ISBN: 978-93-90847-49-5

6. Induction of Defence Related Enzymes in Plant for Sustainable Agriculture

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6.1 Introduction:

Higher plants have a broad range of defense mechanism to protect themselves against various stresses including physical, chemical and biological, such as wounding, exposures to salinity, drought, cold, heavy metals, air pollutants, ultraviolet rays and pathogen attacks. These stresses can induce plant for biochemical and physiological changes, such as physical strengthening of the cell wall through lignification, suberization, and callose deposition; by producing phenolic compounds, phytoalexins and pathogenesis-related (PR) proteins which subsequently protect plant from various stresses. The plants in order to defend themselves against pathogen attack, plants possess a range of constitutive and inducible resistance mechanisms. The most effective type is non-host resistance. This non-host resistance is expressed when a plant comes into contact with a pathogen which is incapable of provoking any disease in this plant (Heath 2000).

In the absence of non-host resistance, the plant is susceptible. The susceptible plants are capable of reacting to the pathogen in such a way that may slow down growth of the pathogen, which is called as basal resistance. Basal resistance is often dependent on the action of one or more of the plant hormones jasmonic acid (JA), ethylene (ET) and salicylic acid (SA). Upon attack, the levels of these hormones are usually enhanced. JA, ET and SA are required in succession for several signal transduction pathways in basal resistance. There are two different signal transduction pathways in plant such as systemic acquired resistance (SAR) and induced systemic resistance (ISR). ISR is called as Jasmonic acid Ethylene (JA-ET) dependant- plant growth promoting rhizobacteria (PGPR) or plant growth promoting fungi PGPF) mediated induced systemic resistance (ISR) which is subjected to the expression of defense related enzymes and defense chemicals for structural and chemical barrier in host plant against pathogen. While, SAR is called as salicylic acid (SA) dependant- necrotizing pathogen mediated systemic acquired resistance (SAR) which is responsible for expression of pathogenesis related proteins (PR-proteins) playing direct defensive role against pathogen attract **(Figure)**. Their expression may be studied by assaying enzymes play role in defense mechanism.

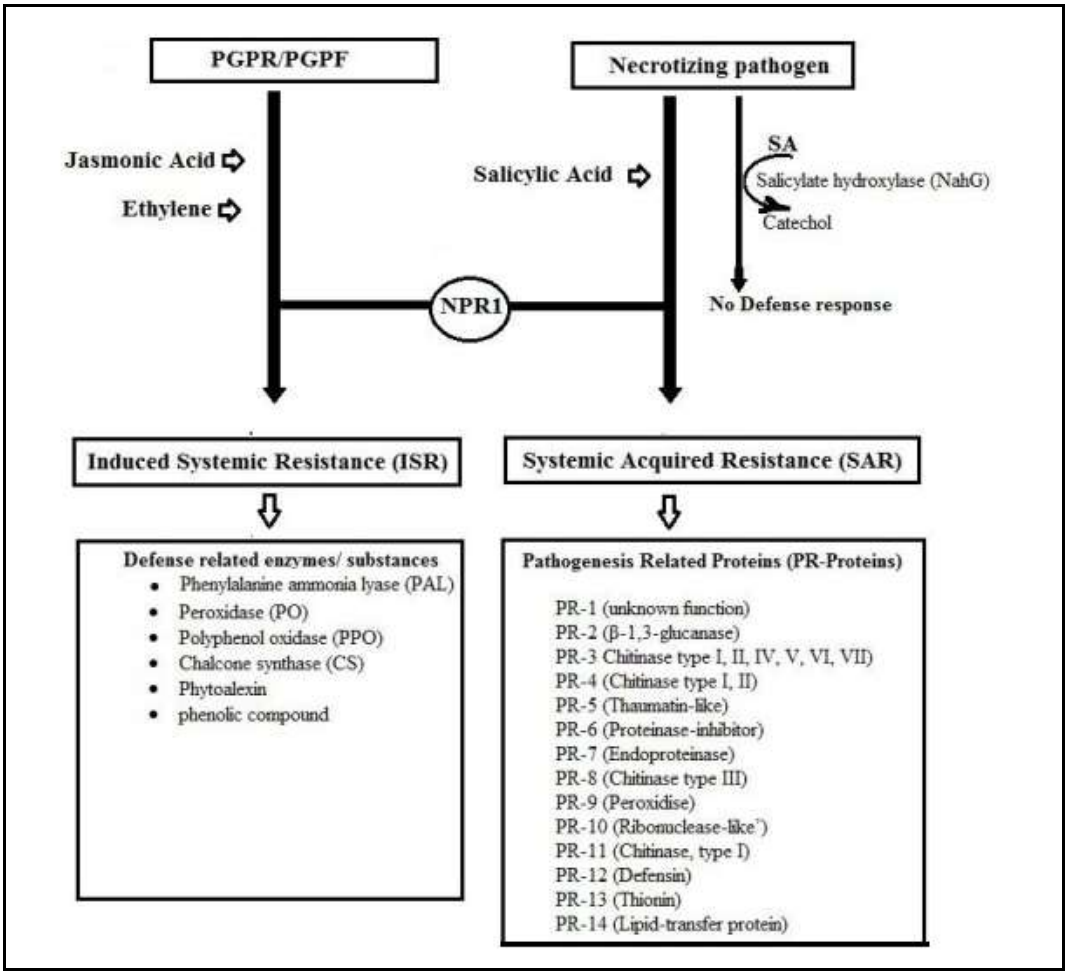

Figure 6.1: Molecular level plant defense related signal transduction pathway: A- Plant Growth promoting rhizobacteria (PGPR)/ Plant Growth promoting fungi (PGPF) mediated

signalling pathway, Induced Systemic Resistance (ISR) which require Jasmonic acid (JA) and Ethylene as elicitor signalling molecules and produce a diverse array of defense related enzymes (such as peroxidase, polyphenol oxidase, chalcone synthase etc.) and defense related substances (such as phytoalexin, anti-microbial phenolic compounds, etc); BNarcotizing pathogen mediated signalling pathway, Systemic Acquired Resistance (SAR) which require endogenous salicylic acid (SA) signalling and produce Pathogenesis-related proteins (PR-proteins).

6.1.1 ISR Related Plant Growth Promoting Rhizobacteria (PGPR):

Root colonizing bacteria (rhizobacteria) that trigger the expression of induced systemic resistance (ISR) and promote growth of plant by several mechanisms are called as plant growth promoting rhizobacteria (PGPR). PGPR primarily include *Serratia marcescens* 90-166, *Bacillus pumilus* Meyer and Gottheil SE34, *Pseudomonas fluorescens* 89B-61, P. fluorescens WCS417r, *P. Putida* 89B -27, *Agrobacterium rubi* A-18, *Bacillus subtilis* OSU-142, *Burkholderia gladioli* OSU-7, *Pseudomonas putida* BA-8.

6.1.2 ISR Related Plant Growth Promoting Fungi (PGPF):

Non-pathogenic, filamentous, saprophytic rhizosphere fungi that significantly enhance the growth of plants and induce expression of induced systemic resistance (ISR) are known as plant growth-promoting fungi (PGPF). PGPF primarily include Ascomycetes (*Penicillium*, *Aspergillus*, *Trichoderma*, *Gliocladium*, *Phoma*, *Fusarium*), Oomycetes (*Pythium*, *Phytophthora*

6.2 Pathogenesis-Related Proteins (PR Proteins):

Pathogenesis-Related Proteins (PR Proteins) accumulate locally in the infected and surrounding tissues, and also in remote uninfected tissues. Production of PR proteins in the uninfected parts of plants can prevent the affected plants from further infection. PR protein in the plants was first discovered and reported in tobacco plants infected by tobacco mosaic virus. Later, these proteins were found in many plants. Most PR proteins in the plant species are acid-soluble, low molecular weight, and protease-resistant proteins. PR proteins depending on their isoelectric points may be acidic or basic proteins but they have similar functions.

Most acidic PR proteins are located in the intercellular spaces, whereas, basic PR proteins are predominantly located in the vacuole. PR-proteins were categorized into 17 families according to their properties and functions, including β-1,3-glucanases, chitinases, thaumatin-like proteins, peroxidases, ribosome-inactivating proteins, defenses, thionins, nonspecific lipid transfer proteins, oxalate oxidase, and oxalate-oxidase-like proteins. Among these PR proteins chitinases and β-1,3-glucanases are two important hydrolytic enzymes that are abundant in many plant species after infection by fungal pathogens. The amount of them significantly increases and play main role of defense reaction against fungal pathogen by degrading cell wall, because chitin and β-1,3-glucan are the major structural component of the cell walls of many pathogenic fungi. β-1,3-glucanases appear to be coordinately expressed along with chitinases after fungal infection. This co-induction of the two hydrolytic enzymes has been described in many plant species, including pea, bean, tomato, tobacco, maize, soybean, potato, and wheat.

Table 6.1: Classification of Pathogenesis Related Proteins (Van Loon and Van Strien, 1999)

Families	Type member	Properties
PR-1	Tobacco PR-1a	Antifungal
PR-2	Tobacco PR-2	β-1,3-glucanase
PR-3	Tobacco P, Q	Chitinase type I,II, IV,V,VI,VII
PR-4	Tobacco 'R'	Chitinase type I,II
PR-5	Tobacco S	Thaumatin- like
PR-6	Tomato Inhibitor I	Proteinase- inhibitor
PR-7	Tomato P69	Endoproteinase
PR-8	Cucumber chitinase	Chitinase type III
PR-9	Tobacco 'lignin forming peroxidase'	Peroxidase
PR-10	Parsley 'PR1'	Ribonuclease like
PR-11	Tobacco 'class V' chitinase	Chitinase, type I
PR-12	Radish Rs- AFP3	Defensin
PR-13	Arabidopsis THI2.1	Thionin
PR-14	Barley LTP4	Lipid- transfer protein
PR-15	Barley OxOa (germin)	Oxalate oxidase
PR-16	Barley OxOLP	Oxalate oxidase-like
PR-17	Tobacco PRp27	Unknown

6.3 Assay of Phenylalanine Ammonialyase (PAL):

It is known that phenylalanine ammonia-lyase (PAL) play a key role in the phenyl-propanoid pathway which catalyses the conversion of L-phenylalanine to trans-cinnamic acid in the first step that lead to the synthesis of defense related compounds like lignin, phytoalexins such as isoflavonoids, and coumarins (Dixon and Lamb, 1999; Mahadevam and Sridhar, 1996).

A. Requirement:

- L- phenylalanine
- Trans-cinnamic acid
- 0.25 M borate buffer
- Polyvinylpyrrolidone (PVP)
- 1M Trichloroacetic acid
- Ice
- Morter and pestle
- Muslin cloths
- Cold centrifuge
- UV-VIS Spectrophotometer

B. Methodology:

PAL activity was determined as the rate of conversion of L- phenylalanine to trans-cinnamic acid at 290 nm as described by Sadasivan & Manickam, 1996. For PAL activity, leaf tissue (1g) was homogenized in 5ml of 0.25 M borate buffer, pH 8.7 containing 0.1g insoluble polyvinylpyrrolidone (PVP) in a morter and pestle at 4^0C.

The homogenate was filtered through muslin cloths and centrifuged at 12000g at 4^0C for 15 minutes. The yellowish green supernatant was used as crude enzyme extract.

The reaction mixture containing 0.2 ml of distilled water and 1.0 ml of 0.1M L-phenylalanine were incubated at 30^0 C for 30minutes.

The reaction was stopped by addition of 0.5 ml of 1M Trichloroacetic acid. The absorbance at 290 nm was recorded by using a UV-VIS Spectrophotometer (Systronics, Model no.118, India). Enzyme activity was expressed on fresh weight basis (μmol min^{-1} g^{-1} using trans-cinnamic acid as standard).

Table 6.2: Phenylalanine Ammonialyase (PAL) activity

Treatments	PAL activity (μmol $min^{-1}g^{-1}$ fresh weight tissue)				
	Days after inoculation				
	0 d	1d	2d	3d	4d
Control	2.5	2.6	2.5	2.6	2.7
A	2.5	3.4	3.6	3.2	3.0
B	2.6	2.8	3.2	3.6	3.5
C	2.4	3.2	4.7	5.6	5.8

6.4 Assay of Polyphenol oxidase (PPO):

Polyphenol oxidase (PPO) is one of the defense related enzymes related to phenyl-propanoid pathway. PPO acts on tannin in phenol form and convert tannin in quinone form, which has inhibitory activity against phytopathogenic microorganisms (Mahadevan and Sridhar, 1996).

A. Requirement:

- mM Tris-HCl buffer, pH 7.2
- M sodium phosphate buffer, pH 6.5
- 0.4 M sorbitol
- mM NaCl
- Ice
- 0.01M catechol
- Mortar and pestle
- Cold centrifuge
- UV-VIS spectrophotometer

B. Methodology:

Polyphenol oxidase activity was determined following the procedure as suggested by Sadasivan & Manickam, 1996. One gram of leaves was homogenized in 5.0 ml of 50 mM Tris-HCl buffer, pH 7.2 containing 0.4 M sorbitol and 1.0 mM NaCl. The homogenate was centrifuged at 12000g at 4^0C for 10 minutes and supernatant was considered as enzyme source for polyphenol oxidase. In a cuvette 2.5 ml of 0.1 M sodium phosphate buffer, pH 6.5 and 200 µl of the extracted enzyme was mixed. The cuvette was placed in a UV-VIS spectrophotometer (Systronics, Model no.118, India) and the initial reading was adjusted to zero at 495 nm. Three hundred µl of 0.01M catechol was added to the cuvette and the changes in absorbance were recorded at 1 minute intervals upto 5 minutes. The enzyme activity was expressed as change in absorbance. Enzyme activity = K x (ΔA min^{-1}) µmol min^{-1} g^{-1} fresh weight tissue (K= 0.272 for polyphenol oxidase).

Table 6.3: Polyphenol Oxidase (PPO) Activity

Treatments	PPO activity = K x (ΔA min^{-1}) µmol min^{-1} g^{-1} fresh weight issue (K= 0.272 for polyphenol oxidase)				
	Days after inoculation				
	0 d	1d	2d	3d	4d
Control	2.72	2.81	3.08	3.33	3.54
A	2.72	3.16	3.50	4.24	5.54
B	2.84	3.54	3.86	4.78	5.34
C	2.90	4.34	4.86	5.64	4.86

6.5 Assay of Chitinase:

Many phytopathogenic fungi contain chitin as major structural cell wall component (Wessels and Sietsma, 1981).

Chitinases commonly known as plant hydrolases, are the key defense enzymes for plant protection against fungal pathogens.

Chitinase degrade chitin present in fungal cell-wall components. Several authors have demonstrated the activity of chitinase as growth inhibitor of fungi (Mauch *et al*., 1988; Elzen and Cornelissen, 1993).

A. Requirement:

- Colloidal chitin
- Ice
- 0.1M sodium acetate buffer, pH 5.2
- PVP

- Mortar and pestle
- Muslin cloth
- Cold centrifuge

B. Methodology:

The colorimetric assay of chitinase was carried out according to the procedure developed by Mahadevan and Sridhar, (1982). Colloidal chitin was prepared as per the method of Berger and Reynolds, (1958). One gram of leaves were extracted in 5.0 ml of 0.1M sodium acetate buffer, pH 5.2 containing 700mg of PVP using mortar and pestle in cold condition. The homogenate was filtered by using four layered muslin cloth, centrifuged at 10000g for 10 minutes and the supernatant was used as crude enzyme source. The assay mixture consisted of 0.5ml crude enzyme, 0.25ml of 0.1M sodium acetate buffer, pH 5.2 and 1ml colloidal chitin (1.8mg/ml) incubated at 37^0 C for 2h. One ml of reaction mixture was taken and then 1ml of distilled water was added to it. The mixture was boiled for 10 minutes and centrifuged at 5000g for 3 minutes to stop the reaction. One ml of the supernatant was added to 0.1ml of 0.8M potassium tetra borate and boiled exactly for 3 minutes. Then hot mixture was cooled and added to 3ml of para-di-methyl amino benzaldehyde (DMAB) reagent. Samples were incubated again at 37^0C for 20 minutes. Immediately after incubation the mixture was cooled and absorbance was recorded within 10 minutes at 585 nm in a UV-VIS Spectrophotometer (Systronics, Model no.118, India). Enzyme activity was expressed on fresh weight basis (mg GlcNAc $h^{-1}g^{-1}$ fresh weight tissue) using N-Acetyl-D Glucosamine as standard.

Table 6.4: Chitinase Activity

Treatments	Chitinase activity (mg GlcNAc $h^{-1}g^{-1}$ fresh weight tissue)				
	Days after inoculation				
	0 d	1d	2d	3d	4d
Control	3.0	3.2	3.5	3.0	3.0
A	3.2	4.0	5.3	4.2	3.5
B	3.0	6.0	5.0	4.4	4.0
C	3.0	5.5	6.8	8.0	7.4

6.6 Assay of β-1,3-Glucanase:

Among the pathogenesis related proteins, β-1,3-glucanase plays an important role in plant defense against phyto-pathogenic fungi. β-1,3-glucanase hydrolyse β-1,3-glucans present in chitin, embedded in matrix (Lawrence *et al*.,1996). Higher β-1,3-glucanase activity in some resistant plants and low in the susceptible plants has been reported by Kini *et al*., 2000.

A. Requirement:

- 0.05M sodium acetate buffer, pH 5.0
- Morter and pestle
- Ice
- Cold Centrifuge

- 4% laminarin
- Dinitrosalicylic acid reagent
- UV-VIS Spectrophotometer
- D-Glucose

B. Methodology:

Activity of β-1,3-glucanase was colorimetrically assayed by the laminarin- dinitrosalicylate method (Pan *et al*., 1991). One gram tea leaves were extracted with 5.0ml of 0.05M sodium acetate buffer, pH 5.0. Extraction was done in a pre-chilled morter and pestle. The extract was centrifuged at 10000g at 4^0 C for 15 minutes. The supernatant was used as crude enzyme extract. The crude enzyme extract of 15.6 µl was added to 15.6 µl of 4% laminarin (Sigma, USA) and then incubated at 40^0 C for 10 minutes. The reaction was stopped by addition of 94 µl of Dinitrosalicylic acid reagent followed by heating for 5 minutes on a boiling water bath. The final colour solution was diluted with 1.0 ml of distilled water and absorbance was recorded at 500 nm in a UV-VIS Spectrophotometer (Systronics, Model no.118, India). Enzyme activity was expressed on fresh weight basis (nmol min^{-1} mg^{-1}) using D-Glucose as standard.

Table 6.5: β-1,3-glucanase activity

Treatments	β-1,3-glucanase activity (nmol $min^{-1}mg^{-1}$ fresh weight tissue)				
	Days after inoculation				
	0 d	1d	2d	3d	4d
Control	10	12	10	10	10
A	10	14	17	20	23
B	10	24	26	25	20
C	10	18	33	42	28
D	10	20	35	45	50

6.7 Assay of Peroxidase:

Peroxidase is a stress related defense enzyme, induced in plants under various environmental changes such as heavy metals, salts, temperature (Kiwan and Lee, 2003), air pollution, (Lee e*t al.*, 2000).

Salicylic acid acts as an endogenous signal in the induction of systemic acquired resistance (SAR) that produces pathogenesis-related proteins (PRs) like peroxidase (PR-9) along with chitinase (PR-3) and β-1,3-glucanase (PR-2).

A. Requirement:

- 0.1M sodium phosphate buffer, pH 6.5.
- Pre-chilled morter and pestle

- 0.05M guaiacol
- Ice
- H_2O_2 (1% v/v)
- Cold Centrifuge
- UV-VIS Spectrophotometer

B. Methodology:

Peroxidase activity was determined according to the procedure given by Hammerschmidt *et al*., 1992. One gram leaves were extracted in 5.0ml of 0.1M sodium phosphate buffer, pH 6.5. The extraction was done in a pre-chilled morter and pestle at 4^0C. The homogenate was filtered through muslin cloth and the filtrate was centrifuged at 6000g at 4^0C for 20 minutes. Supernatant was considered as enzyme source. In a cuvette, 1.5 ml of 0.05M guaiacol and 100 µl of extracted enzyme was mixed. The cuvette was placed in a UV-VIS spectrophotometer (Systronics, Model no.118, India) and the initial reading adjusted to zero at 420 nm. One hundred µl of H_2O_2 (1% v/v) was added to the cuvette and the changes in absorbance were recorded at 1 minute intervals upto 5 minutes. The changes in absorbance (ΔA_{420} min^{-1} g^{-1} fresh weight) of 0.001 were considered as unit of enzyme activity.

Table 6.6: Peroxidase Activity

Treatments	Peroxidase activity (ΔA_{420} min^{-1} g^{-1} fresh weight tissue) 1 unit = 0.001 Absorbance				
	Days after inoculation				
	0 d	1d	2d	3d	4d
Control	60	62	60	60	60
A	60	66	81	83	85
B	60	68	115	135	140
C	60	85	135	155	135
D	60	75	84	95	114

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