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Polyphenol Oxidase In Plants *Vis-À-Vis* Insect And Pathogen Attack

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Abstract

Plants interact with insects and pathogens leading to complex defense approaches against their feeding strategies and disease reactions. Among these defenses, the polyphenol oxidases (PPOs) are the most commonly associated enzymes in both higher plants and fungi.They are significant for entrapment and anti-nutritive defensive mechanisms against these herbivores and micro-organisms. There is increase in the synthesis of polyphenols aided by PPO enzymes under abiotic and biotic stress conditions, which helps the plants to overcome with these environmental or exogenous constraints. PPOs and theirphenolic substrates (mainly quinone compounds) are localized separately within the plants, in plastids and in the vacuoles, respectively, and they get into direct contact only after cellular wounding by external entities like insects and pathogens.In this review, the possible and reported roles or functions of PPOsare discussed in the light of the available literatures. In addition, since PPOs are versatile enzymes, they are expected to have other, newly reported oryet to be discovered functions in plant systems.

Keywords: polyphenol oxidase, enzyme, defense, insects, pathogens

Introduction

Polyphenol oxidases or PPOs are one of the copper (Cu) enzymes in plants, which are also known as tyrosinases or catechol oxidases. In plant systems, the hydroxylation process of monophenols to ortho-diphenols conversion is catalysed by the PPO enzymes in the presence of molecular oxygen (O_2), leading to the subsequentformation of quinones through ortho-diphenols oxidation(Constabel and Barbehenn, 2008; Jukanti, 2017). These PPO-mediated quinone compounds have been reported to alkylate or cross-link proteinswithin the plant system due to their high reactive nature(Mayer, 2006; Bhonwong et al., 2009). This results in the formation of brown pigments, which are commonly observed in damaged plant parts or tissues. PPOs are also responsible for the post-harvestlosses of agricultural perishable produces, mainly fruits and vegetables. Furthermore, taking into account of the importance of browning process in agricultural produces in relation to their economic values, the physico-chemical properties of these enzymes have been widely studied and experimented.

Role of PPOs in plants

PPO enzymes are abundant in the angiosperms, are involved in several plant physiological processes including insect and pathogen defenses (Mayer, 2006) and can be induced by both biotic and abiotic stress conditions (Constabel and Barbehenn, 2008; Fürstenberg-Hägg et al., 2013). Because of the insect-pathogen and wound induced expression, PPOs have been considered as “defense proteins” (Fuerst et al., 2014). Although

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the catalysing action of PPOs in different biochemical reactions are well known, studies or reports on their physiological functions are limited (Constabel et al., 1996; Mayer, 2006). PPO roles have been recognized and well-known in crop physiology and agricultural biochemistryfor several years now, and quite a few hypotheses regarding theirphysiological and biochemical functions in plants have been proposed which includes:

1) Their role in the plant signalling phenyl-propanoid pathway (Araji et al., 2014; Sharma et al., 2019)
2) Wound-mediated oxidative browningof plant tissues resulting insimilar browning processobservedin damaged or cut vegetables and fruits when in contact with the atmospheric oxygen (Eg. browning in freshly cut potato,brinjal, apple, litchi, etc.) (Vaughn and Duke, 1984; Whitaker and Lee, 1995; Jiang et al., 2004)
3) Electron cycling and Mehler reaction–This reaction provides additional photosynthetic electrons when plants experience water-stress conditions (i.e. when carbon digestion diminishes on account of stomatal closure), which may subsequently help in the prevention of excess exhaustion of electrons in direct electron transport. (Vaughn and Duke, 1984; Vaughn et al., 1988; Thipyapong et al., 2004)
4) Oxygen regulation- Photoreduction of molecular oxygen by PSI (Photosystem I) (Steffens et al., 1994; Constabel and Barbehenn, 2008; Jukanti, 2017)
5) Flower petal colouration (eg. In Snapdragon) (Nakayama et al., 2000; Nakayama et al., 2001; Ono et al., 2006)

Inplant systems, PPOs and theirphenoloc substrates (mainly quinones) are reported to be accumulated separately, *i.e.* PPO are expected to be confined in the plastids of chloroplasts (Boeckx et al., 2015), while their phenolic substrateslocalized in the vacuoles (Constabel and Ryan, 1998; Ono et al., 2006; Bhonwong et al., 2009). Thus, these enzymes come into direct contact with their substrates only when cellular decompartmentalization or cell disruption takes place as a result of tissue damage,wounding or attack by insects or pathogens (Constabel et al., 1996; Mayer, 2006; Jukanti, 2017). The potential mechanisms in which PPOs impart defensive responsetowardsinsect herbivores and pathogenic micro-organisms may include (1) toxic effect and antimicrobial activity of quinone substrates, (2) reduction in theavailability of plant proteins and nutrients, (3) formation of physical barriers similar to lignin compound, and (4) generation of ROS, reactive oxygen species (Constabel and Barbehenn, 2008).

Mechanism of PPO defense action against insect herbivores

Extensive studies on polyphenol oxidase enzymes have been carried out by biologists, crop physiologists, plant pathologists and ecologists, who are interested in unravelling the defense mechanism of actions against insect pests and pathogens.In most of the plant species, there isproduction of defensive phenols at the site of wounding or tissue damage, followed by the oxidation of these phenol compounds by the action of PPO enzymes, resulting in the formation of quinones, which are responsible for reducedbio-availability of dietary proteins required for normal growth and development of insect herbivores (Wang *et al.*, 2004; Thipyapong *et al.*, 2007a). After plant wounding by insects, the wounded leaf or damaged tissue releases an 18-amino acid peptide, called systemin, which activates or triggers a lipaseenzymein the receptor cell membrane to release linolenic acid(Fig.1) (Constabel et al., 1996; Bhonwong et al., 2009). Linolenic acid is the precursor of jasmonic acid biosynthesis,

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which serves as a signal for expression of a number of compounds during plant-insect interactions (Bhonwong et al., 2009). Jasmonate-mediated signalling at gene level produce insecticidal compounds like proteinase inhibitors (PI) and enzymes like polyphenol oxidase (PPO), catalase and steroid glycoalkaloids (Constabel and Ryan, 1998; Redman et al., 2001; Alba-Meraz and Choe, 2002), which contributes to plant resistance against many insect pests. These insecticidal compounds may interfere with the normal functioning of insect digestive system, thus deterring the insects from feeding. For these reasons, PPOs has been often termed as "antiherbivore enzymes".The nutritive value of the plant is reduced by the action of PPOs through cross-linking proteins and formation of structural barriers or due to quinone toxicity, which is a result of phenolic metabolites oxidation (Mayer, 2006; Jukanti, 2017). In simple words, the effectiveness of phenolics in plants can be enhanced by oxidation to its polymers (quinones) which will reduce the digestibility, palatability and nutritional valueof the plants conferring resistance against insects. Hence, high levels of PPO correlate with plant resistance against herbivorous insects (Constabel and Ryan, 1998; Mayer, 2006; Wang and Constabel, 2004).Altogether, there are three proposed mechanisms by which PPOs provide defense against insect herbivores:

(1) Decreasing plant nutritional quality through alkylation or cross-linking of importantplant amino acids by the action of PPO-mediatedmain substrates, (2) generation of oxidative stress in the insect gut lumen through redox cycling of quinones, and (3) formation of phenolic oxidation products (generated by quinone redox cycling) like reactive oxygen species (ROS), hydrogen peroxide (H_2O_2), which may be absorbed by the insect herbivores, resulting toxic effectsand death (Constabel and Barbehenn, 2008).

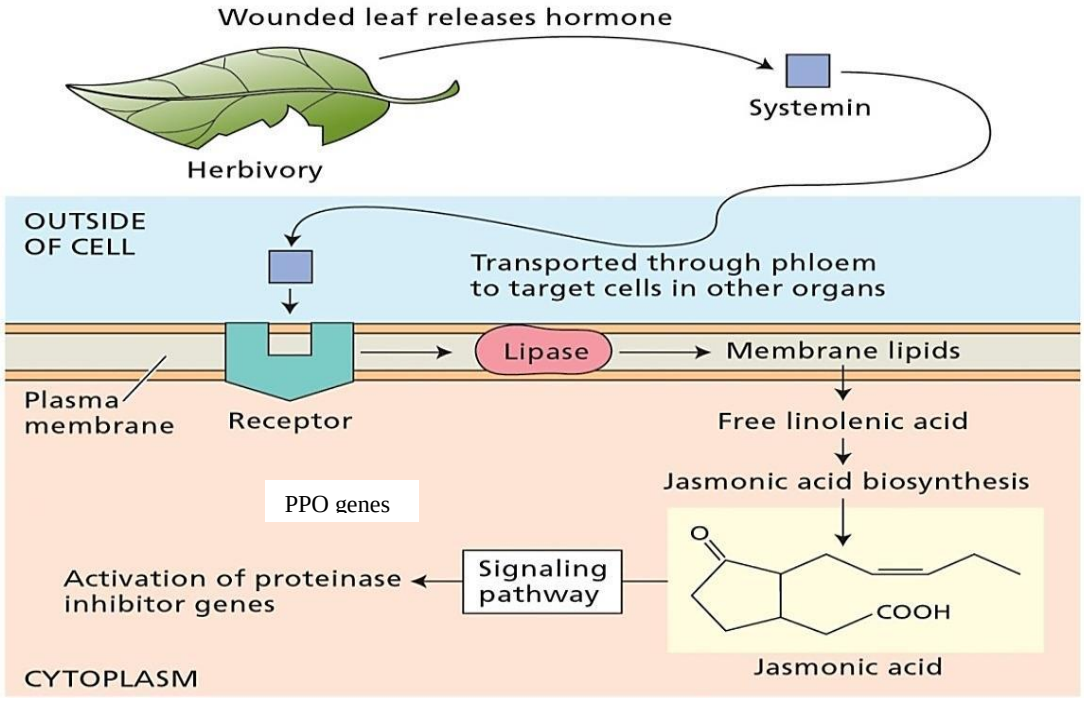

Fig. Series of action involved in PPO enzyme action against insect damage

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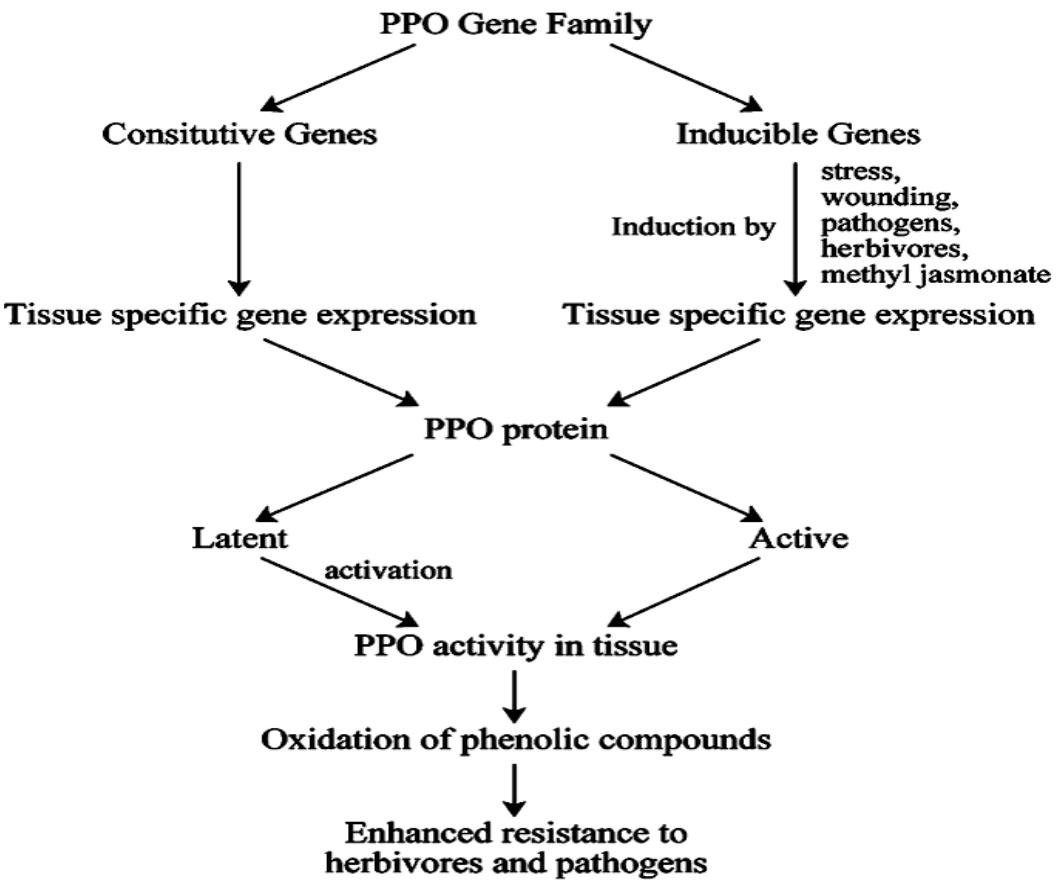

Fig. PPO activity in plant system against insect and pathogens

A very important feature of PPO has been reported to be expression of a variable degree of latency, so that PPOs from some plant species need to be activated with detergents or proteases for its full activity (Vickers et al., 2005; Araji et al., 2014). For instance, PPO from tomato leaf can be extracted in its fully active form without activation (Stout *et al.*., 1998; Thaler et al., 2002), whereas the poplar leaf PPO requires activation with protease or detergent for its full expression (Barbehenn et al., 2007). Reports have suggested that such activating treatments function by removal of a peptide from the active site, *via* proteolysis or partial unfolding of the polypeptide(Thipyapong et al., 2004; Thipyapong et al., 2007a), respectively. The activation of latent PPOs can also be done by treatment with low pH, presumably *via* a conformational change of the active site, thus increasing its accessibility to the phenolic substrates. In addition, it has been proposed that the latency of PPOs may be significant for its defensive functions against different pests (Wang and Constabel, 2004; Thipyapong et al., 2007a).

PPOs for insect resistance in plants:

PPO enzymes are involved in plant defensive response against arthropod herbivores, where their activities have been correlated with resistance to these herbivores (Mayer, 2006; Jukanti, 2017). It has also been reported thatthere is improvement in the growth and developmental rates of herbivoreswhen the activity of these enzymes are inhibited by the action of antioxidants and other chemical inhibitors (when insects are fed on plant-derived diets expressing more PPO) (Fürstenberg-Hägg et al., 2013; Fuerst et al., 2014). In a laboratory experiment conducted to test the PPO activity, the growth and development of

lepidopteran larvae was hinderedwhen artificial diet wasincorporated with both the PPO and their phenolic substrates (Bi et al., 1997; Bhonwong et al., 2009). Moreover, the induction of PPO activity in plants by wounding or plants with induced PPO activity increases thedefense action and resistance against a wide range of insect arthropods.For instance,tissue damage by chewing insects or artificial treatment with jasmonic acid (JA), which is responsible for the induction of PPO activity and other responses (Thipyapong et al., 2004), resulted in enhanced defensive responsestowards a wide range of arthropod herbivores such as biting and chewing insects, sucking pests, mites and even bacterial or fungal pathogens(Thaler *et al*.., 2002; Thipyapong et al., 2004).

In an experiment conducted by Bhonwong et al. (2009), transgenic tomato plants and mutants possessingimproved defense genes expression (including PPO) were observed to have increased levels of insect resistance. For example, it was reported that the transgenic tomatoplant overexpressing *TobpreproHypSys-A* gene (which encodes for systemin precursor protein, hydroxyproline-rich glycopeptide) showed increased resistance againstlarvae of*Helicoverpa armigera*.This was brought about by the accumulation of PPO enzymes and the anti-nutritive factor, proteinase inhibitors (PIs)(Bhonwong et al. (2009). Contrastingly, a study by Stout *et al*.., (1998) revealed that a tomato mutant was found to be insensitive to jasmonic acid(JA),i.e. no expression of PPOs and PIs in response to the artificial tissue damage or JA treatment, anddemonstratedhigh susceptibility against *Tetranychus urticae* (two spotted spider mite).In case of PPO-derived resistance in plants, these defensive enzymes are believed to causeadverse effects on insects by means of diverse mechanisms, although investigations and experimental evidencesmanifesting these proposed mechanisms are missing and yet to be demonstrated.

PPO substrates such as quinone compounds and reactive oxygen species (ROS) may impart direct toxicity to insect arthropods and disease causing micro-organisms (Vaughan et al., 1984; Castanera et al., 1996; Mayer, 2006; Constabel and Barbehenn, 2008; Foyer, 2018; Sharma et al., 2019).Other PPO substrates such as H_2O_2 and ROS may indirectly influence plant resistance against these external factorsby taking part in the plant signalling networks (eg. phenylpropanoid pathway) (Omiadze et al., 2018; Sharma et al., 2019).The mechanism of cross-linking plant cell wall and increased tissue lignification that occursdue to improved PPO activity may inhibit or decrease acquisition of the nutrients by insect arthropods (Mayer, 2006). However, the main PPO mechanism that has gotten the most consideration as for herbivore opposition is the ability of PPO-mediated quinones to alklylate (arylate) dietary protein (Mayer, 2006; Constabel and Barbehenn, 2008; Bhonwong et al., 2009), thuslessening the nutritional value of plant tissues and rendering the availability of plant proteins to herbivores. In the same way, PPOs may be involved in chlorogenic acid oxidation in tomato leaves, thus reducing free NH_2level andplant leaf proteins, and their influence on nutritiveplant proteins were found to be correlated with reduced developmental rates inlarvae of *Spodoptera exigua* (beet armyworm) (Stout et al., 1998; Bhonwong et al., 2009). Derivatization process by PPO-mediated quinone compounds leads to susceptibility of some of thenucleophilic amino acids like methionine, lysine, histidine, and cysteine, which acts as limiting factor in the larval development of lepidopteran insects (Felton and Duffey, 1991).

PPOs for disease resistance in plants:

PPOs haveoften been linked withplant defense against diversedisease causing microbes since their primary substrates (quinones) are strong electrophiles that are capable

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of undergoing various secondary reaction pathways. One such reaction example is theimprovement in the 1, 4-position of *ortho*-quinones to plant cell nucleophile and its turned around conversion in to semi-quinones,resulting in ROS generation (Kuvalekar et al., 2011).Furthermore, PPO-mediated quinones and ROS may havedifferent roles in a wide range of disease defense-related functions in the plant system.In both consistent and inconsistent plant-pathogen associations, PPOs have been reported and accounted to act and respond after cell decompartmentalization due toplant cell death (apoptosis), leading toformation of quinone compounds as one of the end products in the advancement of such defense responses (Francesca et al., 2017; Mao et al., 2019).

In plant systems, disease progression due to pathogen attack may be restricted through direct toxicity *via*quinone-mediated modification (cell alteration) or direct activity of H_2O_2 (hydrogen peroxide) with anti-microbial toxicity (Li and Steffens, 2002; Mao et al., 2019). Moreover, there may be reduction in the nutrient bioavailability to pathogens due to alkylation of plant protein (Fig.2). Similarly, due to the protein alkylation process andimproved lignin generation, there may be creation of a physical barrier against the pathogens.In response to the direct defense against invading microbes, PPO-mediatedH_2O_2substrate (hydrogen peroxide) could also act as one of the components in plant signallingpathwaysthrough induction ofphytoalexin biosynthesis, ethylene, salicylic acid production, and diffusible cellular protectant genes, thus triggering cell death and expression of limited lesions differentiated from the surrounding healthy tissues. This condition is generally referred as hypersensitive response (Duan et al., 2014). Likewise, PPO-generated reactive oxygen species (ROS) may be involved in systemic signalling network through activation of other plant defense genes, resulting in enhanced plant immunity (Li and Steffens, 2002).Although various mechanisms have been reported as a result of PPO enzyme action in the limitationof pathogenic disease progress, confirmatory studies and results are yet to be documented.

PPOs during water stress condition

It is a well-known fact that PPOs are associated with the leaf thylakoid membranes and due to this;there is high Km of PPO for O_2(Lax and Vaughn, 1991; Foyer et al., 2018). Because of the observed relationship between PPO action and oxygen development in detached chloroplasts, the Mehler reaction, which is the photo-reduction of molecular oxygen (O_2) by PS-I (Photosystem I), and regulation of plastidic oxygen levels, has been supposed to be mediated by the mesophyll chloroplast PPO (Upham et al., 1987; Jovanovic, 1998; Lax and Vaughn, 1991). This reaction provides additional photosynthetic electrons under water stress conditions (when carbon digestion diminishes on account of stomatal closure), which may subsequently help in the prevention of excess exhaustion of electrons in direct electron transport. On the other hand, when abundance energy is not disseminated by the defensive systems, cytotoxic ROS are generated. If the collective ROS ability exceeds in the plant system, there will be photo-inhibition and photo-oxidative damage will occur in plants(Akhtar and Mahmoo, 2017). In situations where PPOs are involved in photo-reduction of oxygen (Mehler reaction), plants showing higher PPO levels are likely to increase their tolerance mechanism towards any abiotic stress conditions (Upham et al., 1987; Foyer et al., 2918).

Other possible roles of PPOs

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It is anticipated that PPOs may have progressedconcurrently with the altered oxygenated surrounding, where its functional role is fundamental in the photosynthetic system of terrestrial plants. At the molecular level, there may have been evolution of PPO genes to perform various physiological functions in different plant species in order to adapt and survive with the changing environment. One functional role of PPO was reported in snapdragon forbiosynthesis of the yellow pigment, aurones, responsible for flower colour(Nakayama et al., 2000; Nakayama et al., 2001), and for betalainbiosynthesis (yellow or red pigments) in Caryophyllales plant order (Alphonso et al., 2019). Another report suggested that PPO are involved in the bio-synthesis of lignans in *Larrea tridentate* (creosote bush). Lignans are plant substances having different useful properties includingantioxidant, anticancer and antiviral (Cho et al. (2003). Moreover, due to its capacity to form o-diphenols from mono-phenols, PPO enzymes have been assumed to play a role in the process of p-coumaric acid to caffeic acid conversion through hydroxylation in the phenyl-propanoid signalling pathway (with the action of coumarate 3-hydroxylase enzyme) (Omiadze et al., 2018). However, no apparent differences inp-coumaric acid and caffeic acidlevels were observed, demonstrating that PPOs are not involved in this hydroxylation process in plants. In addition,Omiadze et al. (2018) and Sharma et al. (2019) also reported that PPOs does not contribute in hydroxylation process of coumarate compounds, which may be due to disruption in the plastid PPO uptake.

Defensive role of PPOs in transgenic plants

The first report of the role of PPOs in defense response(mainly anti-nutritive action) against herbivorous insects was experimentedin transgenic tomato plants against *Heliothis zea* by Felton and Duffey (1992), which showed a negative correlation of insect growth and PPO levels. Another convincingreport for the involvement of polyphenol oxidaseenzyme in defense response against herbivorous insects was the discovery of its activation and level of its mRNA in transgenic tomato when induced with systemin and methyljasmonate (MeJA), which are defense-related signalling molecules during herbivore induced wound(Felton and Duffey, 1991). Under normal or natural conditions, because of the simultaneous occurrence of other biochemical and physiological changes in the damaged plant tissues, the determination ofthe expression of induced PPO on insect biology is not feasible.Hence, transgenic plants have beenoften tested for determining the direct effects of PPO activity, where its levels could be controlled or manipulated irrespective of other plant characteristics.

An experiment conducted by Thipyapong et al. (2007b) suggested that the antisense PPO expression (suppressed) and PPO over-expression were found to increase susceptibility and resistance, respectively, of tomato plants to *Pseudomonas syringae*. They further substantiated the functional role of PPO towardsresistance against *P. syringae* by increasing the level of PPO activity up to 5-10 folds, which showed fewer lesions (decrease in 15 fold) caused due to disease infection and reduced growth (reduction up to 100-fold) of the bacterium (*P. syrinagae*)than when experimented on non-transgenic control. Similarly, transgenic plants showing PPO over-expression demonstrated enhanced resistance capacity towards different insects (Li and Steffens, 2002), including common cutworm (*Spodoptera litura*), (Thipyapong et al., 2007a), cotton bollworm (*Helicoverpaarmigera*) (Bhonwong et al., 2009) and beet army worm (*Spodoptera exigua*) (Stout et al., 1998; Bhonwong et al., 2009). In contrast, plants with low or suppressed PPO expression showed higher larval growth and were reported to consume more foliage.Similar experiment done in *Leptinotarsa decemlineata* (Colorado potato beetle) reported higher

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survival, consumption of foliage and increased weight on antisense PPO (suppressed) transgenic tomatoes (Castanera et al., 1996).

Effect of PPO induction in transgenic plants for different insect herbivores:

Plants	Insect Herbivores	Effects	References
Potato	Colorado Potato beetle (*Leptinotarsa decemlineata*)	RGR (-)	Castanera et al., (1996)
Tomato	*Spodiptera exigua*	GR (-)RGR (+)	Stout *et al.*, (1998)
	Spider mite	Population (-)	Stout *et al.*, (1998)
	Manduca sexta	RGR(-)	Redman et al.,(2001)
	Trichoplusia ni	RGR(-)	Thaler et al.,(2002)
	Manduca sexta	None(0)	Thaler et al.,(2002)
Poplar tree	*Malacosoma disstria*	GR(-)	Wang & Constabel (2004)
	Lymantria dispar	GR (-)	Barbehenn et al.., (2007)
Cotton	*Helicoverpa zea*	Not detectable	Bi et al.,(1997)
Oat, Wheat, Barley	*Diuraphis noxia*	None (0)	Ni et al.,(2001)
Soybean	*Ceratoma trifurcate*	None (0)	Bi and Felton(1995)
French bean	*Melanoplus differentialis*	None (0)	Alba-Meraz and Choe (2002)

RGR = Relative Growth Rate, GR = Growth Rate, (+) = increase, (-) = decrease, (0) = no effect

Case studies:

1. A laboratory experimentwas conducted at Tamil Nadu Agricultural University (TNAU) by Khorsheduzzaman et al. (2010) during June to December, 2005 in five brinjal genotypes,to study the biochemical resistance mechanism of fruit and shoot borer (*Leucinodes orbonalis*) in brinjal (*Solanum melongena*) and their correlation with insect infestation. The results suggested that higher amount of polyphenol oxidase (PPO), phenylalanine ammonium lyase (PAL), lignin, and lower amount of reducing sugars were recorded inthe plant parts of less susceptible genotypes. It also revealed thatthere was significant negative correlation between percent shoot and fruit infestation by the insect with PPO, PAL and lignin content. The study clearly indicated the role of PPO in imparting resistance to insect herbivores.

2. A study on the defense responses of rice plants towardsinfestation of small brown planthopper (SBPH) (*Laodelphaxstriatellus* F.) in China (Duanet al., 2014)

In an attempt to unravel the resistance mechanism in rice plants against small brown plant hopper in China, Duan et al.(2014) conducted laboratory experiments on two contrasting rice varieties, Kasalath (SBPH resistant) and Wuyujing (SBPH susceptible). In their experiment, salicylic acid synthesis related genes (*npr1, eds1* andpad4) were induced on both the varieties, and the expression of defense relatedgenes and defense enzymes (such as phenylalanine ammonialyase, PAL;polyphenol oxidase, PPO and peroxidase, POD) were examined. The expression was recorded at 12 hrs post-infestation (hpi) and it was reported that the level of PAL expression in Kasalath increased up to about 7.5-fold than the control treatment. At the same time, the activities and expression ofthese defense enzymes increased manifold in Kasalath rice cultivar, which were also found to be positively correlated with the level of PAL geneexpression.

Conclusion

Polyphenol oxidase enzymes exist extensively in plants, which are responsible for catalyzing oxidation of phenols to quinone compounds in the presence of oxygen, and have beenreported to play functional roles in plant defenses against both biotic and abiotic (environmental) stresses. The mechanism of action of PPO against insect herbivores and plant pathogens is not similar with other compounds in imparting resistance; therefore, they can be exploited and incorporated in both IPM and IDM studies. Moreover, owing to the separate localization of the enzyme and its substrate in plant system, there is a potential to provide both disease and insect resistances through alteration of PPO action in different tissues.However, adverse effects of PPO activity on post-harvest quality of agricultural produce and on the physiology of water-stress conditions should be taken into consideration. Apart from the physiological studiesof PPO conducted on transgenic tomato, the other physiological roles of PPO in non-related plant species may also be studied with the application of antisense/sense technology. Furthermore, transgenic plants that showed lower PPO expression demonstrated favourable water relations and reduced photo-inhibition reaction when compared to transgenic plants with PPO over-expression and normal plants. This suggested that PPO likely have a functional partin plant water-stress conditions, including light-mediated inhibition and oxidation in plants.

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