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Infection mechanisms of grapevine powdery mildew (*Oidium tuckeri*): Comparative studies of the penetration process on artificial membranes and leaf epidermis

by

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Mécanismes d'infection de l'oïdium de la vigne (*Oidium tuckeri*): Etude comparée du processus de pénétration à travers une membrane synthétique et l'épiderme foliaire

R é s u m é : Un modèle d'infection a été établi afin d'étudier les relations hôte-parasite entre la vigne et l'oïdium. Le modèle consiste en une membrane synthétique ($\geq$ 0,4 µm) flottant à la surface d'un substrat liquide; la membrane représente la cuticule de la plante hôte. La préparation des membranes est décrite, ainsi que les méthodes utilisées pour tester leur imperméabilité et leur élasticité. Les meilleurs résultats sont obtenus avec des membranes constituées par des polymères de méthacrylates.

Les conidies d'oïdium qui ont germé sur des membranes recouvrant différents substrats, ont été observées aux microscopes optique et électronique à balayage; une comparaison a été faite avec le processus d'infection dans des conditions naturelles. Sur les membranes la germination et la croissance initiale semblent être contrôlées par l'humidité relative de l'atmosphère ambiante, laquelle dépend de l'osmolarité du substrat liquide. Les suçoirs d'*Oidium tuckeri* sont capables de perforer ces membranes synthétiques, qui sont en principe résistantes aux attaques enzymatiques.

Dans nos conditions expérimentales la pénétration est un processus mécanique. Toutefois, dans des conditions naturelles d'infection, la participation d'enzymes n'est pas à exclure.

K e y w o r d s : oidium, host plant, fungus, epidermis, cell.

Introduction

In studies on host-pathogen interfaces, different suggestions have been made to explain the penetration of powdery mildew and rust fungi through the walls of the host cells.

Many authors assume that penetration is a mechanical process (lit. in DICKINSON 1960). With barley powdery mildew, ultrastructural studies show that the cuticle is displaced, indrawn and ruptured in the penetration hole adjacent to the infection peg of the fungus (STANBRIDGE *et al.* 1971). BRACKER and LITTLEFIELD (1973) observed the displacement of host cell wall fibrils at this site, an effect which should only appear after the application of a mechanical force.

Other observations indicate that penetration is an enzymatical process (KOLATTUKUDY 1985): cutinases were discovered and purified. NICHOLSON *et al.* (1972) attributed to esterases found in appressoria of *Venturia inaequalis* a role in the cuticle digestion.

Theories which involve both physical and chemical mechanisms have been proposed for barley powdery mildew: EDWARDS and ALLEN (1970), MCKEEN and RIMMER (1973) and KUNOH *et al.* (1977) think that a chemical digestion of the cuticle is followed by mechanical pressure on the host cell walls.

No precise analysis has been made yet for grapevine powdery mildew. In order to explore the penetration mechanism involved in this system we tried to establish a model of the infection process and replaced the host cuticle by an artificial membrane. The fact that water inhibits germination of *Oidium tuckeri* conidia (STEIN *et al.* 1985) forced us to use synthetic compounds in order to produce impermeable membranes. Analogous procedures have been used by WELTZIEN-STENZEL (1959) to study the conidial germination of the grapevine powdery mildew and by MERCER *et al.* (1971) to demonstrate mechanical penetration of *Colletotrichum lindemuthianum* into host cells.

In this paper evidence is presented that *Oidium tuckeri* is able to penetrate the host cuticle by mechanical force.

Materials and methods

Plant material: Sporulating leaves of greenhouse plants from various susceptible *Vitis* cultivars (Kerner, Bacchus, Riesling) were used as sources for spores (for details see STEIN *et al.* 1985).

Preparation of membranes: A methacrylate embedding resin (Eukitt), a cellulose nitrate (Matt Zapon lacquer) and a polymethacrylate lacquer (Disbon Universal) were used to prepare the membranes. The substances were diluted with amylacetate, to concentrations ranging from 0.1 to 0.3 v/v. The air volume of a Petri dish containing water or a liquid medium (glucose, mannitol or NaCl solutions) was saturated with amylacetate. For this purpose, the inside of a Petri dish lid was covered with filter paper soaked with amylacetate. A drop of the resin solution was then deposited through a hole in the lid onto the liquid's surface. This drop immediately spread out and dried to form a thin membrane, onto which conidia were brushed. The Petri dish was then closed with a normal lid to insure a high relative humidity (R. H.). The membrane covered only about 50 % of the surface, thus the relative humidity in the air space depended on the osmotic value of the liquid substrate.

After 2 and 7 d incubation, a vital stain (a 0.5 % acetone solution of fluorescein diacetate, diluted in water at 2 µl/ml) was added to the liquid substrate under the membrane. The germinated conidia were observed with a Zeiss Standard microscope equipped with an incandescent UV-light device.

Histological techniques: Young sporulating leaf fragments of the susceptible cv. Kerner (greenhouse plants) were fixed for at least 12 h in a 2 % formaldehyde solution. They were then dehydrated in an alcohol series and embedded in a Spurr epoxyresin mixture (BÖCK 1984). Sections were cut with an ultramicrotome (Porter and Blum) using glass knives. Sections 0.5 µm thick were mounted on glass slides, stained with toluidine blue and observed with a Zeiss photomicroscope, using an aplanatic 100 × immersion objective.

Scanning electron microscopy (SEM): Preparations were freeze-dried, using the technique of BLAICH *et al.* (1984), or freeze-substituted (BLAICH and WIND, in prep.) and then gold-coated with an Edwards sputter coater S 150 B.

Results and discussion

1. Elaboration of the membrane technique

Preparation of the membrane: The substances used to make the membranes have to possess the following properties: they must a) be insoluble in water; b) have a low molecular weight, implying a low viscosity allowing them to spread evenly onto the liquid medium; and c) form an impermeable and elastic polymer. Collodion, for instance, used by WELTZIEN-STENTZEL (1959) is permeable and therefore was omitted.

Eukitt has the advantage of being free of any additive substances like softeners, which could have uncontrolled effects on conidial germination. Moreover, it spreads evenly onto the liquid surface, its thickness varying from 0.2 µm to 0.7 µm, depending on the concentration used (thicknesses measured with the SEM).

As a general rule, concentration of the lacquer used for the membranes (which affects its viscosity) and the liquid substrate's temperature and surface turbulence exert a great influence on form, thickness and evenness of the membranes.

Elasticity tests: A fluorescein diacetate solution (same concentration as specified in 'Material and methods' for the germination studies) was used as liquid medium and droplets of an esterase solution were deposited on the membrane surface. Undamaged membranes bear the weight of these drops (which are 15 times bigger than conidia) without breaking; if there are cracks in the membranes, the drops will come in contact with the vital stain (which only emits fluorescence when degraded by esterases) and become fluorescent (Fig. 1 a). The results of this test largely depend on the membrane material. Eukitt membranes are more fragile than membranes made of Disbon or Zapon lacquers in which softeners are included.

Impermeability tests: The membranes have to be impermeable for two reasons: a) because water or solutions inhibit conidia germination; and b) to make sure that no substance can diffuse from the liquid medium to the surface of the membrane. Super-fine Sephadex particles (G-50), normally used for column-chromatography, have the property to swell when in contact with an aqueous solution. This type of particle was chosen because it is approximatively of the same size (20—80 µm in diameter) as a conidium (20 × 30 µm), thus not breaking the membrane. The particles were stained with fluorescein-Na salt and dehydrated in acetone (dry particles show no fluorescence under UV-light). After preparing the membranes, we brushed the Sephadex particles onto them. Using Eukitt membranes the particles neither increased in volume nor fluoresced. However, with Disbon and Zapon membranes the particles increased and emitted a green fluorescence, demonstrating the permeability of these membranes to aqueous solutions.

As a consequence from these tests, for the following experiments membranes were prepared using Eukitt mixed with 10 % Disbon lacquer (to increase elasticity) and diluted 1 : 4 with amylacetate. Interference colours allow to estimate the quality of the membranes which are not quite uniform, their thinnest areas being, however, below 0.4 µm.

2. Conidial germination

Microscopic studies: Before placing conidia on the membrane, parts of it were covered with cover glasses (1 cm^2) to be used as a control for studying the influence of different substrates on germination and hyphal development. After an incuba-

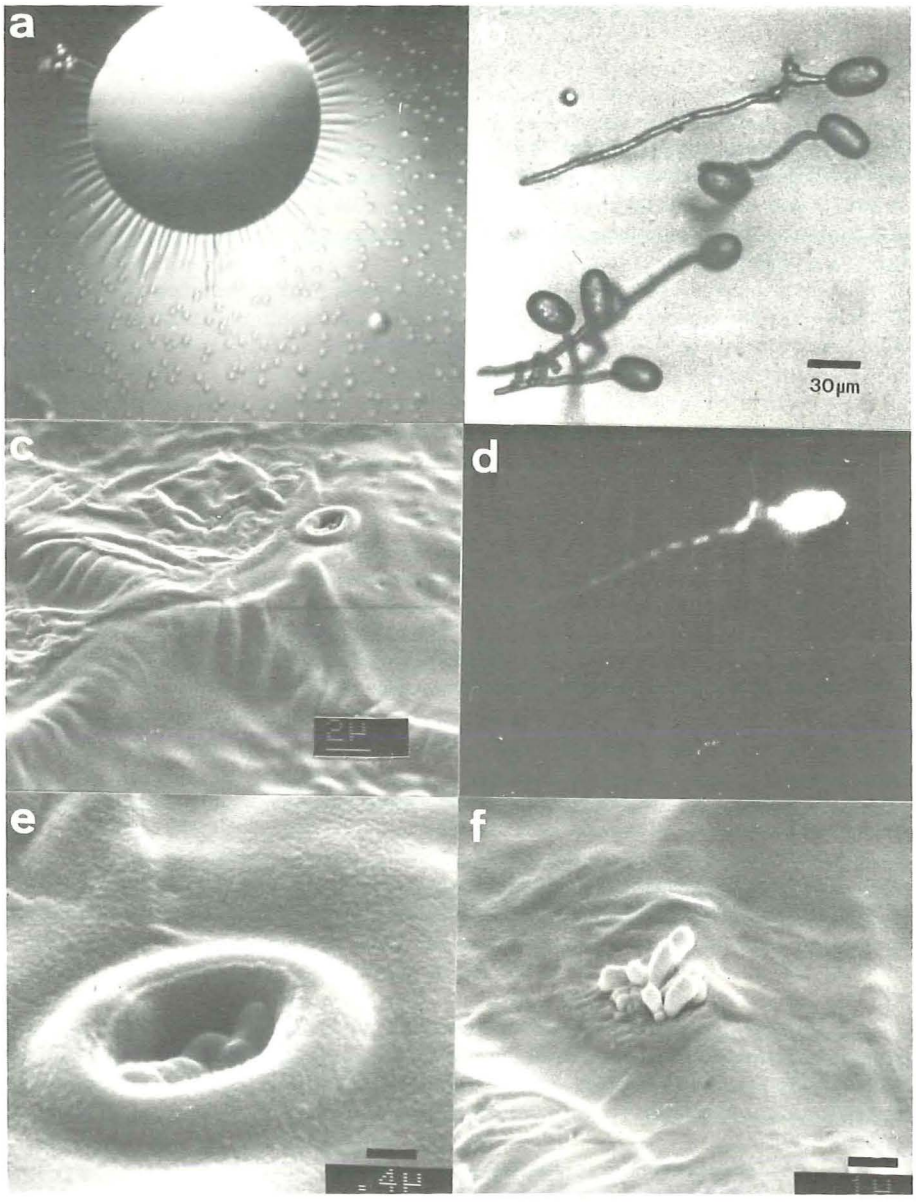

Fig. 1:

a) Elasticity test. Drops of esterases in solution (0.5 mm diameter, about 15 times bigger than conidia) are deposited onto a polymethacrylate membrane (thickness 0.4 µm). A fluorescein diacetate solution is used as liquid substrate under the membrane which bears this drop without breaking (notice the wrinkles near the drop). The tiny droplets are condensed water. × 115.
b and d) Germinated conidia on a membrane. After 5 d incubation, fluorescein diacetate is added to the liquid substrate (here 20 g/l glucose) under the membrane. Only a germinated conidium exhibiting an appressorium and a well developed germtube shows fluorescence in UV-light (d).

tion of 2 d, the germtubes on cover glasses had reached a maximum length of 30 µm. Growth ceased with or without the formation of an appressorium.

On the membrane, however, germtubes reached a length of 120 µm. Different types could be observed: with or without appressorium, some that stopped growth after appressorium formation and others that were branched.

The vital staining technique showed that only germinated conidia were fluorescent in UV-light (Fig. 1 b and d). This indicates that an infection peg had pierced the membrane, since the only possibility for the conidium to absorb the stain was through the appressorium. Germinated conidia with an appressorium which are not fluorescent probably died before or directly after penetration.

It seems that the failure to penetrate the membrane is a limiting factor for the germtube's (primary hypha) further development, since on glass its growth stopped after an appressorium had been formed.

These results clearly show that the penetration of the membrane by infection pegs of *Oidium tuckeri* is achieved mechanically, since fungal pathogens are not expected to be able to digest enzymatically the synthetic substrate. This was corroborated by the following analyses:

SEM studies: Membranes bearing germinated conidia were turned upside down. To accomplish this, cover glasses were placed on the surface of the membrane. The glass with the adhering membrane was then rapidly turned over and lifted with pincers. During this delicate manipulation, the membrane is often torn. Fig. 1 c shows the underside of a membrane covering elongated germtubes like a cloth. A round hole of approximately 1.6 µm can be seen. At higher magnification (Fig. 1 e), the hole appears to have smooth, regular edges, and is surrounded by a swollen ring.

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c, e, f) Scanning electron micrographs:

c) Underside of a membrane, turned over after germination of conidia. Thus the wrinkled membrane covers the germtubes like a sheet. A round hole (1.6 µm in diameter) can be seen.

e) Higher magnification of the hole. Its edges are smooth and regular, it is surrounded by a swollen ring. Some structures appear in the center.

f) Underside of a turned membrane with finger-like structures (haustoria?) emerging from a hole.

a) Test d'élasticité. Des gouttes d'estérases en solution (0,5 mm de diamètre, environ 15 fois plus grandes qu'une conidie) sont déposées sur une membrane de polyméthacrylate (épaisseur 0,4 µm). Une solution de diacétate de fluorescéine est utilisée comme milieu liquide sous la membrane qui porte ces gouttes, sans que celle-ci ne se déchire. (A remarquer les plis autour de la goutte.) Les gouttelettes proviennent de la condensation de l'eau. (× 115.)

b et d) Conidies ayant germé sur une membrane. Le diacétate de fluorescéine est ajouté au milieu liquide (ici 20 g/l de glucose), sous la membrane, après 5 d d'incubation. Sous lumière ultra-violette ne sont fluorescentes que les conidies germées présentant un appressorium et des tubes germinatifs suffisamment développés (d).

c, e, f) Microscopie électronique à balayage:

c) Face inférieure d'une membrane retournée après germination des conidies. De ce fait, la membrane plissée recouvre comme un linge les tubes germinatifs. On aperçoit une perforation circulaire (1,6 µm de diamètre).

e) La perforation à un plus fort grossissement. Les bords sont lisses et réguliers; elle est entourée d'un renflement circulaire. Au centre apparaissent des structures.

f) Face inférieure d'une membrane retournée, présentant des structures digiformes qui émergent d'une perforation (haustorie ?).

These observations are similar to those of BRACKER and LITTLEFIELD (1973), who found evidence on TEM micrographs that the host cuticle was displaced and pushed into the penetration hole made by the powdery mildew, with the infection peg growing out of the center of its appressorium. The swollen ring could correspond to the area where the appressorium is supposed to have adhered to the membrane. In Fig. 1 f, fin-

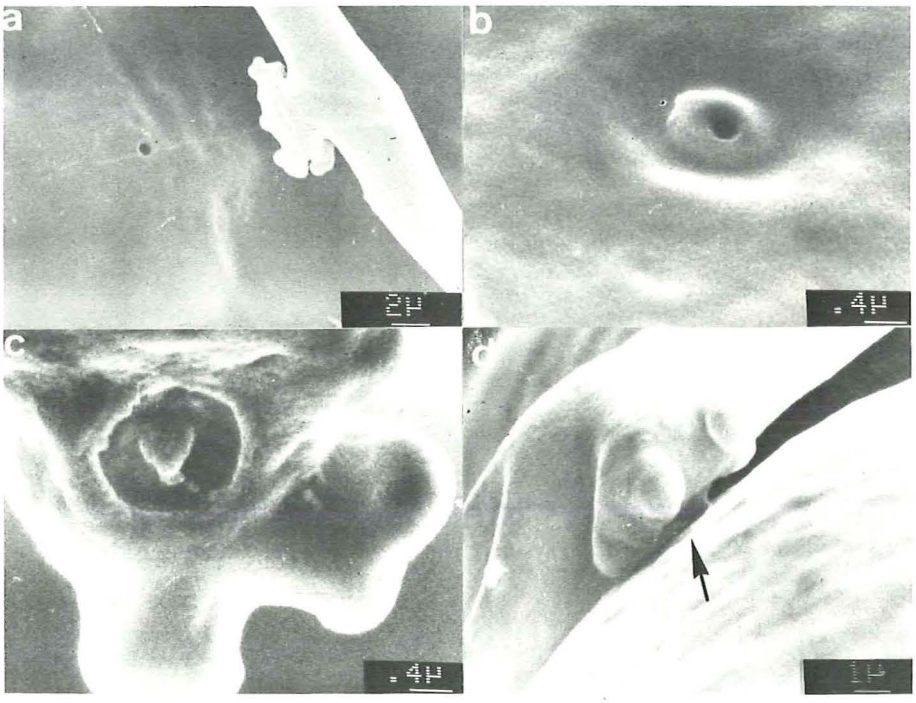

Fig. 2:

a) Powdery mildew hypha and appressorium, accidentally detached from the leaf surface (cv. Kerner), the hole in the host cuticle being caused by the infection peg.

b) Higher magnification of the penetration site in (a). The central hole (0.2 µm in diameter) corresponds to the penetration peg. It is surrounded by a swollen ring, the outer depression corresponds to the area where the appressorial body adhered.

c) Underside of an appressorial body. In the center, part of the infection peg can be seen. The ring and depression appears to be the 'negative' of (b).

d) Hypha and appressorium of *Oidium tuckeri* on a leaf from a susceptible cultivar (Kerner). The appressorial body is lobated and the penetration peg can be seen in the center (arrow).

a) Filament mycélien et appressorium d'oïdium, détachés accidentellement de la surface foliaire (cv. Kerner), la perforation dans la cuticule de l'hôte provenant du suçoir.

b) La région de pénétration de (a), à un plus fort grossissement. Le trou central (0,2 µm de diamètre) correspond au suçoir. Il est entouré par un renflement et la dépression externe correspond à l'endroit où adhérait l'appressorium.

c) Face inférieure d'un appressorium. Au centre on remarque une partie du pédoncule formant le suçoir. Le renflement et la dépression semblent constituer le négatif de (b).

d) Filament mycélien et appressorium d'*Oïdium tuckeri* sur la surface foliaire d'un cultivar sensible (Kerner). L'appressorium est lobé; au centre on aperçoit le pédoncule responsable de la pénétration (flèche).

ger-like structures emerging from one of these holes have a great similarity with the haustorial bodies of *Erysiphe graminis* described by McKeen and Rimmer (1973).

3. Comparison to the natural infection process

SEM micrographs: Fig. 2 a shows a hypha with an appressorium accidentally detached from the host leaf surface together with the corresponding hole made by the infection peg. A higher magnification (Fig. 2 b) of the penetration site (seen from the upper surface) shows the central hole (0.2 µm in diameter) corresponding to the peg. There is a swollen ring where the pore of the appressorium seems to have exerted a strong adhesion onto the cuticle of the host, as described by Dickinson (1960). The outer depression area corresponds to the appressorial body itself (Dickinson 1960).

These traces are the exact 'negative' form of the structures existing on the underside of an appressorium (Fig. 2 c), and are very similar to those on the underside of the artificial membrane (Fig. 1 e), the large swollen area corresponding with the depressed area on the cuticle. The depth of the traces left in the cuticle indicates a strong adhesion which should be sufficient to counteract a mechanical pressure of the penetration peg. The shredded appearance of the appressoria, which can be seen in Fig. 2 c and d, seems to be a structure to enhance adhesion.

The difference of the hole diameters might be explained by the differences between artificial membrane and cell wall, in particular by the absence of defense structures (papillae, see below).

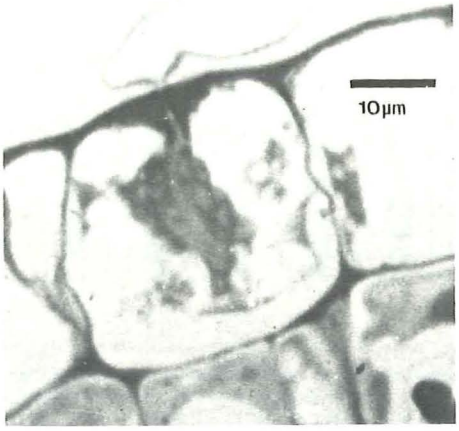

Fig. 3: Light micrograph of a semi-thin section (0.5 µm) through a powdery mildew infected leaf (cv. Kerner), stained with toluidine blue. Hyphae and appressorium can be seen on the host surface, the penetration peg piercing through cuticle and cell wall and the haustorium developing in the cell. Dark-stained deposits accumulate in the penetration site, forming the papilla.

Coupe semi-fine en microscopie optique (0.5 µm) d'une feuille infectée par l'oïdium (cv. Kerner), après coloration au bleu de toluidine. Des filaments mycéliens et un appressorium sont visibles à surface de l'hôte, ainsi que le pédoncule ayant traversé la cuticule et la paroi cellulaire; l'haustorie se développant dans la cellule. Des dépots de couleur sombre s'accumulent dans la zone de pénétration, formant la papille.

Light microscopy: A thin section (0.5 µm) of an infected leaf (Fig. 3) shows that the penetration area is limited to the fine peg piercing its way through cuticle and cell wall. The diameter of the tube seems to increase during this process, until the haustorial body is formed in the cytoplasm. Cuticle and cell wall structures do not seem to be digested or destroyed around the penetration tube. Dark-stained wall-like deposits accumulate in the penetration site, forming the so-called 'papilla'.

This, together with the observations in Fig 2 d, shows that the penetration site is limited to the infection peg, thus allowing the integrity of the host cell to be maintained. This is necessary to a compatible host-obligatory parasite relationship: a larger

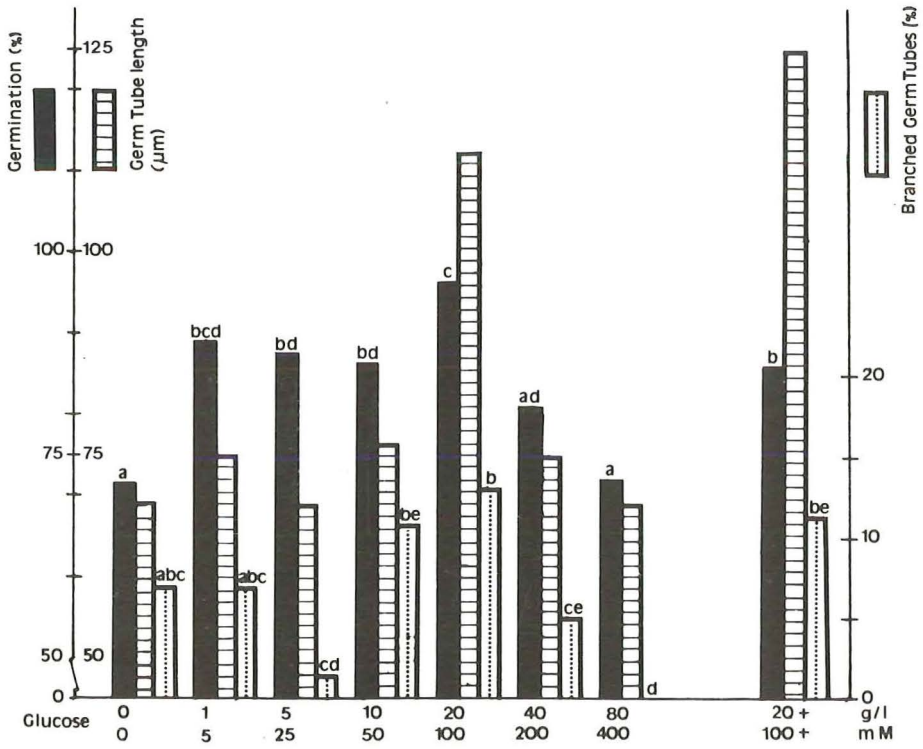

Fig. 4: Influence of the nutrient medium on germination and development of powdery mildew conidia, after an incubation of 7 d on a synthetic membrane system on different glucose substrates. Numbers on abscissa: concentration of glucose in g/l and corresponding molarity; 20 + indicates a 20 g glucose/l substrate with 2 g/l casein hydrolysate. — Black columns: germination in %; striped columns: germtube length in µm; dotted columns: percentage of branched germtubes. Columns indexed by the same letters are not significantly different at the 5 % level. Only columns with identical patterns may be compared.

Influence du milieu nutritif sur la germination et le développement de conidies d'oïdium, après une incubation de 7 d sur une membrane synthétique recouvrant différentes solutions de glucose. En abscisse: les concentrations de glucose en g/l et les molarités correspondantes, 20 + indiquant une solution de glucose (20 g/l) à laquelle on a ajouté 2 g/l d'hydrolysat de caséine. Colonnes noires: pourcentage de germination; colonnes hachurées: longueur du tube germinatif en µm; colonnes pointillées: pourcentage de tubes germinatifs présentant une dichotomie. Les colonnes possédant les mêmes lettres ne sont pas significativement différentes (seuil de 5 %; seules les colonnes présentant les mêmes motifs peuvent être comparées.)

disturbed area around the penetration site would result in loss of the cell contents followed by the destruction of the cell. An obligatory parasite, however, can only infect intact cells (BRACKER and LITTLEFIELD 1973).

4. Membrane tests on different substrates

Upon finding that the fungus was able to penetrate synthetic membranes, some preliminary studies were carried out to analyse the effects of nutritive liquid media on the development of powdery mildew on the membrane surface.

The effects of a carbohydrate source on conidial germination were studied, using glucose solutions at concentrations from 0 g/l (water) to 80 g/l. As shown in Fig. 4, a 20 g/l glucose solution appears to be the optimal concentration for germination rate, germtube length and percentage of branched germtubes of powdery mildew conidia after 7 d incubation onto a membrane. Addition of casein hydrolysate to this solution did not improve these results.

The use of mannitol and NaCl solutions with the same molarity as the glucose solutions did not result in a significant change in the germination rates, branched germtube rates and germtube lengths.

This indicates that these substances act indirectly on conidial germination, through the different relative humidities that each molecular concentration induces. The nature of the substance used seemed to play no role. In its early stage, the process of germination seems only to depend on physical conditions; indeed the conidia of *Oidium tuckeri* need high relative humidities to germinate (STEIN *et al.* 1985), although liquid water inhibits germination. With pure water under the membrane (100 % R. H.), condensation droplets form on the surface of the membrane (Fig. 1 a), inhibiting germination of conidia which are in contact with them. The R. H. shows an optimum for germination at a molarity of 0.1 M (20 g/l) glucose. At higher concentrations the R. H. is probably too low for the conidia to intake enough water molecules for germination.

Conclusion

As for the membrane technique itself it seems that it should allow a step towards an axenic culture of powdery mildew if the culture medium could be adapted. However, it must be pointed out that at present it is far from being a routine method. For instance, the poor reproducibility of the membrane preparation did not allow to determine the limiting thickness preventing penetration by the parasite, although there are indications that membranes thicker than about 1 µm are resistant. Thus the technique must still be enhanced and tests with other lacquers better suited to the present use have to be made in order to improve the quality of the membranes.

On the other hand, the method at its present stage allowed to understand better the different stages in the grape powdery mildew infection process. According to MERCER *et al.* (1971), STANBRIDGE *et al.* (1971) and BRACKER and LITTLEFIELD (1973), we suppose that the penetration of the host plant cuticle is a mechanical process. Form and structure of the appressorial body present all the conditions required for a strong adhesion, which is necessary if mechanical forces are involved.

One cannot discount, though, that *in vivo* the fungus might facilitate mechanical penetration by destabilizing the cuticle enzymatically. Furthermore, the cell wall could be partly digested with enzymes excreted by the penetrating hypha, the hydrophilic property of the cell wall making this more plausible.

Studies on the host-pathogen interface will be undertaken at a higher microscopic resolution in order to find out whether ruptures of the cuticle and/or disturbances of host wall fibrils can be detected, which would corroborate our theory of mechanical (or partly mechanical) penetration under natural conditions.

Summary

In order to study host-pathogen interfaces and relationships between grapevine and powdery mildew, an infection model was established. The model consists of synthetic membranes ($\geq$ 0.4 µm) floating on liquid substrates; the membrane representing the cuticle of the host plant. The preparation of membranes and methods to test their impermeability and elasticity are described. The best results are obtained with membranes consisting of methacrylate polymers.

On membranes layered on various substrates germinating conidia and germtubes were observed with light and scanning electron microscope and compared to the natural infection process on leaf surfaces. On membranes germination and initial growth seem to be controlled by air humidity which depends on the osmotic value of the liquid substrate. As shown the infection pegs of *Oidium tuckeri* are able to perforate synthetic membranes which should be resistant to enzymes. This implies that under our experimental conditions penetration is a mechanical process. Participation of enzymes under natural conditions can, however, not be excluded.

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