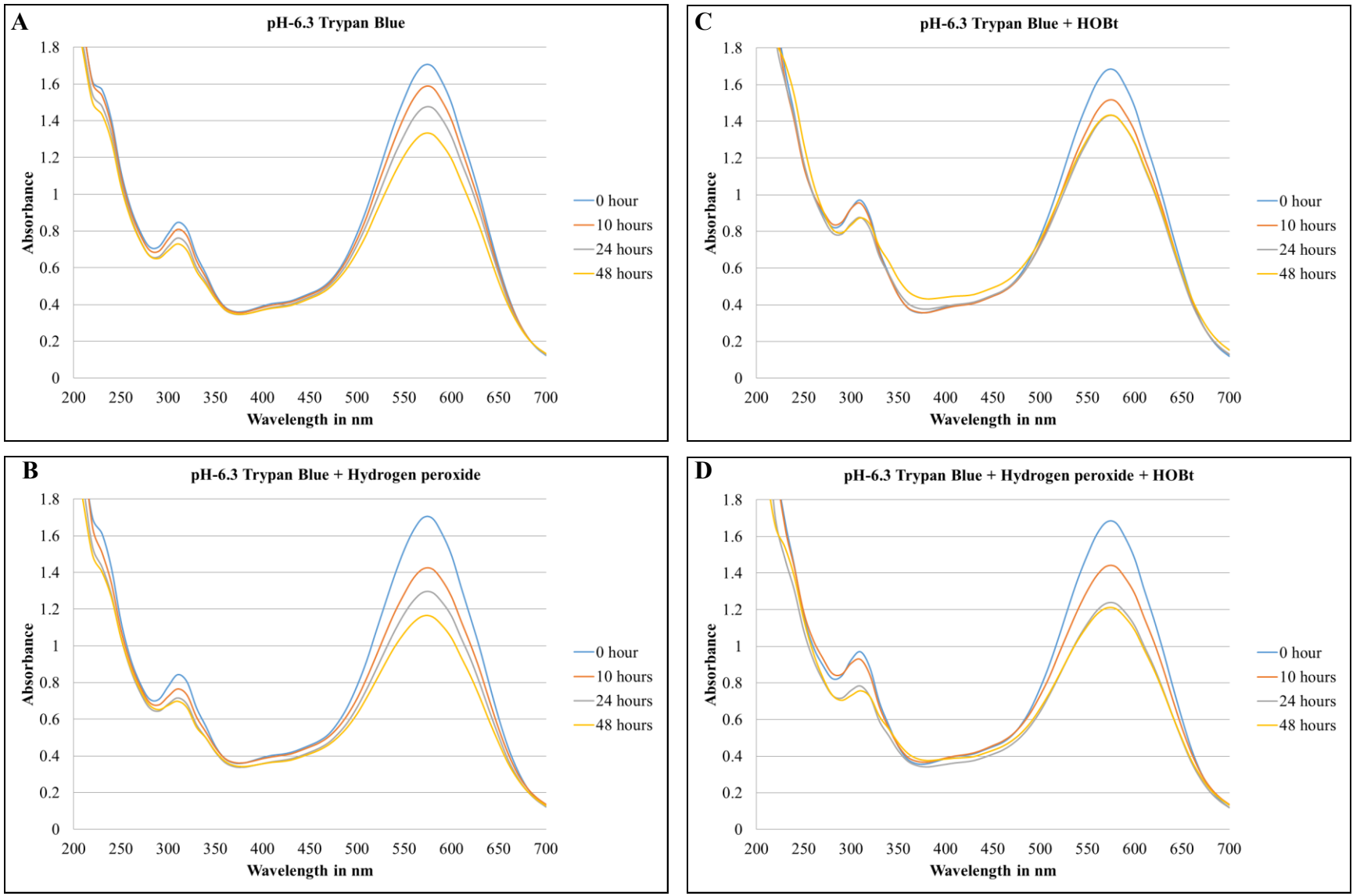


Figure 3.24 Spectra of Trypan Blue exposed to sunflowers in pH 6.3 phosphate buffer containing **A.** no additions; **B.** 1 mM hydrogen peroxide; **C.** 50 µM HOBt and **D.** combination of 1 mM hydrogen peroxide with 50 µM HOBt.

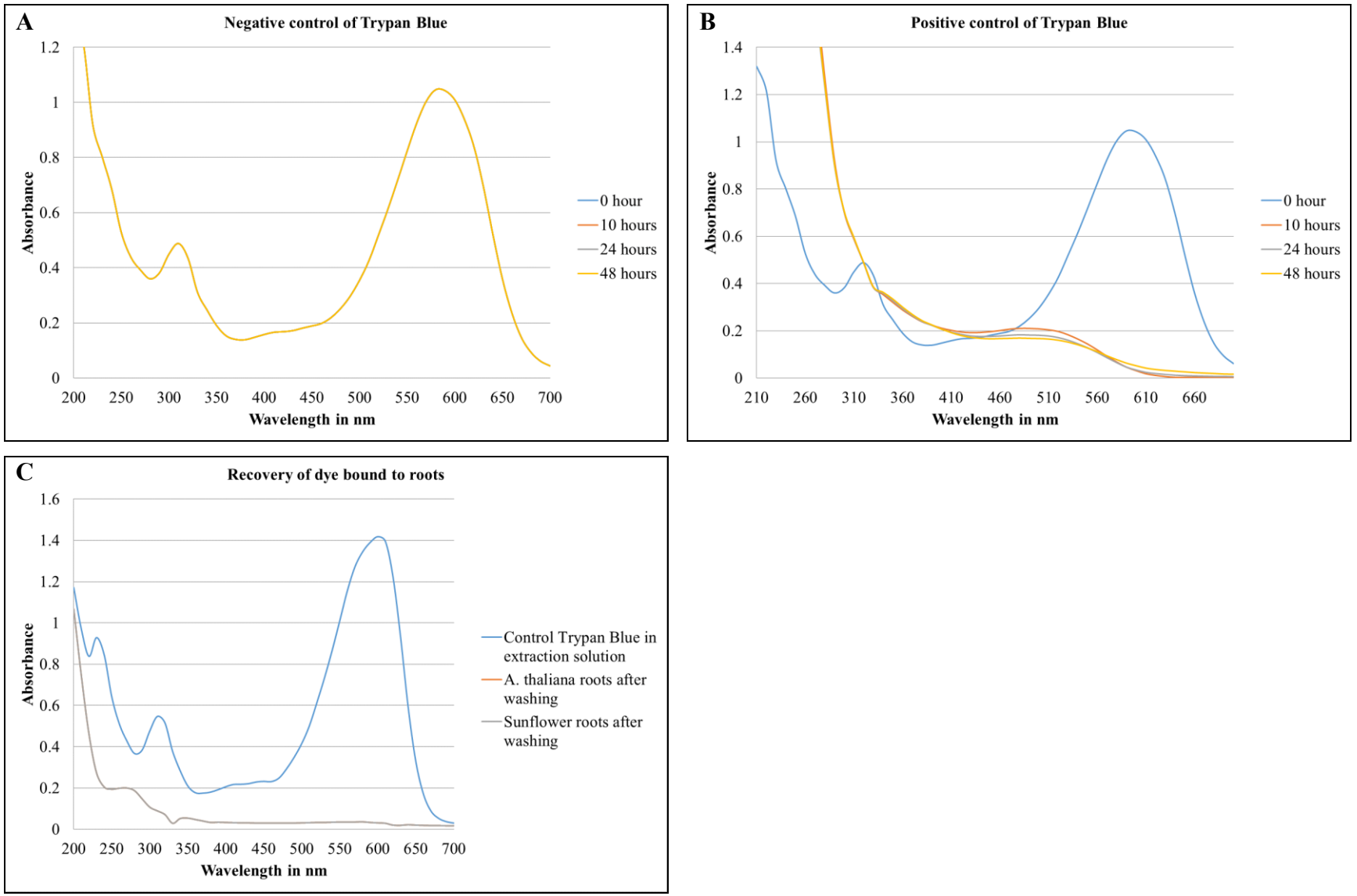


Figure 3.25 Spectra of **A.** Trypan Blue not exposed to plants (Negative control); **B.** Trypan Blue with crushed turnip root extract and 1 mM hydrogen peroxide plus 50 µM HOBt (Positive control) and **C.** Extraction solution of treated roots.

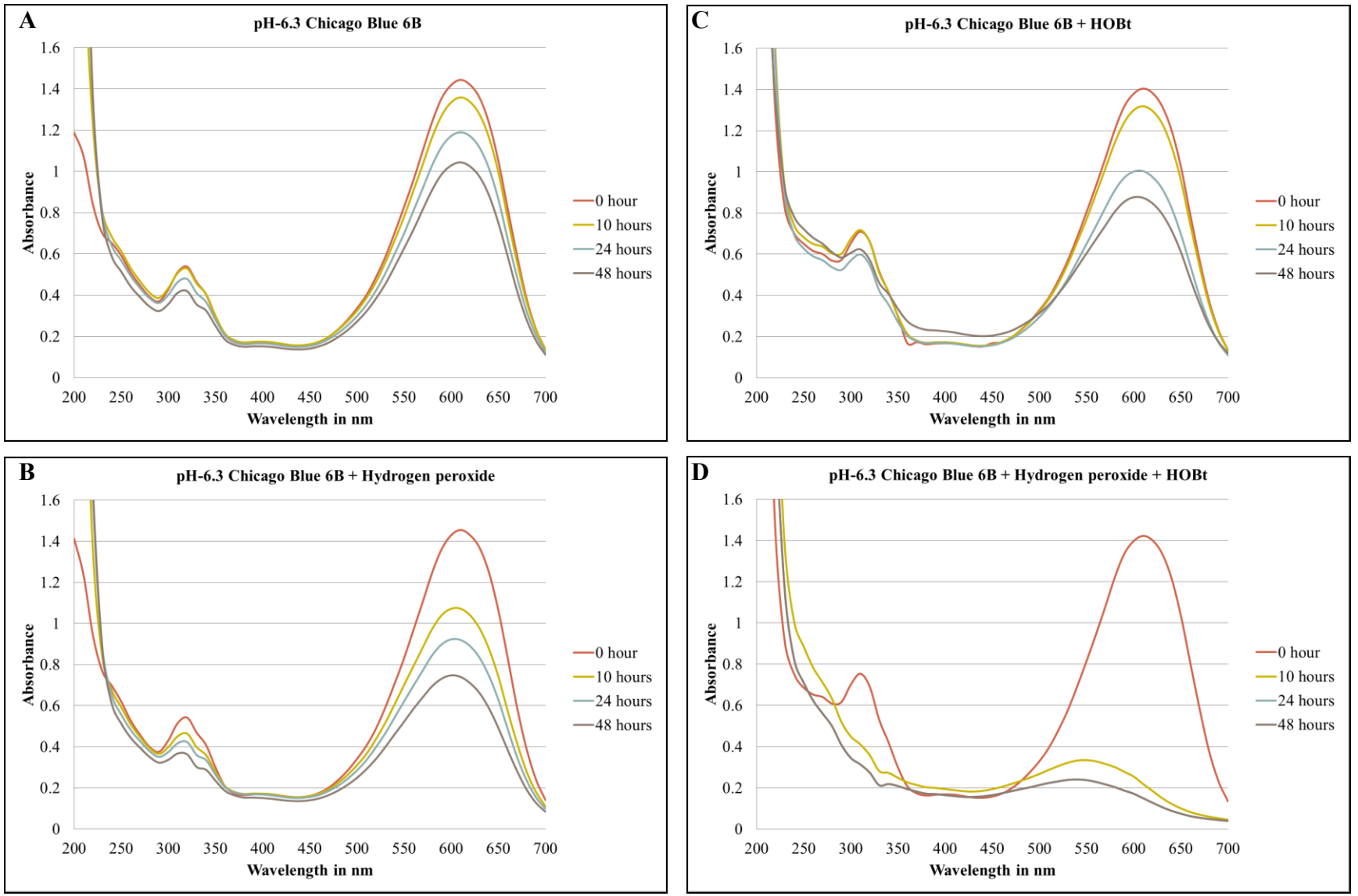


Figure 3.26 Spectra of Chicago Blue 6B exposed to *A. thaliana* plants in pH 6.3 phosphate buffer containing **A.** no additions; **B.** 1 mM hydrogen peroxide; **C.** 50 µM HOBt and **D.** combination of 1 mM hydrogen peroxide with 50 µM HOBt.

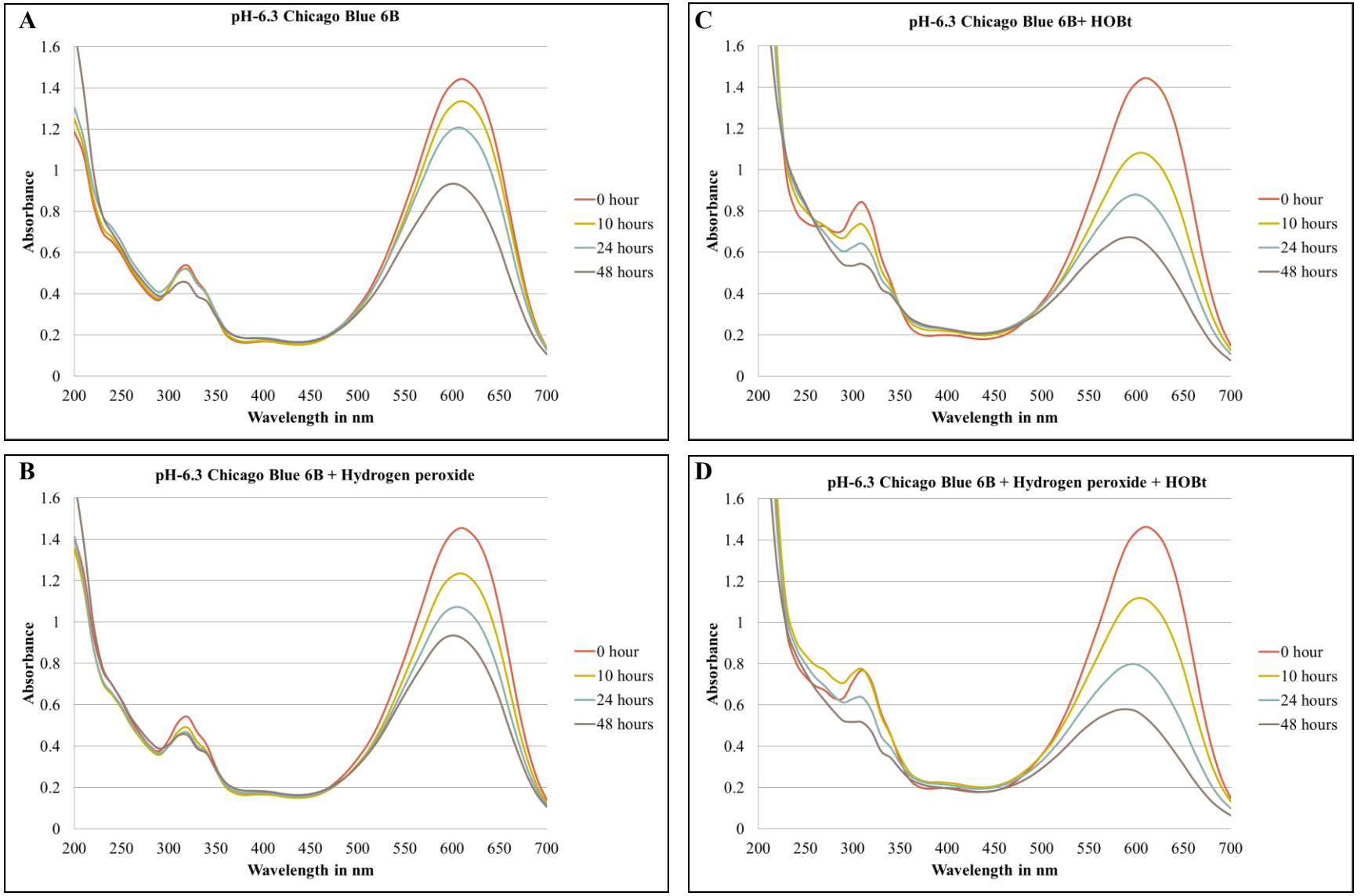


Figure 3.27 Spectra of Chicago Blue 6B exposed to sunflowers in pH 6.3 phosphate buffer containing **A.** no additions; **B.** 1 mM hydrogen peroxide; **C.** 50 µM HOBt and **D.** combination of 1 mM hydrogen peroxide with 50 µM HOBt.

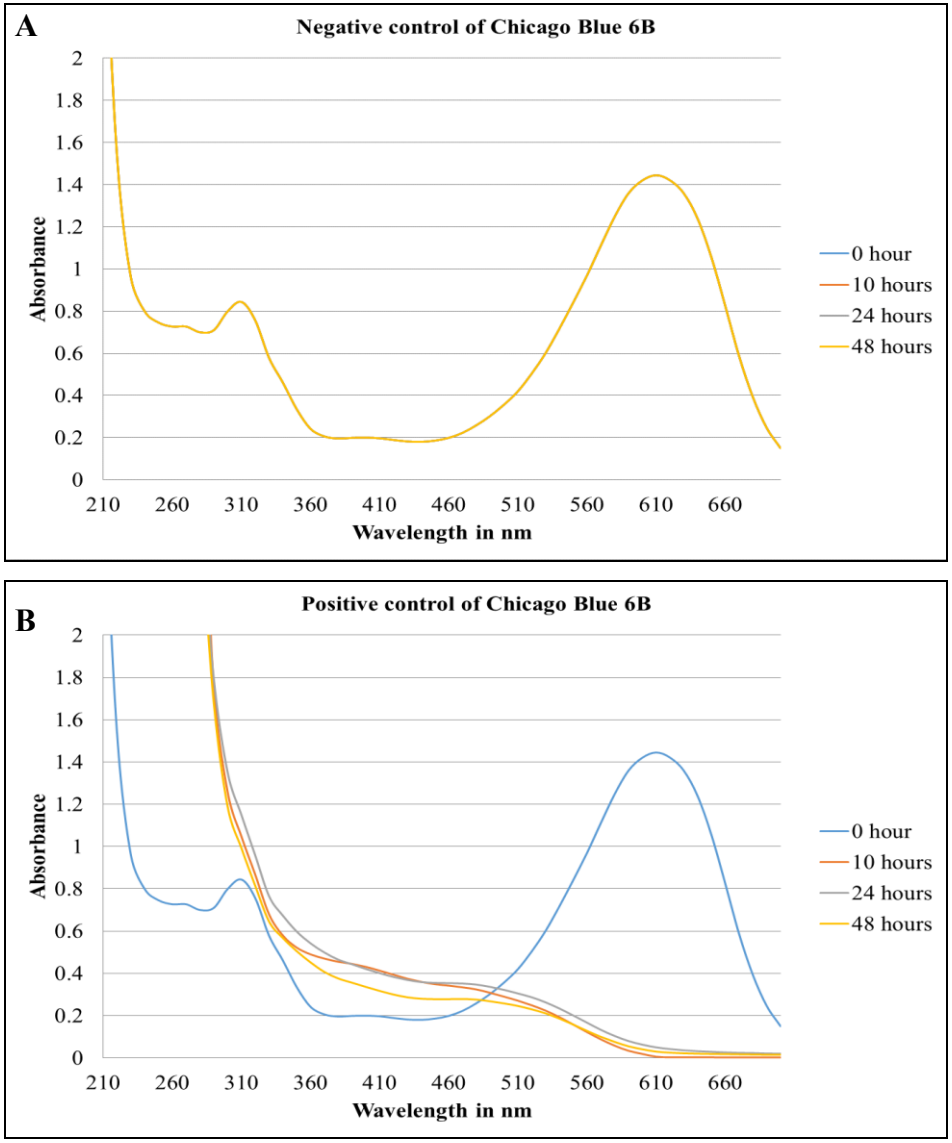


Figure 3.28 Spectra of **A.** Chicago Blue 6B not exposed to plants (Negative control); **B.** Chicago Blue 6B with crushed turnip root extract and 1 mM hydrogen peroxide plus 50 µM HOBt (Positive control).

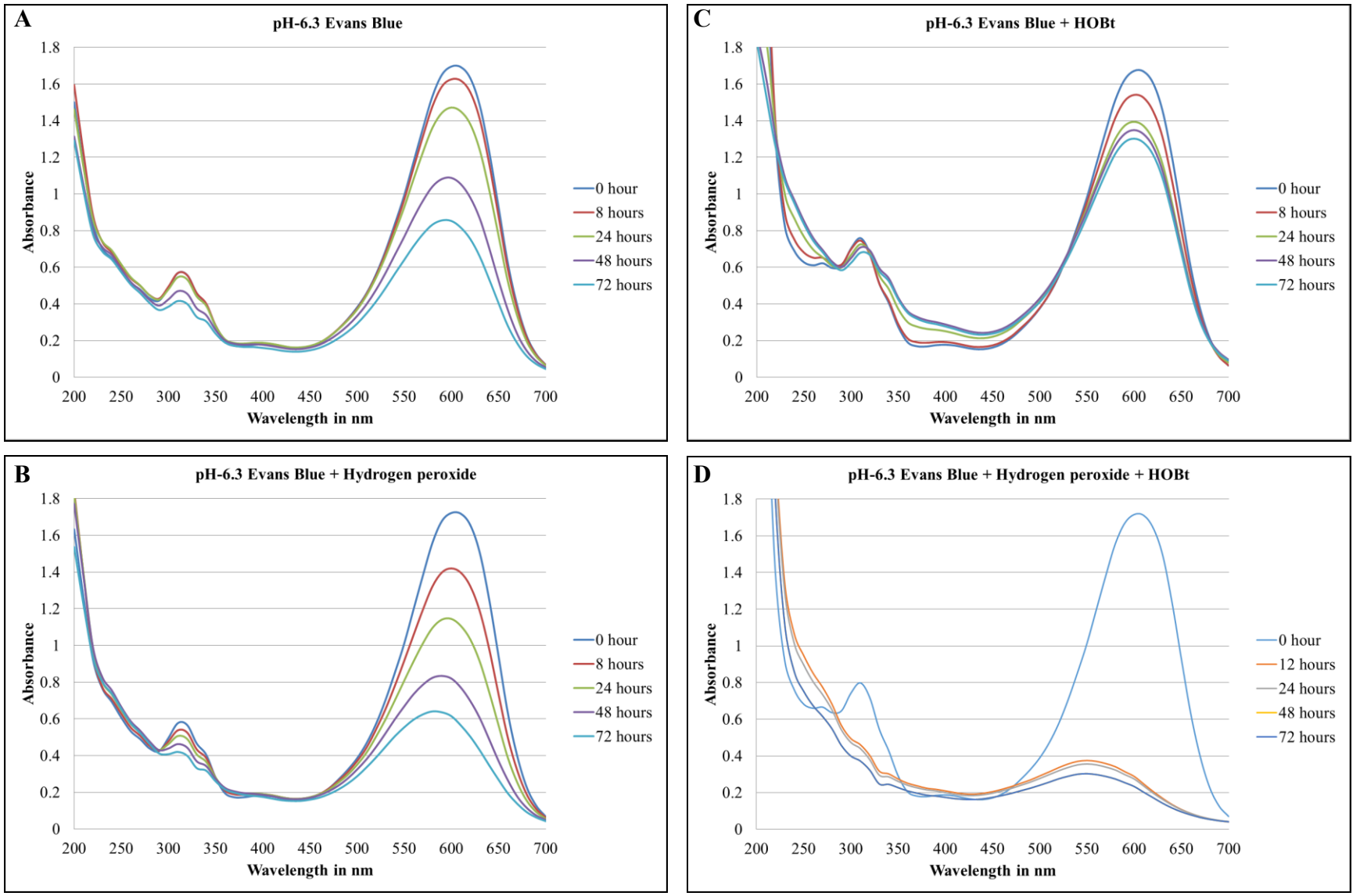


Figure 3.29 Spectra of Evans Blue exposed to *A. thaliana* plants in pH 6.3 phosphate buffer containing **A.** no additions; **B.** 1 mM hydrogen peroxide; **C.** 50 µM HOBt and **D.** combination of 1 mM hydrogen peroxide with 50 µM HOBt.

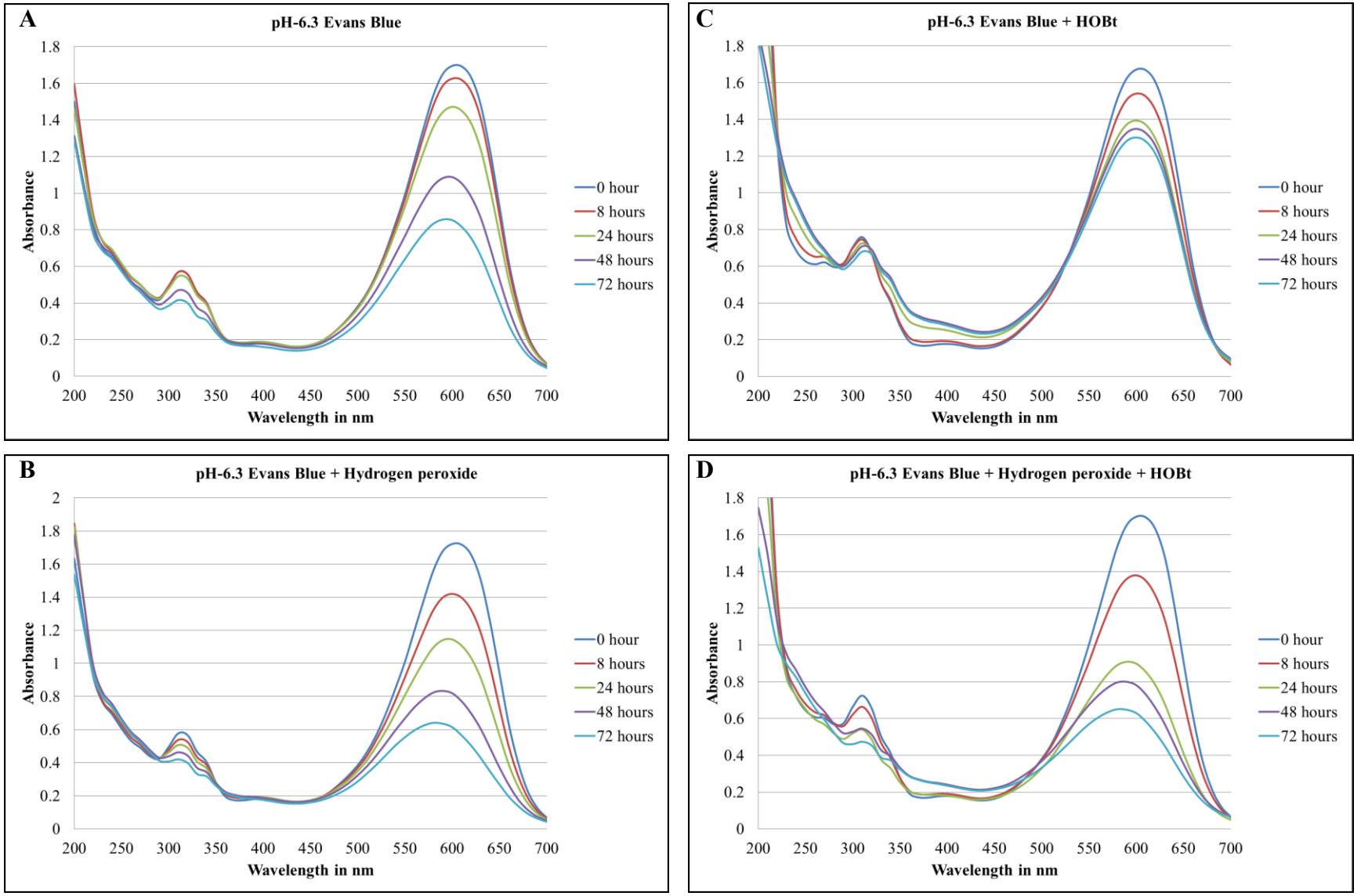


Figure 3.30 Spectra of Evans Blue exposed to sunflowers in pH 6.3 phosphate buffer containing **A.** no additions; **B.** 1 mM hydrogen peroxide; **C.** 50 µM HOBt and **D.** combination of 1 mM hydrogen peroxide with 50 µM HOBt.

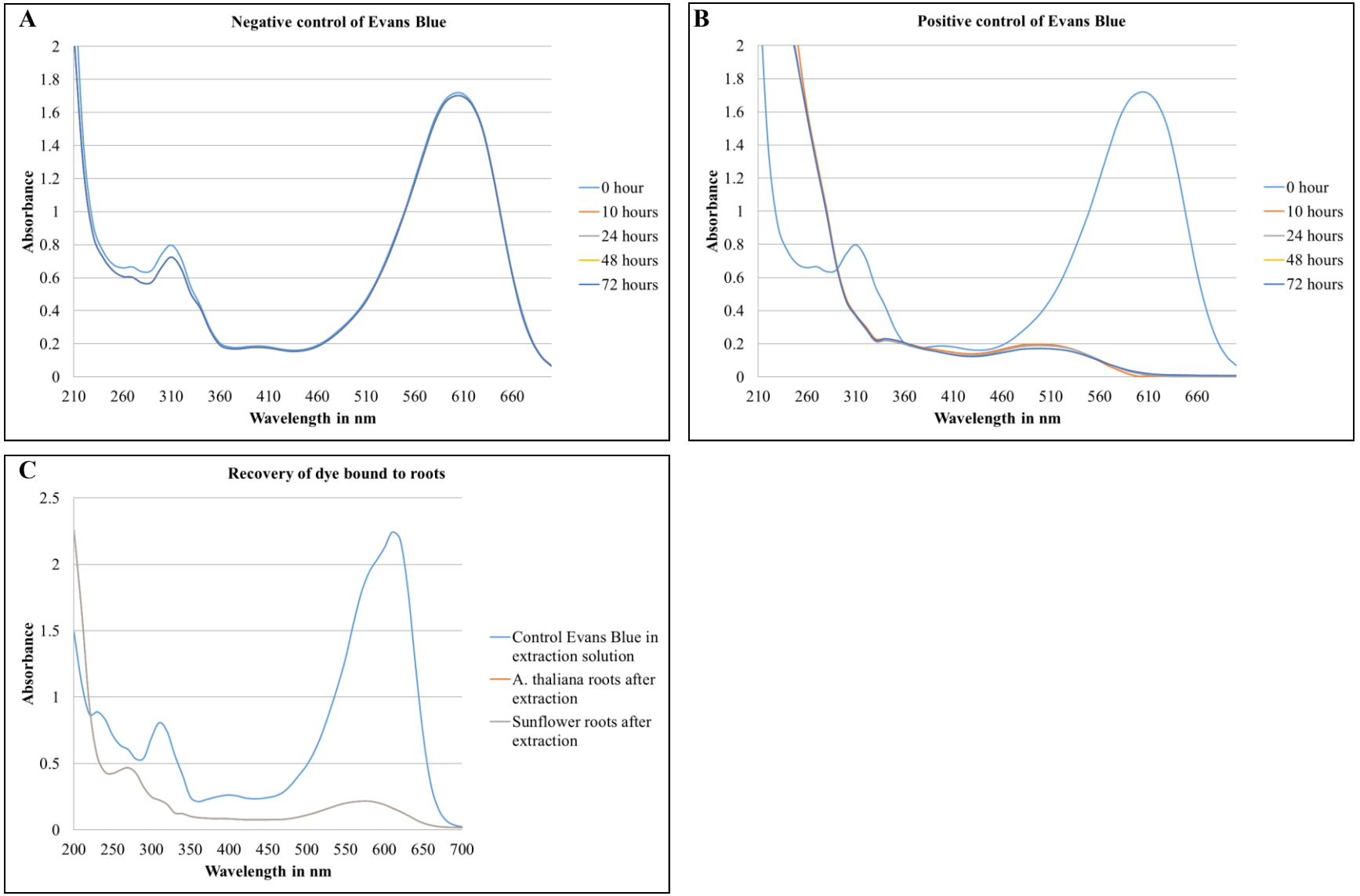


Figure 3.31 Spectra of **A.** Evans Blue not exposed to plants (Negative control); **B.** Evans Blue with crushed turnip root extract and 1 mM hydrogen peroxide plus 50 µM HOBt (Positive control) and **C.** Extraction solution of treated roots.

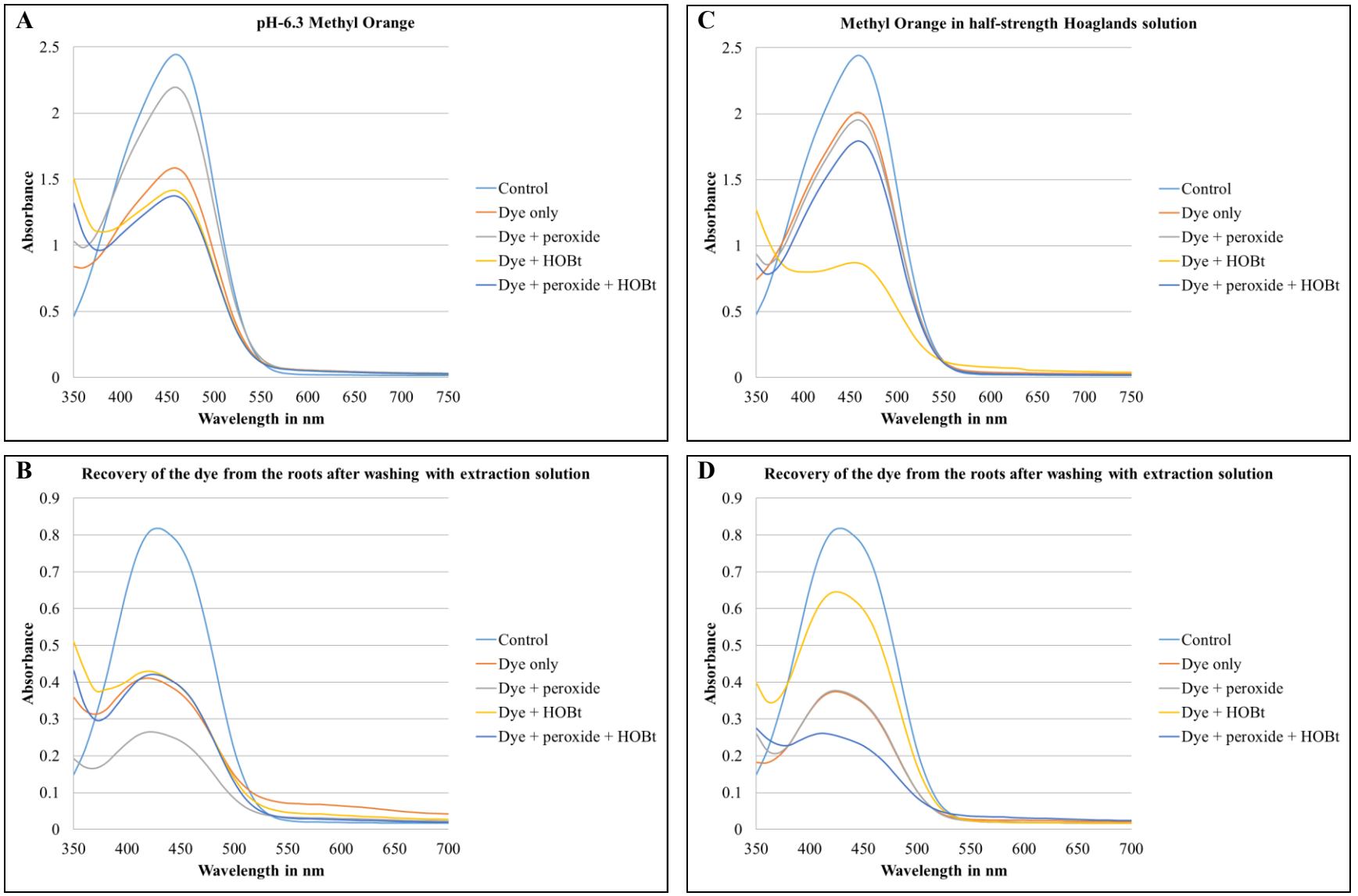


Figure 3.32 A. Exposure of pH 6.3 Methyl Orange to dried sunflower roots and **B.** Washing of roots with extraction solution; **C.** Exposure of Methyl Orange in Hoagland's solution to dried sunflower roots and **D.** Washing of roots with extraction solution.

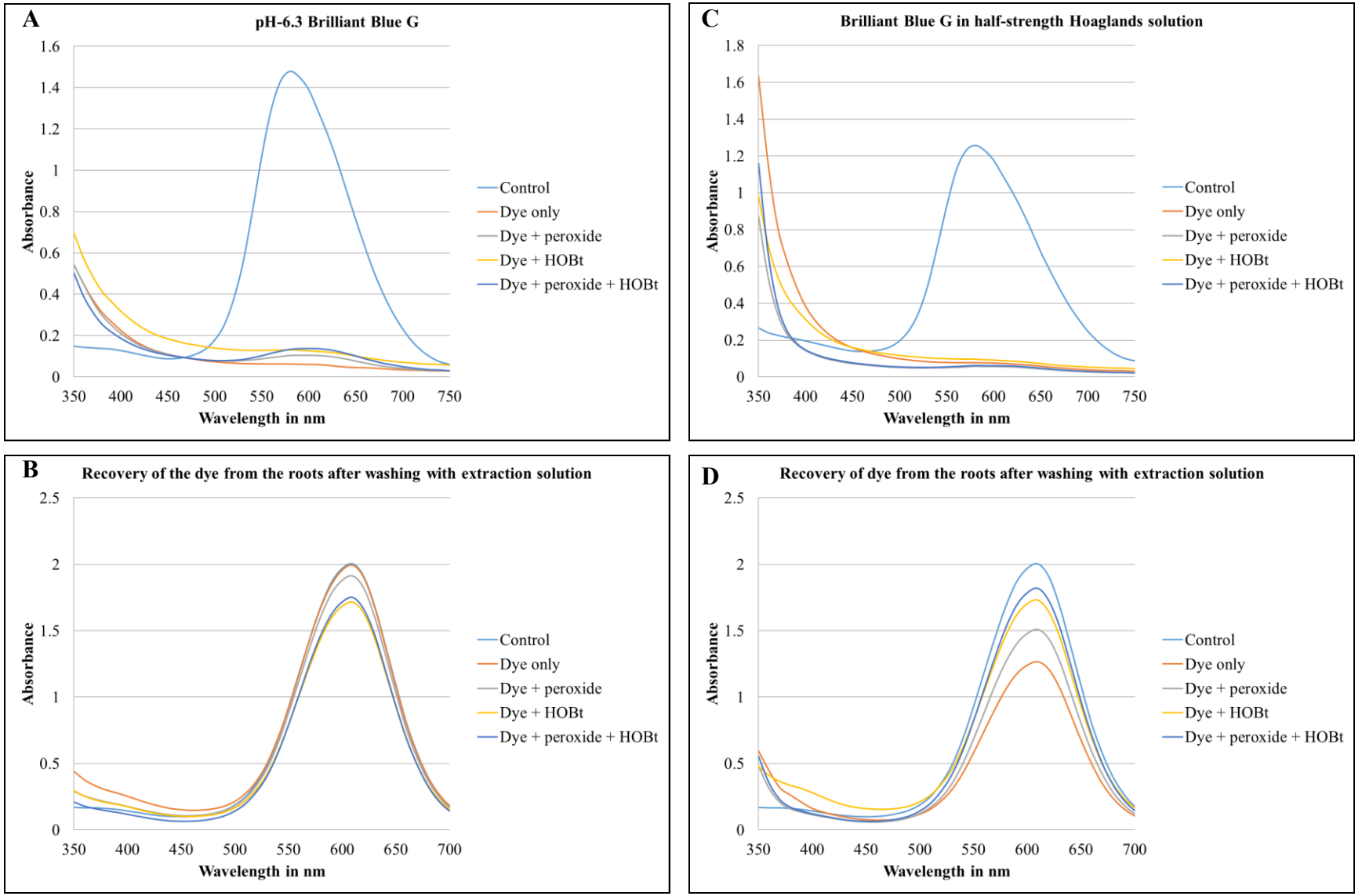


Figure 3.33 A. Exposure of pH 6.3 Brilliant Blue G to dried sunflower roots and **B.** Washing of roots with extraction solution; **C.** Exposure of Brilliant Blue G in Hoagland's solution to dried sunflower roots and **D.** Washing of roots with extraction solution.

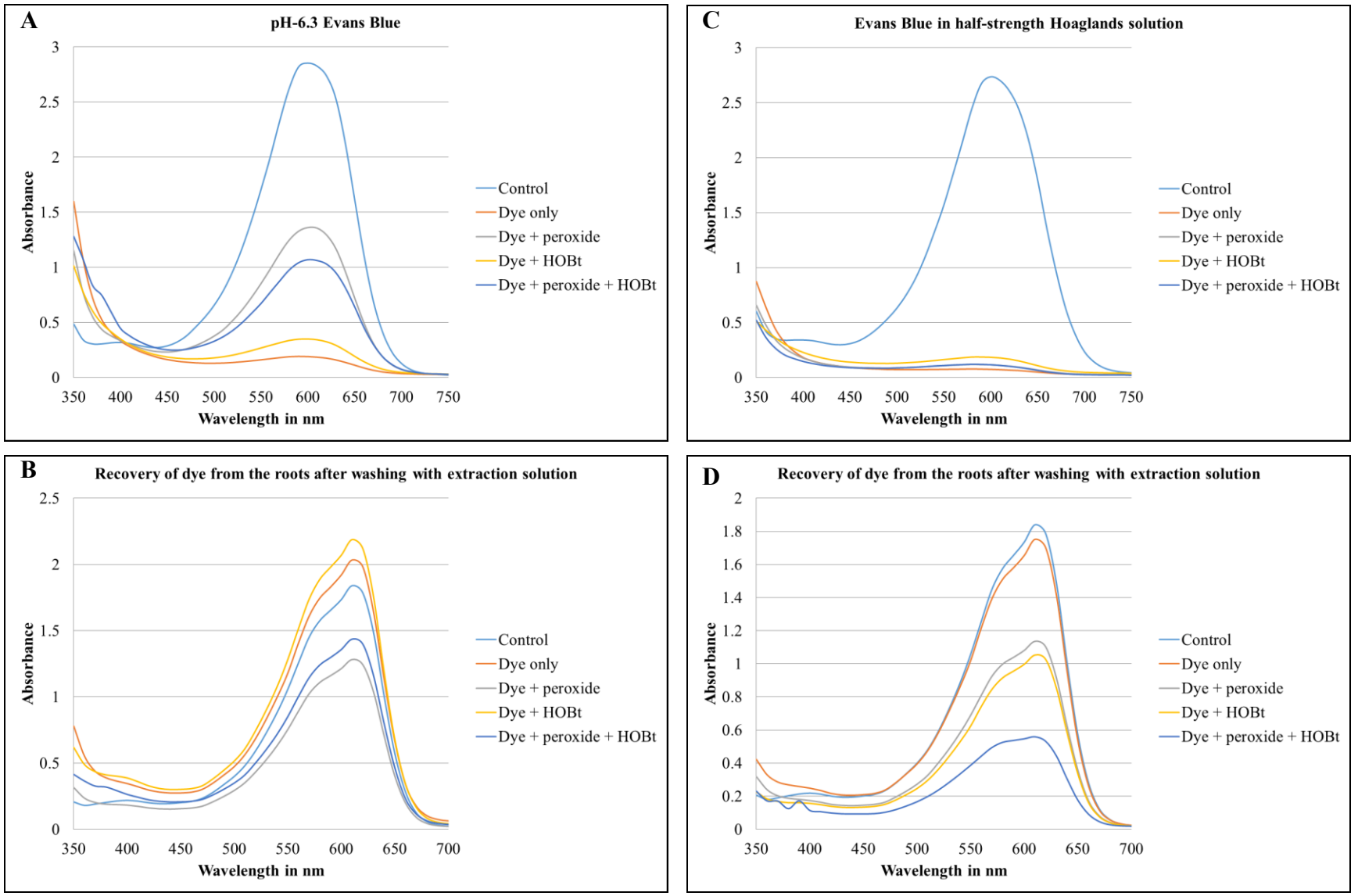


Figure 3.34 A. Exposure of pH 6.3 Evans Blue to dried sunflower roots and **B.** Washing of roots with extraction solution; **C.** Exposure of Evans Blue in Hoagland's solution to dried sunflower roots and **D.** Washing of roots with extraction solution.

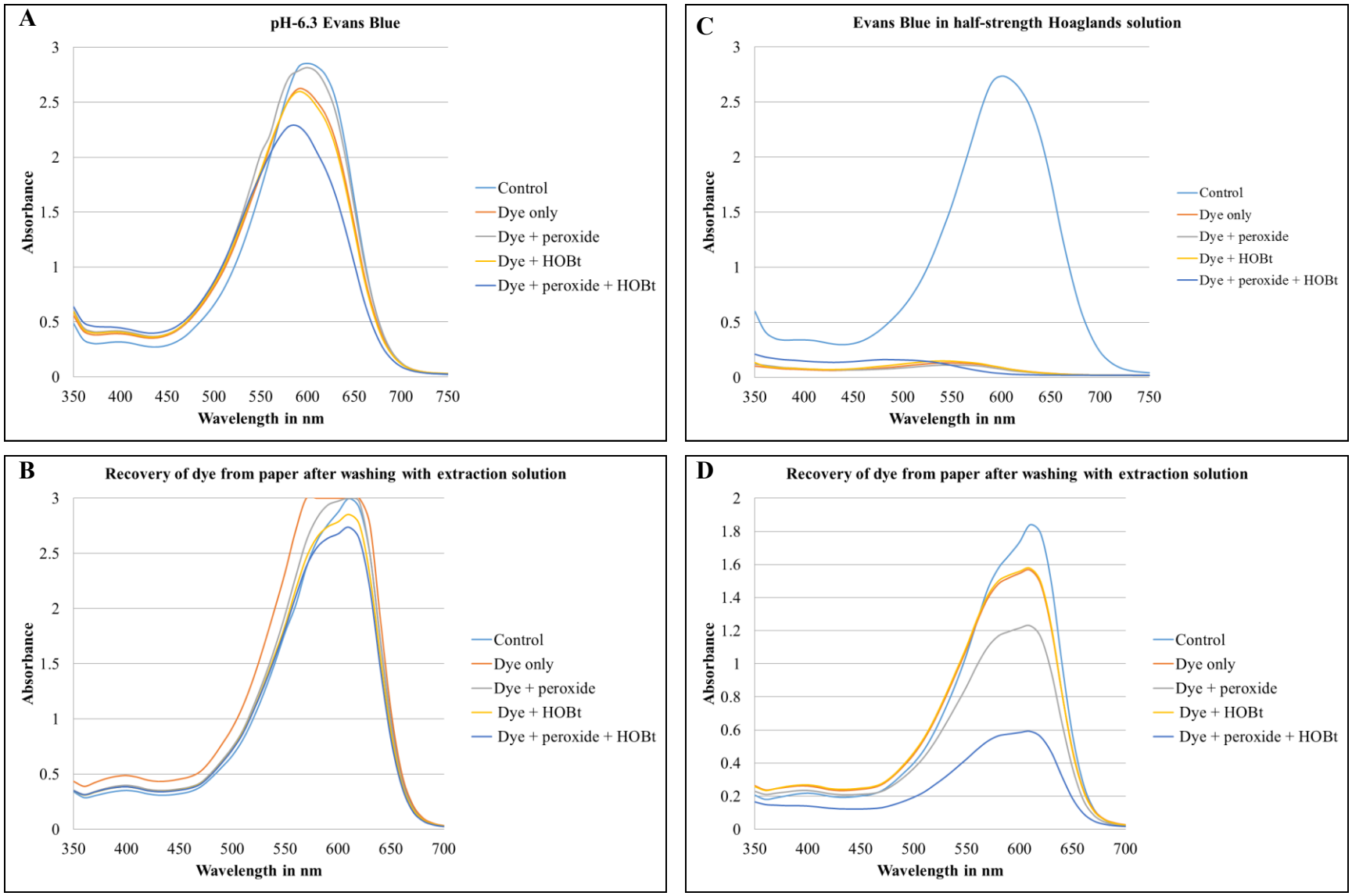


Figure 3.35 A. Exposure of pH 6.3 Evans Blue to Whatman paper # 1 and **B.** Washing of papers with extraction solution; **C.** Exposure of Evans Blue in Hoagland's solution to Whatman paper # 1 and **D.** Washing of papers with extraction solution.

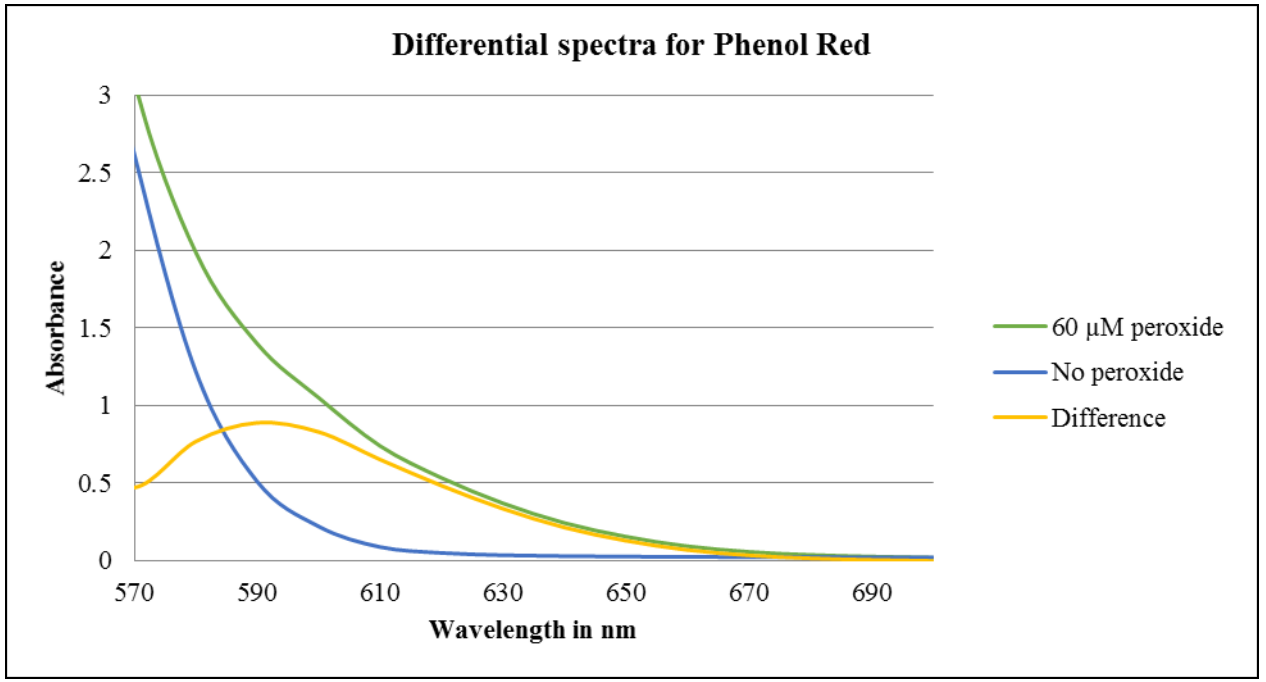


Figure 3.36 Differential spectra from 570-700 nm obtained for interaction of Phenol Red dye with added hydrogen peroxide plus a catalyst-peroxidase. 610 nm was selected as the wavelength to detect the oxidized Phenol Red dye.

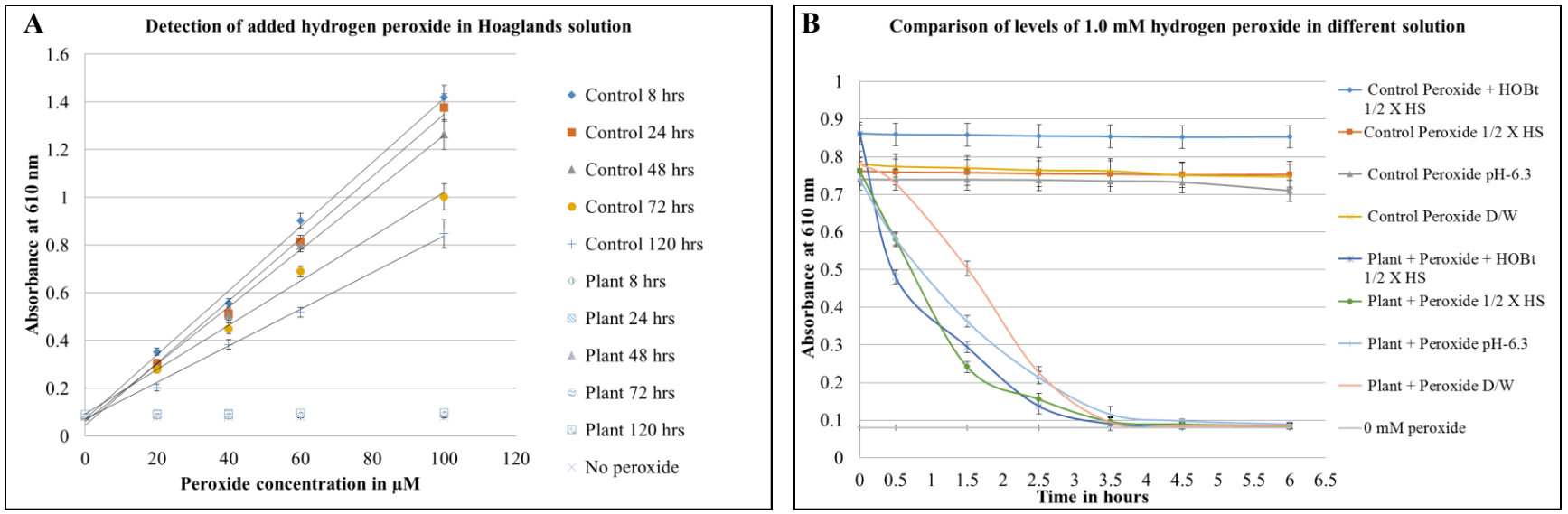


Figure 3.37 A. Trend showing the detection of the different concentrations of hydrogen peroxide in half-strength Hoagland's solution not exposed to plants in comparison to the treatments exposed to plants. **B.** Trend showing the detection of levels of added hydrogen peroxide at set times in different solutions of half-strength Hoagland's solution with 50 µM HOBt (HOBt + 1/2 X HS); half-strength Hoagland's solution only (1/2 X HS); pH 6.3 buffer and distilled water (D/W) containing 1 mM added hydrogen peroxide not exposed to plants in comparison to the treatments exposed to plants.

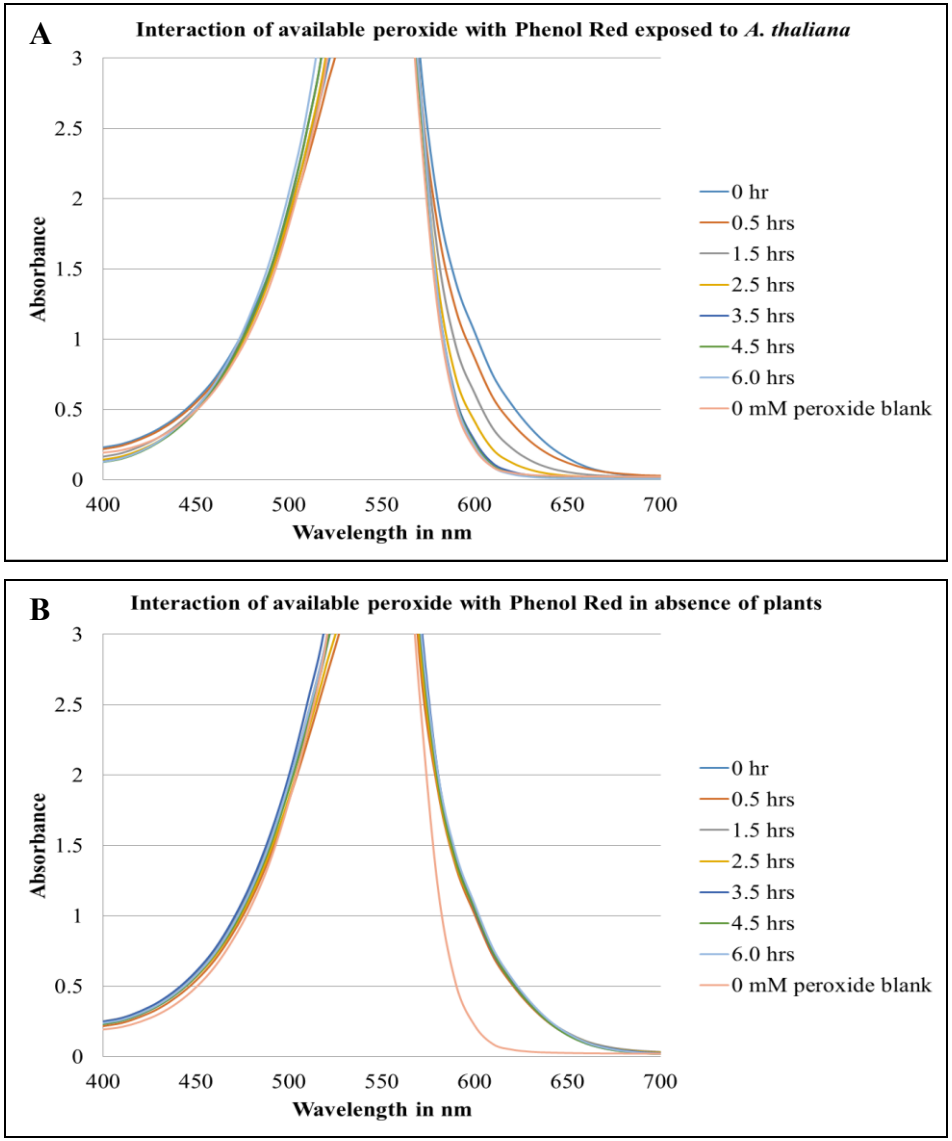


Figure 3.38 Visible spectra of Phenol Red reaction product, indicating levels of available hydrogen peroxide, as function of time when **A.** exposed to live plants and **B.** not exposed to live plants.

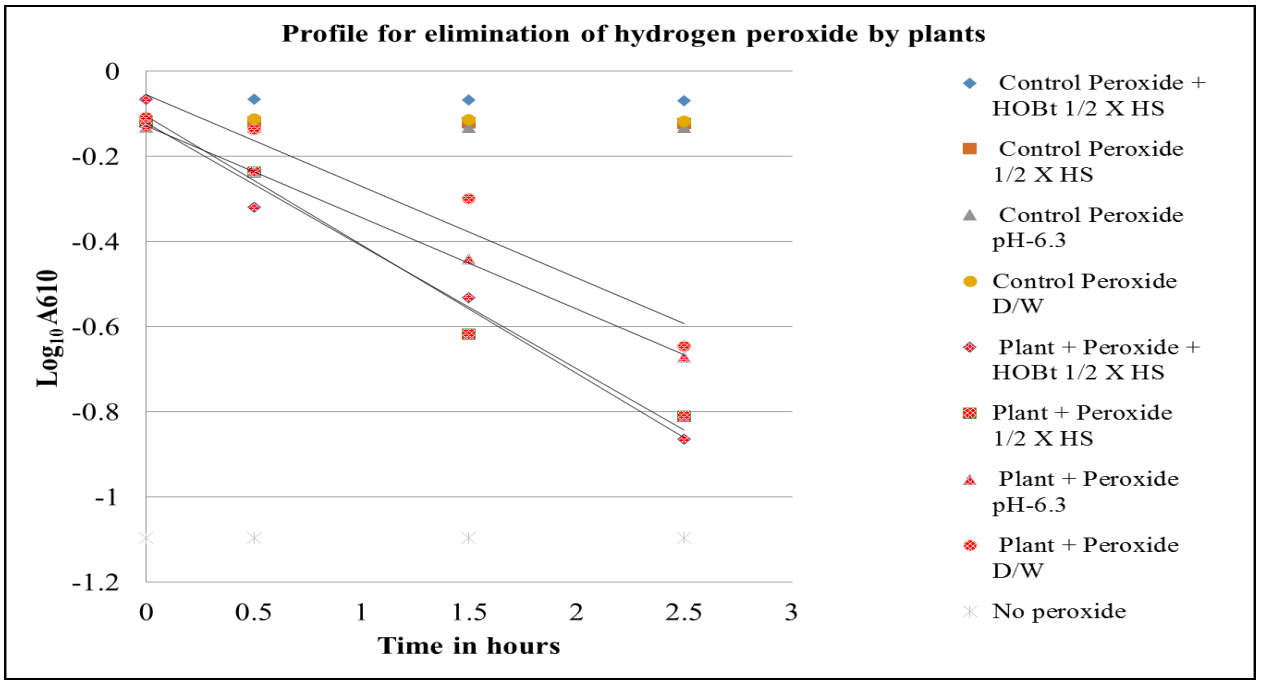


Figure 3.39 Profile showing the elimination kinetics for decrease in the levels of hydrogen peroxide when exposed to *A. thaliana* plants in different solutions of half-strength Hoagland's solution with 50 µM HOBt (HOBt + 1/2 X HS); half-strength Hoagland's solution only (1/2 X HS); pH 6.3 buffer and distilled water (D/W) in comparison to control treatments not exposed to plants.

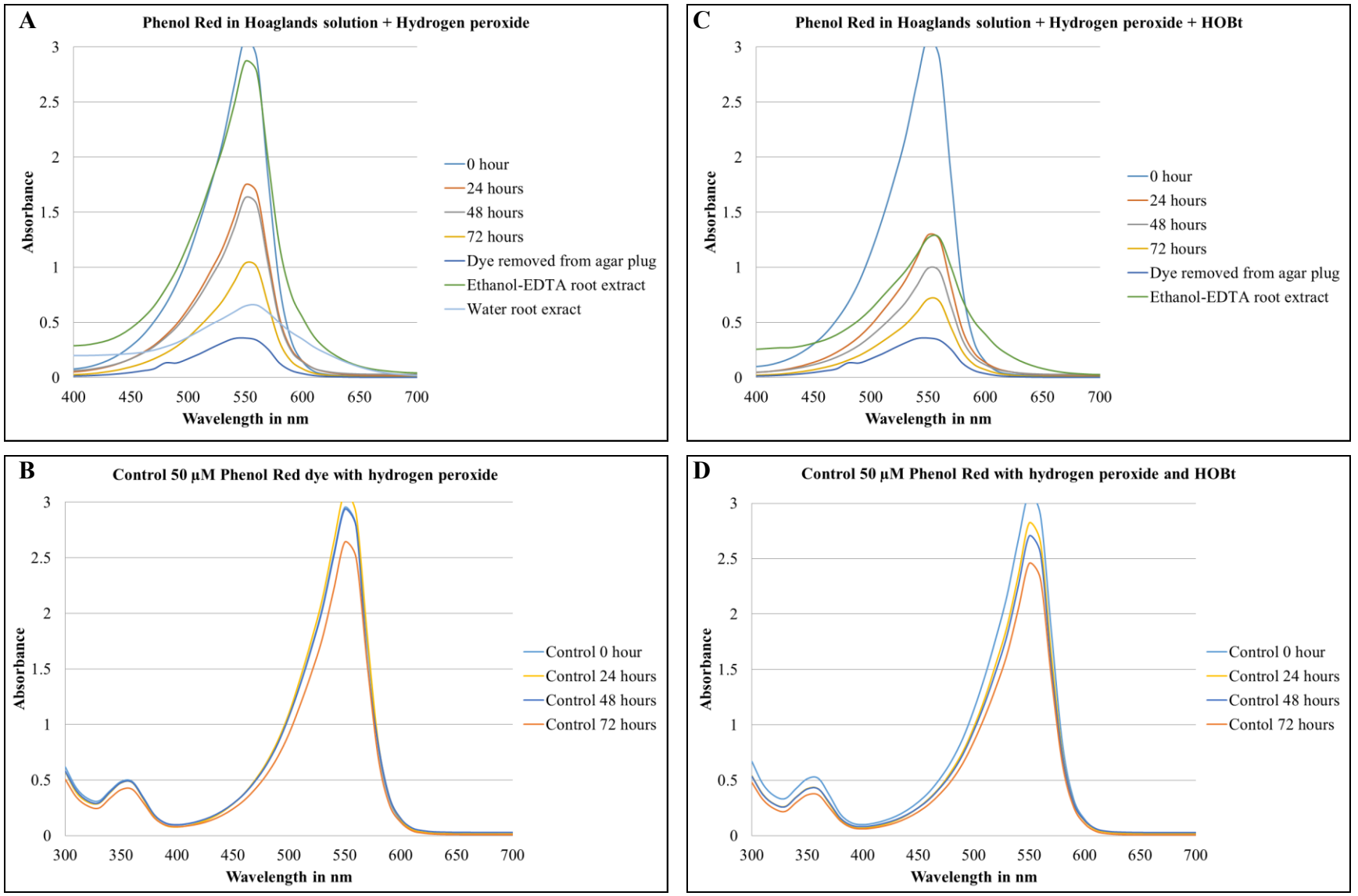


Figure 3.40 Exposure of Phenol Red in half-strength Hoagland's solution to *A. thaliana* plants containing **A.** added 1 mM hydrogen peroxide and **C.** added 1mM hydrogen peroxide and 50 µM HOBt; **B.** and **D.** Respective control not exposed to plants.

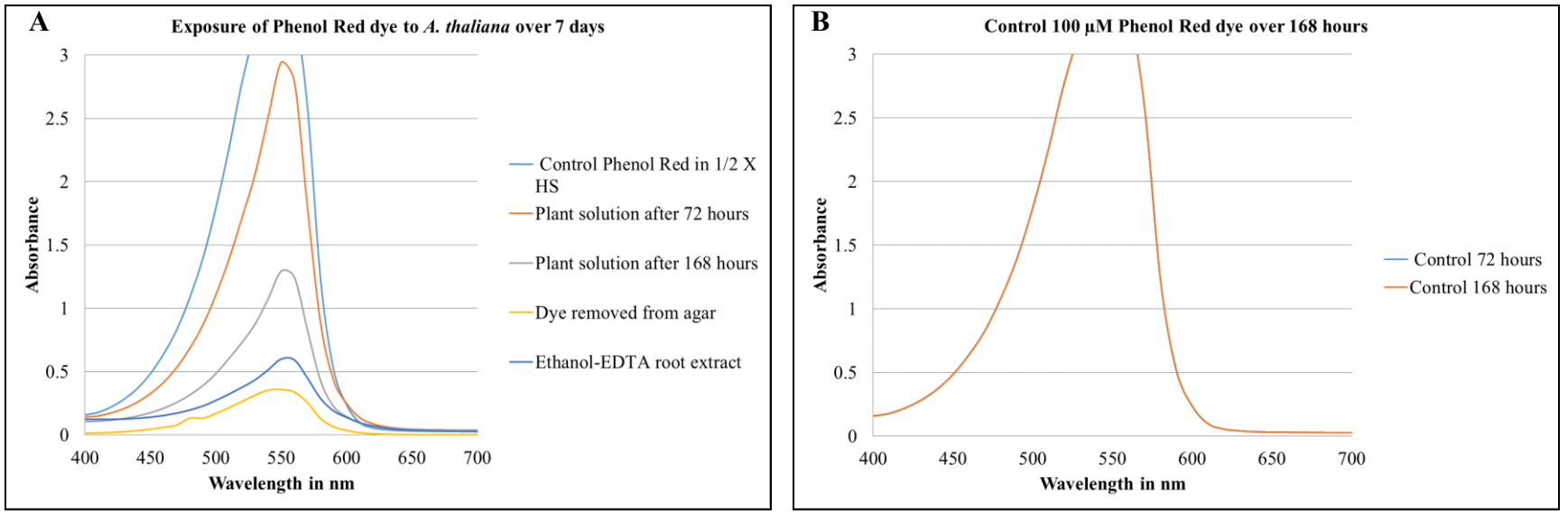


Figure 3.41 A. Exposure of Phenol Red in half-strength Hoagland's solution to *A. thaliana* plants in absence of added hydrogen peroxide and/or HOBt and **B.** control solution of Phenol Red in half-strength Hoagland's solution not exposed to plants.

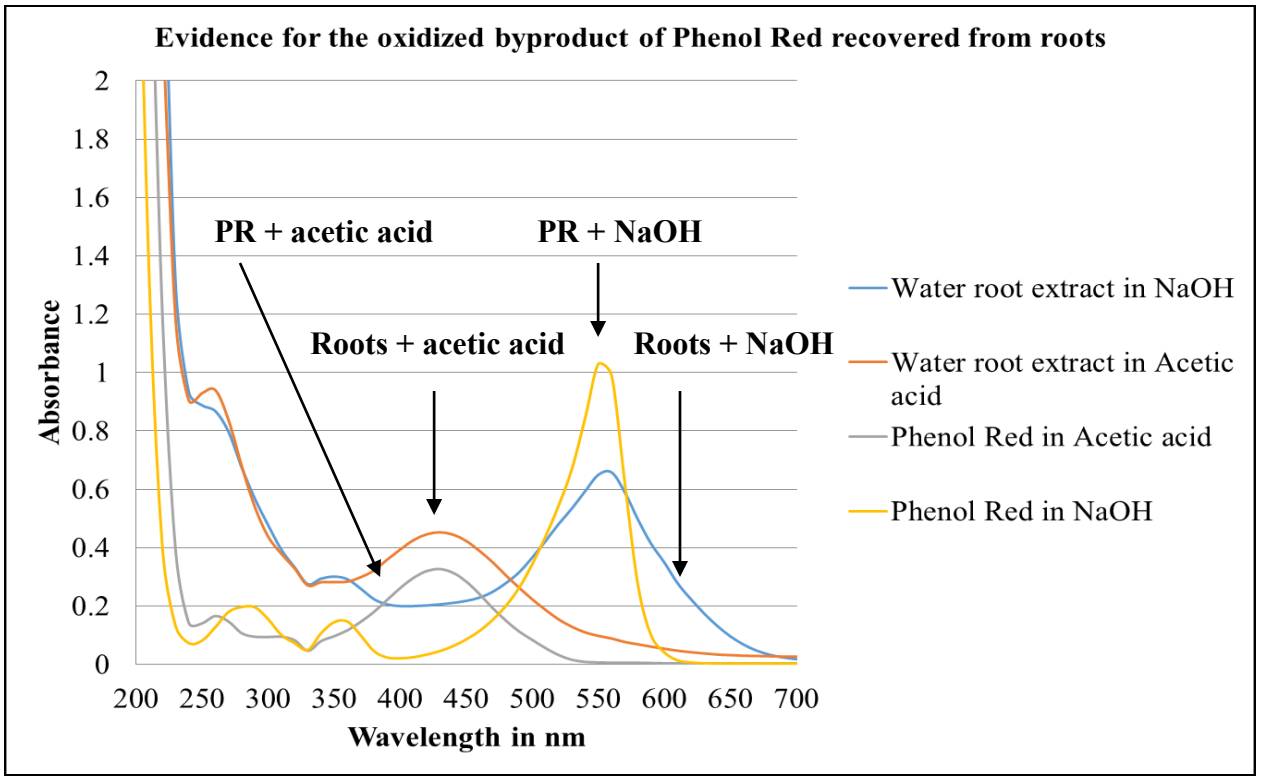
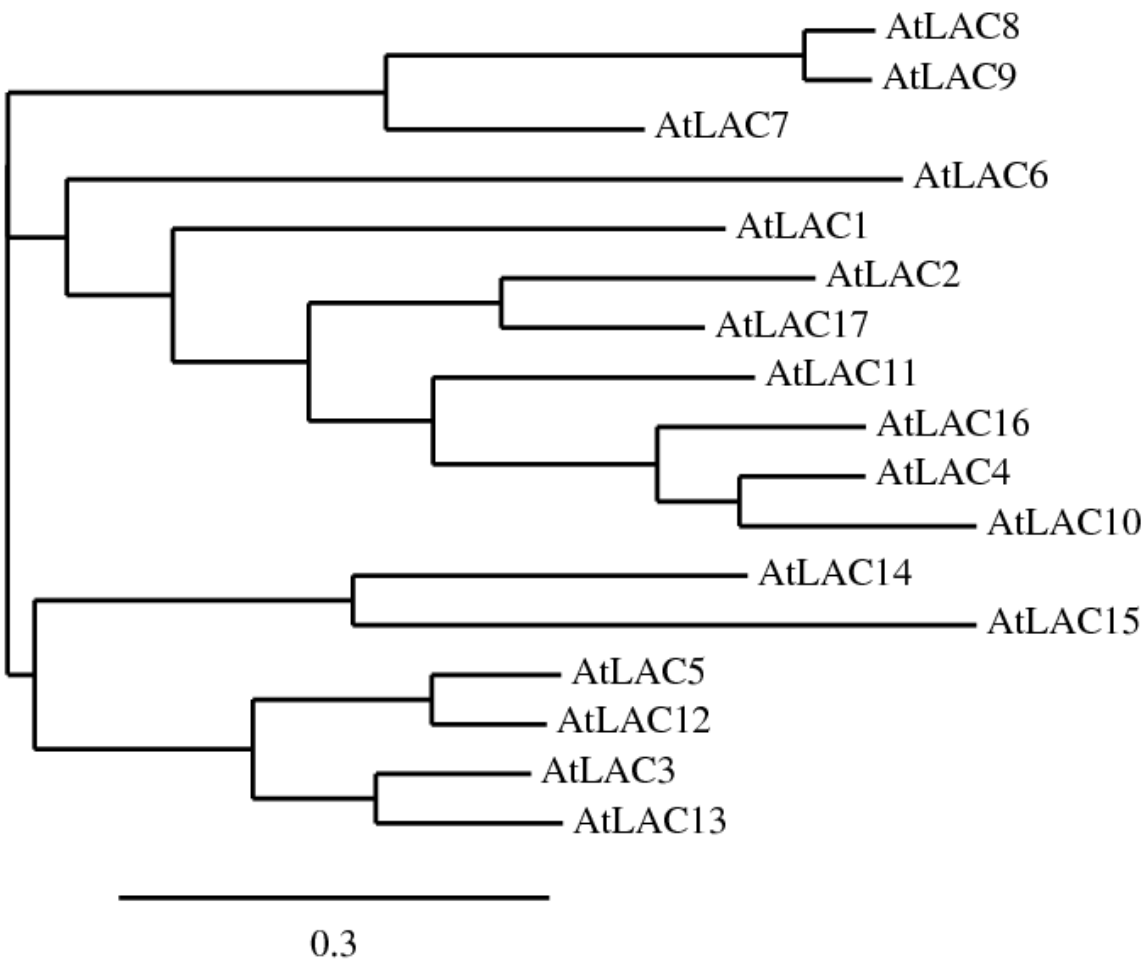


Figure 3.42 Spectra showing evidence for the oxidized Phenol Red dye and its stability under different conditions.



Pairwise % identity comparison

8 vs 9 = 89.21%

9 vs 7 = 58.71%

7 vs 6 = 44.55%

6 vs 1 = 44.75%

1 vs 2 = 50.44%

2 vs 17 = 68.61%

17 vs 11 = 57.22%

11 vs 16 = 59.96%

16 vs 4 = 73.40%

4 vs 10 = 74.37%

10 vs 14 = 43.96%

14 vs 15 = 49.73%

15 vs 5 = 46.91%

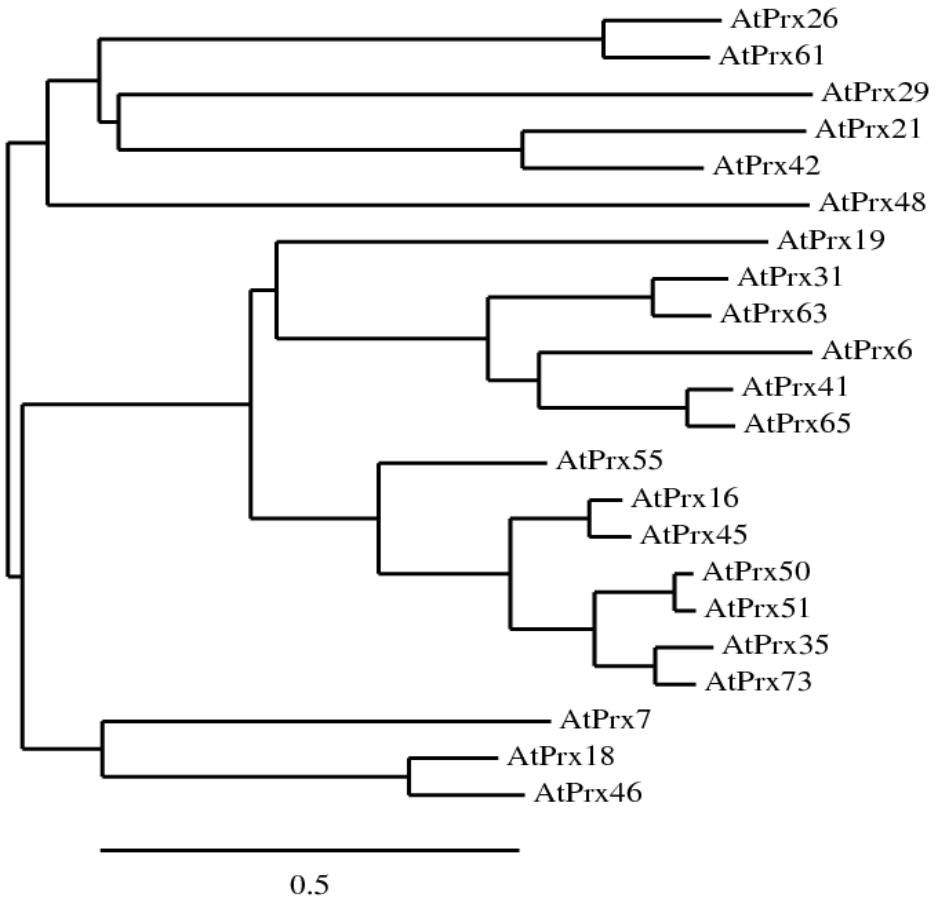
5 vs 12 = 78.23%

12 vs 3 = 64.35%

3 vs 13 = 74.38%

13 vs 8 = 47.27%

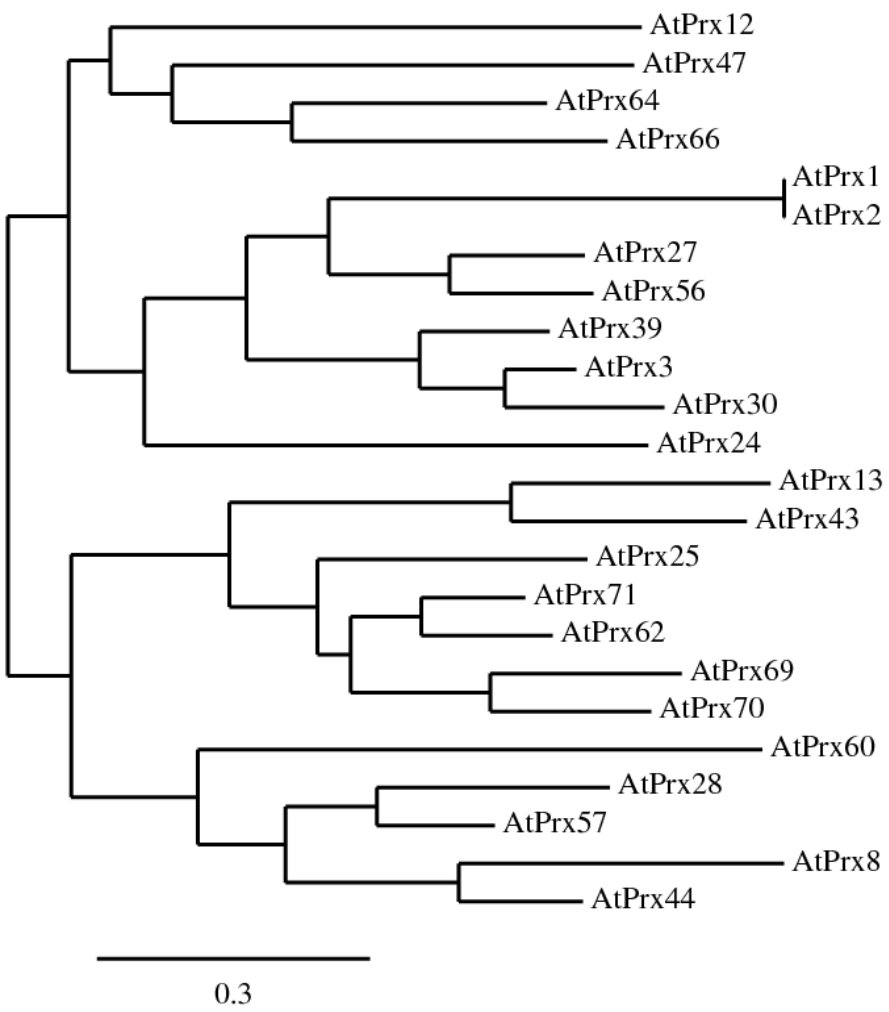
Figure 3.43 Predictions of phylogenetic relationships for laccase proteins of *Arabidopsis* with some pairwise comparisons of percentage similarity in the sequences between the members, down the branch from top to bottom as described in section 2.4.1 of Methods and Materials. Bar value at the bottom represents 0.3 amino acid substitutions per site.



Pairwise % identity comparison

26 vs 61 = 69.46%
61 vs 29 = 30.72%
29 vs 21 = 34.38%
21 vs 42 = 53.85%
42 vs 48 = 32.47%
48 vs 19 = 32.69%
19 vs 31 = 38.85%
31 vs 63 = 80.06%
63 vs 6 = 52.68%
6 vs 41 = 54.66%
41 vs 65 = 79.38%
65 vs 55 = 46.71%
55 vs 16 = 55.62%
16 vs 45 = 81.11%
45 vs 50 = 62.15%
50 vs 51 = 89.36%
51 vs 35 = 68.09%
35 vs 73 = 81.76%
73 vs 7 = 38.29%
7 vs 18 = 43.71%
18 vs 46 = 72.92%
46 vs 26 = 32.90%

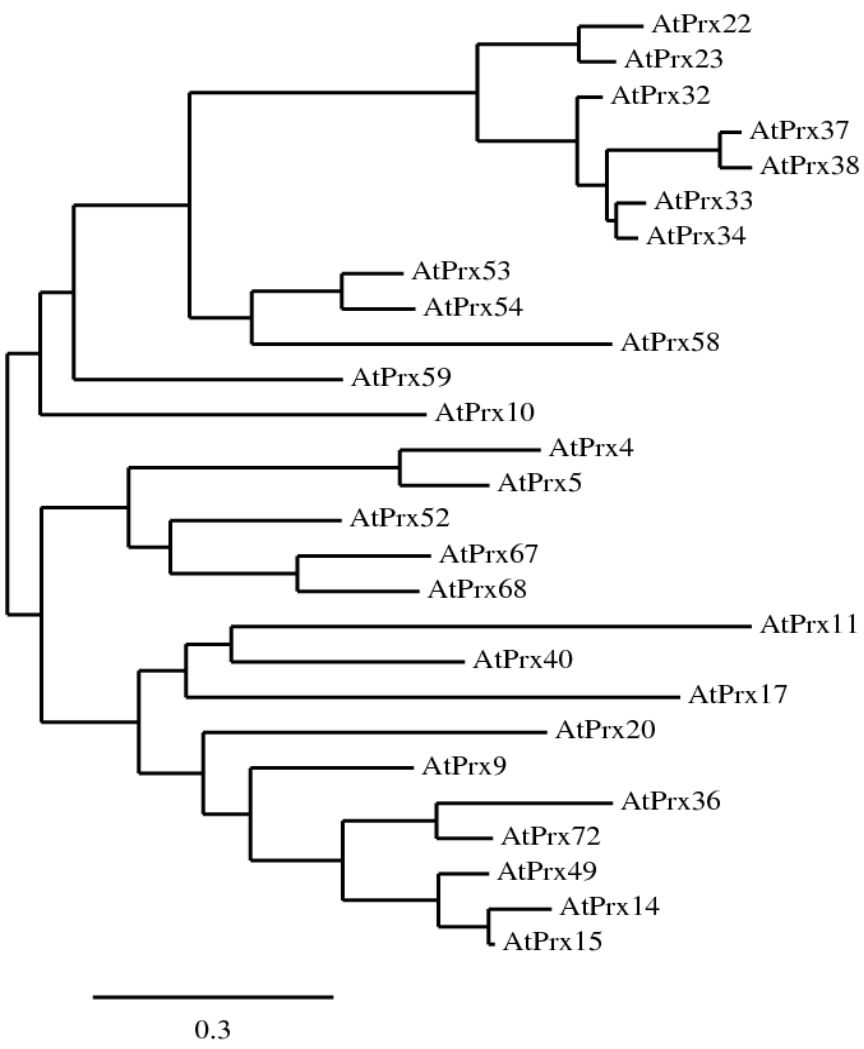
Figure 3.44 Predictions of phylogenetic relationships for peroxidase subfamily A proteins of *Arabidopsis*, with some pairwise comparisons of percentage similarity in the sequences between the members down the branch from top to bottom as described in section 2.4.1 of Methods and Materials. Bar value at the bottom represents 0.5 amino acid substitutions per site.



Pairwise % identity comparison

12 vs 47 = 38.96%
47 vs 64 = 46.43%
64 vs 66 = 51.10%
66 vs 1 = 40.57%
1 vs 2 = 100%
2 vs 27 = 52.50%
27 vs 56 = 72.90%
56 vs 39 = 51.86%
39 vs 3 = 68.94%
3 vs 30 = 72.09%
30 vs 24 = 43.40%
24 vs 13 = 36.08%
13 vs 43 = 56.06%
43 vs 25 = 47.91%
25 vs 71 = 56.47%
71 vs 62 = 76.73%
62 vs 69 = 56.83%
69 vs 70 = 66.77%
70 vs 60 = 38.75%
60 vs 28 = 44.01%
28 vs 57 = 69.87%
57 vs 8 = 52.15%
8 vs 44 = 64.19%
44 vs 12 = 38.83%

Figure 3.45 Predictions of phylogenetic relationships for peroxidase subfamily B proteins of *Arabidopsis*, with some pairwise comparisons of percentage similarity in the sequences between the members down the branch from top to bottom as described in section 2.4.1 of Methods and Materials. Bar value at the bottom represents 0.3 amino acid substitutions per site.



Pairwise % identity comparison
22 vs 23 = 89.40%
23 vs 32 = 68.97%
32 vs 37 = 77.84%
37 vs 38 = 92.77%
38 vs 33 = 75.51%
33 vs 34 = 92.63%
34 vs 53 = 53.31%
53 vs 54 = 84.48%
54 vs 58 = 54.60%
58 vs 59 = 48.28%
59 vs 10 = 49.37%
10 vs 4 = 42.17%
4 vs 5 = 72.70%
5 vs 52 = 56.55%
52 vs 67 = 60.63%
67 vs 68 = 71.52%
68 vs 11 = 42.81%
11 vs 40 = 45.71%
40 vs 17 = 43.57%
17 vs 20 = 43.26%
20 vs 9 = 52.89%
9 vs 36 = 52.98%
36 vs 72 = 68.15%
72 vs 49 = 68.28%
49 vs 14 = 77.34%
14 vs 15 = 88.72%
15 vs 22 = 44.92%

Figure 3.46 Predictions of phylogenetic relationships for peroxidase subfamily C proteins of *Arabidopsis*, with some pairwise comparisons of percentage similarity in the sequences between the members down the branch from top to bottom as described in section 2.4.1 of Methods and Materials. Bar value at the bottom represents 0.3 amino acid substitutions per site.

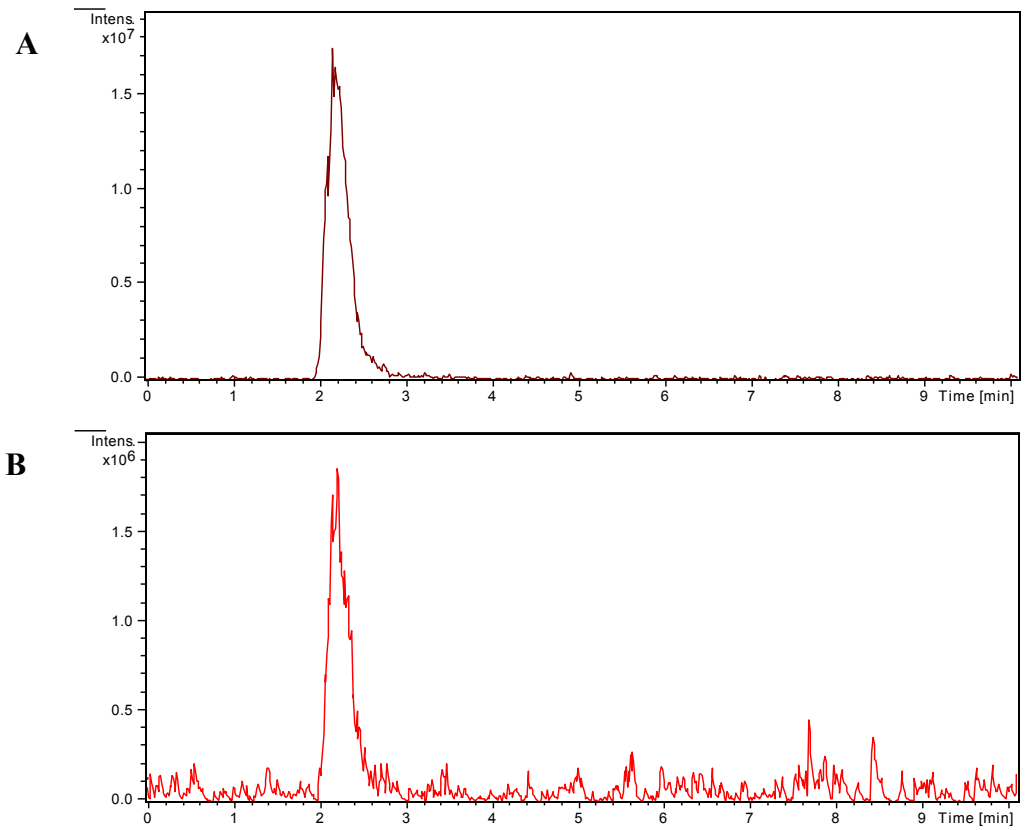


Figure 3.47 Representative reverse phase HPLC elution profile of Methyl Orange dye **A.** for negative control dye solution and **B.** exposed to *A. thaliana* plants in presence of hydrogen peroxide plus HOBt. Note the decrease of peak intensities in B.

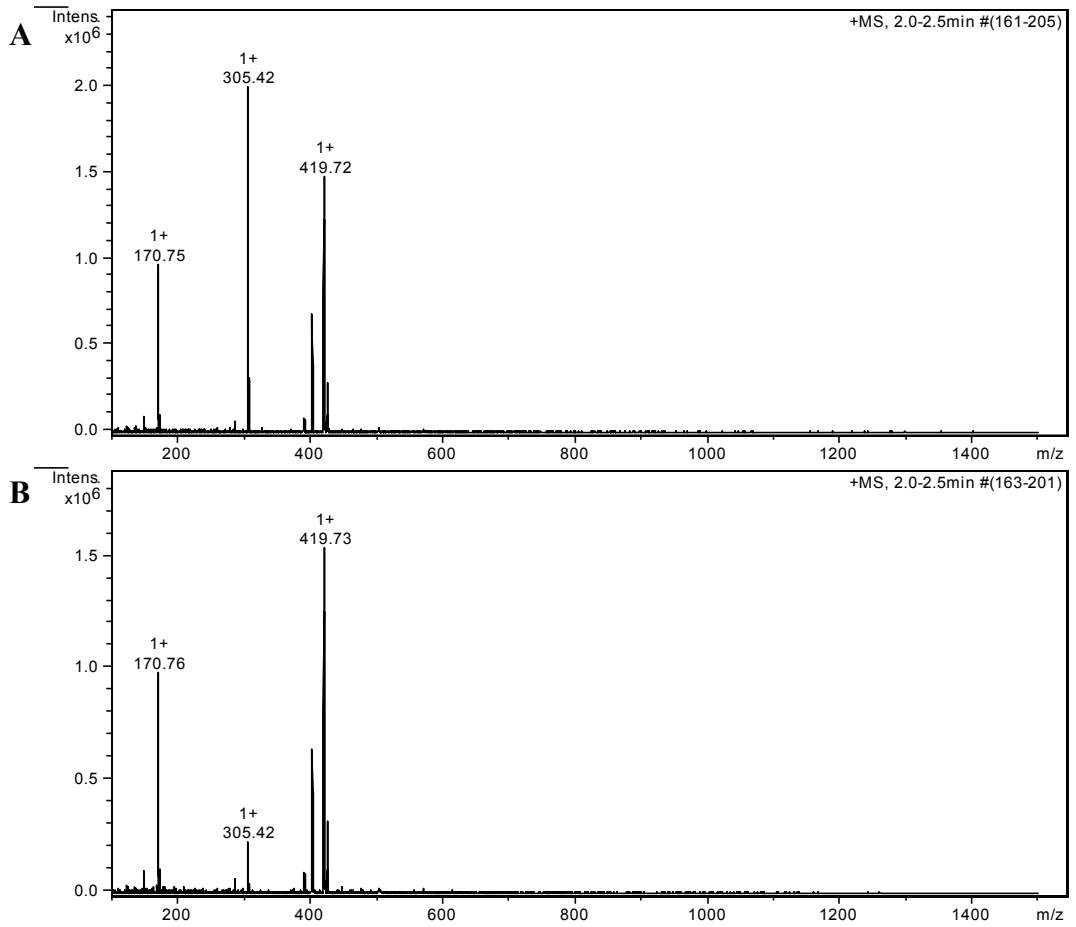


Figure 3.48 Representative ESI/MS profile of Methyl Orange dye **A.** for negative control dye solution and **B.** exposed to *A. thaliana* plants in presence of hydrogen peroxide plus HOBt. Note the decrease of 305 Da peak in B.

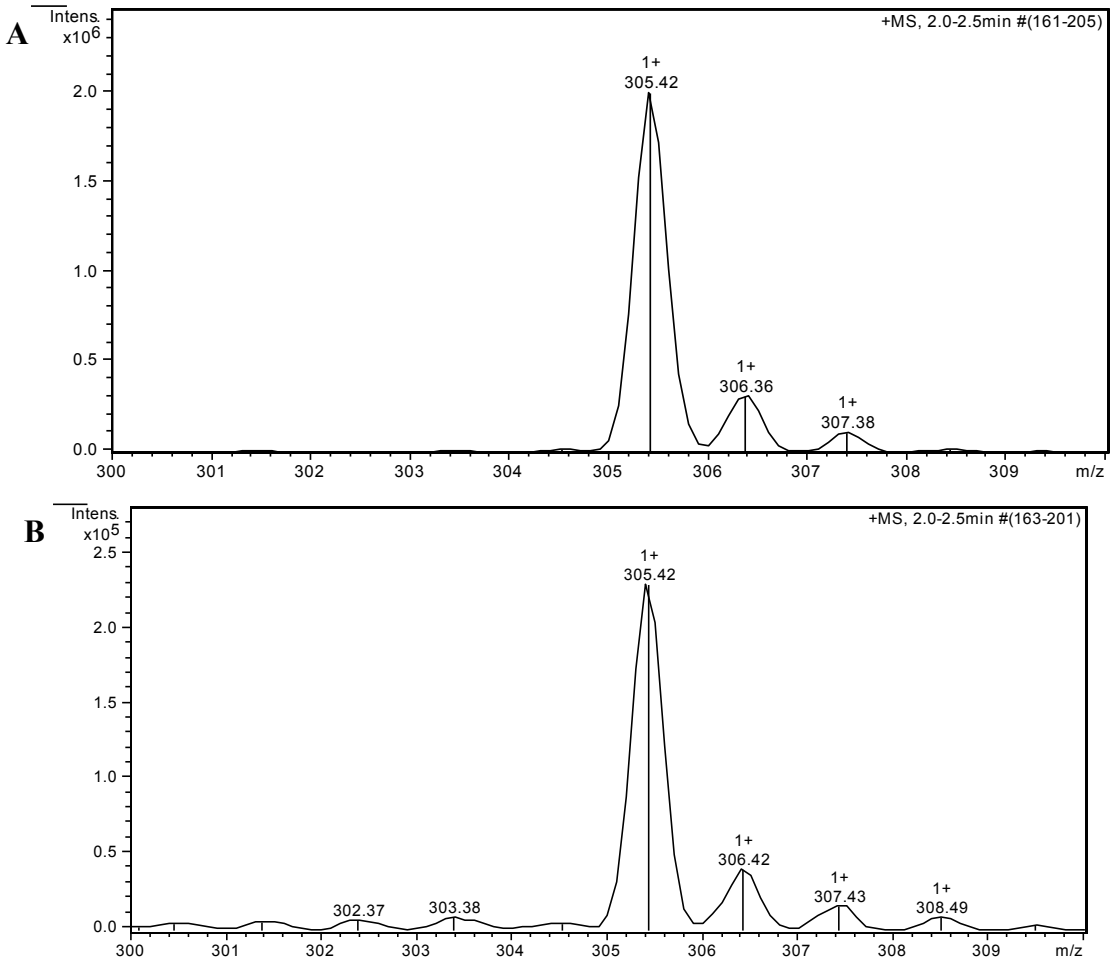


Figure 3.49 Representative peak intensities of Methyl Orange dye **A.** for negative control dye solution and **B.** exposed to *A. thaliana* plants in presence of hydrogen peroxide plus HOBt.

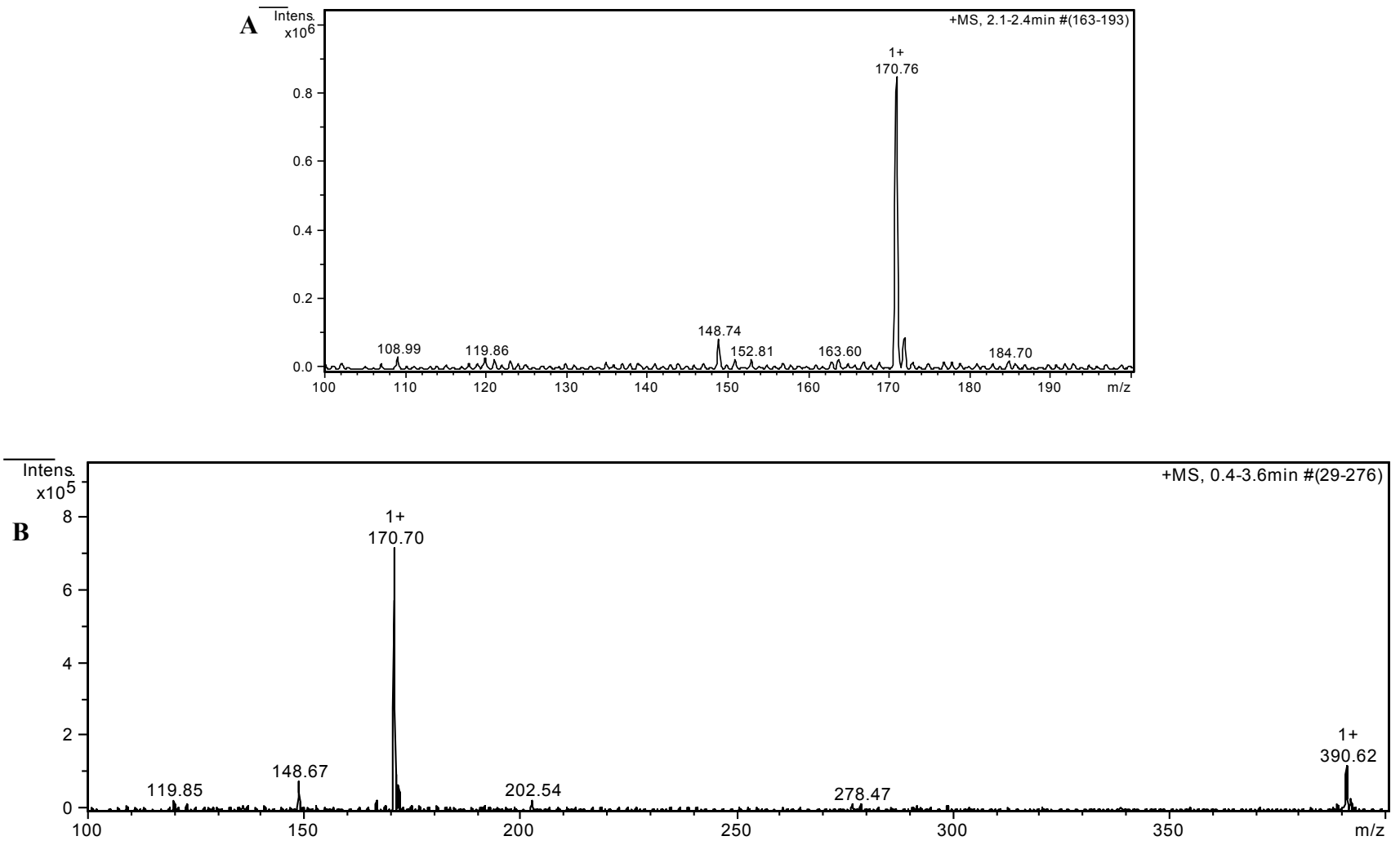


Figure 3.50 Representative ESI/MS profile of Methyl Orange dye **A.** exposed to *A. thaliana* plants in presence of hydrogen peroxide plus HOBt and **B.** for positive control dye solution with crushed turnip extract.

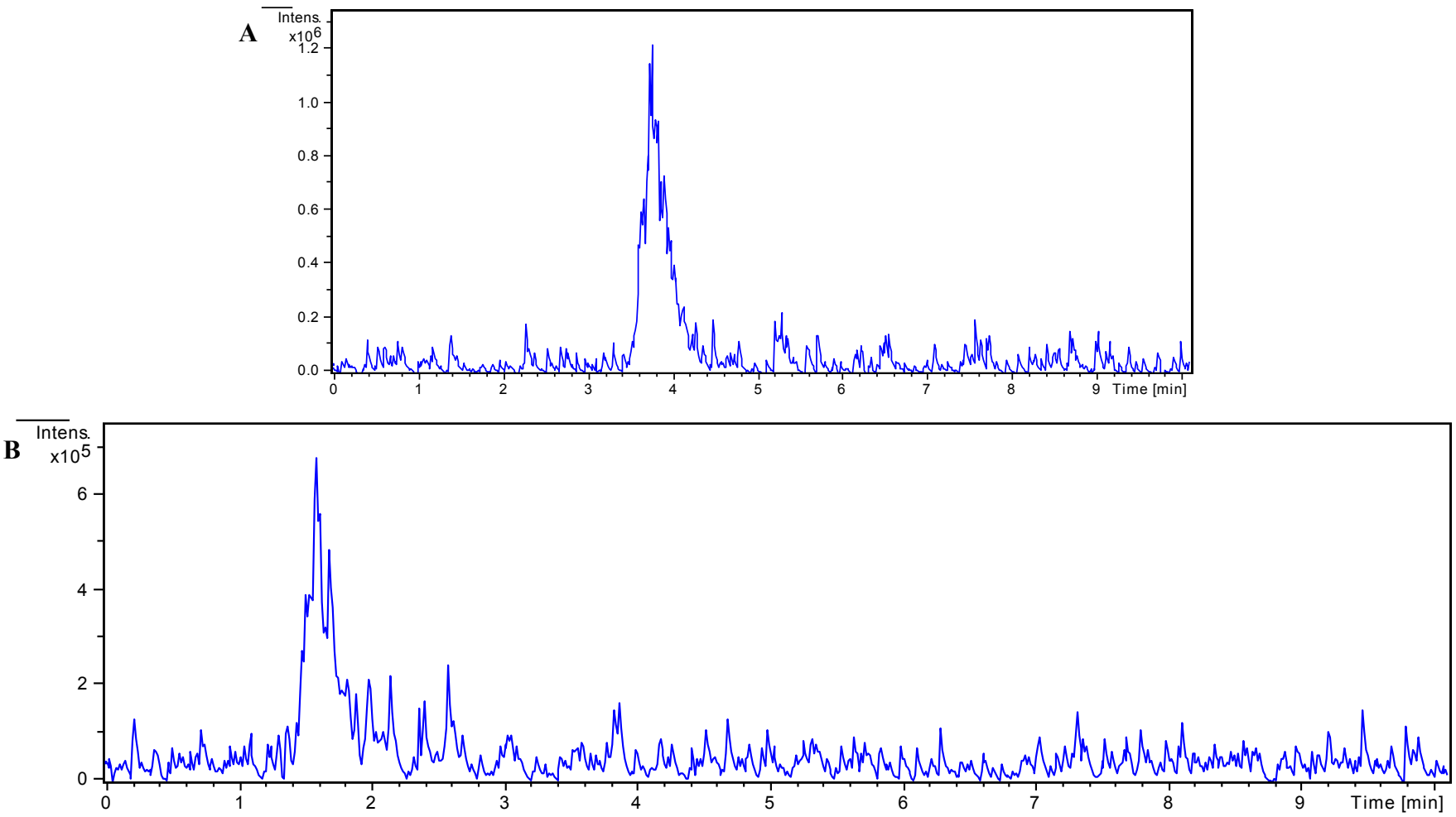


Figure 3.51 Representative reverse phase HPLC elution profile of Bromocresol Green dye **A.** for negative control dye solution and **B.** exposed to *A. thaliana* plants in presence of hydrogen peroxide plus HOBt. Note the decreased peak intensities in B.

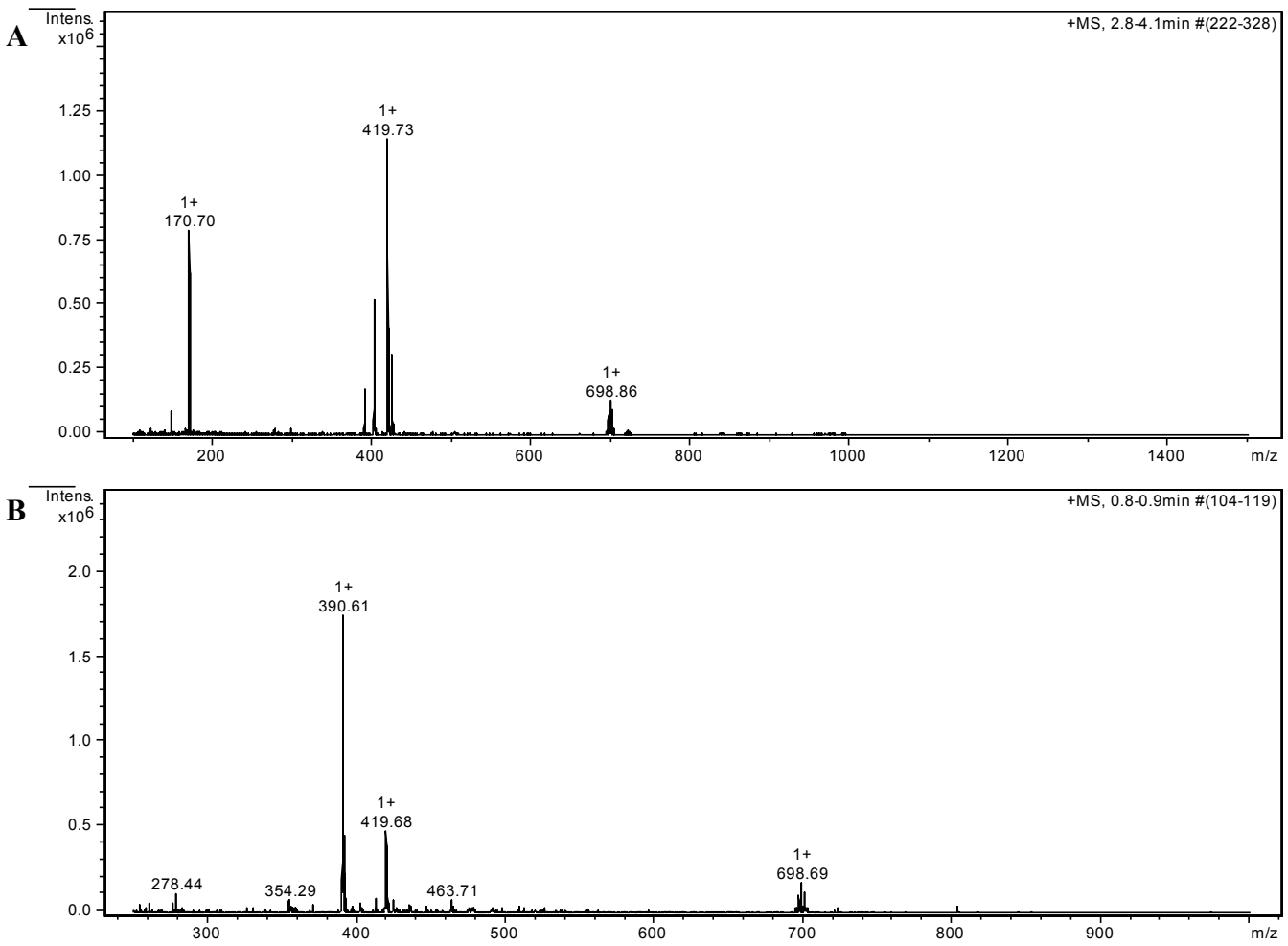


Figure 3.52 Representative ESI/MS profile of Bromocresol Green dye **A.** in negative control dye solution and **B.** exposed to *A. thaliana* plants in presence of hydrogen peroxide plus HOBt.

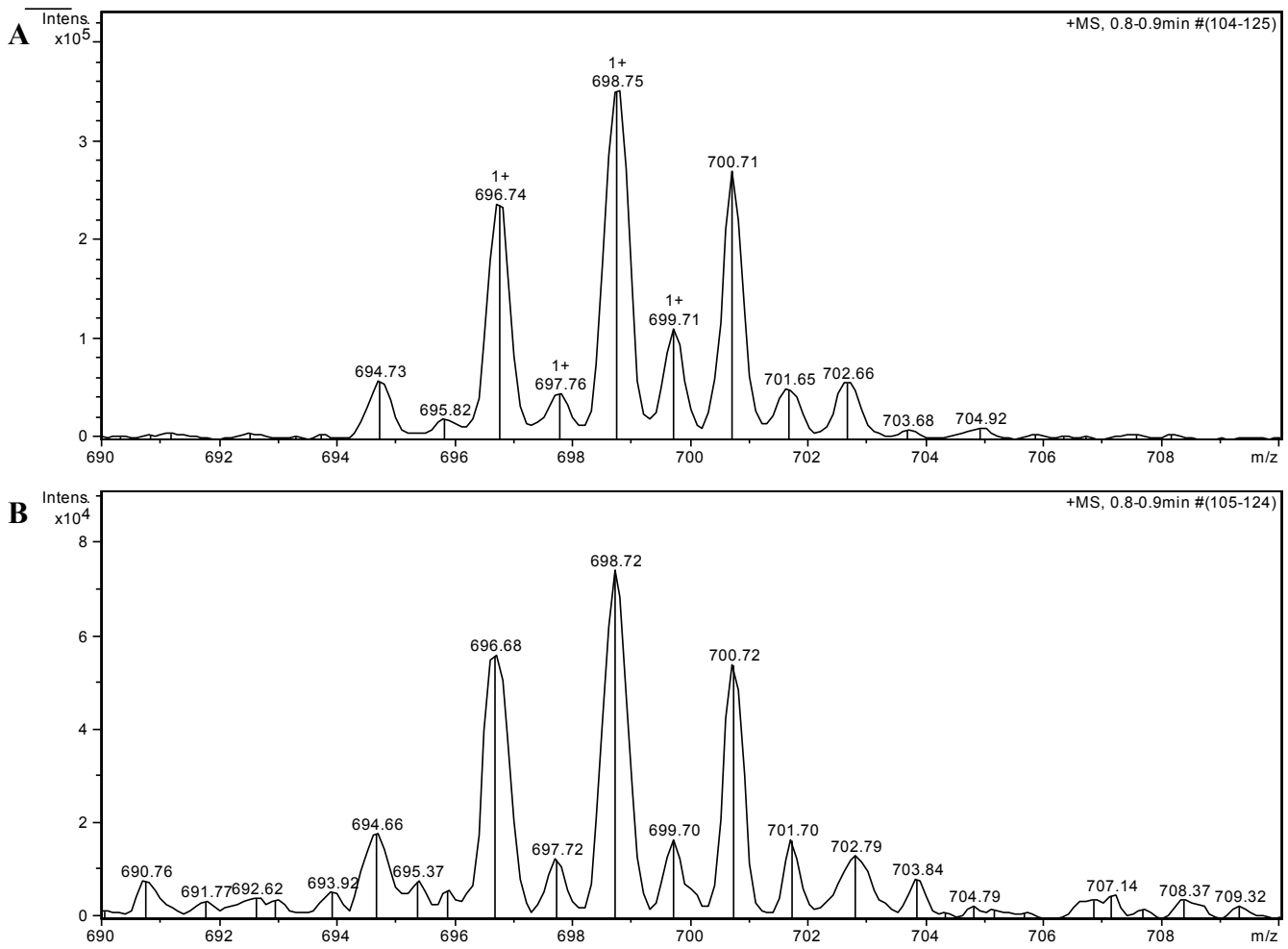


Figure 3.53 Representative peak intensities of Bromocresol Green dye **A.** in negative control dye solution and **B.** exposed to *A. thaliana* plants in presence of hydrogen peroxide plus HOBt. Note the reduced intensity of peaks in B.

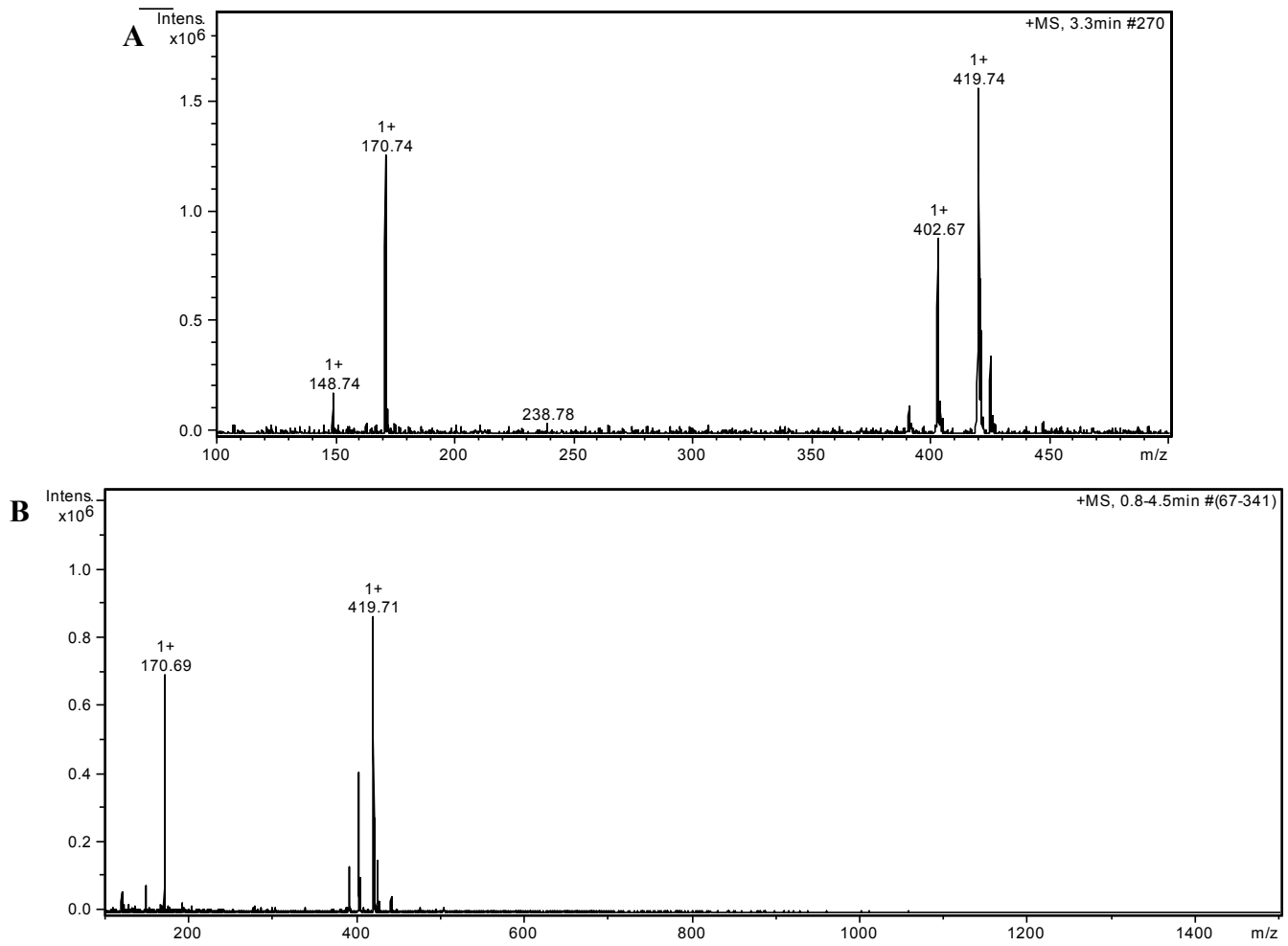


Figure 3.54 Representative ESI/MS profile of Bromocresol Green dye **A.** exposed to *A. thaliana* plants in presence of hydrogen peroxide plus HOBt and **B.** for positive control dye solution with crushed turnip extract.

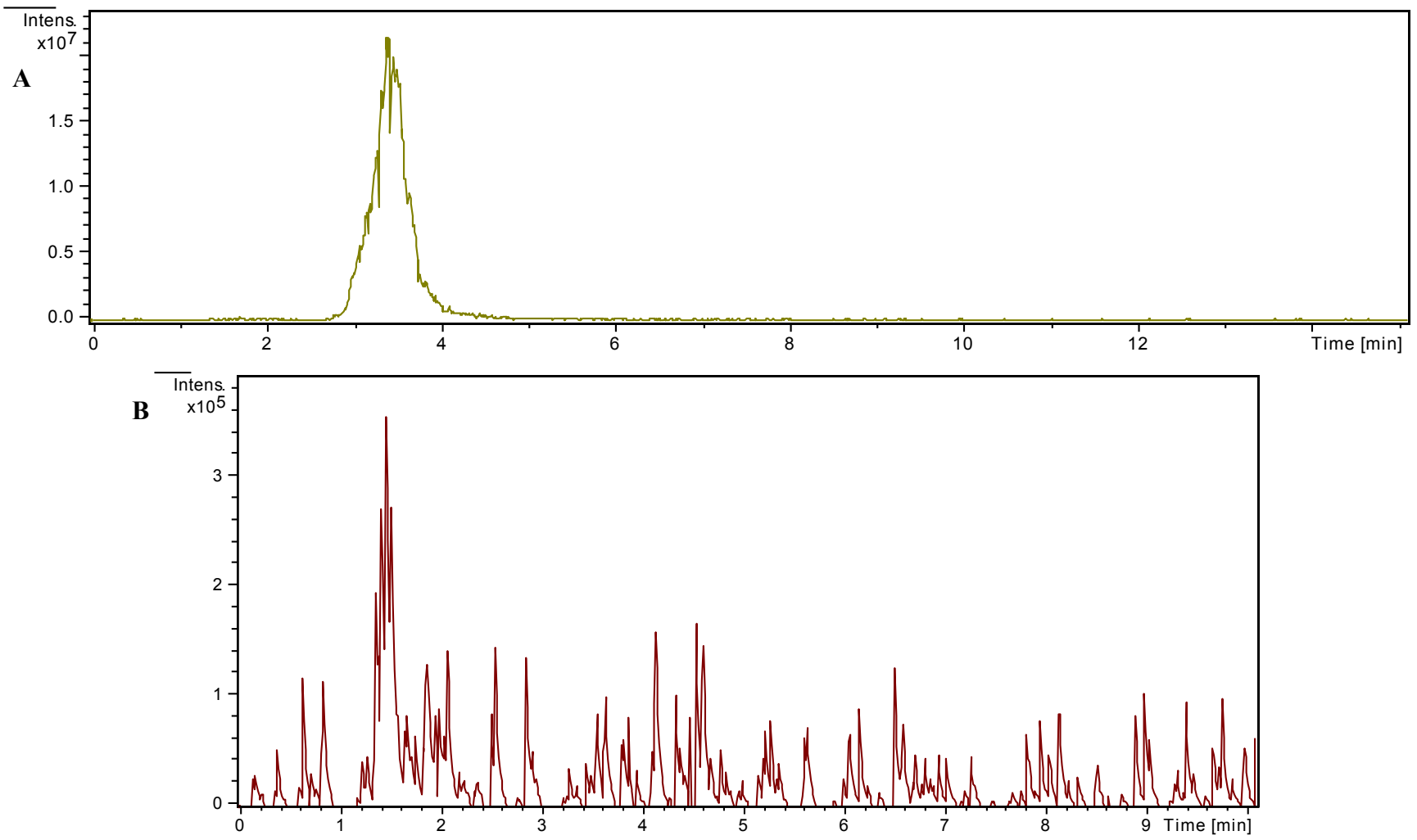


Figure 3.55 Representative reverse phase HPLC elution profile of Brilliant Blue G dye **A.** in negative control dye solution and **B.** exposed to *A. thaliana* plants in presence of hydrogen peroxide plus HOBt. Note the decrease in peak intensities in B.

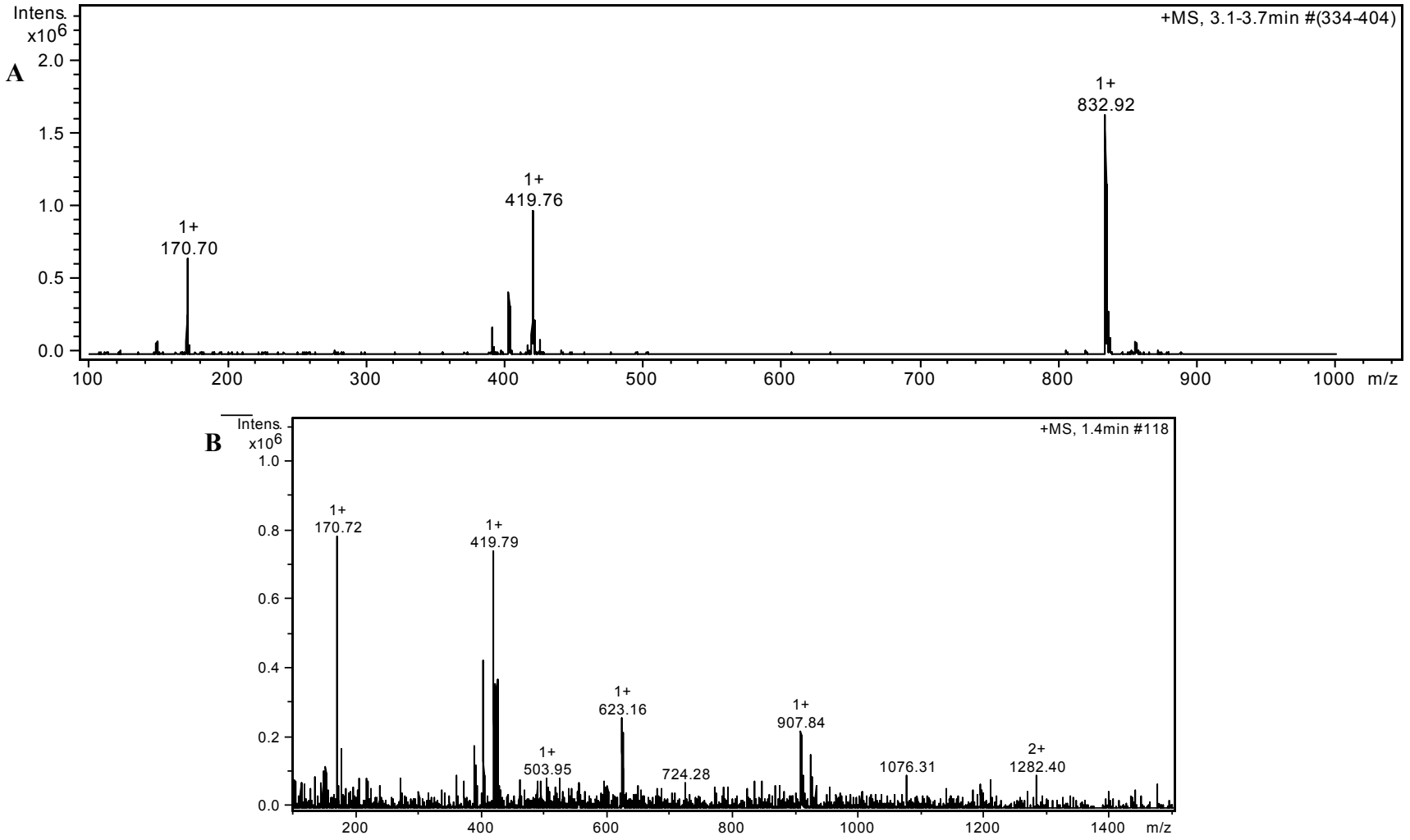


Figure 3.56 Representative ESI/MS profile of Brilliant Blue G dye **A.** in negative control dye solution and **B.** exposed to *A. thaliana* plants in presence of hydrogen peroxide plus HOBt. Note the loss of 832 Da peak in B.

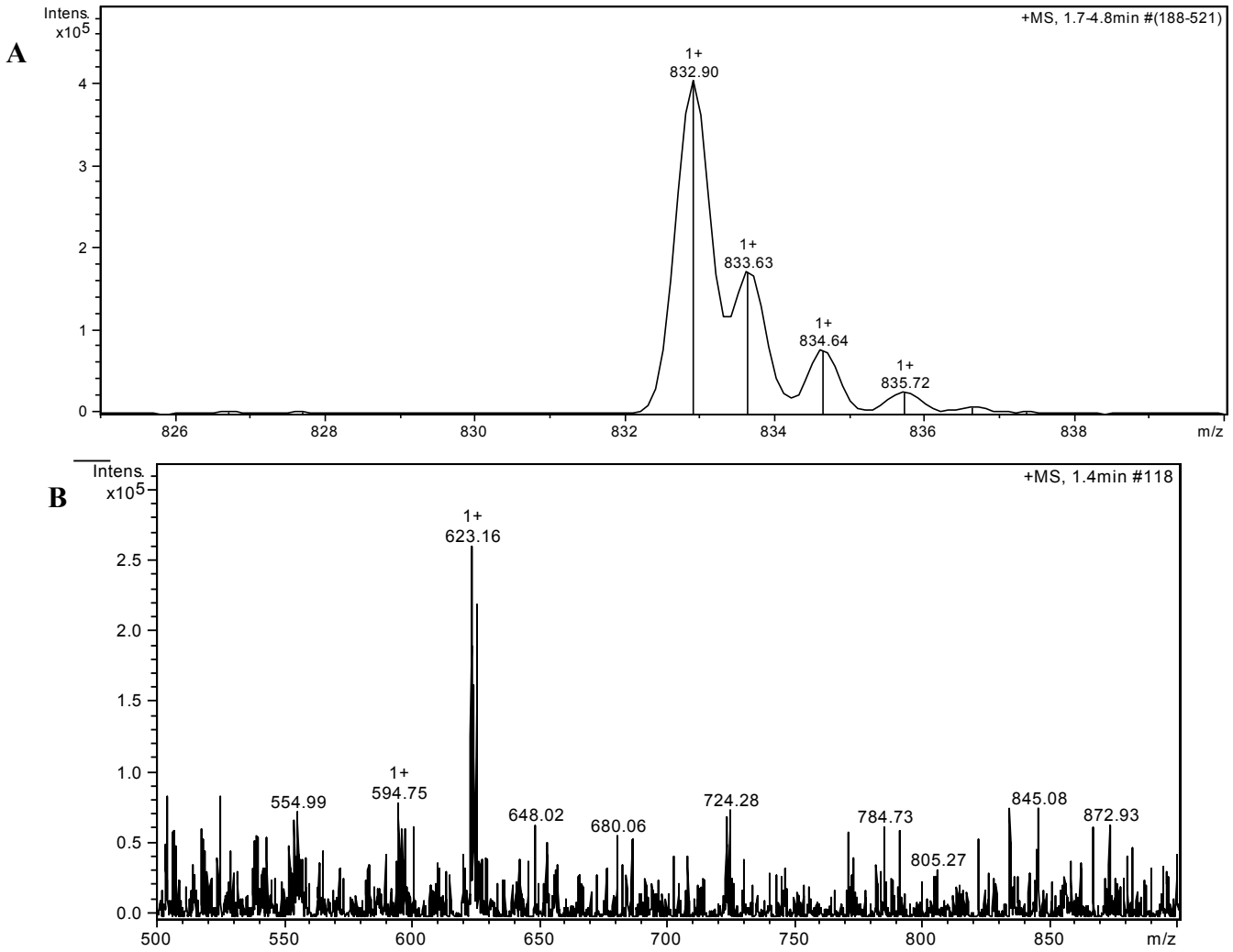


Figure 3.57 Representative peak intensities of Brilliant Blue G dye **A.** in negative control dye solution and **B.** exposed to *A. thaliana* plants in presence of hydrogen peroxide plus HOBt. Note the loss of 832 Da peak in B.

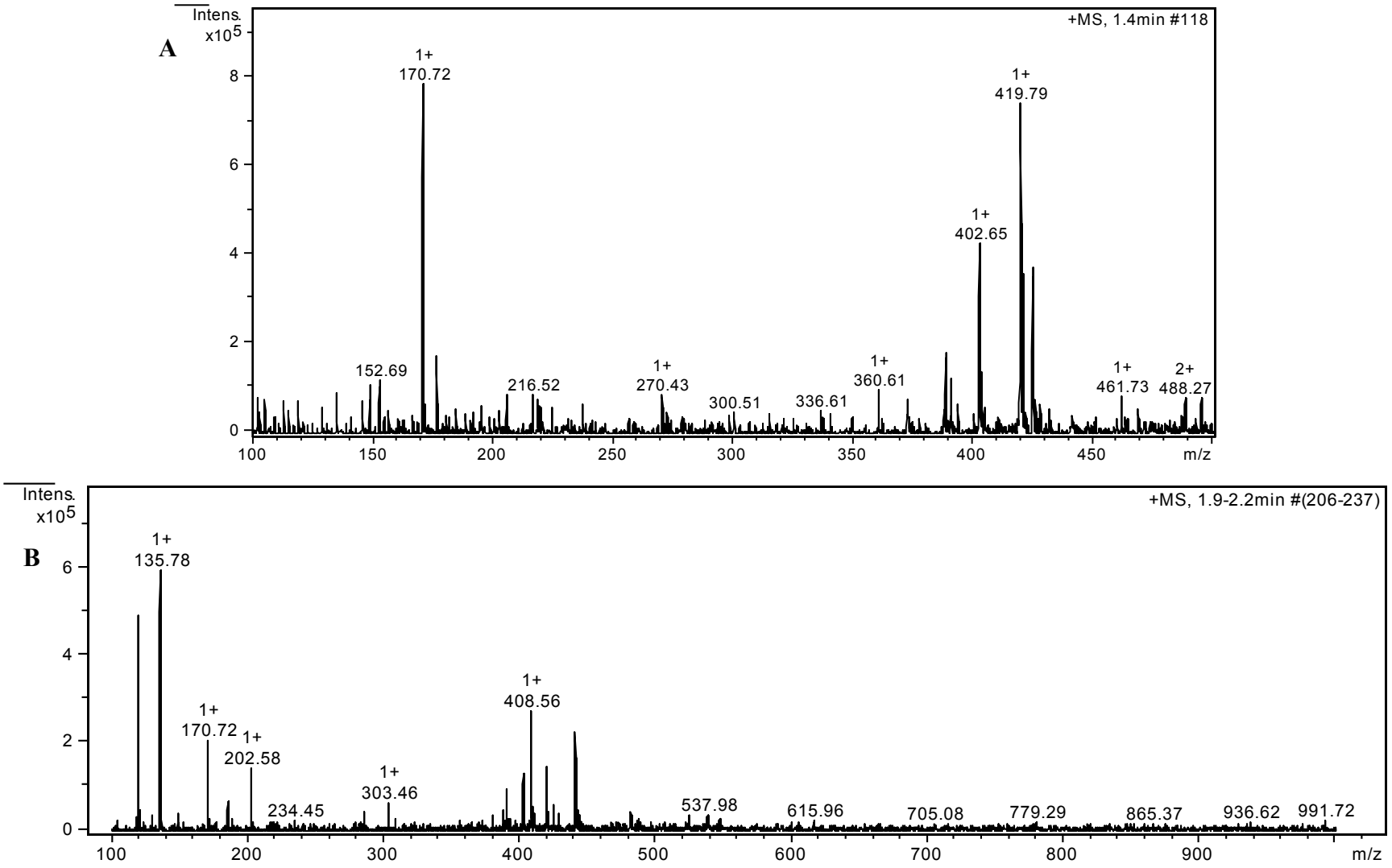


Figure 3.58 Representative ESI/MS profile of Brilliant Blue G dye **A.** exposed to *A. thaliana* plants in presence of hydrogen peroxide plus HOBt and **B.** positive control dye solution with crushed turnip extract.

Table 3.1 Comparison of initial decolorization rate of monoazo dyes between *A. thaliana* and sunflowers under different treatments

Name of dye	Treatment	Initial rate of decolorization in nanomoles per hour per gram fresh root weight	
		A. thaliana	Sunflower
pH 4.6 Methyl Red	No additions	280 ± 33	195 ± 40
	1 mM H_2O_2	370 ± 40	255 ± 18
	50 μM HOBt	245 ± 6	195 ± 25
	1 mM H_2O_2 + 50 μM HOBt	420 ± 18	300 ± 63
pH 6.3 Methyl Red	No additions	229 ± 42	176 ± 25
	1 mM H_2O_2	343 ± 5	244 ± 20
	50 μM HOBt	248 ± 51	181 ± 33
	1 mM H_2O_2 + 50 μM HOBt	393 ± 46	335 ± 31
pH 6.3 Methyl Orange	No additions	28 ± 2	24 ± 4
	1 mM H_2O_2	202 ± 40	205 ± 50
	50 μM HOBt	20 ± 2	13 ± 0.3
	1 mM H_2O_2 + 50 μM HOBt	650 ± 88	426 ± 55

Table 3.2 Comparison of initial decolorization rate of arylmethane dyes between *A. thaliana* and sunflowers under different treatments

Name of dye	Treatment	Initial rate of decolorization in nanomoles per hour per gram fresh root weight	
		A. thaliana	Sunflower
pH 6.3 Brilliant Blue G	No additions	33 ± 7	25 ± 4
	1 mM H_2O_2	90 ± 23	28 ± 6
	50 μM HOBt	38 ± 5	30 ± 6
	1 mM H_2O_2 + 50 μM HOBt	120 ± 35	35 ± 7
pH 4.6 Bromocresol Green	No additions	46 ± 10	25 ± 3
	1 mM H_2O_2	280 ± 50	140 ± 38
	50 μM HOBt	60 ± 23	30 ± 8
	1 mM H_2O_2 + 50 μM HOBt	310 ± 40	150 ± 45
pH 6.3 Bromocresol Green	No additions	20 ± 3	10 ± 2
	1 mM H_2O_2	75 ± 16	40 ± 9
	50 μM HOBt	16 ± 5	5 ± 0.6
	1 mM H_2O_2 + 50 μM HOBt	120 ± 45	45 ± 5
pH 6.3 Malachite Green	No additions	30 ± 7	212 ± 78
	1 mM H_2O_2	250 ± 56	335 ± 105
	50 μM HOBt	25 ± 5	260 ± 84
	1 mM H_2O_2 + 50 μM HOBt	300 ± 75	380 ± 97

Table 3.3 Comparison of initial decolorization rate of disazo dyes between *A. thaliana* and sunflowers under different treatments

Name of dye	Treatment	Initial rate of decolorization in nanomoles per hour per gram fresh root weight	
		A. thaliana	Sunflower
pH 6.3 Trypan Blue	No additions	25 ± 8	15 ± 1
	1 mM H_2O_2	40 ± 12	16 ± 4
	50 µM HOBt	16 ± 4	12 ± 0.8
	1 mM H_2O_2 + 50 µM HOBt	140 ± 35	25 ± 3
pH 6.3 Chicago Blue 6B	No additions	8 ± 1.6	10 ± 3
	1 mM H_2O_2	40 ± 8	12 ± 4.5
	50 µM HOBt	20 ± 4	15 ± 6
	1 mM H_2O_2 + 50 µM HOBt	215 ± 35	35 ± 10
pH 6.3 Evans Blue	No additions	20 ± 6	8 ± 2.2
	1 mM H_2O_2	45 ± 15	22 ± 7
	50 µM HOBt	15 ± 4	13 ± 0.3
	1 mM H_2O_2 + 50 µM HOBt	190 ± 40	30 ± 6.5

Table 3.4 Decolorization of Methyl Red dye in pH 4.6 and pH 6.3 buffer in absence of hydrogen peroxide and HOBt for T-DNA insertion of laccase in *A. thaliana*

Gene annotation	T-DNA insertion line identification number	% decolorization of pH 4.6 Methyl Red	% decolorization of pH 6.3 Methyl Red
At2g29130 (LAC2)	SALK_010994C	85%	83%
At2g30210 (LAC3)	SALK_150071C	88%	75%
At2g38080 (LAC4)	SALK_051892C	88%	89%
At2g40370 (LAC5)	SALK_092440C	84%	77%
At3g09220 (LAC7)	SALK_003265C	87%	76%
At5g01040 (LAC8)	SALK_016487C	88%	90%
At5g01190 (LAC10)	SALK_017722C	88%	87%
At5g03260 (LAC11)	SALK_000192C	87%	80%
At5g05390 (LAC12)	SALK_079491C	82%	75%
At5g07130 (LAC13)	SALK_052328C	88%	87%
At5g48100 (LAC15)	SALK_128292C	83%	75%
At5g58910 (LAC16)	SALK_064093C	88%	85%
WT		95%	95%

Table 3.5 Decolorization of Methyl Orange dye in pH 6.3 buffer in presence of hydrogen peroxide and HOBt for T-DNA insertion of peroxidase in *A. thaliana*

Gene annotation	T-DNA insertion line identification number	% decolorization of pH 6.3 Methyl Orange
At1g05240 (AtPrx1)	SALK_103597C	65%
At1g05250 (AtPrx2)	SALK_101289	74%
At1g05260 (AtPrx3)	SALK_128955C	68%
At1g14540 (AtPrx4)	SALK_044730C	72%
At1g14550 (AtPrx5)	CS840976	65%
At1g24110 (AtPrx6)	SALK_087392C	73%
At1g34510 (AtPrx8)	SALK_130310C	67%
At1g44970 (AtPrx9)	CS877595	68%
At1g49570 (AtPrx10)	SALK_148968	78%
At1g68850 (AtPrx11)	SALK_061576C	70%
At1g71695 (AtPrx12)	SALK_085121C	67%
At2g18140 (AtPrx14)	SALK_127000C	69%
At2g18150 (AtPrx15)	CS849794	66%
At2g18980 (AtPrx16)	SALK_040120C	68%

Gene annotation	T-DNA insertion line identification number	% decolorization of pH 6.3 Methyl Orange
At2g22420 (AtPrx17)	SALK_034684C	72%
At2g24800 (AtPrx18)	CS870388	72%
At2g34060 (AtPrx19)	SALK_051197C	63%
At2g35380 (AtPrx20)	CS900994	62%
At2g37130 (AtPrx21)	SALK_123121C	68%
At2g38380 (AtPrx22)	SALK_144487C	65%
At2g38390 (AtPrx23)	SALK_061249C	62%
At2g41480 (AtPrx25)	SALK_058270C	66%
At2g43480 (AtPrx26)	SALK_018441C	67%
At3g01190 (AtPrx27)	SALK_107573	72%
At3g03670 (AtPrx28)	SALK_069197C	62%
At3g17070 (AtPrx29)	SALK_121694C	70%
At3g21770 (AtPrx30)	SALK_025599C	69%
At3g28200 (AtPrx31)	SALK_089631C	64%
At3g32980 (AtPrx32)	CS879810	69%

Gene annotation	T-DNA insertion line identification number	% decolorization of pH 6.3 Methyl Orange
At3g49110 (AtPrx33)	SALK_056847C	62%
At3g49120 (AtPrx34)	SALK_051825C	68%
At3g49960 (AtPrx35)	SALK_119795C	64%
At3g50990 (AtPrx36)	CS873555	68%
At4g08770 (AtPrx37)	SALK_129122	71%
At4g08780 (AtPrx38)	SALK_142205C	69%
At4g11290 (AtPrx39)	SALK_096147C	68%
At4g16270 (AtPrx40)	SALK_021343C	65%
At4g17690 (AtPrx41)	SALK_087323C	67%
At4g21960 (AtPrx42)	SALK_114615C	67%
At4g25980 (AtPrx43)	SALK_047689C	70%
At4g26010 (AtPrx44)	SALK_148663C	61%
At4g30170 (AtPrx45)	N57202	64%
At4g33420 (AtPrx47)	CS879433	72%

Gene annotation	T-DNA insertion line identification number	% decolorization of pH 6.3 Methyl Orange
At4g33870 (AtPrx48)	SALK_002348C	66%
At4g36430 (AtPrx49)	SALK_129503C	69%
At4g37520 (AtPrx50)	SALK_063662C	68%
At4g37530 (AtPrx51)	SALK_089515C	67%
At5g05340 (AtPrx52)	SALK_081257	72%
At5g06720 (AtPrx53)	SALK_082730	70%
At5g06730 (AtPrx54)	SALK_055496C	68%
At5g14130 (AtPrx55)	SALK_102284	72%
At5g15180 (AtPrx56)	CS163841	67%
At5g17820 (AtPrx57)	SALK_070375	64%
At5g19880 (AtPrx58)	SALK_103878C	66%
At5g19890 (AtPrx59)	SALK_135377C	73%
At5g22410 (AtPrx60)	SALK_114884C	64%

Gene annotation	T-DNA insertion line identification number	% decolorization of pH 6.3 Methyl Orange
At5g24070 (AtPrx61)	SALK_008946C	72%
At5g39580 (AtPrx62)	SALK_151762C	62%
At5g40150 (AtPrx63)	SALK_029351C	73%
At5g51890 (AtPrx66)	SALK_102967C	66%
At5g58390 (AtPrx67)	SALK_129434	61%
At5g58400 (AtPrx68)	SALK_019153C	62%
At5g64100 (AtPrx69)	CS879649	65%
At5g64110 (AtPrx70)	SALK_061298	68%
At5g64120 (AtPrx71)	SALK_121202C	73%
At5g66390 (AtPrx72)	SALK_136893C	70%
At5g67400 (AtPrx73)	SALK_014607C	66%
WT		78%

Chapter 4 - Discussion

The main objective of this research was to determine whether plants could serve as potential tools in remediation of contaminants such as synthetic dyes and thus find future applications outside of agriculture. Comparison in the trends of decolorization of the synthetic dyes from different classes and diverse structures obtained using hydroponically grown *A. thaliana* from the *Brassicaceae* family to those obtained with sunflower (*H. annuus*) from the *Asteraceae* family revealed differences that were quite "subtle" which indicates that the decolorization process of synthetic dyes has common mechanism of operation. The mechanism of synthetic dye decolorization was found to be dependent on many variables but the major contributing factors were dependent on the core structure of the synthetic dye and the additional factors that were added along with the synthetic dye namely hydrogen peroxide plus mediator. While both plants were in general capable of decolorizing the tested synthetic dyes, important differences were noticed not only between the different plants but also based on the class of the dye. In case of the dyes from the monoazo class which are classified by the presence of single azo nucleus the decolorization trends observed were not that different between the plants when exposed to similar treatments and the plants showed enhanced decolorization only in the presence of added hydrogen peroxide and mediator. Major exception was the decolorization of MR. MR is structurally similar to MO but its decolorization was not dependent on the additional factors of added hydrogen peroxide and HOBt or the pH of the solution (Table 2.1; Lillie and Conn, 1969). This behavior was not only different from MO but also different from the representative synthetic dyes from the other classes. Both plants were able to decolorize MR with comparable efficiencies. On the other hand, decolorization of MO was enhanced only when both the plants were exposed to combination of externally added hydrogen peroxide and HOBt while exposure

to external hydrogen peroxide alone did not result in such trends. Similarly for the three structurally related disazo dyes namely EB, CB6B and TB (Table 2.1; Lillie and Conn, 1969) both plants could oxidize and decolorize all of them but only in presence of added hydrogen peroxide and HOBt. As expected the different placement of the functional groups in each of them did contribute to differences in their decolorization and in terms of affinity or preference of substrate. EB and CB6B were relatively more decolorized over TB, but both the plants were able to decolorize the different disazo dyes. Decolorization trends of arylmethane dyes on the other hand revealed that addition of hydrogen peroxide even in the absence of HOBt resulted in relatively better decolorization of the dyes. This was found to be different to exposure of plants to monoazo dyes as well as the disazo dyes where both peroxide and mediator are required to achieve highest observable decolorization while addition of external peroxide alone resulted in relatively lower dye decolorization. The differences in the core structures of arylmethane dyes compared to the azo nucleus in the azo dyes might make the azo dyes more recalcitrant to decolorization. In case of the dyes behaving as pH indicator namely PR and BG, pH of the solution did not seem to affect the decolorization potential of either plants. Exposure to added hydrogen peroxide with or without mediator improved the decolorization over the treatments not containing added hydrogen peroxide. The decolorization of the dyes was also found to be dependent on the surface area of the roots exposed for reaction with the synthetic dyes as well as its composition and structural organization. Such differences in the roots of *A. thaliana* and sunflower plants due to their different ecological niches also might explain the slight differences in the oxidation of ABTS and the Km of the plants for hydrogen peroxide.

Based on the above results a possible mechanism for the decolorization of dyes exposed to plants has been proposed. The overall process of dye decolorization carried out by the plants is

dependent on the environment it is exposed in, along with the presence of factors such as hydrogen peroxide and mediator. It is believed that synthetic dyes behave as abiotic stress factors for the plants especially those grown under hydroponic conditions, and that the plant activates the stress response machinery especially in the roots including generation of reactive oxygen species such as endogenous hydrogen peroxide, superoxide and other free radicals of oxygen (Bolwell *et al.*, 2002; Neill, Desikan and Hancock, 2002; Shin and Schachtman, 2004; Shin, Berg and Schachtman, 2005; Quan *et al.*, 2008; Zhang *et al.*, 2011; Deng *et al.*, 2012; Petrov and Van Breusegem, 2012; Lu *et al.*, 2013). However, the concentration of endogenous hydrogen peroxide and other reactive oxygen species produced in the roots in response to the dye does not seem to be high enough to trigger rapid decolorization of the synthetic dyes. This was found to be true since the dye decolorization trends obtained for both plants which were exposed to the different synthetic dyes with the exception of MR in the absence of external additions of hydrogen peroxide were relatively low as compared to the treatments with added hydrogen peroxide. In order to achieve enhanced decolorization in short time the plants need interaction with higher concentrations of hydrogen peroxide. It has been shown that exposure to exogenous hydrogen peroxide is beneficial for root growth (Deng *et al.*, 2012; Lu *et al.*, 2013). In response to the externally added hydrogen peroxide plant roots initiates a number of events including upregulation of the production of reactive oxygen species as well as release of secretory enzymes (Bolwell *et al.*, 2002; Neill, Desikan and Hancock, 2002; Shin and Schachtman, 2004; Shin, Berg and Schachtman, 2005; Quan *et al.*, 2008; Zhang *et al.*, 2011; Deng *et al.*, 2012; Petrov and Van Breusegem, 2012; Lu *et al.*, 2013). The enzymes secreted in order to neutralize the stress effects of these reactive oxygen species include catalases, different types of peroxidases, glutathione reductases and superoxide dismutases (Apel and Hirt, 2004; Gechev *et al.*, 2006;

Quan *et al.*, 2008; Miller *et al.*, 2010; Petrov and Van Breusegem, 2012; Lu *et al.*, 2013). All these enzymes are capable of using hydrogen peroxide as substrate and bring about its reduction while the secretory class III peroxidases have also been shown to be capable of decolorizing dyes (Husain, 2006; Husain, 2010; Harms, Schlosser and Wick, 2011; Khataee *et al.*, 2013a, 2013b; Watharkar and Jadhav, 2014). In fact it has been shown that the secretion of certain class III peroxidases increases in response to exogenous hydrogen peroxide (Shin and Schachtman, 2004; Shin, Berg and Schachtman, 2005; Almagro *et al.*, 2009; Cosio and Duand, 2010; Tsukagoshi, Busch and Benfey, 2010). On being secreted within the roots these enzymes must be encountering the synthetic dye as well as the mediator HOBt for the dye treatments in which the mediator was added. The roots oxidize the HOBt to its free radical form and oxidized HOBt through radical hydrogen transfer has been shown to bring about enhanced decolorization of the dye (Xu *et al.*, 2000; Fabbrini, Galli and Gentili, 2002; Morozova *et al.*, 2007; Canas and Camarero, 2010). This mechanism of triggering responses in the roots against the added hydrogen peroxide during the initial exposure seem to be true since it was noticed that there was sudden drop in the absorbance values with increased rates of dye decolorization for all the dye treatments involving added hydrogen peroxide, especially in the presence of HOBt in the initial 6-10 hours of exposure to hydrogen peroxide. However, the decolorization values for the dye treatments with the exception of MR in which were exposed to plants no added hydrogen peroxide was not as enhanced. This trend was observed in decolorization of all the representative synthetic dyes in the presence of added hydrogen peroxide and mediator for both plants as well as the positive control of crushed turnip root extract. Exposure to the dye treatments with added HOBt but no added hydrogen peroxide did not trigger the same decolorization behavior indicating the importance of the interactions of external hydrogen peroxide with the plant roots

to enhance the decolorization of dye. These results also provide the first evidence of peroxidase enzymes playing a contributing role in the decolorization of synthetic dyes. Presence of plants with active roots and/or secretory enzymes seem to be necessary for the decolorization since it was seen that there was negligible drop in absorbance values of the dyes for the comparable negative control of the dye containing added hydrogen peroxide over the same time under similar treatment conditions. This indicates that in absence of the active decolorization machinery addition of hydrogen peroxide along with the mediator does not trigger any dye decolorization. The results also suggest that externally added hydrogen peroxide behaves as the limiting factor towards decolorization of dye in presence of plants after the initial time of exposure has passed. This was believed to be true since *A. thaliana* plants which were exposed to externally added 1 mM hydrogen peroxide were shown to reduce its concentration within 6 hours and the type of solution the plants were placed in did not interfere with their ability in diminishing the levels of externally added hydrogen peroxide. This meant that beyond 6 hours the added hydrogen peroxide was no longer available to interact with the plant roots. Thus, it appears that externally added peroxide was no longer the governing factor for the continuation of dye decolorization process beyond 6 hours.

Beyond 6 hours and after the initial exposure to added hydrogen peroxide, it appears that the plants might be adapting and altering the dependence on the added hydrogen peroxide for the dye decolorization since the decolorization process continues though at relatively slower pace. These results strongly suggest the possible role of laccases, secreted within the roots of the plants, in the absence of hydrogen peroxide in bringing about the decolorization of synthetic dyes during the extended times of exposure to the dye.

The batch dye decolorization experiments were however only carried out for 72 hours which is a relatively short time. Moreover, the dyes were prepared in phosphate buffer which made it difficult to study the associations and effects of other salts on the dye decolorization in the presence of plants which are found in real world conditions as well as field studies. The phosphate buffer also stresses the plants due to its lack of nutrients and thus is only good for experiments with short time courses. Half-strength Hoagland's solution which supplies all the nutrients essential for normal and daily plant growth was chosen as the solution for long time experiments since it not only provided all the nutrients which were essential for plant growth but also serves as a good solution to study the interaction and effect of various salts on dye decolorization which replicates real world conditions. It was observed that the dye decolorization trends for most of the dyes prepared in half-strength Hoagland's solution and exposed to the plants under similar treatments as well as reactions were not only comparable but it appeared that with few exceptions the presence of salts did not affect the decolorization potential of the plants. In fact, providing nutrients to the plants seem to reduce the stress on plants so that they would continue to decolorize dyes which included daily exposure to fresh dye. Plants did not require hydrogen peroxide or HOBt to decolorize synthetic dyes when nutrients were provided. It is possible that exposure to the nutrients enhances the growth of the roots and this ensures that the plants continue to decolorize the dyes even on repeated exposure to the dye. Plus the increased surface area of the roots along with the contact time of reaction with the dye ensures that the decolorization of synthetic dyes is not a single cycle but rather a repetitive cycle, even in the absence of added hydrogen peroxide and/or mediator. Both the plants have shown the potential to decolorize synthetic dyes whether added under batch conditions or repeated additions of the

fresh dye and further provides evidence of the potential role of laccases and peroxidases in synthetic dye decolorization.

Reverse phase HPLC-ESI/MS revealed the molecular weight and distinct characteristic peak for MO, BG and BBG in the negative and positive control dye solution along with the residual dye which were exposed to *A. thaliana* plants in presence of added hydrogen peroxide and HOBt. The distinct peaks representative of intact parent dye at the indicated molecular weights were seen for the different dye solution despite the presence of added hydrogen peroxide and HOBt which indicates that the dye on its own was not affected or altered in the presence of these additional components. The relative peak intensities were however, found to be reduced for the dye solutions exposed to plants or were no longer detectable as observed for the positive dye control solutions. These observations also were in good agreement with the relative amount of decolorization calculated using UV-Visible spectrophotometry for the dye treatments exposed to the plants in presence of added hydrogen peroxide and HOBt. The relative amount of dye remaining in the solution after being exposed to plants as calculated using both methods were very close to each other. This showed that not only were the dyes reduced in their concentration in the solution exposed to plants but the process of dye decolorization probably occurs within the roots. To prove this hypothesis the peaks representative of the potential dye break down products that could be possibly enriched in the residual solution treated with plants when compared to the negative dye control using ESI/MS were searched for. However, no peaks indicative of the possible dye by-products could be identified in the residual dye solutions exposed to plants, which favors the notion that the dye decolorization process occurred within the roots. This is an important finding since it has been suggested that reduction of azo dyes results in the formation of amines which have been shown to be carcinogenic and be a potential health hazard if released

into the solution after decolorization (Brown and De Vito, 1993; Robinson *et al.*, 2001; Wallace, 2001). The ES/MSI results indicated that none of the compound from the list of banned compounds as stated in the Directive 2002/61/EC published by the European Commission were identified or enriched in the ESI/MS after the exposure of the dyes to the plants.

In order to confirm that the process of decolorization occurs within the intact plant roots and the by-products of the process are retained within the roots, the representative synthetic dyes were exposed to the crushed root extract. Crushing turnip root and releasing its content would not only serve as a good positive control but the absence of actual physical roots will minimize any interference in the identification of the by-products generated during the dye decolorization process. Exposing crushed turnip root extract under similar conditions as those employed for the plants and led to the complete disappearance of the characteristic peaks indicative of the intact dye, in comparison with the negative dye control. The peaks were no longer detectable for any of the dye solutions exposed to the crushed root extract confirming its ability to decolorize synthetic dyes and behave as the positive control. Still, no new noteworthy by-product of the dye decolorization was detected, apart from the disappearance of the distinct dye peaks. This showed that the break down products of the dye following decolorization somehow escaped the ESI/MS detection. Two possibilities were suggested for the failure to detect these by-products. One possibility was the relatively lower abundance of the break down products as compared to the relative intensity of the intact parent dye which results in their signal being hidden in the background noise. The other possibility was that the ESI/MS analysis was performed only for the dye treatments containing added hydrogen peroxide and mediator, HOBt. HOBt in the presence of laccase and peroxidase enzymes is shown to be oxidized to its free radical whose instability and activity potentially bring about the decolorization of dye by radical hydrogen transfer

mechanism (Xu *et al.*, 2000; Fabbrini, Galli and Gentili, 2002; Morozova *et al.*, 2007; Canas and Camarero, 2010). The oxidation of the dyes by the generated free radicals usually results in the formation of fragments which are quite unstable, leading to their polymerization (Xu *et al.*, 2000; Fabbrini, Galli and Gentili, 2002; Gianfreda and Rao, 2004; Morozova *et al.*, 2007; Canas and Camarero, 2010; Harms, Schlosser and Wick, 2011; Strong and Claus, 2011). Polymerization ultimately leads to the precipitation of the dye from the solution which escapes the detection range of ESI/MS. These results showed that the dye break down products failed to be detected for all the dyes that were run through ESI/MS.

Phylogenetic relationships and amino acid sequence comparisons of the various members of the laccase and peroxidase family revealed that the different laccases and peroxidases are highly evolved and diverse with distinct protein sequences despite certain levels of functional redundancy. This diversity with functional redundancy could also explain the unsuccessful characterization of the mutant plants containing T-DNA insertions in the single laccase and peroxidase genes in terms of their dye decolorization abilities. *Arabidopsis* plants with single T-DNA insertions in the individual laccase and peroxidase genes showed comparable decolorization of MR and MO when treated in similar manner as the wild-type plants. This was in agreement with the results shown by Cosio and Duand, 2010 where they suggested that knocking down single class III peroxidase genes did not result in the complete loss of its function and other peroxidases compensated for the loss by performing the intended function. On the other hand in some instances, peroxidases have high substrate specificity (Chanwun *et al.*, 2013). The results also suggested that the Phenol Red dye may be used in detecting relative levels of exogenous hydrogen peroxide and its interaction with plants and can serve as a candidate dye in identifying the by-product formed due to its interaction with the plant roots. The oxidized PR dye

has an absorbance maximum that was found to be completely different from the parent dye making it simpler to distinguish the two species under alkaline or acidic conditions using UV-Visible spectrophotometry. Of all the dyes that were selected, PR was the only one that showed this potential. Also it was shown that a large percentage of the recovered fraction from the roots was the oxidized product. Thus, was confirmed solely on the basis of the data obtained using UV-Visible spectrophotometry.

Chapter 5 - Conclusions and Future Directions

5.1 Conclusions

This study helps in understanding the basic mechanism by which plants decolorize synthetic dyes. Despite selecting plants from different families, when it came to the decolorization of synthetic dyes both *A. thaliana* as well as sunflower plants showed certain similarities in their ability to decolorize these dyes. Both plants were able to decolorize representative monoazo, disazo and arylmethane dyes under similar batch treatment conditions. There was not much difference observed in their ability to oxidize ABTS and the apparent Km values for hydrogen peroxide that were determined were comparable to each other. Representative dyes from all three classes were decolorized rapidly by plants in the presence of external hydrogen peroxide and mediator. The combination of added hydrogen peroxide with mediator together was found to enhance the decolorization efficiencies and the initial rate of decolorization of the different dyes as compared to the dye treatments with no exposure to externally added hydrogen peroxide. Plants exposed to added hydrogen peroxide alone could also bring about similar levels of arylmethane decolorization. This suggested that peroxidases were the chief group of enzymes that were involved in the initial stages of decolorization. However, for the disazo dyes, *A. thaliana* plants were found to be relatively better at oxidizing the dyes than sunflower when placed under similar treatment conditions. The differences observed between the plants when treated in similar manner are more subtle than significant. There are differences in the surface area of roots available for the reaction with the dyes and the composition of the roots in terms of their cellulose and lignin contents. Structural changes due to different functional groups in the representative dyes from the different classes did not greatly affect the ability of the plants to

decolorize dyes. Added hydrogen peroxide was found to be the limiting factor since the plants exposed to hydrogen peroxide reduced its concentration in 6 hours. Further decolorization of the dyes is seen possibly due to the production of either laccase or peroxidases by the plants as it adapts to the dye conditions. Decolorization of the dyes carried out by plants is repetitive and as long as the nutrients are provided, the plants will continue to decolorize freshly added dye on repetitive basis. Mutant *A. thaliana* plants with T-DNA insertions in the single laccase and peroxidase genes did not seem to affect the dye decolorization potential of the plants as these plants were found to decolorize dyes with similar efficiencies as the wild-type plants. Decolorization of the dyes in the presence of hydrogen peroxide and mediator takes place within the roots. By-products of decolorization are retained in the roots since these by-products were not detected in the solution exposed to either plants or crushed turnip root extract, using reverse phase HPLC-ESI/MS.

5.2 Future Directions

The future objectives would include using molecular tools such as real time RT-PCR and microarray techniques to identify and determine the levels of gene expression upon exposure of the *Arabidopsis* plants to the different dye treatments and comparison to control plants that will not be exposed to the dyes. These gene sets can then be compared to gene sets available for plants under other stresses of abiotic and biotic nature. This can help in studying the particular target genes which have been upregulated in response to the dyes. Individual single knockouts of the genes encoding for enzymes which has been suggested to play a role in dye decolorization did not prove to be informative due to apparent genetic redundancy and overlapping functions of the different peroxidases. Knocking down two or three of the genes simultaneously and then studying the effect of this multiple gene knockouts on the ability of the plants to decolorize dyes

will help give in characterizing the enzymes for their role in decolorization. Alternatively, simultaneous multiple gene silencing of the diverse laccases and peroxidases using either virus induced gene silencing and/or siRNA techniques will help in elucidating the possible roles of these enzymes in dye decolorization. Also screening of the laccases and peroxidases from the roots exposed to dye by purifying the proteins followed by fractionation and separation using ion-exchange chromatography can help reveal the substrate specificity of these enzymes.

Effect of hydrogen peroxide on the regulation of genes in the plants exposed to the different dyes with its comparison to control plants not exposed to the dye will identify new candidate genes and targets involved in stress responses. The complete genome of *Arabidopsis* has been sequenced but the genome of sunflower is still being sequenced and understanding the effect of dyes on the gene and protein expression between various plants including different cultivars of sunflower and monocot plants such as *Miscanthus* will help in identifying the similarities and differences in the behavior of different plants in response to the synthetic dyes. Identifying the particular genes responsible for decolorization can be exploited in expressing it outside the plant cells. This may show potential for a wide variety of applications including the commercial methods of bioremediation.

Exact locations of the enzymes involved in the dye decolorization process including their subcellular localization in the roots still remain elusive. The most probable location for the decolorization is suggested to be the apoplast and the extracellular space close to the cell wall (Cosio and Duand, 2010) since most of the peroxidases have sequences which target them to cell wall and the apoplastic region (Cosio and Duand, 2010). Identifying the location of these enzymes in the plant roots will further aid in elucidating the functional role of these enzymes. Also failure to characterize the potential dye break down products in the solution following

exposure to plants was attributed to the limitations in detection of the ESI/MS instrument and its use of the C18 column. Use of direct injection of the dye in the ESI/MS might potentially overcome this issue. Success in the above objectives can then be extrapolated to crop plants such as rice and legumes with possible implementation of these plants in phytoremediation of synthetic dyes.

The conditions employed in the laboratory settings are far cry from the realistic field conditions. More testing which replicates actual field studies in terms of choice of annual plants versus perennial plants, feasibility of using different levels of hydrogen peroxide in combination with low cost mediators, space and infrastructure involved for setting up an experimental constructed wetland along with monitoring of variables such as temperature, light intensity and contact time and exposure of the plants with not just single dye but a complex mixture of dyes including actual effluents from the textile industry needs to be planned and experimented before the actual implementation of this study for large scale purposes.

The objective of all the above mentioned future directions is to find out newer applications of employing whole plants including commercial plants outside their main role in agriculture and food sources.

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