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Rust Fungus Differentiation on Host Leaves

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INTRODUCTION

Wheat stem rust (*Puccinia graminis* (Pers.) f. sp. *tritici* Eriks and Henn.), an obligate parasitic fungus, causes a serious rust disease of wheat in all wheat growing areas of the world. A study on the behaviour of rust spores on host leaf surface would provide useful information on genetics and biochemistry of primary infection, which could be correlated with the earlier biochemical changes related to susceptibility or resistance. There are a series of distinct steps or stages from the moment a parasite unit is placed on a host until a compatible and functional or incompatible relationship is established. The interaction may be eventually reach a climax in an infection type or an indeterminant status of the diseased condition. Therefore infection type is an indication of whether particular genetic units are involved in the interaction.

Germination on Living Host

A uredospore of fungus germinating on wheat leaf surface characteristically produces a germ tube that elongates to reach a stoma where in forms an appressorium and an infection peg that enters the stomata and develops into a substomatal vesicle; infection hyphae developing from the vesicle spread into the intercellular spaces and absorb food nutrients from the host cell by means of haustoria, which penetrates the host cell (Ehrlich, 1971; Lewis and Day, 1972; and Skipp and Samborki, 1974).

Differention of germ tubes into infection structures is a series of subtle and precisely timed orderly sequence of fungal development, which could be synchronized on leaf surface(Skipp and Samborski, 1974). At the end of dark period of 16 hrs after incubating rust-infected wheat leaves uredospores germinated to produce germ tube which elongated to reach stoma and formed appressorium. In further incubation in darkness, fungal development did not exceed the appressorial stage. By turning light on for 16 hrs and the in darkness for 8 hrs, the fungal development extended to develope infection peg, substomata vesicle, infection hyphae, and finally formed haustorium. Here is a system to compare the same developm-

ental stage of fungal growth on the host regardless of resistant or susceptible lines of wheat, and any biochemical study conducted with these plant materials would be more meaningful than with plant materials with randomly grown fungal development.

With a high degree of synchronization of spore germination, it is possible to pinpoint a molecular change at a particular stage of primary infection and to determine how and when different parasite/host genotypes for compatibility or incompatibility act to affect the interactions between host and parasite.

Physical Factors affecting Germination

Linear growth of the germ tube ceases when infection structures begin to form. The stages in their development are correlated with the position of the guard cells, of stoma, and of the substomatal cavity (Dickinson, 1971; Lewis and Day, 1972; Macko and Staples, 1973; and Maheshwari et al., 1967). Orientation of the germ tube and appressorium formation are considerered to be responses to stimuli from the host(Lewis and Day, 1972).

Two categories of stimuli have been implicated in orientation; hydrotropic response and thigmotropic response. Recently Lewis and Day(1972) furthered the study on thigmotropic response, in which they found that the lattice of wax crystals on wheat leaf surface maximizes the probability of the hyphae contacting a stoma. Germ tubes develop parallel to the short axis of the leaf upon contact with the lattice, which served as an orientation mechanism. When wax layer was disturbed on linear growth of germ tubes were observed, while when germ tubes miss stomata no formation of appressoria was observed.
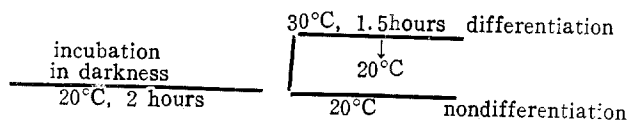
Germination and germ tube formation are affected by a number of factors such as light intensity(no germination occurs at 1,000 ft. candles and the linear growth of germ tube inhibited at 300 ft. candles), temperature (inhibition of germination at 30°C), relative humidity, and carbon dioxide (a little amount of CO_2 is inhibitory although uredospores fix CO_2 in dark reactions and germination occurs in the absence of CO_2 (Shaw, 1974).

GERMINATION PROCEDURE AND TEMPERATURE REGIME

Washing uredospores

A· Shaking 250mg of
   Spores in 250-500ml
   0.01% Triton X-100 or
   double dist. water,
   5 minutes

B· Further washing by
   suction filtration
   with 1,000ml water
   millipore membrane
   sc, 15cm dia., 8μ pore
   size

incubation
in darkness
20°C, 2 hours

30°C, 1.5hours differentiation
   ↓
   20°C

20°C   nondifferentiation

Germination on Artificial Membranes

It has been shown by Dickinson (1971) that the morphology of the germ tube elongating on membranes of nitrocellulose, polystyrene and polymethyl methacrylate depends on the frequency and height of the ridges of these membranes. In response to these membranes, spores produced germ tubes which varied from unbranched to zig-zag growth and differentiation as well. The thigmotropic responses resulted from contact of germ tube with a repetitive series of changes in thickness in plastic membranes or with parallel ridges in nitrocellulose. The stimulus of the membrane is due only to the topographical features of the surface and not to any chemical properties of the surface waxes. Plastics were used to make copies of leaf cuticles in such a way that surface waxes were entirely eliminated. Appressoria were found to be induced over stomata, and it was found that it was the protruding lips of the stomatal guard cells which induced formation of the infection structures (Macko and Staples, 1973).

Uredospores of the rust fungi can be induced *in vitro* to differentiate infection structures (differentiating sporelings), analogous to those formed during the early stages of parasitic invasion of the host plants (Dunkle *et al.*, 1968; Kim, 1971; Maheshwari *et al.*, 1967; and Ramakrishnan and Staples, 1970). In nondifferentiating sporelings, germ tubes continue to grow, but fail to differentiate into infection structures. A variety of techniques has been employed with different rust fungi to elicit this normal course of germ tube development. On moist artificial membranes, uredospores germinate but the sporelings under ordinary conditions fail to differentiate producing only a long unbranched germ tube. However, infection structures are induced by giving an elevated temperature shock treatment (Figure 1). It is
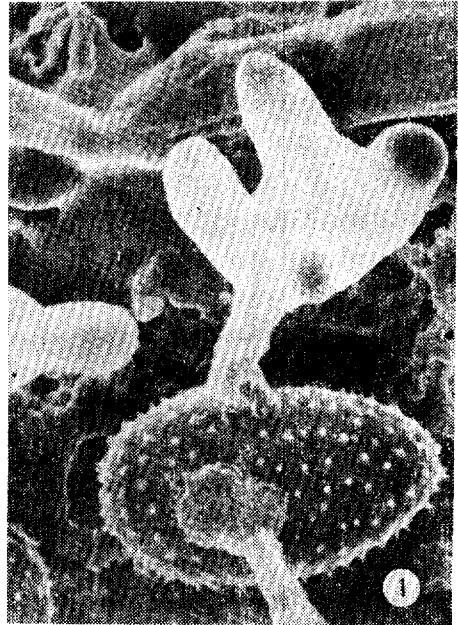

Fig. 1. Germination procedure and temperature regime(ref. Dunkle *et al.*, 1668; Kim, 1971; Kim and Rohringer, 1974; and Maheshwasi *et al.*, 1967)

conceivable that a contact stimulus or heat shock is translated into a chemical message to which the fungus responded. This method of induction of infection structure formation on spores germinating en masse in the absence of the host is quite useful for host/parasite biochemists because the biochemistry of differentiating sporelings obtained in this manner should more closely resemble that of rust infection structures on the host. It is also a convenient *in vitro* system for determining the effect of nutrients and potential inhibitors on development of infection structures.

Chemical Factors affecting Germination

Germination is also controlled by the production of inhibitory (self-inhibitor) or stimulator substances by the spore themselves or by the mycelium(Macko and Staples, 1973 and Shaw, 1964). Spores of many fungi germinate poorly or not at all in dense suspensions or if crowded upon a surface. In many cases it has been demonstrated that inhibitory substance from fungal spores is a primary cause. Germination self-inhibitor from a number of rust spores were isolated and characterized and wheat stem rust uredospores contain methyl cis-4 hydroxy-3-methoxy cinnamate (methyl cis-ferulate). The double bound in the side chain of the cinnamate compounds allow for geometrical cis-trans isomerism. It was shown that cis isomer was made photochemically from the trans isomer synthesized enzymatically by the plants (Macko and Staples, 1973). The cis isomer possesses all of the inhibitory activity, whereas the trans isomer is inactive. Compounds have also been found which stimulate germination of self-inhibited spores. N-nananal was identified as a stimulatory principle in distillates of spores of wheat stem rust. Self-inhibited wheat stem rust uredospores were reported to be stimulated by floating the spores on dilute solutions containing peroxidase and hydrogen peroxide. It was found that methyl cis-ferulate is dimerized by peroxidase action and the dimer is inactive as a germination inhibitor. In nature, the balance between inhibitors and stimulators probably serves as a regulatory factors in germination and plays a role in the colonization on subst-

rate particularly on host plants.

Changes in Fine Structures

A substantial amount of information on the change in fine structures during infection structure formation is available, mainly from electron microscopic studies on rust-infected wheat leaves or from the differentiatings on artificial membranes. Nuclear division was induced normally just prior to formation of the appressorium and involved a transition of the nucleoli from a so-called "unexpanded" to an "expanded" state (Shaw, 1964). This was accompanied by the extrusion of the nucleoli which were not found in expanded nuclei. A second nuclear division takes place in appressorium, and a third division in the substomata vesicle. Normally the haustrial mother cells contain two nuclei and the rest are in the infection hyphae. Nucleoli were found in differentiating sporelings, but only small amorphic nucleoli were found in non-differentiating sporelings, (Ehrlich and Ehrlich, 1971; and Shaw, 1964). These findings have led to considerable speculation on the role of the germ tube nucleus in obligate parasitism since one of the primary function of nucleus is the synthesis of ribosomal RNA needed for protein synthesis. The absence or reduction in size of the nucleolus in uredospores and germ tubes may represent a deficiency in the capacity of the uredospores to achieve a net synthesis of protein during germination, hence the obligate dependence of the rust on the host plants.

Williams and Ledingham (1964) observed that there were more numerous mitochondria in germinating germ tube

than in resting uredospores. There were also migrations of mitochondria, nuclei, and other cellular organelles toward the hyphal tips where is known to be metabolically most active.

Scanning Electron Microscopy

The behaviour of stem rust uredospore germ tubes in relation to the fine structure of wheat leaf surface by scanning electron microscopy was studied by several investigators(Lemis and Day, 1972; Paliwal and Kim, 1974; and Skipp and Samborski, 1974). Appressorium, being exogenous, were the only infection structures observed. The natural topography of the sporelings was altered due to their collapse during specimen preparation for microscopy, Differentiating sporelings(Paliwal and Kim, 1974) show a typical infection structure development with the abundance of anastomosis in the hyphae, while non-differentiating sporelings produce simple unbranched
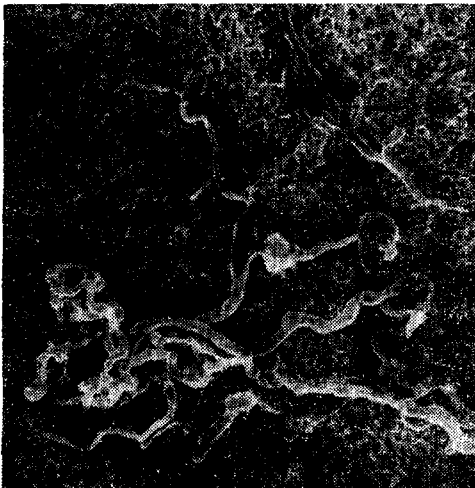

Fig. 2. One of the sporelings shows typical infection structure development. A rather straight germ tube, appressorium (Ap), substomatal-like vesicle(Sv) and infection hyphae(Ih) can be seen ×620(ref. Paliwal and Kim, 1974).

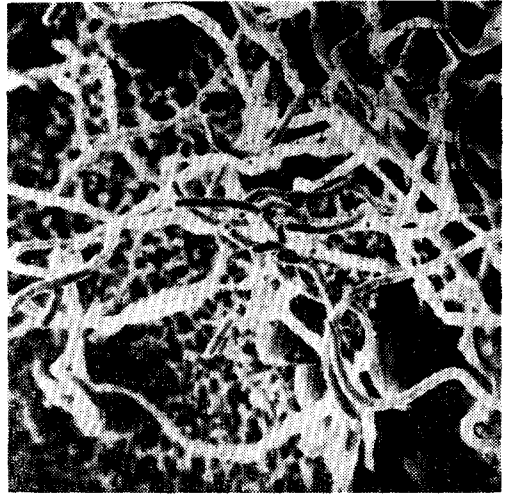Fig. 3. A mass of unbranched, non-differentiated long germ tubes of non-differentiating sporelings on a Millipore membrane ×560 (ref. Paliwal and Kim, 1974).

germ tubes (Figures 2 and 3). The cause of anastomosis around infection structures seems to be due to the layer of mucilagenous material, which is thought to cement the infection structures to the host leaf surface.

Biochemical Changes

The biochemical events of germination and differentiation of rust uredospores are not much studied. It has been suggested that wheat stem rust uredospores depend upon protein synthesis for germination(Dunkle *et al.*, 1968; and Trocha and Daly 1970), which probably is guided by a stored messenger RNA (Dunkle *et al.*, 1968; Kim and Rohringer 1974; Ramakrishnan and Staples 1970; Ramakrishman *et al.*, 1970; and Staples *et al.*, 1970). Differentiation of uredospores seemed to depend on the synthesis of a new messenger RNA as judged by the sensitivity to actinomycin D (Dunkle *et al.*, 1968). The nuclear divisions which occur during differentiation suggest that

nucleic acid metabolism is substantially altered during appressorial formation. It was clear from the work of Dunkle *et al.*(1968) that inhibitors of RNA synthesis interfere with the differentiation of infection structures if present during the heat shock treatment. Inhibitors of protein synthesis prevent differentiation if present following heat treatment. Apparently infection structure formation is accompanied by synthesis of RNA and the completion of infection structure development requires protein synthesis.

It was found from studies on ribosomal RNA of bean rust uredospores that the amount of ribosomes did not change during germination. The RNA contained a fraction with template activity which accumulates in differentiating uredosporelings, but declines in non-differentiating uredosporelings (Ramakrishman and Staples, 1970;and Ramakrishman *et al.*, 1970). Germinating uredosporelings contained a series of polysomes that were at least as heavy as hexamer and probably heavier (Staples *et al.*, 1970). At least 55% of the ribosomes were polymerized until 8 hr when they declined to 35%, regardless of the state of differentiation. It is clear that uredospores have a complete protein synthetic apparatus that is similar to other fungi. However, uredospore ribosomes remain active only for the brief period of germ tube elongation and differentiation.

Uredospores of wheat stem rust synthesize RNA during germination on a non-host substrate, but this coincides with an overall decline of RNA content, primarily because of ribosomal RNA degradation, or possibly because of complex formation with macromolecules as part of normal germination process (Kim and Rohringer, 1974). Such a complex could have accumulated in the interphase during phenol separation. It was found that the interphase was rich in DNA and protein. However, radioisotopic studies revealed that new molecules of RNA were synthesized in germinating and differentiating uredosporelings, but not in non-differentiating sporelings (Kim and Rohringer, 1974). Polyacrylamide gel electrophoresis, sucrose density gradient centrifugation, and RNase T_1 partial digestion of this new type of RNA demonstrated that it was heterogeneous and migrated in the 16-S to 5-S interval on the gels. Some of the RNA present in this fraction may have been preformed in resting uredospores and released from more complex material during the process of differentiation.

RNA species of wheat stem rust uredospores were 25-S, 18-S, 5-S rRNAs, and 4.5-S tRNA, and their approximate molecular weights (in daltons) were 1.65×10^6, 0.80×10^5, 3.6×10^4 and 2.4×10^4, respectively.

During the formation of infection structures DNA also synthesized (Staples, 1974). It appears that the synthesis of mitochondrial DNA is initiated during or shortly after uredospores germinate. Synthesis of this DNA probably is related to early synthesis of mitochondria. The activity of cytochrome oxidase and other electron transport enzymes increase in germinating uredospores. Electron microscopic study has suggested that mitochondria were synthesized during germ tube elongation(Williams and Ledingham, 1964). However, the synthesis of mitochondrial DNA ceases with the initiation of differentiation. As the elongation of

the germ tube ceases with initiation of the appressorium, it is possible that synthesis of mitochondria continues only so long as the germ tube elongates.

Although it has not been studied with wheat stem rust uredospores, it was demonstrated that bean rust uredospores contain multiple forms of DNA-dependent RNA polymerases, suggesting the possible role of these enzymes in the formation of infection structures (Manocha, 1973).

Germinating uredosporelings of wheat stem rust contain RNase which is different from that of the host plants or host/parasite complex in terms of substrate specificity(Chakrauorty et al., 1974), and their functions are considered participating in post-transcriptional process through a selective degradation of certain species of RNA molecules. It was also demonstrated that germinating uredospores leach out RNase into germination medium (Chakrauorty et al., 1974).

Changes in Biochemical Constituents

Biochemical constituents and their changes in content associated with differentiating and non-differentiating sporelings of wheat stem rust were extensively studies. Since folates and polyamines are believed to play an important role in protein synthesis (Kim, 1971), they were measured in germinatings. Differentiating sporelings contained less total folates, less methylated folates, but more formylated folates of low levels of conjugation than did non-differentiating spo-

relings. Stem rust uredospores contained spermidine and smaller amount of an unidentified polyamine. Spermidine content increases after germination, especially after formation of infection structures.

As successful colonization of a host by obligate parasite, such as the rust, depends on the integrity of membranes at the host/parasite interphase, sterols were isolated from uredospores and their identities were determined(Nowak et al., 1972). Uredospores contained trace amount of an unknown sterol, cholesterol, and either ergost-7-enol or stigmasterol, and larger amounts of stigmast-7-enol. After germination, the level of stigmast-7-enol tended to decrease after germination. However, there was no difference in the level of stigmast-7-enol between differentiating and non-differentiating sporelings.

Bioassay

With the increasing awareness of the importance to determine the initial recognition and the specificity in host/parasite interaction, the understanding of precise stages of the spore germination and formation of infection structures in various genotypes is vital, in particular where a bioassay is designed to determine the specificity. Our recent work on the gene-specific RNA determining resistance in wheat to stem rust is partly based on the precise understanding of the behaviour of stem rust uredospores on wheat leaves(Rohringer et al., 1974).

REFERENCES

1. Chakravorty, A.K., M. Shaw, and L.A. Scrubb, 1974. Ribonuclease activity of wheat leaves and rust infection. *Nature* **247** , 577-580.

2. Ehrlich, M. A., and H.G. Ehrlich, 1971. Fine structure of the host-parasite interfaces in mycoparasitism. *Ann. Rev. Phytopathology* 9, 155-184.

3. Dickinson, S., 1971. Studies in the physiology of obligate parasitism, VIII. An analysis of fungal response to thigmotropic stmuli. *Phytopath. Z.* 70, 62-70.

4. Dunikle, L. D., R. Maheshwari, and P.J. Allen, 1968. Infection structures from rust uredospores. Effect of RNA and protein synthesis inhibitors. *Science* 163, 481-482.

5. Kim, W. K., 1971. Folate and polyamine content of undifferentiated and differentiated wheat stem rust uredosporelings. *Can. J. Bot.* 49, 1119—1122.

6. Kim, W. K., and R. Rohringer, 1974. Ribonucleic acids of differentiating and non-differentiating uredosporelings of wheat stem rust. *Can. J. Bot.* 52, 1309—1317.

7. Lewis, B. G., and J. R. Day, 1972. Behaviour of uredospore germ tubes of *Puccinia graminis tritici* in relation to the fine structure of wheat leaf surfaces. *Trans. Br. Mycol. Soc.* 58(1), 139—145.

8. Macko, V., and R. C. Staples, 1973. The Torrey Symposium on current aspects of fungal development. V. Regulation of uredospore germination and germ tube development. *Bull. Torrey Bot. Club* 100(4), 223—229.

9. Maheshwari, R., P. J. Allen, and A. C. Hildebrandt, 1967. Physical and chemical factors controlling the development of infection structures from uredospore germ tubes of rust fungi. *Phytopathology* 57(8), 855—862.

10. Manocha, M. S., 1973. DNA-dependent RNA polymerase in germinating bean rust uredospres. *Can. J. Bot. Microbiol.* 19, 1175—1177.

11. Nowak, R., W. K. Kim, and R.Rohringer, 1972. Sterols of healthy and rust-infected primary leaves of wheat and non-germinated and germinated uredospores of wheat stem rust. *Can. J. Bot.* 50, 185—190.

12. Paliwal, Y. C., and W. K. Kim, 1974. Scanning electron microscopy of differentiating and non-differentiating uredosporelings of wheat stem rust fungus(*Puccinia graminis* f. sp. *tritici*) on an artificial substrate. *Tissue and Cell* 6(3), 391—397.

13. Ramakrishnan, L., and R. C. Staples, 1970. Evidence for a template RNA in resting uredospores of the bean rust fungus. *Contrib. Boyce Thompson Inst.* 24, 197—202.

14. Ramakrishnan, L., and R. C. Staples, 1970. Changes in ribonucleic acids during uredospre differentiation. *Phytopathology* 60, 1087—1091.

15. Rohringer, R., N. K. Howes, W. K. Kim, and D. J. Samborski, 1974. Evidence for a gene-specific RNA determining resistance in wheat to stem rust. *Nature* 249, 585—588

16. Shaw, M, , 1964. The physiology of rust uredospores. *Phytopath. Z.* 50, 159—180.

17. Skipp, R. A., and D. J. Samborski, 1974. The effect of the Sr6 gene for host resistance on histological events during the development of stem rust in near-isogenic wheat lines. *Can. J. Bot.* 52, 1107—1115.

18. Trocha, P., and J.M. Daly, 1970. Protein and ribonucleic acid synthesis during germination of uredospores. *Plant Physiology* 46, 520—526.

19. Staples, R. C., L. Ramakrishnan, and Z. Yaniv, 1970. Membrane bound ribosomes in germinated uredospores. *Phytopathology* 60 (1), 58—62.

20. Staples, R. C., 1974. Synthesis of DNA during differentiation of bean rust uredospores. *Phyisological Plant Pathology* 4, 415—424.

21. Williams, P. G. and G. A. Ledingham, 1964. Fine structures of wheat stem rust uredospores. *Can. J. Bot.* 42, 1503—1508.