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Effects of Aureobasidium pullulans on numbers of lesions on dwarf bean leaves caused by Alternaria zinniae

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Abstract

Inoculating dwarf bean leaves with spore suspensions of *Aureobasidium pullulans* one day before or simultaneously with inoculations made with *Alternaria zinniae* significantly decreased numbers of lesions caused by the latter fungus. Delaying inoculation with *A. pullulans* until 1 day after that with *A. zinniae* lessened the effect on lesion numbers. Increasing numbers of *A. pullulans* spores progressively decreased numbers of *A. zinniae* lesions. Three of five isolates of *A. pullulans* decreased lesion numbers caused by *A. zinniae*. Although *A. pullulans* restricted the germination of *A. zinniae* on dwarf bean leaves, it had no effect when on cherry agar. Few viable propagules of *A. pullulans* were recovered from dwarf bean leaves when examined 7 days after application.

Introduction

Populations of pathogenic and non-pathogenic microorganisms on the surfaces of living leaves have been reviewed by Last and Deighton (1965), Leben (1965) and Sinha (1965). In some instances it seems that non-pathogenic microorganisms influence the development of plant pathogens on the leaves (Wood, 1951; Teliz-Ortiz and Burkholder, 1960; Leben and Daft, 1965), but as yet relatively little is known of this interaction. The present paper describes experiments testing the effects of *Aureobasidium pullulans* (de Bary) Arn. (syn. *Pullularia pullulans* (de Bary) Berkh.) on the infection processes of *Alternaria zinniae* Pape in dwarf beans, *Phaseolus vulgaris* L. The effects have been measured quantitatively and qualitatively in the hope of analysing possible antagonistic influences.

The dwarf bean/*A. zinniae* complex, which is not of economic importance, was chosen because the large-spored pathogen caused discrete lesions on its hosts' primary leaves, dwarf bean being preferred to the usual host, *Zinnia elegans* Jacq., because of the latter's great genetic impurity.

Of several saprophytic microorganisms isolated from bean leaves, *A. pullulans* was retained for the present series of experiments, it occurring on many different substrates in addition to leaves (Cooke, 1959). This yeast-like fungus seems able to decompose leaf epidermal pectin (Smit and Wieringa, 1953) and may therefore influence the development of leaf infecting pathogens in ways other than by antagonism which was shown during in vitro tests done by Baigent and Ogawa (1960).

Materials and methods

Primary leaves of glasshouse-grown 'Irene' dwarf beans were usually inoculated, when 11 days old, with test spore suspensions of (a) a single spore culture of *A.zinniae* isolated from a damping-off *Zinnia elegans* seedling and (b) *A.pullulans* isolated from seemingly healthy leaves of field-grown beans. The spore suspensions were prepared from oatmeal agar cultures of *A.zinniae* when 4 days old, and 7 days-old cherry agar cultures of *A.pullulans*. The suspensions, in 0.1 % Tween 80, were washed by replacing the supernatant after each of three periods of centrifugation. Four types of inocula were prepared:

1. 2.5×10^3 spores of *A.zinniae*/40 µl 0.1 % Tween.
2. As for 1 plus differing numbers of *A.pullulans* spores.
3. *A.pullulans* spores in 0.1 % Tween, their concentrations being the same as tested in 2.
4. 0.1 % Tween without spores (the control).

40 µl of inocula were pipetted to each of the four halves of paired primary leaves, and then by a finger or a glass rod with a flattened end spread over an area of 4.9 cm^2 within a 25 mm diameter glass ring. Each type of inoculum was usually tested on eight replicate half-leaves per test, the four half-leaves on each plant forming a block. The leaves, still attached to their parent plants, were kept horizontal with supports made of PVC during glasshouse incubation at 20 °–25 °C in humid-chambers.

Because numbers rarely increased afterwards, lesions were counted after three days' incubation. Before the effects of different inocula were statistically analysed, using Student's t-test ($P \leqslant 0.05$), lesion numbers were adjusted, allowances being made for lesions developed on half-leaves inoculated with spore-free Tween or Tween with *A.pullulans* spores. The inhibitory effects of *A.pullulans* on numbers of developing *A.zinniae* lesions were calculated as follows:

$$I = 100 \times \left(1 - \frac{1}{n} \sum_{i=1}^{n} a_i\right)$$

where I = percentage inhibition,

$$a_i = \frac{\text{corrected number of lesions formed by inoculum 2}}{\text{corrected number of lesions formed by inoculum 1}}$$

calculated separately for each plant, and n = numbers of plants.

The microscopic development of *A.zinniae* and *A.pullulans* was studied using a slight modification of the method described by Daft and Leben (1966). Pieces of leaves were bleached with 'Stardust' before being stained with Ziehl's carbolfuchsin which colours germ tubes and hyphae.

Results

Effects of different A.pullulans isolates on numbers of lesions caused by A.zinniae

The data from an experiment with isolate A-36 of *A.pullulans* indicate the variation among numbers of lesions caused by *A.zinniae* and counted on eight replicate half-leaves. By itself *A.zinniae* caused 105 to 172 lesions whereas the addition of *A.pullu-*

Table 1. Effects of *Aureobasidium pullulans* (isolate A-36) on the development of lesions by *Alternaria zinniae* when both fungi were inoculated simultaneously to dwarf bean leaves. Inocula contained 2.5×10^3 and/or 5.1×10^7 spores/40 µl of *A. zinniae* and *A. pullulans*, respectively, being prepared in a 0.1% solution of Tween.

Replicate plants	Numbers of lesions/half-leaf			
	Inocula: *A. zinniae*	*A. zinniae* + *A. pullulans*	*A. pullulans*	Spore-free control (0.1% Tween)
1	105	5	3	0
2	115	47	5	2
3	130	7	16	1
4	138	33	6	0
5	147	28	1	0
6	151	17	5	0
7	171	11	8	0
8	172	28	2	0

Tabel 1. Invloed van Aurebasidium pullulans (isolatie A–36) op de vorming van lesies door Alternaria zinniae bij gelijktijdige inoculatie van bonebladeren met beide schimmels. De inocula bevatten $2{,}5 \times 10^3$ *en/of* $5{,}1 \times 10^7$ *sporen van A. zinniae, resp. A. pullulans, per 40 µl 0,1% Tween-oplossing.*

lans decreased the range from 5 to 47 (Table 1). Furthermore, because inoculation with *A. pullulans* was associated with the development of lesions in the absence of *A. zinniae*, it appears that high spore concentrations of the former can induce lesions although those that developed were much smaller than those correlated with *A. zinniae* attack.

When a range of differing isolates of *A. pullulans* were tested, lesion development by *A. zinniae* was inhibited more by A-36 (88.1%) than by similar or more concentrated suspensions of A-127B (38.1%) and A-127A (36.4%). Lesion development was not inhibited by suspensions of A-35 and A-90 (Table 2). Because of its large effect only A-36 was used in the other experiments described in this paper.

Table 2. Effects of five different *Aureobasidium pullulans* isolates on the lesion formation on bean leaves by *Alternaria zinniae* after simultaneous inoculation with *A. pullulans* and *A. zinniae*.

A. pullulans isolate	Spore concentration (number of spores per 40 µl inoculum)	Effects on *A. zinniae* lesions
A–90	1.1×10^7	none
A–35	3.5×10^7	„
A–36	5.1×10^7	inhibitory: 88.1%
A–127B	5.3×10^7	„ 38.1%
A–127A	7.9×10^7	„ 36.4%

Tabel 2. Invloed van vijf verschillende Aureobasidium pullulans-isolaties op de lesievorming op bonebladeren door Alternaria zinniae, na gelijktijdige inoculatie met A. pullulans en A. zinniae.

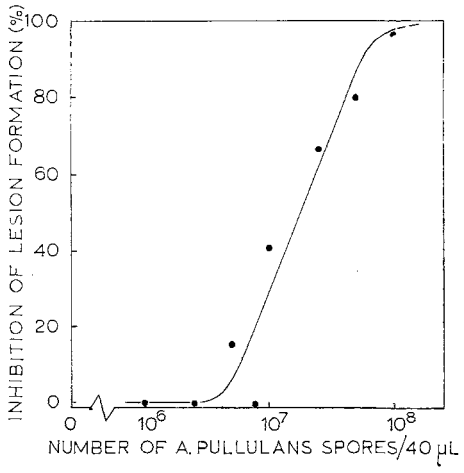

Fig. 1. Inhibitory effects of different concentrations of *Aureobasidium pullulans* spores on the development of dwarf bean leaf lesions caused by *Alternaria zinniae*, after simultaneous inoculation with both fungi.

Fig. 1. Remmende invloed van verschillende sporenconcentraties van Aureobasidium pullulans op de vorming van lesies op bonebladeren door Alternaria zinniae, na gelijktijdige inoculatie met beide schimmels.

Effects of differing concentrations of A.pullulans on the development of lesions by A.zinniae

When differing concentrations of *A.pullulans* spores, ranging from 5.0×10^2 up to 1.0×10^8 spores/40 µl, were tested, lesion development by *A.zinniae* was not affected by 2.5×10^6 or fewer *A.pullulans* spores/40 µl. At greater concentrations numbers progressively decreased; the log-concentration/response curve might be symmetrically S-shaped (Fig. 1).

Effects of differing times of inoculation with A.pullulans relative to those of A.zinniae

Inoculating with *A.pullulans* one day before, instead of simultaneously with *A.zinniae* did not significantly affect its inhibitory effects. On the other hand delaying the application of *A.pullulans* by 24 h significantly decreased the inhibition from 47.4 to 17.1 % (Table 3).

The germination of A.zinniae on leaves

When the germination of *A.zinniae* on leaves was examined by direct microscopy it was found, after $23\frac{1}{2}$ h incubation, that simultaneously inoculating with *A.pullulans* slightly decreased the % germination and appreciably decreased (a) numbers of germ tubes per spore, and (b) germ tube length (Table 4).

Table 3. Effects of the time of inoculation with *Aureobasidium pullulans* (conc. 2.5×10^7 spores/40 µl) on lesion formation by *Alternaria zinniae* (conc. 2.5×10^3 spores/40 µl).

Time of inoculation with *A.pullulans*	Inhibition of lesion formation (%)
1 day before *A.zinniae*	52.3
Simultaneously with *A.zinniae*	47.4
1 day after *A.zinniae*	17.1

Tabel 3. Invloed van het tijdstip van inoculatie met Aureobasidium pullulans (conc. 2,5 × 10⁷ sporen/40 µl) op de lesievorming door Alternaria zinniae (conc. 2,5 × 10³ sporen/40 µl).

Table 4. Effects of *Aureobasidium pullulans* spores (conc. 3.5×10^7 spores/40 µl) on *Alternaria zinniae* spores germinating on the surfaces of dwarf bean leaves, after simultaneous inoculation with both fungi.

Germination characteristics of *A. zinniae* spores	Period of incubation (hours)			
	0		$23\frac{1}{2}$	
	without *A.pull.*	with *A.pull.*	without *A.pull.*	with *A.pull.*
% germination	0[1]	0[2]	100[1]	92[2]
Numbers of germ tubes per spore	0[3]	0[4]	4.9[3]	1.7[4]
Length of germ tubes (µm)	0[5]	0[6]	314[5]	154[6]

[1] 100 spores counted on duplicate half-leaves
[2] 100 „ „ „ one half-leaf
[3] 10 spores examined on duplicate half-leaves
[4] 10 „ „ „ one half-leaf
[5] 10 germ tubes measured on duplicate half-leaves
[6] 10 „ „ „ „ one half-leaf

Tabel 4. Invloed van Aureobasidium pullulans-sporen (conc. 3,5 × 10⁷ sporen/40 µl) op de kieming van Alternaria zinniae-sporen op het oppervlak van bonebladeren, na gelijktijdige inoculatie met beide schimmels.

Development of A. zinniae on agar media

The effects of *A. pullulans* on the in vitro development of *A. zinniae* were tested in two ways. In the first, inverted 8 mm cherry agar discs from 7 days-old *A. pullulans* colonies were seeded with *A. zinniae* spores. After incubating for 5 h at 23 °C similarly large numbers of *A. zinniae* spores (98 and 99%, respectively) had germinated whether inoculated to cherry agar discs with or without *A. pullulans*.

To test effects on mycelial growth, 5 mm agar discs of 7 days-old colonies of *A. pullulans* and *A. zinniae* were placed 30 mm apart on dishes of cherry agar, the stock cultures also being grown on this medium. During subsequent incubation at 23 °C the radial growth of *A. zinniae* was measured along two axes from colony centre to the

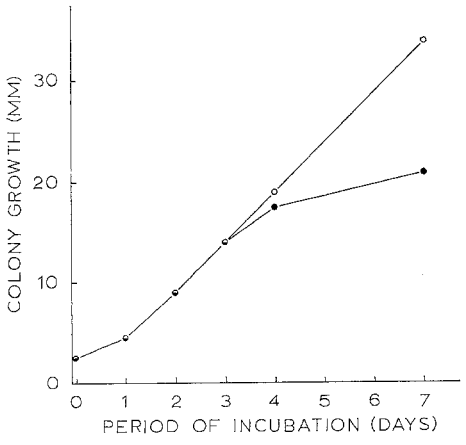

Fig. 2. Mycelial growth of *Alternaria zinniae* when petri dishes of cherry agar were also inoculated with *Aureobasidium pullulans*.
o—o: growth along radius distant from a colony of *A. pullulans*
●—●: growth along radius approaching a colony of *A. pullulans*

Fig. 2. Myceliumgroei van Alternaria zinniae op petrischalen met kersagar die eveneens beënt waren met Aureobasidium pullulans.
o—o: *radiale groei van de kolonie van A. pullulans af*
●—●: *radiale groei naar de kolonie van A. pullulans toe*

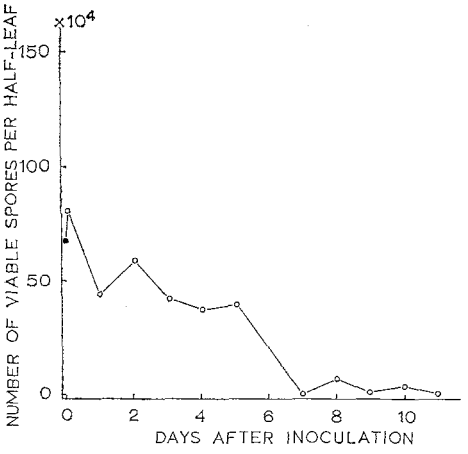

Fig. 3. Numbers of viable *Aureobasidium pullulans* spores washed from bean leaves at different times after inoculation.
●: number of viable *A.pullulans* spores/40 µl just before inoculation

Fig. 3. Aantallen levenskrachtige sporen van Aureobasidium pullulans gespoeld van boneblade-ren op verschillende tijden na inoculatie.
●: *aantal levenskrachtige sporen van A.pullulans/ 40 µl, onmiddellijk vóór inoculatie*

perimeter (a) remote from and (b) adjoining, colonies of *A.pullulans*, the former representing 'normal' and the latter 'influenced' growth (Fig.2).

Growth along the axis remote from *A.pullulans* was linear during 7 days' incubation. Growth along the other axis was retarded between the third and fourth days and by the fifth day colonies of *A.zinniae* and *A.pullulans* were touching indicating that *A.pullulans* influenced the growth of *A.zinniae* at a distance slightly.

Development of A.pullulans on leaves

Numbers of *A.pullulans* spores observed on leaves by direct microscopy decreased with increasing time after inoculation. None were seen to produce germ tubes but some occasionally budded new spores. The decreasing numbers of spores were checked by plating leaf washings, using sterile 0.1 % Tween 80, on cherry agar. By counting colony numbers after incubating at 23 °C for 3 days, it was found that numbers of viable *A.pullulans* spores on leaf surfaces greatly decreased within 7 days of leaf inoculation. The downward trend was sometimes temporarily reversed during the second and third days (Fig. 3).

Discussion

Some isolates of *A.pullulans* decreased numbers of leaf lesions formed on dwarf beans by spore suspensions of *A.zinniae*, whereas others did not. These different effects may be due to the considerable variability of *A.pullulans* reported by Cooke (1959). The inhibitory effect depended on the concentrations of *A.pullulans* and their time of inoculation in relation to that of the plant pathogen. As the decreases were associated with a reduction of numbers and lengths of germ tubes of *A.zinniae* spores, the slower rate of the spore germination may be the principal cause of the inhibition of lesion formation. Inhibition of penetration of hyphae into the leaf or extension in the tissues might be the cause of the slight decrease of lesion formation by *A.pullulans* added 24 h after inoculating with *A.zinniae*.

The curve (Fig. 1) linking effects of different concentrations of *A.pullulans* with numbers of *A.zinniae* lesions resembles a log-normal dosage-response curve suggesting

that *A.pullulans* acts like a fungicide or fungistatic substance. Antibiotic substances produced by living cells or decomposition products released by dying cells might be causal factors. Since *A.pullulans* did not affect the germination of *A.zinniae* on agar but restricted it on leaves, the available nutrients in vitro might have counterbalanced toxic effects of *A.pullulans*. This fungus might also restrict germination of *A.zinniae* spores by creating a nutrient deficiency in infection drops or by inducing leaves to produce inhibitory substances. Which is operative in the present instance is still unknown.

Although cultures of *A.pullulans* were isolated from dwarf bean leaves they seemed unable to re-establish themselves, many spores soon losing their viability. Death may be attributed to a lack of suitable nutrients (Cooke, 1959) or possibly to the effects of inhibitory substances, such as phytoalexins, produced by dwarf bean leaves.

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Samenvatting

De invloed van Aureobasidium pullulans op het aantal lesies op bladeren van stamslabonen veroorzaakt door Alternaria zinniae

Aan de hand van een model, met als waardplant de stamslaboon (*Phaseolus vulgaris* L.) en als parasiet de schimmel *Alternaria zinniae* Pape, werd nagegaan of de vorming van kleine lesies op bonebladeren door deze schimmel beïnvloed kon worden door inoculatie met de schimmel *Aureobasidium pullulans* (de Bary) Arn. geïsoleerd van bonebladeren.

Bij gelijktijdige inoculatie met *A.pullulans* en *A.zinniae* werd het aantal lesies niet beïnvloed door twee van de vijf getoetste isolaties van *A.pullulans*, door twee andere isolaties matig verlaagd en door één isolatie sterk verlaagd (Tabel 1 en 2). De remming uitgeoefend door de laatstgenoemde isolatie is nader onderzocht.

De lesievorming werd geremd door een concentratie van $5{,}0 \times 10^6$ en meer sporen van *A.pullulans*/40 µl; bij stijgende concentraties nam de remming eveneens toe (Fig. 1). Bij inoculatie van bonebladeren met *A.pullulans* 1 dag vóór inoculatie met *A.zinniae* werd het aantal lesies in gelijke mate verlaagd als bij gelijktijdige inoculatie. Werd *A.pullulans* één dag na *A.zinniae* opgebracht, dan was de remming van de lesievorming gering (Tabel 3).

Op het blad werd de sporekieming van *A.zinniae* geremd door *A.pullulans* (Tabel 4), terwijl op een agarbodem de sporekieming niet en de myceliumgroei licht (Fig. 2) geremd werden.

Op de bladeren vond slechts een geringe ontwikkeling van *A.pullulans* plaats; 7 dagen na inoculatie waren nog maar weinig levenskrachtige sporen aantoonbaar (Fig. 3).

Aan de hand van deze resultaten worden aard en mechanisme van de remming besproken.

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