SYNOPSIS
SYNOPSIS OF THE RESEARCH WORK

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BIOCHEMICAL AND MOLECULAR INVESTIGATIONS OF
SOME GALL FORMING RUST FUNGI FROM MAHARASHTRA

Rusts are obligate parasites on plants with relatively complex life cycles and produce a range of different spore forms. During host – pathogen interactions, pathogen produces characteristic symptoms in host. Infections commonly occur on stems, leaf sheaths and leaf blades in the form of small pustules or streaks which at severity become erumpent to liberate spores. However, some rust infections give rise to alterations in the morphological characteristics of host plant tissues and affect the normal development of the host. Uredospores of *Ravenelia tandonii* infect near meristematic tissue of *Acacia catechu*, as a result, there is a loss of apical dominance in host. This, in conjunction with changes in the levels of growth regulators, may give rise to a great proliferation of shoots, traditionally described as witches’ broom. Aeciospores of *Ravenelia esculenta* produces hypertrophy in thorns, inflorescence, flowers and pods of *Acacia eburnea* altering the complete morphology of plant parts to become succulent with bizarre shapes. This hypertrophy or excessive growth may be due to changes in the levels and/or metabolism of growth regulators in infected tissues. Teliospores of *Hapalophragmium ponderosum* form hypertrophy on the stem of *Acacia leucophloea*. Teliospores of *Uromyces hobsonii* and *Cystopsora oleae* form localized swellings called galls on stem, leaves and flowers of *Jasminum malbaricum* and *Olea dioica* respectively and alter morphology of normal host tissue at the site of infection.
A host plant may exhibit a range of responses to challenge by a specific pathogen. Massive disruption to the host occurs when the fungus sporulates.

There are large number of reports indicating the alteration in the physiology and biochemistry of the host during disease development. Some fundamental life processes of plants like photosynthesis are severely affected in the infected organs. In most of the plant – fungus interactions, the host tries to defend itself from the invading pathogen. There are different strategies of the host in which it deals with and arrests the growth of the pathogen. These strategies may range from raising a tough mechanical barrier like lignin to inhibit the entry of the pathogen to several complex biochemical alterations in the infected and surrounding tissues which prove to be unfit for the growth of the pathogen.

The gall forming rust fungi present excellent system for the study of disease development since the traditional type of rust infection leads to formation of hypertrophy or galls in the infected tissues changing the morphology of infected plant parts which might be an outcome of disturbed host physiology due to fungal pathogenesis. Lack of reports on biochemical changes during disease development by rust fungi led us to undertake the present study. In addition, there are scanty reports of the studies on the disease development by gall forming and hypertrophied rust fungi.

Taking into consideration the need for methodical screening of these fungi in addition to their potential as a natural source of phytohormones, the present study was designed for Ph. D. dissertation.

The survey of literature revealed that such types of studies have been carried out in economically important plant – pathogen interactions. But the gall forming rust fungi and their hosts are completely neglected systems which have never been screened before for disease development from initial stages to severe and peak period
of infection. Major work reported earlier on these fungi is mainly concerned with taxonomy. In addition, gall forming rust fungi have never been screened before to check overproduction of phytohormones during disease development as a probable reason for development of hypertrophy.

*Ravenelia esculenta* and *Uromyces hobsonii* are the two most commonly occurring gall forming rust fungi on *A. eburnea* and *J. malbaricum* respectively from Maharashtra. The infection pattern of both of these fungi is very peculiar with variation in the type of malformations produced on the infected parts. In *Uromyces hobsonii*, the infection of the fungus produces localized lesions in the form of typical galls on the infected plant parts. These galls are formed on the leaves, stems and flower buds. The size of galls depends upon developmental stage of the pathogen. In *Ravenelia esculenta*, the infection of the rust produces severe hypertrophy in the infected organs, mainly meristems, thorns, inflorescence and pods get infected. The hypertrophy in the infected organs may even reach to the extent of 30 – 40 times making it difficult to identify the hypertrophied organ. Even a hard and woody structure of thorns turns succulent due to the rust infection and shows bizarre shapes.

The present study, hence, encompasses the biochemical and molecular analysis of these two host – pathogen systems in addition to the comparison of biochemical changes peculiar to uredinal/telial stages and aecial/pycnial stages of the rust.

Host – pathogen complexes from *Ravenelia esculenta* on *Acacia eburnea* and *Uromyces hobsonii* on *Jasminum malbaricum* were screened for biochemical changes during disease development including estimation of Chlorophyll, Reducing Sugars, Phenols, Proline, Free Amino Acids and Proteins. Both the systems were screened to detect the quantitative alterations in the activity of enzymes like polyphenol oxidase.
peroxidase and endo and exocellulases which are known to be involved in modulating the host response to pathogenic challenge. The two complexes were thoroughly screened for quantitative changes in the amount of indole acetic acid (IAA) and free and bound GA along with the activity of indole acetic acid oxidase, an enzyme involved in the breakdown of IAA.

Peroxidase enzymes from both the systems were analysed for their preference towards substrates, their ability to oxidize NADH, role of cofactors in the oxidation of NADH, NADH dependent H$_2$O$_2$ generation, effect of H$_2$O$_2$ on NADH oxidation by peroxidases, their activity in polymerization of monolignols and effect of externally added H$_2$O$_2$ on the polymerization of monolignols. Effect of different cofactors like Mn$^{2+}$ and dichlorophenol (DCP) singly and in combination on these different reactions of peroxidases was also analysed for both the systems.

In *Ravenelia esculenta*, the infected pods, meristems and thorns were analysed whereas in *Uromyces hobsonii* infected leaves and stems were selected for the study. The tissues from both the complexes were selected for study at eight progressive disease stages, starting from healthy to sever infection marked with main morphological variations.

These host pathogen systems have never been analysed at molecular level. For this reason, it was thought to undertake the DNA sequencing of these fungi. The search at NCBI database also revealed that there are no sequences of *Ravenelia* in the database. Our 18S rDNA sequence is the first international report of the DNA sequencing in the genus *Ravenelia* (NCBI Acc. No. DQ145756). There are many sequences of *Uromyces sp.* in NCBI database.

The phylogenetic analysis of *Ravenelia esculenta* revealed its phylogenetic position in the group of related rust fungi, most of which are hypertrophied.
Apart from the studies mentioned above, attempts were made to prepare the pure culture of these fungi by utilizing technique of spore germination in moisture chambers. But all the attempts to culture these fungi failed repeatedly. Instead, the culture of infected leaves of *U. hobsonii* on MS medium with 2mg/l 2,4-D lead to callus formation, which upon microscopic examination revealed the presence of haustoria in the callus cells. But the culture growth was arrested after 6 – 8 weeks.

The biochemical contents of the host – pathogen system showed fluctuations during disease development. The contents of chlorophyll in the infected tissues reduced with progression in the disease. In thorns of *A. eburnea* the chlorophyll decreased at the severe infection stage besides increase during intermediate stages. The thorn being non chlorophyllous tissue showed low chlorophyll content but with progression in the disease the thorns turn succulent and green with concomitant increase in their chlorophyll contents. Pods of *A. eburnea* showed increase in the contents of chlorophyll even at the severe infection stage of the disease. In *J. malbaricum*, the leaves showed initial decrease in chlorophyll but severe infection stages showed increase in chlorophyll content of leaves. The infected stem tissue of *J. malbaricum* showed gradual decrease in chlorophyll contents.

Amount of proteins in *A. eburnea* decrease during disease progression with fluctuations in the contents with progression of the disease, but at any given stage the protein content was observed to be low as compared to that of corresponding healthy tissues (54%, 96% and 66% decrease in protein contents in meristems, pods ad thorns respectively), however, in *J. malbaricum*, the protein content increased during disease development with maximum at stage VI (128% of healthy) in stem and stage VII (52% of healthy) in leaves.
In both the rust systems, contents of phenols showed decrease during disease development with exception of leaves of *J. malbaricum* where the phenolic content increased during the severe infection stage (stage VII with 5% of healthy).

Reducing sugars are seen to be increased during disease progression in both systems, but the infected leaves from *J. malbaricum* showed decrease in the contents of reducing sugars over healthy leaves at all stages of the disease (92% decrease in stage III over healthy).

The proline content in both the systems showed general increase over the corresponding healthy tissues. Only the pods of *A. eburnea* showed decrease in the proline content in severe infection stages (16% decrease over healthy in stage VII). In *A. eburnea* the contents of free amino acids showed increase with progression in the disease with a sharp decline in severe infection stages in all the three tissues studied. In *J. malbaricum*, the free amino acids show variation in the contents with maximum at intermediate disease stages.

Hormonal screening of the host – pathogen systems showed interesting pattern in relation to contents of phytohormones and appearance of hypertrophy. In *A. eburnea* infected with *R. esculenta* the amounts of free GA and bound GA increased in the initial stage of the disease and then the amounts of both decreased with progression in the disease. The contents of bound GA were more in pods and thorns of *A. eburnea* infected with *R. esculenta* as against in meristem where free GA was observed to be more in the initial stage of the disease. In *J. malbaricum* infected with *U. hobsonii*, bound GA showed characteristic increase during intermediate disease stage. IAA content in *A. eburnea* infected with *R. esculenta* decreased with disease progression even though there is severe hypertrophy during disease progression. The significant finding in this host – pathogen system was the appearance of novel indole...
derivative exclusively in severely infected tissues. This novel indole derivative was also observed to have auxin-like activity in plant tissue cultures in the form of initiation of rooting in *Withania somnifera* seedlings which otherwise require presence of NAA and IAA in combination for rooting *in vitro*. In *J. malbaricum* infected with *U. hobsonii*, the IAA contents in infected leaves showed initial increase in the contents but severely infected leaves also contained more amount of IAA as compared to that in healthy. The infected stem tissues showed highest IAA content in stage VI but the severely infected tissues had IAA lower than that in healthy stem tissues.

IAA oxidase (IAAO) activities in these host–pathogen systems were also correlated with the contents of IAA at the particular disease stage. In *A. eburnea* infected with *R. esculenta*, the IAAO unit activity and IAA content at a particular stage could be correlated except for the severe infection stage where both IAAO activities and IAA contents increased in all the three tissues studied. In *J. malbaricum* infected with *U. hobsonii*, the IAA contents and IAAO activities exhibited positive correlation in most of the disease stages.

Peroxidase activities were seen to be increased in all the three tissues studied in *A. eburnea* infected with *R. esculenta*. This increase in the unit activity of peroxidase was observed to be in the range of 23 times to 350 times over healthy during disease progression in these tissues. But in the infected stem tissues of *J. malbaricum* the activity of peroxidase was reported to be always lower than that in healthy tissue whereas the infected leaves of the system exhibited maximum of only 3 times increase in the activity of the enzyme. Similar type of trend was observed in the activity of polyphenol oxidase during disease progression in both the host–pathogen systems. In *A. eburnea* infected with *R. esculenta*, the activity showed increase at
severe disease stages ranging from 2 to 94 times over healthy in different tissues studied. The infected stem tissues of *J. malbaricum* showed decrease in the activity of polyphenol oxidase during all progressive disease stages over healthy tissues whereas the leaf tissues exhibited only 2 time increase in the activity of the enzyme at severe infection stages.

Screening of *A. eburnea – R. esculenta* complex for cellulase activities revealed a characteristic pattern during disease progression in the tissues analysed. The complex showed pronounced activity of endo – β 1,4 glucanase as compared to exo – β 1,4 glucanase. The endo – β 1,4 glucanase activity in meristematic and pod tissues showed initial significant and sharp increase. The enzyme activity in thorns of *A. eburnea* infected with *R. esculenta*, showed increase with disease progression with maximum at severe infection stage. The combined exo – endo – glucanase activities were always increased in pods and thorns whereas the activity in stem tissues exhibited similar pattern of activity as in the case of endo – β 1,4 glucanase. Both endo – β 1,4 glucanase and combined exo – endo – glucanase activities in the leaf and stem tissues showed decrease with progression in the disease in *J. malbaricum* infected with *U. hobsonii*.

The partial characterization of peroxidase form both the complexes revealed change in the pH optima of the enzyme with progression in the disease. The substrate preference of the enzyme also gets altered with disease progression indicating changed substrate specificity of the enzyme during disease development. The ability of the peroxidases to oxidize NADH varied among different disease stages. Concomitantly, NADH dependent H₂O₂ generation varied among disease stages. Externally supplied H₂O₂ had accelerating effect on NADH oxidation by peroxidases at least at one of the concentrations analyzed under study. The NADH oxidation
activity of peroxidases had pronounced accelerating or inhibitory effect of cofactors like Mn$^{2+}$ and dichlorophenol (DCP) supplied individually or in combination during the reaction progress. The NADH oxidation by peroxidases at various disease stages was observed to be comparable but the amount of H$_2$O$_2$ generated increased logarithmically with time. It was hypothesized that this H$_2$O$_2$ generated during the NADH oxidation process might be utilised in the process of lignification in vivo. Hence the cinnamyl alcohol polymerisation by the peroxidases in presence of NADH was analysed and it was found that the activity of the enzyme in polymerizing the monolignols increases with progression in the disease. But externally supplied H$_2$O$_2$ had a strong inhibitory effect on the reaction.

The 18S rDNA sequencing and phylogenetic analysis of *R. esculenta* revealed that the genus *Gymnosporangium* and *Ravenelia* are interrelated and are placed in the same clade within the minimum evolution (ME) tree. The tree can be interpreted in the other way also where there is separation of 14 families of Uredinales depending upon nature of teliospores, nature of aeciospores and structure of pycnia. These studies determine the phylogenetic position of *Ravenelia esculenta* among other rust fungi besides broad separation of Uredinales into two clades. These studies also show that there is phylogenetic correlation between molecular and morphological data. This is the first report of DNA sequencing and phylogenetic positioning in genus *Ravenelia* from India.

The above work on hypertrophied rust fungi is an important contribution to the knowledge of the process of disease development by these fungi. The study highlights the significant changes in the biochemical constituents of the host due to infection of gall forming rust fungi besides the qualitative and quantitative alterations in the contents of growth hormones in the infected tissues with disease development. The
variations in the activity and specificity of different enzymes involved in host defense and metabolism of growth hormones give insights into the host – pathogen interaction. Another significant achievement of the study is the first international report of DNA sequencing in the genus *Ravenelia* and its phylogenetic analysis which has made voluminous addition to the knowledge of evolutionary patterns in rust fungi in general and gall forming rust fungi in particular with respect to transition from primitive to advanced telial hosts and nature of fruiting bodies formed in the advanced rust genera. This study might open the doors for detailed molecular analysis of host – pathogen interactions at molecular level.

The study led to publication of following research papers in the National and International journals of high repute:


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