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Growth of *Verticillium lecanii* in Pustules of Stripe rust (*Puccinia striiformis*)

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With 10 figures

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A number of different fungal hyperparasites have been observed to grow in pustules of rust fungi. Examples of these are *Verticillium* (HASSEBRAUK 1936, KOTTHOFF 1937, GAMS 1971), *Darluca filum* (KRANZ 1973), *Monocillium nordinii* (TSUNEDA and HIRATSUKA 1980), *Alternaria* and *Cladosporium* species (OMAR and HEATHER 1979). *Verticillium lecanii* may be the most interesting fungus for biological control of rust diseases since this fungus not only has a very broad host range in fungi (GAMS 1971), but also attacks different insects (BARSON 1976, HALL 1980). This fungus is part of the natural mycoflora of wheat stem rust (*Puccinia graminis* Pers. f. sp. *tritici*) and stripe rust (*Puccinia striiformis*) infected leaves (MCKENZIE and HUDSON 1976, Dr. EVA FUCHS, pers. communication). *V. lecanii* may be appropriate for the biological control of carnation rust, *Uromyces dianthi* (SPENCER 1980). There are only few studies on the mode of action of the parasitism of *V. lecanii*. Lysis of urediospore germ tubes (GARCIA ACHA *et al.* 1965), bursting of urediospores of coffee rust, *Hemileia vastatrix* (SILVEIRA and RODRIGUES 1971) and penetration into urediospores of stripe rust, *Puccinia striiformis* (SCHROEDER and HASSEBRAUK 1957) by *V. lecanii* has been described. After infection of pustules of bean rust (*Uromyces phaseoli*), the hyperparasite remained restricted to the pustule area and did not penetrate into the bean leaf tissue (MENDGEN and CASPER 1980). There have been no studies on the optimal conditions for growth of *V. lecanii* in the rust pustule. This paper reports the interactions of the germ tubes and the urediospores of *Puccinia striiformis* with *Verticillium lecanii* and the influence of temperature, light and air humidity on the development of the hyperparasite in the rust pustule.

Material and Methods

1. Fungus and plant material

The isolate of *Verticillium lecanii* (Zimm.) Viégas was obtained from Dr. Eva Fuchs, Biologische Bundesanstalt, Braunschweig. It had been isolated from a stripe rust pustule. Its identity was confirmed by Dr. W. Gams (mean spore length: 3.5 μm, mean spore diameter: 2 μm).

The host rust fungus, *Puccinia striiformis* West., race 37 E 132 was also obtained from Dr. Eva Fuchs.

The wheat plants (cultivar Michigan amber) were raised at 15 ± 0.5 °C and illuminated with fluorescent light, 4000 lux, 16 h/days unless otherwise indicated.

Air humidity was controlled by pumping the air that surrounded individual plants through gas washing bottles containing saturated salt solutions (Winston and Bates 1960).

2. Serology

The antiserum against *V. lecanii* was prepared as described earlier (Casper and Mendgen 1979). For the enzyme linked immunosorbent assay (ELISA), the method of Clark and Adams (1977) was adopted. To measure the amount of *Verticillium* hyphae in rust infected plant tissue, 200 mg of the infected leaves were freeze-dried and subjected to the ELISA test. The absorbance values (A_{405}) were used to indicate the amount of *V. lecanii* in the leaf tissue. For immunofluorescence, samples of the infected leaves were fixed in 2 % glutaraldehyde and embedded in paraffin. After sectioning and removal of the paraffin, sections were washed in 0.01 M phosphate buffer, pH 7.2 with 0.15 M NaCl. Incubation of sections was performed in antiserum, diluted 1 : 50 with buffer. After thorough washing in buffer, the reaction with the antiserum was made visible by incubation in fluoresceine-conjugated anti-rabbit-IG from sheep (Miles) diluted with buffer 1 : 20. Photographs were taken with a Leitz fluorescence microscope using filter blocks A, D, I2 and Ilford FP4 film (Mendgen and Casper 1980).

3. Light- and electronmicroscopy

To study the interaction of germ tubes of both fungi, 2 % water agar was spread on a microscope slide and the spores were sprayed on the thin agar layer. The agar was covered with a cover glass. To keep a distance between cover glass and agar surface, bits of glass were put on the agar so that a thin layer of air remained around the spores and their germ tubes.

For electron microscopy of pustules, leaves were sprayed with a spore suspension of *V. lecanii* when the pustules of stripe rust appeared. The plants were then kept under a plastic cover to keep high humidity. Ten days later, when a white weft of sporulating *Verticillium* mycelium could be seen over the uredia, samples were taken for electron microscopy. These samples were fixed in 2 % glutaraldehyde and in 2 % osmium tetroxide, dehydrated in alcohol, and embedded in Spurr's epoxy resin. Sections were made with glass knives, stained with uranylacetate and lead citrate and examined with a Zeiss EM 10 CR electron microscope.

Results

1. The interaction between germ tubes of *Puccinia striiformis* and *Verticillium lecanii*

To determine whether *V. lecanii* was able to attack the rust fungus during its growth on the leaf epidermis, spores of *V. lecanii* and *P. striiformis* were sprayed on a thin agar layer and observed under the microscope for periods of several days at 15 °C and 100 % humidity.

The stripe rust urediospores germinated earlier and the germ tube grew much faster as compared to *V. lecanii*. Therefore, spores of *V. lecanii* were sprayed on agar 24 h in advance of those of *P. striiformis* and were observed during the following 6—8 days. Neither urediospores nor germ tubes of

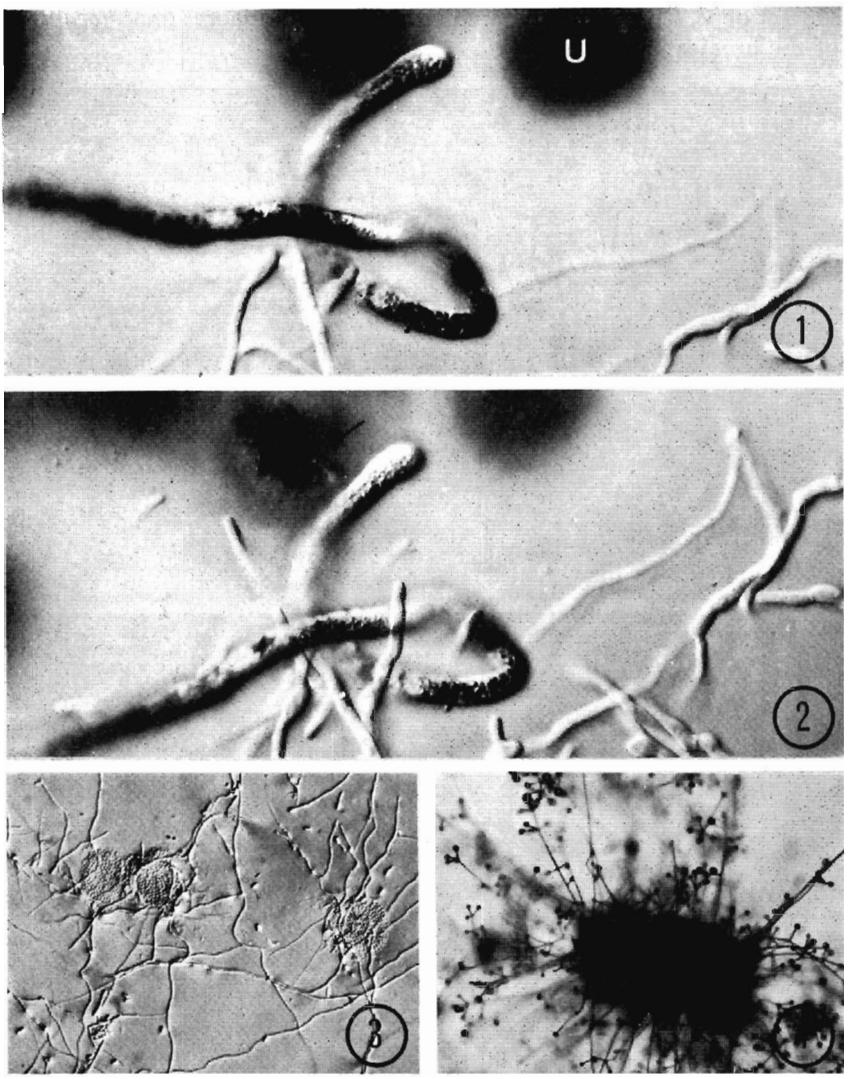

Fig. 1. *Verticillium lecanii*, spores with their germ tubes, 48 h old, on water agar. The germ tube of *Puccinia striiformis* growing between the hyphae of the hyperparasite, is 24 h old. Urediospores (U) of *P. striiformis* (× 400) • Fig. 2. The same preparation as Figure 1, but 24 h later. The germ tube of *P. striiformis* terminated growth. The germ tubes of *V. lecanii* grew past the rust germ tube on to the urediospore (arrow) (× 400) • Fig. 3. A similar preparation as in Figure 1, 4 days later: The urediospores have collapsed (× 160) • Fig. 4. Conidiophores of *V. lecanii* grew out of urediospores about 6 days after the contact between *V. lecanii* and *P. striiformis* (× 160)

P. striiformis attracted the germ tubes of *V. lecanii*. The hyperparasite usually passed the germ tubes of the rust fungus (Fig. 1) and, in case it met a urediospore, tended to branch and to grow on the surface of the urediospores of *P. striiformis* (Fig. 2). Urediospores became disorganized two to three days after they were in contact with the hyperparasite (Fig. 3). About 6 days after the contact of *V. lecanii* and the urediospores, conidiophores of *V. lecanii* grew out of the urediospores (Fig. 4).

Fig. 5. Cross section through a stripe rust (*P. striiformis*) pustule, ten days after inoculation with *V. lecanii*. The section was treated with fluoresceine conjugated *V. lecanii* antibodies. After blue light excitation (340—380 nm) only hyphae and urediospores of the stripe rust fungus can be seen (× 550) • Fig. 6. The same section as in Figure 5, except that the fluoresceine-conjugated antibodies against *V. lecanii* can be seen after selective excitation (450—490 nm) for fluoresceine indicating the hyphae of the hyperparasite (× 550)

2. The interaction of *Verticillium lecanii* and *Puccinia striiformis* within host leaves

Uredia of stripe rust infected with *V. lecanii* generally were covered with a white web of the hyperparasite mycelium four to seven days after inoculation with *V. lecanii*. Ten days after inoculation, cross sections through the pustule area showed large numbers of *Verticillium* hyphae present between the urediospores of the rust fungus. With light microscopy, it was not possible to differentiate between the hyphae of the rust fungus and those of the hyperparasite within the leaf tissue. Therefore, the hyphae of *V. lecanii* were labeled with fluorescing antibodies as described earlier (MENDGEN and CASPER 1980). After this treatment of the paraffin sections, hyphae of *V. lecanii* gave a bright fluorescence and they were observed on the epidermal surface around the pustule and in the pustule, between and within the urediospores. They were not detected within the leaf tissue nor in the sporogenous tissue of the rust fungus. Figure 5 shows such a cross section after blue light excitation (340—380 nm), which demonstrates the hyphae and urediospores of the stripe rust fungus. The same section, after selective excitation for fluoresceine conjugated antibodies, demonstrates the hyphae of the hyperparasite, which were restricted to the urediospore layer (Fig. 6). Cross sections examined with the electron microscope confirmed this observation. *Verticillium* hyphae were, with few exceptions, restricted to the urediospore layer of the uredium. The spore walls of the urediospores became dissolved, following contact with *V. lecanii* hyphae. The dissolution of the wall layer was restricted to the spore wall (Fig. 7).

The pellicle and the spines of the spore were not degraded. The process of wall degradation proceeded centripetally. Some areas in the urediospore layer of the pustule showed spores which were surrounded and penetrated by a large number of *V. lecanii* hyphae (Fig. 8). In these urediospores, the spore wall was completely degraded, but the pellicle and the spines remained intact. The content of the urediospores seemed to be only gradually degraded.

3. Growth conditions for *Verticillium lecanii* in the rust pustule

Since *V. lecanii* is specialized to grow within a rust pustule, it seemed important to define the optimal conditions for the hyperparasite to grow in the uredium. To measure the amount of *V. lecanii* hyphae within the pustule, a serological method was used as proposed by CASPER and MENDGEN (1979). With the enzyme linked immunosorbent assay (ELISA), only *V. lecanii* hyphae reacted. The amount of hyphae of the hyperparasite was measured after variation of air humidity, temperature and light intensity. In all experiments, plants were inoculated with *V. lecanii* when sporulation of *P. striiformis* had begun and samples of the infected wheat leaves were taken seven days later. For every variant 200 mg of the freeze-dried infected leaves were subjected to the ELISA-test. From this test, it was obvious that the growth of the hyperparasite was mainly dependent on air humidity. Greater than 80 % relative humidity was required for growth of *V. lecanii*. Relative humidity of 95 to

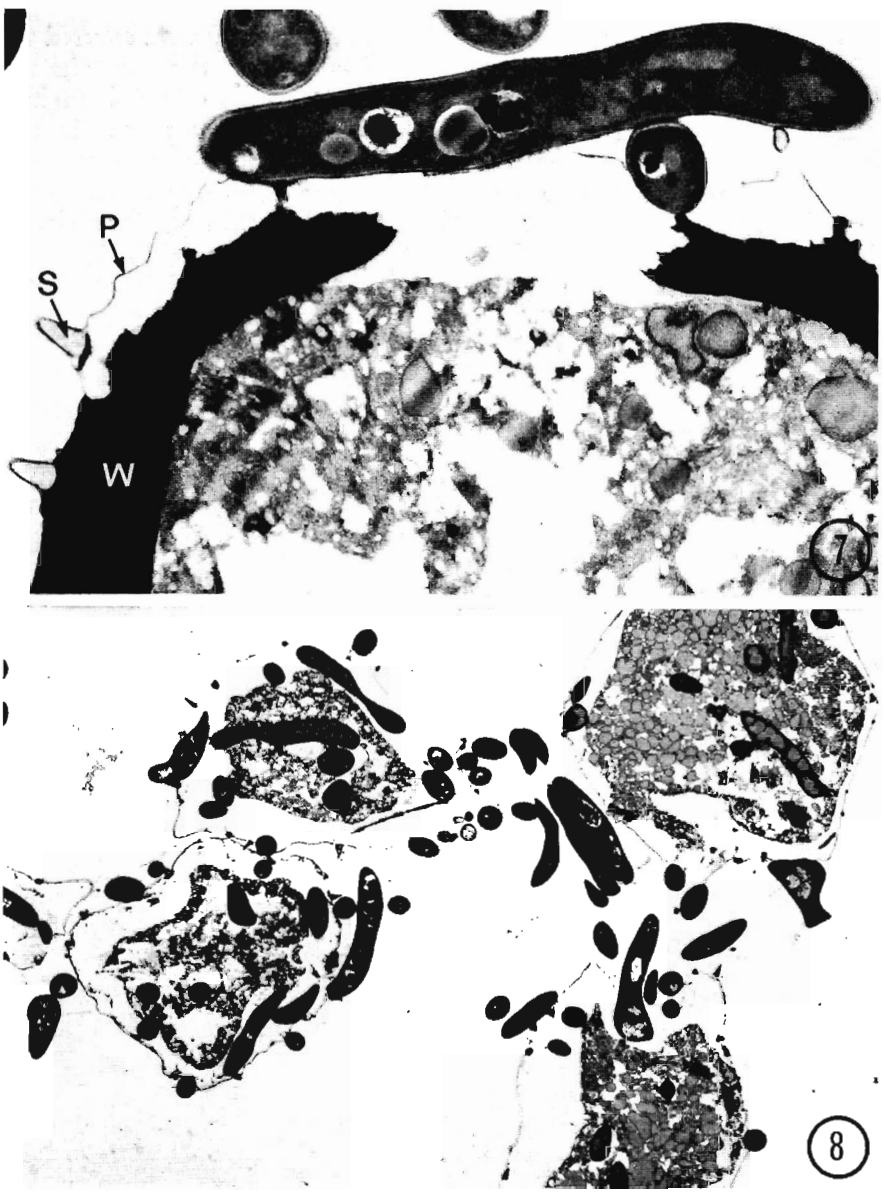

Fig. 7. Partial degradation of a urediospore wall (V) in contact with *V. lecanii* hyphae. The pellicle (P) and the spines (S) remain intact. The sample was taken from a stripe rust pustule ten days after inoculation with *V. lecanii* (× 7000) • Fig. 8. Urediospores of *P. striiformis* and hyphae of *V. lecanii*. A similar sample as in Figure 7, but the urediospore wall is completely degraded. Only a part of the pellicle, the spines and the spore content are still visible. The hyphae of the hyperparasite have penetrated the urediospores at numerous places (× 3500)

100 % was optimal (Fig. 9). The influence of temperature was not very specific. A range of temperatures between 15 °C and 18 °C allowed good growth (Fig. 9). Higher temperatures were not tested as they did not allow good development of stripe rust under the given conditions. Light had a positive effect on the development of *V. lecanii* in the rust pustule (Fig. 10).

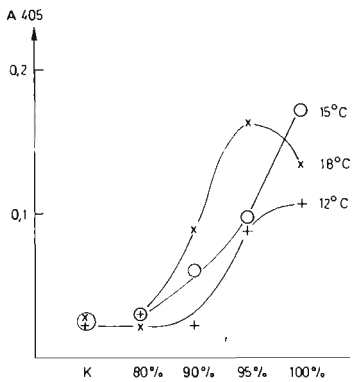

Fig. 9. Influence of temperature and air humidity on the development of *V. lecanii* grown under 4000 lux

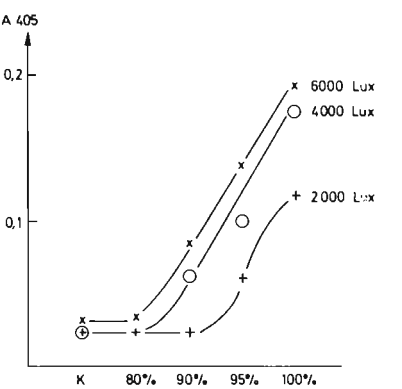

Fig. 10. Influence of light and air humidity on the development of *V. lecanii* grown at 15 °C

Discussion

V. lecanii is specialized to parasitize the urediospores of *P. striiformis*. This observation confirms results in similar host-parasite-hyperparasite systems (Schroeder and Hassebrauk 1957, Garcia Acha 1965, Silveira and Rodriguez 1971). The reason for this specialization may be related to its ability to degrade the spore walls of rust urediospores. The mechanism of wall degradation is still unknown. It is also unknown, why the spore content is broken down to a lesser extent than the urediospore walls. It may be speculated that *V. lecanii* possesses a very efficient chitinase or other wall degrading enzymes. This speculation is supported by the fact that *V. lecanii* attacks both spore walls and insects. Both have chitin as the major structural constituent (Hall 1980).

This study gives some indication on the value of *V. lecanii* for the biological control of rust fungi. The hyperparasite is restricted to the area of the rust uredium and does not penetrate into the leaf of the host plant. This corresponds to observations in bean rust (*Uromyces phaseoli*) infected leaves (MENDGEN and CASPER 1980). Increasing light intensity and temperature favor the growth of the hyperparasite. Since these conditions may also favor the plant's development and consequently the rust fungus development, these results are of minor importance if the hyperparasite's value for biological control is appreciated. More important is *Verticillium*'s requirement of more than 80 % air humidity for growth. This restricts the value of *V. lecanii* as a control agent to geographical areas with high humidity. Our results may explain different findings with *V. lecanii* as a control agent: HASSEBRAUK (1936) had no success with *V. lecanii* in field experiments. However, SPENCER (1980) reported successful control in greenhouse experiments. Obviously, it is important that environmental conditions are rigidly controlled for successfull control of a rust fungus with *V. lecanii*.

Summary

The interaction between *Verticillium lecanii* and *Puccinia striiformis*, growing on wheat leaves, was studied. In agar cultures, germ tubes of *V. lecanii* did not attack germ tubes of *P. striiformis*, however hyphae of *V. lecanii* grew within and around urediospores in sori. Within seven to ten days after inoculation, the hyperparasite did not penetrate into the sporogenous tissue of the rust fungus nor into the leaf tissue. Electron microscopy showed that *V. lecanii* preferably attacked the urediospore walls and spore contents of *P. striiformis*. The pellicle and the spines of the urediospores remained intact. Light intensity had a positive effect on the development of *V. lecanii* in the pustule of *P. striiformis*. Good development of the hyperparasite occurred between 15 °C—18 °C, and 90 %—100 % air humidity.

Zusammenfassung

Das Wachstum von *Verticillium lecanii* in Pusteln des Gelbrosts *(Puccinia striiformis)*

Auf dünnen Agarschichten griffen die Keimschläuche von *Verticillium lecanii* die Keimschläuche der Uredosporen des Gelbrosts nicht an. Wenn *V. lecanii*-Sporen auf ein mit Gelbrost infiziertes Blatt gesprüht wurden, blieb das Wachstum von *V. lecanii* auf die Sporenbereiche beschränkt. Nur die Uredosporen, aber nicht das sporogene Gewebe des Gelbrosts oder das Blattgewebe wurden angegriffen. Im Elektronenmikroskop wurde deutlich, daß *V. lecanii* die Sporenwand, aber nicht die Sporenwarzen und das Sporenhäutchen abbaut. Gute Wachstumsbedingungen für *Verticillium lecanii* waren bei 15 bis 18 °C und 90 bis 100 % Luftfeuchte gegeben.

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