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CASE REPORT

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ENZYMATIC AND HORMONAL STUDIES IN *ACACIA EBURNEA* INFECTED WITH *RAVENELIA ESCULENTA*

K.R. Gandhe¹ and Aniket Kuvalekar²

¹ Reader, PG Research Centre, Department of Botany, Modern College of Arts, Science and Commerce, Shivajinagar, Pune, Maharashtra 411005, India; ² UGC - SRF, PG Research Centre, Department of Botany, Modern College of Arts, Science and Commerce, Shivajinagar, Pune, Maharashtra 411005, India

Email: ¹ kanchan.gandhe@gmail.com (corresponding author)

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ABSTRACT

Ravenelia esculenta Naras. and Thirum. infects *Acacia eburnea* Willd. producing hypertrophy in infected parts, mainly thorns, inflorescence, flowers and fruits. The hypertrophied parts are edible and consumed with relish. The severe stages of the disease are marked by pronounced hypertrophy in the infected parts, frequently 10 times or more. The disease progress is marked by gradual increase in hypertrophy and severe stages show presence of brown aecial cups. The process of disease development was studied to analyse various biochemical changes. Enzymatic studies showed significant shifts in the activities of enzymes like Polyphenol oxidase (PPO) (EC 1.14.18.1), Peroxidase (POX) (EC 1.11.1.7) and IAA Oxidase (IAAO). Activity of PPO was found to be in accordance with the quantity of total phenols. POX activity was found to be maximum during severe infection stage. Maximum IAAO activity was noticed during initiation of hypertrophy. These studies help to reveal the enzymatic changes during disease development by fungi. Changes in IAAO activities are suggestive of possible involvement of IAA, its derivatives and GA interaction in development of hypertrophied structures.

KEYWORDS

Acacia eburnea, disease development, GA, IAA Oxidase, Peroxidase, Polyphenol Oxidase, *Ravenelia esculenta*.

Ravenelia esculenta Naras. and Thirum. is a rust fungus that infects *Acacia eburnea* Willd. (Narasimhan & Thirumalachar, 1961). The infected organs show hypertrophy at various stages

of disease development (Narasimhan & Thirumalachar, 1961). The hypertrophy is marked by presence of brown coloured aecial cups visible on the infected organ (Image 1^w). The hypertrophied structures are edible and are consumed along with liquor. This hypertrophy is an outcome of altered host physiology and hormonal metabolism. There are reports of various host-fungus interactions that lead to alterations in the overall physiology of host. Gandhe *et al.* (2004) have screened the infected host to analyse the host-fungus interaction in *Ravenelia esculenta*. From these studies it was found that besides other metabolic alterations, the infected parts accumulate enormous amounts of aluminium and hence consumption of these infected structures pose potential threat of neural diseases like Alzheimer's disease. Shaw & Samborski (1957) have discussed the physiological changes in mildew and rust infected wheat leaves, initial increase in respiration rate being one of the early physiological responses to pathogen attack. It has been observed that fungal, bacterial and viral diseases also lead to alterations in the biochemical constituents of hosts. These may range from change in chlorophyll contents (Prasanna *et al.*, 2004) due to infection of Citrus Yellow Mosaic virus in leaves of *Citrus sinensis*, accumulation of soluble and reducing sugars due to infection of *Alternaria brassicae* to *Brassica*

^w See Image 1^w in the web supplement at www.zoosprint.org

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juncea (Kiran *et al.*, 2003) to increase in free amino acids and proline contents (Yancey *et al.*, 1982). Chiou (1997) has also observed increase in reducing sugars due to infection of various *Aspergillus* sp. in peanut kernels, especially during later stages of infection. Polyphenols have been detected as preformed antimicrobial compounds in many plants (Osburn, 1996).

There are pronounced hormonal changes due to pathogen infection in plants. Gardan *et al.* (1992) have demonstrated the correlation between the plant species and IAA accumulation in the host plants due to infection of *Pseudomonas syringae* subsp. *Savastanoi* along with confirmation of expression of genes in IAA metabolic pathway during infection process. Auxin production in plant pathogenic *Pseudomonads* and *Xanthomonads* has been detected by Fett *et al.* (1987). Recently Maor *et al.* (2004) have detected *in planta* production of IAA due to infection of *Colletotrichum gloeosporioides* f. sp. *aeschynomene* in *Aeschynomene virginica*.

These biochemical and hormonal changes are associated with changes in enzyme activities of defense enzymes like polyphenol oxidase, peroxidase and other oxidative enzymes (Percival, 2001; Song *et al.*, 2001) in addition to changes in activities of enzymes in hormonal metabolism, like IAA oxidase (Agrios, 1997).

In light of these studies, *Acacia eburnea* infected with *Ravenelia esculenta* was screened for changes in enzymatic activities and in hormonal contents during disease development. The study was undertaken to investigate the correlation between the appearance of hypertrophy and changes in enzymatic activities and hormonal contents during disease development in the host.

MATERIALS AND METHODS

Collection of plant material: Healthy (H) and various disease stages (Initiation of hypertrophy (IHP), Initiation of sporulation (InSp) and severe infection (Inf)) were collected in and around Pune. The material was randomly collected from accessible sites and preserved in brown paper bags until brought in the laboratory. The material was stored at -4°C till it was processed further.

Enzymatic studies:

Polyphenol oxidase (PPO): Isolation and assay of PPO was carried out by a method modified from Esterbauer (1977). Preweighed material of various disease stages was homogenised in chilled 0.1M phosphate buffer (pH 7.0) in chilled mortar and pestle. The ratio of material to phosphate buffer was maintained at 1:5. The homogenate was filtered through Whatman no. 1 filter paper on buchner funnel and the filtrate was centrifuged at 4000rpm for 10min at 4°C. The final volume of the supernatant was adjusted to 10ml and it served as an enzyme source. The reaction mixture contained 1ml 0.1M methyl catechol, 2ml 0.1M phosphate buffer (pH 7.0) and 1ml enzyme. The reaction was detected by increase in the absorbance at 495nm in spectronic 20. Protein concentration from the enzyme extract was determined by Bradford's method (Bradford, 1976).

Peroxidase (POX): Isolation and assay of POX was done as per the method described by Putter (1974). The reaction mixture contained 3ml 0.1M Phosphate buffer (pH 7.0), 0.05ml

20mM guaiacol, 0.03ml 12.3mM H₂O₂ and 0.5ml enzyme. Enzyme activity was detected by increase in absorbance at 436nm in spectronic 20. Protein concentration from the enzyme extract was determined by Bradford's method (Bradford, 1976).

IAA Oxidase (IAAO): IAAO activities at various disease stages was detected by following standard methods (Sadasivam & Manickam, 1996). Reaction mixture contained 2ml 25mM Phosphate buffer (pH6.2), 1ml P-coumaric acid (0.5mg/ml), 1ml 5.9mg/ml manganese chloride and 2 ml enzyme extract. Enzyme reaction in one set was terminated at zero min by addition of 5M perchloric acid and the other set was incubated for 30 min at 37°C with constant shaking. IAA in both 0 min and 30 min reactions was estimated by using Salkowski's reagent (Glickmann *et al.*, 1995). Protein concentration from the enzyme extract was determined by Bradford's method (Bradford, 1976).

Total phenols, IAA and GA estimation: Total phenols were estimated by method of Malick & Singh (1980). Isolated IAA (McDoughall & Hillman, 1978) was estimated by Salkowski's reagent (Glickmann *et al.*, 1995), Isolation and estimation of GA was done by method of Mahadevan (1984).

RESULTS

Enzymatic studies: There is pronounced change in the activities of both PPO and POX during disease development. The graphical representation of the activities of these enzymes is shown in fig. 1 and 2.

The amount of phenols goes on increasing during initial stages of the disease (Table 2). But the amount of phenols is minimum during the severe infection stages. The activity of PPO also goes on increasing with the progress in the disease. The activity of PPO is maximum during the severe infection stages when amount of phenols is also observed to be minimum.

There is initial increase in the activity of POX, at the stage of IHP as compared to that in H. The InSp stage shows decrease in POX activity but still it is more as compared to that in H. The severe infection shows sudden increase in the activity of POX, the increase being very substantial, fifteen times or more. Activity of PPO and POX is shown in terms of enzyme units in Table 1.

Activity of IAAO is shown in Fig. 3. The activity was found to be maximum at IHP stage.

Estimation of total phenols, IAA and GA: The amounts of IAA, free GA (FGA), bound GA (BGA) and phenols are shown in Table 2. There is gradual increase in the amount of phenols during disease progress. However, the phenolic content decreases to its minimum during the severe infection stage. IAA content is seen to decrease as the disease progresses, except at the severe infection stage where there is increase in the IAA content. There is continuous decrease in the amount of bound GA, but amount of free GA decreases initially during IHP stage followed by increase in its amount as the disease progresses, but being lower than that in non-infected host.

DISCUSSION

One of the early events in host-pathogen interaction is the production of activated oxygen radicals (Agrios, 1997). These

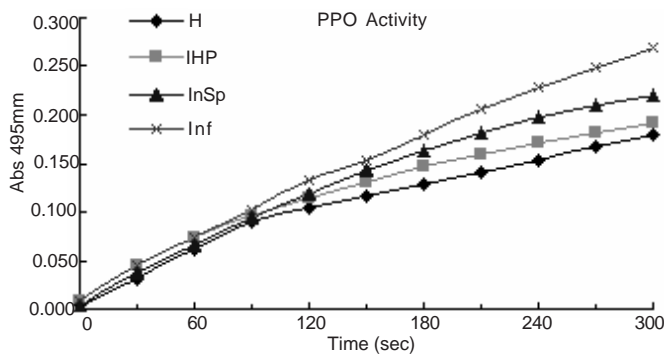


Figure 1. PPO activity during disease development

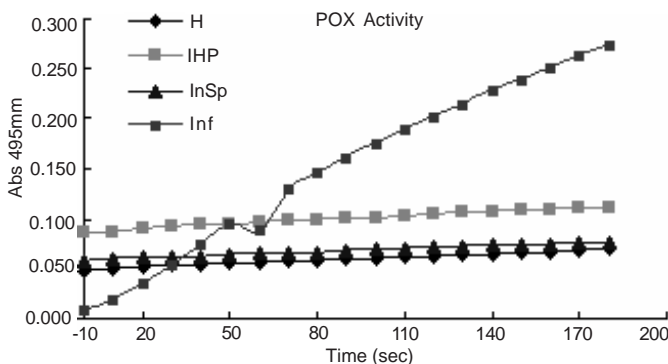


Figure 2. POX activity during disease development

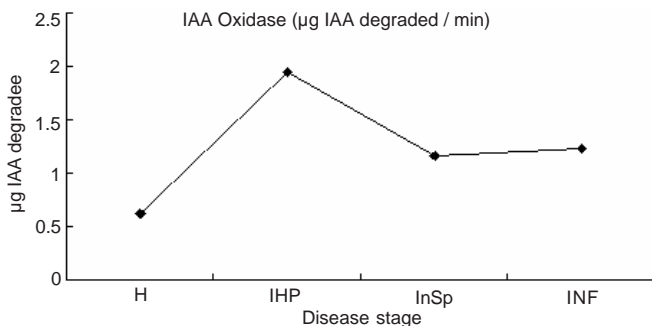


Figure 3. IAA Oxidase activity during disease development

include mainly different species of peroxides. Several studies have reported the nature of metabolites accumulating after infection along with the determination of the newly synthesized enzymes (Agrios, 1997). There are various preformed antimicrobial compounds in plant cells, phenols being one of the important substances playing a key role in conferring the resistance to pathogen attack (Osburn, 1996). It is important for the infecting fungus to overcome this biochemical hurdle to infect and spread within the host. In disease resistant cultivars increase in phenolics is due to high activity of β -glucosidase which converts non-toxic phenolic glycosides to toxic phenolics which are inhibitory to pathogen. These phenolics are converted to quinones by activity of POX enzyme in resistant cultivars. Quinones, the oxidized form of these polyphenols, have been shown to be more toxic to pathogens than the original phenols (Agrios, 1997). Decrease in polyphenols level in

Table 1. Enzyme activity (units/mg) during disease development

Sno.	Enzyme	H	IHP	InSp	Inf
1	POX	4.7×10^2	6.51×10^2	6.48×10^2	7.71×10^3
2	PPO	2.41×10^3	2.71×10^3	4.66×10^3	4.53×10^3

Table 2. Profile of hormonal and phenolic shifts during disease development (All values are expressed as mg/gm)

	H	IHP	InSp	Inf
FGA ^A	1.732 ± 0.13	0.114 ± 0.02	0.119 ± 0.03	0.121 ± 0.01
BGA ^B	1.628 ± 0.04	0.875 ± 0.02	0.596 ± 0.02	0.329 ± 0.03
IAA	1.161 ± 0.03	0.324 ± 0.002	0.144 ± 0.001	0.464 ± 0.01
Phenols	14.603 ± 0.46	62.803 ± 2.52	67.338 ± 1.65	8.139 ± 0.37

^A - Free GA; ^B - Bound GA

sensitive *Brassica juncea* cv Kranti has been also observed after treatment with culture filtrates of *Alternaria brassicae* (Kiran *et al.*, 2003).

Increased PPO activity helps in oxidizing these polyphenols to quinones, to check the spread of infection. The decreased polyphenols level in the infected tissues, in this study, indicates higher PPO activity which is evident from the results. In this study, however, in spite of high amounts of phenols at various stages of disease development, the rust successfully establishes itself within the host. This indicates that either the rust fungus is resistant to the phenols that are produced by the host plant or the fungus itself produces PPO which converts the phenolic substances from the host to some other forms which are no longer toxic to the infecting pathogen. The decrease in the phenols is observed during the severe stage of infection when the rust sporulates profusely and produces large amounts of aeciospores. This decreased phenol level is accompanied by corresponding increase in the PPO activity. This indicates that, even though there is increase in the PPO activity, the rust successfully infects the host and hence it can be said that the enzyme might be produced by the fungus itself and it can have the role as mentioned earlier. However, it is difficult to determine the source of the enzymes, as the infecting fungus being an obligate parasite, is very difficult to culture and is not accessible in axenic form for studies.

Involvement of PPO has been detected to be important in the early interactions between host and pathogen, where the host produces high amounts of PPO as early defense enzymes. This is observed in tomato, for both systemic acquired resistance and induced systemic resistance. Various biochemical changes and role of different enzymes in disease resistance has been reviewed by Vallad *et al.*, (2004). There are reports of production of phenolic substances in response to fungal attack (Agrios, 1997). Accumulation of phenolic compounds during hypertrophy production and initiation of sporulation can be a response to fungal attack.

POX is one of the PR proteins that has been extensively studied. The tight correlation between disease resistance and expression pattern of POX indicated its role in disease resistance. POX belongs to PR-9 group proteins. In case of rice its expression pattern has been studied in response to *Magnaporthe grisea* infection. Defense response in rice against bacterial blight is also characterized by increase in the

expression levels of POX and decrease in the growth rate of the pathogen (Song & Goodman, 2001). In case of tobacco and cucurbits it is observed that the plants with acquired resistance have higher amounts of POX (Vale *et al.*, 2001). POX leads to faster lignification response in systemic acquired resistance protected plants (Percival, 2001) and its expression has also been observed in response to wounding (Cipollini, 1998). In the present studies also the POX activity was found to be substantially higher during the severe infection stages, even though the host is highly susceptible to the rust infection.

Activation of these enzymes indicates the biochemical hurdles posed by the host to inhibit the growth of the pathogen, but the pathogen overcomes these biochemical hurdles and establishes in the host successfully which is indicated by sporulation in the pathogen. In this case the pathogen can be said to be more virulent or the host being more susceptible to the disease.

In the present study the host-pathogen complex was screened for the determination of growth hormones and enzymes involved on their metabolism.

There are several reports which indicate the altered level of growth hormones during fungal or bacterial disease development. The *Agrobacterium tumefaciens* has been extensively studied for disease development, in which increased hormone levels lead to formation of galls in the infected plants (Agrios, 1997).

There are many reports of increased hormonal contents or production of hormones by the pathogens during the process of infection. Kaldorf *et al.* (2000) have detected the biosynthesis of indole -3- butyric acid by infecting AM fungus *Glomus intraradices*. The increased level of IBA leads to altered morphology of roots in maize. The alteration in the morphology is effected ca.10 days after inoculation of the roots by the AM fungus. The inoculate roots produced more lateral roots and this production coincided with increased levels of IBA during initial stages, however, at later stages of infection bound IBA contents increased in the infected roots. Besides this Jentschke *et al.*, (2000) have reported that the infecting mycorrhiza may produce a range of phytohormones or alter the phytohormones levels in their hosts especially by improving the mineral nutrition. In the present studies also there is several folds increase in aluminium contents in infected host. The phytohormones also help in alleviating the metal toxicity (Jentschek *et al.*, 2000). Role of amino acid and sugar conjugates of IAA and IBA during AM development in maize has been described by Fitze *et al.*, (2005). They have demonstrated the complex control mechanisms to regulate the levels of free and conjugated auxins. Frankenberger *et al.* (1987) have also described the production of IAA by ectomycorrhizal fungus *Pisolithus tinctorius* in pines. They have also proposed the role of unidentified indole derivative in the development of root colonization by the mycorrhizal fungus. In the present studies, the infected host showed presence of a distinct indole derivative on TLC which has also been detected by HPLC. Maor *et al.*, (2004), have demonstrated the *in planta* production of IAA by pathogenic fungus *Colletotrichum gloeosporioides* f. sp. *aeschyromene* in *Aeschynomene virginica*. They have demonstrated the role of external tryptophan and indole -3- acetamide in auxin

production and have shown that production of auxins is critical for the initial infection to take place. Leveau *et al.* (2005) have shown that *Pseudomonas putida* strain 1290 utilizes IAA for its growth. Fett *et al.* (1987) have demonstrated the production of IAA by Pseudomonads and Xanthomonads with or without supplement of tryptophan in liquid medium. Gardan *et al.* (1992) have investigated the role of various strains of *Pseudomonas syringae* and the host plants in the production of auxins by the bacteria.

But in all these cases majority of reports indicate either *de novo* production of auxins or increase in auxins during disease development. The present study was undertaken with a hypothesis that production of hypertrophy should be due to increased phytohormones especially IAA concentrations. But the results are contradictory to the proposed hypothesis and also to the earlier studies in various host-pathogen interactions. The screening of *Acacia eburnea* infected with *Ravenelia esculenta* at various disease stages has indicated decrease in the hormone content during disease development. There is decrease in amounts of both IAA and GA. Amount of bound GA goes on decreasing during all disease stages but the free GA contents show initial decrease at initiation of hypertrophy stage followed by increase in Insp and Inf stages. The studies have also shown presence of an indole derivative which is present only in the infected material.

These studies indicate the shifts in the oxidative enzyme activities during disease development. The changes in the hormonal contents with appearance of a novel indole derivative in infected material with an increase in IAA content over initiation of sporulation stage are suggestive of possible role of the indole derivative and IAA-GA interactions in the production of hypertrophy. Studies to isolate and determine the structure of the indole derivative are in progress. The hypertrophy produced in the infected material is 10 times or more over healthy material and if it is seen to be produced due to the indole derivative, it presents a possibility to exploit this derivative for plant tissue culture purposes. The characteristic change in the enzyme activities at initial disease stages can be used for controlling the diseases at early stage thereby minimizing the losses resulting due to established fungal infections in economically important plants.

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Salim Ali Centre For Ornithology and Natural History (SACON)

Invites applications for the following positions in the project
"Environmental Information System (ENVIS)"
 at Hyderabad

Information Officer: Educational qualifications: B.Tech/B.E in Computer Science, Image processing / GIS or M.Sc/MCA with programming abilities in C, C++, Visual Basic, Linux O.S and Developer 2000 with PHP and Postgre SQL, Oracle, RDBMS.; Essential Experience: one or two years experience in website construction, maintenance and Upgradation.

Web Assistant: Educational qualifications: MCA/B.C.A/ B.Sc., (Maths, Physics, Chemistry), HTML, CSS, Java Script, Macromedia Dreamweaver, Flash, Adobe Photoshop, PageMaker; Essential Experience: one year experience in website construction, maintenance.

Note

1. Age below 30 years as on 01.07.2007
2. Duration of the project is for a period of one year. Salary is commensurate with qualifications and, work experience.
3. Application on plain paper with complete bio-data in the following format should reach the **Director In-charge, Salim Ali Centre For Ornithology and natural History, Anaikatty Post, Coimbatore 641108, Tamil Nadu** within 15 days of publication of this advertisement: (1) Name, (2) Age as on 1.1.2007, (3) Permanent address, (4) Present address, (5) details of prescribed qualifications (with grades), (6) other qualifications, if any, with grade obtained, (7) total years of experience in the concerned field, (8) positions held with details of pay and responsibilities, (9) present position with salary scale and responsibilities, (10) any other relevant information. Applications not received in the above format are liable to be rejected.
4. SACON will decide the cut off point for calling the candidates for interview based on the academic qualifications, experience and age.
5. Only those who are highly motivated, creative and committed to the cause of nature conservation and prepared to put in strenuous work need apply.
6. Application should be accompanied by certified copies of documents in support of the qualifications and experience claimed.