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Plant pathology

Enzymatic degradation of isolated plant cuticles and nectarine fruit epidermis by culture filtrates of phytopathogenic fungi

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Summary — *In vitro* degradation of isolated cuticular membranes and *in vivo* degradation of the epidermis of nectarine fruit by *Rhizopus stolonifer* and *Monilia laxa* were investigated. Increases in the permeability of cuticular membranes to ^{86}Rb and of epidermis to ^{45}Ca were used as a measure of degradation. An isolate of *Fusarium solani* f sp *pisi*, which produced a large amount of cutinase, was used as a reference. Culture filtrates of *R stolonifer* and *M laxa* did not change cuticular membrane permeability. Conversely, both fungi caused a marked deterioration in the epidermis of nectarine fruit, probably caused by pectinolytic enzymes. The reaction is thought to probably take place in the cuticular microcracks present on the nectarine fruit surface. Penetration of nectarine fruit epidermis by *M laxa* and *R stolonifer* does not apparently involve cutinolytic enzymes.

***Rhizopus stolonifer* / *Monilia laxa* / *Fusarium solani* / isolated cuticle / cutinase**

Résumé — Dégradation enzymatique de cuticules isolées de plantes et de l'épiderme de nectarines par des filtrats de culture de champignons phytopathogènes. *Dans ce travail, la dégradation* in vitro *de membranes cuticulaires isolées et la dégradation* in vivo *de l'épiderme de nectarine par* Rhizopus stolonifer *et* Monilia laxa *ont été étudiées. Cette dégradation a été mesurée, d'une part par l'augmentation de la perméabilité de membranes cuticulaires au* ^{86}Rb, *et d'autre part par l'augmentation de la perméabilité de l'épiderme au* ^{45}Ca. *Un isolat de* Fusarium solani *f sp* pisi, *a servi de référence. Le traitement de cuticules isolées avec des filtrats de culture de ce champignon augmente la perméabilité cuticulaire au* ^{86}Rb *(figs 2 et 3); par contre, le transfert du* ^{86}Rb *après traitement des cuticules par des surnageants de culture de* R stolonifer *et* M laxa, *dans des conditions comparables d'activité enzymatique, n'est pas modifié (fig 3). Les filtrats de culture de* Fusarium solani *f sp* pisi *causent aussi une perte de poids de cuticules isolées décirées confirmant ainsi la présence de cutinase dans les solutions de culture testées. Les 2 champignons,* R stolonifer *et* M laxa *provoquent une nette dégradation de l'épiderme de nectarine, visible après coloration au bleu de méthylène, qui se traduit par une pénétration plus élevée du* ^{45}Ca *dans les fruits (fig 4). Le traitement avec un filtrat de culture de* Fusarium solani *f sp* pisi *qui ne présente pas d'activité pectinolytique, est sans effet. Les enzymes pectinolytiques produites par* R stolonifer *et* M laxa *sont vraisemblablement à l'origine de l'altération observée avec ces champignons, qui survient sans doute au niveau de microfissures cuticulaires présentes à la surface des nectarines. La pénétration de l'épiderme de nectarines par* M laxa *et* R stolonifer *n'implique probablement pas l'intervention d'enzymes cutinolytiques.*

Rhizopus stolonifer / Monilia laxa / Fusarium solani / *cuticule isolée / cutinase*

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INTRODUCTION

Monilia laxa (Aderh and Ruhl) Honey and *Rhizopus stolonifer* (Ehrenb ex Fr) Lind are the principal fungal pathogens in Europe of peach and nectarine during storage (Anderson, 1925; Byrde and Willetts, 1977; Bompeix *et al,* 1979). Although infection of fruit by both fungi frequently occurs through wounds, direct penetration of germinated conidia of *M laxa* has been demonstrated (Wade, 1956; Hall, 1971). Direct penetration of the plant cuticle by fungal pathogens (Rijkenberg *et al,* 1980; Dickman *et al,* 1982, 1983; Kolattukudy and Koller, 1983) or bacterial pathogens (Bashan *et al,* 1985) requires the production of cutin-degrading enzymes (cutinases). Microcracks on the surface of nectarine fruit (Fogle and Faust, 1975, 1976) might provide alternative sites for fungal penetration. From electron microscopic observations, microcracks can be the main sites of penetration for the mycelium of *M laxa* and *R stolonifer* (Nguyen-The *et al,* 1989). The biochemical composition and the presence or absence of cutinized material associated with these microcracks has not been determined. Therefore it seems important to investigate whether or not cuticular degradation is involved during the penetration processes of *M laxa* and *R stolonifer* in nectarine fruit. An isolate of *Fusarium solani* f sp *pisi* which produces a large amount of cutinase (Baker *et al,* 1982; Kolattukudy, 1985) was used as a reference. Increases in the permeability of nectarine fruit epidermis and of isolated pear cuticular membranes were used to determine the involvement of extracellular enzymes of both fungi upon cuticular penetration.

MATERIALS AND METHODS

Fungal cultures

Strains of *M laxa* and *R stolonifer* were isolated from decaying fruits, as for previous investigations (Nguyen-The *et al,* 1985a, b; 1989). The isolate of *F solani* f sp *pisi* was purchased from the Centraalbureau voor Schimmelcultures (Baarn, The Netherlands) isolate CBS.188.35.

Fruits

Nectarine fruits of the Armking cultivar were grown in experimental orchards (INRA, Alenya, France). Fruits were harvested at the stage of commercial maturity.

Isolated cuticular disks

Cuticular disks (1 cm diameter) were isolated by enzymatic maceration (2% pectinase; 0.2% cellulase, Sigma USA) from fully developed pear leaves (*Pyrus communis* L, cultivar Passe Crassane) and from the skin of mature tomato fruit (*Lypersicon esculentum* Mill) according to the procedure described in Chamel and Bougie (1977). The pear leaf cuticular disks used were obtained from the astomatous upper surface; the absence of any visible hole was checked using a light microscope (x 150). This material has already been used widely as a model in cuticular permeability studies (Chamel, 1986).

Culture solutions

Two liquid culture media were used for the production of cutinase: a V8 medium supplemented with 0.5% (w/v) of cutin powder (Baker *et al,* 1982) and a synthetic medium (Salinas *et al,* 1986: 10 g KNO_3; 5 g KH_2PO_4; 0.25 g Mg SO_4, 7 H_2O; 0.4 mg $FeCl_3$; 3.6 g glucose; 250 mg cutin powder; H_2O dist 1 l). To obtain the cutin powder, tomato fruits were dipped in boiling water and hand-peeled. The skins were twice macerated over a 1-d period in a mixture of 1% pectinase + 1% cellulase (Fluka, Switzerland) diluted in a 0.05 $mol.l^{-1}$ pH 4.8 sodium acetate buffer. The skins were rinsed in distilled water, then in methanol. The cuticular membranes obtained were dewaxed in a mixture of chloroform/methanol (1/1) for 1 h and in chloroform twice for 1 h. The chloroform was decanted and the membranes were ground in methanol, firstly with an Omni-mixer (Sorvall, Newtown, USA) and secondly with a Polytron homogeniser (Kinematica, Switzerland) to obtain a very fine powder. The methanol was decanted and the powder dewaxed in an identical manner to that used for the cuticular membranes. About 0.75 g of cutin powder were obtained from 2 kg of tomato fruits. The production of pectinolytic enzymes was performed in a pectin liquid medium, as described previously (Nguyen-The *et al,* 1984).

Fungi were grown at 25 °C on a shaker in 30 ml of culture solution placed in an Erlenmeyer flask. The inoculum consisted of spores of *R stolonifer* ($\approx 10^5$ spores/flask) or 10 disks (4 mm diameter) of mycelium of *M laxa* or *F solani* f sp *pisi* cut from a culture of the fungus on potato dextrose agar. After 5 d (*R stolonifer* grown on pectin medium), 1 wk (*M laxa* grown on pectin medium), or more generally 3 wk (*R stolonifer, M laxa, F solani* f sp *pisi* grown on V8 + cutin solution or on synthetic medium), the respective liquid cultures were centrifuged for 20 min at 25 000 *g* and the filtrates stored at –20 °C before use.

Enzyme assays

Cutinase was assayed by the degradation of fatty acid esters using *para*-nitrophenyl-butyrate (PNPB) (Sig-

ma, USA) as the substrate. Release of *p*-nitrophenol from *para*-nitrophenol ester of butyric acid is a rapid method to assay cutinase activity, but it is not specific since many fatty acid esterases do not hydrolyse cutin polymers (Kolattukudy, 1985). The production of phenol was detected at 405 nm with a DMS-100 (Varian, USA) spectrophotometer. Reactions were performed at 30 °C in a Tris/HCl 0.1 M pH 8 buffer with 0.01% Triton X-100 (Fluka, Switzerland) 0.1% PNPB using 100 μl culture filtrate in 2.5 ml of the substrate solution. Activity was expressed in absorbance units at 405 nm per min (Au/min).

Pectinolytic activity was measured by a viscosimetric method or by the release of a reducing group and routinely detected in culture filtrates with an agar diffusion assay (Hornewer *et al*, 1987). Assays were conducted at pH 5 (sodium acetate buffer), pH 7 (sodium phosphate buffer) and pH 8 (Tris/HCl buffer) in 0.05-mol.l^{-1} solutions.

Estimation of the permeability of isolated cuticular membranes

The diffusion of ^{86}Rb (a 185 kBq/ml solution which contained 0.1 mmol.l^{-1} Cl^- ion as KCl + RbCl, in a 10-mmol.l^{-1} pH 8 Hepes buffer) was measured by the following procedure. A pear cuticular disk was attached to the end of a thick-walled glass tube (7 mm inner diameter), dipped in a scintillation glass vial containing 10 ml of the non radioactive "receiver" solution (Hepes buffer, 10 mmol.l^{-1} pH 8). The cuticular disk was attached as previously described (Chamel and Bougie, 1977; Chamel, 1980) using RTV 111 (silicone adhesive substance, Rhône-Poulenc), with its external side (*ie* waxy surface of the cuticle) facing the inner space of the tube. System imperviousness was checked by replacing the cuticular disk by a microscope slide (Chamel, 1980). The solution of ^{86}Rb ("donor" solution) was placed inside the glass tube (0.5 ml), and the tube was fixed so that the "donor" and "receiver" solutions were kept at the same level during diffusion (fig 1). The permeability of the cuticular disk was measured by the amount of radioactivity detected in the "receiver" solution after 24 h at room temperature.

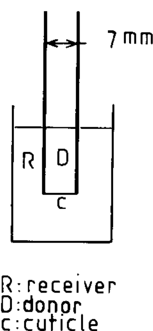

Fig 1. Apparatus for estimating the permeability of isolated cuticular membranes. *D* = 500 μl of "donor" solution, which contained 185 kBq of ^{86}Rb and 0.1 mmol.l^{-1} of KCl + RbCl in a 10 mmol.l^{-1} pH 8 Hepes buffer; *R* = 10 ml of "receiver solution" wich consisted of 10 mmol.l^{-1} pH 8 Hepes buffer; *C* = cuticular membrane isolated from pear leaf and stuck on the opening of a glass tube with waxy side upwards.

The degradation of cuticular disks by culture filtrates was assessed by the same method. Culture filtrates, diluted in pH 8 Hepes buffer (final molarity 10 mmol.l^{-}) supplemented with 0.1% thimerosal (Prolabo, France) to prevent growth of microorganisms, were placed either inside the tube (to attack the waxy side of the cuticle) or both in the tube and in the glass vial (to attack both sides of the cuticle). After a 24 h incubation at room temperature, the cuticular membrane was rinsed several times with distilled water. Diffusion of ^{86}Rb through the isolated cuticular membrane was measured before and after the treatment with culture filtrate and the results are presented by the ratio R = diffusion after/diffusion before. In the case of low fatty esterase activity, the 24-h treatment was repeated 3 times with freshly thawed culture filtrate.

In situ *degradation of nectarine fruit epidermis*

A 20-μl droplet of culture filtrate was deposited on the epidermis in the equatorial area of the nectarine fruit. Culture filtrate was either pure or diluted (1 : 1) in a 0.05-mol.l^{-1} sodium acetate buffer pH 4 or a 0.05 mol.l^{-1} sodium phosphate buffer pH 7. The droplet evaporated within a few h at room temperature and after 24 h the permeability of the epidermis was estimated by 2 methods:

– the fruit surface was stained with a water–ethanol solution of methylene blue (Prolabo, France) supplemented with Triton X-100;

– the penetration of ^{45}Ca inside the fruit was measured at the site previously treated with the culture filtrate. In this latter case, 20 μl of a ^{45}Ca solution (925 kBq/ml, 20 mmol.l^{-1} $CaCl_2$ in a 10 mmol.l^{-1} pH 5 SADH buffer) were deposited on the fruit surface and after 24 h at room temperature, the radioactivity which remained on the fruit surface was washed twice with 2 x 5 ml Tween 20 solution (0.1% in a 10 mmol.l^{-1} pH 5 SADH buffer). As radioactivity counted in the second washing solution was only 4.5% of the radioactivity counted in the first, a third washing was considered unnecessary. Radioactivity which had diffused into the skin of the fruit was measured in a 1-cm diameter disk peeled with a razor blade and dissolved in 1 ml of Lumasolve (Lumac).

Radioactivity measurements

Radioactivity of ^{86}Rb was determined with a gamma counter apparatus (Intertechnique CG 4000, France). Counts were performed on 2-ml aliquots removed from the "receiver" solution and 20-μl aliquots removed from the "donor" solution. The latter were made up to 2 ml with H_2O so that all the samples had the same geometry in the counter.

Radioactivity of ^{45}Ca was determined in 20-µl aliquots of the 925 kBq/ml solution and in 100-µl aliquots of the washing solution with a liquid scintillation counter (spectrometer Packard 4430). Aliquots were mixed with 10 ml of Lumagel (Lumac). Skin samples dissolved in 1 ml Lumasolve were counted in 9 ml of Lipoluma (Lumac).

Weight loss of cuticular material

Dewaxed cuticular disks from tomato fruit were immersed for 24 h in the culture filtrate of *F solani* f sp *pisi* (grown in V8 + cutin solution) mixed with a 20 mmol.l^{-1} pH 8 Hepes buffer (1:1). Disks in heated culture filtrate (10 min, 100 °C) were used as control. The disks (≈ 10 mg) were placed in a scintillation vial with 3 ml of solution. The experiment was performed on 5 vials in each case (active and inactive culture filtrate). The 10 disks of a vial were weighed before and after treatment.

RESULTS

Permeability of isolated Pear leaf cuticles

Production of fatty acid esterase by *F solani* f sp *pisi* and by *R stolonifer* was higher in V8 + cutin solution than in the synthetic + cutin solution (table I). The isolate of *M laxa* that we used in this investigation did not produce any detectable fatty acid esterase. The following experiments were conducted with culture filtrates of fungi grown on V8 + cutin solution, the fatty acid esterase activities of which are presented in table I.

Incubation of both sides of isolated cuticular membranes for 24 h with culture filtrates of *F solani* f sp *pisi* led to an increase in the diffusion of ^{86}Rb. The ratio of increased diffusion *(R)* varied greatly among the different cuticular disks treated with the same culture filtrate (fig 2) with *R* ranging from 1.3 to 5.2. The experiment was carried out on 2 different cuticle samples and the individual variation was much lower in the second sample. It should be noted that treatment of the waxy side of the cuticular disk only did not cause

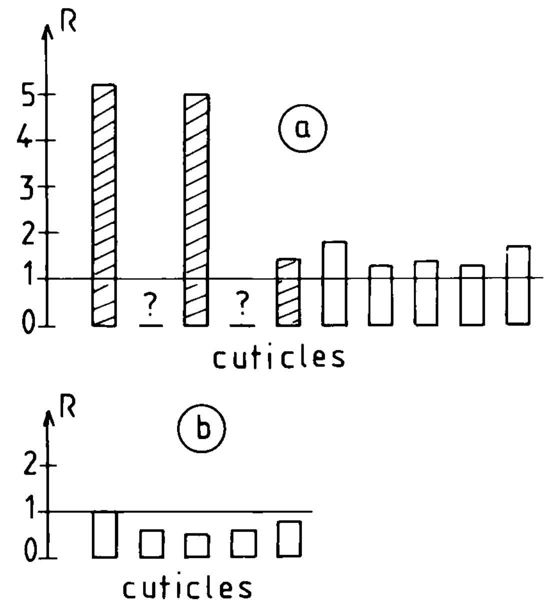

Fig 2. Variation in the diffusion of ^{86}Rb through isolated cuticular membranes before and after a 24-h treatment with the culture filtrate of *F solani* f sp *pisi*. (a) Culture filtrate placed on both sides of the cuticular membrane; (b) culture filtrate placed on the waxy side of the cuticular membrane; R = diffusion after treatment/diffusion before treatment; ? = fissures detected in the cuticular membrane at the end of the experiment. The experiment was performed on 2 samples of 5 cuticles each (▨ and ▭). *F solani* f sp *pisi* was grown in V8 + cutin culture solution, and the filtrate was mixed (1 vol : 1 vol) with a 20 mmol.l^{-1} pH 8 Hepes buffer.

Table I. Fatty acid esterase activities produced by *R stolonifer* and *F solani* f sp *pisi* grown in 2 culture solutions.

Culture Solutions	F solani *f sp* pisi			R stolonifer		
	pH			*pH*		
	6	*7*	*8*	*6*	*7*	*8*
Synthetic + cutin 21 d	NT	NT	0.20 ± 0.05	NT	NT	0
V8 + cutin 21 d	0	0.50 ± 0.125	0.50 ± 0.125	0	0.025 ± 0.006	0.025 ± 0.006

Enzymatic activities were measured by the degradation of para-phenylbutyrate and expressed in A units at 405 nm/min. Each measurement was performed on combined filtrates from 3 flasks, each containing 30 ml of culture filtrate; ± standard deviation. NT = not tested.

any increase in the diffusion of ^{86}Rb (*R* lower than 1). An increase in ^{86}Rb diffusion was still noticeable when the isolated cuticular disks were incubated with *F solani* f sp *pisi* culture filtrate diluted in distilled water (1/5, 1/10, 1/20; fig 3). The 24-h treatment was repeated 3 times before the second diffusion of ^{86}Rb. On the contrary however, although the 1/20 dilution of *F solani* f sp *pisi* culture filtrate and *R stolonifer* culture filtrate (grown on V8 + cutin solution) contained similar fatty acid esterase activity (table I), the latter had no significant effect upon the permeability of cuticular disks (compared to a control).

Weight of dewaxed tomato cuticles

Compared to the increase in permeability of pear cuticular disks, the culture filtrate of *F solani* f sp *pisi* (grown on V8 + cutin) caused a significant loss of weight in dewaxed cuticular membranes (table II). This confirms the presence of cutinase in the culture solutions tested.

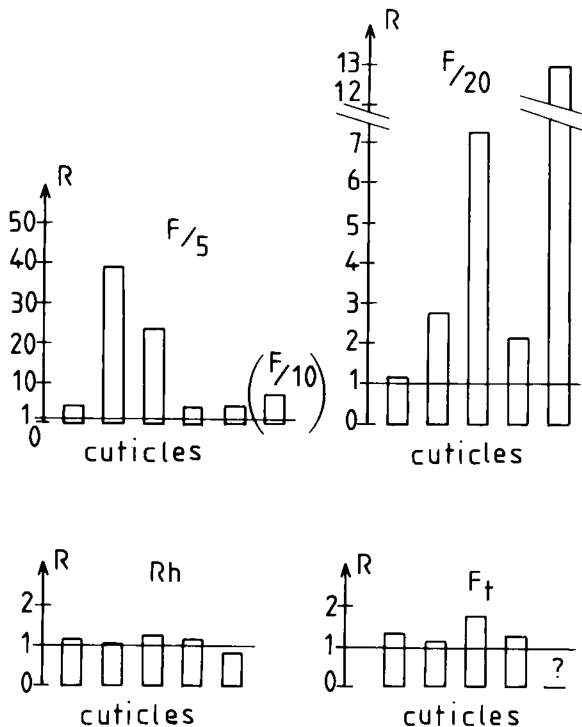

Fig 3. Variations in the diffusion of ^{86}Rb through isolated cuticular membranes before and after treatment with fungal culture filtrate. The treatment consisted of 3 successive 24-h incubations with the culture filtrate. F/5, F/10, F/20 = respectively 1/5, 1/10, 1/20 water dilutions of *F solani* f sp *pisi* culture filtrate; Ft : culture filtrate of *F solani* f sp *pisi* heated 10 min at 100 °C; Rh = culture filtrate of *R stolonifer*, R and ? = see figure 2. Rh and F/20 contained the same fatty acid esterase activity. Fungi were grown in V8 + cutin culture solution and culture filtrates (pure or diluted) were mixed with 100 mmol.l^{-1} pH 8 Hepes buffer (1 vol buffer: 9 vol culture filtrate). Radioactivity of the donor solution was 123 809 ± 2 988 cpm and the amount of radioactivity which diffused into the receiver before treatment never exceeded 10% of this value.

Table II. Weight loss of dewaxed, cuticular membranes isolated from tomato fruit and immersed during 24 h in culture filtrate of *F solani* f sp *pisi*.

	F	*Ft*
W1 (mg)	10.62 ± 0.54	10.58 ± 1.04
W2 (mg)	9.98 ± 0.85	10.35 ± 0.98
W1 – W2 (mg)	0.89 ± 0.45	0.23 ± 0.08
W1 – W2/W1 (%)	8.2 ± 4.0	2.2 ± 0.7

W1: dry weight of 10 disks of cuticular membranes before treatment. W2: dry-weight after treatment. F: treatment with active culture filtrate; Ft: treatment with heated culture filtrate (10 min, 100 °C). Each value is the mean of 5 measures ± standard deviation.

Degradation of nectarine fruit epidermis

Culture filtrate of *R stolonifer* grown on a pectin medium deposited on the surface of nectarine fruit caused a marked alteration after 24 h at room temperature. It could be visualized by staining the surface with methylene blue: after rinsing the dye with tap water, a network of blue-coloured cracks remained. No such alteration occurred when boiled (10 min, 100 °C) filtrate was deposited on the fruit, but it could still be observed when culture filtrate was mixed with 0.1 mol.l^{-1} pH 4 acetate buffer or pH 7 phosphate buffer (1:1). Culture filtrate of *M laxa* grown in the same solution induced a similar alteration, but in a weaker and less reliable manner: alteration occurred in 80% of the fruits treated with *R stolonifer* and 40% of those treated with *M laxa*. The presence of pectinolytic enzymes in the culture filtrate was checked by an agar diffusion assay and *M laxa* culture filtrate contained only 5% of the enzymatic activity in the *R stolonifer* culture filtrate.

Absorption of ^{45}Ca by the surface of nectarine fruit was higher in fruits previously treated with culture filtrate of *R stolonifer* (grown on pectin medium) than with fruits treated with the same culture filtrate heated 10 min at 100 °C. This could be demonstrated both by the lesser amount of ^{45}Ca which remained on the fruit surface (*ie* ^{45}Ca washed with Tween 20 solutions) and by the higher amount detected in the skin (fig 4). The addition of the amounts of ^{45}Ca measured in washing solution and in the skin represented 74.6% ± 5.6 of the total amount deposited on the fruit. Approximately 25% must have penetrated deeper into the mesocarp and

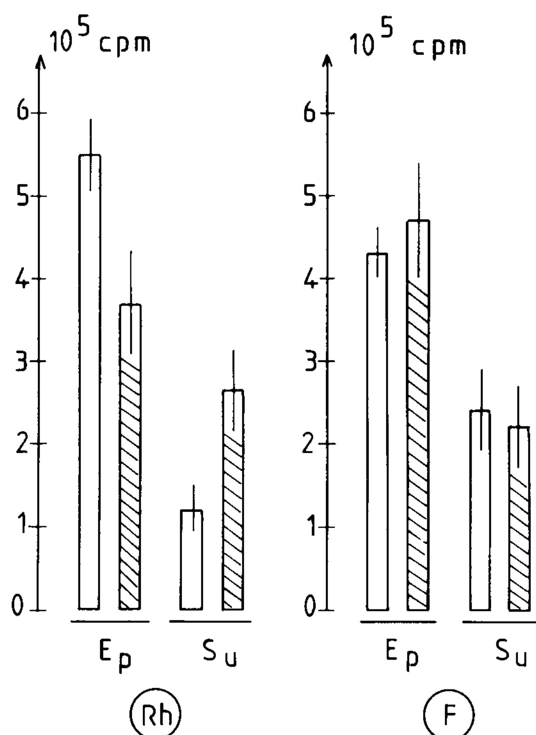

Fig 4. Penetration of ^{45}Ca inside nectarine fruits, the surface of which was previously treated with 20 µl of fungal culture filtrates during 24 h. (Rh) culture supernatant of *R stolonifer* grown on pectin medium (pH 4); (F) culture supernatant of *F solani* f sp *pisi* grown on V8 + cutin (pH 7). Su = radioactivity (cpm) which remained on the surface of fruit after 24 h; Ep = radioactivity (cpm) measured in the skin; ▭ = fruit treated with active culture supernatant (4 measures); ▨= fruit treated with boiled (10 min, 100 °C) culture supernatant (3 measures); ┌┴┐= standard deviation. The ^{45}Ca solution (20 µl) deposited on fruit consisted of a 925 kBq, 20 $mmol.l^{-1}$ $CaCl_2$ solution in a 10 $mmol.l^{-1}$ pH 5 SADH buffer and contained 942 790 ± 524 cpm.

was not counted in our experiment. Crude culture filtrate was used (pH 4), the pectinolytic activity of which (8 nkatal/ml) was measured and characterized in a previous investigation (Hornewer *et al*, 1987). However, no fatty acid esterase could be detected in this pectin medium, either at pH 4, 6, 7 or 8. A similar experiment was conducted with crude culture filtrate of *F solani* f sp *pisi* (pH 7) grown on V8 + cutin medium, the fatty acid esterase activity of which is presented in table I. No significant effect on the penetration of ^{45}Ca could be noticed (fig 4). It should be noted that no pectinolytic activity could be detected in this culture filtrate, at pH 5, pH 7, or pH 8.

DISCUSSION

In vitro, in a culture solution which contained cutin, *F solani* f sp *pisi* and to a lesser extent *R stolonifer*, produced some fatty acid esterases. When convenient dilutions of these culture filtrates were tested, so as to assess the same fatty acid esterase activity in both, *F solani* f sp *pisi* caused a marked increase in the permeability of isolated cuticular membranes, whereas *R stolonifer* did not. The considerable variability observed within the same species has already been reported in several studies related to cuticular penetration (Chamel, 1980; 1986). The variations are attributed to heterogeneity of the physicochemical structure of the cuticle. Baker *et al* (1982) have demonstrated that cutinases purified from a culture solution of *F solani* f sp *pisi* increased the diffusion of glucose and proteins through isolated cuticular disks. The similar results we obtained with a culture filtrate of our isolate of *F solani* f sp *pisi* was probably linked to the presence of a cutinase. The culture filtrate of *R stolonifer* did not increase the permeability of isolated cuticular membranes; the fatty acid esterase produced by the fungus is therefore presumably not a cutinase.

Our results are not sufficient to enable us to state that *R stolonifer* could not produce any cutinase, since:

– only 2 culture media were tested. It might be advisable to confirm the results of the present investigation by using other culture media without a carbon source (like glucose) which could repress the cutinase production. The effect of shaking of the culture should also be considered. Lastly, the permeability assay with isolated cuticles should be tested at pH values above 8;

– the technique we used to detect cutinase may not be as sensitive as conventional methods with radiolabelled cutin powder (Kolattukudy, 1985; Salinas *et al*, 1986). Nevertheless, this provides a good argument for considering that cuticle degradation is not of great importance in the infection of fruit by *R stolonifer*, contrary to *F solani* f sp *pisi* (Koller *et al*, 1982). This assumption is supported by the fact that a culture filtrate of *R stolonifer* (grown on pectin medium) caused a marked increase in the permeability of nectarine fruit epidermis, whereas it did not contain cutinase. The radioactivity of ^{45}Ca measured in the skin is mainly attributable to Ca ions that have penetrated as the greatest fraction of Ca retained by the cuticle (as free ion and exchangeable) was removed by rinsing with an acid solution (10 $mmol.l^{-1}$ buffer, pH 5), as previously shown elsewhere in the case of isolated apple fruit cuticles (Chamel, 1983). Moreover, it has been shown that Ca can penetrate into apple and tomato fruit directly across the fruit cuticle (Chamel, 1989).

Whatever the effect, the results indicate a modification of the fruit surface following its treatment by the culture filtrate of *R stolonifer*. The results obtained with *M laxa* are less consistent, since fatty acid esterase production was not detectable and degradation of nectarine fruit surface (revealed by the staining with methylene blue) was not constant. Nevertheless, these results also suggest that cuticle degradation is not involved in the infection of nectarine by *M laxa*.

Pectinolytic enzymes play an important role in the pathogenicity of *M laxa* and *R stolonifer* (Byrde and Willetts, 1977, Nguyen-The *et al*, 1985a, b), especially during post-penetration processes. From the present investigation, it can be assumed that these enzymes are also important during the penetration of both fungi in nectarine fruit. This would imply that non-cutinized (or poorly cutinized) areas are present on the surface of nectarine fruit. Microcracks that we observed on the cuticle of nectarine fruit (Nguyen-The *et al*, 1989) are possibly the sites of degradation of the epidermis by *R stolonifer* and *M laxa* culture filtrates. Penetration of fungal pathogens in fruits through microcracks has already been observed in the case of apple and *Phytophthora cactorum* (Mourichon and Bompeix, 1979) or in the case of grape and *Botrytis cinerea* (Bessis, 1972; Pucheu-Plante and Mercier, 1983), although a direct penetration of tomato fruit which involved the degradation of cuticle has been demonstrated with *B cinerea* (Rijkenberg *et al*, 1980). Research is in progress to investigate the fine structure of cuticular microcracks on nectarine fruit.

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