Interactions between Alternaria porri and the Saprophytic Mycoflora of Onion Leaves

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ABSTRACT

Infection of onion leaves by *Alternaria porri* was reduced by addition of *Aureobasidium pullulans* cells to the inoculum. Similarly, infection by *Botrytis cinerea* was reduced, while that by *Botrytis squamosa* was not. The common saprophytes of the onion phyllosphere, *A. pullulans*, *Sporobolomyces roseus*, *Cryptococcus luteolus*, and *Cladosporium herbarum* reduced infection of onion leaves by *A. porri* by 55%, 45%, −4%, and 18%, respectively. Superficial mycelial development of the pathogens rather than spore germination was affected by the antagonistic microorganisms. Suppression of infection appeared to be caused neither by depletion of carbohydrates and amino acids in the phyllosphere, nor by pH differences.

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The role of saprophytic hyphal fungi and yeasts colonizing healthy leaves in disease development is only vaguely understood. The increased saprophytic mycoflora on leaves of wind-pollinated plants inhibited the stimulation of certain perthotrophic pathogens in the presence of pollen (6, 7, 19, 21). The saprophytic mycoflora on such leaves probably competes successfully with the pathogens for the nutrients released from the pollen. In addition to instances where the mycoflora plays an antagonistic role in a natural situation of high nutrient concns on the leaf, several possibilities for artifical biological control by common leaf saprophytes have been reported. *Cladosporium herbarum* (Pers.) Link ex Fries and *Aureobasidium pullulans* (De Bary) Arnaud may interfere with infection of strawberries and tomatoes by *Botrytis cinerea* Pers. ex Fries (3, 4, 15). *Candida* sp. reduced leaf infection by *Cochliobolus miyabeanus* (Ito and Kuribay) Drechs. on rice (1).

This study was made to obtain additional information concerning the antagonistic capacities of the main components of the phyllosphere mycoflora and the mechanism(s) involved. The onion (*Allium cepa* L.) was chosen as a host; *A. pullulans*, *Sporobolomyces roseus* Kluijver & V. Niel, *Cryptococcus luteolus* (Saito) Skinner and *Cladosporium herbarum* representing common fungal colonizers in the onion phyllosphere (C. A. Clark, *personal communication*) were tested for possible antagonistic behavior. *Botrytis cinerea* Pers. ex Fries, *Botrytis squamosa* Walker and *Alternaria porri* (Ellis) Cif., each of which produce distinct leaf spots on onion leaves, were utilized as plant pathogens in preliminary experiments. Thereafter, all research was accomplished with *A. porri*.

MATERIALS AND METHODS.—*Culturing methods.*—Onions (cultivar Elba Globe) were grown in the greenhouse at ca. 18 C from bulbs in 10.2-cm (4-inch) clay pots containing an autoclaved mix of sand, peat, soil (1:1:1). Fertilizer (10:10:10) was added via the irrigation water. Additional light was supplied by fluorescent tubes (Sylvania, F96T12-WW-VHO) for 16 h per day. Four weeks after sprouting, the plants, then having ca. five leaves each, were transferred to a controlled environment growth chamber (Cornell University walk-in type) at 21 C(*Botrytis* infection) or 24 C (*Alternaria* infection). Relative humidity (RH) was 75-90%; light intensity was 21,520 lx (2,000 ft-c) during 14 h photoperiods.

A. porri (Isolate 72-1) was isolated from a leaf spot lesion of a field-grown onion. To maintain sporulation and pathogenicity, the isolate frequently was passed through onion. Isolate 64a of *B. squamosa* (2) and 61-34 of *B. cinerea* (8) were utilized in the studies. *B. cinerea* was grown on potato-dextrose agar (PDA) slants. *B. squamosa* was grown on potato agar (50 g potatoes, 20 g agar per liter) slants, which provided good sporulation. Both were grown at 20 C with 12 h fluorescent light per day. *A. porri* initially was grown on PDA slants at 24 C in the dark. Since spore production was poor (5), the inoculum prepared from these cultures consisted mainly of hyphal fragments which proved to be sufficient for obtaining infection. Satisfactory sporulation eventually was achieved on PDA in plastic petri dishes at 24 C with 12 h fluorescent and black light (Sylvania, F15T8-BLB) per day. High RH reported to promote sporulation (5, 14) appeared inhibitory to sporulation. *S. roseus*, *C. luteolus*, *A. pullulans*, and *C. herbarum* were grown on PDA at 23 C in the dark.

Experimental design.—To determine the antagonistic capacities of the saprophytes against the pathogen, onion leaves were inoculated with a mixture of propagules of the pathogen and saprophyte. The consequent infection (the number of lesions) was compared to infection of leaves inoculated with the pathogen only. In several experiments, the saprophytes were applied to the leaves a few days before inoculation with the pathogen.

Propagule suspensions were prepared in sterile distilled water or in 0.025% Triton B-1956 (Rohm and Haas Corporation, Philadelphia, Pa.) by scraping 1 to 2-wk-old cultures with an inoculation needle. Large mycelial

fragments were removed by filtering through cheese cloth. For poorly sporulating cultures of *A. porri*, large mycelial parts were fragmented in a Waring Blendor. The final concn of *S. roseus*, *C. luteolus* and *A. pullulans* in the inoculum varied from 2 to 8×10^7 cells per ml. The concn of *C. herbarum* was 1.2 to 2.8×10^6 and that of *A. porri* propagules was 7 to 14×10^4 per ml. After improving sporulation of *A. porri*, ca. 60% of the propagules consisted of conidia. In some experiments the propagule suspension was washed once with sterile distilled water. Neither the use of Triton nor the washing procedure appeared to affect the results significantly. The third and fourth youngest leaves were rubbed gently with a wet cotton swab before inoculation to increase the wettability of the onion leaf. The inoculum was applied with a De Vilbiss atomizer. After inoculation or application of the saprophytes, the plants were covered with plastic bags to ensure high humidity. Nine to twenty leaves on three to six plants received the same treatment. Six days after inoculation the number of lesions per leaf was counted and expressed per 100 cm^2 of leaf area. The results of each of the experiments were analyzed by Wilcoxon's two-sample test. The antagonistic effect of a saprophyte in a particular experiment was recorded as percent reduction of infection. This is calculated by 100× (1 − no. lesions with saprophyte/no. lesions without saprophyte).

Determination of the phyllosphere population.—Several days after application of the saprophytes to the onion leaves their population was determined. The middle part of each leaf sampled (ca. 20 cm^2) was shaken vigorously in a 250-ml Erlenmeyer flask containing 50 ml sterile distilled water for 1.5 h. Samples (0.1 ml) of the suspension were cultured after appropriate dilutions on agar plates. For this purpose a medium containing 2% agar, 0.1% yeast extract, 1% Bacto-tryptone and 3% glucose was used. Bacterial development was suppressed by addition of Penicillin G Potassium (200 mg/l) and Streptomycin sulfate (100 mg/l) to the medium just before pouring plates. Leaf colonization was

TABLE 1. Effect of *Aureobasidium pullulans* on infection of onion leaves by *Alternaria porri*, *Botrytis cinerea* and *Botrytis squamosa*

Inoculum (propagules/ml)	Mean lesions per leaf (no.)	Reduction of infection (%)
A. porri (10^5/ml)	36	
idem + *A. pullulans* (3.4×10^7/ml)	3[a]	92
A. porri (9.3×10^4/ml)	12	
idem + *A. pullulans* (2.4×10^7/ml)	2[a]	82
B. cinerea (125×10^4/ml)	416	
idem + *A. pullulans* (6×10^7/ml)	172[a]	59
B. cinerea (75×10^4/ml)	51	
idem + *A. pullulans* (7.5×10^7/ml)	24[a]	52
B. squamosa (7×10^4/ml)	536	
idem + *A. pullulans* (2×10^7/ml)	416	22
B. squamosa (3.25×10^4/ml)	477	
idem + *A. pullulans* (5×10^7/ml)	356	25

[a]Significantly different from control treatment, $P = 0.01$.

TABLE 2. Survey of all experiments involving interactions between *Alternaria porri* and selected phyllosphere fungi and yeasts on onion leaves. Saprophytes were applied simultaneously with or before inoculation

Phyllosphere associate	Percentage reduction of infection and colonization in different experiments							
	1	2	3	4	5[a]	6	7[a]	Mean
Aureobasidium pullulans								
% reduction of *A. porri* infection		91**[c]	82**	79**	5	18[+c]	53*[c]	54.7
colonization (propagules/$cm^2 \times 10^3$)[b]				*12.5 (18.2)*		*84*	*45*	
Sporobolomyces roseus								
% reduction of *A. porri* infection	55[+]			43**		50**	32[+]	45.0
colonization (propagules/$cm^2 \times 10^3$)[b]				*23.4 (28.9)*		*225*	*31*	
Cryptococcus luteolus								
% reduction of *A. porri* infection				6		−3	−15	−4.0
colonization (propagules/$cm^2 \times 10^3$)[b]				*7.7 (15.6)*		*241*	*87*	
Cladosporium herbarum								
% reduction of *A. porri* infection						13	23[+]	18.0
colonization (propagules/$cm^2 \times 10^3$)[b]						*16*	*25*	

[a]Saprophytes applied 2 days before inoculation.

[b]Colonization level 2 to 3 days after inoculation, determined by culturing methods. No development of bacteria was observed. For figures in parentheses the colonization was determined directly by microscopic observation of Scotch tape replicas.

[c]* = Reduction significant, $P = 0.05$. ** = Reduction significant, $P = 0.01$. [+] = Reduction significant, $P = 0.10$.

TABLE 3. Effect of phyllosphere fungi and yeasts on the prepenetration development of *Alternaria porri* on onion leaves. Fungi and yeasts used were *Aureobasidium pullulans*, *Sporobolomyces roseus*, *Cryptococcus luteolus* and *Cladosporium herbarum*

Pretreatment	Germination of spores (%)	Germination of hyphal fragments[a] (%)	Germ tubes per germinated spore[b] (no.)	Length of germ tubes per spore[b] (μm)
0.025% Triton	97	64	3.1	1,900
A. pullulans	96	51	2.8	500**[c]
S. roseus	89	43	3.4	600**
C. luteolus	92	61	3.1	1,300
C. herbarum	86	53	3.1	...
Mixture	82	53	2.6	...

[a]Only those with more than three cells were considered.
[b]Thirty spores examined per treatment.
[c]** = Significantly different from the control treatment, $P = 0.01$.

expressed as the number of colonies grown from one cm^2 of leaf surface.

Sampling of leachates.—Leachates from leaves with and without a dense population of the saprophytes were sampled for analysis of the total amount of carbohydrates and amino acids. Either a propagule suspension (2.2 to 14 $\times 10^7$/ml) of the saprophytes or sterile distilled water was sprayed on leaves of onion plants grown at 24 C. After spraying, the plants were covered with a plastic bag as in the experiments with *A. porri*. Three days later, water droplets were applied to detached leaves with a pipette (Experiments 1 and 2, Table 4) or to attached leaves with an atomizer (Experiment 3, Table 4). Evaporation of the droplets was prevented by maintaining high RH. Leaching of exudates into the droplets was maintained for 3 h. Then, within the next hour, the droplets were collected by use of a vacuum aspirator, passed through a Millipore filter (pore diam 0.22 μm) and concd by lyophilization. Total carbohydrate content was determined colorimetrically with anthrone reagent (11), and total amino acid content with the ninhydrin reaction (16).

The density of the saprophyte population on the leaves was determined by plating out leachate droplets, or by shaking three leaves of each treatment in water and culturing samples of the suspension.

RESULTS.—*Sensitivity of three pathogens to antagonistic action by A. pullulans.*—The effect of *A. pullulans* on infection of onion leaves by *A. porri*, *B. cinerea* or *B. squamosa* was compared (Table 1). The number of lesions caused by *B. squamosa* was not reduced significantly by *A. pullulans*. However, a slight antagonistic effect seemed to occur. *B. cinerea* infection was reduced significantly in both experiments to about 50%. *A. porri* was the most sensitive to antagonistic action by *A. pullulans* and, therefore, was selected as the pathogen for detailed studies.

Ability of the several saprophytes to reduce infection by A. porri.—In a number of experiments the saprophytes of the onion phyllosphere were studied for their abilities to reduce *A. porri* infection (Table 2). The reduction of the infection by a particular saprophyte varied considerably in the different experiments. Nevertheless, *A. pullulans* as well as *S. roseus* was able to reduce *Alternaria* infection by ca. 50%. The variation in colonization levels of these saprophytes from 12.5 to 225 $\times 10^3$ colonies/cm^2 did not seem to be correlated with the degree of antagonistic action. The *Cryptococcus* isolate did not reduce infection, though the colonization levels were comparable with those of *S. roseus*. The antagonistic action of *C. herbarum* was slight.

Effect of the saprophytes on prepenetration development of A. porri.—In Experiment 7 (Table 2) the effect of the saprophytes on propagule germination and germ tube development of the pathogen additionally was studied on three leaves of each treatment 3 days after inoculation (Table 3). A section of onion leaf (middle part) was pressed firmly on the adhesive side of clear Scotch tape. After removing the leaf piece the wax layer with the phyllosphere flora was left on the tape. The tape then was mounted on a slide with lactophenol cottonblue for microscopic examination. One hundred propagules per slide (spores and hyphal fragments) were examined at random (Table 3). The ratio between the number of spores and the number of mycelial fragments was approximately 50:50. Generally, the spores germinated more successfully than the hyphal fragments. The saprophytes appeared to cause a slight reduction of germination of both types of propagules, which is too small to account for the differences in infection (Table 2). The number of germ tubes per germinated spore was not significantly affected by the saprophytes.

The total length of all germ tubes (larger than five cells) per germinated spore was measured (Table 3) with the aid of a drawing tube (Wild Heerburgg Ltd.) attached to the microscope and a map measurer (curvimeter). The superficial development of *A. porri* was reduced greatly in the presence of *A. pullulans* and *S. roseus*. *C. luteolus* which did not restrict *A. porri* infection, also did not significantly reduce the superficial development of *A. porri* (Table 3). The effect of *C. herbarum* and the mixture of saprophytes on the mycelial development of *A. porri* could not be determined because it was not possible to distinguish *Cladosporium* mycelium from the superficial mycelium of *A. porri*.

Photomicrographs (Fig. 1, A-F) of the Scotch tape impressions illustrate saprophyte distribution over the leaf surface. It is interesting to note that *A. pullulans* applied as yeast cells has been transformed into thick-walled pigmented cells (Fig. 1-B).

Effect of saprophytes on leachate composition.—Previous experience with the same saprophytic species involving *Helminthosporium* infection (7), and the fact that they did not affect spore

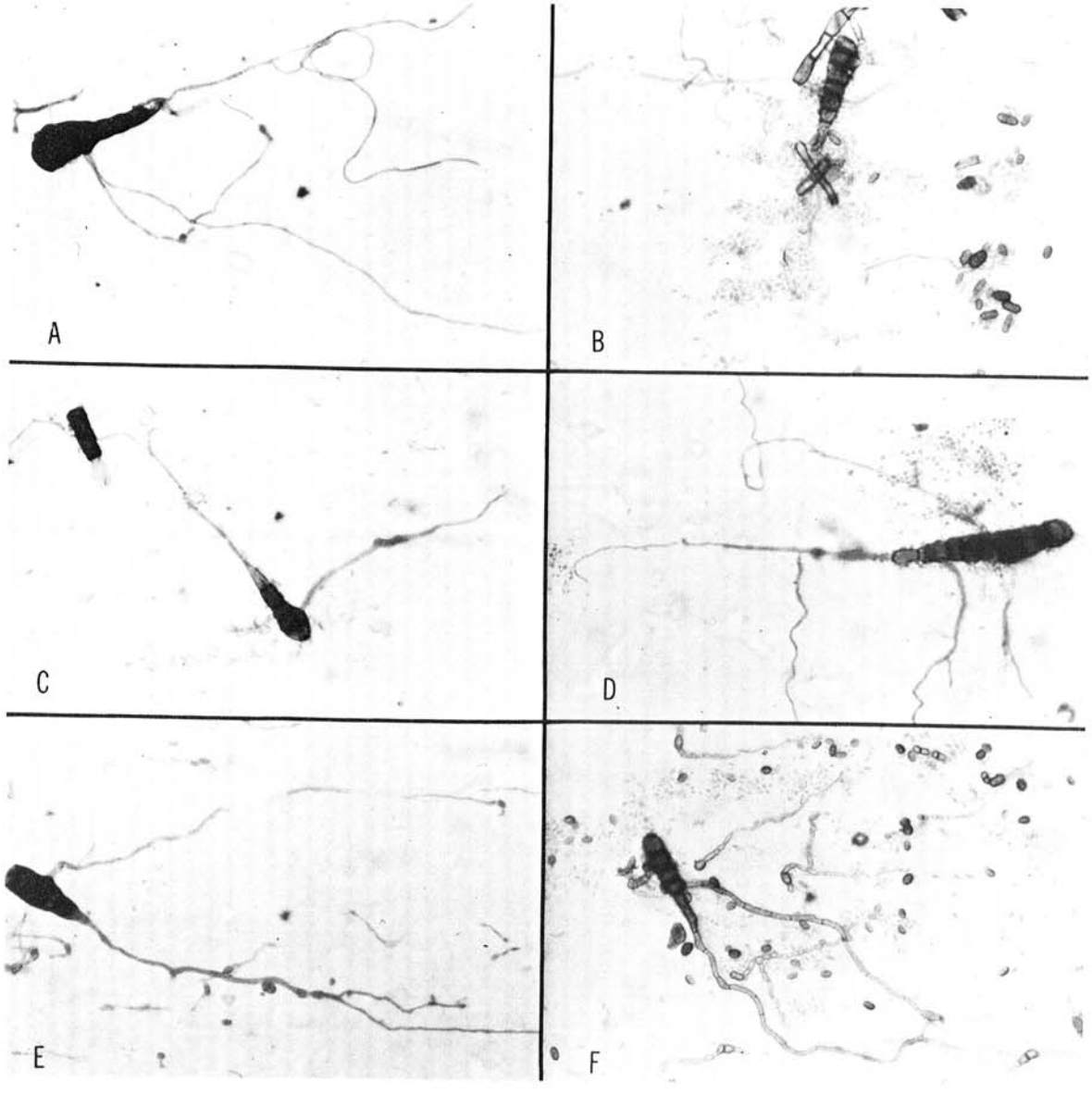

Fig. 1-(A to F). Photomicrographs of Scotch tape replicas of the onion leaf phyllosphere used for studying the effect of saprophytic fungi on the prepenetration development of *Alternaria porri*. **A)** Control, no additions; **B)** *Aureobasidium pullulans* added; **C)** *Sporobolomyces roseus* added; **D)** *Cryptococcus luteolus* added; **E)** *Cladosporium herbarum* added; **F)** Mixture of all the saprophytes added.

germination of *A. porri*, indicated that antibiotic production was not a likely basis for antagonism. Therefore, attempts were made in the present study to explain the antagonistic effects by nutrient competition. In Experiments 1 and 2 (Table 4) the carbohydrate content was slightly higher in the leachates from the control leaves than in leachates from leaves treated with yeasts, whereas in Experiment 3 (Table 4) the saprophyte treatment increased the carbohydrate concn. In this experiment, the concn of the applied saprophyte suspension was 14×10^7 cells/ml, so leaching from those cells, dead or alive, might be responsible for this difference. The amino acid concn either was reduced slightly by the saprophytes (Experiment 1, Table 4) or it was reduced 50% (Experiment 2, Table 4). The differences between carbohydrate and amino acid concns in leachate from leaves colonized with *A. pullulans* and from the control leaves (Experiment 3, Table 4) are not likely to account for the antagonism between *A. pullulans* and *A. porri*. This is supported by the fact that *C. luteolus*, which had no antagonistic effect on *A. porri*, affected the carbohydrate and amino acid concns in the leachates in a similar fashion as did the antagonistic A. *pullulans*.

The colonization levels of the saprophytes in the

TABLE 4. Effect of yeasts on the amount of carbohydrates (expressed as μg glucose/ml) and amino acids (expressed as μmole leucine/ml) in leachates of onion leaves

Phyllosphere associate	Experiment 1		Experiment 2	Experiment 3	
	Carbohydrates[a]	Amino acids[a]	Carbohydrates[a]	Carbohydrates	Amino acids
Water	3.4	0.012	2.8	7	0.050
colonization (propagules/cm^2 × 10^3)[b]	*0.8*[cd]		*0.1*[cd]	*0.2*[dc]	
Aureobasidium pullulans	2.7	0.010	2.4	15	0.025
colonization (propagules/cm^2 × 10^3)[b]	*4.8*[c]		*21.0*[c]	*27.0*[c]	
Cryptococcus luteolus	1.9	0.010	...	20	0.023
colonization (propagules/cm^2 × 10^3)[b]	*5.4*[c]		...	*68.0*[c]	

[a]When multiplied by 18, the concns per 100 cm^2 of leaf surface are found.
[b]Colonization level 3 days after application, determined by culturing methods. No development of bacteria observed.
[c]Cultured from leachate droplets, which appeared half as effective as by culturing from water in which leaves were shaken.
[d]Total mycoflora.
[e]Cultured from water in which leaves were shaken.

leachate experiments were comparable to those used when studying antagonism against *A. porri* (Table 2). For all treatments (Table 4), the amounts of carbohydrates and amino acids detected in Experiment 3 were much higher than those found in Experiments 1 and 2. The atomized leachate droplets in Experiment 3 were much smaller than the pipetted droplets in Experiments 1 and 2 which may have influenced the concn in the droplets. The concns found in Experiment 3 give a better approximation of the concns in the infection droplets, which also were atomized.

The pH of unconcd leachates from leaves with and without the saprophytes was either 6.7 or 6.8. Therefore, it is unlikely that pH differences are responsible for the antagonistic effect.

DISCUSSION.—Infection of onion leaves by *A. porri* was reduced approximately 50% in the presence of *A. pullulans* and *S. roseus*. Although it may be questioned whether the isolates used are representative of the entire natural population of these organisms on onion leaves, the antagonistic behavior of *A. pullulans* and *S. roseus* was in accordance with other reports (7, 9, 10, 12, 21). The antagonistic role of *C. herbarum* evident in other studies (3, 15) was less pronounced in our investigations. Contrary to findings in the present study, *Cryptococcus* isolates were reported to be antagonistic to other pathogens (7). Therefore, before conclusions can be drawn concerning the interaction of this genus with *A. porri*, further study is needed. However, the absence of antagonistic ability of the isolate used in our studies was useful in investigating which stage of the prepenetration development of *A. porri* on the onion leaf was affected.

The spores of *A. porri* germinated equally well in the presence of antagonistic as well as nonantagonistic saprophytes. After germination, however, the superficial growth on the leaf surface was suppressed greatly by *A. pullulans* and *S. roseus*. Reduction of the superficial mycelium of the pathogen may reduce the frequency of penetration and consequently reduce infection. A similar system was found when the above two microorganisms suppressed the increase of superficial mycelium of *Helminthosporium sativum* by pollen (7). The extent of superficial growth of perthotrophic pathogens might determine their sensitivity to antagonistic action by the saprophytes as well as to stimulation by nutrients. This might explain why *B. squamosa*, which penetrates rapidly (8), was not affected by *A. pullulans*.

How the superficial development of *A. porri* on onion leaves is affected by the antagonistic microorganisms still is uncertain. If competition for nutrients is involved, this study demonstrated that the total amount of carbohydrates and amino acids is not likely to be the limiting factor. In this respect, it must be realized that a concn of 10^5 *Aureobasidium* cells per cm^2 of leaf surface will add only 2 mm^2 of propagule surface to that area. It also is possible that consumption of carbohydrates by the antagonistic microorganisms will not result in a reduction of the total amount of carbohydrates in the phyllosphere because of its replacement by translocation processes from internal plant tissue. Nutrients which are difficult to leach (18) are more likely to become limiting by competition. Studies with pollen have revealed that no major nutrients are involved in the stimulation of superficial growth and infection by plant pathogens (6, 17, 20). In the presence or absence of pollen, the antagonistic microorganisms might compete successfully for certain vitamins or trace elements necessary for extensive superficial growth of the pathogen.

The populations of saprophytes used in our studies were high compared to the colonization levels found on unsprayed onion leaves in nature (C. A. Clark, *personal communication*). The waxy onion leaves apparently provide poor conditions for saprophytic colonization compared to leaves of other plants for which colonization levels are much higher (13). If biological control of *A. porri* is attempted in nature with phyllosphere yeasts or other fungi, the greatest difficulty in achieving satisfactory control probably will be the artificial maintenance of the high populations of such organisms without stimulation of the pathogen. When high populations of yeasts or yeastlike organisms occur in nature, they may play a more important antagonistic role than has been recognized previously. Studies on the use of fungicides which conserve such microorganisms and

further exploration of the antagonistic capabilities of these microorganisms warrant increased emphasis in the future.

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