Effect of the Antitranspirant Film Folicote on the *in vitro* Release of Esterase Activity and on the Infection of Bean Leaves with *Botrytis cinerea*

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Abstract

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Bean leaves were protected by the antitranspirant film Folicote against *Botrytis cinerea* infection when the inoculum contained Folicote (5 %) or when leaves were pre-sprayed with Folicote (5%). Infection level was slightly restored with Folicote in the inoculum and highly restored with leaves pre-sprayed with Folicote, upon addition to the inoculum, culture filtrates containing esterase activity induced by juniperic acid (0.05 %) as sole carbon source. Possibility of involvment of this esterase activity in the fungal penetration process is discussed.

Key words: Antitranspirant, Folicote, *Botrytis cinerea,* bean, esterase.

Introduction

The use of antitranspirant films allows plants to save water and protect them against several fungal infections as summarized by Nasraoui (6). In most cases, the mode of action of these films on the fungus is still unknown. Antitranspirant films such as Safe Pack, Colfix, Vapor Gard, Wilt Pruf and Biofilm were found to be fungitoxic to germination and/or growth of *Botrytis cinerea* and *Sphaerotheca fuliginea* (3, 4). Han (5) noticed that the GZM film did not act as a physical barrier in the case of *Colletotrichum gloeosporioides*. In a previous study (9), only Vapor Gard appeared fungitoxic *in vitro* against conidial germination of *B. cinerea*, while Nu Film 17 and Folicote were not. These two antitranspirants did not act as a physical barrier for the fungus when they formed a film on the surface of the culture medium. When they were added to a culture medium containing juniperic acid as sole carbon source, the release of the fungal extracellular esterase activity changed. It increased with Nu Film 17 and was inhibited with Folicote. This follow-up study was conducted to investigate if the antitranspirant Folicote protects the bean host plant against *B. cinerea* infection and if this process is due to an antipenetrant action for which an esterase activity is involved, as Folicote is known to be not fungitoxic.

Materials and Methods

Fungal isolate

The fungal species used for the study was *B. cinerea* (a strain isolated from strawberry in the Plant Pathology Laboratory of Gembloux Faculty of Agronomic Sciences, Belgium). The isolate was routinely grown on oat meal agar medium at 26 °C. Spores collected from ten day-old cultures were used as inoculum.

Host-plant

Bean plants (*Phaseolus vulgaris,* cv. "Princesse") were grown in the greenhouse. Secondary leaves of ten day-old plants were detached, placed on water agar medium in Petri dishes, and then inoculated with the pathogen.

Esterase activity induction

Esterase activity was induced as previousely described (9). *B. cinerea* was grown for six days in a Czapek-Dox's liquid medium (2), pH 6, containing: 3 g $NaNO_3$, 1 g K_2HPO_4, 0.5 g KCl and 0.01 g $FeSO_4.7H_2O$ in 1 liter distilled water. To induce esterase activity, 0.05% (w/v) juniperic acid (16-hydroxyhexadecanoic acid), from Aldrich-Chemie, was added to the medium as sole carbon source. To inhibit this activity, glucose (0.5% w/v) was added to the medium containing the juniperic acid. Spore suspensions of *B. cinerea* were inoculated to the liquid medium (100 ml) in erlenmeyer flasks to reach a final concentration of 10^5 spores/ml. The flasks were incubated for 6 days in a rotary shaker (three replicates each) at 26 °C with a photoperiod of 16 hr light.

At the end of the incubation period, acetone (20% v/v) was added to the culture as an enzyme extractor. The mixture was incubated for further 2 hr and then filtered (0.4 µm) to eliminate the mycelium. Esterase activity was then measured. For the bioassays, the filtrates obtained were dialyzed, lyophilized and redissolved in 1/10 of inital volume in the same buffer used for inoculation. Esterase activity was again measured in that concentrated culture filtrate.

Esterase activity measurement

Esterase activity was evaluated by using *p*-nitrophenyl acetate (PNPA) as substrate and *p*-nitrophenol (PNP) as end product (10). The enzymatic reaction was carried out in 7 ml of 30 mM potassium phosphate buffer (pH 7) mixed with 1 ml enzymatic preparation (the fungal culture filtrate) and 2 ml of 1 mM PNPA (obtained from a stock solution of 100 mM PNPA in methanol). The incubation was carried out at 37 °C for 30 min. PNP was quantified by measuring absorbance at 400 nm.

Biological assays

The detached bean leaves placed in Petri dishes were inoculated with drops of *B. cinerea* spores (50 µl of $5x10^5$ spores/ml) suspended in 60 mM potassium phosphate buffer, pH 6, containing 0.1% (v/v) glycerol and 0.01% (v/v) Tween 20. This suspension was supplemented by Folicote (wax emulsion) from Attraco (Belgium) at different concentrations (0, 1, 2, 3, 4 and 5%, v/v) and/or by the concentrated culture filtrate (half of the inoculum volume). In another bioassay, bean leaves were pre-sprayed with Folicote (5%), 24 hr before inoculation. Petri dishes containing inoculated bean leaves were incubated at 26 °C under 24 µE/sec/m, with a photoperiod of 16 hr light (10 replicates of each treatment). Symptom intensities from inoculum drops on the detached leaves were evaluated 5 days after inoculation using a 0-3 scale (0: no spots, 1: few

small brown necrotic spots, 2: numerous large brown necrotic spots, 3: generalized brown necrosis extending under the whole drop).

Results

Esterase activity

As shown previousely (9), supplementing the mineral solution with 0.05 % of juniperic acid, as sole carbon source, resulted in the release of an extracellular esterase activity by *B. cinerea* mycelium after incubation for 6 days (Fig. 1). This activity was highly reduced upon addition to the medium of 0.5 % glucose. No significant esterase activity was released when the fungus was grown in the mineral solution culture medium. These esterase activities increased nearly 3 to 4 folds when the culture filtrates were lyophilized and then redissolved (Fig. 1).

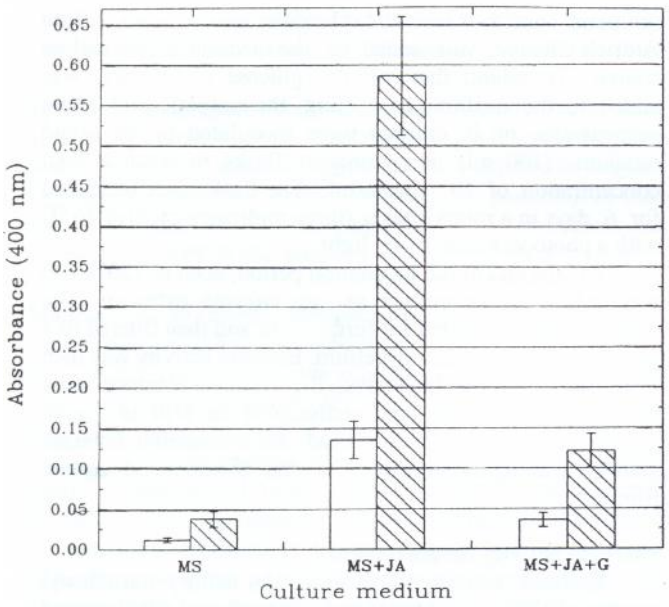

Figure 1. Released esterase activity from *Botrytis cinerea* inoculated to different culture media (MS: Mineral Solution, JA: Juniperic Acid (0.05 %), G: Glucose (0.5 %), ▭: Normal culture filtrate, ▨: Concentrated culture filtrate), (I = 2 x SD).

Biological assays

When detached bean leaves were inoculated, symptoms of the infection by *B. cinerea* appeared 5 days after inoculation. The addition of Folicote in the inoculum resulted in a reduction in the infection symptom, with a direct relationship between the increase in Folicote concentration and the symptom expression. Concentration of Folicote up to 5% in the inoculum resulted in the inhibition of the symptom intensity (Fig. 2).

When the concentrated culture filtrate (containing the highest esterase activity, Fig. 1) was added to the inoculum with Folicote (5%), the infection symptom was slightly restored (Fig. 3 and 4). The addition to the same inoculum of the concentrated culture filtrate with low esterase activity (glucose containing medium), however, did not restore infection (Fig. 3).

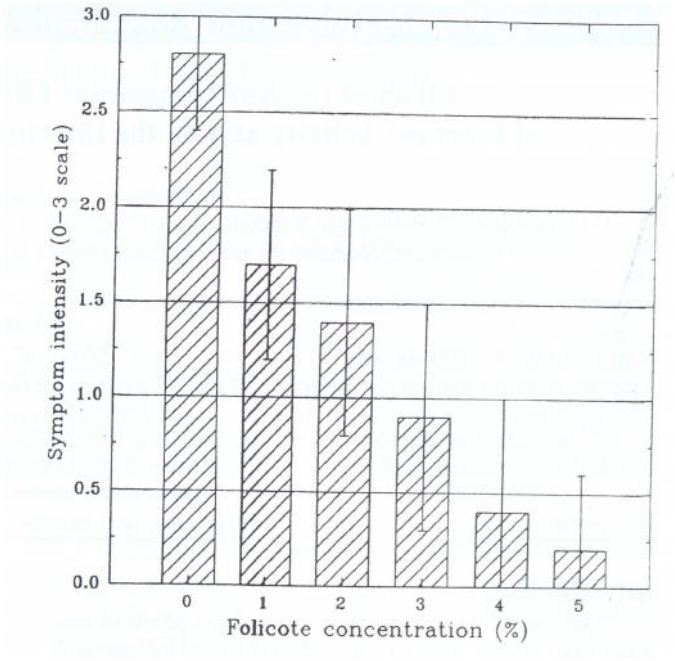

Figure 2. Symptom intensity of the infection of detached bean leaves assessed 5 days after inoculation with *Botrytis cinerea* inoculum containing increasing Folicote concentrations, (I = 2 x SD).

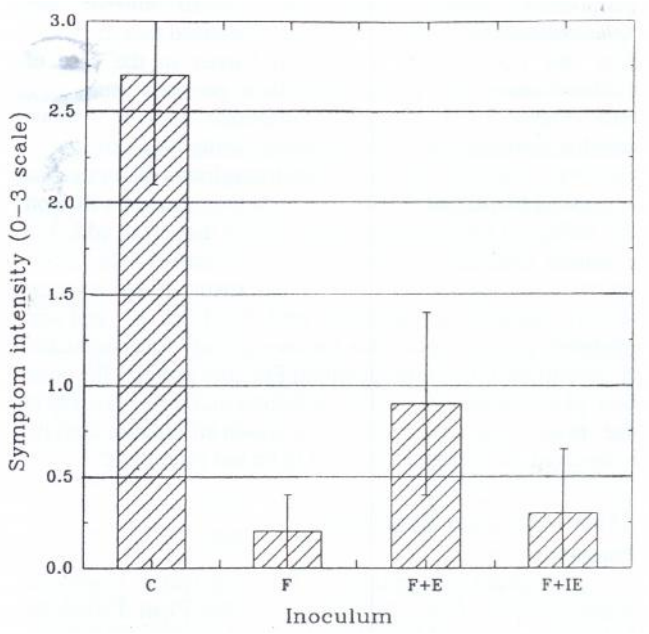

Figure 3. Symptom intensity of the infection of detached bean leaves inoculated with *Botrytis cinerea* inoculum containing Folicote (F), Folicote and Esterase Activity (F+E) or Folicote and Inhibited Esterase Activity (F+IE), C: Control (inoculum alone), (I = 2 x SD).

After the foliar spray of bean leaves by Folicote (5%), inoculation gave a very low infection intensity (Fig. 5 and 6). Similar results were obtained when the inoculum was supplemented by the concentrated culture filtrate with low esterase activity or by the boiled concentrated culture filtrate with high esterase activity. In contrast, when the concentrated culture filtrate containing high esterase activity was added to the inoculum, infection was almost completely restored (Fig. 5).

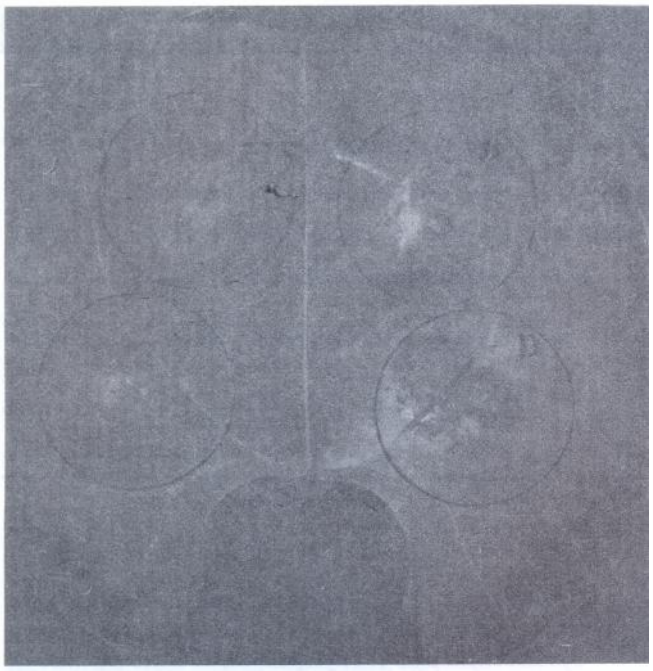

Figure 4. Detached bean leaves inculated with *Botrytis cinerea* inoculum containing Folicote and Inhibited Esterase Activity (A), Folicote and Esterase Activity (B), Folicote (C) or no Folicote (D).

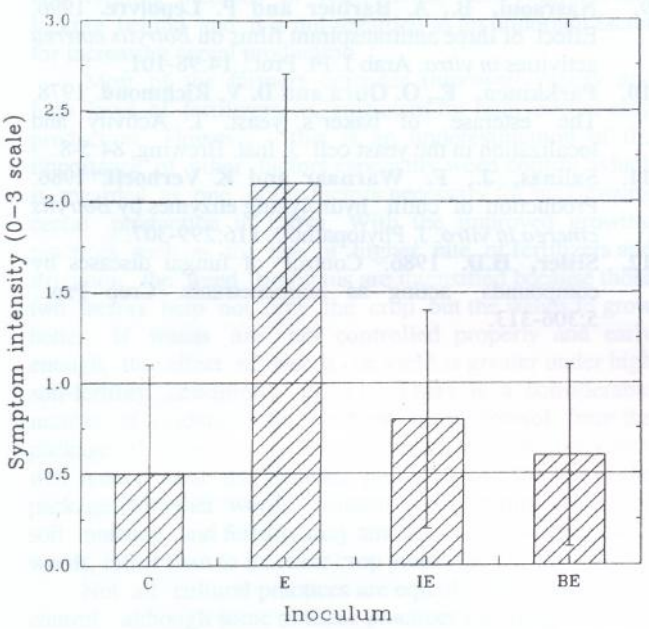

Figure 5. Symptom intensity of the infection of detached bean leaves pre-sprayed with Folicote and inoculated with *Botrytis cinerea* inoculum containing Esterase Activity (E), Inhibited Esterase Activity (IE) or Boiled Esterase Activiy (BE), C: Control, (I = 2 x SD).

Discussion

The use of the antitranspirant film Folicote (3%) has reduced the infection of bean plants inoculated with *B. cinerea* by nearly half (4), while the mode of action is still unknown. In a previous study (9), it was shown that Folicote (5%) was not fungitoxic for *in vitro* conidial germination of *B. cinerea* and did not act as a physical barrier for this fungus when it forms a film on a culture medium. In this study, Folicote (5%) suspended in the inoculum or forming a film on the leaves protected bean plants against infection with *B. cinerea*. The infection was more or less restored when the culture filtrate exhibiting esterase activity was added to the inoculum. Furthermore, the infection was not restored when the culture filtrate was boiled or obtained from medium containing glucose (no esterase activity).

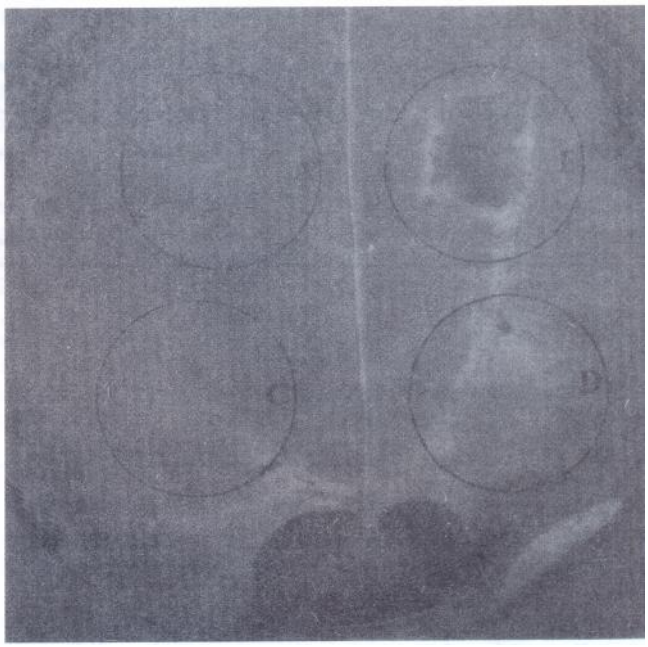

Figure 6. Detached bean leaves pre-sprayed with Folicote and then inoculated with *Botrytis cinerea* inculum containing no Esterase Activity (A), Esterase Activity (B), Boiled Esterase Activity (C) or Inhibited Esterase Activity (D)

These overall results suggest that Folicote acted as an antipenetrant compound like those reported by Sisler (12) and Ali *et al.*, (1), since it did not act as a physical barrier and is not fungitoxic (9). Similar inhibition of the infection caused by Folicote was observed with application of fatty acids in *Mycosphaerella pinodes* (8). In this case, protection depended on the inhibition of penetration in pea leaflets and was related to the inhibition of fungal esterase activity (assumed to be cutinase) upon the addition of fatty acids. The induced esterase activity of *B. cinerea* could be that of its cutinase since it is known that juniperic acid induces a cutinase production in *Ascochyta pisi* (7) and *B. cinerea* produces a cutinase (11) which could be involved in its penetration process in the host plant. Folicote, which is rich in fatty acids, inhibited the release of the fungal esterase activity induced by the culture medium containing juniperic acid (0.05%) as sole carbon source and inhibited the fungal penetration in bean leaves. Thus, as in the case of *M. pinodes* (8), it is possible that the fatty acids of Folicote are involved in the inhibition of the fungal penetration by inhibiting the release of the fungal esterase activity assumed to be cutinase, necessary for the penetration. This evidence, however, still needs validation through more *in situ* experiments. It might lead to the control of the pathogen by nonfungitoxic chemicals.

Acknowledgments

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الملخص

نصـراوي، بوزيـد، كـارين بـالطوس وفيليـب لوبـوافر. 1999. تـاثير الغشـاء المـانع للتعـرق فوليكـوت (Folicote) فـي تحريـر النشـاط الأنزيمـي الإسـتيرازي تحـت ظـروف المختبـر وفـي إصـابة أوراق الفاصـولياء (اللوبيـا) بـالفطر *Botrytis cinerea*. مجلـة وقايـة النبـات العربيـة. 17(2): 121–124.

تبين من خلال هذا البحث أن الغشاء المانع للتعرق Folicote يحمي أوراق الفاصولياء من الإصابة بالفطر *Botrytis cinerea* عندما يكون اللقاح المعدي محتوياً على 5% من فوليكوت أو عندما ترش هذه الأوراق مسبقا بـ 5% من نفس المادة. وتكون الإصابة خفيفة (Folicote مع اللقاح) أو قوية (Folicote مرشوش على الأوراق مسبقاً) عندما تضاف رشاحة المزرعة الفطرية المحتوية على نشاط أنزيمي إستيرازي مستحث بواسطة حمض الشربين Juniperic acid (0.05%) كمصدر وحيد للكربون. ويناقش الباحثون إمكانية تدخل هذا النشاط الإنزيمي الإستيرازي في ظاهرة دخول الفطر داخل العائل.

كلمات مفتاحية: الأغشية المانعة للتعرق،فوليكوت، *Botrytis cinerea*، الفاصولياء، إستيراز.

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