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PRESENT STATUS OF OUR KNOWLEDGE ON MECHANISM OF INFECTION PROCESS IN PATHOGENESIS

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In the process of invasion of host tissue by pathogenic fungi, the first step is penetration of the outer surface. Pathogenic fungi must be able to penetrate the defensive barriers and enter inside the host tissue before establishment of infection. Cuticle constitutes the first barrier in intact aerial organs.

For penetration into host tissue fungal pathogen undergoes particular morphological changes, the first step being germination of spores. Initiation from dormancy to active growth is evidently accompanied by profound structural and biochemical changes. Unfortunately most biochemical studies in the process of spore germination has been made with saprophytic fungi having a low degree of pathogenicity. In those plant pathogenic fungi which depend entirely on the host cells for successful life cycle, it has been noticed that spores of these specialised species remain dormant until an appropriate host plant is near. Existence of specific interactions between spores of a pathogen and corresponding host plant leading to spore germination and differentiation of infection structures has been excellently reviewed by Allen (1976), Macko (1981) and Trione (1981). But the exact mechanism has not yet been fully understood.

In the process of germination endogenous inhibitors have been detected in a wide range of fungal spores. These inhibitors unless they are leached out of the spores during the process of water uptake and hydration, prevent germination presumably by interfering with dissolution of plug of germ pore - a prerequisite condition for germination (Hess *et al*.. 1975). Chemically these inhibitors have been determined to be a group of *cis* - isomers of cinnamic acid derivatives which have a high degree of biological activity with inhibitory concentration of 10^{-8} to 10^{-10}M. Knowledge of the activity of these inhibitors is still rudimentary. It has been contended that chemical stimulus from the host plant may counteract these inhibitors and initiate germination. Our present day knowledge of the mechanisms of signalling which allows to recognize an appropriate host is limited. It has been suggested by Harmann *et al*. (1980) that a variety of carbonyl compounds derived from oxidation

of lipoids may be responsible for regulation of germination of fungal spores in the soil. In the process of infection of aerial organs, apart from germination, production of infection structures is also an essential prerequisite. It is necessary that after germination of spores of pathogenic fungi, the germ tubes must attach themselves to the host plant. Unless the germ tubes make themselves stick to the surface of the plants, on which they have germinated, subsequent developmental changes leading to penetration of the host tissue does not taken place (Wynn and Staples, 1981). As the germ tube grows across the surface of the plant or leaf, it orients its direction of growth perpendicular to the aerial surface of the plant. This orientation helps the pathogen to find probable sites of penetration. Prior to penetration whether through stomata or intact plant surface differentiation into appressorium takes place from which infection peg arises. Saprophytic fungi growing on the plant surface have not been observed to undergo the process of differentiation leading to formation of appressorium and infection structure.

Differentiation of infection structure is considered to be regulated by the mechanical stimulus presented by microstructures of the plant surface (Staples and Hoch, 1982), in some cases chemical stimulus may be involved. The endogenous signal which induces appressorium formation in *Puccinia graminis tritici* in vitro has been identified as acrolein (Macko *et al.*, 1978). This process of differentiation has been claimed as inducible. In *Uromyces appendiculatus*, bean rust, differentiation does not take place on smooth surface. The stimulus appears to be physical. If positive replicas of leaf are made of chemically inert polystyrene and inoculated with spores, the differentiation process will be as on an intact plant surface (Wynn, 1976). However on a smooth surface where physical stimuli are absent, certain chemicals or other treatments, ultrasonic sound or heat shock (Staples and Hoch, 1982) will induce formation of appressorium and infection structures. Certain fungistatic or fungitoxic compounds have been isolated from cuticular waxes of a number of plants which have been found to inhibit germination of spores of pathogenic fungi (Blakeman and Atkinson, 1976, 1981). Only a few of them have been identified, but convincing evidence of their function in nature has not yet been demonstrated.

A pertinent question may arise whether external differentiation into infection structure is accompanied with simultaneous internal change. Evidences so far indicate that several biochemical changes take place as the fungal hyphal cells begin to differentiate into appressorium and infection structure. DNA synthesis begins and nuclei divide (Staples *et al.*, 1975). Several new proteins appear (Huang and Staples, 1982) and a new turn is taken by elements of cytoskeleton (Staples and Macko, 1984). Initiation of these internal changes is presumably based on a receipt of signal from the plant surface and transmission of the same to the nucleus where activation of genes takes place to start the process. In this process, cytoske-

leton is believed to play an important part. Greatest concentration of cytoskeleton takes place near the interface between the plant surface and the pathogen. Indirect evidence is also obtained from the observations that colcemid, griseofulvin and nocadozole, which depolymerize microtubules or podophyllotoxin and cytochalasins, which depolymerize microfilaments in cells of pathogen, induce the formation of infection process even on a smooth surface where differentiation will not normally take place (Staples and Hoch, 1982). Chemicals that lead to stabilization of microtubules, namely deuterium oxide and inhibition of microfilaments e. g. phallotoxin inhibit the process of differentiation of infection structures where differentiation would normally take place (Yoder and Turgeon, 1985).

The actual process at molecular level may be visualized as follows : (a) protein in the extracellular matrix carry out the actual mechanism of sensing and there is a great concentration of proteins at the growing tip of the germ tube. These extracellular proteins are connected to the cytoskeleton, (b) cytoskeletal cables transmit the signal from the tip of the germ tube to the nucleus where activation of genes connected with process of differentiation takes place, and (c) cytoskeleton is most likely to determine the shapes of different infection structures. This postulation at molecular level is largely hypothetical. For determination of events or changes that take place at molecular level, it is necessary that genes, that control production of differentiation-specific proteins and determine their metabolic functions as well as the mechanisms by which are regulated in response to contact stimulus from the host surface, should be identified as further studies.

Actual process of penetration of the host tissue by the pathogen, whether it is strictly mechanical due to physical force of the growth of infection structure or enzymic digestion of the plant cell surface, has always been debated (Van den Ende and Linskens, 1974). Earlier plant pathologists were of the opinion that the proces is entirely mechanical, at least in the first step, enzymes do not play any role in the process (Brown, 1936). Recent evidences indicate that enzyme cutinase plays an important role in the successful entry of the pathogen into the plant surface at least in a few cases.

Outer surface of the plants is covered by cuticle which consists of polymer cutin, embedded in a complex mixture of hydrophobic forms collectively called wax. Waxy materials are present in crystalline form. Electron microscopy shows that the cuticle usually has an amorphous appearance with some reticular structures, the outer region of the cuticle of some plants show lamellar structure. The cuticular wax contains many classes of non-polar C_{20}-C_{32} aliphatic compounds, principally hydrocarbons, wax esters, primary alcohols and fatty acids (Kolattukudy, 1980). Cutin, the structural components of the cuticle is hydroxy-epoxy fatty acid polymer impregnated with waxes (Kolattukudy,.1981).

Cutinase is an esterase which has an active site containing a serine, a histidine, and a carboxyl residue. At least two isoenzymes of cutinase specific for cutin as well as a non specific esterase known to degrade cutin (Purdy and Kolattukudy, 1975). Compounds such as diisopropyl fluorophosphate which bind to active serines, inactivate cutinase.

Cutinase has been isolated from a large number of fungi which include *Fusarium solani pisi*, *F. roseum culmorum*, *F. roseum sambucinum*, *Ulocladium consortiale*, *Colletotrichum gleosporioides*, *Phytocactorum*, *Botrytis cinerea*, *Helminthosporium sativum* and *Streptomyces scabies* (Lin and Kolattukudy, 1980). The inducibility of the enzyme has permitted for isolation of an almost full length of c-DNA clone of the gene that codes for cutinase (Soliday *et al.*, 1984). The primary structure of the enzyme cutinase has been determined from the nucleotide sequence of c-DNA and the amino acid sequence of a portion of cutinase polypeptide.

The fungal cutinases so far isolated from different fungi, on being purified to homogenity, show similarity in size, amino acid composition and similar catalytic properties, namely generation of oligomers and further hydrolysis of oligomers to monomers. Most fungal cutinases contain a single disulphide bridge and no free SH groups. They also contain one methionine, one histidine and one tryptophan per molecule. All of them are glycoproteins containing 3.5—6% carbohydrates which are all attached to proteins of the enzyme. The close similarity in properties of enzymes from all fungal sources so far investigated raises doubt about the functions of the regions of protein where carbohydrates are attached except probably providing stability to the enzyme in the extracellular environment (Kolattukudy and Killer, 1983).

In *Fusarium solani pisi*, the role of cutinase in penetration has been domonstrated (Kolattukudy and Killer, 1983). Furratin-conjugated antibody to cutinase has been used to show the presence of the enzyme at the site of infection (Shaykh *et al.*, 1977). Antibodies to cutinase in the inoculum prevent infection of intact plants, but not wounded plants (Maiti and Kolattukudy, 1979), thereby demonstrating that cutinase is actually required for penetration of the intact host tissue. Inhibitors of the enzyme cutinase such as diisopropyl fluorophosphate also prevent infection, even though they have little or no action on the growth of the fungus. The mode of action of two systemic fungicides, namely hinosan (phosphorodithoate) and benomyl, which prevent the infection of *F. solani pisi* shows that they are inhibitors of cutinase rather than being toxic to pathogen (Killer *et al.*, 1982 a,b). Further evidence on the role of cutinase in infection of host tissue by *Fusarium solani pisi* is obtained from comparative study of the production of enzyme cutinase by highly virulent isolate T-8 and weakly virulent T-30. In isolate T-8, germinating spores have been found to produce a large amount of cutinase, whereas germinating spores of T-30 have been found to produce a very

small amount of cutinase (Killer *et al.*, 1982 c). Similar evidence on the role of cutinase in penetration has been reported in *Colletotrichum gleosporioides* on papaya fruit (Dickman *et al.*, 1982). It has also been observed that mutants of *C. gleosporioides* lacking ability to secrete cutinase are not pathogenic on papaya (Dickman and Patil, 1984).

Direct proof of active involvement of cutinase in penetration of intact host surface is currently available for only two pathogens. It is not yet possible to make any generalization on the role of cutinase in penetr tion.

After penetration of the first barrier of cuticle and cutin layer, either mechanically or with the help of enzymes or both, the pathogen has to encounter the cell wall. Hence successful penetration will require also involvement of cell wall degrading enzymes (CWDE). Although the number and nature of cell wall degrading enzymes produced by germinating spores are as yet unknown, some recent results strongly indicate that cell wall degrading enzymes pectinase, pectinmethyl esterase and cellulase are essential for successful penetration and infection of plant cells with intact cell-wall barrier. Avirulent isolate T-30 of *Fusarium solani pisi* can be made to attack the host when supplemented with exogenous cutinase, pectinase, pectinmethyl esterase and cellulase (Killer *et al.*, 1981). All the four enzymes are considered to be essential for enhancement of virulence of avirulent T-30 isolate of *F. solani pisi*. Detection of pectinase in germinating spores of *Botrytis cinerea* confirms the view.

Regarding penetration of suberized periderm layers which protect underground parts of plants, ultrastructural studies have shown that fungi can affect breach periderm or suberized layers (Peek *et al.*, 1972) Enzymology of suberin degradation is poorly understood and therefore it is not yet possible to test directly whether such enzymes are involved in penetration by pathogens into structures protected by periderm.

The cell-wall degrading enzymes playing vital role may be classified as degradation of pectin polysaccharides. On the basis of specificity of polymers with esterified (methoxy) or non esterified carboxyl groups which are known as pectins and poly galactouronic acid respectively, the corresponding enzymes are known as polymethyl galactouronase (PMG), polygalactouronase (PG), pectinlyase (PL) and polygalactouronide lyase (PGL). The methoxy group of pectin is removed by pectin methyl esterase (PME) which acts in conjunction with PG or PGL. Besides enzymes may be divided as (-endo) when they attack internal regions of chains at random or terminally as (-exo).

Cellulose degradation is usually restricted by its insoluble and partly crystalline form. Conversion into glucose requires a complex of enzymes originally designated by Reese (1977) as C_1, Cx and cellobiase. The number of components and

precise modes of action, particularly of C_1 are in dispute, but overall concept of a synergistic system in which the individual enzymes are relatively ineffective against native cellulose is well established for various microorganisms including *Fusarium solani* (Wood, 1969). In nature tissue cellulose break down is often limited by presence of lignin. It is interesting to note that a wood decay fungus simultaneously degrades two polymers in cell wall with enzyme cellobiase and quinone oxidoreductase (Eriksson, 1977). An indication of the range and characteristics of cell wall degrading enzymes can be obtained from cultures of pathogenic fungi grown on host cell-walls, although the properties occassionally differ from forms detected *in vivo* (Cooper, 1977).

It is necessary that activity of CWDE should be present and detected in diseased tissue before appearance of or coincident with symptoms. However lack of activity does not discount involvement as plants contain inhibitors of CWDE, particularly oxidized phenolics and wall bound PG inhibitors (Bryde and Archer, 1977).

Increase in CWDE activity may not always be derived from pathogen. Healthy plants possess a number of CWDE involved in processes such as germination, pollination, dissolution of xylem vessel end walls, abscission, fruit ripening etc. Wall degradation by a parasite may release host-bound host enzymes, as would be evident from increased PME activity in comparisons of natural isolates for pathogenicity and production of CWDE usually reveals a great degree of variability within a species for numerous factors many of which may be unrelated to synthesis of CWDE. In this context, studies with induced mutants with a common genetic background deficient, superproductive and constitutive for several CWDE would be profitable line of approach to elucidate the mechanisms. (Howell, 1976; Cooper, 1977). Such studies will obviously rely on correlation based on numbers amenable to statistical analysis (Howell, 1975) as loss of pathogenicity can reflect mutations other than CWDE production ; nevertheless maintained pathogenicity of a single mutant deficient in a specific CWDE is strong evidence against involvement of that enzyme.

Transmission electron microscopy can give valuable informations in connection with participation of CWDE. Displacement of wall fibrils indicates mechanical penetration, localized degradation is due to well bound or rapidly inactivated enzymes, whereas extensive wall dissolution signifies action of fully diffusible extracellular CWDE. Loss of middle lamella implies action by pectic enzymes (Baker *et al.*,1980).

Botrytis cinerea, a necrotroph, causes extensive change in wall during or even prior to penetration where endo-PG occurs in ungerminated conidia of the pathogen (Verhoeff & Liem,1978)and is secreted on the surface of onion leaves before penetration by *Botrytis squamosa* (Hancock *et al.*, 1963). In organisms with high pectolytic activity e.g. *Rhizoctonia solani*, *Erwinia carotovora*, damage to cell walls of the host plants occurs well ahead of penetration. However this process of killing in advance

of penetration is likely to be arrested by possible host resistance induced by endo-PG and endo-PL, both of which can elicit phytoalexin (West, 1981).

Extensive degradation of cell wall is invariably accompanied by cytoplasmic disruption. Studies with homogeneous preparations of endo-pectic hydrolyases and lyases from various fungi and bacteria have clearly established the unique roles of CWDE as agents for maceration of eell walls as well as simultaneous damage to protoplant, a characteristic symptom of soft rot disease (Bateman and Basham, 1976). Apple tissue treated with pure endo-PL shows features of typical invasion, namely degradation of cell wall, retraction of plasmalemma, dilation and vesiculation of endoplasmic reticulum (Hislop *et al.*, 1979). Cells with considerable alterations are dead while those with lesser alterations are injured. Damage to plasmalemma is considered to be due to weakening of cell wall unable to bear the turgor pressure, which ultimately leads to death. Besides leakage of ions from cells may take place due to exposure to endo-pectic enzymes (Hislop *et al.*, 1979). Leakage of ions is indicative of cell injury due to a toxic component which may be released from the degraded cell walls. Cell normally contain many enzymes and molecules of physiological and damaging effects, which are normally released on degradation. These include peroxidase, glucose oxidase, hydrogen peroxidase, ehtylene, acid phosphatase, PME, glycosidases, lectins, PG inhibitors, and wall carbohydrate fragments acting as elicitors of phytoalexin (Lund and Mapson, 1973 ; Mussell and Strand, 1977 ; Cooper *et al*, 1961 ; Hahn *et al*, 1981 ; Moneil *et al*, 1982).

Polygalactouroxidases have been implicated with wilted vessels due to occlusion of xylem vessels by pectic gels arising from pit membranes, resulting in water stress (Cooper and Wood, 1980), other symptoms resulting from breakdown products of cell wall. Evidences regarding role of pectolytic enzymes in the disease syndrome of wilt are contradictory except in *Verticillium* wilts of tomato and lucrene where PL and in cotton PG have been found to be present before the appearance of symptoms. Howell's (1976) observation, that pathogenicity of UV-induced mutants of *Verticillium dahliae* deficient for either PG or PL, are retained, have cast doubt regarding the role of these enzymes. However positive evidences regarding involvement of pectolytic enzymes in wilting are very limited.

Cellulases are usually active at later stages of infection except in a few diseases namely lesions incited by *Rhizoctonia solani* and *Sclerotinia sclerotiorum* or tobacco stems affected by *Pseudomonas solanacearum* where pathogenicity of isolates has been found to have a positive correlation with cellulase production (Kelman and Cowling, 1965). Insoluble nature and location of fibrilo in cell walls may explain the infrequent or limited participation of cellulase in pathogenesis (Cooper, 1983). Question now arises whether CWDE has any role in biotrophs, where there should be minimal changes in host cell walls in the process of penetration and infected cell

and pathogen should remain alive for a considerable period of time. Infection process in biotrophs was previously considered to be entirely mechanical, but ultra-structural studies of the cell walls of infected host cells have indicated involvement of localized action of CWDE. In *Bremia lactucae*, microfibrils are not distorted around infection pegs which may be highly convoluted and lacking cell wall (Ingram *et al.*, 1976), on the other hand exceptionally marked wall disruption in haustorial formation has been noticed in *Peronospora spinaciae* (Cooper, 1981).

Recent studies on urediospores of *Uromyces spp.* and sporidia of *Ustilago maydis* in culture suggest production of CWDE in very small amounts and bounds form. Endo polygalacto uronidases which are most effective in dissolution of cell wall is however, absent (Baker *et al*, 1980). Cellulases are absent suggesting there by that primary wallls only are affected.

The primary walls of different host plants infected by the pathogen show similarity in structure and composition, in terms of major polysaccharides and glycosidic linkages (Albersheim, 1976 ; Darvill *et al.*, 1980). Walls isolated from cultivars with different levels of resistance are equally susceptible to CWDE, besides *in vitro*, they induce similar types of cell wall dissolving enzymes (Cooper, 1977). Epidermis of resistant and susceptible cultivars are equally penetrated by pathogenic fungi (Cooper *et al.*, 1981).

In non-host plants, resistance to invasion of pathogen sometimes noticed in the cell walls, in as much as cell walls from host and non-host species have a differential rate on production of pectic enzymes. No consistent relationship with resistance and susceptibility with supporting synthesis of cell wall dissolving enzymes *in vitro* has been observed except in a few cases namely *Botrytis fabae* on *Vicia faba* (Cooper *et al.*, 1981). In this context a comparative study of pathogens attacking monocotyledons and dicotyledons and cell walls of monocotyledons and dicotyledons are enteresting. There is a distinct difference in the cell wall composition of monocotyledons and dicotyledons. Cell wall disso'ving enzymes of pathogens attacking monocotyledonous plants namely *Helminthosporium maydis* causing corn blight will have much less effect on bean, conversely cell wall dissolving enzymes of *Colletrotrichum lindemuthianum*, bean anthracnose pathogen, will have on maize (Baker *et al.*, 1977, 1980). One plausible explanation may be that pathogens of either group have evolved their range of cell wall degrading enzymes on the basis of cell wall composition probably through long adaptation. Xylanase, α-xyloside, β-glucanase, β-galactanase are much higher cell wall dissolving enzymes of *Helminthosporium maydis* or *Ustilago maydis*, where as endo-PG, endo-PL, cellulase are more active in *Verticillium alboatrum* (Anderson, 1978).

In conclusion it may be stated that the first defensive barriers, namely cuticle, primary cell-walls of most plants show similar structural features. It is unlikely that the fungal enzymes which degrade them would have host species specificity

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